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Protons Regulate Vesicular Glutamate Transporters through an Allosteric Mechanism

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Abstract

The quantal nature of synaptic transmission requires a mechanism to transport neurotransmitter into synaptic vesicles without promoting non-vesicular efflux across the plasma membrane. Indeed, the vesicular transport of most classical transmitters involves a mechanism of H^+ exchange which restricts flux to acidic membranes such as synaptic vesicles. However, vesicular transport of the principal excitatory transmitter glutamate depends primarily on membrane potential, which would drive non-vesicular efflux, and the role of protons is unclear. Adapting electrophysiology to record currents associated with the vesicular glutamate transporters (VGLUTs), we characterize a chloride conductance that is gated by lumenal protons and chloride and supports glutamate uptake. Rather than coupling stoichiometrically to glutamate flux, lumenal protons and chloride allosterically activate vesicular glutamate transport. Gating by protons serves to inhibit what would otherwise be substantial non-vesicular glutamate efflux at the plasma membrane, thereby restricting VGLUT activity to synaptic vesicles.

Introduction

For classical neurotransmitters, quantal release by exocytosis depends on their transport into synaptic vesicles. The transporters responsible for this activity reside primarily on synaptic

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Author contributions

J.E. designed and performed most of the experiments. He generated most of the data, figures and text of the manuscript. R.C. performed experiments in oocytes to test the interaction between glutamate and chloride and a subset of the pharmacology experiments and compare isoforms. M.M. contributed to the comparison of the isoforms in oocytes. K.S. helped to prepare synaptic vesicles and T.S. provided the E. coli strain DK8/pTR19-ASDS. R.H.E. conceived the idea, supervised the work and wrote the manuscript with J.E.

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vesicles, but appear at the plasma membrane with exocytosis. In addition, a stable pool of cell surface transporter may facilitate rapid recycling, and previous work has indeed identified a 'readily retrievable pool' of synaptic vesicle protein at the plasma membrane (Fernandez-Alfonso et al., 2006; Hua et al., 2011). At the cell surface, vesicle transporters have the potential to mediate non-vesicular efflux, thereby degrading the quantal output, conferring a tonic signal or influencing receptor expression. Indeed, pioneering studies at the neuromuscular junction demonstrated the nonquantal leakage of acetylcholine (ACh) from motor nerve terminals predicted to exceed spontaneous quantal release in total amount by two orders of magnitude (Katz et al., 1977, 1981). Although the mechanism remains unknown, motor neurons recycle ACh in the form of choline rather than ACh, implicating by default the vesicular ACh transporter (VAChT) in non-vesicular efflux of ACh. However, most vesicular transporters including VAChT couple transmitter uptake to H^+ exchange, requiring expression on intracellular membranes acidified by the vacuolar H^+-ATPase, such as synaptic vesicles, and limiting activity at the cell surface, which exhibits little or no pH gradient (ΔpH) (Edwards, 2007). Nonetheless, all of the vesicular transporters depend to some extent on lumenally positive membrane potential (Δψ), which could in principle continue to drive efflux across the plasma membrane. This possibility pertains especially to vesicular uptake of the principal excitatory transmitter glutamate, which relies primarily on Δψ rather than ΔpH (Maycox et al., 1988; Carlson et al., 1989a; Edwards, 2007).

Is there a mechanism that limits the non-vesicular efflux of glutamate? Although the vesicular glutamate transporters might couple glutamate uptake to the exchange of lumenal H^+, similar to other vesicular transporters, dissipation of the pH gradient (ΔpH) has actually been shown to increase glutamate uptake into synaptic vesicles (Shioi et al., 1989; Maycox et al., 1990; Goh et al., 2011), arguing against a mechanism involving H^+ exchange. Apparently, dissipation of ΔpH can promote glutamate uptake by relieving inhibition of the H^+ pump to increase the main driving force Δψ. On the other hand, under different conditions, dissipation of ΔpH has been shown to inhibit vesicular glutamate transport (Tabb et al., 1992; Wolosker et al., 1996). The role of H^+ in glutamate transport has thus remained unclear despite important implications for non-vesicular release as well as the lumenal concentration of glutamate achieved at steady-state (Edwards, 2007).

Vesicular glutamate transport also exhibits complex interactions with Cl^-. Transport shows a biphasic dependence on Cl^-, with activation by 2–10 mM Cl^- and inhibition at higher concentrations (Maycox et al., 1988; Carlson et al., 1989a). At low concentrations, Cl^- appears to act allosterically (Hartinger et al., 1993; Juge et al., 2010) whereas at high concentrations, it inhibits glutamate uptake presumably by dissipating the driving force Δψ. However, the site of allosteric activation by Cl^- remains unclear, with different studies favoring a cytosolic site, a lumenal site or both (Hartinger et al., 1993; Schenck et al., 2009; Preobraschenski et al., 2014). Although cytosolic Cl^- regulates quantal glutamate release in synaptic transmission (Price et al., 2006; Hori et al., 2012), the rationale for physiological regulation by Cl^- also remains unknown.

Chloride provides charge compensation for the electrogenic H^+-ATPase, enabling the acidification of synaptic vesicles (Maycox et al., 1988; Gras et al., 2008; Hnasko et al., 2010). This has generally been attributed to specific Cl^- carriers on the synaptic vesicle

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membrane (Stobrawa et al., 2001; Riazanski et al., 2011) similar to those responsible for the acidification of other endosomal membranes (Stauber et al., 2013). Surprisingly, the vesicular glutamate transporters (VGLUTs) have been shown to promote the acidification of synaptic vesicles by Cl^-, suggesting that they also exhibit a Cl^- conductance (Bellocchio et al., 2000; Schenck et al., 2009; Preobraschenski et al., 2014). Rather than distinct carriers dedicated to Cl^-, the VGLUTs may in fact account for most of the Cl^- permeation into synaptic vesicles (Schenck et al., 2009). However, another report has failed to identify any Cl^- permeability associated with the VGLUTs (Juge et al., 2010), and the basis for this discrepancy has remained unclear. Since Cl^- entry could dissipate the same $\Delta\psi$ that drives glutamate uptake, it also remains unclear why the VGLUTs would promote the flux of both anions.

The multiple, complex interactions of the VGLUTs with H^+ and Cl^- have been difficult to integrate into a model for synaptic vesicle filling. This reflects an incomplete understanding of their basic function, due in large part to limitations of the available radiotracer flux assays. In contrast to the vesicular monoamine transporters which exhibit robust, high affinity transport, the VGLUTs confer weak radiotracer uptake, particularly in heterologous expression systems. To overcome these limitations, we have now developed the use of electrophysiology to study the VGLUTs. We show that they exhibit a Cl^- conductance allosterically activated by lumenal H^+ as well as Cl^-. In addition, lumenal H^+ and Cl^- activate glutamate transport, with no evidence for H^+ exchange. The allosteric requirement for H^+ efficiently prevents non-vesicular efflux across the plasma membrane, thereby restricting VGLUT activity to acidic membranes such as synaptic vesicles.

Results

VGLUT2 exhibits a Cl^- conductance gated by H^+ and Cl^-

The dependence of VGLUTs on $\Delta\psi$ suggested that it might be possible to record their activity using electrophysiology. However, the VGLUTs localize to intracellular membranes both in native synaptic vesicles and after heterologous expression, making them inaccessible to standard electrophysiological approaches that monitor currents across the plasma membrane. To study the VGLUTs using electrophysiology, we produced a version of VGLUT2 with increased cell surface expression. Specifically, we mutated dileucine-like motifs at both N- and C-termini that we had previously found to influence the endocytosis of closely related VGLUT1 (Figure 1A) (Foss et al., 2013). The resulting VGLUT2 mutant was readily detected at the plasma membrane of *Xenopus* oocytes with an antibody that recognizes the HA epitope inserted into the lumenal loop between the first two transmembrane domains (Figure 1B,C).

Using two-electrode voltage clamp to record from oocytes expressing the internalization-defective VGLUT2, we detected no currents in physiological external solution at pH 7.4. However, lowering external pH (pH_o) to resemble the acidic synaptic vesicle lumen produced a large inwardly rectifying current that was not observed in water-injected oocytes (Figure 1C,D). To characterize this current, we substituted external Na^+ and K^+ with the impermeable cation choline. Since currents of similar magnitude were still observed in choline chloride at pH_o 5.0 (Figure 1E), Na^+ and K^+ are not required. Direct comparison of

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Na^+, K^+ and choline further demonstrated that these cations do not affect the currents (Fig. S1). However, substituting external Cl^- (Cl^-_o) with the impermeant anion gluconate greatly reduced the VGLUT2-associated currents (Figure 1E). Thus, the currents depend on external H^+ and Cl^-.

To understand how H^+ activate the currents associated with VGLUT2, we examined the response to a range of pH_o. The currents show a steep dependence on pH_o with an EC_{50} of pH 5.53 ± 0.06 (Figure 2A,B), well within the range reported for endosomes and synaptic vesicles (Mitchell et al., 2004). However, changing pH_o does not influence the reversal potential of the currents (Figures 2A,C and S2A). Thus, H^+ activate without contributing to the currents observed.

The currents also depend on the concentration of Cl^-_o. Present even at 2 mM Cl^-_o, the currents increase linearly with higher concentrations (Figure 2D,E). Since external Cl^- produces inward currents, they cannot reflect only permeation by external Cl^-, and Cl^-_o thus gates the conductance. However, varying Cl^-_o does influence the reversal potential of the currents, indicating that Cl^- also permeates (Figure 2F). Although the strong rectification complicates measurement of the reversal potential, it shifts almost as much as predicted by the Nernst equation for the Cl^- concentrations tested (Figures 2F and S2B): 36.0 ± 4.54 mV per 10-fold change in Cl^- gradient, or 40 mV per 10-fold change in Cl^- gradient when corrected for the activity of choline Cl. The electrophysiology thus indicates two distinct roles for Cl^-, one on the lumenal surface of the protein in allosteric activation, and another in permeation.

