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Delineation of an endogenous zinc-binding site in the human dopamine transporter

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The molecular basis for substrate translocation in the Na^+/Cl^--dependent neurotransmitter transporters remains elusive. Here we report novel insight into the translocation mechanism by delineation of an endogenous Zn^{2+}-binding site in the human dopamine transporter (hDAT). In micromolar concentrations, Zn^{2+} was found to act as a potent, non-competitive blocker of dopamine uptake in COS cells expressing hDAT. In contrast, binding of the cocaine analogue, WIN 35,428, was markedly potentiated by Zn^{2+}. Surprisingly, these effects were not observed in the closely related human norepinephrine transporter (hNET). A single non-conserved histidine residue (His193) in the large second extracellular loop (ECL2) of hDAT was discovered to be responsible for this difference. Thus, Zn^{2+} modulation could be conveyed to hNET by mutational transfer of only this residue. His375 conserved between hDAT and hNET, present in the fourth extracellular loop (ECL4) at the top of transmembrane segment VII, was identified as a second major coordinate for Zn^{2+} binding. These data provide evidence for spatial proximity between His193 and His375 in hDAT, representing the first experimentally demonstrated proximity relationship in an Na^+/Cl^--dependent transporter. Since Zn^{2+} did not prevent dopamine binding, but inhibited dopamine translocation, our data suggest that by constraining movements of ECL2 and ECL4, Zn^{2+} can restrict a conformational change critical for the transport process.

Keywords: dopamine/membrane protein structure/metal ion site/Na^+/Cl^--dependent transporters/transmembrane transport

Introduction

The dopamine transporter (DAT) together with the closely related norepinephrine and serotonin transporters forms a subfamily within the large family of Na^+/Cl^--dependent transporters (Giros and Caron, 1993). These transporters all share a predicted topology with 12 transmembrane segments (Figure 1) and are functionally characterized by co-transport of substrate with Na^+ and Cl^- (Amara and Kuhar, 1993; Giros and Caron, 1993). The DAT is localized presynaptically in the nerve terminals and regulates the amount of available dopamine in the synapse by mediating rapid re-uptake of released dopamine (Horn, 1990; Amara and Kuhar, 1993). 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 (Horn, 1990; Amara and Kuhar, 1993; Giros *et al*., 1996). Moreover, DAT is the principal target for the psychostimulatory, addictive drugs, cocaine and amphetamine (Horn, 1990; Giros *et al*., 1996).

The tertiary structure of Na^+/Cl^--dependent transporters is not yet known, and the nature of the molecular processes responsible for the translocation mechanism has not been elucidated. Several studies have attempted to define functional domains both in DAT and in other Na^+/Cl^--dependent transporters. Site-directed mutagenesis has led to the identification of some residues that are important for either the transport process or for substrate/blocker binding (Kitayama *et al*., 1992; Pantanowitz, *et al*., 1993; J.B.Wang *et al*., 1995; Barker and Blakely, 1996; Bismuth *et al*., 1997; Chen *et al*., 1997a,b). In addition, several groups have constructed chimeric molecules between homologous, but pharmacologically distinct transporter subtypes (Barker *et al*., 1994; Buck and Amara, 1994, 1995; Giros *et al*., 1994; Stephan *et al*., 1997). These studies have provided important information about domains determining substrate and blocker selectivity; however, due to the inherent limitation of the chimeric approach, they have not given insight into the translocation process, which is believed to be evolutionarily conserved among this class of transporters.

Zn^{2+} is required for the function of numerous proteins, serving both as a part of the active site in, for example, metallo-enzymes, and acting to stabilize protein domains, such as the Zn^{2+} finger-binding motif in transcription factors (Vallee and Falchuk, 1993; Schwabe and Klug, 1994). The structure of many Zn^{2+}-binding sites is known from X-ray crystallography of Zn^{2+}-binding proteins and, thus, the geometry of the interaction between Zn^{2+} and different coordinating residues is well characterized (Vallee and Falchuk, 1993; Schwabe and Klug, 1994). This, together with the small size of the zinc(II) ion, makes artificially generated Zn^{2+}-binding sites a highly useful approach for probing structure–function relationships in other proteins (Elling *et al*., 1995; He *et al*., 1995; Elling and Schwartz, 1996; Sheikh *et al*., 1996). In G protein-coupled receptors, for example, construction of bis-His Zn^{2+}-binding sites has led to important information about both the organization of the transmembrane helices and their potential movements during receptor activation (Elling *et al*., 1995; Elling and Schwartz, 1996; Sheikh *et al*., 1996).

