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Bioenergetics of Neurotransmitter Transport

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Neurotransmitter transporters are essential components in the recycling of neurotransmitters released during neuronal activity. These transporters are the targets for important drugs affecting mood and behavior. They fall into at least four gene families, two encoding proteins in the plasma membrane and two in the synaptic vesicle membrane, although the known vesicular transporters have not all been cloned. Each of these transporters works by coupling the downhill movement of small ions such as Na^+, Cl^-, K^+, and H^+ to the uphill transport of neurotransmitter. Plasma membrane transporters move the transmitter into the cytoplasm by cotransport with Na^+. Many transporters also couple Cl^- cotransport to transmitter influx and these all belong to the NaCl-coupled family, although within the family the coupling stoichiometry can vary. Transporters for glutamate couple influx of this excitatory amino acid to Na^+ and H^+ influx and K^+ efflux. Transporters in synaptic vesicles couple H^+ efflux to neurotransmitter transport from the cytoplasm to the vesicle lumen.

KEY WORDS: Ion coupling; stoichiometry; vesicle; plasma membrane.

INTRODUCTION

Neurotransmitter transporters are responsible for the uptake and storage of chemical transmitters. These neurotransmitters are released by exocytosis of synaptic vesicles with the neuronal plasma membrane in response to a depolarizing action potential. They act at receptors on the external surface of other nerve cells and also with autoreceptors on the cell from which they are released. This process of neurotransmission is terminated by a number of processes including diffusion and metabolic degradation of the neurotransmitter, and desensitization of the receptors. However, the most common inactivation process is transport, or reuptake, into the nerve cell that released the transmitter, or into neighboring neurons or glial cells. After transport into the cytoplasm, the neurotransmitter is further transported into synaptic vesicles. Neurotransmitter transporters on synaptic vesicle and plasma membrane play important roles in the normal and pathological physiology of the brain. Drugs that interfere with these transport processes have profound effects on mood and behavior. They include the antidepressants, such as imipramine and fluoxetine, and stimulants such as amphetamine, methylphenidate, and cocaine.

Most of the neurotransmitter transporters belong to a large family of plasma membrane proteins that couple solute movement to the inward flux of sodium and chloride ions (Amara and Kuhar, 1993; Borowsky and Hoffman; 1995, Lester *et al.*, 1996; Nelson and Lill, 1994; Uhl and Johnson, 1994). Approximately 25% of the amino acid sequence of these proteins is conserved throughout the family. Within this family is a group of transporters for the biogenic amines dopamine (DA), norepinephrine (NE), and serotonin (5-HT). These transporters share a high degree of sequence identity (about 50%) and a common sensitivity to cocaine (Rudnick and Clark, 1993). Another group of transporters are responsible for accumulation of γ-aminobutyric acid (GABA). These transporters, which share about 50% sequence identity, include the GABA transporters GAT-1, GAT-2, and GAT-3 as well as the betaine transporter BGT-1 (Nelson and Lill,

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1994). The NaCl-coupled transporter family also includes transporters for the amino acids glycine (Smith *et al.*, 1992), proline (Fremeau *et al.*, 1992), and taurine (Uchida *et al.*, 1992), a creatine transporter (Guimbal and Kilimann, 1993), and a few homologous orphan proteins for which no transport function has been demonstrated.

The glutamate transporters represent a separate family, distinct in structure and properties from the NaCl-coupled transporter family (Kanner, 1993). They include five independently isolated clones, GLAST (EAAT1), GLT-1 (EAAT2), EAAC-1 (EAAT-1), EAAT-4, and EAAT-5, all of which transport glutamate and aspartate. In addition to these glutamate transporters, two amino acid exchangers, ASCT-1 and ASCT-2, belong to the same family (Utsunomiyatate *et al.*, 1996).

Neurotransmitters that are accumulated by nerve terminals are further concentrated within synaptic vesicles by vesicular transport systems. These transporters are not known to be related either structurally or functionally to any of the plasma membrane families. The biogenic amines and acetylcholine are transported into synaptic vesicles by a family of related transporters VMAT-1 (Liu *et al.*, 1992), VMAT-2 (Peter *et al.*, 1994), and VAChT (Erickson *et al.*, 1994). The two VMAT clones are highly similar in sequence, and transport the biogenic amines DA, NE, and 5-HT. VMAT-2 additionally transports histamine (Peter *et al.*, 1995), and VAChT is selective for acetylcholine (Erickson *et al.*, 1994). A distinctly different protein (R. Edwards, personal communication) is responsible for GABA accumulation in synaptic vesicles (Hell *et al.*, 1990). This transporter bears little sequence homology to VMAT and VAChT. Finally, an independent vesicular transporter for glutamate has been characterized (Maycox *et al.*, 1988) but not identified at the molecular level.

The function of neurotransmitter transporters has been thought of as analogous to that of acetylcholinesterase. This enzyme terminates the action of acetylcholine at the neuromuscular junction by hydrolyzing it to choline and acetate. Other neurotransmitters (aside from peptides) are not inactivated enzymatically. Rather they are removed from the extracellular space by the transport systems (Fig. 1). However, recent evidence suggests that the transporters are not concentrated at the sites of neurotransmitter release, like acetylcholinesterase, but are separated by some distance from the release sites. Immunological localization of the dopamine transporter (DAT) shows that it is close to sites of release on axons and dendrites, but is excluded from the vesicular fusion zone (Hersch *et al.*, 1995; Nirenberg *et al.*, 1996). Furthermore, the primary location for brain glutamate transporters appears to be on cells receiving glutamatergic input and on glial cells but not on cells releasing glutamate (Lehre *et al.*, 1995). Many neuronal neurotransmitter receptors are rapidly desensitized by their agonist, calling into question the importance of transporters in terminating transmitter action. However, the profound effects on mood and behavior by drugs that interfere with neurotransmitter transport indicate that they are important for normal brain function.

Some neurotransmitter transporters are located on cells outside the nervous system, while others are restricted to neurons. The glutamate transporter EAAT-3, for example, is found on neurons in the brain, but it is also quite abundant in kidney as well as other tissues (Arriza *et al.*, 1994). Among the GABA transporters, GAT-1 and GAT-3 are neuronal, but GAT-2 and BGT-1 are found in kidney and liver (Jursky *et al.*, 1994). The placenta expresses both norepinephrine and serotonin transporters (NET and SERT, respectively) (Balkovetz *et al.*, 1989, Ramamoorthy *et al.*, 1993), and SERT is also found in the adrenal medulla, and on platelets, mast cells, and basophils (Hoffman, 1994). These transporters clearly serve many physiological functions, both in the central nervous system and in the periphery.

