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REVIEW

Serotonin transport in the 21st century

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Serotonin (5-hydroxytryptamine [5-HT]) is accumulated within nerve endings by the serotonin transporter (SERT), which terminates its extracellular action and provides cytoplasmic 5-HT for refilling of synaptic vesicles. SERT is the target for many antidepressant medications as well as psychostimulants such as cocaine and ecstasy (3,4-methylenedioxymethamphetamine). SERT belongs to the SLC6 family of ion-coupled transporters and is structurally related to several other transporter families. SERT was studied in the 1970s and 1980s using membrane vesicles isolated from blood platelets. These studies led to a proposed stoichiometry of transport that has been challenged by high-resolution structures of SERT and its homologues and by studies of SERT electrophysiology. Here, we review the original evidence alongside more recent structural and electrophysiological evidence. A self-consistent picture emerges with surprising insights into the ion fluxes that accompany 5-HT transport.

Introduction

In 1978, Peter Mitchell was awarded the Nobel Prize in Chemistry for the chemiosmotic theory of energy transduction by biological membranes (Mitchell, 1979). The prize confirmed what many had already accepted: that the difference in concentration of an ion or any other solute between one side of a membrane and the other could be used by biological systems for doing work and that the electrical potential difference across the membrane could increase or decrease the amount of work that a chemical gradient could perform. This work could take the form of spinning the rotary motor of the bacterial flagellum, driving the synthesis of ATP or moving a second solute from one side of the membrane to the other, even against a preexisting gradient of that solute. Mitchell coined the word "proticity" for when an electrochemical potential of protons was the driving force and extended that to "chemicalicity" when another ion, such as sodium, was doing the work. The idea that a transmembrane gradient of H^+ or Na^+ could drive accumulation of solutes within a cell excited the imagination of many biologists studying transport systems in prokaryotes and eukaryotes.

The 1970s were fruitful years for applying the principles of chemiosmosis to transport processes. A popular system employed to study how transporters worked was to make "membrane vesicles," a preparation championed by H. Ronald Kaback in bacteria (Kaback, 1972) and Ulrich Hopfer and Heini Murer in mammalian cells (Murer and Hopfer, 1974). These vesicles were devoid of intracellular sources of energy and were able to accumulate solutes internally only when the appropriate energy sources were supplied. In bacterial membrane vesicles, this energy was supplied by enzymes, intrinsic to the membrane, that pumped protons out of the vesicle, while in mammalian plasma membrane vesicles, the energy came from imposing a gradient of Na^+ by diluting Na^+-free vesicles into a medium containing Na^+. Kaback showed that the electrochemical H^+ potential served to drive amino acid and lactose accumulation (Ramos et al., 1976), and Murer and Hopfer showed that a Na^+ gradient would drive glucose transport into intestinal brush border membrane vesicles (Murer and Hopfer, 1974), as had been proposed by Crane et al. (1965).

The reuptake of neurotransmitters after their release into the synapse was potentially another one of these ion-coupled transport systems. Indeed, transport of one neurotransmitter, serotonin (5-hydroxytryptamine [5-HT]), was studied in platelets by Sneddon and Lingjaerde and found to require both Na^+ and Cl^- (Lingjaerde, 1969; Sneddon, 1969), raising the prospect that both ions were coupled to 5-HT transport. Membrane vesicles from platelets verified the Na^+ and Cl^- requirements (Rudnick, 1977), and membrane vesicles made from synaptosomes (pinched-off nerve endings) were shown to accumulate two other neurotransmitters, GABA (γ-aminobutyric acid) and glutamate, when a Na^+ gradient was imposed (Kanner, 1978; Kanner and Sharon, 1978).

Membrane vesicles were, at the time, the best system for understanding the energetics and stoichiometry of transporters because they provided a way to identify the requirements for ions and to test the ability of ion gradients to influence substrate

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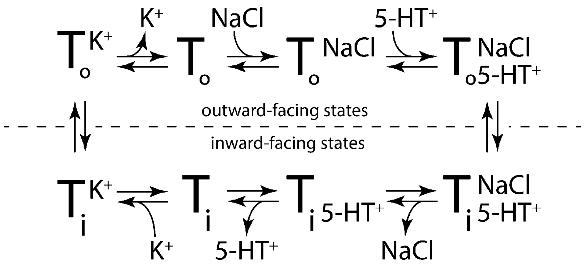

Figure 1. **Schematic diagram of the SERT reaction cycle as proposed in 1979 (Nelson and Rudnick, 1979).** T_o represents outward-facing conformations of SERT and T_i inward-facing conformations.

accumulation. In the case of serotonin transporter (SERT), these studies led to a picture where 5-HT was cotransported together with one Na^+ and one Cl^-, and a K^+ ion was transported in the opposite direction (Fig. 1). That transport stoichiometry, and the energy coupling that it implied, was established >40 yr ago (Rudnick and Nelson, 1978) and has not been seriously challenged until recently. However, there has been tremendous progress in the field since that time, particularly in the biophysical and structural understanding of these proteins. How well does the picture from 1978 hold up today? This review will attempt to answer that question and examine the stoichiometry, energetics, and conformational changes of SERT in the cold, hard light of the 21st century.

20th century SERT: Stoichiometry in the age of vesicles

The earliest work on ion coupling to 5-HT transport sought to find a good model system and determine which ions were required. Platelets, those circulating blood cells that participate in hemostasis, were known to take up 5-HT and release it when the cells aggregated at sites of vascular injury (Holmsen et al., 1969; Shepherd and Vanhoutte, 1979). 5-HT accumulated by platelets is stored within subcellular organelles called dense granules. The ability of platelets to accumulate, store, and release 5-HT mirrored the corresponding process in serotonergic neurons, which store 5-HT in synaptic vesicles, release it during neuronal activity, and then take it up to terminate the signal. Taking on faith that the similarity of function reflected similarity of the components, several researchers decided to study platelet 5-HT uptake with the expectation that the same protein was responsible for 5-HT uptake into platelets and neurons (Lingjaerde, 1969; Sneddon, 1973). As it turns out, the assumption was correct (Giannaccini et al., 2010; Hohmann et al., 2011), and platelets proved to be a useful model system.

One advantage of platelets was that they could be purified relatively easily, in contrast with synaptosomes, pinched-off nerve endings, which derive mostly from nonserotonergic neurons. Purified platelets could be lysed by osmotic shock (Barber and Jamieson, 1970), and the resulting plasma membranes spontaneously resealed to form relatively homogeneous membrane vesicles devoid of cytoplasmic contents or energy sources but were remarkably impermeable to the ions that were required for 5-HT transport. When these vesicles were first used for transport studies, their sidedness was not determined due to the lack of appropriate tools, and the issue was never revisited when those tools became available. Loaded with K^+, and diluted into NaCl, these vesicles would accumulate radiolabeled 5-HT to concentrations several hundred-fold above that of the external medium (Rudnick, 1977; Fig. 2 A). Addition of an ionophore, such as gramicidin, which would dissipate the Na^+ and K^+ concentration gradients, caused the accumulated 5-HT to leave the vesicles, demonstrating that the energy in the concentration differences between intravesicular and extravesicular Na^+ and/or K^+ was used to generate and maintain a transmembrane concentration gradient of 5-HT. One advantage of platelet plasma membrane vesicles, unlike vesicles prepared from many other mammalian cells, was their low ionic permeability, allowing 5-HT to come into equilibrium with the driving forces before imposed ion gradients dissipated.

The following subsections discuss findings from 5-HT uptake in membrane vesicles from platelets and cover the coupling between 5-HT transport and transmembrane gradients of Na^+, K^+, H^+, and Cl^-, efflux and exchange of 5-HT, and the ability of SERT to conduct ionic currents uncoupled to 5-HT transport.

Na^+ and K^+ stoichiometry

Because platelet plasma membrane vesicles could establish an equilibrium between 5-HT accumulation and the Na^+ and K^+ gradients that were driving it, we were able to measure the thermodynamic coupling between 5-HT uptake and gradients of the two ions (Rudnick, 1977; Talvenheimo et al., 1983). This coupling could be directly translated into a stoichiometry for ion-coupled 5-HT transport. The equations that describe the relationship between the electrochemical potentials of a substrate and a coupled ion for symport or antiport are

$$ns\frac{RT}{F}ln\frac{[S]_i}{[S]_O} = n_X\frac{RT}{F}ln\frac{[X]_O}{[X]_i} - \Delta\psi(n_S Z_S + n_X Z_X)$$

for symport (movement in the same direction) and

$$ns\frac{RT}{F}ln\frac{[S]_i}{[S]_o} = n_X\frac{RT}{F}ln\frac{[X]_i}{[X]_o} - \Delta\psi(n_X Z_X\text{-}n_S Z_S)$$

for antiport (movement in opposite directions), where S is the substrate with charge Z_S, X is the ion with charge Z_X, and n_S is the number of substrate molecules moving with n_X ions. $[S]_i$ and $[S]_o$ are internal and external substrate concentrations and similarly for the ion concentrations. R is the gas constant, F is the Faraday, and T is the temperature in degrees kelvin. Early on, we discovered that 5-HT accumulation was functionally electroneutral (Rudnick and Nelson, 1978), meaning that transport did not drive electrical charge across the membrane, so the terms of these equations that related transport to charge movement, $\Delta\psi(n_S Z_S + n_X Z_X)$ and $\Delta\psi(n_X Z_X\text{-}n_S Z_S)$, could be ignored. That allows simplification of the equations to

$$n_S 60\log\frac{[S]_i}{[S]_o} = n_X 60\log\frac{[X]_o}{[X]_i}$$

and

$$n_S 60\log\frac{[S]_i}{[S]_o} = n_X 60\log\frac{[X]_i}{[X]_o}\epsilon\ 29.4°C$$

at 29.4°C. Thus, a plot of the log of the accumulated 5-HT gradient $[5\text{-HT}]_i/[5\text{-HT}]_o$ against the log of the Na^+ gradient $[Na^+]_o/$

