Vesicular and Plasma Membrane Transporters for Neurotransmitters

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The regulated exocytosis that mediates chemical signaling at synapses requires mechanisms to coordinate the immediate response to stimulation with the recycling needed to sustain release. Two general classes of transporter contribute to release, one located on synaptic vesicles that loads them with transmitter, and a second at the plasma membrane that both terminates signaling and serves to recycle transmitter for subsequent rounds of release. Originally identified as the target of psychoactive drugs, these transport systems have important roles in transmitter release, but we are only beginning to understand their contribution to synaptic transmission, plasticity, behavior, and disease. Recent work has started to provide a structural basis for their activity, to characterize their trafficking and potential for regulation. The results indicate that far from the passive target of psychoactive drugs, neurotransmitter transporters undergo regulation that contributes to synaptic plasticity.

The speed and potency of synaptic transmission depend on the immediate availability of synaptic vesicles filled with high concentrations of neurotransmitter. In this article, we focus on the mechanisms responsible for packaging transmitter into synaptic vesicles and for reuptake from the extracellular space that both terminates synaptic transmission and recycles transmitter for future rounds of release. Collectively, we refer to this entire process as the neurotransmitter cycle.

The recycling of neurotransmitter illustrates a general, conceptual problem for the mechanism of vesicular release. At the plasma mem-

brane, more active reuptake should help to replenish the pool of releasable transmitter, but may also reduce the extent and duration of signaling to the postsynaptic cell. Conversely, loss of reuptake increases the activation of receptors but results in the depletion of stores (Jones et al. 1998). At the vesicle, steeper concentration gradients release more transmitter per vesicle but reduce the cytosolic transmitter available for refilling, whereas more shallow gradients facilitate refilling but reduce the transmitter available for release. The way in which the nerve terminal balances these competing factors thus has profound consequences for synaptic transmission.

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NEUROTRANSMITTER SYNTHESIS AND TRANSPORT INTO SECRETORY VESICLES

The ability of vesicles to concentrate neurotransmitter was suspected by Bernard Katz and coworkers when identifying quantal events associated with the release of acetylcholine (ACh) at the neuromuscular junction. The subsequent identification of synaptic vesicles by electron microscopy then provided a structural basis for this phenomenon (Katz 1971). Julius Axelrod, Arvid Carlsson, and others extended the concept of vesicular storage, finding that radio-labeled catecholamines became “stabilized” after uptake (Carlsson 1963; Axelrod 1971).

The amount of neurotransmitter released per vesicle can also influence the postsynaptic response. The extent of vesicle filling is particularly important for volume transmission by neuromodulators such as monoamines, ACh, and neuropeptides, in which release often occurs at a distance from receptors. However, the amount of transmitter released per vesicle influences signaling even at classical synapses, where receptors lie immediately under the release site. Even high affinity NMDA receptors for glutamate are not saturated by the release of a single synaptic vesicle at many synapses (Mainen et al. 1999; McAllister and Stevens 2000), indicating the potential for changes in vesicle filling to influence synaptic transmission, particularly for lower affinity ionotropic receptors or G protein-coupled receptors outside the synapse. There are three major determinants of vesicle filling: the cytosolic concentration of transmitter, the H^+ electrochemical driving force across the vesicle membrane, and intrinsic properties of the vesicular transporter such as its ionic coupling.

Neurotransmitter Biosynthesis

The availability of cytosolic neurotransmitter depends on specific biosynthetic enzymes. The enzyme tyrosine hydroxylase has a rate-limiting role in catecholamine biosynthesis, and normally functions at a small fraction of its potential capacity because of allosteric feedback inhibition by L-Dopa and its downstream product dopamine (DA) (Zigmond et al. 1989). Phos-

phorylation regulates tyrosine hydroxylase activity through modulation of this feedback inhibition, and the enzyme undergoes stringent regulation at transcriptional as well as post-translational levels (Kaneda et al. 1991). Much less is known about the closely related enzyme tryptophan hydroxylase, which is involved in serotonin (5-hydroxytryptamine, 5-HT) production. The biosynthetic enzyme for ACh, choline acetyltransferase (ChAT), has similarly received little attention, but the gene resides at the same chromosomal locus as the vesicular ACh transporter (VACHT), and indeed shares some of the same promoters (Erickson et al. 1994; Cervini et al. 1995), indicating highly conserved, coordinate regulation of the two proteins. ChAT also appears to undergo regulation by phosphorylation (Dobransky and Rylett 2005), but under most circumstances, the plasma membrane choline transporter (CHT) is thought to be rate-limiting (Ferguson et al. 2004; Sarter and Parikh 2005).

GABA production relies on two, distinct biosynthetic enzymes, glutamic acid decarboxylase (GAD) of 65 kD and 67 kD. An autoantigen in diabetes, the 65 kD isoform associates directly with vesicles through palmitoylation (Christgau et al. 1992), whereas the 67 kD isoform is cytosolic. However, the 67 kD isoform appears much more important for GABAergic neurotransmission than the 65 kD-animals lacking the 67 kD isoform die shortly after birth because of cleft palate, but also show up to 80% reduction in GABA levels (Asada et al. 1997; Condie et al. 1997). In contrast, loss of the 65 kD isoform has minimal effect on inhibitory neurotransmission. Surprisingly, the amplitudes of evoked and spontaneous inhibitory postsynaptic currents (IPSCs) show no difference from controls, but the knockouts show increased synaptic depression to sustained synaptic activation (Tian et al. 1999), raising the interesting possibility that vesicle filling might influence release probability.

Considering its general role in intermediary metabolism and protein synthesis, glutamate can be synthesized from multiple sources. However, the inability to regenerate most of these sources (such as α -ketoglutarate from the tri-

carboxylic acid cycle) would result in the depletion of glutamate stores by prolonged stimulation. For this reason, the glutamate released as a transmitter is generally considered to derive from glutamine, which can be regenerated from glutamate through the so-called glutamine-glutamate cycle (Fig. 1). In contrast to most other classical transmitters which undergo reuptake directly into the nerve terminal, glutamate appears to recycle indirectly: after uptake into

astrocytes through the excitatory amino acid transporters (EAATs), glutamate is converted into glutamine by glutamine synthetase, then transferred back to neurons where it is converted to glutamate and ammonia through the action of glutaminase (Fig. 1) (Albrecht et al. 2007). Consistent with this cycle, inhibition of glutamine synthetase results in the shift of glutamate immunoreactivity from neurons to astrocytes in the retina (Pow and Robinson 1994).

