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Review

The Neurotransmitter Cycle and Quantal Size

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Changes in the response to release of a single synaptic vesicle have generally been attributed to postsynaptic modification of receptor sensitivity, but considerable evidence now demonstrates that alterations in vesicle filling also contribute to changes in quantal size. Receptors are not saturated at many synapses, and changes in the amount of transmitter per vesicle contribute to the physiological regulation of release. On the other hand, the presynaptic factors that determine quantal size remain poorly understood. Aside from regulation of the fusion pore, these mechanisms fall into two general categories: those that affect the accumulation of transmitter inside a vesicle and those that affect vesicle size. This review will summarize current understanding of the neurotransmitter cycle and indicate basic, unanswered questions about the presynaptic regulation of quantal size.

The analysis of postsynaptic responses at the neuromuscular junction originally suggested that a single vesicle filled with neurotransmitter is the elementary unit (or "quantum") of synaptic transmission (Katz, 1971). Although quantal size has generally been considered invariant, it has become clear that the response to release of a single vesicle can change as a function of stimulation and contributes to forms of plasticity such as long-term potentiation. However, this modulation of quantal size is postsynaptic, involving changes in receptor sensitivity or number.

It has been less widely appreciated that the amount of transmitter released per vesicle can also vary. The high affinity of many receptors has suggested that they may be saturated under physiological conditions, in which case changes in the amount of transmitter per vesicle would have no impact on synaptic transmission. However, considerable evidence now shows that even high-affinity receptors are not saturated at many if not most synapses. Changes in vesicle filling thus have great potential to influence synaptic transmission. The aim of this review is to summarize our current understanding of the factors that regulate vesicle filling and to identify open questions about regulation of quantal size by the neurotransmitter cycle. The first part reviews evidence that high-affinity receptors are not saturated and addresses the diverse observations that indicate presynaptic regulation of quantal size under physiological circumstances. The main part of the review discusses the mechanisms that influence vesicle filling, from factors that influence the cytosolic concentration of transmitter, such as biosynthesis and recycling, to the H^+ electrochemical gradient that drives transport, the vesicle conductances that regulate the expression of this gradient, and the neurotransmitter transporters themselves. In general, changes in quantal size may reflect changes in the lumenal concentration of transmitter or changes in vesicle size, and we consider several models to account for these possibilities.

A Single Synaptic Vesicle Does Not Saturate Postsynaptic Receptors

In many cases, neurotransmitter can activate receptors at a considerable distance from the release site. Spillover from "private" synapses or volume transmission by "social" neuromodulators such as monoamines, acetylcholine, and neural peptides is particularly susceptible to regulation by presynaptic changes in quantal size because the receptors involved are almost by definition not saturated under physiological conditions. The activation of G protein-coupled metabotropic receptors by essentially any classical transmitter, including glutamate and GABA as well as monoamine, falls into this category. Changes in the amount of transmitter released per vesicle will influence both the extent and potentially the duration of signaling by these receptors. However, it has become increasingly clear that presynaptic changes in quantal size also influence the activation of ionotropic receptors immediately postsynaptic to a release site.

The large amount of transmitter in a synaptic vesicle might be expected to saturate all the ionotropic receptors at a synapse, reducing if not eliminating the effect of changes in vesicle filling. Receptor saturation should reduce the variation in quantal size measured at an individual synapse. However, the substantial variation in quantal size observed even at the neuromuscular junction suggests that postsynaptic receptors are not saturated by the contents of a single synaptic vesicle (Fatt and Katz, 1952; Van der Kloot, 2003). In the central nervous system, it has been more difficult to assess variation in quantal size because synapses reside at different distances from the recording electrode, and electrotonic filtering should produce inherent variation in quantal size, even if the postsynaptic response to a single quantum were identical at all synapses. In addition, synapses vary in release probability and the number of postsynaptic receptors (Murthy et al., 1997; Nusser et al., 1997). To eliminate these sources of variability, investigators have used hypertonic media to stimulate directly only one or a small number of

presynaptic boutons, or loose patch recording to measure a local dendritic response. Under these conditions, the fluctuation in quantal size appears just as large as with electrical stimulation that activates many boutons (Bekkers et al., 1990a; Liu and Tsien, 1995; Liu et al., 1999a; Forti et al., 1997), indicating that the variation is intrinsic to an individual synapse. The imaging of local calcium transients in dendrites further supports the large variability in response at a single synapse (Mainen et al., 1999). The intrinsic variability in quantal size in turn suggests that receptors are not always saturated by a single quantum of glutamate.

More recently, the ability to patch directly onto the presynaptic calyx of Held in the auditory pathway has shown that increasing the concentration of glutamate in the pipette can increase quantal size measured postsynaptically, directly excluding receptor saturation at that synapse (Ishikawa et al., 2002). Although it might have been expected that a quantum of glutamate would not saturate low-affinity AMPA receptors, synaptically released glutamate also fails to saturate high-affinity NMDA receptors (Mainen et al., 1999; McAllister and Stevens, 2000), possibly because although high, the peak concentration of synaptic glutamate is very short lived and hence activates only a fraction of receptors. AMPA and NMDA responses are highly correlated at a given synapse (McAllister and Stevens, 2000), indicating a presynaptic source for the variation. At inhibitory synapses, $GABA_A$ receptors also do not appear to be saturated by a quantum of transmitter (Frerking and Wilson, 1996; Barberis et al., 2004). The drug zolpidem increases the affinity of $GABA_A$ receptors and does not influence quantal size at inhibitory synapses onto pyramidal cells in layer 2/3 of the cortex, indicating receptor saturation, but does increase the response at many other synapses in hippocampus and cortex (Hajos et al., 2000). Distinct inhibitory synapses onto the same neuron may even differ in the extent of saturation. Changes in the amount of transmitter released per vesicle thus have great potential to influence the ionotropic response at central synapses as well as at the neuromuscular junction.

Presynaptic Regulation of Quantal Size by Activity

If changes in the amount of transmitter released per vesicle can influence the postsynaptic response, is this regulated under physiological conditions? At the neuromuscular junction, prolonged high-frequency stimulation reduces quantal size by approximately 20% (Doherty et al., 1984; Naves and Van der Kloot, 2001). This decline and the recovery that ensues do not follow a time course expected for desensitization and resensitization of acetylcholine receptors, suggesting a presynaptic mechanism. Consistent with this, impairment of release delays the onset of decline in quantal size, as if there were a pool of synaptic vesicles filled with more transmitter that release first but are eventually exhausted. Further, activation of cAMP-dependent protein kinase (PKA) by hypertonic media can increase quantal size presynaptically by up to 4-fold at the neuromuscular junction (Van der Kloot, 1987a), and the stimulation of either protein kinase C or nicotinic receptors blocks this increase (Van der Kloot, 1993). The release of the calcitonin gene-related peptide (CGRP) by motor neurons may influence this regulation under physiological conditions (Van der Kloot et al., 1998). Blockade of neural activity for 1 week at the rat neuromuscular junction substantially increases quantal size, apparently through a presynaptic mechanism (Wang et al., 2005). The lack of correlation with activity in muscle further suggests that this mechanism does not involve retrograde signaling from the postsynaptic cell.

Growing evidence indicates the presynaptic regulation of quantal size under physiological conditions. In *Drosophila*, quantal size correlates with the rate of foraging. Immediately after shift from a plate with food to one without, the flies begin to crawl faster, and those that belong to the fastest subgroup show an increase in quantal size and evoked release that begins 35 min after the shift and appears to have a presynaptic origin (Steinert et al., 2006). In synaptosomal preparations from the rat brain, depolarization increases the vesicular stores of glutamate (Bole et al., 2002), and stimulation of chromaffin cells increases quantal size (Pothos et al., 2002). Further, suppression of activity acts presynaptically to reduce the amplitude of miniature inhibitory events in both developing and mature hippocampal neurons, perhaps as part of a homeostatic mechanism to regulate brain excitability (Hartman et al., 2006).

The Neurotransmitter Cycle

The amount of transmitter released per vesicle can be regulated either before or after fusion. Regulation *after* vesicle fusion involves closure of the fusion pore before the full release of vesicle contents. Although demonstrated quite convincingly for large dense-core vesicles by a variety of methods, this "kiss-and-run" mechanism remains controversial for synaptic vesicles and has been reviewed elsewhere (Fernandez-Peruchena et al., 2005; Harata et al., 2006). Interestingly, the extremely rapid release of transmitter through a flickering fusion pore would place extraordinary demands on vesicle refilling (Staal et al., 2004). In this review, we focus on the regulation of quantal size *before* fusion with the plasma membrane, due directly to changes in vesicle filling.

The vesicular storage of transmitter poses several problems inherent in the mechanism of exocytotic release. First, the activation of postsynaptic receptors generally depends on the release of large amounts of lumenal transmitter, but the high rates of firing by many neurons would then deplete the terminal of vesicular stores. To solve this problem, most transmitters undergo recycling, either directly back into the nerve terminal or indirectly into glia. Second, large amounts of lumenal transmitter will require significant time for refilling, particularly if the transport mechanism is slow, and this may also limit release. High rates of transport and a high cytoplasmic concentration of transmitter will tend to circumvent this problem, and most vesicular transport activities (for acetylcholine,

GABA, and glutamate) exhibit a low apparent affinity for transmitter, consistent with high cytoplasmic concentrations. However, transmitters with intrinsic toxicity, such as dopamine, may require low cytoplasmic concentrations. In this case, the transport mechanism needs to generate a steep gradient of transmitter from cytoplasm to vesicle lumen, and the limited supply of cytosolic substrate will further slow the rate of filling. Along with transport mechanism, transmitter recycling and the cytosolic concentration of transmitter thus have very important roles in vesicle filling. The concentration of transmitter inside synaptic vesicles depends on the concentration of substrate available in the cytoplasm, the transport mechanism, and nonspecific leakage across the vesicle membrane. We will thus first address factors that regulate the cytosolic concentration of transmitter, starting with those involved in recycling.

Plasma Membrane Transporters

After exocytotic release, most classical transmitters are cleared from the synapse by transport across the plasma membrane (Figure 1). In the case of essentially all transmitters other than glutamate, reuptake occurs back into the nerve terminal and is mediated by a family of transporters dependent on cotransport of both Na^+ and Cl^- (Table 1). The subject of considerable interest for their role in controlling synaptic transmitter, extrasynaptic spillover, and the response to antidepressants and addictive psychostimulants (Mortensen and Amara, 2003; Blakely et al., 2005), these transporters also have a crucial role in transmitter recycling. Loss of the dopamine transporter in knockout mice increases extracellular dopamine in the striatum by ~5-fold, but the animals also show a 95% reduction in whole-brain dopamine (Giros et al., 1996; Jones et al., 1998). Because the vast majority of dopamine resides in vesicular stores, the analysis shows that de novo synthesis via tyrosine hydroxylase cannot keep up with transmitter release. Indeed, striatal slices from wild-type mice will continue to release dopamine in response to stimulation for several hours, but slices from dopamine transporter knockouts fail to release after only a few stimuli. The vesicular stores of dopamine thus rely heavily on reuptake through the dopamine transporter, at least in the striatum, which contains a high density of dopaminergic terminals.

