A Conserved Salt Bridge between Transmembrane Segments 1 and 10 Constitutes an Extracellular Gate in the Dopamine Transporter*

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Background: The presence of an extracellular gate in the dopamine transporter has been proposed.

Results: We use rescue mutations, zinc site engineering, and cysteine-reactive chemistry to establish the presence of the gate.

Conclusion: Arg-85 and Asp-476 constitute a functional thin gate in the dopamine transporter.

Significance: This gains further insight into the molecular mechanisms behind substrate transport by mammalian neurotransmitter transporters.

Neurotransmitter transporters play an important role in termination of synaptic transmission by mediating reuptake of neurotransmitter, but the molecular processes behind translocation are still unclear. The crystal structures of the bacterial homologue, LeuT, provided valuable insight into the structural and dynamic requirements for substrate transport. These structures support the existence of gating domains controlling access to a central binding site. On the extracellular side, access is controlled by the “thin gate” formed by an interaction between Arg-30 and Asp-404. In the human dopamine transporter (DAT), the corresponding residues are Arg-85 and Asp-476. Here, we present results supporting the existence of a similar interaction in DAT. The DAT R85D mutant has a complete loss of function, but the additional insertion of an arginine in opposite position (R85D/D476R), causing a charge reversal, results in a rescue of binding sites for the cocaine analogue [3H]CFT. Also, the coordination of Zn2+ between introduced histidines (R85H/D476H) caused a \( \sim 2.5 \)-fold increase in [3H]CFT binding (\( B_{\text{max}} \)). Importantly, Zn2+ also inhibited [3H]dopamine transport in R85H/D476H, suggesting that a dynamic interaction is required for the transport process. Furthermore, cysteine-reactive chemistry shows that mutation of the gating residues causes a higher proportion of transporters to reside in the outward facing conformation. Finally, we show that charge reversal of the corresponding residues (R104E/E493R) in the serotonin transporter also rescues [3H](S)-citalopram binding, suggesting a conserved feature. Taken together, these data suggest that the extracellular thin gate is present in monoamine transporters and that a dynamic interaction is required for substrate transport.

The neurotransmitter:sodium symporters (NSSs)\(^2\) encompass a family of secondary active transporters that use the \( \text{Na}^+ \) gradient across the plasma membrane as a driving force to transport solutes against their concentration gradient. Within the NSS family, we find the transporters for the monoamines dopamine (DA), norepinephrine, and serotonin. They are localized to the presynaptic terminals where they mediate rapid reuptake of the respective neurotransmitter and thereby control synaptic signaling tonus. Because of their central role in monoamine signaling, it is not surprising that the transporters are important pharmacological targets; the classical tricyclic antidepressants inhibit both the transporters for norepinephrine and serotonin (SERT), the selective serotonin reuptake inhibitors inhibit SERT, and the stimulatory effects of cocaine and amphetamine are caused by the interaction of these compounds with SERT, norepinephrine transporter, and the dopamine transporter (DAT).

Despite their physiological and pharmacological importance, there are still many unanswered questions relating to the structural basis and molecular mechanisms behind substrate transport. Attempts to determine the tertiary structure of mammalian NSS proteins have so far proven unsuccessful mainly because of problems with obtaining sufficient amounts of purified protein of appropriate stability. These problems have been overcome by expression of either bacterial homologues to the mammalian NSS proteins, such as LeuT from Aquifex aeolicus (1), or invertebrate transporters, such as the Drosophila melanogaster DAT (dDAT) (2). LeuT is a Na+ -coupled transporter with specificity for the hydrophobic amino acids glycine, alanine, methionine, and leucine (1, 3). High resolution structures of LeuT have revealed a protein with 12 transmembrane segments (TMs) organized in a pseudo 2-fold symmetry axis.

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2 The abbreviations used are: NSS, neurotransmitter:sodium symporter; DAT, dopamine transporter; dDAT, Drosophila DAT; synDAT, synthetic human DAT; SERT, serotonin transporter; MTSET, [2-(trimethyl-ammonium)ethyl]-methanethiosulfonate; TM, transmembrane segment; CFT, 2-carbomethoxy-3-(4-fluorophenyl)tropane; S-CIT, (S)-citalopram; GAT-1, GABA transporter-1; [3H]DA, 3,4-(Ring-2,5,6-\(^3\)H)-dihydroxyphenylethylamine; UB, uptake buffer.
between TM1–5 and TM6–10 with a binding site for substrate localized central in the protein (S1 site) flanked by TM1, TM3, TM6, and TM8 (1). So far, LeuT has been crystallized in three distinct conformations: outward open, outward occluded, and an inward open conformation (1, 4). Although significant homology exists between LeuT and the mammalian NSS proteins, there are also divergent structures, including the loop domains and the much longer N and C termini found in the mammalian transporters. Despite these differences, LeuT has been used as a model protein for studying the dynamics and conformational changes that underlie substrate translocation in the mammalian NSS proteins (5–8). The recent crystallization of dDAT in an outward open conformation revealed a structural fold very similar to LeuT, supporting that LeuT is a valid model for eukaryotic NSS proteins at least for structural inferences (2).

