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3 **Ion-coupling mechanism of excitatory amino acid transporters**

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17

18 **Abstract**

19 Human excitatory amino acid transporters (EAATs) maintain glutamate gradients in
20 the brain essential to neurotransmission and to prevent neuronal death. They use ionic
21 gradients as energy source, and co-transport transmitter into the cytoplasm with Na^+
22 and H^+ , while counter-transport K^+ to re-initiate the transport cycle. However, the
23 molecular mechanisms underlying ion-coupled transport remain incompletely
24 understood. Here, we present 3D structures and thermodynamic analysis of EAAT1 in
25 different ion-bound conformations, including elusive counter-transport ion bound
26 states. Binding energies of Na^+ and H^+ , and unexpectedly Ca^{2+} , are coupled to
27 neurotransmitter binding. Ca^{2+} competes for a conserved Na^+ site suggesting a
28 regulatory role of Ca^{2+} in glutamate transport at the synapse, while H^+ binds to a
29 conserved glutamate residue stabilizing substrate occlusion. The counter-transported
30 ion binding site overlaps with that of glutamate, revealing the K^+ mechanism to exclude
31 the transmitter during the transport cycle, and to prevent its neurotoxic release on the
32 extracellular side.

33

34 Keywords: cryo-EM/Neurotransmitter transport/ permeation and transport/solute
35 carrier/X-ray crystallography

36 Human excitatory amino acid transporters (EAATs) belong to the solute carrier 1
37 (SLC1) family, and catalyze active transport of excitatory transmitters using energy
38 stored in ionic transmembrane gradients (Erecinska *et al*, 1983; Kanner & Sharon,
39 1978; Nelson *et al*, 1983). In each transport cycle, EAATs stoichiometrically co-
40 transport one molecule of transmitter with 3 Na⁺ and 1 H⁺, while counter-transport 1
41 K⁺ to re-orient the transmitter binding site to the extracellular side, and re-initiate the
42 cycle (Barbour *et al*, 1988; Zerangue & Kavanaugh, 1996). EAAT1-2 are highly
43 expressed on the plasma membrane of astrocytic glia (Lehre & Danbolt, 1998), where
44 they are essential components of tripartite synapses and contribute to clear the
45 transmitter from the cleft, as well as to regulate extracellular glutamate concentration
46 in the brain (Zhou & Danbolt, 2014). Consistently, their dysfunctions are linked to
47 several neurodegenerative disorders (Pajarillo *et al*, 2019).

48 SLC1 proteins are homo-trimers (Canul-Tec *et al*, 2017; Garaeva *et al*, 2018; Yernool
49 *et al*, 2003; Yernool *et al*, 2004), and their individual subunits catalyze transport
50 independently (Grewer *et al*, 2005; Koch & Larsson, 2005; Leary *et al*, 2007; Ruan *et al*,
51 2017). Each subunit contains two structural and functional domains (Reyes *et al*,
52 2009): a relatively rigid (Groeneveld & Slotboom, 2007) scaffold domain (scaD) that
53 forms the inter-subunit interfaces; and a highly dynamic (Akyuz *et al*, 2013; Erkens *et al*,
54 2013; Matin *et al*, 2020) transport domain (tranD) that binds substrate as well as
55 thermodynamically-coupled ions (Boudker *et al*, 2007; Guskov *et al*, 2016; Larsson *et al*,
56 2010; Seal & Amara, 1998; Tao *et al*, 2010; Zhang *et al*, 1998), and shuttles across
57 the membrane in an “elevator-like” manner to translocate the cargo (Crisman *et al*,
58 2009; Reyes *et al*, 2009).

59 Biophysical analyses of Na⁺-dependent EAAT archaeal homologs, Glt_{Ph} (Boudker *et al*
60 *et al*, 2007) and Glt_{TK} (Guskov *et al*, 2016), showed that co-transported sodium ions bind

61 three conserved sites in the tranD (Na1-3), and that binding energy of the three co-
62 transported Na⁺ ions is coupled to substrate binding and occlusion, rather than directly
63 driving its translocation (Reyes *et al.*, 2013). In the absence of substrate, two Na⁺ bind
64 cooperatively to Na1 and Na3, and contribute to form the substrate binding site, while
65 subsequent binding of substrate and Na⁺ to Na2 lead to substrate occlusion under
66 helical-hairpin 2 (HP2) (Alleva *et al.*, 2020; Ewers *et al.*, 2013; Guskov *et al.*, 2016;
67 Reyes *et al.*, 2013; Verdon *et al.*, 2014). HP2 dynamics controls substrate access to its
68 binding site on opposite sides of the membrane, and HP2 closure enables elevator-like
69 movements of the tranD, and isomerization between outward- and inward-facing states
70 (Alleva *et al.*, 2020; Boudker *et al.*, 2007; Garaeva *et al.*, 2019; Reyes *et al.*, 2009).
71 However, archaeal homologs are limited molecular models, as they are H⁺ and K⁺
72 independent transporters (Ryan *et al.*, 2009). Moreover, archaeal Na⁺-coupling
73 mechanism has not been confirmed experimentally in EAATs. In addition, the folding
74 instability of purified mammalian EAAT orthologs has precluded so far similar
75 crystallographic and thermodynamic analyses of human transporters, and recent cryo-
76 electron microscopy (cryo-EM) structures of EAAT3 fell short of revealing the
77 complete ion-coupled mechanism (Qiu *et al.*, 2021).

78 In this work, we set out to study EAAT ion-coupled transport mechanism using
79 thermostabilized human EAAT1 mutants, namely EAAT1_{CRYST} and EAAT1_{CRYST-II}
80 (Canul-Tec *et al.*, 2017; Cirri *et al.*, 2018), as well as wild type EAAT1 (EAAT1_{WT}).
81 EAAT1_{CRYST} constructs are nearly identical to EAAT1_{WT} at the core of the tranD,
82 where transmitter and coupled-ions bind. Consistently, they retain intact Na⁺, H⁺, and
83 K⁺ coupled transport mechanism after purification in detergent solutions and
84 reconstitution in synthetic liposomes. Importantly, detergent-purified EAAT1_{CRYST}
85 proteins are stable under different substrate and ionic conditions, and enable both

86 thermodynamic and X-ray crystallographic analysis of the neurotransmitter transport
87 cycle, while EAAT1_{WT} was amenable to structural analysis by cryo-EM.

88 **Conserved SLC1 sodium-coupling mechanism**

89 L-glutamate uptake by purified EAAT1_{CRYST} is strictly dependent on opposite
90 transmembrane gradients of sodium and potassium (Canul-Tec *et al.*, 2017). To
91 quantify binding and ion-substrate thermodynamic coupling, we measured changes in
92 intrinsic tryptophan fluorescence of purified EAAT1_{CRYST} in detergent solutions. Both
93 Na⁺ and transmitter binding, but not K⁺, induced robust tryptophan-fluorescence
94 changes (> 15%) that enable titration of ligands (Fig. 1A,B). EAAT1_{CRYST} contains two
95 tryptophan residues, W287₂₆₇ in the scaD, and W473₄₅₃ in the tranD (herein, amino acid
96 number refers to EAAT1_{WT} sequence, and the corresponding number in EAAT1_{CRYST}
97 is in subscript). Individual phenylalanine mutants W287₂₆₇F and W473₄₅₃F decreased
98 (~ 5%), and abolished, respectively, Na⁺/transmitter induced fluorescence signal
99 (Appendix Figure S1A). Although the structural details underlying tryptophan
100 fluorescence changes in EAAT1_{CRYST} are unclear, these experiments show that the two
101 tryptophan residues are required to probe Na⁺ binding, and that changes in tranD
102 dynamics induced by Na⁺ and K⁺ binding are significantly different.

103 In the absence of substrate, sodium ions bind EAAT1_{CRYST} in a cooperative manner
104 with an apparent dissociation constant (K_D) of 21.6 ± 0.4 mM, and a hill coefficient
105 (n_H) of 2.2 ± 0.1 (Fig. 1C), consistent with binding of two sodium ions to the apo tranD
106 at Na1 and Na3. In agreement with this, asparagine mutation of strictly conserved
107 D400₃₈₀ in Na3 greatly impaired sodium binding ($K_D > 100$ mM). Moreover, in the
108 presence of allosteric inhibitor UCPH₁₀₁ (Jensen *et al.*, 2009), which traps the
109 transporters in outward-facing states (Canul-Tec *et al.*, 2017), Na⁺ titrations of
110 EAAT1_{CRYST} yielded similar binding parameters ($K_D = 14.6 \pm 0.3$ mM; $n_H = 1.6 \pm 0.1$).

111 These results indicate that tryptophan-fluorescence changes report on conformational
112 changes associated to binding, rather than membrane translocation of the tranD, and
113 further that Na⁺ binds outward- and inward facing states with comparable affinities.
114 Substrate binding to EAAT1_{CRYST} was strongly sodium-dependent, and the logarithmic
115 plot of the substrate K_D versus sodium concentration yielded a straight line with a slope,
116 or coupling efficiency (C_E) value of -2.6 ± 0.3 (Fig. 1D). C_E is the apparent number of
117 ions thermodynamically coupled to binding of one substrate molecule, and our results
118 show that in EAAT1_{CRYST} the binding energy of the three co-transported sodium ions
119 is coupled to neurotransmitter binding. Moreover, at 1 mM Na⁺, transmitter K_D values
120 in the presence (0.9 ± 0.1 mM) and absence of UCPH₁₀₁ (2.8 ± 0.8 mM) were
121 comparable.

122 Indeed, similarities in Na⁺, and Na⁺-transmitter coupled binding properties between
123 archaeal homologs (Reyes *et al.*, 2013) and EAAT1_{CRYST} strongly suggests
124 conservation of the Na⁺-coupling mechanism.

125 To gain structural insight on the location of the sodium binding sites, we extended the
126 reported crystallographic atomic model of Na⁺/transmitter bound EAAT1_{CRYST} (Canul-
127 Tec *et al.*, 2017) in complex with UCPH₁₀₁ (see methods). Notably, the extended model
128 includes an N-terminal helix that lies nearly parallel to the membrane plane (TM1a'),
129 and has not been noted before in structures of SLC1 proteins (Fig. 2A,B). Interestingly,
130 amino acid deletions in this region slow down substrate uptake by EAAT1_{WT} expressed
131 in cells (Appendix Figure S1B), suggesting a modulatory role of TM1a' on transport
132 kinetics. Notably, EAAT1_{CRYST} Fo-Fc electron density map omitting sodium ions
133 shows three distinct density peaks ($> 4\sigma$) within the tranD (Fig. 2C). Several lines of
134 structural and functional evidence indicate that the observed peaks correspond to three
135 co-transported Na⁺ bound to the tranD: the peaks accurately localize to the three

136 conserved sodium binding sites (Na1-Na3) previously observed in X-ray structures of
137 archaeal homologs (Alleva *et al.*, 2020; Boudker *et al.*, 2007; Guskov *et al.*, 2016).
138 Accordingly, three sodium ions modeled in EAAT1_{CRYST} structure reveal nearly
139 identical coordination compared to the homologs (Fig. 2D); ion coordination in
140 EAAT1_{CRYST} at the three sites is exclusively done by oxygen atoms, as expected for a
141 weak acid like Na⁺, while water coordination is expected to have additional
142 contributions by nitrogen atoms, reflecting its H-bonding capacity (Nayal & Di Cera,
143 1996); Finally, reported mutagenesis of residues that contribute side chains to Na1 and
144 Na3, including D400₃₈₀N (Fig. 1C), impaired Na⁺-dependent function in both EAATs
145 and prokaryotic homologs (Bastug *et al.*, 2012; Boudker *et al.*, 2007; Tao *et al.*, 2010).
146 Overall, the above structural and thermodynamic results are in excellent agreement with
147 the sodium-coupling mechanism of archaeal transporters (Alleva *et al.*, 2020; Ewers *et*
148 *al.*, 2013; Guskov *et al.*, 2016; Reyes *et al.*, 2013; Verdon *et al.*, 2014), and confirm its
149 conservation in human SLC1 proteins. In this mechanism, cooperative Na⁺ binding to
150 apo transporters leads to occupancy of Na1 and Na3 sites and contributes to the
151 formation of the substrate binding site, while subsequent binding of substrate and Na⁺
152 to Na2 lead to substrate occlusion under HP2, and enables transmembrane movements
153 of the tranD. Na⁺, as well as Na⁺-transmitter coupled binding affinities to outward- and
154 inward-facing states are similar.

