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Serotonin-transporter mediated efflux: A pharmacological analysis of amphetamines and non-amphetamines

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Abstract

The physiological function of neurotransmitter transporter proteins like the serotonin transporter (SERT) is reuptake of neurotransmitter that terminates synaptic serotoninergic transmission. SERT can operate in reverse direction and be induced by SERT substrates including 5-HT, tyramine and the positively charged methyl-phenylpyridinium (MPP^+), as well as the amphetamine derivatives *para*-chloroamphetamine (pCA) and methylene-dioxy-methamphetamine (MDMA). These substrates also induce inwardly directed sodium currents that are predominantly carried by sodium ions. Efflux via SERT depends on this sodium flux that is believed to be a prerequisite for outward transport. However, in recent studies, it has been suggested that substrates may be distinct in their properties to induce efflux. Therefore, the aim of the present study was a pharmacological characterization of different SERT substrates in uptake experiments, their abilities to induce transporter-mediated efflux and currents. In conclusion, the rank order of affinities in uptake and electrophysiological experiments correlate well, while the potencies of the amphetamine derivatives for the induction of efflux are clearly higher than those of the other substrates. These discrepancies can be only explained by mechanisms that can be induced by amphetamines. Therefore, based on our pharmacological observations, we conclude that amphetamines distinctly differ from non-amphetamine SERT substrates.
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Keywords: Carrier-mediated efflux; Amphetamine; Serotonin; Facilitated exchange diffusion model; Channel properties

1. Introduction

The presynaptic monoamine transporters clear extracellular serotonin, dopamine and norepinephrine (5-HT, DA, NE) after release from presynaptic terminals (Rudnick and Clark, 1993). The respective transport proteins that mediate this reuptake are part of a larger family of neurotransmitter:sodium symporters (NSS 2.A.22; Busch and Saier, 2002) and named according to their substrate 5-HT, DA and NE transporter (SERT, DAT, NET). SERT and NET are targets for widely prescribed medications such as the tricyclic antidepressants (e.g. imipramine) and the serotonin-selective-reuptake inhibitors (SSRI, e.g. fluoxetine, Prozac®; Barker and Blakely, 1995; Schloss and Williams, 1998) or norepinephrine-selective-reuptake inhibitors (NSRI, e.g. reboxetine). Psychostimulants and cocaine (Seiden et al., 1993; Kuhar et al., 1991) also act at monoaminergic neurotransmitter transporters. While the action of cocaine is a mere inhibition of the uptake transporter, a reversal of the transport direction of SERT (Wall et al., 1995; Cinquanta et al., 1997; Crespi et al., 1997) constitutes the action of SERT substrates such as methylene-dioximethamphetamine (MDMA, better known as ecstasy; Green et al., 2003). Reverse operation has also been termed calcium-independent, carrier-mediated efflux or release (Attwell et al., 1993; Levi and Raiteri, 1993). Many models attempt to explain the releasing action of transporter substrates including the facilitated exchange diffusion model (Fischer and Cho, 1979; Bönisch and Trendelenburg, 1989).

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The models explain the releasing action as a consequence of the translocation of the releasing agent by the plasmalemmal transporter into the cell that is followed by a conformational change of the protein facilitating outward transport of the monoamine.

In this strict model, influx determines the rate of efflux. The faster the carrier can flip to the inside, the faster it can reverse to the outside and expel the substrate. In such a model one would expect influx and efflux rates to be modulated in a stoichiometrically coupled fashion (Fischer and Cho, 1979). Recent findings of our group show inconsistencies with this hypothesis as reverse transport rates in SERT did not seem to be strictly coupled to ratios of inward transport (Sitte et al., 2001). Furthermore, Pifl and Singer (1999) demonstrated a lack of a positive correlation of uptake and release rates in experiments exploiting DAT- and NET-expressing human embryonic kidney (HEK) 293 cells. Taken together, these findings question the hypothesis of facilitated exchange diffusion where uptake of the releasing substrate is considered to be the sole prerequisite for release of the preloaded substrate.

In recent years, electrophysiological approaches uncovered the existence of a conductance in several monoamine transporters that is uncoupled from the transport cycle (Mager et al., 1994; Sonders et al., 1997; Galli et al., 1996). This 'current in excess' has been suggested to be carried by sodium ions (Adams and DeFelice, 2002). Based on direct comparison of transport rates and charge transfer induced by amphetamine, we concluded that substrate efflux induced by D-amphetamine in DAT correlates better to D-amphetamine induced current than to substrate influx (Sitte et al., 1998). Galli's group confirmed and extended this view most elegantly by a combination of electrophysiological patch-clamp recordings and amperometry (Khoshbouei et al., 2003). Their findings revealed the dependence of amphetamine-triggered dopamine efflux on intracellular sodium and depolarization. Furthermore, amphetamines are capable of depolarizing DAT-expressing oocytes (Meinild et al., 2004). Most recently, we communicated that amphetamine-induced efflux by SERT is contingent on the spatial proximity of transporter proteins in an oligomeric complex (Seidel et al., 2005). We concluded that SERT substrates differ in their propensity to induce efflux either via the dimeric partner protein or according to the alternate access scheme via the very same transporter subunit within the oligomeric complex (Seidel et al., 2005). Kahlig et al. (2005) examined the interaction of DAT and substrates like DA and amphetamine by noise analysis combined with amperometry; they observed that only amphetamine, but not dopamine, is capable of inducing a transporter channel that allows for burst-like events releasing distinct amounts of DA.

