The Role of Zinc Ions in Reverse Transport Mediated by Monoamine Transporters*

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The human dopamine transporter (hDAT) contains an endogenous high affinity Zn2+ binding site with three coordinating residues on its extracellular face (His193, His375, and Glu396). Upon binding to this site, Zn2+ causes inhibition of [3H]1-methyl-4-phenylpyridinium ([3H]MPP+) uptake. We investigated the effect of Zn2+ on outward transport by superfusing hDAT-expressing HEK-293 cells preloaded with [3H]MPP+. Although Zn2+ inhibited uptake, Zn2+ facilitated [3H]MPP+ release induced by amphetamine, MPP+, or K+-induced depolarization specifically at hDAT but not at the human serotonin and norepinephrine transporter (hNET). Mutation of the Zn2+ coordinating residue His375 to Lys (the corresponding residue in hNET) eliminated the effect of Zn2+ on efflux. Conversely, the reciprocal mutation (K189H) conferred Zn2+ sensitivity to hNET. The intracellular [3H]MPP+ concentration was varied to generate saturation isotherms; these showed that Zn2+ increased Vmax for efflux (rather than KM_efflux-intracellular). Thus, blockage of inward transport by Zn2+ is not due to a simple inhibition of the transporter turnover rate. The observations provide evidence against the model of facilitated exchange-diffusion and support the concept that inward and outward transport represent discrete operational modes of the transporter. In addition, they indicate a physiological role of Zn2+, because Zn2+ also facilitated transport reversal of DAT in rat striatal slices.

The principal physiological action of the monoamine transporters is the removal of synaptically released neurotransmitter by a sodium-driven secondary-active transport mechanism (1). The transporters are situated primarily in the presynaptic membrane and include the transporters for dopamine, norepinephrine, and serotonin (hDAT, hNET, and hSERT, respectively) (1). These transporters form a subfamily within the large class of Na+/?Cl−-coupled transporters (2). Monoamine transporters have been the focus of intensive research, in particular because they represent targets for clinically important therapeutics, e.g. antidepressant drugs (3), which increase the availability of serotonin and/or norepinephrine by blocking reuptake. Moreover, drugs of abuse such as amphetamine and cocaine target these transporters (4).

It has been well known that the monoamine transporters are not only capable of sodium-dependent transmembrane uptake but also of reverse transport of their substrate (5). Substrate efflux can be observed upon membrane depolarization, if the transmembrane sodium gradient is abrogated, or it can be elicited by extracellular substrates. The latter mechanism is thought to underlie the addictive and reinforcing properties of amphetamine derivatives (4). Furthermore, excitation of glutamatergic receptors at dopaminergic neurons of the substantia nigra induces reverse operation of hDAT; this contributes to important autoinhibitory effects mediated by the dopamine D2-receptors to regulate overstimulatory inputs of the subthalamic nucleus (6). This novel evidence indicates a critical physiological role for reverse transport; thus, monoamine transporters mediate both inwardly and outwardly directed fluxes of monoamine in the brain.

The bivalent cation Zn2+ is widely distributed in the central nervous system (7). Zn2+ serves as a chelated counter ion for stored neurotransmitters in synaptic vesicles (8) and, upon nerve stimulation, Zn2+ is co-released with the neurotransmitter (9). The same can be observed under pathological conditions, i.e. in brain ischemia. Physiologically, the extracellular concentration of Zn2+ may reach 10–20 μM (10), and these levels may further rise up to 300 μM in pathological situations (9, 11, 12). The release of Zn2+ is very interesting, because the activity of several neurotransmitter receptors and transporters are modulated by micromolar concentrations of Zn2+ (13–16). For example, Zn2+ has recently been reported to block transport-associated ion currents through glutamate transporter subtypes (salamander excitatory amino acid transporter (17) and EAAT1 (15)). Moreover, Zn2+ was found to enhance binding of cocaine analogues and to inhibit uptake of dopamine by synaptosomal membranes (18), an effect that is accounted for by direct binding of Zn2+ to hDAT (19). The high affinity Zn2+ binding site in wild type hDAT was mapped to three coordinating residues situated on the extracellular face of the transporter, His193 in the large extracellular loop between transmembrane segment (TM) 3 and 4, His375 at the external end of TM 7 and Glu396 at the external end of TM 8 (19, 20). The inhibitory effect on uptake suggests that, by binding to the transporter, Zn2+ constrains relative movements between extracellular loop 2, TM 7, and TM 8 that are critical for the translocation process. Moreover, due to the strict
geometric requirements for binding the small zinc(II) ion, and based on several additional engineered Zn$^{2+}$-binding sites in hDAT, it became possible to deduce a model of the tertiary structure in a putative TM 7/8 microdomain (21). Finally, a simple exchange of corresponding amino acids resulted in the transfer of the high affinity binding properties to the hNET (19, 20) and to the more distantly related rat $\gamma$-aminobutyric acid (GABA) transporter (22). This reveals strong support toward an evolutionary conserved motif in the tertiary structure of Na$^+/Cl^-$-dependent transporters highly relevant for the translocation process.

