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# 3 Structure and allosteric inhibition mechanism of 4 excitatory amino acid transporter 1

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#### 22

#### 23 Abstract

24 Human members of the solute carrier 1 (SLC1) family of transporters take up 25 excitatory neurotransmitters in the brain and amino acids in peripheral organs. 26 Dysregulation of their functions is associated to neurodegenerative disorders and 27 cancer. Here we present the first crystal structures of a thermostabilized human SLC1 28 transporter, the excitatory amino acid transporter 1 (EAAT1), with and without 29 allosteric and competitive inhibitors bound. The structures show novel architectural 30 features of the human transporters, including intra- and extracellular domains with 31 potential roles in transport function, as well as regulation by lipids and post-32 translational modifications. The coordination of the inhibitor in the structures and the 33 change in the transporter dynamics measured by hydrogen-deuterium exchange mass 34 spectrometry, reveal an allosteric mechanism of inhibition, whereby the transporter is 35 locked in the outward-facing states of the transport cycle. Our results provide 36 unprecedented insights into the molecular mechanisms of function and pharmacology 37 of human SLC1 transporters.

39	SLC1 transporters constitute a large family of ion-coupled amino acid
40	transporters present in all kingdoms of life <sup>1</sup> . In humans, there are seven SLC1
41	transporters that share 40-70% amino acid identity (Extended Data Fig. 1) and have
42	evolved to serve two specialized functions <sup>2</sup> : in the central nervous system, SLC1
43	excitatory amino acid transporters (EAAT1-5) take up the neurotransmitter glutamate
44	into the cell. In peripheral organs, EAATs take up glutamate and aspartate, while
45	neutral amino acid transporters (ASCT1-2) exchange small amino acids between the
46	extra- and intracellular compartments, contributing to the cellular solute homeostasis.
47	Glutamate is the most important excitatory transmitter in the mammalian brain
48	and is involved in most aspects of brain physiology, from development to cognition <sup>3</sup> .
49	Notably, most of the glutamate in the brain is intracellular, and it has to be
50	continuously pumped into the cytoplasm to allow for rounds of transmission and
51	prevent cytotoxicity. This essential neurological function is done by the EAAT1-5
52	isoforms expressed at the plasma membrane of astrocytes and neurons. In particular,
53	astroglial EAAT1 and EAAT2 orthologs are highly expressed in the hind- and
54	forebrain, respectively, and are responsible for most of the glutamate uptake in the
55	rodent brain <sup>4</sup> . EAATs are powerful molecular pumps capable of maintaining up to

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10<sup>4</sup>-fold glutamate gradients by using energy stored in sodium, proton and potassium

gradients<sup>5</sup>. Remarkably, their dysregulation has been associated with several

neurological diseases, including amyotrophic lateral sclerosis<sup>6</sup>, ataxia<sup>7,8</sup>, stroke<sup>9</sup>,

depression<sup>10</sup> and glioma<sup>11</sup>, making them important drug targets. Moreover, they are

also expressed in intestine and kidney, where mutations in EAAT3 have been

associated to dicarboxylic aminoaciduria<sup>12</sup>.

ASCTs are structurally similar to EAATs, and function as sodium-dependent neutral amino acid exchangers at the plasma membrane<sup>13</sup>. They are highly expressed in intestine, kidney and testis, where they play a key role in maintaining the amino acid cellular homeostasis. Importantly, ASCT2 is up-regulated in several forms of cancer, including melanoma<sup>14</sup>, lung<sup>15</sup>, prostate<sup>16</sup> and breast cancer<sup>17</sup>, and it is a key drug target for the treatment and diagnosis of these diseases.

68 Despite the need for small compounds that selectively and allosterically 69 modulate SLC1 human transporters, most of their pharmacology is based on substrate-analogs that inhibit transport with rather low selectivity among EAAT<sup>18</sup> and 70 ASCT<sup>19</sup> isoforms, respectively. Notably, the only known selective allosteric 71 72 modulators of SLC1 transporters are a series of non-competitive EAAT1-selective 73 inhibitors. of which 2-Amino-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-74 5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (UCPH<sub>101</sub>) is the best studied<sup>20,21</sup>. 75 However, its mechanism of action is still poorly understood at the molecular level.

76 In structural terms, most our knowledge of the molecular mechanism of 77 transport and pharmacology of SLC1 transporters comes from the prokaryotic 78 homolog GltPh that has been crystallized in the main conformational states of the transport cycle, outward-<sup>22</sup> and inward-facing states<sup>23,24</sup>, as well as in complex with a 79 and competitive inhibitor of the EAATs<sup>25</sup>, DL-threo-β-80 non-selective 81 benzyloxyaspartic acid (TBOA). However, the presence of amino acid insertions and 82 deletions, as well as important differences in the transport function and pharmacology 83 of GltPh, make this homolog a limited structural model to understand the molecular 84 mechanism of the human SLC1 proteins.

