ABSTRACT

The membrane transporters for the monoamines serotonin (SERT) and dopamine (DAT) are prominent targets of various psychoactive substances, including competitive inhibitors, such as tricyclic antidepressants, methylphenidate, and cocaine. Upon rapid application of a substrate, SERT and DAT display an inwardly directed current comprised of a peak and a steady-state component. Binding of a competitive inhibitor to the transporter leads to reduction of the peak current amplitude because occupancy of the transporter isomerizes from its outward-facing conformation to the inward-facing conformation. The conceptual framework was developed for the substrate such that the substrate (and cosubstrates) is released into the cell. It is gratifying to note that the predictions of the alternating access model have been verified (Forrest et al., 2008). LeuTα, a bacterial relative of eukaryotic monoamine transporters/solute carrier family 6 family members, has been crystallized in various conformational intermediates (Yamashita et al., 2005; Krishnamurthy and Gouaux, 2012), including an antidepressant-bound state (Singh et al., 2007; Zhou et al., 2007). Similarly, the structure of the dopamine transporter from Drosophila melanogaster has been solved in a complex with antidepressants (Pomatsa et al., 2013). The latter structure confirmed that 1) the binding sites of inhibitors and substrate overlap and 2) the binding site of inhibitors is accessible via the outward-facing conformation. Monoamine transporters are closely related. Their substrate specificity is, in part, overlapping. Moreover, they bind an amazingly large collection of chemically diverse inhibitors. Some inhibit all three monoamine transporters, whereas others show exquisite selectivity (Kristensen et al., 2011). The basis for this selectivity has been explored by a combination of tools, including mutagenesis, docking, and molecular modeling studies (see, e.g., Andersen et al., 2011, 2014; Severinsen et al., 2014). However, the insights provided by these studies are limited since inhibitor binding has mostly been described by equilibrium parameters (e.g., $K_i$ or $IC_{50}$). In kinetic terms, the equilibrium dissociation constant $K_D$ can be described as the dissociation rate constant divided by the association rate constant (i.e., $K_D = k_{off}/k_{on}$). Given that $k_{on}$ has been thought to be close to the diffusion-controlled limit, selectivity for one binding site over another has been implicitly assumed to result from differences in $k_{off}$.

Introduction

Reuptake of the biogenic amines dopamine (DA), norepinephrine, and serotonin [5-hydroxytryptamine (5-HT)] by their cognate transporters [dopamine transporter (DAT), norepinephrine transporter, and serotonin transporter (SERT), respectively] is a prerequisite for homeostatic control of neurotransmission (Kristensen et al., 2011). Accordingly, inhibition of monoamine transport reshapes the presynaptic and postsynaptic responses by both short-term increases of neurotransmitter levels in the synaptic cleft and long-term depletion of vesicular stores. These changes contribute to the therapeutic action of antidepressants and methylphenidate (MPH) and to the toxicity of recreational drugs (Kristensen et al., 2011).

Membrane transporters are thought to operate by an alternating access mechanism. The conceptual framework was proposed by Jardetzky in 1966 (Jardetzky, 1966). The alternating access model posits that the transporter presents a substrate binding site to the extracellular space. Upon binding of the substrate and, in many instances, cosubstrates (i.e., ions), the transporter isomerizes from its outward-facing conformation to its inward-facing conformation. In this state, an exit pathway is created for the substrate such that the substrate (and cosubstrates) is released into the cell. It is gratifying to note that the predictions of the alternating access model have been verified (Forrest et al., 2008). LeuTα, a bacterial relative of eukaryotic monoamine transporters/solute carrier family 6 family members, has been crystallized in various conformational intermediates (Yamashita et al., 2005; Krishnamurthy and Gouaux, 2012), including an antidepressant-bound state (Singh et al., 2007; Zhou et al., 2007). Similarly, the structure of the dopamine transporter from Drosophila melanogaster has been solved in a complex with antidepressants (Pomatsa et al., 2013). The latter structure confirmed that 1) the binding sites of inhibitors and substrate overlap and 2) the binding site of inhibitors is accessible via the outward-facing conformation.

