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Na^+ and the substrate permeation pathway in dopamine transporters

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Abstract

Advances have been made in characterizing the relationship between Na^+ and the substrate permeation pathway in the dopamine transporter. This review covers the role of Na^+ in co-transport with dopamine as well as in the recognition of dopamine. Apparent recognition depends on the preparation studied: it differs between intact cells heterologously expressing the dopamine transporter and membranes prepared from these cells. In our search for amino acid residues in the transporter involved in Na^+ action, W84 and D313 were found to play a special role in cation interaction, with evidence for regulation of both Na^+ and H^+ sensitivity. Mutation of D313 to N appeared to decrease the affinity for the dopamine transporter in intact cells, not by altering recognition per se. A model is proposed in which *access* of dopamine, not *recognition itself*, is regulated by D313 and Na^+. Thus, the role of external Na^+ in intact cell preparations is to turn dopamine transporters to the externally facing form, allowing access of dopamine to its binding site.

Keywords: DAT (dopamine transporter); Dopamine recognition; Cation; Na^+; Access model

1. Introduction

The monoamine transporters or carriers, located on the plasma membrane of nerve terminals, transport monoamine substrates across the membrane. By taking up substrate into neurons, they play a critical role in terminating biogenic amine neurotransmission and in maintaining monoamine homeostasis in the central nervous system (Giros et al., 1996; Jones et al., 1998; Whitby et al., 1960; Coyle and Snyder, 1969; Iversen, 1971; Shaskan and Snyder, 1970). The monoamine carriers include transporters for dopamine. Evidence has accumulated that dopaminergic transmission occurs, for a large part, by volume transmission involving migration of transmitter through the extracellular space, nonjunctional aminergic innervation of neurons, and location of biogenic amine transporters outside synapses (Zoli et al., 1999; Descarries et al., 1991; Carlsson et al., 1969; Garris and Wightman, 1994). Many substances, such as the psychostimulant amphetamine, the dopaminergic neurotoxin 1-methy-4-phenylpyridinium (MPP^+) and various sympathomimetic amines, structurally resemble dopamine. They are thus substrates for the dopamine transporter and can be transported (Jones et al., 1998; Millier et al., 1999). The dopamine transporter is also a major molecular target for the addictive drug cocaine and, to a lesser extent, antidepressants (Tatsumi et al., 1997; Amara and Sonders, 1998). Therefore, interactions with dopamine transporter proteins can have profound neurobiological, pathophysiological, and pharmacological consequences. In the past years, with the help of the cloned transporter (Kilty et al., 1991; Shimada et al., 1991; Giros et al., 1992; Vandenbergh et al., 1992), advances have been made in characterizing the relationship between Na^+ and the substrate permeation pathway in the dopamine transporter. The focus of this review is on the recent progress in this area.

2. Na^+ is co-transported with dopamine but what is its role in dopamine recognition?

2.1. Co-transport of Na^+ with dopamine translocation

As other members of the Na^+- and Cl^--dependent plasma membrane transporters, the dopamine transporter enables the transport of substrate together with Na^+ and Cl^- as co-substrates (McElvain and Schenk, 1992; Gu et al., 1994). Another plasma membrane protein, Na^+,K^+-ATPase, main-

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tains an inwardly directed transmembrane Na^+ gradient by pumping Na^+ ions out of, and K^+ into, the cell. This transmembrane gradient is important, because it allows the uphill transport of dopamine to be driven by the downhill Na^+ flux that accompanies dopamine translocation (Rudnick, 2002). The electrogenic activity of the Na^+,K^+-ATPase also contributes to the maintenance of the membrane potential, interior negative, which in turn causes Cl^- ions to be lost from the cell. Both the potential and Cl^- gradient contribute to dopamine uptake because transport is electrogenic and Cl^--dependent, bringing in positive charge as well as Cl^- along with dopamine translocation (Rudnick, 2002). The positive charge comes from the co-transport of two Na^+ ions with one dopamine molecule, as determined by initial rate measurements; the theoretically more reliable thermodynamic approach has not yet been applied to the dopamine transporter because membrane vesicle preparations containing the dopamine transporter are not available (Rudnick, 2002). At physiological pH, the dopamine molecule exists mostly in the positively charged protonated form, and this form, possibly together with the zwitterionic form, is the likely substrate for the dopamine transporter (Berfield et al., 1999). The overall stoichiometry of the dopamine uptake process likely is dopamine$^+$/Na^+/Cl^- = 1:2:1, and therefore two positive charges enter with each dopamine molecule taken up.

