Defining Proximity Relationships in the Tertiary Structure of the Dopamine Transporter

IDENTIFICATION OF A CONSERVED GLUTAMIC ACID AS A THIRD COORDINATE IN THE ENDOGENOUS Zn\(^{2+}\)-BINDING SITE

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Recently, we have described a distance constraint in the unknown tertiary structure of the human dopamine transporter (hDAT) by identification of two histidines, His\(^{193}\) in the second extracellular loop and His\(^{375}\) at the top of transmembrane (TM) 7, that form two coordinates in an endogenous, high affinity Zn\(^{2+}\)-binding site. To achieve further insight into the tertiary organization of hDAT, we set out to identify additional residues involved in Zn\(^{2+}\) binding and subsequently to engineer artificial Zn\(^{2+}\)-binding sites. Ten aspartic acids and glutamic acids, predicted to be on the extracellular side, were mutated to asparagine and glutamine, respectively. Mutation of Glu\(^{396}\) (E396Q) at the top of TM 8 increased the IC\(_{50}\) value for Zn\(^{2+}\) inhibition of \(^{[3H]}\)dopamine uptake from 1.1 to 530 \(\mu\)M and eliminated Zn\(^{2+}\)-induced potentiation of \(^{[3H]}\)WIN 35,428 binding. These data suggest that Glu\(^{396}\) is involved in Zn\(^{2+}\) binding to hDAT. Importantly, Zn\(^{2+}\) sensitivity was preserved following substitution of Glu\(^{396}\) with histidine, indicating that the effect of mutating Glu\(^{396}\) is not an indirect effect because of the removal of a negatively charged residue. The common participation of Glu\(^{396}\), His\(^{193}\), and His\(^{375}\) in binding the small Zn\(^{2+}\) ion implies their proximity in the unknown tertiary structure of hDAT. The close association between TM 7 and 8 was further established by engineering of a Zn\(^{2+}\)-binding site between His\(^{375}\) and a cysteine inserted in position 400 in TM 8. Summarized, our data define an important set of proximity relationships in hDAT that should prove an important template for further exploring the molecular architecture of Na\(^{+}\)/Cl\(^{-}\)-dependent neurotransmitter transporters.

The dopamine transporter (DAT) tightly controls the amount of available dopamine in the synaptic cleft by mediating rapid reuptake of released dopamine into the presynaptic nerve terminal (1, 2). In this way, DAT plays a critical role in modulating the physiological effects of dopamine, including regulation of locomotor activity, cognitive functions, and neuroendocrine systems (1, 2). The DAT, together with the closely related norepinephrine (NET) and serotonin transporters, forms a subfamily within a larger family of neurotransmitter and amino acid transporters that are characterized functionally by their dependence on the presence of both Na\(^{+}\) and Cl\(^{-}\) in the extracellular fluid (3, 4). Of particular interest, DAT, NET, and serotonin are transporters targets for both antidepressants drugs and several commonly abused psychostimulatory drugs, such as cocaine and amphetamine (1, 2, 4). Although cocaine and amphetamine inhibit all three transporters with similar potencies, it is generally assumed that inhibition of DAT is primarily responsible for the reinforcing and locomotor stimulatory effects of these drugs (5–7).

Na\(^{+}\)/Cl\(^{-}\}-dependent transporters are believed to share a common topology characterized by 12 transmembrane segments and an intracellular location of the amino and carboxyl termini (2–4). Many studies have been carried out to characterize functional domains both in DAT and other Na\(^{+}\)/Cl\(^{-}\}-dependent transporters. Generation of chimeric transporter molecules has provided insight into the domains determining the subtype-specific pharmacological properties of the different transporters (8–12). Moreover, point mutagenesis (13–18) and a variety of other mutagenesis-based approaches, such as the substituted cysteine accessibility method (19, 20) and random mutagenesis techniques (21), have been applied and lead to identification of some residues thought to be involved in the transport process or binding of substrate and/or blockers. However, little is yet known about the tertiary structure of this important class of transporters, and the nature of the molecular processes responsible for the translocation mechanism remains unknown.

Recently, we have reported an important distance constraint in the tertiary structure of the hDAT based on the discovery of an endogenous high affinity Zn\(^{2+}\)-binding site (22). Initially, we observed that Zn\(^{2+}\), in micromolar physiological concentrations, acts as a potent noncompetitive blocker of dopamine uptake. Furthermore, it was found that micromolar concentrations of Zn\(^{2+}\) markedly potentiate binding of the cocaine-like blocker, WIN 35,428 (22). Systematic mutation of histidines, predicted to be on the extracellular face of the transporter, identified two histidines, His\(^{193}\) in the large second extracellular loop 2 outside transmembrane segment (TM) 3 and His\(^{375}\) at the top of TM 7, as two coordinates in this endogenous Zn\(^{2+}\)-binding site (Fig. 1) (22). The two residues are separate in the primary structure, but their common participation in Zn\(^{2+}\) binding designated their spatial proximity in the tertiary structure (22).