The mechanistic basis for transport reversal is poorly understood. Earlier models compared the transporter to a revolving door, which mediates influx or efflux provided that there is a driving force, i.e. the gradient of Na$^+$ and substrate (23). In this model of facilitated exchange-diffusion, inward and outward transport are stoichiometrically linked events and, thus, strictly coupled. Hence, the model predicts that inhibition of influx must result in reduced efflux. Here, we have exploited the ability of Zn$^{2+}$ to bind specifically to the dopamine transporter to demonstrate that inward and outward transport represent discrete operational modes of a sodium-coupled transporter. Our results show that physiologically relevant concentrations of Zn$^{2+}$ enhance reverse transport by hDAT (but not by hNET or hSERT), although uptake is blocked. This presumably has physiological implications, because a regulation of dopamine transport by Zn$^{2+}$ can be recapitulated in striatal slices.

**MATERIALS AND METHODS**

**Molecular Biology and Transfection**—cDNA encoding the hDAT and hNET in pRL-CMV were kindly provided by Dr. R. D. Blakely (Vanderbilt University, Nashville, TN). A detailed description of the hDAT mutant as well as the hNET mutant is given in the recent publications of Norregaard et al. (19) and Loland et al. (20). Stable and/or transient expression of the desired cDNAs in HEK-293 cells was achieved by transfection using the CaPO$_4$-precipitation method (26). Alternatively, Lipofectamine Plus was used according to the manufacturer’s description (Invitrogen). Suitable transfected cell lines were grown essentially as described previously (27). At least two different stable cell lines were tested to exclude clonal effects.

**Uptake Experiments**—The experiments were performed as described previously (28). In brief, 5 x 10$^5$ HEK-293 cells transiently or permanently expressing the hDAT, hNET, hSERT, or mutants were seeded onto 48-well plates and left in normal culture medium for 4 days after plating. Each well was washed once with 0.5 ml of KRH buffer (Krebs-Ringer-Hepes buffer; Hepes 10 mM, NaCl 120 mM, KCl 3 mM, CaCl$_2$ 2 mM, MgCl$_2$ 2 mM, glucose 20 mM, final pH 7.4, room temperature). The cells were incubated with $[^3]$H]-methyl-4-phenylpyridinium ($[^3]$HMP) (0.2 $\mu$Ci, 88.5 Ci/mmol) and various concentrations of unlabeled MPP$^+$ (range: 0.63–300 $\mu$M; final volume: 0.1 ml), a well-known substrate of monoamine transporters (Refs. 29–31; see Table I). This uptake was temperature-dependent and sensitive to co-incubation with the non-selective uptake blocker cocaine (100 $\mu$M) as well as to specific uptake blockers like nomifensine, nisoxetine, and paroxetine (hDAT, hNET, and hSERT, respectively). After 8 min at room temperature, uptake was terminated rapidly by removal of buffer and washing with 0.5 ml of ice-cold buffer. Cells were lysed with 0.5 ml of 1% SDS and transferred into scintillation vials for liquid scintillation counting. The experiments shown in Fig. 1 were conducted after a preincubation period of 5 min using a constant concentration of $[^3]$HMP$^+$ (50 nM).

**Superfusion Experiments**—We used a superfusion system, which allows for the continuous monitoring of the efflux of substrate from appropriately transfected cells after preloading with radiolabeled substrate (32). Because released substrate is washed away immediately, the confounding effects of ongoing reuptake are minimized by the superfusion system (28). In brief, cells were grown overnight on round glass coverslips (5-mm diameter, 4 x 10$^5$ cells per coverslip) then incubated with $[^3]$HMP$^+$ (0.8 $\mu$Ci, final concentration 10 $\mu$M) for 20 min at 37°C in 0.1 ml of KRH. Coverslips were then transferred to small superfusion chambers (0.2 ml) and superfused with KRH buffer (25°C, 0.7 ml min$^{-1}$) as described (27). A washout period of 40 min established a stable baseline for efflux of radioactivity; thereafter, the experiment was started with the collection of fractions (2 or 4 min). At the end of the experiment, cells were lysed in 1% SDS.

To obtain quantitative data on efflux, the cells were incubated with a range of different $[^3]$HMP$^+$ concentrations (2–128 $\mu$M). Intracellular $[^3]$HMP$^+$ concentrations were calculated using the accumulated radioactivity in the cells, a cell number of 27,000 per glass coverslip, and a cell volume of 1.08 pl/cell ± 0.12 (mean ± S.E. of four independent determinations; $[^3]$H$_2$O$_2$-Gujinil method (27)).