Coupling

The neurotransmitter transporters are all ion-coupled carriers which mediate the accumulation of the neurotransmitter substrate in response to the downhill movement of one or more ion gradients. They are not directly coupled to the hydrolysis of ATP. However, they are indirectly coupled through the ion gradients generated by ion-pumping ATPases. The plasma membrane transporters are indirectly driven by the Na^+/K^+-ATPase which generates gradients of Na^+(out > in) and K^+(in > out) and in the process creates a membrane potential ($\Delta\psi$, inside negative) (Fig. 2). Chloride ion passively equilibrates with this $\Delta\psi$, leading to a Cl^- concentration gradient (out > in). Various plasma membrane transporters utilize the Na^+, Cl^-, and K^+ gradients and the $\Delta\psi$ as driving forces for uphill neurotransmitter transport. In the synaptic vesicle membrane, a vacuolar ATPase pumps H^+ ions into the vesicle lumen, making it acidic and positive with

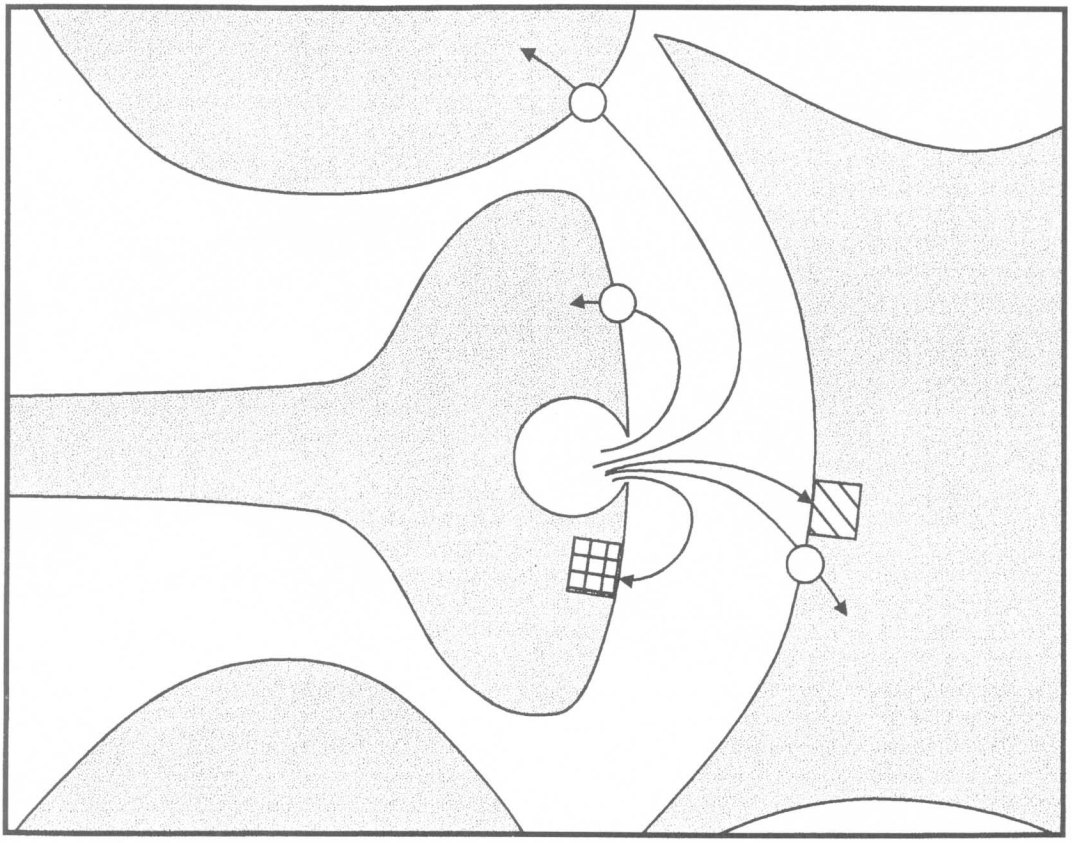

Fig. 1. Neurotransmitter action and reuptake. Neurotransmitters released by exocytosis act at receptors on the postsynaptic neuron (striped box) and on the presynaptic neuron (hatched box). Reuptake occurs by specific transporters (circles) on presynaptic cells, postsynaptic cells, and surrounding glial cells.

respect to the cytoplasm. Vesicular neurotransmitter transporters use the pH difference (ΔpH) and membrane potential ($\Delta\psi$) to drive accumulation of neurotransmitters within the vesicle.

Gradients

The driving forces responsible for accumulation vary depending on the specific neurotransmitter transporter. Part of this variation has to do with the particular family that the transporter belongs to. The NaCl-coupled transporters are all coupled to Na^+ and Cl^- symport, although the Na^+ stoichiometry and the coupling of transport to other ions varies even within this family. The glutamate transporters EAAT-1 to EAAT-5 are all coupled to Na^+ symport and K^+ antiport, and Cl^- is not symported with glutamate. Finally the vesicular transporters apparently do not use Na^+, K^+, or Cl^-, but transport is coupled, instead, to antiport of H^+ and to the $\Delta\psi$. Within each family, there can be differences in ion coupling due to differing charge or protonation of the substrate, differences in Na^+ stoichiometry, or the participation of other ions.

STOICHIOMETRY

SERT

In the NaCl-coupled family, the best studied transporters are SERT, GAT-1, and NET. In all three cases, the stoichiometry has been determined using plasma membrane vesicles, and all three transporters have different stoichiometry (Fig. 3). SERT was first characterized in plasma membrane vesicles isolated from platelets (Rudnick, 1977). Previous studies used intact cell preparations that contained intracellular amine storage organelles (dense granules) that sequester most of the intracellular 5-HT. Platelet membrane vesicles

