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 Zn^{2+} between introduced histidines (R85H/D476H) caused a ~2.5-fold increase in [3H]CFT binding (B_{max}). Importantly, Zn^{2+} 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) encompass a family of secondary active transporters that use the 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-(3-fluorophenyl)trimethoxy-3-(4-fluorophenyl)propane; 5-CIT, (S)-citalopram; GAT-1, GABA transporter-1; [3H]DA, 3,4-(ring-2,5,6-^3H)-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-156 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 [3H]DA uptake and dramatically decrease $B_{max}$ for binding of the cocaine analogue ((-)-2β-carbomethoxy-3β-(4-fluorophenyl)tropane ([3H]CFT). Next, we find that charge reversal at both sites partially restores [3H]CFT

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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 sulfhydryl-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 pUBi1z (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 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 pUBi1z (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 (HPGDSSGDSSG) 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/Xbal 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 M sucrose.

**Uptake Experiments**—Uptake assays were performed on intact cells essentially as described (25) using 3,4-[Ring-2,5,6-$^{3}$H]-dihydroxyphenylethylamine ([$^{3}$H]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 [$^{3}$H]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 μM 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 [$^{3}$H]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.

**[$^{3}$H]CFT Binding Experiments**—Binding assays were carried out essentially as described for the uptake experiments on whole cells only using [$^{3}$H]β-carbomethoxy-3β-(4-fluorophenyl)tropane ([$^{3}$H]CFT or [$^{3}$H]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 [$^{3}$H]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.
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[^3]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[^3]H]CFT were initiated by the addition of 4 nM[^3]H]CFT (76.6 Ci/mmol) in 450 μL of UB and placed at 4°C. After 1 h the medium was aspirated, cells were washed twice with UB (room temperature) and 0.5 ml of UB (room temperature) with 50 μM nomifensine (to inhibit reassociation of[^3]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.

[^3]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[^3]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 washed, and the antibody was detected and quantified instantaneously by chemiluminescence using Supersignal ELISA femto maximum sensitivity substrate (Pierce) and a Wallac Victor2 luminescence counter (PerkinElmer Life Sciences). 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 Digidata 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[^3]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[^3]H]DA transport or[^3]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, asparagin, 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[^3]H]DA uptake and[^3]H]CFT binding. Mutation of Arg-85 to aspartate (R85D) caused a complete loss of any measurable[^3]H]CFT binding, as well as[^3]H]DA transport (Fig. 2, A and B, and Table 1). Likewise,[^3]H]DA uptake could not be measured in the D476R mutant. However, we did observe significant[^3]H]CFT binding in D476R, although it was only ~10% of DAT WT but with a similar affinity (Table 1). Interestingly, introduc-
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FIGURE 2. Reversal of the presumed salt bridge residues can partly rescue DAT function. A, [3H]DA uptake capacity of the DAT WT and mutants with either a single (R85D and D476R) or both charges (R85D/D476R) reversed. Neither of the mutants were able to accumulate [3H]DA distinguishable from the background signal. The data are means ± S.E. from the pIC50 estimates (Table 1). B, transporter surface expression as quantified by ELISA on intact COS7 cells transiently transfected with a DAT background construct that has a HA tag inserted into the second extracellular loop. The data are means ± S.E. with the DAT WT expression set as 100% in all experiments. The surface expression of the indicated DAT mutants were 22 ± 3, 21 ± 3, and 28 ± 5% for R85D, D476R, and R85D/D476R, respectively, relative to the WT transporter. C, inhibition of [3H]CFT binding by DAT WT (black squares) and DAT R85D/D476R (red circles). DAT is able to displace [3H]CFT binding both in the DAT WT (K_i = 0.91 (0.66; 1.26) μM, mean (S.E. interval), n = 5) and in the DAT R85D/D476R (K_i = 15 (11, 20) μM, mean (S.E. interval), n = 4). All experiments are performed in triplicate with a concentration range of CFT (0.1 nM to 100 μM) or DA (1 nM to 1 μM) in 12 determinations on intact COS7 cells transiently transfected with DAT WT or indicated mutant.

