Neuron, Volume *84*

Supplemental Information

Vesicular Glutamate Transporters Use Flexible Anion and Cation Binding Sites for Efficient Accumulation of Neurotransmitter

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

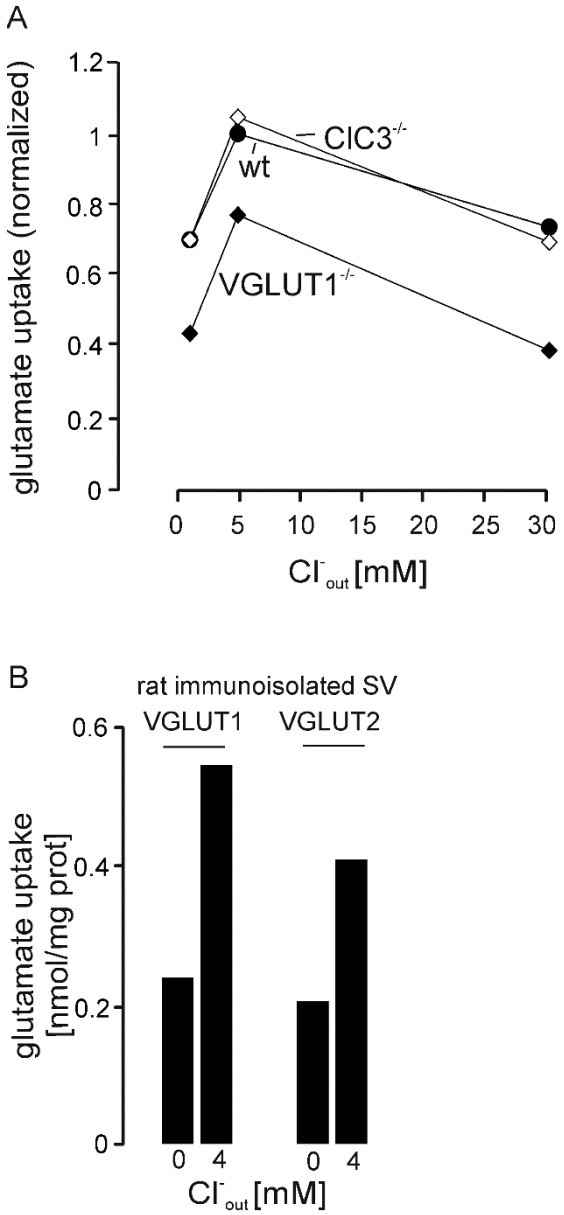

Figure S1: Cl^- dependence of VGLUT-mediated glutamate uptake is independent of the isoform and does not require the Cl^- channel ClC3, related to Figure 1. A: Cl^-- dependence of glutamate uptake by SV isolated from wild type, $VGLUT1^{-/-}$, or $ClC3^{-/-}$ mice. Data represent FCCP-sensitive uptake and are normalized to uptake at 4 mM chloride of the respective wild type.* B: Glutamate uptake by SV immunoisolated from rat brain using antibodies specific for VGLUT 1 or 2, respectively. The immunoisolated vesicles are highly enriched for their respective antigens, with only very limited overlap (Zander et al., 2010). Values are expressed as nmol/mg protein.* (*n=1).