Figure 1. Role of plasma membrane and vesicular neurotransmitter transporters in synaptic transmission. After the exocytotic release from synaptic vesicles, neurotransmitter is transported back into the terminal by Na^+ and Cl^- -dependent plasma membrane transporters (PMT), thereby regenerating the vesicular pools required to sustain release. In the case of glutamate, excitatory amino acid transporters (EAATs) are generally found on cells other than those directly involved in glutamate release; most of the uptake occurs into astrocytes, mediated by EAAT1 and 2, which do not couple stoichiometrically to the flux of Cl^- . Nonetheless, other isoforms such as EAAT3 can be expressed by neurons, although generally not at presynaptic sites or not by glutamate neurons. The glutamate taken up by glia undergoes conversion to glutamine and is then thought to recycle to neurons through the system N transporters expressed by glia and the system A transporters expressed by neurons, with conversion back to glutamate by phosphate-activated glutaminase (PAG) within neurons. Synaptic vesicles fill with neurotransmitter through a process driven by the vacuolar-type H^+ -ATPase. However, different transmitters depend on different components of the H^+ electrochemical gradient produced by this pump. The vesicular monoamine transporter (VMAT) and closely related vesicular acetylcholine transporter depend primarily on the chemical component, ΔpH , whereas vesicular glutamate transporters (VGLUTs) depend predominantly on the membrane potential, $\Delta\psi$. The entry of anions such as Cl^- (but also glutamate) promote the formation of ΔpH by dissipating $\Delta\psi$ and hence allowing the H^+ pump to generate ΔpH , although the factors that promote $\Delta\psi$ have remained unexplored.

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The identification and characterization of system N and A transporters has suggested a mechanism for the transfer of glutamine from astrocyte to neuron (Fig. 1). The system N transporters SN1 and SN2 exchange 1 Na⁺ and 1 glutamine for 1 H⁺, resulting in electroneutrality (Chaudhry et al. 1999). As a result, they depend only on the Na⁺ gradient across the plasma membrane (not the membrane potential) and hence produce a very shallow gradient of glutamine that enables efflux simply by lowering the extracellular concentration of glutamine (Chaudhry et al. 1999; Broer et al. 2002). In contrast, the system A transporters SA (or SAT, SNAT) 1–3 couple the movement of neutral amino acids such as glutamine only to the flux of Na⁺, and hence depend on membrane potential as well as the Na⁺ gradient, producing much steeper concentration gradients that can take up into neurons the glutamine released by astrocytes (Chaudhry et al. 2002b). Interestingly, the system N and A transporters are closely related to each other in sequence, differ primarily in the coupling to H⁺, and presumably contribute to nitrogen metabolism by the liver through a similar cycle (Haussinger and Schliess 2007).

On the other hand, the physiological role of these transporters and even glutamine itself in excitatory transmission requires further characterization. Although some work has suggested that the system A inhibitor methylaminoisobutyric acid (MeAIB) can impair glutamatergic transmission (Armano et al. 2002), it has been very difficult to influence baseline synaptic transmission by depleting extracellular glutamine (Kam and Nicoll 2007). Increasing extracellular glutamine can increase glutamate release, but endogenous levels appear adequate to sustain release under normal conditions. Indeed, the most recent work indicates a role for the glutamine-glutamate cycle primarily under pathologic conditions, such as seizures (Tani et al. 2007), and we still do not know the actual role of different glial and neuronal glutamine transporters.

After uptake by the neuron, glutamine is converted to glutamate by the mitochondrial enzyme phosphate-activated glutaminase (Curthoys and Watford 1995), but we again under-

stand little of its role in synaptic transmission. A knockout of the main isoform expressed in brain results in neonatal death because of impaired respiration, but baseline excitatory transmission appears normal, indicating the presence of an alternate biosynthetic pathway, presumably caused by expression of the other glutaminase isoform (Masson et al. 2006). Despite the normal mEPSC amplitude, knockout animals still show increased synaptic rundown in response to high frequency stimulation, indicating that the kidney/brain isoform nonetheless contributes to release.

We know little about the actual cytosolic concentration of most classical transmitters. Labeling with antibodies has suggested cytosolic levels of glutamate in the low millimolar range (Gundersen et al. 1995). The direct manipulation of cytosolic contents at the calyx of Held also suggests low millimolar concentrations of glutamate, and further indicates the potential for changes in vesicle filling to influence quantal size (Ishikawa et al. 2002). In the case of DA, the insertion of a carbon fiber electrode directly into the cell has enabled detection of cytosolic levels (Mosharov et al. 2003). In chromaffin cells, the concentrations of cytosolic catecholamine can reach 50 μM, but in midbrain DA neurons, endogenous levels are undetectable (<0.1 μM) unless boosted with the biosynthetic precursor L-Dopa (Mosharov et al. 2009). However, these levels could be greatly affected by activity-dependent alterations in DA synthesis, as noted above. Furthermore, the psychostimulant amphetamine can quickly release DA from the cytosol through the plasma membrane DA transporter (DAT) prior to depleting intracellular vesicle stores, suggesting that sufficient intracellular DA may be available to support transporter-mediated DA efflux (Jones et al. 1998). Inhibition of the intracellular, metabolic enzyme monoamine oxidase (MAO) further increases cytosolic DA, suggesting that multiple mechanisms serve to balance the potential for cytoplasmic toxicity with the need for vesicle refilling. Indeed, transport into the cell as well as into the vesicle are additional, important determinants of cytosolic transmitter concentration (Torres et al. 2003b).

Proton Electrochemical Driving Force

The transport of all classical transmitters into neurosecretory vesicles depends on a H^+ electrochemical driving force ($\Delta\mu_{H^+}$) generated by the vacuolar H^+ -ATPase (Fig. 1). The H^+ pump produces a $\Delta\mu_{H^+}$ similar in magnitude to if not greater than that achieved across the plasma membrane for Na^+ , K^+ , and Cl^- , providing a similar force to drive transport. However, the actual concentrations of H^+ are many orders of magnitude lower: pH 7 is 0.1 μM H^+ whereas the other ions are in the high millimolar range. In contrast to the relatively stable ionic gradients across the plasma membrane, $\Delta\mu_{H^+}$ can thus be made and dissipated with the flux of many fewer H^+ , ideal for synaptic vesicles, which undergo repeated exocytosis and endocytosis that respectively dissipate and regenerate $\Delta\mu_{H^+}$.

The H^+ -ATPase uses the energy released by ATP hydrolysis to pump protons into the vesicle lumen. Composed of a membrane sector V0 and a peripheral sector V1 that resemble the F0 and F1 sectors of the mitochondrial ATP synthase, the vacuolar H^+ pump also uses a rotary mechanism (Forgac 2007). However, the two H^+ pumps function in different directions under physiological conditions, the vacuolar pump hydrolyzing ATP to move H^+ , whereas the mitochondrial ATP synthase uses H^+ flux to produce ATP. Interestingly, the V0 sector has also been implicated in membrane fusion. Mutations in the V0 but not V1 sector disrupt vacuole fusion in yeast, and because both of these impair vacuole acidification, it suggests a direct role for the V0 sector in fusion, independent of acidification (Bayer et al. 2003). Similarly, mutation of a neuron-specific $\alpha 1$ subunit of the *Drosophila* V0 sector affects the evoked release of transmitter, but not the amplitude of spontaneous release events, again suggesting a specific role in fusion independent of the acidification required for vesicle filling (Hiesinger et al. 2005), although the mechanism remains unclear.