Monoamine transporters are closely related. Their substrate specificity is, in part, overlapping. Moreover, they bind an amazingly large collection of chemically diverse inhibitors. Some inhibit all three monoamine transporters, whereas others show exquisite selectivity (Kristensen et al., 2011). The basis for this selectivity has been explored by a combination of tools, including mutagenesis, docking, and molecular modeling studies (see, e.g., Andersen et al., 2011, 2014; Severinsen et al., 2014). However, the insights provided by these studies are limited since inhibitor binding has mostly been described by equilibrium parameters (e.g., $K_i$ or $IC_{50}$). In kinetic terms, the equilibrium dissociation constant $K_D$ can be described as the dissociation rate constant divided by the association rate constant (i.e., $K_D = k_{off}/k_{on}$). Given that $k_{on}$ has been thought to be close to the diffusion-controlled limit, selectivity for one binding site over another has been implicitly assumed to result from differences in $k_{off}$.
(i.e., longer half-life of the inhibitor in the preferred binding site translates into higher affinity). However, information on the rate constants that govern the binding and unbinding reactions of monoamine transporter ligands is only accessible for a limited set of high-affinity radioligands.

Here, we present an electrophysiological approach to study binding of SERT and DAT inhibitors, with high temporal precision and without the need of any radioligands as the surrogate. We show that this approach is suitable to study rapidly dissociating inhibitors, the kinetics of which are otherwise currently not measureable. Importantly, our analysis shows that the association rate constant $k_a$ can be more influential than $k_{diss}$ in determining the selectivity of inhibitors for closely related binding sites. We exemplify this using 1) the DAT selective inhibitor MPH, which binds very slowly to SERT and 2) desipramine, which binds rapidly to SERT but slowly to DAT. Thus, access of the inhibitor and/or selection of its binding pose can be the rate-limiting step contributing to selectivity.

Materials and Methods

Modification of Plasmids and Transfection. Mutants were created with the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) with the cDNA encoding yellow fluorescent protein–tagged human SERT as a template and confirmed by sequencing. HEK-293 cells were transiently transfected with plasmids (4 μg/75-cm² culture flask) encoding SERT mutants or wild-type SERT using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Cells were assayed 48 hours after transfection.

Whole-Cell Patch Clamp. Patch-clamp recordings were performed with HEK-293 cells stably expressing hSERT and hDAT or transiently expressing SERT mutants I172A and I172V. In all instances, the cells were seeded at a low density 24 hours before measuring currents. Substrate-induced hSERT and hDAT currents were recorded under a voltage clamp using the whole-cell patch-clamp technique. Briefly, glass pipettes were filled with a solution consisting of 133 mM K-glucolate, 5.9 mM NaCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2 with KOH. Substrate-induced peak currents were isolated using an internal solution composed of 152 mM NaCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES (pH 7.2 with NaOH), which eliminated the steady-state current component.

The cells were continuously superfused with an external solution (140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 2 mM MgCl₂, 20 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH). Currents were recorded at room temperature (20–24°C) using an Axopatch 200B amplifier and pClamp 10.2 software (MDS Analytical Technologies, Sunnyvale, CA). Cells were voltage clamped to a holding potential of 0 mV, and the washout period following 5-HT/DA application was 30 seconds in all cases. Current traces were filtered at 1 kHz and digitized at 2 kHz using a Digidata 1320A (MDS Analytical Technologies). Drugs were applied using DAD-12 (Adams & List, Westbury, NY), which permits complete solution exchange around the cells within 100 milliseconds. Current amplitudes were determined using Clampfit 10.2 software (MDS Analytical Technologies). Passive holding currents were subtracted, and the traces were filtered using a 100-Hz digital Gaussian low-pass filter.

