Title
ROLE OF CHLORIDE IN VESICULAR GLUTAMATE TRANSPORT

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ROLE OF CHLORIDE
IN VESICULAR GLUTAMATE TRANSPORT

by

ROGER CHANG, BS

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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of the

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Thank you.

Abstract

Synaptic transmission relies on the transport of neurotransmitters into specialized endocytic compartments, synaptic vesicles. Vesicular glutamate transporters (VGLUTs) package glutamate, the major excitatory neurotransmitter, primarily using the membrane potential. Knockout of either of the two major VGLUT isoforms results in perinatal lethality. Two peculiar and controversial properties of VGLUTs are the extraordinary dependence on Cl and a putative Cl conductance. This study investigated this essential transport process and the relationship of Cl, the Cl conductance, and protons using precise electrophysiology techniques.

Two-electrode voltage clamping of Xenopus Oocytes expressing internalization-defective VGLUTs revealed a pH- and Cl-dependent Cl conductance. This Cl conductance was sensitive to both a drug that inhibits vesicular glutamate transport as well as glutamate. Although vesicular glutamate transport was not observed in Xenopus Oocytes, patching on HEK293T endolysosomes expressing VGLUTs revealed both glutamate and Cl conductances. These conductances required luminal Cl and were potentiated by cytoplasmic Cl. Using site-directed mutagenesis of conserved, charged amino acids, we identified key transmembrane residues that regulate anion and glutamate conductances including one responsible for activation by luminal Cl. This finding demonstrates that the mechanism for allosteric activation by Cl is distinct from permeation. In addition, Cl competes with vesicular glutamate transport. This competition is modulated by luminal protons. This study is the first to electrophysiologically reveal the regulatory mechanisms behind the transport of a neurotransmitter, and can be used as a model for the study of other vesicular neurotransmitter transporters.

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1 Introduction

1.1 Regulation of Neurotransmitter Release

Synaptic transmission is a highly regulated process fundamental to all information processing by the brain. Chemical neurotransmitters are packaged into specialized endocytic compartments, synaptic vesicles, released into the synaptic cleft, and bind to postsynaptic receptors (Figure 1.1). The physiological ability to regulate the output response, or synaptic plasticity, account for many forms of learning and memory.[1-3] Defects in synaptic transmission also appear to underlie many neurological and psychiatric disorders from epilepsy and Parkinson's disease to autism spectrum disorders and schizophrenia.[4-7] Although regulation of postsynaptic receptors (r) is a relatively well-characterized mechanism for plasticity, presynaptic factors such as release probability (p), pool size, and the amount of neurotransmitter per synaptic vesicle contribute to other forms of plasticity (Figure 1.1).

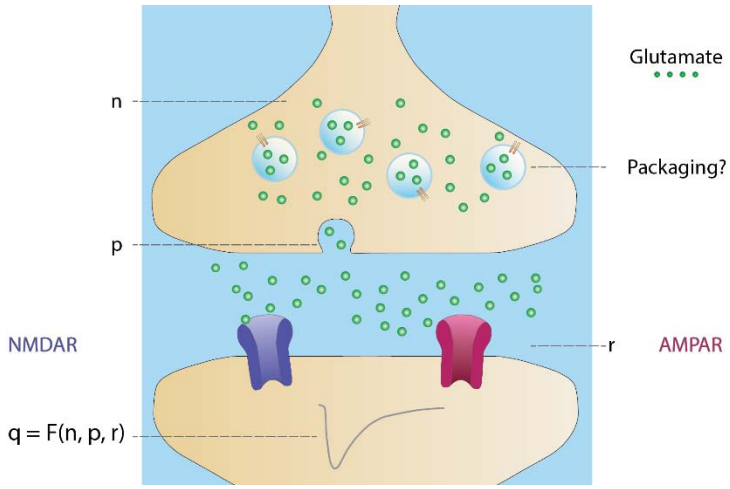

Figure 1.1 Overview of Synaptic Neurotransmission.
Factors affecting the output, postsynaptic response (q), include the number of synapses (n), the probability of release (p), the number of receptors (r), and potentially the amount of neurotransmitter packaged into synaptic vesicles.

The response to release of a single synaptic vesicle (q, quantal size) is generally assumed to reflect postsynaptic receptor expression; but it has been shown that the transmitter released by a single synaptic vesicle does not fully activate or saturate postsynaptic receptors (Figure 1.1).[8-10] Thus, changes in quantal size may also reflect presynaptic changes in the amount of transmitter released per vesicle.[9, 11, 12] In fact, changes in neural activity produce homeostatic changes in the expression of vesicular neurotransmitter transporters.[13] Overexpression of the vesicular glutamate transporter 1 (VGLUT1) also increases quantal size.[14, 15] However, decreased

expression reduces the number of vesicles filled with glutamate rather than the amount stored per vesicle, suggesting that one transporter may be sufficient to fill a synaptic vesicle.[16] Theoretically, changes in expression would be expected to affect the rate of vesicular transmitter filling and not the gradient eventually achieved at equilibrium. In contrast, changes in the ionic driving force would impact the amount of transmitter stored at equilibrium. A number of mechanisms may indeed regulate this driving force and have been either suggested or shown to influence quantal size. [17, 18]

1.2 Vesicular Glutamate Transport

The transport of all classical transmitters into secretory vesicles relies on a H^+ electrochemical gradient ($\Delta\mu_{H+}$) generated by the vacuolar-type H^+-ATPase (Figure 1.2).[19] Whereas the vesicular transport of neurotransmitters such as monoamines and acetylcholine relies predominantly on the chemical component ΔpH, vesicular transport of the major excitatory neurotransmitter, glutamate, depends more on the membrane potential, $\Delta\psi$.[19-25] Vesicular glutamate transport also does not have a clear dependence on protons.[26, 27] The transport activity is very specific, recognizing glutamate, but not the closely related amino acid aspartate.[20, 21, 28, 29]

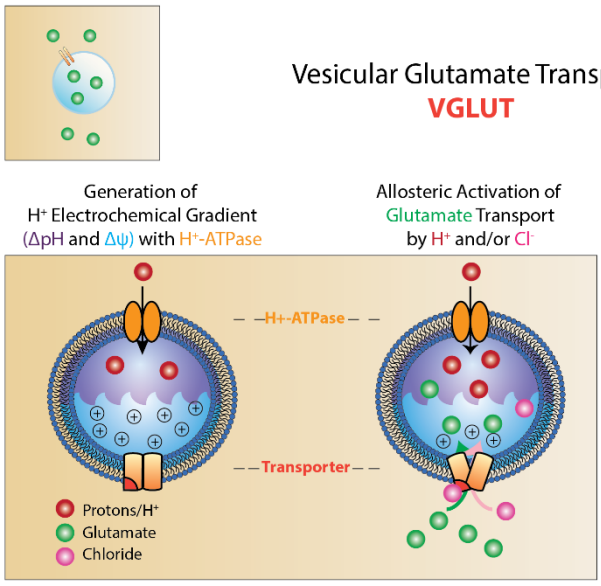

Figure 1.2 Known Properties of VGLUT.
Vesicular neurotransmitter transporters rely on the generation of a proton electrochemical gradient by the proton pump in synaptic vesicles. Vesicular glutamate transporters (VGLUTs) primarily rely on the membrane potential ($\Delta\psi$); protons and Cl are implicated in regulating their activity.

The Edwards lab and others have identified a family of three closely related vesicular glutamate transporters (VGLUTs) in the SLC17 branch of the major facilitator superfamily.[28, 30, 31] Abolishing individual VGLUT isoforms

effectively inactivates excitatory neurotransmission in different parts of the brain. In mice, VGLUT knockouts result in severe phenotypes from deafness to seizures and perinatal lethality, demonstrating the importance of VGLUTs for brain function. [32-34] The two main isoforms VGLUT1 and 2 exhibit mutually exclusive distribution patterns in the brain, with VGLUT1 predominantly in the telencephalon (cerebral cortex, hippocampus) and VGLUT2 in the diencephalon (thalamus) and brainstem.[31] The third, less widely expressed isoform, VGLUT3, is found in neurons generally thought to release a transmitter other than glutamate.[31] In addition to differences in expression, the VGLUT isoforms also rely on different trafficking pathways.[35, 36] However, it is unclear whether the VGLUT isoforms differ in transport activity.

Chloride (Cl) plays an important, interesting role in vesicular glutamate transport (Figure 1.2). It is well-established that Cl allosterically activates vesicular glutamate transport in a biphasic manner. [20, 21, 24, 28, 37] In addition to the well-established allosteric activation by Cl, the Edwards lab discovered a Cl conductance associated with VGLUT1.[28] Additional work confirmed and extended this finding, demonstrating that loss of VGLUT1 impairs the acidification of synaptic vesicles by Cl.[24] Therefore VGLUT1 is the main Cl conductance on synaptic vesicles and affects the electrochemical driving force in synaptic vesicles. Ultimately, this Cl conductance could affect the amount of neurotransmitter packaged into synaptic vesicles.

1.3 Membrane Transporter Assays

Most of our understanding about vesicular glutamate transport derives from the analysis of native synaptic vesicles isolated from the brain (Figure 1.3).[20-22, 37] However, the heterogeneity of vesicle types and the presence of multiple membrane proteins including pumps, channels and transporters make it challenging to characterize the properties of a single vesicular neurotransmitter transporter in any detail. Heterologous expression of VGLUTs in neuroendocrine cells, including PC12 cells, confers low levels of transport activity, making the basic characterization of vesicular transport very difficult. More recently, several groups have purified the recombinant transporters in eukaryotic cells and reconstituted the pure protein into artificial

membranes.[38, 39] However, some of the results obtained using this preparation have conflicted, including the presence of a Cl conductance. [25, 38, 39]

These conflicting results may reflect a number of factors. First, defects in the structural integrity of liposomes reconstituted with the transporter may produce artifacts.[25, 38] Variation in membrane leakiness may account for discrepancies in the reported role of Cl using artificial membranes reconstituted with VGLUT2.[24, 25, 39] Second, heterologous expression systems do not confer reliable vesicular glutamate transport activity.[20]

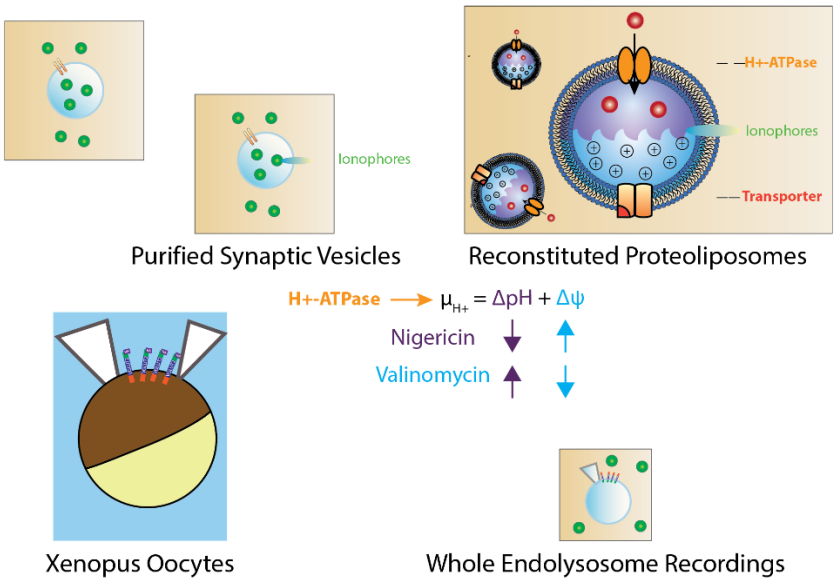

Figure 1.3 Membrane Transport Assays.
Earlier assays to study vesicular neurotransmitter transporters including purified synaptic vesicles and reconstituted proteoliposomes had several advantages including the presence of the vesicular neurotransmitter transporter in a native or defined protein phospholipid environment. Mislocalizing VGLUTs to the plasma membrane of Xenopus Oocytes, enabled robust measurements of currents using two electrode voltage clamp. Whole endolysosome recordings is an approach that enables direct, precise control of the chemical and electrical gradient as well as the chemical composition.

In addition, it is difficult to investigate the individual, separate impact of the electrical or chemical gradients on the activity of membrane transport proteins. This difficulty arises from the inability to precisely control the membrane potential and the luminal chemical composition at the same time. As a workaround, purified synaptic vesicle, reconstituted proteoliposome, and heterologously-expressing PC12 cell assays use ionophores like valinomycin and nigericin that dissipate the $\Delta\psi$ and ΔpH, respectively (Figure 1.3). Unfortunately, dissipation of either component of the electrochemical gradient compromises control of the other component, further complicating interpretation of the data.