It is believed that the accessibility to the central substrate binding site from either side of the membrane is controlled by the concerted movements of specific gating domains within the transporters (9–16). The constellation of the gating domains should be mediated by a network of dynamic interaction between specific residues. The existence of such “gating residues” has been confirmed by inferences from the crystal structures of LeuT. In the outward occluded conformation of LeuT, access to the S1 site from the extracellular side is blocked in part by a water-mediated salt bridge formed by an arginine in TM1 (Arg-30) and an aspartate in TM10 (Asp-404). In the outward facing conformation, the Arg-30/Asp-404 salt bridge is broken, resulting in the exposure of the substrate binding site to the extracellular aqueous environment (4), whereas the ionic interaction is direct without a water molecule present in the inward facing conformation (1, 4). Thus, according to the current crystal structures of LeuT, it is likely that Arg-30/Asp-404 forms a functionally important so-called “thin gate” as opposed to the “thick gate” on the intracellular side of the outward occluded conformation, which consists of 22 Å densely packed protein.

Sequence alignment of the NSS members shows that the positive and negative charge in the two positions are almost completely conserved within the family (17), substantiating the importance of this putative gate in the function of this class of proteins. However, to date most inferences about the role of the interaction rely on the solved crystal structures of LeuT and molecular modeling of mammalian transporters based on the LeuT (18–20); hence, whether the two residues also interact in mammalian NSS proteins and what role they have in substrate transport and inhibitor binding are yet to be established. Importantly, the role of the aspartate has been studied in the GABA transporter-1 (GAT-1), where it was shown that Asp-451, the cognate residue to Asp-404 in LeuT, only can be replaced by a glutamate if activity must be retained and that the D451E mutant shifts the conformational equilibrium of GAT-1 toward a more outward facing configuration (21). Further, the same group elegantly showed that the impaired transport efficiency in D451E can be rescued by a similar mutation in a presumed intracellular gating residue (D410E), suggesting a functional connectivity between the two (22).

In DAT, it has been proposed that, upon binding of dopamine, a hydrogen bond is formed between Asp-79 and Tyr-165 in TM3 (19). These apparent gating residues are located right above the binding site and could cause the initial closure of the external side, but this interaction is not conserved in LeuT and has no apparent connection to the more extracellularly located Arg-85 and Asp-476 (equivalent to Arg-30 and Asp-404 in LeuT). Here, we provide experimental data demonstrating the existence in DAT of a functional interaction between the conserved residues Arg-85 in TM1 and Asp-476 in TM10 (Fig. 1). First, we substantiate the functional importance of the two residues by showing that individual charge-reversing mutations at the two loci eliminate $[^3]$H]DA uptake and dramatically decrease $B_{max}$ for binding of the cocaine analogue ((-)-2β-carbethoxy-3β-(4-fluorophenyl)tropane ($[^3]$H)CFT). Next, we find that charge reversal at both sites partially restores $[^3]$H)CFT
binding, consistent with an interaction between the two residues. In further support of a structural and functional importance of the interaction between the two residues, we show that coordination of Zn$^{2+}$ between inserted histidines in the two positions potentiates $[^{3}H]$CFT binding and block $[^{3}H]$DA uptake. Finally, we provide evidence for the presence of the corresponding salt bridge in the homologous SERT, and we demonstrate that mutational disruption of the putative interaction renders an inserted cysteine in position 159 (I159C) in TM3 more exposed to inactivation by the sulphydryl-reactive agent, MTSET (2-(trimethylammonium)ethyl)methane thiosulfonate), This suggests a shift of the transporter toward a more outward open configuration and supports an important role of the TM1/TM10 interaction in regulating conformational transitions in the transport cycle.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—We used a synthetic human DAT (synDAT) gene kindly provided by J. Javitch (Columbia University, New York, NY) that encodes a protein with an amino acid sequence identical to that of human DAT WT, but the nucleotide sequence was altered to increase the number of unique restriction sites and to optimize codon utilization (23). The synDAT were subcloned into pcDNA3 (Invitrogen) for expression in COS7 cells. The human SERT was cloned into the mammalian expression vector pUbiz (18). The E2C background construct was generated by mutations in the synDAT background of Cys-90 and Cys-306 to alanine. This construct was designated “E2C,” because the two extracellular cysteines were mutated. Introduction of an HA (human influenza hemagglutinin) antibody tag into the second extracellular loop of DAT WT (HA-DAT) was performed by introducing the HA sequence YPYDVPDYASL (one-letter amino acid codes) into the second extracellular loop by replacing the sequence (HPGDSSGDSS) in positions 193–203. All mutations were generated by the QuikChange method (adapted from Stratagene, La Jolla, CA) or two-step PCR using Pfu polymerase with either synDAT WT, synDAT H193K, or synDAT E2C as template. Subcloning in synDAT were performed using Clal/KpnI for mutations in position 85 and Nhel/Xba for substitutions of Asp-476. Subcloning into HA-DAT was performed with KpnI and Xhol. Similarly, in human SERT, NotI/PvuI or Nhel/Xba combinations were used for R104D and D493R, respectively. For expression in Xenopus laevis oocytes, mutants were transferred from pcDNA3 synDAT to pXOOM (24) synDAT vector using a BamHI (New England Biolabs) dual site. cRNA was generated as run-off transcripts from pXOOM synDAT vector linearized with Pmel using T7 mMESSAGE-mMACHINE (Ambion) and purified using Megaclear (Ambion) following the manufacturer’s instructions. All mutations were confirmed by restriction enzyme mapping and DNA sequencing (MWG Eurofins). Positive clones were amplified by transformation into XL1 blue competent cells (Stratagene), and the positive colony was picked and grown in LB medium overnight at 37 °C in an orbital incubator (Infors) at 200 rpm. Plasmids were harvested using the maxi prep kit provided by Qiagen.