Transport Inhibition Assays. HEK-293 cells were seeded on 48-well plates and precoated with poly-d-lysine (0.5 × 10⁵ cells/well) 24 hours prior to the experiment. Each well was washed with 500 μl Krebs-HEPES buffer (KHP) (130 mM NaCl, 1.3 mM KH₂PO₄, 1.5 mM CaCl₂, and 0.5 mM MgSO₄, pH 7.4 with NaOH). The cells were incubated in 0.2 ml KHP buffer containing 0.1 μM [³H]5-HT. The incubation time for [³H]5-HT was 1 minute. Uptake inhibition was assessed by incubating the cells with increasing concentrations of MPH 5 minutes prior and during incubation. Nonspecific uptake was estimated in the presence of paroxetine (10 μM). After incubation at room temperature, the cells were washed with 0.5 ml of ice-cold KHP buffer. Finally, cells were lysed with 0.5 ml 1% SDS, transferred into 2 ml of scintillation cocktail (Rotiszint eco plus LSC, Art. 00163; Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and counted in a Packard 2300TR TriCarb liquid scintillation analyzer (PerkinElmer, Waltham, MA).

Statistics. Uncertainties are shown as 95% confidence intervals (square brackets in main text). Nonoverlapping 95% confidence intervals were considered as significant differences.

Results

Electrophysiological Approach to Determine Association and Dissociation Rate Constants of Monoamine Transporter Inhibitors. Currents through SERT and DAT have two components: 1) an inwardly directed peak current, triggered by rapid substrate application, and 2) a steady-state current (Erreger et al., 2008; Schicker et al., 2012) (Fig. 1A). The peak current is associated with the transition that carries the substrate through the membrane. Hence, the induction of the peak current requires substrate binding to the outward-facing conformation of the transporter and can serve as a probe of inhibitor occupancy at the binding site. However, the presence of the steady-state current component makes it difficult to reliably measure the peak current. The available models for the transport cycle of SERT and DAT predict that raising the intracellular Na⁺ concentration suppresses the

![Fig. 1](https://example.com/fig1.png)
steady-state current without affecting the peak current (Erreger et al., 2008; Bulling et al., 2012; Schicker et al., 2012). Here, we take advantage of this predicted suppression to isolate the peak current (compare Fig. 1, A and B).

Substrate binding triggers the peak current. Cocaine competes with the substrate for binding to SERT and DAT in their outward-facing conformations (Forrest et al., 2008; Bulling et al., 2012). Hence, occupation of the binding site by cocaine ought to progressively reduce the peak current amplitude. We developed a recording protocol to test this prediction (Fig. 1C). We first elicited a peak current using a fast solution exchange protocol to rapidly apply and remove 10 μM 5-HT at the cell. The resulting amplitude of the peak current reflected the maximum number of transporters in the outward-facing conformation that were not occupied by the inhibitor. Subsequently, cocaine was applied for 0.2, 0.5, 1, 2, 5, and 10 seconds prior to application of 10 μM 5-HT. A time-dependent decrease in peak current amplitude is evident in Fig. 1C. The time course of current decline reflects the process of cocaine binding and was well described by a monoexponential function, allowing us to calculate the apparent rate of cocaine association (k_{app}).

Furthermore, we designed a complementary protocol to directly measure k_{off}, as shown in Fig. 1D. Cocaine (1 μM) was applied for 5 seconds, which was long enough to reach binding equilibrium (compare Fig. 1C). Then, the cell was washed for a period of 0.2, 0.5, 1, 2, 5, 10, and 20 seconds to allow for cocaine dissociation prior to application of 10 μM 5-HT and recording of the peak current to assess the fraction of transporters that had remained available for 5-HT binding. As predicted, the peak current recovered over time (Fig. 1D). The time course was again adequately described by a monoexponential function, yielding an estimate for the k_{off} of cocaine.

