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Authors

Eriksen, Jacob
Li, Fei
Edwards, Robert H

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The Mechanism and Regulation of Vesicular Glutamate Transport: Coordination with the Synaptic Vesicle Cycle

Jacob Eriksen, **Fei Li**, **Robert H. Edwards**

Departments of Physiology and Neurology, UCSF School of Medicine

Abstract

The transport of classical neurotransmitters into synaptic vesicles generally relies on a H^+ electrochemical gradient ($\Delta\mu_{H+}$). Synaptic vesicle uptake of glutamate depends primarily on the electrical component $\Delta\psi$ as the driving force, rather than the chemical component ΔPH. However, the vesicular glutamate transporters (VGLUTs) belong to the solute carrier 17 (SLC17) family, which includes closely related members that function as H^+ cotransporters. Recent work has also shown that the VGLUTs undergo allosteric regulation by H^+ and Cl^-, and exhibit an associated Cl^- conductance. These properties appear to coordinate VGLUT activity with the large ionic shifts that accompany the rapid recycling of synaptic vesicles driven by neural activity. Recent structural information also suggests common mechanisms that underlie the apparently divergent function of SLC17 family members, and that confer allosteric regulation.

Introduction: The Synaptic Vesicle Cycle

Glutamate serves as the principal excitatory neurotransmitter. Release by exocytosis requires transport into synaptic vesicles by the vesicular glutamate transporters (VGLUTs), which are members of the solute carrier 17 (SLC17) gene family. In contrast to most plasma membrane transporters and channels which rely for their driving force on a relatively stable ionic gradient of Na^+, K^+ or Cl^-, the VGLUTs, like other endosomal transporters including the vesicular neurotransmitter transporters for monoamines and acetylcholine (SLC18) and for the inhibitory transmitters GABA and glycine (SLC32), rely on a H^+ electrochemical gradient ($\Delta\mu_{H+}$) [1]. Made by the vacuolar-type H^+-ATPase, $\Delta\mu_{H+}$ provides an ideal driving force for rapidly recycling synaptic vesicles. Under physiological conditions, free H^+ occur at concentrations (pH 7 is 0.1 μM) much lower than the other major inorganic ions (high millimolar), enabling transmembrane gradients of a similar magnitude at much lower absolute concentrations. The regeneration of $\Delta\mu_{H+}$ after endocytosis thus requires translocation of fewer H^+ and consumes less energy. At the same time, these rapid changes

Address correspondence to R.H. Edwards at, Departments of Neurology and Physiology, UCSF School of Medicine, 600 16th St., GH-N272B, San Francisco, CA 94158-2517, (415) 502-5687, robert.edwards@ucsf.edu.

Declaration of interests

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in driving force and associated ionic conditions impose a series of constraints on the process of vesicle filling, particularly at high rates of release. How do synaptic vesicles fill rapidly despite these changing conditions? The high lumenal concentrations of transmitter achieved also require displacement of the external ions trapped during endocytosis. These constraints are particularly problematic for the VGLUTs.

In contrast to the SLC18 and SLC32 families of vesicular neurotransmitter transporters which exchange lumenal H^+ for cytosolic transmitter and hence depend primarily on the chemical component of $\Delta\mu_{H+}$ (ΔPH), the VGLUTs depend primarily on the membrane potential ($\Delta\psi$) [2, 3], The absence of ΔPH limits the activity of SLC18 and 32 proteins after exocytosis, but resting membrane potential should in principle drive non-vesicular glutamate efflux [4], potentially reducing the high signal:noise conferred by quantal release. Thus, the VGLUTs require a mechanism to regulate their activity at the cell surface.

The mammalian SLC17 gene family comprises 9 members (SLC17A1-9). All transport anions, but both substrates and transport mechanism diverge widely. SLC17A1-4 were originally identified as Na^+-coupled phosphate transporters [5] but subsequently shown to recognize organic anions (e.g., penicillin, probenecid and uric acid) [6, 7]. SLC17A5 (known as sialin) shows high sequence similarity to the VGLUTs but transports sialic acid and other monocarboxylic acids out of the lysosome driven by the electroneutral cotransport of one H^+[8-11].

Mammals express three VGLUT isoforms--VGLUT1 (SLC17A7), VGLUT2 (SLC17A6) and VGLUT3 (SLC17A8). The three isoforms differ in their distribution, with VGLUT1 expressed primarily in the cortex and hippocampus, at synapses with a low probability of release, and VGLUT2 by cells in the diencephalon and brainstem, with a higher release probability [12]. There have been no reports suggesting that the isoforms differ substantially in transport mechanism, consistent with high sequence similarity in the transmembrane domains. However, their sequences diverge at cytosolic N- and C-termini, suggesting differences in trafficking or regulation. Indeed, the isoforms appear to differ in both endocytosis and exocytosis [13-15]. Originally thought only to correlate with the probability of neurotransmitter release, or to reflect localization on different synaptic vesicles, the isoforms have more recently been suggested to regulate release probability [13]. Closely related in function to the VGLUTs, SLC17A9 encodes a vesicular ATP transporter that is also driven by $\Delta\psi$ [16].

Since the demonstration of glutamate uptake by synaptic vesicles 35 years ago [2, 17, 18], many properties have been ascribed to the VGLUTs. Studies using synaptic vesicles and heterologous expression systems have reported multiple substrates and allosteric effectors, but the basic mechanism of transport has proven elusive until the recent development of methods to characterize their function as well as structure.

Substrate Recognition by SLC17

The Km for glutamate uptake by synaptic vesicles has generally been reported ~1 mM [2, 19-22], a relatively high Km that is nonetheless in the range of the glutamate concentration

in the presynaptic cytoplasm [23]. In this respect, the VGLUTs differ from the Na^+-coupled plasma membrane excitatory amino acid transporters (EAATs) that belong to a structurally different family (SLC1) [24]. The EAATs have a low micomolar Km for glutamate, allowing them to efficiently remove released glutamate from the synapse to nanomolar concentrations. However, a few studies have suggested that under certain conditions, the VGLUTs may have a much lower (low micromolar) Km [17, 25]. In addition, the VGLUTs do not recognize aspartate [19, 26-28], which is transported by the EAATs. Interestingly, recent work using purified protein and *in vitro* reconstitution has suggested that, in contrast to the VGLUTs, Sialin can transport aspartate as well as glutamate, suggesting a role in excitatory synaptic transmission [29]. However, loss of VGLUT1 essentially eliminates excitatory transmission in the hippocampus, and the residual transmission shows no alteration in the AMPA:NMDA ratio [30], which would be predicted since aspartate preferentially activates NMDA receptors. The ability of Sialin to function as a VGLUT-like transporter under physiological conditions thus remains unclear. Other work has suggested the storage of aspartate in synaptic vesicles, but this was independent of Sialin [31], and the mechanism responsible remains unknown.

Consistent with their high specificity, the VGLUTs recognize multiple features of the substrate. The -NH2 group of glutamate has a critical role since the VGLUTs do not recognize linear dicarboxylic acids lacking this group such as glutarate and alpha-ketoglutarate, even as inhibitors [19]. Similarly, replacing the side chain carboxylate with an amide (in glutamine) or removal of the α–carboxylate (in GABA) also eliminate recognition. Thus, all three charged functional groups have an important role. The distance between them is also important since aspartate is not recognized. However, addition of a methyl or methylene group off the carbon backbone is tolerated, with little change in apparent affinity relative to glutamate [19, 27, 32]. In addition, a series of conformationally restricted cycloglutamates are all transported by the VGLUTs. Synaptic vesicles accumulate 1-aminocyclobutane-trans-1,3-dicarboxylic acid, 1-aminocyclopentane-trans-1,3-dicarboxylic acid, and 1-aminocyclohexane-trans-1,3-dicarboxylic acid with an apparent affinity higher than for glutamate [27, 32, 33]. On the other hand, the cis configuration of these compounds is not recognized. The VGLUTs thus require the trans conformation as well as a length that equals or exceeds 5 carbons.

Until recently, we knew little about the recognition of glutamate by individual residues in the VGLUTs. Only a few mutational studies have been carried out and the limited signal:noise of most available assays makes it difficult to ascribe defects in transport to substrate recognition [25, 34, 35]. However, we recently obtained two crystal structures of DgoT, a bacterial member of the SLC17 transporter family showing 24% sequence identity with the mammalian VGLUTs [36]. This protein was shown to catalyze galactonate:H^+ symport, similar to Sialin and distinct from the VGLUTs. Two structures were determined: an inward open apo structure (i.e., without ligand) and an outwardly oriented, occluded structure with D-galactonate. Like other major facilitator superfamily (MFS) proteins, DgoT contains two six-helix bundles (N- and C-domains) which reorient to expose binding sites to the two sides of the membrane [36].