In experiments using high Zn$^{2+}$ was used. NCt was obtained by the washout of KC1 or choline chloride, respectively. Female Sprague-Dawley rats (200–250 g, Forschungsanstalt für Versuchstierzucht, Himberg, Austria) were used to perform experiments (see also Ref. 33). In brief: After decapitation and removal of the brain, striata were prepared and cut into 0.3-mm-thick slices using a McIlwain tissue chopper. The slices were then incubated for 60 min at 37°C in 0.5 ml of KRH containing 0.25 $\mu$M $[^3]$HMP$^+$, washed twice, and inserted into the superfusion chambers; KRH was supplemented with 10 $\mu$M EDTA, and the slices were superfused at 25°C and at a flow of 0.7 ml/min. This washout period of 60 min served to establish a basal efflux of radioactivity and to chelate free zinc; superfusate samples were collected at 2-min intervals. Under the given conditions, Zn$^{2+}$ was concentrated at 20 $\mu$M resulting in a concentration of 10 $\mu$M free Zn$^{2+}$. At the end of the experiment, the slices were homogenized in 1.2 ml of KRH by sonication (Branson sonifier B 15; Branson Sonic Power, Danbury, CT).

Tritium in the superfusate fractions, the SDS cell lysates, and slice homogenates was determined by liquid scintillation counting. Release of $[^3]$H label is expressed as a fractional rate, i.e. the radioactivity released during a fraction was expressed as the percentage of the total radioactivity present in the cells at the beginning of that fraction.

**Chemicals**—Tissue culture reagents were from Invitrogen Life Technologies. $[^3]$HMP$^+$ was from Invitrogen (Boston, MA). D-Amphetamine was kindly donated by SmithKline & French (Welwyn Garden City, Herts, UK). Cocaine HCl was from Dolda AG (Basel, Switzerland). Unlabeled MPP$^+$ was from Research Biochemicals International (Natick, MA). All other chemicals were from commercial sources.

**Data Collection**—$V_{max}$, $K_{m}$, $EC_{50}$, and $IC_{50}$ values were calculated by performing non-linear regression analysis using Prism 3.02 fitting and plotting software (GraphPad, San Diego, CA). Statistical significance was evaluated by Student’s t test (paired or unpaired as appropriate).

**RESULTS**

Zinc Inhibits Uptake of MPP$^+$ at the hDAT wt Only with High Affinity—Previous observations showed that Zn$^{2+}$ blocked uptake of $[^3]$Hamphetamine by hDAT, because it interacted with an endogenous Zn$^{2+}$ binding site (19). The present study, we used $[^3]$HMP$^+$ as a substrate rather than dopamine for the following reasons: (i) $[^3]$HMP$^+$ is metabolically stable, i.e. resistant to degradation by monoamine oxidase, and hydrophilic such that it is trapped within the cell; back diffusion does not confound the interpretation of uptake and, more importantly, of release experiments from preloaded cells. (ii) Because diffusion is minimal, it is, in addition, possible to preload the cells and to determine the intracellular concentration, an advantage that is instrumental if the kinetics of reverse transport are to be analyzed (see below). (iii) $[^3]$HMP$^+$ is a substrate for all monoamine transporters (i.e. DAT, NET, SERT); thus, it is ideally suited for a comparative analysis. Fig. 1 illustrates the effect of Zn$^{2+}$ on uptake of $[^3]$HMP$^+$ by hDAT (Fig. 1A), hNET (Fig. 1B) and hSERT (Fig. 1C). As expected, Zn$^{2+}$ only blocked hDAT with high affinity (squares in Fig. 1A) whereas uptake by wild type hNET (circles in Fig. 1B) and hSERT (Fig. 1C) resulted in concentration-dependent inhibition of both hNET and hSERT. At the concentration of Zn$^{2+}$ that exceeded 100 $\mu$M, the endogenous Zn$^{2+}$ binding site of hDAT was previously mapped, and the residue found in the corresponding position of hNET, high affinity inhibition was abrogated and Zn$^{2+}$ sup-
Because Zn2+ also safely concludes from the available evidence that Zn2+ transporters use their endogenous substrates (19) it is previously obtained by evaluating the Zn2+ sensitivity of monamine transporters and mutant constructs. HEK-293 cells expressing wild type monamine transporters and mutant constructs were distributed in 48-well plates (5 × 10^5 cells). For determination of the K_m values for [3H]MPP+ transport as well as IC_50 values for Zn2+ inhibition by Zn2+ a monophasic low affinity inhibition curve was fitted to the data (Fig. 1). Surprisingly, this amphetamine-elicited efflux was markedly enhanced, rather than inhibited, by the addition of 10 μM Zn2+ to the superfusion buffer (Fig. 2A, open squares). We stress that Zn2+ per se did not affect basal efflux (Fig. 2A). The modulatory effect of Zn2+ was lost upon mutational exchange of all three coordinating residues (hDAT-H193K-H375A-E396Q, hSERT (3) 19.7 0.01 0.6 346 0.23 0.01 313 ± 1.2 169 ± 0.5 12.15 ± 0.1 34.6 ± 0.9 0.06 ± 0.01 3684 ± 16 2682 ± 12