2.2. Na^+ and dopamine binding in membrane preparations

Clearly, Na^+ is required for the translocation of dopamine by its transporter, and the first step initiating the uptake cycle is thought to be the recognition of dopamine as a reversible binding interaction with the transporter (Bönisch, 1998). However, the role of Na^+ in this first recognition step is under debate. Kinetic analysis of Na^+-dependent dopamine uptake by human embryonic kidney (HEK) cells expressing the human dopamine transporter (DAT) suggest a sequential binding order of dopamine and Na^+ with Na^+ binding before dopamine (Chen et al., 1999; Earles and Schenk, 1999). Electrophysiological measurements on oocytes expressing the human DAT show blockade of transporter-mediated leak currents by dopamine in the complete absence of Na^+ (Sonders et al., 1997). Although the later observation negates an absolute requirement for Na^+ in dopamine binding, a stimulatory role for Na^+ is still a possibility. Thus, cocaine also blocked leak currents in the absence of Na^+ (Sonders et al., 1997), even though a stimulatory role for Na^+ in cocaine analog binding to the dopamine transporter has been shown repeatedly (Reith and Coffey, 1993; Chen et al., 1997a,b; Li and Reith, 1999; Corera et al., 2000). In biochemical studies on the role of Na^+ in dopamine binding, the measuring tool is the dopamine-induced inhibition of binding of a radiolabeled blocker because the dissociation of the dopamine-transporter complex is too rapid, ruling out successful application of commonly used techniques for separating bound from free ligand (see Li and Reith, 1999). With this approach applied to membrane preparations, the common finding has been that Na^+ can be stimulatory for dopamine binding in the presence of inhibitory cations such as $Tris^+$, K^+, Li^+, Ca^{2+}, or choline$^+$ (Bonnet et al., 1988; Amejdki-Chab et al., 1992a; Reith and Coffey, 1993; Chen et al., 1997a, 2002; Li and Reith, 1999), whereas in the absence of such cations, Na^+ concentrations of 10 mM and up had little or no effect with Br^- as the co-varying anion (Amejdki-Chab et al., 1992a,b; Li and Reith, 1999). When Na^+ concentrations of 2 mM and up, again with Br^-, were examined for their effects on the binding of D-amphetamine, DL-octopamine, and *p*-tyramine, no increases in binding upon elevating [Na^+] were observed, and models assuming low binding in the absence of Na^+ did not provide fits as good as models in which substrate binds in the absence of Na^+ with an affinity close to that observed at 2 mM Na^+ (Li and Reith, 2000). All studies taken together indicate a concentration-dependent *inhibitory* activity of Na^+ on the binding of DL-octopamine and *p*-tyramine but not dopamine and D-amphetamine; this inhibition can be offset by a stimulatory effect of Cl^-, and a partially stimulatory effect of Br^-; Cl^- stimulation is observed for all four substrates (Amejdki-Chab et al., 1992a,b; Li and Reith, 1999, 2000).

The above studies prompted us to look for a *stimulatory* effect upon raising [Na^+], perhaps occurring at Na^+ concentrations lower than 2 mM and perhaps not uncoverable in the modeling approaches applied so far. The radioligand was [^{3}H]2β-carbomethoxy-3β-(4-fluorophenyl) tropane (CFT), a phenyltropane analog of cocaine, and the membrane preparations were from HEK-293 cells expressing human dopamine transporter. First, the Na^+ dependency of binding was monitored in a sodium phosphate buffer with [Na^+] ranging between 2 and 20 mM (Fig. 1F). These experiments were carried out at pH 7.0 in conjunction with sets performed at pH 7.4 reported previously (Li et al., 2002). This was done because substrates in brain can bind, theoretically, to either inside-facing transporter protein, where the ambient intracellular pH is 7.0, or outside facing transporter, where the extracellular pH is closer to 7.3–7.4 (Siesjo et al., 1985). In addition, multiple anions were examined as co-varying counterions for Na^+ because of the potential problems associated with drawing conclusions based on the use of only one anion which might affect binding by itself. Increasing [Na^+] by adding NaBr to a 2-mM NaPhos buffer dramatically enhanced [^{3}H]CFT binding, and had little or no effect on the binding of dopamine, D-amphetamine, *p*-tyramine, or DL-octopamine (Fig. 1F). We addressed the possibility that a stimulatory effect of Na^+ between 0 and 2 mM Na^+ might have been missed in the above experiments. Na^+ curves were examined in the presence of 74 mM $Tris^+$, an inhibitory cation that could shift a Na^+ stimulation curve to the right, uncovering Na^+ stimulation now occurring at higher Na^+ concentrations. While dopamine and D-amphetamine showed dramatic increases in binding upon raising [NaBr] in either Tris–