The Cl^- conductance shares properties with vesicular glutamate transport

To characterize further the Cl^- conductance associated with VGLUT2, we tested the sensitivity to known anion channel blockers. 5-Nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) does not inhibit the currents, and niflumic acid (NFA) inhibits the currents only at high micromolar concentrations (Figure 3A). On the other hand, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) potently inhibits the currents with IC_{50} of 194 nM [119–318 nM] (Figure 3A). Interestingly, DIDS has been shown to inhibit synaptic vesicle glutamate uptake in the same concentration range (Hartinger et al., 1993). In addition, the more specific but membrane impermeant VGLUT inhibitor Evans Blue inhibits the currents when injected into oocytes (Figure S3A). Addition to the outside of the oocyte inhibits the currents as well, but with a latency up to minutes that varied with the concentration of dye used (data not shown), suggesting that it acts from the cytoplasmic face of the membrane. The pharmacology thus indicates parallels between the VGLUT2-associated Cl^- conductance and vesicular glutamate transport.

Activation by Cl^- suggests another similarity between the Cl^- conductance and vesicular glutamate transport. To assess the specificity of this allosteric activation, we therefore tested several other anions. Bromide but not iodide, nitrate or isothiocyanate activates the conductance associated with VGLUT2 (Figure 3B). Remarkably, previous work using native brain synaptic vesicles and reconstituted VGLUT2 showed a very similar selectivity for the allosteric activation of vesicular glutamate transport by inorganic anions (Naito et al., 1985;

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Juge et al., 2010). In addition, we now find that external Br^{--} shifts the reversal potential to more negative values, indicating that it also permeates (Figure S3B).

To assess further a direct role for VGLUT2 in the Cl^- conductance, we replaced the highly conserved Arg-322 in transmembrane domain 7 with alanine. This residue has been predicted to form part of the central binding site in the VGLUTs, and to have an important role in vesicular glutamate transport (Almqvist et al., 2007; Herman et al., 2014). Oocytes injected with cRNA encoding R322A VGLUT2 show no pH-induced currents despite plasma membrane expression equivalent to VGLUT2 (Figure 3C,D). The Cl^- conductance thus represents a property of VGLUT2.

To determine the contribution of endogenous, cytosolic glutamate to the currents, we recorded from HEK293T cells expressing internalization-defective VGLUT2 using the whole cell patch clamp configuration to define cytosolic composition. With a glutamate-free pipette solution containing 145 mM TMA gluconate and 5 mM Cl^-, we observed inwardly rectifying currents in cells expressing VGLUT2 and EGFP but not in control cells expressing EGFP alone (Figures 4A,B and S4). Very similar to the results in *Xenopus* oocytes, the currents depend on low pH_o and high Cl^-_o (Figure 4A–C). The currents in HEK293T cells also show sensitivity to DIDS, with no effect of DIDS on control cells (Figures 4D and S4). Parallel experiments with 5 mM glutamate added to the pipette solution showed very similar currents to those without glutamate (Figure 4E). Glutamate may affect the VGLUT-associated currents, but the effect is too small to detect by comparing different transfected cells. Regardless, glutamate is not required for the currents, which must therefore be stoichiometrically uncoupled from glutamate flux.

On the other hand, we found that cell surface VGLUT2 can mediate glutamate transport as well as the Cl^- conductance. In control HEK293T cells, reducing external pH produces no detectable increase in the low level of bulk glutamate efflux. In contrast, cells expressing internalization-defective VGLUT2 release substantially more glutamate in response to lowering external pH, and this increase is sensitive to DIDS whereas DIDS has no effect on efflux from control cells (Figure S5). Although cytosolic glutamate is not required for the currents associated with VGLUT2, the protein thus retains transport activity at the plasma membrane, and the dependence of glutamate efflux on low pH establishes another similarity between the Cl^- conductance and vesicular glutamate transport. It also supports a role for the allosteric regulation by H^+ in suppression of non-vesicular glutamate efflux across the plasma membrane.

To determine whether external glutamate can influence the currents associated with VGLUT2, we added a range of concentrations directly to *Xenopus* oocytes expressing the internalization-defective transporter. External glutamate inhibits the currents only partially at the highest concentration tested (96 mM) (Figure 3D). However, the effect is clearly dose-dependent and the closely related amino acid aspartate has no discernible effect (Figure 3D), consistent with the known specificity of ligand recognition by synaptic vesicle glutamate transport (Naito et al., 1985). To assess the relationship between glutamate and Cl^-, we repeated the experiment in different concentrations of Cl^- (10 and 40 mM). Glutamate

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inhibits the currents more potently in 10 mM Cl^- than in 40 mM, indicating competition between the two anions (Figure 3E).

VGLUT1–3 express the Cl^- conductance, but with different properties

To determine whether related transporters in the SLC17 family also exhibit a Cl^- conductance, we generated similar internalization-defective versions of VGLUT1, VGLUT3 and the closely related sialic acid transporter sialin (Morin et al., 2004; Wreden et al., 2005). In VGLUT1 and 3, we introduced mutations into the three dileucine-like motifs conserved in all members of the family (Figure S6A). Expressed in *Xenopus* oocytes, VGLUT1 and 3 exhibit inwardly rectifying currents dependent on low pH_o, similar to VGLUT2 (Figures 5A). Indeed, the Cl^- dependence of VGLUT1- and VGLUT3-associated currents shows no difference from those of VGLUT2 (Figure S6B,C). However, the VGLUT3-associated current displayed a small but significant difference in pH dependence from VGLUT1 and 2, requiring lower pH for maximal activation (Figure 5C). For these experiments, we used transporters without an HA tag because the currents associated with VGLUT3 were sensitive to this modification, a phenomenon not observed for VGLUT1 and 2 (compare to Figures 1 and 2). We did however use the HA-tagged constructs to estimate surface expression of the different isoforms, and observed higher levels of VGLUT3 than VGLUT1 and 2 (Figure S6D). We also tested an internalization-defective version of the closely related sialic acid transporter sialin (Wreden et al., 2005), but observed no currents associated with its expression in oocytes despite high cell surface expression (Figure 5B and S6D,E). Thus, the Cl^- conductance appears specifically associated with the VGLUTs. All three VGLUT isoforms mediate vesicular glutamate transport with very similar properties including a common requirement for $\Delta\psi$ and biphasic dependence on Cl^- (Bellocchio et al., 2000; Takamori et al., 2000; Fremeau et al., 2001; Takamori et al., 2001; Fremeau et al., 2002; Gras et al., 2002; Schafer et al., 2002) and the Cl^- conductance now also appears shared, indicating a conserved function. However, VGLUT3 differs in the H^+ requirements for maximal activation.

Implications for vesicular glutamate transport

The similarity between Cl^- conductance and vesicular glutamate transport predicts that transport will exhibit many of the same properties observed for the conductance. We have therefore purified recombinant VGLUT2 from insect cells (Figure S6A,B) and coreconstituted it into liposomes with a bacterial ATP synthase (TF_0F_1) to create the required H^+ electrochemical driving force (Schenck et al., 2009; Preobraschenski et al., 2014). Addition of ATP to the proteoliposomes confers substantial glutamate uptake that depends on reconstitution with VGLUT2 and shows inhibition by Evans Blue (Figures 6A and S7C). Using this system, we tested the prediction that lumenal Cl^- activates vesicular glutamate transport. In the presence of 5 mM external Cl^-, liposomes containing 160 mM KCl indeed confer much higher transport activity than liposomes containing 160 mM Kgluconate (Figure 6A), as shown previously for VGLUT1 (Schenck et al., 2009; Preobraschenski et al., 2014). Second, we tested the prediction that lumenal H^+ activate vesicular glutamate transport. Consistent with gating of the Cl^- conductance by low pH, alkalinization of lumenal pH with the H^+/K^+ ionophore nigericin effectively reduces transport by VGLUT2 (Figure 6A). Dissipation of the predicted driving force $\Delta\psi$ with the

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K^+ ionophore valinomycin also impairs uptake. Similar to the Cl^- conductance, vesicular glutamate transport thus appears to depend on lumenal Cl^- and H^+ as well as the driving force $\Delta\psi$. These results using reconstituted transporter are also consistent with the suppression of non-vesicular glutamate efflux at neutral pH (Figure S5).

Since the Cl^- conductance associated with VGLUT3 differs from those associated with the other isoforms, we also reconstituted VGLUT3 into liposomes. Proteoliposomes with VGLUT3 and the bacterial H^+ pump display ATP-dependent, Evans Blue-sensitive glutamate uptake very similar to that of VGLUT2, and the transport activity also depends on lumenal Cl^- and low lumenal pH (Figure 6B). VGLUT3 also shows a consistent trend toward lower uptake activity than VGLUT2. In more striking contrast to VGLUT2, however, dissipation of $\Delta\psi$ with valinomycin fails to inhibit glutamate uptake by VGLUT3 at this time point even though the ionophore clearly promotes acidification of proteoliposomes with VGLUT3 + TF0F1 (Figure S7D).

The use of a H^+ pump makes it difficult to control lumenal pH independent of $\Delta\psi$ (or ΔpH), and the changes in multiple variables limit interpretation of the experiments. To define better the conditions, we have therefore reconstituted the VGLUTs alone, with lumenal KCl at pH 7.3. Using external K gluconate also at pH 7.3, we detect glutamate uptake only marginally above background (defined in Evans Blue) (Figure 6C). However, lowering external pH to 6 in the presence of nigericin, which lowers lumenal pH as well, greatly increases glutamate transport (Figure 6C). In the absence of a pH gradient, lowering pH thus activates vesicular glutamate transport, consistent with allosteric activation by H^+. The sensitivity of glutamate uptake to valinomycin further indicates the electrogenic nature of the transport, and the only ionic gradient available to produce $\Delta\psi$ is the outwardly directed Cl^- gradient. Indeed, the presence of high external Cl^- effectively abolishes transport (Figure 6C). If the Cl^- gradient produces $\Delta\psi$, there must also be a pathway for Cl^- flux, and the conductance associated with VGLUT2 presumably provides this mechanism.