### TABLE 1

| DAT construct | CFT B_{max}^{\text{final/10^5 cells}} | CFT K_{i} | DA K_{i} | N
|----------------|----------------------------------|----------|-----------|--|
| WT             | 284 ± 31 (18; 21)                 | 0.91 (0.66; 1.26) | 9 | 5
| R85D           | ND                               | 8 | 4
| D476R          | 32 ± 3 (5.2; 8.5)                 | 12 (8.7; 19) | 6 | 4
| R85D/D476R     | 37 ± 4 (5.1; 11)                 | 15 (11; 20) | 14 | 4

### TABLE 2

| DAT construct | B_{max}^{\text{final/10^5 cells}} | CFT K_{i} | DA K_{i} | N
|---------------|----------------------------------|----------|-----------|--|
| WT            | 210 ± 74 (9.8; 13)               | 9.8 (7.5; 13) | 3 |
| HA-R85D       | ND                               | ND       | 3 |
| HA-D476R      | 43 ± 9 (31; 47)                  | 31 (21; 47) | 4 |
| HA-R85D/D476R | 35 ± 8 (20; 36)                  | 20 (12; 36) | 4 |

Introduction of 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 transports showed that all mutations, R85D, D476R, and R85D/D476R, decreased the surface expressed DAT protein to ~20%
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.

**Mutation of the Salt Bridge Causes a Complete Inhibition of the Leak Currents**—To assess whether the lack of uptake was due to a massive depolarization of the cell membrane, e.g. due to an increased leak current, we performed an electrophysiologically characterized DAT. Accordingly, we evaluated DAT-mediated currents using two-electrode voltage clamp on *Xenopus* oocytes expressing DAT WT, R85D, D476R, or R85D/D476R. As previously reported (29), the WT possessed a minor cocaine-sensitive outward rectifying leak current at negative potentials (Fig. 3A; 6.1 ± 3.5 nA (mean ± S.E., \( n = 5 \)) at −60 mV). We were not able to determine similar leak currents in either of our mutant transporters (Fig. 3B–D). When substituting Na\(^+\) (130 mM) with Li\(^+\), the DAT-mediated leak current increased substantially to −255 ± 35 nA (mean ± S.E., \( n = 5 \)) at −60 mV. A similar effect has previously been reported for the mouse DAT (26) and the GAT-1 (30, 31). Interestingly, again we were not able to measure any leak current by any of the reverting mutants, suggesting an abolishment of the leak current induced conformation in all three mutants. Thus, the lack of \([3H]\)DA uptake in the is not due to an increased leak current causing a massive depolarization of the cell membrane. Namely, the holding potential at unclamped conditions was not different between the oocytes expressing WT and mutants, suggesting that the lack of transport is not due to a change in the membrane potential; also we were not able to measure any DA-induced current in any of the mutants either (data not shown).

**Conversion of the Salt Bridge to a Zn\(^{2+}\) 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 Zn\(^{2+}\) in proteins is well established from crystal structures of multiple Zn\(^{2+}\)-binding proteins such as DNA binding Zn\(^{2+}\) finger proteins and enzymes (32, 33). We hypothesized that if an arginine in position 85 in TM1 and an arginine at position 476 in TM10 can interact in WT DAT, it should be possible to engineer a bidentate Zn\(^{2+}\) binding site between the two helices with a Zn\(^{2+}\) 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 Zn\(^{2+}\) coordination (Fig. 4A) (34). Histidine residues were accordingly inserted in positions 85 and 476 in a DAT background construct where the endogenous Zn\(^{2+}\) 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 Zn\(^{2+}\) on \([3H]\)CFT binding was measured in both R85H/H193K and H193K/D476H and in the bis-His mutant. As shown in Fig. 4B, Zn\(^{2+}\) 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 Zn\(^{2+}\) was present (Fig. 4B; mean ± S.E., \( n = 8 \)). The potentiation of \([3H]\)CFT binding by Zn\(^{2+}\) was only observed for the bis-His mutant and not for mutants with only a single histidine inserted into the Zn\(^{2+}\)-insensitive background, *i.e.* R85H/H193K and H193K/D476H (Fig. 4B). This suggests that both histidine residues must be present to coordinate Zn\(^{2+}\) binding between the two positions in order for Zn\(^{2+}\) to potentiate \([3H]\)CFT binding. The effect was only seen with Zn\(^{2+}\); neither Cu\(^{2+}\), Ni\(^{2+}\), Ag\(^{2+}\), nor Cu\(^{2+}\)-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 Zn\(^{2+}\) or any other of the tested divalent cations (data not shown). Thus, the potentiating effect of Zn\(^{2+}\) 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 Zn\(^{2+}\) binding site between R85H and D476H, we investigated the effect of Zn\(^{2+}\) 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\(^{2+}\) (Fig. 4C). In the absence of Zn\(^{2+}\), \(^{3}H\)CFT dissociates from the DAT bis-His mutant with a dissociation rate constant \((K)\) 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.