Recent work on the neuronal glycine transporter GlyT2 further supports a role for plasma membrane reuptake in transmitter recycling (Table 1). The two related glycine transporters GlyT1 and GlyT2 differ in the stoichiometry of coupling to Na^+, with GlyT2 predicted to generate particularly steep concentration gradients (Roux and Supplisson, 2000). GlyT2 is also expressed by neurons, and GlyT1 by glia (Zafra et al., 1995). The knockout of GlyT1 produces a phenotype of excessive inhibition that involves respiratory muscles and causes neonatal mortality, suggesting that this transporter has a primary role in glycine clearance from the synapse (Gomeza et al., 2003a) (Table 1). In contrast, inactivation of GlyT2 produces a startle

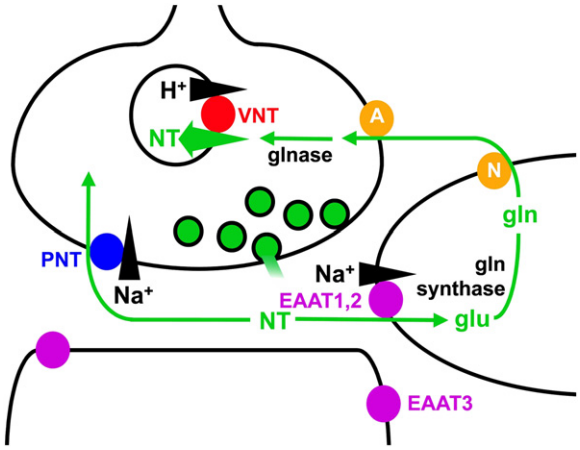

Figure 1. The Neurotransmitter Cycle
Most classical transmitters other than glutamate are recaptured by Na^+-/Cl^--dependent plasma membrane neurotransmitter transporters (PNTs) located at the nerve terminal. However, glutamate is taken up by excitatory amino acid transporters (EAAT1, -2) expressed on astrocytes, converted to glutamine (gln) by the glial enzyme gln synthase, and transferred back to neurons by the sequential action of glial system N transporters SN1 and -2, and neuronal system A (SA) transporters before conversion back to glutamate by glutaminase and packaging into synaptic vesicles by the vesicular glutamate transporters (VGLUTs). EAAT3 is expressed postsynaptically, by neurons.

phenotype similar to that caused by mutations in glycine receptors (Gomeza et al., 2003b). Indeed, the loss of GlyT2 reduces quantal size at inhibitory glycinergic synapses, indicating an important role for this transporter in glycine recycling.

In the case of ACh, hydrolysis by acetylcholinesterase terminates signaling, and the product choline is then recycled into the nerve terminal. The choline transporter does not belong to the same family of proteins as the dopamine and glycine transporters (Okuda et al., 2000), but does depend on Na^+ and appears to have an equally important role in recycling transmitter (Table 1). Inactivation of the choline transporter results in death within 1 hr after birth, due to respiratory failure (Ferguson et al., 2004). Interestingly, the choline transporter as well as the glycine transporters undergo activity-dependent trafficking at the nerve terminal (Aragon and Lopez-Corcuera, 2003). The choline transporter in particular resides on a subset of synaptic vesicles and thus undergoes exocytosis at precisely the time when choline uptake is needed (Ferguson et al., 2003). Because many plasma membrane transporters serve to recycle transmitter, it is surprising that more do not exhibit a similar form of regulation. A number show regulated cell-surface trafficking, but few reside on synaptic vesicles (Blakely et al., 2005). Perhaps constitutive cell-surface expression has a distinctive role in clearance, or constitutive uptake of choline is detrimental.

Glutamine-Glutamate Cycle

The mechanism by which glutamate recycles remains less clear. After synaptic release, glutamate is taken up

Table 1. Expression, Ionic Coupling, and Synaptic Phenotype Produced by the Loss of Plasma Membrane Neurotransmitter Transporters

PNT	Family	Location	Ionic Coupling	Loss of Function Synaptic Phenotype
NET	SLC6	NE cells intracellular	$1Na^+$:$1Cl^-$:NE^+ [1] cotransport	↑ extracellular NE, ↓ intracellular stores [2]
DAT	SLC6	DA cells cell surface	?$2Na^+$:$1Cl^-$:DA^+ [3] cotransport	↑ extracellular DA [4] ↓ intracellular stores [5]
SERT	SLC6	5HT cells cell surface	$1Na^+$:$1Cl^-$:$5HT^+$ ↔ $1K^+$ [6] electroneutral [7]	↑ extracellular 5HT, ↓ intracellular stores [8]
GAT-1	SLC6	GABA neurons	$2Na^+$:$1Cl^-$:GABA [9] cotransport	↑ tonic signaling, ↓ phasic transmission [10]
GAT-2	SLC6	neurons glia		
GAT-3	SLC6	glia		
GAT-4	SLC6	neurons glia		
GLYT1	SLC6	glia	$2Na^+$:$1Cl^-$:gly [11]	↑ synaptic glycine [12]
GLYT2	SLC6	gly neurons	$3Na^+$:$1Cl^-$:gly [11]	↓ glycine release [13]
GLAST (EAAT1)	SLC1	glia	$3Na^+$:$1H^+$:glu^- ↔ $1K^+$ [14] chloride conductance [16]	↑ spillover [15]
GLT1 (EAAT2)	SLC1	glia	$3Na^+$:$1H^+$:glu^- ↔ $1K^+$ [17]	↑ extracellular glu, degeneration [18]
EAAC1 (EAAT3)	SLC1	neurons dendritic	$3Na^+$:$1H^+$:glu^- ↔ $1K^+$ [19] chloride conductance [16]	↑ spillover [20] degeneration (↓cys/GSH)[21]
EAAT4	SLC1	neurons cerebellum	chloride conductance [22]	↑ spillover [23]
EAAT5	SLC1	retinal presynaptic	chloride conductance [24]	
CHT	SLC5	cholinergic presynaptic	Na^+-dependent	minimal ACh release[25]

NET, norepinephrine (NE) transporter; DAT, dopamine (DA) transporter; GAT, GABA transporter; GlyT, glycine (gly) transporter; EAAT, excitatory amino acid transporter; CHT, choline transporter.
[1](Gu et al., 1996), [2](Xu et al., 2000), [3](Sonders et al., 1997), [4](Giros et al., 1996), [5](Jones et al., 1998), [6](Rudnick and Nelson, 1978), [7](Quick, 2003), [8](Bengel et al., 1998), [9](Lu and Hilgemann, 1999), [10](Jensen et al., 2003), [11](Roux and Supplisson, 2000), [12](Gomeza et al., 2003a), [13](Gomeza et al., 2003b), [14](Owe et al., 2006), [15](Tong and Jahr, 1994; Huang et al., 2004b; Glowatzki et al., 2006), [16](Wadiche et al., 1995), [17](Levy et al., 1998), [18](Rothstein et al., 1996; Tanaka et al., 1997), [19](Zerangue and Kavanaugh, 1995), [20](Otis et al., 1997; Brasnjo and Otis, 2001; Huang et al., 2004b), [21](Aoyama et al., 2006), [22](Fairman et al., 1995), [23](Huang et al., 2004a; Takayasu et al., 2005), [24](Eliasof and Jahr, 1996; Arriza et al., 1997), [25](Ferguson et al., 2004).

primarily by the Na^+-dependent excitatory amino acid transporters EAAT1 and -2 (Rothstein et al., 1996; Tanaka et al., 1997; Huang et al., 2004b; Takayasu et al., 2005) (Figure 1 and Table 1). EAAT1 and -2 are expressed almost exclusively by astrocytes (Rothstein et al., 1994; Chaudhry et al., 1995), precluding direct reuptake of glutamate into the nerve terminal. Recent work has demonstrated expression of an alternatively spliced EAAT2 isoform at nerve terminals in the hippocampus (Chen et al., 2004), but its physiological role there remains uncertain.

Neurochemical studies have shown that most of the glutamate released as transmitter derives from the precursor glutamine (Hamberger et al., 1979b). Where does this glutamine come from? After release from neurons, the glutamate taken up by astrocytes is converted into glutamine by glutamine synthetase (Rothstein and Tabakoff, 1984; Pow and Robinson, 1994; Pow and Crook, 1996; Winkler et al., 1999) (Figure 1). Glutamine is then transferred back to neurons, apparently through the sequential action of amino acid system N and A transporters.

System N and A transporters were originally described as Na^+-dependent glutamine transport activities in the liver (Kilberg et al., 1980; Weissbach et al., 1982). Despite its Na^+ dependence, the system N transporter SN1 mediates efflux under physiological conditions (Chaudhry et al., 1999), and its expression by astrocytes suggests a role in the efflux of glutamine from glia required for the glutamine-glutamate cycle (Figure 1). The system A transporters are closely related to system N but appear to mediate the unidirectional uptake of glutamine by neurons. Blockade of system A transporters with the high-affinity substrate methyl-aminoisobutyric acid has been reported to reduce quantal size at excitatory synapses formed in culture (Armano et al., 2002), but the data about localization have

been conflicting, and both SAT1 and -2 may be expressed in dendrites rather than at the nerve terminal (Armano et al., 2002; Mackenzie et al., 2003). Interfering with glutamine production and transfer can apparently reduce epileptiform discharges (Bacci et al., 2002; Tani et al., 2007), but it has been difficult to demonstrate a role for glutamine uptake in sustained glutamate release (Kam and Nicoll, 2007).

How can closely related transporters mediate glutamine efflux from astrocytes but glutamine uptake by neurons? SN1 mediates flux reversal under physiological conditions because although a Na^+-cotransporter, it is also coupled to H^+ exchange, and the resulting electroneutrality eliminates membrane potential as a driving force, leaving only the concentration gradient of Na^+ to produce a shallow gradient of glutamine that allows bidirectional glutamine flux around thermodynamic equilibrium (Chaudhry et al., 2001; Broer et al., 2002). SN2 also couples to H^+ exchange (Nakanishi et al., 2001) and appears to have an overlapping pattern of expression in the mammalian brain (R.H.E., unpublished data). In contrast to SN1 and -2, the system A transporters are not coupled to the translocation of H^+ and are therefore electrogenic as well as Na^+ dependent (Sugawara et al., 2000a, 2000b; Yao et al., 2000; Reimer et al., 2000), producing steeper concentration gradients of neutral amino acids, including glutamine. The system A transporters are also expressed by neurons, but SAT1/SNAT1 appears to be expressed primarily by inhibitory neurons (Chaudhry et al., 2002a) and SAT2/SNAT2 by excitatory neurons (Varoqui et al., 2000) (F.A. Chaudhry, personal communication). SAT1 was originally reported to be more selective for glutamine, but both isoforms appear to recognize many neutral amino acids (Mackenzie et al., 2003).