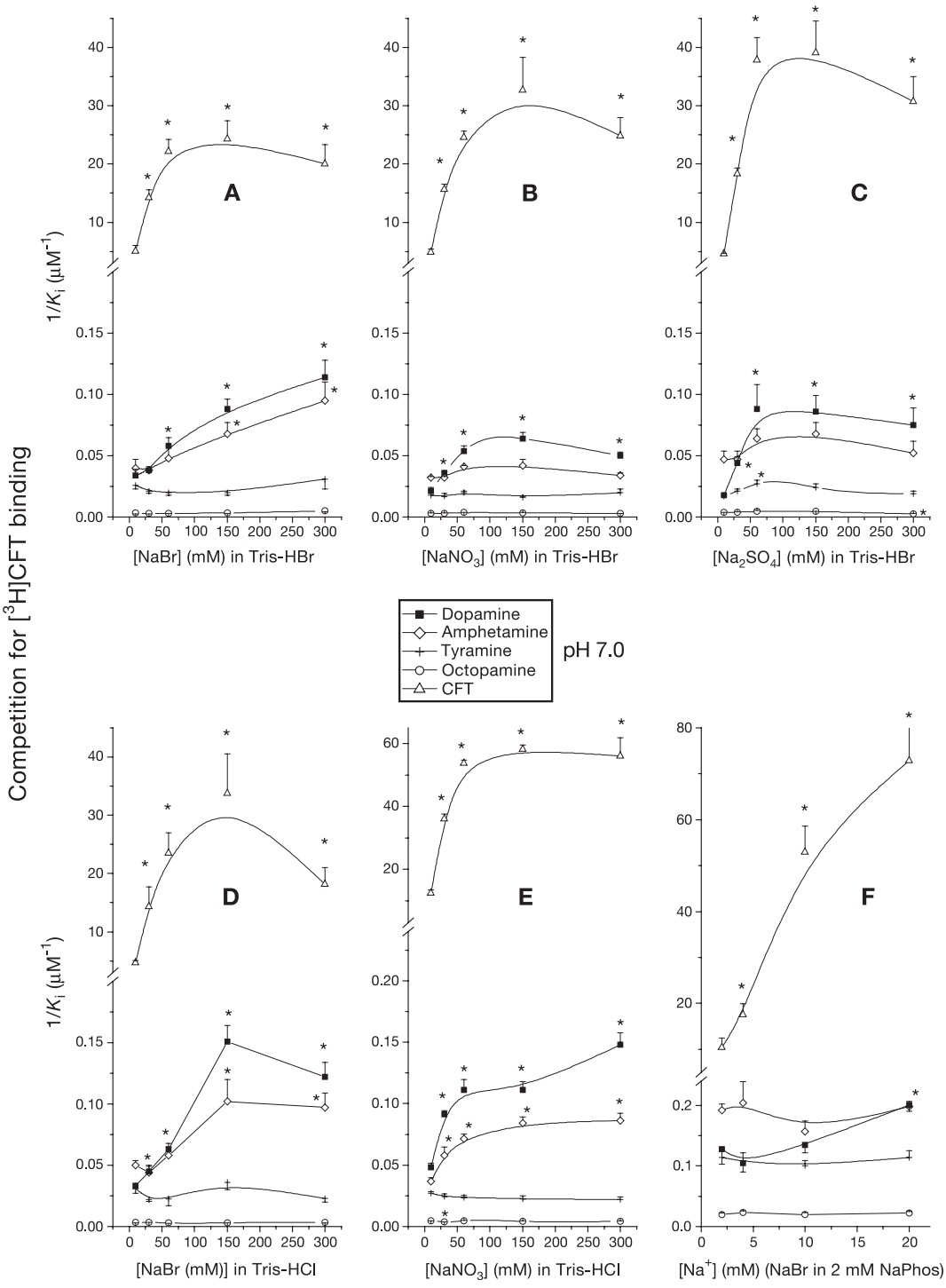

Fig. 1. Effect of varying $[Na^+]$ with different anions on potency of substrates and CFT in inhibiting $[^3H]$CFT binding. In (A–E), $[Na^+]$ (10, 30, 60, 150, or 300 mM) was varied in tandem with $[Br^-]$, $[NO_3^-]$, and $[SO_4^{2-}]$ in Tris (74 mM) buffer, adjusted to pH 7.0 by HCl or HBr. In (F), a lower range of Na^+ concentrations was achieved by including 0, 2, 8, or 18 mM NaBr in 2 mM NaPhos, pH 7.0. Each inhibition curve was obtained in three to six independent experiments carried out in triplicate. Values shown are means ± S.E. For each curve, differences between points were tested by one-way ANOVA followed by Dunnett Multiple Comparison Test for comparing each point with the lowest $[Na^+]$ in the curve ($*P<0.05$).

HBr or Tris–HCl buffer (Fig. 1A and D, respectively), little or no change was observed for the binding of *p*-tyramine or DL-octopamine. $[^3H]$CFT binding showed a biphasic Na^+ dependency with initial Na^+ stimulation followed by inhibition. Similar findings were obtained with NO_3^- or SO_4^{2-} as the anion co-varying with Na^+ (Fig. 1B, C, and E). The important exception was that the dopamine and D-amphetamine curves started to level off at Na^+ concentrations of 60