Interestingly, evidence indicates the presence of naturally occurring Zn^{2+}-binding sites in several receptor

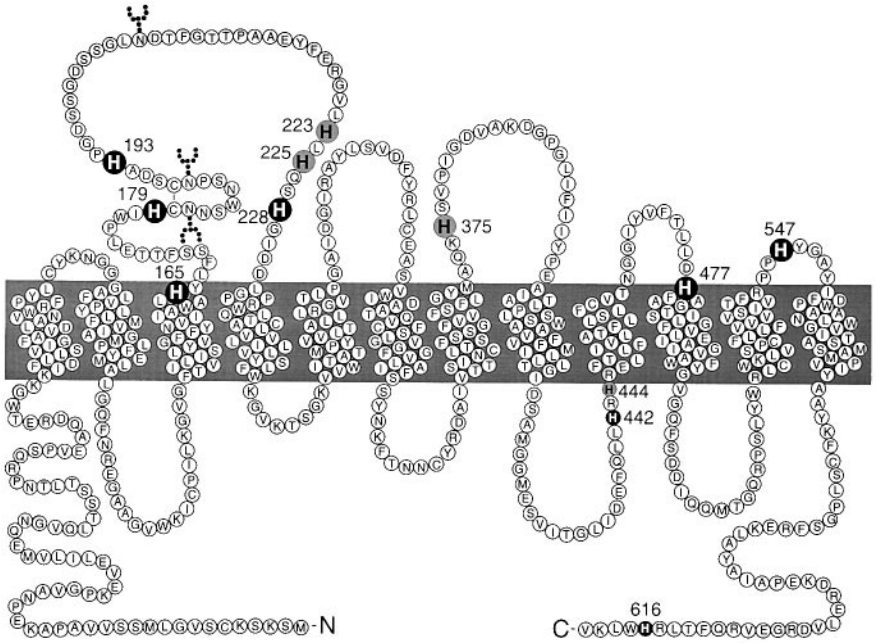

Fig. 1. Two-dimensional representation of hDAT (Giros and Caron, 1993). The transporter contains 12 histidine residues of which eight are non-conserved between hDAT and hNET: His165, His179, His193, His228, His442, His477, His547 and His616 (black circles). Four histidines are conserved between hDAT and hNET: His223, His225, His375 and His444 (shaded circles). The histidine residues examined for coordination of Zn^{2+} are shown as enlarged circles.

proteins in the brain. These include ionotropic glutamate receptors, some γ-aminobutyric acid (GABA) receptor subtypes and the strychnine-sensitive glycine receptor (Peters *et al*., 1987; Westbrook and Mayer, 1987; Hollmann *et al*., 1993; Bloomenthal *et al*., 1994; Laube *et al*., 1995). Moreover, previous data have indicated that Zn^{2+} can modulate dopamine uptake and DAT blocker binding in rat brain preparations (Richfield, 1993; Bonnet *et al*., 1994). In the present study, we provide direct evidence that the human DAT (hDAT), but not the closely related human norepinephrine transporter (hNET), possesses an endogenous, high-affinity Zn^{2+}-binding site. By identifying two major coordinating histidines residues in this binding site, we obtain interesting new insight into the translocation mechanism and map an important distance constraint in an Na^+/Cl^--dependent transporter.

Results

Zn^{2+} inhibits dopamine transport and stimulates WIN 35,428 binding at the dopamine transporter

Zn^{2+} inhibited [^{3}H]dopamine uptake in COS-7 cells expressing hDAT in a biphasic manner (K_i, high affinity = 0.8 μM, and K_i, low affinity >1000 μM) (Figure 2A; Table I). The effect of micromolar concentrations of Zn^{2+} on [^{3}H]dopamine uptake was reversible; thus, 10 min preincubation with 10 μM Zn^{2+} followed by washing the cells resulted in unchanged [^{3}H]dopamine uptake and Zn^{2+} inhibition curves as compared with control cells (data not shown). In contrast to hDAT, [^{3}H]dopamine uptake mediated by hNET expressed in COS-7 cells was affected only by millimolar concentrations of Zn^{2+} (Figure 2A; Table I). This indicates that hDAT but not hNET possesses an endogenous high-affinity Zn^{2+}-binding site. This was supported further by investigating the effect of Zn^{2+} on binding of [^{3}H]WIN 35,428, a cocaine-like