The aim of the present study was to characterize the relationship between inward and outward transport as well as transport-associated currents induced by different non-amphetamine substrates of SERT, like 5-HT, tyramine and MPP^+. In comparison, we analysed the amphetamine derivatives, *para*-chloroamphetamine (pCA) and MDMA.

2. Materials and methods

2.1. Cell line transfection and cell culture

Since HEK293 cells stably expressing the SERT, alter their level of expression depending on time in culture (V_{max} values decrease about 80% within a few weeks; Sitte et al., 2001), we established a HEK293 T-REx cell line stably expressing SERT under the control of a tetracycline-regulated expression system (T-REx™, INVITROGEN; Tet-on) which is supposed to have a constant level of expression.

The cDNA for the human plasmalemmal SERT (Ramamoorthy et al., 1993) was a generous gift of Dr R.D. Blakely (Department of Pharmacology and Center for Molecular Neuroscience, School of Medicine, Vanderbilt University, Nashville, TN). The coding region was subcloned into pEGFP-C1 (CLONTECH, Palo Alto, CA) removing the GFP coding region as described previously (Scholze et al., 2000). From this construct the SERT-coding region was excised using *Xho*I and cloned into the multiple cloning site of the inducible expression vector pcDNA4/TO. The resulting construct (hS4TO) was co-transfected with the regulatory plasmid pcDNA6/TR into the T-REx-293 cell line, a mammalian cell line that stably expresses the Tet repressor, which is commercially available (Invitrogen, Carlsbad, CA).

To create stable transfectants, hS4TO-DNA was transfected into the T-REx-293 cell line using the calcium phoshate precipitation method (Chen and Okayama, 1987). One day after transfection, zeocin (300 μg/ml) was added and the surviving cells were grown for 10 days. Then, single colonies were picked and tested for their transport activity. The best clone was selected and used throughout all experiments.

SERT expression was initiated by addition of tetracycline (1 μg/ml) at least 24 h before an experiment.

2.2. Uptake experiments

The experiments were performed in poly-D-lysine-coated 24-well plates 2 days after plating 1.5×10^5 cells/well. Each well was washed with 2 ml of buffer (Krebs-HEPES buffer containing 10 mM HEPES, 130 mM NaCl, 1.3 mM KH_2PO_4, 1.5 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$, 34 mM dextrose, final pH 7.35). The cells were incubated in a final volume of 0.2 ml of buffer containing 0.5 μCi of [^{3}H]5-HT (25.5 Ci/mmol) or 0.9 μCi of [^{3}H]MPP^+ (85 Ci/mmol) or 0.4 μCi of [^{3}H]tyramine (40 Ci/mmol) and various concentrations of unlabeled substrate.

To prevent diffusion (Scholze et al., 2001), the uptake buffer was rapidly aspirated after an incubation period of 1 min at room temperature and the cells were washed twice with 2 ml of ice-cold buffer. Cells were lysed with 0.5 ml of 1% sodium dodecyl sulfate (SDS) and transferred into vials for liquid scintillation counting. Non-specific uptake was defined as uptake in the presence of 30 μM clomipramine and amounted to less than 20% of total uptake in the presence of 130 mM NaCl.

2.3. Superfusion experiments

The cells were grown overnight on poly-D-lysine coated round glass coverslips (5-mm diameter; 4×10^4 cells/coverslip) and incubated with 30 μM [^{3}H]5-HT (0.4 μCi/well), 80 μM [^{3}H]MPP^+ (0.8 μCi/well) or 200 μM [^{3}H]tyramine (0.8 μCi/well) for 20 min at 37 °C in a final volume of 0.1 ml buffer. Coverslips were then transferred to small superfusion chambers (0.2 ml) and superfused with Krebs-HEPES buffer at 25 °C and a flow through rate of 0.7 ml/min.

After a washout period of 45 min to establish a stable efflux of radioactivity, the collection of 2-min fractions of superfusate was started. Drugs were added 6 min after commencement of sample collection for a 10-min period. At the end of experiments cells were lysed in 1% SDS. Tritium in the superfusate fractions and in the SDS-lysates was determined by conventional liquid scintillation counting. The release of ^{3}H 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. Drug-induced release was calculated by subtracting estimated basal efflux from total

efflux during minute 6 to minute 10 of drug exposure and expressed as percentage of radioactivity in the cell at the beginning of drug exposure.

2.4. Electrophysiological experiments

About 4×10^4 cells were split into poly-D-lysine coated 35-mm diameter cell culture dishes 48 h prior to the recording of I_{Na}. The external (bathing) solution has been described above (Krebs-HEPES buffer) and had an osmolality of 300 mosmol/l. Patch pipettes were filled either with 120 mM KCl, 10 mM HEPES, 1.1 mM EGTA, 0.1 mM $CaCl_2 \cdot 2H_2O$, 2 mM $MgCl_2 \cdot 6H_2O$ and 30 mM dextrose or 110 mM $CsCl_2$, 20 mM tetraethylammonium chloride, 1.1 mM EGTA, 0.1 mM $CaCl_2 \cdot 2H_2O$, 2 mM $MgCl_2 \cdot 6H_2O$ and 30 mM dextrose (270 mosmol/l, respectively), to block voltage activated potassium currents; both solutions were adjusted to pH 7.35 with KOH.

