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Review

From synapse to vesicle: the reuptake and storage of biogenic amine neurotransmitters

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Contents

I. Overview

The Na^+- and Cl^--coupled neurotransmitter transporters represent a fascinating group of integral membrane proteins encoded by a closely related family of recently cloned cDNAs [1–20]. These carrier proteins couple the transmembrane movement of Na^+, Cl^- and in some systems K^+, to the reuptake of neurotransmitters released into the synaptic cleft [21] (Fig. 1). As such, they function to regulate neurotransmitter activity by removing extracellular transmitter. Inhibitors that interfere with this regulation include antidepressant drugs and stimulants, such as the amphetamines and cocaine. Of the plasma membrane transporters which have been studied, three proteins responsible for catalyzing transport of serotonin (5-hydroxytryptamine (5-HT) [3,4] and the catecholamines norepinephrine (NE) [2] and dopamine (DA) [5–7,17]) stand out as a distinct subfamily. These biogenic amine transporters are all inhibited by cocaine, and share other structural and mechanistic properties.

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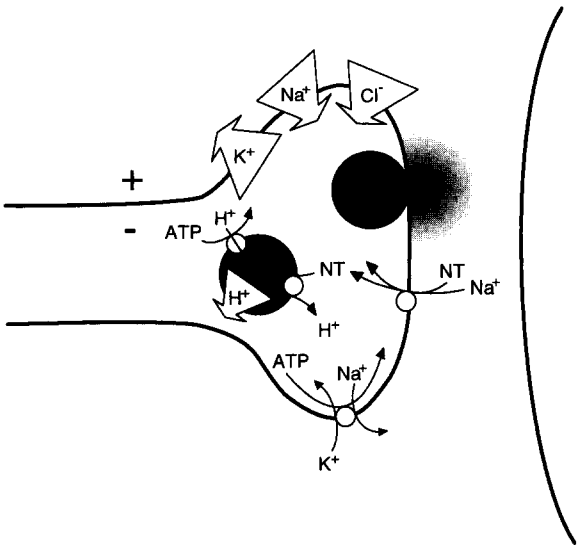

Fig. 1. Neurotransmitter recycling. Gradients of Na^+, K^+ and Cl^- generated by the plasma membrane Na^+/K^+-ATPase drive influx of released neurotransmitter (NT) across the plasma membrane. Once inside the nerve terminus, the transmitter is accumulated within synaptic vesicles by exchange with intravesicular H^+ ions supplied by the vacuolar H^+-pumping ATPase.

Recapture of the released biogenic amine neurotransmitter is only the first of two steps required for efficient neurotransmitter recycling [22]. A second transport process sequesters cytoplasmic transmitters within synaptic vesicles, in preparation for their release by exocytosis (Fig. 1). This second step is catalyzed by a vesicular neurotransmitter transporter driven by an electrochemical H^+ gradient [23]. The vesicular transporters found in cells transporting various biogenic amines seem to be very similar, if not identical, in their functional properties. Other vesicular transporters, with different specificities, are found in neurons that release amino-acid neurotransmitters and acetylcholine [24–30].

II. What do the transporters do?

The plasma membrane biogenic amine transporters, like all neurotransmitter transporters, are involved in regulating the level of biogenic amines in the synaptic cleft. Blocking the action of a transporter increases the level of its cognate neurotransmitter in the cleft. The degree of pre- and post-synaptic receptor occupation is consequently increased, as is the extent to which the transmitter is subject to diffusion and oxidation, processes that interefere with normal neurotransmitter recycling. Early experiments demonstrated that peripheral biogenic amine stores were depleted by stimulation if reuptake was blocked [31,32]. More recent results using in vivo microdialysis demonstrate that neuronal activity increases extracellular biogenic amine concentration and that transport blockers potentiate this increase [33,34].

Drugs that interfere with biogenic amine transporter function have profound behavioral effects, leading to their therapeutic use [35–37], as well as their abuse as stimulants [38–40]. Compounds that block the reuptake of 5-HT or NE have proven useful in treating clinical depression [37]. The therapeutic effect seems to be indirect, since transporter blockade occurs as soon as the drug enters the brain, but symptomatic relief requires weeks of treatment [41]. Following the discovery that imipramine, the prototypical tricyclic antidepressant, inhibited 5-HT transport, much effort centered on synthesizing specific inhibitors of 5-HT transport. The most specific of these, such as fluoxetine and sertraline, have proven their effectiveness in alleviating depression [42–44]. Nevertheless, the link between inhibition of 5-HT or NE reuptake and the ultimate therapeutic effects of transport inhibitors remains elusive.

Stimulants such as cocaine and methylphenidate are believed to exert their behavioral effects by binding to, and inhibiting, the DA transporter [38,45]. The increased level of DA in specific brain regions, for example the ventral tegmental area, is believed to underlie the reinforcing property of cocaine that leads to its abuse [46]. Amphetamines represent another class of stimulants that increase extracellular levels of biogenic amines. Their mechanism differs from that of cocaine, although it also involves biogenic amine transporters. Amphetamine derivatives are apparently substrates for biogenic amine transporters, and lead to transmitter release by a process of transporter-mediated exchange [47,48]. Not only catecholamines but also 5-HT is released by amphetamines. In particular, compounds such as *p*-chloroamphetamine and 3,4-methylenedioxymethamphetamine (MDMA, also known as 'ecstasy') not only preferentially release 5-HT, but also cause degeneration of serotonergic nerve endings [49].

III. How do they do it?

The best studied of the three plasma membrane biogenic amine transporters is the 5-HT transporter. Much of the available data on 5-HT transport comes from studies using platelet plasma membrane vesicles as a model system [50]. The availability of membrane vesicles from platelets [51], rat basophilic leukemia cells [52] and placenta [53] has allowed a detailed understanding of the 5-HT transporter's mechanism. Less is known about the mechanism of NE transport and still less about DA transport, due to the lack of similar membrane vesicle systems. The situation is now likely to improve for the catecholamine transporters

with the availability of systems for expression of the cloned transporter cDNAs [54].

III-A. Ionic requirements

All of the plasma membrane biogenic amine transporters, like other neurotransmitter transporters in this gene family, require both Na^+ and Cl^- in the external medium for neurotransmitter influx. Early studies by Sneddon and Lingjaerde [55,56] on the 5-HT transporter of intact platelets led to the suggestion that both Na^+ and Cl^- are co-transported with 5-HT. Studies in both intact platelets and in platelet plasma membrane vesicles demonstrated that while Cl^- could be replaced by Br^-, and to a lesser extent by SCN^- or NO_2^-, Na^+ could not be replaced by other cations [55–58]. Similar results were obtained with NE and DA transport into synaptosomes. Kuhar and Zarbin [59] demonstrated the Na^+ and Cl^- requirements for many synaptosomal neurotransmitter transporters, including those for NE and DA.

Evidence that Na^+ and Cl^- were actually co-transported with 5-HT came from studies using platelet plasma membrane vesicles [50]. When a Na^+ concentration gradient (out > in) was imposed across the vesicle membrane in the absence of other driving forces, this gradient was sufficient to drive 5-HT accumulation [57]. In this experiment, internal Na^+ was replaced with Li^+, a cation found to be inert for the 5-HT transporter in membrane vesicles [57]. Coupling between Na^+ and 5-HT transport is an inescapable consequence of the fact that Na^+ could drive transport only if its own gradient is dissipated. Thus, Na^+ influx must accompany 5-HT influx. Na^+ coupled 5-HT transport into membrane vesicles is insensitive to inhibitors of other Na^+ transport processes, such as ouabain and furosemide, supporting the hypothesis that Na^+ and 5-HT fluxes are coupled directly by the transporter [57,60]. Many of these results have been reproduced in membrane vesicle systems from cultured rat basophilic leukemia cells [52], mouse brain synaptosomes [61] and human placenta [53].