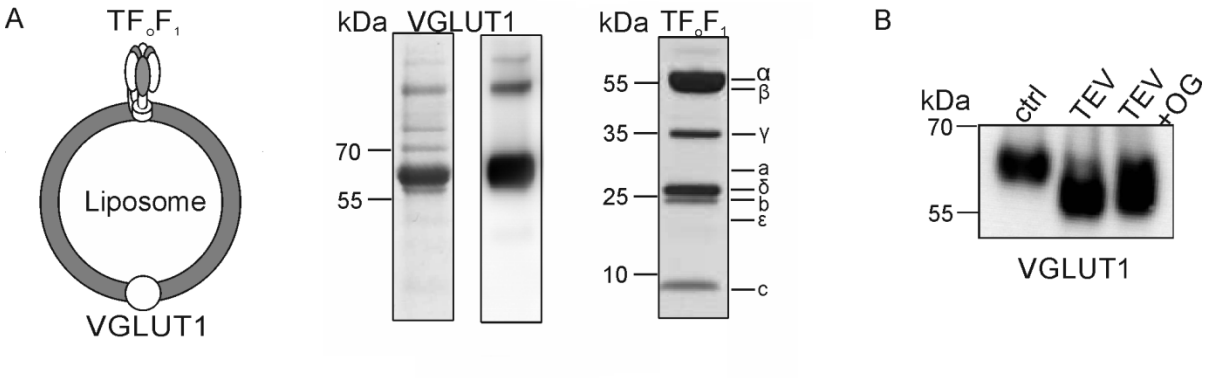

Figure S2: Characterization of proteoliposomes containing purified recombinant VGLUT1 and the proton ATPase TF_oF_1, related to Figure 2. A: Coomassie Blue-stained SDS-polyacrylamide gels (10%) of the purified proteins (5µg protein/lane) and an immunoblot for VGLUT1 (1µg). B: After reconstitution, VGLUT1 is predominantly oriented with the cytoplasmic side facing outward. VGLUT1 containing an N-terminal streptavidin binding peptide tag was reconstituted in liposomes and incubated with TEV protease (TEV) in the absence or presence of the detergent n-Octyl-β-D-glucopyranoside (OG) (TEV+OG). Note that an almost quantitative shift is observed under both conditions.

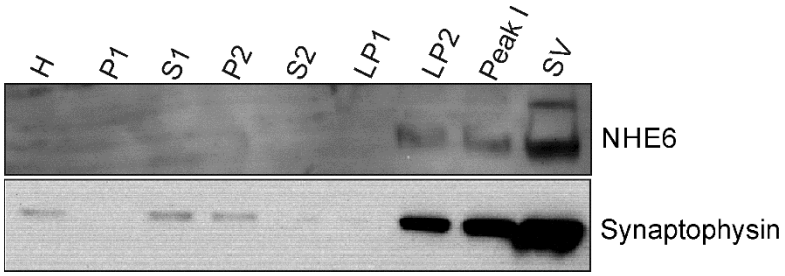

Figure S3: The cation/proton exchanger NHE6 co-purifies with synaptic vesicles, related to Figure 4. Synaptic vesicles were purified from rat brain homogenate (H) using a standard protocol involving the isolation of synaptosomes (P2), followed by lysis of synaptosomes and stepwise enrichment of SVs using differential centrifugation (LP2) sucrose density gradient centrifugation, and size-exclusion chromatography on controlled-pore glass beads (Huttner et al., 1983). Two peaks elute from the column, with peak I containing mainly contaminating membranes and peak II (SV) containing synaptic vesicles at over 90% purity. Equal amounts of protein (20µg/fraction) were analyzed by immunoblotting for NHE6 and the synaptic vesicle marker synaptophysin. Both proteins are highly enriched in the purified SV fraction.