**TABLE I**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>K_m (µM)</th>
<th>IC_50 (Zn2+)</th>
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<tbody>
<tr>
<td>hDAT wt</td>
<td>18.0 ± 3.5</td>
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</tr>
<tr>
<td>hDAT-H193K</td>
<td>16.4 ± 2.3</td>
<td>169 ± 0.5</td>
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<tr>
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<td>17.8 ± 2.4</td>
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<tr>
<td>hNET wt</td>
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<td>34.6 ± 0.9</td>
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<tr>
<td>hNET-K189H</td>
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**FIG. 1. Effects of Zn2+ on the uptake of [3H]MPP+ by monoamine transporters and mutant constructs.** HEK-293 cells expressing wild type monoamine transporters and mutant constructs were distributed in 48-well plates (5 × 10^5 cells). The washed cells were preincubated in Krebs-Ringer-Hepes (KRH) buffer (0.1 ml) containing Zn2+ in the concentrations indicated. After 5 min, the buffer was replaced by KRH containing Zn2+ and [3H]MPP+ (50 nM). After 8 min at room temperature, uptake was terminated and radioactivity was determined by liquid scintillation counting. For K_m values and IC_50 values see Table I. A: hDAT wt, B: hDAT-H193K, C: hNET wt, D: hNET-K189H, E: hSERT wt, F: hDAT-H193K-V377H. Data represent means ± S.E. of three to six experiments performed in triplicate.

**pressed uptake with a monophasic low affinity inhibition curve** (triangles in Fig. 1A). Conversely, the corresponding mutation in hNET (i.e. replacing Lys189 with His) conferred high affinity inhibition by Zn2+ to hNET (triangles in Fig. 1B). The K_m values for [3H]MPP+ transport as well as IC_50 values for Zn2+ are summarized in Table I. Based on these data we conclude that transport of [3H]MPP+ faithfully reproduces the data previously obtained by evaluating the Zn2+ sensitivity of monamine transporters using their endogenous substrates (19); it is also safe to conclude from the available evidence that Zn2+ suppresses the transport of all substrates by hDAT.

**Zinc Enhances Efflux Induced by Amphetamine at hDAT**—Because Zn2+ inhibited uptake of [3H]MPP+ at the hDAT, a similar blockage was to be anticipated for release if efflux simply reflected reverse of transport. This was not the case. Cells that expressed hDAT were preloaded with [3H]MPP+. Upon challenge with a maximally effective concentration of amphetamine (10 μM), transport reversal was induced and resulted in release of [3H]MPP+ (Fig. 2A). Surprisingly, this amphetamine-elicited efflux was markedly enhanced, rather than inhibited, by the addition of 10 μM Zn2+ to the superfusion buffer (Fig. 2A, open squares). We stress that Zn2+ per se did not affect basal efflux (Fig. 2A). The modulatory effect of Zn2+ was lost upon mutational exchange of all three coordinating residues (hDAT-H193K-H375A-E396Q, hSERT (3) 19.7 0.01 0.6 346 0.23 0.01 313 ± 1.2 169 ± 0.5 12.15 ± 0.1 34.6 ± 0.9 0.06 ± 0.01 3684 ± 16 2682 ± 12

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In contrast to wild type hDAT, release from cells expressing the hNET was not affected by co-application of Zn2+ (Fig. 2C). We have exploited this insensitivity to ask if Zn2+-enhanced outward transport was conferred to hNET upon replacement of Lys189 by the appropriate Zn2+ coordinating ligand (i.e. His). In fact, in cells expressing hNET-K189H, Zn2+ promoted efflux that had been induced by amphetamine (open symbols in Fig. 2D); although the effect was less pronounced than that seen with hDAT wt. Finally, Zn2+ did not affect amphetamine-induced release of [3H]MPP+ via hSERT; and cocaine (100 μM) completely inhibited amphetamine-driven efflux mediated by wild type and mutant transporters irrespective of the presence or absence of Zn2+ (data not shown).

We tested Zn2+ over a wide concentration range; up to 300 μM, Zn2+ did not affect basal release of [3H]MPP+ from preloaded cells that expressed hDAT, hNET, or hSERT (see inset in Fig. 3). Because the physiological significance of higher concentrations is questionable, we did not further investigate the discrepancy between basal release through hSERT and hNET-K189H, which was not affected by 1 mM Zn2+ (see Fig. 3, inset) and efflux through hDAT wt, hNET wt, and hDAT-H193K, which was stimulated to some extent by 1 mM Zn2+ (see Fig. 3, inset).

In the presence of amphetamine, the efflux-enhancing effect...
of Zn$^{2+}$ at hDAT-expressing cells was concentration-dependent; maximum enhancement was observed at 3 to 30 μM Zn$^{2+}$; higher concentrations caused inhibition of efflux resulting in a bell-shaped concentration-response curve (squares in Fig. 3). A reasonably similar bell-shaped curve was observed if the effect of Zn$^{2+}$ was evaluated on amphetamine-induced efflux through hNET-K189H (downward triangles in Fig. 3). In contrast, over a similar concentration range, Zn$^{2+}$ did not enhance efflux in HEK-293 cells expressing hDAT-H193K, hNET wt, or hSERT wt (upward triangles, circles, and diamonds, respectively, in Fig. 3).