Since the VGLUTs and DgoT show considerable sequence similarity at the substrate binding pocket, the galactonate-bound structure of DgoT suggests which residues are most likely to interact with glutamate in the VGLUTs (Figure 1). In DgoT, the carboxyl group of galactonate points towards the N-domain where it interacts with three residues: R47 (TM1), through an electrostatic interaction, and two surrounding tyrosines (Y44 in TM1 and Y79 in TM2), through hydrogen bonds. All three of these residues are conserved in Sialin, consistent with recognition of the carboxyl group of sialic acid and related substrates. In the VGLUTs, the arginine in TM1 and tyrosine in TM2 are conserved but the TM1 tyrosine of DgoT and Sialin is replaced by the phenylalanine, which cannot form a hydrogen bond with the substrate carboxyl. This might help to explain the lower apparent affinity of the VGLUTs relative to DgoT and Sialin. Previous work supports the importance of the TM1 arginine, as replacement of this residue eliminates transport by VGLUT1-3 [25] and Sialin [37]. Similarly, replacement of the TM1 arginine inactivates DgoT [36]. Interestingly, SLC17A3 and A4 contain an asparagine and glutamine at this position rather than arginine, and at least several of their substrates lack a carboxyl group [38, 39].

In addition to residues interacting with the carboxyl group of galactonate, DgoT contains four residues that make 6 hydrogen bonds with hydroxyl groups on the substrate [36]. These include glutamines in TM5 and 7, a serine in TM10 and asparagine in TM11. This tight coordination presumably contributes to the relatively high apparent affinity of DgoT for galactonate (Km ~20 μM) [36]. Remarkably, DgoT fails to recognize low millimolar concentrations of the epimer gluconate even as an inhibitor, demonstrating extraordinary substrate selectivity.

In contrast to the substrates of DgoT and sialin, glutamate contains two carboxyl groups. Consistent with this, the VGLUTs contain an arginine in TM7 of the C-domain at the level of the substrate binding pocket, a residue highly conserved among the VGLUTs but absent from other SLC17 family members. This residue is required for VGLUT activity [25, 34], presumably to recognize one of the two carboxyls.

Using a VGLUT homology model based on the outward occluded structure of DgoT, the lowest energy state of VGLUT2 with glutamate docked involves coordination of the α-carboxyl by R322 and of the amine group by hydrogen bonding to S323, which is specific to the VGLUTs. The side chain carboxyl is coordinated by both R88 and Y135, similar to galactonate. The predicted distance between R88 and R322 presumably accounts for the inability of VGLUTs to recognize aspartate.

Other VGLUT Ligands

In contrast to vesicular monoamine and ACh transporters, we lack potent, specific inhibitors of the VGLUTs. Azo dyes such as Evans blue, Trypan Blue, and Brilliant Yellow potently and competitively inhibit the VGLUTs, but seem likely to interact with other proteins as well [40-42]. Indeed, multiple members of SLC17 show sensitivity to Evans Blue [16, 43]. These dyes are also cell impermeant, limiting their use with cells. They are thus used primarily for uptake assays with purified synaptic vesicles or proteoliposomes where they can be added directly to the cytoplasmic face of the vesicle membrane. The cell-permeant

dye Rose Bengal also inhibits vesicular glutamate transport but since it is not a competitive inhibitor [44], it remains unclear whether it acts specifically on the VGLUTs. Clodronate potently inhibits the purified, reconstituted vesicular nucleotide transporter [45] but at high concentrations also inhibits the VGLUTs.

The VGLUTs were originally identified as phosphate (Pi) transporters due to their sequence similarity to NaPi-1 (SLC17A1) which had been shown to confer Na^+-dependent Pi uptake when expressed in *Xenopus* oocytes [5]. VGLUT1 and 2 (previously known as brain-specific and differentiation-specific Pi transporters) apparently conferred Na^+-dependent Pi uptake across the plasma membrane [46, 47]. However, the VGLUTs reside primarily on intracellular membranes, and even NaPi-1 was subsequently shown to recognize organic anions [6, 7], suggesting that all SLC17 family members transport organic anions rather than Pi. The VGLUTs are clearly required for glutamatergic neurotransmission [48, 49]. It was therefore surprising that purified VGLUT2 reconstituted into liposomes mediates Pi uptake [35]. In this case, Pi transport was also found to depend on Na^+. Remarkably, however, this activity was not sensitive to mutation of any residues known to be important for glutamate transport [35]. Another more recent study has reported inhibition of synaptic vesicle glutamate uptake and acidification by high concentrations of Pi [50]. Further, they showed that synaptic vesicles and purified, reconstituted VGLUT take up Pi through a mechanism dependent on $\Delta\mu_{H+}$, and heterologous expression of internalization-defective VGLUT confers Pi uptake across the plasma membrane dependent on a Na^+ gradient [50]. However, inhibition of glutamate uptake by Pi should not be accepted as unequivocal evidence for recognition of Pi as substrate since Pi is a product of the V-type ATPase and high concentrations may thus impair ATP hydrolysis and generation of$\Delta\mu_{H+}$. In addition, we found no inhibition of glutamate uptake by Pi in a heterologous expression system [26] and currents due to Pi flux were not detected by patch clamp recording of endosomes expressing VGLUT1 [25], a preparation that shows currents for glutamate. Although Pi uptake has now been reported for multiple SLC17 family members (SLC17A1, A4 and several plant transporters) [51, 52], the physiological role of Pi transport by these and other SLC17 family members remains unclear.

Multiple Modes of Interaction with Chloride

Allosteric Activation from the Cytosol

Early studies on synaptic vesicles showed stimulation of glutamate uptake by the inclusion of low millimolar (2-10 mM) chloride in the reaction buffer [19]. However, the effect of chloride is biphasic, with higher concentrations inhibiting glutamate uptake [19, 21, 27, 53]. After identification of the VGLUTs, the same stimulatory effect of chloride was observed for heterologous expression [20, 26] and for purified VGLUT reconstituted into liposomes [35, 54-56], suggesting that it reflects a direct interaction with the transporters. The interaction with cytosolic Cl^- was further defined as overlapping with a 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)-binding site on the VGLUTs [57]. A generic anion carrier blocker, DIDS inhibits glutamate uptake into synaptic vesicles and this inhibition can be prevented by incubation in high Cl^-, suggesting that the two anions compete for the same site which is presumably allosteric with respect to the substrate

binding site [57]. Endogenous ketone bodies have been proposed to inhibit VGLUT activity by preventing allosteric activation by Cl^-, suggesting a mechanism for use of the ketogenic diet in refractory epilepsy [55]. Dependence on Cl^- and sensitivity to DIDS as well as Evans Blue also appear to define the SLC17 family, including recently described members from plants [58]. However, it is important to note that both vesicular monoamine and GABA transporters also exhibit a biphasic response to Cl^- [59, 60]. In these cases, increasing Cl^- may activate transport by promoting the formation of ΔPH and high Cl^- may inhibit transport by dissipating required $\Delta\psi$.

The inhibition of VGLUTs by high Cl^- has also been thought to reflect loss of the driving force $\Delta\psi$. However, several lines of evidence raise the possibility that high Cl^- may also have a direct effect on the VGLUTs. First, the biphasic effect of Cl^- depends on the glutamate concentration. With higher glutamate, the response to Cl^- shifts to higher concentrations and the inhibitory effect of chloride becomes less pronounced [19, 21], which would not be expected if Cl^- affected only the driving force. In addition, whole endosome recording of VGLUT currents in transfected HEK cells, under voltage clamp conditions that control $\Delta\psi$, shows that increasing the cytosolic Cl^- from 1 to 10 mM increases the Km for glutamate [25]. The analysis of glutamate uptake by synaptic vesicles also supports an effect of Cl^- independent from the driving force. High Cl^- (80 mM) increases the Km for glutamate relative to 4 mM Cl^-, despite the use of a H^+/K^+ ionophore to maintain constant $\Delta\psi$ across experiments [21]. Thus, in addition to indirect effects of Cl^- through $\Delta\mu_{H+}$, the inhibitory effect of high Cl^- on glutamate transport reflects at least in part competition with glutamate for binding, an effect distinct from the allosteric effect of cytosolic Cl^-.