mM and up, as opposed to the apparent stimulatory effect of Br^- at higher [Na^+] (Fig. 1A and D). In the case of dopamine and D-amphetamine, it was possible that Na^+ removed an inhibitory action of $Tris^+$ rather than being stimulatory by itself. To address this possibility, a buffer was used completely free of Na^+, allowing the construction of Na^+ curves truly starting at 0 mM. [Na^+] was varied with SO_4^{2-} as the co-varying anion. [^{3}H]CFT binding increased upon raising Na^+ from 0 to 50 mM; in contrast, no stimulation of dopamine binding was observed when Na^+ was raised from 0 to 5 mM, and no Na^+ dependency was apparent upon further raising Na^+ to 50 mM; there was also no stimulation of the binding of *p*-tyramine and DL-octopamine upon raising Na^+ from 0 to 5 mM (Li et al., 2002). This makes it unlikely that the Na^+ stimulation of binding seen for dopamine and D-amphetamine in the presence of 74 mM $Tris^+$ reflects a direct binding enhancing effect of Na^+ uncovered by the presence of inhibitory $Tris^+$. More likely, $Tris^+$ exerts a binding inhibitory effect by binding to a site overlapping with the substrate binding domain, and Na^+ removes the $Tris^+$ inhibition by conformationally changing the $Tris^+$ binding site. Evidence has been advanced previously for direct interference by cations of the binding of not only blockers (Heron et al., 1996; Chen et al., 1997a; Li and Reith, 1999; Corera et al., 2000) but also substrates (Li and Reith, 1999, 2000; Chen et al., 2002), and $Tris^+$ is likely to be one of these cations interacting with Na^+ as monitored by the binding of 1-(2-(diphenylmethoxy)-ethyl)-4-(3-phenyl-2-[^{3}H]propenyl) piperazine ([^{3}H]GBR 12783) (Bonnet et al., 1988). Overall, the results point to a lack of stimulatory effect of Na^+ on the binding of substrates including dopamine to the dopamine transporter in membrane preparations.

2.3. Na^+ and dopamine binding in intact cells expressing dopamine transporter

Dopamine transporter characteristics may differ between membrane preparations where both sides of membrane face the same ion environment and intact cells where ion gradients across the membrane exist along with the presence

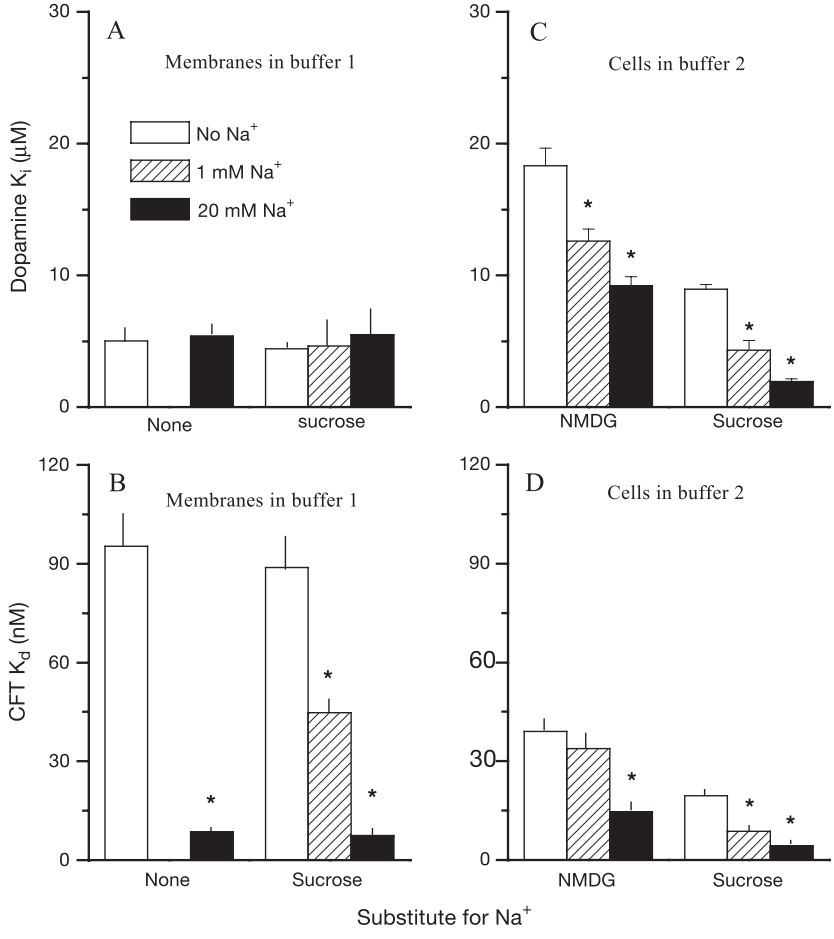

Fig. 2. Whole cells (C and D) stably expressing the human dopamine transporter or their membrane preparations (A and B) were incubated with 2 or 4 nM [^{3}H]CFT and various concentrations of unlabeled CFT or dopamine for 15 min at 25 °C in HEPES–Tris buffer ("buffer 1" for membranes) or Krebs–Ringer–HEPES ("buffer 2" for cells) containing substitutes for Na^+ as indicated (240 mM sucrose or 130 mM *N*-methyl-D-glucamine (NMDG). See Section 2.3 for description of buffers 1 and 2. The indicated Na^+ concentrations were achieved by adding NaCl (to buffer 2) or sodium isethionate (to buffer 1) directly to the assay mixture. Values shown are means ± S.E. for six independent experiments carried out in triplicate. *$P<0.05$ versus the corresponding group in the absence of Na^+ (Dunnett's test).