**Cell Culture and Transfection**—COS7 cells were maintained at 37 °C in 10% CO$_2$ in DMEM (in house) supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 0.01 mg/ml gentamicin (all from Invitrogen). One day prior to transfection, 6 × 10$^6$ cells were seeded in 175-cm$^2$ flasks. All constructs were transiently transfected into COS7 cells using Lipo2000 according to the manufacturer’s protocol (Invitrogen). Stage V defolliculated Xenopus laevis oocytes were ordered from EcoCyte BioScience. Oocytes were injected with ~50 ng of cRNA and incubated in Kulori’s medium (88 mM NaCl, 1 mM KCl, 1 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM Hepes-Tris) at 18 °C for ~5 days, or until use.

**Membrane Preparation**—Membranes were prepared from COS7 cells 2 days after transient transfection with human SERT WT or mutant plasmid using the Lipo2000 transfection protocol (Invitrogen) as described previously (18). After detachment, cells were lysed with two ultrasound bursts (Branson Sonifier with microtip) in membrane buffer (120 mM NaCl, 5 mM KCl, 1.2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 25 mM Hepes, pH 7.5), pelleted (4900 × g for 10 min (Sigma SK15 swing-out rotor)), and resuspended in membrane buffer containing 0.3 mM sucrose.

**Uptake Experiments**—Uptake assays were performed on intact cells essentially as described (25) using 3,4-[Ring-2,5,6-3H]-dihydroxyphenylethylamine ([3H]DA) (30–60 Ci/mmol) (PerkinElmer Life Sciences). Briefly, transfected COS7 cells were plated in either 24-well dishes (10$^5$ cells/well) or 12-well dishes (3 × 10$^5$ cells/well) coated with polyornithine (Sigma) to achieve an uptake level of no more than 10% of total added [3H]DA. The uptake assays were carried out 2 days after transfection in uptake buffer (UB) (25 mM Hepes, 130 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1 mM L-ascorbic acid, 5 mM d-glucose, and 1 mM of the catechol-O-methyltransferase inhibitor Ro 41-0960 (Sigma), pH 7.4). Prior to the experiment, the cells were washed once in 500 µl of UB, and the nonlabeled compound was added to the cells in the indicated concentrations in a total volume of 500 µl. The assay was initiated by the addition of 6–10 nM [3H]DA. Nonspecific uptake was determined with 1 µM nomifensine (Sigma-Aldrich). After 5 min of incubation at room temperature, the cells were washed twice with 500 µl of ice-cold UB, lysed in 250 µl (24 wells) or 300 µl (12 wells) of 1% SDS, and left for >30 min at 37 °C. All samples were transferred to 24-well counting plates (PerkinElmer Life Sciences), and 500 µl (24 wells) or 600 µl (12 wells) of Optiphase Hi Safe 3 scintillation fluid (PerkinElmer Life Sciences) was added followed by counting of the plates in a Wallac TriLux β-scintillation counter (PerkinElmer Life Sciences). All experiments were carried out with 12 determinations of DA or Zn$^{2+}$ concentrations ranging from 10 nM to 1 mM performed in triplicate.

**[3H]CFT Binding Experiments**—Binding assays were carried out essentially as described for the uptake experiments on whole cells only using [3H]β-carbomethoxy-3β-(4-fluorophenyl)tropane ([3H]CFT or [3H]WIN 35,428) (76 – 87 Ci/mmol) (PerkinElmer Life Sciences). Previous to the binding experiment, cells were washed once in ice-cold UB, and after the addition of unlabeled compound in the indicated concentrations and [3H]CFT, the reactions were incubated at 5 °C until equilibrium were obtained (>100 min). All experiments were carried out with 12 determinations of CFT (concentration range, 0.1 nM to 0.1 mM) or Zn$^{2+}$ (concentration range, 10 nM to 1 mM) performed in triplicate.
Extracellular Gating Residues in Human Dopamine Transporter

[^H]CFT Dissociation Rate Experiments—Prior to the assay, COS7 cells were treated and transfected with the indicated DAT mutants as described above for the uptake and binding experiments. Binding of[^H]CFT were initiated by the addition of 4 nM[^H]CFT (76.6 Ci/mmol) in 450 μl of UB and placed at 4 °C. After 1 h the medium was aspirated, was washed twice with UB (room temperature) and 0.5 ml of UB (room temperature) with 50 μM nomifensine (to inhibit reassociation of[^H]CFT) were added at t = 0. For the Zn2+ experiments, Zn2+ (200 μM) were added, and the plates were incubated for 10 min (room temperature) prior to the initiation of dissociation. The UB also contained 200 μM Zn2+ during the dissociation period. The reaction was stopped by the aspiration of UB at the indicated time points, and the cells were lysed and counted as previously described. All determinations were performed in triplicate.

[^H](S)-Citalopram Binding Experiments—The (S)-citalopram (S-CIT) binding to SERT WT and mutants was determined by the addition of 3–5 nM[^H]S-CIT in binding buffer together with increasing concentrations of S-CIT in the concentration range from 0.01 to 10,000 nM (12 determinations in triplicate) in 96-well plates. Subsequently, membranes expressing human SERT WT or mutants were added to a total volume of 400 μl. The binding mixture was incubated for 1 h at room temperature and subsequently filtered, washed, and counted as described for the dissociation rate assay. Nonspecific binding was determined by adding 5 μM paroxetine.

ELISA for Quantification of Cell Surface Expression—Two days after transfection, cells were washed twice with PBS and fixed in 4% paraformaldehyde. After 30 min of blocking of unspecific sites with PBS supplemented with 5% fetal calf serum, anti-HA antibody coupled to the horseradish peroxidase (80 milliunits/ml, clone 3F10; Roche) was applied for 30 min at room temperature and subsequently filtered, washed, and counted as described. All experiments were performed at least in triplicate.