Kinetics of Cocaine Binding to SERT and DAT. From these two experimental approaches, one can extract independent estimates of k_{off} and k_{on} for inhibitor binding to SERT and DAT, as illustrated for cocaine in Fig. 2. As expected for a simple bimolecular reaction, the time-dependent decrease in peak current amplitude accelerated with increasing cocaine concentrations (Fig. 2, A and B). Accordingly, a plot of the k_{app} of cocaine binding over the cocaine concentration yielded straight lines (Fig. 2C). Their slopes correspond to k_{on}. The lines are parallel; hence, cocaine binds to DAT and SERT with comparable association rates (1.53 [1.43–1.63] × 10^{6} M^{-1}s^{-1} and 1.65 [1.46–1.84] × 10^{6} M^{-1}s^{-1}, respectively). On the other hand, the y-intercepts, which correspond to k_{off}, differ (0.35 [0.28–0.39] s^{-1} versus 0.88 [0.68–1.08] s^{-1} for DAT and SERT, respectively), suggesting that the difference in affinity is attributable to a difference in k_{off}. We verified that cocaine dissociates more rapidly from SERT by directly measuring cocaine dissociation using the time-dependent recovery of the peak current as a readout (Fig. 2D) (SERT k_{off} = 0.66 [0.48–0.83] s^{-1}; DAT k_{off} = 0.35 [0.29–0.41] s^{-1}), independently confirming the difference in the y-intercepts. Finally, we also determined the equilibrium dissociation constant (K_{D}) for cocaine binding from the peak amplitude in the presence and absence of cocaine. For example, after cocaine (100 nM) binding reached equilibrium, the peak amplitude triggered by 5-HT application was ~70% of the control current (Fig. 2B), translating into a cocaine occupancy of ~30%. Over a range of cocaine concentrations, inhibition of the peak current yields a titration curve (Fig. 2E), from which K_{D} values can be estimated. These were in reasonable agreement with the

\[ K_{D} = k_{off}k_{on} \]
TABLE 1
Kinetic and steady-state binding parameters of cocaine, MPH, and desipramine

The $k_{on}$ and $k_{off}$ values are derived from the plot of $k_{app}$ over inhibitor concentration, where the slope and y-intercept of the regression line yield $k_{on}$ and $k_{off}$, respectively. The kinetic dissociation constant $K_{on}$ is $k_{off}/k_{on}$. The equilibrium dissociation constant $K_{Dapp}$ was estimated by a fit of one site-binding model to the amplitudes of the currents that remained unblocked in steady state as a function of increasing inhibitor concentrations.

<table>
<thead>
<tr>
<th></th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>$K_{on}$</th>
<th>$K_{Dapp}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
<td>DAT</td>
<td>1.53 [1.43–1.63] × 10^6</td>
<td>0.35 [0.28–0.39]</td>
<td>2.2 [1.9–2.6] × 10^7</td>
</tr>
<tr>
<td></td>
<td>SERT</td>
<td>1.65 [1.46–1.84] × 10^6</td>
<td>0.88 [0.68–1.08]</td>
<td>5.3 [4.0–6.7] × 10^7</td>
</tr>
<tr>
<td>MPH</td>
<td>DAT</td>
<td>8.32 [7.31–9.33] × 10^8</td>
<td>0.45 [0.39–0.51]</td>
<td>5.4 [4.5–6.3] × 10^7</td>
</tr>
<tr>
<td></td>
<td>SERT</td>
<td>4.30 [4.10–4.50] × 10^9</td>
<td>0.49 [0.37–0.60]</td>
<td>11.4 [8.5–14.2] × 10^6</td>
</tr>
<tr>
<td>Desipramine</td>
<td>DAT</td>
<td>9.27 [8.41–10.10] × 10^4</td>
<td>0.25 [0.09–0.42]</td>
<td>2.7 [0.9–4.4] × 10^6</td>
</tr>
<tr>
<td></td>
<td>SERT</td>
<td>6.10 [5.35–6.85] × 10^6</td>
<td>0.55 [0.42–0.66]</td>
<td>9.0 [6.7–1.1] × 10^8</td>
</tr>
</tbody>
</table>

Differences in Association Rate Constants as Determinants of Inhibitor Selectivity

We explored this hypothesis by examining the kinetic basis for transporter inhibition by MPH. MPH is highly selective for DAT, which it blocks in the nanomolar range. In contrast, the inhibition of SERT requires MPH concentrations in the micromolar range (Tatsumi et al., 1997). This selectivity was recapitulated by measuring MPH-mediated inhibition of the peak current under steady-state conditions (Fig. 3A) ($K_D$ = 0.20 [0.17–0.25] and 7.75 [5.27–11.40] μM for DAT and SERT, respectively). Binding of MPH to DAT was considerably faster than to SERT, as judged by the rate of association to SERT. Hence, DAT binds MPH much more rapidly than SERT, but both release it at a comparable rate, suggesting that access to and/or formation of the binding site rapidly than SERT, but both release it at a comparable rate, suggesting that access to and/or formation of the binding site.