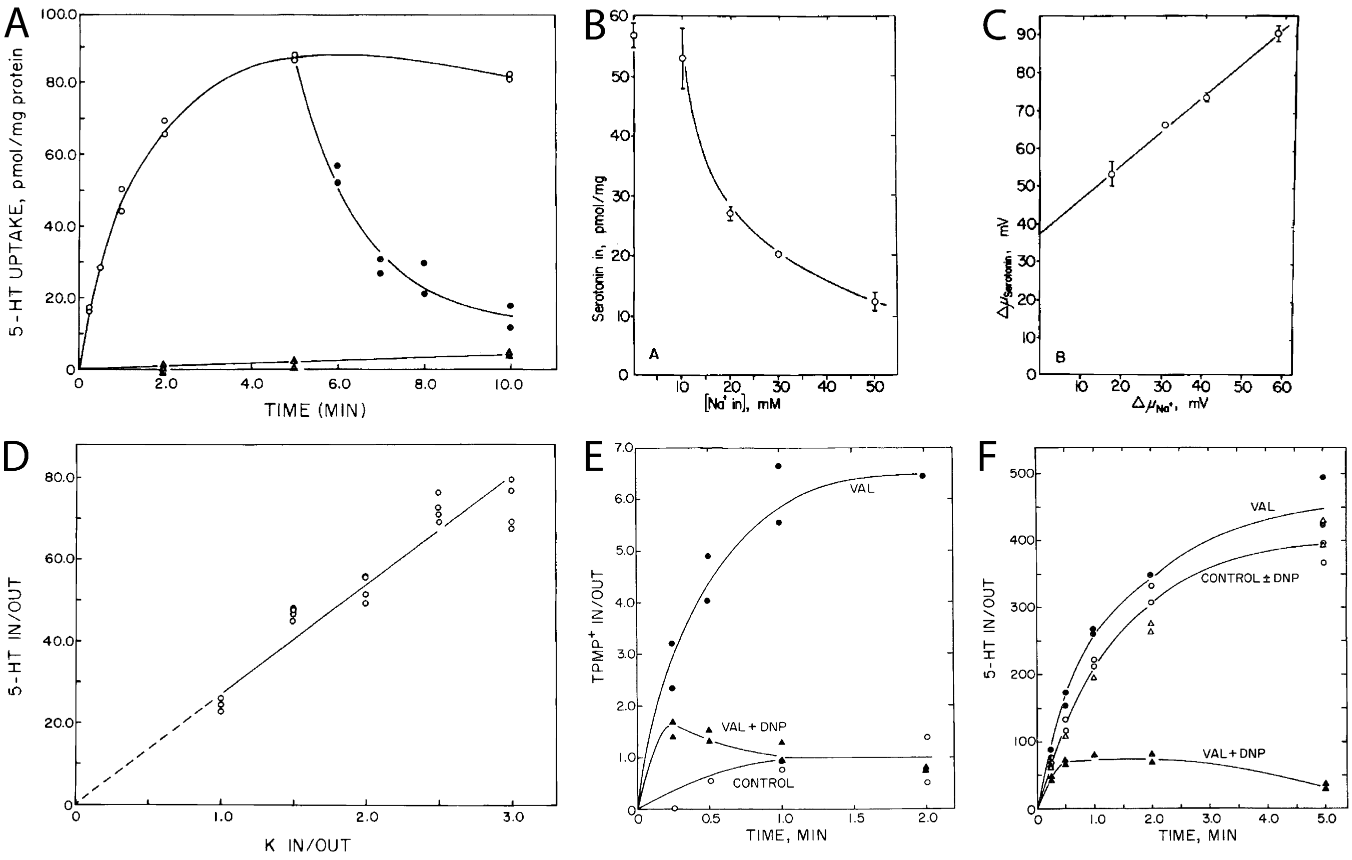

Figure 2. **Transport measurements in platelet plasma membrane vesicles. (A)** Time course of [^{3}H]-5-HT accumulation. Intracellular buffer contained K-phosphate (circles) or NaCl (triangles). External buffer contained NaCl. Gramicidin was added at 5 min (filled circles). From Rudnick (1977), reprinted with permission from the *Journal of Biological Chemistry*. **(B)** Increasing intravesicular [Na^+] (with constant [Na^+] out) decreased intravesicular 5-HT accumulated in response to transmembrane Na^+ and K^+ gradients. Error bars indicate SD. **(C)** Replot of data from B as the chemical potential of the accumulated 5-HT gradient vs. the chemical potential of the imposed Na^+ gradient. From (Talvenheimo et al., 1983), reprinted with permission from the *Journal of Biological Chemistry*. **(D)** Accumulation of intravesicular 5-HT vs. the imposed K^+ gradient (both expressed as the ratio of solute in/out). From Rudnick (1977), reprinted with permission from the *Journal of Biological Chemistry*. **(E)** Imposition of a K^+ gradient (in > out) does not generate a membrane potential (measured by uptake of the lipophilic cation $TPMP^+$) in the absence of valinomycin (VAL). Addition on the proton ionophore 2,4-dinitrophenol (DNP) allowed H^+ influx to dissipate the K^+ diffusion potential established by valinomycin. **(F)** Accumulation of 5-HT was driven by imposition of a K^+ gradient and addition of valinomycin did not significantly enhance accumulation. However, 2,4-dinitrophenol addition dramatically inhibited accumulation in the presence of valinomycin, probably due to acidification of the vesicle interior. In both E and F, the K^+ gradient was generated by dilution of vesicles equilibrated in NaCl + KCl into NaCl + LiCl. From (Rudnick and Nelson, 1978), reprinted with permission from *Biochemistry*.

$[Na^+]_I$ for symport or the K^+ gradient $[K^+]_I/[K^+]_o$ for antiport should give a straight line with a slope equal to the coupling stoichiometry Na^+:5-HT or K^+:5-HT. In the special case of a 1:1 stoichiometry, a simple linear plot of $[5\text{-HT}]_i/[5\text{-HT}]_o$ vs. $[X^+]_i/[X^+]_o$ would give a straight line.

Measuring the accumulated 5-HT gradient as a function of the imposed Na^+ gradient (Fig. 2 B) and plotting $\Delta\mu_{5\text{-HT}}$, the chemical potential of the 5-HT gradient, against $\Delta\mu_{Na}{}^+$, gave a straight line with a slope of 0.9 (Fig. 2 C), strongly suggesting a 1:1 5-HT/Na^+ stoichiometry (Talvenheimo et al., 1983). For K^+, the transmembrane 5-HT gradient ($[5\text{-HT}]_i/[5\text{-HT}]_o$) was a linear function of the K^+ gradient ($[K^+]_I/[K^+]_o$) suggesting 1:1 stoichiometry also for K^+ (Rudnick, 1977; Fig. 2 D). This led to the proposal that one Na^+ ion was transported into the cell with each 5-HT molecule and one K^+ ion was transported out in the same transport cycle. Because 5-HT exists predominantly in the positively charged form at neutral pH, this cycle would predict one positive charge also entering on each cycle, at odds with the finding of electroneutral transport. However, Cl^- ion was also required for 5-HT transport (Lingjaerde, 1971; Rudnick, 1977), leading to the suggestion that 5-HT entered the cell as a cation in symport with one Na^+ and one Cl^- and with antiport of one K^+ ion (Fig. 1).

A role for protons

SERT requires extracellular Na^+ and Cl^- to transport 5-HT (Lingjaerde, 1969; Sneddon, 1969; Rudnick, 1977). Although cytoplasmic K^+ stimulates 5-HT transport by SERT, it is not required (Nelson and Rudnick, 1979). How could an ion be directly coupled to transport and yet not required? As stated above, 5-HT transport is electroneutral. This observation ruled out the possibility that a transmembrane K^+ gradient generated a diffusion potential that drove transport electrically (Rudnick, 1977) and instead favored a mechanism in which K^+ efflux was mediated by SERT and directly coupled to 5-HT transport (Rudnick and Nelson, 1978).

The evidence against electrical coupling between K⁺ and 5-HT uptake came from experiments in which inside negative membrane potentials were monitored by accumulation of triphenylmethylphosphonium (TPMP⁺). Like other lipophilic cations, TPMP⁺ distributes across lipid membranes according to the membrane potential, accumulating on the electrically negative side of the membrane (Bakeeva et al., 1970). An example of this use of TPMP⁺ is to detect a diffusion potential for K⁺. In a vesicle with an internal K⁺ concentration higher than that of the medium, K⁺ efflux through a permeable membrane would lead to the interior becoming electrically negative due to the exit of positive charge. In response, TPMP⁺, if present, would concentrate in the vesicle lumen. However, the K⁺ permeability of biological membranes varies widely, and if K⁺ does not flow out of the vesicle to generate a potential, TPMP⁺ would not be accumulated.

We found that imposing a K⁺ gradient (in > out) did not lead to TPMP⁺ accumulation within platelet plasma membrane vesicles, suggesting that these membranes were not permeable to K⁺ ions. Adding the K⁺-specific ionophore valinomycin allowed K⁺ to exit, down its concentration gradient, and thus generate an electrical potential that drove accumulation of TPMP⁺ (Fig. 2 E). The ability of a K⁺ gradient (in > out) to stimulate 5-HT accumulation without valinomycin indicated that generation of a membrane potential was not required for the K⁺ gradient to drive 5-HT transport (Fig. 2 F). Moreover, the membrane potential that resulted from valinomycin addition had little added effect on 5-HT transport (Rudnick and Nelson, 1978; Nelson and Rudnick, 1979; Fig. 2 F), arguing that the equilibrium accumulation of 5-HT is electroneutral and directly stimulated by K⁺ antiport.

How, then, do we understand why intravesicular K⁺ is coupled to 5-HT transport without being required? The answer came from observations that a transmembrane pH difference (ΔpH, acid inside) could stimulate transport and serve as a driving force for 5-HT accumulation even in the absence of a Na⁺ gradient (Keyes and Rudnick, 1982). The stimulation by ΔpH was enhanced in the absence of K⁺. Conversely, stimulation by internal K⁺ was inhibited at low pH. The implication is that the cytoplasmic site where K⁺ binds could also accommodate a proton and that the two cations competed at that site. Physiologically, the site is likely to be saturated with K⁺, but in a nonphysiological low-K⁺ environment, H⁺ apparently replaces K⁺ in the transport reaction. The effect of ΔpH was clearly through SERT, because 5-HT accumulation in response to ΔpH required Na⁺ and was blocked by imipramine, a SERT inhibitor. Transport of the deprotonated neutral form of 5-HT together with Na⁺ and Cl⁻ would also provide a way for ΔpH to drive accumulation but would not explain why low pH inhibits stimulation of 5-HT uptake by internal K⁺. The kinetic competition between the two cations makes it likely that H⁺ can replace K⁺ in the transport cycle (Keyes and Rudnick, 1982; Fig. 3).