Electrophysiology—*Xenopus* oocytes were voltage clamped similar to previously reported methods (26) using Dagan CA1-B (Dagan Corporation), connected to a PC with Digitada 1440A (Axon Instruments) and controlled with pClamp 9.2 (Axon Instruments). An Ag/AgCl grounding electrode was connected to the recording chamber via a 2 M KCl, 2% agarose bridge to minimize liquid junction potential offsets. Current was acquired every 0.1 ms, and 50 Hz interference was off-line filtered using the built-in functions of Clampfit 9.2 (Axon Instruments). All oocytes were clamped in 20-mV steps between −100 mV (250 ms) and +40 mV (250 ms) and returned to the holding potential of −60 mV (250 ms) prior to each voltage step. All buffers were gravity perfused, and current voltage relationships (I/V) were analyzed on steady-state currents using Origin 8 (Origin lab). The cocaine-sensitive current is defined as (I – I_COC). All recording buffers include: 2.5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 1 mM Hepes-Tris.

MTSET Labeling Experiments—Two days after transfection, COS7 cells expressing either the DAT E2C background construct or mutations herein and seeded in 12- or 24-well plates were washed once with 500 μl of UB. Subsequently, MTSET ([2-(trimethylammonium)ethyl]-methanethiosulfonate) (Toronto Research Chemicals, Toronto, Canada) was added at a final concentration of 0.5 mM, and the cells were incubated at room temperature for 5 min. The stock MTSET solution was freshly prepared in H2O and immediately diluted 10-fold by application to the transfected cells into a final volume of 500 μl of UB. After incubation, the cells were washed three times in 500 μl of UB at room temperature before initiation of[^H]DA uptake, performed as described above, but here only the maximal uptake (without unlabeled DA) and the nonspecific uptake (in the presence of 1 μM nomifensine) were determined, both in triplicate. The reaction was stopped, and uptake was counted as described above. The effects of the added compound on MTSET reactivity were determined by calculating the effect of preincubation with the compound alone and with MTSET all performed in parallel on the same plate using triplicate determinations.

RESULTS

Reversal of the Proposed Salt Bridge Residues Partly Restores Binding—To evaluate a possible interaction between Arg-85 and Asp-476 in DAT, we first mutated either Arg-85 or Asp-476 into alanines and measured the impact on DAT functionality in terms of DA transport capacity and binding of the high affinity cocaine analogue CFT on intact COS7 cells transiently transfected with DAT WT or mutants. In agreement with a critical role of both residues, neither DAT R85A nor DAT D476A possessed any measurable[^H]DA transport or[^H]CFT binding activity (data not shown). This result concurs with previous data in GAT-1 showing that mutation of the residue corresponding to Asp-476 (Asp-451) to either a cysteine, asparagine, or serine renders the transporter devoid of any measurable transport (21).

To further investigate the possible relationship between the residues, we tested the consequences of reversing the charges at the two loci (R85D and D476R). Accordingly, we constructed the single mutants R85D and D476R, as well as the double mutant with reverted residues, R85D/D476R. The mutants were transiently transfected into COS7 cells and assessed for[^H]DA uptake and[^H]CFT binding. Mutation of Arg-85 to aspartate (R85D) caused a complete loss of any measurable[^H]CFT binding, as well as[^H]DA transport (Fig. 2, A and B, and Table 1). Likewise,[^H]DA uptake could not be measured in the D476R mutant. However, we did observe significant[^H]CFT binding in D476R, although it was only ~10% of DAT WT but with a similar affinity (Table 1). Interestingly, introduc-
ing D476R into the nonfunctional R85D mutant resulted in a marked increase in binding capacity relative to the R85D single mutant, up to ~10% of WT (Fig. 2B and Table 1). However, we were not able to measure any [3H]DA transport in R85D/D476R, showing that charge reversal is only compatible with [3H]CFT binding but not with transport (Fig. 2A).

The rescue of [3H]CFT binding in R85D/D476R compared with R85D alone could be due either to an increase in surface expression or to a re-establishment of intact binding sites in the expressed transporters. To determine whether the observed differences were an effect of a change in surface expression, we introduced an HA antibody tag into the second extracellular loop of DAT WT as described (27). Insertion of the HA tag permitted quantification of DAT surface expression by ELISA. In agreement with previous investigations (28), the HA tag insertion did not perturb [3H]DA uptake and [3H]CFT binding in DAT WT (Table 2). Furthermore, we observed the same phenotype of the charge reversal mutations, i.e. introducing D476R into HA-DAT R85D, resulted in rescue of [3H]CFT binding to the same extent as without HA tag present (Table 2).

The ELISA data on HA-tagged DAT WT and mutant transporters showed that all mutations, R85D, D476R, and R85D/D476R, decreased the surface expressed DAT protein to ~20%

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**TABLE 1**

<table>
<thead>
<tr>
<th>DAT construct</th>
<th>CFT $B_{\text{max}}$ (fmol/10^5 cells)</th>
<th>Kd (nM)</th>
<th>N</th>
<th>DA $B_{\text{max}}$ (fmol/10^5 cells)</th>
<th>Kd (nM)</th>
<th>N</th>
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<tbody>
<tr>
<td>WT</td>
<td>285 ± 31</td>
<td>18 (16; 21)</td>
<td>9</td>
<td>0.91 (0.66; 1.26)</td>
<td>5</td>
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<tr>
<td>R85D</td>
<td>ND</td>
<td>ND</td>
<td>8</td>
<td>ND</td>
<td>4</td>
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<tr>
<td>D476R</td>
<td>32 ± 3</td>
<td>6.7 (5.2; 8.5)</td>
<td>6</td>
<td>12 (8.7; 19)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>R85D/D476R</td>
<td>37 ± 4</td>
<td>7.6 (5.1; 11)</td>
<td>4</td>
<td>15 (11; 20)</td>
<td>4</td>
<td></td>
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**TABLE 2**

