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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 Zn^{2+} binding site with three coordinating residues on its extracellular face (His^{193}, His^{375}, and Glu^{396}). Upon binding to this site, Zn^{2+} causes inhibition of [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP^+) uptake. We investigated the effect of Zn^{2+} on outward transport by superfusing hDAT-expressing HEK-293 cells preloaded with [^{3}H]MPP^+. Although Zn^{2+} inhibited uptake, Zn^{2+} facilitated [^{3}H]MPP^+ release induced by amphetamine, MPP^+, or K^+-induced depolarization specifically at hDAT but not at the human serotonin and the norepinephrine transporter (hNET). Mutation of the Zn^{2+} coordinating residue His^{193} to Lys (the corresponding residue in hNET) eliminated the effect of Zn^{2+} on efflux. Conversely, the reciprocal mutation (K189H) conferred Zn^{2+} sensitivity to hNET. The intracellular [^{3}H]MPP^+ concentration was varied to generate saturation isotherms; these showed that Zn^{2+} increased V_{max} for efflux (rather than $K_{M\text{-}Efflux\text{-}intracellular}$). Thus, blockage of inward transport by Zn^{2+} 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 Zn^{2+}, because Zn^{2+} 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,[1] 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 D_2-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 Zn^{2+} is widely distributed in the central nervous system (7). Zn^{2+} serves as a chelated counter ion for stored neurotransmitters in synaptic vesicles (8) and, upon nerve stimulation, Zn^{2+} 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 Zn^{2+} 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 Zn^{2+} is very interesting, because the activity of several neurotransmitter receptors and transporters are modulated by micromolar concentrations of Zn^{2+} (13–16). For example, Zn^{2+} has recently been reported to block transport-associated ion currents through glutamate transporter subtypes (salamander excitatory amino acid transporter (17) and EAAT1 (15)). Moreover, Zn^{2+} was found to enhance binding of cocaine analogues to and inhibit uptake of dopamine by synaptosomal membranes (18), an effect that is accounted for by direct binding of Zn^{2+} to hDAT (19).

The high affinity Zn^{2+} binding site in wild type hDAT was mapped to three coordinating residues situated on the extracellular face of the transporter, His^{193} in the large extracellular loop between transmembrane segment (TM) 3 and 4, His^{375} at the external end of TM 7 and Glu^{396} at the external end of TM 8 (19, 20). The inhibitory effect on uptake suggests that, by binding to the transporter, Zn^{2+} constrains relative movements between extracellular loop 2, TM 7, and TM 8 that are critical for the translocation process. Moreover, due to the strict

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[1] The abbreviations used are: hDAT, human dopamine transporter; hNET, human norepinephrine transporter; hSERT, human serotonin transporter; HEK-293, human embryonic kidney 293 cells; MPP^+, 1-methyl-4-phenylpyridinium; wt, wild type; TM, transmembrane segment; CMV, cytomegalovirus.

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 γ-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 pRC/CMV were kindly provided by Dr. M. G. Caron (Duke University, Durham, NC (24)); cDNA encoding the hSERT in pcDNA3 (25) was a generous gift of 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). Stable 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, 5×10^5 HEK-293 cells transiently or permanently expressing the hDAT, hNET, hSERT, or mutants were seeded onto poly-D-lysine-coated 48-well plates, and influx was measured 1–2 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]1-methyl-4-phenylpyridinium ([^{3}H]MPP^+, 0.2 μCi, 88.5 Ci/mmol) and various concentrations of unlabeled MPP^+ (range: 0.03–300 μM; 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 μM) as well as more 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}H]MPP^+ (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, 4×10^5 cells per coverslip) then incubated with [^{3}H]MPP^+ (0.8 μCi, final concentration 10 μM) 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}H]MPP^+ concentrations (2–128 μM). Intracellular [^{3}H]MPP^+ 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]H_2O-[^{14}C]inulin method (27)).

In experiments in which high K^+ was used or Na^+ was omitted, NaCl was iso-osmotically replaced by KCl or choline chloride, respectively.

Female Sprague-Dawley rats (200–250 g, Forschungsanstalt für Versuchstierzucht, Himberg, Austria) were used to perform *ex vivo* 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 μM [^{3}H]MPP^+, washed twice, and inserted into the superfusion chambers; KRH was supplemented with 10 μM 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 stable basal efflux of radioactivity and to chelate free zinc; superfusate samples were collected at 2-min intervals. Under the given EDTA concentration, Zn^{2+} was added at 20 μM resulting in a concentration of 10 μM 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}H]MPP^+ 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 Calculation—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}H]dopamine by hDAT, because it interacted with an endogenous Zn^{2+} binding site (19). In the present study, we used [^{3}H]MPP^+ as a substrate rather than dopamine for the following reasons: (i) [^{3}H]MPP^+ 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}H]MPP^+ 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}H]MPP^+ by hDAT (Fig. 1*A*), hNET (Fig. 1*B*) and hSERT (Fig. 1*C*). As expected, Zn^{2+} only blocked hDAT with high affinity (*squares* in Fig. 1*A*) whereas uptake by wild type hNET (*circles* in Fig. 1*B*) and hSERT (Fig. 1*C*) required concentrations of Zn^{2+} that exceeded 100 μM. The endogenous Zn^{2+} binding site of hDAT was previously mapped, and His^{193} on the extracellular loop 2 was shown to be of critical importance in forming the coordination sphere. If the binding of Zn^{2+} to hDAT was impeded by substituting His^{193} with Lys (*i.e.* the residue found in the corresponding position of hNET), high affinity inhibition was abrogated and Zn^{2+} sup-