The electrogenic nature of vesicular glutamate transport and the putative associated channel activity suggest that electrophysiology might provide another way to assess VGLUT

function (Figure 1.3). Two-voltage electrode clamping of Xenopus Laevis oocytes is a routine method to determine the biophysical properties of plasma membrane ion channels.[40, 41] Mutations of dileucine-like motifs mislocalize the VGLUTs to the plasma membrane, enabling the use of this robust technique.[35] Unfortunately, it is still impossible to control the luminal contents and define the chemical gradient. In addition, the orientation of the VGLUTs is opposite to that in its more native endocytic compartment. Whole endolysosomal recordings would enable the electrophysiological characterization of VGLUTs in a more native, phospholipid environment.[42, 43] This direct approach enables access to the luminal contents and control of the chemical gradient.

1.4 Purpose of this Study

The central hypothesis is that the Cl conductance associated with VGLUTs and Cl allosteric activation regulates the transport of glutamate into synaptic vesicles, and thus quantal size. Therefore, the purpose of this study is to use heterologous expression systems including Xenopus Oocytes and HEK293T endolysosomes, and determine the role of protons and Cl as well as identify residues critical for the biophysical regulation of both the Cl conductance and vesicular glutamate transport.

2 Materials and Methods

2.1 Constructs

Internalization-defective, mammalian, *Rattus Norvegicus* VGLUT1-3 (NCBI Reference Sequences: NM_053859.2, NM_053427.1, NM_153725.1) constructs were made with a luminal HA-tag or without the tag.[35] The HA-tag is located between transmembrane domains 1 and 2 in a conserved region of the three isoforms. Since the VGLUTs normally target to endocytic compartments, we have introduced a small number of point mutations at the N- and C-termini that disrupt endocytosis of these transporters and retain VGLUTs at the plasma membrane. The luminal HA epitope tag, was used to verify expression at the plasma membrane. These internalization-defective mutations should not affect transport activity.[44] For example, N- and C-terminal deletions of the vesicular monoamine transporters redistribute them to the plasma membrane without eliminating their transport activity.[45] In addition, we have transfected the same endocytosis-defective VGLUT construct into HEK cells, and observed pH-sensitive glutamate efflux. Site-directed mutagenesis of internalization-defective constructs was done by QuickChange. These constructs were subcloned into a mammalian pGEMHE-M vector backbone with SacI and EcoRI.

Wild-type *Rattus Norvegicus* VGLUT1-3 constructs were subcloned into the pEGFP-N1 vector. Forward and reverse primers were designed for all 3 isoforms to include a Kozak sequence (5'-CGCCACC-3') at the 5' end of the VGLUT-EGFP constructs as well as the necessary restriction sites to subclone. The position of the Arg-322, Arg-88, and Arg-184 corresponded to that of the human VGLUT2 (UniProt: Q9P2U8.1). After aligning all 3 highly homologous isoforms of the *Rattus Norvegicus* with the human VGLUT2 using the Clustal Omega algorithm, the R322A, R88A, and R184A mutations was introduced into the wild-type versions of the *Rattus Norvegicus* VGLUTs using either QuickChange (pVGLUT2 R322A-EGFP and pVGLUT3 R322A-EGFP) or New England Biolabs' Q5® Site-Directed Mutagenesis Kit

(Catalog No. E0554), and confirmed by DNA sequencing.[46] To enlarge endolysosomes, the Rab5 Q79L-mCherry construct (AddGene Plasmid No. 35138), was obtained.

2.2 Xenopus Oocytes Plasma Membrane Recordings

cRNA (50 ng per oocyte) encoding internalization-defective WT and mutant VGLUTs were injected into defolliculated Xenopus Laevis oocytes (Nasco, Fort Atkinson, WI. and EcoCyte Bioscience, Austin, TX.). After incubation in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH = 7.4) with 50 mg/ml tetracycline and gentamicin at 16˚C for 5-6 days, oocytes were recorded by two-electrode voltage clamp. The recording solutions were Ca^{2+} free ND96, 96 mM Choline Cl (or Gluconate) containing 1 mM $MgCl_2$ and 5 mM buffer, pH = 7.4 or 5.0, or as indicated. Steady-state current-voltage (I-V) relationships were obtained using a protocol of 300-ms voltage steps from -120 to +60 mV with a holding potential of -20 mV. Gap-free recordings were performed holding Xenopus Oocytes at -70 mV. Two electrode voltage clamp recordings were performed with an Axon Instruments GeneClamp 500B amplifier and a DigiData 1322A digitizer, using pCLAMP software (Molecular Devices, Sunnyvale, CA.). MATLAB (Mathworks, Natick, MA.), Adobe Illustrator (Adobe, San Jose, CA.), and Prism (GraphPad, LaJolla, CA.) were used to analyze and plot out the traces.

Measurements of expression of the HA-tag at the plasma membrane of Xenopus Laevis oocytes as previously described.[47, 48] Oocytes were incubated in ND96 with 1% BSA at 4˚C for 30 minutes, and then for 1 hour in ND96 with 1% BSA and anti-HA antibody (HA.11 or 3F10 at 1:1000 dilution). Oocytes were then washed using ND96 with 1% BSA and incubated in secondary mouse or rat horseradish peroxidase-coupled secondary antibody. Oocytes were then thoroughly washed again for 10 times, 5 minutes each and plated in individual chambers on a plate reader. 50 µL of SuperSignal ELISA Femto Maximum substrate (Thermo Fisher Scientific, Waltham, MA.) was then added to each oocyte, and the resulting absorbance signal was quantified with a Veritas Luminometer (Promega, Madison, WI).

2.3 Whole Endolysosomal Recordings

Patch-clamp in the whole-endolysosome configuration was used to measure currents from pVGLUT WT or R322A, R88A, and R184A-EGFP and Rab5 Q79L-mCherry expressing HEK293T endolysosomes. HEK293T cells were cultured in Dulbecco's Modified Eagle Media (ThermoFisher, Catalog No. 11965-092, Waltham, MA.) with 10% Defined Fetal Bovine Serum (GE Healthcare Life Sciences HyClone, Catalog No. SH30070.0, Pittsburgh, PA.). Rab5 Q79L-mCherry DNA and the Wild Type or Mutants of the pVGLUT-EGFP (1 µg each) were cotransfected onto a mono-layer of these HEK293T cells (~70-90% confluency) in 6-well tissue-culture plates using the PolyJetTM transfection reagent (SignaGen Catalog No. SL100688, Rockville, MD.) at a ratio of 3:1 (PolyJetTM (µL):DNA (µg)). Per manufacturer's recommendation, incubation of the PolyJet™ transfection complex was allowed to occur for 12-18 hours, after which fresh serum-containing media was replaced. After 4-5 hours, transfected cells were trypsinized and replated onto 35 mm tissue culture dishes containing PLL-coated coverslips and the aforementioned serum-containing media, and incubated until recording the next day.

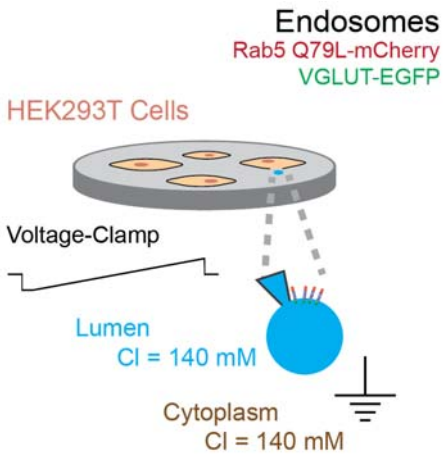

Figure 2.1 Whole Endolysosomal Recording Setup.
Mammalian HEK293T cells plated on PLL-coated glass coverslips and transfected with Rab5 Q79L with and without the desired VGLUT WT/mutant construct. One pipette was used to dissect the fluorescently-tagged endolysosome from the cell. Another was then used to patch and break into the endolysosome, enabling direct chemical control of the luminal contents of the endosome. Ramp protocols running from -100 mV to +100 mV with a holding potential of 0 mV were then run to set the membrane potential. Different bath solutions were then perfused, enabling acute control of the cytoplasmic environment as well as chemical gradients.

Patch-pipettes made of borosilicate glass (Sutter Instruments, Catalog No. BF150-75-10) were pulled with a Sutter Instrument P-87 puller (Novato, CA.) and fire-polished with the Narishige Microforge MF-830 (Amityville, NY.) to a final resistance of 6-10 MΩ. Similar to Cang et al. 2013[43], the cell membrane of HEK293T cells containing enlarged endolysosmes was ruptured with a glass pipette (Figure 2.1).

Fluorescently-tagged endolysosomes were then pushed out using the same pipette tip. Another clean pipette tip was then used to patch on and form a gigaseal with the isolated endolysosome. Only one enlarged endolysosome was recorded from each coverslip.

Patch recordings were performed with an Axon Instruments Axopatch 200B amplifier and a DigiData 1440A data acquisition system in conjunction with pCLAMP software (Molecular Devices, Sunnyvale, CA.). The recordings obtained from the amplifier were not inverted. The standard voltage ramp protocol was from -100 mV to +100 mV over 800 msecs. Before commencing the ramp, the holding potential at 0 mV was dropped to -100 mV for 50 msec. The step protocol to measure steady-state current-voltage (I-V) relationships were obtained using a protocol of 300-ms voltage steps from -120 to +60 mV with a holding potential of -20 mV. The holding potential was similarly 0 mV. The standard bath (cytosolic) solution contained (in mM) 140 NMDG Gluconate, 2 $MgSO_4$, 20 HEPES, 5 EGTA, 10 Glucose, pH to 7.2. The standard pipette (luminal) solution contained (in mM) 140 NMDG Cl, 1 $MgCl_2$, 20 MES, 10 Glucose, 2 $CaCl_2$, pH to 5.0. Various substitutions to these standard solutions are noted in the figure legends particularly to the pH (substitute with 20 mM HEPES and pH to 7.2 or 20 mM MES and pH to 5.0), addition of L-Glutamic Acid or L-Aspartic Acid (20 mM replacing 20 mM NMDG Gluconate), and substitution of Gluconate with Chloride, Bromide, or Phosphate. Figures were plotted with MATLAB (Mathworks, Natick, MA.), Adobe Illustrator (Adobe, San Jose, CA.), and Prism (GraphPad, LaJolla, CA.). Nonlinear regression functions in Prism were used to fit dose-response curves, constraining the top and bottom values in some cases. Reversal potentials were estimated from the ramp recordings by measuring the voltage at the current closest to 0 pA. Reversal potentials were estimated from step recordings by plotting average current vs voltage traces, drawing a best-fit first-order line, and retrieving the voltage from this fitted-line closest to 0 pA.

3 Results

3.1 Internalization-Defective VGLUTs

3.1.1 pH and Cl Dependent Cl Conductance

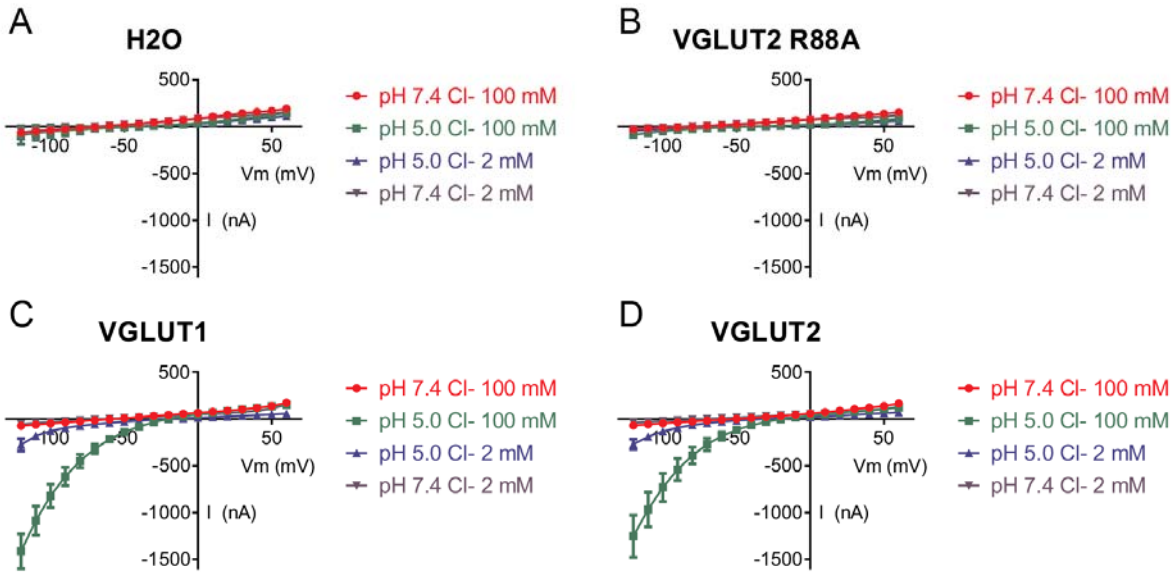

Figure 3.1 pH- and Cl-dependent Cl Conductance in VGLUT-expressing Oocytes.
Oocytes were injected with water or mRNA encoding internalization-defective, HA-tagged VGLUT1, VGLUT2 and a mutant of VGLUT2, R88A. After 5 days of expression, oocytes were recorded with varying external pH and Cl in bath solutions with 98 mM Choline Cl-/Gluc, 1 mM MgCl2, 5 mM HEPES/MES pH = 7.4/5.0. Representative IV curves were plotted (Mean ± SEM, n = 3 oocytes).