The structures of many Zn\(^{2+}\)-binding sites are known from x-ray crystallography of Zn\(^{2+}\)-binding proteins, and therefore,
FIG. 1. Two-dimensional representation of the hDAT. On the predicted extracellular face, the hDAT contain 10 aspartic acids and glutamic acids that are conserved between hDAT and the hNET (large black circles). The histidines previously shown to be involved in Zn2+ binding to hDAT, His193, and His375 (22) are indicated as larger white circles.

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco's modified Eagle's medium 041 01885 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 0.01 mg/ml gentamicin. Wild type and mutant constructs in pBLCMV were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as described previously (27, 28).

[^H]Dopamine Uptake Experiments—Uptake assays were performed modified from Giros et al. (29) using 2,5,6[^H]Dopamine (7–21 Ci/mmol) (Amersham Pharmacia Biotech). Transfected COS-7 cells were plated in either 24-well dishes (10^5 cells/well) or 12-well dishes (2–3 × 10^5 cells/well) to achieve an uptake level of 5–10% of total added[^H]Dopamine. The uptake assays were carried out 2 days after transfection. Prior to the experiment, the cells were washed once in 500 µl of uptake buffer (5 mM Tris base, 7.5 mM HEPES, 120 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, pH 7.1). The compound to be tested was added to the cells, and uptake was initiated by addition of 10 nM[^H]dopamine in a final volume of 500 µl. After 10 min of incubation at 37 °C, the cells were washed twice with 500 µl of uptake buffer, lysed in 500 µl 1% SDS and left 1 h at 37 °C. All samples were transferred to 24-well counting plates (Wallac, Turku, Finland), 500 µl of Opti-phase Hi Safe 3 scintillation fluid (Wallac) was added followed by counting of the plates in a Wallac Tri-Lux β-scintillation counter (Wallac). Nonspecific uptake was determined in the presence of 1 mM unlabeled dopamine (Research Biochemicals International, Natick, MA). All determinations were performed in triplicate.

Ligand Binding—Binding assays were carried out on whole cells using[^H]WIN 35,428 (83.5 Ci/mmol) (NEN Life Science Products) as radioligand. One day after transfection, cells were seeded in either 24-well dishes (10^5 cells/well) or 12-well dishes (2–3 × 10^5 cells/well) to achieve a binding level of 5–10% of total added[^H]WIN 35,428. Two days after transfection, competition binding assays were performed in a final volume of 500 µl of uptake buffer containing 2–4 nM[^H]WIN 35,428 and indicated concentrations of compound to be tested. Binding was terminated after 2 h at 4 °C by washing the cells twice in 500 µl of uptake buffer prior to lysis in 500 µl 1% SDS for 1 h at 37 °C. All samples were transferred to 24-well counting plates (Wallac, Turku, Finland), and 500 µl of Opti-phase Hi Safe 3 scintillation fluid (Wallac) was added followed by counting of the plates in a Wallac Tri-Lux β-scintillation counter (Wallac). Nonspecific binding was determined in the presence of 10 µM WIN 35,428 (RB). Determinations were made in triplicate.

Calculations—Uptake and binding data were analyzed by nonlinear regression analysis using Prism 2.0 from GraphPad Software (San Diego, CA).

RESULTS

Glu396 Is a Putative Third Coordinate in the hDAT Zn2+-binding Site—Zn2+ potently inhibits[^H]dopamine uptake in a biphasic manner with a high affinity component (IC90 = 1.1 µM) and a low affinity component (IC90 > 1000 µM) in COS-7 cells transiently expressing the wild type (WT) hDAT (Fig. 2A...
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Mutation of Glu^{396} in the Homologous hNET—We have shown earlier that introducing a histidine in position 189 of the hNET (hNET-K189H), corresponding to His^{193} in hDAT, converts Zn^{2+} into a potent inhibitor of [^{3}H]dopamine uptake mediated by the hNET (Ref. 22 and shown in Fig. 2B for comparison). We demonstrated also that mutation of His^{372} in hNET-K189H, corresponding to His^{375} in hDAT, essentially eliminated the high affinity component in hNET-K189H, suggesting that Zn^{2+} is interacting with the same site in the hNET-K189H mutant as in WT hDAT (22). This was further supported by mutating Glu^{393} to glutamine in hNET-K189H, corresponding to Glu^{396} in the hDAT. As shown in Fig. 2B, Zn^{2+} was only a rather poor inhibitor of [^{3}H]dopamine uptake in this mutant (hNET-K189H-E393Q), displaying a 30-fold increase in IC_{50} value as compared with hNET-K189H (Fig. 2B and Table I). These data indicate that Glu^{393} is involved in Zn^{2+} binding in hNET-K189H. The effect of mutating Glu^{393} was, however, not as dramatic as the effect of mutating Glu^{396} in the hDAT, where a 500-fold increase in IC_{50} value was observed for Zn^{2+} (Table I and Fig. 2A). Most likely, this difference is due to some subtle structural differences between hDAT and hNET. Similar to what was observed following mutation of Glu^{396} in hDAT, mutation of Glu^{393} in hNET-K189H did not cause changes in the K_{D} for [^{3}H]dopamine uptake and the K_{m} for [^{3}H]WIN 35,428 binding (Table I).