<table>
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<tr>
<th>DAT construct</th>
<th>CFT $B_{\text{max}}$ (fmol/10^5 cells)</th>
<th>Kd (nM)</th>
<th>N</th>
<th>DA $B_{\text{max}}$ (fmol/10^5 cells)</th>
<th>Kd (nM)</th>
<th>N</th>
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<tr>
<td>HA-WT</td>
<td>210 ± 74</td>
<td>9.8 (7.5; 13)</td>
<td>3</td>
<td>31 (21; 47)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HA-R85D</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td>31 (21; 47)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HA-D476R</td>
<td>43 ± 9</td>
<td>31 (21; 47)</td>
<td>4</td>
<td>20 (12; 36)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>HA-R85D/D476R</td>
<td>35 ± 8</td>
<td>20 (12; 36)</td>
<td>4</td>
<td>20 (12; 36)</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
of WT expression (surface expression as a percentage of WT: R85D, 22 ± 3; D476R, 21 ± 3; and R85D/D476R, 28 ± 5; Fig. 2C). Although there was a tendency for R85D/D476R to express more than the single mutants, the difference was not significant (Student’s unpaired t test, R85D/D476R versus R85D and D476R, p = 0.15 and 0.18, respectively). When comparing the degree of surface expression to the observed $B_{\text{max}}$, values, it is clear that the restored binding in R85D/D476R, as compared with R85D alone, cannot be solely the result of restored surface expression, but rather from a reestablishment of the CFT binding site by the introduction of the D476R mutation. It is important to note that full rescue of $[^3H]$CFT binding would not be expected because the putative interaction between the two residues is likely stabilized by other neighboring residues that presumably only will be able to form suboptimal interactions when the residues are swapped.

To further characterize the double mutant, we wanted to assess whether the loss of $[^3H]$DA transport was due to the loss of DA binding or whether it was a mechanistic perturbation of the translocation mechanism. Accordingly, we assessed the potency of DA to inhibit binding of $[^3H]$CFT to R85D/D476R. This showed that DA was able to displace the bound $[^3H]$CFT albeit with a 16-fold lower potency than in the WT (Fig. 2D and Table 1). This suggests that DA is still able to bind to the double mutant but that the actual translocation mechanism is perturbed.

**Conversion of the Salt Bridge to a Zn2+−Binding Site Restores Binding Capacity**—The partial rescue of $[^3H]$CFT binding in the double mutant is consistent with the presence of a direct interaction between Arg-85 and Asp-476, but we cannot exclude a conformational rescue because of indirect allosteric effects by the inserted residues. To determine whether the two positions in TM1 and TM10 indeed are in close proximity of each other and thereby likely to interact, we employed zinc site engineering. The coordination of Zn2+ in proteins is well established from crystal structures of multiple Zn2+-binding proteins such as DNA binding Zn2+−finger proteins and enzymes (32, 33). We hypothesized that if an arginine in position 85 in TM1 and an aspartate in position 476 in TM10 can interact in WT DAT, it should be possible to engineer a bidentate Zn2+−binding site between the two helices with a Zn2+−coordinating residue in each of the two positions (e.g. His, Cys, Asp, or Glu). Indeed, substituting Arg-85 and Asp-476 with histidine residues in a DA-bound DAT model (19), based on the outward occluded structure of LeuT, shows that the possible distance between the histidine nitrogens can be below 4 Å, which is within range for a tight Zn2+−coordination (Fig. 4A) (34). Histidine residues were accordingly inserted in positions 85 and/or 476 in a DAT background construct where the endogenous Zn2+ binding site (35–37) has been removed (DAT H193K). In contrast to the charge reversal mutants, both R85H/H193K and H193K/D476H retained $[^3H]$CFT binding properties similar to WT. Also, the $K_m$ value for $[^3H]$DA was retained, but the transport activity ($V_{\text{max}}$) was reduced with the highest effect observed on the R85H/H193K mutant (Table 3). This suggests that a charged pair of residues is not required per se to maintain the $[^3H]$CFT binding site but has a critical impact on DA transport. In the double histidine mutant (R85H/H193K/D476H), $B_{\text{max}}$ for $[^3H]$CFT binding was reduced ~50% (Table 3), whereas $[^3H]$DA transport activity was reduced to ~10% of WT (Table 3). As for the single His mutants, the $K_m$ value for R85H/H193K/D476H was similar to what was observed for the DAT H193K background mutant.

The effect of Zn2+ on $[^3H]$CFT binding was measured in both R85H/H193K and H193K/D476H and in the bis-His mutant. As shown in Fig. 4B, Zn2+ caused a dose-dependent increase in $[^3H]$CFT binding in the R85H/H193K/D476H mutant with a maximum of 243 ± 19% compared with when no Zn2+ was present (Fig. 4B; mean ± S.E., n = 8). The potentiation of $[^3H]$CFT binding by Zn2+ was only observed for the bis-His mutant and not for mutants with only a single histidine inserted into the Zn2+-insensitive background, i.e. R85H/H193K and H193K/D476H (Fig. 4B). This suggests that both histidine residues must be present to coordinate Zn2+−binding between the two positions in order for Zn2+−to potentiate $[^3H]$CFT binding. The effect was only seen with Zn2+; neither Cu2+, Ni2+, Ag2+, nor Cu2+-phenanthroline displayed any potentiating effect on $[^3H]$CFT binding (data not shown). The insertion of cysteine residues, either in combination with a histidine (R85H/D476C or R85C/D476H) or at both sites (R85C/D476C) in the DAT H193K background did not result in any $[^3H]$CFT binding potentiation by Zn2+ or any other of the tested divalent cations (data not shown). Thus, the potentiation effect of Zn2+ in the bis-His mutant is specific for the histidines and unlikely caused by unspecific effects caused by the mutation of the two residues.