Perfusing low external pH and high external Cl on injected VGLUT-expressing oocytes resulted in an inwardly rectifying current (Figure 3.1). Water-injected control oocytes showed no such pH- or Cl- activated current. Importantly, the pH required is within the range of physiological pH observed for the synaptic vesicle lumen (~ 5.8). Since the currents are inwardly rectifying, the high external Cl seems to gate this conductance. Changes in external Cl also shift the reversal potential, suggesting that Cl is the permeant ion (Figure 3.2). The shift in reversal potential is slightly less than predicted by the Nernst equation for exclusive permeation by Cl. However, the strong inward rectification and requirement for external Cl to activate the conductance complicate the measurement of reversal potential. In contrast, changes in external pH have no effect on reversal potential (Figure 3.2). Thus, external protons gate, but do not permeate this conductance.

In addition to Cl, external Br, but not other halides or inorganic anions gate the conductance. Importantly, Cl and Br, but not other anions allosterically activate vesicular

glutamate transport. The similar anion selectivity for allosteric activation of both these processes suggests that the conductance and vesicular glutamate transport share a common underlying mechanism.[20]

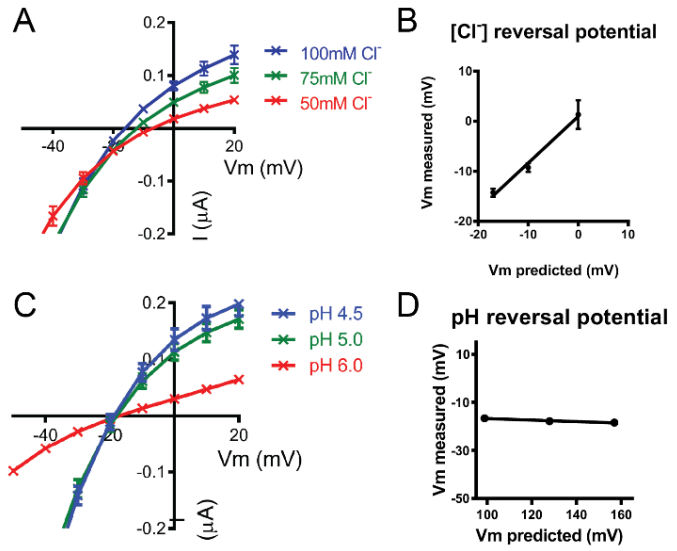

Figure 3.2 Cl and Proton Changes in Reversal Potential. Oocytes were injected with mRNA encoding internalization-defective, HA-tagged VGLUT2. After 5 days of expression, oocytes were recorded with different Cl and proton concentrations. The IV curves (left panels) and measurements of reversal potential (right panels) were plotted. (Mean ± SEM, n = 3 oocytes) (Data from Dr. Jacob Eriksen).

We then sought to determine whether cytoplasmic glutamate might contribute to or even be required for this inwardly rectifying conductance. To address this possibility, we expressed the internalization-defective VGLUT2 in HEK293 cells and observed Cl- and pH-dependent inwardly rectifying currents very similar to those observed in oocytes. However, the currents occurred regardless of whether the patch pipette contained glutamate. Since current magnitude varies in different cells, we cannot conclude that cytoplasmic glutamate does not modulate the conductance, but we can say that cytoplasmic glutamate is not required for the conductance.

3.1.2 Cl and Glutamate Competition

To determine whether glutamate modulates the conductance, external glutamate was added to oocytes expressing VGLUT2 (Figure 3.3). Glutamate inhibits the currents in a dose-dependent manner. Aspartate, which is not recognized by the VGLUTs, does not have an effect on these currents. Further, inhibition by glutamate appears to be more potent at low than high external Cl, indicating competition between glutamate and Cl (Figure 3.3). These results establish a connection between the Cl conductance and vesicular glutamate transport.

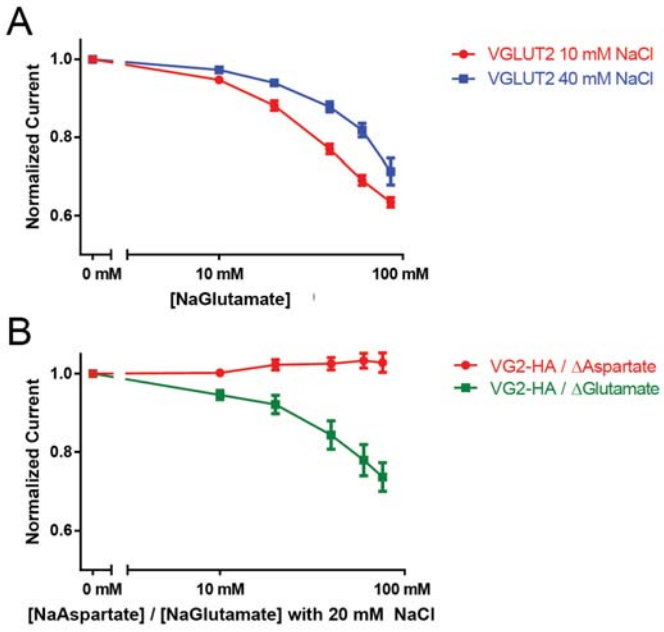

Figure 3.3 Cl and Glutamate Competition.
Oocytes were injected with mRNA encoding internalization-defective, HA-tagged VGLUT2. After 5 days of expression, oocytes were constantly held at -70 mV, and perfused with two different NaCl concentrations (10 mM and 40 mM) varying the NaGlutamate concentration (top panel) or one NaCl concentration (20 mM) varying the NaAspartate and NaGlutamate (bottom panel). All solutions had 5 mM MES, 2 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2, pH = 5.0. The steady-state currents were measured, normalized, and plotted (Mean ± SEM, n > 5 oocytes).

3.1.3 Site-Directed Mutagenesis

Many conserved, charged amino acid residues were identified within the transmembrane domains (TMDs) of VGLUT2 as potential residues that serve as the structural basis for the pH and Cl-dependence of this inwardly-rectifying Cl conductance. These experiments identified two residues in VGLUT2 required for the Cl conductance (Arg-88 in TMD1 and Arg-322 in TMD7, Figure 3.1). Mutating these residues to a neutrally charged residue (Ala) had no effect on surface expression. Unfortunately, the exhaustive screen yielded no residues that affected the Cl or pH dependence of this inwardly rectifying conductance.

3.1.4 Pharmacology

Evans Blue is an inhibitor of vesicular glutamate transport in purified synaptic vesicles and reconstituted proteoliposome.[24, 28, 49, 50] When applied to either the external (luminal) face of the oocyte or injected into the oocyte, Evans Blue inhibited the VGLUT-associated Cl conductance (Figure 3.4). Interestingly, the kinetics of external Evans Blue inhibition was slow, suggesting that the drug crossed the plasma membrane, and inhibited VGLUTs on the cytoplasmic face of the membrane.

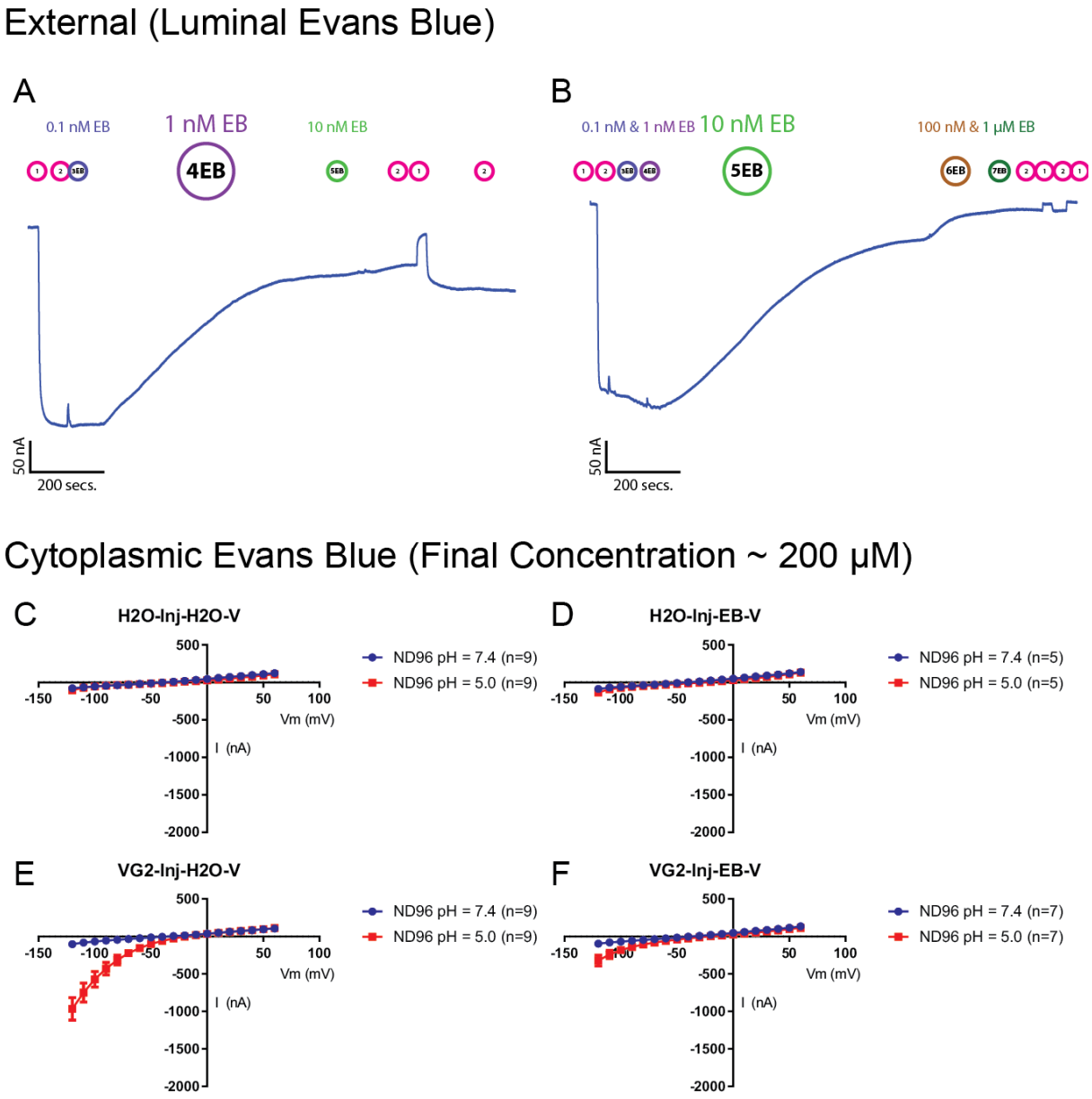

Figure 3.4 Effects of External and Cytoplasmic Evans Blue.
Oocytes were injected with mRNA encoding internalization-defective, HA-tagged VGLUT2. After 5 days of expression, occytes were recorded. Different concentrations of Evans Blue (EB) diluted in ND96 pH = 5.0 (Solution 2) were added externally (Panel A, 1 nM and B, 10 nM Evans Blue) while the oocyte was clamped at – 70mV. Oocytes were perfused initially or washed out with ND96 pH = 7.4 (Solution 1). Approximately 200 µM water or Evans Blue were injected on the vegetal side, into the cytoplasm, of previously injected water or VGLUT2 oocytes and recorded 30 minutes afterward (Panel C-F).

3.1.5 VGLUT Isoforms

Initially, only internalization-defective VGLUT1 and 2-HA expressed in oocytes exhibited an inwardly rectifying pH- and Cl-dependent Cl conductance. However, further investigation with endolysosomal recordings hinted that VGLUT3 may also possess this conductance (Figure 3.6). After removing the HA-tag of internalization-defective VGLUT3 and expressing it in oocytes, there was a Cl conductance in VGLUT3 as well. Interestingly, the pH and Cl dependence of this conductance is nearly identical across all 3 isoforms (Figure 3.5, data not shown).[51] This stark similarity in the presence and regulation of this Cl conductance suggests a shared transport mechanism amongst all the VGLUT isoforms.