155 **Proton coupling**

156 L-glutamate uptake by purified EAAT1_{CRYST} decreases steeply at basic pH values (Fig.
157 3A), resembling transport pH-dependence observed in EAAT mammalian orthologs
158 expressed in cells (Watzke *et al.*, 2000; Zerangue & Kavanaugh, 1996), and
159 demonstrating that the proton-coupled transport mechanism of the thermostable mutant
160 is intact.

161 To gain thermodynamic insight on this mechanism, we measured Na⁺, as well as
162 Na⁺/transmitter coupled binding to apo transporters as a function of pH using the
163 tryptophan-fluorescence assay. There was no significant change in Na⁺ apparent K_D in
164 a pH range from 6 to 10 ($C_E = 0.09 \pm 0.03$), while substrate K_D significantly increased
165 with pH ($C_E=0.55 \pm 0.02$) at constant [Na⁺] (Fig. 3B). These results demonstrate that
166 neurotransmitter binding is thermodynamically coupled to binding of one H⁺, and argue
167 that cooperative Na⁺ binding to apo transporters, at Na1 and Na3, is H⁺-uncoupled. We
168 next asked if the apo transporters are able to bind protons. Indeed, total tryptophan-
169 fluorescence changes associated to saturating Na⁺/transmitter coupled binding
170 significantly decreased at pH values below neutral (Fig. EV1A,B), indicating that
171 protons change tryptophan-fluorescence of apo transporter upon binding.

172 To find ionizable side chains that could act as proton acceptors in the tranD, we
173 compared amino acid sequences from representative vertebrate species of two divergent
174 branches of SLC1 transporters (Gesemann *et al*, 2010): pH-dependent EAATs, and pH-
175 independent neutral amino acid exchangers (so-called ASCTs) (Fig. EV1C,D). The
176 tranD of EAAT1_{CRYST} contains over 30 ionizable side chains, but only Y127₁₂₇ (TM3),
177 Y405₃₈₅ (TM7b), E406₃₈₆ (TM7b), and R479₄₅₉ (TM8b) are strictly conserved among
178 EAAT orthologs, and not among ASCT ones. R479₄₅₉ is in close proximity to
179 conserved acidic residues (E406₃₈₆, D472₄₅₂, and D476₄₅₆), while Y127₁₂₇ backbone is
180 part of Na3, where Na⁺ arguably binds in a pH-independent fashion. Hence, it is highly
181 unlikely that those two residues exchange protons with the bulk during the transport
182 cycle, and we focused our analysis on Y405₃₈₅ and E406₃₈₆. Indeed, conservative single
183 mutations bearing non-ionizable sidechains had minor effects on pH-dependence of
184 substrate binding in Y405₃₈₅F ($C_E=0.37 \pm 0.06$), but abolished this dependence in
185 E406₃₈₆Q ($C_E=0.00 \pm 0.04$) (Fig. 3C). Our results agree well with early studies of rodent

186 ortholog EAAC1 showing that equivalent mutation to E406₃₈₆Q (E373Q) impairs pH-
187 dependence of apparent glutamate-binding (Greuer *et al*, 2003), as well as with
188 molecular dynamic simulations of transmitter binding (Heinzelmann & Kuyucak,
189 2014), and support the role of E406 carboxylate as the main proton acceptor in the
190 transport cycle.

191 From a structural viewpoint, E406₃₈₆ sidechain is occluded within the tranD core at
192 hydrogen-bond distance of G447₄₂₇ backbone-carbonyl oxygen, T450₄₃₀ sidechain
193 hydroxyl, and M451₄₃₁ backbone amide in HP2b in the Na⁺/transmitter-bound
194 EAAT1_{CRYST} structure (Fig. 3D). Consistent with its proton acceptor role, protonation
195 of E406₃₈₆ carboxylate group would expand its H-bonding capacity and enable
196 formation of a H-bond network with HP2b, contributing to maintain HP2 in a close
197 position with substrate occluded underneath, and explaining at least partly the
198 thermodynamic coupling between H⁺ and transmitter binding. Because it is not possible
199 to determine the protonation state of amino acid sidechains in the EAAT1_{CRYST} crystal
200 structure, we determined the structure of EAAT1_{CRYST}-E406₃₈₆Q mutant, mimicking
201 the protonated state of E406₃₈₆. Indeed, the position of E406₃₈₆Q side chain is identical
202 within experimental error to that of E406₃₈₆ (Appendix Figure S2A,B), consistent with
203 E406₃₈₆ being protonated in the Na⁺/transmitter-bound state.

204 All functional and structural results converge to show that Na⁺/transmitter bound
205 structure represents the transmitter translocation complex of the tranD with 3 Na⁺, 1
206 H⁺, and 1 neurotransmitter molecule bound.

207 **Counter-transported ion binding-site**

208 To understand the structural basis underlying ion counter-transport in EAATs, we
209 solved the crystal structure of transporters purified in Rb⁺-based buffer, in the absence
210 of Na⁺ and transmitter, and in complex with UCPH₁₀₁. Rb⁺ is a K⁺ analog, and the

211 advantage for crystallographic experiments is that Rb⁺ yields robust anomalous X-ray
212 scattering signals to unambiguously determine the position of bound ion(s) to the
213 transporter, at the resolution of our structure (~3.9 Å). Importantly, liposome uptake
214 experiments show that Rb⁺ is counter-transported by EAAT1_{CRYST}, demonstrating that
215 it is a functional K⁺ analog for EAAT1 structural studies (Fig. 4A).

216 Rb⁺ and UCPH₁₀₁ bound EAAT1_{CRYST} structure adopts an outward-facing open state,
217 and the anomalous difference map with the X-ray beam tuned at a Rb⁺ absorption
218 maximum (0.815 Å) shows two anomalous-difference peaks (> 5σ), namely P1 and P2.
219 P1 localizes to the substrate binding site near conserved D476₄₅₆, and P2 to Na3 (Fig.
220 4B). As negative control, we collected diffraction data from a similar crystal, but with
221 the X-ray beam tuned off the Rb⁺-absorption maximum (0.998 Å), and observed P2 (>
222 5σ), but not P1 in the anomalous-difference map (Fig. EV2). These results demonstrate
223 that Rb⁺, a functional counter-transported ion by EAAT1, is bound to the transporter at
224 the substrate binding site. Notably, this counter-transported ion binding site, namely
225 K_{CT}, overlaps with the position of the substrate amide-nitrogen atom in the
226 Na⁺/transmitter-bound structure, and it is coordinated by the carboxylate side chain of
227 D476₄₅₆ (TM8b), backbone carbonyl oxygen of S363₃₄₃ (HP1), and possibly its
228 sidechain, as well as that of T480₄₆₀ (TM8b) and water molecules (Fig. 4B). The
229 striking overlap with the transmitter binding site makes K_{CT} an optimal site for the
230 counter-transported ion to exclude the transmitter during the transport cycle.

231 Consistently, early computational (Holley & Kavanaugh, 2009), functional (Wang *et*
232 *al.*, 2013) and crystallographic (Verdon *et al.*, 2014) studies of EAAT homologs
233 predicted mutually exclusive transmitter and K⁺ binding sites at similar positions.

234 **Calcium binding to Na3 site**

235 Our anomalous scattering analysis reveals a second ion bound to Na3 in the Rb⁺ bound
236 EAAT1_{CRYST} structure. In addition to Rb⁺ (at ~100 mM), crystallization conditions
237 contain Ba²⁺ and Ca²⁺ (at ~25 mM), and only the former could generate significant
238 anomalous peaks off the Rb⁺ absorption maximum (0.998 Å). To shed light on this
239 problem, we solved the structure of transporters purified in the absence of substrate,
240 Na⁺, and Rb⁺, and using choline as a substitute for monovalent cations, but otherwise
241 under similar crystallization conditions containing Ba²⁺ and Ca²⁺. In these crystals, we
242 found a single anomalous-difference peak that corresponds to P2 (> 8σ) at Na3 (Fig.
243 5A), consistent with Ba²⁺ binding at this sodium site. From a structural point of view,
244 Ba²⁺ binding to Na3 would be possible through conformational changes around the
245 ³⁹⁸NMDG motif, particularly at the level of N398₃₇₈ that moves away from the center
246 of the site compared to the transmitter bound structure, enabling occupancy by a larger
247 cation than Na⁺ (Fig. EV3).

248 From a functional perspective, Ba²⁺ binding to a conserved Na⁺-binding site in EAATs
249 raises the interesting possibility that physiologically-relevant analog Ca²⁺ would also
250 bind there. Ca²⁺ and Ba²⁺ binding to EAAT1_{CRYST} transporters was confirmed by
251 changes in intrinsic-tryptophan fluorescence, and showed several important features:
252 Ca²⁺ binds apo transporters with K_D = 2.9 ± 0.4 mM in a non-cooperative manner (n_H
253 = 0.9 ± 0.1), and mutation D400₃₈₀N increased its K_D by at least an order of magnitude
254 (Fig. 5B), consistent with binding of one calcium ion to Na3; Ca²⁺ binding parameters
255 were not significantly affected by allosteric inhibitor UCPH₁₀₁ (K_D = 1.4 ± 0.1 mM;
256 n_H = 0.9 ± 0.03), implying that Ca²⁺ affinities to outward- and inward-facing states are
257 comparable; Ca²⁺ (and Ba²⁺), but not K⁺ significantly increased Na⁺ apparent K_D (Fig.
258 EV4), indicating competitive binding between Ca²⁺ (or Ba²⁺) and Na⁺, as well as lack
259 of K⁺ affinity for Na1 and Na3; binding of substrate was thermodynamically coupled

260 to one Ca^{2+} ($C_E = -0.8 \pm 0.1$) (Fig. 5C), as expected for a cation that binds to Na3. In
261 addition, reconstituted transmitter uptake in liposomes demonstrates that Ca^{2+} is not
262 counter-transported (Fig. 4A), at least not at rates comparable to K^+ or Rb^+ , suggesting
263 that a cation bound to Na3 is not able to form a translocation complex to re-locate the
264 substrate binding site. However, Ca^{2+} weakly inhibited transport with half-maximal
265 inhibitory concentrations (IC_{50}) ~ 5 mM, when added to K^+ -containing intra-liposomal
266 side, but it lacked effect when added to the Na^+ /transmitter-containing extracellular
267 solution in cells (Fig. EV5A,B). Lack of extracellular Ca^{2+} effect on steady-state
268 transport is likely due to competition to much stronger Na^+/H^+ /transmitter coupled
269 binding, while intra-liposomal Ca^{2+} inhibition could be due to facilitation of transmitter
270 re-binding inside the liposome, as Ca^{2+} and transmitter binding are thermodynamically
271 coupled.

272 From a physiological perspective, Ca^{2+} /transmitter coupling could play a regulatory
273 role of Ca^{2+} in glutamate transport at tripartite synapses. Fine astrocytic processes at
274 the synapse undergo transient increases in intracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$), and modulate
275 neuronal activity by increasing the amount of extracellular neurotransmitter available
276 (Bazargani & Attwell, 2016). One potential mechanism linking these two phenomena
277 is that at physiological glutamate intracellular-concentrations in astrocytes (mM),
278 $[\text{Ca}^{2+}]_i$ transients could promote re-binding of the transmitter to EAATs, slowing down
279 glutamate transport and increasing the amount of transmitter available at the synapse.
280 Testing that and other hypotheses regarding the effect of extra- and/or intracellular Ca^{2+}
281 on transmitter transport will require further experimentation, including time-resolved
282 techniques.

283 **Conformational changes associated to ion counter transport**

284 Rb⁺/Ba²⁺ bound EAAT1_{CRYST} structure shows extensive and concerted conformational
285 changes on the extracellular half of the tranD compared to the Na⁺/transmitter bound
286 state (Fig. 4B). The helical arms of HP2 and TM8a, and to a lesser extent the
287 extracellular part of TM3, TM6, and TM7 move outward and towards the scaD, while
288 the tip of HP2 is in an open position stabilized by a ~90-degree rotation of M399₃₇₉
289 sidechain towards the extracellular side, and separated from the tip of HP1 by as much
290 as ~11 Å. These movements expose the tranD core to the bulk, and enable hydration of
291 transmitter, Na⁺, and H⁺ binding sites, as well as distort sodium and transmitter
292 coordination (Fig. EV3): backbone and sidechain movements of N398₃₇₈ and M399₃₇₉,
293 at the conserved ³⁹⁸NMDG motif, break Na⁺ coordination at Na1-3, while those of HP2,
294 T402₃₈₂, and R479₄₅₉ disrupt transmitter's binding site.