We also reconstituted VGLUT3 alone, again with high lumenal Cl^-. In contrast to VGLUT2, we observe no specific glutamate uptake by VGLUT3 at pH 7.3 (Figure 6D). Lowering external pH in the presence of nigericin does confer glutamate uptake, but the stimulation is much smaller than observed for VGLUT2 ($p=0.0025$ by unpaired t-test) (Figure 6C,D). Nonetheless, transport remains dependent on $\Delta\psi$ and low pH, consistent with the expression of a Cl^- conductance by VGLUT3, although with properties different from the other isoforms.

We then determined whether the activation of glutamate uptake by H^+ extends into the pH range relevant for synaptic vesicles. Reconstituting VGLUT2 without a H^+ pump but with high lumenal Cl^-, we varied external pH in the presence of nigericin. Glutamate transport increases progressively from pH 7.3 to 6.0, and then declines at lower pH (Figure 6G). Since synaptic vesicles have a pH ~5.8 (Mitchell et al., 2004; Egashira et al., 2015), the sensitivity of vesicular glutamate transport to pH seems appropriately tuned to the physiological conditions. The activation of glutamate uptake by H^+ also resembles activation of the Cl^- conductance, with the decrement in transport but not conductance at extremely low pH

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presumably reflecting the reduction in pH on both sides of the membrane in the biochemical reconstitution experiments.

The role of H^+ as allosteric activator rather than as coupled ion in turn predicts an effect of H^+ on the rate of glutamate transport. To test this prediction, we examined the time course of glutamate uptake by VGLUT2 coreconstituted with the bacterial H^+ pump and high lumenal Cl^-. Dissipation of either ΔpH (with nigericin) or Δψ (with valinomycin) greatly reduces glutamate transport at all late time points (10–40 min) (Figure 6E). However, dissipation of Δψ with valinomycin has a less dramatic effect at early time points. Remarkably, at the earliest time point (1 min), dissipation of the driving force actually increases transport. We similarly reconstituted VGLUT3. In this assay, VGLUT3 also exhibits less transport activity than VGLUT2 reconstituted in the same way (Figure 6E,F). However, valinomycin stimulates uptake by VGLUT3 at early time points (1 and 2 min) even more dramatically than the effect on VGLUT2 (Figure 6E,F). In the case of VGLUT2 and particularly VGLUT3, allosteric activation by H^+ thus potently stimulates the rate of vesicular glutamate transport. At late times, dissipation of Δψ with valinomycin does inhibit glutamate uptake by VGLUT3, but the effect is minor relative to the more severe inhibition of VGLUT2 (Figure 6E,F). The allosteric activation by lumenal H^+ that results from dissipation of Δψ must therefore compensate in part for the loss of driving force. Further, uptake by VGLUT3 reaches a plateau by 20 minutes whereas uptake by VGLUT2 remains linear to almost 40 minutes (Figure 6E,F).

To determine whether lumenal H^+ and Cl^- promote glutamate transport into native membranes, we preloaded synaptic vesicles with KCl or K gluconate and measured uptake in the absence of ATP. In the presence of nigericin, lowering pH from 7.3 to 6.0 enabled us to detect uptake by KCl^- loaded synaptic vesicles that was sensitive to valinomycin and hence dependent on Δψ (Figure 6H). Preloading with K gluconate does not support the increase in uptake at pH 6.0. Thus, lumenal H^+ and Cl^- can support electrogenic synaptic vesicle glutamate transport in the absence of ΔpH.

Neutral pH suppresses non-vesicular glutamate efflux from neurons

To determine whether physiological extracellular pH suppresses non-vesicular efflux by the VGLUTs, we expressed internalization-defective VGLUT2 in cultured hippocampal neurons (Figure 7C) and used whole cell recording to monitor glutamate receptor currents at pH 7.4 and pH 6.0. In control neurons, lowering the pH to 6.0 did not produce any detectable AMPA receptor currents (Figure 7A, B). When recording from cultures expressing plasma membrane (pm) VGLUT2, however, low pH produced a large inward current that was almost completely blocked by the AMPA receptor antagonist CNQX (Figure 7A,B). Neutral pH thus limits the non-vesicular efflux of glutamate mediated by transporters at the plasma membrane.

Discussion

The results provide definitive evidence for the association of a Cl^- conductance with the VGLUTs. They also reveal the requirement for allosteric activation of the conductance by H^+ and Cl^- itself. Both ions act from the extracellular or lumenal face of the membrane but

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only Cl^- permeates and the inward currents activated by external Cl^- show two roles for Cl^-, in gating as well as permeation. The two roles for Cl^- also involve different faces of the membrane, with gating from the lumenal surface and permeation primarily from the cytoplasmic surface due to the rectification, indicating mechanisms for the effects of both lumenal and cytosolic Cl^- reported previously (Hartinger et al., 1993; Schenck et al., 2009; Preobraschenski et al., 2014). In addition, activation of the VGLUT-associated Cl^- conductance by H^+ suggests a simple explanation for previous, discrepant observations. Work that observed a Cl^- conductance associated with the VGLUTs involved vesicle acidification, providing the required allosteric activation by H^+ (Bellocchio et al., 2000; Schenck et al., 2009; Preobraschenski et al., 2014). In contrast, work that did not detect the conductance used membranes at neutral pH (Juge et al., 2010). Although at least one other member of the SLC17 family exhibits a Cl^- conductance (Busch et al., 1996; Bröer et al., 1998), the closely related sialin does not, indicating some specificity for the VGLUTs. Interestingly, the structurally distinct plasma membrane excitatory amino acid transporters also exhibit a Cl^- conductance but this is not conserved in all isoforms, and glutamate activates rather than inhibits the conductance associated with these carriers (Tzingounis et al., 2007).

Activation of the VGLUT-associated Cl^- conductance by lumenal H^+ and Cl^- suggests a positive feedback mechanism to acidify synaptic vesicles. Chloride entry through the conductance provides charge compensation to disinhibit the H^+ pump and promote vesicle acidification, further activating the conductance. Inactive at the plasma membrane due to the neutral extracellular pH, the conductance will thus develop only as lumenal pH begins to drop after endocytosis. In this way, the positive feedback that results from allosteric activation of the Cl^- conductance by lumenal H^+ and Cl^- has the potential to act as a switch for synaptic vesicle acidification, with implications for the corelease of glutamate with dopamine, serotonin and acetylcholine, all of which rely on ΔpH as the driving force for vesicular transport (Gras et al., 2008; Varga et al., 2009; Hnasko et al., 2010; Higley et al., 2011; Nelson et al., 2014; Shabel et al., 2014). Importantly, the conductance activates only at pH less than 7.4, enabling clear discrimination of endocytic from plasma membrane VGLUT.

The Cl^- conductance also shares requirements with vesicular glutamate transport. Both activities require allosteric activation by Cl^- (Maycox et al., 1988; Carlson et al., 1989a; Hartinger et al., 1993; Juge et al., 2010). Remarkably, this activation begins in the same low millimolar range for both and shows the same specificity for Br^- as well as Cl^-. In addition, glutamate inhibits the Cl^- conductance but aspartate has much less effect, consistent with the lack of aspartate recognition by the VGLUTs. Further, glutamate inhibits the conductance more potently in low than high Cl^-, indicating competition between the ions. The organic anion glutamate and inorganic anion Cl^- may thus use a similar permeation pathway through the transporters. Alternatively, they may compete at the site of allosteric activation. Supporting the relationship between conductance and glutamate transport, DIDS and Evans Blue potently inhibit both activities.

Similarity to the Cl^- conductance predicts one property of vesicular glutamate transport that has not previously been appreciated—allosteric activation by H^+. Vesicle acidification has

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been suggested to limit the driving force Δψ by inhibiting the H^+ pump, or to couple directly to glutamate flux through a mechanism of H^+ exchange similar to other vesicular neurotransmitter transporters. Extrapolating from the strictly allosteric activation of Cl^- currents by H^+, however, we now find that lumenal H^+ also activate vesicular glutamate transport and this effect occurs independent of a pH gradient. Although we cannot exclude the possibility that Δψ drives H^+ movement coupled to glutamate flux even in the absence of ΔpH, the results are consistent with an allosteric mechanism similar to that activating the Cl^- conductance. In fact, a role for lumenal H^+ in vesicular glutamate transport has been suggested previously, but these experiments were performed using a H^+ pump and pump activity varied with pH (Tabb et al., 1992). We now show that the activation by lumenal H^+ can occur in the absence of a H^+ pump, and extends into the range of physiological pH for synaptic vesicles.

The allosteric activation by H^+ helps to establish a functional relationship between the VGLUTs and other members of the SLC17 family. In contrast to the VGLUTs, which are driven by Δψ, other SLC17 family members such as sialin mediate electroneutral H^+ cotransport (Morin et al., 2004; Wreden et al., 2005), and it has been difficult to understand how such different functions could arise from proteins so similar in sequence. The allosteric regulation of VGLUTs now indicates an interaction with H^+ that may involve the same sites which couple H^+ movement to substrate flux by other SLC17 family members.

In addition, the allosteric activation by H^+ suggests a physiological role for the Cl^- conductance associated with VGLUTs. Although the Cl^- conductance may dissipate the Δψ that drives glutamate uptake, it also acidifies the synaptic vesicle, thereby conferring the required allosteric activation of VGLUTs by lumenal H^+. Allosteric control predicts an effect on the rate of vesicle filling, and we find that dissipation of Δψ with valinomycin increases the initial rate of glutamate uptake by both VGLUT2 and 3. Thus, allosteric activation by H^+ can compensate at least initially for the loss of driving force. The inverse relationship between Δψ and ΔpH further suggests that a trade-off between the rate (determined by ΔpH) and extent (determined by Δψ) of glutamate transport controls vesicle filling.