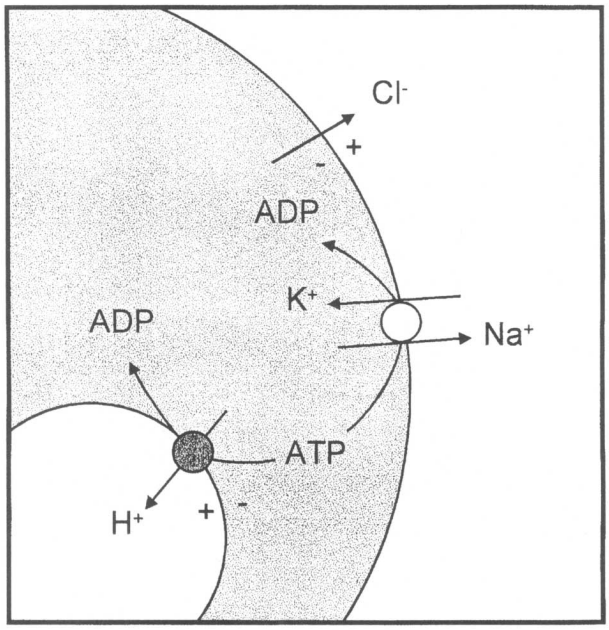

Fig. 2. ATP generates ion gradients. Cytoplasmic ATP acts at the plasma membrane Na^+/K^+-ATPase (white circle) to pump Na^+ out of the cell and K^+ into the cell. Since 3 Na^+ ions are pumped per 2 K^+ ions, a membrane potential ($\Delta\psi$, interior negative) is generated. Cl^- ion is driven out of the cell by $\Delta\psi$, generating a Cl^- gradient. At the synaptic vesicle membrane, the vacuolar ATPase (gray circle) uses cytoplasmic ATP to pump H^+ ions into the vesicle, generating a $\Delta\psi$ (interior positive) and a pH difference (interior acid).

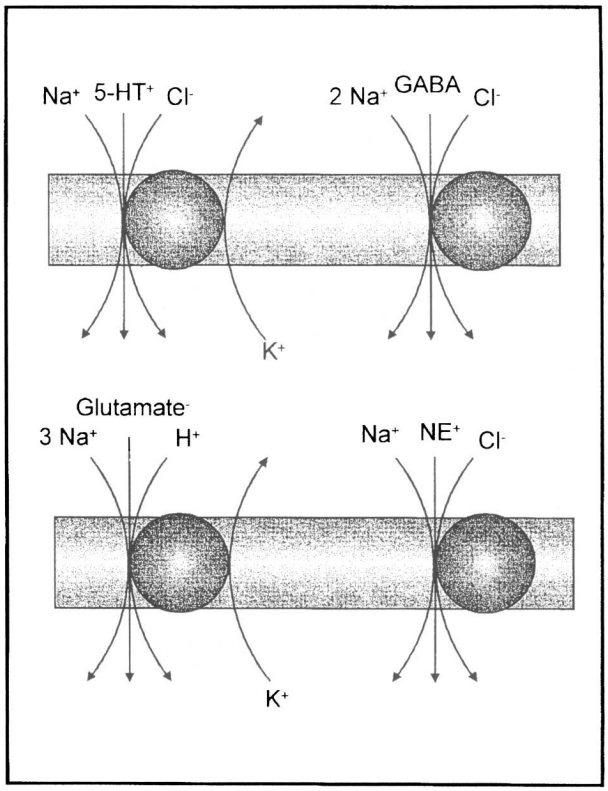

Fig. 3. Ion coupling stoichiometry for plasma membrane neurotransmitter reuptake. Coupling stoichiometries are shown for 5HT, NE, GABA, and glutamate transport.

were shown to accumulate internal 5-HT to concentrations hundreds of times higher than the external medium when appropriate transmembrane ion gradients were imposed (Rudnick, 1977). These vesicle experiments demonstrated conclusively that the plasma membrane transporters generated gradients of their substrate amines using the energy of transmembrane Na^+, Cl^-, and K^+ ion gradients.

When a Na^+ concentration gradient (out > in) was imposed across the platelet plasma vesicle membrane in the absence of other driving forces, this gradient was sufficient to drive 5-HT accumulation (Rudnick, 1977). Coupling between Na^+ and 5-HT transport follows from 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 (Nelson and Rudnick, 1981, Rudnick, 1977). Many of these results have been reproduced in membrane vesicle systems from cultured rat basophilic leukemia cells (Kanner and Bendahan, 1985), mouse brain synaptosomes (O'Reilly and Reith, 1988), and human placenta (Balkovetz *et al.*, 1989).

Chloride symport with 5-HT is somewhat less directly supported, 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, raising internal Cl^- decreases the Cl^- gradient, and inhibits 5-HT uptake. External Cl^- is required for 5-HT uptake, and Cl^- can be replaced by Br^-; to a lesser extent, by SCN^-; NO_3^-, and NO_2^-, and not at all by SO_4^{2-}, PO_4^{2-}, and isethionate$^-$ (Nelson and Rudnick, 1982). Efflux of 5-HT, in contrast, requires internal but not external Cl^- (Nelson and Rudnick, 1982). The possibility that Cl^- stimulated transport by electrically compensating for electrogenic (charge moving) 5-HT transport 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 (Nelson and Rudnick, 1982).

Initially, it was proposed that a $\Delta\psi$ generated by K^+ diffusion was responsible for driving electrogenic 5-HT transport (Rudnick, 1977). This conclusion was based, in part, on the observation that internal K^+ stimulated 5-HT transport but K^+ was not required. Subsequent studies, however, showed that K^+ stimulated transport even if $\Delta\Psi$ was close to zero (Nelson and Rudnick, 1979; Rudnick and Nelson, 1978). In the absence of a K^+ gradient, the addition of 30 mM K^+ simultaneously to both the internal and external medium increased the transport rate 2.5-fold. (Nelson and Rudnick, 1979). Moreover, hyperpolarization of the membrane by valinomycin in the presence of a K^+ gradient had little or no effect on transport. There were two conclusions from these results. First, the transport process was likely to be electrically silent. Second, since the K^+ gradient did not seem to act indirectly through $\Delta\Psi$, it was likely to act directly by exchanging with 5-HT.

The reason 5-HT transport still occurred in the absence of K^+ became apparent in a study of the pH dependence of 5-HT transport. In the absence of K^+, internal H^+ ions apparently fulfill the requirement for an antiported cation (Keyes and Rudnick, 1982). Even when no other driving forces were present (NaCl in = out, no K^+ present), a transmembrane pH difference (ΔpH, interior acid) could serve as the sole driving force for transport. ΔpH-driven 5-HT accumulation required Na^+ and was blocked by imipramine (an antidepressant that specifically blocks SERT) or by high K^+ (in = out), indicating that it was mediated by the 5-HT transporter, and not due to nonionic diffusion. 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.