of a membrane potential. We therefore initiated experiments systematically comparing the two situations, using buffer systems compatible with both. In one system (buffer 1), HEPES–Tris was used not containing any cations besides Na^+, allowing the comparison of results from cells with those from membrane preparations. Another buffer (#2) was modified Krebs–Ringer–HEPES–*N*-methyl-D-glucamine chloride (NMDG) containing physiological concentrations of Ca^{2+}, Mg^{2+}, and K^+, allowing, in cell binding assays, the comparison of results with sucrose as a Na^+ substitute with those with NMDG as the substitute. Sucrose was fixed at 240 mM or NMDG at 130 mM, and Na^+ concentration was varied between 0 and 20 mM by adding sodium isethionate (to buffer 1) or NaCl (to buffer 2) directly. In membranes, the K_d of CFT binding was sensitive to Na^+ (Fig. 2B). At 20 mM, a concentration producing a plateau in stimulating CFT binding, Na^+ reduced the CFT K_d by 10-fold (Fig. 2B), but had no effect at all on the dopamine K_i, regardless of the presence of sucrose (Fig. 2A). Further raising Na^+ to 150 mM or using buffer 2 with membranes also did not modify the K_i for dopamine (experiments not shown). Different from what was observed in membrane preparations, in cells, increasing Na^+ concentration considerably reduced the dopamine K_i for inhibiting CFT binding (Fig. 2C). Noticeably, in most cases, this effect became statistically significant at the concentration as low as 1 mM. Moreover, the dopamine K_i-reducing effect of Na^+ was consistently observed regardless of substitutes (sucrose or NMDG) and ion components in the system (buffer 1 or 2). Interestingly, at 4 °C where a substrate is not substantially transported, in both buffer systems, addition of Na^+ up to 20 mM also decreased the dopamine K_i. We also found that Na^+-induced changes in dopamine K_i for inhibiting CFT binding are in parallel to those in K_m for dopamine uptake, but not accompanied with changes in intracellular Na^+ concentration or membrane potential (Chen et al., 2003). Taken together, these results are consistent with the idea that Na^+ enhances the inhibition of CFT binding by dopamine mainly via enhancing dopamine interactions at binding-associated steps and that this Na^+ effect does not require the occurrence of inward translocation cycles coupled with external Na^+ or alterations in cellular milieu associated with extracellular Na^+.

3. Search for amino acid residues in the dopamine transporter involved in Na^+ action

3.1. Selection of W84 and D313 among tryptophan and acidic residues in the dopamine transporter

As a cation, Na^+ can be thought to interact in several ways with amino acid residues in the dopamine transporter. It could directly interact with negatively charged side chains of acidic amino acids (Martin et al., 1999), or be coordinated by a cluster of electronegative residues such as oxygen atoms of the acidic amino acid side chains (Poolman et al., 1996). An alternative electronegative source for cation coordination is presented by aromatic amino acid residues via their π electrons at the face of aromatic side chains (Kumpf and Dougherty, 1993; Miller, 1993). To identify their possible involvement in cation interaction with the dopamine transporter, we decided to focus on acidic (glutamate, aspartate) and aromatic (tryptophan) residues in the transporter. We also limited our selection of residues for study to those that are absolutely or conservatively retained in the family of Na^+, Cl^--dependent neurotransmitter transporters. The reasoning is that highly conserved residues are more likely involved in features common for all members of this family, such as Na^+ binding or conformational changes linked to Na^+-binding, We systematically substituted these residues by π-electron- or charge-removing mutation. The following mutants were generated: D63L, D68N, W84L, E117Q, W132L, W177L, W184L, E215Q, W267L, W311L, D313N, D345N, E428Q, D436N, D476N, W520L, and W556L. These mutants were prepared as stably transfected HEK-293 cell lines and examined for dopamine transporter function. Out of these 17 mutants, 4 mutants, i.e. E117Q, W132L, W177L, and W184L, showed little dopamine transporter immunostaining on the plasma membranes of the expressing cells, and also little uptake and binding activity (Chen et al., 2001). Among the remaining 13 mutants, 2 mutants were especially interesting: W84L and D313N. Thus, they responded to substitution of 130 mM LiCl or KCl for NaCl in the buffer (reducing final $[Na^+]$ to 20 mM) with an appreciably smaller decrease in $[^3H]$CFT binding to intact cells than wild type. W84L and D313N also responded with a smaller decrease upon lowering the pH of the buffer to 5, suggesting that the two mutants could interact with cations in a more general way. In addition, W84L and D313N displayed a shift in the relative selectivity towards cocaine analogs as compared with dopamine (Chen et al., 2001), which is of interest because Na^+ binding is crucial for cocaine (analog) binding to the dopamine transporter (Reith and Coffey, 1993; Chen et al., 1997a,b; Li and Reith, 1999).