We know little about the regulation of V-ATPase activity, but much evidence suggests an indirect form of regulation by chloride (Fig. 1).

In the absence of Cl^- , the ATPase can in fact transport very few H^+ because the development of a lumen-positive membrane potential ($\Delta\Psi$) opposes the activity of the pump. Indeed, synaptic vesicles develop a pH gradient (ΔpH) only after the addition of an anion such as Cl^- . Entry of the anion dissipates $\Delta\Psi$, secondarily activating the H^+ pump to produce ΔpH , and ATPase activity can increase several orders of magnitude in response to the addition of Cl^- (Forgac 2007). It is also generally considered that intracellular members of the ClC channel family account for the entry of Cl^- . However, it has become clear that many of the intracellular ClCs, including the prototypic *Escherichia coli* protein, function as Cl^-/H^+ exchangers rather than as channels (Accardi and Miller 2004; Picollo and Pusch 2005; Scheel et al. 2005), and although counterintuitive, this coupling increases ΔpH (Novarino et al. 2010). Synaptic vesicles contain multiple ClC isoforms, and it remains unclear which—if any—of the ClCs confer the chloride flux required for synaptic vesicle acidification.

Anions other than Cl^- may also play a role in synaptic vesicle acidification. In addition to ATP, which may promote monoamine uptake independent of its role as a substrate for the H^+ pump (Bankston and Guidotti 1996), glutamate itself acidifies vesicles. Indeed, multiple monoamine cell populations express a VGLUT isoform that appears to promote vesicle filling with monoamine, presumably because of an increase in ΔpH . DA neurons express VGLUT2 and the conditional inactivation of VGLUT2 in DA neurons reduces the effect of psychostimulants (Birgner et al. 2010) by impairing DA storage and release (Hnasko et al. 2010). Similarly, the loss of VGLUT3 from cholinergic interneurons in the striatum reduces ACh storage and release (Gras et al. 2008). However, glutamate appears to promote a more stable ΔpH than Cl^- , and the mechanism appears to reflect the stoichiometry of coupling by VGLUTs (Hnasko et al. 2010).

It is important to note that the glutamate released by monoamine neurons also serves to activate postsynaptic glutamate receptors, independent of its role in vesicle acidification and

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monoamine storage. As predicted from previous work showing glutamate responses to the stimulation of midbrain DA input to the striatum (Chuhma et al. 2004), the expression of channelrhodopsin specifically in DA or 5-HT neurons (which express high levels of VGLUT3 [Fremeau et al. 2002; Gras et al. 2002; Schafer et al. 2002]) has enabled the detection of robust glutamatergic responses in, respectively, the postsynaptic medium spiny or hippocampal neurons (Varga et al. 2009; Stuber et al. 2010; Tecuapetla et al. 2010). Habenula neurons similarly corelease glutamate and ACh, with drastically different postsynaptic effects (Ren et al. 2011). In general, the role of the two signals released by a single neuron remains unclear, but recent work has shown a role for differential release of ACh and GABA from the same retinal starburst amacrine cells involved in direction selectivity (Lee et al. 2010).

Vesicular Neurotransmitter Transport

The expression of $\Delta\mu_{H^+}$ as ΔpH or $\Delta\Psi$ has particular significance for transmitter release because different vesicular transporters depend to differing extents on the two components of the gradient. Vesicular monoamine and ACh transport rely predominantly on ΔpH , vesicular glutamate transport primarily on $\Delta\Psi$, and GABA transport more equally on both chemical and electrical components of $\Delta\mu_{H^+}$ (Fig. 1). Consistent with these differences in bioenergetics, the vesicular monoamine and ACh transporters show close sequence similarity to each other, whereas the vesicular glutamate and GABA transporters comprise entirely distinct families.

Classical studies in chromaffin granules showed that vesicular monoamine transport involves the exchange of two luminal protons for one cytosolic, protonated amine (Johnson et al. 1981; Knoth et al. 1981), and this stoichiometry accounts for the greater reliance on ΔpH than $\Delta\Psi$. With a vesicle pH ~ 5.8 and $\Delta\Psi \sim 60$ mV, the stoichiometry in turn predicts monoamine gradients of up to 10^5 (lumen relative to cytosol), consistent with the high concentrations achieved inside chromaffin granules.

There are two vesicular monoamine transporters (VMATs) in mammals-VMAT1 expressed by nonneural cells such as adrenal chromaffin cells, and VMAT2 by neurons. However, both isoforms recognize multiple monoamines as substrates, with a slightly higher apparent affinity for VMAT2 (Peter et al. 1994; Erickson et al. 1996). Consistent with this, VMAT2 is expressed by multiple central monoamine populations (Weihe et al. 1994; Peter et al. 1995).

The VMATs have a K_m in the low to sub-micromolar range, whereas the vesicular ACh, GABA, and glutamate transporters all show K_m s in the low millimolar range. This high apparent affinity may contribute to the robust VMAT activity detected by radiotracer flux assays. In addition, it presumably enables the VMATs to achieve maximal transport at low cytosolic levels of substrate. Indeed, the vesicular monoamine transporter was cloned on the basis of its ability to protect against the parkinsonian neurotoxin MPP⁺, presumably by sequestering the toxin inside secretory vesicles and away from its primary site of action in mitochondria (Liu et al. 1992). VMAT may similarly protect against the toxicity of endogenous DA, and a defect may contribute to the degeneration of DA neurons in Parkinson's disease (Caudle et al. 2007; Mosharov et al. 2009). However, many factors limit the accumulation of DA in the cytoplasm-in the absence of VMAT activity, the brain content of monoamines declines by $>90\%$ – 95% due in part to cytoplasmic metabolism by monoamine oxidase (Fon et al. 1997; Takahashi et al. 1997; Wang et al. 1997).

On the other hand, the VMATs have a relatively low V_{max} , with an estimated rate of 5–20/sec depending on the substrate (Peter et al. 1994). Vesicle filling may therefore limit monoamine release from neurons with high rates of firing. The loss of VMAT2 in mice causes neonatal lethality because of impaired movement and feeding (Fon et al. 1997; Takahashi et al. 1997; Wang et al. 1997). Little is known about the regulation of intrinsic VMAT activity and its role in monoamine release.