295 The outward-facing open states seen in the structures of apo transporters (Rb⁺/Ba²⁺ and
296 Ba²⁺ bound, respectively) are nearly identical (Fig. 5A), suggesting that the presence of
297 divalent cations in the crystallization buffer and/or crystal contacts might restrict
298 conformational changes of the tranD. Moreover, the open position of HP2 should
299 preclude transmembrane movements of the tranD and therefore, these structures likely
300 represent intermediates of the transport cycle that do not correspond to the K⁺
301 translocation complex. To overcome these limitations, we determined the cryo-EM
302 structure of EAAT1_{WT} purified in a K⁺-based buffer (in the absence of other
303 monovalent and divalent coupled cations, as well as transmitter and UCPH₁₀₁) at an
304 overall ~ 4.0 Å resolution (Fig. 6A and Appendix Figure S3). The quality of the cryo-
305 EM map was better in the scaD than in tranD, likely reflecting the dynamic nature of
306 the latter (Appendix Figure S4), and we observed non-protein density for lipid or
307 detergent molecules at the scaD-tranD, as well as at inter-subunit interfaces suggesting
308 a regulatory role of lipids in tranD dynamics and trimer stability, respectively.

309 The cryo-EM structure shows a symmetric EAAT1_{WT} trimer in an inward-facing state
310 with TM1a and TM1a' laying nearly parallel to the membrane, as well as two long beta-
311 strands between TM4b-c in the scaD protruding outside the membrane plane,
312 resembling the ones observed in human ASCT2 (Garaeva *et al.*, 2018). Consistent with
313 an elevator-like mechanism, the entire tranD undergoes a large movement that
314 translocates ligand binding-sites across the membrane, moving them by as much as 18
315 Å into the cytoplasmic side (Fig. 6B). Notably, tranD backbone adopts a different
316 conformation compared to that in Na⁺/transmitter and Rb⁺/Ba²⁺ crystal structures,
317 respectively (Fig. 6C). The extracellular half of the tranD, including HP2 and TM8a,
318 moves outward and towards the scaD expanding the tranD core compared to the
319 Na⁺/transmitter bound state, but to a lesser extent than in the Rb⁺/Ba²⁺ bound state. In
320 the cytoplasmic part of the tranD, TM7a moves away from HP1b at the level of Na1.
321 Yet, the tip of HP2 remains in contact with that of HP1 in a close position. The
322 concerted backbone movements of the tranD have several important mechanistic
323 implications: separation of HP2a, HP1b, and TM7a away from each other disrupts
324 backbone-atom coordination at Na1, Na2, and transmitter binding sites; concomitantly,
325 close proximity of HP1 and HP2 tips suggests that backbone carbonyl oxygen atoms of
326 I423₄₀₃ and/or P424₄₀₄ (HP2 tip) could contribute to counter-transported ion
327 coordination and occlusion at K_{CT} and therefore, that the tranD adopts an occluded state
328 competent for K⁺ translocation.

329 To gain further insight into K⁺-induced conformational changes outside the context of
330 the crystal lattice, we compared hydrogen-deuterium exchange linked to mass
331 spectrometry (HDX-MS) profiles of transporters at 25°C in Na⁺/transmitter, and K⁺
332 buffers, respectively (Fig. 6D and Appendix Figure S5, S6). EAAT1_{WT} was not
333 amenable to HDX-MS analysis due rapid and irreversible unfolding at 25°C (Cirri *et*

334 *al.*, 2018). Hence, we probed thermostabilized EAAT1_{CRYST} using short deuterium-
335 labeling time (1 min) to avoid unfolding events, and in the presence of UCPH₁₀₁ to
336 restrict the conformational sampling to outward-facing states. Indeed, transporters in
337 K⁺ compared to Na⁺/transmitter buffers showed a significant increase in HDX that
338 localizes to the tip of HP1 and HP2, as well as the NMDG motif. These regions lack
339 secondary structure, in both transmitter and counter-transported ion bound states, and
340 contain key residues for Na⁺ and transmitter coordination. Therefore, the observed
341 changes in HDX imply that K⁺ induces a significant increase in backbone hydration
342 around the ligand binding sites, which is in excellent agreement with the
343 conformational expansion of the tranD core observed in both crystal and cryoEM
344 structures of apo transporters. Importantly, K⁺-induced HDX changes reverted fully
345 upon adding back Na⁺/transmitter (Appendix Figure S6, control condition),
346 demonstrating that transporters remain folded and respond to addition of ligands, and
347 that observed HDX changes were not due to unfolding events of purified protein
348 assayed at 25°C.

349 **Discussion**

350 Based on our structural and thermodynamic analyses, we propose a complete Na⁺, H⁺,
351 K⁺ coupled neurotransmitter transport mechanism that unifies a wealth of structural and
352 biophysical data on SLC1 proteins (Fig. 7). In this mechanism, Na⁺ binding to Na1-
353 Na3 and protonation of E406₃₈₆ are thermodynamically coupled to transmitter binding
354 and occlusion, and lead to the formation of the transmitter translocation complex,
355 represented by EAAT1_{CRYST} Na⁺/transmitter bound crystal structure (Fig. 2A-D). In
356 turn, K⁺ binding to K_{CT} promotes self-occlusion, and formation of a K⁺ translocation
357 complex that excludes the transmitter, and it is represented by EAAT1_{WT} cryo-EM
358 structure (Fig. 6A,B).

359 Cooperative Na⁺ binding to Na1 and Na3 in apo EAAT1_{CRYST} enables substrate binding
360 through re-arrangements of the NMDG signature motif that resemble those observed in
361 SLC1 archaeal homologs (Guskov *et al.*, 2016; Verdon *et al.*, 2014), and this process
362 is independent of both H⁺ and K⁺. Sodium-saturated apo-transporters undergo three key
363 thermodynamically-coupled events that lead to substrate occlusion under HP2, and the
364 formation of transmitter translocation complex: Na⁺ binding to Na2 stabilizes HP2a
365 against TM7a; protonation of E406₃₈₆ in TM7b enables hydrogen-bonding with HP2b;
366 and transmitter binding secures closure of HP2 tip through direct coordination.

367 What are then the events that lead to formation of the K⁺ translocation complex? Our
368 results demonstrate counter-transported ion binding to K_{CT}, as well as hydration of the
369 tranD core associated to K⁺, strongly suggesting that these are key events to form the
370 translocation complex. Importantly, K_{CT} fulfills two essential requirements as counter-
371 transported ion binding site, first it precludes transmitter binding preventing futile
372 transport cycles and potentially-cytotoxic release of glutamate and second, it promotes
373 formation of a competent occluded state that enables elevator-like movements of the
374 tranD, as suggested by the closed position of HP2 in EAAT1_{WT} cryo-EM structure.

375 Early reports predicted counter-transported ion binding sites similar to K_{CT} and
376 mutually-exclusive transmitter and K⁺ binding mechanisms (Holley & Kavanaugh,
377 2009; Verdon *et al.*, 2014; Wang *et al.*, 2013), in excellent agreement with our results.

378 However, this view has been challenged by computational and functional studies that
379 highlighted the role of Na1, as a likely counter-transported ion binding site (Kortzak *et*
380 *al.*, 2019; Wang *et al.*, 2020). Several lines of experimental evidence presented here
381 argue against that proposal: first, crystals grown in ~100 mM counter-transported ion
382 (Rb⁺) do not show anomalous-scattering peaks at Na1, revealing lack of ion occupancy
383 at this site (Fig. 4B and Fig. EV2); second, K⁺ concentrations up to 1.5 M have no

384 significant effect on cooperative Na⁺ binding to apo transporters, arguably at Na1 and
385 Na3, consistent with lack of K⁺ affinity for these sites. Moreover, Ca²⁺ binds to Na3
386 with a K_D value in the low mM range, and induces a large increase in Na⁺ apparent K_D
387 (Fig. EV4). This supports the intuitive idea that significant occupancy of sodium sites
388 by other cations impacts the apparent Na⁺-binding parameter. Finally, in EAAT1_{WT}
389 cryo-EM structure, solved in the presence of 100 mM K⁺ and absence of divalent
390 cations, backbone movements of TM7a away from HP1 distort Na1 geometry, and it is
391 not consistent with ion coordination at this site (Fig. 6C).

392 The close proximity of conserved R479₄₅₉ to K_{CT} argues that electrostatic shielding of
393 its sidechain is required for K⁺ occlusion and translocation, as suggested by reported
394 apo EAAT3 structure (Qiu *et al.*, 2021) and MD simulations (Kortzak *et al.*, 2019), and
395 hydration of the tranD core is likely key to this process. Both our crystal and cryo-EM
396 structures of apo transporters show expansions of the tranD core around the ligand
397 binding sites, and HDX-MS experiments confirmed increased hydration in this region.
398 Water penetration in the tranD core should directly contribute to shield R479₄₅₉ charge,
399 and more importantly induce deprotonation of E406₃₈₆, enabling ionic interactions
400 between the two residues. Hydrogen-bonding between R479₄₅₉ and T402₃₈₂ could also
401 contribute to electrostatic shielding (Fig. 4B). Consistently, E406₃₈₆ and T402₃₈₂ are
402 highly conserved in EAAT orthologs, but are glutamine and alanine, respectively, in
403 ASCT ones. Therefore, we propose a dual role of E406₃₈₆ and R479₄₅₉ in transport: in
404 the transmitter translocation complex protonated E406₃₈₆ contributes to transmitter
405 occlusion through hydrogen bonding with HP2, while R479₄₅₉ coordinates the gamma
406 carboxylate group of the substrate. In the K⁺ translocation complex, deprotonated
407 E406₃₈₆ engages in ionic interactions with R479₄₅₉, enabling K⁺ occlusion at K_{CT}.
408 Equivalent mutations to E406Q in mammalian orthologs impair both H⁺ and K⁺

409 coupled transmitter transport (Greuer *et al.*, 2003; Kavanaugh *et al.*, 1997), further
410 supporting the dual role of E406 in H⁺ and K⁺ coupling. Moreover, the cryo-EM
411 structure of inwardly-open EAAT3 in apo conditions shows equivalent residues to
412 E406 and R479 forming a salt bridge, although their roles in substrate occlusion seem
413 different (Qiu *et al.*, 2021), likely reflecting evolutionary differences among EAAT
414 isoforms.

415 Our findings shed light on controversial and important aspects of EAATs transport
416 cycle, and suggest novel mechanisms to regulate glutamate levels at tripartite synapses.

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418 **Conflict of interest**

419 The authors declare no competing financial interests.

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584 **Methods**

585 **Protein expression and purification**

586 Genes encoding EAAT1 and thermostabilized EAAT1_{CRYST} constructs were cloned
587 into the pcDNA3.1(+) plasmid (Invitrogen) as previously described with N-terminal
588 Strep-tag II affinity tag, eGFP and PreScission site(Canul-Tec *et al.*, 2017). Protein was
589 expressed in HEK293F cells (Thermo Fisher Scientific) grown in Freestyle293 medium
590 (Invitrogen) at 37 °C to densities of 2.5×10^6 cells ml⁻¹. Cells were transiently
591 transfected using 7.5 mg ml⁻¹ polyethylenimine (PEI) (Polysciences) and 2.5 mg ml⁻¹
592 of DNA. Transfected cells were diluted with an equivalent volume of Freestyle293
593 medium 8 h post-transfection and treated with 10 mM of sodium butyrate (Sigma) 12
594 h after dilution. Cells were harvested by centrifugation 48 h after transfection,
595 resuspended at 1:4 (w/v) ratio in cold buffer containing 50 mM HEPES/Tris-base, pH
596 7.4, 50 mM NaCl, 1 mM L-asp, 1 mM EDTA, 1 mM Tris(2-carboxyethyl) phosphine
597 (TCEP), 1 mM Phenylmethylsulfonyl fluoride (PMSF) and 1:200 (v/v) dilution of
598 mammalian protease inhibitor cocktail (Sigma), and flash-frozen in liquid nitrogen for
599 storage at -80 °C.