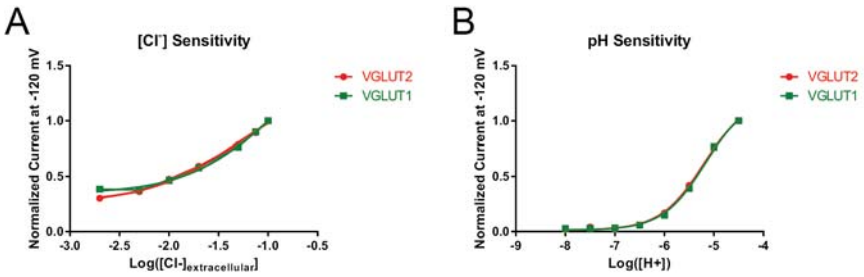

Figure 3.5 pH and Cl sensitivity among VGLUT isoforms.
Oocytes were injected with mRNA encoding internalization-defective, HA-tagged VGLUT2. After 5 days of expression, oocytes were recorded. The maximal currents for different Cl (Panel A) and proton (Panel B) concentrations were recorded and normalized to the maximal current for each oocyte (Vm = -120 mV) (Mean ± SEM, n = 3 oocytes).

3.2 Endolysosomal VGLUTs

3.2.1 Cl Conductance

Control endolysosomes expressing Rab5 Q79L exhibited an inward Cl conductance with high luminal Cl in the pipette. Several Cl channel blockers were subsequently used to eliminate this confounding endogenous Cl conductance. Luminal 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) successfully eliminated these background conductances. In the presence of cytoplasmic Cl, but not the bulky anion gluconate (0 mM Cl), all 3 mammalian VGLUT isoforms cotransfected with Rab5 Q79L revealed both an inward and outward conductance. This finding, in contrast to those in Xenopus Oocytes, suggests that Cl permeates in both directions, into the lumen (outward conductance in endolysosomes, inward conductance in Xenopus Oocytes) as well as out of the lumen (inward conductance in endolysosomes). This inward Cl conductance in

the VGLUTs could serve as an exchange mechanism. The movement of Cl out of the synaptic vesicles facilitates glutamate movement into the lumen, recorded as an outward glutamate conductance.

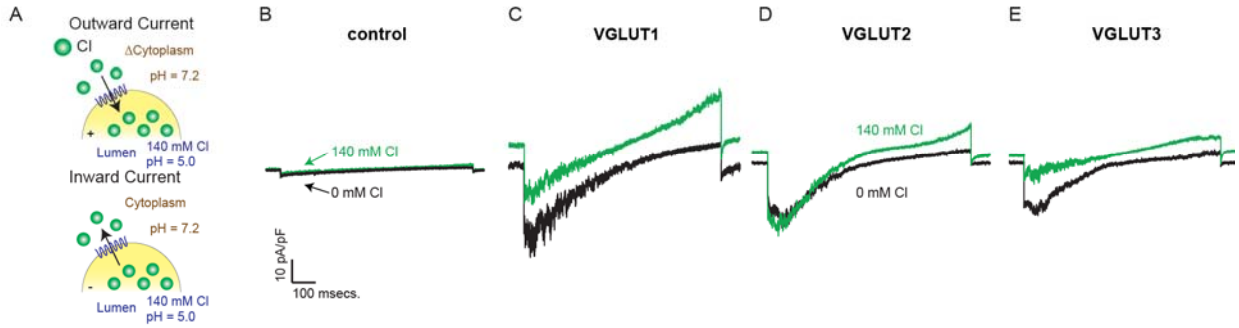

Figure 3.6 Cl Conductance in VGLUT Endolysosomes.
(A) Whole endolysosome patch-clamp recording setup for Cl conductances. (B, C, D, and E) Representative recordings with ramp protocols from -100 mV to +100 mV in 800 msecs. Endolysosomes were enlarged with Rab5 Q79L and co-transfected with VGLUT1 (C), VGLUT2 (D), or VGLUT3 (E) (n > 3 recordings).

3.2.2 Glutamate Conductance

Remarkably, we were able to observe an outward conductance in the VGLUTs after perfusing on glutamate, but not aspartate, which the VGLUTs do not recognize. [21, 28, 29] In both bath solutions, 20 mM NMDG Gluconate was replaced with 20 mM NMDG Glutamate or Aspartate (Figure 3.7). Most endolysosomes recorded had both Cl and glutamate conductances. To our knowledge, these results with endolysosomes are the first electrophysiology recordings of a vesicular neurotransmitter transporter with its known substrates.

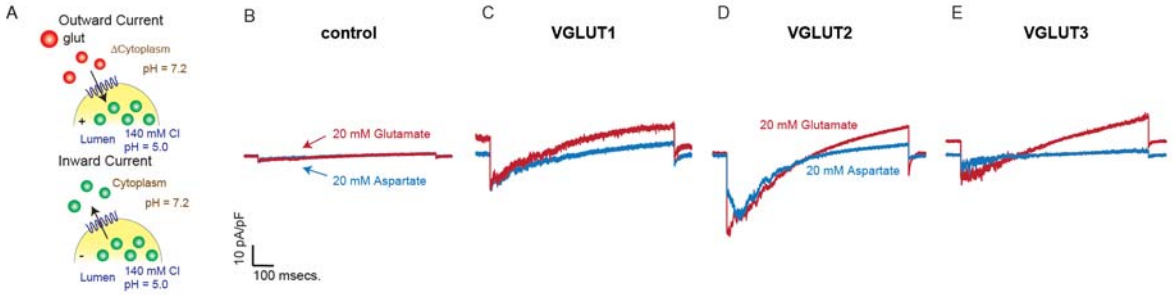

Figure 3.7 Glutamate Conductance in VGLUT Endolysosomes.
(A) Whole endolysosome patch-clamp recording setup for glutamate conductances. (B, C, D, and E) Glutamate and aspartate solutions were perfused onto the same endolysosomes from Figure 3.6 (n > 3 recordings).

3.2.3 Dose-Dependence of Cl and Glutamate

Varying the cytoplasmic Cl and glutamate concentrations revealed an interesting difference between the Cl and glutamate conductances. Cytoplasmic glutamate saturated outward glutamate conductances in VGLUT1-expressing endolysosomes with an EC50 similar to what was previously reported.[28] In contrast, cytosolic Cl did not saturate outward Cl conductances (Figure 3.8). This finding suggests that the associated Cl conductance acts more like an ion channel while vesicular glutamate transport operates more like a transporter with saturating kinetics. The difference in dose-dependence suggests that these two conductances have different transport mechanisms, although further evidence (Figure 3.9, Figure 3.10, Figure 3.11, Figure 3.12, Figure 3.14, Figure 4.1) suggests shared regulation.

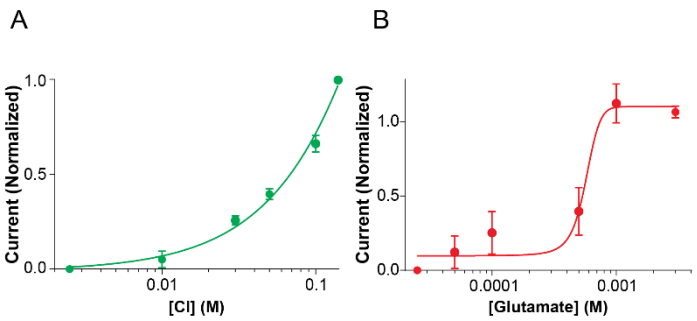

Figure 3.8 Dose Dependence of Cl and Glutamate Conductances in VGLUT1 Endolysosomes.
HEK293T Endolysosomes co-transfected with Rab5 Q79L and VGLUT1 were patched. Different concentrations of Cl (A) were perfused on the same endolysosome during the same recording protocol described in Figure 2.1. The maximal current from each recording was normalized to the maximum current measured for different concentrations of Cl. A similar approach was done with Glutamate (B) in the presence of 10 mM cytosolic Cl (n = 4 recordings).

3.2.4 Site-Directed Mutagenesis – Abolish Transport

To further establish that the Cl and glutamate conductance observed was through the VGLUTs, the same mutations that abolished the Cl conductance in the Xenopus Oocytes (R322A and R88A in TMD7 and TMD1 respectively) were introduced in all three VGLUT isoforms (aligned and numbered to VGLUT2 with Clustal Omega).[46] These VGLUT mutants had similar fluorescent expression to the VGLUT WT when cotransfected with Rab5 Q79L in endolysosomes. All 3 VGLUT R322A-expressing endolysosomes lacked a significant Cl conductance, similar to control Rab5 Q79L-expressing endolysosomes. Interestingly, VGLUT R88A did not have a distinct outward Cl conductance, but had an inward Cl conductance. This

finding further establishes the presence of an inward Cl conductance, which was not present as an outward Cl conductance in Xenopus Oocytes. Both the VGLUT R322A and R88A-EGFP expressing endolysosomes did not have vesicular glutamate transport.

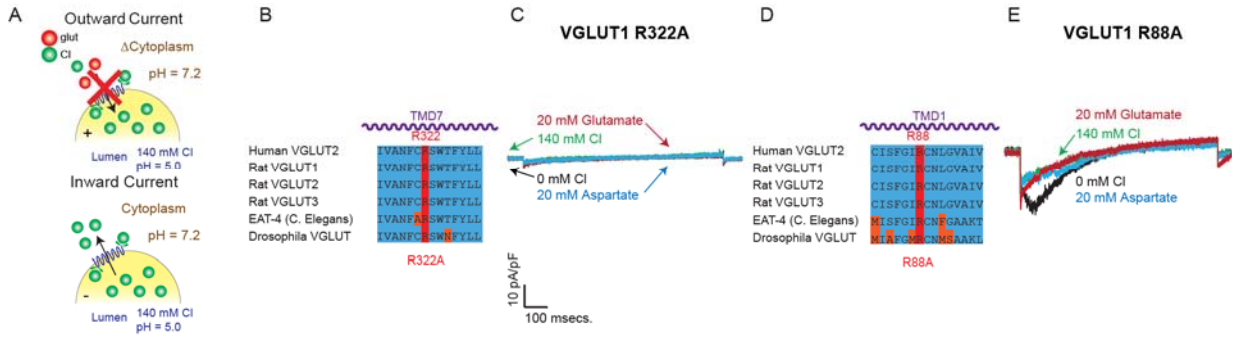

Figure 3.9 Mutations that Abolish Transport in VGLUT Endolysosomes.
HEK293T Endolysosomes co-transfected with Rab5 Q79L and VGLUT1 R322A or VGLUT1 R88A were patched. Both Cl and Glutamate were perfused on the cytoplasmic side with high Cl in the pipette (A). These residues (B and D) conserved amongst species and isoforms exhibited either no outward and inward Cl and Glutamate conductances (C, VGLUT1 R322A) or only inward conductances (E, VGLUT1 R88A) (n = 4 recordings).

3.2.5 Luminal and Cytoplasmic Cl

Since there was a putative Cl binding site of VGLUTs, we used this direct approach to determine which side of the membrane Cl acts on to activate vesicular glutamate transport.[37, 51] VGLUT-expressing endolysosomes did not require cytosolic Cl to activate vesicular glutamate transport; however, luminal Cl was required to activate vesicular glutamate transport as well as the associated Cl conductance. In agreement with previous reports, cytosolic Cl at low concentrations potentiated vesicular glutamate transport, serving as a potential mechanism to regulate the flow of glutamate into synaptic vesicles.[28]

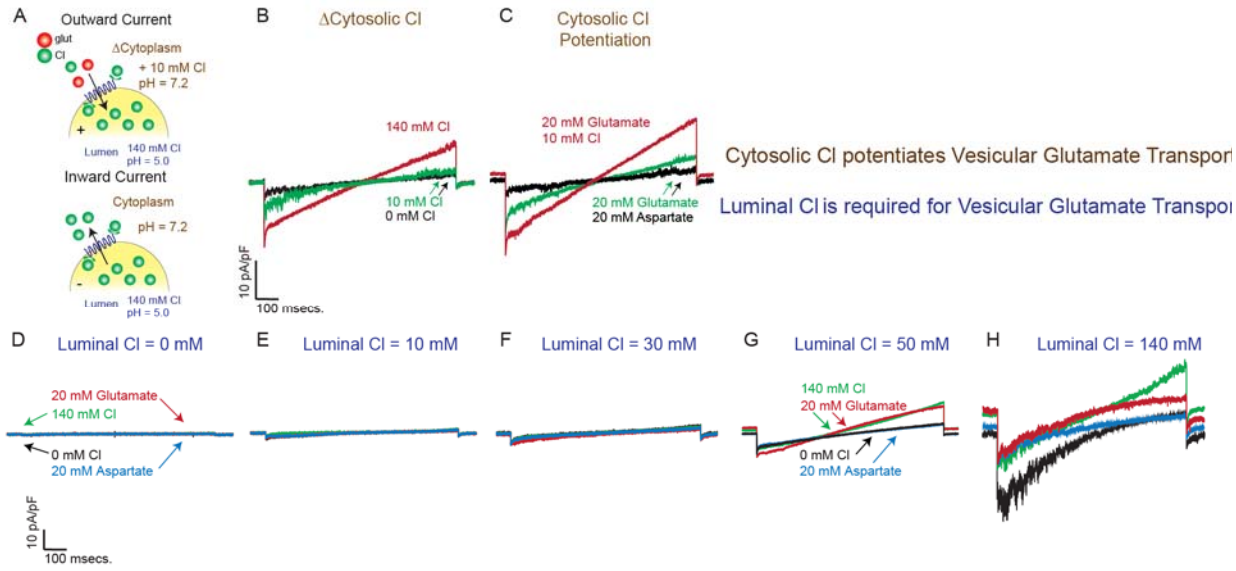

Figure 3.10 Effects of Luminal and Cytoplasmic Cl in VGLUT1 Endolysosomes.
HEK293T endolysosomes co-transfected with VGLUT1 and Rab5 Q79L were patched. (A) A basal amount (10 mM) of Cytoplasmic Cl (B) was added to the same endolysosome in the presence of 20 mM Cytoplasmic Glutamate (C) (n = 5 recordings). Whole endolysosomes expressing VGLUT1 were recorded using different concentrations of Cl in the pipette (luminal) (D-H) (n = 4 recordings).

