

# Probing dopamine transporter structure and function by $\text{Zn}^{2+}$ -site engineering

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## Abstract

The biogenic amine transporters belong to the class of  $\text{Na}^+/\text{Cl}^-$ -coupled solute carriers and include the transporters for dopamine (DAT), norepinephrine (NET), and serotonin (SERT). These transporters are the primary targets for the action of many psychoactive compounds including the most commonly used antidepressants as well as widely abused drugs such as cocaine and amphetamines. In spite of their pharmacological importance, still little is known about their higher structural organization and the molecular mechanisms underlying the substrate translocation process. In this review, it will be described how we have used  $\text{Zn}^{2+}$ -binding sites as a tool to probe the structure and function of  $\text{Na}^+/\text{Cl}^-$ -coupled biogenic amine transporters with specific focus on the human DAT (hDAT). The work has not only led to the definition of the first structural constraints in the tertiary structure of this class of transporters, but also allowed inferences about conformational changes accompanying substrate translocation and residues critical for regulating the equilibrium between different functional states in the transport cycle.

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## 1. Introduction

The availability in the synaptic cleft of dopamine, serotonin, and norepinephrine (referred to as the biogenic amines) is tightly regulated by specific transmembrane transport proteins that mediate rapid reuptake of the released transmitter and thereby terminate their effect at pre- and postsynaptic receptors. These transport proteins belong to the class of  $\text{Na}^+/\text{Cl}^-$ -coupled solute carriers and include the transporters for dopamine (DAT) (Fig. 1), norepinephrine (NET), and serotonin (SERT). The biogenic amine transporters have received particular attention because they are targets for the action of several drugs. This includes both the most commonly used antidepressants and widely abused psychostimulants, such as cocaine, amphetamine, and 'ecstasy' (Norregaard and Gether, 2001; Torres et al., 2003).

Intensive research efforts in recent years have provided significant new insight into the molecular organization of the biogenic amine transporters and how these proteins

operate at the molecular level (Norregaard and Gether, 2001). A high-resolution structure is, however, still not available for a  $\text{Na}^+/\text{Cl}^-$ -dependent transporter or for any other transport protein utilizing a transmembrane ion gradient as the driving force for the transport process (secondary active transporters). It is of interest that low-resolution three-dimensional structures have now been published for the bacterial  $\text{Na}^+/\text{H}^+$ -antiporter NhaA (Williams, 2000; Williams et al., 1999) and of the bacterial oxalate transporter OxlT based on cryo-electron microscopy of two-dimensional crystals (Heymann et al., 2001; Hirai et al., 2002). These structures have provided the first information concerning the higher structural organization of 12 transmembrane secondary active transport proteins, but to what degree the structure of these very different proteins may resemble that of the  $\text{Na}^+/\text{Cl}^-$ -dependent transporters to which they share no sequence homology remains elusive.

In the absence of a high-resolution structure, a broad spectrum of non-crystallographic strategies have proven highly useful for obtaining insight into structure/function relationships in the family of  $\text{Na}^+/\text{Cl}^-$ -dependent neurotransmitter transporters (for review, see Norregaard and

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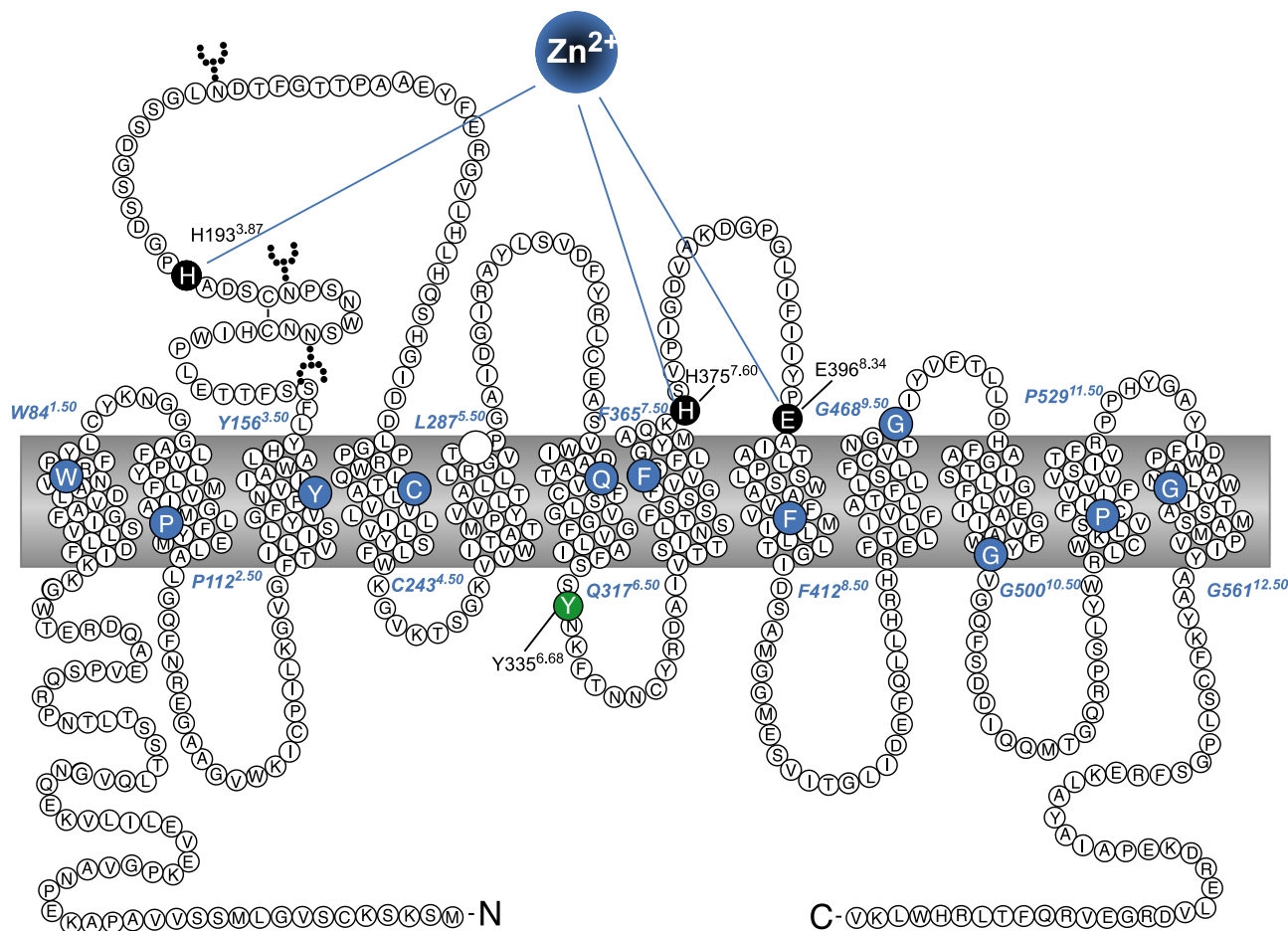