### Efflux Induced by the Substrate MPP$^+$ Is Also Enhanced by Zinc

At a concentration that caused a substantial inhibition of inward transport by hDAT wt (10 μM, see Fig. 1A), Zn$^{2+}$ promoted outward transport. Thus, the data presented so far suggested that, in the presence of Zn$^{2+}$, inward transport of amphetamine was not a prerequisite for release. In other words, we surmised that sole binding of substrate on the extracellular side, rather than binding and inward transport, sufficed to initiate transport reversal (provided that there is enough substrate on the intracellular side). However, amphetamine is notorious for its ability to accumulate in cells by diffusion. Because the pK$_a$ of amphetamine is close to the extracellular pH, the modest transmembrane pH gradient is sufficient to trap protonated amphetamine within the cell.

Zn$^{2+}$-promoted efflux may thus also result from an additional effect of amphetamine that arises from an intracellular site of action. To rule out this possibility, we have employed MPP$^+$ to verify our conjecture. Because MPP$^+$ is a substrate for monoamine transporters, the compound can also induce reverse transport albeit less efficiently than amphetamine (34). In cells that had been preloaded with [3H]MPP$^+$, Zn$^{2+}$ markedly enhanced efflux induced by MPP$^+$ only if the cells expressed hDAT wt (open symbols in Fig. 4A) or hNET-K189H (open symbols in Fig. 4D). It is, however, evident from Fig. 4 (B and C) that efflux through hDAT-H193K and hNET wt, respectively, was essentially identical in the absence and presence of Zn$^{2+}$. The same was true for hSERT wt (data not shown). Thus, the results obtained with MPP$^+$-induced outward transport of [3H]MPP$^+$ also supported the interpretation that release does not require inward transport of substrate.

![Image of graphs showing efflux induced by the substrate MPP$^+$ and enhanced by zinc](image_url)
Enhanced by Zinc

— Although widely studied, drug-induced transport reversal does not reflect a physiological phenomenon. Under physiological conditions, transport reversal may be induced by Na\(^{+}\) influx (e.g. due to membrane depolarization). Alternatively, in brain ischemia, the transmembrane Na\(^{+}\) gradient dissipates because of ATP depletion, which prevents Na\(^{+}\) extrusion, and glutamate release, which promotes Na\(^{+}\) entry. To test if facilitation of efflux by Zn\(^{2+}\) is physiologically relevant, we removed extracellular Na\(^{+}\), and we depolarized the cells by eliminating the K\(^{+}\) gradient. Efflux induced by isosmotic replacement of Na\(^{+}\) by choline was neither enhanced by Zn\(^{2+}\) (10 \(\mu M\)) at the hDAT wt nor at the mutant hDAT-H193K (data not shown). In contrast, the efflux elicited by elevating the extracellular K\(^{+}\) concentration (from 3 to 120 \(mM\)) was significantly enhanced in the presence of Zn\(^{2+}\) (10 \(\mu M\)) at the hDAT wt (Fig. 7A); as expected, this facilitation of transport reversal was not seen with the mutant form hDAT-H193K (Fig. 7B).

Amphetamine-induced Efflux of MPP\(^{+}\) Is Also Enhanced by Zinc in Rat Striatal Slices—The endogenous Zn\(^{2+}\) binding site of the DAT is highly conserved among species orthologues. We exploited this fact to explore if Zn\(^{2+}\) also facilitated transport reversal by a DAT expressed in its native environment. Striatal slices were prepared from rat brain and superfused with KRH in the presence of EDTA (10 \(\mu M\)) to chelate Zn\(^{2+}\) released due to tissue trauma. As predicted from the experiments carried out on transfected cells (cf. baselines in Figs. 2–4), Zn\(^{2+}\) alone (free concentration ~10 \(\mu M\)) did not enhance basal [\(^{3}H\)]MPP\(^{+}\) efflux (Fig. 8, open squares, min 2–14). In contrast, efflux elicited by amphetamine (10 \(\mu M\)) was enhanced by Zn\(^{2+}\) (Fig. 8, min 14–24). A comparison of four separate experiments (with slices from two animals) showed that the difference in area under the curve (mean \pm S.D. = 5.85 \pm 2.56 and 8.39 \pm 3.56% for amphetamine and amphetamine plus Zn\(^{2+}\), respectively) was significantly different (\(p = 0.02, t\) test for paired data). Slice preparations have technical limitations; transport reversal cannot be studied under depolarizing conditions in slices, because Ca\(^{2+}\)-induced exocytosis occurs upon depolarization (due to opening of voltage-dependent Ca\(^{2+}\) channels and of glutamatergic and nicotinic ligand-gated ion channels). Hence, it has not been possible to document the combined effect of depolarization and Zn\(^{2+}\) in rat striatal slices.