600 Cell suspension was thawed and diluted 1:2 volumes in buffer complemented with 350
601 mM NaCl, 10% glycerol, 1% dodecanoyl sucrose (DDS, Anatrace) and 0.2%
602 Cholesteryl Hemisuccinate Tris Salt (CHS, Anatrace). After 1 h detergent extraction,
603 cell debris was removed (4,500 g, 0.5 h), and the supernatant cleared (186,000 g for 1
604 h). Clear lysate was incubated with Strep-Tactin Sepharose High Performance resin
605 (GE Healthcare) previously equilibrated with buffer containing 50 mM HEPES/Tris-
606 base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM TCEP, 5% glycerol, 0.05% DDS and
607 0.01% CHS, for 2 h. In crystallization experiments of apo transporters in Rb⁺ or choline
608 buffers (see below) resin incubation was preceded by an ultracentrifugation membrane-

609 wash step, as described before (Canul-Tec *et al.*, 2017). Resin was washed with 20
610 column volumes (CV) of equilibration buffer supplemented with 1:1:1 lipid mixture of
611 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-
612 glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-
613 phosphoglycerol (POPG) (Avanti Polar Lipids) at total concentration of 25 μ M. Protein
614 was eluted with buffer supplemented with 2.5 mM D-desthiobiotin (Sigma), and
615 subjected to N-term PreScission cleavage during 2 h. The sample was concentrated with
616 100-kDa cutoff Amicon centrifugal filter unit (Millipore) and injected into a size-
617 exclusion chromatography (SEC) column Superose 6 10/300 (GE Healthcare),
618 equilibrated in different buffers (see below). All purification steps were carried out at
619 4 °C.

620 **Intrinsic-tryptophan fluorescence binding assay**

621 PreScission-treated EAAT1_{CRYST} and mutant transporters were further purified by SEC
622 in buffer containing: HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM
623 TCEP, 5% glycerol, 0.05% DDS, 0.01% CHS and 25 μ M lipid mix. Peak fractions were
624 concentrated using a 100-kDa cutoff Amicon centrifugal filter, and then diluted 10-fold
625 in buffer: 50 mM HEPES/Tris-base, pH 7.4, 200 mM ChCl, 1 mM TCEP, 0.05% DDS
626 and 0.01% CHS. This procedure was repeated three times to ensure buffer exchange.
627 Binding experiments started by further diluting transporters ~ 100-fold in a 1-ml quartz
628 cuvette filled with the above-mentioned Ch⁺-based buffer to reach 2-4 μ M final
629 protomer concentration. In L-asp titrations, final NaCl or CaCl₂ concentrations were
630 added to the cuvette as indicated in the Figures. To assay binding of ligands in the
631 presence of UCPH₁₀₁, 20 μ M final concentration was added to both protein sample and
632 cuvette, and allowed for ~30 min equilibration time. Binding experiments at different

633 pH values were done by substituting HEPES/tris-base with MES/tris-base (pH 5 and
634 6), or Tris/HCl (pH 9 and 10) in the cuvette buffer.

635 Experiments were done at 25 °C under stirring. Intrinsic tryptophan fluorescence was
636 excited at 280 nm, and emission recorded at 325 nm using a QuantaMaster 40
637 spectrofluorometer (Horiba). Protein samples were equilibrated for 2-min in the
638 cuvette, before ligand addition at different concentrations, and fluorescence emission
639 was averaged over the last 10s after equilibration. Fractional fluorescence changes were
640 fitted to the Hill equation. Data analysis, fitting, and figures were done with SigmaPlot
641 12 (Systat Software, Inc).

642 **Crystallization and structure determination**

643 PreScission-treated transporters were supplemented with 100 μM UCPH₁₀₁ (Abcam)
644 for 30 minutes at 4 °C, and further purified by SEC in buffers at pH 7.4 (HEPES/Tris-
645 base), containing 1 mM TCEP, 5% glycerol, 100 μM UCPH₁₀₁, 0.25% decanoyl
646 sucrose (DS, Merck Millipore), 0.05% CHS, 25 μM lipid mix, and one of the following:
647 200 mM Na and 1 mM L-asp (Na⁺/asp-bound state); 200 mM RbCl (Rb⁺/Ba²⁺-bound
648 state); or 200 mM ChCl (Ba²⁺-bound state). Peak fractions were concentrated to 4 mg
649 ml⁻¹, supplemented with 0.2% n-Octyl-β-D-glucopyranoside (BOG, Anatrace) and
650 0.04% CHS, and used immediately for crystallization. Vapor-diffusion hanging-drop
651 crystallization and cryogenic conditions were as previously described (Canul-Tec *et al.*,
652 2017), using equal volumes of sample and a reservoir solution containing 100 mM Tris,
653 pH 8.2, 28-32% (v/v) PEG 400, 50 mM CaCl₂ and 50 mM BaCl₂.

654 Complete and highly-redundant X-ray diffraction datasets were collected at tunable
655 beamlines PROXIMA-1 (SOLEIL synchrotron, St. Aubin, France), as well as ID29 and
656 ID30B (European Synchrotron Radiation Facility, Grenoble, France). EAAT1_{CRSYT} and
657 EAAT1_{CRYST-II} crystals purified in Rb⁺- or Ch⁺-based buffers were smaller and typically

658 diffracted at significant lower resolutions ($> 6\text{\AA}$) than those purified in Na^+ /asp-based
659 buffers ($< 4\text{\AA}$), and they required larger crystal-screening efforts to collect datasets at
660 the reported resolutions.

661 Diffraction datasets were processed as described before (Canul-Tec *et al.*, 2017) using
662 XDS package (Kabsch, 2010) and AIMLESS (Evans & Murshudov, 2013). All crystals
663 belonged to the same space group ($P6_3$), and diffracted anisotropically. Anisotropy
664 correction was made with STARANISO (Global Phasing Lim.). Appendix Table S1
665 and S2 include comparison of reflection statistics before and after anisotropic
666 correction reflecting complete sampling of reciprocal space, as well as refinement
667 statistics of reflections along crystallographic axes. Corrected anisotropic amplitudes
668 were used for molecular replacement in PHASER (McCoy *et al.*, 2007), using TranD
669 and ScaD of EAAT1_{CRYST} or EAAT1_{CRYST-II} (PDB codes 5LLM and 5LM4,
670 respectively) as independent search models. Final electron density maps were obtained
671 through rounds of manual building in COOT (Emsley *et al.*, 2010) and refinement in
672 Buster (Blanc *et al.*, 2004), until reaching good crystallographic statistics and
673 stereochemistry (Appendix Table S1 and S2). Anomalous difference maps were
674 calculated with SHELX and ANODE (Thorn & Sheldrick, 2011) using diffraction data
675 with high-resolution cutoff of 6\AA , and without anisotropic correction.

676 In the Na^+ /asp-bound EAAT1_{CRYST} structure, we modelled extra electron density in the
677 tranD (TM7-HP2 loop, residues 397-403), and in the scaD N-terminus (TM1a' helix,
678 residues 26-36). Rounds of refinement with extended transporter models incrementally
679 improved the quality of 2Fo-Fc maps, and yielded three peaks in the Fo-Fc omit maps
680 that localize to conserved Na1-Na3. We modelled 3 Na^+ bound to the transporter at
681 these peaks. We also calculated anomalous differences maps, and observed a single
682 anomalous peak on the surface of the ScaD, nearby residue Q245 (TM5), that we

683 modeled as a bound barium ion with low occupancy (0.35). Our functional and
684 structural data do not support functional roles of this ion on the transport mechanism.
685 Rb⁺/Ba²⁺ and Ba²⁺ bound electron density maps of apo transporters, respectively, were
686 lower resolution compared to Na⁺/asp-bound. This is likely due to the observed
687 conformational re-arrangements of the tranD that break or weaken crystal-lattice
688 contacts between TM1a' and extracellular tranD surface of a crystallographic-
689 symmetry mate. Consistently, in Rb⁺/Ba²⁺ and Ba²⁺ bound structures, respectively,
690 TM7-HP2 loop and TM1a' were not modeled due to lack of density in these regions.
691 Rb⁺ at K_{CT}, as well as Ba²⁺ at Na3 were modeled based on anomalous difference maps
692 from datasets at maximum and off-maximum Rb⁺ absorption wavelengths,
693 respectively.

694 Atomic model validation was performed with Molprobity (Chen *et al*, 2010). Structural
695 figures were prepared with PyMOL Molecular Graphics System (Schrodinger, LLC).

696 **Cryo-EM sample preparation, data collection and processing**

697 EAAT1_{WT} (Uniprot P43003) sequence was mutated at predicted N-glycosylation sites
698 (N206T, and N216T), and expressed and purified as mentioned above, with SEC buffer
699 containing: 50 mM HEPES/Tris-base, pH 7.4, 100 mM KCl, 1 mM TCEP, 0.0084%
700 glycol-diosgenin (GDN), and 0.0017% CHS. Peak fractions were concentrated at 4-5
701 mg ml⁻¹ and spotted immediately on glow-discharged Quantifoil R1.2/1.3 Au grids
702 (Quantifoil Micro Tools GmbH), and plunge frozen using a Vitrobot Mark IV (FEI) at
703 4°C under 100% humidity. Grids were stored in liquid nitrogen.

704 Cryo-EM micrographs were recorded on a Titan Krios electron microscope (Thermo
705 Fisher Scientific) operated at 300 kV, equipped with GatanK2 direct electron detector
706 (Appendix Table S3). Movies were collected on counting mode automatically using
707 SerialEM (Mastrorade, 2005) with a pixel size of 0.814 Å. The defocus range was -

708 0.8 to -2.0 μm and each movie contained 40 frames with a dose per frame of 1.03
709 electrons/ \AA^2 , and total exposure time 8 s.

710 Single-particle data processing was done in cryoSPARCv3.1.0 (Punjani *et al*, 2017)
711 (Appendix Figure S3). Beam-induced patch-motion correction, and patch contrast
712 transfer function estimation were done with in-built cryoSPARC routines, respectively.

713 2,298,481 auto-picked particles were 2x binned and extracted from 13,607 manually-
714 selected movies. Initially, 517,319 selected from 2D-classification were subjected to
715 several rounds of *ab initio* classification. This yielded a 3D reconstruction from 71,296
716 particles in which the transmembrane helices of the transporter were evident, but
717 refined at $> 6 \text{ \AA}$ using unbinned particles for non-uniform refinement (Punjani *et al*,
718 2020). We then input those *ab initio* volumes to re-classify the original set of 2,298,481
719 2x-binned particles using several rounds of heterogenous refinement. In the first round
720 of heterogenous refinement, two unrelated cryo-EM maps were included as templates
721 to aid discarding bad particles. From this, 433,972 particles were selected and unbinned
722 for further rounds of heterogenous refinement and *ab initio* classification. An *ab initio*
723 3D reconstruction from a final set of 34,433 particles yielded a map at an overall ~ 4.0
724 \AA , after non-uniform refinement with C3 symmetry imposed. The map quality was
725 significantly better in the region corresponding to the scaD than that of the tranD. This
726 is somehow expected, since the three tranDs undergo independent conformational
727 changes both relative to the three scaDs, as well as locally.

728 For model building, the scaD and tranD of $\text{Rb}^+/\text{Ba}^{2+}$ bound X-ray structure were fitted
729 into the EM map as separate rigid-bodies using Chimera. This initial model was then
730 adjusted manually in COOT preserving the secondary structure observed in the crystal.

731 We also used the cryoEM structure of ASCT2 (PDB 6GCT) (Garaeva *et al.*, 2018) as
732 a reference for regions of the tranD that were not modeled in the crystal structure and

733 for which we had density in the cryo-EM map. We omitted conserved residues
734 ³⁹⁸NMD⁴⁰⁰ in the final cryo-EM model due to lack of density in this region. The final
735 model was refined in PHENIX (Adams *et al*, 2010), and figures made with UCSF
736 Chimera (Pettersen *et al*, 2004), UCSF ChimeraX (Goddard *et al*, 2018), and PYMOL.