Fig. 1. Schematic representation of the human dopamine transporter. The large second extracellular loop contains three sites for N-linked glycosylation and two cysteines that are conserved among all biogenic amine transporters and believed to form a disulfide bridge (Chen et al., 1997; Wang et al., 1995; Sur et al., 1997). The three residues coordinating  $\text{Zn}^{2+}$  binding to the hDAT wild type are highlighted (enlarged black circles) (Norregaard et al., 1998; Loland et al., 1999). The most conserved residue in each transmembrane segment is also highlighted (grey circles). To generate a generic numbering scheme allowing direct comparison of positions between the individual members of the transporter family, these residues have arbitrarily been assigned by the number of the helix and the number 50. Other residues are then numbered according to their position relative to this conserved residue. For example, 1.55 indicates a residue in TM1 five residues carboxyterminal to the most conserved residue in this TM (Trp1.50). Throughout the chapter, all residues are indicated both by their actual amino acid number in the transporter and in superscript by this generic numbering scheme (Goldberg et al., 2003).

Gether, 2001; Chen and Reith, 2000; Torres et al., 2003). In this review, it will be described how we have utilized endogenous and engineered  $\text{Zn}^{2+}$ -binding sites to explore the structure and molecular function of  $\text{Na}^+/\text{Cl}^-$ -dependent neurotransmitter transporters. The work has not only allowed the definition of the first structural constraints in the tertiary structure of this class of transporters but has also provided new insights into both conformational changes accompanying substrate translocation and mechanisms governing conformational isomerization in the translocation cycle.

## 2. The use of $\text{Zn}^{2+}$ as a tool for probing structure/function relationships

$\text{Zn}^{2+}$ -binding sites are found in numerous soluble proteins including in particular enzymes and DNA binding

transcription factors (Frederickson, 1989; Vallee and Falchuk, 1993; Alberts et al., 1998; Auld, 2001). In enzymes,  $\text{Zn}^{2+}$  may either have a direct catalytic role (*catalytic sites*) or  $\text{Zn}^{2+}$  may stabilize the active site structure of the enzyme (*structural sites*) (Auld, 2001). In DNA binding transcription factors,  $\text{Zn}^{2+}$  purely serves a structural role stabilizing the geometry of the so-called  $\text{Zn}^{2+}$  finger motif (Frederickson, 1989; Alberts et al., 1998).

The availability of the three-dimensional structure of now around 200 soluble  $\text{Zn}^{2+}$ -binding proteins has provided profound insight into the structural basis for the interaction of  $\text{Zn}^{2+}$  with polypeptides and defined the strict structural constraints for coordination of the zinc(II) ion (Alberts et al., 1998; Auld, 2001). The physical properties of the small  $\text{Zn}^{2+}$  make it highly useful as a tool for investigation of structure/function relationships in various proteins. As for other metal ions, the residues that can act as ligands for  $\text{Zn}^{2+}$  are those that contain electron-donating atoms (S, O or

N) or have an amino acid side chain with ionizable groups (Schwabe and Klug, 1994). Although this includes serine, tyrosine, arginine and lysine, the strongest interactions are with the imidazole side chain of histidine, the sulfhydryl side chain of cysteine and the carboxylate side chains of glutamate and aspartate (Schwabe and Klug, 1994).

The predominant coordination geometry found among  $\text{Zn}^{2+}$ -binding sites in soluble proteins is tetrahedral (Alberts et al., 1998; Auld, 2001). For catalytic sites, this involves the side chains of three residues plus a water molecule with histidine being the most abundant ligand and only occasional involvement of cysteines, aspartates and glutamates (Alberts et al., 1998; Auld, 2001). More infrequently, the  $\text{Zn}^{2+}$ -binding sites may have five or six coordinates involving a variable number of water molecules (Auld, 2001). The sites having five coordinates adopt most often a trigonal bipyramidal geometry and less often a square-based pyramidal geometry, whereas sites with six ligands are found to adopt octahedral geometry (Auld, 2001). The average distance between the coordinating ligand and  $\text{Zn}^{2+}$  is in the range of 2.0–2.3 Å (Alberts et al., 1998). Accordingly, two residues involved in coordinating the same zinc(II) ion must be in close proximity in the tertiary structure of the protein with the  $\alpha$ -carbons being approximately 13 Å apart (Alberts et al., 1998).

The crystal structures of  $\text{Zn}^{2+}$ -binding proteins did not only reveal well-defined tertiary structure constraints to accommodate  $\text{Zn}^{2+}$  binding but also well-defined constraints at the secondary structure level. For example, coordination of  $\text{Zn}^{2+}$  between two histidines that are located in the same  $\alpha$ -helix requires that the two histidines are positioned as  $i$  and  $i-4$  with  $i$  assuming the *gauche*-rotamer and  $i-4$  the *trans*-rotamer (Alberts et al., 1998; Norregaard et al., 2000). If the two residues instead are positioned as either  $i$  and  $i-3$  or  $i$  and  $i-5$ , binding of  $\text{Zn}^{2+}$  will involve a substantial distortion of the helix (Alberts et al., 1998; Norregaard et al., 2000). A similar strict pattern was observed for pairs of  $\text{Zn}^{2+}$ -binding histidines in  $\beta$ -strands. Two histidines within a  $\beta$ -strand must be positioned as  $i$  and  $i-2$  to bind  $\text{Zn}^{2+}$  without distortion of the strand (Alberts et al., 1998; Norregaard et al., 2000). As described later in this review, this information may allow useful inferences about secondary and tertiary structure relationships from endogenous and engineered  $\text{Zn}^{2+}$ -binding sites.

Due to the well-defined structural constraints,  $\text{Zn}^{2+}$ -binding sites have been artificially introduced into proteins in many cases (Berg and Shi, 1996).  $\text{Zn}^{2+}$  is particularly well-suited for this purpose: Divalent zinc is in contrast to, e.g.  $\text{Cu}^{2+}$  not redox active; neither the potential oxidized form,  $\text{Zn}^{3+}$ , nor the potential reduced form,  $\text{Zn}^+$ , is accessible under physiologic conditions. Also,  $\text{Zn}^{2+}$  is relatively labile in kinetic terms meaning that it undergoes ligand exchange reactions relatively rapidly (Berg and Shi, 1996).  $\text{Zn}^{2+}$ -binding sites have been artificially introduced into proteins to assist their purification, crystallization and X-ray

crystal structure determination (Berg and Shi, 1996). Moreover,  $\text{Zn}^{2+}$ -binding sites have been artificially introduced into receptor and transporter proteins with the purpose of defining structural constraints in the secondary, tertiary and quaternary structure of these proteins (Norregaard et al., 2000; Loland et al., 1999, 2002; Elling et al., 1995, 1997; Elling and Schwartz, 1996; Thirstrup et al., 1996; Voss et al., 1995a,b; Ballesteros et al., 2001; MacAulay et al., 2001; Norgaard-Nielsen et al., 2002).