The vesicular ACh transporter (VAChT) resembles the VMATs in primary sequence (Alfonso et al. 1993), but shows a much higher

Km (Parsons 2000). Despite this, it has a relatively slow turnover $\sim 1/\text{sec}$ (Varoqui and Erickson 1996). A large body of work has shown presynaptic regulation of quantal size at the neuromuscular junction (Van der Kloot 2003), possibly involving VACHT (Song et al. 1997; Lima Rde et al. 2010).

The vesicular GABA transporter (VGAT) recognizes both inhibitory transmitters GABA and glycine (Wojcik et al. 2006). Identified by genetic studies in *C. elegans* (McIntire et al. 1993b; McIntire et al. 1997), VGAT defined a family of proteins structurally unrelated to VMAT, consistent with a greater dependence on $\Delta\Psi$. Nonetheless, dependence on ΔpH had suggested that VGAT still functions as a H^+ exchanger. Surprisingly, purified VGAT reconstituted into artificial membranes may require Cl^- for active transport (Juge et al. 2009). Importantly, the Cl^- is cotransported, and with a Hill coefficient of 2, predicting strong dependence on the Cl^- gradient as well as $\Delta\Psi$.

VGAT has the lowest apparent affinity of any vesicular transporter ($K_m \sim 5 \text{ mM}$ for GABA, $\sim 25 \text{ mM}$ for glycine), and probably relies on high cytosolic concentrations of substrate produced either by GAD in the case of GABA or by the plasma membrane glycine transporter GLYT2 in the case of glycine (Gomez et al. 2003a). Among the vesicular neurotransmitter transporters, VGAT is so far the only one known to require a specific sorting protein, identified in *C. elegans* as UNC-46, a lysosome-associated membrane protein (LAMP) family member (Schuske et al. 2007).

The VGLUTs depend on $\Delta\Psi$ to a greater extent than the other vesicular transporters (Fig. 1), and belong to a distinct family of transport proteins. Although originally identified as Na^+ -dependent phosphate transporters, their localization to synaptic vesicles and glutamate uptake on heterologous expression indicated a role for the VGLUTs in vesicular glutamate transport (Bellocchio et al. 2000; Takamori et al. 2000). Disruption of the VGLUT genes in worms and mice has confirmed their role in synaptic glutamate release (Lee et al. 1999; Fremeau et al. 2004; Wojcik et al. 2004; Wallen-Mackenzie et al. 2006; Gras et al. 2008; Seal et al. 2008; Hnasko

et al. 2010). Indeed, other members of the so-called type I phosphate transporter family also appear to recognize organic anions (Busch et al. 1996; Verheijen et al. 1999). However, the coupling mechanisms vary considerably among family members, with sialin using H^+ cotransport to drive the electroneutral efflux of sialic acid out of the lysosome (Morin et al. 2004; Wreden et al. 2005) whereas the VGLUTs and the more distantly related vesicular nucleotide transporter VNUT (Sawada et al. 2008) exchange rather than cotransport H^+ . Although inorganic phosphate does not inhibit glutamate uptake by the VGLUTs (Bellocchio et al. 2000), reconstitution of the purified transporter confers Na^+ -dependent phosphate transport (Juge et al. 2006). Even more remarkably, sialin, an electroneutral H^+ cotransporter, has been suggested to transport both glutamate and aspartate through a mechanism dependent on $\Delta\Psi$ (Miyaji et al. 2008). Because VGLUT1-3 recognize glutamate but not aspartate, it will be important to determine whether sialin in fact contributes to the evoked release of aspartate.

The VGLUTs also show at least two distinct interactions with Cl^- . First, they show an uncoupled Cl^- conductance that may influence vesicle acidification, as described above. This conductance would serve to dissipate the $\Delta\Psi$ that drives glutamate transport, but glutamate appears to inhibit this conductance (Bellocchio et al. 2000). Second, Cl^- regulates vesicular glutamate transport through an allosteric mechanism. VGLUT activity shows a biphasic dependence on Cl^- , with an optimum $\sim 2\text{--}10 \text{ mM}$. Although originally considered to reflect changes in the expression of $\Delta\mu_{\text{H}^+}$, it has become clear that Cl^- operates independently of the driving force to influence intrinsic VGLUT activity (Hartertinger and Jahn 1993; Wolosker et al. 1996; Juge et al. 2010). However, manipulation of cytosolic Cl^- at the calyx of Held has remarkably little effect on quantal size (Price and Trussell, 2006), suggesting that vesicle filling in vivo may reflect the thermodynamic equilibrium reached by the system more than the kinetics of transport.

In the brain, VGLUT1-3 define neurons as glutamatergic, and show an almost mutually

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exclusive pattern of expression, particularly in adulthood. VGLUT1 is expressed by glutamatergic neurons in the hippocampus and cortex, VGLUT2 in thalamus and brainstem and VGLUT3 in scattered neurons often not associated with the release of glutamate. For example, VGLUT3 is expressed by a subset of GABAergic interneurons as well as cholinergic interneurons of the striatum and serotonergic raphe neurons (Fremeau et al. 2002; Gras et al. 2002, 2008; Amilhon et al. 2010). In these cells, glutamate transport by VGLUT3 (and by VGLUT2 in DA neurons) both promotes vesicle filling with the other transmitter, and its release activates postsynaptic receptors, as discussed above. Notably, a variety of neuronal populations express VGLUT2 or 3 transiently during development. In the cortex, transient expression of VGLUT2 presumably accounts for the appearance of a phenotype in VGLUT1 knockout mice only around two weeks after birth (Fremeau et al. 2004). In the auditory brainstem, transient expression of VGLUT3 also contributes to the strengthening and tonotopic refinement of synapses (Noh et al. 2010).

What difference in vesicle filling is conferred by the different VGLUT isoforms? The crude transport assays currently available reveal no clear difference in uptake activity. Rather, the isoforms appear to differ in trafficking. Although VGLUT1 and 2 are almost exclusively axonal, VGLUT3 localizes to dendrites as well as axons in a number of cell populations (Harkany et al. 2004). VGLUT1 contains two polyproline motifs that interact with proteins involved in endocytosis such as endophilin, and influence recycling of the transporter (Voglmaier et al. 2006). Such motifs presumably influence the sorting of VGLUTs to distinct endocytic pathways, and recent work suggests that these pathways may generate synaptic vesicles with a different probability of release (Weston et al. 2011).

Regulation of Vesicular Transporters

Despite the potential for vesicle filling to regulate quantal size, we still know little about the physiological role for regulation of vesicular

transport activity. Regulation could occur at the level of the driving force, through changes in the expression of $\Delta\mu_{H^+}$ as ΔpH or $\Delta\Psi$. Indeed, the differences in ionic coupling by different vesicular neurotransmitter transporters might be expected to require differences in the expression of $\Delta\mu_{H^+}$. However, recent work has shown that glutamatergic and GABAergic synaptic vesicles differ primarily in the expression of vesicular neurotransmitter transporter, i.e., VGLUT and VGAT (Gronborg et al. 2010).