3.2.6 Site-Directed Mutagenesis – Luminal Cl Binding Site

The conserved Arg-184 in transmembrane domain 4 was a candidate as the luminal Cl binding site for allosteric activation of the VGLUTs. In HEK293T cells expressing the internalization-defective VGLUT2 R184A, there was an inward Cl conductance in the absence of external, luminal Cl (data not shown, from Dr. Jacob Eriksen). Unfortunately, we were unable to confirm this finding in Xenopus Oocytes (data not shown). In a more native, physiological phospholipid environment though, VGLUT R184A-expressing endolysosomes did not require luminal Cl for both the outward Cl and glutamate conductances (Figure 3.11). All 3 VGLUT R184A isoforms exhibited this finding suggesting the conservation of this luminal Cl binding site, Arg-184 in TMD 4. Together these three mutations (R322A, R88A, and R184A), identify critical residues that define vesicular glutamate transport and further strengthen the role of the associated Cl conductance.

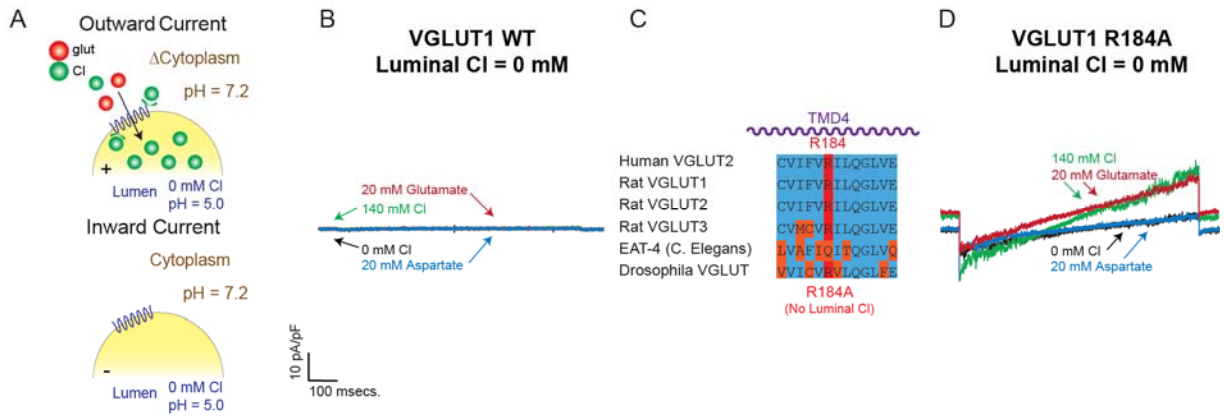

Figure 3.11 Luminal Cl Binding Site of VGLUTs.
HEK293T endolysosomes expressing VGLUT1 or VGLUT1 R184A were patched with no Cl in the pipette (A). Similar to Figure 3.10D, no Cl or glutamate conductances were observed in VGLUT1 WT-EGFP (n = 4 recordings). Arg-184, a conserved residue amongst the VGLUTs (C) was mutated to a neutral Ala. Luminal Cl-independent Cl and glutamate conductances were observed (n = 7 recordings).

3.2.7 Pharmacology

To further verify electrogenic Cl and glutamate transport observed in these VGLUT-expressing endolysosomes, Evans Blue was also used. The drug inhibited vesicular glutamate transport and its associated Cl conductance with similar, submicromolar potency (Figure 3.12). Of note, the inward Cl conductance, not previously observed in Xenopus Oocyte or HEK293T cell assay, was also inhibited by Evans Blue. Although not represented in the traces, Evans Blue inhibited both glutamate and Cl conductances in VGLUT-expressing endolysosomes within the time-frame of a standard recording, unlike in the internalization-defective VGLUT-expressing Xenopus Oocytes.

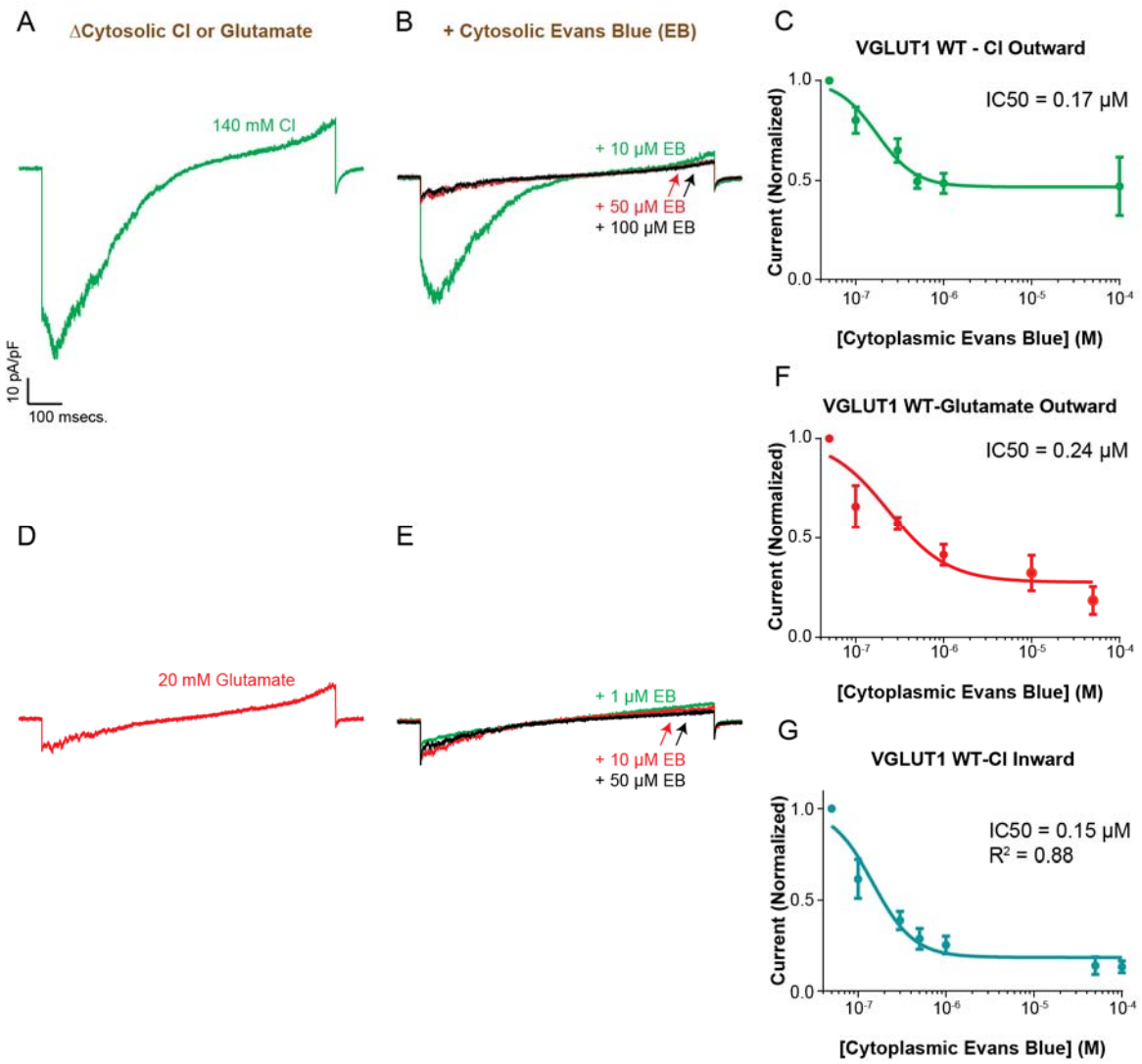

Figure 3.12 Effect of Luminal and Cytoplasmic Evans Blue in VGLUT Endolysosomes.
Cl conductance observed in VGLUT1-expressing endolysosomes before (A) and after (B and C) increasing amounts of Cytoplasmic Evans Blue (n = 4). Glutamate conductance observed in VGLUT1-expressing endolysosomes before (D) and after (E, F, and G) increasing amounts of Cytoplasmic Evans Blue (n > 3 recordings).

3.2.8 Other Anion Conductances and Activation

The VGLUTs were originally identified as sodium-dependent phosphate transporters, and experiments with reconstituted proteoliposomes also suggested that they transported phosphate.[25, 28, 30, 52, 53] However, VGLUT1-expresing endolysosomes did not exhibit sodium-independent or -dependent phosphate conductances of similar magnitude as the Cl conductance (Figure 3.13A-C). This finding is consistent with that observed in Xenopus Oocytes.

Since bromide, but not other halides or organic anions activated the VGLUTs in Xenopus Oocytes and synaptic vesicular preparations, bromide was added to both the pipette and bath of VGLUT-expressing endolysosomes (Figure 3.13D-F). In all 3 VGLUT isoforms, bromide also activated and permeated the VGLUT-expressing endolysosomes, similar to Cl.

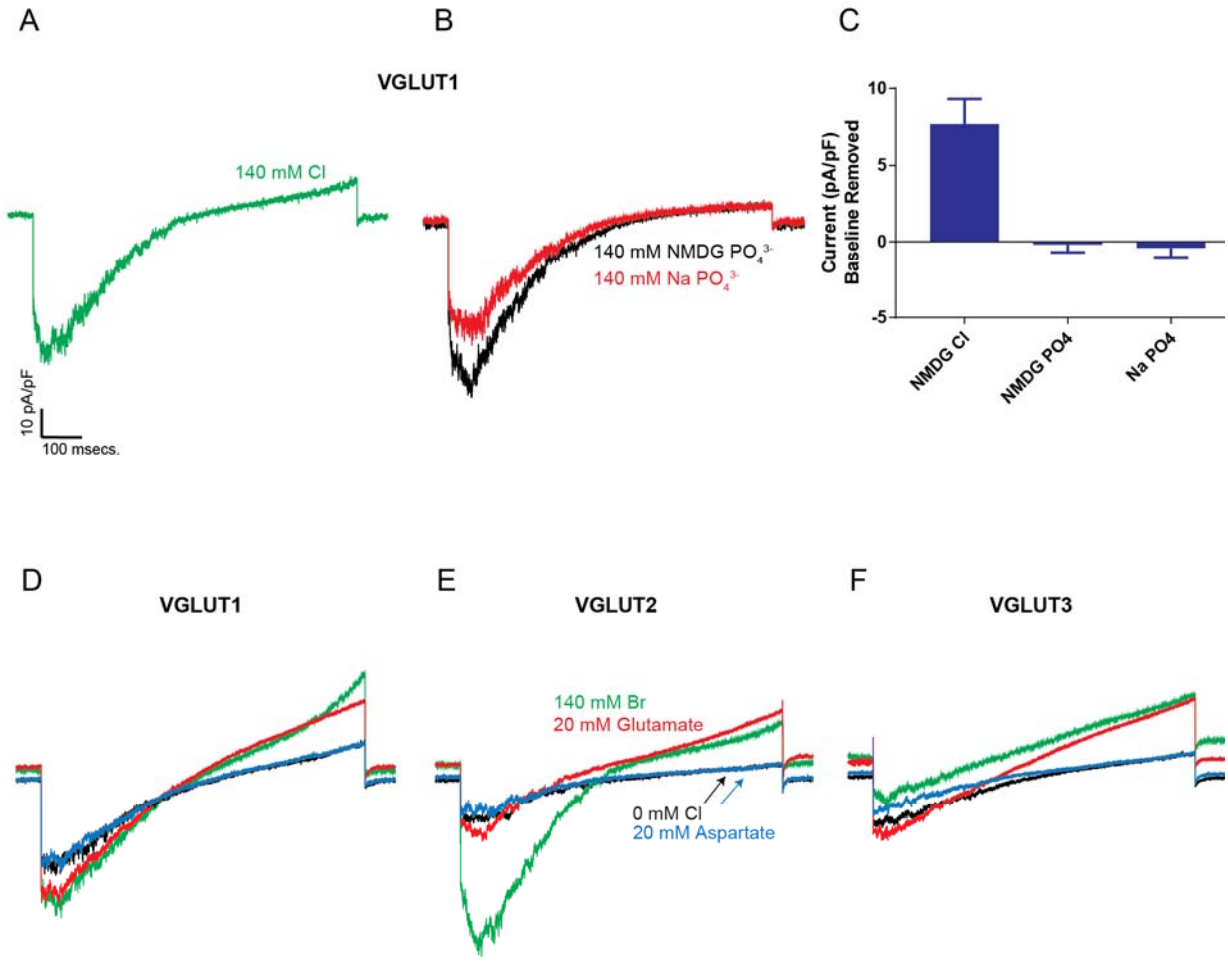

Figure 3.13 Effect of Phosphate and Bromide on VGLUT Endolysosomes.
(A-C) Representative recordings and statistics (C) of VGLUT1 expressing endolysosomes in the presence of cytosolic NMDG Cl (A), NMDG $PO4_3^-$ (B), and Na $PO4_3^-$ (B) with 140 mM NMDG Cl in the lumen (n = 5 recordings). (D-G) Representative recordings of VGLUT-EGFP expressing endolysosomes with 140 mM NMDG Br in the pipette, in the presence of cytosolic gluconate, bromide, glutamate, and aspartate (n > 3 recordings).