Ass say E

In our analysis, we tested desipramine, which is selective for SERT over DAT. It blocks SERT and DAT, with $K_I$ values in the range of 100 nM and 10 μM, respectively (Tatsumi et al., 1997). This selectivity was reproduced by measuring the inhibition of the peak current at equilibrium ($K_D$ = 56.0 [47.7–65.7] nM for DAT versus 3.93 [3.26–4.47] μM for SERT) (Fig. 4A). Our kinetic analysis showed that desipramine binds slowly to DAT but rapidly to SERT; specifically, we calculated a 60-fold difference in association rate constants from the plots of $k_{app}$ versus [desipramine] (9.27 [8.41–10.10] × 10^4 μM for DAT versus 6.10 [5.35–6.85] × 10^6 μM for SERT) (Fig. 4B–E). In contrast, the $k_{off}$ values did not differ (0.33 [0.21–0.45] s^-1 for DAT versus 0.29 [0.24–0.34] s^-1 for SERT) (Fig. 4F).

Our analysis unequivocally shows that monoamine transporters can discriminate between inhibitors by differences in the rate of association.

Affinity Changes Induced by Site-Directed Mutagenesis Resolved Kinetically

Drug-receptor interactions are frequently investigated by site-directed mutagenesis, and

Fig. 3. Kinetics of MPH binding to SERT and DAT. (A) Titration curve for MPH-mediated, steady-state inhibition of the peak current elicited by 10 μM substrate through DAT (black squares) and SERT (blue squares). (B–E) The association rate constant of MPH binding to SERT (B) and DAT (C) was determined as outlined in Fig. 1C. The $k_{app}$ values were calculated from the monoeponential fit and plotted over the MPH concentration to estimate the values of $k_{on}$ and $k_{off}$ from the slope and y-intercept of the regression lines, respectively. (D and E) Each data point represents the respective $k_{app}$ value of a single cell at a given concentration. (F) The time course of MPH dissociation from DAT (black squares) and SERT (blue squares) was determined as outlined in Fig. 1D, and the $k_{off}$ was estimated from a fit to a monoeponential equation to obtain an independent estimate of $k_{off}$. All peak currents were normalized to the amplitude of the peak current in the absence of MPH. The data are mean ± S.D. from six to nine independent measurements.
conclusions are drawn from changes in affinity (Henry et al., 2006; Andersen et al., 2011). However, these ligand affinity changes do not discriminate between differences in \( k_{\text{on}} \) and \( k_{\text{eff}} \), thereby ignoring important information that can indicate involvement of the mutated residue in direct binding site interactions, the access pathway to that site, or protein conformational changes required to form the site. Therefore, we attempted to accelerate the binding of MPH to SERT by site-directed mutagenesis and subjected candidate mutants to our experimental analysis. We identified residue Ile172, where mutation to valine (I172V) decreased affinity and where mutation to alanine (I172A) increased affinity (Fig. 5A; see Supplemental Material and Supplemental Figs. 1 and 2). We could decipher the kinetic basis of these affinity changes and found that they are attributable to differences in \( k_{\text{on}} \), whereas \( k_{\text{eff}} \) was unchanged (Fig. 5, B and C) (\( k_{\text{off}} \) of SERT-I172A versus SERT-I172V was 8.66 [6.68–10.06] × 10^4 M\(^{-1}\)s\(^{-1}\) versus 2.42 [2.12–2.72] × 10^4 M\(^{-1}\)s\(^{-1}\); see Supplemental Material).

These results further validate the accuracy of our approach since we resolved differences in \( k_{\text{on}} \) as small as a factor of two. However, we want to stress that the modest increase in \( k_{\text{on}} \) by 2-fold cannot explain the 20-fold difference that is seen between SERT and DAT (Fig. 3). Therefore, it will be up to future investigations to identify the structural determinants that govern the forward binding reaction of monoamine transporter ligands (see Discussion).