Mutation of Arg-85/Asp-476 Renders DAT in a More Open to Out Conformation—We hypothesize that the putative Arg-85/Asp-476 salt bridge is involved in the conformational isomer-
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 deter-
mine such a change in the conformational equilib-
rium of DAT is exemplified as an interaction between the π (61) and τ (e2) nitrogens, is 3.8 Å, which is within Zn2⁺-coordinating distance.

All mutations were performed in the background of the Zn2⁺-insensitive DAT H193K background or mutants herein. The
binding and uptake characteristics for histidine mutants introduced into the DAT H193K background

| DAT construct | CFT | DA |
|---------------|-----|-----|
|               | B<sub>max</sub> | K<sub>a</sub> | N | V<sub>max</sub> | K<sub>m</sub> | N |
| H193K         | 191 ± 12 | 15 (12; 190) | 3 | 6300 ± 1100 | 1.7 (0.9; 3.5) | 4 |
| H193K/R85H    | 209 ± 28 | 36 (25; 51)  | 3 | 430 ± 119  | 3.1 (1.7; 5.6) | 5 |
| H193K/D476H   | 205 ± 28 | 44 (36; 54)  | 3 | 1421 ± 131 | 2.0 (1.3; 3.1) | 3 |
| H193K/R85H/D476H | 89 ± 11  | 13 (11; 16)  | 4 | 463 ± 33  | 3.7 (2.5; 5.4) | 8 |
| H193K/R85H/D476H + 200 µM Zn<sup>2+</sup> | 201 ± 21  | 17 (15; 19)  | 4 | ND         | ND           | 3 |

Experiments performed on transiently transfected COS7 cells expressing the Zn<sup>2+</sup>-insensitive DAT H193K background or mutants herein. The K<sub>a</sub> and B<sub>max</sub> values of CFT binding and the K<sub>m</sub> and V<sub>max</sub> 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. Non-specific [³H]DA uptake or [³H]CFT binding were determined with 1 µM nomifensine. The IC<sub>50</sub> values used in the estimation of K<sub>a</sub> and K<sub>m</sub> values were calculated from means of pIC<sub>50</sub> values and the S.E. interval from the pIC<sub>50</sub> = S.E. The B<sub>max</sub> and V<sub>max</sub> values are shown as means ± S.E. ND, not detectable.

All mutations were performed in the background of the Zn<sup>2+</sup>-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

![Figure 4](image-url)
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 the extracellular vestibule of DAT is 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 DAT with MTSET (0.5 mM) and measuring the effect on subsequent uptake of 12.0 \textsuperscript{3}H]DA by MTSET 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 the 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 \textsuperscript{3}H]-S-CIT binding. Accordingly, we generated the SERT mutants R104E, E493R, and R104E/E493R; expressed them in COS7 cells; and measured \textsuperscript{3}H]-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 glutaminase 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 an opposing position. The D476R/D85D, by the insertion of an arginine residue (D476R) in the binding site for the cocaine analogue, CFT.