3.2. Na^+ coupling of cocaine analog but not dopamine binding in membranes of W84L and D313N

When membrane preparations of wild type, W84L, and D313N were examined in HEPES–Tris buffer (buffer 1 as above in Section 2.3, without cations other than Na^+), addition of Na-isethionate steeply increased $[^3H]$CFT binding and maximal levels were reached already at 20 mM (Fig. 3B). D313N and W84L responded with increases in binding at lower $[Na^+]$ than wild type, indicating a greater sensitivity to Na^+. When the K_d of CFT binding was assessed at varying $[Na^+]$, lower $[Na^+]$ stimulated CFT binding affinity to a greater extent in the mutants than in wild type, with affinity in the mutants always far exceeding that in wild type (Chen et al., 2002), suggesting the mutations did not simply promote Na^+ binding. Rather, they appeared to enhance the coupling between Na^+ binding and CFT binding.

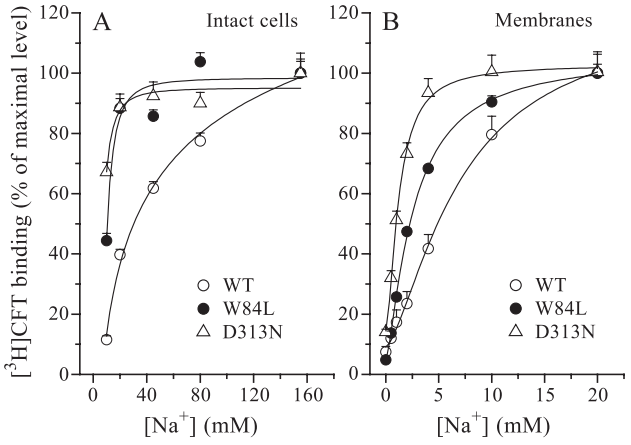

Fig. 3. Stimulation by Na^+ of [^{3}H]CFT binding and effect of mutations. (A) Intact cells, NaCl was isotonically replaced by LiCl in Krebs–Ringer–HEPES buffer; (B) membrane preparations, sodium isethionate was added to HEPES–Tris buffer without substitution. Binding assays were conducted for 15 min at 21 °C. Results are shown as the percentage of the maximal binding activity. For wild-type (WT), W84L, and D313N, the respective maximal binding to intact cells was 1.77 ± 0.12, 1.45 ± 0.06, and 0.52 ± 0.02 pmol/mg; the respective maximal binding to membranes was 2.81 ± 0.16, 3.38 ± 0.06, or 0.89 ± 0.13 pmol/mg. Values represent means ± S.E. of more than eight experiments performed in triplicate.

In contrast, under the same conditions, the affinity of dopamine binding to membranes was not affected by increasing [Na^+] in either mutants or wild type (Chen et al., 2002), suggesting that the mutations did not alter dopamine binding to the Na^+-bound dopamine transporter. Thus, dopamine binds equally well to Na^+-free and Na^+-bound dopamine transporters.

3.3. Effect of Na^+ and H^+ on cocaine analog and dopamine binding, and dopamine uptake, in intact cells expressing W84L and D313N

The greater sensitivity of CFT binding to Na^+ in membranes of the mutants compared with wild type was perhaps surprising in view of the *reduced* sensitivity described above under 3.1. The reason for this apparent discrepancy became clear when studying the effect of replacing NaCl by LiCl in KRH (see Section 2.3) which contains a mixture of cations normally present extracellularly (Fig. 2A). While wild type was still on the ascending part of the Na^+ curve for CFT binding at 20 mM Na^+, the level chosen in the experiments of Section 3.1, D313N and W84L had already approached their maximal binding at this level of Na^+. Thus, reducing [Na^+] from 150 to 20 mM affected wild type more than the mutants, not because of *less*, but because of *greater*, sensitivity to Na^+.