After transfer from glia, glutamine undergoes conversion by the neuronal enzyme glutaminase (also known as the phosphate-activated or kidney glutaminase [PAG]) back to glutamate prior to exocytotic release (Conti and Minelli, 1994; Laake et al., 1995) (Figure 1). Another isoform originally identified in the liver may also contribute (Curthoys and Watford, 1995; Kvamme et al., 2001). Indeed, a recent knockout of the kidney glutaminase showed remarkably little impairment of excitatory transmission (Masson et al., 2006). The animals die shortly after birth, but the metabolic function of this glutaminase isoform in nitrogen metabolism or pH balance by the kidney may contribute to lethality, and baseline excitatory transmission appears normal. The animals do show a moderate defect in the response to prolonged high-frequency stimulation and a complete loss of glutaminase activity in brain extracts, although the conditions of the enzyme assay may have precluded detection of the liver isoform. The full role of glutaminase and indeed the glutamine-glutamate cycle in excitatory transmission thus remains uncertain.

In contrast, the glutamine-glutamate cycle has a clear role at particular inhibitory synapses. In the hippocampus, inhibition of the EAATs reduces quantal size at inhibitory synapses (Mathews and Diamond, 2003), presumably by blocking uptake of the glutamate ultimately converted to GABA by glutamic acid decarboxylase. Interestingly, the effect of EAAT inhibition on inhibitory transmission does not require exocytosis and vesicle refilling, suggesting that it operates on a pool of vesicles that are already available. More recently, it has been shown that inhibition of glutamine production in astrocytes and transfer to neurons reduces the size of both evoked and miniature inhibitory currents in the hippocampus, supporting a role for the glutamine-glutamate cycle (Liang et al., 2006). In this case, however, moderate synaptic activity was required to trigger the reduction. It is also possible that the glutamine-glutamate cycle is particularly important at inhibitory synapses that lack a presynaptic GABA transporter for direct recycling.

Why do glutamate and GABA retrieval involve a mechanism as apparently complex as the glutamine-glutamate cycle? The cycle may have evolved from very similar pathways in the liver and kidney, where the components also undergo metabolic regulation. In the liver, periportal hepatocytes express glutaminase and target the ammonia derived from glutamine to the urea cycle. Perivenous cells then conjugate any excess ammonia not captured by periportal cells back to glutamine through the action of glutamine synthetase. Periportal cells thus resemble neurons and perivenous cells astrocytes (Chaudhry et al., 2002b). In the kidney, metabolic acidosis upregulates both glutaminase and SN1, helping to buffer and eliminate the acid load (Gstraunthaler et al., 2000; Solbu et al., 2005). The observations in liver and kidney raise the possibility of parallel mechanisms governing the glutamine-glutamate cycle in the nervous sytem. The encephalopathy produced by liver failure may involve a disturbance of this cycle in the brain.

Biosynthesis and Metabolism

Similar to any enzymatic reaction, transport into vesicles concentrates lumenal transmitter (the product) to an extent dependent directly on the cytosolic concentration (substrate). In addition to the mechanisms involved in recycling, biosynthetic enzymes thus influence the cytosolic concentration of transmitter and hence quantal size. In the case of glutamatergic transmission, kidney and liver glutaminase may be redundant, accounting for the relatively mild effect of losing the kidney isoform (Masson et al., 2006). However, both isoforms appear to undergo regulation by inorganic phosphate (Curthoys and Watford, 1995), with phosphate converting the inactive monomeric form of the enzyme to the active tetramer. Because the Km for phosphate exceeds cytosolic concentrations of phosphate, changes in phosphate levels have the potential to influence glutamate production, but the physiological role of this mechanism in transmitter release remains unknown.

At inhibitory synapses, the role of the biosynthetic enzyme glutamic acid decarboxylase (GAD) in regulation of quantal size has been controversial. GAD upregulates in response to seizures (Feldblum et al., 1990; Esclapez and Houser, 1999) and downregulates with deafferentation

(Gierdalski et al., 1999). In addition, pharmacologic inhibition of GAD has been reported to reduce quantal size in mammals (Murphy et al., 1998; Engel et al., 2001), but to impair only the probability of release in crustaceans (Golan and Grossman, 1996). Consistent with the latter result, the knockout of GAD65 has no clear effect on quantal size, but rather influences the probability of release, particularly after prolonged stimulation (Tian et al., 1999). This effect suggests a role for vesicle filling or at least GAD65 itself in the regulation of exocytosis. Alternatively, loss of GAD may result in the fusion of "empty" synaptic vesicles not detectable by postsynaptic recording. Enzymes that metabolize GABA might also be expected to influence quantal size at inhibitory synapses. Although predicted to have robust effects on GABA stores (Axmacher et al., 2004), inhibition of the metabolic enzyme GABA transaminase with the anticonvulsant vigabatrin has had conflicting effects on inhibitory transmission and quantal size (Engel et al., 2001; Overstreet and Westbrook, 2001).

Tyrosine hydroxylase, the rate-limiting step in catecholoamine biosynthesis, has received considerable attention for its feedback inhibition by L-Dopa and dopamine and for its regulation by phosphorylation (Zigmond et al., 1989; Fitzpatrick, 2000). Activating the enzyme increases dopamine content and release from a variety of cells and has been used to increase dopamine production by transplants for Parkinson's disease (Chang et al., 2002). Using direct, electrochemical measurements of release made possible by the oxidation of dopamine by a carbon fiber electrode held at characteristic potentials (Wightman et al., 1991; Albillos et al., 1997; Mosharov and Sulzer, 2005), addition of L-Dopa, the product of tyrosine hydroxylase, increases quantal size in adrenal chromaffin cells, PC12 cells, and dopamine neurons, whereas tyrosine hydroxylase inhibition mediated by autoreceptors decreases quantal size (Colliver et al., 2000; Pothos et al., 1996, 1998a, 1998b).

Electrochemistry has also begun to provide the first direct measurements of cytosolic monoamine concentration. In comparison to vesicular stores, the amount of cytosolic transmitter is very small and hence may not correlate well with total cellular contents or quantal release, particularly if transport into the vesicle is regulated. Although the cytosolic concentration of most classical transmitters is not known, immunocytochemistry for glutamate has suggested concentrations in the 1–10 mM range (Storm-Mathisen and Ottersen, 1990), consistent with the results obtained with dialyzing glutamate into the nerve terminal (Ishikawa et al., 2002). However, the insertion of a carbon fiber electrode into a patch pipette enables direct access to the cytosol, and either measurement of dopamine levels by cyclic voltammetry or measurement of total catechol by amperometry (Mosharov et al., 2003). In adrenal chromaffin cells, cytosolic dopamine concentrations range from 5 to 50 μM. Although it has always been presumed that inhibition of vesicular monoamine transport would increase cytosolic catecholamine levels, direct measurement by intracellular patch electrochemistry shows no change in cytosolic dopamine after inhibition of the vesicular monoamine transporter with reserpine, although total catechol levels do rise presumably as a result of metabolism (Mosharov et al., 2003). Indeed, simultaneous inhibition of both the metabolic enzyme monoamine oxidase and the vesicular monoamine transporter increases cytosolic dopamine, as does L-Dopa alone. Remarkably, the cytosolic concentration of dopamine in dopamine neurons is undetectable (<0.1 μM) unless augmented by exogenous L-Dopa (E. Mosharov and D. Sulzer, personal communication). The cytosolic concentration of at least monoamine transmitters thus undergoes extremely tight regulation.

Vesicular Neurotransmitter Transport

Because essentially all classical transmitters are synthesized in the cytoplasm or appear there after reuptake, exocytotic release requires transport into secretory vesicles, and native vesicle preparations exhibit multiple, distinct transport activities. Transport into the vesicle generally involves the exchange of lumenal H^+ for cytoplasmic transmitter and hence depends on a H^+ electrochemical gradient, which is produced by the vacuolar (yeast lysosome) H^+-ATPase. In addition to the cytosolic concentration of substrate, factors that influence vesicle filling thus include the activity of the transporter, the magnitude of the driving force, and the extent of nonspecific leakage across the vesicle membrane. However, the extremely rapid recycling of synaptic vesicles along with their small size imposes a number of other, surprising constraints on the concomitant recycling of neurotransmitter. First, unlike plasma membrane channels and transporters, which rely on large, stable gradients of ions such as Na^+, K^+, and Cl^-, the filling of synaptic vesicles depends on a H^+ electrochemical gradient that is both dissipated and regenerated with each cycle of exo- and endocytosis. Further, a lumenal pH of 6 (only 1 μM) corresponds to less than one free H^+ per vesicle, although many lumenal H^+ are presumably buffered by vesicle proteins, ATP, or transmitter itself. At the same time, internalizing synaptic vesicles contain millimolar concentrations of Na^+ and Cl^-, which presumably leave the vesicle as H^+ enter and transmitter accumulates. Although we still understand little about how these events are coordinated, the machinery involved in vesicle filling must operate within their constraints.

H^+ Pump

The driving force for neurotransmitter transport into secretory vesicles is provided by the vacuolar H^+-ATPase (Figure 2). Similar in overall structure and function to the F0/F1-ATPase from mitochondria and bacteria, the vacuolar H^+ pump mediates H^+ flux in the opposite direction under physiological conditions (Inoue et al., 2005). Rather than using the energy stored in a H^+ electrochemical gradient to produce ATP, the vacuolar pump uses the energy released by ATP hydrolysis to make a H^+ electrochemical gradient, and this activity is responsible for acidification of endosomes and lysosomes as well as

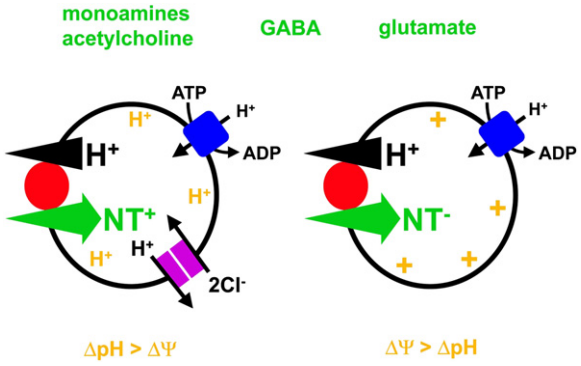

Figure 2. Vesicular Neurotransmitter Transporters Vary in Their Dependence on the Chemical and Electrical Components of the H^+ Electrochemical Gradient