GAT

In the case of GAT-1 mediated GABA transport, early results using synaptosomes and brain slices were compromised by the levels of endogenous GABA. In 1978, Kanner and coworkers demonstrated [^{3}H]GABA transport into plasma membrane vesicles isolated from synaptosomes (Kanner, 1978). GABA transport in these vesicles was driven by an imposed Na^+ concentration gradient and was insensitive to inhibitors of the Na^+,K^+-ATPase. Imposition of a diffusion potential (interior negative) with an H^+ gradient and CCCP or a K^+ gradient and valinomycin stimulated GABA uptake, indicating that the transport system was electrogenic. In addition to Na^+, external Cl^- also stimulated GABA accumulation, and a gradient of Cl^- (out > in) served as a driving force for uptake (Kanner and Kifer, 1981; Radian and Kanner, 1983).

The possibility that Cl^- stimulated GABA influx merely by dissipating a $\Delta\psi$ (interior positive) generated by electrogenic Na^+-GABA symport was tested using an imposed K^+ diffusion potential. The requirement for external Cl^- persisted even in the presence of a $\Delta\psi$ (interior negative) generated by valinomycin-mediated K^+ efflux (Kanner, 1978, Radian and Kanner, 1983). Thus, the Cl^- requirement was independent from any ability of Cl^- to permeate the membrane. Furthermore, internal Cl^- stimulated GABA efflux, demonstrating the vectorial nature of the Cl^- requirement as expected for a symported ion.

The number of Na^+ and Cl^- ions symported with GABA determines the overall electrogenicity of the process. GABA is predominantly a neutral zwitterion at neutral pH. If one Na^+ ion and one Cl^- ion were symported, there would be no net charge movement. The fact that GABA transport is driven by an imposed $\Delta\psi$ suggests that this is not the case and that more Na^+ ions than Cl^- ions were symported. Measurements of the influence of the Na^+ gradient on GABA accumulation suggested that two Na^+ ions were symported (Radian and Kanner, 1983), and this was later confirmed by direct flux measurements with reconstituted purified GAT-1 in proteoliposomes (Keynan and Kanner, 1988). In these experiments, the amount of Na^+ entering the liposomes was twice the amount of Cl^- or GABA, supporting a stoichiometry of two sodium ions and one chloride ion symported per molecule of GABA.

NET

Two plasma membrane vesicle systems have emerged for studying NE transport: the placental brush border membrane (Ramamoorthy *et al.*, 1992) and cultured PC-12 cells (Harder and Bonisch, 1985). Harder and Bonisch (1985) concluded that NE transport into PC12 vesicles was coupled to Na^+ and Cl^-, and was electrogenic. Ganapathy and coworkers (Ramamoorthy *et al.*, 1992) studied NET mediated transport of both NE and DA into placental membrane vesicles [DA is utilized by NET as a substrate (Gu *et al.*, 1994)]. They reached similar conclusions regarding

ion coupling, but both groups were left with some ambiguity regarding K^+.

Gu *et al.* (1994) established LLC-PK_1 cell lines stably expressing the biogenic amine transporters SERT, NET, and DAT as well as the GABA transporter GAT-1. One attractive advantage of LLC-PK_1 cells is that it has been possible to prepare plasma membrane vesicles that are suitable for transport studies (Brown *et al.*, 1984). Membrane vesicles should have identical composition except for the heterologously expressed transporter. Moreover, these vesicles are suitable for estimating equilibrium substrate accumulation in response to imposed ion gradients. This property allowed definition of the ion coupling stoichiometry for NET using the known stoichiometries for GAT-1 and SERT mediated transport as internal controls.

For NET, accumulation of [^{3}H]dopamine (DA) was stimulated by imposition of Na^+ and Cl^- gradients (out > in) and by a K^+ gradient (in > out). To determine the role that each of these ions and gradients play in NET mediated transport, the influence of each ion on transport was measured when that ion was absent, present at the same concentration internally and externally, or present asymmetrically across the membrane. The presence of Na^+ or Cl^-, even in the absence of a gradient, stimulated DA accumulation by NET, but K^+ had little or no effect in the absence of a K^+ gradient. Stimulation by a K^+ gradient was markedly enhanced by increasing the K^+ permeability with valinomycin, suggesting that net positive charge is transported together with DA. The cationic form of DA is likely to be the substrate for NET, since varying pH did not affect the K_M of DA for transport. The Na^+:DA stoichiometry was estimated by measuring the effect of internal Na^+ on peak accumulation of DA. Taken together, the results suggested that NET catalyzes symport of one cationic substrate molecule with one Na^+ ion and one Cl^-ion, and that K^+ does not participate directly in the transport process (Gu *et al.*, 1996).

EAAT

The glutamate transporter GLT-1 (EAAT-2) was, like GAT-1, studied in synaptosomal plasma membrane vesicles (Kanner and Sharon, 1978). As with other plasmalemmal neurotransmitter transporters, a sodium ion gradient (out > in) could serve as a driving force for glutamate uptake, but unlike GABA and NE transporters, glutamate accumulation was strictly dependent on the presence of internal K^+. Furthermore, a gradient of K^+ (in > out) provided a driving force for glutamate uptake. The requirement for internal K^+ remained even in the presence of an inwardly directed SCN^- gradient imposed to create a $\Delta\psi$ (interior negative) (Kanner and Sharon, 1978). Thus, the K^+ requirement did not simply satisfy electrical neutralization of electrogenic glutamate influx but, rather, reflected the direct antiport of K^+ for glutamate.

Addition of valinomycin to vesicles with a K^+ gradient (in > out) increased glutamate accumulation as expected if net positive charge entry was coupled to glutamate influx (Kanner and Sharon, 1978). However, if glutamate is transported in its predominant form, as a monoanion, and if K^+ efflux is coupled to glutamate influx, then negative charge should enter with glutamate if only one Na^+ is symported with glutamate. For this reason, Kanner and co-workers proposed that at least three Na^+ ions are symported (Kanner and Sharon, 1978). Subsequently it was found that the glutamate transporter of kidney, probably EAAT-3, symported glutamate with H^+ ions, in addition to Na^+ (Nelson *et al.*, 1983). This property has been confirmed with glutamate transporters also from other tissues. It is not clear if H^+ is symported or if the true substrate is zwitterionic glutamic acid, since these alternatives are stoichiometrically equivalent. It has even been proposed that glutamate is antiported with OH^- ions (Bouvier *et al.*, 1992), although this conclusion was based on transport currents that are now known to represent a separate activity of the transporter (Fairman *et al.*, 1995) (see below). More recently, a careful analysis of glutamate accumulation by *Xenopus* oocytes expressing EAAT-3 demonstrated that glutamate is symported with one H^+ ion and three Na^+ ions and antiported with one K^+ ion (Zerangue and Kavanaugh, 1996).