737 **Radioactive substrate transport assays**

738 Liposomes were formed at 5:1 molar ratio of POPC and Cholesteryl Hemisuccinate
739 (Avanti Polar Lipids) and resuspended at 8 mg ml⁻¹ in buffer containing 50 mM
740 HEPES/Tris, pH 7.4 and 200 mM NaCl, and pre-treated with 1.3% DDS and 0.26%
741 CHS for 1 h. eGFP-EAAT1_{CRYST} fusion construct was purified by SEC in buffer
742 containing: HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM TCEP, 5%
743 glycerol, 0.05% DDS, 0.01% CHS and 25 μM lipid mix. Purified eGFP-EAAT1_{Cryst}
744 was mixed with destabilized liposomes at a 1:40 (w/w, protein/lipid ratio) for 1 h.
745 Detergent removal was repeated three times with Biobeads SM-2 (BioRad) at 100 mg
746 ml⁻¹ and at 4 °C. Proteoliposomes were loaded with internal buffer (50 mM
747 HEPES/Tris-base, pH 7.4 buffer, 200 mM KCl and 1.25% glycerol) through 10 freeze-
748 thaw cycles followed by 10 extrusion cycles through 400-nm polycarbonate
749 membranes (Avanti Polar Lipids). Proteoliposomes were centrifuged (164,300 g, 30
750 min at 4 °C) and resuspended at 100 mg of lipids ml⁻¹ in buffer containing 50 mM
751 HEPES/Tris-base, pH 7.4, 200 mM choline chloride (ChCl) and 1.25% glycerol for
752 immediate use.

753 Substrate uptake was performed at 37°C for 30 min, and initiated by diluting
754 proteoliposomes 10-fold into buffer containing 50 mM HEPES/Tris-base, pH 7.4, 200
755 mM NaCl, 1.25% glycerol, 50 μM L-glutamate and 10 μM L-[¹⁴C(U)]-glutamate
756 (PerkingElmer). Uptake was stopped with 5-fold dilution of proteo-liposome mix into
757 ice-cold buffer (50 mM HEPES/Tris-base, pH 7.4, 200 mM ChCl and 1.25% glycerol)

758 followed by immediate filtration and wash on nitrocellulose 0.22- μ m filters (Merck
759 Millipore). Background radioactivity was estimated on each sample using
760 proteoliposomes diluted in buffer containing 200 mM ChCl, instead of NaCl, and
761 subtracted during analysis. Filter membranes were transferred to scintillation cocktail
762 Ultima Gold (PerkinElmer), and radioactivity quantified using Tri-Carb 3110TR
763 counter (PerkinElmer). This setup was used to calibrate different batches of radioactive
764 L-glutamate source. Protein quantification was done in an Infinite M1000Pro
765 microplate reader (Tecan) using calibrated eGFP fluorescence intensity.

766 Uptake experiments at different pH values were done with modified external buffers
767 containing: 50 mM MES/Tris-base pH 6.0, HEPES/Tris-base pH 7.4, or 50 mM
768 Tris/HCl pH 9.0 or 10.0. Counter-transported ions were probed by exchanging KCl in
769 the intraliposomal buffer for equal concentrations of RbCl, or ChCl, or 125 mM ChCl
770 and 50 mM CaCl₂ through freezing-thawing cycles. Reported values are means of at
771 least three independent experiments, each one measured at least in triplicates.

772 In HEK293-cells uptake experiments, cells were collected 36-44 h after transfection,
773 washed two times, and resuspended at a density of 50×10^6 cells ml⁻¹ in buffer 11 mM
774 HEPES/Tris-base, pH 7.4, 140 mM ChCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂,
775 and 10 mM D-glucose, for immediate use. Substrate uptake was performed as described
776 in liposomes with some variations. For experiments shown in Appendix Figure 1,
777 reaction time was 1 min in buffer containing 11 mM HEPES/Tris-base, pH 7.4, 140
778 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 10 mM D-glucose, 50 μ M L-
779 glutamate, and 5 μ M [¹⁴C] L-glutamate. For experiments shown in Fig. EV5, reaction
780 time was 15 min in buffer containing 11 mM HEPES/Tris-base, pH 7.4, 140 mM NaCl,
781 4.7 mM KCl, 5 mM CaCl₂, 10 mM D-glucose, and either 50 μ M L-glutamate/10 μ M

782 [¹⁴C] L-glutamate, or 15 μM L-glutamate/5 μM [¹⁴C] L-glutamate. In Mg²⁺-containing
783 conditions, all Ca²⁺ was substituted for Mg²⁺.

784 Cells were filtered using 0.8-μm nitrocellulose filters, and background radioactivity
785 was estimated from cells transfected with empty vector in NaCl reaction buffer, and
786 subtracted from the uptake data. Reported values are means of three independent
787 experiments measured in duplicates.

788 **Hydrogen-deuterium exchange mass spectrometry (HDX-MS)**

789 PreScission-treated EAAT1_{CRYST} was further purified by SEC in buffer containing:
790 HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM TCEP, 5% glycerol,
791 0.05% DDS, 0.01% CHS, 25 μM lipid mix, and 100 μM UCPH₁₀₁. This sample
792 constitutes reference Na⁺/L-asp-bound state. K⁺-bound state was obtained by two
793 cycles of 10-fold dilution-concentration of reference samples in buffer: 50 mM
794 HEPES/Tris-base, pH 7.4, 200 mM KCl, 1 mM TCEP, 100 μM UCPH₁₀₁, 0.05% DDS
795 and 0.01% CHS. To probe reversibility of K⁺-induced HDX changes, a Na⁺/L-asp-
796 bound control state was generated by addition of 200 mM NaCl and 1 mM L-asp to the
797 K⁺-bound state. Final concentration of all samples for HDX-MS analysis was ~0.8 mg
798 ml⁻¹.

799 Peptide map was generated using nanoLC-MS/MS. 20 pmol of reference transporters
800 were digested for 2min in 0.75% formic acid using an immobilized pepsin column (2.1
801 x 20 mm, Affipro). Peptic peptides were collected and purified onto C18 Stage-Tips
802 before nanoLC-MS/MS analysis using an EASY-nLCTM 1200 system (Thermo-
803 Scientific) coupled to the nanoelectrospray ion source of an Orbitrap Q-Exactive Plus
804 mass spectrometer (Thermo-Scientific). Peptides were loaded on an in-house packed
805 nano-HPLC column (75 μm x 25 cm) with C18 resin (Aeris PEPTIDE XB-C18, 1.7μm
806 particles, 100 Å pore size, Phenomenex) and separated by reverse-phase

807 chromatography at 250 nL/min using a gradient of acetonitrile with 0.1% formic acid.
808 The Orbitrap mass spectrometer was set up in data-dependent acquisition mode. After
809 a survey scan in the Orbitrap (resolution 60,000 at m/z 400), the 10 most intense
810 precursor ions were selected for HCD fragmentation with a normalized collision energy
811 set up to 28 (resolution 30,000). Only charge states between 1 and 10 were selected and
812 a dynamic exclusion of 20 s was set. NanoLC-MS/MS data were processed
813 automatically using Mass Spec Studio v1.3.2(Rey *et al*, 2014) to identify peptides with
814 the following parameters: 4-40 amino acid length, charge states between 1 and 5, mass
815 accuracy of 7 ppm for both MS and MS/MS, and a false discovery rate (FDR) of 5%.
816 Deuterium exchange was initiated by diluting 64 pmol of purified transporter with the
817 appropriate labeling buffers at 25°C. These buffers reached final level of 80% D₂O and
818 otherwise were as described above for reference, K⁺-bound and control states,
819 respectively. 30 pmol transporter aliquots were removed at 60s, and immediately
820 quenched with a cold acidic solution (1.25% formic acid) to decrease the pH to 2.5.
821 Samples were then snap-frozen in liquid nitrogen and stored at -80°C until LC-MS
822 analysis.
823 Quenched samples were rapidly thawed and injected into a cooled ACQUITY UPLC
824 M-Class HDX system (Waters) maintained at 4°C. 15 pmol of transporter were online
825 digested for 3 min at 20°C and 80 μL/min of solvent A (0.15% formic acid (v/v) in
826 water) using an immobilized pepsin column (2.1 x 20 mm, Affipro). Peptic peptides
827 were desalted onto a C18 trap column (Kinetex® EVO C18, 2.6μm, 100Å, 2.1 x 20
828 mm, Phenomenex) at a flow rate of 80 μL/min of solvent A and then separated at 70
829 μL/min by a linear gradient from 10 to 60% of solvent B (0.15% formic acid (v/v) in
830 acetonitrile) in 11 min using a C18 analytical column (Kinetex® EVO C18, 1.7μm,
831 100Å, 1 x 100 mm, Phenomenex). The pepsin column was washed with 1.5M

832 guanidinium chloride / 5% acetonitrile / 1% formic acid. Blank injections were
833 performed between each run to ensure the absence of carry-over. The LC flow was
834 directed to a Synapt™ G2-Si HDMS™ mass spectrometer (Waters) equipped with an
835 electrospray ionization (ESI) source. Mass spectra were acquired in positive and
836 resolution mode over the m/z range of 300-1500 with 0.5 s scan time.

837 Data analysis was performed on biological triplicates using at least five replicates per
838 transporter state. Deuterium uptake values were calculated for each peptide using Mass
839 Spec Studio v1.3.2 and no adjustment was made for back-exchange. Student t-tests and
840 Woods plots were performed for each transporter state using the embedded statistical
841 module in Mass Spec Studio v1.3.2 with the following parameters: standard deviation
842 cutoff = 2 and 1-p cutoff = 0.99.

843 **Data availability**

844 Atomic coordinates of crystal structures were deposited in the RCSB Protein Data Bank
845 (PDB) under the following accession numbers: Na⁺/transmitter bound EAAT1_{CRYST}
846 (7AWM; <https://doi.org/10.2210/pdb7AWM/pdb>); Na⁺/transmitter bound
847 EAAT1_{CRYST}-E386Q (7AWQ; <https://doi.org/10.2210/pdb7AWQ/pdb>); Rb⁺/Ba²⁺
848 bound EAAT1_{CRYST} (7AWN; <https://doi.org/10.2210/pdb7AWN/pdb>); Rb⁺/Ba²⁺
849 bound EAAT1_{CRYST-II} (7AWP; <https://doi.org/10.2210/pdb7AWP/pdb>); and Ba²⁺
850 bound EAAT1_{CRYST-II} (7AWL; <https://doi.org/10.2210/pdb7AWL/pdb>). Atomic
851 coordinates and cryo-EM map of EAAT1_{WT} structure were deposited in the PDB
852 (7NPW; <https://doi.org/10.2210/pdb7NPW/pdb>), and Electron Microscopy Data Bank
853 (EMD-12524; <https://www.emdataresource.org/EMD-12524>).

854 **Code availability**

855 In-house script to facilitate reflection processing with STARANISO (P.L.) is available
856 at

857 (https://raw.githubusercontent.com/legrandp/xdsme/master/bin/noarch/run_xds2staran
858 [iso.sh](#))

859

860 **Acknowledgements**

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863 European Synchrotron Radiation Facility for assistance with data collection; The IECB
864 cryoEM imaging facility is acknowledged for support in cryo-EM sample screening
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866 Platform for support in cryoEM data collection. The work was funded by the European
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868 Pasteur-Roux Postdoctoral Fellowship. Further support from Institut Pasteur, INCA
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870 and J.C.-R. is acknowledged.

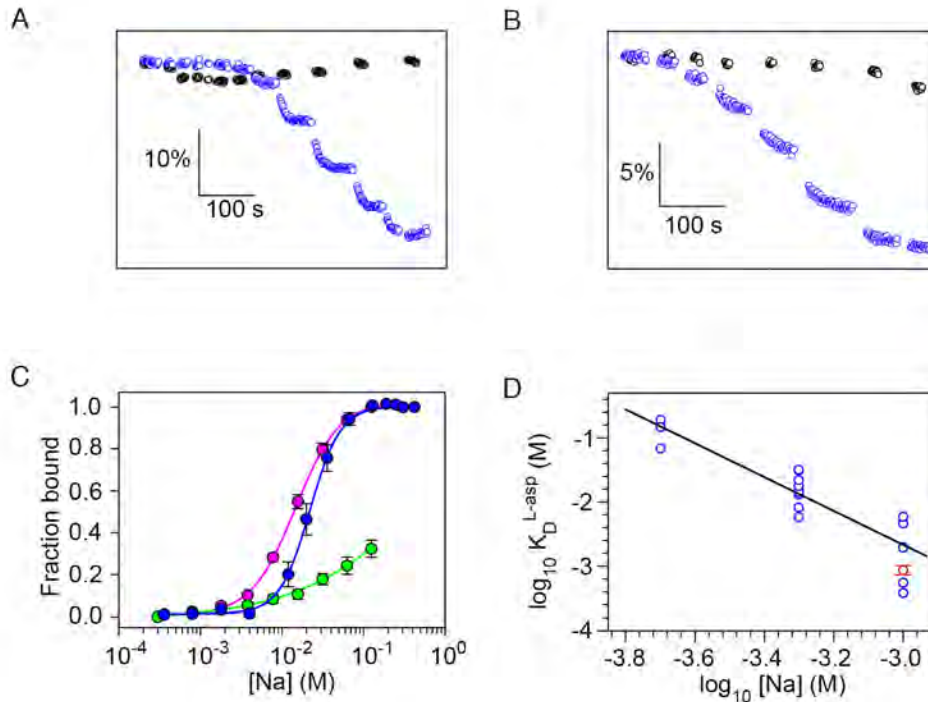
871 **Author contribution**

872 J.C.C.-T. optimized and performed protein expression, purification, and
873 crystallization, as well as binding and transport assays; A.K. prepared and optimized
874 cryo-EM grids and collected cryo-EM data. A.K and N.R. analyzed cryo-EM data
875 and built the structure. R.A. performed molecular biology and uptake experiments
876 in cells; J.C.C.-T and N.R. designed binding and uptake experiments, and analyzed
877 those data; J.D. performed and analyzed HDX-MS experiments; M.R. and J.C.-R.
878 designed and analyzed HDX-MS experiments.; J.C.C.-T. collected crystallographic
879 data; J.C.C.-T., P.L., and N.R. analyzed diffraction data; J.C.C.-T and N.R.
880 analyzed structures and prepared the manuscript, with contributions and edits

881 related to HDX from J.D., M.R., and J.C.-R., and those related to X-ray

882 crystallography from P.L.; N.R. conceived and supervised the project.