To further validate the existence of a bidentate Zn2+−binding site between R85H and D476H, we investigated the effect of Zn2+ on dissociation of prebound $[^3H]$CFT. Our previous data have demonstrated that the binding site for cocaine and cocaine analogues like CFT is situated in the primary substrate binding
cavity (S1) overlapping with the binding site of DA (19). Thus, the CFT binding site is situated below the putative thin gate presumably formed by the Arg-85/Asp-476 interaction. We hypothesized accordingly that forcing a structural constraint between position 85 and 476 with Zn$^{2+}$/H11001 should trap prebound $[^3]$H CFT in S1 and thus decrease the dissociation rate of $[^3]$H CFT from DAT. COS7 cells expressing R85H/H193K/D476H was preincubated with $[^3]$H CFT, before measuring the dissociation rate in the absence and presence of 200 $\mu$M Zn$^{2+}$/H11001 (Fig. 4C). In the absence of Zn$^{2+}$, $[^3]$H CFT dissociates from the DAT bis-His mutant with a dissociation rate constant ($K_{o}$) of 0.076 ± 0.0038 min$^{-1}$ (mean ± S.E., $n$ = 7; Fig. 4C) in agreement with previous results (19). In the presence of Zn$^{2+}$, however, the dissociation rate constant decreased 5-fold ($K = 0.015 ± 0.0015$ min$^{-1}$, mean ± S.E., $n$ = 7; Fig. 4C), suggesting that the coordination of Zn$^{2+}$ between His-85 and His-476 occludes the path from the CFT binding site to the extracellular environment. We did not observe any significant effect of Zn$^{2+}$ on $[^3]$H CFT dissociation from the DAT H193K background or from the single His mutants (fold change in $[^3]$H CFT dissociation rate constants between DAT mutants incubated with or without the presence of 200 $\mu$M Zn$^{2+}$/buffer:Zn$^{2+}$ ratio): H193K, 1.25 ± 0.17 min$^{-1}$; R85H/H193K, 1.15 ± 0.13 min$^{-1}$; and H193K/D476H, 0.80 ± 0.09 min$^{-1}$ mean ± S.E., $n$ = 3).

We also tested the effect of Zn$^{2+}$ on $[^3]$H DA transport in R85H/H193K/D476H. Whereas no effect of Zn$^{2+}$ is seen in the background mutant H193K (36) or when R85H and D476H are individually inserted into H193K (Fig. 4D), Zn$^{2+}$ potently inhibited $[^3]$H DA uptake in R85H/H193K/D476H (IC$_{50}$ = 2.6 [2.1–3.1] $\mu$M; Fig. 4D). In addition, to further substantiate that Zn$^{2+}$ can coordinate between R85H and D476H, the results support that dynamic TM1/TM10 interactions and not simple static proximity between the two helices are required for transport. Mutations to the proposed salt bridge do not permit Li$^{+}$-sensitive leak in the transporter. A–D, $I$/V plots of steady-state cocaine sensitive leak currents in WT, R85D, D476R, and the double mutant R85D/D476R assessed in 130 mM NaCl (open black squares) and 130 mM LiCl (open red circles) means ± S.E. ($n$ = 4–5). The inwardly rectifying leak current observed in WT when substituting NaCl with LiCl is not observed when mutating residues in the proposed outer gate (B–D). The $I$/V plots were generated in 20-mV steps from −100 to +40 mV. We hypothesize that the putative Arg-85/Asp-476 salt bridge is involved in the conformational isomer-
Extracellular Gating Residues in Human Dopamine Transporter

FIGURE 4. Functional analysis of the TM1/TM10 interaction by Zn$^{2+}$ binding to the histidine substituted mutant DAT R85H-D476H. A, the molecular docking model of DAT from Fig. 1 here with inserted histidines in positions 85 and 476 (orange sticks). The distance between possible coordinating histidines, here exemplified as an interaction between the π (61) and τ (ε2) nitrogens, is 3.8 Å, which is within Zn$^{2+}$-coordinating distance. B, the addition of Zn$^{2+}$ to R85H/D476H (red circles) dose-dependently increases [3H]CFT binding to 243 ± 19% (mean ± S.E., n = 8) in 1 mM Zn$^{2+}$ relative to no Zn$^{2+}$ present. The effect was observed when applying subsaturating concentrations of [3H]CFT together with increasing concentrations of Zn$^{2+}$ up to a final concentration of 1 mM. No effect of Zn$^{2+}$ on [3H]CFT binding was observed on either R85H (black squares) or D476H (black triangles) single mutants (n = 7). C, Zn$^{2+}$ decreases off rate of bound [3H]CFT to the R85H/D476H mutant. The dissociation rate constant (K) for [3H]CFT from R85H/D476H shows a 5-fold decrease by the addition of 200 μM Zn$^{2+}$, from 0.076 ± 0.0038 min$^{-1}$ (black circles) to 0.015 ± 0.0015 min$^{-1}$ (cyan squares) (mean ± S.E., n = 7). No effect of Zn$^{2+}$ on dissociation was observed in the R85H, D476H, or H193K background mutants (data in text). D, transport of [3H]DA by R85H/D476H is inhibited by the addition of Zn$^{2+}$. The uptake capacity by R85H/D476H is markedly decreased relative to the DAT H193K background construct but still within the detection limit of the assay (Table 3). The addition of Zn$^{2+}$ to R85H/D476H (red circles) results in a dose-dependent inhibition of [3H]DA uptake (K_i = 2.6 [1.7; 3.1] μM, mean[S.E. interval], n = 3). The effect of Zn$^{2+}$ on the single mutants R85H (squares) and D476H (triangles) were indistinguishable from the Zn$^{2+}$ effect on the background mutant H193K reported previously (36). All mutations were performed in the background of the Zn$^{2+}$-insensitive mutant DAT H193K. The data are means ± S.E. performed in triplicate on COS7 cells transiently transfected with the indicated mutants.