VMAT

In synaptic vesicles, the transmembrane pH difference (ΔpH, interior acid) and membrane potential ($\Delta\psi$, interior positive) provide driving forces for neurotransmitter accumulation. The best studied vesicular system is the vesicular monoamine transporter, VMAT, which is also found in chromaffin granules (Fig. 4). These organelles are relatively abundant and easy to purify, so most of the early work on VMAT was performed with chromaffin granules and with membrane vesicles formed by osmotic lysis of these granules.

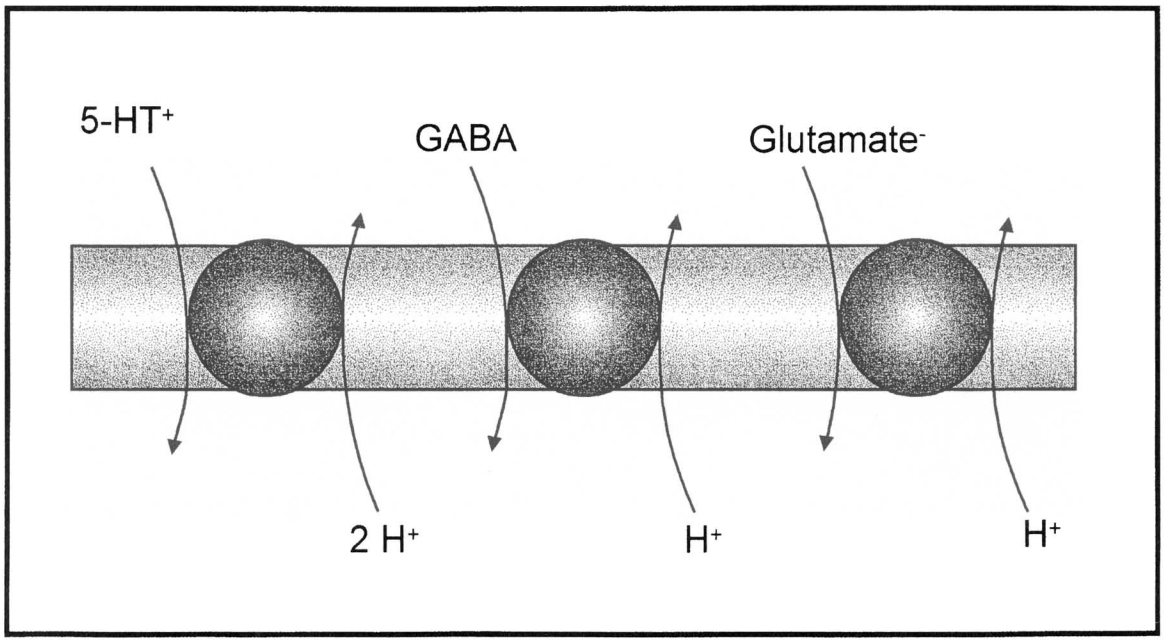

Fig. 4. *Ion coupling stoichiometry for synaptic vesicle neurotransmitter transport. Coupling to the electrochemical H^+ potential varies for VMAT (left) and vesicular transporters for GABA and glutamate. VMAT is driven primarily by ΔpH, GABA transport by ΔpH and Δψ equally, and glutamate primarily by Δψ.*

The vacuolar ATPase remains active in chromaffin granule vesicles, and ATP addition leads to generation of an H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$) (Johnson and Scarpa, 1979). In the presence of ATP, these vesicles take up 5-HT or DA to high internal concentrations (Kirschner, 1962). Uptake is a direct response to generation of $\Delta\tilde{\mu}_{H^+}$ by the ATPase. This conclusion rests on three observations: (1) ATP hydrolysis generates both components of $\Delta\tilde{\mu}_{H^+}$. (2) Artificial generation of ΔpH, Δψ, or both drives amine accumulation. (3) Agents that dissipate $\Delta\tilde{\mu}_{H^+}$ uncouple ATP hydrolysis from amine transport.

In the absence of permeant anions in the medium, ATP-driven proton pumping generates a Δψ in chromaffin granules. Addition of a permeant ion like Cl^- dissipates the Δψ by electrophoretic influx, and allows further H^+ pumping until a significant ΔpH is generated. By varying the Cl^- concentration, the relative contribution of ΔpH can be manipulated (Johnson *et al.*, 1979). In the absence of ATP, imposition of ΔpH by dilution provides a driving force for transient amine accumulation (Schuldiner *et. al.*, 1978). In the absence of permeant anions, addition of ATP to chromaffin granules has essentially no effect on ΔpH but dramatically increases amine uptake, pointing to the importance of Δψ (Holz, 1978). Confirmation of the ability of Δψ to drive transport came from studies in which a diffusion potential was artificially imposed using an inwardly directed K^+ gradient and valinomycin (Njus and Radda, 1979). Finally, ionophores that dissipate either ΔpH or Δψ, or both, inhibit transport driven by ATP (Kanner *et al.*, 1980).

By varying the components of $\Delta\tilde{\mu}_{H^+}$ and measuring ΔpH, Δψ, and amine accumulation, it has been possible to calculate the stoichiometry of H^+/amine antiport (Johnson *et al.*, 1981; Knoth *et al.*, 1981b). The results are consistent with exchange of a protonated amine substrate with two internal H^+ ions or a neutral amine with one H^+ (Johnson *et al.*, 1981; Knoth *et al.*, 1981b). These two alternatives are impossible to distinguish from each other with the thermodynamic methods used. There has been much interest in the form of the substrate transported, but results have been contradictory and no consensus has been reached (Daniels and Reinhard, 1988; Knoth *et al.*, 1981a; Kobold *et al.*, 1985; Ramu *et al.*, 1983; Scherman and Henry, 1981). The bioenergetic consequences of either possibility are the same. Two net H^+ ions leave the vesicle but only one net positive charge leaves for each amine molecule taken in. Thus, amine uptake is coupled more strongly to the ΔpH than it is to the Δψ generated by the vacuolar ATPase.