Mutation of Glu^{396} Eliminates Zn^{2+}-induced Potentiation of [^{3}H]WIN 35,428 Binding—As illustrated in Fig. 3, Zn^{2+} causes a marked potentiation of [^{3}H]WIN 35,428 binding to the hDAT with maximal potentiation in the presence of 10 μM Zn^{2+}. In our previous study we have shown that this potentiation is due mainly to an increase in the apparent number of binding sites (22). Mutation of His^{193} or His^{375} eliminates the potentiation by Zn^{2+}, indicating that both inhibition of dopamine uptake and potentiation of [^{3}H]WIN 35,428 binding by Zn^{2+} are due to the interaction of Zn^{2+} with the same site (22). Mutation of Glu^{396} to glutamine also eliminated Zn^{2+}-induced potentiation of [^{3}H]WIN 35,428 binding (Fig. 3). This provides additional support that Glu^{396} is a third coordinate in the hDAT Zn^{2+}-binding site. Following mutation of the other nine acidic residues (Asp^{191}, Glu^{212}, Glu^{218}, Asp^{232}, Asp^{301}, Glu^{307}, Asp^{381}, Asp^{385}, and Glu^{476}), Zn^{2+}-induced potentiation was at least partially preserved (Table I).

A Histidine Can Substitute for Glutamate in Position 396—It is possible that the loss of Zn^{2+} sensitivity upon mutating Glu^{396} to glutamine in hDAT is due to an indirect structural effect caused by the removal of a negative charge rather than the disruption of a direct interaction with Zn^{2+}. To exclude this possibility we substituted the glutamate with another residue capable of coordinating Zn^{2+}. As shown in Fig. 4, histidine could fully substitute for the glutamate (hDAT-E396H mutant) in respect of Zn^{2+} inhibition of [^{3}H]dopamine uptake. In fact, the inhibition curve was monophasic with a Hill slope of −2.2, and micromolar concentrations of Zn^{2+} were able to inhibit [^{3}H]dopamine uptake entirely in contrast to the WT hDAT, where saturation of the apparent high affinity site only results in 60−70% inhibition of [^{3}H]dopamine uptake (Fig. 2A). Regarding Zn^{2+} potentiation of [^{3}H]WIN 35,428 binding the histidine could substitute only partly for the glutamic acid (15% increase in binding in E396H versus 55% in WT; Table II).

To ensure that Zn^{2+} binding to hDAT-E396H involved His^{193} and His^{375} we mutated His^{393} and His^{375} by themselves (hDAT-H193K-E396H and hDAT-H375A-E396) and combined (hDAT-H193K-H375A-E396H) (Table II). Mutation of His^{375} (hDAT-H193K-H375A-E396H) eliminated the high affinity

and Table I and Ref. 22). Previously, we have shown evidence that the high affinity component is due to the interaction of Zn^{2+} with at least two residues, His^{193} and His^{375}, on the predicted extracellular face of the transporter (Fig. 1 and Ref. 22). His^{193} is conserved between hDAT and hNET, whereas His^{375} is conserved between the two transporters. Full Zn^{2+} susceptibility can be conveyed to the hNET by mutational transfer of only His^{193} (22); therefore, Zn^{2+}-coordinating residues in addition to His^{193} and His^{375} can be expected to be conserved between hDAT and hNET. To examine the possible participation of aspartates and glutamates in binding of Zn^{2+}, we mutated the 10 conserved aspartates and glutamates predicted to face the extracellular environment to asparagine and glutamine, respectively (Fig. 1). All mutant transporters were functional and displayed K_{m} values for [^{3}H]dopamine uptake and K_{D} values for [^{3}H]WIN 35,428 binding comparable with WT hDAT (Table I). Mutation of Glu^{215}, Glu^{218}, Asp^{232}, Asp^{301}, Glu^{307}, Asp^{381}, Asp^{385}, and Glu^{476} did not change the ability of Zn^{2+} to inhibit [^{3}H]dopamine uptake, whereas mutation of Glu^{191} to glutamine lead to a small 2−3-fold increase in the IC_{50} value for Zn^{2+} inhibition (Table I). In contrast, mutation of Glu^{396} to glutamine caused an almost 500-fold increase in the IC_{50} value for Zn^{2+} inhibition of [^{3}H]dopamine uptake (Fig. 2A and Table I). This suggests that Glu^{396} could be a third coordinate in the hDAT Zn^{2+}-binding site. Notably, the effect on Zn^{2+} inhibition by mutating Glu^{396} is similar to the effect observed by mutating the previously identified Zn^{2+}-coordinate, His^{193} and His^{375} (Ref. 22 and shown in Fig. 2A for comparison).