Chloride Conductance and Allosteric Activation from the Lumen

In addition to the allosteric effects of Cl^- on VGLUT activity, competition with glutamate for flux and indirect effects on the driving force for glutamate transport, several studies have now shown that the VGLUTs exhibit an associated Cl^- conductance. This possibility was first suggested by the association of a Cl^- conductance with NaPi-1 (SLC17A1) [7]. With identification of the VGLUTs, it then became possible to show that heterologous expression increased the chloride-and H^+-ATPase-dependent acidification of PC12 cell vesicles [26]. Corroborating the associated Cl^- conductance, synaptic vesicles from VGLUT1 KO mice showed less acidification by Cl^- relative to wild-type synaptic vesicles [54]. In addition, purified VGLUT conferred a Cl^- permeability when reconstituted into proteoliposomes with a H^+-ATPase [54, 56]. Furthermore, uptake of glutamate into VGLUT proteoliposomes was stimulated by high luminal chloride [34, 54, 56]. However, the reliance on coreconstitution with a H^+-ATPase makes it difficult to infer direct ionic effects on the VGLUTs: ionic gradients influence the expression of $\Delta\mu_{H+}$ as ΔPH and $\Delta\psi$. The efflux of lumenal Cl^- may thus promote the formation of the driving force $\Delta\psi$.

To disentangle the role of ions in driving force and allosteric regulation, we used voltage clamp recordings from cells overexpressing the VGLUTs. Voltage clamp eliminates the potential for indirect effects of both H^+ and Cl^- on $\Delta\psi$. In *Xenopus* oocytes, the VGLUTs were targeted to the plasma membrane by inactivating multiple endocytosis motifs at both N- and C-termini [34], exposing the luminal face of VGLUT to the extracellular medium.

Expression of all three mammalian isoforms conferred robust currents with multiple interesting features. First, the conductance required low external (topologically equivalent to lumenal) pH for activation [34]. The currents activated at just below pH 7 and the EC_{50} for H^+ was ~pH 5.5, very close to the pH of synaptic vesicles [61]. Second, the conductance was also activated by external Cl^-. In synaptic vesicles, endocytosis presumably traps the external Cl^- that confers this activation. However, lumenal Cl^- seems less important than H^+ since some currents persist at low pH in the absence of Cl^-. Third, the conductance was specific for Cl^-, not H^+: the reversal potential shifted only with a change in Cl^- [34]. However, the conductance is both activated by and permeable to Br^- as well as Cl^-. Previous work had indeed shown that among halides, only these two stimulate glutamate uptake by synaptic vesicles [19] and purified, reconstituted VGLUT [35]. For VGLUTs expressed at the plasma membrane, the conductance also rectified, with little outward current (due to Cl^- influx) at positive cytoplasmic potentials and progressively larger inward currents (due to Cl^- efflux) at negative cell membrane potentials. At the synaptic vesicle, this rectification would correspond to greater rates of Cl^- influx than efflux. However, the actual direction of flux would depend on both the Cl^- gradient and electrical potential across the synaptic vesicle membrane and might be directed out of the vesicle, driven by the Cl^- trapped in the lumen during endocytosis.

Although demonstrated using the associated Cl^- conductance, the gating by H^+ and Cl^- corresponds closely with that required for activation of vesicular glutamate transport. The common properties of the Cl^- conductance and glutamate transport suggest overlap between the two permeation pathways but their relationship remained unclear because we could not detect outward currents produced by the uptake of external glutamate by *Xenopus* oocytes, and we could not control cytosolic glutamate to assess the role of glutamate efflux in the inward currents [34]. However, parallel patch clamp experiments with VGLUT misexpressed at the surface of HEK cells showed the chloride conductance even in the absence of cytosolic glutamate. The Cl^- conductance is thus not obligatorily coupled to glutamate flux. However, high concentrations of external glutamate inhibited the VGLUT-associated currents in oocytes, consistent with competition between the organic and inorganic anions.

To detect currents due to glutamate flux, we expressed the wild-type transporters in HEK cells and made patch clamp recordings from enlarged endosomes. This enabled us to manipulate the solution facing the cytosolic side of the membrane and revealed glutamate as well as Cl^- currents. These currents showed properties similar to those found in *Xenopus* oocytes: dependence on both cytosolic and lumenal Cl^- for allosteric activation, specificity for Br^- as well as Cl^- and sensitivity to known VGLUT inhibitors such as Evans Blue and DIDS [25]. However, direct comparison of Cl^- and glutamate currents illustrated one important difference. The glutamate currents saturated at low millimolar concentrations of glutamate. In contrast, the Cl^- currents did not saturate through the high millimolar concentrations tested [25]. This difference in saturation suggests that glutamate permeates through the alternating access mechanism of a transporter, and Cl^- through the pore of a channel. Although competitive, and hence likely to involve related conduction pathways, the two anions may thus use distinct mechanisms for translocation. The ability to detect Cl^- but not glutamate flux at the plasma membrane further supports channel-like activity for Cl^-.

In addition, experiments with both whole-cell and endosome patch clamp recordings have identified the site required for allosteric regulation by lumenal Cl^-. In contrast to wild-type VGLUT, which shows no currents in the absence of lumenal Cl^-, the replacement by alanine of a highly conserved TM4 arginine (R184 in VGLUT2, Figure 1) shows both Cl^- and glutamate currents in the absence of lumenal anion [25]. Thus, neutralization of this residue eliminates the requirement for lumenal Cl^-, providing strong evidence that lumenal Cl^- confers allosteric activation by binding to this site. The electrophysiology (in oocytes as well as whole endosomes) also showed that the highly conserved arginine in TM7 of the VGLUTs (R322 in VGLUT2, Figure 1) is required for both glutamate and Cl^- currents [25, 34]. As noted above, this residue was predicted by the modelling to bind the α-carboxyl of glutamate (Figure 1).

Although demonstrated for NaPi-1 (SLC17A1) as well as the VGLUTs, a Cl^- conductance is not a common feature of the entire SLC17 family. In particular, the close VGLUT relative Sialin does not produce any currents coupled to transport (due to the electroneutral cotransport of sialic acid and a single H^+) or uncoupled currents [34]. In the VGLUTs, the similar selectivity for allosteric activation and permeation (Cl^- and Br^-) suggest that the site of allosteric activation (e.g., the TM4 arginine) may lie on the conduction pathway. In the DgoT structures, the TM4 arginine lies within the N-domain but near the main cavity. We do not know its role in galactonate transport, but DgoT activity does not require Cl^- [36]. The DgoT structure nonetheless suggests two water-permeable pathways. The substrate translocation pathway lies in the main cavity between N- and C-domains of DgoT. In addition, the N-domain contains a tunnel from the periplasm to a polar pocket within the N-domain [36]. Although glutamate and Cl^- compete for permeation, the Cl^- currents observed with mutation of the TM4 arginine suggest that this residue does not lie along the conduction pathway. The TM4 arginine thus appears to exert a strictly allosteric effect. In contrast, mutation of both the TM1 and TM7 arginines eliminates the Cl^- conductance as well as glutamate transport, suggesting that Cl^- permeates through the main cavity. In this regard, it is interesting to note that the structurally unrelated EAATs also have a Cl^- conductance, but the pathway appears separate from that involved in glutamate translocation [62, 63].

Taken together, the VGLUTs appear to interact with Cl^- in three distinct modes. First, cytosolic Cl^- activates glutamate transport with an optimum ~2-10 mM. This allosteric mode of activation has been observed with synaptic vesicles, functional reconstitution of purified protein into proteoliposomes, and whole endosome recording. Second, the VGLUTs exhibit an associated Cl^- conductance that indirectly influences vesicle acidification but has also been documented by direct voltage clamp recording in oocytes and patch clamp recording in whole cells and endosomes. Third, a lower affinity site involving the TM4 arginine mediates allosteric activation by lumenal Cl^- of both glutamate transport and the Cl^- conductance.

What do the interactions with Cl^- tell us about the regulation of vesicular glutamate transport during the synaptic vesicle cycle? Indeed, direct manipulation of presynaptic Cl^- at the calyx of Held shows the same biphasic effect of Cl^- observed biochemically *in vitro* [22]. In addition, the cytoplasmic Cl^- concentration at a central synapse has been estimated

at 21 mM [64], around the concentration optimal for VGLUT activity. Changes in presynaptic Cl^- will thus change the filling of synaptic vesicles with glutamate and for the many synapses where receptors are not saturated, the response to a single vesicle, or quantal size [65, 66]. Cytoplasmic Cl^- changes during neural development, but we do not know whether presynaptic Cl^- changes as a function of activity.

In contrast, the luminal side of the synaptic vesicle experiences large changes in Cl^- concentration as the recycling synaptic vesicle membrane captures the high concentrations of Cl^- found outside the cell. Since glutamate fills synaptic vesicles to similar concentrations (100-300 mM), the Cl^- trapped during endocytosis must leave the vesicle to prevent hyperosmotic conditions due to the influx of glutamate, and in non-neural cells, the lumenal chloride concentration has been shown to drop at endocytosis [67, 68]. The VGLUT-associated Cl^- conductance may enable this effective exchange of anions, and recent work monitoring synaptic vesicle acidification has supported this possibility: efforts to inactivate selectively either glutamate transport or the associated Cl^- conductance perturb vesicle acidification in a way that suggests the equal and opposite flux of Cl^- and glutamate, consistent with replacement of lumenal Cl^- by glutamate [69]. However, the pharmacology used to distinguish between these two functions of the VGLUTs remains poorly understood and the selectivity uncertain. Elucidating the physiological role of the Cl^- conductance associated with vesicular glutamate transport thus awaits tools to manipulate the two activities independently and monitor directly the effects on glutamate release. Further, we do not know whether other channels influence lumenal Cl^- and in the absence of information about lumenal Cl^- and membrane potential, it is also difficult to predict the direction of Cl^- flux mediated by the VGLUTs.