The vacuolar-type H^+-ATPase (named after the yeast vacuole or lysosome, blue) produces the H^+ electrochemical gradient that drives the transport of all classical neurotransmitters into secretory vesicles. In all cases, the movement of H^+ down their electrochemical gradient is coupled to the transport of transmitter in the opposite direction. Despite this common H^+ exchange mechanism, different vesicular neurotransmitter transporters (red) rely to differing extents on the two components of the H^+ electrochemical gradient, the chemical gradient ΔpH and the electrical gradient ΔΨ. Vesicular monoamine and ACh transport, which involves the exchange of protonated cytosolic transmitter for two lumenal H^+, involves more H^+ than charge movement and hence depends more on ΔpH than ΔΨ. Although the precise stoichiometry of ionic coupling by vesicular glutamate transport remains unknown, the transport of anionic glutamate involves the movement of n H^+ and n + 1 charge. The VGLUTs thus depend more on ΔΨ than ΔpH. Transport of the zwitterion GABA with no net charge involves the movement of equal number of H^+ and charge, making VGAT equally dependent on both ΔpH and ΔΨ. These differences suggest that vesicles storing monoamines might exhibit a larger ΔpH than ΔΨ, and those storing glutamate a larger ΔΨ than ΔpH. Whether vesicles differ in the extent to which they express the two components of the H^+ electrochemical gradient remains unknown, however intracellular chloride carriers such as the synaptic vesicle ClC-3 promote vesicle acidification by dissipating the charge developed by the vacuolar H^+ pump, secondarily activating the H^+ pump to make a larger ΔpH. Recent work has also suggested that the intracellular ClCs function as exchangers of two Cl^- for one H^+ (Picollo and Pusch, 2005; Scheel et al., 2005).

dense-core vesicles and synaptic vesicles. The pump is composed of two domains, a peripheral V1 domain that catalyzes ATP hydrolysis and an integral membrane V0 domain that translocates H^+. Each domain contains multiple subunits, and crystallographic studies are beginning to characterize the structure (Drory and Nelson, 2006). Similar to the F-ATPases, the v-ATPase uses a rotary mechanism, with V1 rotating with respect to V0 during H^+ translocation (Nakanishi-Matsui and Futai, 2006). Multiple observations in yeast also indicate the potential for regulation of vacuolar H^+ pump activity. However, the role for direct regulation of the H^+ pump in mammals has not been explored.

Remarkably, biochemical studies in yeast and genetic studies in *Drosophila* have suggested that the vacuolar H^+ pump and in particular the V0 domain has a role in membrane fusion, independent of its well-established role in vesicle acidification (Peters et al., 2001; Hiesinger et al., 2005). A mutation in the V0 a1 subunit inhibits synaptic vesicle exocytosis without affecting acidification. Although the mechanism for this apparent effect remains unknown, it is remarkable that another subunit of V0 was previously suggested to mediate the nonvesicular release of transmitter (Dunant and Israel, 2000), and the same subunit has been implicated in the homotypic fusion of vacuoles in yeast (Peters et al., 2001).

pH Gradient versus Membrane Potential and the Role of Chloride

In contrast to the paucity of information about regulation of intrinsic vacuolar H^+ pump activity, considerable evidence indicates an important role for other ions in secondary activation of the pump. Isolated synaptic vesicles (and other acidic intracellular membranes including endosomes and lysosomes) show minimal acidification with addition of only ATP. However, the subsequent addition of chloride results in dramatic acidification, presumably because the entry of chloride dissipates any developing membrane potential (ΔΨ) and so enables the H^+ pump to generate a larger ΔpH (Figure 2). The electrical (ΔΨ) and chemical (ΔpH) components of the H^+ electrochemical gradient made by the vacuolar pump can thus be regulated independently (Johnson, 1988).

Previous work has identified a family of intracellular chloride channels responsible for vesicle acidification (Jentsch et al., 2005) (Figure 2). The analysis of knockout mice has confirmed the importance of these channels in the function of the endocytic pathway in kidney and bone (Piwon et al., 2000; Kornak et al., 2001). However, the knockout of ClC-3, one of the main isoforms expressed in brain, shows an early, severe degenerative phenotype affecting primarily the hippocampus and retina (Stobrawa et al., 2001). Synaptic vesicles from the ClC-3 knockout show impaired acidification, but the effect on transmitter release and in particular quantal size was difficult to assess, presumably because the synapses expressing ClC-3 degenerate, and the remaining synapses express another isoform and hence show no impairment.

Interestingly, recent work has suggested that the intracellular ClCs may function as Cl^-/H^+ exchangers rather than Cl^- channels (Figure 2). This possibility was originally suggested by the analysis of a bacterial protein conferring Cl^- flux. Reconstitution of the purified protein into artificial membranes showed a current-voltage relationship that did not fit the Nernst equation for chloride, but was consistent instead with a transport mechanism involving the exchange of one H^+ for two Cl^- (Accardi and Miller, 2004). Following up on this observation, it was subsequently found that two intracellular ClCs in mammals, ClC-4 and ClC-5, similarly exhibit Cl^-/H^+ exchange activity when expressed at the plasma membrane (Picollo and Pusch, 2005; Scheel et al., 2005). Although ClC-3, a major isoform on synaptic vesicles, has not itself been shown to mediate Cl^-/H^+ exchange, the strong evidence for closely related proteins suggests that ClC-3 behaves similarly. How then can Cl^- entry promote vesicle acidification through a H^+ exchange mechanism? The answer lies in the

stoichiometry of ionic coupling by these carriers, which involves the exchange of two Cl^- for each H^+ and hence the movement of much more charge (3+) than H^+ (1). This stoichiometry in turn predicts greater dissipation of $\Delta\Psi$ than ΔpH, still resulting in secondary activation of the H^+ pump and vesicle acidification. On the other hand, the advantages of a Cl^-/H^+ exchanger over a simple Cl^- channel remain unclear.

ClCs may also contribute to the efflux of lumenal chloride immediately after synaptic vesicle endocytosis. In contrast to the small number of free lumenal H^+ generated by the H^+ pump, endocytosis traps huge amounts of extracellular Na^+ and Cl^- in the lumen of endosomes and recycling synaptic vesicles. The intracellular ClCs exhibit a strong outward rectification when expressed at the plasma membrane (Friedrich et al., 1999), suggesting that they mediate chloride efflux from the vesicle much more rapidly than influx. It is therefore possible that chloride may exit recycling synaptic vesicles immediately after endocytosis, then enter more slowly later, as the vesicle acidifies (Sonawane et al., 2002; Sonawane and Verkman, 2003). Despite the evidence that Cl^- flux enables the development of ΔpH at the expense of $\Delta\Psi$, it is less clear how a synaptic vesicle or other intracellular compartment might increase $\Delta\Psi$ at the expense of ΔpH.

It is important to note that $\Delta\Psi$ has been much more difficult to assess than ΔpH. Nonetheless, purified chromaffin granules exhibit a substantial ΔpH even before the addition of ATP (Holz, 1979), suggesting substantial buffering at low pH, presumably by lumenal protein, and the addition of ATP increases selectively $\Delta\Psi$, from substantial negative to positive potentials. In most other membranes and particularly synaptic vesicles, the magnitude of $\Delta\Psi$ remains unknown. However, recent work has begun to suggest that synaptic vesicles contain cation channels that might control membrane potential. The trp channel TRPM7 appears to reside on synaptic vesicles, and its downregulation by siRNA reduces quantal size (Krapivinsky et al., 2006). A dominant-negative version of the protein dramatically impairs evoked release, but this may reflect interactions with the exocytotic machinery rather than effects on vesicle filling.

Pharmacologic and Physiologic Regulation of the H^+ Electrochemical Gradient

The effect of many psychostimulants indicates the importance of the H^+ electrochemical gradient for neurotransmitter storage and quantal size. In addition to the effects on plasma membrane transport, amphetamine promotes the efflux of dopamine from vesicular stores. Amphetamine can interact specifically with the vesicular monoamine carrier (Peter et al., 1994), but efflux involves at least in part dissipation of the H^+ electrochemical gradient across the vesicle membrane. Indeed, amphetamine appears to act like a number of other weak bases, such as chloroquine, to dissipate the driving force for vesicle filling with monoamine (Sulzer and Rayport, 1990; Sulzer et al., 1993). Consistent with this role at the vesicle, amphetamine also reduces quantal size measured by amperometry, and increases cytosolic dopamine (Sulzer et al., 1995; Mosharov et al., 2003).

But is the vesicle H^+ electrochemical gradient regulated under physiological circumstances? In parafollicular cells of the thyroid gland, stimulation of the plasma membrane calcium receptor increases vesicle acidification, apparently by activating the vesicle chloride conductance (Barasch et al., 1988; Tamir et al., 1996). Prolonged stimulation of adrenal chromaffin cells by depolarization or secretagogue also produces increased vesicle acidification, a greater proportion of larger ''active'' vesicles that contain a halo around the dense core, and larger quantal size (Pothos et al., 2002). A similar mechanism may account for the remarkable, graded increase in quantal size observed with increasing strength of stimulation, although this has been attributed to regulation of the fusion pore (Elhamdani et al., 2001). These observations support the potential to regulate quantal size through changes in H^+ electrochemical gradient as well as cytosolic transmitter concentration. However, it is important to note that the mechanisms responsible for activity-dependent acidification and increased quantal size remain poorly understood.

Transient alkalinization has also been observed immediately before the exocytosis of mast cell granules (Williams and Webb, 2000). Although this may represent an entirely distinct phenomenon, such as the opening of a fusion pore with concomitant alkalinization just prior to full fusion with the plasma membrane, other work has indicated that alkalinization can actually precede exocytosis (Han et al., 1999). The mechanisms responsible for vesicle alkalinization and its relationship to the acidification observed in other systems remain unknown.

Vesicular Neurotransmitter Transporters

The transport of all classical neurotransmitters into secretory vesicles depends on the H^+ electrochemical gradient generated by the vacuolar H^+ pump, but the transport of different transmitters relies to different extents on the two components of the gradient: ΔpH and $\Delta\Psi$ (Figure 2). Vesicular monoamine and acetylcholine transport depend primarily on ΔpH and vesicular glutamate transport on $\Delta\Psi$ (Johnson, 1988; Maycox et al., 1988; Carlson et al., 1989a). Vesicular GABA transport depends more equally on both ΔpH and $\Delta\Psi$ (Kish et al., 1989; Hell et al., 1990). However, it is important to note that all of the activities are electrogenic and hence rely at least in part on $\Delta\Psi$. Although the distinction between these activities may seem to depend on subtle differences in energetics, the proteins responsible belong to three entirely distinct families. We will now discuss the properties of these proteins, focusing on their role in synaptic transmission.