3.2.9 Luminal H^+

Multiple studies suggested a role for protons in vesicular glutamate transport, either as a switch, chemical driving force, or direct coupling to glutamate transport.[21, 22, 51] In this unique

system, we can characterize the exact proton contribution by further separating the chemical proton gradients from the electrical membrane potential and defining the absolute luminal and cytoplasmic proton concentration.

When VGLUT was expressed at the plasma membrane in Xenopus Oocytes and HEK293T cells, luminal protons allosterically activated the VGLUT associated Cl conductance; at neutral pH the Cl conductance shut down.[51] Surprisingly, at neutral pH and without a pH gradient, in HEK293T endolysosomes glutamate and Cl conductances were still recorded in all 3 VGLUT isoforms. Thus, luminal protons at physiological concentrations do not affect the presence of Cl and glutamate conductances through the VGLUTs in a more native phospholipid environment.

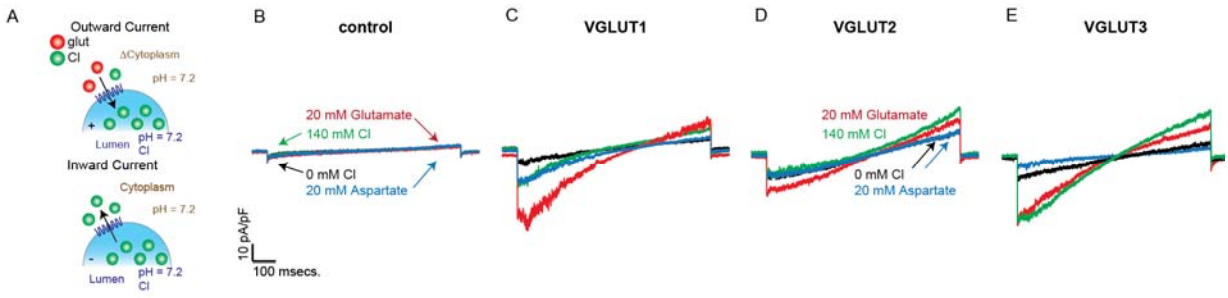

Figure 3.14 Luminal Neutral pH in VGLUT Endolysosomes.
Representative recordings of HEK293T endolysosomes enlarged with Rab5 Q79L, and co-transfected with VGLUT (n > 4) with a pipette solution containing 140 mM NMDG Cl - pH = 7.2, dissipating the pH gradient.

3.2.10 H^+-Coupling Differences

As previously mentioned, other vesicular neurotransmitter transporters like VMAT and VAChT rely more on a pH gradient than a membrane potential. To further distinguish the VGLUTs in this unique, direct assay, the effects of the pH gradient on the VGLUT glutamate conductance were investigated (Figure 3.15). When the pH gradient was acutely dissipated, the outward Cl conductance increased, but had little effect on the estimated reversal potential. In contrast, dissipating the pH gradient did not have an effect on the observed outward glutamate conductance, but surprisingly shifted the estimated reversal potential from ramp recordings.

Step recordings recorded a similar shift (VGLUT1: $E_{RevShift}$ for Cl = -8.65 ± 7.89 mV, $E_{RevShift}$ for Glutamate = 32.87 ± 6.05 mV, mean ± SEM, data not shown). The VGLUT R322A and Rab5 Q79L control endolysosomes did not demonstrate any conductances in the presence of solutions that contained glutamate, Cl, or protons. However, this observed shift in the reversal potential does not match the expected Nernst equation, suggesting an additional unaccounted for proton conductance, potentially unrelated to the VGLUTs.

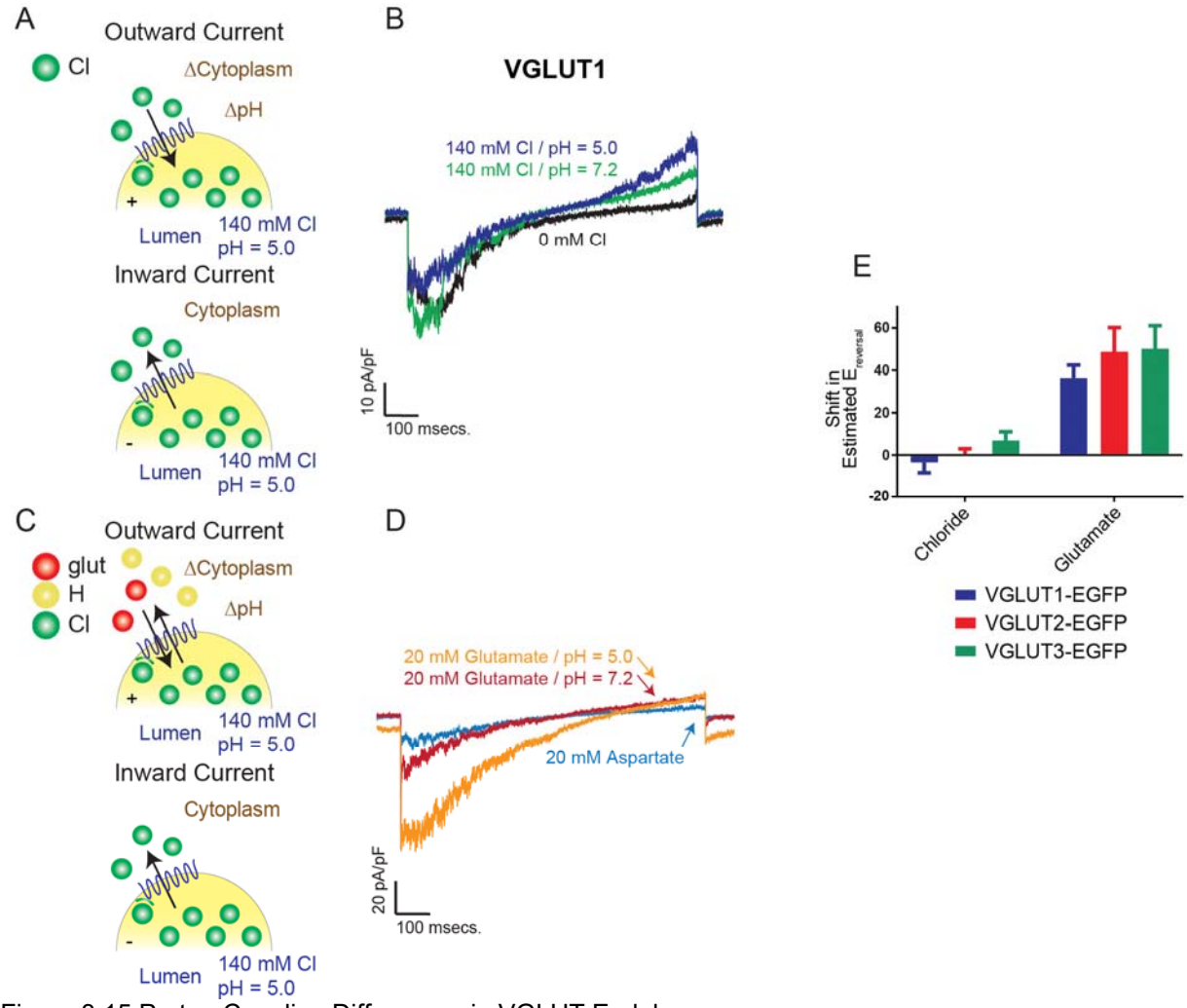

Figure 3.15 Proton Coupling Differences in VGLUT Endolysosomes.
Representative recordings of the Cl (A-B) and glutamate (C-D) conductance in HEK293T endolysosomes expressing VGLUT1. The Cl conductance (Blue) and Glutamate conductance (Gold) after acutely dissipating the pH gradient were recorded. The shift in the estimated reversal potential after dissipating the pH gradient ($E_{rev,\ estimated\ pH\ =\ 5.0} - E_{reversal,\ estimated\ pH\ =\ 7.2}$) was measured (E) (n = 6-9 recordings).

3.2.11 Cl and Glutamate Competition – Role for luminal pH.

In contrast to the VGLUTs mislocalized to the plasma membrane, the VGLUT-expressing endosomes had both the Cl and glutamate conductances. To further understand the effect of the Cl conductance on vesicular glutamate transport, a similar competition assay in endolysosomes was done, this time with glutamate dose-responses. The glutamate currents were recorded in the presence of 1 mM Cl and 10 mM Cl. The normalized glutamate dose-response showed a dramatic shift, three orders of magnitude compared to the imposed one order of magnitude difference for cytoplasmic Cl (Figure 3.16a). This finding suggests an interaction between Cl and glutamate although they share distinct transport mechanisms.

Even though there was no obvious effect of luminal H^+ on the magnitude of the Cl and glutamate conductance (Figure 3.14), we hypothesized that luminal H^+ may serve to regulate the interaction between Cl and glutamate. The glutamate dose-dependence in the presence of 1 mM Cl and 10 mM Cl was repeated, this time with neutral luminal pH (Figure 3.16). Surprisingly, we observed a much smaller shift with luminal neutral pH. Although a two-way ANOVA failed to show an effect of protons, the difference in the shift of the EC50 was 10 times more with luminal acidic pH compared to luminal neutral pH. Thus, luminal protons affect the extent of this competition between Cl and glutamate.

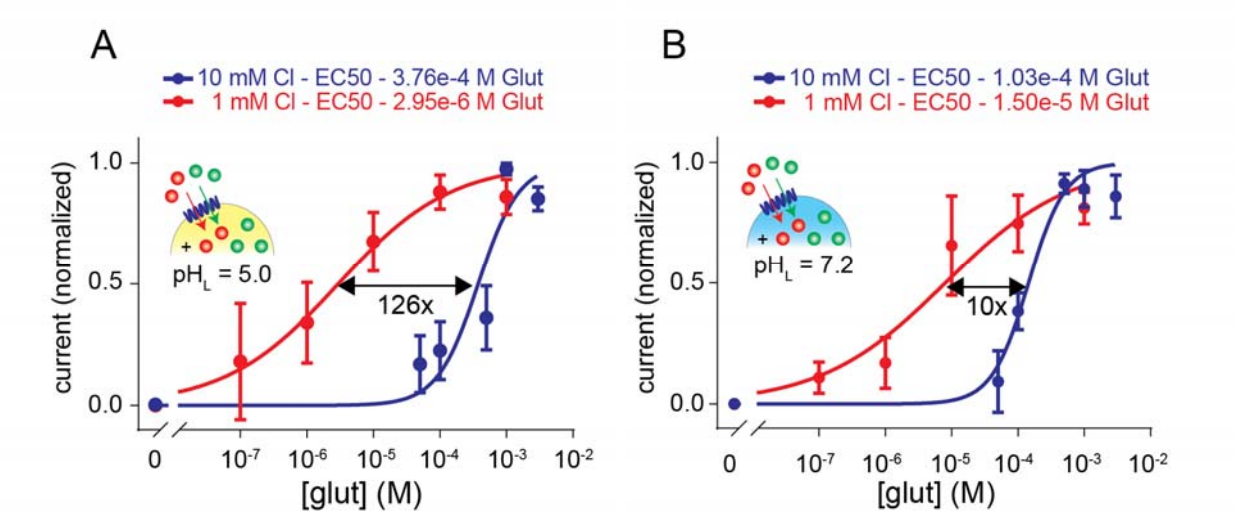

Figure 3.16 Role for Luminal H^+ in Cl and Glutamate Competition.
Dose-response curves of glutamate in the presence of 1 mM Cl (red) and 10 mM Cl (blue) at either acidic luminal pH (A) or neutral luminal pH (B) in VGLUT1-expressing endolysosomes (n = 3-7).