The binding of Zn$^{2+}$ to DAT R85H/H193K/D476H causes an increase in $B_{\text{max}}$ for $[^{3}H]$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 $[^{3}H]$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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REFERENCES
1. Yamashita, A., Singh, S. K., Kawate, T., Jin, Y., and Gouaux, E. (2005) Crystal structure of a bacterial homologue of Na+ /Cl−-dependent neurotransmitter transporters. Nature 437, 215–223
2. Penmatsa, A., Wang, K. H., and Gouaux, E. (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. Nature 503, 85–90
3. Singh, S. K., Piscitelli, C. L., Yamashita, A., and Gouaux, E. (2008) A competitive inhibitor traps LeuT in an open-to-out conformation. Science 322, 1655–1661
4. Krishnamurthy, H., and Gouaux, E. (2012) X-ray structures of LeuT in substrate-free outward-open and apo inward-open states. Nature 481, 469–474
5. Kazmier, K., Sharma, S., Quick, M., Islam, S. M., Roux, B., Weinstein, H., Javitch, J. A., and McHaourab, H. S. (2014) Conformational dynamics of ligand-dependent alternating access in LeuT. Nat. Struct. Mol. Biol. 21, 472–479
6. Zhao, Y., Terry, D. S., Shi, L., Quick, M., Weinstein, H., Blanchard, S. C., and Javitch, J. A. (2011) Substrate-modulated gating dynamics in a Na+-coupled neurotransmitter transporter homologue. Nature 474, 109–113
7. Claxton, D. P., Quick, M., Shi, L., de Carvalho, F. D., Weinstein, H., Javitch, J. A., and McHaourab, H. S. (2010) Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter/sodium symporters. Nat. Struct. Mol. Biol. 17, 822–829
8. Zhao, Y., Terry, D., Shi, L., Weinstein, H., Blanchard, S. C., and Javitch, J. A. (2010) Single-molecule dynamics of gating in a neurotransmitter transporter homologue. Nature 465, 188–193
9. Kniazeff, J., Loland, C. J., Goldberg, N., Quick, M., Das, S., Sitte, H. H., Javitch, J. A., and Gether, U. (2005) Intramolecular cross-linking in a bacterial homolog of mammalian SLC6 neurotransmitter transporters suggests an evolutionary conserved role of transmembrane segments 7 and 8. Neuropharmacology 49, 715–723
10. Loland, C. J., Gränäs, C., Javitch, J. A., and Gether, U. (2004) Identification of intracellular residues in the dopamine transporter critical for regulation of transporter conformation and cocaine binding. J. Biol. Chem. 279, 3228–3238
11. Loland, C. J., Norregaard, L., Litman, T., and Gether, U. (2002) Generation of an activating Zn2+ switch in the dopamine transporter: mutation of an intracellular tyrosine constitutively alters the conformational equilibrium of the transport cycle. Proc. Natl. Acad. Sci. U.S.A. 99, 1683–1688
12. Forrest, L. R., Zhang, Y. W., Jacobs, M. T., Gesmonde, J., Xie, L., Honig, B. H., and Rudnick, G. (2008) Mechanism for alternating access in neurotransmitter transporters. Proc. Natl. Acad. Sci. U.S.A. 105, 10338–10343
13. Zhang, Y. W., and Rudnick, G. (2006) The cytoplasmic substrate permeation pathway of serotonin transporter. J. Biol. Chem. 281, 36213–36220