The arguement that Cl^- is co-transported with 5-HT is somewhat less direct, as it has been difficult to demonstrate 5-HT accumulation with only the Cl^- gradient as a driving force. However, the transmembrane Cl^- gradient influences 5-HT accumulation when a Na^+ gradient provides the driving force. Thus, internal Cl^- decreases the Cl^- gradient and inhibits 5-HT uptake. In contrast, 5-HT efflux requires internal Cl^-, but external Cl^- is not needed for efflux [58]. One possible explanation for stimulation by Cl^- is that Cl^- might be required to electrically compensate for rheogenic (charge moving) 5-HT transport. This possibility was ruled out by the observation that a valinomycin-mediated K^+ diffusion potential (interior negative) was unable to eliminate the external Cl^- requirement for 5-HT influx [58].

Perhaps the greatest surprise during the early studies of 5-HT transport was the discovery that K^+ efflux was directly coupled to 5-HT influx. Previously, K^+ countertransport had been invoked for other transport systems to explain stimulation by internal K^+ [62–64]. In those other cases, however, it became clear that a membrane potential generated by K^+ diffusion was responsible for driving rheogenic transport processes. Stimulation of 5-HT transport into platelet plasma membrane vesicles by intravesicular K^+ was also initially attributed to a K^+ diffusion potential since internal K^+ stimulates transport but is not absolutely required [57]. Subsequent measurements, however, showed that K^+ stimulated transport even if the membrane potential was close to zero [65]. When valinomycin was added to increase the K^+ conductance of the membrane, a K^+ diffusion potential (inside negative) was generated. Transport was essentially the same whether or not a diffusion potential was imposed in addition to the K^+ concentration gradient [66]. The K^+ gradient did not seem to act indirectly through the membrane potential, but rather, directly by exchanging with 5-HT.

Evidence relating the membrane potential to serotonin transport has been mixed in other systems. Bendahan and Kanner [52] found that serotonin transport into plasma membrane vesicles from rat basophilic leukemia cells was stimulated by a K^+ diffusion potential. However, other workers studying plasma membrane vesicles from mouse brain and human placenta concluded that serotonin transport in these tissues was not driven by a transmembrane electrical potential ($\Delta\psi$, interior negative) [67,68]. Further evidence that K^+ acts directly on the transporter came from measurements of K^+ effects on 5-HT transport which occur even in the absence of a K^+ gradient. The addition of 30 mM internal K^+ increased the transport rate 2.5-fold even when 30 mM K^+ was added simultaneously to the external medium [66].

The results described above argue that K^+ is countertransported during 5-HT influx. However, transport still occurred in the absence of K^+. The reason for this became apparent in a study of the pH dependence of 5-HT transport. In the absence of K^+, internal H^+ ions apparenty fulfill the requirement for a countertransported cation. For example, when the interior of platelet plasma membrane vesicles was acidified to pH 5.5, and the vesicles were diluted into a medium of pH 7.5, 5-HT influx was stimulated, and the stimulation by internal K^+ was significantly blunted [69]. Conversely, when internal K^+ was high, internal H^+ ions could no longer stimulate. Even when no other driving forces were present (NaCl in = out, no K^+ present), a transmembrane pH difference (ΔpH, interior acid) could

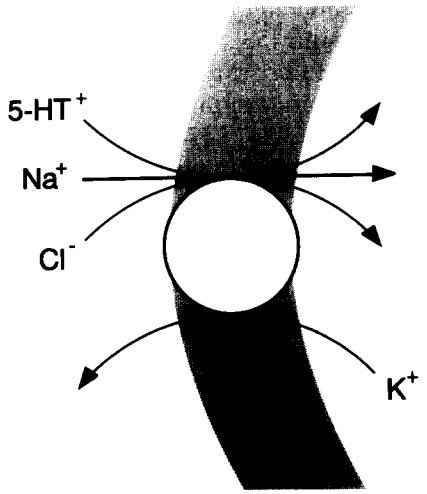

Fig. 2. Driving forces for 5-HT transport. Inwardly-directed gradients of Na^+ and Cl^- are coupled by the 5-HT transporter to serotonin influx by a process of symport (or co-transport). The K^+ gradient also serves as a driving force for the transporter, which catalyzes exchange (countertransport or antiport) of internal K^+ for external 5-HT.

serve as the sole driving force for transport. ΔpH-driven 5-HT accumulation required Na^+ and was blocked by imipramine or by high K^+ (in = out), indicating that it was mediated by the 5-HT transporter, and not due to non-ionic diffusion [69]. From all of these data, it was concluded that inwardly-directed Na^+ and Cl^- gradients, and outwardly-directed K^+ or H^+ gradients served as driving forces for 5-HT transport (Fig. 2).

Although no membrane vesicle systems have been described that transport DA, two plasma membrane vesicle systems have emerged for studying NE transport: The placental brush-border membrane [70] and cultured PC-12 cells [71]. In both systems, NE accumulation requires external NaCl and is stimulated by internal K^+. In neither system, however, was the role of internal K^+ definitely attributed to direct participation of K^+ in the NE transport process. Moreover, NE transport in both placental and PC-12 membrane vesicles was stimulated by imposition of $\Delta\psi$, raising the possibility that a passive K^+ conductance of the vesicle membrane results in $\Delta\psi$ generation and indirectly stimulates rheogenic NE transport [70,71].

III-B. Stoichiometry

Two general techniques have been used to assess the Na^+, Cl^- and K^+ stoichiometry for 5-HT, NE and DA transport. The first of these, which is technically easier, is to measure the dependence of transport rate (or its kinetic determinants K_m or V_{max}) on Na^+, Cl^-, or K^+ concentration, and to fit the rate dependence to an equation of the following form, where solving for n gives and estimate of the Na^+ stoichiometry for transport:

$$R = \frac{R_{max} \cdot [Na^+]^n}{K^n_{Na^+} + [Na^+]^n} \quad (1)$$

where n is the number of Na^+, Cl^-, or K^+ ions participating in the reaction, R_{max} and K_{Na+} represent the maximal effect and the half-maximal concentration for Na^+, respectively, and R is the measured rate function, either absolute rate, apparent affinity ($1/K_m$) or V_{max}.