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886 **Figure 1 Na⁺ and transmitter binding**

887 A. Tryptophan-fluorescence time course associated to Na⁺ (blue) and K⁺ (black)
 888 binding, respectively, to detergent-purified apo EAAT1_{CRYST} at pH 7.4. Horizontal and
 889 vertical scale bars represent time and $\Delta F/F_0$, respectively.

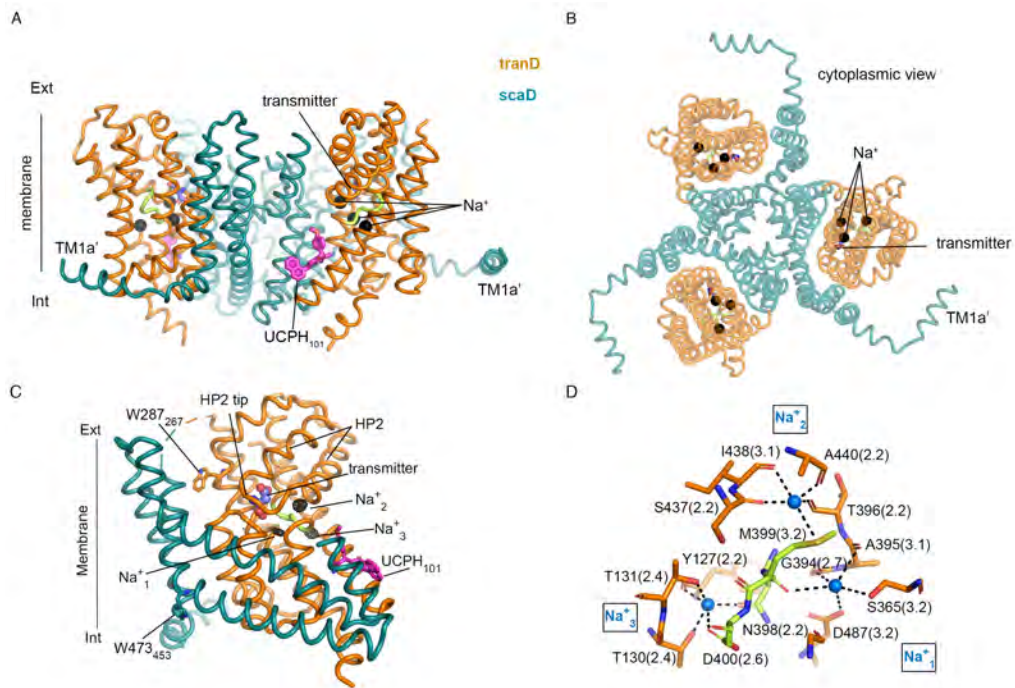
890 B. Tryptophan-fluorescence time course associated to L-asp binding in presence of 1
 891 mM (blue) and absence (black) of Na⁺, respectively, at pH 9.

892 C. Na⁺ binding isotherms of EAAT1_{CRYST} at pH 7.4 in the presence (pink) and absence
 893 (blue) of UPCH₁₀₁. Mutation D400₃₈₀N at Na3 strongly impairs Na⁺ binding (green).
 894 Solid lines represent fits of Hill equation.

895 D. Log-log plot of L-asp K_D as a function of Na⁺ concentration. Empty circles are K_D
 896 values (n=20) at pH=9.0 in the absence of UCPH₁₀₁ (blue), and solid line is the fit of a
 897 straight line. For comparison, average L-asp K_D at 1 mM Na⁺ in the presence pf of
 898 UPCH₁₀₁ is also shown (n=3; red).

899 Data information: in (C) symbols represent average and s.e.m. values of at least three
 900 independent titrations. In (D), blue symbols represent individual K_D values from n
 901 number of independent titrations, and red symbol represents average and s.e.m values
 902 from three experiments.

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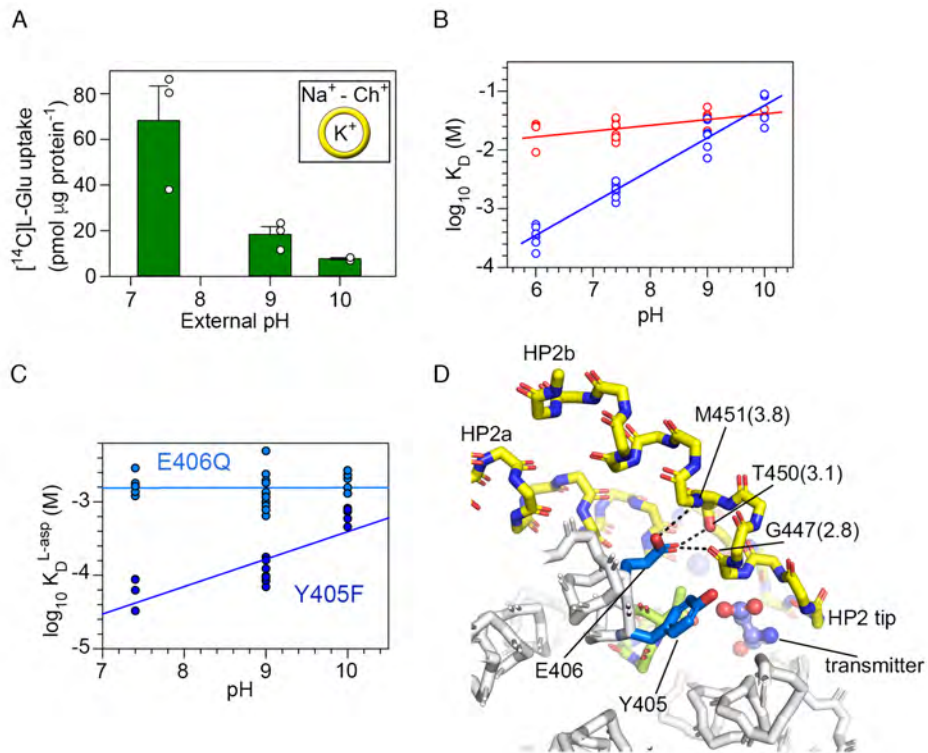
906 **Figure 2 EAAT1_{CRYST} Na⁺/transmitter bound structure**

907 A, B. Views of EAAT1_{CRYST} trimer in outward-facing Na⁺/transmitter-bound state,
908 including N-term helix TM1a'.

909 C. EAAT1_{CRYST} protomer viewed from the membrane with tranD orange, scaD teal
910 (TM4a,b omitted), and ³⁹⁸NMDG motif green. Fo-Fc Na⁺-omit map contoured at 3.5σ
911 (black mesh) around the tranD core.

912 D. Coordination details of three Na⁺-bound (blue sphere) to EAAT1_{CRYST}. Residue
913 numbering corresponds to EAAT1_{WT}, and the coordination distance (angstrom) is in
914 parenthesis.

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Figure 3 H⁺ and transmitter binding

918 A. Na⁺-induced L-glutamate uptake by purified EAAT1_{CRYST} reconstituted in
 919 liposomes loaded with K⁺. Choline (Ch⁺) condition was subtracted to the Na⁺ condition.

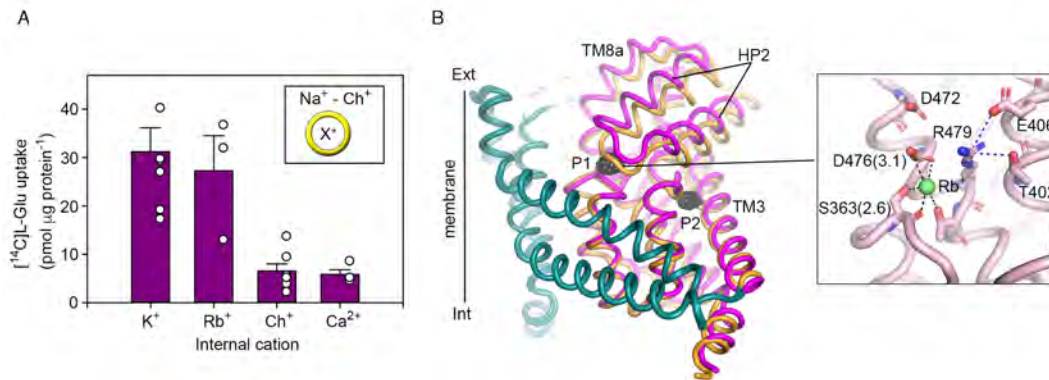
920 B. Log-log plots of Na⁺ (red, n=19) and L-asp (blue, n=27) K_D, as a function of pH at
 921 [Na⁺]=0.5 mM. Na⁺ (n=10) and L-asp (n=7) titration data at pH 7.4 and 9.0,
 922 respectively, are also reported in Fig. 1. Solid lines are fits of straight lines.

923 C. Log-log plots of L-asp K_D for EAAT1_{CRYST}-E406₃₈₆Q (light blue, n=24) and
 924 EAAT1_{CRYST}-Y405₃₈₅F (dark blue, n=18), as a function of pH at [Na⁺]=0.5 mM..

925 D. Potential H⁺-bond network between protonated E406₃₈₆ (TM7b) and residues in
 926 HP2b in EAAT1_{CRYST} transmitter-bound structure. HP2 is depicted in yellow and the
 927 ³⁹⁸NMDG motif in green. Residue numbering corresponds to EAAT1_{WT}, and the
 928 interatomic distance (angstrom) is parenthesis.

929 Data information: in (A) bar plots depict averages and s.e.m. values of three
 930 independent experiments (empty circles) performed in triplicate. In (B,C) symbols
 931 represent individual K_D values from *n* number of independent titrations.