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![Graph showing 3H-dopamine uptake (%) vs log[Zn2+] (M)](image)

*Fig. 2. The effect of Zn^{2+} on [^{3}H]dopamine uptake in hDAT, hNET, and mutant transporters.* A. Zn^{2+}-inhibition of [^{3}H]dopamine uptake in COS-7 cells transiently expressing WT hDAT (□) and hDAT-E396Q (▲). The Zn^{2+}-inhibition curves observed when mutating His^{193} to Lys (dashed line) and His^{375} to Ala (dotted line) are shown for comparison (22). Data are the means ± S.E. of 15 (hDAT) and 4 (E396Q) experiments performed in triplicate. B. Zn^{2+}-inhibition of [^{3}H]dopamine uptake in COS-7 cells transiently expressing WT hNET (□), hNET-K189H ( ●), and hNET-K189H-E393Q ( ●). Data are the means ± S.E. of 14 (hNET), 3 (hNET-K189H), and 5 (hNET-K189H-E393Q) experiments performed in triplicate.
An Engineered Zn$^{2+}$-binding Site between the TM 7 and 8—Our data suggest that His$^{375}$ at the top of TM 7 is facing non-cysteine residues were inserted in positions (resulting in hDAT-H193K-H375A-E396Q-T400C) eliminated $\text{Zn}^{2+}$-induced potentiation of [3H]dopamine uptake in COS-7 cells transiently expressing hDAT-E396H (●), hDAT-H193K-E396H (△), hDAT-H375A-E396H (□), and hDAT-H193K-H375A-E396H (■). The $\text{Zn}^{2+}$-induced curve for WT hDAT is shown for comparison as a dotted line. Data are the means ± S.E. of 3–6 experiments performed in triplicate.

**binding of Zn$^{2+}$**, which confirmed a crucial role of His$^{375}$ (Table II and Fig. 4). However, mutation of His$^{396}$ (hDAT-H193K-E396H) did only increase the IC$_{50}$ value for Zn$^{2+}$ inhibition 2-fold, suggesting that His$^{396}$ only has a minor role in Zn$^{2+}$ inhibition of [3H]dopamine uptake in the hDAT-E396H mutant (Table II and Fig. 4). Possibly, insertion of a histidine in position 396 causes a structural change of the Zn$^{2+}$-binding site that could lead to involvement of as yet unidentified residues in addition to His$^{375}$ and the histidine in position 396. Nonetheless, the data still support the close proximity between His$^{375}$ and Glu$^{396}$ and are consistent with Glu$^{396}$ being a third coordinate in the endogenous Zn$^{2+}$-binding site.

**An Engineered Zn$^{2+}$-binding Site between the TM 7 and 8**—Our data suggest that His$^{375}$ at the top of TM 7 is facing Glu$^{396}$ at the top of TM 8. To further elaborate this spatial proximity between TM 7 and 8, we next wished to engineer artificial metal ion-binding sites between the two domains. Assuming that Glu$^{396}$ is situated in an α-helical environment, it could be expected that His$^{375}$ also is close to the i+4 or i+3 position from Glu$^{396}$ in TM 8 and thus that a bidentate Zn$^{2+}$-binding site could be generated between His$^{375}$ and either the i+4 or i+3 position, whereas the i+2 position would be expected to be located on the opposite side of the helix (Fig. 5A). Accordingly, three mutant transporters were generated in which His$^{356}$ and Glu$^{396}$ were removed, His$^{375}$ was preserved, and cysteines residues were inserted in positions $i+2$ (hDAT-H193K-E396Q-I398C), $i+3$ (hDAT-H193K-E396Q-A399C) and $i+4$ (hDAT-H193K-E396Q-T400C). Cysteines were chosen because their smaller side chain would be expected to be better tolerated in the transmembrane regions as compared with histidines. As illustrated in Fig. 5B, Zn$^{2+}$ was a potent inhibitor of [3H]dopamine uptake in hDAT-H193K-E396Q-T400C (i+4), displaying an IC$_{50}$ value of 24 μM in contrast to 660 μM for the background mutant, hDAT-H193K-E396Q (Table III and Fig. 5B). This almost 30-fold increase in apparent Zn$^{2+}$ affinity was not only dependent on the presence of a cysteine in position 400 but was also dependent on the presence of His$^{375}$. Mutation of His$^{375}$ to alanine in the hDAT-H193K-E396Q-T400C construct (resulting in hDAT-H193K-H375A-E396Q-T400C) eliminated