Using this analysis for the serotonin transporter yields an n of 1 for both Na^+ and Cl^- in platelet plasma membrane vesicles. Thus, plotting V_{max} or $1/K_m$ for 5-HT vs. either $[Na^+]$ or $[Cl^-]$ yields a simple rectangular hyperbola (Fig. 3). Similar results were obtained for NE transport into placental brush-border membrane vesicles [70] and intact PC-12 cells [72], where initial rates of transport showed a simple hyperbolic dependence on Na^+ or Cl^- – consistent with a Na^+/Cl^-/NE stoichiometry of 1:1:1. The DA transporter, however, has a different ion dependence. While

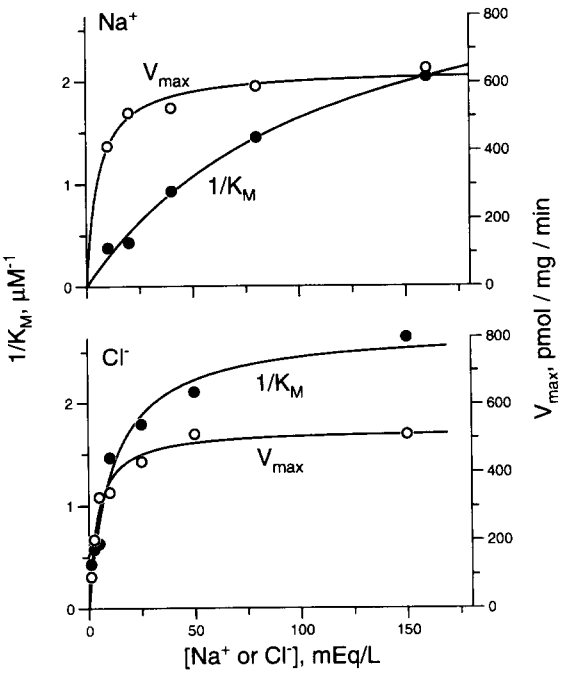

Fig. 3. Effect of Na^+ and Cl^- on 5-HT transport into platelet plasma membrane vesicles. The rate of 5-HT transport into membrane vesicles was determined over a range of 5-HT concentrations at each of the indicated Na^+ (upper panel) and Cl^- concentrations (lower panel). When Na^+ was varied, it was replaced isotonically with Li^+, and Cl^- was held constant. Similarly, when Cl^- was varied, it was replaced with isethionate and Na^+ was held constant. From each 5-HT concentration curve, K_m (●) and V_{max} values (○) were calculated by nonlinear regression. The lines represent the best fits of V_{max} and $1/K_m$ to the data using nonlinear regression and a simple saturation model where only one Na^+ and one Cl^- ion are cotransported with 5-HT.

initial rates of DA transport were found to be dependent on a single Cl^-, two Na^+ ions were apparently involved in the transport process [73]. Thus, the initial rate of DA transport into suspensions of rat striatum was a simple hyperbolic function of $[Cl^-]$ but depended on $[Na^+]$ in a sigmoidal fashion. These data are consistent with a $Na^+/Cl^-/DA$ stoichiometry of 2:1:1. These differences in Na^+ stoichiometry have been reproduced with the cloned transporter cDNAs stably expressed in LLC-PK_1 cell lines, indicating that they are intrinsic properties of the transporters and not artifacts due to the different cell types used (Gu, H., Wall, S. and Rudnick, G., unpublished data).

It is important at this point, to note that steady-state kinetics do not necessarily provide accurate information on co-transport stoichiometry. It is possible that more than one Na^+ ion is required for DA binding or even translocation (as reflected in the rate measurements), but that only one of those Na^+ ions is actually co-transported. It is also possible that NE is co-transported with two Na^+ ions, but that the affinities or rates of association of the two Na^+ ions is so disparate that the initial rate of transport is dependent on only the weaker binding or slower associating of the two. For this reason, it is essential to confirm the stoichiometry by an independent method. For transport systems, thermodynamic measurements of coupling between solute fluxes has proven useful. In the case of the GABA transporter, where both thermodynamic and kinetic data exist, both methods indicate a $Na^+/Cl^-/GABA$ stoichiometry of 2:1:1 [54,74,75]. Thus, the dependence of transport rate on a given ion may suggest a transport stoichiometry, but cannot provide proof for

$nA_{out} + B_{out} = nA_{in} + B_{in}$	Equation 2. Mass balance for cotransport of B with nA
$K_{eq} = 1 = \frac{[A_{in}]^n \cdot [B_{in}]}{[A_{out}]^n \cdot [B_{out}]}$	Equation 3. Equilibrium for A/B cotransport
$n \ln \frac{[A_{in}]}{[A_{out}]} = \ln \frac{[B_{out}]}{[B_{in}]}$	Equation 4. Relationship between transmembrane chemical potential of A and B.

In the thermodynamic method, a known Na^+, Cl^-, or K^+ concentration gradient is imposed across the plasma membrane as a driving force, and the substrate concentration gradient in equilibrium with that driving force is measured. By varying the concentration gradient of the driving ion, and measuring the effect on substrate accumulation, the stoichiometry can be calculated. For a simple system where two solutes, A and B, are cotransported, a plot of $\ln(A_{in}/A_{out})$ vs. $\ln(B_{in}/B_{out})$ gives the A/B stoichiometry as its slope. As a special case, if the stoichiometry is 1:1, then a plot of A_{in}/A_{out} vs. B_{in}/B_{out} will be a straight line. If more than one solute is coupled to substrate transport, then the above analysis still holds as long as the gradients of all other co- or countertransported solutes (as well as $\Delta\psi$ for a rheogenic process) are held constant. Using this method, a stoichiometry of 1:1 was determined for 5-HT transport with respect to both Na^+ [76] and K^+ [57]. The Cl^- stoichiometry was deduced from the fact that 5-HT transport was not affected by imposition of a $\Delta\psi$ (interior negative), and was, therefore, likely to be electroneutral. Given that 5-HT is transported in its cationic form [69,77], only a $5\text{-}HT^+/Na^+/Cl^-/K^+$ stoichiometry of 1:1:1:1 is consistent with all the known facts. Obviously, the above analysis requires an experimental system, like membrane vesicles, where the composition of both internal and external media can be controlled.

III-C. Mechanism

It is interesting to consider how the biogenic amine transporters, with molecular masses in the 60–70 kDa range, are able to couple the fluxes of substrate, Na^+,

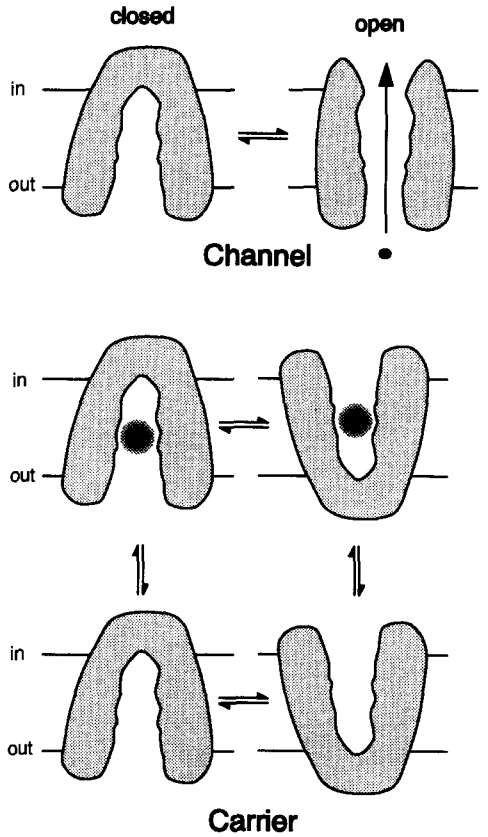

Fig. 4. The difference between channel- and carrier-mediated transport. In this schematic depiction of channel and carrier activity, the structural similarities of channel and carriers, as well as the functional differences, are emphasized. In the upper part, the interconversion of closed and open (conducting) states of a channel are shown. The lower part of the figure, shows solute transport by a prototypical carrier. The two carrier forms on the left represent the carrier with its binding site facing the external medium and the two on the right show the binding site facing the cytoplasm. The two upper carrier forms show solute bound to the transporter.