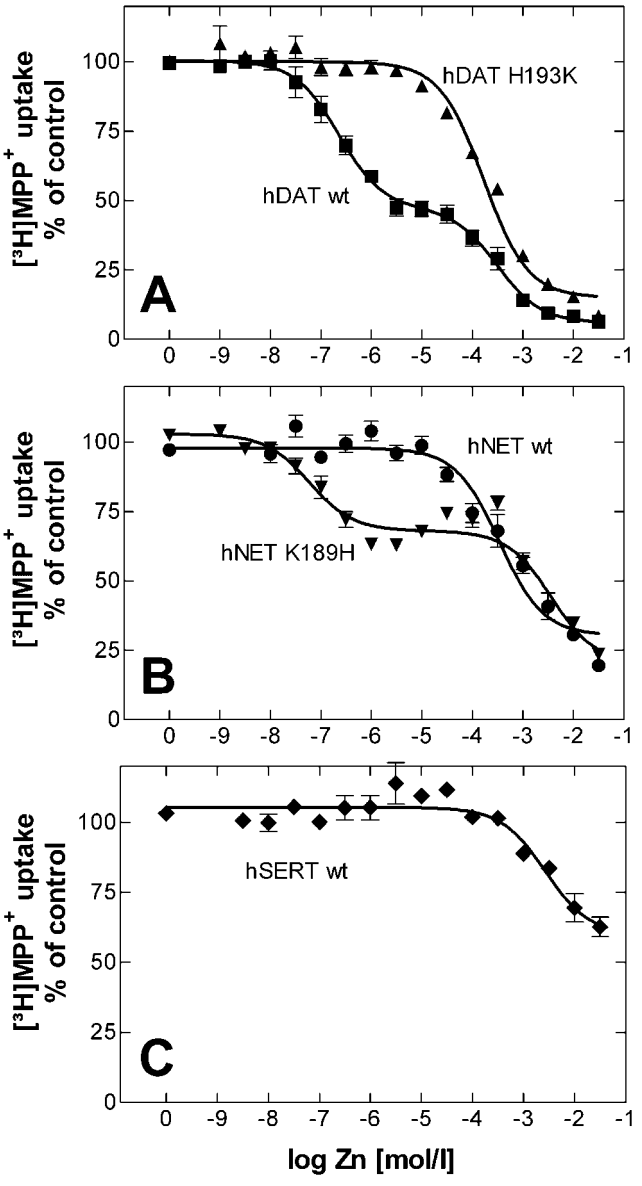

FIG. 1. **Effects of Zn^{2+} on the uptake of [^{3}H]MPP^+ by monoamine transporters and mutant constructs.** HEK-293 cells expressing wild type monoamine transporters and mutant constructs were distributed in 48-well plates (5×10^5 cells). The washed cells were preincubated in Krebs-Ringer-Hepes (KRH) buffer (0.1 ml) containing Zn^{2+} in the concentrations indicated. After 5 min, the buffer was replaced by KRH containing Zn^{2+} and [^{3}H]MPP^+ (50 nM). After 8 min at room temperature, uptake was terminated and radioactivity was determined by liquid scintillation counting. For $K_{m,\ \mathrm{Influx}}$ and IC_{50} values see Table I. *A*: hDAT wt, ■; hDAT-H193K, ▲. *B*: hNET wt, ●; hNET-K189H, ▼. *C*: hSERT wt, ♦. 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. 1*A*). Conversely, the corresponding mutation in hNET (*i.e.* replacing Lys^{189} with His) conferred high affinity inhibition by Zn^{2+} to hNET (*triangles* in Fig. 1*B*). The K_m values for [^{3}H]MPP^+ transport as well as IC_{50} values for Zn^{2+} are summarized in Table I. Based on these data we conclude that transport of [^{3}H]MPP^+ faithfully reproduces the data previously obtained by evaluating the Zn^{2+} sensitivity of monoamine transporters using their endogenous substrates (19); it is also safe to conclude from the available evidence that Zn^{2+} suppresses the transport of all substrates by hDAT.

Zinc Enhances Efflux Induced by Amphetamine at hDAT—Because Zn^{2+} inhibited uptake of [^{3}H]MPP^+ at the hDAT, a similar blockage was to be anticipated for release if efflux simply reflected reversal of transport. This was not the case. Cells that expressed hDAT were preloaded with [^{3}H]MPP^+. Upon challenge with a maximally effective concentration of amphetamine (10 μM), transport reversal was induced and this resulted in release of [^{3}H]MPP^+ (Fig. 2*A*). Surprisingly, this amphetamine-elicited efflux was markedly enhanced, rather than inhibited, by the addition of 10 μM Zn^{2+} to the superfusion buffer (Fig. 2*A*, *open squares*). We stress that Zn^{2+} *per se* did not affect basal efflux (Fig. 2*A*). The modulatory effect of Zn^{2+} was lost upon mutational exchange of all three coordinating residues (hDAT-H193K-H375A-E396Q, $n = 3$, data not shown). In fact, mutation of a single residue, namely His^{193} to Lys (the corresponding residue found in hNET), sufficed to abolish the enhancing effect of Zn^{2+}; the extent of amphetamine-elicited release was comparable in hDAT-H193K-expressing cells (*cf. closed symbols* in Fig. 2, *A* and *B*). However, Zn^{2+} did not affect transport reversal to any appreciable extent (*open symbols*, Fig. 2*B*).

In contrast to wild type hDAT, release from cells expressing the hNET was not affected by co-application of Zn^{2+} (Fig. 2*C*). We have exploited this insensitivity to ask if Zn^{2+}-enhanced outward transport was conferred to hNET upon replacement of Lys^{189} by the appropriate Zn^{2+} coordinating ligand (*i.e.* His). In fact, in cells expressing hNET-K189H, Zn^{2+} promoted efflux that had been induced by amphetamine (*open symbols* in Fig. 2*D*); although the effect was less pronounced than that seen with hDAT wt. Finally, Zn^{2+} did not affect amphetamine-induced release of [^{3}H]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 Zn^{2+} (data not shown).

We tested Zn^{2+} over a wide concentration range; up to 300 μM, Zn^{2+} did not affect basal release of [^{3}H]MPP^+ from preloaded cells that expressed hDAT, hNET, or hSERT (see *inset* to 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 Zn^{2+} (see Fig. 3, *inset*) and efflux through hDAT wt, hNET wt, and hDAT-H193K, which was stimulated to some extent by 1 mM Zn^{2+} (see Fig. 3, *inset*).