Patch electrodes were pulled from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) with a programmable Flaming-Brown micropipette puller (P-97; Sutter Instruments Co., Novato, CA, USA) and heat-polished to a final tip resistance of 4—8 MΩ. Whole cell currents were recorded at room temperature (22 ± 2 °C), using an Axopatch 200B amplifier and the PClamp 6.0 software (Axon Instruments, Foster City, CA, USA). Currents were low-pass filtered at 1 kHz and stored on an IBM compatible computer. Traces were analysed off-line by the Clampfit program (Axon). Drugs were applied via a drug application device (DAD-12; Adams & List, Westbury, NY, USA) which permits exchange of solutions surrounding the cell under investigation within less than 100 ms. Cells were continuously superfused with bathing solution or with solutions containing different concentrations of the substrates 5-HT, tyramine, MPP^+, *para*-chloroamphetamine (pCA) and methylene-dioximethamphetamine (MDMA), or with the SSRI paroxetine. The cells were voltage-clamped at a holding potential of −70 mV. Alternatively, substances were examined using 150 ms voltage steps from −20 mV to potentials between −120 and +20 mV. Peak currents were normalised to cell capacitance and displayed as substrate inward currents minus leak currents ($I_{tot} - I_{leak}$).

2.5. Calculations and statistics

V_{max} and K_m values were calculated using nonlinear regression fits performed with Prism (GraphPad, San Diego, CA). The values for concentrations eliciting half maximal 5-HT efflux or inward currents were calculated using the nonlinear curve fitting computer program Prism (Graph PAD Software, San Diego, CA). All results are expressed as means ± SEM values. To determine statistical differences between drug effects, one-way ANOVA with subsequent Bonferroni's multiple comparison test was applied.

2.6. Materials

Tissue culture reagents were from Gibco Life Technologies. $[^3H]$5-HT (25.5 Ci/mmol) and $[^3H]MPP^+$ (85 Ci/mmol) was from PerkinElmer Life Science Products (Boston, MA) and $[^3H]$tyramine (40 Ci/mmol) were from American Radiolabeled Chemicals Inc. (St. Louis, MO). 5-HT, *para*-chloroamphetamin (PCA) and MDMA were from Sigma-Aldrich Handels GmbH (Vienna, Austria). Tyramine was from FLUKA (Bucha, Switzerland), paroxetine was from SB SmithKline & French (Welwyn Garden City, Herts, UK) and MPP^+ was from RBI/Sigma, Natick, MA). All other chemicals were obtained from Merck or Sigma-Aldrich.

3. Results

The hS4TO cell line exhibited a clomipramine-sensitive, time-, temperature-, and concentration-dependent accumulation of $[^3H]$5-HT, $[^3H]MPP^+$ and $[^3H]$tyramine (see Table 1 for V_{max} and K_m values).

Table 1
Average K_m (in μM) and V_{max} values (in pmol/min per 10^6 cells) of uptake of SERT substrates in hS4TO cells and, in italic letters, HEK293 SERT cells for PCA, see Seidel et al. (2005)

	K_m	V_{max}
$[^3H]$5-HT	3.99 ± 0.57	801 ± 84
$[^3H]MPP^+$	47.51 ± 11.86	1011 ± 72
$[^3H]$Tyramine	52.7 ± 15.87	991 ± 51
PCA	*5.00 ± 2.90*	*390 ± 77*

Values were derived using nonlinear fitting, and represent mean ± SEM of 4—11 observations; one observation = one substrate analysis performed in triplicate.

3.1. Substrate-induced $[^3H]$5-HT efflux

Basal $[^3H]$5-HT efflux rate from hS4TO cells stably expressing the SERT preincubated with $[^3H]$5-HT amounted to 4.38 ± 0.16%/min (246 ± 10 dpm) during min 2—6 (N = 50, randomly chosen experiments). Addition of the SERT substrates 5-HT and tyramine to the superfusion buffer caused a concentration- and time-dependent increase in $[^3H]$5-HT efflux rate (Fig. 1A), reaching a maximum of about 7%/min after 4 min of drug exposure at saturating concentrations of 5-HT (300 μM; Fig. 1A).

Concentrations of 100 μM tyramine and above induced a concentration-dependent increase. The maximal effect was reached at 3 mM representing an efflux rate of about 7.5%/min following 4 min of drug exposure (Fig. 1C).

By contrast, the SERT substrate MPP^+ caused a decrease of $[^3H]$5-HT efflux (~1%/min) up to a concentration of 30 μM. Higher concentrations caused increases with a maximal effect similar to those of tyramine or 5-HT (Fig. 1B).

Likewise, both amphetamine-derivatives pCA and MDMA led to a concentration dependent increase of $[^3H]$5-HT efflux rate (Fig. 1D and E). Moreover, the extent of tritium overflow was the same as those observed with the other substrates.

3.2. Electrophysiological experiments

Application of SERT substrates to hS4TO cells clamped at a holding potential of −70 mV (shown in Fig. 2A for 5-HT, trace 1) induced an inwardly directed current. Co-application of the antagonist paroxetine (1 μM) completely blocked the substrate evoked current (Fig. 2A, trace 2). 5-HT, MPP^+ and tyramine led to concentration-dependent increases in inward currents (Fig. 2B—D) that showed saturation at high concentrations. The amphetamine derivatives MDMA and pCA also induced inward currents, but the relaxation of the current was distinct from those induced by other SERT substrates (Fig. 2E and F).

In a separate set of experiments, the current—voltage relation of currents evoked by different substrates was investigated using the step protocol described in Section 2. hS4TO cells displayed an outwardly rectifying potassium current of considerable amplitude at test pulses positive to −20 mV (Fig. 3A) that was completely abolished by replacing KCl in

the pipette solution by CsCl (data not shown). The amplitudes of both the leak and the 5-HT-induced currents were paroxetine- and voltage-dependent as shown in Fig. 3A and reversed between −40 mV and −20 mV, respectively. Determination of the net current for other substrates revealed similar behaviors and reversal potentials (Fig. 3B). Due to differences in platings and current sizes among cells, we attempted to determine maximally induced currents in single cells (Fig. 3C). Tyramine induced significantly higher currents as compared to those of 5-HT; in comparison, MPP^+ as well as the amphetamine derivatives elicited slightly lower current fluxes as compared to those evoked by 5-HT (Fig. 3C).