What then is the role of allosteric regulation by lumenal Cl^-? This remains to be determined, but the efflux that occurs as synaptic vesicles fill with glutamate may reduce Cl^- below the amounts required for glutamate transport activity. Inactivation of glutamate transport may then set the upper limit of lumenal glutamate, and also prevent the energetically wasteful efflux of loaded glutamate, a possibility supported by whole endosome recording [25].

Protons

Despite considerable progress, the role of pH in vesicular glutamate uptake remains a mystery. The closest mammalian relative to the VGLUTs, Sialin catalyzes electroneutral H^+:sialic acid symport [10, 11]. DgoT has a similar mechanism, although dependence on a negative membrane potential suggests a H^+:galactonate stoichiometry >1:1 [36]. The VGLUTs must operate differently since they transport glutamate into synaptic vesicles against a H^+ gradient. However, whether the VGLUTs couple the flux of H^+ to glutamate remains unresolved.

Multiple studies have reported that a pH gradient and low lumenal pH are not required for vesicular glutamate uptake. Agents that dissipate ΔPH (e.g., the K^+/H^+ ionophore nigericin or ammonium chloride) and hence alkalinize the lumen stimulate glutamate uptake by synaptic vesicles, supporting the role of $\Delta\psi$ (rather than ΔPH) as driving force, in contrast to other vesicular transporters that exchange H^+ for cytosolic transmitter [2, 27, 70, 71].

Purified, reconstituted VGLUT has also been reported to require only $\Delta\psi$ for activity [55]. This mechanism has been suggested to make sense because a lumenal glutamate concentration ~200 mM and cytosolic glutamate ~10 mM predict a gradient ~20-fold which could be accommodated by a $\Delta\psi$ ~+80 mV. In contrast, glutamate/H^+ exchange with $\Delta\psi$ ~ +80 mV and ΔPH ~2 predicts a much larger concentration gradient ~40,000-fold that would cause osmotic stress.

On the other hand, several studies support a role for lumenal H^+ in vesicular glutamate transport. In experiments using both synaptic vesicles [21, 53] and purified, reconstituted VGLUT [34, 35, 54], dissipation of ΔPH reduces glutamate uptake. Indeed, one of the protonatable residues required for galactonate transport by DgoT (glutamate 133 in TM4, fig 1E) shows conservation to Sialin and the VGLUTs [36].

Several studies suggest that Cl^- regulates the role of lumenal H^+ in vesicular glutamate transport. At low millimolar cytosolic Cl^-, dissipation of ΔPH stimulates glutamate uptake whereas at higher Cl^-, loss of ΔPH inhibits [21, 53, 54]. Cytosolic Cl^- is well known to function as a shunt to dissipate the $\Delta\psi$ formed by the V-ATPase, thereby disinhibiting the H^+ pump to increase ΔPH. In this respect, ΔPH affects glutamate transport differently depending on the magnitude of ΔPH. At low Cl^-, with small ΔPH, dissipation of ΔPH serves mainly to increase the driving force $\Delta\psi$, thereby promoting glutamate uptake. At high Cl^- (large ΔPH), dissipation of ΔPH inhibits glutamate uptake. A large pH gradient thus appears to promote glutamate transport but it has been difficult to quantify $\Delta\psi$. Since dissipation of ΔPH also increases lumenal pH, absolute pH rather than a pH gradient may be responsible for the stimulatory effect of vesicle acidification on glutamate uptake. Consistent with this possibility, we recently found that luminal H^+ regulate VGLUT activity through an allosteric mechanism [34]. Several lines of evidence support this. First, as noted above, external (i.e., lumenal) H^+ activate the Cl^- conductance associated with expression of VGLUTs at the surface of *Xenopus* oocytes [34]. In this preparation, low external pH activates the currents without affecting their reversal potential (i.e., without permeation). Thus, in contrast to Cl^- that both allosterically activates and permeates the VGLUTs, luminal H^+ only allosterically activate. Importantly, the currents develop only as external pH drops below physiological, suggesting activation only in acidic, intracellular membranes such as synaptic vesicles. In addition, we found that glutamate transport by VGLUT reconstituted into liposomes depends on low lumenal pH even in the absence of ΔPH [34]. In particular, transport was maximal at pH 6.0 but $\Delta\psi$ was still required to drive transport. Further supporting this conclusion, dissipation of ΔPH was shown to stimulate synaptic vesicle glutamate uptake to a greater extent at pH 6.8 than pH 7.4 [53]. Lumenal pH thus has an important allosteric role, along with $\Delta\psi$ as the driving force.

What is the physiological role of allosteric activation by lumenal H^+? Since the resting membrane potential of the cell provides a strong driving force for glutamate transport by the VGLUTs, regulation by pH might be a way to prevent transport activity at the cell surface, after synaptic vesicle exocytosis and before endocytosis. To test this possibility, we expressed an internalization-defective form of VGLUT on the surface of hippocampal neurons and recorded postsynaptic currents [34]. At neutral pH, we detected no efflux of glutamate but lowering external pH to 6.0 evoked large AMPA receptor currents. Allosteric

activation by H^+ may thus serve to prevent non-vesicular glutamate efflux by the VGLUTs, which would degrade the quantal signal produced by exocytic release.

However, the requirement for allosteric activation by H^+ is not consistent with observations made using SVs, in particular the increase in transport with dissipation of ΔPH. One possibility is that synaptic vesicles can sustain a larger $\Delta\psi$ than reconstituted proteoliposomes. Thus, the conversion from ΔPH to $\Delta\psi$ may be more efficient in synaptic vesicles. In principle, the V-ATPase can make ΔPH of 4.6 [72], far in excess of that observed in synaptic vesicles with a lumenal pH ~5.6 [61, 73, 74] and hence ΔPH ~1.7. A pH gradient ~4.6 corresponds to $\Delta\psi$ ~270 mV, but we do not know the membrane potential actually achieved by synaptic vesicles in neurons or by proteoliposomes *in vitro*. In other major facilitator superfamily transporters such as *E. coli* lac permease, the stoichiometry of H^+ coupling increases at higher pH [75, 76], setting a precedent for changes in the role of H^+ that might contribute to the anomalous behavior of VGLUTs. It is also possible that lumenally positive $\Delta\psi$ can promote protonation despite high pH, a phenomenon observed in the H^+ symporter cystinosin [77, 78].

Since the driving force for VGLUTs derives primarily if not exclusively from $\Delta\psi$, the mechanism appears to involve uniport, i.e., the carrier mediates net flux of only glutamate and its associated negative charge. Comparison of individual glutamatergic and GABAergic synaptic vesicles indeed supports H^+ exchange by the vesicular GABA but not glutamate transporters [70]. However, it is at this point difficult to exclude a role for H^+ exchange: at high glutamate and/or Cl^-, dissipation of ΔPH reduces glutamate uptake [21]; the effect of ΔPH is pronounced in proteoliposomes [34, 54]; even the glutamate efflux triggered by lowering external pH is consistent with a H^+ exchange mechanism [34]. Comparison to other vesicular neurotransmitter transporters shows the VGLUTs are more electrogenic but even a H^+: glutamate stoichiometry of 1:1 would move +2 net charge out of the synaptic vesicle, in contrast to the vesicular monoamine and GABA transporters that move +1 charge, so a strong dependence on membrane potential does not unequivocally demonstrate uniport. It is also difficult to interpret the inhibition by high external Cl^- (which has been suggested to indicate a lack of coupling to H^+) because Cl^- competes with glutamate for permeation [25, 26]. Recent work has inferred H^+ exchange from the apparent acceleration of synaptic vesicle acidification in the absence of VGLUT1 [69]. However, glutamatergic synaptic vesicles have a substantially lower pH (~5.8) than GABAergic vesicles (~6.4), which do exhibit GABA:H^+ exchange [70, 79], suggesting that the VGLUTs do not mediate H^+ exchange. To analyze acidification directly, Martineau et al. used acid to quench surface reporter and observed what they thought was immediate acidification of the internalizing membrane [69]. However, loss of VGLUT1 impairs the endocytosis of VAMP2, the v-SNARE used as a reporter in these experiments [80], and immediate quenching of the increased cell surface VAMP2 by external acid provides a more likely explanation than instantaneous acidification of internalized membrane. As a result, there is no evidence that VGLUTs mediate H^+ exchange although we also cannot exclude this possibility.