The simple principle of this ' $\text{Zn}^{2+}$ -site engineering' approach is based on the ability of properly oriented  $\text{Zn}^{2+}$  coordinating residues to chelate  $\text{Zn}^{2+}$  in low-micromolar concentrations. As  $\text{Zn}^{2+}$  ligands, histidine should in most cases be preferred over cysteine, aspartate and glutamate. Even though cysteines can bind  $\text{Zn}^{2+}$  with high affinity, they have several disadvantages: The presence of a free sulfhydryl might interfere with protein folding due to spontaneous formation of disulfide bridges between a pair of inserted cysteines. The oxidation state of cysteine may be difficult to control in the presence of metal ions. Histidines should be preferred over aspartates and glutamates simply due to the higher affinity of  $\text{Zn}^{2+}$  for the imidazol side chain.

To achieve  $\text{Zn}^{2+}$  binding and accordingly generate a successful  $\text{Zn}^{2+}$ -binding site between inserted histidines, several criteria must be satisfied: As mentioned, the distances between the  $\alpha$ -carbons of the substituted side chains should be less than 13 Å to allow the corresponding imidazole side chains to bind  $\text{Zn}^{2+}$ . In addition, the residues substituted must be exposed to the aqueous environment to allow  $\text{Zn}^{2+}$  binding. Finally, binding of  $\text{Zn}^{2+}$  must be measurable. In membrane proteins that cannot be purified in sufficiently large quantities to allow direct structural analyses, the only applicable way of detecting  $\text{Zn}^{2+}$  binding is indirectly via a putative effect of  $\text{Zn}^{2+}$  on the function of the protein. In the biogenic amine transporters, we have accomplished this by assessing  $\text{Zn}^{2+}$ -induced inhibition of substrate uptake, impairment of ion currents or changes in the binding properties of transport inhibitors (Loland et al., 1999, 2002; MacAulay et al., 2001; Norregaard et al., 1998, 2000; Norgaard-Nielsen et al., 2002).

### 3. Structural inferences from $\text{Zn}^{2+}$ -binding sites on the dopamine transporter

By investigating the susceptibility of the unmodified human dopamine transporter (hDAT) and the homologous human norepinephrine transporter (hNET) to  $\text{Zn}^{2+}$ , we obtained evidence that the hDAT but not the hNET contains an endogenous high-affinity  $\text{Zn}^{2+}$ -binding site (Norregaard et al., 1998). As illustrated in Fig. 2A, it was found that  $\text{Zn}^{2+}$  in micromolar concentrations is a potent inhibitor of [ $^3\text{H}$ ]dopamine uptake in hDAT. This effect was not observed in the hNET (Norregaard et al., 1998). The inhibition in hDAT was biphasic with an  $\text{IC}_{50}$  value for the high-affinity

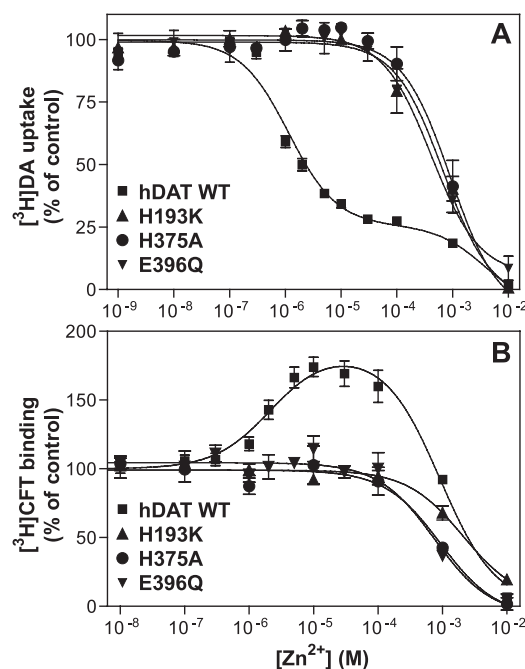


Fig. 2. Evidence for an endogenous Zn<sup>2+</sup>-binding site in the human dopamine transporter. (A) Zn<sup>2+</sup> potentially inhibits [<sup>3</sup>H]dopamine uptake in COS-7 cells transiently expressing hDAT wild type (■) in a biphasic manner with a high-affinity component around (EC<sub>50</sub> = 1 μM) and a low-affinity component. Mutation of either His193<sup>3,87</sup> (hDAT H193K (▲)), His375<sup>7,60</sup> (hDAT H375A (●)) or Glu396<sup>8,34</sup> (hDAT E396Q (▼)) abolishes the high-affinity component, suggesting their common participation in the endogenous Zn<sup>2+</sup>-binding site (Loland et al., 1999; Norregaard et al., 1998). (B) Mutation of either hDAT H193K (▲), hDAT H375A (●) or hDAT E396Q (▼) eliminates the Zn<sup>2+</sup>-induced potentiation of [<sup>3</sup>H]CFT (2β-carbomethoxy-3β-(4-fluorophenyl)tropane) binding observed in the hDAT wild type (■). Reproduced from Loland et al. (1999) and Norregaard et al. (1998).

phase of ~1 and >1000 μM for the low affinity phase (Norregaard et al., 1998). In the hNET, only low affinity inhibition was observed. The low affinity inhibition by Zn<sup>2+</sup> at millimolar concentrations most likely represents nonspecific toxic effects of Zn<sup>2+</sup>; however, the high-affinity inhibition in hDAT conceivable reflected interaction of Zn<sup>2+</sup> with specific site within the hDAT (Norregaard et al., 1998). The presence of an endogenous Zn<sup>2+</sup>-binding site in the hDAT was further supported by the observation that Zn<sup>2+</sup> in micromolar concentrations potentiated binding of the cocaine analog [<sup>3</sup>H]CFT (2β-carbomethoxy-3β-(4-fluorophenyl)tropane or WIN 35,428) to the hDAT (Fig. 2B) but not the hNET (Norregaard et al., 1998).