Other Vesicular Transporters

Vesicular transport systems for other neurotransmitters have not been as well characterized. A transporter for acetylcholine, VAChT, has been cloned and has strong sequence similarity to VMAT (Erickson *et al.*, 1994). In either native ACh storage vesicles or vesicles from cells transfected with VAChT cDNA transport is dependent on ATP and is inhibited by H^+ ionophores such as FCCP and nigericin (Nguyen and Parsons, 1995; Varoqui and Erickson, 1996). A vesicular GABA transporter VGAT was originally identified as an UNC-47 mutant in *C. elegans* (McIntire *et al.*, 1993). This transporter has been expressed in mammalian cells and appears to be specific for GABA. Other similar sequences have been identified that may encode transporters for glycine or glutamate (R.H. Edwards, personal communication). Each of these transport activities has been characterized in synaptic vesicles, and although GABA and glycine transport show similar dependence on ΔpH and $\Delta\psi$ (Burger *et al.*, 1989), both processes have different characteristics from glutamate transport and also from monoamine transport (Hell *et al.*, 1990).

The differences in H^+ coupling between vesicular transporters center around the ability of protonophores such as nigericin and FCCP to inhibit transport. Agents such as nigericin (in the presence of external K^+ or Na^+) and weakly basic amines selectively dissipate ΔpH with little effect on $\Delta\psi$. This results in major inhibition of biogenic amine transport but less effect on GABA or glycine accumulation, and virtually no effect on glutamate transport (Hell *et al.*, 1990). In contrast, agents that selectively dissipate $\Delta\psi$, such as valinomycin (in vesicles loaded with K^+) or permeant anions such as SCN^- have a much more dramatic effect on glutamate transport and relatively little effect on amine accumulation.

Comparing the response to nigericin and SCN^- suggests a different H^+-charge coupling for the different transporters. GABA and glycine are zwitterionic substrates and carry no net charge at physiological pH. Exchange of one amino acid molecule for one or more H^+ ions is expected to be driven equally well by ΔpH and $\Delta\psi$. The amine substrates of VMAT, however, are positively charged (protonated) at neutral pH. Exchange with one H^+ ion would be driven only by ΔpH and not by $\Delta\psi$. Evidence suggests, however, that two H^+ ions are exchanged for each amine molecule (Johnson *et al.*, 1981; Knoth *et al.*, 1981b). As a consequence, both ΔpH and $\Delta\psi$ drive amine uptake, although ΔpH is a stronger driving force since more net H^+ ions than charges move in the transport process. Glutamate is negatively charged at physiological pH. Glutamate accumulation has been proposed to be a simple uniport driven by $\Delta\psi$ (Maycox *et al.*, 1988). As such, ΔpH would have no effect on glutamate accumulation. Other evidence favors an exchange of glutamate for H^+ ions (Tabb *et al.*, 1992). However, because of the negative charge on glutamate, $\Delta\psi$ provides more energy than ΔpH, since more net charges than H^+ ions cross the membrane in the transport process.

MECHANISM

The mechanism by which transport proteins couple the movement of their substrates with ion movements has been the subject of much speculation but little experimental verification. Most workers have embraced the notion of a binding site that is alternately accessible to the two membrane surfaces. Immediately after binding at such a site, a substrate could be released only to the side from which it bound, but the transporter has the capacity to convert the binding site so that it is accessible from the other side of the membrane. At all times, a permeability barrier exists between the binding site and one side of the membrane (Fig. 5), but that barrier can move from one side of the binding site to the other, giving the site alternating access to the two aqueous compartments that the membrane separates (Mitchell, 1990).

For effective coupling between substrate and Na^+ or Cl^- influx, the interconversion between alternate forms of the transporter must occur only under specific circumstances. For example, NET mediated NE transport requires symport of Na^+ and Cl^- (Gu *et al.*, 1996). Therefore, the interconversion between external access to the NE site and internal access should occur only when NE, Na^+, and Cl^- are all bound at the site. Following dissociation of these solutes to the cytoplasm, the reverse interconversion could occur, regenerating an external NE site. Thus, the interconversion would be allowed only when the substrate site was empty, or when Na^+, Cl^-, and NE were all bound. This type of mechanism would ensure the coupling between NE, Na^+, and Cl^- fluxes. It also accounts for the phenomenon of counterflow, which results from the fact that movement of substrate in one direction does not impede movement in the opposite direction (Stein, 1967). Accumulation of substrate against a concentration gradient will be driven by the differences