The results also suggest another role for the Cl^- conductance in vesicular glutamate transport. In the case of VGLUTs reconstituted without a H^+ pump, an outwardly directed Cl^- gradient suffices to drive glutamate transport that depends on Δψ. In addition to dissipation of Δψ by Cl^- influx, Cl^- efflux can thus produce Δψ, and the different directions of flux will naturally depend on the Cl^- gradient across the synaptic vesicle membrane as well as the Δψ set by the H^+ pump. Indeed, the role of the Cl^- conductance in production of Δψ might become particularly important when accumulating ΔpH late in the course of vesicle filling inhibits the H^+ pump. The Cl^- conductance can therefore regulate vesicular glutamate transport in two distinct ways: by promoting the allosteric activation by H^+ that affects the rate of filling, and by contributing to the driving force Δψ that affects the extent. Conservation of the Cl^- conductance among all three isoforms further attests to its functional significance.

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However, we find that VGLUT3 differs from the other isoforms in several respects. First, the Cl^- conductance associated with VGLUT3 requires lower pH for maximal activation. Second, VGLUT3 consistently exhibits lower glutamate uptake activity than VGLUT2, both when reconstituted alone, reliant on a Cl^- gradient to generate $\Delta\psi$, and with a H^+ pump. Glutamate transport by VGLUT3 is also much less sustained than by VGLUT2. Third, and most remarkably, dissipation of the driving force $\Delta\psi$ has relatively little effect on glutamate uptake by VGLUT3. Interestingly, one previous study reported some of the same distinctive features for glutamate transport by VGLUT3 but ascribed them to differences in expression (Gras et al., 2002). The differences in Cl^- conductance now suggest a mechanism for the differences in glutamate transport. In particular, the requirement for lower pH to activate the Cl^- conductance associated with VGLUT3 presumably results in smaller $\Delta\psi$ to drive glutamate uptake in the membranes reconstituted without a H^+ pump. If the altered pH dependence extends to glutamate transport itself, this may account for reduced uptake in the presence of a H^+ pump. The modest effect of valinomycin on glutamate uptake by VGLUT3 presumably also reflects the potency of valinomycin to lower lumenal pH into the range required for maximal allosteric activation of VGLUT3. In contrast, valinomycin strongly inhibits VGLUT2 because allosteric activation by H^+ is already maximal, and valinomycin serves only to dissipate the driving force $\Delta\psi$.

Why then do specific cell populations express VGLUT3 rather than a different isoform? Cholinergic interneurons in the striatum and a large proportion of serotonin neurons in the raphe nuclei corelease glutamate and express VGLUT3 (Gras et al., 2008; Varga et al., 2009; Liu et al., 2014; Nelson et al., 2014). The partial activation of VGLUT3 at the usual synaptic vesicle of pH 5.8 may restrain glutamate transport and thereby facilitate uptake of the other, cationic transmitter. However, many neurons expressing VGLUT3 release glutamate without another classical transmitter, and the rationale for expression of this isoform remains unclear.

Finally, the requirement for activation of the VGLUTs by H^+ provides a mechanism to suppress the non-vesicular efflux of glutamate by VGLUTs at the plasma membrane. Low external pH is required to promote glutamate efflux across the plasma membrane of transfected HEK cells, as suggested previously although interpreted then to reflect H^+ exchange (Mackenzie et al., 2008). Our data now show that the requirement of VGLUTs for H^+ is allosteric. In addition, we use plasma membrane-targeted VGLUT2 to show that low pH is required to activate non-vesicular glutamate efflux from hippocampal neurons. Thus, transporter at the plasma membrane is inactivated under physiological conditions. Ischemia or traumatic brain injury that reduce extracellular pH may therefore promote glutamate efflux and excitotoxicity through this mechanism (McDonald et al., 1998; Chesler, 2003; Timofeev et al., 2013).

In summary, the results identify novel properties intrinsic to the VGLUTs that have the potential to promote vesicle acidification, then use the lumenal H^+ to activate glutamate uptake, thereby restricting glutamate flux to synaptic vesicles and enabling quantal release.