Pharmacologic characterization by uptake experiments in the presence and absence of micromolar concentrations of Zn<sup>2+</sup> suggested that dopamine can bind to the Zn<sup>2+</sup>-occupied transporter but that translocation is blocked (Norregaard et al., 1998). The most likely explanation for this is that Zn<sup>2+</sup> restricts—but not completely prevents—movements critical for dopamine transport causing the transporter to translocate at a lower efficacy. Another possibility that we cannot exclude is that in the presence of Zn<sup>2+</sup>, the translo-

cation process occurs at unchanged rate but that dopamine is less likely to be released to the intracellular environment, thus, Zn<sup>2+</sup> apparently increases the chance that the transporter reorients from its putative inward facing conformation to its outward facing conformation with the dopamine still bound.

The structural basis for the high-affinity interaction with hDAT was investigated by application of a systematic mutagenesis approach aimed at identifying Zn<sup>2+</sup> coordinating residues within the transporter molecule. Since the hNET in contrast to hDAT was insensitive to Zn<sup>2+</sup> at least one coordinating residue could be expected to be non-conserved between the hDAT and the hNET. Moreover, since Zn<sup>2+</sup> is unlikely to penetrate the plasma membrane, it would be expected that the coordinating residues would be on the extracellular face of the transporter molecule. Indeed, a systematic knock-out of all non-conserved histidine residues at the extracellular side of the hDAT did identify a histidine (His193<sup>3,87</sup>)<sup>1</sup> in the second extracellular loop (ECL2) (Fig. 1) that upon mutation to lysine (the corresponding residue in hNET) eliminated high-affinity Zn<sup>2+</sup> susceptibility and the potentiated binding of [<sup>3</sup>H]CFT (Fig. 2) (Norregaard et al., 1998). The involvement of His193<sup>3,87</sup> was further supported by the fact that if a histidine was inserted into the corresponding position in the hNET (hNET K189H), hDAT-like Zn<sup>2+</sup> susceptibility was transferred to this transporter (Norregaard et al., 1998). This observation also gave the important information that the remaining coordinate(s) should be found among residues conserved between hDAT and hNET. In full agreement with this prediction, subsequent systematic mutation of all the conserved extracellular histidines identified an additional coordinating residue (His375<sup>7,60</sup> at the top of transmembrane segment (TM)7, Fig. 1) that upon mutation into an alanine revealed the same dramatic decrease in Zn<sup>2+</sup> susceptibility as observed for the H193K mutation (Fig. 2A) (Norregaard et al., 1998). In the search of a third coordinate, subsequent mutation of multiple cysteines predicted to be accessible from the extracellular side did not indicate their participation in Zn<sup>2+</sup> binding (Norregaard, Ferrer, Javitch and Gether, unpublished observation); however, a mutational screen of all extracellular aspartates and glutamates identified a glutamate at the extracellular end of TM8 at position 396<sup>8,34</sup> (Fig. 1) that upon mutation to alanine revealed the same phenotype as that observed for H193K and H375A and thus likely represented a third Zn<sup>2+</sup>-coordinating residue (Fig. 2) (Loland et al., 1999). The involvement of both His375<sup>7,60</sup> and Glu396<sup>8,34</sup> in Zn<sup>2+</sup> binding was additionally supported by the possibility of substituting either of the residues with a

<sup>1</sup> All residues are indicated in superscript by the generic numbering scheme described by Goldberg et al. (2003). According to this scheme the most conserved residue in each transmembrane segment has been given the number 50, and each residue is numbered according to its position relative to this conserved residue. For example, 1.55 indicates a residue in TM1 five residues carboxyterminal from the most conserved residue (Trp1.50).



cysteine (Norregaard et al., 2003). Moreover,  $\text{Zn}^{2+}$  inhibition of [ $^3\text{H}$ ]dopamine uptake could be eliminated in the cysteine mutants upon reaction with the positively charged sulfhydryl-reactive compound [2-(trimethylammonium)ethyl]-methanethiosulfonate (MTSET) (Norregaard et al., 2003). In addition to support the involvement of the residues in  $\text{Zn}^{2+}$  binding, this observation supports the notion that  $\text{Zn}^{2+}$  inhibition of uptake is due to the ability of  $\text{Zn}^{2+}$  to diminish possible movements of TM7 and TM8 relative to one another rather than due to a change in local electrostatic potential caused by the presence of a positive charge (Norregaard et al., 2003).

The identification of the three residues involved in  $\text{Zn}^{2+}$  binding to the endogenous  $\text{Zn}^{2+}$ -binding site has defined the first set of distance constraints in the tertiary structure of the hDAT. According to the hydrophobicity plot, His375<sup>7.60</sup> and Glu396<sup>8.34</sup> are predicted to be located right at the extracellular end of TM7 and TM8, respectively (Fig. 1). They are separated with a large loop of 20 residues, allowing TM7 and TM8 to be rather far apart in the tertiary structure. However, their common participation in the  $\text{Zn}^{2+}$ -site outlines the close association between the two transmembrane segments. Moreover, the participation of His193<sup>3.87</sup> in binding of  $\text{Zn}^{2+}$  to the hDAT outlines the association of the outer portion of TM7 and TM8 with the large extracellular loop connecting TM3 and TM4.

To further explore this spatial proximity between TM7 and TM8, a series of engineered  $\text{Zn}^{2+}$ -binding sites were established (Loland et al., 1999; Norregaard et al., 2000). If Glu396<sup>8.34</sup> is situated in an  $\alpha$ -helical environment, it could be expected that His375<sup>7.60</sup> was also close to the residue situated one helical turn from Glu396<sup>8.34</sup>, i.e. the residues in the  $i+4$  or  $i+3$  positions, while the  $i+2$  position would be expected to be located on the other side of the helix. Accordingly, three mutant transporters were generated in which His193<sup>3.87</sup> and Glu396<sup>8.34</sup> were removed, His375<sup>7.60</sup> preserved and cysteine residues inserted in positions  $i+2$ ,  $i+3$ , and  $i+4$  relative to position 396<sup>8.34</sup> in TM8 (Loland et al., 1999). The insertion of cysteines was chosen in favor of the stronger binding histidines because these residues are smaller and therefore likely better tolerated in the transmembrane domains where space can be limited. Indeed,  $\text{Zn}^{2+}$  could inhibit [ $^3\text{H}$ ]dopamine uptake when a cysteine was inserted in position 400<sup>8.38</sup> ( $i+4$ ) displaying an  $\text{IC}_{50}$  value of 24  $\mu\text{M}$  as compared to 660  $\mu\text{M}$  for the background mutant (hDAT H193K-E396Q) (Loland et al., 1999). By probing cysteine residues in TM8 against His375<sup>7.60</sup> in TM7, our results strongly support that the top of TM8 adopts an  $\alpha$ -helical configuration.