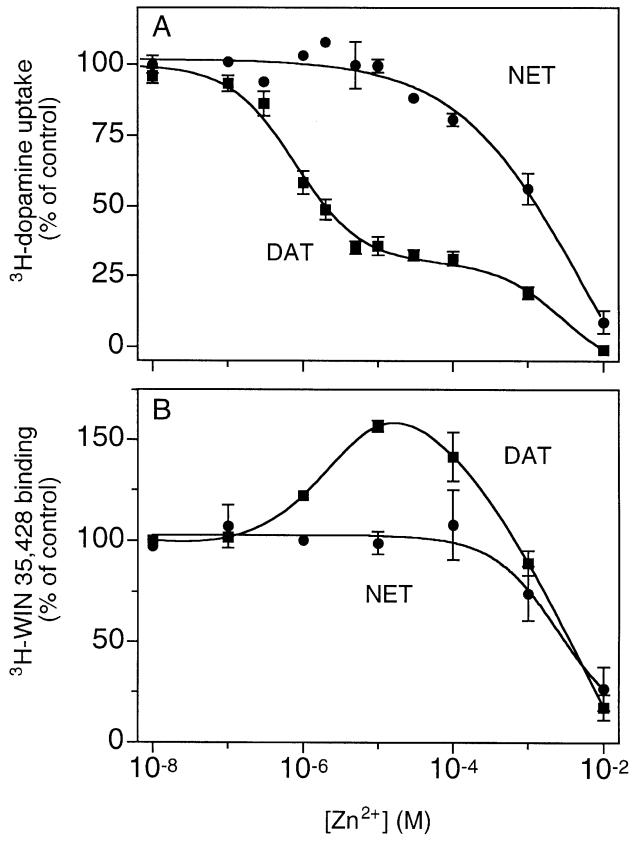

Fig. 2. Evidence for an endogenous Zn^{2+}-binding site in hDAT but not in hNET. (**A**) Zn^{2+} inhibition of [^{3}H]dopamine uptake in COS-7 cells transiently expressing hDAT (■) and hNET (●). (**B**) Effect of Zn^{2+} on binding of [^{3}H]WIN 35,428 to hDAT (■) and hNET (●). Data are means ± SE of 3–8 experiments performed in triplicate.

Table I. Uptake and binding characteristics of hDAT and hNET mutant transporters

	[^{3}H]Dopamine					[^{3}H]WIN 35,428		
	K_m (μM) (SE interval)	V_{max} (pmol/min/10^5 cells) ± SE	IC_{50} (Zn^{2+}) (μM) (SE interval)	F_{MUT} (Zn^{2+})	High-affinity Zn^{2+} sites (%) ± SE	K_d (nM) SE interval	B_{max} (fmol/10^5 cells) ± SE	Zn^{2+} ratio (% B_0) ± SE
hDAT	4.0 (3.7–4.4)	11 ± 1	0.79 (0.67–0.93)	1	70 ± 3	9.1 (6.5–12.5)	160 ± 30	157 ± 3
hNET	2.4 (2.2–2.6)	4 ± 1	1100 (900–1360)	1390	–	23 (19–28)	81 ± 18	99 ± 6[a]
hDAT-H165Y	5.7 (4.1–8.0)	5 ± 2	1.03 (0.81–1.32)	1.3	45 ± 3	9.1 (6.8–12.1)	83 ± 25	165 ± 11
hDAT-H179D	2.7 (2.2–3.2)	3 ± 1	1.29 (1.01–1.65)	1.6	71 ± 4	13.0 (9.4–17.6)	84 ± 19	168 ± 15
hDAT-H179A	6.2 (5.9–6.4)	4 ± 1	0.61 (0.49–0.76)	0.8	82 ± 6	11.8 (10.8–12.8)	88 ± 12	172 ± 9
hDAT-H228S	7.8 (6.9–8.7)	7 ± 1	1.64 (1.40–1.93)	2.1	74 ± 2	8.7 (6.1–12.2)	76 ± 21	137 ± 17
hDAT-H477T	5.4 (4.4–6.6)	11 ± 1	0.71 (0.55–0.91)	0.9	40 ± 6	13.1 (12.4–13.9)	202 ± 16	162 ± 8
hDAT-H547T	5.9 (4.9–7.1)	19 ± 5	1.54 (1.20–1.97)	1.9	64 ± 6	11.7 (10.3–13.2)	302 ± 58	134 ± 6
hDAT-H193K	5.2 (4.4–6.3)	9 ± 2	520 (380–720)	660	–	13.1 (11.6–14.7)	163 ± 45	92 ± 3[a]
hDAT-H223A-H225A	2.1 (1.7–2.6)	0.6 ± 0.1	1.08 (0.80–1.46)	1.4	88 ± 7	ND	ND	ND
hDAT-H375A	6.9 (6.6–7.3)	6 ± 1	790 (560–1110)	1000	–	10.6 (9.0–12.5)	91 ± 11	103 ± 14[a]
hDAT-H193K-H375A	5.5 (4.8–6.6)	9 ± 3	1040 (750–1450)	1320	–	8.0 (6.8–9.4)	122 ± 23	87 ± 7[a]
hNET-K189H	2.2 (1.7–3.0)	3 ± 1	0.41 (0.26–0.64)	0.5	56 ± 6	16.7 (11.9–23.1)	94 ± 26	117 ± 3[a]
hNET-H372A	2.1 (1.9–2.4)	5 ± 1	2070 (1680–2560)	2620	–	n.d.	n.d.	n.d.
hNET-K189H-H372A	1.1 (1.0–1.2)	3 ± 1	1210 (930–1580)	1530	–	n.d.	n.d.	n.d.