3.2.12 Specificity of Inward Conductance for Cl

To separate the mechanisms between Cl and glutamate for permeation further, we investigated the specificity of the inward conductances. Both luminal Cl and Br, but not gluconate, activated and permeated, resulting in an inward conductance in endosomes expressing VGLUTs (Figure 3.6, Figure 3.10, Figure 3.13, Figure 3.17). However, luminal glutamate failed to do so (Figure 3.17). In addition, luminal glutamate failed to activate the outward Cl conductance, emphasizing that Cl itself activates the VGLUTs. The inward conductance in endosomes expressing VGLUTs was specific to Cl, further demonstrating the differences between the two conductances despite the competition through the VGLUTs (Figure 3.3 and Figure 3.16).

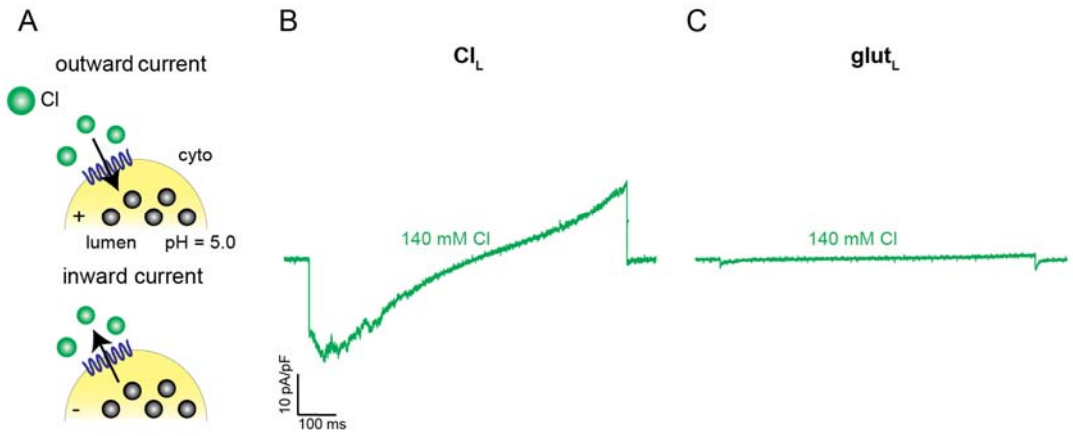

Figure 3.17 Inward Conductance Mediated by Cl and not Glutamate.
Representative recordings and compiled data of endosomes expressing VGLUT1 with either high lumenal (B) Cl or (C) glutamate in the presence of external Cl (n = 4 each).

4 Discussion

4.1 Whole Endolysosomal Recordings and Membrane Transporters

When measuring electrogenic glutamate transport through the VGLUTs, two major issues arise: (1) the VGLUTs are transporters, which as a class of membrane proteins have slow kinetics, and (2) the expression of VGLUTs in mammalian systems like PC12 cells is low. Fortunately, this study was able to elicit measurable glutamate conductances in VGLUT-expressing endolysosomes and define its regulation by Cl, protons, and critical amino acid residues.

Several of the properties observed using whole endolysosomal recordings match those of vesicular glutamate transport activity in synaptic vesicles. There is activation by low basal amounts of cytoplasmic Cl, a reliance on membrane potential more so than a pH gradient, similar activation by halides, and a K_m of 1-3 mM Glutamate.[20, 28, 37, 54] In addition, several novel properties emerged including proton coupling of vesicular glutamate transport as well as an inward Cl conductance that underlies maximal VGLUT activity.[21, 24, 55, 56]

Although technically complex, whole endolysosomal recordings of HEK293T cells enabled rapid generation of data with different conditions and constructs. Unlike the use of mRNA-injected Xenopus Laevis oocytes, cell culture of HEK293T cells was reproducible and quick (2 days of transfection and plating versus 5-6 days of mRNA expression). This feature enabled the identification of conserved VGLUT residues critical for VGLUT activity, in all 3 isoforms. The access to the luminal environment that this system afforded enabled the identification of the requirements for luminal Cl, potentiation by cytoplasmic Cl, potential differences in proton coupling between the Cl and glutamate conductances, the role of luminal protons in regulating the competition between Cl and glutamate, and specificity of the inward conductance for Cl and not glutamate. To ensure high copy numbers of VGLUTs in endosomes, HEK293T cells were only used up to 3 weeks or 12 passages after thawing. However, lipid-based transfection of stable Rab5 Q79L-mCherry and VGLUT-EGFP constructs was less tricky

and more reproducible than the generation of labile mRNA from linearized VGLUT constructs for injection of Xenopus Laevis oocytes.

The use of fluorescent tag enabled the quick visualization of VGLUT mutants that did not show conductances, but presumably correctly folded in the endolysosomes. Although currents were much bigger in two-electrode voltage clamping of Xenopus Laevis oocytes, VGLUT constructs required an HA-tag and time-intensive ELISAs to ensure proper surface expression. In addition, the correct orientation and expression of the VGLUT in its more native protein-phospholipid environment took place in whole endolysosomal recordings. In reconstituted proteoliposomes, the orientation is not guaranteed and oftentimes can be a mixed population; in Xenopus Laevis oocytes and plasma-membrane targeted VGLUTs in HEK293T cells, the orientation is opposite to that of a synaptic vesicle.

In conclusion, the use of whole endolysosomal recordings for this vesicular neurotransmitter transporter was reliable, reproducible, and quick. This technique can be adopted to investigate other membrane transport proteins such as the Vesicular GABA Transporter (VGAT).

4.2 Cl in Synaptic Neurotransmission

Sodium and Potassium, two major cations, are involved in the regulated depolarization and repolarization, respectively, of excitable tissues like mitochondria, cardiomyocytes, and neurons. In contrast, the anion Cl plays a unique role in tuning the extent and rate of excitability in synaptic vesicles containing vesicular glutamate transport. At the synapse level, work in the Calyx of Held revealed that, after depleting the synaptic vesicle pool, Cl was found to directly affect the vesicle refilling rate and recovery of EPSCs.[57] There was a biphasic relationship for cytoplasmic Cl on both these factors, similar to the allosteric activation of vesicular glutamate transport in synaptic vesicles and reconstituted proteolipososmes.[20, 24, 28]

Chloride's ability to modulate how much and how fast synaptic vesicles fill by allosteric activation and permeation through the VGLUTs is unique. In HEK293T endolysosomes

expressing VGLUTs, there exists both an inward and outward Cl conductance, activated by luminal Cl. The inward Cl conductances creates a positively-charged membrane potential, which promotes vesicular glutamate transport. In contrast, the outward Cl conductance competes with the outward glutamate conductance for entry into the synaptic vesicle. In general, the inward Cl conductances of HEK293T endolysosomes expressing VGLUTs are larger in absolute magnitude than the outward Cl conductances, suggesting that modulating the contributions of either can determine the extent of synaptic vesicle filling of glutamate. Cytoplasmic Cl also does allosterically activate vesicular glutamate transport in HEK293T endolysosomes (Figure 3.10) as previously reported.[20, 24, 28] Thus the regulation of Cl in neurons through transporters like the electroneutral K-Cl co-transporter, KCC2, has a highly significant role for excitatory neurotransmission.[58]

4.3 Cl in Vesicular Glutamate Transport

As previously mentioned, Cl allosterically activates vesicular glutamate transport and there is a putative Cl conductance in VGLUTs that affects the membrane potential, the major driving force for vesicular glutamate transport.[20, 24, 28, 37] Although previous evidence argued that VGLUTs do not have a Cl conductance, this study and others strongly demonstrates that there exists both an anion allosteric binding site and conductance (Figure 3.1, Figure 3.3, Figure 3.6, Figure 3.8, Figure 3.9, Figure 3.10, Figure 4.1).[24, 28, 37, 39] We further identify a conserved, positively-charged, binding site, Arg-184 in transmembrane domain 4, which when mutated to a neutral residue, Alanine, does not require luminal Cl to activate vesicular glutamate transport and the associated Cl conductance (Figure 3.11). This R184A mutation switches the VGLUT to a constitutively active state, mimicking the result of Cl, a negatively charged anion, binding and neutralizing Arginine, a positively-charged residue.

4.4 Conservation of VGLUT Isoforms and Critical Residues

The two main isoforms VGLUT1 and 2 exhibit mutually exclusive distribution patterns in the brain, with VGLUT1 predominantly in the telencephalon (cerebral cortex, hippocampus) and

VGLUT2 in the diencephalon (thalamus) and brainstem.[31] The third, less widely expressed isoform, VGLUT3, is found in neurons generally thought to release a transmitter other than glutamate.[31] In addition to differences in expression, the VGLUT isoforms also rely on different trafficking pathways.[35, 36]

Despite differences in expression pattern, multiple lines of evidence suggest that the VGLUT isoforms share similar transport mechanism and regulation. Initial evidence using Xenopus Oocytes revealed that the Cl conductance exhibited similar pH and Cl sensitivity amongst all 3 VGLUT isoforms (Figure 3.5).[51] All three isoforms suffice to confer glutamate release by neurons and in HEK293T endolysosomes (Figure 3.7).[28, 30, 31, 52-54] Unpublished work in the lab used conditional VGLUT1 and VGLUT2 double knockout hippocampal cultures. These conditional hippocampal cultures didn't have any mEPSC activity. However, individually infecting with lentiviruses encoding all three VGLUT WT isoforms, all exhibiting Cl conductances, resulted in rescue of mEPSC amplitude and frequency. Herman et al. also rescued VGLUT1 knockout hippocampal autaptic cultures and successfully rescued mEPSC activity with VGLUT2.[59]

Interestingly, Herman et al. also mutated the same sites (VGLUT2 Arg-88 and Arg-322) we identified with Xenopus Oocytes that abolished the Cl conductances (Figure 3.1) as well as the luminal Cl independent site, VGLUT2 Arg-184. Infecting the VGLUT1 knockout hippocampal autpatic cultures with these constructs using either double (R184K, R88Q) or triple (R184K, R88Q, and R322A)-point mutations resulted in decreases in mEPSC frequency and amplitude.[59]

In HEK293T endolysosomes, we extended these findings by mutating all 3 VGLUT isoforms with the same 3 residues to Ala (R88A, R184A, R322A). Like VGLUT1 R88A and R322A, VGLUT2 and VGLUT3 R88A and R322A all abolish outward glutamate conductances (Figure 3.9). All 3 VGLUT R322A isoforms abolished both outward and inward Cl conductances. All 3 VGLUT R88A isoforms abolished only outward Cl conductances. Although the inward Cl conductances were somewhat variable in all the HEK293T endolysosomes, the presence of an

inward Cl conductance in all 3 VGLUT R88A isoform-expressing endolysosomes, but not in all 3 VGLUT R322A isoform-expressing endolysosome, strongly suggests that the inward Cl conductance is through the VGLUTs. The luminal Cl binding site, Arg-184, is also conserved amongst all 3 isoforms as well. There was constitutive activation without luminal Cl of the Cl and Glutamate conductance in all 3 VGLUT R184A isoforms-expressing endolysosome. Given the similarities in Cl and Glutamate conductances affected by not only 1, but 3 residues, amongst all 3 VGLUT isoforms, there is strong evidence that the VGLUT isoforms do not differ in the regulation of their transport mechanism.

4.5 Differences between Plasma Membrane and Endolysosomal VGLUTs

The conflicting findings between internalization-defective and WT VGLUTs highlights the importance of the protein-phospholipid environment and regulation of VGLUT activity through the synaptic vesicle cycle. In the same mammalian HEK293T cells, recordings of the plasma membrane targeted VGLUTs revealed different regulation than the endocytic membranes. At the plasma membrane of both HEK293T cells and Xenopus Oocytes, a pH- and Cl-dependent inward Cl conductance was observed through VGLUTs; however, there was no outward Cl conductance, luminal pH-dependence, nor, more importantly, any electrogenic vesicular glutamate transport like in VGLUT-expressing endolysosomes.

An unidentified protein or phospholipid in plasma membranes could inhibit vesicular glutamate uptake, enabling the expeditious release of glutamate into the synaptic cleft. High proton concentrations, unlike those measured at the synaptic cleft, are required to activate VGLUTs in Xenopus Oocytes, HEK293T cells, and neurons.[51] Artificially protonating these proteins or phospholipids may overcome this negative regulation.

In synaptic vesicles, a specialized type of endosome, VGLUTs need to activate when fully endocytosed to ensure efficient packaging of glutamate. Both low and neutral luminal pH activate the VGLUTs (Figure 3.6, Figure 3.7, Figure 3.14). High luminal Cl achieved through endocytosis of extracellular fluid is required for VGLUT activity, serving as an activating signal.

Low cytoplasmic Cl (4 mM) maximally activates VGLUT activity.[20, 28, 54, 57] The physiological concentrations of cytoplasmic (110 mM) and extracellular (luminal, 10 mM) Cl matches these optimal requirements observed for VGLUT activity in endolysosomal recordings (Figure 3.10). Thus, proteins and phospholipids at the plasma membrane in conjunction with the intrinsic properties of the VGLUTs, would ensure tight regulation of VGLUT activity throughout the synaptic vesicle life cycle.