In another set of experiments, the secondary structure at the external end of TM7 was probed by engineering sites with two coordinating residues within the same hypothetical helix. It was attempted specifically to take advantage of the finding described in the previous section that coordination of  $\text{Zn}^{2+}$  between two histidines located in an  $\alpha$ -helix requires that the two histidines are positioned as  $i$  and  $i-4$

position (Norregaard et al., 2000). First, His193<sup>3.87</sup> in ECL 2 was changed to lysine to create a  $\text{Zn}^{2+}$  insensitive background (hDAT H193K). In this construct, a histidine was inserted in  $i-4$  position relative to His375<sup>7.60</sup> (H193K-M371H).  $\text{Zn}^{2+}$  was a potent inhibitor of [ $^3\text{H}$ ]dopamine uptake in this mutant, indicating coordination of  $\text{Zn}^{2+}$  between His375<sup>7.60</sup>, Glu396<sup>8.34</sup> and M371H and thus supporting an  $\alpha$ -helical configuration in the top of TM7 (Norregaard et al., 2000). This was further corroborated by the observation that no increase in the apparent  $\text{Zn}^{2+}$  affinity was found upon introduction of histidines at positions  $i-2$ ,  $i-3$  and  $i-5$  relative to His375<sup>7.60</sup>. Interestingly, a different pattern was observed when histidines were introduced at positions  $i+2$  (V377H),  $i+3$  (P378H),  $i+4$  (I379H), and  $i+5$  (G380H) relative to His375<sup>7.60</sup>. Thus, a marked increase in the apparent  $\text{Zn}^{2+}$  affinity was observed by introducing a histidine not only at the  $i+4$  position from His375<sup>7.60</sup> but also at the  $i+2$  and  $i+3$  positions (Norregaard et al., 2000). These data are *not* consistent with an  $\alpha$ -helical configuration and indicate the absence of a well-defined secondary structure between residue 375 and 379. Thus, the data suggest an approximate boundary between the end of the TM7 helix and the succeeding loop around position 375 (Norregaard et al., 2000).

Altogether, our identification of the coordinating residues in the endogenous hDAT  $\text{Zn}^{2+}$ -binding site followed by the engineering of several artificial sites did not only define a series of proximity relationships in the tertiary structure but also secondary structure relationships. Moreover, the data provided information about the orientation of TM7 relative to TM8. The structural inferences from our observations were used to develop the molecular model shown in Fig. 3 of the 'TM7/8 microdomain' (Norregaard et al., 2000). The model is an important example of how structural insight obtained by ' $\text{Zn}^{2+}$ -site engineering' can provide sufficient information for at least an initial structural mapping of a selected protein domain.

The fact that the endogenous  $\text{Zn}^{2+}$ -binding site in hDAT could be transferred to the homologous hNET (Norregaard et al., 1998) suggests the possibility that  $\text{Zn}^{2+}$ -site engineering also can be used as an 'evolutionary fingerprint', meaning that if a  $\text{Zn}^{2+}$ -binding site can be transferred from one protein to another by mutation of the corresponding residues, it provides strong support that the two proteins adopt a similar tertiary structure even if their primary amino acid sequences are different. This hypothesis was further supported by our ability to transfer two engineered  $\text{Zn}^{2+}$ -sites from the hDAT to the rat  $\gamma$ -amino butyric acid (GABA) transporter, rGAT-1 (MacAulay et al., 2001). Introduction of a histidine in position 349<sup>7.60</sup> (position 375 in hDAT) together with either a histidine in position 370<sup>8.34</sup> (position 396 in hDAT) or a cysteine in position 374<sup>8.38</sup> (position 400 in hDAT) resulted in bidentate  $\text{Zn}^{2+}$ -binding sites where  $\text{Zn}^{2+}$  binding lead to potent inhibition of [ $^3\text{H}$ ]GABA uptake (MacAulay et al., 2001).

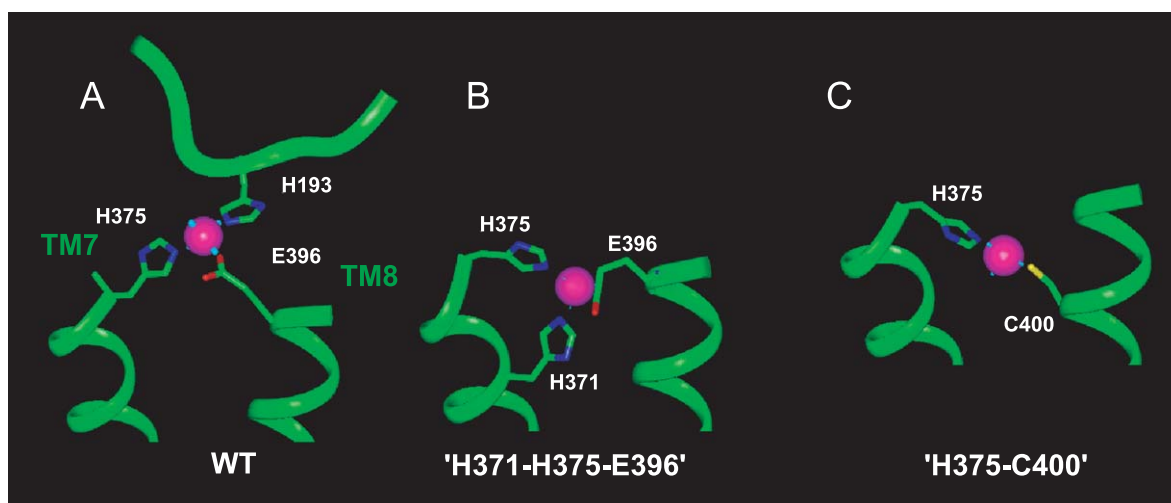


Fig. 3. Molecular Model of the TM 7/8 microdomain. The model incorporates the constraints defined by data achieved in Loland et al. (1999), and Norregaard et al. (1998, 2000). Three different  $\text{Zn}^{2+}$ -binding sites were included in the modeling procedure. (A) The endogenous  $\text{Zn}^{2+}$ -binding site in hDAT (His193<sup>3.87</sup>–His375<sup>7.60</sup>–Glu396<sup>8.34</sup>) (Loland et al., 1999; Norregaard et al., 1998, 2000); (B), the engineered site His375<sup>7.60</sup>–Cys400<sup>8.38</sup> (Loland et al., 1999; Norregaard et al., 1998, 2000); (C), the engineered site His371<sup>7.54</sup>–His375<sup>7.60</sup>–Glu396<sup>8.34</sup> (Loland et al., 1999; Norregaard et al., 1998, 2000). The three  $\text{Zn}^{2+}$ -binding sites were all modeled assuming tetrahedral coordination geometry. Fragments Ile347<sup>7.32</sup>–His375<sup>7.60</sup> (TM7) and Glu396<sup>8.34</sup>–Leu401<sup>8.39</sup> (TM8) were modeled as two ideal anti-parallel  $\alpha$ -helices with a predicted crossing angle of 161.3° between the axes and a distance between the  $\alpha$ -carbon of His375<sup>7.60</sup> and Glu396<sup>8.34</sup> of 10.4 Å (Norregaard et al., 2000). This relative backbone orientation of TM7 and TM8 satisfies all three  $\text{Zn}^{2+}$ -binding sites through side chain rotations alone without changes in the position of the two backbones. Reproduced from Norregaard et al. (2000).