ND, not detectable; n.d., not determined; [a]$P < 0.01$ compared with hDAT; two-tailed *t*-test.

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 non-linear 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_{50} values for Zn^{2+} are for the high-affinity binding site. The estimated fraction of high-affinity Zn^{2+}-binding sites is shown as a percentage of total binding sites. The IC_{50} values used in the estimation of K_m and K_d values were calculated from means of pIC_{50} values and the SE interval from the pIC_{50} ± SE The Zn^{2+} ratio (means ± SE) were calculated as $(B_{Zn}/B_0)\times100\%$ (B_{Zn}, [^{3}H]WIN 35,428 binding in the presence of 10 μM Zn^{2+}; B_0, control binding). F_{mut} (Zn^{2+}) is defined as IC_{50} (Zn^{2+}; mutant)/IC_{50} (Zn^{2+}; hDAT wild-type). All experiments were performed three to eight times.

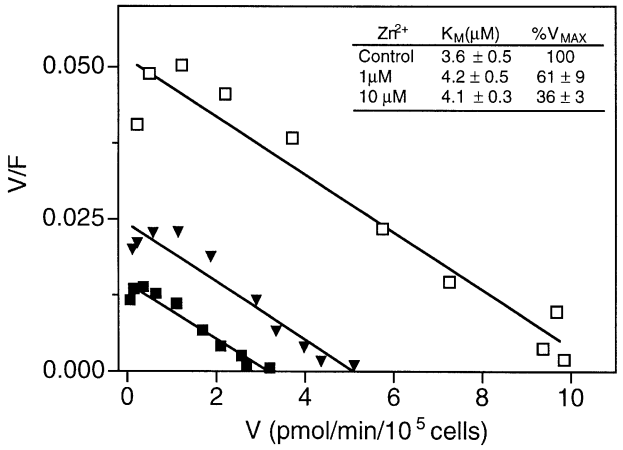

Fig. 3. Zn^{2+} acts as a non-competitive blocker of [^{3}H]dopamine uptake. Scatchard plot of [^{3}H]dopamine uptake by COS-7 cells expressing hDAT in the absence (□) and presence of 1 μM (▼) and 10 μM (■) Zn^{2+}. Cells were incubated with 10 nM [^{3}H]dopamine for 10 min in the presence of various concentrations of dopamine ± Zn^{2+}. The experiment shown is representative of three identical experiments. The shown K_m and V_{max} (% of control) for [^{3}H]dopamine are means ± SE of three experiments.

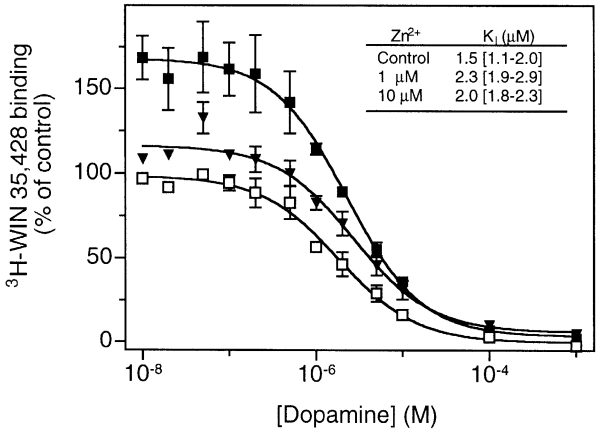

Fig. 4. Inhibition of [^{3}H]WIN 35,428 binding by dopamine in COS-7 cells transiently expressing hDAT in the absence (□) and presence of 1 μM (▼) and 10 μM (■) Zn^{2+}. Data are means ± SE (n = 3) as a percentage of [^{3}H]WIN 35,428 control binding to hDAT in the absence of Zn^{2+}. The K_i values shown for dopamine are means ± SE interval (n = 3).

blocker. While [^{3}H]WIN 35,428 binding to intact COS-7 cells expressing hNET was only affected at millimolar concentrations of Zn^{2+}, a biphasic response was again observed for hDAT (Figure 2B; Table I). However, contrary to the effect on [^{3}H]dopamine uptake, micromolar concentrations of Zn^{2+} increased apparent [^{3}H]WIN 35,428 binding up to a maximum of 50–60% in the presence of 10 μM Zn^{2+} (Figure 2B; Table I). Notably, in COS-7 cell membranes, a similar biphasic effect of Zn^{2+} on [^{3}H]WIN 35,428 binding was observed for hDAT (data not shown).