In the presence of amphetamine, the efflux-enhancing effect

TABLE I

Kinetic constants of [^{3}H]MPP^+ uptake in HEK-293 cells expressing 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). For determination of the $K_{M\ \mathrm{Influx}}$ values, the washed cells were incubated in Krebs-Ringer-Hepes buffer (0.1 ml) containing [^{3}H]MPP^+ (0.2 μCi, specific activity 88.5 Ci/mmol) and various concentrations of unlabeled MPP^+ (range: 0.03–300 μM). For determination of $IC_{50\ \mathrm{zinc}}$ values, Zn^{2+} was added 5 min prior to [^{3}H]MPP^+ (50 nM). After 8 min at room temperature, uptake was terminated and radioactivity was determined by liquid scintillation counting. $K_{M\ \mathrm{Influx}}$ and IC_{50} values were calculated from non-linear regression analysis of uptake data and represent mean values ± S.E. (*numbers* in *parentheses* denote number of experimental observations performed in triplicate).

cDNA	$K_{M\ \mathrm{Influx}}$	IC_{50} (Zn^{2+})
		μM
hDAT wt (6)	18.0 ± 3.5	0.23 ± 0.01
		313 ± 1.2
hDAT-H193K (3)	16.4 ± 2.3	169 ± 0.5
hDAT-H193K-V377H (3)	17.8 ± 2.4	12.15 ± 0.1
hNET wt (4)	1.7 ± 0.6	346 ± 0.9
hNET-K189H (4)	2.3 ± 0.9	0.06 ± 0.01
		3684 ± 16
hSERT (3)	19.7 ± 1.0	2682 ± 12