**Discussion**

A drug is clinically useful if it is selective and displays high affinity for its target, thereby limiting off-target effects. In general, high affinity is thought to result from tight binding, i.e., slow dissociation of the drug from its binding site. Related proteins sample a similar chemical space of inhibitors due to their structural similarity. For instance, SERT, norepinephrine transporter, and DAT are all blocked by cocaine. Conversely, selective inhibitors of these transporters have been identified, but these were discovered by serendipity (Hyman, 2014). In fact, even to date, the relation between high affinity and selectivity is poorly understood. Inhibitor binding has mostly been described by surrogate parameters of the equilibrium-dissociation constant \( K_D \) (e.g., IC\(_{50}\) converted to \( K_I \)). In addition, direct measurement of \( k_{\text{on}} \) and \( k_{\text{eff}} \) has only been possible for a limited set of high-affinity radioligands. It has been implicitly assumed that a selective ligand dissociates slowly from the preferred target, but is rapidly released from the low-affinity interaction site (Copeland et al., 2006; Pan et al., 2013).

In the present study, we have subjected this assumption to experimental scrutiny and show that, in fact, the association rate constant \( k_{\text{on}} \) is not the dissociation rate constant \( k_{\text{off}} \), can specify selective discrimination between binding sites in SERT and DAT.

To the best of our knowledge, this is the first report of reliable kinetic measurement of the rapidly dissociating monoamine transporter inhibitors cocaine, MPH, and desipramine.

We show that cocaine binds SERT and DAT with similar association rate constants but differs concerning its dissociation rate constants. In contrast, desipramine and MPH discriminate between SERT and DAT by means of the forward binding reaction. When directly recorded at different concentrations, the on rate of MPH binding to DAT was ~20 times faster than that to SERT, whereas the respective dissociation rate constants differed only by a factor of two. The ability of desipramine to discriminate between SERT and DAT was even accounted for by a ~60-fold difference in the association rate constants, whereas the dissociation rate constants were essentially unchanged. Furthermore, we show that with the described approach, one can directly resolve affinity changes induced by site-directed mutagenesis kinetically.

How can we understand the kinetic differences of inhibitor binding in structural terms? The contribution of \( k_{\text{eff}} \) to binding affinity is intuitively grasped in the induced-fit model (Vogt and Di Cera, 2012). The binding site accommodates the ligand in an open state and closes down on the compound by forming a network of bonding interactions. These can be visualized or inferred from crystal structures (Pennmatsa et al., 2013). The
Fig. 5. Mutations of I172 change the affinity of SERT for MPH by affecting $k_{\text{on}}$ but not $k_{\text{off}}$. Inhibition of (A) peak current at steady state in wild-type (WT) SERT (blue), SERT-I172A (green), and SERT-I172V (red) expressed in HEK-293 cells. (B) The association rate constant for MPH binding to SERT-I172A and SERT-I172V (see Supplemental Fig. 2) was determined from the time-dependent block of the peak current as outlined in Fig. 1C (main text), and the extracted $k_{\text{app}}$ values were plotted against MPH to estimate the values of $k_{\text{on}}$ and $k_{\text{off}}$ from the slope and $y$-intercept of the regression lines. (C) MPH dissociation was directly measured, and $k_{\text{off}}$ was estimated from a fit to a monoexponential function. All peak currents were normalized to the amplitude of the peak current in the absence of MPH. The data are mean ± S.D. from four to eight independent measurements. Wild-type data shown in this figure were taken from Fig. 3 and added for the sake of comparison.
Perform data analysis: Hasenhuetl, Sandtner.
Wrote or contributed to the writing of the manuscript: Hasenhuetl, Schicker, Koenig, Freissmuth, Sandtner.

References


Address correspondence to: Walter Sandtner, Institute of Pharmacology, Center of Physiology and Pharmacology, Medical University Vienna, Waehringerstr. 13a, A-1090 Vienna, Austria. E-mail: walter.sandtner@meduniwien.ac.at.
Supplementary Information

Ligand selectivity among the dopamine and the serotonin transporter specified by the forward binding reaction

Peter S Hasenhuetl, Klaus Schicker, Xaver Koenig, Yang Li, Subhodeep Sarker*, Thomas Stockner, Sonja Sucic, Harald H Sitte, Michael Freissmuth, Walter Sandtner †

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

*Present address: Clinical Division of Fish Medicine, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria

†To whom correspondence should be addressed: Walter Sandtner, E-mail: walter.sandtner@meduniwien.ac.at
Supplementary Text

Differences in the association rate of compounds may be a general feature that contributes to the discrimination between closely related binding sites. If this was the case, we hypothesized that it could be possible to accelerate binding of the inhibitor by mutagenesis of the binding site or the access pathway of the ligand. Accordingly, we searched for mutations in SERT that rendered the protein more or less susceptible to inhibition by methylphenidate (MPH).