Electrophysiological analysis of these rGAT-1 mutants upon expression in *Xenopus laevis* oocytes showed that the inhibition of uptake was accompanied by a corresponding inhibition of the substrate-induced current (MacAulay et al., 2001). Interestingly, analysis of the uncoupled  $\text{Li}^+$  conductance found in the rGAT-1 showed that  $\text{Zn}^{2+}$  strongly inhibited the leak conductance in the rGAT-1 T349H-E370H mutant whereas no inhibition by  $\text{Zn}^{2+}$  was observed in rGAT-1 T349H-Q374C (MacAulay et al., 2001). This differential effect provided strong evidence that the leak conductance is a unique operational mode of the transporter involving conformational changes distinct from those associated with substrate translocation (MacAulay et al., 2001). Moreover, it reflects yet another example of how engineering of  $\text{Zn}^{2+}$ -binding sites can be helpful for the dissection of not only structural relationships but also specific functional mechanisms in membrane proteins.

#### 4. Intracellular residues critical for regulating transporter conformation and cocaine binding

The mechanism by which the biogenic amine transporters couple movement of substrate with ion movement has been the subject of much speculation though little experimental verification. Most workers have embraced the model first proposed by Jardetzky (1966) that suggests the binding site to be alternate accessible to the two membrane surfaces. The model predicts that the transporter, in the presence of  $\text{Na}^+$  but in the absence of substrate, primarily resides in the outward facing conformation, ready

to bind extracellular substrate. Upon binding of substrate, the transporter has the capacity to change conformation and initiates translocation causing transition of the transporter to the inward facing conformation followed by release of substrate and  $\text{Na}^+$  to the intracellular environment. According to this model, an impermeable barrier exists at all times between the binding site and one side of the membrane, but the barrier can change from one side of the binding site to the other, giving the site alternate access to the two aqueous compartments that the membrane separates (Jardetzky, 1966; Mitchell, 1990; Rudnick, 1997; Veenhoff et al., 2002). For effective coupling between substrate and  $\text{Na}^+$  translocation, the changes in conformation performed by the transporter must occur only under specific conditions. For example, when the DAT requires dopamine,  $\text{Na}^+$ , and  $\text{Cl}^-$  for transport, the change in conformation between the external access and internal access to the dopamine binding site must only take place when both dopamine and ions are bound to their respective sites. Following dissociation of the solutes to the cytoplasm, the reverse action could take place rendering the empty binding site for dopamine exposed to the external environment. A prerequisite for this model is that the transporter holds external and internal ‘gates’, i.e. protein domains that are capable of occluding access to the binding site of substrate from the external and internal domains, respectively. Little is known about such domains in this family of transporters as well as the molecular mechanisms governing the cooperative function of the putative gating domains. However, It would be predicted that stabilization of the transporter in the outward facing conformation requires a network of constraining intramo-

lecular interactions, possibly in the gating domains themselves, which is released upon substrate binding to the transporter and thus controls the conformational equilibrium of the translocation cycle. It follows that mutation of residues that are part of this stabilizing network of intramolecular interactions may cause spontaneous changes in the distribution between different conformational states in the translocation cycle.

We have recently identified a tyrosine in the third intracellular loop, Tyr335<sup>6,68</sup>, which is conserved throughout the entire family of Na<sup>+</sup>-coupled transporters from bacteria to mammals, that might be part of such a network of constraining intramolecular interactions. This conclusion was based on the observation that mutation of this tyrosine completely altered the effect of Zn<sup>2+</sup> at the endogenous Zn<sup>2+</sup>-binding site in the hDAT from potent inhibition of transport to potent stimulation of transport (Fig. 4). In the absence of Zn<sup>2+</sup>, transport capacity was reduced to less than 1% of that observed for the wild type, however, the presence of Zn<sup>2+</sup> in only micromolar concentrations caused a close to 30-fold increase in uptake (Loland et al., 2002). We inferred from these data that the mutation of Tyr335<sup>6,68</sup> to an alanine led to disruption of intramolecular interactions critical for stabilizing the transporter in a conformation in which extracellular substrate can bind and initiate transport. As a consequence, the transporter might accumulate in the inward facing conformation and/or putative intermediate

states between the inward and outward facing conformation. Such a change in the conformational equilibrium upon mutation of Tyr335<sup>6,68</sup> could explain the reduced transport capacity of the Y335A mutant since only a small fraction of transporters would reside in the outward facing resting conformation and thus be available for transport. Since it is likely that Zn<sup>2+</sup> stabilizes an outward facing conformation of the transporter (Loland et al., 1999; Norregaard et al., 1998), it is conceivable that Zn<sup>2+</sup> is capable of reestablishing a conformational equilibrium similar to the wild type hDAT allowing substrate binding and translocation to occur. In this context, it is important to note that occupancy of the endogenous high-affinity Zn<sup>2+</sup>-binding site in the hDAT wild type does not result in full inhibition of uptake but only in maximum around 75% inhibition (Norregaard et al., 1998). Thus, the Zn<sup>2+</sup>-occupied transporter is still capable of translocating substrate albeit with reduced efficiency.