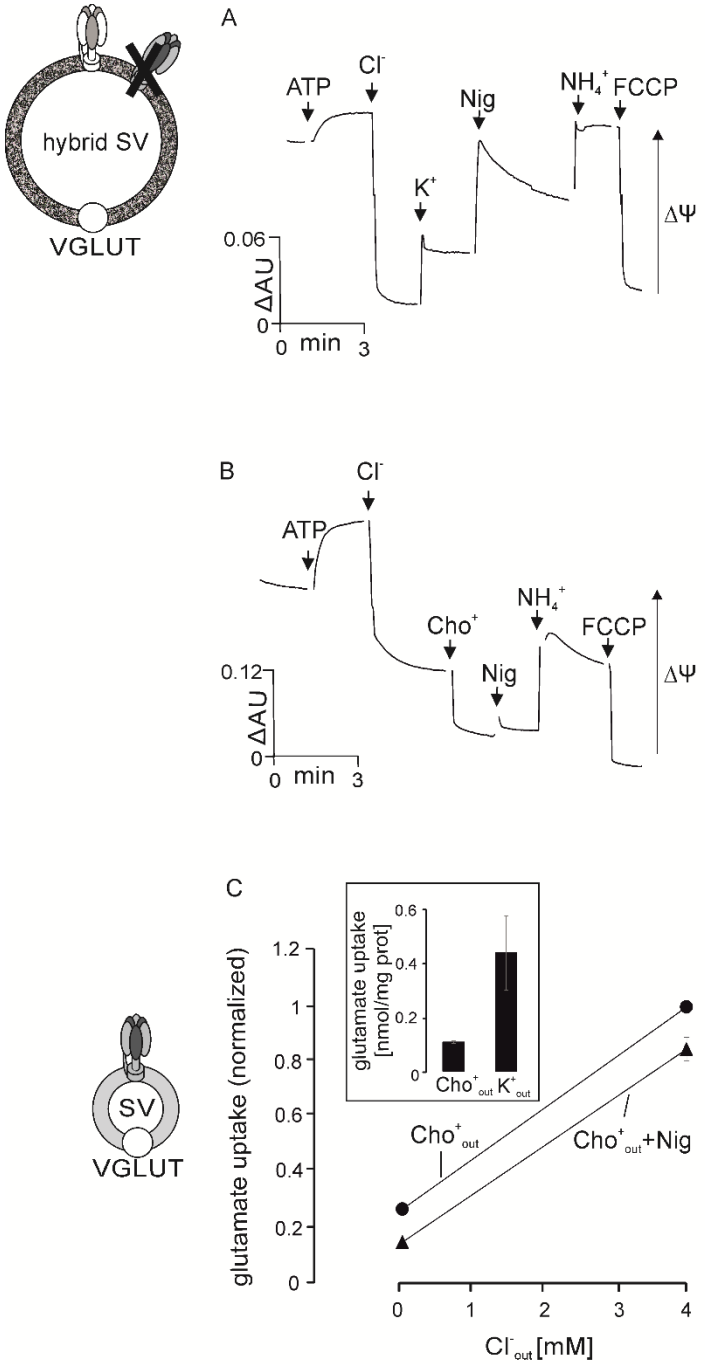

Figure S4: K^+ enhances glutamate uptake by increasing ΔΨ in hybrid vesicles by H^+ exchange, related to Figure 5. A: Changes in ΔΨ, measured with the dye Oxonol VI, in hybrid vesicles preloaded with 150 mM choline gluconate in 300 mM external glycine buffer. The additions were ATP, 30 mM choline chloride (Cl^-) and K-gluconate (K^+), 5 nM nigericin (Nig), 5 mM $(NH_4)_2SO_4$ (NH_4^+) and FCCP. Addition of ATP results in proton pumping causing an increase in ΔΨ. Addition of Cl^- results in Cl^- import, neutralizing the positive charges (net import of HCl), resulting in a drop of ΔΨ. Subsequent addition of K^+ results in proton export, increasing proton pumping and thus resulting in an increase of in ΔΨ that is further enhanced by the Nig that operates as K^+/H^+ exchanger under these conditions. NH_4^+ abolishes the pH-gradient and thus increases ΔΨ. FCCP dissipates both components of the electrochemical potential. Arrows are indicative of the substrate addition time points. B: Same as in A, but K^+ was replaced with the impermeant cation choline (Cho^+). Neither Cho^+ nor nigericin increased ΔΨ as expected. C: Glutamate uptake by synaptic vesicles (LP2 fraction) at 0 and 4 mM choline chloride was performed at 150mM external choline gluconate and with (Cho^+_{out}+Nig) and without 50 nM nigericin (Cho^+_{out}). Note that nigericin did not stimulate glutamate uptake in absence of K^+. The inset shows glutamate uptake at 4 mM Cl^- in presence of K-gluconate (K^+_{out}) and choline gluconate (Cho^+_{out}). Mean values with bars representing the experimental range. n=1-2.