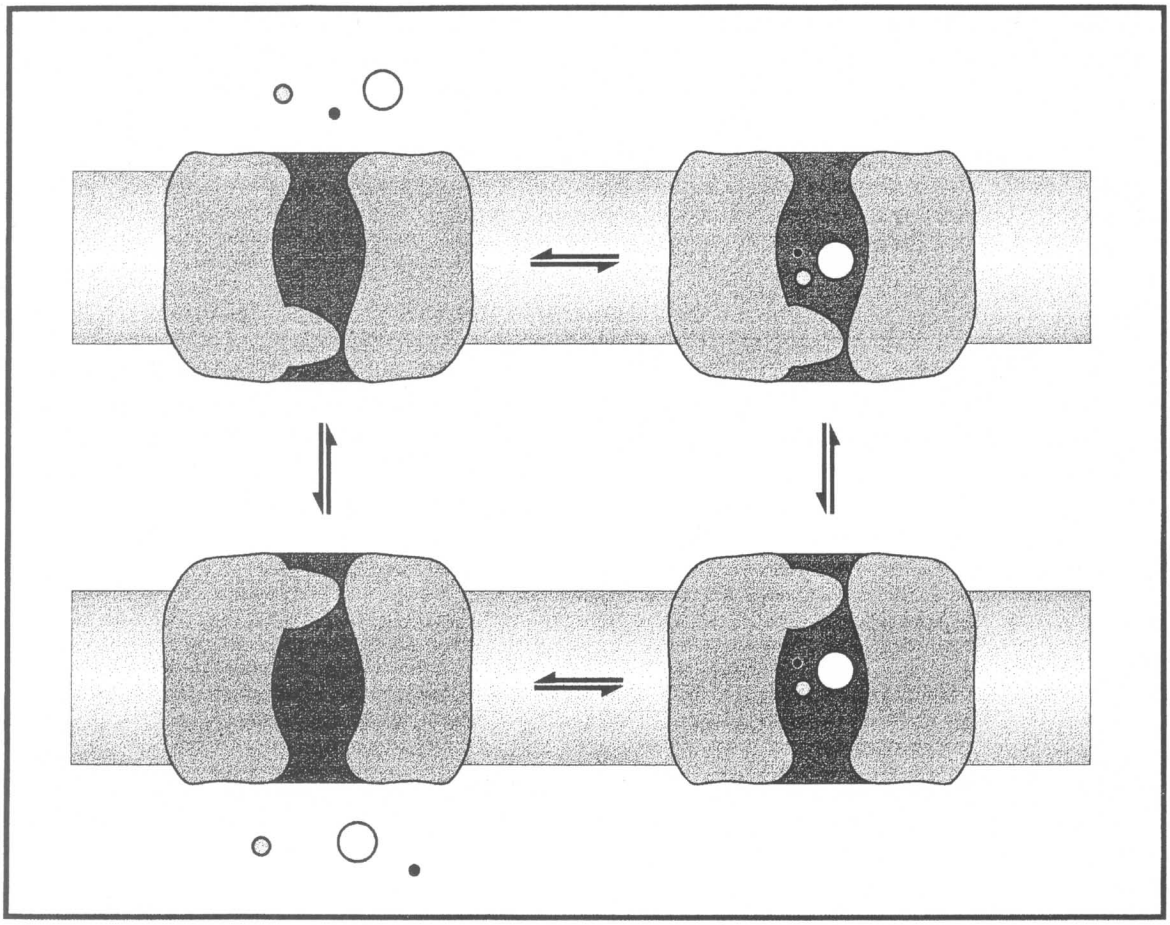

Fig. 5. Mechanism for ion-coupled neurotransmitter transport. Extracellular substrate together with cotransported ions (upper left) bind to the transporter binding site (upper right) but cannot dissociate to the cytoplasm because of the barrier. After a conformational change, a barrier prevents dissociation to the outside, but the cytoplasm is accessible to the binding site (lower right). After dissociation of bound solutes (lower left), a second conformational change returns the transporter to its original state.

in Na^+ and Cl^- concentration on the two sides of the membrane. These differences favor formation of the fully loaded binding site on the cell exterior and favor Na^+ and Cl^- dissociation to the cytoplasm.

Neurotransmitter transport into synaptic vesicles is coupled to antiport of H^+. This type of coupling is also accommodated by an alternating access mechanism. The rules for interconversion of transporter forms, however, are different for antiport. Interconversion should not occur, in antiport, unless either substrate or an H^+ ion is bound. Thus, at low intravesicular pH, H^+ will bind to the site, allowing interconversion, followed by dissociation of H^+ to the neutral cytoplasm. The empty site, facing the cytoplasm, waits until substrate binds and allows interconversion to the inward facing form. Once inside, the high H^+ ion concentration displaces the substrate from the binding site.

For transporters like SERT and EAAT, both Na^+ symport and K^+ antiport occur. The alternating accessibility model also accommodates these systems. Substrate symport with Na^+ could result from a requirement for substrate to bind together with Na^+ (and Cl- for SERT) before the accessibility of the site could change from external to internal. After substrate, Na^+ and Cl^- dissociate to the cytoplasm; return to the external form requires binding of internal K^+ [or H^+ (Keyes and Rudnick, 1982)] to the site before it becomes accessible to the cell exterior.

This model predicts that symported ions will bind to the transporter at the same time as substrate, and that will be transported together. Antiported ions, in contrast, should cross the membrane in a separate step from the substrate transport step. Studies of Na^+, Cl^-, and 5-HT binding to SERT suggest that

all three solutes are bound to the same form of the transporter (Humphreys *et al.*, 1994), and other work indicates that K^+ is transported in a separate step (Nelson and Rudnick, 1979). For VMAT, studies with reserpine binding suggest that H^+ is transported in a separate step prior to reserpine or substrate binding (Rudnick *et al.*, 1990). Thus, the alternating access mechanism seems to be a suitable model for neurotransmitter transporters.

CHANNEL ACTIVITY

Some neurotransmitter transporters, in addition to their ability to couple the uphill fluxes of their neurotransmitter substrate to the downhill movement of Na^+ and other ions, also catalyze an uncoupled ion flux (Sonders and Amara, 1996). This phenomenon was first observed with the serotonin transporter (SERT), for which most evidence suggested an electroneutral exchange of internal K^+ with Na^+, Cl^-, and 5-HT^+ (Nelson and Rudnick, 1979; Rudnick and Nelson, 1978). When expressed in *Xenopus* oocytes SERT gave rise to cation currents in the presence of the substrate, 5-HT (Mager *et al.*, 1994). These currents had all of the hallmarks of a transporter mediated ion flux; it required the symported ions Na^+ and Cl^- and it was inhibited by transport blockers (Mager *et al.*, 1994). The voltage dependence of this current, however, was distinct from that of 5-HT transport. The current had an ohmic character, increasing in magnitude as the membrane was hyperpolarized, but transport was insensitive to the same voltage changes (Mager *et al.*, 1994). Single channel studies on excised patches of oocyte membrane demonstrated discrete channel openings which occurred at a low frequency relative to the rate of 5-HT flux (Lin *et al.*, 1996). In each of these conductance events, many hundreds of ions would flow, but the events would occur only approximately once per hundred transport cycles, assuming that the transport cycles were distributed more or less evenly with time (Lin *et al.*, 1996).

In the glutamate transporter EAAT-4, chloride currents were observed to accompany glutamate transport (Fairman *et al.*, 1995). Because Cl^- is not required for transport, it was possible to identify the relative contribution of the current carried by electrogenic glutamate flux and the current carried by uncoupled Cl-flux (Wadiche *et al.*, 1995). The two activities are clearly different and are characteristic of all transporters in this family. Although single channels were not observed, noise analysis in cells expressing endogenous glutamate transporters suggests that the Cl-current is composed of discrete electrical events as expected for an ion channel (Picaud *et al.*, 1995). One important similarity between the uncoupled currents passed by EAAT and SERT is that in both cases, the currents are triggered by substrate transport.

Uncoupled ion flux is a common property of neurotransmitter transporters. In addition to the glutamate and 5-HT transporters, currents have been observed with NET and DAT and with GAT-1 (Cammack and Schwartz, 1996; Galli *et al.*, 1996; Sonders *et al.*, 1997). Noise analysis suggests that the currents associated with GAT and NET are due to brief channel openings (Cammack and Schwartz, 1996; Galli *et al.*, 1996), and an estimate of the frequency of channel openings for NET suggests that they occur about as often as transport events (Galli *et al.*, 1996). Each transporter seems to have a characteristic frequency relative to transport events. With NET, that frequency is very high (Galli *et al.*, 1996), with SERT it is lower (Lin *et al.*, 1996), and with GAT the channel openings are very infrequent and are not stimulated by GABA transport (Cammack and Schwartz, 1996).