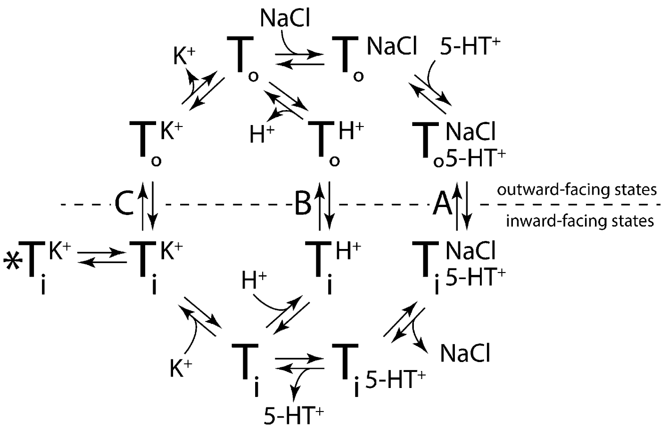

Figure 3. **Updated 1982 mechanism of 5-HT transport accounting for antiport with H⁺.** In path A, 5-HT is transported across the membrane together with Na⁺ and Cl⁻. Return of the transporter to the outward facing state would occur by outward transport of H⁺ by path B, or K⁺ by path C. *T represents a hypothetical conductive transporter state in equilibrium with T_iK^+.

What about Cl⁻?

Stimulation of 5-HT transport by Cl⁻ was first observed in platelets (Lingjaerde, 1969). Several other neurotransmitter and amino acid transporters had also been reported to require Cl⁻, including choline, norepinephrine, and γ-aminobutyrate (Kanner, 1978; Kuhar and Zarbin, 1978). For 5-HT transport into platelet membrane vesicles, Cl⁻ could be replaced by Br⁻ and, to a lesser extent, by SCN⁻ and NO_2^- (Nelson and Rudnick, 1982). Consistent with the symport of Cl⁻ with 5-HT, it was required in the extracellular medium for 5-HT influx but not in the vesicle lumen. However, unlike Na⁺, K⁺, or H⁺, a Cl⁻ gradient alone was not sufficient to drive 5-HT accumulation. Nevertheless, a reasonable argument for Cl⁻ symport was drawn from the fact that the overall transport process was electroneutral. If one Na⁺ was transported into the cell together with a positively charged 5-HT molecule, two positive charges would enter the cell, which would be neutralized by one K⁺ leaving and one Cl⁻ entering. Cotransport of 5-HT⁺ and Na⁺ with one Cl⁻ and exchanging with one K⁺ would thus balance the charge movement and preserve the electroneutrality of the transport cycle (Fig. 3).

5-HT efflux and exchange

If the ion or substrate gradients are reversed, SERT can cycle through the same intermediates in the opposite direction, leading to substrate efflux (Rudnick, 1977; Rudnick and Wall, 1992b). SERT can also catalyze substrate exchange. Exchange is favored when the transporter is forced into a limited number of states, for example in the presence of an elevated intracellular Na⁺ concentration (Hasenhuetl et al., 2016). These conditions disfavor the K⁺-bound forms responsible for return to the outward-facing conformation. Under these conditions, SERT oscillates between the substrate-bound outward- and inward-facing conformations and rarely completes a full transport cycle. It is worth noting that the action of amphetamines relies on the ability of SERT to catalyze this exchange between cytoplasmic 5-HT and extracellular amphetamines. The effect of

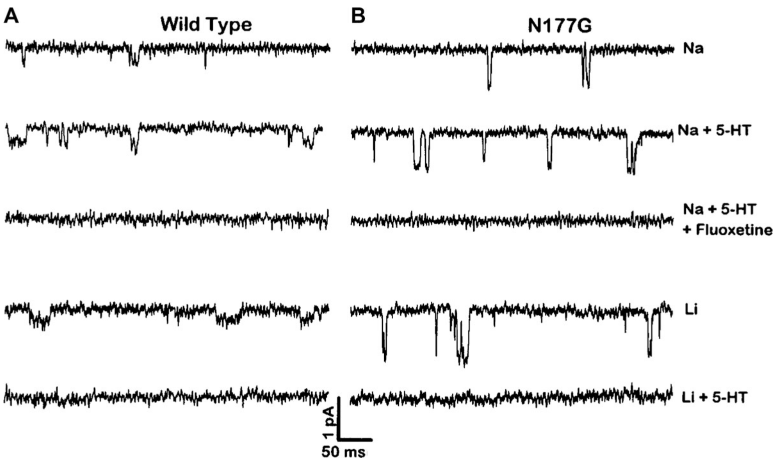

Figure 4. **SERT-mediated single channel currents.** Channel activities from oocytes expressing the wild-type (A) and N177G mutant (B) rat SERT in outside-out patches at −100 mV. Traces labeled Na and Li were recorded in the absence of 5-HT, showing the leakage states. Traces labeled Na + 5-HT represent the channel activities induced by 5-HT (10 mM), showing the 5-HT–induced state. The channel activities were blocked by the uptake inhibitor fluoxetine (10 mM). 5-HT–induced channel activities require Na^+ as a permeable ion; only a few openings are observed in the presence of Li^+ plus 5-HT. All traces in A are from a single patch; those in B are from another patch. Both experiments were performed with normal Cl^- concentrations on both sides of the membrane. The single-channel conductance was much larger for the SERT N177G mutant, demonstrating that they originated from SERT. Figure 4 is reprinted from Lin et al. (1996) with permission from *Biophysical Journal*.

amphetamine derivatives on SERT and 5-HT transport was explored in platelet membrane vesicles (Rudnick and Wall, 1992a,b, 1993; Schuldiner et al., 1993) and in more recent studies using patch-clamp recording (Hilber et al., 2005; Sandtner et al., 2014; Hasenhuetl et al., 2018).

Uncoupled currents in a coupled transporter

The idea that SERT catalyzed electroneutral transport was challenged in 1994 by measurement of 5-HT–dependent currents by Henry Lester, Sela Mager, and their coworkers in *Xenopus* oocytes expressing rat SERT (Mager et al., 1994). These inward currents were, like transport, dependent on Na^+ and Cl^- and inhibited by the transport blocker fluoxetine (Prozac). An electroneutral mechanism, consistent with the results from platelet vesicles, does not predict that charge movement would accompany 5-HT transport (Rudnick and Nelson, 1978). Why, then, did the oocyte studies find that 5-HT transport was associated with a transmembrane current?

The resolution of this apparent discrepancy lay in how the measurement of electrogenicity was made in the two preparations. In platelet vesicles, imposition of a membrane potential had no effect on accumulation of 5-HT (Rudnick and Nelson, 1978; Fig. 2 F), but in oocytes expressing SERT, transmembrane current was measured by voltage clamp, where it was shown that addition of 5-HT increased the inward current, caused by positive charge moving into the cell along with 5-HT (Mager et al., 1994). When influx of radiolabeled 5-HT was measured in oocytes, it was found to be insensitive to changes in membrane potential, just as in platelets (Mager et al., 1994). Thus, transport was consistent with an electroneutral stoichiometry in both experimental systems, but the oocyte experiments had revealed that associated with transport was a current that was not coupled to transport (Mager et al., 1994). This current, unlike transport, was highly voltage sensitive. Subsequent studies detected channel-like openings in oocytes expressing SERT (Lin et al., 1996; Fig. 4). The frequency of these openings was increased two- to fourfold by 5-HT, an effect that required Na^+ and was blocked by fluoxetine. Although the frequency of channel openings was hundreds of times lower than the rate of 5-HT transport, thousands of charges crossed the membrane in each event (Lin et al., 1996) resulting in over seven charges crossing the membrane for each 5-HT molecule transported (Mager et al., 1994).

Summarizing the results from platelet membrane vesicles and SERT-expressing oocytes, these early studies established the electroneutral transport of 5-HT coupled to Na^+ (and probably Cl^-) symport and antiport of either K^+ or H^+. However, in addition to these coupled ion fluxes, SERT also carried another current that was associated with 5-HT transport but energetically uncoupled from the transport cycle.

21st century SERT: Structures and surprises

The last decade of the 20th century witnessed the cloning of numerous transporter genes that segregated into many apparently distinct transporter families. Sequences for SERT, norepinephrine transporter (NET), and dopamine transporter (DAT) were similar to those of GAT-1, the GABA transporter, and the glycine transporters GlyT1 and GlyT2 (Guastella et al., 1990; Blakely et al., 1991; Giros et al., 1991; Hoffman et al., 1991; Pacholczyk et al., 1991; Liu et al., 1992; Smith et al., 1992; Kim et al., 1994). Because many neurotransmitter transporters were found in this group of closely related genes, the transporter family was named NSS (neurotransmitter sodium symporter; Saier et al., 2016) or SLC6 in the human genome classification (~20 human members at this writing; Kristensen et al., 2011; Rudnick et al., 2013). Despite the name, many other transporters that do not transport neurotransmitters, including many sequences from prokaryotes, belong to this family. The vast majority of NSS transporters are amino acid transporters, although a few, notably SERT, NET, DAT, and an octopamine transporter (Caveney et al., 2006), transport amines. Many of the mammalian members of the family, like SERT, are stimulated by, or require, Cl^- for transport.

In 2005, the laboratory of Eric Gouaux published a high-resolution structure for LeuT, a bacterial member of the NSS family (Yamashita et al., 2005). The structure was unique at that time and contained an inverted structural repeat in which the first five transmembrane helices (TM1–TM5) were similar in structure to the following five helices (TM6–TM10), but the two repeats were in opposite topological orientations. TM11 and TM12 were not part of the repeat structure and are likely to fulfill a peripheral role in the function of these proteins. In fact, several bacterial transporters seem to function well despite lacking TM12 (Androutsellis-Theotokis et al., 2003; Quick et al., 2006). Publication of the LeuT structure ushered in a new era for NSS transporters. From that time forward, functional and mechanistic studies of this family gained a structural context that could not be ignored in subsequent mechanistic and functional studies (Penmatsa and Gouaux, 2014).

The following sections describe the structural details of SERT and other members of the NSS family of transport proteins, the structural similarity with other transporter families previously thought to be unrelated, the conformational mechanism by which NSS transporters move their substrates through the membrane, and how these discoveries changed the way we think about SERT stoichiometry.