Mutation of Tyr335<sup>6,68</sup> was not only accompanied by an altered response to Zn<sup>2+</sup> but also by substantial changes in apparent affinities for both substrate and inhibitors. Whereas the apparent affinity for substrates increased up to 20-fold upon mutation of Tyr335<sup>6,68</sup>, the apparent affinity for cocaine and cocaine-like inhibitors decreased up to 150-fold (Loland et al., 2002). To the best of our knowledge, this is to date the most dramatic alteration in affinity for cocaine and cocaine-like inhibitors such as CFT and RTI-55 reported upon mutation of a single residue in the biogenic amine

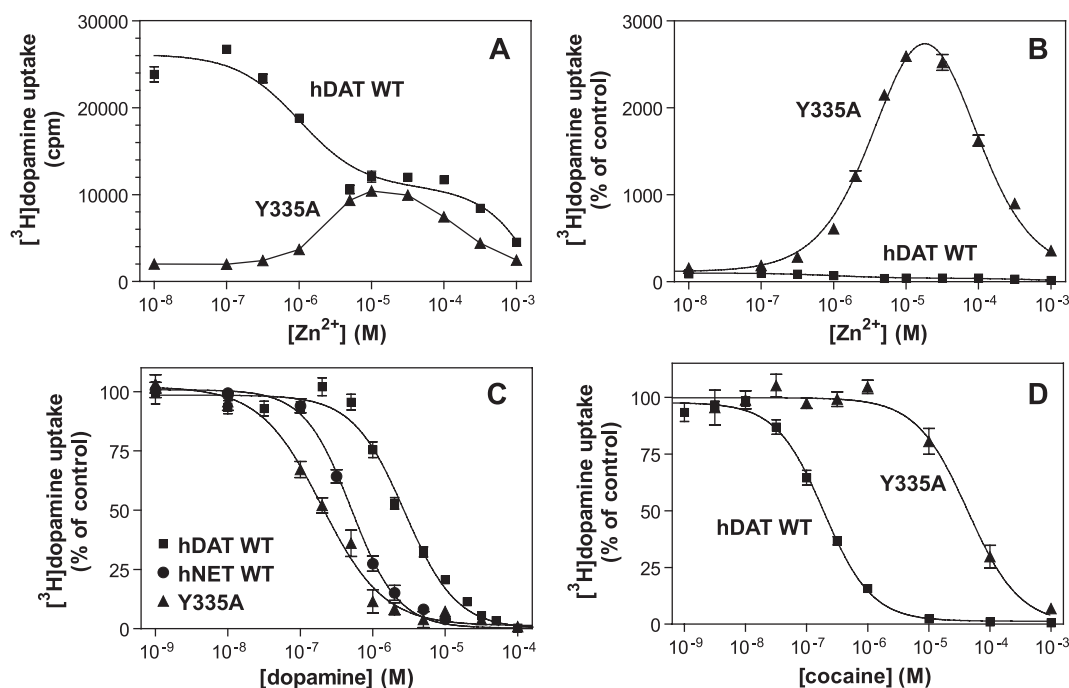


Fig. 4. Conversion of an inhibitory Zn<sup>2+</sup> switch to a stimulatory Zn<sup>2+</sup> switch by mutation of Tyr335<sup>6,68</sup>. (A) The effect of Zn<sup>2+</sup> on [3H]dopamine uptake in COS-7 cells transiently expressing hDAT Y335A (▲) or hDAT wild type (■). Each data point is expressed as mean ± S.E. in CPM of triplicate determinations from a representative experiment. (B) Normalized data for the Zn<sup>2+</sup> effect on [3H]dopamine uptake in COS-7 cells transiently expressing hDAT Y335A (▲) or hDAT wild type (■). Reproduced from Loland et al. (2002). (C) Evidence for a higher apparent affinity for dopamine in the hDAT Y335A (▲) mutant compared to hDAT wild type (■) and hNET wild type (●). (D) The hDAT-Y335A (▲) mutant exhibits an almost 150-fold decrease in apparent cocaine affinity as compared to hDAT wild type (■). Modified from Loland et al. (2002).

transporters. The dramatic changes in apparent affinities for the inhibitors as well as for substrates were partially restored, however, by the application of  $\text{Zn}^{2+}$  (Loland et al., 2002). Thus, we would infer that the observed dramatic changes in apparent inhibitor affinity in Y335A are not necessarily indicative of a direct involvement of the mutant residue in inhibitor binding, but is more likely a direct consequence of a changed conformational equilibrium in which the transporter only rarely assumes the high-affinity binding state for cocaine and other inhibitors. Hence, binding of the inhibitors requires a major change in conformational distribution and thus more energy as compared to binding to the wild type transporter as directly reflected in the lowered apparent affinities. It is interesting to note that binding of the structurally distinct inhibitor GBR 12,909 was affected much less by mutation of Tyr335<sup>6,68</sup> than cocaine, RTI-55, CFT, and mazindol (Loland et al., 2002). One possibility is that GBR 12,909 is binding to a different site of the transporter. However, another intriguing possibility is that the interaction between GBR 12,909 and the hDAT is less conformationally dependent; hence, this compound may bind to several conformations in contrast to cocaine analogs, which would be predicted to highly depend on the conformational state of the transporter. Nevertheless, it is clear that this interpretation of our data further underlines the necessity of being extremely cautious when concluding from the growing body mutational data showing changes in apparent affinities for, e.g. cocaine (Itokawa et al., 2000; Lin et al., 2000a,b; Lin and Uhl, 2002; Wu and Gu, 2003; Kitayama et al., 1992). Thus, any inferences about direct interactions must be supported by, for example, photo affinity labeling strategies (Zou et al., 2001; Dutta et al., 2001).

If our hypothesis regarding the Y335A mutation is correct, mutation of other residues would be expected to display a similar phenotype, i.e. other residues would be predicted in conjunction with Tyr335<sup>6,68</sup> to be part of the same stabilizing network of intramolecular interactions responsible for regulation of transporter conformation. Indeed a screen of possible candidates has revealed three additional residues that upon mutation give rise to similar phenotype as that of Y335A, i.e. potentiation of uptake by  $\text{Zn}^{2+}$  as well as decreased apparent affinity for inhibitors and increased apparent affinity for substrates (Loland et al., submitted for publication). Interestingly, these residues are situated either in the same loop as Tyr335<sup>6,68</sup>, intracellular loop (ICL) 3, or in the “adjacent” ICL2 and ICL4 (Loland et al., submitted for publication). To further explore these Y335A-like mutants and accordingly substantiate our hypotheses, we established a structural read-out of the conformational state of the mutant transporters by using the reactivity of a cysteine engineered into position 159<sup>3,53</sup> in TM3 (Loland et al., submitted for publication). This position was chosen based on recent observations by Chen and Rudnick (2000) in the homologous rat SERT and hNET indicating that the accessibility of the corresponding residue (position 155<sup>3,53</sup> in hNET and 179<sup>3,53</sup> in rat SERT) to the

positively charged sulfhydryl-reactive compound MTSET is dependent on whether the transporter assumes an outward facing conformation or an inward facing conformation. Consistent with our hypothesis, the accessibility data provided direct structural support for an alteration in the conformational equilibria of the mutant transporters. The accessibility data further suggested that the addition of  $\text{Zn}^{2+}$  caused a restoration of the conformational equilibrium. This restoration of the conformational equilibrium was, however, only partial and interestingly resulted in an equilibrium that resembled that of the hNET more than that of the hDAT (Loland et al., submitted for publication).