SERT and MPH are ideally suitable for these experiments because MPH binds SERT slower than DAT (Fig. 3, see main text). Hence, it may be possible to accelerate MPH binding to SERT by site-directed mutagenesis. We mutated several residues in the substrate binding site of SERT that differ between DAT and SERT. Of the positions (Supplementary Fig. 1 and 2A), which we mutated, we identified one - I\textsuperscript{172} – that, when mutated to alanine, lowered, and when mutated to valine, increased the IC\textsubscript{50} of MPH in an uptake inhibition assay (1.65 [1.37-1.98] vs. 4.35 [3.62-5.23] vs. 14.72 [11.41-18.97] \(\mu\)M, respectively; Supplementary Fig. 2A). The same rank order in affinity was observed, when occupancy by MPH was measured by recording its effect on the peak current (Fig. 5A). Thus, this pair of mutants provided an opportunity to examine whether we could ascribe mutation-induced affinity changes to changes in \(k_{\text{on}}\) and \(k_{\text{off}}\). The observations were unequivocal: MPH bound more rapidly to SERT-I\textsuperscript{172}A (\(k_{\text{on}} = 8.66 [6.68-10.06] \times 10^{4} \text{M}^{-1}\text{s}^{-1}\) - Fig. 5B and Supplementary Fig. 2B) than to SERT-I\textsuperscript{172}V (\(k_{\text{on}} = 2.42 [2.12-2.72] \times 10^{4} \text{M}^{-1}\text{s}^{-1}\) - Supplementary Fig. 5B and Supplementary Fig. 2C), whereas direct and indirect determination of \(k_{\text{off}}\) revealed no difference in dissociation rates (0.55 [0.38-0.73] s\(^{-1}\) vs. 0.43 [0.28-0.58] s\(^{-1}\) - Fig. 5C).
Supplementary Fig. 1. Uptake inhibition by methylphenidate (MPH) assessed in HEK-293 cells expressing wild-type SERT and several binding site mutants thereof. In SERT\textsuperscript{Y95F}, SERT\textsuperscript{G100A} and SERT\textsuperscript{F170A}, the respective residues were mutated to the equivalent residues in DAT. The IC\textsubscript{50} values for uptake inhibition for the mutants were: 12.83[9.54-17.24] \(\mu\)M, 8.00[6.01-10.66] \(\mu\)M and 4.72[3.65-6.10] \(\mu\)M, respectively. None of these mutants showed an increase in affinity to MPH – \textit{i.e.} the IC\textsubscript{50} for wild-type SERT was 4.35[3.62-5.23] \(\mu\)M. The SERT\textsuperscript{F95A} mutant displayed a moderate loss in affinity for MPH -5.95[4.67-7.60] \(\mu\)M- compared to the DAT-like SERT\textsuperscript{Y95F} mutant.
**Supplementary Fig. 2.** Inhibition of (A) [3H]5-HT uptake in wild-type SERT (blue), SERT-I172A (green) and SERT-I172V (red), expressed in HEK-293 cells. Data in (A) are means ± S.D of three independent experiments conducted in triplicate and from 5 experiments, respectively. (B and C) The association rate constant for MPH binding to SERT-I172A (B) and SERT-I172V (C) was determined from the time-dependent block of the peak current as outlined in Fig. 1C (main text). The k_{app}-values from these curves were plotted against [MPH] to estimate the values of k_{on} and k_{off} from the slope and the y-intercept of the regression lines (see Fig. 5 B main text). All peak currents were normalized to the amplitude of the peak current in the absence of MPH. The data are means ± S.D from 4 to 8 independent measurements.