Differences in the number of transporters per vesicle would influence the rate of vesicle filling, but not necessarily the equilibrium eventually achieved. Indeed, one VGLUT protein appears sufficient to fill a single synaptic vesicle (Daniels et al. 2006). VGLUT1 heterozygotes show no obvious deficit in synaptic transmission (Fremeau et al. 2004). On the other hand, behavioral differences were reported in heterozygous VGLUT mice (Leo et al. 2009; Schallier et al. 2009), suggesting that the transporter may become rate-limiting with higher levels of activity. In this regard, overexpression of VACHT can increase quantal amplitudes (Song et al. 1997), suggesting that in the cholinergic system as well, transporter expression can limit the rate of vesicular filling. The VGLUTs were also identified as activity-regulated genes (Ni et al. 1994; Aihara et al. 2000), that along with VGAT show homeostatic changes in expression with altered neural activity (De Gois et al. 2005), providing circumstantial evidence for a role in regulation of transmitter release. However, the trafficking of neurotransmitter transporters also has great potential to influence their number on synaptic vesicles. Interestingly, psychostimulants have been shown to influence the localization and function of VMAT2 (Volz et al. 2007; Farnsworth et al. 2009).

A number of reports describe the regulation of vesicle filling by heterotrimeric G proteins, in particular $G\alpha_2$ (Ahnert-Hilger et al. 1998; Holtje et al. 2003; Winter et al. 2005). Although the molecular mechanism of modulation remains uncertain, it may involve a direct interaction of G protein with neurotransmitter transporter (Winter et al. 2005; Brunk et al. 2006).

Not All Transporters on Synaptic Vesicles Transport Substrates into the Vesicular Compartment

Several transporters found on synaptic vesicles cannot use vesicular ion and voltage gradients to concentrate solutes across vesicular membranes. The high-affinity, Na^+ and Cl^- dependent, L-proline transporter (PROT) (Freneau et al. 1992) resides on synaptic vesicles in a subset of terminals that release L-glutamate (Crump et al. 1999; Renick et al. 1999). Although little evidence supports a role for proline as neurotransmitter, PROT activity is easily detectable at the plasma membrane in brain synaptosomes (Nadler 1987) where it may modulate glutamate release (Cohen and Nadler 1997a), glutamate receptor function (Cohen and Nadler 1997b), or perhaps ensure the availability of cytoplasmic glutamate for vesicular uptake and release (Atlante et al. 1996). A second example is the hemicholinium-sensitive, choline transporter (CHT), a Na^+ and Cl^- -dependent transporter that provides for uptake of the precursor at the plasma membrane of cholinergic nerve terminals (Macintosh et al. 1956; Yamamura and Snyder 1972; Maire and Wurtman 1985). CHT localizes to a subset of VAcHT-positive synaptic vesicles (Ferguson et al. 2003; Nakata et al. 2004). As with PROT, the vesicular location provides a mechanism for the coupling of choline transport to exocytotic transmitter release through coincident insertion of transporters into the plasma membrane.

PLASMA MEMBRANE NEUROTRANSMITTER TRANSPORTERS

All small molecule neurotransmitters require a mechanism to terminate signaling, and this usually involves transporter-mediated reuptake. Enzymatic conversion of ACh by acetylcholinesterase is perhaps the only exception to this rule. In many cases, neurotransmitter reuptake occurs into the terminal from which it was released, but in other instances, particularly for amino acid neurotransmitters, these molecules can be cleared by transporters located on astrocytes (Fig. 1) (Bergles et al. 1999) or even on terminals that release another neurotransmitter (Gresch

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et al. 1995; Zhou et al. 2005). After release, diffusion of neurotransmitter within the extracellular space is extremely rapid, with levels predicted to drop in milliseconds below the concentrations needed to activate receptors (Clements 1996), much faster than expected from the rate at which transporters can move neurotransmitters across the membrane ($\sim 1\text{--}10$ transfer events/sec). Thus, binding of neurotransmitters to their transporters ("buffering") is suspected to be just as or more important in clearing neurotransmitter than the actual transport process.

As with the concentrative uptake of neurotransmitter into vesicles, all plasma membrane neurotransmitter transporters use transmembrane ion or voltage gradients to energize the clearance process (Pastuszko et al. 1982; Zeranque and Kavanaugh 1996; Rudnick 1997). This is particularly important for the amino acid transmitters that exist at very high concentrations in the cytosol. In this case, transporters that only equilibrate substrate across the membrane would result in efflux rather than uptake. Plasma membrane neurotransmitter transporters thus rely on Na^+ and K^+ gradients across the plasma membrane established by the ubiquitous Na^+/K^+ ATPase.

Studies with knockout mice have shown the physiological importance of transporters for proper synaptic function (Giros et al. 1996; Rothstein et al. 1996; Bengel et al. 1998; Gomez et al. 2003a,b; Murphy and Lesch 2008). In addition to the expected reductions in transmitter clearance, these models have revealed a profound contribution of transporters to presynaptic neurotransmitter homeostasis, with significantly reduced pools (particularly for DA, NE, and 5-HT) in the absence of reuptake (Fig. 1). Profound changes in pre- and postsynaptic receptors also occur in these models, underscoring the functional significance of transporters for both sides of the synapse.

Pharmacology of Plasma Membrane Neurotransmitter Transport

Pharmacology has played a crucial role in the study of neurotransmitter transporters. In the late 1950s and early 1960s, the availability of

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highly sensitive fluorescence and radiotracer flux assays for epinephrine, norepinephrine (NE), DA and 5-HT enabled Julius Axelrod and his colleagues to identify reuptake as the major mechanism that terminates synaptic transmission (Axelrod 2003). In particular, the group showed that the actions of multiple psychotropic drugs, including cocaine and tricyclic antidepressants, derive from their inhibition of biogenic amine reuptake (Whitby et al. 1960; Axelrod 1961; Hertting et al. 1961; Glowinski and Axelrod 1964). Two decades later, scientists at Eli Lilly would use the same assay to identify the blockbuster, 5-HT-selective reuptake inhibitor (SSRI), fluoxetine (ProzacTM) (Fuller and Wong, 1990). More recently, antagonists that target the norepinephrine transporter (NET) and dopamine transporter (DAT) more selectively (e.g., the NET inhibitor atomoxetine or the DAT inhibitor methylphenidate) have found clinical uses in the treatment of attention-deficit hyperactivity disorder (ADHD). It has also become clear that OCT-3, a transporter that confers low-affinity but high-capacity uptake, contributes to 5-HT clearance (Daws et al. 2006; Baganz et al. 2008). OCT-3 is highly expressed in midbrain raphe nuclei (see Allen Brain Atlas at <http://www.brain-map.org/>), but its presynaptic role remains unknown. Indeed, the contribution of OCT-3 to 5-HT clearance is most evident in cases of SERT deficiency (Baganz et al. 2008). In addition, GABA transporter subtypes continue to receive attention for the treatment of epilepsy (Madsen et al. 2010), and transporters for glycine (a coagonist with glutamate at NMDA receptors) for the treatment of schizophrenia (Hashimoto 2010). EAATs have been the target of far less drug discovery, no doubt reflecting the concern over excessive elevation of extracellular glutamate, which can be excitotoxic. Potentiation of EAAT activity remains an interesting, but largely unexplored area of neurotransmitter transporter pharmacology.