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Materials and Methods

Electrophysiology

Oocyte recording—cRNA (50 ng/oocyte) encoding internalization-defective rat VGLUT1–3 and sialin with the lumenal hemagglutinin (HA) tag (Figures 1–3 and S1–3), or without the tag (Figure 5A,C and S6) (Wreden et al., 2005; Voglmaier et al., 2006) were injected into defolliculated *X. laevis* oocytes. The tag is located at the position indicated below between transmembrane domains 1 and 2 of the three isoforms:

```
                    v
VGLUT1          MVNNSTTHRG GHVVVQKAQ
VGLUT2          MVNNSTIHRG GKVIKEKAK
VGLUT3          MVNNSTVYVD GKPEIQTAQ
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After incubation in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) with 50 μg/ml tetracycline and gentamicin at 16° C for 5–6 days, the cells were recorded by standard two-electrode voltage clamp in Ca^{++}-free ND96, 98 mM choline Cl (or gluconate) containing 1 mM $MgCl_2$ and 5 mM buffer, or as indicated. Steady-state current/voltage (I-V) relations were obtained using a protocol of 300-ms voltage steps from a holding potential of –20 mV to –120 - +60 mV in steps of 10 mV.

Whole-cell patch clamp recording: HEK cells—HEK293T cells were transfected with pIRES2-EGFP or pIRES-EGFP FI/AA 2xGG VGLUT2 24–48 h before recording. The pipette solution contained (in mM) 145 TMA-gluconate, 30 HEPES pH 7.5, 5 EGTA, 2.5 $MgCl_2$, +/– 5 mM glutamate. Currents were recorded from a holding potential of 0 mV using either a 800 ms ramp protocol to −100 mV - +60 mV or a step protocol to –120 mV - +60 mV, in the following bath solutions: Chloride solution pH 7.5 (in mM, 145 choline Cl^-, 30 HEPES, 2 Mg gluconate), Chloride solution pH 5 (145 choline Cl^-, 50 MES, 2 Mg gluconate), Gluconate solution pH 7.5 (145 choline gluconate, 30 HEPES, 2 Mg gluconate), Gluconate solution pH 5 (145 choline gluconate, 50 MES, 2 Mg gluconate).

Whole cell recording: neurons—Recordings were made from dissociated hippocampal neurons on days 15–17 *in vitro* (DIV 15–17). The pipette solution solution consisted of (in mM): 115 Cs-$MeSO_3$, 10 CsCl, 5 NaCl, 10 HEPES (pH 7.35), 0.6 EGTA, 20 TEA-Cl, 4 Mg-ATP, 0.3 Na3GTP, 10 QX-314 (mOsm 300) (Nosyreva et al., 2013). Bath solutions contained (in mM): 145 NaCl, 4 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 0.001 tetrodotoxin, 0.1 picrotoxin, 0.05 APV and 10 HEPES for pH 7.4 or 10 MES for pH 6.0 (mOsm 310). Neurons with a pyramidal shape were recorded in the whole cell configuration while clamped at –70 mV and maintained in 100 μM cyclothiazide (Tocris) to avoid proton-induced AMPA receptor desensitization (Lei et al., 2001), 500 μM amiloride (Tocris) to inhibit acid-sensitive ion channel (ASIC) currents and 100 μM TBOA (Tocris) to inhibit plasma membrane glutamate transporters. Currents (low pass filtered at 2 kHz and sampled at 10 kHz) were recorded for 2 minutes in bath solution at pH 7.4 to establish baseline current, then for 2 minutes at pH 6.0 to record low pH-induced current and finally for 2

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minutes at pH 6.0 + 10 μM CNQX to inhibit AMPA receptor currents and thereby define the proton-dependent AMPA receptor currents. The difference in current was determined by subtracting the baseline current at pH 7.4 from the current in low pH.

Surface expression

The detection of cell surface HA-tagged protein was performed essentially as described (Zerangue et al., 1999). Briefly, oocytes were incubated in ND96 with 1% BSA (ND96 + BSA) at 4° C for 30 min, then for one hour in ND96 + BSA and HA.11 (1:1000). After washing in ND96 + BSA at 4° C, the oocytes were incubated at 4° C for 1 hour with sheep anti-mouse antibody conjugated to horseradish peroxidase (GE) diluted 1:5000 in ND96 + BSA, washed again, transferred to a 96-well plate containing 50 μl ND96, 50 μl SuperSignal ELISA Femto Maximum substrate and the mixture incubated for 5 minutes at room temperature before quantification of the emitted light in a Veritas luminometer.

Protein purification and reconstitution

F0F1-ATP synthase—His-tagged ATP synthase holoenzyme (TF0F1) from the thermophilic Bacillus sp. PS3 was constitutively expressed in *E. coli* DK8 (native *unc* operon deleted) from the plasmid pTR19ASDS29 (Suzuki et al., 2002). Membranes from the bacteria were resuspended in buffer A (100 mM KCl, 20 mM HEPES pH 7.5, 20 mM imidazole, 5 mM $MgCl_2$), n-dodecyl-β-D-maltoside (DDM) added to a final concentration of 2% for solubilization, insoluble material removed by sedimentation and the supernatant incubated with Talon Superflow resin (Clontech) at 4° C for 2 hours. The resin was washed with 20 column volumes (CV) Buffer A + 0.08% DDM and the protein eluted in buffer A + 0.08% DDM and 250 mM imidazole. The eluted protein was desalted on a 10DG column (Bio-Rad) with buffer A containing 0.08% DDM and 10% glycerol and frozen in liquid nitrogen and stored at −80° C.