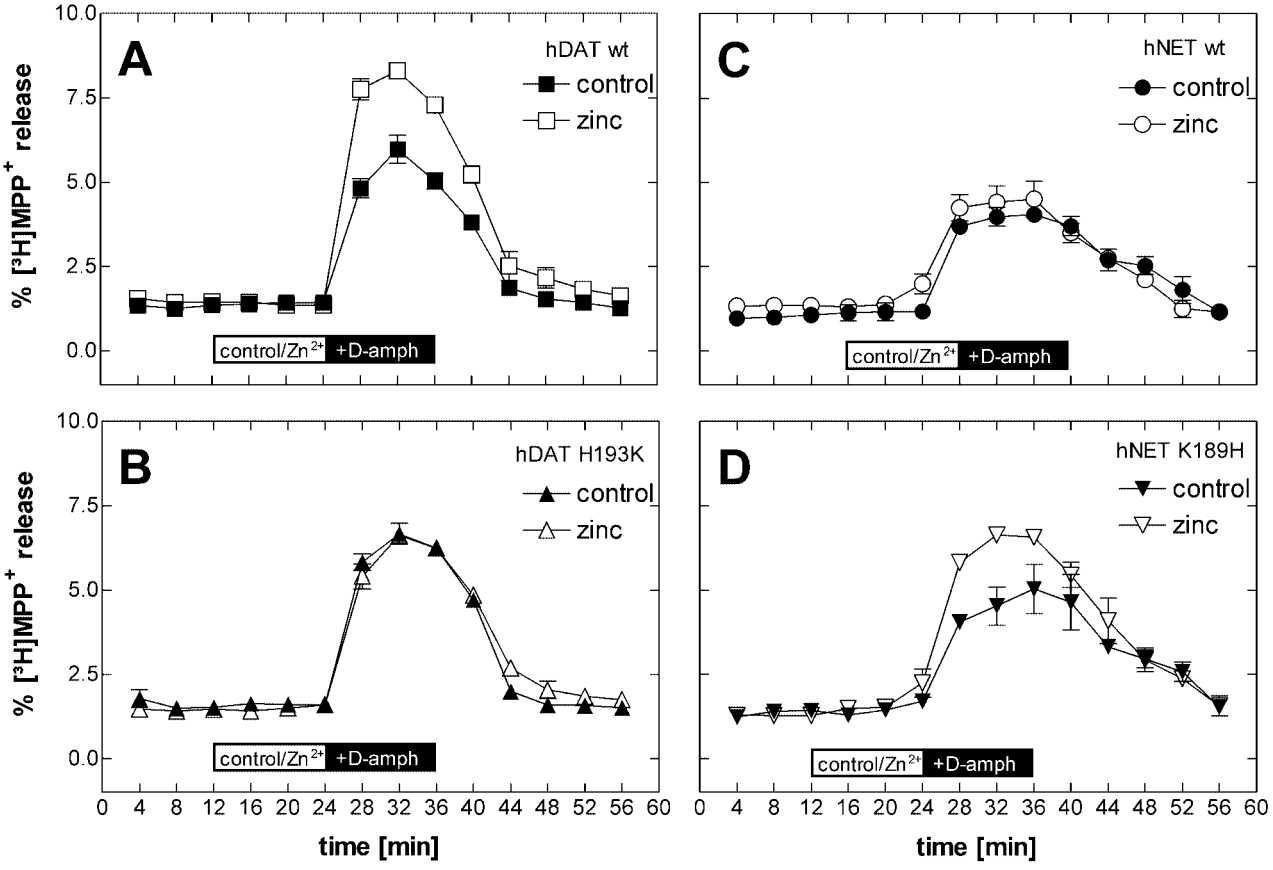

FIG. 2. **Influence of Zn^{2+} on amphetamine-induced [^{3}H]MPP^+ efflux.** Cells were preloaded with [^{3}H]MPP^+ and superfused upon reaching a stable baseline (basal efflux: mean of the three fractions before drug addition; hDAT wt: *panel A*, ■/□, basal efflux: 0.247 ± 0.004%·min^{-1}, *i.e.* 245.6 ± 6.7 dpm·min^{-1}, n = 60 observations of randomly chosen experiments performed on different days; hDAT-H193K: *panel B*; ▲/△, basal [^{3}H]MPP^+ efflux: 0.433 ± 0.08%·min^{-1}, *i.e.* 181.2 ± 7.1 dpm·min^{-1}, n = 47; hNET wt: *panel C*, ●/○, basal [^{3}H]MPP^+ efflux: 0.087 ± 0.004%·min^{-1}, *i.e.* 125.9 ± 5.3 dpm·min^{-1}, n = 60; hNET-K189H: *panel D*, ▼/▽, basal [^{3}H]MPP^+ efflux for hNET-K189H: 0.147 ± 0.006%·min^{-1}, *i.e.* 185.2 ± 8.2 dpm·min^{-1}, n = 56). The experiment was started with the collection of 4-min fractions. After three fractions (12 min) of basal efflux, cells were exposed to Zn^{2+} (10 μM), or left at control conditions as indicated. After six fractions (from 24 min and onward), amphetamine (*panels A* and *B*: 10 μM, *panels C* and *D*: 1 μM) was added to all superfusion channels. After nine fractions (from 36 min and onward), all channels were switched back to control conditions. 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 six to twelve observations (one observation equals one superfusion chamber; all experiments were performed in triplicate).

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. 1*A*), 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 [^{3}H]MPP^+, Zn^{2+} markedly enhanced efflux induced by MPP^+ only if the cells expressed hDAT wt (*open symbols* in Fig. 4*A*) or hNET-K189H (*open symbols* in Fig. 4*D*). 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 [^{3}H]MPP^+ also supported the interpretation that release does not require inward transport of substrate.

An Artificially Introduced Zinc Binding Site Cannot Replace the Endogenous Binding Site in hDAT wt—The effect of Zn^{2+} could be conferred hNET by introducing a Zn^{2+} binding site; however, this Zn^{2+} binding site was created at a position homologous to that of the endogenous Zn^{2+} binding site of hDAT. We have therefore also addressed the specificity of the endogenous Zn^{2+} binding site by testing if Zn^{2+} promoted release indiscriminately, provided that it was bound on the extracellular surface of hDAT. We analyzed a mutant hDAT that con-

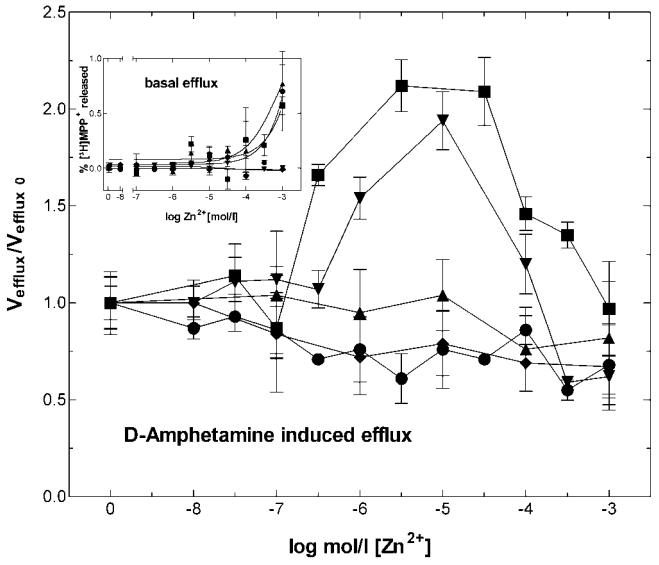

FIG. 3. **Concentration-response relationship of amphetamine and Zn^{2+} induced [^{3}H]MPP$^+$ release.** HEK-293 cells expressing hDAT wt (■; [amphetamine]: 10 μM), hDAT-H193K (▲; [amphetamine]: 10 μM), hNET wt (●; [amphetamine]: 1 μM), hNET-K189H (▼; [amphetamine]: 1 μM), or hSERT wt (♦; [amphetamine]: 10μM; basal [^{3}H]MPP$^+$ efflux: 0.224 ± 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. $V_{efflux}/V_{efflux\ 0}$ 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 ± S.E. of six to ten observations (one observation equals one superfusion chamber; all experiments were performed in duplicate).

tained an engineered Zn^{2+} binding site: In this mutant, His^{193} was changed to Lys and a new Zn^{2+} coordinating histidine was inserted in position 377 resulting in a tridentate Zn^{2+} binding site with His^{375}, His^{377}, and Glu^{396} as coordinating residues (21). This mutant transported [^{3}H]MPP$^+$ with a K_m that was comparable to wt hDAT (Table I). Contrary to hDAT wt, in this hDAT-H193K-V377H, the inhibition curve for Zn^{2+} was monophasic (*triangles* in Fig. 5*A*). This discrepancy possibly reflects the fact that the engineered Zn^{2+} binding site had a less favorable geometry; thus the low affinity, inhibitory component was less resolved from the newly created high affinity component to allow for a robust biphasic curve fitting and hence resolution of two sites. Nevertheless, a fit to the overall inhibition curve gives a minimum estimate for the inhibitory potency of Zn^{2+} on [^{3}H]MPP$^+$ uptake (IC_{50} = 12.4 μM; see Table I). Most importantly, the engineered Zn^{2+} binding site failed to support enhancement of amphetamine induced efflux (Fig. 5*B*).

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 μM) in HEK-293 cells that expressed hDAT wt, hDAT-H193K, and hNET wt. The resulting saturation isotherm showed that Zn^{2+} (10 μM) clearly enhanced the V_{max} of outwardly directed transport induced by amphetamine only at the hDAT wt (Fig. 6*A*). In contrast, the $K_{m,\ EFFLUX}$ was not affected by Zn^{2+} (Table II). As expected, Zn^{2+} did neither affect V_{max} for outward transport of [^{3}H]MPP$^+$ nor $K_{m,\ EFFLUX}$ in hDAT-H193K and hNET wt (Fig. 6, *B* and *C*).

Efflux of MPP$^+$ Induced by Depolarizing Conditions Is Also Enhanced by Zinc—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 isoosmotic replacement of Na^+ by choline was neither enhanced by Zn^{2+} (10 μM) 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 μM) at the hDAT wt (Fig. 7*A*); as expected, this facilitation of transport reversal was not seen with the mutant form hDAT-H193K (Fig. 7*B*).