The mechanism by which Zn^{2+} affects dopamine uptake was explored by [^{3}H]dopamine uptake assays in the presence and absence of Zn^{2+}. Scatchard analysis of the data showed that Zn^{2+} did not change the K_m for dopamine uptake, but caused a dramatic decrease in the V_{max} value. In the presence of 10 μM Zn^{2+}, the V_{max} value was reduced to ~30% of the control level (Figure 3). This indicates that Zn^{2+} acts like a non-competitive blocker of dopamine uptake. We also investigated the mechanism underlying Zn^{2+} potentiation of [^{3}H]WIN 35,428 binding by performing saturation binding experiments with [^{3}H]WIN 35,428 in the absence and presence of 10 μM Zn^{2+} (data not shown). The experiments indicated an increase in the apparent B_{max} (148 ± 10%, mean ± SE, n = 3, in the presence of 10 μM Zn^{2+} as compared with control). In contrast, we did not observe a change in K_d (K_d = 10 ± 2 nM, mean ± SE, n = 3, in the presence of 10 μM Zn^{2+} as compared with 12 ± 3 nM, n = 3, in the absence of Zn^{2+}).

To investigate whether Zn^{2+} inhibition of dopamine uptake was due to non-competitive inhibition of either dopamine binding or dopamine translocation, we compared the ability of dopamine to compete for [^{3}H]WIN 35,428 binding in the absence and presence of 1 and 10 μM Zn^{2+}. If Zn^{2+} prevents dopamine binding, then the ability of dopamine to compete for [^{3}H]WIN 35,428 binding in the presence of Zn^{2+} should be impaired. However, Zn^{2+} changed neither the K_i value for dopamine nor the ability of dopamine to displace [^{3}H]WIN 35,428 fully (Figure 4). Thus, dopamine can apparently bind with unchanged affinity to the Zn^{2+}-occupied transporter, indicating that Zn^{2+} specifically inhibits the translocation process.

Mutation of non-conserved histidine residues

Histidines, cysteines, glutamic acids and aspartic acids are most commonly found as coordinating residues in Zn^{2+}-binding sites (Vallee and Falchuk, 1993). In order to investigate the possible nature of the residues involved in Zn^{2+} binding to hDAT, a series of different cations were tested for their ability to inhibit dopamine uptake. The rank order of potency was consistent with the presence of at least one histidine residue in the Zn^{2+}-binding site: $Zn^{2+}>Ni^{2+}>Co^{2+}>Co^{3+}>Cd^{2+}>Mn^{2+}$ (data not shown; Glusker, 1991). We therefore decided to mutate histidines not conserved between hDAT and hNET (Figure 1). Mutation of His165, His179, His228, His477 and His547 on the extracellular side of hDAT to the corresponding residue in hNET (hDAT-H165Y, -H179D, -H228S, -H477T and -H547T; Table I) did not change the K_i value for Zn^{2+} inhibition of [^{3}H]dopamine uptake, nor did it significantly change potentiation of [3H]WIN 35,428 binding (Table I). Since His179 is a Glu in hNET and possibly could substitute for the histidine in coordination of Zn^{2+}, we also mutated this residue to an Ala (hDAT-H179A). Again, no change in Zn^{2+} sensitivity was found (Table I). However, mutation of His193 in the second extracellular loop, ECL2, (Figure 1) to Lys present in hNET (hDAT-H193K) increased the K_i value for Zn^{2+} inhibition of [^{3}H]dopamine uptake 660-fold (Figure 5A; Table I). Moreover, the potentiation of [^{3}H]WIN 35,428 binding was eliminated (Figure 5C; Table I). In both hDAT-H193K and the other histidine mutants, we observed K_m values for [^{3}H]dopamine uptake and K_d values for [^{3}H]WIN 35,428 binding similar to the wild-type (Table I). It should be noted that mutation of His165 and His477 reduced the percentage of apparent high-affinity Zn^{2+}sites from ~70 to 40–45% (Table I). However, we interpret this as an indirect structural effect, since the high-affinity K_i value for Zn^{2+} inhibition did not change (Table I). Overall, the data imply that His193 is a major coordinate in the

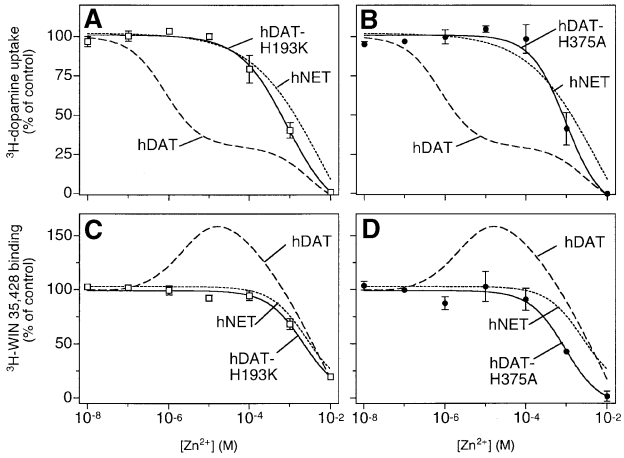

Fig. 5. Mutation of His193 and His375 in hDAT eliminates the effects of Zn^{2+} in micromolar concentrations. (**A**) Zn^{2+} inhibition of [^{3}H]dopamine uptake by hDAT-H193K (□). (**B**) Zn^{2+} inhibition of [^{3}H]dopamine uptake by hDAT-H375A (●). (**C**) Effect of Zn^{2+} on [^{3}H]WIN 35,428 binding to hDAT-H193K (□). (**D**) Effect of Zn^{2+} on [^{3}H]WIN 35,428 binding to hDAT-H375A (●). The long-dashed line indicate the wild-type hDAT inhibition curve, the short-dashed line the wild-type hNET curve (see Figure 2). Data are means ± SE of three to four experiments performed in triplicate.