How are the currents carried by neurotransmitter transporters related to substrate transport? Before attempting to answer this question, it is important to distinguish between currents due to ion-coupled substrate movement and those resulting from uncoupled ion flux. These two currents are not always easy to distinguish from one another since uncoupled ion flux is frequently stimulated by substrate transport. In the case of SERT, where transport is electroneutral, and EAAT, where the transport current can be isolated by removing Cl^-, it has been possible to measure the uncoupled current (Wadiche *et al.*, 1995). In NET the uncoupled current is so large that the transport current can be ignored (Galli *et al.*, 1996), but in other cases it is difficult to evaluate the relative contribution of transport and uncoupled currents.

The existence of an uncoupled current suggests that the transporter is capable of forming some sort of aqueous transmembrane pore. It is tempting to believe that this pore, through which ions can freely cross the membrane, is the same pathway through which transport substrates travel during normal transport.

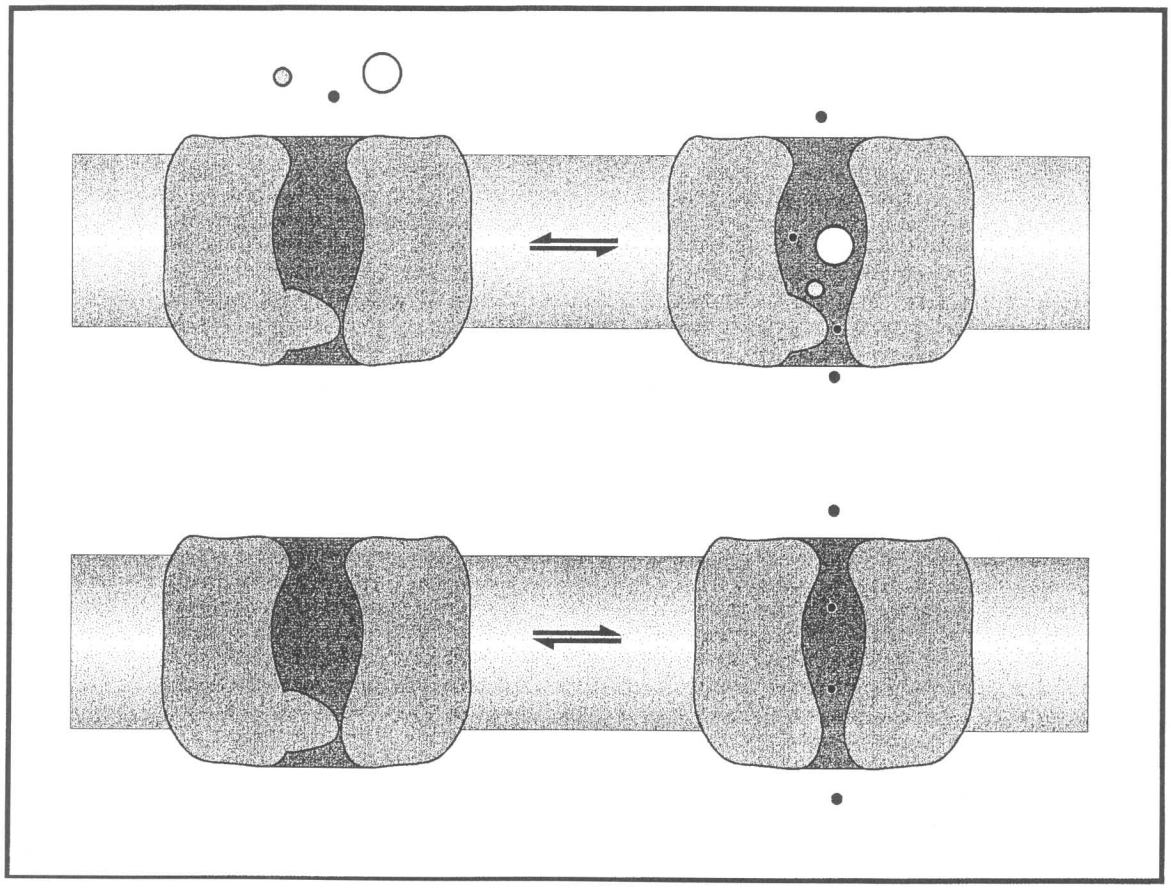

Fig. 6. Channel formation. Two ways for a transporter to conduct ions are shown. Top, the barrier that prevents substrate dissociation to the cytoplasm could allow ions to pass (upper right), perhaps as a consequence of substrate binding. Bottom, the transporter might assume a conformation with no barrier to dissociation from the binding site. Other mechanisms are also possible.

Transport, however, cannot be coupled to ion flux through a simple channel. Mechanisms to explain transport as a channel-mediated process (Su *et al.*, 1996) fail to account for important properties of carrier-mediated transport such as counterflow (Stein, 1967).

During transport, we expect there to be a barrier preventing bound substrate from dissociating to one side of the membrane. One way that a transporter could form a channel is if the barrier that prevented substrate movement did not completely block ion movement (Fig. 6). However, we know that for some transporters, channel activity is a rather rare event (Lin *et al.*, 1996) relative to transport. It may be that the channel is formed when the barriers on each side of the substrate site are both open. If the substrate is accessible to both sides of the membrane simultaneously, ions could diffuse from one side to the other through the binding site. For coupled transport, it is disadvantageous for both barriers to be open simultaneously. However, the cell might gain some advantage from uncoupled ion flux (Sonders and Amara, 1996), or it may represent an unproductive side reaction whose energetic costs have been tolerated during evolution.

In all examples of uncoupled ion flux mediated by neurotransmitter transporters, the flux is stimulated by substrate transport. This finding suggests that it is the conformational changes during transport that trigger the channel to open. If so, the channel properties may provide an insight into those changes. If ion flux through these transporters follows the same physical path that substrates travel, then the individual residues that make up the substrate binding site could also influence the ion selectivity of the channel. Alternatively, the channel activity may represent a different pathway through the membrane that opens with some

frequency that depends on substrate transport. In any case, the ability to measure ion flux in real time with sophisticated electrophysiological techniques provides an additional way to look at the individual events that make up the transport cycle.

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