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 μM) 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 μM) did not enhance basal [^{3}H]MPP$^+$ efflux (Fig. 8, *open squares*, min 2–14). In contrast, efflux elicited by amphetamine (10 μM) 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 ± S.D. = 5.85 ± 2.56 and 8.39 ± 3.56%·min 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, methylenedioximethamphetamine ("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-exocytotic 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 refuted, 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-

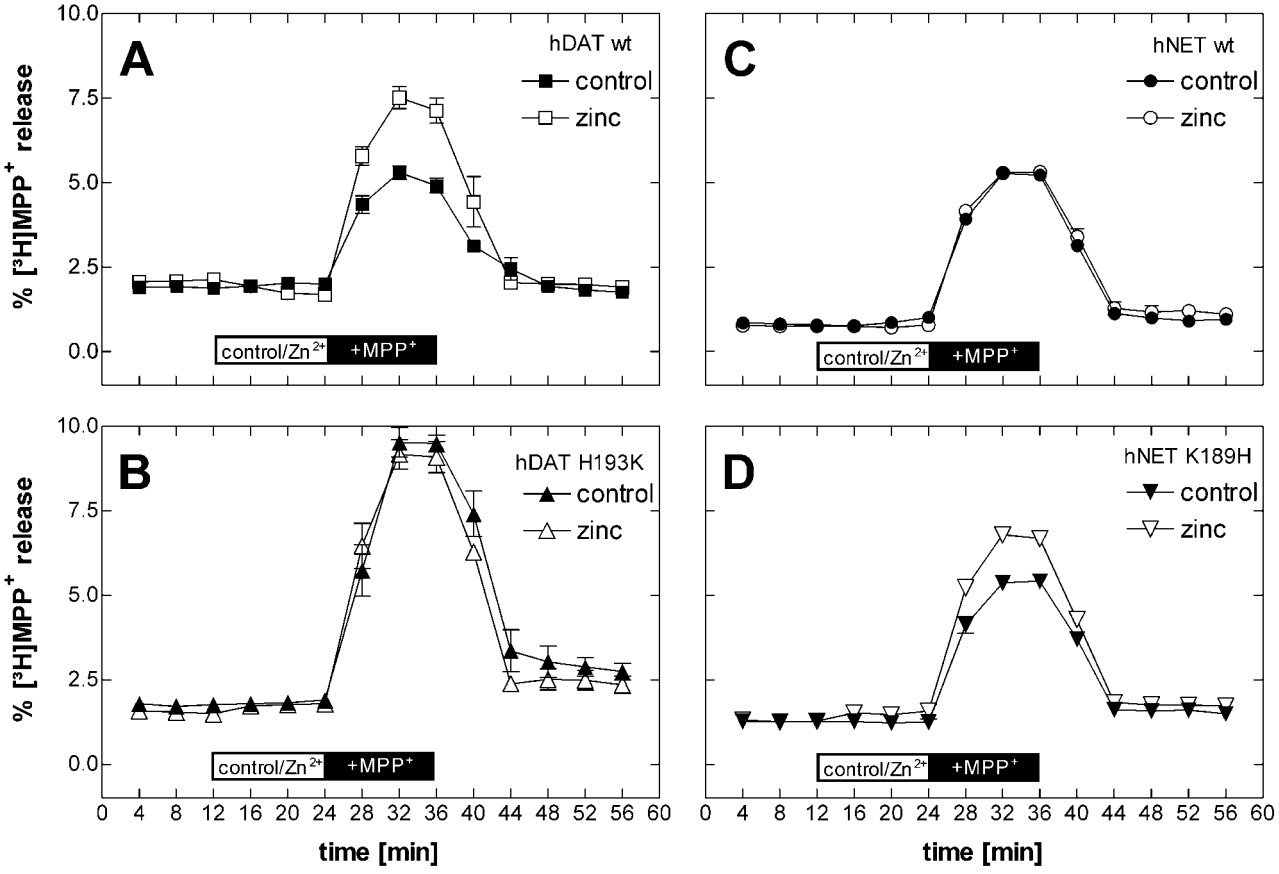

FIG. 4. **Influence of Zn^{2+} on MPP^+ induced [^{3}H]MPP^+-efflux.** Time course of the effects of Zn^{2+} on MPP^+-induced efflux from HEK-293 cells expressing hDAT wt (*A*, ■/□), hDAT-H193K (*B*, ▲/△), hNET wt (*C*, ●/○), or hNET-K189H (*D*, ▼/▽). Cells were preloaded with [^{3}H]MPP^+ and superfused, and 4-min fractions were collected. After three fractions (12 min) of basal efflux, cells were exposed to Zn^{2+} (10 μM), or left at control conditions as indicated. After six fractions (from 24 min onward), MPP^+ (100 μM) was added to all superfusion channels. After nine fractions (from 36 min and onward) all channels were switched back to control conditions. 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 six to twelve observations (one observation equals one superfusion chamber; all experiments were performed in triplicate).

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+}, 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