Monovalent cations

Monovalent cations stimulate glutamate uptake into synaptic vesicles [71]. This has been shown to occur through a proton cation exchange mechanism that converts ΔPH into Δψ, thereby increasing the driving force for glutamate uptake. Taking advantage of the ability to manipulate presynaptic conditions at the calyx of Held, Huang and Trussell have also shown that cytoplasmic cation concentration influences quantal size [71, 81]. Further, presynaptic Na^+ can change due to influx through HCN channels, suggesting a mechanism for activity-dependent regulation of vesicle filling. Single synaptic vesicle fluorescent measurements have since corroborated the effect of cations on the synaptic vesicle electrochemical gradient [70]. This synaptic vesicle cation/H^+ exchange activity pharmacologically resembles an intracellular member of the Na/H exchanger family of transporters [71]. However, the effect of NHE inhibition on single synaptic vesicles has been ambiguous because it blocked only the effect of Na^+, not K^+, on ΔPH [70].

The molecular identity of the synaptic vesicle cation/H^+ exchanger remains unclear. NHE6-9 localize to intracellular membranes, and NHE6 and 9 to endosomes. NHE9 has been implicated in a recessive form of autism spectrum disorder [82], but the knockout does not affect stimulation of vesicular glutamate uptake by cations [83]. NHE6 localizes more specifically to synaptic vesicles [56, 84]. However, stimulation of glutamate uptake by cations has been observed with purified VGLUT after reconstitution with a bacterial F0F1-ATPase [56], suggesting a direct effect of monovalent cations on the glutamate transporter although it remains possible that cations activate the coreconstituted H^+-ATPase directly. On the other hand, we did not observe any effect of either Na^+ or K^+ on the VGLUT-associated Cl^- conductance in *Xenopus* oocytes [34].

Structural insight into mechanism

The recent structures of DgoT have begun to provide insight into the mechanism of vesicular glutamate transport. The SLC17 family contains a highly conserved glutamate in TM4 (Figure 1E). We presume that this glutamate mediates both the H^+ cotransport of sialin and DgoT. Its proximity to the TM1 arginine that recognizes substrate suggests a mechanism for coupling: protonation should disrupt the electrostatic interaction, liberating the arginine to recognize the substrate carboxyl. Conversely, deprotonation of this residue presumably enables the unloaded carrier to recycle. However, the VGLUTs must have lost the coupling to H^+ symport of DgoT (and Sialin) because the outwardly directed H^+ gradient would otherwise transport glutamate out of the synaptic vesicle. Since the TM4 glutamate shows conservation to the VGLUTs, it may serve as the site of allosteric activation by H^+. Interestingly, the SLC17 NPTs and vesicular nucleotide transporter (VNUT) lack a glutamate in TM4 and like the VGLUTs, appear driven by Δψ rather than ΔPH [7, 38]. We presume that they differ from VGLUTs and do not require allosteric activation by H^+ although this remains unknown. In addition to the TM4 glutamate, DgoT contains an aspartate in TM1 required for transport [36] that is missing from both Sialin and the VGLUTs. Since Sialin, like DgoT, mediates H^+ cotransport, we hypothesize that this aspartate contributes to translocation of the second H^+ by DgoT that confers net charge movement. In contrast, Sialin cotransports 1 H^+ with sialic acid, resulting in

electroneutrality [10, 11]. The arginine in TM4 that confers activation of the VGLUTs by Cl^- is also highly conserved in the SLC17 family. Since many other family members including DgoT and Sialin do not require Cl^-, it remains unclear what role this residue plays in those proteins. However, the TM4 arginine lies directly above the conserved TM4 glutamate and the TM1 arginine involved in substrate recognition. Interaction with Cl^- may therefore titrate the pKa of these residues, influencing protonation and vice versa.

In the VGLUT transport cycle, the binding of lumenal H^+ and Cl^- seem well positioned to liberate the TM1 arginine required for substrate recognition. In the case of DgoT and Sialin, the H^+ and substrate both translocate to the cytoplasmic face of the membrane, where they dissociate and the unloaded carrier then reorients to the lumen. In the case of VGLUTs, however, the H^+ do not translocate and dissociate with substrate, conferring only allosteric regulation.

Regulation of vesicular glutamate transport through the synaptic vesicle cycle

How do the properties of the VGLUTs coordinate vesicular glutamate flux with the synaptic vesicle cycle? Upon exocytosis, the reliance of VGLUTs on $\Delta\psi$ would result in non-vesicular glutamate flux, thereby degrading the signal conferred by quantal release (Fig. 2). Allosteric regulation of the VGLUTs by lumenal H^+ appears to prevent this by limiting their activity to acidic membranes such as synaptic vesicles. The binding of lumenal Cl^- has an equally important but distinct role. After endocytosis, the declining levels of lumenal Cl^- should limit the flux of glutamate late in vesicle filling, preventing energetically wasteful leakage. The anion conductance enables the Cl^- efflux required for its replacement by glutamate.

The mechanistic information obtained from structural and biophysical analysis of the VGLUTs now provides us with tools to explore the role of these regulatory mechanisms in the synaptic vesicle cycle and the quantal release of glutamate required for excitatory neurotransmission.

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References

[1]. Schuldiner S, Shirvan A, Linial M, Vesicular neurotransmitter transporters: from bacteria to humans., Physiol. Rev, 75 (1995) 369–392. [PubMed: 7724667]

[2]. Maycox PR, Deckwerth T, Hell JW, Jahn R, Glutamate uptake by brain synaptic vesicles. Energy dependence of transport and functional reconstitution in proteoliposomes., J. Biol. Chem, 263 (1988) 15423–15428. [PubMed: 2902091]

[3]. Shioi J, Ueda T, Artificially imposed electrical potentials drive L-glutamate uptake into synaptic vesicles of bovine cerebral cortex, Biochem. J, 267 (1990) 63–68. [PubMed: 1970243]

[4]. Mackenzie B, Illing AC, Morris ME, Varoqui H, Erickson JD, Analysis of a vesicular glutamate transporter (VGLUT2) supports a cell-leakage mode in addition to vesicular packaging, Neurochem. Res, 33 (2008) 238–247. [PubMed: 18080752]

[5]. Werner A, Moore ML, Mantei N, Biber J, Semenza G, Murer H, Cloning and expression of cDNA for a Na/Pi cotransport system of kidney cortex., Proc. Natl. Acad. Sci. USA, 88 (1991) 9608–9612. [PubMed: 1946375]

[6]. Bröer S, Schuster A, Wagner CA, Bröer A, Forster I, Biber J, Murer H, Werner A, Lang F, Busch AE, Chloride conductance and Pi transport are separate functions induced by the expression of NaPi-1 in Xenopus oocytes, J. Memb. Biol, 164 (1998) 71–77.

[7]. Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Broer S, Biber J, Murer H, Lang F, Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in Xenopus oocytes permeable for organic and inorganic anions, Proc. Natl. Acad. Sci. USA, 93 (1996) 5347–5351. [PubMed: 8643577]

[8]. Mancini GM, Beerens CE, Galjaard H, Verheijen FW, Functional reconstitution of the lysosomal sialic acid carrier into proteoliposomes, Proc. Natl. Acad. Sci. USA, 89 (1992) 6609–6613. [PubMed: 1631163]

[9]. Mancini GM, de Jonge HR, Galjaard H, Verheijen FW, Characterization of a proton-driven carrier for sialic acid in the lysosomal membrane. Evidence for a group-specific transport system for acidic monosaccharides, J. Biol. Chem, 264 (1989) 15247–15254. [PubMed: 2768261]

[10]. Morin P, Sagne C, Gasnier B, Functional characterization of wild-type and mutant human sialin, EMBO J., 23 (2004) 4560–4570. [PubMed: 15510212]

[11]. Wreden CC, Wlizla M, Reimer RJ, Varied mechanisms underlie the free sialic acid storage disorders, J. Biol. Chem, 280 (2005) 1408–1416. [PubMed: 15516337]

[12]. Fremeau RT Jr., Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, Bellocchio EE, Fortin D, Storm-Mathisen J, Edwards RH, The expression of vesicular glutamate transporters defines two classes of excitatory synapse, Neuron, 31 (2001) 247–260. [PubMed: 11502256]

[13]. Weston MC, Nehring RB, Wojcik SM, Rosenmund C, Interplay between VGLUT isoforms and endophilin A1 regulates neurotransmitter release and short-term plasticity, Neuron, 69 (2011) 1147–1159. [PubMed: 21435559]

[14]. Foss SM, Li H, Santos MS, Edwards RH, Voglmaier SM, Multiple dileucine-like motifs direct VGLUT1 trafficking, J. Neurosci, 33 (2013) 10647–10660. [PubMed: 23804088]

[15]. Li H, Santos MS, Park CK, Dobry Y, Voglmaier SM, VGLUT2 Trafficking Is Differentially Regulated by Adaptor Proteins AP-1 and AP-3, Front Cell Neurosci, 11 (2017) 324. [PubMed: 29123471]

[16]. Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H, Yamamoto A, Moriyama Y, Identification of a vesicular nucleotide transporter, Proc. Natl. Acad. Sci. U S A, 105 (2008) 5683–5686. [PubMed: 18375752]

[17]. Disbrow JK, Gershten MJ, Ruth JA, Uptake of L-[3H] glutamic acid by crude and purified synaptic vesicles from rat brain., Bioc. Biop. Res. Comm, 108 (1982) 1221–1227.