**DISCUSSION**

It has generally been assumed that amphetamine, methylenedioxyamphetamine ("ecstasy") and related drugs of abuse induce reversal of transport in the monoamine transporters, because they are substrates for inward transport. The transporter-mediated influx of such substrate has been considered a prerequisite for non-exocytic release of neurotransmitter by reversed transport. Thus, the core assumption of the facilitated exchange-diffusion model (23) predicts that the transporter operates like a revolving door. If this model is correct, inward transport of amphetamine (or congeners), of the Cl\(^{-}\) counterion and of Na\(^{+}\) is tightly coupled to efflux of neurotransmitter (34). Conversely, the model of facilitated exchange-diffusion has to be refruted, if efflux and influx can be separated. In several previous studies, the kinetics of transport were difficult to reconcile with a tight coupling of inward and outward transport, but it has proven difficult to obtain un-

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**Figure 3. Concentration-response relationship of amphetamine and Zn\(^{2+}\)-induced [\(^{3}H\)]MPP\(^{+}\) release.** HEK-293 cells expressing hDAT wt (○; [amphetamine]: 10 \(\mu M\)), hDAT-H193K (△; [amphetamine]: 10 \(\mu M\)), hNET wt (●; [amphetamine]: 1 \(\mu M\)), hNET-K189H (▼; [amphetamine]: 1 \(\mu M\)), or hSERT wt (▲; [amphetamine]: 10 \(\mu M\)); basal [\(^{3}H\)]MPP\(^{+}\) efflux: 0.224 \(\pm\) 0.007%·min\(^{-1}\); i.e. 243.8 ± 10.9 dpm·min\(^{-1}\), \(n = 40\) were preloaded with [\(^{3}H\)]MPP\(^{+}\) and superfused, and 2·min fractions were collected. After three fractions (6 min) of basal efflux, cells were exposed to Zn\(^{2+}\) or left at control conditions. After seven fractions (from 14 min onward), amphetamine was added to all superfusion channels for the following five fractions. \(V_{\text{max}}/V_{\text{max}}\) values were generated by division of the value (mean of the last 6 min of fraction collection) in the presence of amphetamine and Zn\(^{2+}\) by the value in the absence of Zn\(^{2+}\). Inset, influence of Zn\(^{2+}\) on basal [\(^{3}H\)]MPP\(^{+}\) efflux. The fractional rates of Zn\(^{2+}\)-related [\(^{3}H\)]MPP\(^{+}\) efflux were generated by subtraction of the value of basal efflux (mean of the first 6 min of fraction collection under control conditions) from the value of efflux in the presence of Zn\(^{2+}\). Symbols represent means \pm S.E. of six to ten observations (one observation equals one superfusion chamber; all experiments were performed in duplicate).

**Quantitative Aspects of Zinc Enhancing Amphetamine-induced Efflux of MPP\(^{+}\)**—The enhancement exerted by Zn\(^{2+}\) may reflect a change in substrate affinity at the intracellular binding site of the transporter. Alternatively, Zn\(^{2+}\) may enhance the efficiency of reverse transport. To differentiate between these two possibilities, we preloaded the cells with varying amounts of [\(^{3}H\)]MPP\(^{+}\) and estimated the intracellular concentration by correcting for the cellular water space. Efflux of [\(^{3}H\)]MPP\(^{+}\) was induced by amphetamine (10 \(\mu M\)) in HEK-293 cells that expressed hDAT wt, hDAT-H193K, and hNET wt. The resulting saturation isotherm showed that Zn\(^{2+}\) (10 \(\mu M\)) clearly enhanced the V\(_{\text{max}}\) of outwardly directed transport induced by amphetamine only at the hDAT wt (Fig. 6A). In contrast, the K\(_{\text{m efflux}}\) was not affected by Zn\(^{2+}\) (Table II). As expected, Zn\(^{2+}\) did neither affect V\(_{\text{max}}\) for outward transport of [\(^{3}H\)]MPP\(^{+}\) nor K\(_{\text{m efflux}}\) in hDAT-H193K and hNET wt (Fig. 6, B and C).

Efflux of MPP\(^{+}\) Induced by Depolarizing Conditions Is Also Enhanced by Zinc—As expected, this facilitation of transport reversal was not seen with the mutant form hDAT-H193K (Fig. 7B).
equivocal evidence against the facilitated exchange-diffusion model, because it was not possible to separate inward and outward transport (27, 35).