Vesicular Monoamine Transport

Vesicular monoamine transport involves the exchange of cytoplasmic transmitter for two lumenal protons (Johnson et al., 1981; Knoth et al., 1981) (Table 2). Because monoamines are recognized in their protonated state, the

Table 2. Expression, Ionic Coupling, and Synaptic Phenotype Produced by the Loss of Vesicular Neurotransmitter Transporters

VNT	Family	Location	Ionic Coupling	Loss of Function Synaptic Phenotype
VMAT2	SLC18	monoamine neurons	$amine^+ \leftrightarrow 2H^+$ (VMAT1) [1]	↓ monoamine stores, no release [2]
VAChT	SLC18	cholinergic neurons	$ACh^+ \leftrightarrow 2H^+$ [3]	partial reduction: reduced ACh release [4]
VGAT/VIAAT	SLC32	inhibitory neurons	GABA/gly $\leftrightarrow nH^+$	no release of GABA or gly [5]
VGLUT1	SLC17	glutamate neurons	$glu^- \leftrightarrow nH^+$ Cl^- dependent	no glutamate release at certain synapses [6]
VGLUT2	SLC17	glutamate neurons	$glu^- \leftrightarrow nH^+$ Cl^- dependent	no glutamate release at certain synapses [7]
VGLUT3	SLC17	non-glutamate cells	$glu^- \leftrightarrow nH^+$ Cl^- dependent	

VMAT2, vesicular monoamine transporter 2; VAChT, vesicular ACh transporter; VGAT, vesicular GABA and glycine (gly) transporter, also known as VIAAT, vesicular inhibitory amino acid transporter; VGLUT, vesicular glutamate transporter.
[1](Knoth et al., 1981; Johnson et al., 1981), [2](Takahashi et al., 1997; Fon et al., 1997; Wang et al., 1997), [3](Nguyen et al., 1998), [4](Prado et al., 2006), [5](Wojcik et al., 2006), [6](Fremeau et al., 2004; Wojcik et al., 2004), [7](Wallen-Mackenzie et al., 2006; Moechars et al., 2006).

reaction involves the movement of only 1+ charge for every two H^+, accounting for the greater dependence on ΔpH than $\Delta\Psi$. Why is this stoichiometry important? The ionic coupling is important because it confers the ability to package huge concentrations of monoamine at equilibrium. For a lumenal pH ~5.5 and hence ΔpH ~2, coupling to two H^+ will concentrate monoamines 10^4-fold. An additional $\Delta\Psi$ ~60 mV (although this is generally not known) would increase the gradient by an extra 10-fold, for a total monoamine gradient ~10^5. Indeed, chromaffin granules contain up to molar concentrations of monoamine, although much of it is insoluble in a morphologically recognizable aggregate, or dense core.

Originally characterized in chromaffin granules, molecular cloning of the vesicular monoamine transporters (VMATs) revealed similar properties for both the nonneural isoform VMAT1 (expressed by chromaffin and other endocrine cells) and neuronal VMAT2. Both VMAT1 and -2 recognize dopamine, norepinephrine, and serotonin as substrates, but VMAT2 has a slightly (2- to 3-fold) higher apparent affinity and recognizes histamine as well (Peter et al., 1994; Erickson et al., 1996). Consistent with this, VMAT2 is expressed by dopaminergic, noradrenergic, serotonergic, and histaminergic neurons in both the central nervous system and the periphery (Weihe et al., 1994; Peter et al., 1995).

How does the loss of vesicle transporter influence monoamine release? In the absence of VMAT2, exocytotic release is eliminated, and the stores of brain monoamine are dramatically reduced (Takahashi et al., 1997; Fon et al., 1997; Wang et al., 1997). The animals can move, but feed poorly and die within a few days after birth. Consistent with a specific deficiency in vesicular storage, amphetamines can rescue feeding and prolong survival, presumably by promoting efflux directly across the plasma membrane and thus circumventing the defect in exocytotic release. Inhibition of the metabolic enzyme monoamine oxidase (MAO) also rescues behavior and improves survival, apparently by simply increasing the amount of monoamine (Fon et al., 1997). Indeed, MAO inhibition restores serotonin levels to above wild-type, but has little effect on dopamine levels, suggesting important differences in the regulation of serotonin and dopamine production, possibly due to differences in the feedback inhibition of tryptophan and tyrosine hydroxylase by their products. In addition, the loss of VMAT2 does not appear to affect the number or recycling of synaptic vesicles that remain in dopamine neurons (Croft et al., 2005), suggesting that empty vesicles undergo exocytosis and that the synaptic vesicle and neurotransmitter cycles are not directly coupled.

The VMATs also protect against the parkinsonian neurotoxin MPTP by sequestering the active metabolite inside secretory vesicles, away from its primary site of action in mitochondria (Liu et al., 1992b; Takahashi et al., 1997; Gainetdinov et al., 1998). The submicromolar Km of VMAT1 and -2 presumably contributes to the sequestration of toxin and suggests that VMAT2 may also protect against cytoplasmic toxicity of the normal transmitter dopamine itself. Dopamine can indeed injure cells through a receptor-independent mechanism that involves spontaneous oxidation and the production of free radicals. This mechanism apparently contributes to the toxicity of amphetamines (Cubells et al., 1994), and VMAT2 has been shown to protect against the accumulation of melanin, an oxidation product of dopamine, and against other forms of degeneration (Sulzer et al., 2000; Sang et al., 2007). On the other hand, low cytosolic levels may also limit the pool of monoamine available to refill rapidly cycling synaptic vesicles.

How do the properties of VMAT2 contribute to monoamine release evoked by high-frequency stimulation? In addition to a low Km, which may reduce the available cytosolic monoamine, the VMATs exhibit a relatively slow maximal turnover rate for saturating concentrations of substrate. Using radiolabeled versions of the VMAT inhibitors tetrabenazine or reserpine to determine the number of transporters, heterologous expression of VMAT1 and -2 confers a rate at 29°C of ~5/s/transporter for serotonin (Schuldiner et al., 1993; Peter et al., 1994), comparable if not faster than the rates observed using native chromaffin granules and brain synaptic vesicles (Scherman, 1986; Scherman and Boschi, 1988). The rate would presumably be higher at 37°C and the lower apparent affinity substrate dopamine appears to be transported up to four times more rapidly than serotonin. Nonetheless, the rates may be lower in cells such as neurons where substrate is not saturating. As a result, it may take minutes to refill rapidly cycling synaptic vesicles, and hence limit quantal size. Transport activity would be expected to influence quantal size under these circumstances, but it is very difficult to isolate effects on quantal size in the context of strong stimulation. Indeed, the analysis of quantal size by postsynaptic recording has generally focused on spontaneous rather than evoked release. Amperometry can detect all evoked release but the difficulty identifying reliable release sites complicates its application.

Vesicular Acetylcholine Transport

Originally described at the *Torpedo* electric organ, the vesicular acetylcholine transporter (VAChT) resembles the VMATs in its primary dependence on ΔpH (Nguyen et al., 1998). Consistent with this ionic coupling, the protein eventually identified in *C. elegans* as required for ACh release closely resembles the VMATs in sequence (Alfonso et al., 1993). In contrast to the VMATs, VAChT exhibits a millimolar Km, suggesting a higher cytoplasmic concentration of acetylcholine than monoamines that might be expected to facilitate vesicle filling. Despite its role at the neuromuscular junction, however, the vesicular acetylcholine transporter appears even slower than the VMATs, with a turnover ~1/s (Varoqui and Erickson, 1996). This slow rate may contribute to the defects in ACh release and cognitive impairment observed in mice with only 45%–65% reduction in VAChT expression (Prado et al., 2006). In addition, inhibition of VAChT with the relatively specific compound vesamicol does not affect vesicle cycling (Parsons et al., 1999), similar to the observations in VMAT2 knockout mice.

Vesicular GABA Transport

Identified through genetic analysis in *C. elegans*, the vesicular GABA transporter (VGAT) defines a family of mammalian proteins entirely distinct from the VMATs and VAChT (McIntire et al., 1993b, 1997). Similar to VAChT, however, VGAT exhibits a very low apparent affinity (Km ~5 mM). VGAT (also known as the vesicular inhibitory amino acid transporter [VIAAT]) is expressed by glycinergic as well as GABAergic neurons, and glycine inhibits the transport of GABA by VGAT, but the IC_{50} is so low (~25 mM) that it has been difficult to demonstrate glycine transport directly in biochemical assays (McIntire et al., 1997; Chaudhry et al., 1998). Genetic disruption in mice has provided some of the first evidence that VGAT loads glycine as well as GABA into synaptic vesicles and is indeed required for the release of both transmitters (Wojcik et al., 2006). Although the mechanism of transport involves H^+ exchange, the stoichiometry for H^+ remains unknown. If it involves one H^+ (and since GABA is a neutral zwitterion, 1+ charge), this would produce a gradient of only ~1000-fold for ΔpH ~2 and $\Delta\Psi$ ~+60 mV, substantially less than the VMATs. On the other hand, the cytosolic concentration of GABA presumably exceeds that of dopamine, and even a cytosolic GABA concentration ~100 μM would confer high millimolar lumenal concentrations. In the case of glycine, which is recognized at low apparent affinity, VGAT may rely on particularly high cytosolic concentrations of glycine generated by the neuronal plasma membrane glycine transporter GLYT2 (Roux and Supplisson, 2000). Interestingly, the GAD65 isoform associates with synaptic vesicles and may interact specifically with VGAT—inhibition of GAD appears to reduce VGAT activity (Jin et al., 2003). For a variety of reasons, VGAT may thus not need to produce a particularly large gradient of GABA across the vesicle membrane.

Little is known about how VGAT might contribute to synaptic plasticity. The protein undergoes phosphorylation, but this does not appear to change its transport activity (Bedet et al., 2000). On the other hand, VGAT mRNA and protein downregulate in response to suppression of neural activity (De Gois et al., 2006), and this has also been observed in association with a reduction in quantal size (Hartman et al., 2006). Recent work in *C. elegans* has also demonstrated a requirement for the protein UNC-46 (related to the lysosome-associated membrane proteins) in the trafficking of VGAT to the nerve terminal (Schuske et al., 2007), providing some of the first information about associated proteins required for the targeting of any synaptic vesicle protein.

Vesicular Glutamate Transport

Properties

The transport of glutamate into synaptic vesicles exhibits several unusual properties. In contrast to the plasma membrane excitatory amino acid transporters, vesicular glutamate transport recognizes glutamate but not aspartate (Naito and Ueda, 1985). In addition, it depends predominantly on $\Delta\Psi$ rather than ΔpH and has been suggested to function as an ion channel (dependent only on $\Delta\Psi$) rather than a H^+ antiporter (Maycox et al., 1988; Carlson et al., 1989a). In large part, the dependence of vesicular glutamate transport on the two components of the H^+ electrochemical gradient will depend on which component predominates in the membranes under the conditions studied. However, the difference between channel and transporter mechanism has extremely important

consequences for the amount of glutamate per vesicle because a channel would concentrate glutamate only ~10-fold for $\Delta\Psi$ ~60 mV. In contrast, the exchange of cytosolic glutamate for one lumenal H^+ would result in the movement of 2+ charge as well as one H^+. For ΔpH ~2 and $\Delta\Psi$ ~60 mV, this coupling would produce a much larger glutamate gradient ~10^4. Although a cytoplasmic glutamate concentration ~10 mM would require a gradient only ~10-fold to produce 100 mM lumenal glutamate, considerable evidence supports the H^+ exchange mechanism. It is true that dissipation of ΔpH can have minimal effect on vesicular glutamate transport or sometimes even increase it (Tabb et al., 1992), presumably by increasing $\Delta\Psi$. However, the dissipation of $\Delta\Psi$ leaves some residual activity that is abolished by dissipation of ΔpH (Tabb et al., 1992; Bellocchio et al., 2000), strongly supporting the mechanism of H^+ exchange.