[18]. Naito S, Ueda T, Adenosine triphosphate-dependent uptake of glutamate into protein I-associated synaptic vesicles., J. Biol. Chem, 258 (1983) 696–699. [PubMed: 6130088]

[19]. Naito S, Ueda T, Characterization of glutamate uptake into synaptic vesicles, J. Neurochem, 44 (1985) 99–109. [PubMed: 2856886]

[20]. Bai L, Xu H, Collins JF, Ghishan FK, Molecular and functional analysis of a novel neuronal vesicular glutamate transporter., J. Biol. Chem, 276 (2001) 36764–36769. [PubMed: 11432869]

[21]. Wolosker H, de Souza DO, de Meis L, Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient, J. Biol. Chem, 271 (1996) 11726–11731. [PubMed: 8662610]

[22]. Hori T, Takahashi T, Kinetics of synaptic vesicle refilling with neurotransmitter glutamate, Neuron, 76 (2012) 511–517. [PubMed: 23141063]

[23]. Ishikawa T, Sahara Y, Takahashi T, A single packet of transmitter does not saturate postsynaptic glutamate receptors, Neuron, 34 (2002) 613–621. [PubMed: 12062044]

[24]. Bridges RJ, Esslinger CS, The excitatory amino acid transporters: pharmacological insights on substrate and inhibitor specificity of the EAAT subtypes, Pharmacol. Ther, 107 (2005) 271–285. [PubMed: 16112332]

[25]. Chang R, Eriksen J, Edwards RH, The dual role of chloride in synaptic vesicle glutamate transport, Elife, 7 (2018).

[26]. Bellocchio EE, Reimer RJ, Fremeau RTJ, Edwards RH, Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter., Science, 289 (2000) 957–960. [PubMed: 10938000]

[27]. Moriyama Y, Yamamoto A, Vesicular L-glutamate transporter in microvesicles from bovine pineal glands. Driving force, mechanism of chloride anion activation, and substrate specificity, J. Biol. Chem, 270 (1995) 22314–22320. [PubMed: 7673214]

[28]. Takamori S, Rhee JS, Rosenmund C, Jahn R, Identification of differentiation-associated brain-specific phosphate transporter as a second vesicular glutamate transporter., J. Neurosci, 21 (2001) RC182. [PubMed: 11698620]

[29]. Miyaji T, Omote H, Moriyama Y, Functional characterization of vesicular excitatory amino acid transport by human sialin, J. Neurochem, 119 (2011) 1–5. [PubMed: 21781115]

[30]. Herring BE, Silm K, Edwards RH, Nicoll RA, Is aspartate an excitatory neurotransmitter?, J. Neurosci, 35 (2015) 10168–10171. [PubMed: 26180193]

[31]. Morland C, Nordengen K, Larsson M, Prolo LM, Farzampour Z, Reimer RJ, Gundersen V, Vesicular uptake and exocytosis of L-aspartate is independent of sialin, FASEB J., 27 (2013) 1264–1274. [PubMed: 23221336]

[32]. Winter HC, Ueda T, Glutamate uptake system in the presynaptic vesicle: glutamic acid analogs as inhibitors and alternate substrates, Neurochem Res, 18 (1993) 79–85. [PubMed: 8096630]

[33]. Winter HC, Ueda T, The glutamate uptake system in presynaptic vesicles: further characterization of structural requirements for inhibitors and substrates, Neurochem Res, 33 (2008) 223–231. [PubMed: 17940888]

[34]. Eriksen J, Chang R, McGregor M, Silm K, Suzuki T, Edwards RH, Protons regulate vesicular glutamate transporters through an allosteric mechanism, Neuron, 90 (2016) 768–780. [PubMed: 27133463]

[35]. Juge N, Yoshida Y, Yatsushiro S, Omote H, Moriyama Y, Vesicular glutamate transporter contains two independent transport machineries, J. Biol. Chem, 281 (2006) 39499–39506. [PubMed: 17046815]

[36]. Leano JB, Batarni S, Eriksen J, Juge N, Pak JE, Kimura-Someya T, Robles-Colmenares Y, Moriyama Y, Stroud RM, Edwards RH, Structures suggest a mechanism for energy coupling by a family of organic anion transporters, PLoS Biol, 17 (2019) e3000260. [PubMed: 31083648]

[37]. Ruivo R, Sharifi A, Boubekeur S, Morin P, Anne C, Debacker C, Graziano JC, Sagne C, Gasnier B, Molecular pathogenesis of sialic acid storage diseases: insight gained from four missense mutations and a putative polymorphism of human sialin, Biol. Cell, 100 (2008) 551–559. [PubMed: 18399798]

[38]. Jutabha P, Kanai Y, Hosoyamada M, Chairoungdua A, Kim do K, Iribe Y, Babu E, Kim JY, Anzai N, Chatsudthipong V, Endou H, Identification of a novel voltage-driven organic anion transporter present at apical membrane of renal proximal tubule, J. Biol. Chem, 278 (2003) 27930–27938. [PubMed: 12740363]

[39]. Togawa N, Miyaji T, Izawa S, Omote H, Moriyama Y, A Na+-phosphate cotransporter homologue (SLC17A4 protein) is an intestinal organic anion exporter, Am. J. Physiol. Cell Physiol, 302 (2012) C1652–1660. [PubMed: 22460716]

[40]. Fykse EM, Fonnum F, Amino acid neurotransmission: dynamics of vesicular uptake., Neurochem. Res, 21 (1996) 1053–1060. [PubMed: 8897468]

[41]. Chaudhry FA, Edwards RH, Fonnum F, Vesicular neurotransmitter transporters as targets for endogenous and exogenous toxic substances, Ann. Rev. Pharmacol. Toxicol, 48 (2008) 277–301. [PubMed: 17883368]

[42]. Tamura Y, Ozkan ED, Bole DG, Ueda T, IPF, a vesicular uptake inhibitory protein factor, can reduce the Ca(2+)-dependent, evoked release of glutamate, GABA and serotonin, J. Neurochem, 76 (2001) 1153–1164. [PubMed: 11181835]

[43]. Miyaji T, Echigo N, Hiasa M, Senoh S, Omote H, Moriyama Y, Identification of a vesicular aspartate transporter, Proc. Natl. Acad. Sci. USA, 105 (2008) 11720–11724. [PubMed: 18695252]

[44]. Ogita K, Hirata K, Bole DG, Yoshida S, Tamura Y, Leckenby AM, Ueda T, Inhibition of vesicular glutamate storage and exocytotic release by Rose Bengal, J. Neurochem, 77 (2001) 34–42. [PubMed: 11279259]

[45]. Kato Y, Hiasa M, Ichikawa R, Hasuzawa N, Kadowaki A, Iwatsuki K, Shima K, Endo Y, Kitahara Y, Inoue T, Nomura M, Omote H, Moriyama Y, Miyaji T, Identification of a vesicular ATP release inhibitor for the treatment of neuropathic and inflammatory pain, Proc. Natl. Acad. Sci. U S A, 114 (2017) E6297–E6305. [PubMed: 28720702]

[46]. Ni B, Rosteck PR, Nadi NS, Paul SM, Cloning and expression of a cDNA encoding a brain-specific Na+-dependent inorganic phosphate cotransporter., Proc. Natl. Acad. Sci. USA, 91 (1994) 5607–5611. [PubMed: 8202535]

[47]. Aihara Y, Mashima H, Onda H, Hisano S, Kasuya H, Hori T, Yamada S, Tomura H, Yamada Y, Inoue I, Kojima I, Takeda J, Molecular cloning of a novel brain-type Na(+)-dependent inorganic phosphate cotransporter, J. Neurochem, 74 (2000) 2622–2625. [PubMed: 10820226]

[48]. Fremeau RT Jr., Voglmaier S, Seal RP, Edwards RH, VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate, Trends Neurosci., 27 (2004) 98–103. [PubMed: 15102489]

[49]. Wojcik SM, Rhee JS, Herzog E, Sigler A, Jahn R, Takamori S, Brose N, Rosenmund C, An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size, Proc. Natl. Acad. Sci. U S A, 101 (2004) 7158–7163. [PubMed: 15103023]