Summarized, the Y335A mutation has defined a paradigm of mutations causing spontaneous changes in the distribution between individual functional states in the translocation cycle. The paradigm has received additional support by our subsequent identification of yet additional intracellular residues that upon mutation display a Y335A-like phenotype. Moreover, it is important to note that residues critical for maintaining this equilibrium of the transport cycle might obviously not only be found in the intracellular domains of the transporter. Recent electrophysiological analysis of two mutations at the top of TM7 in the GAT-1 indicates the presence of major changes in distribution of conformational states in the translocation cycle and accordingly that residues on the extracellular face of the transporter also can be critical for regulating the conformational distribution (MacAulay et al., 2003). Our interpretation of both the hDAT and the rGAT-1 mutants shows parallels to, for example, the concept underlying constitutively activating mutations in G protein-coupled receptors (Samama et al., 1993; Lefkowitz et al., 1993; Gether, 2000). Substantial experimental evidence suggests that such mutations promote the release of intramolecular constraining interactions in the receptor molecule, leading to spontaneous formation of the active receptor state and, thus, a change in the conformational equilibrium between inactive and active receptor conformations (Gether, 2000; Lefkowitz et al., 1993; Samama et al., 1993). Constitutively activating mutations in G protein-coupled receptors have proven to be highly valuable tools for gaining insight into the molecular function of this family of membrane proteins (Gether, 2000). Thus, the possibility to turn the transport of substrate on and off and switch between conformations as can be done in the Y335A mutant with  $\text{Zn}^{2+}$  represents a new valuable tool for the study of the translocation process in the family of  $\text{Na}^+/\text{Cl}^-$ -coupled transporters.

## 5. Physiological role of $\text{Zn}^{2+}$ -binding sites

Apart from the hDAT, evidence has been obtained indicating the presence of  $\text{Zn}^{2+}$ -binding sites also in many other membrane proteins. This includes: ligand-gated ion channels such as the GABA<sub>A</sub> receptor (Draguhn et al., 1990; Horenstein and Akabas, 1998), the glycine receptor



(Bloomenthal et al., 1994; Laube et al., 1995) and the NMDA receptors (Peters et al., 1987; Westbrook and Mayer, 1987; Hollmann et al., 1993; Choi and Lipton, 1999; Fayyazuddin et al., 2000); G protein-coupled receptors such as the melanocortin MC<sub>1</sub> and MC<sub>4</sub> receptor (Holst et al., 2002), the tachykinin NK<sub>3</sub> receptor (Rosenkilde et al., 1998) and the  $\beta_2$  adrenoceptor (Swaminath et al., 2002, 2003); and neurotransmitter transporters such as the glutamate transporter, EAAT-1 (Vandenberg et al., 1998). The three-dimensional structures of these proteins have not been solved yet; thus, as described here for the hDAT, the presence of endogenous Zn<sup>2+</sup>-binding sites has been discovered indirectly by the ability of low-micromolar concentrations of Zn<sup>2+</sup> to modulate the function of these proteins. The physiological role of the Zn<sup>2+</sup>-binding sites identified in these membrane proteins remains unclear. The extracellular free Zn<sup>2+</sup> concentration is generally kept at a low level in the brain (estimated to be in the range from 1 pM to 10 nM, Frederickson, 1989); however, in glutamatergic nerve terminals, especially at the mossy fiber synapses in the hippocampus, Zn<sup>2+</sup> is found in very high concentration in the synaptic vesicles (3–30 mM) (Frederickson and Bush, 2001). Upon the arrival of an action potential at the terminal, Zn<sup>2+</sup> has been shown to be released concomitantly with glutamate and based on studies using hippocampal slice preparations, this has been estimated to result in free Zn<sup>2+</sup> concentration that transiently might reach 10–30  $\mu$ M (Vogt et al., 2000; Li et al., 2001a,b). Older studies have even estimated that the concentration may reach 100–300  $\mu$ M upon stimulation of glutamatergic neurons (Vogt et al., 2000). It is, therefore, likely that Zn<sup>2+</sup>, in sufficiently high concentrations, at least transiently may be present in the synapses to *modulate* the function of transporters and receptors containing endogenous Zn<sup>2+</sup>-binding sites. Final evidence for this will have to await the application of, for example, transgenic strategies involving homologous substitution with Zn<sup>2+</sup> insensitive mutants (knock-ins).

In the context of a potential physiological function of the hDAT Zn<sup>2+</sup>-binding site, it is of interest to mention our studies on the effect of Zn<sup>2+</sup> on reverse transport. The biogenic amine transporters are, like other Na<sup>+</sup>-coupled transporters, not only capable of Na<sup>+</sup>-dependent uptake but also of reverse transport of their substrate(s) (Levi and Raiteri, 1993). An interesting observation was, that in contrast to substrate uptake of the hDAT wild type where Zn<sup>2+</sup> act as an inhibitor, Zn<sup>2+</sup> enhances efflux of substrate; when cells are preloaded with [<sup>3</sup>H]MPP<sup>+</sup> (1-methyl-4-phenylpyridinium) and then challenged with amphetamine, the addition of Zn<sup>2+</sup> causes a substantial increase in [<sup>3</sup>H]MPP<sup>+</sup> efflux (Scholze et al., 2002). A similar effect of Zn<sup>2+</sup> in response to amphetamine was observed in striatal slices (Scholze et al., 2002). The augmentation of efflux mediated by Zn<sup>2+</sup> was importantly found to be a result of Zn<sup>2+</sup> binding to the same endogenous Zn<sup>2+</sup>-binding site as that mediating inhibition of uptake (Scholze et al., 2002). These data represent the first example of allosteric modulation of

hDAT that differentially modulates inward and outward transport. This might be interesting in vivo since reverse transport of dopamine via the hDAT has recently been suggested to be responsible for non-exocytotic, Ca<sup>2+</sup>-independent release of dopamine in the Substantia Nigra upon excitation of glutamatergic neurons projecting from the subthalamic nucleus (Falkenburger et al., 2001). This release contributes to the autoinhibitory effects mediated by the dopamine D2 receptors to regulate overstimulatory inputs from the subthalamic nucleus (Falkenburger et al., 2001). Since Zn<sup>2+</sup> in many brain regions is stored in synaptic vesicles and possibly co-released together with glutamate—leading transiently to a free Zn<sup>2+</sup> concentration in the higher micromolar range (Frederickson and Bush, 2001)—it is a conceivable possibility that Zn<sup>2+</sup> may modulate dopamine release in Substantia Nigra. Thus, our observations may not merely be a biochemical peculiarity but could be physiologically relevant.

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