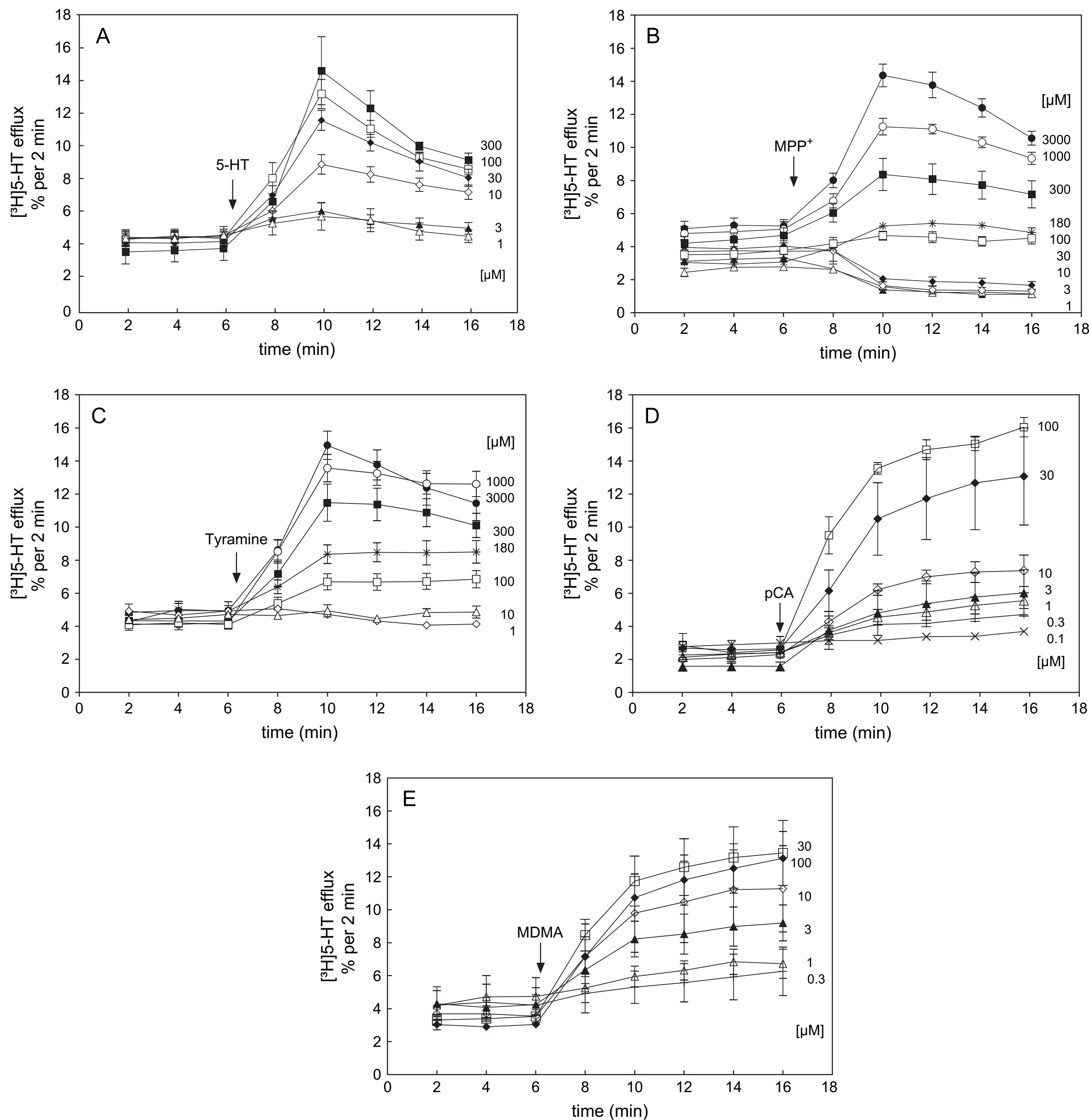

Fig. 1. Time course of the effects of SERT substrates on [^{3}H]5-HT efflux from hS4TO cells. The cells were loaded with 30 μM [^{3}H]5-HT and superfused, and 2-min fractions were collected. After three fractions (6 min) of basal efflux, the buffer was switched to a buffer containing different concentrations of SERT substrates: (A) 5-HT; (B) MPP^+; (C) tyramine; (D) pCA; (E) MDMA. 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. The graphs in panels (A)–(E) show typical superfusion experiments performed in duplicate or triplicate that are representative of 3–6 experiments.

3.3. Uptake experiments and concentration–response curves

Saturation isotherms are given in Fig. 4A for the substrates [^{3}H]5-HT, [^{3}H]MPP$^+$ and [^{3}H]tyramine, respectively. For comparison, we included the values for uptake of pCA (gray symbols, dotted line; Seidel et al., 2005). Calculated K_m and V_{max} values are given in Table 1 (values for PCA are from Seidel et al., 2005).