[50]. Preobraschenski J, Cheret C, Ganzella M, Zander JF, Richter K, Schenck S, Jahn R, Ahnert-Hilger G, Dual and Direction-Selective Mechanisms of Phosphate Transport by the Vesicular Glutamate Transporter, Cell Rep., 23 (2018) 535–545. [PubMed: 29642010]

[51]. Guo B, Jin Y, Wussler C, Blancaflor EB, Motes CM, Versaw WK, Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters, New Phytol., 177 (2008) 889–898. [PubMed: 18086223]

[52]. Pavon LR, Lundh F, Lundin B, Mishra A, Persson BL, Spetea C, Arabidopsis ANTR1 is a thylakoid Na+-dependent phosphate transporter: functional characterization in Escherichia coli, J Biol Chem, 283 (2008) 13520–13527. [PubMed: 18353780]

[53]. Tabb JS, Kish PE, Van Dyke R, Ueda T, Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH, J. Biol. Chem, 267 (1992) 15412–15418. [PubMed: 1353494]

[54]. Schenck S, Wojcik SM, Brose N, Takamori S, A chloride conductance in VGLUT1 underlies maximal glutamate loading into synaptic vesicles, Nat. Neurosci, 12 (2009) 156–162. [PubMed: 19169251]

[55]. Juge N, Gray JA, Omote H, Miyaji T, Inoue T, Hara C, Uneyama H, Edwards RH, Nicoll RA, Moriyama Y, Metabolic control of vesicular glutamate transport and release, Neuron, 68 (2010) 99–112. [PubMed: 20920794]

[56]. Preobraschenski J, Zander JF, Suzuki T, Ahnert-Hilger G, Jahn R, Vesicular glutamate transporters use flexible anion and cation binding sites for efficient accumulation of neurotransmitter, Neuron, 84 (2014) 1287–1301. [PubMed: 25433636]

[57]. Hartinger J, Jahn R, An anion binding site that regulates the glutamate transporter of synaptic vesicles., J. Biol. Chem, 268 (1993) 23122–23127. [PubMed: 8226829]

[58]. Miyaji T, Kuromori T, Takeuchi Y, Yamaji N, Yokosho K, Shimazawa A, Sugimoto E, Omote H, Ma JF, Shinozaki K, Moriyama Y, AtPHT4;4 is a chloroplast-localized ascorbate transporter in Arabidopsis, Nat Commun, 6 (2015) 5928. [PubMed: 25557369]

[59]. Johnson RG, Pfister D, Carty SE, Scarpa A, Biological amine transport in chromaffin ghosts. Coupling to the transmembrane proton and potential gradients, J. Biol. Chem, 254 (1979) 10963–10972. [PubMed: 40978]

[60]. Hell JW, Maycox PR, Jahn R, Energy dependence and functional reconstitution of the gamma-aminobutyric acid carrier from synaptic vesicles., J. Biol. Chem, 265 (1990) 2111–2117. [PubMed: 1688846]

[61]. Egashira Y, Takase M, Takamori S, Monitoring of vacuolar-type H+ ATPase-mediated proton influx into synaptic vesicles, J. Neurosci, 35 (2015) 3701–3710. [PubMed: 25716867]

[62]. Cater RJ, Vandenberg RJ, Ryan RM, The domain interface of the human glutamate transporter EAAT1 mediates chloride permeation, Biophys. J, 107 (2014) 621–629. [PubMed: 25099801]

[63]. Machtens JP, Kortzak D, Lansche C, Leinenweber A, Kilian P, Begemann B, Zachariae U, Ewers D, de Groot BL, Briones R, Fahlke C, Mechanisms of anion conduction by coupled glutamate transporters, Cell, 160 (2015) 542–553. [PubMed: 25635461]

[64]. Price GD, Trussell LO, Estimate of the chloride concentration in a central glutamatergic terminal: a gramicidin perforated-patch study on the calyx of Held, J. Neurosci, 26 (2006) 11432–11436. [PubMed: 17079672]

[65]. Liu G, Choi S, Tsien RW, Variability of neurotransmitter concentration and nonsaturation of postsynaptic AMPA receptors at synapses in hippocampal cultures and slices [see comments], Neuron, 22 (1999) 395–409. [PubMed: 10069344]

[66]. Wilson NR, Kang J, Hueske EV, Leung T, Varoqui H, Murnick JG, Erickson JD, Liu G, Presynaptic regulation of quantal size by the vesicular glutamate transporter VGLUT1, J. Neurosci, 25 (2005) 6221–6234. [PubMed: 15987952]

[67]. Saha S, Prakash V, Halder S, Chakraborty K, Krishnan Y, A pH-independent DNA nanodevice for quantifying chloride transport in organelles of living cells, Nat. Nanotechnol, 10 (2015) 645–651. [PubMed: 26098226]

[68]. Sonawane ND, Thiagarajah JR, Verkman AS, Chloride concentration in endosomes measured using a ratioable fluorescent Cl- indicator: evidence for chloride accumulation during acidification, J. Biol.Chem, 277 (2002) 5506–5513. [PubMed: 11741919]

[69]. Martineau M, Guzman RE, Fahlke C, Klingauf J, VGLUT1 functions as a glutamate/proton exchanger with chloride channel activity in hippocampal glutamatergic synapses, Nat. Comm, 8 (2017) 2279.

[70]. Farsi Z, Preobraschenski J, van den Bogaart G, Riedel D, Jahn R, Woehler A, Single-vesicle imaging reveals different transport mechanisms between glutamatergic and GABAergic vesicles, Science, 351 (2016) 981–984. [PubMed: 26912364]

[71]. Goh GY, Huang H, Ullman J, Borre L, Hnasko TS, Trussell LO, Edwards RH, Presynaptic regulation of quantal size: K(+)/H(+) exchange stimulates vesicular glutamate transport, Nat. Neurosci, 14 (2011) 1285–1292. [PubMed: 21874016]

[72]. Grabe M, Wang H, Oster G, The mechanochemistry of V-ATPase proton pumps, Biophys J, 78 (2000) 2798–2813. [PubMed: 10827963]

[73]. Mitchell SJ, Ryan TA, Syntaxin-1A is excluded from recycling synaptic vesicles at nerve terminals, J. Neurosci, 24 (2004) 4884–4888. [PubMed: 15152049]

[74]. Atluri PP, Ryan TA, The kinetics of synaptic vesicle reacidification at hippocampal nerve terminals, J. Neurosci, 26 (2006) 2313–2320. [PubMed: 16495458]

[75]. LeBlanc G, Rimon G, Kaback HR, Glucose 6-phosphate transport in membrane vesicles isolated from Escherichia coli: effect of imposed electrical potential and pH gradient, Biochemistry, 19 (1980) 2522–2528. [PubMed: 6992861]

[76]. Kaczorowski GJ, Kaback HR, Mechanism of lactose translocation in membrane vesicles from Escherichia coli. 1. Effect of pH on efflux, exchange and counterflow., Biochemistry, 18 (1979) 3691–3697. [PubMed: 38836]

[77]. Forrest LR, Kramer R, Ziegler C, The structural basis of secondary active transport mechanisms, Biochimica et biophysica acta, 1807 (2011) 167–188. [PubMed: 21029721]

[78]. Ruivo R, Bellenchi GC, Chen X, Zifarelli G, Sagne C, Debacker C, Pusch M, Supplisson S, Gasnier B, Mechanism of proton/substrate coupling in the heptahelical lysosomal transporter cystinosin, Proc. Natl. Acad. Sci. U S A, 109 (2012) E210–217. [PubMed: 22232659]

[79]. Egashira Y, Takase M, Watanabe S, Ishida J, Fukamizu A, Kaneko R, Yanagawa Y, Takamori S, Unique pH dynamics in GABAergic synaptic vesicles illuminates the mechanism and kinetics of GABA loading, Proc. Natl. Acad. Sci. U S A, 113 (2016) 10702–10707. [PubMed: 27601664]

[80]. Pan PY, Marrs J, Ryan TA, Vesicular glutamate transporter 1 orchestrates recruitment of other synaptic vesicle cargo proteins during synaptic vesicle recycling, J. Biol. Chem, 290 (2015) 22593–22601. [PubMed: 26224632]

[81]. Huang H, Trussell LO, Presynaptic HCN channels regulate vesicular glutamate transport, Neuron, 84 (2014) 340–346. [PubMed: 25263752]

[82]. Morrow EM, Yoo SY, Flavell SW, Kim TK, Lin Y, Hill RS, Mukaddes NM, Balkhy S, Gascon G, Hashmi A, Al-Saad S, Ware J, Joseph RM, Greenblatt R, Gleason D, Ertelt JA, Apse KA, Bodell A, Partlow JN, Barry B, Yao H, Markianos K, Ferland RJ, Greenberg ME, Walsh CA, Identifying autism loci and genes by tracing recent shared ancestry, Science, 321 (2008) 218–223. [PubMed: 18621663]