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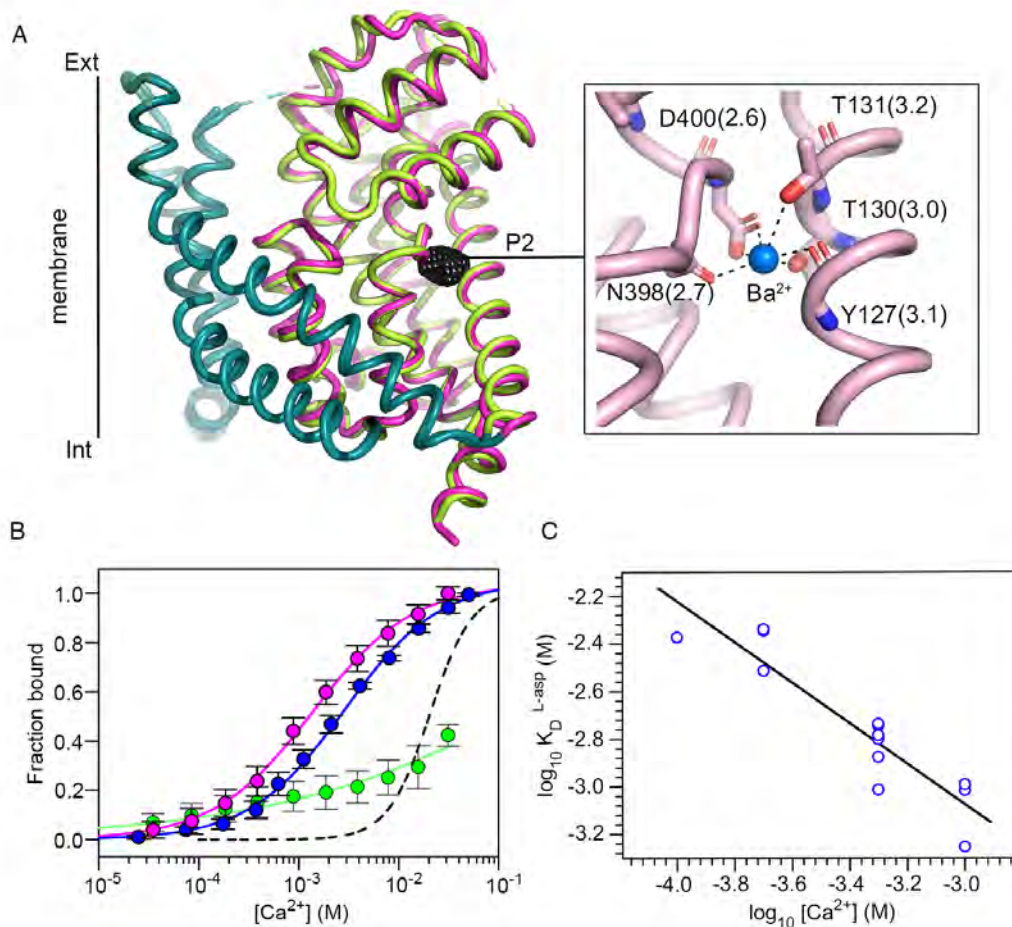
Figure 4 Counter-transported ion binding site

935 A. Purified EAAT1_{CRYST} takes up L-glutamate in liposomes loaded with K⁺ or Rb⁺, but
 936 not with choline (Ch⁺), or with Ch⁺ and Ca²⁺. Plots depict data as in Fig. 3A.

937 B. Superimposition of Na⁺/transmitter and Rb⁺ bound EAAT1_{CRYST} structures,
 938 respectively, with tranD depicted in orange (Na⁺/transmitter) and pink (Rb⁺). TM4 and
 939 UCPH₁₀₁ are omitted for clarity. Black mesh depicts anomalous-difference map
 940 contoured at 3.5σ around the tranD. Rb⁺ coordination is shown in the inset. Residue
 941 numbering corresponds to EAAT1_{WT}, and the coordination distance (angstrom) is
 942 parenthesis.

943 Data information: in (A) bar plots depict averages and s.e.m. values of three
 944 independent experiments (empty circles) performed in triplicate.

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Figure 5 Divalent cation binding

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A. Superimposition of Rb⁺/Ba²⁺ and Ba²⁺ bound EAAT1_{CRYST} structures with tranD depicted in pink and green, respectively, and TM4 removed for clarity. Black mesh depicts anomalous-difference map in the absence of Rb⁺ contoured at 4σ around the tranD, and inset shows details of Ba²⁺ coordination at Na3. Residue numbering corresponds to EAAT1_{WT}, and the coordination distance (angstrom) is parenthesis.

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B. Ca²⁺ binding isotherms of EAAT1_{CRYST} at pH=7.4 in the presence (pink) and absence (blue) of UPCH₁₀₁. Mutation D400₃₈₀N at Na3 strongly impairs Ca²⁺ binding (green). Symbols represent average and s.e.m. values of at least three independent experiments. Solid lines represent fits of Hill equation and dashed line is the fit in Fig. 1C (blue line) corresponding to Na⁺ binding.

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C. Log-log plot of L-asp K_D as a function of Ca²⁺ (n=13).

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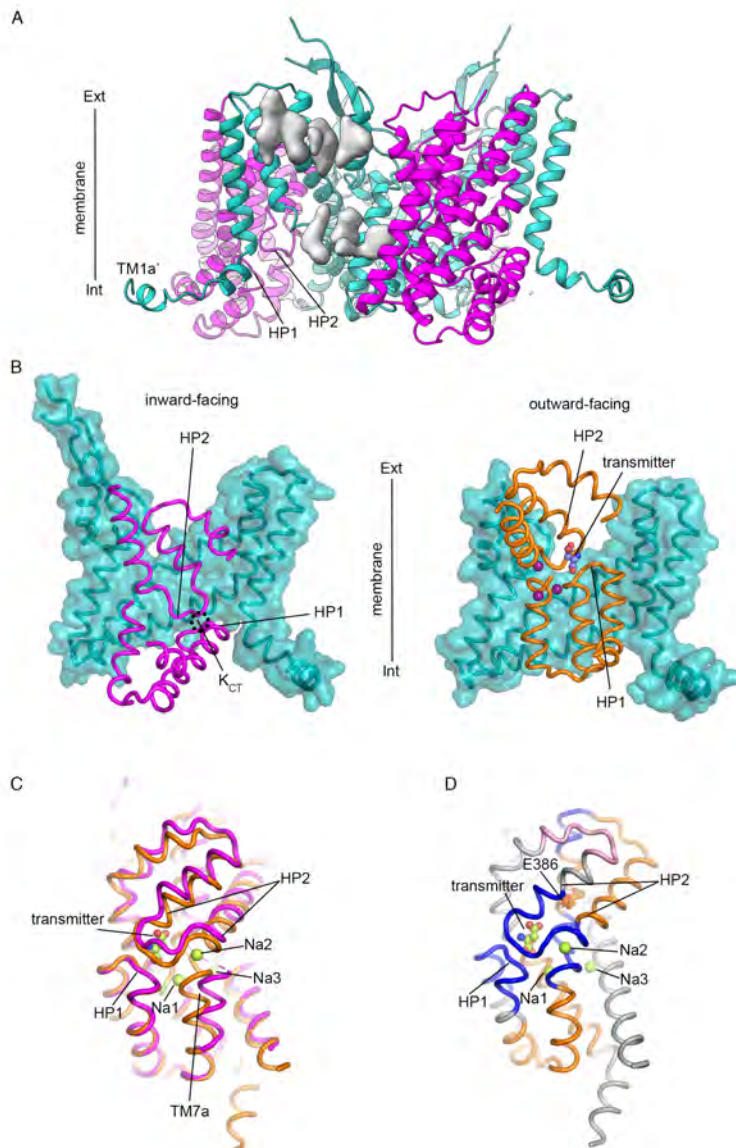
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Data information: in (B) symbols represent average and s.e.m. values of at least three independent titrations. In (C), blue symbols represent individual K_D values from *n* number of independent titrations.

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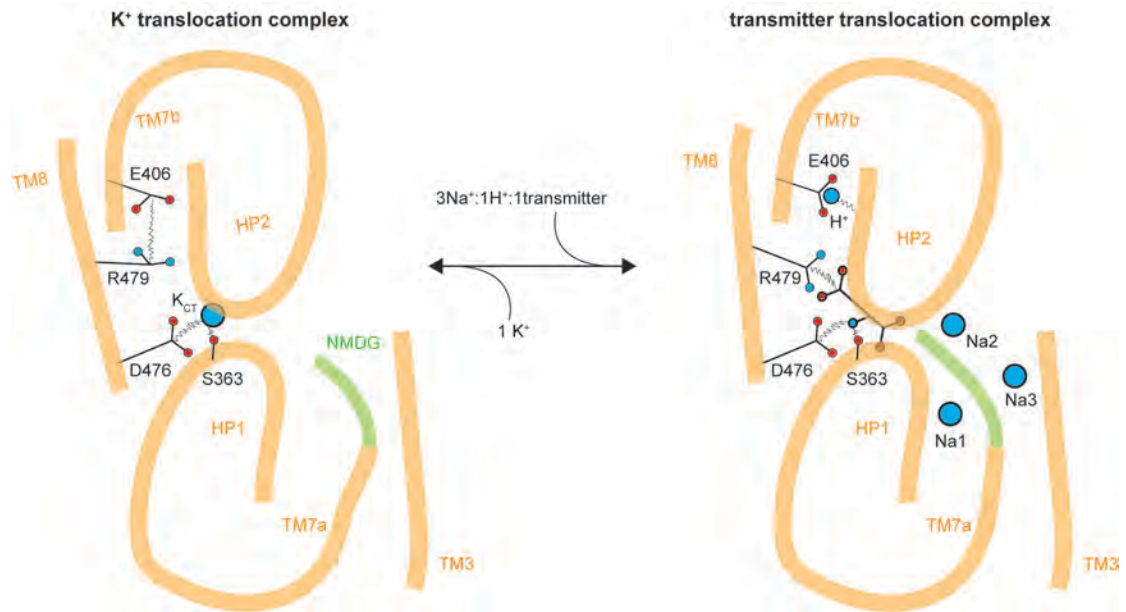
Figure 6 CryoEM structure and HDX-MS changes in K⁺ buffer

A. CryoEM structure of EAAT1_{WT} trimer with tranDs and scaDs depicted in magenta and teal, respectively. Non-protein extra density corresponding to lipid/detergent molecules is depicted in grey.

B. Comparison of EAAT1_{WT} (left) and EAAT1_{CRYST} (right) structures in inward- and outward-facing states, respectively. Several TMs were removed for clarity.

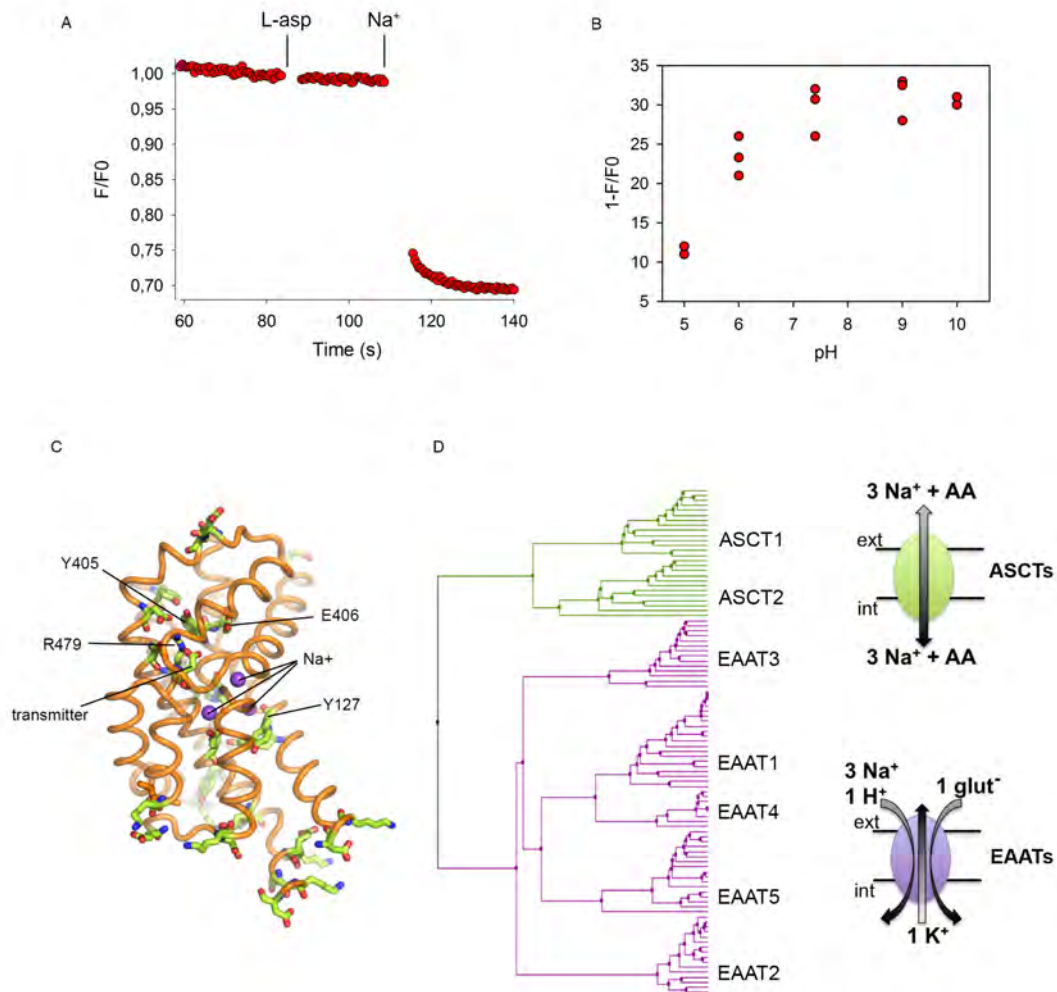
C. TranDs from EAAT1_{WT} (magenta) and EAAT1_{CRYST} (orange) structures are overlapped using HP1 as reference.