Concentration–response curves (CRC) for the effects of the SERT substrates on [^{3}H]5-HT efflux are shown in Fig. 4B. The calculated EC_{50} values are 10.18 ± 1.7 μM for 5-HT, 145.80 ± 1.6 μM for tyramine and 175.50 ± 1.4 μM for MPP$^+$; the values for pCA and for MDMA are 6.727 ± 1.305 μM and 2.872 ± 0.631 μM, respectively (see also Table 2). The maximum effects expressed as a percentage of radioactivity released during the last 6 min of drug exposure, were approximately 2%/min for MDMA, 3%/min for PCA, 5-HT and MPP$^+$, and about 4%/min for tyramine.

CRCs for current normalized to maximally induced current in each cell are calculated from steady-state measurements and given Fig. 4C.

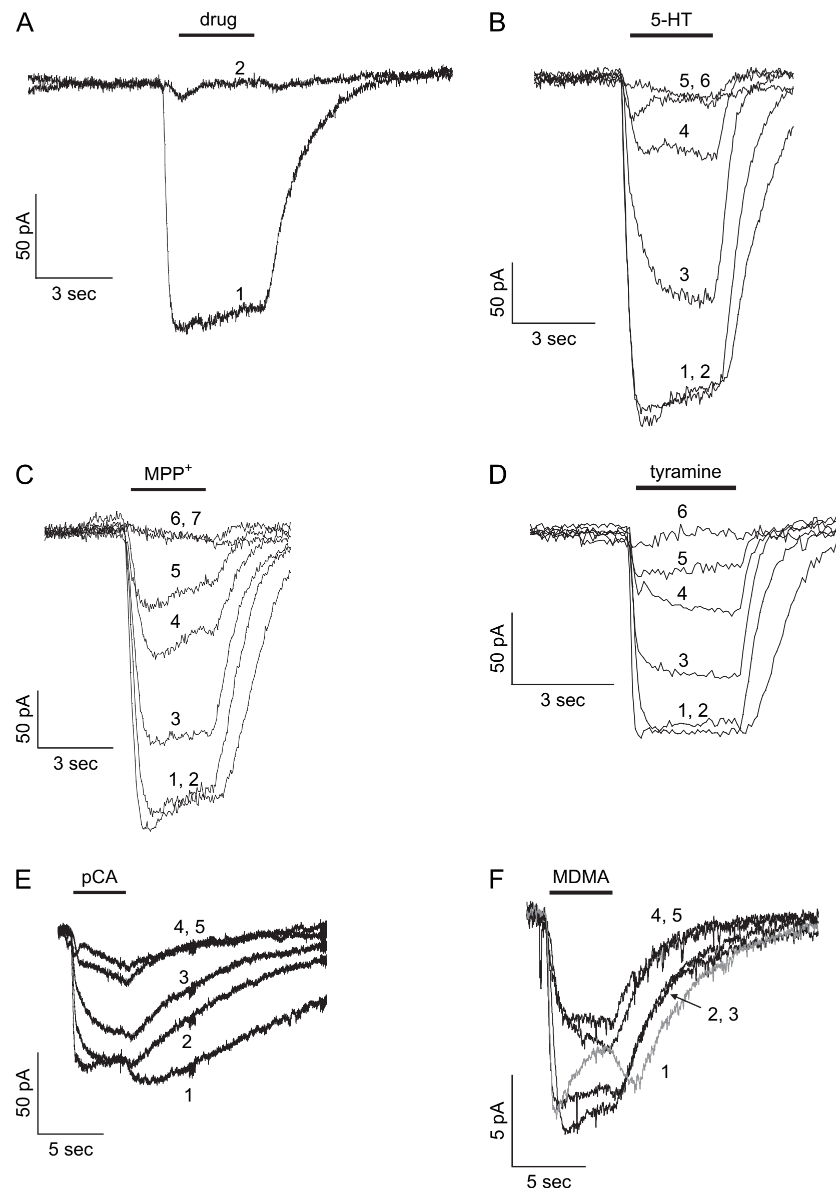

Fig. 2. Whole-cell patch-clamp recordings of hS4TO cells. The holding potential was −70 mV. The solution used to fill the patch pipette was made up with KCl. (A) The bar indicates superfusion with 30 μM 5-HT in the absence and presence of 1 μM paroxetine (traces 1 and 2, respectively). (B)–(F) display the concentration-dependent effects of 5-HT, MPP$^+$, tyramine, pCA and MDMA. The numbers denote the different concentrations used: 5-HT (B): 30 μM (1), 10 μM (2), 1 μM (3), 0.3 μM (4), 0.1 μM (5) and 0.01 μM (6); tyramine (C): 1 mM (1), 100 μM (2), 30 μM (3), 10 μM (4), 3 μM (5) 0.3 μM (6); MPP$^+$ (D): 300 μM (1), 100 μM (2), 30 μM (3), 10 μM (4), 3 μM (5), 1 μM (6) and 0.3 μM (7); pCA (E): 10 μM (1), 3 μM (2), 2 μM (3), 1 μM (4), 3 μM (5); MDMA (F): 30 μM (1), 10 μM (2), 3 μM (3), 2 μM (4), 1 μM (5).

4. Discussion

The aim of the present study was to test the conjecture that amphetamine- and non-amphetamine substrates differ in the way they elicit efflux. We characterized the pharmacological properties of two amphetamine-like substrates, pCA and MDMA, and compared them with those of the non-amphetamine substrates 5-HT, MPP^+ and tyramine. For this purpose, we employed HEK293 cells stably transfected with the human SERT under the control of a tetracycline inducible expression system (hS4TO cells). We studied the effects of the different substrates in radiotracer release experiments, whole-cell patch-clamp recordings and, radiotracer uptake assays.