Cl^- and K^+ in a stoichiometric manner. The problem faced by a coupled transporter is more complicated than that faced by an ion channel, since a channel can function merely by allowing its substrate ions to flow across the lipid bilayer. Such uncoupled flux will dissipate the ion gradients and will not utilize them to concentrate another substrate. However, the structural similarities between transporters and ion channels may give a clue to the mechanism of transport (Fig. 4). Just as an ion channel may have a central aqueous cavity surrounded by amphipathic membrane-spanning helices, a transporter may have a central binding site which accomodates Na^+, Cl^- and substrate. The difference in mechanism between a transporter and a channel may be that, while a channel assumes open (conducting) and closed (non-conducting) states, a transporter can also assume two states which differ only in the accessibility of the central binding site. In each of these states, the site is exposed to only one face of the membrane, and the act of substrate translocation represents a conformational change to the state where the binding site is exposed on the opposite face (Fig. 4). Thus, the transporter may behave like a channel with a gate at each face of the membrane, but only one gate may be open at any point in time.

For this mechanism to lead to co-transport of ions with substrate molecules, the transporter must obey a set of rules governing the conformational transition between its two states (Fig. 5). For cotransport of Na^+, Cl^- and 5-HT, the rule would allow a conformational change only when the binding site was occupied with Na^+, Cl^- and substrate or when the site was completely empty. To account for K^+ countertransport with 5-HT, the conformational change could occur when the binding site contained either K^+ or Na^+, Cl^- and 5-HT. This simple model of a binding site exposed alternately to one side of the membrane or the other can explain most carrier-mediated transport. However, it makes specific predictions about the behavior of the transport system.

In particular, this model requires that 5-HT is transported in the same step as Na^+ and Cl^- but in a different step than K^+. The evidence that 5-HT and K^+ are transported in different steps comes from efflux and exchange experiments with plasma membrane vesicles. In vesicles containing NaCl, [^{3}H]5-HT efflux is supported by external K^+ in a reversal of the normal influx reaction. In the absence of external K^+, radiolabel efflux is inhibited, but it can be accelerated by external unlabeled 5-HT [66]. This reaction represents an exchange of internal and external 5-HT which does not require the K^+-dependent rate-limiting step for net 5-HT efflux. Thus, the steps required for 5-HT binding, translocation and dissociation do not include the steps (where K^+ is translocated) that become rate-limiting in the absence of K^+.

Another prediction of the model for biogenic amine transport is that Na^+, Cl^- and substrate should all be bound to the transporter prior to translocation. Since it has been difficult to directly measure substrate binding to the biogenic amine transporters, the most direct data relating to this point comes from studies of the

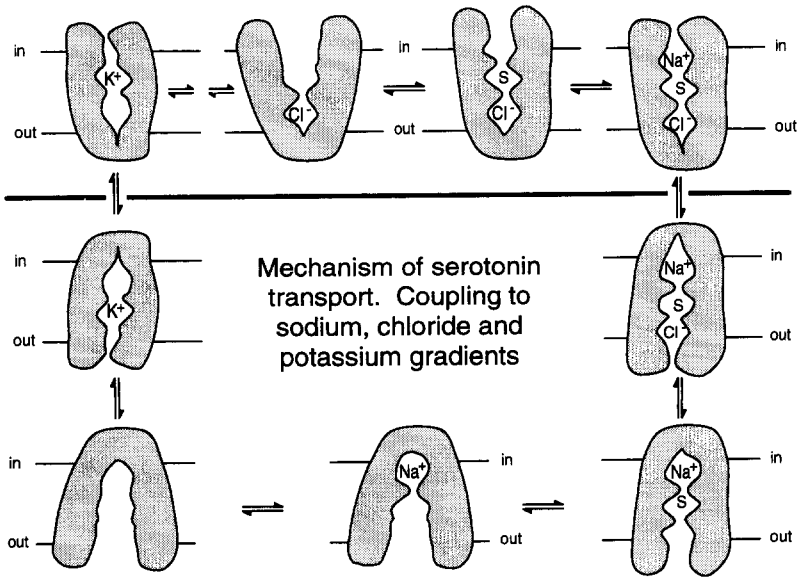

Fig. 5. Model for Na^+, Cl^- and K^+ coupling to 5-HT transport. Counter-clockwise from the lower left, the transporter is shown binding external Na^+, 5-HT (S) and Cl^- and then interconverting to a form (above the line) in which the binding sites for these ligands is accessible from the cytoplasm. This interconversion represents the transport process. Following dissociation of Na^+, 5-HT and Cl^-, the transporter binds internal K^+ (upper left) and converts to a form which can release K^+ to the external medium (left side) to return to the starting point.

Na^+ and Cl^- dependence of inhibitor binding. The tricyclic antidepressants imipramine and desipramine are high-affinity ligands for the 5-HT and NE transporters, respectively. Na^+ and Cl^- both increase the affinity of the 5-HT transporter for imipramine [78] and the NE transporter for desipramine [79,80]. To the extent that imipramine and desipramine binding reflect the normal process of substrate binding, these results indicate that Na^+ and Cl^- are bound together with substrate prior to translocation.

This conclusion is likely to extend also to 5-HT and NE binding, but there are differences between substrate transport and inhibitor binding. For example, the Na^+ dependence of transport rate is a simple hyperbolic function consistent with the involvement of one Na^+ ion. In contrast, the Na^+ dependence of imipramine and desipramine binding is sigmoidal, indicating that more than one Na^+ ion is involved [76,80]. Furthermore, other ligands have different binding requirements. Neither paroxetine nor analogs of cocaine, for example, require Cl^- for binding [81,82], and cocaine analogs exhibit a pH dependence not seen with imipramine [81,83]. Finally, the rate of imipramine binding is much too slow to reflect the 5-HT binding step in transport. However, more indirect experiments, using 5-HT or DA to displace either [^{3}H]imipramine [76] or an ^{125}I-labeled cocaine analog [83], indicated that both Na^+ and Cl^- increase the affinity of substrate binding. Thus, it is likely that all three are bound together to the transporter and are transported together.

IV. Identification and characterization of Na^+-dependent biogenic amine transporter cDNAs

As an attempt to identify biogenic amine transporters, a number of groups have attempted to purify the 5-HT transporter to homogeneity. The protein has been solubilized in an active form using digitonin [84]. Using this solubilized preparation, two groups achieved significant purification of the 5-HT transporter from platelet and brain using affinity resins based on citalopram, a high-affinity ligand for the transporter [85,86]. In neither case was the protein purified to homogeniety or reconstituted in liposomes to recover transport activity. Launay et al. [87] have apparently purified the transporter to homogeneity using affinity colums based on 5-HT itself or 6-fluorotryptamine, but also did not reconstitute transport activity with the purified protein. Reconstitution of the transporter has, however, been demonstrated with a urea-cholate extract of placental membranes [88]. As these studies were in progress, molecular cloning of other Na^+-dependent transporter cDNAs was providing an independent approach for studying their structure.