**TABLE I**

Uptake and binding characteristics of hDAT, hNET, and Asp/Glu mutant transporters

| hDAT Mutants | [3H]Dopamine uptake | IC$_{50}$ (Zn$^{2+}$) [S.E. interval] | P$_{MUT}$ (Zn$^{2+}$) binding | [3H]WIN 35,428 binding | K$_{S}$ (S.E. interval) | B$_{max}$ | Zn$^{2+}$ ratio |
|--------------|---------------------|------------------------------------|----------------------------|---------------------|---------------------|---------|-----------------|
| hDAT         | 13                   | 1.3 [1.1–1.8]                     | 2200 ± 315                  | 1.1 [1.0–1.2]      | 1                   | 15      | 11 [10–12]      |
| hNET         | 15                   | 0.6 [0.5–0.7]                     | 762 ± 90                    | 0.9 [0.8–1.0]      | 0.8                 | 3       | 38 [33–44] |
| hDAT D191N   | 4                    | 3.6 [3.2–4.0]                     | 7146 ± 339                  | 2.9 [2.5–3.5]      | 2.6                 | 3       | 21 [18–25] |
| hDAT E215Q   | 3                    | 0.8 [0.7–1.0]                     | 792 ± 69                    | 0.9 [0.8–1.0]      | 0.8                 | 3       | 7 [6–8] |
| hDAT E218Q   | 3                    | 0.63 [0.57–0.69]                  | 33 ± 6                      | 1.3 [1.1–1.6]      | 1.2                 | 3       | 44 [34–50] |
| hDAT D232N   | 3                    | 2.0 [1.8–2.2]                     | 4038 ± 1800                 | 0.7 [0.6–1.0]      | 0.6                 | 3       | 36 [22–31] |
| hDAT D301N   | 3                    | 0.96 [0.90–1.02]                  | 747 ± 51                    | 1.0 [0.7–1.4]      | 0.9                 | 3       | 11 [10–12] |
| hDAT E307Q   | 3                    | 2.2 [2.0–2.5]                     | 1580 ± 180                  | 0.7 [0.6–0.0]      | 0.6                 | 3       | 41 [34–44] |
| hDAT D381N   | 5                    | 0.82 [0.75–0.88]                  | 1198 ± 11                   | 0.7 [0.6–0.8]      | 0.6                 | 4       | 22 [18–27] |
| hDAT D385N   | 3                    | 1.7 [1.3–2.1]                     | 1425 ± 44                   | 1.2 [0.9–1.5]      | 1.3                 | 3       | 51 [45–57] |
| hDAT E396Q   | 4                    | 1.9 [1.4–2.4]                     | 726 ± 183                   | 530 [407–690]      | 451                 | 4       | 15 [11–21] |
| hDAT D476N   | 3                    | 7.7 [6.4–9.1]                     | 2000 ± 200                  | 0.9 [0.8–1.0]      | 0.8                 | 3       | 35 [30–42] |
| hNET K198H   | 3                    | 0.6 [0.5–0.8]                     | 516 ± 58                    | 0.6 [0.5–0.7]      | 0.5                 | 3       | 17 [15–20] |
| hNET E393Q   | 3                    | 0.6 [0.5–0.7]                     | 693 ± 3                     | >1000              | >1000               | 3       | 29 [25–34] |
| hNET K189H   | 4                    | 0.45 [0.41–0.49]                  | 315 ± 31                    | 18 [11–28]         | 16                  | 4       | ND |

$^a$ Significantly different from hDAT wild type ($p < 0.01$).
proximity relationships in the dopamine transporter

The $K_m$ and $V_{max}$ for $[^{3}H]$dopamine uptake and the $K_d$ and $B_{max}$ for $[^{3}H]$WIN 35,428 binding were calculated from nonlinear regression analysis of uptake and binding data, respectively. $Zn^{2+}$ inhibition of $[^{3}H]$dopamine uptake was fitted to either a two-site or a one-site model. When fitted to a two-site model, the indicated $IC_{so}$ values for $Zn^{2+}$ are for the high affinity binding site. The $IC_{so}$ values used in the estimation of $K_m$ and $K_d$ values were calculated from means of $pIC_{so}$ values and the S.E. interval from the $pIC_{so}$ = S.E. The $Zn^{2+}$ ratio ($\%$ $B_{max}$) were calculated as $\left(\frac{B_{max}}{B_{max}}\right) \times 100\%$, where $B_{max}$ $[^{3}H]$WIN 35,428 binding in the presence of 10 $\mu M$ $Zn^{2+}$; $B_{max}$ control binding. $F_{MUT} (Zn^{2+})$ is defined as $IC_{so} (Zn^{2+}; mutant)/IC_{so} (Zn^{2+}; hDAT WT)$.