Vesicular glutamate transporters—Rat VGLUT2 and VGLUT3 cDNAs were cloned into pFastBac with the N-terminal Twin-strep-tag and the Bac-to-Bac system used to produce recombinant baculovirus. To express the transporters, High Five cells were infected with baculovirus and grown for 48 hr. Membranes were collected and resuspended in buffer B (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM DTT, 10 μg/ml pepstatin A, 10 μg/ml leupeptin), rapidly frozen in liquid nitrogen and stored at –80° C until further use. Membranes from 1 L cells were thawed, DDM added to a final concentration of 2% and the membranes solubilized by incubation at 4° C for 2 h. Detergent-insoluble material was removed by sedimentation and the supernatant incubated with 0.5 ml Strep-Tactin Superflow beads (IBA) at 4° C for 2 h. The beads were then washed with ten CV of buffer B containing 0.08% DDM and ten CV of buffer B containing 1% OG and 0.5 mg/ml reconstitution lipid mix (1,2-dioleoylsn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine and cholesterol at a molar ratio of 65:10:25). The transport protein was then eluted in buffer B with 1% OG, 0.5 mg/ml reconstitution lipids and 2.5 mM D-desthiobiotin, and reconstituted immediately into artificial membranes.

Reconstitution—VGLUT was reconstituted into liposomes essentially as described before (Schenck et al., 2009; Preobraschenski et al., 2014). Briefly, the protein:reconstitution lipid

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ratio (mol/mol) was adjusted to 1:40,000 for TF_0F_1, and 1:2000 for VGLUT. After mixing, the lipid and protein were dialyzed overnight at 4° C in either high Cl^- liposome buffer (150 mM KCl, 5 mM $MgCl_2$, HEPES pH 7.3) or buffer lacking Cl^- (150 mM K gluconate, 5 mM Mg gluconate, and 2 mM HEPES pH 7.3). After dialysis overnight, the buffer was replaced and dialysis continued for an additional 2 hours. To remove any residual detergent, the sample was incubated with 100 mg/ml biobeads SM2 (Bio-Rad) for 2 hours and used for experiments the same day.

Transport assays

Proteoliposomes—To measure uptake driven by TF0F1, 25 μl proteoliposomes containing 2–3.5 μg VGLUT were diluted into 775 μl uptake buffer (150 mM K gluconate, 5 mM HEPES-KOH pH 7.3, 5 mM Mg gluconate, 0.2 mM glutamate, 10 μCi/ml L-[3,4-^{3}H]-glutamic acid (PerkinElmer) and 2 mM MgATP), for a final Cl^- concentration of 5 mM. To measure uptake in the absence of TF0F1 (VGLUT only), the uptake buffer at pH 7.3 contained 150 mM K gluconate, 10 mM HEPES-KOH pH 7.3 and 5 mM Mg gluconate (KGluc pH 7.3). At pH 6.0, the buffers contained 0.2 mM glutamate and 10 μCi/ml L-[3,4-^{3}H]-glutamic acid with either 150 mM K gluconate, 10 mM MES-KOH pH 6.0 and 5 mM Mg gluconate (KGluc pH 6) or 160 mM KCl, 10 mM MES-KOH pH 6.0 and 5 mM $MgCl_2$ (KCl pH 6). The reactions were incubated at 30° C for the times indicated and stopped by rapid filtration through 0.45 μm HAWP discs (Millipore). The filters were washed 3 times with cold wash buffer (150 mM K gluconate, 5 mM HEPES-KOH, pH 7.3, 5 mM Mg gluconate, dried, solubilized in CytoScint (MP Biomedicals) and the bound radioactivity measured using an LS 6000SC scintillation counter (Beckman). All experiments were performed in duplicate or triplicate and repeated at least three times. Glutamate uptake was normalized to the mass of reconstituted VGLUT added to each reaction.

Synaptic vesicles—Rat brain synaptic vesicles were prepared as previously described (Hell et al., 1994; Goh et al., 2011). Glutamate uptake was measured in reaction buffers containing 1 mM K glutamate, 20 μCi/ml L-[3,4-^{3}H]-glutamic acid and 500 nM Bafilomycin A, 100 nM nigericin with either 150 mM K gluconate, 10 mM HEPES-KOH pH 7.3 or 150 mM K gluconate, 10 mM MES-KOH pH 6.0. The final chloride concentration was 4 mM in all conditions. 100 μg LP2 protein was added to the reaction buffer at 30° C and the reaction stopped 2 minutes later by rapid filtration through Supor 200 0.2 μm discs (PALL). The filters were washed 4 times with cold wash buffer (150 mM K gluconate, 10 mM HEPES-KOH pH 7.3), dried and the bound radioactivity measured as above. After subtraction of the background determined in 50 μM Evans Blue, uptake was normalized to the mass of LP2 protein added to the reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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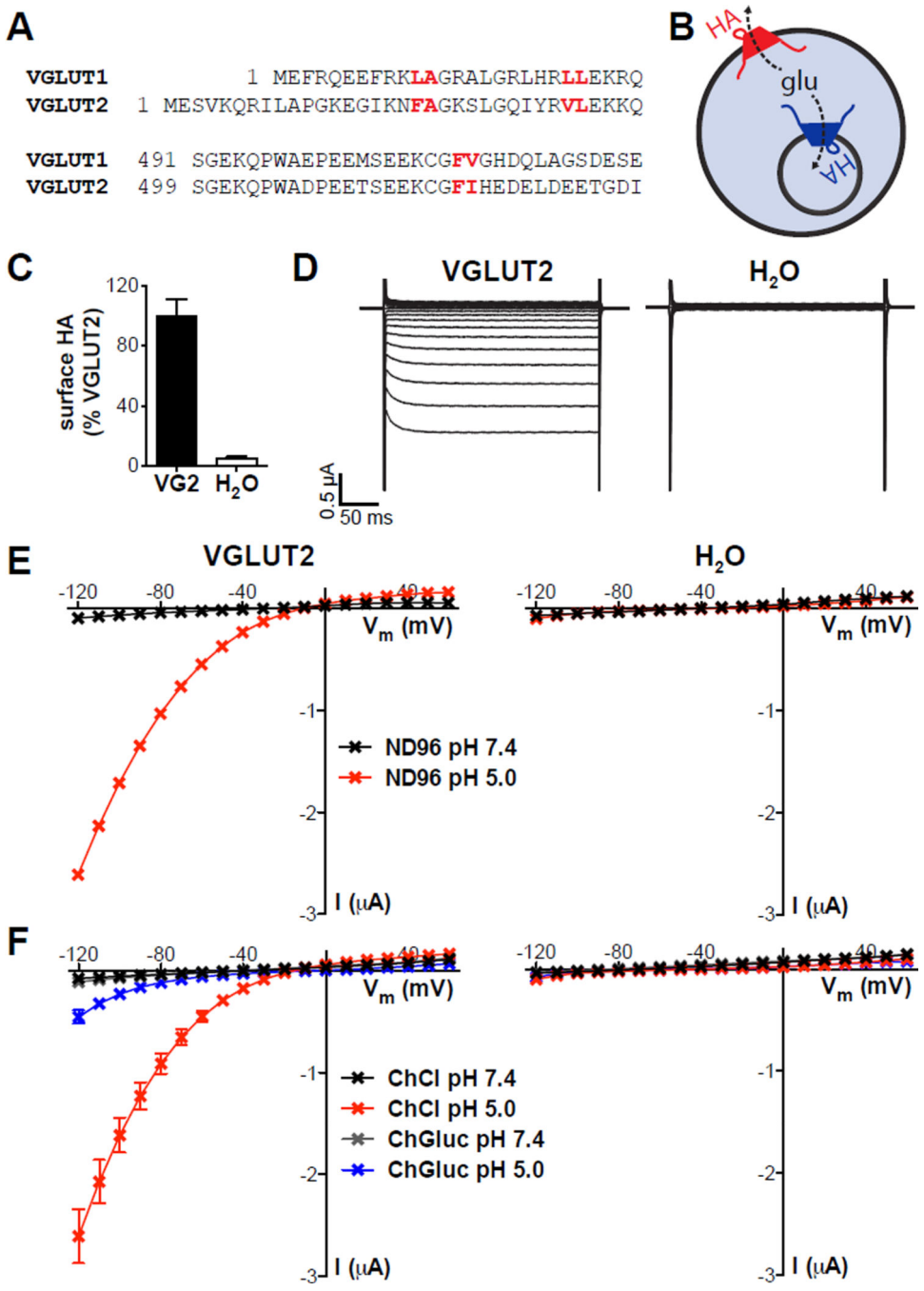