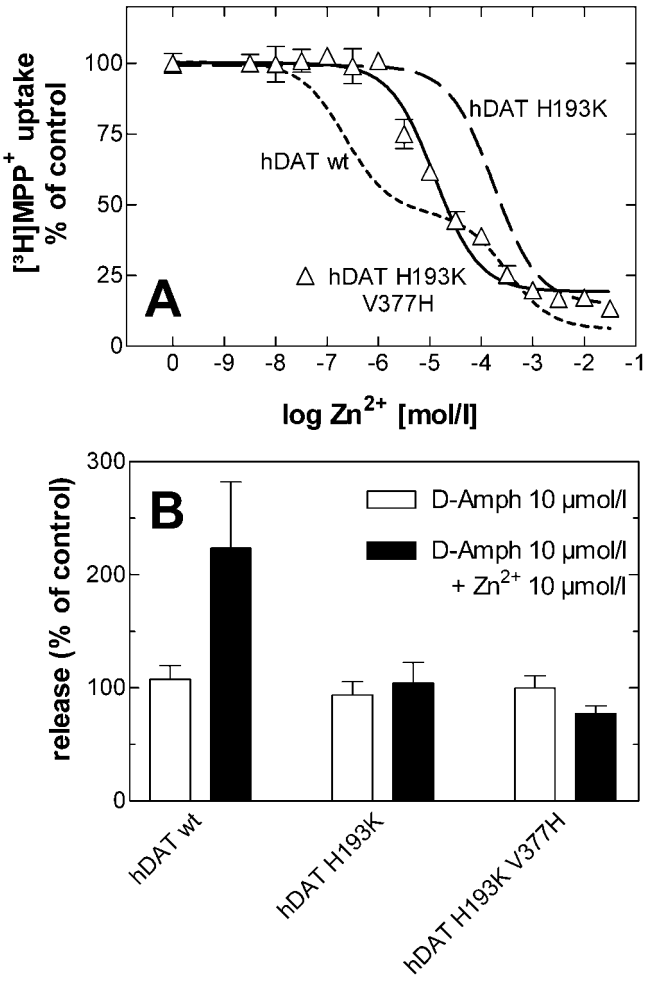

FIG. 5. **The influence of Zn^{2+} on uptake and efflux of [^{3}H]MPP^+ in a mutant dopamine transporter that contains an artificially engineered Zn^{2+} binding site.** *A*, uptake of [^{3}H]MPP^+ by HEK-293 cells expressing hDAT-H193K-V377H in the presence of Zn^{2+}; for comparison, see uptake inhibition by Zn^{2+} at hDAT wt (*dotted line*) and hDAT-H193K (*dashed line*). For experimental details, see legend to Fig. 1*A*. *Symbols* represent data obtained in three independent determinations ± S.E. (performed in duplicate). *B*, amphetamine (10 μM) induced efflux from HEK-293 cells expressing hDAT wt, hDAT-H193K-V377H, and hDAT-H193K in the presence or absence of Zn^{2+}. The cells 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. Release is expressed as the percentage of that induced in the presence of amphetamine alone. *Bars* represent means ± S.E. of six observations (one observation equals one superfusion chamber; all experiments were performed in duplicate).

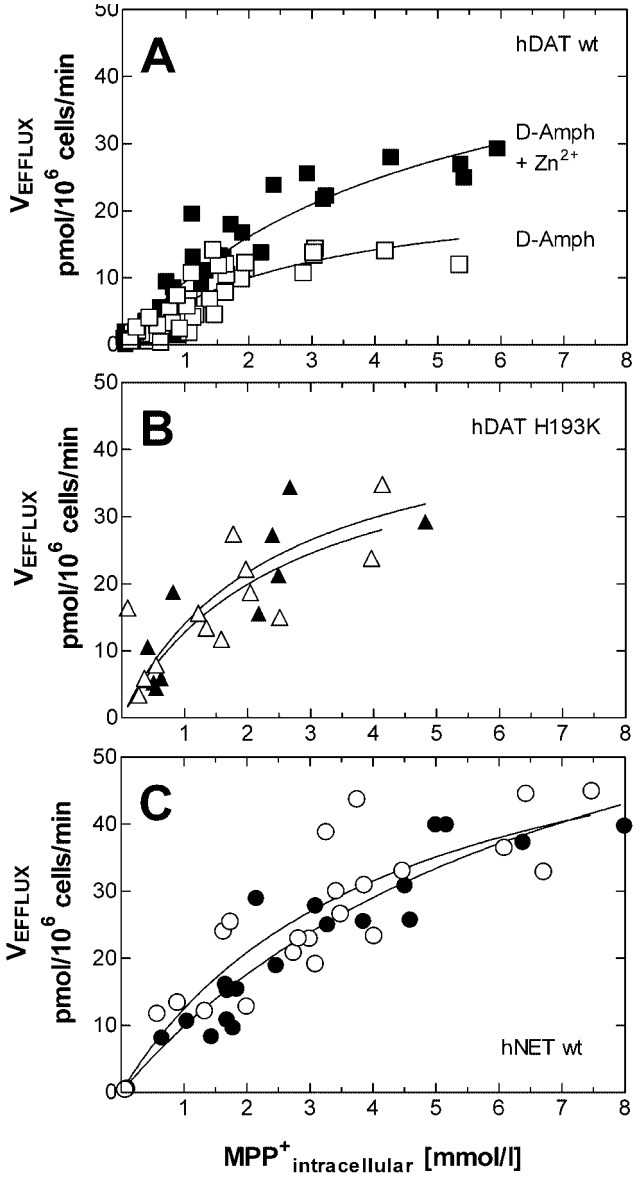

FIG. 6. **Quantitative measurements of amphetamine induced [^{3}H]MPP^+ release in the presence or absence of Zn^{2+}.** Cells were preloaded with different concentrations of [^{3}H]MPP^+ (2–128 μM) and superfused. According to the experimental protocol given in the legend to Table II, the cells were challenged with amphetamine in the presence or absence of Zn^{2+}. Efflux rates (V_{EFFLUX}, pmol/10^6 cells/min) were calculated from the mean value of the last three fractions with subtraction of basal efflux (first three fractions), with a number of 27,000 cells/coverslip and an intracellular volume of 1.1 pl (see "Methods"). *A*, hDAT wt (in the presence (■) or absence (□) of Zn^{2+}). *B*, hDAT-H193K (in the presence (▲) or absence (△) of Zn^{2+}). *C*, hNET wt (in the presence (●) or absence (○) of Zn^{2+}). Each *symbol* represents one observation of three to six experiments (all experiments were performed in duplicate).

uptake), covered this physiologically relevant range, with maximum stimulation occurring at 3–30 μM. It is therefore conceivable that the action of Zn^{2+} 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 Zn^{2+} can also be demonstrated in rat striatal slices. Furthermore, we showed, in transfected cells, that efflux induced by membrane depolarization is also enhanced by Zn^{2+}. Interestingly, reversal of the Na^+ gradient *per se* (by iso-osmotic replacement of Na^+ with choline) was not enhanced by Zn^{2+}. 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 Zn^{2+} 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 Zn^{2+} binding site; the engineered Zn^{2+} binding site in which a His residue was introduced in TM7 at position 377, *i.e.* on top of coordinating His^{371} (21). Thus, two of the three coordinating residues (*i.e.* His^{371} and Glu^{396} in TM8) were the same as in the endogenous Zn^{2+} 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