| hDAT Mutants | $[^{3}H]$ Dopamine uptake | $K_m$ [S.E. interval] | $V_{max}$ [fmol/min/10^5 cells] | $IC_{so} (Zn^{2+})$ [S.E. interval] | $F_{MUT} (Zn^{2+})$ | $[^{3}H]$WIN 35,428 binding | $K_d$ [S.E. interval] | $B_{max}$ | $Zn^{2+}$ ratio |
|--------------|---------------------------|----------------------|-----------------------------|---------------------------------|-------------------|--------------------------|-------------------|-----------|--------------|
| hDAT         | 13                        | 1.5 [1.3–1.8]        | 2200 ± 315                  | 1.10 [1.0–1.2]                  | 1                 | 110–12                  | 86 ± 13           | 155 ± 10   |              |
| hDAT E396H   | 4                         | 0.7 [0.5–0.9]        | 72 ± 13                     | 0.64 [0.54–0.78]                | 0.6               | 19–12                   | 39 ± 10           | 115 ± 5    |              |
| hDAT H193K + | 3                         | 1.4 [1.1–1.6]        | 339 ± 64                    | 1.43 [1.36–1.51]                | 1.3               | 32[30–34]               | 93 ± 27           | 102 ± 12   |              |
| E396H        |                           |                      |                             |                                 |                   |                         |                   |           |              |
| hDAT H375A + | 6                         | 1.0 [0.7–1.5]        | 124 ± 20                    | 593[466–754]                    | 539               | 3                      | 16[13–21]        | 110 ± 3    |              |
| E396H        |                           |                      |                             |                                 |                   |                         |                   |           |              |
| hDAT H193K + | 9                         | 0.9 [0.8–1.2]        | 48 ± 9                      | 469[405–543]                    | 426               | 2                      | 16[13–19]        | 17 ± 6     | 100 ± 4     |
| H375A + E396H|                           |                      |                             |                                 |                   |                         |                   |           |              |
| hDAT H193K + | 5                         | 3.5 [3.0–4.0]        | 4229 ± 813                  | 664[611–721]                    | 604               | 3                      | 22[20–25]        | 142 ± 43   | 89 ± 9      |
| E396Q        |                           |                      |                             |                                 |                   |                         |                   |           |              |
| hDAT H193K + | 5                         | 3.4 [3.0–3.9]        | 917 ± 131                   | >1000                           | >1000             | 3                      | 17[15–20]        | 112 ± 30   | 97 ± 7      |
| H375A + E396Q|                           |                      |                             |                                 |                   |                         |                   |           |              |

$^{*}$Significantly different from hDAT wild type ($p < 0.01$).

**Fig. 5.** Evidence for an engineered $Zn^{2+}$-binding site between TM 7 and TM 8. A, two-dimensional representation of the TM 7/TM 8 region with indication of residues chosen for substitution with cysteine. B, $Zn^{2+}$ inhibition of $[^{3}H]$dopamine uptake in COS-7 cells transiently expressing hDAT-H193K-E396Q-T400C (●) and control mutant hDAT-H193K-E396Q (∆). C, $Zn^{2+}$ inhibition of $[^{3}H]$dopamine uptake in COS-7 cells transiently expressing hDAT-H193K-E396Q-A399C (●), hDAT-H193K-E396Q-A399C (∆), and control mutant hDAT-H193K-E396Q (○). Data are the means ± S.E. of 3–6 experiments performed in triplicate.

High affinity $Zn^{2+}$ inhibition of $[^{3}H]$dopamine uptake (IC<sub>so</sub> = 995 nM; Table III). This indicates that an inhibitory bidentate $Zn^{2+}$-binding site had been generated between His<sup>375</sup> and T400C. For hDAT-H193K-E396Q-A399C (i+3) we observed a small 2–3-fold decrease in the IC<sub>so</sub> value for $Zn^{2+}$, whereas a 5-fold decrease was found for hDAT-H193K-E396Q-I398C (i+2) (Fig. 5C and Table III). Importantly, this 5-fold decrease in the IC<sub>so</sub> value for $Zn^{2+}$ in hDAT-H193K-E396Q-I398C (IC<sub>so</sub> = 122 µM) as compared with control, hDAT-H193K-E396Q (IC<sub>so</sub> = 664 µM; Table III) was found to be independent of the presence of His<sup>375</sup> because a similar IC<sub>so</sub> value was observed in the control mutant hDAT-H193K-H375A-E396Q-I398C (IC<sub>so</sub> = 137 µM; Table III).

**Discussion**

In our previous study we demonstrated that the hDAT contains an endogenous high affinity $Zn^{2+}$-binding site and that $Zn^{2+}$ by binding to this site acts as a potent noncompetitive blocker of dopamine uptake (22). Moreover, we identified two residues on the extracellular face of the transporter, His<sup>193</sup> and His<sup>375</sup> (Fig. 1) forming two coordinates in this binding site (22). In this study, we show evidence that a highly conserved glutamic acid, Glu<sup>396</sup>, is a third coordinate in the hDAT $Zn^{2+}$-binding site. The presence of a third coordinating residue in the hDAT $Zn^{2+}$-binding site is supported by the estimated affinities of $Zn^{2+}$ for different $Zn^{2+}$-binding sites. The affinity of $Zn^{2+}$ for binding sites involving three residues has generally been observed to range from $10^{-8}$ to $10^{-6}$ M, whereas sites involving four coordinates ranges from $10^{-7}$ to $10^{-5}$ M and sites with two coordinates from $10^{-3}$ to $10^{-2}$ M (30–32). The wide affinity ranges reflect the fact that the $Zn^{2+}$ affinity depends on a broad variety of factors in addition to the ability of the coordinating side chain to chelate $Zn^{2+}$ (32); however, the observed apparent affinity of $Zn^{2+}$ for the hDAT (IC<sub>so</sub> = 1 µM; Table I) is mostly consistent with a binding site containing three coordinating residues. It is therefore also our estimate that no additional residues are involved, in agreement with the fact that we at present have mutated the majority of candidate $Zn^{2+}$-binding residues in the hDAT (this study and Ref. 22).