Cocaine and D-amphetamine (AMPH), two highly addictive psychostimulants, interact with DAT, NET, and SERT proteins rather non-selectively and rapidly elevate synaptic levels of their corresponding biogenic amine substrates, although through distinct mechanisms. Cocaine

is a competitive transporter inhibitor (Carroll et al. 1997), whereas AMPH and its congeners are competitive substrates, again acting on all three biogenic amine transporters. By competing with the uptake of DA, AMPH would be expected to increase extracellular biogenic amine. However, the mechanisms by which AMPH raises synaptic neurotransmitter are far more complex (Blakely et al. 2005). First, external amphetamine can exchange with cytosolic monoamine, then diffuse back out of the cell for repeated rounds of exchange and hence cause the efflux of monoamine. AMPH can also elevate cytosolic monoamine by competing for uptake into vesicles, or by dissipation of the H⁺ electrochemical driving force for vesicle storage (Sulzer et al. 1993). The elevated cytosolic concentrations of DA, NE, and 5-HT can then not only retard uptake, but promote transporter reversal (“efflux”) across the plasma membrane. The amphetamine analog methylenedioxymethamphetamine (MDMA, “ecstasy”) has a similar effect, but limited to the serotonin transporter SERT (Rudnick and Wall 1992). Interestingly, AMPH produces a rise in intracellular Ca²⁺ (Gnegy et al. 2004), possibly by reducing the pH gradients needed to sequester Ca²⁺. AMPH also leads to serine phosphorylation at the amino terminus of DAT mediated by Ca²⁺/calmodulin dependent kinase CaMKII, and phosphorylation at these sites appears to promote a state permissive for efflux (Fog et al. 2006). It is reasonable to wonder whether a mechanism that shifts the susceptibility of the transporters between influx and efflux represents a disturbance in transporter regulation because of a foreign substance, or a process used under physiological conditions. Further studies are needed to understand the physiological and pathophysiological relevance of transporter-mediated neurotransmitter efflux.

Early electrophysiological studies of heterologously expressed NET, DAT, and SERT revealed significant neurotransmitter-gated ion flow, which exceeded that needed for coupling to neurotransmitter uptake (Blakely et al. 1994). These observations suggest multiple conductance states for neurotransmitter transporters, one that is stoichiometrically coupled to ion

movement, and another that resembles the uncoupled flow of ions seen with ligand-gated channels (DeFelice and Blakely 1996; Su et al. 1996). Galli and colleagues detected NE-gated conductances in NET-transfected HEK-293 cells (Galli et al. 1996), and by carbon fiber amperometry identified NE flux states that coincided with channel openings (Galli et al. 1998), suggesting that at least under some circumstances, channel events can support neurotransmitter uptake. But are these findings an artifact of heterologous expression? The cells used lack a number of NET-interacting proteins (e.g., syntaxin 1A, SYN) that can alter NET activity and NET currents. However, Carvelli and coworkers have also detected neurotransmitter-gated channel states in cultured DA neurons (Carvelli et al. 2004). In addition, DAT-mediated currents can influence the excitability of mammalian DA neurons (Ingram et al. 2002), which seems unlikely if changes in membrane potential depend on the small amount of ion flow arising from the traditional uptake process. SERT-dependent currents can also be detected in neurons, in which charge flux is developmentally regulated and dependent on the interactions of SERT with SYN (Quick 2003). Remarkably, the coupling stoichiometry predicted from 5-HT uptake studies ($1 \text{ Na}^+ / 1 \text{ Cl}^- / 1 \text{ 5-HT}^+$ inward and 1 K^+ moving on the reverse cycle) predicts no net currents coupled to 5-HT uptake, indicating that charge movement must be uncoupled from flux. PKC activation with phorbol esters dissociates SYN from GAT, SERT, NET, and DAT, and when unbound, NET can generate channel activity (Sung et al. 2003). Thus, non-stoichiometric charge movement may reflect transporter regulation, enabling transporters to influence neuronal excitability.

Structure of Plasma Membrane Neurotransmitter Transporters

The cloning of GABA, glycine, NE, DA, and 5-HT transporters showed that these molecules belong to a larger family of Na^+ and Cl^- coupled transporters (*SLC6* family) with predicted 12 transmembrane domains (TMDs) and cytosolic amino and carboxyl termini. Shortly after,

cloning and sequence analysis of multiple EAAT isoforms showed that these transporters lie within a distinct gene family (*SLC1*) (Amara 1992) with 8–10 TMDs. Sedimentation and immunoprecipitation studies have supported a homomultimeric structure for both families, but each monomer appears to have the capacity for transport (Horiuchi et al. 2001; Hahn et al. 2003; Torres et al. 2003a; Yernool et al. 2003).

Transporter structure-function studies took a major leap forward with the elucidation of high resolution X-ray crystal structures for bacterial members of the *SLC1* and *SLC6* families. The first structure of an *SLC1* family member, a glutamate transporter from *Pyrococcus horikoshii*, (GltP(Ph)) revealed a “bowl-shaped” trimer with a large solvent vestibule and three distinct substrate binding sites, one per monomer (Yernool et al. 2004). The authors suggested that helical hairpins cradling the substrate binding sites serve as gates that control glutamate access to the cytosol (Fig. 2). The structure of the first *SLC6* representative, a leucine transporter from *Aquifex aeolicus* (LeuT), revealed a monomeric structure of 12 TMDs with two groups of helices related to each other via a pseudo two-fold axis of symmetry and a set of helices with inverted topology (TMs 1–5 and 6–10) (Fig. 3) (Yamashita et al. 2005). However, LeuT was crystalized with a single putative substrate bound at a site organized in the plane of the membrane by 4 TMDs (1, 3, 5, and 8), in a closed conformation. A potential dimer interface between LeuT monomers was also identified involving TMDs 10 and 11. Since the solution of GltP(Ph) and LeuT crystal structures, additional structures of other bacterial homologs and related transporters have been obtained in different conformations and with different ligands bound, providing essential information about substrate recognition, ionic coupling (e.g., by Cl^-) (Zomot et al. 2007) and conformational changes during the transport cycle, as well as opportunities for homology modeling of the vertebrate neurotransmitter transporters (Andersen et al. 2009; Krishnamurthy et al. 2009; Sinning et al. 2010). Recent single molecule studies have also helped to characterize the movement of internal and external gates predicted by the structures (Zhao et al. 2010).