Substrate- and ion-binding sites

In addition to the inverted repeat, which is a feature common to many transporters (Forrest, 2015), binding sites for substrate and two Na$^+$ ions were observed in the LeuT x-ray structure. Bound in the center of the protein was a molecule of leucine, and the protein was accordingly named LeuT and referred to as a leucine transporter (Yamashita et al., 2005). Ironically, leucine is one of the worst substrates for LeuT, which prefers alanine but also transports several additional amino acids (Singh et al., 2008). The two bound Na$^+$ ions were found at sites with very different properties. One of these sites, called Na1, was coordinated by the carboxyl group of the bound leucine molecule, suggesting an important role in substrate binding and transport. The other Na$^+$ ion was bound at a site (Na2) between TM1 and TM8, where it would be expected to stabilize the protein structure. Although an aqueous pathway led from the extracellular (periplasmic) face of the protein toward the binding site, the bound leucine and Na$^+$ ions were insulated from this pathway by the side chains of several residues. The structure was accordingly classified as an outward-facing occluded conformation (Fig. 5 B).

Siblings and cousins: Transporters with the LeuT fold

Shortly after the release of the LeuT structure, additional transporter structures began to appear. Remarkably, many of these closely resembled LeuT in structure despite having little or no sequence homology. Several transporter families are now known to contain the LeuT fold, and they constitute a superfamily of structurally related transporters referred to as APC (amino acid-polyamine-organocation superfamily) (Vastermark et al., 2014) or FIRL (five transmembrane-helix inverted-topology repeat, LeuT-like; Khafizov et al., 2012). There are two general explanations for why proteins unrelated by sequence would have very similar structures. One explanation is convergent evolution, by which the same structure evolved many times independently. The other possibility is that all these structurally related families evolved from a common ancestor and that the loss of sequence similarity reflected the relative unimportance of a specific sequence, as opposed to the structure, which was retained because it was intimately related to the function of these proteins as transporters.

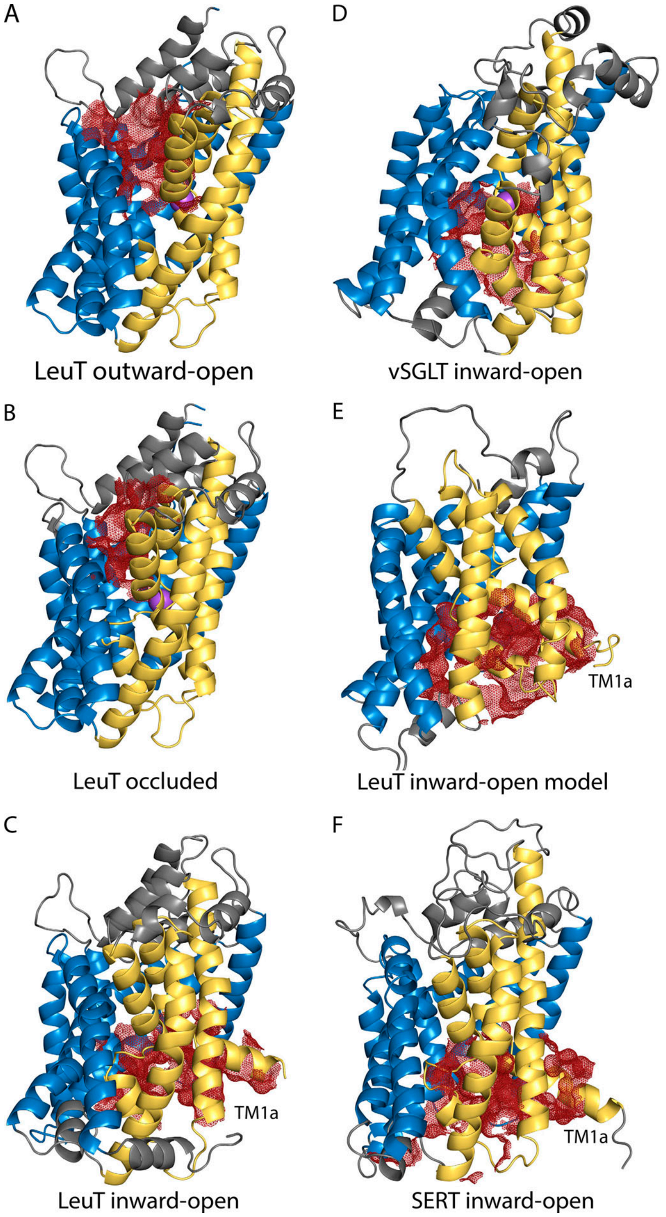

Figure 5. **Structures of LeuT and related transporters.** Structures of LeuT in (A) an outward-open conformation (PDB accession no. 3TT1), (B) an outward-occluded state (PDB accession no. 2A65), (C) an inward-open conformation (PDB accession no. 3TT3), (D) vSGLT in an inward-open conformation (PDB accession no. 3DH4), (E) a repeat-swapped model of the inward-open conformation of LeuT (Forrest et al., 2008), and (F) SERT in an inward-open conformation (PDB accession no. 6DZZ). Bundle helices are in gold, scaffold in blue, loops in gray, and Na2 in violet. For clarity, only TM helices 1–10 are shown. Red mesh indicates the open permeation pathways.

The first LeuT-like structure to appear, after LeuT itself, was for vSGLT, a bacterial galactose transporter (Faham et al., 2008). Unlike LeuT, the vSGLT structure was in an inward-open conformation. Next was Mhp1, a nucleobase transporter, solved in an outward-open conformation (Weyand et al., 2008) and later in an inward-open conformation (Shimamura et al., 2010). Although both vSGLT and Mhp1 were in related APC/FIRL families, neither are members of the NSS/SLC6 family. Both of these transporters catalyze substrate symport with Na$^+$, and both of them contain residues in TM8 corresponding with those in LeuT that coordinate Na^+ in the Na2 site. The Na2 site seems conserved in Na^+-dependent transporters throughout the APC/FIRL superfamily (Yamashita et al., 2005; Faham et al., 2008; Weyand et al., 2008; Ressl et al., 2009; Penmatsa et al., 2013; Malinauskaite et al., 2014; Coleman et al., 2016), although not all transporters in the superfamily are Na^+ dependent.

Conformational mechanisms of LeuT and SERT

The two repeats in the structures of APC/FIRL family transporters are similar in structure, but not identical. Had they been identical, the transporter structure would be symmetrical, at least over the 10-helix repeat region. The structure is asymmetric, because two helices in each repeat come together to form a four-helix bundle that sits at an angle relative to the rest of the protein. The bundle packs closely against the rest of the repeating helices in the cytoplasmic half of the structure but is separated from them in the extracellular half, creating the aqueous pathway leading toward the binding sites (Forrest and Rudnick, 2009; Fig. 5, A and B). Lucy Forrest predicted that swapping the conformations of the two repeats would generate a structure representing LeuT in an inward-facing conformation (Forrest et al., 2008). In the resulting repeat-swapped model (Fig. 5 E), the four-helix bundle was at a different angle relative to the rest of the repeat helices (referred to, along with TM11 to TM12, as the scaffold). The new position of the bundle closed the extracellular pathway and opened a pathway from the cytoplasm to the substrate and Na^+-binding sites, similar to its position in the inward-open vSGLT (Fig. 5 D). Because the principal motion involved in going between these conformations was the tilt of the bundle, it was referred to as the "rocking bundle" mechanism (Forrest and Rudnick, 2009).

In agreement with the predictions of the rocking bundle hypothesis were measurements in SERT that mapped out residues lining the cytoplasmic permeation pathway (Forrest et al., 2008). Cysteine scanning mutagenesis in the predicted cytoplasmic half of SERT identified one face in each of TMs 1, 5, 6, and 8 containing residues that were accessible to the water-soluble reagent MTSEA (2-Aminoethyl methanethiosulfonate hydrobromide). In SERT homology models based on outward-facing LeuT (and the subsequent outward-open structure of SERT; Coleman et al., 2016), these residues were buried in the protein structure. Cocaine, an inhibitor that stabilized SERT in outward-facing conformations, decreased the accessibility of these cytoplasmic pathway positions while ibogaine, which stabilized inward-facing conformations (Jacobs et al., 2007), increased accessibility. Significantly, the corresponding positions in LeuT were predicted to be accessible in the repeat-swapped model of the inward-open conformation (Forrest et al., 2008; Fig. 5 D).

The repeat-swapped model of inward-open LeuT was similar to inward-open structures of vSGLT (Fig. 5 D; Faham et al., 2008) and Mhp1 (Shimamura et al., 2010), providing further evidence for the rocking bundle hypothesis throughout the APC/FIRL superfamily. However, for LeuT, the story proved more complicated. When a structure for the inward-open state of LeuT was finally obtained (Fig. 5 C; Krishnamurthy and Gouaux, 2012; Grouleff et al., 2015), it agreed with many of the repeat-swapped model's predictions, especially in the extracellular pathway, but differed in that the cytoplasmic permeation pathway opens primarily by movement of the cytoplasmic half of TM1 (TM1a) away from the scaffold without the coordinated movement of TM2, TM6, and TM7 (Fig. 5 C, also observed in the recent inward-open structure of SERT [Fig. 5 F; Coleman et al., 2019]). The conformational change in LeuT and SERT would therefore follow the structures shown in Fig. 5 A (outward-open) to Fig. 5 B (occluded) and Fig. 5 C (inward-open). These conformational changes are consistent with recent Hydrogen-Deuterium exchange studies of LeuT and SERT (Adhikary et al., 2017; Möller et al., 2019). This movement exposes the same residues predicted by the rocking bundle model to become accessible when the cytoplasmic pathway opens (Krishnamurthy and Gouaux, 2012; Penmatsa and Gouaux, 2014). Ironically, the repeat-swapped model of inward-open LeuT was a better predictor of inward-open structures of the related APC/FIRL families represented by vSGLT and Mhp1 (Faham et al., 2008; Shimamura et al., 2010) than it was for the NSS family.

Do LeuT and SERT structures agree with transport stoichiometry?

Studies in platelet membrane vesicles suggested that SERT transported 5-HT together with one Na^+ and one Cl^- ion and that a K^+ (or H^+) ion was transported in the opposite direction in the same transport cycle (Keyes and Rudnick, 1982). Does this stoichiometry make sense, now that we know the binding-site structures of SERT and LeuT? The answer is complex but very interesting. We can certainly interpret aspects of the structure to make sense of SERT stoichiometry, but such a reading of the structure for clues about stoichiometry raises additional questions.