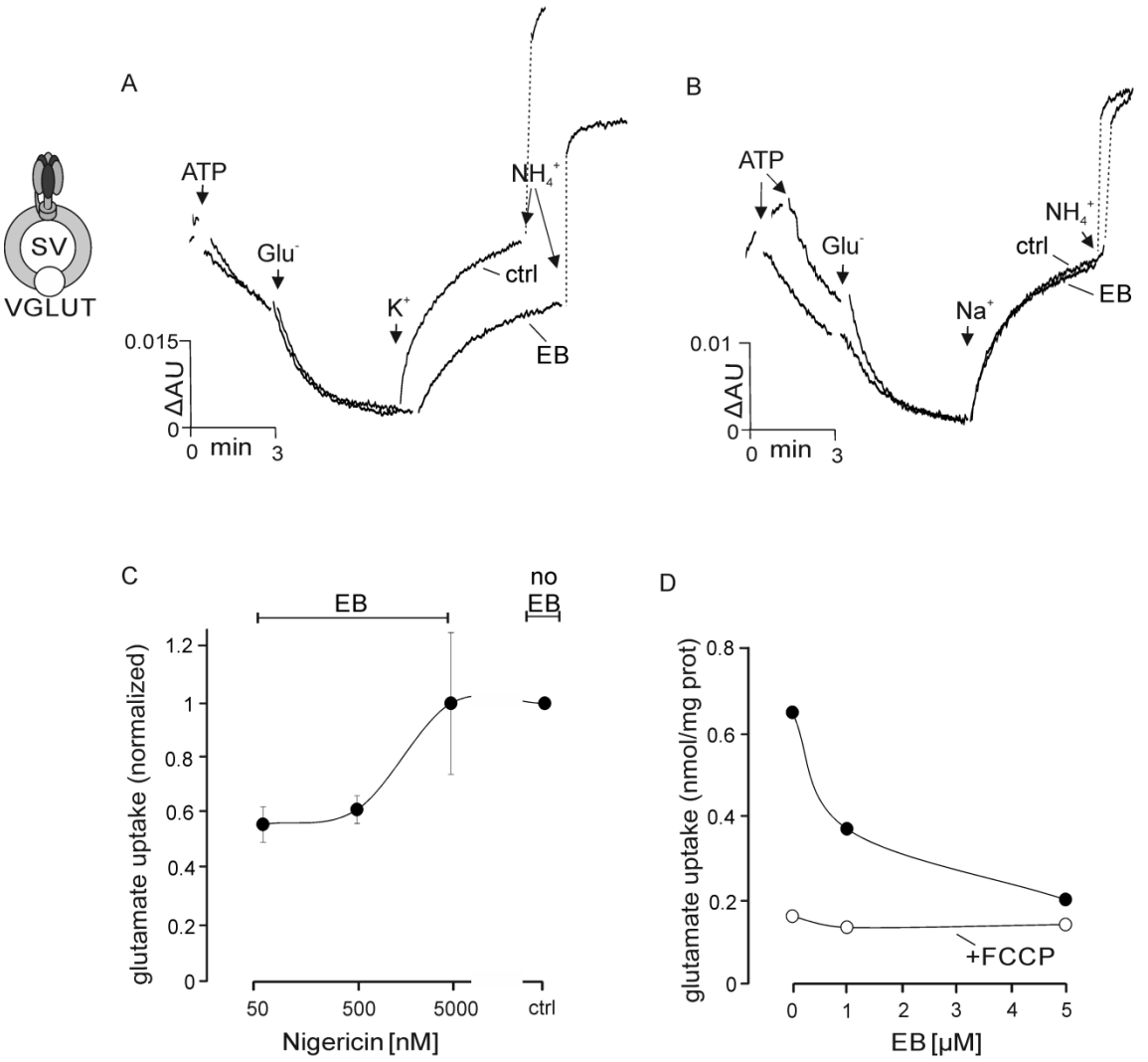

Figure S5: Evans Blue inhibits glutamate by binding to the cation binding site of VGLUT, related to Figure 6. A, B: Glutamate induced acidification of SVs (SV-fraction) is not affected by Evans Blue (EB, 1.5 µM) but reversal by K^+ (A) but not by Na^+ (B) is inhibited (see figure 2 for details of the assay). The additions were ATP (1.2 mM) to start the reaction, choline glutamate (Glu^-) (10 mM), which induces acidification, K^+ and Na^+ (added at 30 mM as gluconate salts), and finally 20 mM $(NH_4)_2SO_4$ (NH_4^+) to disperse ΔpH. Note that EB partially blocks reversal of ΔpH by K^+ but not by Na^+, in agreement with the selective stimulation of glutamate uptake by K^+. A dual wavelength spectrophotometer was used which enables detection of changes in acridine orange absorbance despite a high background absorbance caused by the presence of EB. C: Nigericin reverses inhibition of glutamate uptake by Evans Blue (1µM) in a dose-dependent manner. In the assay, synaptic vesicles (LP2-fraction) were used. Data were normalized to the value obtained in the absence of inhibitor and represent mean values of n=2 with bars indicating the experimental range. D: Glutamate uptake by synaptic vesicles (LP2-fraction) at varying concentrations of Evans Blue (EB) in the absence and presence of the proton ionophore FCCP shows that Evans Blue does not completely inhibit proton-gradient dependent glutamate uptake (data from a single experiment).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunoisolation

Immunoisolations of synaptic vesicles were performed according to previously published protocols (Gronborg et al., 2010; Takamori et al., 2000; Zander et al., 2010). In brief, paramagnetic beads (Dynabeads Pan Mouse IgG, Life Technologies) containing covalently linked antibodies specific for all mouse IgG subclasses were first incubated with the respective primary mouse antibodies (Synaptic Systems) suspended in coating buffer [PBS, pH 7.4, 0.1% BSA (w/v)]. Affinity-purified mouse antibodies were supplied at a concentration of 0.5 – 1 µg of IgG per 10^7 beads and rotated for 2 h at 4°C. The coated beads were washed four times in coating buffer. Immunoisolation was performed overnight at 4°C under rotation using a suspension of the coated beads and an LS1 fraction diluted with incubation buffer [PBS, pH 7.4, 2 mM EDTA, and 5% BSA (w/v)] to yield a ratio of 75 µg protein to 1.4 x10^7 beads. The beads were washed three times in incubation buffer, and three times in coating buffer. Beads without primary antibodies were subjected to the same procedures and served as control for non-specifically bound material (Zander et al., 2010).

Preparation of light membrane fractions from PC12 cells

VGLUT2-PC12 and mock-PC12 cells (kindly provided by Lutz Birnbaumer, Department of Neurobiology,Division of Intramural Research, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina USA) were grown (DMEM, 5 % FCS, 10 % HS, 2 mM L-glutamate and PenStrep, 37 °C, 10 % CO_2) to a confluency of ~60 %. Cells of five 10 cm dishes were resuspended in 1 ml sucrose buffer (320 mM sucrose, 10 mM MOPS-Tris, pH 7.3, 2 mM $MgSO_4$) and homogenized using a ball-bearing homogenizer (10 µm clearance) (Barysch et al., 2010; Takamori et al., 2000). To remove debris and larger particles including mitochondria, the homogenate was centrifuged for 10 min at 10,000xg. The resulting supernatant containing the light membranes was submitted to ultracentrifugation at 200,000xg for 20 min (S140AT rotor, Sorvall). The resulting membrane pellet was resuspended in ~ 100-150 µl sucrose buffer.