Radiotracer release experiments (Pifl et al., 1995) were carried out by preincubation of hS4TO cells with radioactively labeled 5-HT and monitoring subsequent efflux of radioactivity. Interestingly, hS4TO cells displayed a marked basal efflux after a 45 min wash-out period (Fig. 1). All substrates elicited concentration-dependent increases in $[^3H]$5-HT efflux (Fig. 1). However, MPP^+ induced a decrease of $[^3H]$5-HT efflux at low concentrations (Fig. 1B). This finding is interesting in itself as it is difficult to explain within the rigid body of a classic alternate access model (Jardetzky, 1966). Such models assume that a transporter is occupied by only one substrate molecule at a time and that the cycle speed is determined by the affinity of the substrate species binding the outward or the inward conformation, respectively. The only kind of interaction between different substrate molecules being assumed in such models is competition for a binding site. Our data indicate that an interaction in the transport of two different substrate species (MPP^+ and $[^3H]$5HT) is taking place, because MPP^+ is capable of changing the transport rates of $[^3H]$5-HT. Competition is very unlikely to be the cause of that, because if this were the case administration of MPP^+ should reduce the reuptake rate of $[^3H]$5-HT thereby increasing the apparent net efflux rate at any concentration (Scholze et al., 2001); conversely we see a decrease at low concentrations of MPP^+.

Therefore, we conclude that any alternate access model accounting for the ‘undulating behavior’ found in this study will have to be extended to allow for allosteric interactions between different substrate molecules; for instance bound MPP^+ may induce a conformational change that hinders the conformational change allowing for efflux of cytoplasmatically located $[^3H]$5-HT.

In an earlier report (Sitte et al., 1998), we have shown that amphetamine-induced dopamine efflux correlates much better with the induction of transporter-related current than its uptake; therefore, we examined the inwardly directed current in hS4TO cells elicited by various SERT substrates and compared it with their uptake and their effect on $[^3H]$5-HT efflux. The current was inwardly directed, inhibited by 5-HT

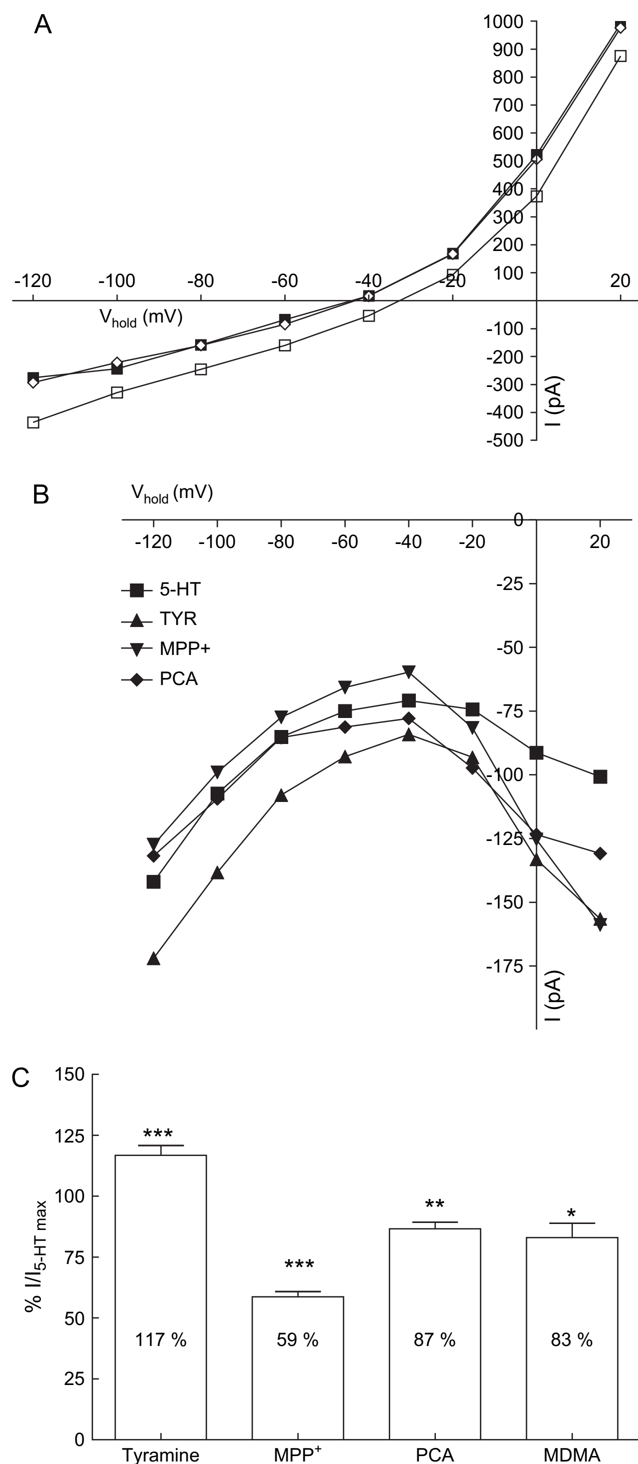

Fig. 3. Voltage-dependence of substrate-induced currents in hS4TO-cells. (A) Representative *I–V* plot of a hS4TO cell patched with an internal solution containing KCl. Current in the absence (■) or presence of 30 μM 5-HT (□); 1 μM paroxetine was added to 30 μM 5-HT to test for inhibitor-sensitivity (◇). Symbols show a single cell representative of four experiments with similar results. (B) *I–V* plot of hS4TO cells patched with an internal solution containing KCl. Cells were voltage-clamped at a holding potential of −20 mV and stepped to the indicated levels of test potentials as described in Section 2. Cells were superfused with 30 μM 5-HT (■), 300 μM MPP^+ (●), 300 μM tyramine (▼) or 10 μM PCA (◆). Symbols show single cells representative of three experiments with similar results. (C) Substrate induced inward currents with saturating concentrations as percentage of the current induced by 5-HT in single cells. Statistical analysis was achieved by ANOVA followed by Bonferoni's post-hoc test for multiple comparisons calculated with GraphPad Prism 4 software (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