All NSS structures contain two Na^+ sites (shown for LeuT and SERT in Fig. 6), but SERT was found to transport only one Na^+ with 5-HT (Talvenheimo et al., 1983). Other transporters in the family transport two or more Na^+ ions with their substrate. The Na^+ stoichiometry of the transporters for GABA (GAT-1) and glycine (GlyT1) were determined to be 2:1 (Radian and Kanner, 1983; Roux and Supplisson, 2000; another glycine transporter, GlyT2, apparently transports three Na^+ ions with glycine; Roux and Supplisson, 2000). The Na^+ stoichiometry of LeuT has never been determined, but LeuT-mediated amino acid transport was stimulated when a membrane potential (interior negative) was imposed by a K^+ diffusion potential (Yamashita et al., 2005; Ryan and Mindell, 2007). This result might be expected if either one or two Na^+ ions are symported with a neutral substrate. However, in the bacterial transporter Tyt1, an H^+ ion was proposed to be antiported with substrate (Zhao et al., 2010a), and LeuT is likely to be similar, so if only one Na^+ was symported, there would be an electroneutral exchange of one Na^+ for one H^+ (Fig. 7). Therefore, the stimulation of transport by a membrane potential implies symport of two or more Na^+ ions, consistent with the number of Na^+-binding sites.

How, then, can the SERT Na^+ stoichiometry of 1:1 be explained? One clue comes from measurement of the NET Na^+ stoichiometry (Gu et al., 1996), which also appeared to be 1:1, like SERT. Although dopamine transport by DAT has commonly been

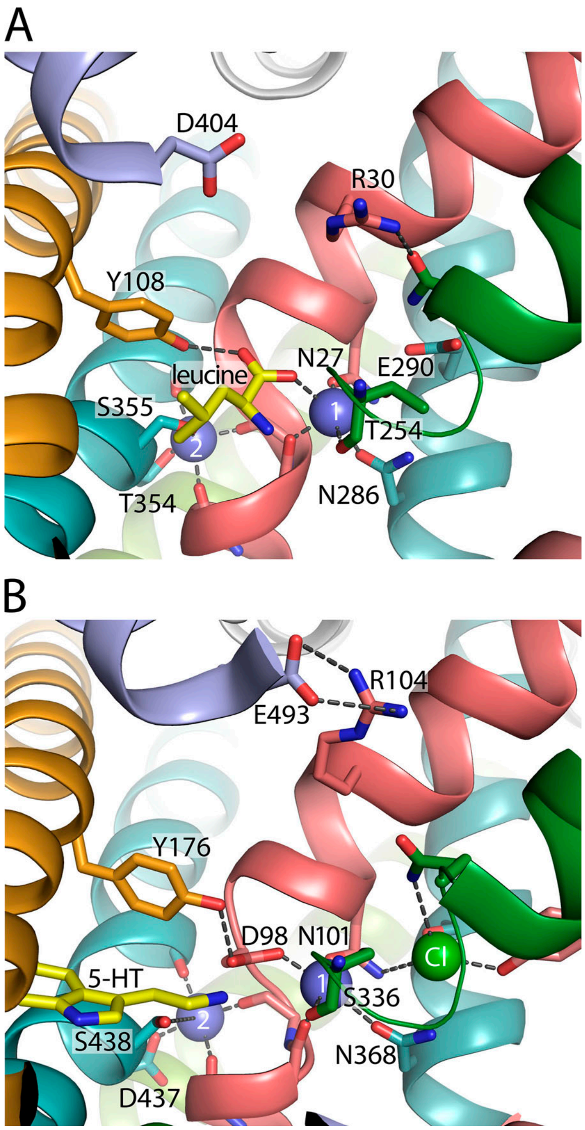

Figure 6. **Central binding sites of LeuT and SERT with a bound molecule of substrate, showing binding sites for Na⁺ that are preserved in all known NSS structures. (A)** Outward occluded structure of LeuT (PDB accession no. 2A65) showing a molecule of leucine (yellow) bound at the central site, interacting with Na1 (blue sphere 1) and Y108 (TM3) through its carboxyl group. In addition to the substrate molecule, the Na1 site is formed by side chains from N27 (TM1, T254 [TM6], and N286 [TM7]) as well as main-chain carbonyl oxygen atoms from TM1 and TM6. Na^+ (blue) at the Na2 site interacts with side chains from T354 and S355 (TM8) and main-chain carbonyls from TM1 and TM8. D404 (TM10) and R30 (TM1) are separated in this structure but form an ion pair in the inward-open LeuT structure (PDB accession no. 3TT3). **(B)** Outward-occluded model of SERT (Hellsberg et al., 2019) aligned to LeuT in A and showing a molecule of 5-HT (yellow) bound at the central site. The carboxyl group of D98 (TM1) occupies a location corresponding to that of the substrate carboxyl in LeuT and makes similar interactions with Na1 and Y176 (TM3). In addition to D98, side chains from N101 (TM1), S336 (TM6), and N368 (TM7) coordinate the Na^+ ion. The Na2 site is formed by residues corresponding to those in LeuT. The Cl^- ion (green) is bound in SERT at a position corresponding to the carboxyl group of E290 in LeuT and an ion pair is formed between R104 (TM1) and E493 (TM10). Parts of TM6 and TM10 were removed for clarity. TM regions are colored as follows: TM1, salmon; TM3, orange; TM5, lime; TM6, forest; TM7, light teal; TM8, aquamarine; TM10, light blue.

described to occur together with two Na^+ ions, the data supporting that stoichiometry were from kinetic experiments showing participation of two Na^+ ions (Krueger, 1990; McElvain and Schenk, 1992). However, evidence that two Na^+ ions are symported with dopamine was not shown. Such an experiment is difficult because of uncoupled ion fluxes in DAT (Sonders et al., 1997).

Together with DAT, NET and SERT transport amines and not amino acids like most members of the NSS family. In LeuT, the Na^+ ion bound at Na1 interacts with the substrate carboxyl group (Fig. 6 A; Yamashita et al., 2005; Singh et al., 2008), which is missing in the substrates for SERT, NET, and DAT. However, these amine transporters all have an aspartate residue in TM1 (Asp98 in SERT) at a position where NSS amino acid transporters have a conserved glycine (Barker et al., 1999). In SERT and DAT x-ray structures, the side-chain carboxyl of this aspartate is positioned close to Na1, restoring the missing carboxyl group present in amino acid NSS substrates (Fig. 6 B; Penmatsa et al., 2013; Coleman et al., 2016).

Replacing the substrate carboxyl group with one derived from the protein might lead to the difference in transport stoichiometry. When NSS transporters transition to the inward-open conformation, the increased distance between TM1 and TM8 disrupts the Na2 site, allowing Na^+ to dissociate to the cytoplasm (Fig. 8). Dissociation of an amino acid substrate from this conformation also disrupts the Na1 site by removing the carboxyl group that interacts with Na^+ (Zhao et al., 2012; Zomot et al., 2015). However, an amine substrate such as 5-HT can dissociate from SERT (or NET or DAT) without disrupting Na1, because the protein supplies the carboxyl ligand to Na^+, not the substrate. Therefore, it is possible that the Na^+ ion at Na1 remains bound to SERT, NET, and DAT during the process of substrate dissociation and returns to an outward-open conformation, leading to a 1:1 stoichiometry of Na^+/substrate transport (Zhang et al., 2018). A recent structure of inward-open SERT suggests that the Na1 site may retain affinity for Na^+ in this conformation (Coleman et al., 2019).

If Na1 does not dissociate from SERT, NET, or DAT, then what happens with Cl^-? Electrostatic interaction between bound Na^+ and Cl^- may be preserved in the inward-open state, and Cl^- has no apparent pathway for dissociation unless the Na1 site is vacant. If this is true, then Cl^- also would remain bound throughout the transport cycle and would not be symported with substrate. The structures of NSS amino acid and amine transporters are different in subtle ways. Integrating our appreciation of these differences with the conformational mechanism of transport has led us to an enhanced understanding of the interaction of substrates and ions at their binding sites. This new understanding casts doubt on the long-held expectation that Cl^- symport occurs in 5-HT transport.

Following the transport reaction in real time

Many insights into the function of SERT have been obtained by measuring accumulation of radiolabeled substrate by cells expressing the transporter or vesicles prepared from those cells. However, a major disadvantage of this approach is its low sampling rate. At room temperature SERT can transport two or

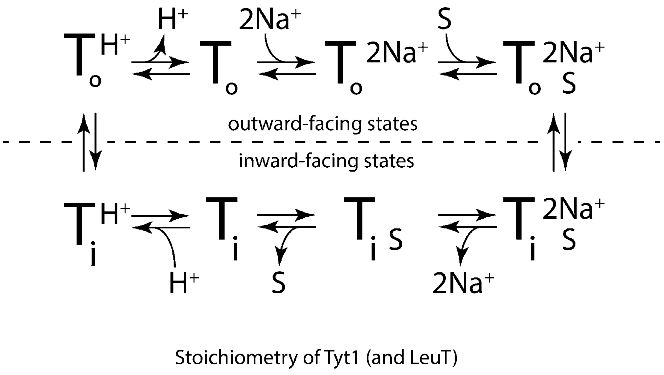

Figure 7. **Transport scheme for Tyt1 and LeuT.** This scheme assumes that one proton is transported out for each substrate molecule transported in with two Na⁺ ions. Based on results with Tyt1 (Quick et al., 2006).

three molecules of 5-HT per second; it takes SERT less than half a second to complete a full transport cycle (Talvenheimo et al., 1979). This is too short a period to be adequately sampled by radiolabeled substrate accumulation. However, it is possible to track currents from transmembrane charge movement associated with transport using electrophysiological tools, which can resolve events that occur on the millisecond time scale (Grewer et al., 2013).

Electrophysiology can provide a more detailed understanding of transporter kinetics, but these techniques have been helpful also in assessing the stoichiometry of many other transport systems. If transport is an electrogenic process in which charges move across the membrane, electrophysiological measurements of transport currents and reversal potentials can help determine transport stoichiometry.

As mentioned earlier, the current that accompanies 5-HT transport was interpreted as an uncoupled ion flux that was a feature of some intermediate in the SERT transport cycle but was suppressed when the transporter was at rest. The transmembrane voltage drives this current but has little effect on transport rate (Mager et al., 1994). This insensitivity of transport rate to voltage was interpreted as support for an electroneutral transport cycle. However, a measurement of the initial transport rate reflects only the slowest step in the transport cycle. For example, a hypothetical transporter with an electrogenic cycle might transport at a rate insensitive to voltage, thus appearing to be electroneutral, if the rate-determining step did not move charge across the membrane. Conversely, a voltage-sensitive transport rate might be misinterpreted as evidence for an electrogenic cycle. If the rate-determining step carried charge across the membrane but that charge was compensated by equal and opposite charge movement in another part of the cycle, the overall cycle would be electroneutral despite the rate being voltage sensitive.