The identification of cDNAs for Na^+-dependent biogenic amine transporters has contributed greatly to our understanding of the molecular structure and function of these important proteins. The first cDNA encoding a biogenic amine carrier was identified by Pacholczyk et al. [2] using an expression cloning strategy in COS cells. A norepinephrine transporter cDNA (NET) was isolated from an SK-N-SH human neuro-

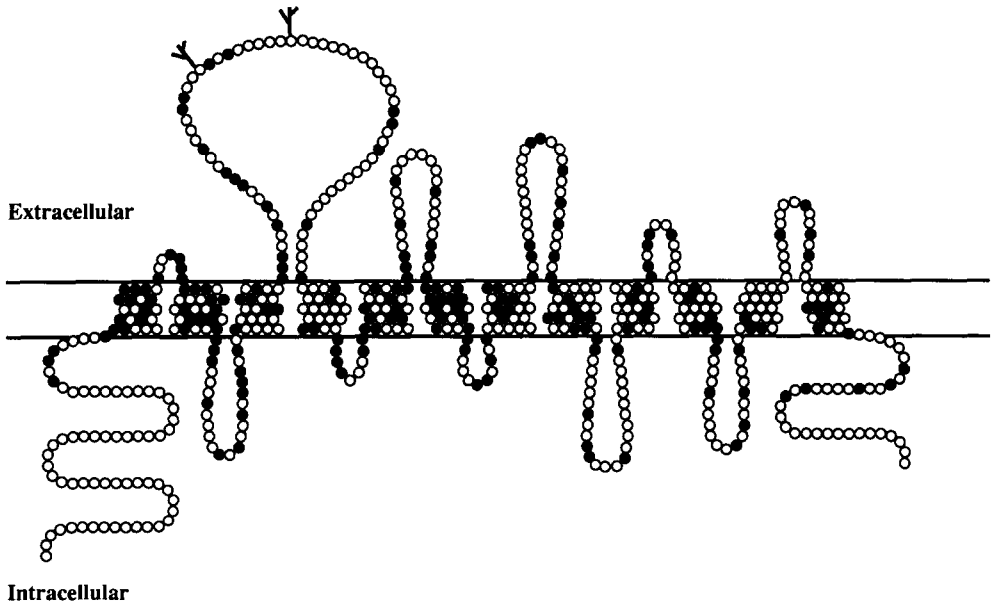

Fig. 6. Schematic representation of the serotonin transporter. Proposed model of the serotonin transporter with twelve transmembrane domains, cytoplasmic amino and carboxy-termini, proposed *N*-linked glycosylation sites and residues conserved in dopamine and norepinephrine transporters (●).

blastoma library on the basis of its ability to direct the transport of ^{125}I-labeled *m*-iodobenzylguanidine, a norepinephrine analog. Comparison of the predicted amino-acid sequence for NET with that for the Na^+-dependent γ-aminobutyric acid (GABA) transporter, GAT-1, previously identified by Guastella et al. [1] revealed that these transporters are members of a multigene family. Given the significant degree of homology between the two transporters, several groups designed degenerate oligonucleotide primers and have used them in homology-based polymerase chain reaction strategies for identifying additional members of the family. Several members of this transporter gene family have been identified, including carriers for GABA [13,14], taurine [12,15], the kidney osmolyte betaine [18], glycine [16,89] and proline [11], in addition to biogenic amine transporters for dopamine [5–8,17] and serotonin [3,4]. Hydropathy analyses of these transporter sequences are virtually superimposable, and predict proteins with 12 transmembrane domains having both their amino and carboxy-termini on the cytoplasmic side of the plasma membrane (Fig. 6). There is a significant degree of identity across this family of transporters (25%) despite their very different substrate specificities. Homology is highest in the proposed transmembrane domains (TM) and particularly sparse in the extracellular loop connecting TM3 and TM4. This large extracellular loop is the location of from 1–4 potential *N*-linked glycosylation sites in each transporter sequence.

Alignment of the amino-acid sequences for the biogenic amine transporters (Fig. 7) shows that 41% of their residues are identical. This homology increases to a total of 54% when conservative substitutions are considered. The various dopamine transporter cDNAs encode virtually identical proteins with the exception of a few amino-acid differences between species [5–8,17]. Similarly, the two rat serotonin transporter

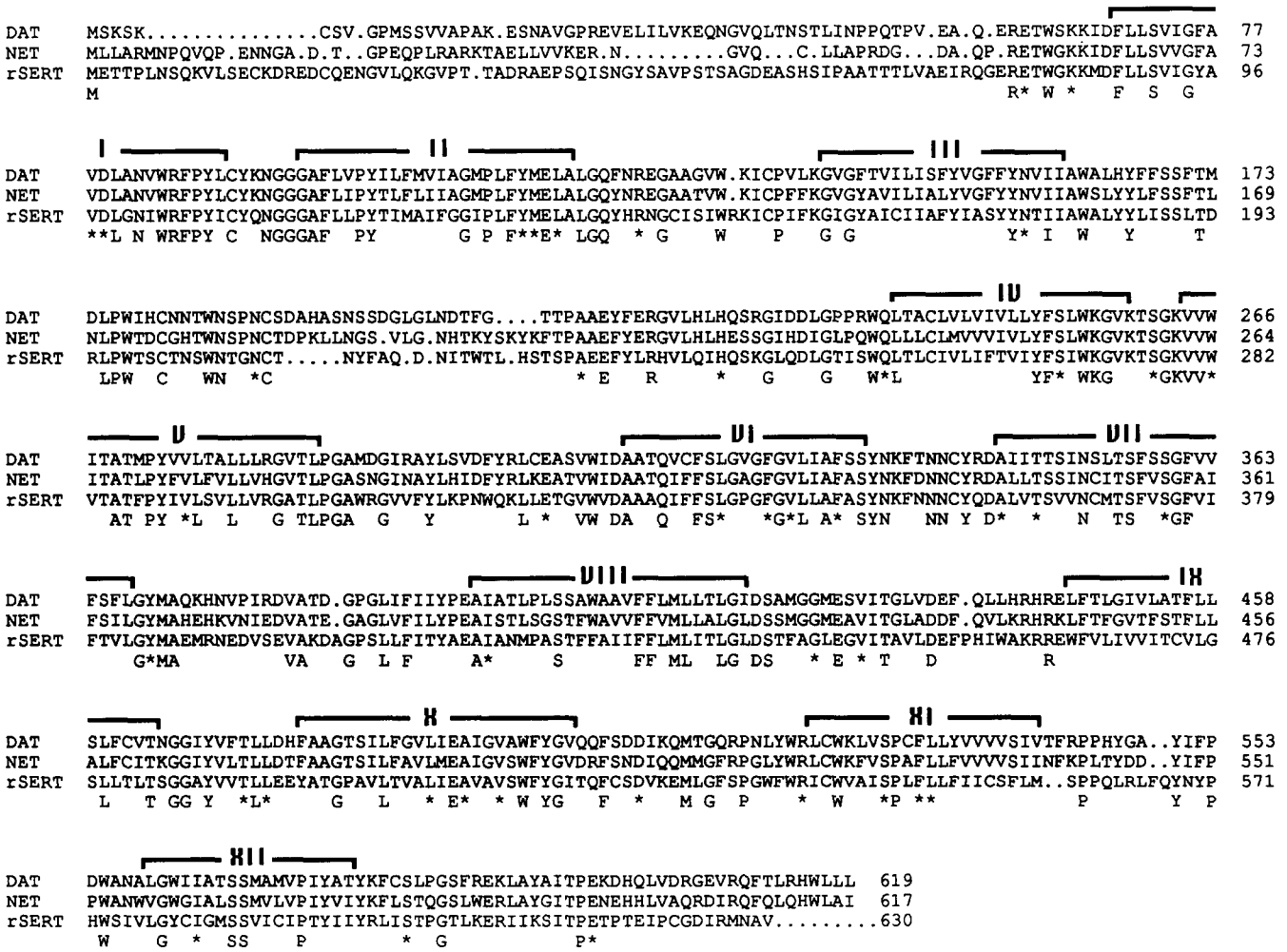

Fig. 7. Alignment of the amino-acid sequences of rat DAT, human NET and rat rSERT. Transmembrane domains for DAT are bracketed above the appropriate residues. Residues that are common to all members of this transporter gene family are designated in the fourth line. Asterisks identify residues that are unique to the biogenic amine carriers. DAT, dopamine transporter [6]; NET, norepinephrine transporter [2]; rSERT, serotonin transporter [4].

cDNAs encode identical proteins [3,4,90]. There are 42 residues that are uniquely conserved in the biogenic amine carriers and are not conserved in other members of the neurotransmitter transporter family (Fig. 7). Several of these unique residues are charged or protonatable, and are found clustered in the transmembrane domains. It has been suggested that these may play a role in the binding and/or transport of several polar amino groups that are found in inhibitors and substrates for the biogenic amine transporters [4,20]. Binding to the transporter is likely to involve interaction of ligands with residues scattered throughout the proposed transmembrane domains.