Expression and purification of recombinant proteins

VGLUT1: VGLUT1 was expressed in insect cells using the baculovirus expression system (Hitchman et al., 2009; Luckow et al., 1993; Smith et al., 1983). Streptavidin binding peptide (SBP)-tagged (Keefe et al., 2001; Schenck et al., 2009) *mus musculus* VGLUT1 cDNA (in pDEST8 vector, Gateway® baculovirus vector, Invitrogen) was inserted into the bacmid EMBacY (baculovirus coding DNA with an integrated yellow fluorescent protein (YFP) expression marker) via Tn7 transposition in DH10 bacterial strain (Bieniossek et al., 2008; Luckow et al., 1993). The VGLUT1 cDNA carrying bacmid was transfected in *Spodoptera frugiperda* cells (Sf9, in Sf900 media, Invitrogen) and cultured at 27 °C in suspension culture until a sufficient viral titer was obtained. *Trichoplusia ni* (High5, Invitrogen) cells in suspension culture (0.7 * 10^6 cells/ml, Express Five media, Invitrogen) were used for protein expression. VGLUT1 expression was monitored by measurement of YFP fluorescence and was maximal 36 h post-infection with 10 mg/l protein expressed. High5 cells were harvested 36-48 h post-infection and processed for VGLUT1 isolation.

The VGLUT1 purification procedure followed a protocol given earlier with some modifications (Schenck et al., 2009). Briefly, High5 cells from 1 l culture were resuspended in 50 ml of ice cold

resuspension buffer (40 mM Tris, pH 7.3, 300 mM KCl, 2 mM EDTA) with 1 x Protease Inhibitor Cocktail (EDTA free, Merck) and 5 mM β-mercaptoethanol. N-dodecyl-β-D-maltopyranoside (DDM, Glycon) was added to a final concentration of 2 % (w/v) and cells were lysed for 1 h at 4 °C under constant rotation. Subsequently, cell debris were removed by centrifugation at 250,000 xg (70Ti rotor, Beckman Coulter) for 20 min at 4 °C. The supernatant was supplemented with 1 ml streptavidin beads (Pierce) and rotated for 3 h at 4 °C. The beads were washed with 2 x 10 resin volumes of wash buffer (resuspension buffer containing 0.1 % DDM) and the protein was finally eluted with 5 x 1 resin volume of elution buffer (15 mM Tris, pH 7.3, 100 mM KCl, 0.6 mM EDTA, 2 mM (+) biotin, 5 mM β-mercaptoethanol and 0.05 % DDM). The beads were incubated for 5 min on ice for each elution step. The elution fractions were pooled and concentrated using a 30 kDa MWCO VivaSpin concentrator (Sartorius) to yield a final protein concentration of ~1 mg/ml (~ 3-5 fold). Due to its large micelle size (~50 kDa) DDM accumulated in the concentrated protein sample to a final concentration of ~0.25 %. Concentrated VGLUT1 was snap frozen in aliquots and stored at -80 °C. 1 l of High5 expression culture yielded ~ 0.7 mg of purified VGLUT protein.

Proton ATPase TF_oF_1: The pTR19-ASDS plasmid carrying TF_oF_1 with a His_6-tagged β-subunit was kindly provided by M. Yoshida (Suzuki et al., 2002). TF_oF_1 was expressed in *E.coli* DK8 (Suzuki et al., 2002) and purified as given earlier (Schenck et al., 2009) with slight modifications. The affinity purification with Talon beads (Clontech) was performed at 4 °C followed by anion exchange chromatography at room temperature using the ÄKTA system (GE Healthcare). The protein was supplemented with 10% glycerol, snap frozen and stored at -80 °C.

SNARE proteins: The ΔN complex constructs consist of the pETDuet-1 vector carrying syntaxin-1A (183-288) and the C-terminal fragment of synaptobrevin 2 (49-96) and the pET28a vector carrying His_6-tagged SNAP-25A (Pobbati et al., 2006; Stein et al., 2007). All cDNAs originated from *Rattus norvegicus* and were used for co-expression. The components of the ΔN complex, syntaxin-1A (183-288), synaptobrevin 2 (49-96) and SNAP-25A were co-expressed in *E.coli* BL21(DE3) and purified as previously described (Pobbati et al., 2006; Stein et al., 2007). Ni^{2+}-NTA affinity purification was followed by anion exchange chromatography using the ÄKTA system (GE Healthcare).