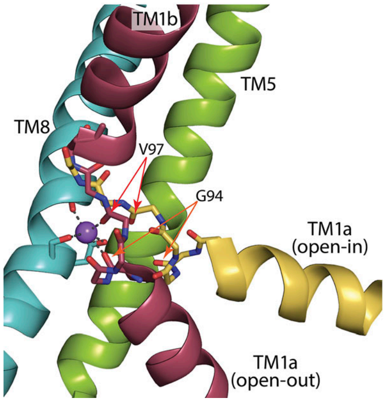

Figure 8. **The SERT Na2 site is disrupted in the inward-open conformation.** Aligned structures of SERT in outward-open (PDB accession no. 5I6X) and inward-open (PDB accession no. 6DZZ) conformations showing the movement of TM1a away from TM8 in the inward open conformation, which disrupts the Na2 site. Note the distances between the Na⁺ ion and the coordinating backbone carbonyl oxygen atoms of V97 and G94 in TM8, which increase from 2.4 Å and 2.5 Å, respectively, in the outward-open structure to 3.9 Å and 6.3 Å in the inward-open conformation.

Because the SERT stoichiometry is electroneutral (Rudnick and Nelson, 1978) and the currents that SERT carries are uncoupled from transport (Mager et al., 1994), it would appear unlikely that electrophysiological analysis could shed much light on SERT. However, some steps in the SERT transport cycle do move charge across the membrane, and the uncoupled current reflects occupation of a specific intermediate in the cycle. So, even in this apparently uninviting context, we have learned a lot about SERT by studying the transmembrane currents associated with SERT function.

The following subsections describe an electrophysiological approach to understanding 5-HT transport that allow insight into the binding of substrate and ions from the extracellular medium, the influence of cytoplasmic ions and 5-HT, the rate-determining return of SERT from inward- to outward-facing conformations, and how these findings force a reevaluation of SERT ion-coupling stoichiometry.

Currents carried by SERT reflect a partial reaction and occupation of a K⁺-bound state

In HEK293 cells expressing SERT, whole-cell patch clamp allows control of the membrane potential and the ionic composition both inside and outside the cell. With NaCl outside the cell and K⁺ inside, rapid addition of external 5-HT causes a brief peak of inward current followed by a steady inward current that lasts as long as external 5-HT is present (Fig. 9 A). The peak current represents the synchronous binding of 5-HT to SERT molecules that have already bound Na⁺ and Cl⁻, transition of this complex to an inward-open conformation, and intracellular dissociation of 5-HT and Na⁺ (Fig. 9 B; Schicker et al., 2012; Hasenhuetl et al., 2016). The steady current represents the uncoupled flux of charge into the cell from SERT molecules that have dissociated Na⁺ and 5-HT and bound K⁺ (Figs. 3 and 9 E; Adams and DeFelice, 2003; Schicker et al., 2012).

The peak current, which we observed upon rapid application of 5-HT, is a coupled current that is produced by partial reactions within the transport cycle that carry substrate and ions through the membrane (Fig. 9, A and B; Schicker et al., 2012). Under physiological intra- and extracellular ionic conditions, but in the absence of substrate, SERT does not cycle through outward- and inward-facing conformations but rather is trapped in an outward-facing conformation with Na⁺ and Cl⁻ bound. Addition of

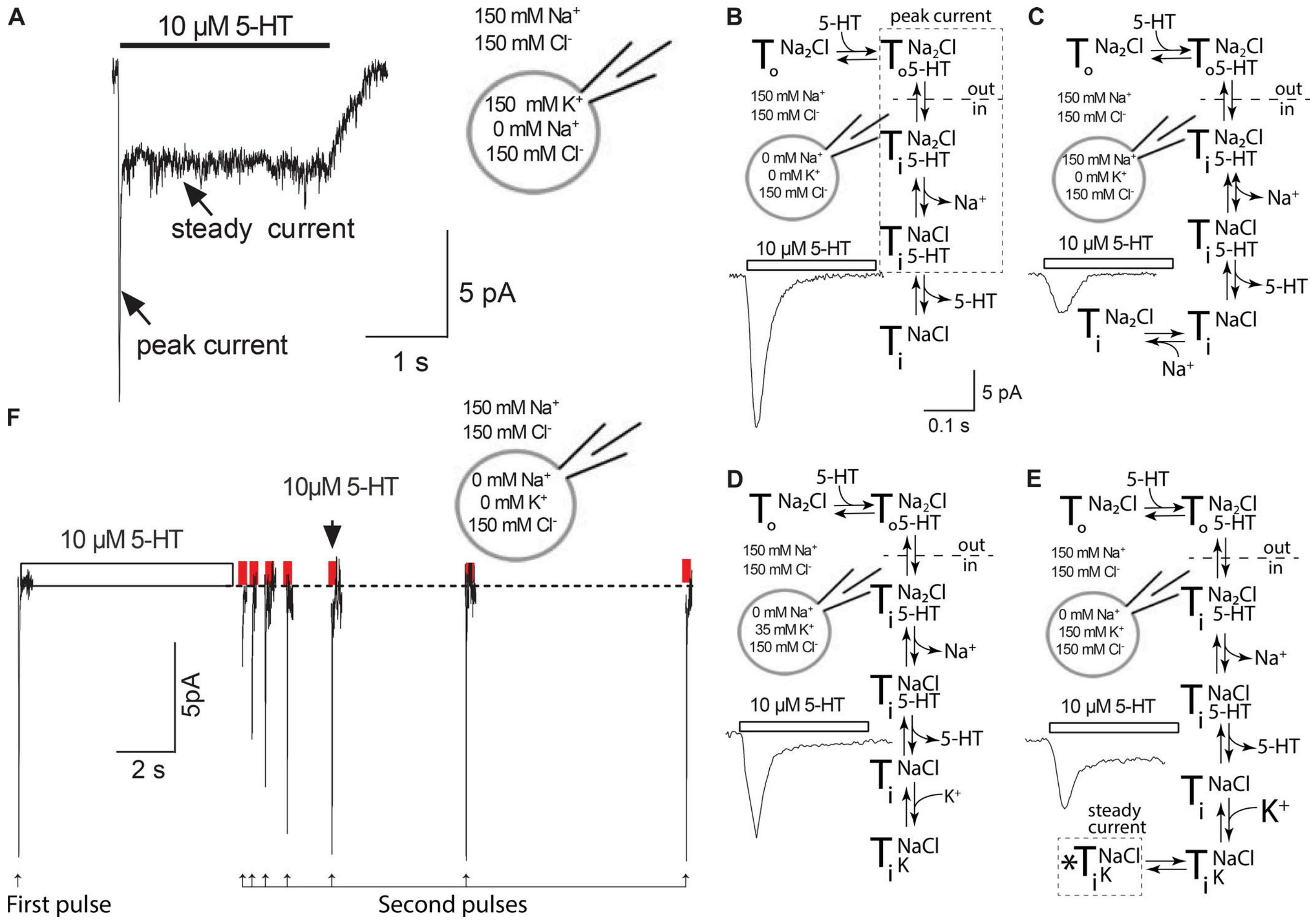

Figure 9. **Peak and steady SERT currents induced by rapid application of 5-HT. (A)** Application of 5-HT to a SERT-expressing HEK293 cell perfused with KCl in the absence of internal Na+. The peak current was induced on application of 5-HT and the steady current continued as long as 5-HT was present. **(B–E)** Current traces obtained under the conditions shown, with the partial reaction cycle proposed to be associated with those traces. **(B)** In the absence of internal alkali cations, a strong peak current was observed with no steady current. The dashed box shows the steps responsible for the peak current. **(C)** Addition of high internal Na+ suppressed the peak current. The peak current was smaller than in A, because at high intracellular Na+, the Na2 site was mostly occupied, and thus, there was little net flux of Na+ into the cytosol. **(D and E)** Addition of internal K+ at intermediate (D) or high (E) concentrations progressively increased the steady current and decreased the peak current. The dashed box shows a hypothetical intermediate responsible for the steady current. **(F)** Two-pulse protocol for measuring recovery of the outward-facing conformation of SERT. Traces from six trials are shown, with arrows indicating additions of extracellular 5-HT. Red boxes indicate when the second pulse was applied. The current recovered with a monoexponential time course. The rate of current recovery is a measure of the turnover rate.

extracellular 5-HT causes a synchronous transition that converts SERT to an inward-facing state that releases Na+ and substrate to the cytoplasm. These events all occur rapidly, even on the electrophysiological time scale, and cannot be distinguished kinetically, but together, they result in the peak current (Fig. 9, A and B).

The actual peak current measured results from movement of charges within the transmembrane electrical field and is composed of currents associated with binding of extracellular charges, their dissociation to the cytoplasm, and movement of the electrical field in response to SERT conformational changes. Importantly, the peak current not only provides a kinetic estimate of these reactions but also serves as a tool to study other kinetic processes, such as substrate and inhibitor binding and the transporter's turnover rate, with high temporal precision. The peak current indicates that a subset of intermediate steps moves charge through the membrane upon substrate addition. Electroneutrality of the overall reaction implies that an equal and opposite charge movement must occur somewhere else in the cycle. The peak current also allowed us to confirm that the turnover rate of SERT is voltage independent (this has been verified for voltages ranging from −80 mV to +50 mV), reinforcing observations that the rate-determining steps are unlikely to move charge across the membrane (Hasenhuetl et al., 2016). Additional study of this process allowed us to determine the rates for most of the partial reactions for this transporter operating in the forward direction (Schicker et al., 2012; Hasenhuetl et al., 2018).

The first steps in transport: Extracellular 5-HT, Na+, and Cl− binding to SERT

Previous experiments demonstrated that Na+, 5-HT, and Cl− were all required for conversion of SERT to an inward-open conformation (Zhang and Rudnick, 2006). At high concentrations of substrate and ions, the kinetics of charge movement is independent of 5-HT and ion concentration, indicating that the rate of this substrate transport step was limited by steps other than binding, such as conversion from outward- to the inward-facing conformation. Because the peak current requires binding of all three ligands, it can be slowed down by decreasing the concentration of any one of the three (Schicker et al., 2012; Hasenhuetl et al., 2016). Under these conditions, the rate of charge movement is limited by the rate at which that limiting substrate or ion binds. Importantly, at rate-limiting concentrations of Na^+, 5-HT, or Cl^-, this binding rate was unaffected by the transmembrane electric potential over a range from −60 mV to +30 mV. Therefore, substrate and ion binding to the outward-facing conformation is a voltage-independent reaction. This is different from several other transporters in the NSS and related Solute:Sodium Symporter (Faham et al., 2008) families. For instance, Na^+ binding to the outward facing conformation of SGLT1, GAT1, and the GlyTs was shown to be highly voltage dependent (Mager et al., 1993; Panayotova-Heiermann et al., 1995; López-Corcuera et al., 1998). These studies show that the initial binding of extracellular $5\text{-}HT^+$, Na^+, and Cl^- does not require movement of these ions through the transmembrane electric field.