Recent work examined the binding properties of a cocaine analog 2β-2-carbomethoxy-3-(4-[^{125}I]iodophenyl)tropane (β-[^{125}I]CIT) to the serotonin and dopamine transporters in rat striatal membranes and platelet plasma membranes [83]. Many similarities between the two transporters were found. For example, in contrast to the requirements for substrate interaction with these transporters, β-[^{125}I]CIT binding was pH-dependent and Cl^--independent. These findings suggest that there are multiple sites on the transport protein that contribute to substrate and inhibitor interactions, and suggest that there is a protonatable residue involved with the binding of cocaine to both the serotonin and dopamine transporters. One difference between the two transporters is that, while β-[^{125}I]CIT binding to both is stimulated by Na^+, the Na^+ dependence is sigmoidal for DAT and hyperbolic for SERT. This behavior is similar to the Na^+ dependence of transport by the two systems [72,76] and may reflect different Na^+ transport stoichiometries.

Kitayama et al. [20] have demonstrated that mutation of a single Asp residue at position 79 in TM1 of the dopamine transporter to Ala or Gly significantly reduces its ability to transport both [^{3}H]dopamine and the neurotoxin [^{3}H]1-methyl-4-phenylpyridinium ([^{3}H]MPP$^+$). The same mutations decrease the transporter's ability to bind the cocaine analog [^{3}H]2-carbomethoxy-3-(4-fluorophenyl)tropane ([^{3}H]CFT). Binding of [^{3}H]CFT is affected less by mutation of Asp-79 to Glu. In this same study mutation of Ser-356 to Gly or Ala significantly altered transport while having much less of an effect on binding. In the future, further examination of residues unique to the biogenic amine carriers should allow one to discern the sequence elements involved with both binding and substrate transport, as well as ion interactions.

Functional expression of biogenic amine transporter cDNAs in heterologous systems has led to the determination of their transport kinetics and inhibitor sensitivities that may be compared with native carriers. NET was isolated from a SK-N-SH human neuroblastoma cell library and expressed in HeLa cells for transport studies [2]. HeLa cells expressing NET exhibited a saturable and Na^+-dependent uptake of norepinephrine, with a K_m of 457 nM. Transport of norepinephrine was inhibited by a number of agents with a rank order of potency identical to that observed for the native carrier in SK-N-SH cells and cortical synaptosomes. Among the most potent inhibitors of norepinephrine transport were the tricyclic antidepressants desipramine and nortryptilene, the psychostimulants (+)-amphetamine and cocaine, and dopamine. Northern blot analysis with an NET probe showed two hybridizable species of 5.8 and 3.6 kb in SK-N-SH cells, PC-12 cells, rat brainstem and adrenal gland. A single 3.6 kb species was found in rat midbrain, forebrain, cortex, cerebellum and basal ganglia. The predominant 5.8 kb species is thought to represent the cloned transporter, while the significance of the ubiquitous 3.6 kb species is not understood.

TABLE I

Characteristics of heterologously expressed biogenic amine transporter cDNAs

cDNA	Species	Substrate	K_m (nM)	mRNA) size (kb)
NET [2]	Human	Norepinephrine	457	5.8 3.6
DAT [6]	Rat	Dopamine	885	3.6
DAT1 [5]	Rat	Dopamine	1190	3.7
DAT [7]	Bovine	Dopamine	31500	3.0
DAT [8]	Rat	Dopamine	300	n.d.
DAT [17]	Human	Dopamine	1200	n.d.
5-HTT [3]	Rat	Serotonin	529	3.1
rSERT [4]	Rat	Serotonin	320	3.7

n.d., not determined.

To date several dopamine transporter cDNAs have been isolated from rat, bovine and human brain libraries [5–8,17]. The various heterologous systems which have been used for the functional characterization of these transporters all demonstrate saturable, high-affinity, Na^+-dependent transport of dopamine with K_m values ranging from 300 nM to 31.5 μM (Table I). These values are somewhat higher than those determined for [^{3}H]DA transport into striatal synaptosomes [91,92]. Although the K_i values determined for a number of dopamine transport inhibitors varied considerably between studies, the rank order of potency was the same (GBR 12909 > mazindol > cocaine > nomifensine > desipramine), with the exception that the relative order of cocaine and nomifensine is sometimes reversed [5,7,8]. Probes made to the rat DAT hybridized to mRNAs of 3.6 to 3.7 kb [5,6], while a band for bovine DAT appeared at 3.0 kb [7] by Northern blot analysis. In situ hybridization has shown DAT mRNA concentrated in substantia nigra pars compacta and ventral tegmental area of both rat and bovine brain [7,8]. While the transcript size of the human

DAT was not determined, previous studies with low stringency hybridization using a bovine DAT probe showed a 4.5 kb mRNA hybridizing in human substantia nigra RNA [7]. Chromosomal hybridization with human DAT probes has resulted in the identification of the corresponding gene on the distal end of chromosome 5 [17].

Serotonin transporter cDNAs have been isolated independently from rat basophilic leukemia cells, rat brainstem, human placenta and mouse [3,4,93,94]. Transport by the serotonin transporter expressed in transfected CV-1 and HeLa cells was high affinity and saturable with a K_m from 300 to 500 nM (Table I) [3,4]. Serotonin transport was shown to depend on both Na^+ and Cl^-, which is characteristic of all members of this family examined thus far. As a result, this group of carriers has come to be known as the Na^+-and Cl^--dependent neurotransmitter transporter family (for review, see Ref. 95). Several tricyclic and heterocyclic antidepressants inhibited the heterologously expressed serotonin transporter with a rank order of potency similar to that observed for the native brain serotonin transporters [96], indicating that the expressed protein posseses the ability to both transport serotonin and to bind antidepressants. The anorectic drug fenfluramine and the neurotoxin MDMA were extremely effective at blocking serotonin uptake [3]. Northern blot analyses reveal a single mRNA species of 3.1 to 3.7 kb in size expressed in rat midbrain and brainstem, as well as several peripheral tissues including rat gut, lung, adrenal, spleen, stomach, uterus and kidney [3,4]. In situ hybridization histochemistry with serotonin transporter probes revealed mRNA in serotonergic neurons of the rat brain, in addition to the lamina propria of the stomach and duodenum, and the chromaffin cells of the adrenal [4].

To allow for more detailed comparison between biogenic amine transporters, the NE and DA transporter cDNAs have been used to construct cell lines stably expressing these transporters (Gu, H., Wall, S. and Rudnick, G., unpublished data). In LLC-PK_1 cells, these catecholamine transporters display dramatically different requirements for Na^+ and Cl^-. The Na^+ dependence of DA accumulation by LLC-PK_1 cells stably expressing the DA transporter is sigmoidal, suggesting that more than one Na^+ ion is involved. In contrast, DA accumulation by the NE transporter expressed in LLC-PK_1 cells displays a hyperbolic Na^+ dependence. The Na^+ dependence mirrors the behavior of each system in synaptosomes, and indicates that the differences reflect intrinsic properties of the transporters. The Cl^- dependence of transport also differs markedly between the two catecholamine transporters. The NE transporter requires only approx. 5 mM Cl^- for maximal activity, but the DA transporter requires concentrations over 20-times higher.