In the present study we demonstrate that Zn$^{2+}$ discriminated between inward and outward transport mediated by hDAT; Zn$^{2+}$ blocked uptake of substrates but promoted efflux. The actions of Zn$^{2+}$ were specific and not accounted for by any site of action other than the transporter molecule hDAT per se, because (i) it was abrogated by mutation of a single His residue Zn$^{2+}$ (His$^{193}$) that participates in the coordination of the transition metal. (ii) Based on the Zn$^{2+}$ resistance of hSERT and hNET, we rule out an action on other cellular Zn$^{2+}$-binding proteins that are relevant for establishing the driving force for transport (e.g. Na$^+$/K$^+$-ATPase (36)). (iii) In hNET, mutational replacement of Lys$^{189}$, the residue homologous to His$^{193}$ in hDAT, is predicted to result in the formation of a tridentate coordination sphere for Zn$^{2+}$ (His$^{193}$), because the other two residues are conserved at the homologous positions (19, 20). Accordingly, $[^{3}H]$MPP$^+$ release was enhanced by Zn$^{2+}$ in hNET-K189H. Although qualitatively similar (i.e. of comparable affinity), the effect of Zn$^{2+}$ on hNET-K189H was less pronounced in magnitude than that on wild type hDAT. Most likely, this difference can be rationalized by subtle structural and functional differences between the two transporters. Thus, the observation that Zn$^{2+}$ can enhance substrate release while simultaneously blocking substrate uptake invalidates the facilitated exchange-diffusion model. Zn$^{2+}$ enhanced MPP$^+$-induced efflux; contrary to amphetamine, MPP$^+$ cannot enter the cells by simple diffusion to any appreciable extent. It is therefore conceivable that binding of the substrate to the extracellular site of the transporter rather than substrate influx suffices to induce transport reversal. Accordingly, influx and efflux must represent discrete operational modes of the transporter.

It has been generally assumed that, under physiological conditions, monoamine transporters operate exclusively in the inward mode; their main physiological task is to rapidly remove exocytotically released neurotransmitter from the synapse (37). The importance of reverse transport, which results in non-exocytotic, Ca$^{2+}$-independent release of neurotransmitter (38), has long been relegated to pathophysiological situations (e.g. ischemia, see Ref. 38) or to drug abuse (e.g. amphetamine derivatives and other psychostimulants, see Ref. 34). However, most recently, a physiological role was reported for the reverse mode of operation: Upon excitation of glutamatergic input from the subthalamic nucleus, dopaminergic neurons in the substantia nigra release dopamine due to transport reversal. This non-exocytotically released dopamine suffices to support dendrodendritic autoinhibition (6). In many brain regions, Zn$^{2+}$ is stored in synaptic vesicles and co-released together with glutamate; under basal conditions, the extracellular levels of Zn$^{2+}$ are low (∼10 nM; see Refs. 39, 40). Upon neuronal stimulation, however, Zn$^{2+}$ is co-released with the neurotransmitters and, consequently, the free Zn$^{2+}$ concentration may transiently reach values that range from 10–20 μM (10) up to 300 μM (11). The concentrations of Zn$^{2+}$ shown in this study, required for the stimulation of dopamine release (as well as inhibition of
uptake), covered this physiologically relevant range, with maximum stimulation occurring at 3–30 μM. It is therefore conceivable that the action of Zn²⁺ on hDAT does not merely reflect a biochemical peculiarity but that it is physiologically relevant. This conjecture is supported by the finding that the facilitating action of Zn²⁺ can also be demonstrated in rat striatal slices. Furthermore, we showed, in transfected cells, that efflux induced by membrane depolarization is also enhanced by Zn²⁺. Interestingly, reversal of the Na⁺ gradient per se (by iso-osmotic replacement of Na⁺ with choline) was not enhanced by Zn²⁺. The mechanistic basis for this discrimination remains obscure, and its implication is unclear, because in neurons, where DAT is expressed physiologically, there is no reversal of the Na⁺ gradient without depolarization. Transport reversal by depolarization has recently been documented in neurons ex vivo; it was suggested that the release of glutamate depolarized the dendrites resulting in transport reversal and subsequent release of dopamine non-exocytotically from the dendritic region (6). Thus, when Zn²⁺ is co-released with glutamate, it may greatly augment the efflux of dopamine.

Interestingly, the enhancement of substrate-induced release was only observed for the endogenous Zn²⁺ binding site; the engineered Zn²⁺ binding site in which a His residue was introduced in TM7 at position 377, i.e. on top of coordinating His³⁷¹ (21). Thus, two of the three coordinating residues (i.e. His³⁷¹ and Glu³⁹⁶ in TM8) were the same as in the endogenous Zn²⁺ binding site. Because of the distance constraints, the transition metal ion cannot assume a position that differs vastly from that
TABLE II
Kinetic constants of \( ^{3}H\)MPP\(^{+}\) efflux performed with HEK-293 cells expressing monoamine transporters and mutant constructs