4.6 Cl and Glutamate Conductances in VGLUTs

HEK293T endolysosomes expressing VGLUTs revealed both Cl and glutamate conductances in the same patch-clamp recording. This feature enabled investigation of the similarities and differences between the Cl and Glutamate properties and conductances in the VGLUTs. Both the Cl and Glutamate conductances were activated by cytosolic Cl and required luminal Cl (Figure 3.8 and Figure 3.10). All 3 VGLUT isoforms with the same 3 mutations (R88A, R322A, and R184A) had similar effects on both the Cl and Glutamate conductances (Figure 3.9 and Figure 3.11). In addition, both Cl and Glutamate conductances were inhibited by submicromolar concentrations of Evans Blue, a known inhibitor of VGLUT activity in purified synaptic vesicles and reconstituted proteoliposome, at sub-millimolar concentrations (Figure 3.12).[24, 28]

However, there were two notable differences between these two conductances. The dose-dependence of cytoplasmic Cl did not saturate in HEK293T endolysosomes expressing VGLUTs (Figure 3.8), similar to what was observed with Xenopus Oocytes expressing internalization-defective VGLUTs.[51] In contrast, the Glutamate conductance saturates at low millimolar concentrations as previously reported (Figure 3.8).[20, 24, 28] These findings suggest that the Cl conductance in VGLUTs, similar to the EAATs, acts like an ion channel, while the Glutamate conductance acts like a transporters which has saturating kinetics.[60-62] Glutamate conductances may also be coupled to protons while Cl conductances are not (Figure 3.15).[55, 56]

However, the shift in the reversal potential observed does not fit the expected Nernst equation, suggesting a confounding, additional proton conductance in this system.

In summary, the Cl and Glutamate conductances exhibit similar regulation, but permeate through the VGLUTs through different transport mechanisms.

4.7 Role of the Cl Conductance in Vesicular Glutamate Transport

The VGLUT expressing endosomes and internalization defective VGLUTs on the plasma membrane of Xenopus Oocytes both demonstrated inward movement of Cl into the lumen or synaptic vesicle (Figure 3.1 and Figure 3.6). To demonstrate a role for this associated Cl conductance, competition assays between Cl and glutamate were performed. The Xenopus Oocyte data demonstrated that high concentrations of glutamate inhibits this inward movement of Cl into the synaptic vesicle, suggesting an interaction between the independent Cl and glutamate pathways (Figure 3.3). Since glutamate conductances were measured in VGLUT-expressing endosomes, the next question was whether or not cytoplasmic Cl conversely inhibited movement of glutamate into synaptic vesicles. Using two physiologically relevant concentrations of cytoplasmic Cl, the glutamate dose response in VGLUT-expressing endolysosomes revealed that cytoplasmic Cl, which permeates through the VGLUTs, also competes with glutamate (Figure 3.16). This finding suggests that the inward movement of Cl (outward conductance) inhibits glutamate transport and regulates the rate of synaptic vesicle filling.

In addition, luminal protons may also play a role in regulating synaptic vesicle filling by determining the extent of this Cl and glutamate competition. When the synaptic vesicle buds off the plasma membrane, the lumen matches that of extracellular fluid – high Cl concentrations (100 mM) and neutral pH (7.4). As the synaptic vesicle matures and fills with glutamate, the proton pump on synaptic vesicle acidifies, lowering the luminal pH (5.5). Although the VGLUT-expressing endosomes packaged both Cl and glutamate into synaptic vesicles at neutral and low pH, cytoplasmic Cl was more effective at competing with vesicular glutamate transport at

more acidic luminal pH (Figure 3.16). Thus, the accumulation of luminal protons serves to regulate vesicular glutamate transport by favoring Cl entry as the synaptic vesicle matures.

To add further credence to the role of the outward movement of Cl in generating the necessary membrane potential for vesicular glutamate transport, the specificity of the inward conductances in VGLUT-expressing endosomes was investigated. Previously, luminal Cl and Br both activated and permeated out of VGLUT-expressing endosomes into the cytoplasm. Luminal glutamate in VGLUT-expressing endosomes failed to exhibit a similar inward conductance and also did not activate an outward Cl (or glutamate) conductance (Figure 3.17). The concentration gradient of Cl across synaptic vesicles favors movement of Cl out of the synaptic vesicle (100 mM Luminal vs 20 mM Cytoplasmic Cl). Previous work in reconstituited proteoliposomes suggested that this movement of Cl out of synaptic vesicles underlies maximal vesicular glutamate transport.[24] Since luminal Cl is required to activate vesicular glutamate transport (50 mM Cl), the outward movement of Cl could eventually lower luminal Cl below this threshold and halt further vesicular glutamate transport. The specificity of this inward conductance through the VGLUT for a physiologically relevant anion and not the packaged glutamate substrate further supports this electrogenic mechanism of vesicular glutamate transport.

4.8 Model of Vesicular Glutamate Transport

Based on findings with Xenopus Oocytes, HEK293T endolysosomes, synaptic vesicles, and reconstituted proteoliposomes expressing VGLUT WT and mutant isoforms, a model was generated for vesicular glutamate transport and the roles of protons, Cl, and the associated Cl conductances (Figure 4.1). There are multiple interpretations of the data shown; however, this model is used to shed some light on the controversial process of synaptic vesicle filling.

All vesicular neurotransmitter transporters utilize the synaptic vesicle electrochemical gradient. The VGLUTs primarily rely on a membrane potential to fill the synaptic vesicle with

glutamate, unlike other classical neurotransmitters like VMAT and VAChT that rely on a chemical gradient to drive dopamine and acetylcholine, respectively.

The VGLUTs achieve this regulation by membrane potential as well as activation and inhibition through Cl. After endocytosis of extracellular fluid with high Cl concentrations, luminal Cl activates the VGLUT by neutralizing the conserved, positively-charged Arg-184 site in transmembrane domain 4 (Figure 4.1-1). Arg-88 and Arg-322 are two key residues that affect both the Cl and Glutamate conductances (Figure 3.9). To promote vesicular glutamate transport, Cl leaves the synaptic vesicle (inward Cl conductance) down its concentration gradient and generate the necessary membrane potential to enable glutamate packaging into the synaptic vesicle (Figure 4.1-2). This inward conductance is specific to Cl, and not to the substrate, glutamate (Figure 3.17). Since high luminal Cl is required to activate vesicular glutamate transport, the loss of luminal Cl and corresponding vesicular glutamate transport ultimately stops once luminal Cl reaches a certain threshold (50 mM Cl, Figure 3.11). This data and interpretation is in line with another study that observed in reconstituted proteoliposomes, maximal glutamate conductance was reported with high luminal Cl and a putative inward Cl conductance.[24] Vesicular glutamate transport may be coupled to protons unlike the Cl conductance; however the supporting data is not as concrete (Figure 3.15).[21] Although the mechanism for permeation is different for vesicular glutamate transport and the Cl conductance (Figure 3.8), both compete and inhibit the other in two different heterologous systems and approaches (Figure 3.3 and Figure 3.16). Thus, the outward Cl conductance inhibits vesicular glutamate transport (Figure 4.1-3).

Modulating the outward Cl and glutamate conductances would potentially regulate the final glutamate concentration in synaptic vesicles. The role of luminal protons in this process was investigated. There was more competition between Cl and glutamate at acidic pH than at neutral pH. This finding suggests that when the synaptic vesicle initially buds off the plasma membrane, vesicular glutamate transport is favored. As the synaptic vesicle matures, like the

loss of luminal Cl, the accumulation of luminal protons inhibits further vesicular glutamate transport and favors the inward movement of Cl into the synaptic vesicle (Figure 4.1-3).

In addition, cytoplasmic Cl has been shown to allosterically activate vesicular glutamate transport (Figure 3.10). Any regulation of cytoplasmic Cl through transporters like KCC2 and NKCC1 can also modulate excitatory neurotransmission.[58] In fact, experiments in the Calyx of Held demonstrated a dependence on cytoplasmic Cl for EPSC recovery (both amplitude and rate).[57]

In conclusion, regulation of VGLUT activity in specialized endosomes like synaptic vesicles relies on Cl (activation and permeation), key critical residues (Arg-88, Arg-184, and Arg-322), and protons. The importance of Cl regulation for excitatory neurotransmission is exhibited at the synapse level in the Calyx of Held.[57] The use of Xenopus Oocytes and HEK293T endolysosomes revealed intrinsic properties of VGLUTs that enable Cl and protons to regulate excitatory neurotransmission.

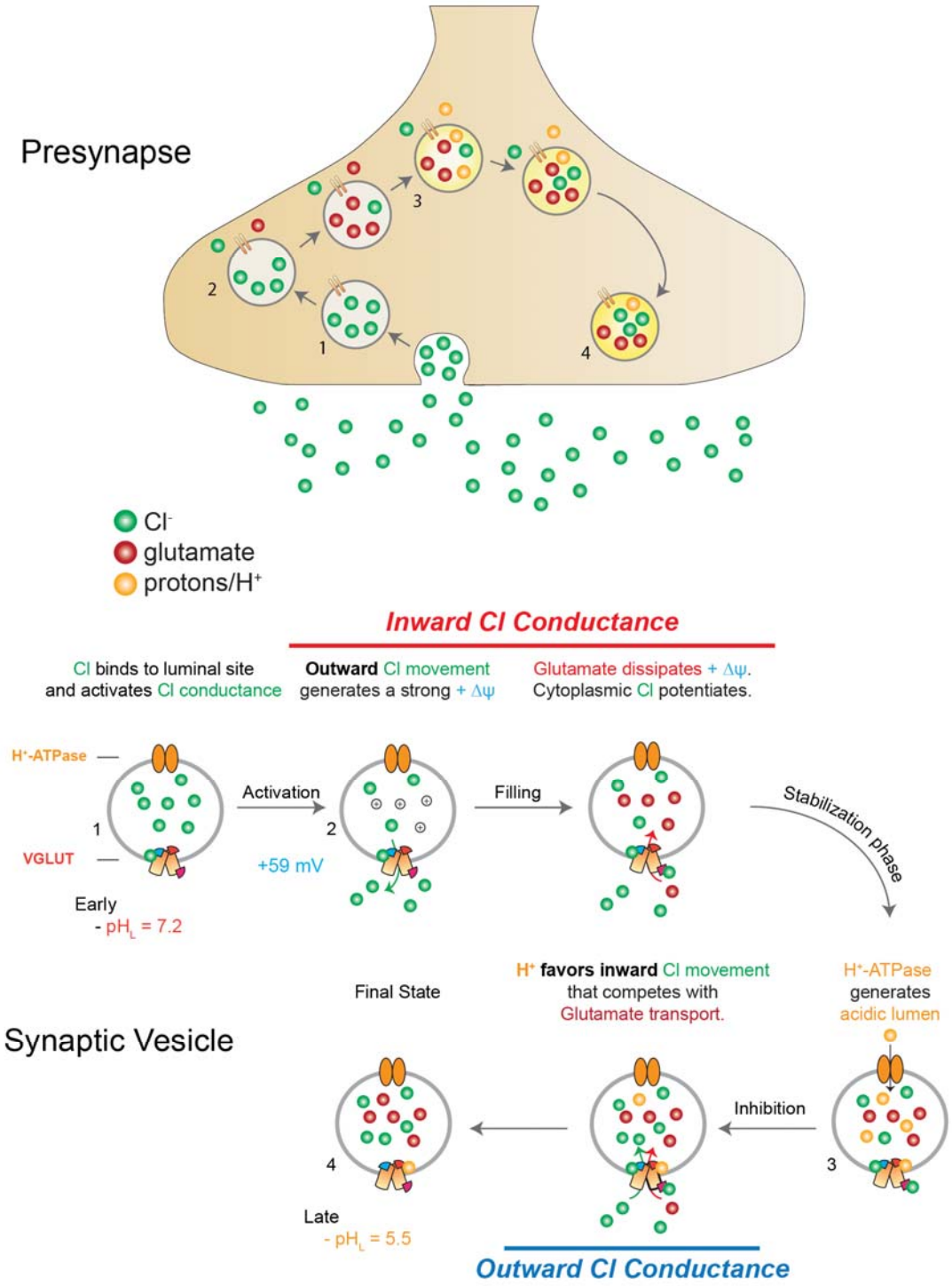

Figure 4.1 Model of Vesicular Glutamate Transport.
Top Panel, The synaptic vesicle life cycle starting from endocytosis as the synaptic vesicle pinches off with high Cl^- and high pH from the synaptic cleft (Step 1) and ending when the vesicle acidifies to low pH and fills with glutamate (Step 4).
Bottom Panel, Corresponding steps of synaptic vesicle during the synaptic vesicle life cycle are shown to illustrate the effects of lumenal Cl^- (Step 1), the inward Cl^- conductance (Step 2), the glutamate conductance, acidification (Step 3), and competing outward Cl^- conductance (Step 4).

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