Preparation of proteoliposomes

Proteoliposomes were generated by detergent removal via dialysis from a mixture of the detergent-solubilised components (Rigaud and Levy, 2003; Rigaud et al., 1995). In detail, the synthetic lipid mix consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) (both Avanti Polar Lipids) and Cholesterol (Chol) (from sheep wool, Avanti Polar Lipids) at a molar ratio of DOPC:DOPS:Chol 65:10:25 was formed by evaporating the organic solvent from the lipids and dissolving the dried lipid film in buffer (300 mM Glycine, 10 mM MOPS-Tris, pH 7.3, 2 mM $MgSO_4$, 5% n-Octyl-β-D-glucopyranosid) to a concentration of 10mg/ml lipids. The protein:lipid ratio (mol/mol) was adjusted to ~1:40000 for TF_oF_1, to ~1:500 for the ΔN complex and to ~1:2000 for VGLUT1. The final lipid concentration was adjusted to 3.6 mM. After mixing, the respective solutions were dialysed in Slide-A-Lyzer dialysis cassettes (2 kDa MWCO, Thermo Scientific) over night at 4 °C in 300 mM glycine, 150 mM choline gluconate or choline chloride, 10 mM MOPS-Tris, pH 7.3, and 2 mM $MgSO_4$ if not otherwise indicated. The dialysis buffer was additionally supplemented with 2 g BioBeads (BioRad) to adsorb detergent monomers. After dialysis, remaining

DDM in the VGLUT1/TF_oF_1 liposomes was complexed with 2,6-di-O-methyl-β-cyclodextrin (β-CD, Sigma) (Degrip et al., 1998; Schenck et al., 2009) by adding a few crumbs of solid β-CD to the liposomes and incubating them on ice for 1 h by occasionally inverting the tubes. External buffer exchange was performed by size exclusion chromatography (PD10, GE Healthcare). Up to 1 ml of liposome suspension was applied to one column. The liposome fractions were identified by their viscous and turbid appearance and collected separately. The samples were diluted 1.5-2 fold. The size distribution of the formed liposomes was measured using Dynamic Light Scattering.

Generation of hybrid SVs

TF_oF_1/ΔN liposomes were fused with SVs for 45 min at room temperature (Holt et al., 2008). Per data point, typically 30 µg of LP2 were mixed with 12 µl of liposomes and adjusted to 50 µl with reconstitution buffer (150 mM choline gluconate or choline chloride, 10 mM MOPS, 2 mM $MgSO_4$, pH 7.3). For external buffer exchange the samples were loaded on pre-packed size exclusion columns (PD10, GE Healthcare) (see proteoliposomes). The dilution factor due to gel filtration was 1.5-2. Prior to assaying the hybrid SVs were supplemented with 0.2 µM bafilomycinA1 (Calbiochem).

Measurement of glutamate uptake

Glutamate uptake was performed as previously published (Hell et al., 1990; Maycox et al., 1988; Takamori et al., 2000). The uptake was measured with 2 µCi ^{3}H-glutamic acid (GE Healthcare, Hartmann Analytik GmbH) per data point in the presence of 2 mM ATP, 50 µM choline glutamate, 0 - 30 mM choline chloride in uptake buffer (150 mM choline gluconate, 10 mM MOPS-Tris, pH 7.3, 2 mM $MgSO_4$ and either 50 mM choline gluconate, K-gluconate or Na-gluconate). 90 µl LP2 (15-20 µg total protein/sample), proteoliposomes (6-8 µg VGLUT1/sample), hybrid SVs (~ 15 µg total protein /sample) or PC12-LMFs (~60 µg total protein/sample) were mixed with 10 x uptake buffer and incubated for 15 min at 32 °C. Glutamate uptake in Figures 1, S1, S3C and S4C was performed according to (Winter et al., 2005; Zander et al., 2010). ~10 µg LP2 and 2 µCi ^{3}H-glutamic acid (Hartmann Analytik GmbH and GE Healthcare) were used per data point in 150 mM K-gluconate (KGC) or choline gluconate, 20 mM 1, 4-piperazinediethanesulfonic acid; 4 mM EGTA; 2.9 mM $MgSO_4$ (corresponding to1 mM free Mg^{2+}); and 2 mM ATP, adjusted to pH 7.0 with KOH. Uptake was corrected by subtracting non-specific uptake using 30-60 µM FCCP if not stated otherwise.