HEK-293 cells expressing wild type monoamine transporters and mutant constructs were seeded onto glass-coverslips in 96-well plates (4×10^5 cells), loaded with different concentrations of [^{3}H]MPP$^+$ (final concentration range, 2–128 μM), and superfused. Upon reaching a stable baseline, the experiment was started with the collection of 4-min fractions. After three fractions (12 min) of basal efflux, cells were exposed to Zn^{2+} (10 μM), or left at control conditions. After six fractions (from 24 min and onward), amphetamine (hDAT wt, hDAT-H193K: 10 μM, hNET wt: 1 μM) was added to all superfusion channels. Efflux rates (V_{Efflux}; pmol/10^6 cells/min) were calculated from the mean value of the last three fractions with subtraction of basal efflux (first three fractions), with a number of 27,000 cells/coverslip and an intracellular volume of 1.1 pl (see "Methods"). $V_{max,Efflux}$ and $K_{M\ Efflux}$ values were calculated from non-linear regression analysis of release data and represent mean values ± S.E. (three to six experimental observations performed in duplicate).

cDNA	Control		Zinc (10 μM)	
	$V_{max\ Efflux}$	$K_{M\ Efflux}$	$V_{max\ Efflux}$	$K_{M\ Efflux}$
	pmol/min/10^6 cells	μM	*pmol/min/10^6 cells*	μM
hDAT wt	24.3 ± 4.9	2.9 ± 0.9	52.8 ± 8.0	4.6 ± 1.1
hDAT-H193K	45.5 ± 20.7	2.6 ± 2.2	47.8 ± 15.6	2.4 ± 1.5
hNET wt	64.4 ± 13.7	4.2 ± 1.7	83.2 ± 19.1	7.5 ± 2.7

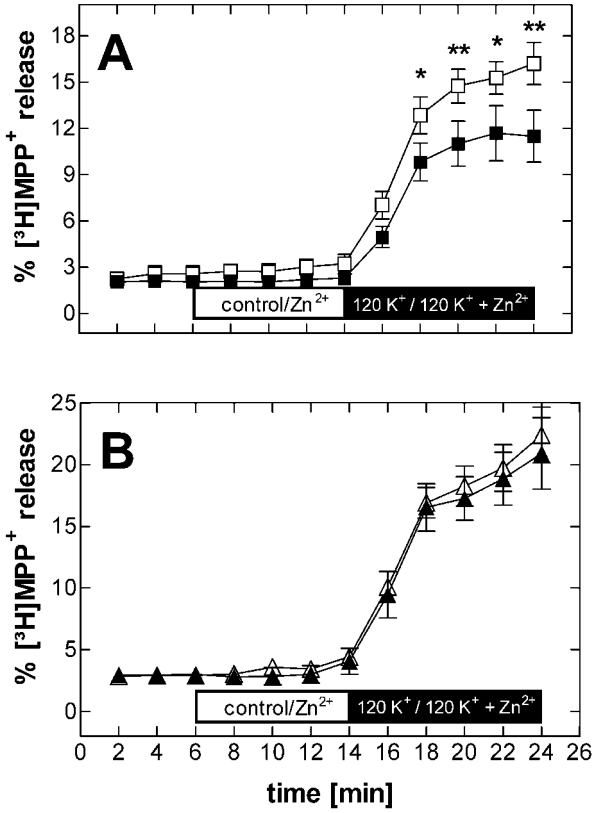

FIG. 7. **Influence of Zn^{2+} on [^{3}H]MPP$^+$ efflux-induced high potassium conditions.** Time course of the effects of Zn^{2+} on MPP$^+$-induced efflux from HEK-293 cells expressing hDAT wt (*A*, ■/□) or hDAT-H193K (*B*, ▲/△). Cells expressing hDAT wt (*A*, ■/□) or hDAT-H193K (*B*, ▲/△) were preloaded 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 μM), or left at control conditions as indicated. After seven fractions (from 14 min onward), buffer conditions were changed from KRH containing NaCl to KCl. 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 (*, $p < 0.05$; **, $p < 0.01$).

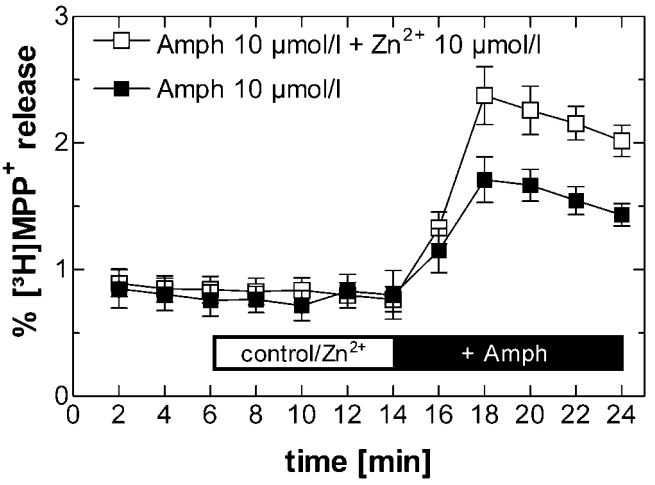

FIG. 8. **Influence of Zn^{2+} on amphetamine-induced [^{3}H]MPP$^+$ efflux in rat striatal slices.** Rat striatal slices were preloaded with [^{3}H]MPP$^+$ and superfused using KRH supplemented with EDTA (10 μM), and 2-min fractions were collected. After three fractions (6 min) of basal efflux, cells were exposed to KRH containing Zn^{2+} (20 μM, □), or left at control conditions (■). After seven fractions (from 14 min onward) amphetamine (10 μM) 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 *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