endogenous hDAT Zn^{2+}-binding site and may determine the distinct Zn^{2+} sensitivity of hDAT and hNET.

Mutation of conserved histidine residues

Three histidines conserved between hDAT and hNET on the extracellular side were mutated to alanines to identify additional coordinates (Figure 1). Mutation of His223 and His225 in ECL2 together (hDAT-H223A-H225A) did not change Zn^{2+} susceptibility; however, mutation of His375 to Ala (hDAT-H375A) in ECL4 at the top of transmembrane segment (TM) VII caused a 1000-fold increase in the K_i value for Zn^{2+} inhibition of [^{3}H]dopamine uptake and eliminated potentiation of [^{3}H]WIN 35,428 binding (Figure 5B and D; Table I). Mutation of His193 and His375 together (hDAT-H193K-H375A) caused a slight further decrease in apparent Zn^{2+} affinity (Table I). Neither the single point mutation of His375 nor the double mutation (DAT-H193K-H375A) changed the uptake and binding properties of the transporter (Table I). Mutation of His223 and His225 together did not change the K_m for [^{3}H]dopamine, but reduced the V_{max} 20-fold. This most likely reflects reduced expression, explaining our inability to measure [^{3}H]WIN 35,428 binding to this mutant (Table I). Taken together, the data suggest that His375 is a second coordinate in the hDAT high-affinity Zn^{2+}-binding site.

Mutational transfer of Zn^{2+} susceptibility to the hNET

Introducing His193 in hDAT into the corresponding position in hNET (hNET-K189H) resulted in transfer of full Zn^{2+} susceptibility to the hNET with respect to inhibition of [^{3}H]dopamine uptake (Figure 6). To probe whether we had generated a Zn^{2+}-binding site in hNET identical to the site in hDAT, we also mutated His372 in hNET corresponding to the conserved His375 in hDAT (hNET-H372A) and combined this mutant with the hNET-K189H mutant (hNET-K189H-H372A). Mutation of His372 in

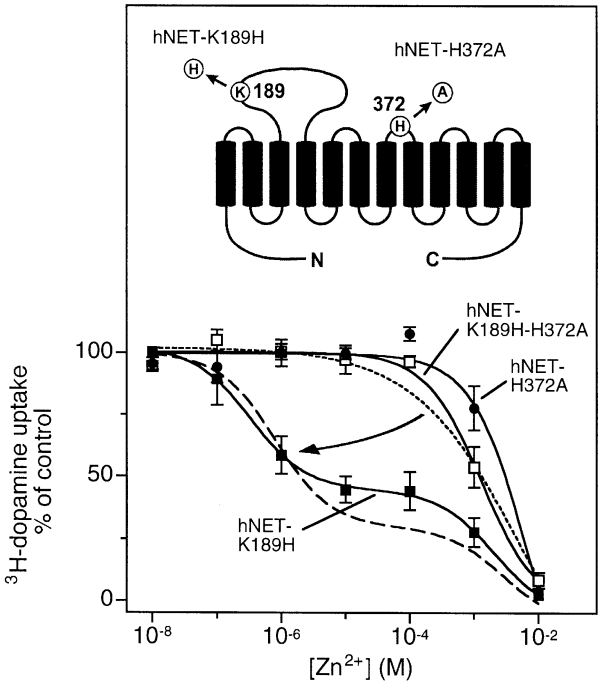

Fig. 6. Transfer of Zn^{2+} susceptibility to the hNET. Inhibition of [^{3}H]dopamine uptake by the hNET mutants hNET-K189H (■), hNET-H372A (●) and hNET-K189H-H372A (□) expressed in COS-7 cells. The long-dashed line indicates the wild-type hDAT inhibition curve, the short-dashed line the wild-type hNET curve (see Figure 2). Data are means ± SE of three to four experiments performed in triplicate.

hNET-K189H had the same effect as mutating His375 in hDAT (Figure 6). Figure 6 also shows that in the absence of both histidines (hNET-H372A), Zn^{2+} sensitivity is slightly lower than in the presence of one of them (hNET and hNET-K189H-H372A). These data strongly support the hypothesis that His193 and His375 in hDAT are coordinates in the same Zn^{2+}-binding sites. Furthermore, they show that the non-conserved residue, His193, determines the distinct Zn^{2+} sensitivity of hDAT and hNET with respect to inhibition of [^{3}H]dopamine uptake. It should be noted that introducing His193 in hNET (hNET-K189H) resulted in only weak potentiation of [^{3}H]WIN 35,428 binding. A possible explanation could be that subtle differences in the binding mode of [^{3}H]WIN 35,428 to hDAT and hNET, as indicated by the 2- to 3-fold difference in affinity (Table I), attenuate the apparent allosteric effect of Zn^{2+} on [^{3}H]WIN 35,428 binding.