[83]. Ullman JC, Yang J, Sullivan M, Bendor J, Levy J, Pham E, Silm K, Seifikar H, Sohal VS, Nicoll RA, Edwards RH, A mouse model of autism implicates endosome pH in the regulation of presynaptic calcium entry, Nat Commun, 9 (2018) 330. [PubMed: 29362376]

[84]. Gronborg M, Pavlos NJ, Brunk I, Chua JJ, Munster-Wandowski A, Riedel D, Ahnert-Hilger G, Urlaub H, Jahn R, Quantitative comparison of glutamatergic and GABAergic synaptic vesicles unveils selectivity for few proteins including MAL2, a novel synaptic vesicle protein, J. Neurosci, 30 (2010) 2–12. [PubMed: 20053882]

[85]. DeLuca S, Khar K, Meiler J, Fully flexible docking of medium sized ligand libraries with RosettaLigand, PLoS One, 10 (2015) e0132508. [PubMed: 26207742]

Highlights:

- Protons and chloride allosterically activate the VGLUTs
- The VGLUTs exhibit an associated chloride conductance
- Allosteric regulation coordinates glutamate transport with the SV cycle
- The SLC17 family includes transporters with diverse activities
- Structure of a bacterial homologue suggests the mechanism for allosteric activation

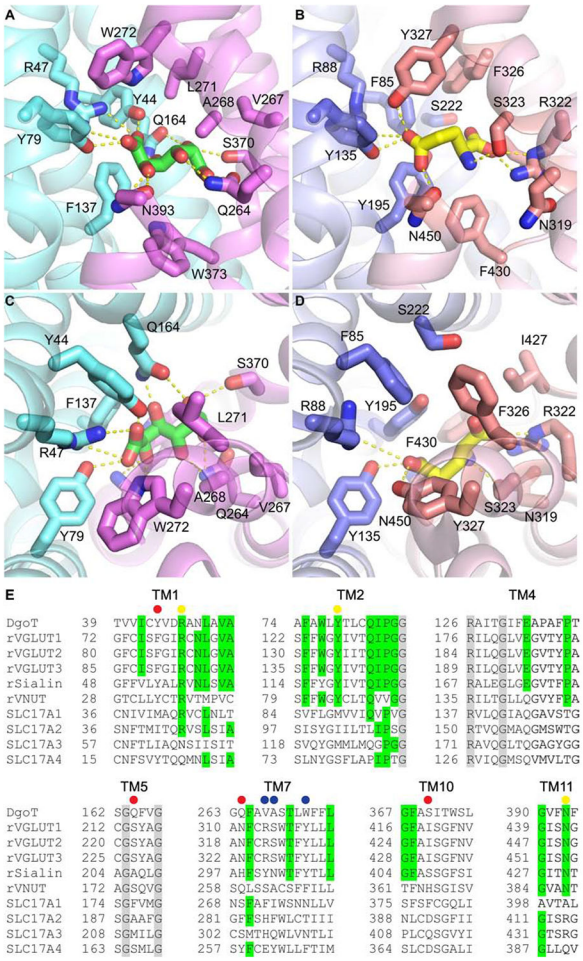

Figure 1: Substrate binding site of DgoT and VGLUT modeled on DgoT.

Cross-sectional side (A) and top view (C) of galactonate (green) in the substrate binding pocket of DgoT E133Q (PDB: 6E9O), and the corresponding side (B) and top view (D) of vGlUT2 modeled on DgoT E133Q with glutamate docked in the binding site. N-domains are blue and C-domains are purple. E, Alignment of the mammalian SLC17 family members with DgoT. Residues conserved among all family members are shown in grey, and residues conserved between DgoT and the VGLUTs are highlighted in green. Red dots indicate residues specifically involved in galactonate binding by DgoT, blue dots indicate residues specifically involved in glutamate binding by VGLUTs, and yellow dots indicate residues involved in substrate binding by both DgoT and the VGLUTs. Glutamate was docked using the Rosetta Ligand Docking protocol on the Rosie server [85]. The ligand docking with the lowest interface dG and highest docking score was chosen.

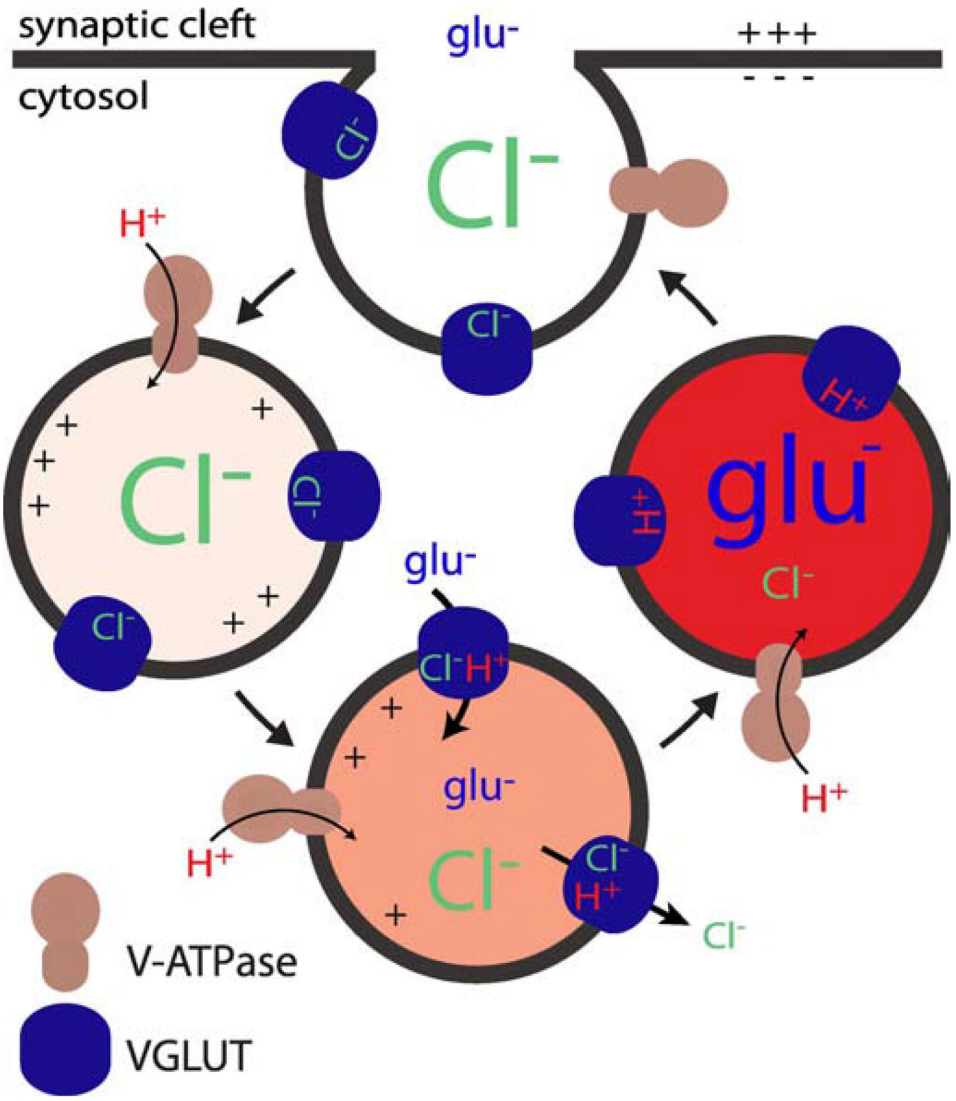

Figure 2: Glutamate uptake in the synaptic vesicle cycle

Cartoon illustrating the potential regulation of the VGLUTs during the synaptic vesicle cycle. *Top*, Glutamate is released into the synaptic cleft by exocytosis, and the VGLUTs are presumed not to function at the plasma membrane due to the lack of allosteric activation by H^+. Subsequently, the VGLUTs and V-ATPase internalize, trapping the high extracellular concentration of Cl^- (and neutral pH) in the lumen. *Left*, After endocytosis, the V-ATPase pumps H^+, creating a lumenally positive membrane potential and lowering lumenal pH (indicated by red color). *Bottom*, The drop in luminal pH together with the high luminal [Cl^-] should allosterically activate glutamate (glu^-) transport and the VGLUT-associated Cl^- conductance to increase the lumenal concentration of glutamate and lower that of Cl^-. *Right*, Late in the cycle, synaptic vesicles should have filled with glutamate and the efflux of Cl^- through the conductance should eliminate the allosteric activation required for vesicular glutamate transport. The reduced lumenal Cl^- should thus help to stabilize the glutamate content of synaptic vesicles by preventing its efflux.