Vesicular glutamate transport also exhibits an unusual biphasic dependence on chloride. Transport is optimal at 2–10 mM Cl^-, with some but less activity at 0 and 150 mM (Naito and Ueda, 1985). Presumably, high Cl^- concentrations dissipate the $\Delta\Psi$ required for transport. However, the increase in activity produced by low concentrations of chloride is not simply due to the production of ΔpH because the activation persists despite clamping ΔpH (Wolosker et al., 1996). Indeed, Cl^- appears to interact at an allosteric site on the transporter that regulates both forward and reverse flux (Hartinger and Jahn, 1993). Despite these reproducible biochemical observations, recent work has shown that quantal size does not vary with intracellular chloride concentration (Price and Trussell, 2006). Paired whole-cell recordings from pre- and postsynaptic neurons at the calyx of Held, where glutamate receptors are not saturated, shows that spontaneous events do not vary in size when the presynaptic terminal is dialyzed with 5–100 mM chloride. How can this result be reconciled with biochemical observations? The answer seems to be that measurements of radiotracer flux examine only the earliest phase of uptake and are therefore kinetic measurements. In contrast, the analysis of quantal size almost certainly occurs closer to a thermodynamic equilibrium. It is thus possible that chloride concentration affects only the rate of vesicle filling and not the final gradient of substrate achieved. Indeed, chloride has been shown to have much less effect on steady-state measurements of transport, using high concentrations of glutamate (Wolosker et al., 1996).

Proteins

The vesicular glutamate transporters have also been suggested to perform an entirely distinct cellular function. They were originally identified as type-I Na^+-dependent inorganic phosphate transporters (Ni et al., 1994), but it has since become clear that most members of this family subserve a function other than phosphate transport. Sialin exports sialic acid from lysosomes (Verheijen et al., 1999; Morin et al., 2004; Wreden et al., 2005), and the founding member NaPi-1 has been shown to recognize organic anions (Busch et al., 1996; Bröer et al., 1998). Consistent with a primary role for type-I phosphate transporters in the transport of organic anions, the VGLUTs also belong to this family. It remains less clear whether any of these proteins actually mediate phosphate transport. Glutamate transport by the VGLUTs does not depend on Na^+, and high concentrations of inorganic phosphate do not inhibit glutamate uptake by VGLUT1 (Bellocchio et al., 2000). Indeed, heterologous expression of the VGLUTs appears to upregulate endogenous, low-affinity phosphate transport (which can be Na^+-independent) through unknown mechanisms (R.H.E., unpublished data), very similar to NaPi-1 (Bröer et al., 1998). On the other hand, the recent purification and functional reconstitution of VGLUT2 has shown that this protein can catalyze Na^+-dependent phosphate transport in addition to glutamate/H^+ exchange (Juge et al., 2006). Further, a series of mutations that disrupt glutamate transport do not affect phosphate uptake, suggesting that the two substrates translocate through different mechanisms. Although a role for phosphate transport by the VGLUTs remains uncertain, it might contribute to activation of the phosphate-activated glutaminase and hence glutamate production at the nerve terminal (Bellocchio et al., 1998). Because the VGLUTs reside on synaptic vesicles, this might confer a mechanism to couple glutamate production to exocytotic glutamate release.

Heterologous expression of the VGLUTs confers essentially all of the properties previously reported for glutamate uptake by brain synaptic vesicles, including specificity for glutamate and a biphasic dependence on chloride (Bellocchio et al., 2000; Takamori et al., 2000; Fremeau et al., 2001; Takamori et al., 2001; Herzog et al., 2001; Fremeau et al., 2002; Takamori et al., 2002; Gras et al., 2002; Varoqui et al., 2002). Interestingly, the related NaPi-1 exhibits a large chloride conductance inhibited by organic anions (Busch et al., 1996), raising the question whether other type-I phosphate transporters do as well. VGLUT1 exhibits a chloride conductance, and this is inhibited by glutamate, but not aspartate (Bellocchio et al., 2000). The relationship between glutamate transport and chloride conductance also remains unclear, and might contribute to the unusual chloride dependence of VGLUTs. The poor understanding of vesicular glutamate transport derives from the very limited assays currently available, particularly after heterologous expression.

Distribution and Synaptic Role

The three known VGLUT isoforms show almost mutually exclusive patterns of expression in the adult brain. VGLUT1 is expressed by excitatory neurons in the cortex, hippocampus, and cerebellar cortex (granule cells), whereas VGLUT2 is expressed by cells in the thalamus and brainstem (Bellocchio et al., 2000; Takamori et al., 2000; Fremeau et al., 2001; Takamori et al., 2001; Herzog et al., 2001; Fremeau et al., 2002; Gras et al., 2002; Varoqui et al., 2002). The loss of VGLUT1 indeed eliminates release at mature synapses that express VGLUT1 in adulthood (Fremeau et al., 2004). During development, however, the transient, early expression of VGLUT2 in hippocampus and cerebellum confers residual, VGLUT1-independent

glutamate release that appears to be entirely normal in terms of baseline characteristics and to originate from distinct synapses. Nonetheless, VGLUT2 synapses that remain in the developing hippocampus of VGLUT1 knockout mice show more rapid synaptic depression than wild-type, suggesting either that these synapses recycle their vesicles more slowly than the VGLUT1 synapses of wild-type mice or that VGLUT1 and -2 differ in their recycling. Indeed, more recent work has shown that VGLUT1 but not VGLUT2 interacts with proteins involved in synaptic vesicle endocytosis, and these interactions confer differences in the rate of recycling (Voglmaier et al., 2006). The VGLUT1 knockout also shows abnormal endocytic structures (Fremeau et al., 2004), suggesting effects of the transporter on aspects of the synaptic vesicle cycle. Like VMAT2 and VAChT, however, inhibition of vesicular glutamate transport with the H^+ pump inhibitor bafilomycin does not affect vesicle recycling (Zhou et al., 2000), and analysis of the VGLUT1 knockout confirms the release of empty vesicles (Wojcik et al., 2004). The VGLUT2 knockout shows a severe impairment of glutamate release at synapses in the brainstem responsible for respiration, and hence dies shortly after birth (Wallen-Mackenzie et al., 2006; Moechars et al., 2006).

In contrast to VGLUT1 and -2, VGLUT3 is expressed by cells not traditionally associated with glutamate, such as serotonergic neurons in dorsal and medial raphe nuclei, cholinergic interneurons in the striatum, and a subset of GABAergic interneurons in cortex and hippocampus (Gras et al., 2002; Fremeau et al., 2002; Schafer et al., 2002). VGLUT3 has also been detected outside the nervous system, and unlike VGLUT1 and -2, heterologous expression has not thus far been found to confer glutamate release (Takamori et al., 2002), raising questions about its physiological role. In addition to a potential role as transmitter, the glutamate stored by VGLUT3 might simply modulate storage and/or release of the other classical transmitter usually associated with VGLUT3$^+$ neurons (e.g., serotonin in the raphe, ACh in striatal interneurons, and GABA in cortical and hippocampal interneurons). Alternatively, the glutamate stored by VGLUT3 might serve a metabolic role. However, several observations support a role for VGLUT3 in glutamate release.

VGLUT3 exhibits a subcellular location distinct from VGLUT1 and -2. In projection neurons such as serotonergic neurons in the raphe nuclei, VGLUT3 appears restricted to axon terminals like VGLUT1 and -2, but localizes to the cell body and dendrites as well as axon of cholinergic interneurons in the striatum, and GABAergic interneurons in hippocampus and cortex (Fremeau et al., 2002). VGLUT3 also localizes to the dendrites of pyramidal neurons in the cortex, where it contributes to dendritic glutamate release, a novel form of retrograde synaptic signaling (Harkany et al., 2004). The trafficking of VGLUT3 thus appears to confer novel modes of glutamate release not possible with the other isoforms.

The transient expression of VGLUT3 during development has suggested a particular role for this isoform in plasticity. In the auditory system, neurons in the medial nucleus of the trapezoid body (MNTB) form synapses that transiently release GABA onto cells of the lateral superior olive (LSO) early in postnatal development, then switch to glycine (Gillespie et al., 2005). At the same time, these synapses undergo tonotopic refinement and strengthening that contribute to sound localization. During early development, MNTB neurons also release glutamate that activates postsynaptic NMDA receptors, suggesting a role in synaptic plasticity, and this glutamate release coincides with the transient expression of VGLUT3. However, it remains to be determined whether this glutamate release actually contributes to synapse development and sound localization.

SV2 and Other Vesicular Carriers

Membrane proteins other than the H^+ pump, ion channels, and neurotransmitter transporters may also contribute to the lumenal contents of synaptic vesicles and hence to quantal size. In addition to its role as a transmitter, anionic ATP apparently promotes the costorage of cationic transmitters such as serotonin (Bankston and Guidotti, 1996), but the transporter responsible for ATP uptake by synaptic vesicles remains unknown. Conversely, the function of many identified synaptic vesicle membrane proteins remains poorly understood. In particular, the SV2 family of polytopic membrane proteins shows strong sequence similarity to a wide range of carbohydrate transporters, but its biochemical function has eluded characterization. Knockout of the SV2A isoform results in seizures and death within weeks after birth, and physiological analysis has alternatively suggested roles in calcium regulation or the readily releasable pool of synaptic vesicles (Crowder et al., 1999; Janz et al., 1999). Interestingly, recent work has shown that SV2A is a direct target for the major clinical anticonvulsant levetiracetam (Keppra) (Lynch et al., 2004). Despite their sequence similarity to transporters, however, the SV2 proteins have not been reported to influence quantal size.

The Determinants of Vesicle Filling

Considering all of the factors involved in the transport of neurotransmitter into synaptic vesicles, how is filling actually determined under physiological conditions? A simple equilibrium based on the factors described above does not account for the experimental observations, suggesting that additional forces are operative. We now present several models for vesicle filling, discuss the evidence for and against them, and consider their implications. All of these have the potential to influence both the lumenal concentration of transmitter and vesicle size, but we will consider these two outcomes separately.