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Control</th>
<th>Zinc (10 ( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max, efflux}} )</td>
<td>( K_{M, \text{efflux}} )</td>
</tr>
<tr>
<td></td>
<td>pmol/min/10(^{6}) cells</td>
<td>( \mu )M</td>
</tr>
<tr>
<td>hDAT wt</td>
<td>24.3 ± 4.9</td>
<td>2.9 ± 0.9</td>
</tr>
<tr>
<td>hDAT-H193K</td>
<td>45.5 ± 20.7</td>
<td>2.6 ± 2.2</td>
</tr>
<tr>
<td>hNET wt</td>
<td>64.4 ± 13.7</td>
<td>4.2 ± 1.7</td>
</tr>
</tbody>
</table>

Fig. 8. Influence of Zn\(^{2+}\) on amphetamine-induced \( ^{3}H\)MPP\(^{+}\) efflux in rat striatal slices. Rat striatal slices were preloaded with \( ^{3}H\)MPP\(^{+}\) and superfused using KRH supplemented with EDTA (10 \( \mu\)M), or left at control conditions (■). After seven fractions (from 14 min onward) amphetamine (10 \( \mu\)M) was added. Data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the slices at the beginning of that fraction. Symbols represent means ± S.E. of twelve observations (one observation equals one superfusion chamber; all experiments were performed in triplicate).

in the endogenous Zn\(^{2+}\) binding site. Nevertheless, Zn\(^{2+}\) failed to enhance outward transport, although inward transport was potently suppressed. This discrepancy points to a critical role for extracellular loop 2, which carries His\(^{193}\) in mediating the action of Zn\(^{2+}\) on reverse transport. But how can Zn\(^{2+}\) binding concomitantly lead to inhibition of inward transport and facilitation of outward transport? Previous data have shown that Zn\(^{2+}\) inhibits translocation but not substrate binding to the transporter (19). This led to the conjecture that Zn\(^{2+}\) imposed a conformational constraint on the transporter, which impeded movements critical for the translocation process (19). As a result, the turnover rate was predicted to be diminished with stabilization of the transporter mainly in a conformation with the substrate binding site open to the extracellular environment (outward facing conformation (19)). Clearly, the present data are inconsistent with a simple inhibition of the transporter turnover rate in the presence of Zn\(^{2+}\). Rather they suggest that Zn\(^{2+}\) binding facilitates the return step of the transporter in a way that increases the chances that a substrate molecule is carried with the transporter from the intracellular to the extracellular environment. As a net result, the transporter still primarily accumulates in the outward facing conformation. However, when assessed in a quantitative manner, efflux of MPP\(^{+}\) did not increase due to an altered affinity of MPP\(^{+}\) for the transporter from the intracellular side; the increased probability of outward transport is solely accounted for by the higher efficiency with which substrate is carried to the extracellular side in the presence of Zn\(^{2+}\) (Fig. 6). However, Zn\(^{2+}\) cannot \textit{per se} induce efflux; thus, substrate needs to be bound simultaneously at the outward and the inward facing end of the permeation pathway. This is difficult to envisage if the transporter is viewed as a monomeric unit that contains a single permeation pore. It may therefore be interesting to consider the possibility that the transporter does not operate by a simple alternating access scheme. For example, the conformational constraint that Zn\(^{2+}\) imposes on the tertiary structure may facilitate the opening of the alternative pathway that allows intracellular substrate to permeate through the hydrophobic core of the transporter. Alternatively, a parsimonious

**Fig. 7. Influence of Zn\(^{2+}\) on \( ^{3}H\)MPP\(^{+}\) efflux-induced high potassium conditions.** Time course of the effects of Zn\(^{2+}\) on MPP\(^{+}\)-induced efflux from HEK-293 cells expressing hDAT wt (A, ■), or hDAT-H193K (B, ▲). Cells expressing hDAT wt (A, ■) or hDAT-H193K (B, ▲) were preloaded with \( ^{3}H\)MPP\(^{+}\) and superfused, and 2-min fractions were collected. After three fractions (6 min) of basal efflux, cells were exposed to Zn\(^{2+}\) (10 \( \mu\)M), or left at control conditions (■). After seven fractions (from 14 min onward) amphetamine (10 \( \mu\)M) was added. Data are presented as fractional efflux, i.e. each fraction is expressed as the percentage of radioactivity present in the cells at the beginning of that fraction. Symbols represent means ± S.E. of nine observations (one observation equals one superfusion chamber; all experiments were performed in triplicate). The statistical significance was assessed by Student’s t test for paired samples (*, \( p < 0.05\); **, \( p < 0.01\).
explanation of the data is the hypothesis that there is only a single path for substrate but that the functional unit is not the monomeric transporter. Precedent for oligomeric channels or transporters with more than one pore per functional unit include Cl⁻ channels (two pores/homodimer, Ref. 41) and aquaporins (four pores/tetramer, Ref. 42). In the presence of Zn²⁺, binding of extracellular substrate to one transporter favors translocation of intracellular substrate by the second transport unit in the oligomer. This scheme is supported by the fact that transporters constitutively form oligomers (43, 44). Based on this model, mutants that are defective in oligomerization ought to be incapable of supporting reverse transport. This prediction is currently being explored.

REFERENCES