A more complicated situation appears to exist for the sensitivity of [^{3}H]CFT binding to H^+ as measured in experiments with lowering pH of KRH buffer bathing intact cells, with only D313N being *less* sensitive to pH decreases down to pH 6, and both W84L and D313N responding with smaller CFT binding decreases upon reducing pH into the 4.5–5.5 range. In contrast, we found the inhibition by H^+ of dopamine uptake into intact cells to be substantially enhanced at W84L and D313N compared with wild type in experiments with pH ranging from 8.5 down to 4.5. This was further examined in intact cells as follows (Table 1). We determined the effect of a one-unit pH shift on kinetic parameters of dopamine uptake as well as the potency of dopamine in inhibiting [^{3}H]CFT binding. The pH values (pH 7 and 8 for wild type, and pH 7.5 and 8.5 for the mutants) were chosen to allow significant reduction in uptake from its maximal value at optimal pH (8 for wild type and 8.5 for the mutants). B_{max} and K_d of [^{3}H]CFT binding were determined in parallel to allow estimation of dopamine K_i and turnover rate. In the tested range of pH (7–8.5), H^+ had no significant effect on B_{max} and had modest inhibitory effect (~ 173%, 68%, and 19% for wild type, W84L, and D313N, respectively) on the K_d for CFT binding to intact cells. For wild type, decreasing pH slightly affected the apparent affinity of dopamine, as indicated by the modest increase in both dopamine K_i for inhibiting CFT binding and dopamine K_m for uptake (Table 1). For W84L, lowering pH significantly reduced the K_m for dopamine uptake but had little effect on the K_i for dopamine in inhibiting CFT binding, indicating an impact of events after dopamine binding on the K_m value. Mutation of D313N decreased both K_i and K_m upon lowering pH (Table 1). In a separate experiment, the effect of H^+ on dopamine K_i for inhibiting CFT binding to intact cells was examined at pH 6.4 and 7.4. Again, lowering pH significantly decreased dopamine K_i at D313N, but significantly increased dopamine K_i at wild type and W84L. To exclude the possibility that the increased dopamine K_m at the two mutants was due to accelerated dopamine degradation at higher pH, we also examined the K_m for wild type at pH 8.5 (K_m = 590 ± 75 nM, n = 4), which was not different from the value at pH 8 (Table 1). The results taken together indicate that the mutations did not enhance the inhibitory effect of H^+ on

Table 1
Effect of pH on dopamine binding and translocation

hDAT	pH	K_i (μM)	K_m (μM)	V_{max} (pmol/mg/min)	Turnover (min^{-1})
Wild type	7	6.31 ± 0.79	0.828 ± 0.124	4.41 ± 0.31	1.13 ± 0.08
	8	5.81 ± 0.67	0.583 ± 0.007	8.29 ± 0.52[a]	2.13 ± 0.13[a]
W84L	7.5	8.66 ± 2.45	0.754 ± 0.087	1.25 ± 0.10	0.50 ± 0.04
	8.5	7.94 ± 1.07	1.900 ± 0.267[a]	3.70 ± 0.70[a]	1.48 ± 0.28[a]
D313N	7.5	30.2 ± 1.3	2.387 ± 0.206	0.48 ± 0.05	0.30 ± 0.03
	8.5	73.5 ± 9.3[a]	6.876 ± 0.610[a]	4.14 ± 0.99[a]	2.54 ± 0.61[a]

Cells were incubated with 10 nM [^{3}H]dopamine or [^{3}H]CFT (4 nM) in Ca^{2+}-free Krebs–Ringer–HEPES buffer at indicated pH for 5 min or 15 min at 21 °C. Because pH (7–8.5) has no significant effect on B_{max} of CFT binding, for each cell line, the B_{max} data at two pH levels were combined and averaged: 3.90 ± 0.68, 2.51 ± 0.36, and 1.62 ± 0.38 pmol/mg for wild type, W84L, and D313N, respectively. The averaged B_{max} data were used to calculate the turnover rate (V_{max}/B_{max}).
Data are expressed as means ± S.E. for four experiments performed in triplicate.
[a] $P < 0.05$ versus the value at lower pH (paired t-test).

dopamine binding to intact cells. In contrast, the mutations enhanced the inhibitory effect of H^+ on dopamine translocation as measured by V_{max} and turnover rate (Table 1). When raising pH to the optimal level (8.5), the turnover rate was near normal at D313N and improved at W84L. Thus, the enhanced H^+ inhibition of dopamine uptake by the mutants partially explains their poor function at physiological pH (7.4), as the translocation effect is relieved upon raising the pH. In addition, a link can be seen between these events and the Na^+ sensitivity of the two mutants: the two residues, in addition to their involvement of Na^+ regulation of CFT binding, also contribute to molecular events associated with H^+ interactions at translocation steps, and in this respect, mutation of these residues enhances both Na^+ and H^+ sensitivity.

4. Dopamine *access* is facilitated by Na^+ on the extracellular side and by D313 in the dopamine transporter

4.1. Role of D313 in dopamine binding in intact cells but not membranes expressing dopamine transporter

As shown in Table 1, the dopamine K_i at intact cells with D313N was ~ 4-fold greater than in cells with wild type. We also observed a similar difference between intact cells expressing D313N and membrane preparations prepared from these cells, with membranes having the higher affinity. Furthermore, there was no difference between membranes prepared from wild-type cells and D313N cells. Thus, the possibility can be entertained that dopamine recognition per se is not affected by the D313 mutation. Rather, it is possible that something else, connected with dopamine recognition, is different in cells as compared with membranes.