TABLE 3

Binding and uptake characteristics for histidine mutants introduced into the DAT H193K background

Experiments performed on transient transfected COS7 cells expressing the Zn$^{2+}$-insensitive DAT H193K background or mutants herein. The K_d and B_max values of CFT binding and the K_m and V_max values for DA uptake were calculated from nonlinear regression analysis of accumulated [3H]radioligand in the presence increasing concentrations of unlabeled ligand (CFT or DA) using 11 consecutive concentrations performed in triplicate. Nonspecific [3H]DA uptake or [3H]CFT binding were determined with 1 μM nornisensine. The IC_{50} values used in the estimation of K_d and K_m values were calculated from means of pIC_{50} values and the S.E. interval from the pIC_{50} ± S.E. The B_max and V_max values are shown as means ± S.E. ND, not detectable.

<table>
<thead>
<tr>
<th>DAT construct</th>
<th>CFT</th>
<th>DA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B_max</td>
<td>K_d</td>
</tr>
<tr>
<td></td>
<td>fmol/10^6 cells</td>
<td>nM</td>
</tr>
<tr>
<td>H193K</td>
<td>191 ± 12</td>
<td>15 (12; 190)</td>
</tr>
<tr>
<td>H193K/R85H</td>
<td>209 ± 28</td>
<td>36 (25; 51)</td>
</tr>
<tr>
<td>H193K/D476H</td>
<td>205 ± 28</td>
<td>44 (36; 54)</td>
</tr>
<tr>
<td>H193K/R85H/D476H</td>
<td>89 ± 11</td>
<td>13 (11; 16)</td>
</tr>
<tr>
<td>H193K/R85H/D476H + 200 μM Zn$^{2+}$</td>
<td>201 ± 21</td>
<td>17 (15; 19)</td>
</tr>
</tbody>
</table>

ization of the transporter by operating as an external thin gate that occludes the accessibility to the substrate binding site from the extracellular environment. It would therefore be expected that breakage of the proposed salt bridge results in a changed isomerization, presumably by shifting the conformational equilibrium toward a higher fraction of transporters residing in the outward open conformation at any given time. A way to determine such a change in the conformational equilibrium of DAT...
is to use cysteine-reactive compounds directed toward a cysteine incorporated into the extracellular vestibule of DAT. When the cysteine-reactive and cell-impermeable compound MTSET reacts with the cysteine residue, the transport process is inhibited. It has been shown for DAT (10, 38), norepinephrine transporter (14), and SERT (14) that a cysteine inserted into TM3 (corresponding to Ile-159 in DAT) is differentially exposed to the extracellular environment depending on the conformational state of the transporters; when the extracellular gate is open, MTSET can react and thus inhibit subsequent transport, whereas when the gate is closed, Cys-159 is protected, and transport is preserved (10, 38). For the experiments, we used a DAT background construct (E2C) with two endogenous extracellular cysteines (C90A and C306A) removed, rendering DAT insensitive to MTSET reactivity. We expressed the construct in COS7 cells and monitored its reactivity by incubating for 5 min with MTSET (0.5 mM) and measuring the effect on subsequent [3H]DA transport. In agreement with previous data (10), the [3H]DA transport by E2C I159C was inhibited by MTSET to a significantly increased uptake inhibition by MTSET to 43 ± 8, 37 ± 10, and 32 ± 7%, respectively. The data are means ± S.E. (n = 5–8) performed in triplicate on COS7 cells transiently transfected with the indicated mutants.

The TM1/TM10 Interaction Is Also Present in SERT—We then investigated whether the interaction between Arg-85 and Asp-476 was present also in other mammalian NSS proteins. The arginine residue is conserved within the mammalian NSS family, whereas the cognate position of Asp-476 is a glutamate residue in SERT (Glu-493). Analogous to the DAT experiments, we generated charge reversal mutations of the aligned residues in SERT and measured their functionality in terms of possible rescue of high affinity [3H]-S-CIT binding. Accordingly, we generated the SERT mutants R104E, E493R, and R104E/E493R; expressed them in COS7 cells; and measured [3H]-S-CIT binding on membrane preparations of transiently transfected cells. Interestingly, for the single mutants, the binding properties were opposite as those seen for the DAT mutants. In SERT, it was R104E that possessed remaining binding activity of ~30% compared with WT, whereas E493R had no measurable binding. This could be due to the fact that SERT also has a glutamine residue in position 494, which could substitute for the missing Glu-493 as has been observed in GAT-1 (21). Otherwise, the pattern was similar to our observations in DAT, namely that introducing R104E in E493R mutant restored binding to ~30% of WT capacity (Fig. 6 and Table 4) with a less than 2-fold decrease in affinity. These data suggest that the cognate residues in SERT also interact in a way similar to our observations in DAT, only with a switched impact on binding for the two residues.