Equilibrium

At equilibrium, the ionic coupling of vesicular neurotransmitter transporters together with the H^+ electrochemical driving force should dictate the concentration gradient of transmitter across the vesicle membrane (Figure 3). The

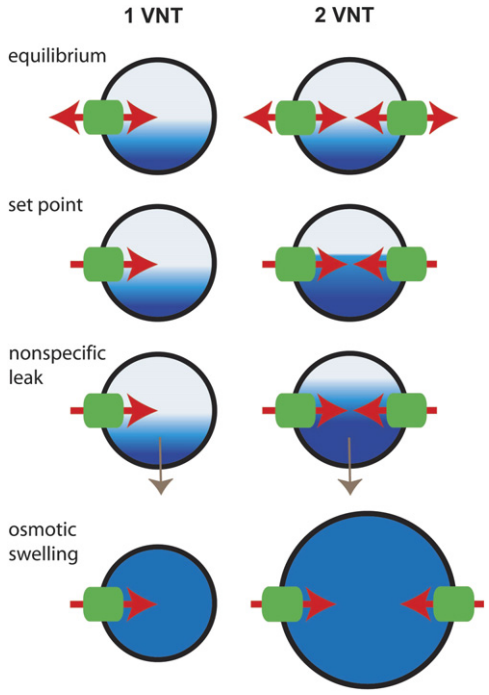

Figure 3. The Relationship between Neurotransmitter Transport Activity and Vesicle Filling

Depending on the circumstances, increasing the number of vesicular neurotransmitter transporters (green) is predicted to have different effects on vesicle filling (blue).

Equilibrium model. If a vesicle containing one transporter (left column) fills to an equilibrium dictated solely by the H^+ electrochemical driving force and the cytosolic concentration of transmitter, then increasing the number of transporters (right column) will have no effect on lumenal transmitter concentration. The minimal effects of reduced VGLUT expression on quantal size in VGLUT1$^{+/-}$ heterozygous mice as well as the *Drosophila* neuromuscular junction (Fremeau et al., 2004; Daniels et al., 2006) suggest that vesicular glutamate transport exhibits these properties. On the other hand, if the rate of filling is slow relative to the cycling of synaptic vesicles, then more transporters will increase the extent of filling achieved before exocytosis (not shown).

Set point model. Secretory vesicles may also fill to a fixed set point not related directly to the transport mechanism. According to this model, increased transporter expression increases filling, but only up to a set point, which may be substantially less than expected from transporter expression. (At the two extremes, a relatively high set point might have no effect on the increase in vesicle filling, and a relatively low set point might prevent any increase.) Although lumenal transmitter may provide feedback to the transport machinery through the activation of heterotrimeric G proteins (Brunk et al., 2006b), there is little physiological evidence for an independent set point that limits vesicle filling.

Nonspecific leak (*leaky bathtub*) *model.* If vesicles exhibit a nonspecific leak (i.e., not mediated by the neurotransmitter transporter), then the number of transporters will influence filling by offsetting this leak, even at equilibrium. This mechanism presumably accounts for the observed effects of VMAT and VAChT expression on quantal size (Song et al., 1997; Pothos et al., 2000) and is consistent with the relatively high nonspecific permeation of cationic transmitters through the largely anionic phospholipid bilayer.

Osmotic swelling model. Because the cytosolic concentration of transmitter, driving force, and ionic coupling of transport often predict lumenal concentrations of transmitter that exceed physiological osmolarity, vesicle filling may also be limited by the development of an osmotic gradient. This gradient presumably accounts for the swelling of chromaffin vesicles after loading with exogenous L-Dopa, and their shrinkage after inhibition of VMAT, as well as the uniform concentration of lumenal transmitter despite substantial changes in vesicle size (Pothos et al., 1996; Colliver et al., 2000; Bruns et al., 2000; Gong et al., 2003). Although vesicles that vary in size but not the concentration of lumenal transmitter might also result from changes in vesicle biogenesis, this mechanism seems unlikely to account for the acute changes in quantal size that result from manipulation of cytosolic transmitter or the inhibition of transport, particularly in the case of nonrecycling vesicles such as chromaffin granules. Nonetheless, it is important to note that despite the data supporting it, this model violates current thinking about the inelasticity of cell membranes.

number of transporters would influence quantal size only if there were insufficient time to reach equilibrium, a situation that might be expected to occur only if filling were slow relative to the recycling of synaptic vesicles. Indeed, vesicular monoamine and ACh transport have low turnover numbers that could limit vesicle filling. However, the number of transporters apparently influences quantal size independent of the rate of vesicle recycling, implicating additional forces.

Transporter Expression and Leak

In chromaffin cells as well as dopamine neurons, overexpression of VMAT2 increases quantal size measured by amperometry (Pothos et al., 2000). Because these cells are relatively quiescent in culture, the effects of VMAT overexpression appear independent of activity. Overexpression of VAChT also increases quantal size at the developing neuromuscular junction (Song et al., 1997). Conversely, a 50% reduction in VMAT2 (in mice heterozygous for the null allele) both reduces monoamine release (Fon et al., 1997; Travis et al., 2000) and influences the behavioral response to amphetamine as well as the sensitivity to MPTP (Wang et al., 1997; Gainetdinov et al., 1998). A reduction in VAChT by only 45% has major effects on cognitive function and appears to involve a reduction in quantal size (Prado et al., 2006).

If secretory vesicles have sufficient time to fill (which is presumably the case at least for spontaneously released vesicles), why then does the level of transporter expression make a difference? Although not established definitively, most of the data are consistent with the presence of a nonspecific leak of transmitter across the vesicle membrane (i.e., not mediated by the vesicular transporter) (Figure 3). Synaptic vesicles and other intracellular membranes used for heterologous expression all exhibit a substantial nonspecific accumulation of monoamine driven by a H^+ electrochemical gradient, even in the absence of a functional transporter. It is presumed that the unprotonated amine diffuses across the vesicle membrane, then undergoes protonation inside the vesicle, preventing its efflux. Conversely, a substantial nonspecific efflux must also exist, and previous work has shown that the efflux of monoamine from chromaffin granules induced by dissipation of ΔpH cannot be blocked by specific inhibitors such as reserpine (Maron et al., 1983). Synaptic vesicles also exhibit a substantial nonspecific leak not blocked by inhibitors (Floor et al., 1995). The expression of more transport protein presumably serves to offset this efflux even under physiological conditions. In addition to

a nonspecific leak of transmitter, secretory vesicles can exhibit a significant leak of H^+, possibly through the H^+ pump itself (Nelson et al., 2002). A H^+ leak should reduce transmitter accumulation simply by dissipating the driving force for transport, but might also promote nonspecific leak of monoamine by increasing the amount of unprotonated transmitter in the vesicle lumen.

Recent work has suggested that the level of VGLUT expression also influences quantal size. In biochemical experiments, the available inhibitors reduce the steady-state accumulation of glutamate as well as the rate of its accumulation (Wilson et al., 2005). Overexpression of the VGLUTs in dissociated hippocampal culture and in autapses (single neurons forming synapses onto themselves) also appears to increase quantal size by a variety of criteria (Wojcik et al., 2004; Wilson et al., 2005). Conversely, the loss of VGLUT1 and -2 reduces quantal size in autaptic culture (Wojcik et al., 2004; Moechars et al., 2006). In addition, the VGLUTs undergo considerable regulation, both during development and in response to activity. Neurons in the cortex, hippocampus, and cerebellar granule cells that make only VGLUT1 in adulthood transiently express VGLUT2 during early development (Miyazaki et al., 2003; Fremeau et al., 2004; Boulland et al., 2004). Originally identified as genes induced by kainic acid and growth factor (Ni et al., 1994; Aihara et al., 2000), VGLUT1 and -2 respond to manipulation of activity in culture. Consistent with a homeostatic role, increased activity downregulates VGLUT1 at both the level of RNA and protein (De Gois et al., 2006). Surprisingly, increased activity also upregulates VGLUT2. Circadian rhythm also appears to regulate the expression of VGLUT1, although at the level of the protein rather than its mRNA (Yelamanchili et al., 2006).

On the other hand, several observations suggest that VGLUT expression may not normally limit quantal size. First, mice heterozygous for a wild-type VGLUT1 allele do not differ from wild-type in hippocampal field potentials (Fremeau et al., 2004). Any reduction in quantal size should be reflected in this evoked response, so the lack of difference from wild-type effectively excludes an alteration in quantal size when VGLUT expression is reduced by half. Second, a reduction in VGLUT expression in *Drosophila* does not reduce quantal size—only the frequency of miniature events drops as the level of VGLUT falls below 10%–20% wild-type (Daniels et al., 2006), presumably due to the release of "empty" vesicles. Indeed, a single VGLUT appears sufficient to fill a vesicle at this synapse. On the other hand, VGLUT overexpression in *Drosophila* apparently increases quantal size (Daniels et al., 2004). Interestingly, increased VGLUT expression in *Drosophila* also produces a homeostatic reduction in quantal content. Although a similar homeostatic mechanism might account for the unchanged quantal size observed in heterozygous VGLUT1 knockouts, the hippocampal slice preparation does not generally exhibit the same robust homeostasis as the *Drosophila* neuromuscular junction.

How can the observations in vivo be reconciled with the biochemical results and studies in culture? In the transport assay, it is important to note that steady-state measurements may reflect limitations of the assay (such as the consumption of ATP or proteolysis) rather than a true thermodynamic equilibrium, and this is an inherent shortcoming of many radiotracer flux assays. In the autapses made from VGLUT1 knockouts, the miniscule amounts of residual transport activity may well account for a reduction in quantal size, but the results from *Drosophila* suggest that more physiological changes in transport activity might not have produced detectable changes. It is more difficult to understand how VGLUT overexpression increases quantal size while the loss of VGLUT has no effect until it is almost eliminated. Perhaps VGLUT expression influences vesicle size during biogenesis, rather than the extent of filling. The work in *Drosophila* indeed shows increased vesicle size with VGLUT overexpression (Daniels et al., 2004). And if the amount of VGLUT expressed does not influence vesicle filling, why would this differ from the situation with monoamines and acetylcholine, where transporter expression does apparently affect quantal size (Fon et al., 1997; Pothos et al., 2000; Song et al., 1997)? The difference may again reflect nonspecific leakage across the membrane, with cationic transmitter more permeant than anionic through a bilayer with a high proportion of acidic phospholipid. In addition, it is important to point out that even if quantal size assessed from spontaneous events is not obviously affected by changes in VGLUT expression, the transporter may become limiting with high rates of synaptic activity and vesicle recycling, when it is not possible to measure quantal size directly. A reduction in VGLUT expression can indeed impair behavior dramatically with only modest effect on synaptic transmission (Smear et al., 2007).

Nonspecific leakage may also account for the differential effects of transport inhibitors on vesicular storage. In the absence of stimulation, an inhibitor would be expected to deplete the stores of transmitter only if there is a leak—in this case, maintenance of the stores requires ongoing transport activity. In contrast, stimulation should be required to deplete the stores of a transmitter that does not leak. The permanently charged ACh should exhibit less nonspecific leak than reversibly protonated monoamines. Consistent with minimal if any leak, the VAChT inhibitor vesamicol does not deplete vesicular stores in the absence of stimulation (Cabeza and Collier, 1988). Vesamicol appears to have no effect until ~15% of the ACh stores are released. Preformed vesicles must therefore retain their contents until released, and the requirement for functional VAChT applies only to recycling vesicles. In contrast, the VMAT inhibitor reserpine depletes monoamines in nonrecycling chromaffin granules within minutes, in the absence of strong stimulation (Kozminski et al., 1998). Monoamine vesicles must thus exhibit a substantial leak, accounting for the large effects of VMAT2 expression on quantal size. It is more difficult to reconcile the apparent lack of ACh leak in the presence of vesamicol

with the observed effects of VAChT expression on quantal size (Song et al., 1997).