Importantly, several $Zn^{2+}$-binding sites in soluble proteins have been described involving two histidines and a glutamate (25). In these sites, the geometry is most often found to be tetrahedral with the fourth coordinate being a water molecule.
TABLE III
Uptake characteristics of mutants with inserted cysteine residues in the top of TM 8

| hDAT mutants          | [3H]Dopamine uptake | $K_m$ [S.E. interval] | $V_{max}$ [fmol/min/10^6 cells] | IC$_{50}$ (Zn$^{2+}$) [S.E. interval] |
|-----------------------|----------------------|----------------------|----------------------------------|--------------------------------------|
| hDAT H193K + E396Q   | 5                    | 3.5 [3.0–4.0]        | 4229 ± 313                       | 664 [611–721]                      |
| hDAT H193K + E396Q   | 5                    | 0.44 [0.38–0.50]     | 297 ± 59                         | 122 [104–144]                      |
| + I398C               | 5                    | 1.7 [1.4–2.1]        | 1004 ± 132                       | 280 [234–334]                      |
| hDAT H193K + E396Q   | 3                    | 1.2 [1.0–1.4]        | 439 ± 76                         | 24 [21–27]                         |
| + A399C               | 5                    | 0.74 [0.69–0.78]     | 68 ± 19                          | 995 [899–1102]                     |
| hDAT H193K + H375A + | 3                    | 0.8 [0.7–1.0]        | 495 ± 89                         | 137 [127–146]                      |
| E396Q + T400C         | 5                    | 0.44 [0.38–0.50]     | 297 ± 59                         | 122 [104–144]                      |
| hDAT H193K + H375A + | 5                    | 1.7 [1.4–2.1]        | 1004 ± 132                       | 280 [234–334]                      |
| E396Q + T400C         | 3                    | 1.2 [1.0–1.4]        | 439 ± 76                         | 24 [21–27]                         |
| hDAT H193K + H375A + | 5                    | 0.74 [0.69–0.78]     | 68 ± 19                          | 995 [899–1102]                     |
| E396Q + I398C         | 3                    | 0.8 [0.7–1.0]        | 495 ± 89                         | 137 [127–146]                      |

According to known structures of Zn$^{2+}$-binding proteins, the distance between the Zn$^{2+}$ ion and the coordinating nitrogen of the imidazole side chain vary from 2.0 Å-2.3 Å and is largely independent of the coordination geometry (25). Hence, the identification of three residues involved in Zn$^{2+}$-binding to the endogenous hDAT Zn$^{2+}$-binding site and the engineering of a Zn$^{2+}$-binding site between TM 7 and 8 impose an important set of distance constraints in the tertiary structure of hDAT (Fig. 6). Glu$^{396}$ is predicted to be situated right at the extracellular end of TM 8, which is separated from TM 7 by a large loop of approximately 20 residues, allowing TM 7 and 8 to be rather far apart in the tertiary structure. However, the present data outline the close association between the two transmembrane segments, which is further documented by the engineered Zn$^{2+}$-binding site between His$^{375}$ and the cysteines inserted in position 400. Moreover, the participation of His$^{375}$ in binding of Zn$^{2+}$ to the hDAT outline the association of the outer portion of TM 7 and 8 with the large second extracellular loop connecting TM 3 and 4. In summary, the data from the present study together with our previous data (22) provide the first crude insight into the tertiary organization of a Na$^+/Cl^-$ dependent transporter. It is important to emphasize that the data, moreover, are consistent with the originally proposed topology of hDAT (shown in Fig. 1) and thus support recent studies based on site-directed chemical labeling of cysteines or lysines (20, 34).

Engineering of artificial Zn$^{2+}$-binding sites has previously been used as a powerful tool to probe the structure of both G protein-coupled receptors and the Lac permease of *Escherichia coli* (30, 31, 35, 36). In this study, we have engineered a Zn$^{2+}$-binding site between His$^{375}$ and a cysteine inserted in position 400 (Fig. 5). The possibility of Zn$^{2+}$ to coordinate not only between His$^{375}$ and Glu$^{396}$ but also between His$^{375}$ and a cysteine inserted in position 400 further defines the orientation of His$^{375}$, and thus TM 7, in relation to TM 8. To accommodate the data, His$^{375}$ must be facing TM 8, positioned between His$^{375}$ and Thr$^{400}$ (Fig. 6). Our data are moreover consistent with Glu$^{396}$ and Thr$^{400}$ being located in an α-helical environment. The apparent affinity, as indicated from the IC$_{50}$ value for Zn$^{2+}$ inhibition of [3H]dopamine uptake, increased more than 30-fold by inserting the cysteine in position 400 (i–4 from Glu$^{396}$), whereas only smaller increases were observed for positions 399 and 398 (Table III). The apparent increase in affinity for Cys$^{396}$ was also observed in the absence of His$^{375}$ (mutant hDAT-H193K-H375A-E396Q-I398C) and therefore cannot be attributed to coordination of Zn$^{2+}$ between these two residues.