reuptake inhibitors and reverted to baseline after cessation of the drug application (Fig. 2A). As shown by recent publications of Adams and DeFelice (2002) and Quick (2003), these currents are carried by Na^+. This was also the case in our experiments, since: (i) isoosmotically lowering of the NaCl concentration considerably reduced the inwardly directed current (not shown) and (ii) according to the Hodgkin–Goldman–Katz equation, the substrate-induced current shifted to the right in comparison to the leak current (Fig. 3B). The currents of all substrates saturated at higher concentrations (Fig. 2B–F). Similar to the experiments shown in a recent publication (Seidel et al., 2005), the results obtained reveal that the 5-HT induced current is slightly higher than the current observed with the amphetamines and MPP^+; tyramine is the only substrate capable of inducing higher current than 5-HT (Fig. 3C).

When we compared the effects of the substrates on efflux and current with their transporter mediated uptake (Fig. 4), a more complex image evolved. The most potent inducer of efflux was pCA (rank order of potencies in induction of efflux: pCA > MDMA > 5-HT > MPP^+ > tyramine; Fig. 4B). In contrast, 5-HT showed the highest affinity in uptake experiments (rank order of affinities in uptake experiments: 5-HT > PCA > tyramine > MPP^+; Fig. 4A, results for PCA are taken from Seidel et al., 2005, for comparison, MDMA has not been determined in uptake experiments). Concentration–response curves obtained from the electrophysiological experiments reveal a picture that resembles the situation in the uptake experiments (Fig. 4C). Again, 5-HT shows the highest affinity to induce current (rank order of potencies in induction of currents: 5-HT > pCA > MDMA > MPP^+ > tyramine; Fig. 4C).

A direct comparison with the data obtained in DAT (Sitte et al., 1998) reveals distinct differences between the two transport proteins. In DAT, a perfect match was obtained between affinities of the substrates in uptake and current; this, however, is not the case in SERT; there is only some correlation with respect to the rank order of potencies. In DAT, the maximally inducible current correlated much better with the maximally inducible efflux than with the uptake rate. There is somehow a resemblance in SERT; the maximally inducible current was highest with tyramine, followed by 5-HT, and then