Identification of cDNAs for the biogenic amine transporters has contributed greatly to the molecular understanding of these proteins. Future studies on transporter structure and function will be needed to understand how substrates and inhibitors interact with the protein and cause their physiological effects. The information obtained from such studies will aid in understanding how altered transport function may contribute to the psychopathology of affective disorders, and will be helpful to further rational drug design.

V. How is Na^+-dependent biogenic amine transport regulated?

Alterations in Na^+-dependent biogenic amine transport have been identified in both neurological [97] and psychiatric disorders [98–105]. Although the underlying mechanism(s) of these alterations are not yet understood, they seem to involve changes in the substrate concentration dependence of transport (K_m) or its maximal velocity (V_{max}). Biogenic amine transport regulation under normal conditions has been shown for serotonin transport in response to both circadian and seasonal changes [102,105]. Studies which compare normals to clinically depressed patients have shown alterations in platelet serotonin transport [98,99,101, 106]. In one such study, patients showed seasonal alterations in both K_m and V_{max} for serotonin transport throughout a 2-year period, yet these alterations were different in patients as compared to controls [105]. It has been suggested that changes in the normal fluctuation of serotonin transport may contribute to the susceptibility of an individual to depression [102]. Although many studies report a correlation between depression and altered platelet and/or brain serotonin transport, in general the field is not in agreement as to whether these changes exist and are significant. A recent study examining imipramine binding in brain from post mortem suicide victims as compared to controls [104] reported no difference in the K_d or B_{max} for binding in the frontal cortex. Others, however, have identified decreases in serotonin transport sites in brain from post mortem human depressed patients, as evidenced by a decrease in the B_{max} for imipramine binding (for review, see Ref. 107). It is likely that experimental variability across studies is responsible for the conflicts that exist in the literature. Alterations in platelet serotonin transport have been found in patients with multiple sclerosis as well [97]. The observed increase in K_m for platelet serotonin transport was positively correlated with the disability status of the patients.

In addition to depression and multiple sclerosis, aberrant biogenic amine transport has been found in patients with schizophrenia [101,103]. Examination of post mortem brain tissue from schizophrenic patients

and controls showed alterations in both norepinephrine and dopamine transport, but not in serotonin transport [103]. There was an increase in the K_m and V_{max} for dopamine and norepinephrine transport in the nucleus accumbens and nucleus caudatus. It is evident from these studies and others that alterations in biogenic amine transport are associated with the pathogenicity of these and other disorders. Discerning how these transporters may be regulated will aid in the understanding of how transport function becomes altered in neurological and psychiatric disease states.

Much of the research to date on the regulation of biogenic amine transport has examined the effects of agents that activate several components of second messenger systems on serotonin transport in cell lines or platelets [108–115]. Potential mechanisms for transport regulation by second messengers are shown in Fig. 8. Serotonin transport in the JAR human placental choriocarcinoma cell line (JAR cells) is enhanced by incubation of the cells in cholera toxin [110]. Cholera toxin treatment resulted in an increase in the V_{max} and a decrease in the K_m for serotonin transport. The effects of cholera toxin could be mimicked by isobutylmethylxanthine (IBMX), dibutyryl cAMP and forskolin, and antagonized by the protein kinase inhibitor *N*-(2-aminoethyl)-5-isoquinolone sulfonamide. A second study compared the effects of cholera toxin on serotonin transport in JAR cells and PC12 rat adrenal pheochromocytoma cells (PC12 cells) [111]. Incubation of cells in cholera toxin resulted in differential regulation of serotonin transport in JAR and PC12 cells. Cholera toxin stimulated serotonin transport in JAR cells, yet potently inhibited transport in PC12 cells. Once again the effects of cholera toxin were mimicked by forskolin and IBMX in both cell lines. Assuming that the serotonin transporters in these two cell lines are the same, these findings suggest that cellular components may play a role in determining how transport will be regulated in different cell types. Regulation by second messengers has also been reported for dopamine transport. In a rat hypothalamic cultured cell line, DA transport is enhanced in a dose-dependent manner by treating the cells with dibutyryl cAMP and forskolin [112].

In vivo and in vitro experiments have shown that activation of the inositol trisphosphate (IP_3) cascade by agents such as phorbol 12-myristate 13-acetate (PMA) can alter serotonin transport [108,109,113,114]. Intravenous injection of PMA into anesthetized dogs resulted in a decrease in serotonin removal from the blood [113]. Similarily, PMA treatment of a perfused rabbit lung preparation resulted in a significant decrease in serotonin removal [108]. Although the decrease in serotonin uptake that was observed in these studies may be the result of any number of harmful effects that PMA may have on cells, more recent studies have shown that these effects are likely to result from activation of the IP_3 cascade and protein kinase C (PKC) [109,115]. Blood platelet serotonin transport is inhibited by treatment with PMA and mezerein, a phorbol ester analog [115]. Treatment with these phorbol esters has no effect on the K_m, but causes a consistent reduction in V_{max} values. The reduction in V_{max} was blocked by staurosporine, a PKC inhibitor, and was not associated with changes in transporter number or ion gradients. These results suggest that the transporter activity is regulated by activation of PKC. In a separate study, PMA treatment of bovine artery endothelial cells resulted in a concentration-dependent decrease in serotonin transport that could be mimicked by the PKC stimulants phorbol-12,13-dibutyrate and mezerein, but not with the inactive isomer phorbol-12,13-didecanoate. Cells pretreated with the PKC inhibitor staurosporine were resistant to the effects of PMA. Of particular interest is the finding that PMA treatment did not increase total PKC activity in these cells, but caused a translocation of active PKC from the cytosol to the plasma membrane. PMA treat-

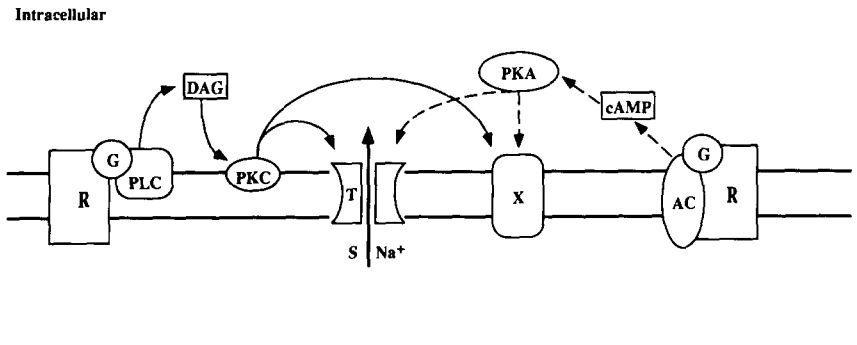

Fig. 8. Diagram of potential modes of transport regulation by second messenger systems. Direct regulation by phosphorylation of the transport protein (solid lines). Indirect regulation of transport by phosphorylation of a membrane protein (×) (dashed lines), such as Na^+/K^+ATPase, which may regulate ion gradients necessary for transport. R, receptor; G, guanine nucleotide binding protein; PLC, phospholipase C; T, transporter; S, substrate; PKA, cAMP-dependent protein kinase; AC, adenylyl cyclase.

ment may affect serotonin uptake by translocating activated PKC to the membrane. There it may act on the transporter or other membrane proteins, such as Na^+/K^+-ATPase, which regulate ion gradients required for transport.