Acidification

Acidification measurements were performed according to previous publications (Hell et al., 1990; Maycox et al., 1988) using acridine orange (AO, Molecular Probes) as a pH sensitive dye (Palmgren, 1991). Changes in absorbance at 492 nm (ΔAU) were monitored in an Aminco dual-wavelength spectrophotometer using absorbance at 530 nm as reference, giving a read-out of lumenal pH-changes (Hell et al., 1990; Maycox et al., 1988). Usually, 600 - 650 µl buffer (300 mM glycine, 10 mM MOPS, pH 7.3, and 2 mM $MgSO_4$) were mixed in a 1 ml glass cuvette with 50 - 100 µl of VGLUT/TF_oF_1 liposomes (preloaded with 300 mM glycine, 1-3 mM MOPS-Tris, pH 7.3, and 2 mM $MgSO_4$), hybrid SVs (preloaded with 150 mM choline gluconate, 3 mM MOPS-Tris, pH 7.3, and 2 mM $MgSO_4$) or ~50 µg SV fraction (in 320 mM sucrose, 10 mM MOPS-Tris, pH 7.3, and 2 mM $MgSO_4$) containing 10 µM AO and measured at 32 °C. Substrates were added at the following final concentrations: 1.2 mM ATP, 5 nM valinomycin, 20 mM $(NH_4)_2SO_4$, 10 mM choline glutamate, 30 mM

K-gluconate, Na-gluconate, choline gluconate and 1.5 µM Evans Blue. Representative traces are shown in the figures.

Membrane potential

Measurements of changes in membrane potential were carried out using VGLUT1/TF_oF_1 liposomes and hybrid SVs using Oxonol VI (Molecular Probes), an anionic dye used to detect changes in ΔΨ (Hell et al., 1990; Russell, 1984; Shioi et al., 1989). Absorbance changes at 625 nm (ΔAU) and a reference wavelength at 587 nm were detected using the same Aminco dual wavelength spectrophotometer as for AO measurements. Measurements were performed at 32 °C in 600 - 650 µl buffer (300 mM glycine, 1-3 mM MOPS-Tris, pH 7.3, 2 mM $MgSO_4$) with 100-200 µl of VGLUT1/TF_oF_1 liposomes or hybrid SVs and 15 µM OxonolVI. Applied concentrations: 1.5 mM ATP, 5 nM valinomycin, 5 nM nigericin, 5 mM $(NH_4)_2SO_4$, 50 µM FCCP and 30 mM choline chloride, K-gluconate or choline gluconate. Representative traces are shown in the figures.

SPQ measurements

Chloride flux measurements were performed using 6-Methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) (Santa Cruz Biotechnology) as a chloride sensitive fluorescent dye, which is collisionally quenched by chloride ions (Biwersi et al., 1994; Verkman, 1990). +/-VGLUT1 liposomes were preloaded with 150 mM KCl/30 mM K-gluconate, pH 7.3 and SPQ (10 mM before dialysis) and fluorescence changes at 443 nm (AU) were monitored using a FluroMax-2® Spectrofluorometer with an excitation wavelength of 344 nm. Measurements were carried out with 150 µl liposomes at 32°C in either 180 mM K-gluconate or 150 mM KCl/30 mM K-gluconate, pH 7.3. The applied concentration of valinomycin was 2 nM. Representative traces are shown in the figures.

SUPPLEMENTAL REFERENCES

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