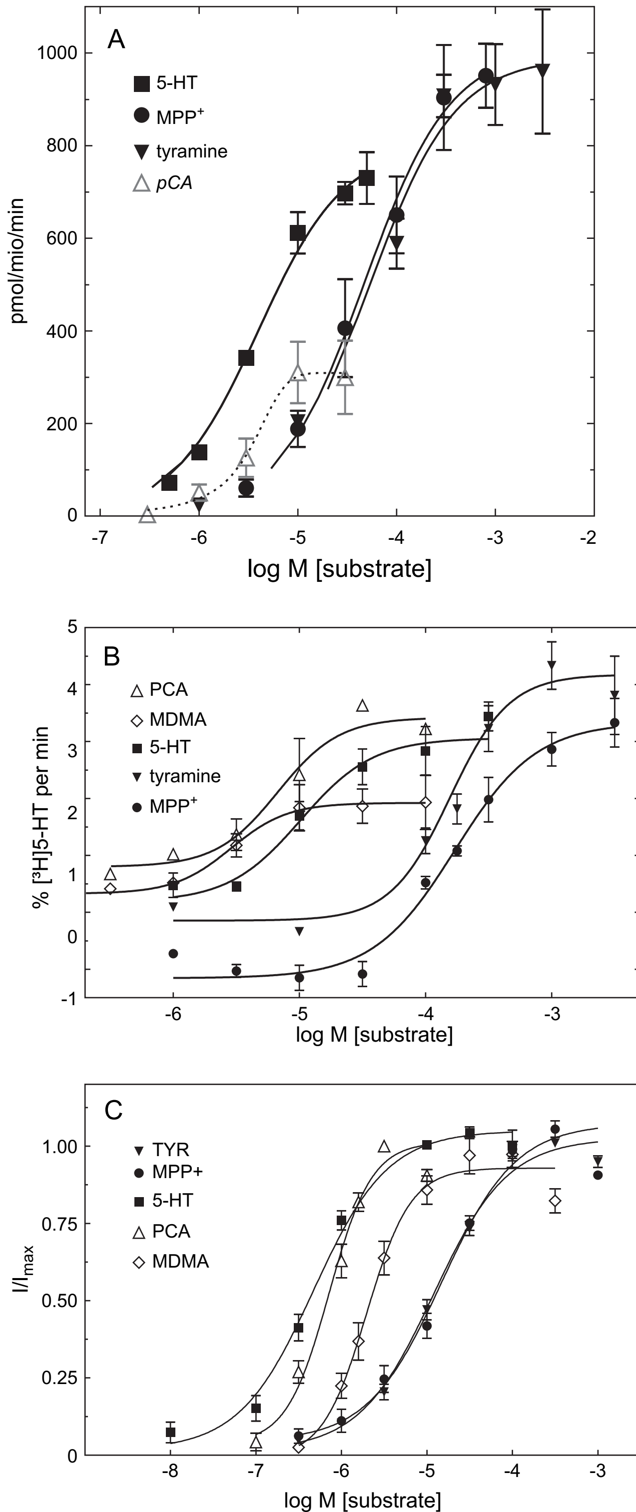

Fig. 4. Concentration–response curves of uptake, efflux and electrophysiological data. (A) Saturation isotherms of substrate transport; 5-HT (■), $[^3H]MPP^+$ (●), $[^3H]$TYR (▼). pCA uptake (△) is shown as gray symbols and with dotted line (reproduced from Seidel et al., 2005). hS4TO cells were distributed in 24-well plates as described in Section 2 (1.5×10^5 cells) and incubated in the presence of different concentrations of $[^3H]$-labeled substrate for 1 min, washed, solubilized, and the tritium activity was determinated by liquid scintillation counting. Nonspecific uptake was defined in the presence of 30 μM clomipramine. One of 11 experiments, performed in triplicate is shown. (B) Effects of SERT substrates on efflux of $[^3H]$5-HT in T-REx hS4TO cells. Concentration–response curves of SERT substrates pCA (△), MDMA (◇), 5-HT (■), MPP^+ (●) and TYR (▼) for their effects on $[^3H]$5-HT efflux. For experimental details see Section 2. Drug induced efflux was calculated from total radioactivity released during the last 6 min of drug exposure minus estimated baseline efflux and expressed as percentage of radioactivity present in the cells at the beginning of drug addition. Symbols represent mean ± SEM of six observations (one observation = one superfusion chamber; duplicate determinations from at least three independent experiments). The data were fitted by nonlinear regression. (C) Concentration–response curves for the net currents ($I_{substrate} - I_{leak}$) of the SERT substrates 5-HT (■), pCA (△), MDMA (◇), MPP^+ (●), tyramine (▼) are given. The resulting EC_{50} values are: 5-HT 0.47 ± 1.1 μM, tyramine 11.95 ± 1.1 μM, MPP^+ 14.82 ± 1.2 μM, pCA 1.49 ± 0.5 μM, and for MDMA 1.88 ± 0.32 μM.

Table 2
Comparison of the potencies/affinities of different SERT substrates in this study

	5-HT (μM)	PCA (μM)	MDMA (μM)	MPP^+ (μM)	Tyramine (μM)
K_m	3.99 ± 0.57	*5.00 ± 2.90*	n.d.	47.5 ± 11.9	52.7 ± 15.9
$ED_{50\ current}$	0.47 ± 0.06	0.51 ± 0.10	1.32 ± 0.12	14.8 ± 0.07	12.0 ± 0.04
$EC_{50\ efflux}$	10.25 ± 1.44	5.35 ± 1.2	2.87 ± 1.63	170.7 ± 1.3	145.8 ± 1.3

PCA, MDMA and MPP^+. In contrast, uptake experiments revealed that MPP^+ was the substrate transported at the fastest rate, followed by tyramine, 5-HT and PCA (Seidel et al., 2005). Hence, there is a similarity in that the correlation between maximal uptake velocity and maximally inducible current fails. In DAT, the maximally inducible efflux differs considerably between substrates and correlates much better with the E_{max} values of current induction; in SERT, we did not observe significant differences between the E_{max} values in efflux induction (Fig. 4C; see also Scholze et al., 2000) and no clear-cut correlation with the E_{max} values of current induction (Fig. 3C).

For DAT, we concluded that the increase in intracellular sodium by a presumptive channel mode of operation (Sonders and Amara, 1996) was the sole trigger for outward transport of preloaded substrate (Sitte et al., 1998). Nonetheless, sodium is important for SERT in the present set of data. However, not only sodium is needed for induction of efflux—as the main prerequisite—but also a change in the membrane potential, namely a depolarization (Khoshbouei et al., 2003) that can be induced by transporter substrates (Meinild et al., 2004). Recent observations from Galli's laboratory on DAT as well as from our laboratory on SERT point to an even more complex situation; the substrates of these transporters seem to differ in the way they induce carrier-mediated efflux. Kahlig et al. (2005) explain that amphetamines induce a distinct channel pore in DAT that mediates solely outward flux of dopamine (DA); most importantly, the authors conclude from their experiments that this channel induction cannot similarly be observed when DA is used. Hence, DA, but not amphetamine, induces reverse transport in the transporter-like mode, and that is according to the facilitated exchange diffusion model (Fischer and Cho, 1979), while amphetamines induce both the transporter-like and the channel-like mode. In the present experiments, all the amphetamine-like substrates were found to be more potent in induction of efflux as compared to the non-amphetamine substrates (Fig. 4C). This distinct enhancement of this higher potency in induction of efflux must be viewed in light of these recent publications discussed before. The only way to explain the higher potency is to hypothesize an additive effect that adds on the transporter-like efflux elicited by non-amphetamine-like substrates and produces the proposed channel-like mode of efflux also in SERT.

In a recent publication on SERT, our laboratory concluded from experiments that employed the protein kinase C (PKC)-inhibitor GF109203X (bisindolyl-maleimide I; Seidel et al., 2005) the importance of phosphorylation of the transporter prior to reverse operation: only amphetamine but not non-amphetamine substrates of SERT can directly induce the activity of PKC (Giambalvo, 1992; Kramer et al., 1997). These observations are in line with studies employing heterologously expressed DAT (Khoshbouei et al., 2004) as well as a striatal preparation (Kantor and Gnegy, 1998), and the norepinephrine transporter (Kantor et al., 2001; Kantor et al., 2002). And the influence of PKC or probably other kinases is currently being explored in our laboratory.

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