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Figure 2. Crystal structure of Gltph (PDB ID2NWX), a prokaryotic member of the SLC1 family of neurotransmitter transporters. Ribbon diagram of the Gltph trimer viewed in the membrane plane (*upper panel*) and from the extracellular surface (*lower panel*), with the protomers colored cyan, magenta, and green. L-aspartate is shown as a stick model with carbon, nitrogen, and oxygen atoms colored yellow, blue, and red, respectively. The two sodium ions identified in the structure are depicted as blue spheres. The substrates are bound at an occluded site located halfway across the membrane bilayer, near HP2, TM8, and the unwound region of TM7. (Figure courtesy of Dr. Satinder K. Singh, Department of Cellular and Molecular Physiology, Yale University School of Medicine.)

Regulation of Neurotransmitter Transporters: Transcription

Regulation of neurotransmitter transporters by transcription and splicing remains much less well understood than posttranscriptional mechanisms. The most highly studied transcriptional

regulatory sequence for a neurotransmitter transporter is a repeat in the SERT promoter, termed the 5HTTLPR (Lesch et al. 1996). Fewer repeats, in the so-called short (s) forms of the promoter, appear to reduce mRNA and protein expression relative to promoters with a greater number of repeats, (the long (l) promoter variant). In humans, the (s) variant has been associated with neuroticism and anxiety traits (Lesch et al. 1996), and in some studies, with aspects of mood disorders (Kim et al. 2000; Pollock et al. 2000), particularly the response to adverse events (Caspi et al. 2003) and antidepressants (Zalsman et al. 2006). Although much remains to be learned regarding the transcriptional regulation of both SLC1 and SLC6 family members, small molecule modulators of transporter gene expression can be identified even in the absence of detailed mechanistic information. For example, Rothstein and workers (2005) identified molecules related to β -lactam antibiotics that enhance expression of the EAAT2/GLT1 gene, thereby conferring neuroprotection in cultured cells and animal models.

Regulation of Neurotransmitter Transporters: Trafficking and Phosphorylation

Like all membrane proteins involved in cell signaling, neurotransmitter transporters are known to undergo both constitutive and regulated trafficking to and from the cell surface, a process influenced by multiple protein kinases (Rama-moorthy et al. 2011). In addition, neurotransmitter transporters have been found to undergo phosphorylation-dependent catalytic activation (Steiner et al. 2008).

Only a few phosphorylation sites in neurotransmitter transporters have been carefully identified and the importance of none have been established *in vivo*. PKC can phosphorylate glutamate transporter GLT1/EAAT2 (Casado et al. 1993), and activation of PKC reduces surface expression of GLT-1 (Kalandadze et al. 2002), but the relationship between phosphorylation and trafficking remains unclear. On the other hand, PKC activation results in the phosphorylation of EAAT3 at Ser465, and this event correlates with enhanced activity and plasma

Figure 3. Crystal structure of LeuT (PDB ID 2A65), a prokaryotic member of the SLC6 family of neurotransmitter transporters. Ribbon diagram of LeuT viewed in the plane of the membrane (*upper panel*) and from the extracellular surface (*lower panel*). TMs 1,3,6,8 are colored magenta, orange, green, and blue, respectively. L-leucine is shown as a stick model with carbon, nitrogen, and oxygen atoms colored yellow, blue, and red, respectively. The two sodium ions are depicted as cyan spheres. The substrates are bound at an occluded site at the center of the membrane bilayer, near TMs 3 and 8 and the unwound sections of TMs 1 and 6. (Figure courtesy of Dr. Satinder K. Singh, Department of Cellular and Molecular Physiology, Yale University School of Medicine.)

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membrane trafficking (Baik et al. 2009). In the case of the SLC6 family, PKC activation both phosphorylates and down-regulates DAT (Vaughan et al. 1997), but the phosphorylation does not appear important for trafficking, implicating the phosphorylation of other proteins in the effects on trafficking of DAT (Granas et al. 2003). By contrast, the phosphorylation of N-terminal serines by CaMKII and PKC promotes efflux, such as that triggered by AMPH (Foster et al. 2002; Fog et al. 2006). DAT also undergoes phosphorylation by MAPK in vitro and in vivo (Gorentla et al. 2009), and recent studies of D2 DA receptor modulation of DAT trafficking have been linked to ERK activation (Bolan et al. 2007).

SERT also undergoes phosphorylation by a range of kinases (Blakely et al. 1998; Ramamoorthy et al. 1998; Samuvel et al. 2005). Interestingly, phosphorylation and endocytosis of SERT by PKC can be suppressed by 5-HT (Ramamoorthy and Blakely 1999), suggesting a mechanism for the transmitter to regulate its own clearance. PKG activation also stimulates SERT trafficking and activity (Zhu et al. 2004, 2005; Ramamoorthy et al. 2007; Steiner et al. 2008).

Neurotransmitter Transporter-Associated Proteins

Previous work has identified three major classes of protein interactions made by plasma membrane neurotransmitter transporters: with SNARE proteins, in particular syntaxin 1A (SYN1A), with kinases and phosphatases, including PKC, PKG, CaMKII, and protein phosphatase 2A (PP2A); and with cell surface receptors (e.g., D2 DA receptors and integrin $\beta 3$ -containing adhesion receptors (ITGB3)).

PKC increases cell surface expression of the GABA transporter GAT1 when the transporter is expressed in *Xenopus* oocytes along with brain mRNA, suggesting a role for other proteins (Quick et al. 1997). Indeed, coexpression of the SNARE protein SYN1A reproduced the effect of brain mRNA. Subsequent work has revealed that GAT1 and SYN1A associate directly in a higher order complex, and this complex can be regulated by PKC activation (Beckman et al. 1998). In cultured hippocampal neurons, SYN1A

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inhibits GAT1 activity and clostridial toxins reverse this effect (Deken et al. 2000), consistent with a role for SYN1A interactions in regulation of GAT1 trafficking.

Interactions with SYN1A have since been described for many neurotransmitter transporters, and show three main features. First, intracellular calcium inhibits SYN1A/NET association, and SYN1A/NET interactions inhibit both NE uptake and associated currents in detached patches (Sung et al. 2003). A rise in intracellular Ca^{2+} that promotes vesicular fusion is thus predicted to cause dissociation of SYN1A from NET, increasing NE transport activity to clear transmitter released at the same time. Second, although SYN1A can suppress entry of DA/NE and inward channel states, it may also promote the outward movement of DA through both coupled and uncoupled pathways. (Binda et al. 2008). Indeed, AMPH itself stimulates DAT/SYN association, mediated by the first 33 amino acids of the DAT amino terminus and by CaMKII activation (Fog et al. 2006).