4.2. A model for Na^+ effects on the interaction of dopamine with its transporter in intact cells vs. membrane preparations

We propose that the factor altering apparent dopamine affinity in cells is *access* of dopamine to its binding site, rather than the *recognition itself*. Thus, it can be speculated that dopamine transporters in intact cells with a resting membrane potential in the absence of Na^+ mostly face inwardly (Fig. 4). Addition of external Na^+ turns most transporters into the externally facing form, allowing access of dopamine to its binding site from the outside (dopamine cannot easily penetrate membranes and bind from the inside). In membrane preparations without membrane potential, we speculate most transporters are in the inward-facing form, with or without Na^+ bound to its site depicted in the normally external portion of the protein. However, in these preparations, dopamine can access its binding site from the side normally facing the interior. Even if outward-facing forms of the transporters were to be generated in the presence of Na^+ in membrane preparations, it would not alter dopamine binding because dopamine can access those transporters from the normal external side. Measured dopamine affinity is a time-averaged value of the binding to the different forms (states) of the transporter which continuously interconvert in spontaneous conformational changes as is commonly accepted for receptor proteins (Gether, 2000).

5. Concluding remarks

A wealth of information has been obtained regarding Na^+ modulation of Na^+/Cl^--dependent neurotransmitter transporters, inspiring speculations regarding potential mechanisms underlying Na^+ effects on the dopamine transporter. Recent studies suggest that reversal of monoamine transport is elicited by lowering extracellular Na^+ (Pifl et al., 1997), or increasing intracellular Na^+ (Chen et al., 1998; Pifl and Singer, 1999; Scholze et al., 2000; Khoshbouei et al., 2003). Thus, it is possible that intracellular Na^+ has an effect opposite to that of external Na^+, i.e., promoting the formation of inward-facing state, thereby reducing the binding of external substrates. In this context, Na^+ stimulation of dopamine binding from the side facing the exterior relies on the asymmetric distribution of Na^+ across the membrane, and would not happen in cell-free preparations where Na^+ is applied equally to both sides of the membrane.

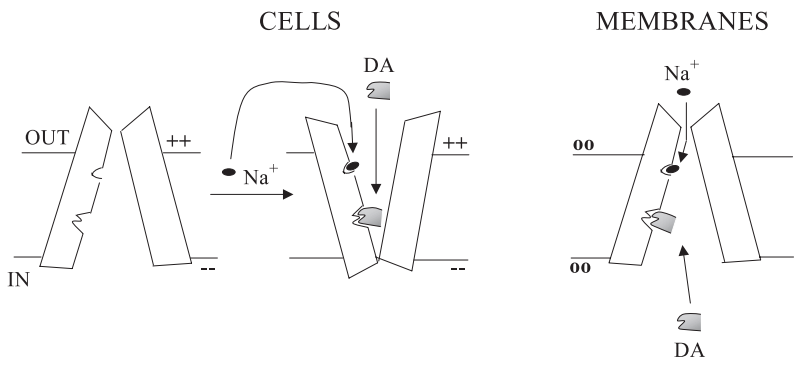

Fig. 4. Model for Na^+ effects on the interaction of dopamine with its transporter in intact cells vs. membrane preparations. See Section 4.2 for description.

Studies with chimeric dopamine–norepinephrine transporters suggest that three different segments of the dopamine transporter are involved in the Na^+-dependence of dopamine transport: the region from the NH_2 terminal through the first two transmembrane domains, the junction between transmembrane domains 5 and 6, and the region encompassing transmembrane domain 9 through the COOH terminal (Syringas et al., 2000). Replacement of each segment with the corresponding norepinephrine transporter segments alters the Hill number for Na^+-dependent dopamine uptake. Interestingly, the first two segments are also regions containing W84 (in transmembrane domain 1) and D313 (in top of transmembrane domain 6), respectively. In the γ-aminobutyric acid (GABA) transporter, disruption of transient current linked to Na^+ binding by mutation of W68, a residue aligned with W84 of the dopamine transporter, is related to alterations in the propensity of the GABA transporter to exist in a certain conformational state (Mager et al., 1996; Li et al., 2000). In serotonin transporters, impairment of the Na^+-dependence of serotonin uptake by mutations in transmembrane domain 7 is involved in perturbation of the propagation of the conformational changes after Na^+ binding; this conformational change can alter the accessibility of an endogenous cysteine in transmembrane domain 1 to methanethiosulfonate reagents (Penado et al., 1998; Kamdar et al., 2001). These findings are consistent with the view that Na^+ modulates substrate permeation with the involvement of a network of intramolecular interactions, propagating conformational interconversions associated with the transport cycle.

The accompaniment of an alteration in Na^+ action with an alteration in the apparent sensitivity of substrate transport to H^+ is not specific to the dopamine transporter. It has also been reported for two other members of the Na^+/Cl^--dependent transporter family, the GABA transporter (Forlani et al., 2001) and the serotonin transporter (Barker et al., 1999). Such a common connection indicates that another aspect of Na^+ modulation could be to maintain a proper environment for pH-sensitive residues that are important for substrate translocation.

Clarification of these mechanisms will improve our understanding of the molecular basis governing Na^+ modulation of dopamine transport. It will also help identifying structural determinants conferring different Na^+ dependence for the binding of dopamine and cocaine analogs. Such knowledge could open up the possibility to develop compounds with unique ion susceptibility in the development of medications for cocaine addiction.

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