Figure 1. VGLUT2 targeted to the plasma membrane confers pH-dependent inwardly rectifying currents in *Xenopus* oocytes

(A) Alignment of VGLUT1 and 2 N-terminus (above) and C-terminus (below) shows the three conserved dileucine-like endocytosis motifs (red) that were replaced by glycines and alanines, respectively. (B) Diagram showing the orientation and location of wild type HA-VGLUT2 on endosomes (blue) and of internalization-defective HA-VGLUT2 at the plasma membrane (red). (C) Surface expression of mutant VGLUT2 was detected with an antibody directed against an HA epitope tag in the first extracellular loop of VGLUT2 (n=11 oocytes for VGLUT2 and 8 for water). (D) Two-electrode voltage clamp recordings in Ca^{++}-free

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ND96 at pH 5.0 of oocytes injected with VGLUT2 RNA (left) or water (right). From a holding potential of −20 mV, the membrane was stepped from −120 mV to +60 mV in 10 mV increments. (E) Current-voltage (I-V) relationships of the steady-state currents as in C, from VGLUT2-expressing (left) and water-injected oocytes (right) in ND96 at pH 7.4 (black) or pH 5.0 (red) (n=7). (F) I-V curves from oocytes injected with VGLUT2 cRNA or water, recorded in 98 mM choline chloride (ChCl) at either pH 7.4 (black) or pH 5.0 (red), and in 98 mM choline gluconate (ChGluc) at either pH 7.4 (grey) or pH 5.0 (blue) (n=10–13). Data indicate mean ± s.e.m. See also Figure S1.

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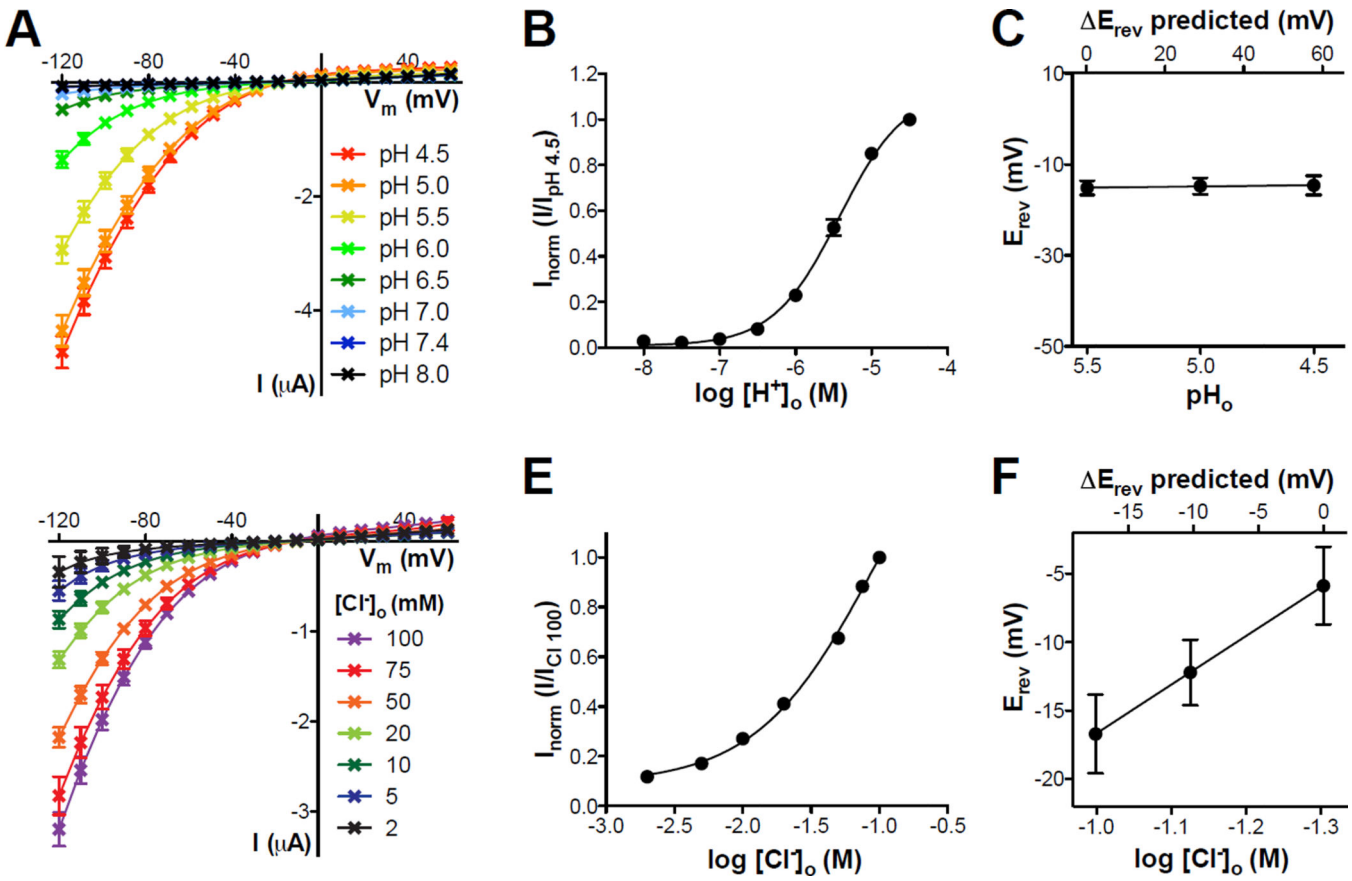

Figure 2. External H^+ and Cl^- activate the VGLUT2 conductance but only Cl^- permeates

(A) I-V relationships from oocytes expressing VGLUT2. Bath solutions contained 98 mM choline Cl and either 5 mM MES (pH 4.5–6.5) or 5 mM HEPES (pH 7.0–8.0). (B) Dose response of the currents at −120 mV to increasing external H^+ shows an EC_{50} pH 5.43 ± 0.04 (n=8). (C) Reversal potential (E_{rev}) of currents at external pH (pH_o) 5.5, 5.0 and 4.5 are plotted against the predicted change in E_{rev} for a H^+-selective conductance (n=8). (D) I-V curves from $VGLUT2^+$ oocytes in 2–100 mM external choline Cl (substituting with choline gluconate), all at pH_o 5.0. (E) Dose response of currents at −120 mV to increasing external Cl^- (Cl^-_o). (F) E_{rev} for currents are plotted against predicted E_{rev} for Cl^- at 100, 75 and 50 mM Cl^-_o (n=8). Data indicate mean ± s.e.m. See also Figure S2.

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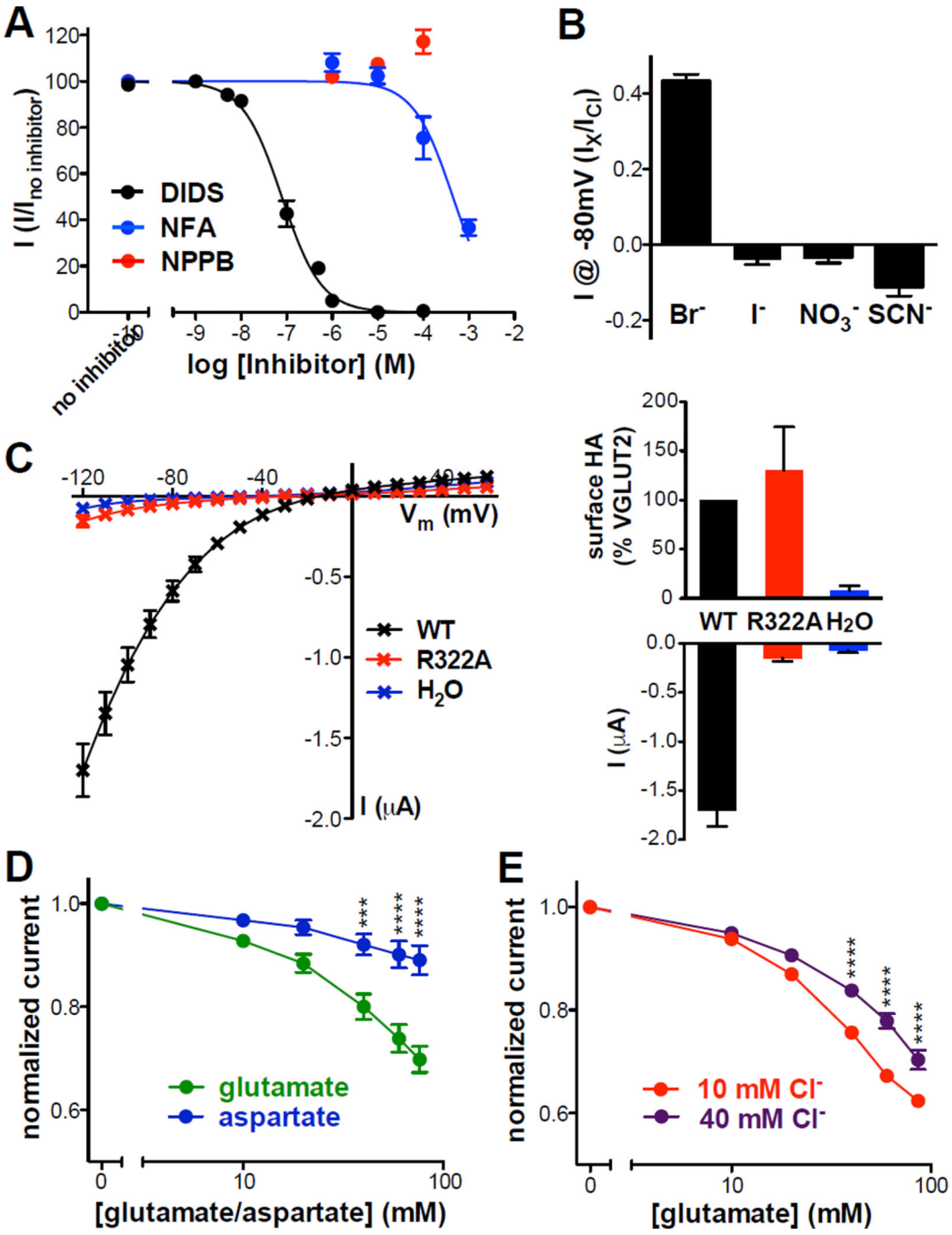

Figure 3. Similarity of the Cl^- conductance to vesicular glutamate transport

(A) Dose response of the VGLUT2 currents at −80 mV in ND96 at pH 5 shows an IC_{50} for DIDS (black) 194 nM [119; 318nM] (n=10), for NFA (blue) 96.2 μM [37.0; 250 μM] (n=4) and N.D. for NPPB (red) (n=4). (B) Activation of currents at −80 mV by ND96 in which NaCl has been replaced with NaBr, NaI, $NaNO_3$ or NaSCN, all at pH 5 (n=6–17). (C) Left, I-V curves from oocytes expressing internalization-defective but otherwise wild type (WT) or R322A VGLUT2, or from water-injected controls, all performed in ND96 at pH 5 (n=6 oocytes per condition). Upper right panel shows surface expression of WT and R322A

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VGLUT2 as in Figure 1 (n=3, 7–11 oocytes per experiment). Lower right panel shows the mean current ± s.e.m. at −120 mV (n=3 with 3–5 oocytes per experiment). (D) Glutamate inhibits the currents (recorded at −70 mV) more potently than aspartate in 20 mM NaCl at pH 5. (E) Inhibition of the currents by glutamate in either 10 mM or 40 mM NaCl, all at pH 5. In A, B, D and E, the steady-state currents were normalized and plotted as mean ± s.e.m. (n = 19–24 oocytes) ***, $p<0.001$ and ****, $p<0.0001$ by 2-way ANOVA with multiple comparisons See also Figure S3.

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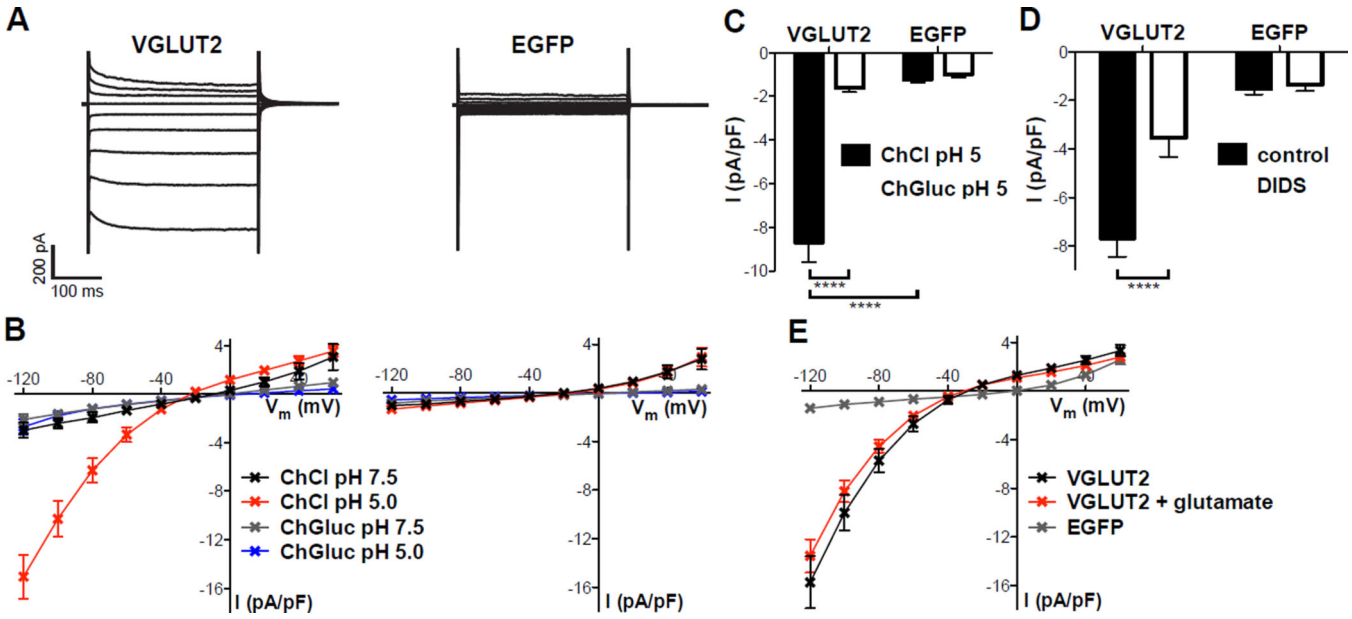

Figure 4. The Cl^- conductance associated with VGLUT2 does not depend on glutamate