The peak current: Conformational change and cytoplasmic Na^+ dissociation

During the conformational change from outward- to inward-open LeuT (and SERT), the aqueous pathway between the central binding sites and the extracellular medium closes and a pathway opens up between the binding sites and the cytoplasm. This transition is expected to reshape the electrical field across the protein (Fig. 10), which should move outward from the cytoplasmic half of the protein to the extracellular half. Any charges in the binding site effectively move through the field during this transition. Rapid dissociation of ions from within the field also would contribute to the current, in addition to any other charge movements in the protein that accompany conformational change.

Experimentally, the peak current observed upon 5-HT addition was mostly eliminated by raising intracellular Na^+ (Fig. 9 C). This could result from Na^+ dissociating and rebinding from within the electrical field. However, we consider it more likely that these binding events incorporate a concomitant conformational change. The Na2 site, from which Na^+ dissociates, is formed by TM1 and TM8, which are close in outward-facing x-ray structures with Na^+ bound (Yamashita et al., 2005; Singh et al., 2008; Krishnamurthy and Gouaux, 2012) but separate during the process of intracellular Na^+ dissociation, which leads to the inward-open structures of Na^+-free LeuT (Krishnamurthy and Gouaux, 2012) or SERT with Na1 and Cl^- sites occupied (Coleman et al., 2019; Fig. 8). Binding of cytoplasmic Na^+ (to Na2) may induce a reverse conformational change in SERT by stabilizing the cytoplasmic pathway in a closed state, leading to an occluded conformation. The possibility that Na^+ binds at Na2 and thereby closes the cytoplasmic pathway and reshapes the electrical field through SERT is consistent with a mechanism for coupling intracellular ion binding to conformational change as a concerted process.

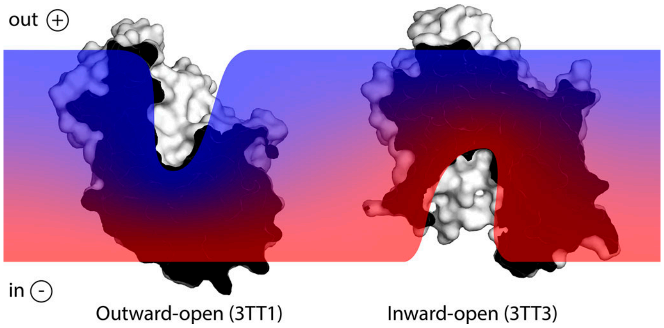

Figure 10. **Representation of the expected change of the electrical field through a transporter accompanying a change in conformation.** LeuT structures 3TT1 and 3TT3 are shown, as an example, in cross section to reveal the extracellular and cytoplasmic pathways, respectively, and how conformational change could shift the binding site from being at the relatively positive electrical potential of the external medium (blue) to the relatively negative potential of the cytoplasm (red).

The relationship of the peak current to voltage indicates the valence, or the amount of charge transferred across the membrane. The voltage dependence of the peak current measured in the nominal absence of intracellular Na^+ was consistent with slightly more than one full charge moving through the membrane electric field upon conversion to an inward-facing conformation and dissociation of Na^+ (Hasenhuetl et al., 2016). We found that raising cytoplasmic Na^+ decreased the current, consistent with the essential involvement of Na^+ dissociation in this process (Fig. 9 C). Although cytoplasmic Na^+ decreased the peak current, it did not eliminate it. At saturating Na^+, the peak current that remained (Fig. 9 C) corresponded to ~0.15 charges that represent additional charge movements relative to the field. In contrast, changing the intracellular Cl^- or 5-HT concentration did not change the peak current. This implied that these two other ligands either do not dissociate internally or that their binding reactions are voltage independent.

K^+ binding to inward-open SERT

Although neither intracellular 5-HT nor Cl^- affected the charge moving in the peak current, increasing intracellular K^+ decreased the peak current almost as much as Na^+ (Fig. 9 E) and similarly decreased the valence of the peak current (Hasenhuetl et al., 2016). This suggested that K^+ binding to inward-open SERT was a voltage-dependent process, possibly involving conformational changes that compensate electrically for the entry of charge with Na^+. The decreased peak current at high intracellular K^+ was consistent with K^+ ions binding essentially as fast as Na^+ ions dissociate and with the full charge of that K^+ ion replacing the charge of the dissociating Na^+ ion. This result also raises the possibility that the K^+ ion might bind at the same site from which Na^+ dissociates, leading to a similar conformational change, although the possibility that K^+ binds at a different site within the electrical field cannot be excluded.

The steady current that follows the peak current requires intracellular K^+ (Adams and DeFelice, 2002; Schicker et al., 2012;

Fig. 9, B, D, and E). In a recent study, we reasoned that the channel-like state of SERT that carried the uncoupled current (Fig. 3) was in equilibrium with the K^+-bound inward-facing conformation (Schicker et al., 2012). This conclusion provides a framework for studying the transport cycle of SERT using the uncoupled current. In the absence of intracellular Na^+, high intracellular 5-HT concentrations (>1 mM) fully suppressed the SERT steady currents but did not affect the peak current (Hasenhuetl et al., 2016). Raising the intracellular K^+ concentration could not abolish this inhibitory effect of 5-HT. These results suggest that 5-HT binds and dissociates from inward-open SERT at least as fast as K^+ binds and that 5-HT and K^+ do not compete for the same binding site. The sequence of events that follows the 5-HT–induced conformational change, therefore, would begin with dissociation of Na^+ followed by K^+ binding. If 5-HT remains bound (under conditions with high intracellular 5-HT), the process would not proceed to the point where the K^+-dependent steady-state current begins, but at low intracellular 5-HT, this current begins immediately after the peak current, consistent with fast dissociation of 5-HT (as fast as that of Na^+).

Because high intracellular 5-HT does not affect the peak current, 5-HT binding and dissociation must not be associated with movement of charge through the electric field. This observation is consistent either with intracellular 5-HT binding in its neutral form within the electrical field or cytoplasmic 5-HT^+ binding to a substrate site that is at the same potential as the cytoplasm. In the latter case, 5-HT binding to the inward-facing substrate site might not reshape the electric field (like Na^+ binding at the Na2 site) by inducing conformational changes. In contrast to Na^+, K^+, and 5-HT, intracellular Cl^- did not affect the steady current, nor did it affect the ability of either Na^+ or K^+ to decrease the peak current, consistent with the proposal that Cl^- does not bind or dissociate intracellularly.

Return of K^+-bound SERT to an outward-open state

The return of the K^+ loaded transporter is the rate-limiting step in the transport cycle of SERT (Nelson and Rudnick, 1979; Hasenhuetl et al., 2016). We measured the rate of the return step, which is equivalent to the rate-determining step, using a two-pulse protocol (Otis and Kavanaugh, 2000). In this experiment, we applied the first pulse: 10 µM extracellular 5-HT (a saturating concentration; open bar, Fig. 9 F). This forced essentially all the SERT molecules into the transport cycle and induced the peak current, which gives a measure of the total number of available transporters. When we applied a second pulse after a time interval that was short relative to the turnover time, the resulting peak current was very small, because most of the transporters had not yet returned to a state capable of binding extracellular 5-HT. The amplitude of the induced second peak current reflected the fraction of transporters that had gone through the transport cycle and returned to the outward facing state. After a sufficiently long interval between pulses, the second peak current returned to the amplitude of the first peak, because all transporters had completed the transport cycle. Using a series of intervals between the pulses, we established the entire time course of the recovery, which represents the process of inward-facing SERT returning to the outward-facing conformation (Fig. 9 F). This time course was accelerated by intracellular K^+ as expected if K^+ binding to the inward-facing conformation facilitated its return to the outward-facing state (Figs. 1 and 3). Conversely, internal 5-HT (2 mM) slowed down the recovery by binding to SERT and decreasing the amount of free SERT capable of returning to the outward-facing conformation (Figs. 1 and 3). We also measured the time course of the recovery over a wide range of voltages, finding that it was essentially voltage independent and confirming that the rate limiting return step does not move charge across the membrane.

This return step, the rate-determining step in the transport cycle, is also the least understood. We do not know where K^+ binds in this process, nor do we know whether K^+ binding, like Na^+ binding, induces a conformational change. What we do know, however, is that the intermediate with K^+ bound is likely the one responsible for the steady current. During the time that intracellular K^+ is bound, SERT has some probability of opening a conductance responsible for the steady current (*T; Figs. 3 and 9 E). The frequency of channel openings observed by Lin et al. (1996) was much lower than the turnover number, indicating that the probability of channel opening is low. We also know that intracellular 5-HT has opposite effects on inward-facing SERT with Na^+ vs. K^+ bound. 5-HT is essential for the reversal of the transport step with Na^+, but it blocks the return step with K^+.

As described earlier in this review, H^+ was able to replace K^+ as a driving force for 5-HT accumulation in platelet plasma membrane vesicles (Keyes and Rudnick, 1982). To examine the role of protons in more detail, we tested the ability of low internal pH (5.5) to replace internal K^+. In the absence of internal K^+, the steady-state current is absent at an internal pH of 7.2 but is restored by lowering internal pH to 5.5 (Hasenhuetl et al., 2016). As stated above, intracellular K^+ stimulates the rate of the return step as measured by the recovery time course. In the absence of internal K^+, lowering the internal pH to 5.5 also restores the recovery rate. In both cases (internal K^+ or low internal pH), recovery is insensitive to membrane potential, indicating that the steps following K^+ or H^+ binding do not move charge across the membrane. Additionally, low internal pH, like internal K^+, decreased the amount of charge crossing the membrane with the peak current, consistent with a site that could bind K^+ or H^+ as Na^+ dissociates (Hasenhuetl et al., 2016). These results explain how a transmembrane pH difference (internally acidic) could drive 5-HT accumulation (Keyes and Rudnick, 1982). Intracellular K^+ binds to counter the charge movement associated with the peak current (conformational change and Na^+ dissociation). In this step, which allows the substrate-free return of SERT to an outward-facing conformation (and concomitantly stimulates the steady current), H^+ apparently replaces K^+ in every facet of its action, suggesting that they may bind at the same site on inward-open SERT.