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 Zn^{2+}, binding of extracellular substrate to one transporter favors translocation of intracellular substrate by the second transporter 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

1. Rudnick, G. (1997) in *Neurotransmitter Transporters: Structure, Function, and Regulation* (Reith, M. E. A., ed) pp. 73–100, Humana Press Inc., Totowa, NJ
2. Nelson, N. (1998) *J. Neurochem.* **71,** 1785–1803
3. Iversen, L. (2000) *Mol. Psychiatry* **5,** 357–362
4. Seiden, L. S., Sabol, K. E., and Ricaurte, G. A. (1993) *Annu. Rev. Pharmacol. Toxicol.* **33,** 639–677
5. Levi, G., and Raiteri, M. (1993) *Trends. Neurosci.* **16,** 415–419
6. Falkenburger, B. H., Barstow, K. L., and Mintz, I. M. (2001) *Science* **293,** 2465–2470
7. Perez-Clausell, J., and Danscher, G. (1985) *Brain Res.* **337,** 91–98
8. Berneis, K. H., Pletscher, A., and Da Prada, M. (1969) *Nature* **224,** 281–282
9. Howell, G. A., Welch, M. G., and Frederickson, C. J. (1984) *Nature* **308,** 736–738
10. Vogt, K., Mellor, J., Tong, G., and Nicoll, R. (2000) *Neuron* **26,** 187–196
11. Assaf, S. Y., and Chung, S. H. (1984) *Nature* **308,** 734–736
12. Koh, J. Y., Suh, S. W., Gwag, B. J., He, Y. Y., Hsu, C. Y., and Choi, D. W. (1996) *Science* **272,** 1013–1016
13. Westbrook, G. L., and Mayer, M. L. (1987) *Nature* **328,** 640–643
14. Laube, B., Kuhse, J., Rundstrom, N., Kirsch, J., Schmieden, V., and Betz, H. (1995) *J. Physiol.* **483,** 613–619
15. Vandenberg, R. J., Mitrovic, A. D., and Johnston, G. A. (1998) *Mol. Pharmacol.* **54,** 189–196
16. Schetz, J. A., Chu, A., and Sibley, D. R. (1999) *J. Pharmacol. Exp. Ther.* **289,** 956–964
17. Spiridon, M., Kamm, D., Billups, B., Mobbs, P., and Attwell, D. (1998) *J. Physiol.* **506,** 363–376
18. Richfield, E. K. (1993) *Mol. Pharmacol.* **43,** 100–108
19. Norregaard, L., Frederiksen, D., Nielsen, E. O., and Gether, U. (1998) *EMBO J.* **17,** 4266–4273
20. Loland, C. J., Norregaard, L., and Gether, U. (1999) *J. Biol. Chem.* **274,** 36928–36934
21. Norregaard, L., Visiers, I., Loland, C. J., Ballesteros, J., Weinstein, H., and Gether, U. (2000) *Biochemistry* **39,** 15836–15846
22. MacAulay, N., Bendahan, A., Loland, C. J., Zeuthen, T., Kanner, B. I., and Gether, U. (2001) *J. Biol. Chem.* **276,** 40476–40485
23. Fischer, J. F., and Cho, A. K. (1979) *J. Pharmacol. Exp. Ther.* **208,** 203–209
24. Giros, B., el Mestikawy, S., Bertrand, L., and Caron, M. G. (1991) *FEBS Lett.* **295,** 149–154
25. Ramamoorthy, S., Bauman, A. L., Moore, K. R., Han, H., Yang, F. T., Chang, A. S., Ganapathy, V., and Blakely, R. D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2542–2546
26. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
27. Sitte, H. H., Hiptmair, B., Zwach, J., Pifl, C., Singer, E. A., and Scholze, P. (2001) *Mol. Pharmacol.* **59,** 1129–1137
28. Scholze, P., Sitte, H. H., and Singer, E. A. (2001) *Neurosci. Lett.* **309,** 173–176
29. Javitch, J. A., and Snyder, S. H. (1985) *Eur. J. Pharmacol.* **106,** 455–456
30. Buck, K. J., and Amara, S. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12584–12588
31. Cesura, A. M., Ritter, A., Picotti, G. B., and Da Prada, M. (1987) *J. Neurochem.* **49,** 138–145
32. Pifl, C., Drobny, H., Reither, H., Hornykiewicz, O., and Singer, E. A. (1995) *Mol. Pharmacol.* **47,** 368–373
33. Valenta, B., Drobny, H., and Singer, E. A. (1988) *J. Pharmacol. Exp. Ther.* **245,** 944–949
34. Sitte, H. H., Huck, S., Reither, H., Boehm, S., Singer, E. A., and Pifl, C. (1998) *J. Neurochem.* **71,** 1289–1297
35. Pifl, C., and Singer, E. A. (1999) *Mol. Pharmacol.* **56,** 1047–1054
36. Hexum, T. D. (1974) *Biochem. Pharmacol.* **23,** 3441–3447
37. Povlock, S. L., and Amara, S. G. (1997) in *Neurotransmitter transporters: Structure, Function, and Regulation* (Reith, M. E. A., ed) pp. 1–28, Humana Press, Totowa, NJ
38. Attwell, D., Barbour, B., and Szatkowski, M. (1993) *Neuron* **11,** 401–407
39. Frederickson, C. J., Howell, G. A., and Kaserkis, E. J. (1984) *Neurology and Neurobiology*, Alan R. Liss, Inc., New York
40. Frederickson, C. J. (1989) *Int. Rev. Neurobiol.* **31,** 145–238
41. Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T., and MacKinnon, R. (2002) *Nature* **415,** 287–294
42. Sui, H., Han, B. G., Lee, J. K., Walian, P., and Jap, B. K. (2001) *Nature* **414,** 872–878
43. Schmid, J. A., Scholze, P., Kudlacek, O., Freissmuth, M., Singer, E. A., and Sitte, H. H. (2001) *J. Biol. Chem.* **276,** 3805–3810
44. Hastrup, H., Karlin, A., and Javitch, J. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10055–10060