Third, presynaptic DAT/SYN interactions have now been shown to regulate DA signaling in vivo. Like its mammalian counterparts, *C. elegans* DAT (DAT-1) interacts with the SYN1A ortholog UNC-64 (Carvelli et al. 2008) and disrupting this interaction leads to excessive channel activity and tonic depolarization. DA uptake appears normal and the animals show a phenotype characteristic of excessive DA release (McDonald et al. 2007). If SYN cannot interact normally with DAT-1, the transporter may thus enter into a state that depolarizes DA terminals and produces increased DA release.

Neurotransmitter transporters can form stable associations with protein phosphatases and kinases that enable the rapid, local modulation of transporter trafficking and function. In one example, SERT associates with the catalytic subunit of Ser/Thr phosphatase PP2Ac (Bauman et al. 2000), with similar association observed for NET and DAT. Interestingly, the transport of 5-HT attenuates the phosphorylation and endocytosis of SERT triggered by phorbol esters (Ramamoorthy and Blakely 1999), and this might reflect either reduced association of PKC (or a PKC-regulated SERT kinase) or enhanced

dephosphorylation. The carboxyl terminus of DAT also harbors a PDZ recognition sequence that interacts with PICK1, a PDZ domain protein implicated in DAT trafficking (Torres et al. 2001).

Do the receptors that functionally modulate transporter signaling also physically associate with transporters? D2 DA receptors have been found to associate with DAT (Lee et al. 2007); the interaction appears to be agonist-independent and results in enhanced transporter surface expression and DA uptake. A3 adenosine receptors (A3ARs) may associate with SERT (Zhu et al. submitted). Activation of A3ARs rapidly increases endogenous SERT function (Zhu et al. 2004, 2007), through a mechanism involving PKG1 and p38 MAPK effects on SERT trafficking and catalytic activity. In contrast to D2 DA receptor interactions with DAT, A3AR agonist enhances the association of SERT with A3AR and surface expression of SERT. NOS, the enzyme that produces cGMP to activate PKG1 and SERT (Zhu et al. 2004), also appears to associate physically with SERT in vivo (Chanrion et al. 2007). Thus, multiple components of a signaling system that links adenosine receptor modulation to 5-HT clearance appear to form a complex.

Human Diseases Linked to Plasma Membrane Neurotransporter Dysfunction

The first reported human disease produced by mutation in a neurotransmitter transporter is the Ala457Pro substitution in NET, found in two sisters with orthostatic intolerance (OI), also known as POTS for postural orthostatic tachycardia syndrome) (Shannon et al. 2000). OI produces syncope on standing, and is associated with elevated plasma NE and DHPG/NE ratios. Genetically, the NET Ala457Pro mutation appears to be the major determinant of the OI phenotype in this family, and studies in vitro show that the Ala457Pro NET variant shows greatly reduced surface expression and a consequent, dramatic reduction in NE uptake (Hahn et al. 2003). Moreover, the mutant shows a dominant negative effect on wild type NET, trapping the wild type protein inside the cell.

Although the Ala457Pro mutation is rare, loss of NE clearance and/or NET has been observed in OI subjects more broadly, suggesting that other mechanisms can produce the syndrome (Jacob et al. 1997; Lambert et al. 2008). Interestingly, OI subjects also show cognitive impairments that may reflect NET activity and NE signaling in the brain (Raj et al. 2009). Supporting this possibility, a promoter variant in NET has been found to recruit a transcriptional repressor, leading to reduced NET expression, and influencing risk of ADHD (Kim et al. 2006) as well as systolic blood pressure in healthy subjects (Kohli et al. 2011). A loss-of-function variant in EAAT1—also a putative dominant negative—has been found in a patient with episodic ataxia, seizures, migraine, and alternating hemiplegia (Jen et al. 2005).

Genetic variation in SERT has been reported to contribute to obsessive-compulsive (OCD) and autism spectrum disorders (ASD). A rare coding variant of SERT (Ile425Val) (Glatt et al. 2001; Ozaki et al. 2003) is enriched in subjects with OCD, Asperger's syndrome, anorexia and major depression in two unrelated families. In vitro studies subsequently showed that the Ile425Val variant confers elevated SERT activity attributable to catalytic activation (Kilic et al. 2003; Prasad et al. 2005). Five additional SERT variants were then also identified in ASD, and all confer a gain of function (Sutcliffe et al. 2005; Prasad et al. 2009). One of the SERT variants—Gly56Ala—is hyperphosphorylated and occupies a high-affinity, hyperactive state that occludes further stimulation by PKG and P38 MAPK (Prasad et al. 2005). In contrast, the other hyperactive SERT variants identified by Sutcliffe et al. show increased cell surface expression.

Because drugs that act on DAT—methylphenidate (RitalinTM) and AMPH (AdderallTM)—can be effective for treatment of ADHD, a cohort of ADHD subjects was screened for DAT mutations, identifying the Ala559Val DAT variant (Mazei-Robison and Blakely 2005). Functional studies of Ala559Val DAT initially failed to detect changes in inward DA flux or DAT surface expression, but when the cells were loaded with DA, amperometric recordings revealed spontaneous efflux of DA that was enhanced by depolariza-

tion (Mazei-Robison et al. 2008). Remarkably, AMPH no longer caused DA efflux but, rather, antagonized the outward movement of DA. Further, the phenotype of the Ala559Val variant depended on D2 DA receptor stimulation, CaMKII activity and amino-terminal phosphorylation sites, very similar to the effects of AMPH (Bowton et al. 2010). Drugs targeting DAT thus interact differently with altered transport proteins, whether incurred by mutation or by regulation. Although this DAT variant was not observed in hundreds of controls, it was reported in a girl with bipolar disorder (Grunhage et al. 2000). The comorbidity of ADHD and bipolar disorder (Klassen et al. 2010) suggests that the variant may affect more general behavioral traits that underlie multiple DA-linked disorders.

CONCLUSION

Recent work has shown that although originally identified as the targets of psychoactive drugs, neurotransmitter transporters have a crucial role in synaptic transmission. They also undergo regulation at the level of catalytic activity and trafficking in response to physiological as well as pharmacological stimuli. As a result, neurotransmitter transporters contribute not only to the acute effect of drugs, but to the long-term adaptation that underlies normal physiology as well as drug addiction and other neuropsychiatric disease. However, we still understand relatively little about the fundamental properties of many neurotransmitter transporters, such as their associated ionic conductances. Future work thus still needs to elucidate their basic function as well as the structural basis for transport, the mechanisms of regulation, and their physiological role in neural systems and in behavior.

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