14. Chen, J. G., and Rudnick, G. (2000) Permeation and gating residues in serotonin transporter. Proc. Natl. Acad. Sci. U.S.A. 97, 1044–1049
15. Chen, N., Rickey, J., Berfield, J. L., and Reith, M. E. (2004) Aspartate 345 of the dopamine transporter is critical for conformational changes in substrate translocation and cocaine binding. J. Biol. Chem. 279, 5508–5519
16. Chen, N., Zhen, J., and Reith, M. E. (2004) Mutation of Trp84 and Asp313 of the dopamine transporter reveals similar mode of binding interaction for GBR12909 and benztpine as opposed to cocaine. J. Neurochem. 89, 853–864
17. Beuming, T., Shi, L., Javitch, J. A., and Weinstein, H. (2006) A comprehensive structure-based alignment of prokaryotic and eukaryotic neurotransmitter/Na+ symporters (NSS) aids in the use of the LeuT structure to probe NSS structure and function. Mol. Pharmacol. 70, 1630–1642
18. Plenge, P., Shi, L., Beuming, T., Te, J., Newman, A. H., Weinstein, H., Gether, U., and Loland, C. J. (2012) Sertic hindrance mutagenesis in the conserved extracellular vestibule impedes allosteric binding of antidepressants to the serotonin transporter. J. Biol. Chem. 287, 39316–39326
19. Beuming, T., Kniazeff, J., Bergmann, M. L., Shi, L., Gracia, L., Raniszewska, K., Newman, A. H., Javitch, J. A., Weinstein, H., Gether, U., and Loland, C. J. (2008) The binding sites for cocaine and dopamine in the dopamine transporter overlap. Nat. Neurosci. 11, 780–789
20. Sinning, S., Musgaard, M., Jensen, M., Severinsen, K., Celik, L., Koldsha, H., Meyer, T., Bols, M., Jensen, H. H., Schiatt, B., and Wiborg, O. (2010) Binding and orientation of tricyclic antidepressants within the central substrate site of the human serotonin transporter. J. Biol. Chem. 285, 8363–8374
21. Ben-Yona, A., and Kanner, B. I. (2012) An acidic amino acid transmembrane helix 10 residue conserved in the neurotransmitter-sodium: symporters is essential for the formation of the extracellular gate of the γ-amino butyric acid (GABA) transporter GAT-1. J. Biol. Chem. 287, 7159–7168
22. Ben-Yona, A., and Kanner, B. I. (2013) Functional defects in the external and internal thin gates of the γ-amino butyric acid (GABA) transporter GAT-1 can compensate each other. J. Biol. Chem. 288, 4549–4556
23. Saunders, C., Ferrer, J. Y., Shi, L., Chen, J., Merrill, G., Lamb, M. E., Leeb-Lundberg, L. M., Carvelli, L., Javitch, J. A., and Galli, A. (2000) Amphetamine-induced loss of human dopamine transporter activity: an internalization-dependent and cocaine-sensitive mechanism. Proc. Natl. Acad. Sci. U.S.A. 97, 6850–6855
24. Jespersen, T., Grunnet, M., Angelo, K., Klærke, D. A., and Olesen, S. P. (2002) Dual-function vector for protein expression in both mammalian cells and Xenopus laevis oocytes. BioTechniques 32, 536–538, 540
25. Loland, C. J., Mereu, M., Okunola, O. M., Cao, J., Priszinzano, T. E., Mazier, J., and McHaourab, H. S. (2010) Ion/substrate-dependent conformational dynamics of a bacterial homolog of neurotransmitter/sodium symporters. Nat. Struct. Mol. Biol. 17, 822–829