D. K⁺-induced HDX increase (blue) and decrease (pink) in EAAT1_{CRYST} is mapped on Na⁺/transmitter-bound tranD structure. Unchanged regions (orange) and those outside HDX-MS sequence coverage (grey) are also shown.



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Figure 7 EAAT1 $3Na^+/1H^+/1K^+$ coupled transport mechanism
 Cartoon representation of $Na^+/H^+/transmitter$, and K^+ translocation complexes, respectively.



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Figure EV1 Proton binding to conserved ionizable sidechain in EAAT_{CRYST}

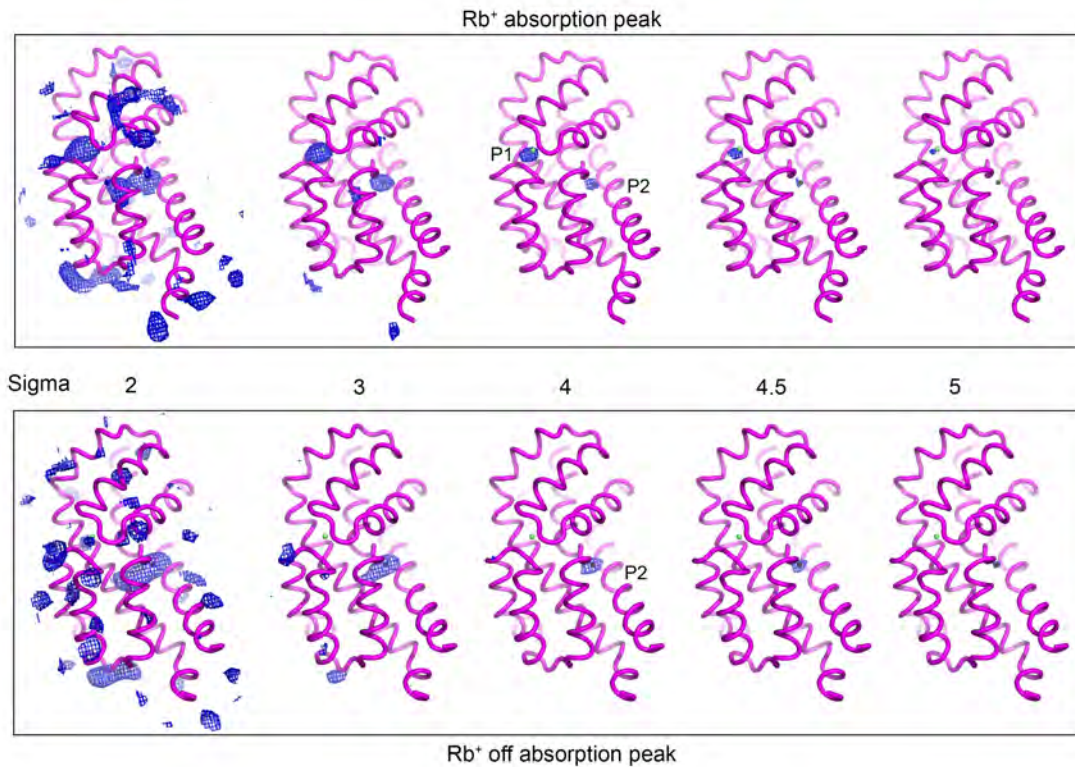
A. EAAT1_{CRYST} tryptophan-fluorescence time-course at pH 10 upon addition of saturating concentrations of L-asp, and Na⁺.

B. Percentage of L-asp/Na⁺ induced total fluorescence change (1-F/F₀) decreases at pH values below neutral.

C. Out of >30 ionizable sidechains (green sticks) in the tranD of EAAT1_{CRYST} only four (labelled with residue number) are strictly conserved in EAATs, and not in ASCTs.

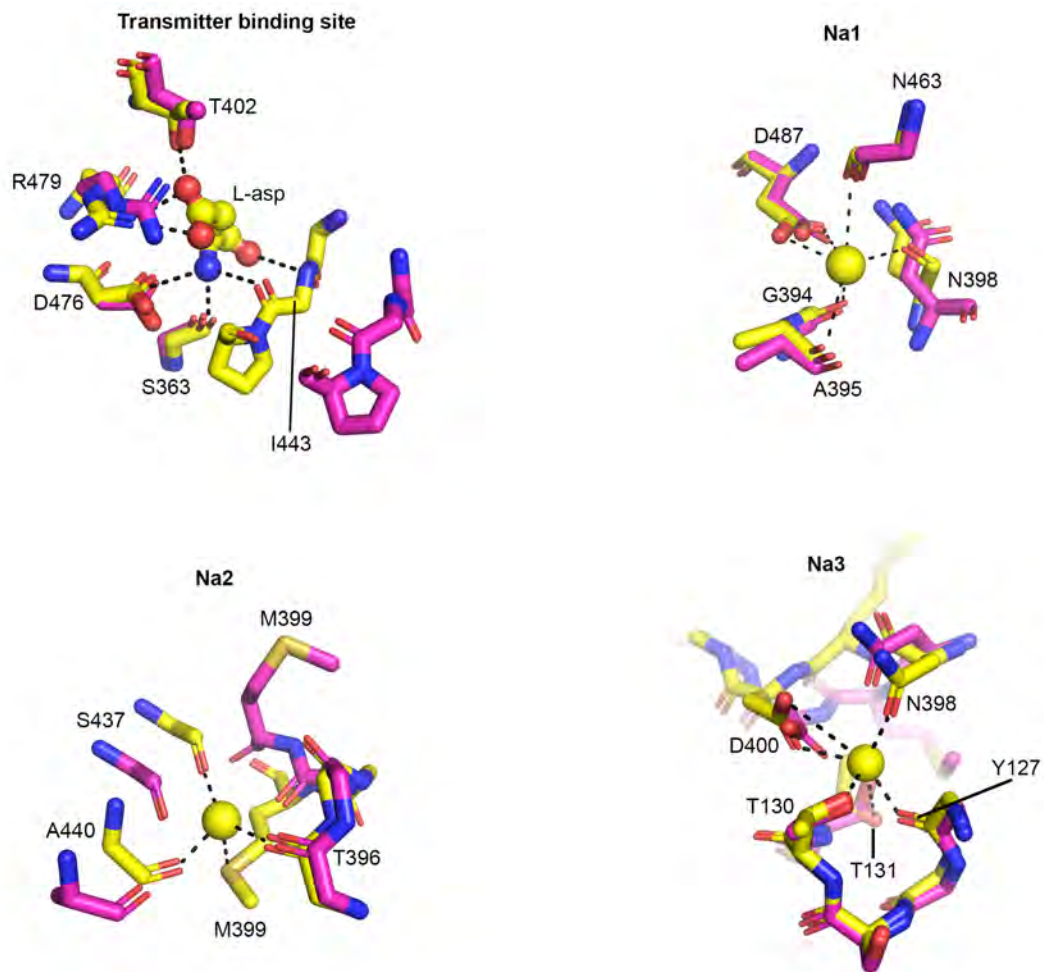
D. Phylogenetic tree from 101 tranD sequences of representative vertebrate species (Gesemann *et al.*, 2010) calculated with Jalview (BLOSUM62 average distance).

Transport stoichiometry of EAATs and ASCTs vertebrate proteins is depicted in cartoon representation.



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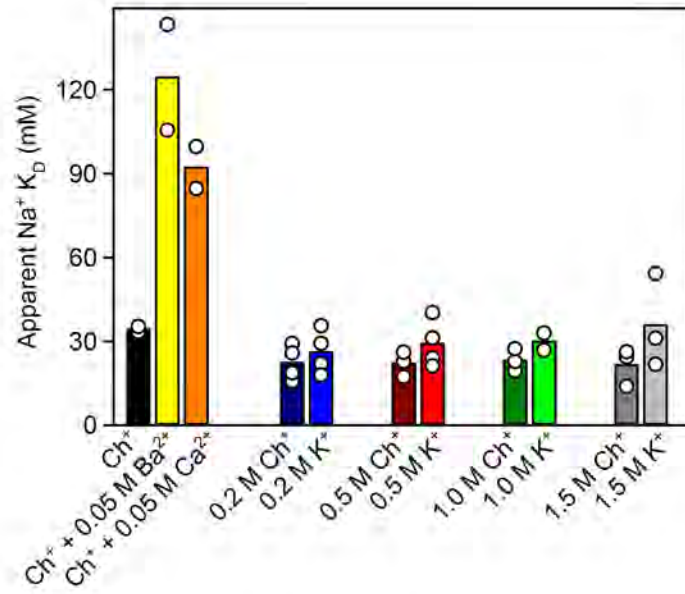
Figure EV2 Anomalous difference maps of Rb⁺/Ba²⁺-bound structure. Comparison of anomalous difference maps of Rb⁺/Ba²⁺-bound EAAT_{CRYST} structure at the Rb⁺ absorption maximum (0.815 Å), and that of EAAT_{CRYST-II} off the Rb⁺ absorption maximum (0.998 Å). Maps are contoured at increasing sigma levels around the tranD. Rb⁺ (green sphere), and Ba²⁺ (black sphere) bound to the transporters are depicted.



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Figure EV3 Transmitter and sodium binding sites in Na^+ /transmitter- and Rb^+ -bound structures

Conformational re-arrangements around the transmitter and three Na^+ binding sites in Na^+ /transmitter- (yellow) and Rb^+ -bound (pink) EAAT_{CRYST} structures.



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1013 **Figure EV4 Na^+ titrations in different ionic conditions**

1014 EAAT1_{CRYST} apparent $\text{Na}^+ K_D$ significantly increased upon addition of 50 mM Ba^{2+}

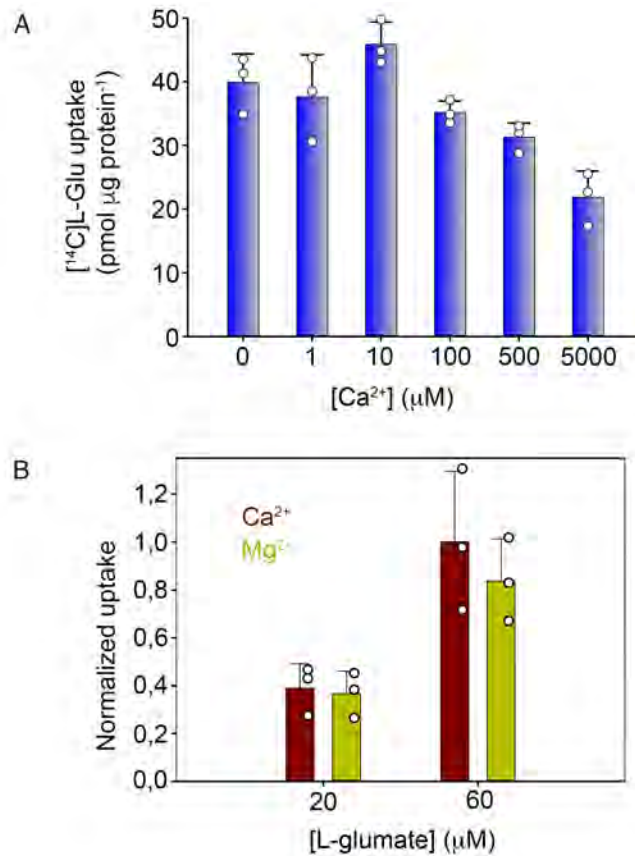
1015 or Ca^{2+} to a choline (Ch^+)-based cuvette buffer, but were not modified when Ch^+ in

1016 the buffer was substituted for K^+ at concentrations up to 1.5 M. Bar plots present

1017 average of at least two independent experiments (empty circles).

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1021 **Figure EV5 Ca²⁺ effect on steady-state transmitter transport**

1022 A. Effect of intra-liposomal Ca²⁺ on L-glutamate transport in the presence of opposite
1023 gradients of Na⁺ and K⁺.

1024 B. Effect of 5mM extracellular Ca²⁺ on L-glutamate transport in cells expressing
1025 EAAT1_{CRYST}. There was not significant change in Na⁺-induced L-glutamate uptake
1026 upon substitution of extracellular Ca²⁺ for Mg²⁺, a divalent cation that does not induce
1027 changes in Trp fluorescence or compete for Na⁺ (not shown).

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