Earlier, we have shown evidence that Zn$^{2+}$ by binding to the endogenous high affinity binding site of the hDAT noncompeti-
tivitively prevents dopamine translocation without interfering with dopamine binding to the transporter (22). It is conceivable that Zn\(^{2+}\), by keeping His\(^{193}\), His\(^{375}\), and Glu\(^{396}\) in close spatial proximity, inhibits a conformational change critical for the translocation process. Therefore, our data provide indirect evidence for an important role of extracellular loop 2 and TM 7/8 in the conformational changes accompanying translocation of dopamine. It was interesting to observe that substitution of Glu\(^{396}\) with His allows Zn\(^{2+}\) to fully inhibit the translocation process (Fig. 4) in contrast to our observation in the WT hDAT where saturation of the high affinity Zn\(^{2+}\)-binding site (in presence of around 100 \(\mu\)M of Zn\(^{2+}\)) only resulted in approximately 60–70% inhibition of uptake (Fig 2A and Ref. 22). However, with respect to Zn\(^{2+}\) potentiation of \(^{3}H\)[WIN 35,428 binding, the histidine was only able to substitute partly for the glutamic acid (15% increase in binding in E396H versus 55% in WT; Table II). One possible explanation is a change in the Zn\(^{2+}\) coordination geometry in hDAT-E396H. This is supported by the observation that His\(^{193}\) is only playing a minor role for the ability of Zn\(^{2+}\) to inhibit \(^{3}H\)dopamine uptake in hDAT-E396H, i.e. mutation of His\(^{193}\) in hDAT-E396H (hDAT-H193K-E396H) only increases the IC\(_{50}\) value for Zn\(^{2+}\) around 2-fold (Fig 4) in contrast to mutation of His\(^{375}\) (hDAT-H375A-E396H), which eliminated apparent high affinity binding of Zn\(^{2+}\) (Table III). It could therefore be hypothesized that substitution of glutamate with histidine in position 396 may lead to interaction between Zn\(^{2+}\) and so far unidentified residues in addition to His\(^{375}\) and His\(^{396}\). The steep inhibition curve with a Hill slope of \(-2.2\) in hDAT-E396H is another indication of a more complex pattern of interaction between Zn\(^{2+}\) and the transporter (Fig. 4). Nevertheless, it is also possible that fixation of TM 7 and 8 is the key element in Zn\(^{2+}\)-induced inhibition of dopamine uptake whereas the interaction with His\(^{193}\) in extracellular loop 2 is of less importance. Thus, a histidine in position 396 could be envisioned to be more optimal for Zn\(^{2+}\) coordination than a glutamic acid and accordingly facilitate the ability of Zn\(^{2+}\) to constrain motions of TM 7 relative to TM 8. An increasing number of receptors and transporters in the brain have been shown to be regulated by Zn\(^{2+}\) in low micromolar concentrations. In addition to the DAT, these include ionotropic glutamate receptors, some \(\gamma\)-aminobutyric acid receptor subtypes, the strychnine-sensitive glycine receptor, dopamine D2 receptors, and the EAAT1 glutamate transporter (37–43). This is consistent with the increasing amount of evidence pointing to Zn\(^{2+}\) as an important neuromodulator in the brain (44). It is highly likely that free Zn\(^{2+}\) is present in the synaptic clefts in concentrations required to modulate the function of the proteins. The basal concentration of Zn\(^{2+}\) in the brain is approximately 10 nM (45, 46). However, upon neuronal stimulation Zn\(^{2+}\) has been shown to be released in concentrations of approximately 300 \(\mu\)M (47). Although substantial information is available about Zn\(^{2+}\)-binding sites in soluble proteins, including transcription factors and a variety of enzymes, little is known about Zn\(^{2+}\)-binding sites in membrane proteins. High resolution structural information is not available for any of these proteins, and at present, residues involved in Zn\(^{2+}\) binding have been identified only in the \(\gamma\)-aminobutyric acid \(\delta1\) subunit (48), in the \(\gamma\)-aminobutyric acid \(\beta1\) subunit (49) and in the glutamate transporter EAAT1 (42). In the EAAT1, two histidines were identified in the predicted large second extracellular loop (42). Evidently, additional insight into the specific structural and regulatory role of Zn\(^{2+}\) at neurotransmitter transporters and receptors is a prerequisite for further clarifying the role of Zn\(^{2+}\) as a neuromodulator in the human brain.

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