Back to the stoichiometry

In the electroneutral transport scheme we proposed over 35 yr ago (Keyes and Rudnick, 1982), Na^+ and Cl^- were symported with cationic 5-HT^+ and K^+ was antiported to complete the cycle

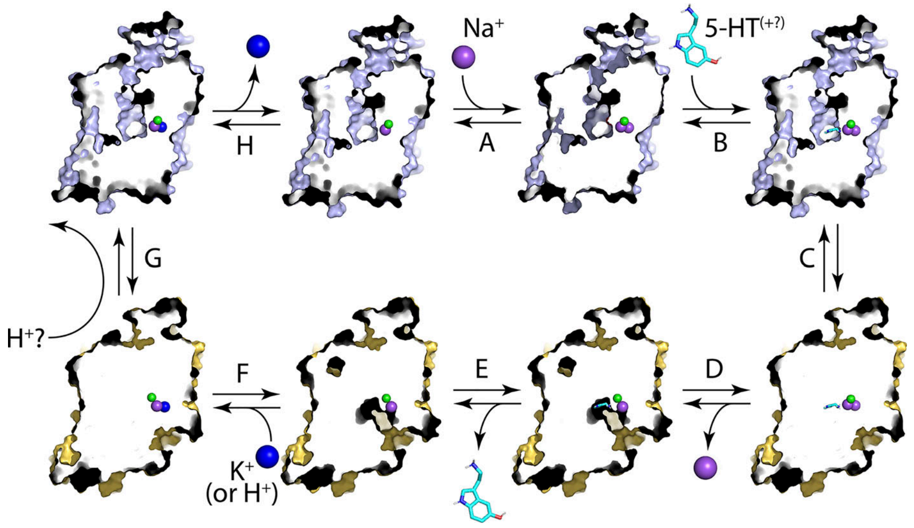

Figure 11. **An overall scheme for 5-HT transport by SERT.** In step A, the transporter, with Na1 and Cl sites occupied, binds an extracellular Na$^+$ ion at the Na2 site, which stabilizes the open-out conformation. Subsequent binding of a 5-HT molecule in step B allows a conformational change that closes the extracellular pathway (step C) and allows the concerted opening of the cytoplasmic pathway and dissociation of the ion bound at Na2 (step D) along with the peak current (Fig. 9 B). Dissociation of 5-HT (step E) and intracellular K$^+$ binding (step F) might not occur in an ordered sequence, but the presence of bound 5-HT prevents formation of the conductive state responsible for the steady current (Fig. 9, A and E; this current may be a property of the intermediate shown between steps F and G). Binding of K$^+$ reverses the charge movement that occurs in step D, possibly because it induces a conformational change that closes the cytoplasmic pathway. With K$^+$ bound, SERT undergoes a conformational transition to an outward-open state (step G), followed by K$^+$ dissociation to the extracellular medium (step H). For charge movements to be balanced, the entry of Na$^+$ and 5-HT$^+$ must be offset by efflux of a cation (shown as exit of a proton in step G) or by the transport of 5-HT in its neutral rather than charged form.

(Fig. 3). There is compelling support for an electroneutral scheme (Rudnick and Nelson, 1978; Keyes and Rudnick, 1982; Mager et al., 1994; Quick, 2003), but the inability of internal Cl$^-$ to alter the peak current or to influence K$^+$ binding (Hasenhuetl et al., 2016) argues that Cl$^-$ (like the Na$^+$ ion bound to the Na1 site) does not dissociate to the cytoplasm and is not symported with 5-HT. Therefore, another aspect of the original proposal must be reevaluated, because uptake of Na$^+$ and 5-HT$^+$ coupled to K$^+$ efflux would be electrogenic, moving one positive charge into the cell with each 5-HT molecule. The 1:1 transport stoichiometry for Na$^+$ (Talvenheimo et al., 1983) and K$^+$ (Rudnick, 1977; Fig. 3) with 5-HT agrees well with the valence of the Na$^+$-dependent peak current and its reversal by intracellular K$^+$ (Hasenhuetl et al., 2016). To reconcile these observations with an electroneutral transport cycle, 5-HT could be transported in the neutral form (Fig. 11). Another possibility that 5-HT is transported as a cation and that a proton (possibly bound to Asp98) leaves the cell with K$^+$ in the return step (Fig. 11). Low internal pH can substitute for K$^+$ (Keyes and Rudnick, 1982; Hasenhuetl et al., 2016), supporting H$^+$ antiport, and we cannot exclude the possibility that an additional H$^+$ ion is antiported. The alternative, that the true substrate for SERT is unprotonated, neutral 5-HT, would be surprising. Proposed binding poses for 5-HT (Celik et al., 2008) show its amino group close to Asp98 and, at pH 7, neutral 5-HT accounts for <1% of 5-HT in solution.

Thus, after over 40 yr of research, some aspects of the transport cycle—symport with 1 Na$^+$ ion and antiport with one K$^+$ or H$^+$—have stood the test of time, while symport with Cl$^-$ is likely to be incorrect and transport of the cationic form of 5-HT is up for debate. Despite some lingering questions, the revised transport cycle (Fig. 11) is consistent with more recent structural and electrophysiological data and with almost all the older observations. Future developments may require the application of experimental methods and computational approaches that have yet to be developed.

How are ion and substrate transport coupled?

Despite our understanding that transmembrane gradients of Na$^+$ and K$^+$ are responsible for energetically driving 5-HT accumulation, we do not really know how SERT transforms the energy in these ion gradients into the energetically uphill accumulation of 5-HT. Indeed, the mechanistic details of ion–substrate coupling are only now just being revealed for any transporters. Fortunately, one of these transporters is LeuT. Can our understanding of coupling in LeuT help us understand the process in SERT?

The coupling mechanism for LeuT was first suggested by some experiments using another bacterial NSS transporter, Tyt1 (Quick et al., 2006). They implied that the transporter adopts an outward-facing conformation in the presence of Na$^+$ and that subsequent binding of substrate allowed conversion back to inward-open conformations. The same Na$^+$ and substrate effects on conformation were observed also in LeuT and SERT (Zhao et al., 2010b, 2011; Tavoulari et al., 2016; Terry et al., 2018; Zhang et al., 2018). These results led to a strategy employed by Tyt1, LeuT, and potentially all NSS transporters where Na$^+$ binding, apparently at the Na2 site, stabilizes an outward-facing conformation, thereby preventing uncoupled transport of Na$^+$ (Tavoulari et al., 2016). Only after substrate binds is the transporter able to convert to an inward-facing conformation, from which it can release Na$^+$ and substrate. To prevent uncoupled transport of substrate, LeuT (and presumably all NSS amino acid transporters), binds substrate very poorly in the absence of Na$^+$.

For the two conformational changes, the Na$^+$-dependent stabilization of outward-facing conformations and the substrate-dependent relaxation back to inward-facing states, we have identified the responsible structural components in LeuT. Na$^+$, binding at the Na2 site, fosters interaction between TM1 and TM8 in the cytoplasmic half of LeuT that closes the cytoplasmic pathway (Tavoulari et al., 2016). Additional interactions near the cytoplasmic face of LeuT and other NSS transporters help to stabilize the inward-closed state (Yamashita et al., 2005; Kniazeff et al., 2008; Ben-Yona and Kanner, 2013). These interactions are antagonized by substrate binding, which closes the extracellular pathway and allows the cytoplasmic pathway to open. Our recent studies have identified an interaction in LeuT that is essential for substrate binding to initiate the conformational change that ultimately delivers substrate and Na^+ to the cytoplasm (Zhang et al., 2018). In this interaction, the carboxyl group of the amino acid substrate connects a Na^+ ion bound at Na1 on one side and the phenolic hydroxyl group of Tyr108 on the other (Fig. 6 A). The conformational change also requires interactions in the extracellular pathway that stabilize the closed state of that pathway and allow the cytoplasmic pathway to open (Shi et al., 2008; Zhang et al., 2018).

Although this mechanism can be extended to other NSS amino acid transporters, SERT and the other amine transporters must use a modification of the mechanism because their substrates lack a carboxyl moiety. Instead, the NSS amine transporters contain an aspartate (Asp98 in SERT, Fig. 6 B) where their amino acid transporting counterparts contain a glycine residue (Gly24 in LeuT). With an amine as a substrate, SERT (along with NET and DAT) should be less dependent on Na^+ for substrate binding, because the direct interaction of Na^+ with the substrate is missing. Indeed, 5-HT binds to SERT even in the absence of Na^+ (Humphreys et al., 1994). Despite the presence of a protein carboxyl group to replace the missing substrate carboxyl in 5-HT, the conformational change requires 5-HT in addition to Na^+ and Cl^- (Zhang and Rudnick, 2006), indicating that another mechanism, different from that of LeuT, is responsible for 5-HT to initiate conformational change.

Nonetheless, similar interactions seem to be important in SERT. The γ-carboxyl of Asp98 is located between the Na1 site and a tyrosine residue (Tyr176) corresponding to Tyr108 in LeuT. Moreover, mutation of Tyr176 in SERT blocks the ability of substrate (5-HT) to activate the conformational transition of SERT from outward- to inward-facing conformations, similar to the way that mutating Tyr108 blocked the corresponding substrate-induced conformational change in LeuT (Zhang et al., 2018). We still do not understand how 5-HT binding in SERT activates the interactions among Tyr176, Asp98, and Na^+, and this activation is a focus of our current research.

Where do we go from here?

In addition to understanding how 5-HT activates conformational change in SERT, we are still in the dark concerning other fundamental aspects of the mechanism. Even if Cl^- is not released intracellularly, it is still required for 5-HT–dependent conformational change, and we have little understanding of what it does. It may play a vestigial role in SERT as part of the Na1 site, or it could have a more active role. The site at which K^+ binds has not been identified. Does K^+ bind at the Na2 site? If so, why does SERT behave so differently when K^+ is bound there compared with Na^+? Is 5-HT bound and transported in the neutral form, which would be consistent with recent electrophysiological results, or is a proton antiported with K^+ to compensate for the influx of 5-HT^+? It seems that despite major advances in the understanding of SERT mechanism, this transporter is still holding on to some of its secrets, for the time being, at least.

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