Discussion

This study provides direct evidence that hDAT possesses an endogenous Zn^{2+}-binding site involving two histidine residues on the extracellular face of the transporter, His193 and His375. Our data demonstrate that Zn^{2+} bound to this site acts as a non-competitive blocker of substrate translocation. Concurrently, Zn^{2+} potentiates binding of the cocaine-like blocker WIN 35,428. These observations support earlier studies in rat brain preparations showing potentiation of blocker binding and inhibition of dopamine uptake by Zn^{2+} (Richfield, 1993; Bonnet *et al.*, 1994). Previous studies have also suggested that several other complex membrane proteins in the central nervous system contain endogenous Zn^{2+}-binding sites, such as ionotropic

glutamate receptors, some GABA receptor subtypes and the strychnine-sensitive glycine receptor (Peters *et al*., 1987; Westbrook and Mayer, 1987; Hollmann *et al*., 1993; Bloomenthal *et al*., 1994; Laube *et al*., 1995). To date, histidine residues coordinating Zn^{2+} binding have been identified only in the GABA ρ1 subunit (T.L.Wang *et al*., 1995) and the GABA β1 subunit (Horenstein and Akabas, 1998); otherwise, residues coordinating Zn^{2+} binding in these receptor proteins have, to our knowledge, not yet been identified. Interestingly, artificial Zn^{2+}-binding sites have recently been generated in other families of membrane proteins and have been shown to be highly useful for probing structure–function relationships (Elling *et al*., 1995; He *et al*., 1995; Elling and Schwartz, 1996; Sheikh *et al*., 1996).

From crystal structures of Zn^{2+}-binding proteins, it is known that the average distance between the zinc(II) ion and the coordinating nitrogen of a histidine residue averages 2 Å (Glusker, 1991). Hence, the identification of two histidines involved in Zn^{2+} binding suggests that His193 is in close proximity to His375, imposing an important distance constraint in the unknown tertiary structure of the hDAT (Glusker, 1991). This represents, to our knowledge, the first experimentally supported proximity relationship in an Na^+/Cl^--dependent transporter. According to the two-dimensional model of hDAT shown in Figure 1, we would predict that His375 is located at the N-terminus of ECL4 (top of TM VII) and His193 is ~26 residues from the top of TM III. Nearly half of the 26 residues between His193 and the top of TM III are believed to form a loop because of the presumed disulfide bridge formed by Cys180–Cys189. This significantly constrains the possible conformational space for His193.

Both Zn^{2+} inhibition of dopamine uptake and the effect of Zn^{2+} on [^{3}H]WIN 35,428 binding were biphasic (Figure 2). Interestingly, biphasic responses to Zn^{2+} have also been observed at the strychnine-sensitive glycine receptor with potentiation of the glycine-induced Cl^- current by micromolar concentrations and inhibition by millimolar concentrations of Zn^{2+} (Bloomenthal *et al*., 1994). The data were proposed to result from the interaction of Zn^{2+} with two distinct metal ion-binding sites (Bloomenthal *et al*., 1994). In parallel, it is likely that the inhibitory effects of Zn^{2+} in millimolar concentrations at both hNET and hDAT are due to interaction with a site different from that involved in the effects observed at micromolar concentrations of Zn^{2+} in hDAT. It should also be considered, however, that the inhibitory effects of Zn^{2+} in millimolar concentrations may be non-specific, caused by the non-physiological toxic concentrations of Zn^{2+} (Frederickson, 1989).

Our data provide clear evidence that the effects of Zn^{2+} in micromolar (and physiological) concentrations are due to coordination of Zn^{2+} to at least two histidines in the hDAT. However, Zn^{2+} concentrations that would be expected to saturate this apparent high-affinity Zn^{2+}-binding site (~100 μM) only cause 60–70% inhibition of dopamine uptake (Figure 2). One explanation could be that the transporter can still translocate substrate even when Zn^{2+} is bound, but only at reduced efficacy (30–40% of normal efficacy). Alternatively, it may reflect the existence of two conformational states of the transporter with distinct Zn^{2+} sensitivity. We cannot distinguish between the two hypotheses; however, they are both consistent with the major implication from this study, that Zn^{2+} non-competitively inhibits substrate translocation by maintaining His193 in ECL2 and His375 in ECL4 at the top of TM VII in close spatial proximity. In other words, our data suggest that by restraining relative movements of ECL2/ECL4, Zn^{2+} may inhibit a conformational change critical for the translocation process. Interestingly, a critical role for ECL2 in the substrate translocation was proposed recently based on a chimeric exchange of a part of the ECL2 loop between the serotonin transporter (SERT) and the NET (Stephan *et al*., 1997).