(A) Whole cell recordings from HEK293T cells expressing internalization-defective VGLUT2 (left) or EGFP alone (right). The bath solution contained 145 mM ChCl at pH 5 and the membrane potential was stepped in 20 mV increments from −120 to +60 mV from a holding potential of 0 mV. (B) I-V relationships in patch clamp recordings from HEK293T cells expressing VGLUT2 (left) or empty vector (EGFP) controls (right). Bath solutions contained ChCl at pH 7.4 (black), ChCl at pH 5 (red), ChGluc at pH 7.4 (grey) and ChGluc at pH 5 (pink) (n=7 cells). (C) Maximal inward current normalized to cell capacitance from voltage ramps of VGLUT2 or EGFP-only HEK 293T cells in external ChCl pH 5.0 (filled) or ChGluc pH 5.0 (open) (n=15–19 cells). (D) Maximal inward current in ChCl pH 5.0 before (filled) and after (open) application of 50 μM DIDS (n=6–8 cells). ****, $p<0.0001$ by 2-way ANOVA with multiple comparisons (E) I-V relationship as in B from VGLUT2 cells in external ChCl pH 5.0 with (red) or without (black) 5 mM glutamate in the pipette (n=6 cells). In all HEK293T cell experiments, the pipette solution contained 145 mM TMA gluconate and 2.5 mM $MgCl_2$ at pH 7.5. Data indicate mean ± s.e.m. See also Figures S4 and S5.

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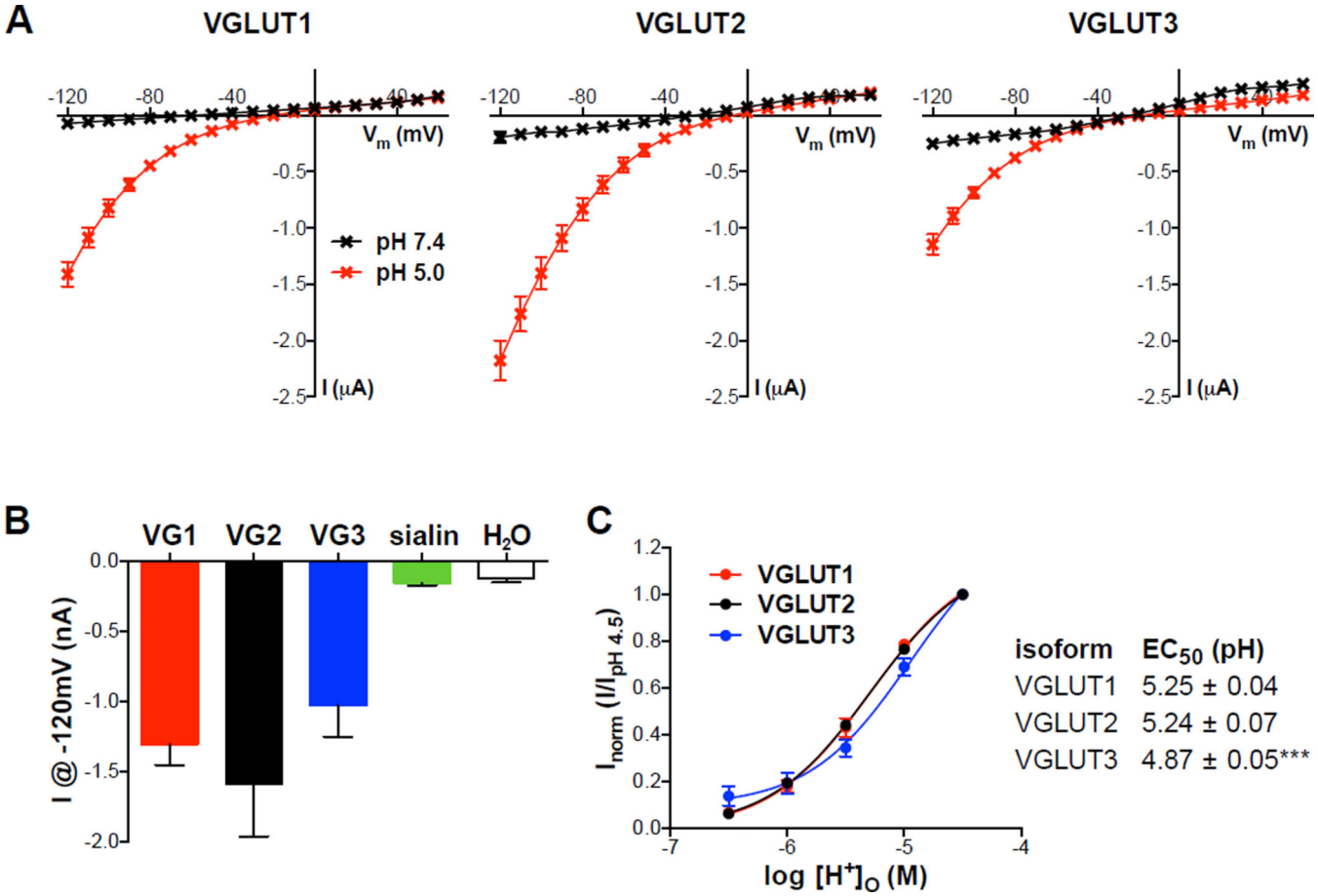

Figure 5. VGLUT1-3 all exhibit a H^+-activated Cl^- conductance

(A) I-V relationship of currents from oocytes expressing untagged VGLUT1-3 in ND96 at pH 7.4 or 5.0. (B) Currents at −120 mV in ND96 at pH 5.0 from oocytes injected with VGLUT1-3, sialin cRNA or water (n=3–5 experiments with 3–5 oocytes per experiment). (C) pH dependence of currents associated with untagged VGLUT1-3, as in Figure 2A,B (***, $p<0.001$ by one-way ANOVA, n=6–8 oocytes per isoform). Data indicate mean ± s.e.m. See also Figure S6.

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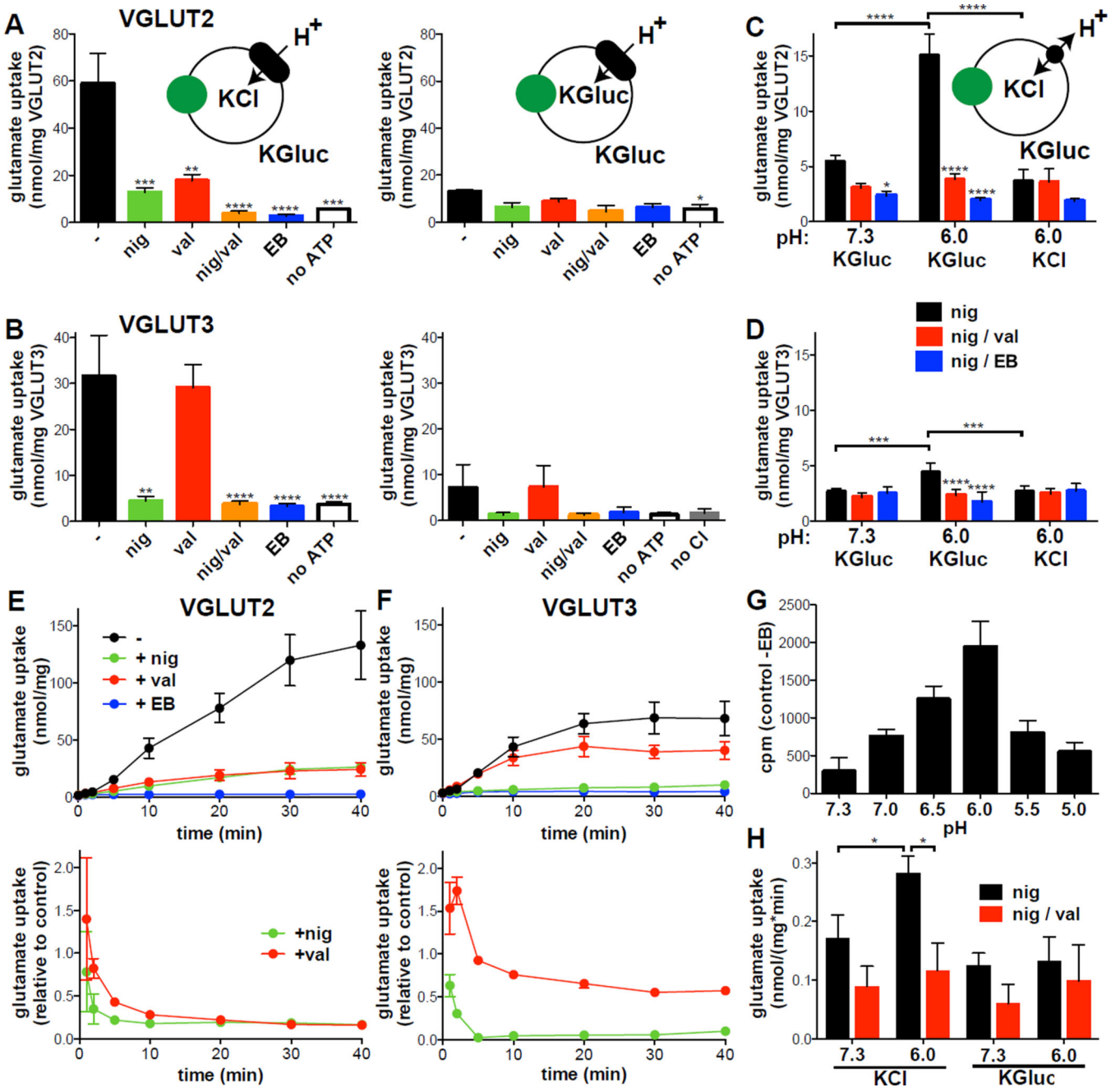

Figure 6. Functional reconstitution shows allosteric activation of vesicular glutamate transport by H^+ and different properties of transport by VGLUT2 and 3

(A,B) ATP-driven glutamate uptake by liposomes reconstituted with TF_0F_1 and either VGLUT2 (A) or VGLUT3 (B) that contain 160 mM KCl (left) or KGluc (right). Uptake for 10 minutes was measured in the presence of 155 mM KGluc, 5 mM KCl and either nigericin (100 nM), valinomycin (100 nM), both ionophores, Evans blue (EB, 50 μM) or no added ATP (n=3). (C,D) Glutamate uptake into liposomes reconstituted with VGLUT2 (C) or VGLUT3 (D) alone that contain 160 mM KCl at pH 7.3. Uptake was measured at 5 minutes in 155 mM KGluc containing 5 mM KCl at either pH 7.3 or 6.0. Alternatively, uptake was

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measured in 160 mM KCl at pH 6.0. In all conditions, nigericin was used to equilibrate internal and external pH, valinomycin to dissipate $\Delta\psi$ and Evans Blue to define the VGLUT-dependent uptake (n=4 for VGLUT2, 3 for VGLUT3). (E,F) Time course of ATP-driven glutamate uptake into liposomes reconstituted with TF0F1 and either VGLUT2 (E) or VGLUT3 (F) that contain 160 mM KCl. Uptake was monitored in 155 mM KGluc with 5 mM KCl and either nigericin, valinomycin or Evans Blue (n=3). Uptake in the presence of either nigericin or valinomycin is expressed relative to control at same time in the panels below. (G) Glutamate uptake at 5 minutes by liposomes reconstituted with VGLUT2 alone as described in C and D above, in the presence of 155 mM KGluc, 5 mM KCl, nigericin and the external pH indicated (n=3). (H) Glutamate uptake by synaptic vesicles preloaded with 150 mM KCl or KGluconate (4 mM KCl) was measured at pH 7.3 or pH 6.0 in the presence of nigericin (100 nM) and Bafilomycin A (500 nM) with or without valinomycin (200 nM). Uptake was measured after 2 minutes and background defined in the presence of Evans Blue subtracted. The comparisons in A and B are all to untreated control with ATP, and in C and D to the same conditions with nigericin alone, unless otherwise indicated. Data indicate mean ± s.e.m. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ by two-way ANOVA See also Figure S7.

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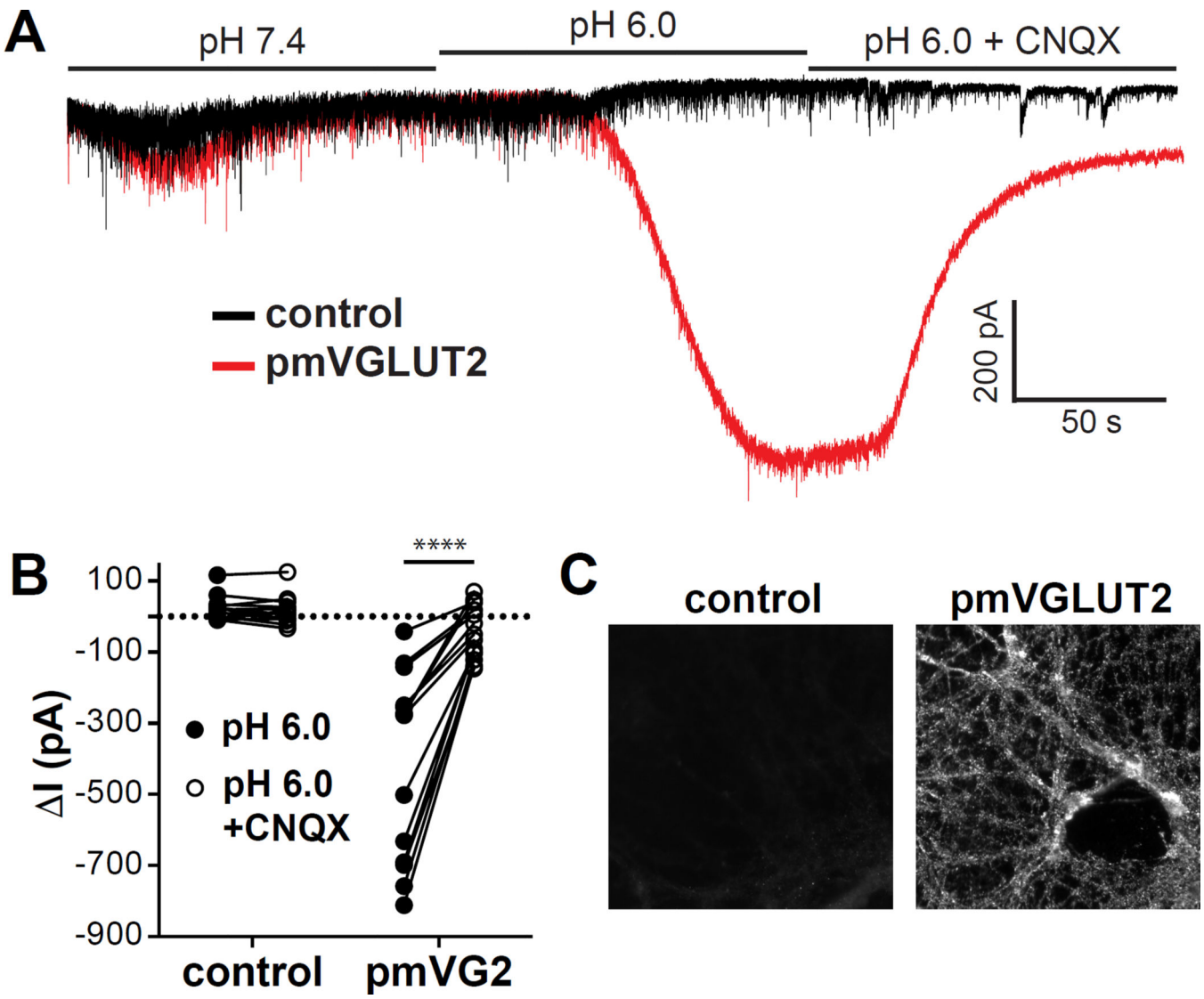

Figure 7. Low extracellular pH enables non-vesicular glutamate efflux by VGLUT2, activating AMPA receptors

(A) Traces from whole cell recording of rat hippocampal neurons expressing internalization-defective HA-tagged VGLUT2 (pmVGLUT2) and controls during perfusion first in pH 7.4, then pH 6.0 and finally pH 6.0 with the AMPA receptor antagonist CNQX (10 μM). (B) Change in the currents (ΔI) due to shift in extracellular pH from 7.4 to 6.0, without and with CNQX (n=14 cells from 3 independent cultures). ****, $p<0.0001$ by two-way ANOVA with multiple comparisons. (C) Unpermeabilized cultures were immunostained for the HA epitope.