Chronic and acute exposure to drugs such as cocaine or nicotine have also been shown to alter biogenic amine transport function [116–118]. The nucleus accumbens from rats receiving a single injection of cocaine daily for three days showed a decrease in dopamine uptake, resulting from an increase in the K_m for transport, and a supersensitivity to the inhibitory effects of cocaine [116]. The effect of cocaine on dopamine transport was selective for the nucleus accumbens as no change was seen in the striatum. In contrast to cocaine, nicotine does not inhibit dopamine transport by direct interaction with the transport protein [117]. Inhibition by nicotine cannot be blocked by GBR 12935, a highly-selective inhibitor of dopamine transport, but is mimicked by the nicotinic agonists carbachol and 1,1-dimethyl-4-phenylpiperazinium iodide and blocked by nicotinic antagonists. Nicotine has no effect on synaptosomal dopamine transport, suggesting that post synaptic elements are required for the regulation of dopamine transport in these chopped tissue preparations. Regulation of dopamine uptake by nicotine was proposed to involve the nicotine-stimulated release of a neurotransmitter from a postsynaptic element that interacts with a presynaptic receptor, stimulating a second messenger system that is capable of affecting transport [117].

Although it is clear from the literature that Na^+-dependent transport is regulated by a number of agents, the mechanisms by which this regulation occurs remain to be elucidated. Regulation might be the result of direct phosphorylation of transport proteins by various kinases (Fig. 8). Consensus sequences for phosphorylation sites recognized by various kinases have been identified in biogenic amine transporter amino-acid sequences [4,7,8,17]. A canonical site for phosphorylation by cAMP-dependent protein kinase was identified in the serotonin transporter sequence located in the amino-terminus near the inititation codon [4]. Consensus phosphorylation sequences were identified for both cAMP-dependent protein kinase and PKC in several of the dopamine transporter sequences [7,8,17]. Potential residues for phosphorylation in the dopamine transporter sequences were found in predicted intracellular sequence regions throughout the protein.

Since the studies described above did not measure phosphorylation states of the regulated transport proteins, one must consider all possible modes of regulation which might contribute to alterations in transporter function. Regulation of transport may be indirect via the phosphorylation of another plasma membrane protein, such as an ion channel or Na^+/K^+-ATPase, which would modify ion gradients necessary for transport (Fig. 8). Other modes of regulation may include transcriptional or translational modulation of transporter expression, stabilization of transporter against degradation, or recruitment of transporters from an intracellular compartment. The insulin-responsive glucose transporter is regulated by recruitment from intracellular vesicles to the plasma membrane [119]. It is not yet clear what role regulation of transport processes plays in vivo. However, understanding the various mechanisms by which regulation may occur will help in discerning how transport regulation may contribute to modulating neuronal activity.

VI. How do storage vesicles transport amines?

Accumulation of biogenic amines within secretory organelles is similar in a variety of cell types storing NE, DA, 5-HT, Epi and histamine. The transport system consists of two components: a vacuolar ATP-driven H^+ pump that acidifies the organelle interior [23] and a vesicular amine transporter that exchanges these internal H^+ ions with cytoplasmic biogenic amines [120,121]. Among the organelles that contain this system are synaptic vesicles, adrenal chromaffin granules, platelet dense granules and mast cell and basophil secretory granules [52,121–124]. In the absence of ATP, imposition of a transmembrane pH difference (ΔpH) provides a driving force for biogenic amine accumulation [120,125,126]. A consequence of ATP-driven H^+ pumping into these organelles is the development of a transmembrane electrical potential difference ($\Delta\psi$) [127]. This membrane potential is also a driving force for amine accumulation [128–132]. Studies from a number of laboratories have led to the understanding that for every protonated amine molecule taken into the vesicle, two H^+ ions are released into the cytoplasm [123,133,134]. Either of two mechanisms of transport account equally well for this overall stoichiometry. In one, the protonated amine molecule is the true substrate which is exchanged for two H^+ ions [135]. A stoichiometrically and thermodynamically equivalent mechanism has also been proposed in which the amine dissociates prior to binding as the neutral free base. The free base is subsequently exchanged for a single H^+ ion [136]. Either way, amine accumulation is dependent on both the ΔpH and the $\Delta\psi$. Because the amine substrate is exchanged for the equivalent of two H^+ ions, a 10-fold H^+ concentration gradient (one pH unit) will lead to a 100-fold gradient of substrate. Membrane potential is not as strong a driving force, since only one charge crosses the membrane with each catalytic cycle.

Biogenic amine accumulation into chromaffin granules has been used as a model for amine storage within all monoaminergic secretory organelles. In these gran-

ules, biogenic amine accumulation is blocked by reserpine and tetrabenezine, two inhibitors that have been used to identify the vesicular amine transporter. Henry and co-workers [137] used [^{125}I]iodoazidoketanserin to label a polypeptide of approx. 80 kDa. Schuldiner and co-workers took advantage of the slow dissociation of reserpine from the transporter [138] to isolate the transporter in an active form from detergent solubilized bovine chromaffin granule membranes [139]. The purified preparation consisted of an 80 kDa glycoprotein that could reconstitute tetrabenizine-sensitive H^+/5-HT exchange in proteoliposomes.

VII. Cloning the vesicular amine transporter cDNA

Two expression cloning strategies led to the isolation of cDNAs encoding vesicular amine transporters. Edwards and co-workers [140] were investigating the mechanism by which PC-12 cells resist the toxic action of the neurotoxin MPP^+ [141], which was known to be accumulated by chromaffin granules [142]. Transfection of CHO fibroblasts, normally sensitive to MPP^+, with cDNA from PC-12 cells led to the appearance of MPP^+-resistant transfectants which concentrated DA into vesicular structures. When the PC-12 cDNA responsible for this property was isolated and sequenced [143], it was found to predict peptide sequences highly homologous to those of peptides derived from the purified bovine chromaffin granule amine transporter [139].

Independently, Hoffman and co-workers [144] transfected CV-1 cells with cDNA prepared from rat basophilic leukemia (RBL) cell mRNA. RBL cells take up 5-HT using the plasma membrane 5-HT transporter, and store it in secretory granules containing a vesicular amine transporter. Some of the cDNA clones that conferred increased 5-HT uptake on CV-1 cells encoded a vesicular transporter which functioned to accumulate 5-HT in acidic organelles. The sequence of the RBL vesicular amine transporter is highly homologous to the vesicular transporter cloned from PC-12 cells, and almost identical to a vesicular amine transporter cloned from rat brain [143]. Despite the similarity among cloned vesicular transporters, there is essentially no homology between vesicular and plasma membrane transporters for biogenic amines. Limited homology, however, was found between vesicular amine transporters and bacterial drug resistance proteins [140,144].

Apparently, expression of the amine transporter in CHO and CV-1 cells leads to its localization to acidic organelles. Using the existing ΔpH, the transporter sequesters MPP^+, 5-HT and DA within the organelle and decreases their cytoplasmic concentration. In the case of MPP^+, this represents a detoxifying mechanism that rescues the transfected CHO cells.

VIII. Summary

Biogenic amine transport systems in the presynaptic plasma membrane and the synaptic vesicle provide a mechanism for rapidly terminating the action of released transmitters and for recycling neurotransmitters. Alterations in the activity of these transporters, either by endogenous regulatory mechanisms or by drugs, affect the regulation of synaptic transmitter levels. For drugs such as antidepressants and stimulants that interact with these transport systems, the therapeutic and behavioral consequences are profound. Now that the cDNAs encoding the transporters have been isolated, we can expect rapid progress in understanding how the individual proteins work at the molecular level to couple ion gradients to the reuptake and storage of biogenic amine neurotransmitters.

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