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S., Kopajtic, T., Shi, L., Katz, J. L., Tanda, G., and Newman, A. H. (2012) R-modafinil (armodafinil): a unique dopamine uptake inhibitor and potential medication for psychostimulant abuse. *Biol. Psychiatry.* 72, 405–413

26. Borre, L., Andreassen, T. F., Shi, L., Weinstein, H., and Gether, U. (2014) The second sodium site in the dopamine transporter controls cation permeation and is regulated by chloride. *J. Biol. Chem.* 289, 25764–25773

27. Sorkina, T., Miranda, M., Dionne, K. R., Hoover, B. R., Zahniser, N. R., and Sorkin, A. (2006) RNA interference screen reveals an essential role of Nedd4-2 in dopamine transporter ubiquitination and endocytosis. *J. Neurosci.* 26, 8195–8205

28. Kniazeff, J., Shi, L., Loland, C. J., Javitch, J. A., Weinstein, H., and Gether, U. (2008) An intracellular interaction network regulates conformational transitions in the dopamine transporter. *J. Biol. Chem.* 283, 17691–17701

29. Sonders, M. S., Zhu, S. J., Zahniser, N. R., Kavanaugh, M. P., and Amara, S. G. (1997) Multiple ionic conductances of the human dopamine transporter: the actions of dopamine and psychostimulants. *J. Neurosci.* 17, 960–974

30. Kanner, B. I. (2003) Transmembrane domain I of the /H9253-amino- butyric acid transporter GAT-1 plays a crucial role in the transition between cation leak and transport modes. *J. Biol. Chem.* 278, 3705–3712

31. MacAulay, N., Zeuthen, T., and Gether, U. (2002) Conformational basis for the Li+/H11001-induced leak current in the rat /H9253-amino- butyric acid (GABA) transporter-1. *J. Physiol.* 544, 447–458

32. Schwabe, J. W., and Klug, A. (1994) Zinc mining for protein domains. *Nat. Struct. Biol.* 1, 345–349

33. Alberts, I. L., Nadassy, K., and Wodak, S. J. (1998) Analysis of zinc binding sites in protein crystal structures. *Protein Sci.* 7, 1700–1716

34. Karlin, S., and Zhu, Z. Y. (1997) Classification of mononuclear zinc metal sites in protein structures. *Proc. Natl. Acad. Sci. U.S.A.* 94, 14231–14236

35. Loland, C. J., Norregaard, L., and Gether, U. (1999) Defining proximity relationships in the tertiary structure of the dopamine transporter. Identification of a conserved glutamic acid as a third coordinate in the endogenous Zn2+/binding site. *Biochemistry.* 39, 15836–15846

36. Norregaard, L., Frederiksen, D., Nielsen, E. O., and Gether, U. (1998) De-}

37. Stockner, T., Montgomery, T. R., Kudlacek, O., Weissensteiner, R., Ecker, G. F., Freissmuth, M., and Sitte, H. H. (2013) Mutational analysis of the high-affinity zinc binding site validates a refined dopamine transporter homology model. *PLoS. Comput. Biol.* 9, e1002909

38. Loland, C. J., Desai, R. I., Zou, M. F., Cao, J., Grundt, P., Gerstbrein, K., Sitte, H. H., Newman, A. H., Katz, J. L., and Gether, U. (2008) Relationship between conformational changes in the dopamine transporter and cocaine-like subjective effects of uptake inhibitors. *J. Biol. Chem.* 283, 813–823

39. Schicker, K., Uzelac, Z., Gesmonde, J., Bulling, S., Stockner, T., Freissmuth, M., Boehm, S., Rudnick, G., Sitte, H. H., and Sandtner, W. (2012) Unifying concept of serotonin transporter-associated currents. *J. Biol. Chem.* 287, 438–445

40. MacAulay, N., Bendahan, A., Loland, C. J., Zeuthen, T., Kanner, B. I., and Gether, U. (2001) Engineered Zn2+/switches in the /H9253-amino- butyric acid (GABA) transporter-1: differential effects on GABA uptake and currents. *J. Biol. Chem.* 276, 40476–40485

41. Norregaard, L., Visiers, I., Loland, C. J., Ballesteros, J., Weinstein, H., and Gether, U. (2000) Structural probing of a microdomain in the dopamine transporter by engineering of artificial Zn2+/binding sites. *Biochemistry.* 39, 15836–15846

42. Andersen, J., Olsen, L., Hansen, K. B., Taboureau, O., Jørgensen, F. S., Jørgensen, A. M., Bang-Andersen, B., Egebjerg, J., Stromgaard, K., and Kristensen, A. S. (2010) Mutational mapping and modeling of the binding site for (S)-citalopram in the human serotonin transporter. *J. Biol. Chem.* 285, 2051–2063

43. Wang, H., Goehring, A., Wang, K. H., Penmatsa, A., Ressler, R., and Gouaux, E. (2013) Structural basis for action by diverse antidepressants on biogenic amine transporters. *Nature.* 503, 141–145