DISCUSSION

The family of NSS proteins is thought to function by an alternating access mechanism, which requires the presence of gates that mediate alternated accessibility to the central substrate binding site from either the extracellular or intracellular environment. Inferences from crystal structures have provided valuable information about the likely nature of these gates, but their presence and implication in the translocation mechanism...
in the mammalian NSS proteins are still to be determined in detail. Here, we provide experimental data suggesting that a pair of two highly conserved residues in DAT (Arg-85 in TM1 and Asp-476 in TM10) forms a dynamic interaction that is critical for the translocation of substrate, as well as for stabilizing the binding site for the cocaine analogue, CFT.

The charge reversal experiments show that it is possible to restore binding in the completely nonfunctional mutant, DAT R85D, by the insertion of an arginine residue (D476R) in the opposing position. The D476R per se does also perturb function; therefore in a nonrescue situation, one would expect a opposing position. The D476R, by the insertion of an arginine residue (D476R) in the binding site for the cocaine analogue, CFT.

\begin{table}
\centering
\caption{B$_{\text{max}}$ and affinity for S-CIT on SERT WT and mutants}
\begin{tabular}{|c|c|c|c|}
\hline
SERT construct & B$_{\text{max}}$ & K$_{d}$ & N \\
\hline
WT & 468 ± 32 & 2.8 (2.5; 3.3) & 3 \\
R104D & 139 ± 24 & 4.8 (3.2; 7.2) & 3 \\
D493R & ND & ND & 3 \\
R104D/D493R & 152 ± 10 & 4.5 (3.5; 5.6) & 3 \\
\hline
\end{tabular}
\end{table}

The binding of Zn$^{2+}$ to DAT R85H/H193K/D476H causes an increase in B$_{\text{max}}$ for $[^{3}\text{H}]$CFT, but interestingly it apparently does not increase its affinity. This contrasts with the classical pharmacological concept that, at saturating conditions, all possible binding sites should be occupied which should result in similar B$_{\text{max}}$ but with different affinities for CFT in the two conditions. A similar effect of Zn$^{2+}$ on $[^{3}\text{H}]$CFT binding was observed in the DAT WT with Zn$^{2+}$ binding to the endogenous site (35, 36). One possible explanation is that CFT alone is unable to change the conformational equilibrium of DAT toward the conformation prone to bind CFT, it is only the conformational fluctuation of the protein per se that determines whether a CFT binding site is formed. Thus, irrespective of the added CFT concentration, the DAT fraction prone to bind CFT would be the same. In contrast, Zn$^{2+}$ is able to change the conformational equilibrium of DAT shifting it toward the CFT prone conformation by the coordination of R85H and D476H (or by binding to the endogenous Zn$^{2+}$ binding site). Accordingly, the CFT affinity would remain the same, and the B$_{\text{max}}$ will increase. Further experiments will have to elucidate the nature of Zn$^{2+}$ binding to DAT. Taken together, these Zn$^{2+}$ data substantiate the close proximity between Arg-85 and Asp-476 and support the role of dynamic TM1/TM10 interactions for stabilizing inhibitor binding and promoting transport.

In the present study, we show that the formation of the thin gate facilitates the binding of the tested ligands to DAT (CFT) and SERT (S-CIT). This is in agreement with our previous published molecular docking models of the same setup, DAT:CFT and SERT:S-CIT, based on the structure of LeuT (18, 19). The interaction was also observed by other groups in molecular docking models of SERT with bound imipramine (20) or (S)-citalopram (42). Thus, the data presented here support previously published docking models, suggesting that the binding of inhibitors induces the outward occluded conformation. This is in contrast to the published crystal structure of the dDAT with nortriptyline bound (2). Here the Arg-Asp interaction is broken, suggesting that nortriptyline binds to outward open conformation. Interestingly, the same outward open conformation was shown to bind both selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors, clomipramine, and the stimulant mazindol to a LeuT:biogenic amine transporter hybrid (LeuBAT) (43). Further investigations are necessary to elucidate the binding conformations induced by the different inhibitors in the mammalian monoamine transporters.

The MTSET experiments support the hypothesis that mutation of the Arg-85/Asp-476 interaction shifts the conformational equilibrium toward a higher fraction of transporters residing in the outward open conformation. The probing for
changes in conformational stages of the outer gate by investigating the MTSET reactivity toward an inserted cysteine into position 159 has become a well established method used in both DAT, the norepinephrine transporter, and SERT (10, 14, 38). Accordingly, we show here that even relatively conserved mutations (R85H or D476N) change the susceptibility of Cys-159 for reacting with MTSET, suggesting an increased accessibility of the cysteine residue toward the extracellular aqueous environment (Fig. 5).

Finally, we show that the shift of the charges in the corresponding residues in SERT results in a similar rescue pattern as we observed in DAT. As opposed to DAT, it is the R104E that possesses residual binding capacity in SERT. The Asp-476 in DAT is a glutamate in SERT, and mutation to arginine completely abolishes [3H]S-CIT binding (Fig. 6). Analogous to the charge reversal in DAT, the loss of binding in E493R is partly rescued by the R104E mutation. Despite the difference in binding pattern of the single mutants, the data do suggest that the two residues also interact in SERT.

In all, we here provide evidence that the DAT residues Arg-85 and Asp-476, shown to form the thin gate in LeuT, also interact in DAT. The interaction between the two positions promotes the binding of [3H]CFT but, more importantly, the data also show that constraining the residues with Zn2+ inhibits the transport of DA, suggesting that the interaction is dynamic and must be continuously broken and formed during substrate translocation. Moreover, the presence of the charged pair throughout NSS family proteins and the presumed conserved functional role from LeuT to DAT presented here strongly supports an evolutionary conserved functional role for the two residues among all NSS proteins.

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