Even though our data are obtained *in vitro*, it is tempting to suggest that Zn^{2+} is a modulator of dopamine transporter activity *in vivo*. This is consistent with the increasing amount of evidence pointing to Zn^{2+} as a neuromodulator (Huang, 1997). It is highly likely that free Zn^{2+} is present in the synaptic cleft in concentrations required to modulate the function of the hDAT. The basal concentration of Zn^{2+} in the brain is ~10 nM (Frederickson *et al*., 1984; Frederickson, 1989); however, upon neuronal stimulation, Zn^{2+} has been shown to be released in concentrations of ~300 μM (Assaf and Chung, 1984). Intriguingly, free Zn^{2+} has been localized in synaptic vesicles and, recently, a Zn^{2+} transporter (ZnT-3) that sequesters Zn^{2+} into synaptic vesicles has been cloned (Palmiter *et al*., 1996; Wenzel *et al*., 1997). Moreover, it is interesting to note that Zn^{2+} levels in patients with Parkinson's disease are significantly elevated in substantia nigra, caudate nucleus and lateral putamen (Dexter *et al*., 1989). Nevertheless, further studies are required to elucidate the physiological and/or pathophysiological relevance of the endogenous Zn^{2+} site in hDAT.

In summary, we have defined two coordinating residues in an endogenous Zn^{2+}-binding site in hDAT and have thereby mapped an important distance constraint in the tertiary structure of a Na^+/Cl^--dependent transporter. Identification of further coordinates in the Zn^{2+}-binding site may add additional distance constraints that all together may prove an important scaffold for designing new artificial Zn^{2+} sites. Hence, Zn^{2+} site engineering may represent a significant new means for depicting the tertiary organization of this class of transporters.

Materials and methods

Site-directed mutagenesis

The cDNAs encoding hDAT and hNET were kindly provided by Dr Marc G.Caron (Duke University, Durham, NC). Mutant transporters were constructed by PCR-derived mutagenesis using *Pfu* polymerase according to the manufacturer's instructions (Stratagene, La Jolla, CA). The generated PCR fragments were digested with the appropriate enzymes, purified by agarose gel electrophoresis and cloned into the eukaryotic expression vector pRc/CMV, containing either hDAT or hNET (Pifl *et al*., 1993; Giros *et al*., 1994). All mutations were confirmed by restriction enzyme mapping and DNA sequencing using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP on an Alfexpress™ DNA sequencer according to the manufacturer's instructions.

Expression of transporters in COS-7 cells

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) 041-31885 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 0.01 mg/ml gentamicin. Wild-type and mutant constructs in pRc/CMV were transiently transfected into COS-7 cells by the calcium phosphate precipitation method as previously described (Johansen *et al*., 1990; Gether *et al*., 1992).

[^{3}H]Dopamine uptake experiments

Uptake assays were performed modified from Giros *et al*. (1992) using 2,5,6-[^{3}H]dopamine (7–21 Ci/mmol) (Amersham, Little Chalfont, UK). Transfected COS-7 cells were plated in either 24-well dishes (10^5 cells/well) or 12-well dishes ($2–3\times10^5$ cells/well) to achieve an uptake level of 5–10% of total added [^{3}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 [^{3}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 of 1% SDS and left for 1 h at 37°C. All samples were counted by liquid scintillation counting. Non-specific uptake was determined in the presence of 1 mM dopamine (RBI, Natick, MA). All determinations were performed in triplicate.

[^{3}H]WIN 35,428 binding experiments

Binding assays were carried out on whole cells using [^{3}H]WIN 35,428 (83.5 Ci/mmol) (Dupont-NEN, Boston, MA) as radioligand. One day after transfection, cells were seeded in 12-well dishes ($2–3\times10^5$ cells/well) or in 6-well dishes (5×10^5 cells/well) to acheive a binding level of 5–10% of total added [^{3}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 nM [^{3}H]WIN 35,428 and the 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 of 1% SDS for 1 h at 37°C. All samples were counted by liquid scintillation counting. Non-specific binding was determined in the presence of 10 µM WIN 35,428 (RBI, Natick, MA). Determinations were made in duplicate or triplicate. Saturation binding experiments were performed as above with increasing concentrations (2–150 nM) of 1:4-diluted [^{3}H]WIN 35,428 in the absence or presence of 10 µM Zn^{2+}.

Calculations

Uptake and binding data were analysed by non-linear regression analysis using Inplot 4.0 or Prism 2.0 from GraphPad Software, San Diego, CA.

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