Set Point

In addition to regulation at the level of transcription, vesicular neurotransmitter transporters have been suggested to undergo direct regulation at the level of the protein or its activity. A fragment of the cytoskeletal protein fodrin has been purified as an inhibitor of vesicular GABA and glutamate transport, but we still know little about the mechanism or its physiological role (Ozkan et al., 1997; Tamura et al., 2001). Heterotrimeric G proteins have also been shown to inhibit both vesicular monoamine and glutamate transport in a variety of systems. Multiple heterotrimeric G proteins associate with secretory vesicles, and activation of the G protein αo_2 with nonhydrolyzable forms of GTP inhibits uptake of monoamine and glutamate by 30%–50%, affecting primarily Vmax rather than Km (Pahner et al., 2003; Ahnert-Hilger et al., 1998; Höltje et al., 2000; Winter et al., 2005). The mechanism does not obviously involve a change in vesicle acidification, suggesting a direct interaction with the transport protein. In platelet granules that store serotonin and express VMAT2, α_q serves a similar role, and inhibition appears to depend on lumenal transmitter: in the absence of serotonin (using mice that lack platelet tryptophan hydroxylase), activated α_q no longer inhibits transport. Inhibition is restored by the addition of monoamine, both in the serotonin-deficient platelets and in heterologous expression systems (Holtje et al., 2003). Thus, G proteins may serve to relay a signal about lumenal transmitter back to the transporter and provide a set point for vesicle filling (Figure 3). The lumenal sensor remains unknown, and it seems improbable that an internalized receptor would be activated specifically by high lumenal concentrations of transmitter. However, deleting part of the large first lumenal loop in VMAT1 and -2 dramatically reduces if not abolishes the inhibition, implicating this domain in the mechanism (Brunk et al., 2006a). Remarkably, $G\alpha o_2$ also appears to alter the chloride dependence of synaptic vesicle glutamate transport, eliminating it in αo_2 knockout mice and shifting it to lower concentrations when activated by a nonhydrolyzable form of GTP, although the mechanism remains unclear (Winter et al., 2005).

The Relationship between Neurotransmitter and Synaptic Vesicle Cycles

Recent work has further suggested that VAChT may interact directly with the v-SNARE synaptobrevin involved in synaptic vesicle fusion. A mutation that replaces a glycine in the ninth transmembrane domain of *C. elegans* VAChT ortholog *unc-17* with arginine essentially eliminates transport activity, and the resulting uncoordinated phenotype is rescued by a mutation in the transmembrane domain of synaptobrevin that replaces an isoleucine with aspartate (Sandoval et al., 2006). This allele-specific supression strongly suggests a direct interaction of the two proteins and indicates a connection between the synaptic vesicle and neurotransmitter cycles that has not previously been considered. However, acidic residues introduced into other vesicle proteins also give partial rescue, raising the alternative possibility that the interaction might only serve to stabilize the mutant transport protein. As noted above, the inhibition of filling does not appear to influence synaptic vesicle exocytosis or recycling in cholinergic, aminergic, or glutamatergic neurons (Parsons et al., 1999; Croft et al., 2005; Zhou et al., 2000; Wojcik et al., 2004), but the converse remains unexplored. From extensive work reviewed elsewhere (Liu et al., 1999b; Fleckenstein and Hanson, 2003), we know that changes in membrane trafficking have the potential to influence transporter number at both the plasma membrane and on secretory vesicles, but we are only beginning to understand its physiological regulation.

The Control of Vesicle Volume

In addition to changes in the concentration of lumenal contents, secretory vesicles can vary in size. This may occur during vesicle biogenesis, but several of the changes observed are acute, suggesting the distention of vesicles already formed. Because biological membranes are generally not considered elastic, this is very surprising.

Variation in vesicle size was originally appreciated in the *Torpedo* electric organ, which contains two biochemically distinct populations of synaptic vesicles that can be separated by density gradient fractionation (Zimmermann and Denston, 1977). The larger vesicles, termed VP1, contain 3- to 5-fold more ACh than the smaller VP2 vesicles, but VP2 exhibit substantially higher VAChT activity (Gracz et al., 1988). In contrast to chromaffin granules, which increase in size with stimulation (Pothos et al., 2002), VP1 vesicles predominate at rest, and the smaller VP2 appear with stimulation (Giompres et al., 1981). Although vesicle populations with distinct size have not been resolved at the neuromuscular junction, the appearance of vesicles resembling VP2 may account for the reduction in quantal size observed at the neuromuscular junction after strong stimulation (Doherty et al., 1984; Naves and Van der Kloot, 2001). Indeed, preformed vesicles of larger quantal size have been observed at frog, rat, and snake neuromuscular junctions (reviewed in Prior and Tian, 1995; Van der Kloot, 2003). After stimulation of the electric organ in *Torpedo*, VP2 is slowly replaced by VP1. It has been speculated that VP1 corresponds to the reserve pool of vesicles observed at many synapses, and VP2 to the recycling pool (Rizzoli and Betz, 2005). In contrast to the apparently interconvertible VP1 and -2, however, imaging experiments with styryl FM dyes suggest that reserve and recycling pools are functionally if not morphologically and biochemically distinct. Remarkably, the membrane of VP2 appears to be twice the thickness of VP1 (Van der Kloot, 2003), suggesting that the vesicles may indeed simply swell with transmitter accumulation. Regardless of whether VP1 and -2 are biochemically distinct vesicle populations, or interconvertible due to changes in filling, the observations suggest general principles that may also pertain at other synapses.

The analysis of monoamine storage and release has supported the influence of filling on vesicle size. After supplementation of pheochromocytoma PC12 cells with L-Dopa, the total amount of dopamine released per large dense-core vesicle increases, but remarkably, the concentration does not change (Colliver et al., 2000; Gong et al., 2003). (The two modes of electrochemical recording, amperometry and voltammetry, permit this distinction to be made, as does capacitance, which measures vesicle membrane surface area after exocytosis.) Consistent with a selective effect on the amount but not the concentration of transmitter released, dense-core vesicle size increases after addition of L-Dopa, and only the halo surrounding the dense core enlarges, without an effect on the dense core itself (Colliver et al., 2000). Increasing cytosolic transmitter thus has the potential to increase vesicle size independent of an increase in lumenal concentration, suggesting that existing vesicles may swell to accommodate more contents. Further supporting this hypothesis, variation in quantal size correlates well with variation in vesicle volume in the leech, and the average concentration of transmitter released per vesicle seems not to differ despite major differences in vesicle size (Bruns et al., 2000).

How can the lumenal concentration of transmitter be so precisely controlled? One simple possibility is that the development of an osmotic gradient will tend to oppose filling and so impose a relatively uniform constraint on all vesicles (Figure 3). The H^+ electrochemical gradient may thus serve to produce both a gradient of transmitter and an osmotic gradient that will eventually limit the lumenal accumulation of transmitter. The ionic coupling of many vesicular neurotransmitter transporters, along with the known or estimated cytosolic concentration of transmitter, are predicted to generate hyperosmolar lumenal contents, which, if they do not precipitate, might be expected to distend the vesicle, presumably up to some limit set by the membrane lipids and the H^+ electrochemical driving force. The selective effect of L-Dopa on the amount but not the concentration of released dopamine and the correlation of quantal size with vesicle volume assessed by capacitance (Colliver et al., 2000; Gong et al., 2003) are both consistent with this hypothesis.

Alternatively, changes in vesicle size may reflect differences in biogenesis and hence intrinsic biochemical differences in vesicle composition. Several *Drosophila* mutants show an increase in quantal size at the neuromuscular junction that correlates with increased vesicle volume (Zhang et al., 1999; Karunanithi et al., 2002). Interestingly, one of the mutations affects the *lap* gene encoding a *Drosophila* homolog of clathrin adaptor protein AP180 (Zhang et al., 1998) and another the lipid phosphatase synaptojanin involved in clathrin uncoating (Dickman et al., 2005), strongly suggesting that the increased size results from a defect in biogenesis. Quantal size and vesicle volume both show increased variation in the *lap* mutant, consistent with a role for the gene product in the production of homogeneous vesicles.

Under physiological circumstances, chromaffin cells have been reported to contain two discrete populations of dense-core vesicles, and the adaptor protein AP-3 appears to control their production (Grabner et al., 2005; Grabner et al., 2006). Overexpression of the neuronal AP-3 isoform reduces quantal size, and the loss of AP-3 increases quantal size, both changes accompanied by parallel changes in vesicle size. Interestingly, synaptic vesicles at 1b and 1s synapses of the wild-type *Drosophila* neuromuscular junction also differ substantially in both quantal size and vesicle volume (Karunanithi et al., 2002), further supporting a role for the control of vesicle volume even under physiological circumstances. The increase in quantal size associated with increased activity in *Drosophila* appears to reflect an increase in vesicle volume (Steinert et al., 2006). In this case, the ability to influence the appearance of larger quantal events through manipulations previously shown to affect recruitment of the reserve and recycling synaptic vesicle pools suggests that these two, distinct pools account for the variation in quantal size, although it remains unclear whether they actually differ in vesicle volume.

An elegant study at the calyx of Held has recently provided a crucial test for the hypothesis that variation in quantal size reflects variation in vesicle volume. By averaging a large number of events at many synapses, the authors were able to measure simultaneously the capacitance change associated with fusion of a single vesicle and quantal size detected postsynaptically (Wu et al., 2007). The results show no correlation between quantal size and capacitance jump, strongly suggesting variation in the concentration of lumenal transmitter rather than vesicle volume. However, it is important to note that capacitance detects only the surface area of the plasma membrane and so can detect differences in vesicle surface area only *after* fusion with the plasma membrane. If vesicles swell as a result of osmotic stress during transmitter accumulation, their surface area after fusion presumably returns to its original, undistended state. Vesicle surface area *before* fusion cannot be detected by capacitance measurements at the plasma membrane, but rather requires other methods such as electron microscopy.

Vesicle Filling and Synaptic Plasticity

How might changes in vesicle filling contribute to synaptic plasticity? The diversity of mechanisms that influence the cytosolic concentration of transmitter, the H^+ electrochemical driving force, and transport into the vesicle suggests many possibilities. In addition, components of the release machinery and synaptic vesicle membrane proteins of unknown function may influence this process. However, we still understand little about these basic mechanisms and do not as yet have the appropriate biophysical methods to study them directly. As a result, we understand very little about how quantal size is actually controlled under physiological conditions and how it changes in synaptic plasticity. In the future, progress will depend on a combination of molecular and physiological

approaches to explore such basic questions as how vesicle filling influences vesicle volume and the relationship between different synaptic vesicle pools, which was originally suggested by classical studies in the *Torpedo* electric organ.

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