Here we present 3.1-3.3 Å X-ray crystal structures of thermostable EAAT1 variants in complex with a substrate (L-aspartate), and the allosteric inhibitor

 $UCPH_{101}$ . The structures, and supporting functional data, show new architectural features of the EAATs and ASCTs, and unravel the allosteric mechanism of UCPH<sub>101</sub>-like inhibitors in atomic detail. Taken together, these structural data can prove useful for the design of novel allosteric compounds with improved selectivity for both EAATs and ASCTs.

#### 92 EAAT1 engineering and crystallization

93 Purified wild-type EAAT1 shows a poly-disperse size-exclusion 94 chromatogram in detergent solutions, and lacks transport activity upon reconstitution 95 in synthetic liposomes (Fig. 1a). To confer stability to EAAT1, we introduced consensus mutations<sup>26</sup> in the predicted transmembrane helices, and obtained a 96 97 biochemically stable and functional transporter (Extended Data Fig. 1). As the protein 98 was still refractory to crystallization, we hypothesized that the extracellular region of 99 the transporter between the predicted transmembrane helix 3 (TM3) and the 100 cytoplasmic half of TM4 (TM4c) could preclude crystal growth due to the presence of 101 a long amino acid insertion compared to GltPh. We therefore engineered a chimeric 102 transporter substituting this region with the corresponding amino acid sequence from 103 ASCT2, which is the shortest in this region among human SLC1 transporters 104 (Extended Data Fig. 1). The resulting transporter, called EAAT1<sub>crvst</sub>, shares an overall 105  $\sim$ 75% sequence identity with wild type EAAT1, and up to  $\sim$ 90% identity at the C-106 terminal core of the protein, where the transported substrate and coupled ions are expected to bind<sup>25,27-31</sup>. Importantly, purified EAAT1<sub>crvst</sub> reconstituted in liposomes 107 108 showed robust glutamate uptake that depended on opposite gradients of sodium and 109 potassium ions across the bilayer (Fig. 1a and Extended Data Fig. 2a), and was 110 inhibited by the EAAT1-selective compound UCPH<sub>101</sub> in a concentration-dependent 111 fashion (IC<sub>50</sub> of  $4.5\pm0.3 \mu$ M, Hill coefficient 0.92±0.07) (Fig. 1b). These data show

that the transport mechanism and pharmacological selectivity are conserved in
EAAT1<sub>crvst</sub>.

114 Notably, EAAT1<sub>crvst</sub> formed crystals in the presence of UCPH<sub>101</sub> that diffract 115 X-rays anisotropically and up to 3.25 Å resolution, and we solved its inhibitor-bound 116 structure by molecular replacement (see Methods and Extended Data Table 1). As 117 EAAT1<sub>crvst</sub> was refractory to crystallization in the absence of UCPH<sub>101</sub>, we reasoned 118 that mutations in the inhibitor-binding pocket could aid with the crystallization of the 119 transporter. A construct carrying M231I and F235I mutations (EAAT1<sub>cryst-II</sub>; Extended 120 Data Fig. 1), crystallized both in the presence and absence of the inhibitor, and 121 diffracted X-rays up to 3.1 and 3.32 Å resolution, respectively (Extended Data Table 122 1). Remarkably, purified EAAT1<sub>cryst-II</sub> reconstituted in liposomes also showed robust 123 sodium- and potassium-dependent glutamate uptake, while the UCPH<sub>101</sub>  $IC_{50}$ 124 increased >30-fold (>131 $\pm$ 38  $\mu$ M, Hill coefficient 0.92 $\pm$ 0.0; Fig. 1a,b), as expected 125 for mutations in the binding pocket of the inhibitor (see below).

#### **Domain organization**

127 The structure of EAAT1<sub>cryst</sub> shows a symmetric homotrimer in a substrate- and 128 UCPH<sub>101</sub>-bound outward-facing conformation (Fig. 1c-e). EAAT1<sub>cryst</sub> adopts an overall GltPh-like fold<sup>22,23</sup> (Extended Data Fig. 3), in which each monomer is 129 130 composed of two domains: a trimerization or scaffold domain (ScaD), including 131 TM1-2 and TM4-5; and a transport domain (TranD), including TM3, TM6-8 and re-132 entrant helical loops 1-2 (HP1-2; Fig. 1f). The ScaD forms all inter-subunit contacts through residues in TM2, TM4 and TM5, that include six salt bridges and bury 133  $\sim$ 3,000 Å<sup>2</sup> from each subunit (Extended Data Fig. 4). Hence, the three ScaDs form a 134 135 compact central structure with a propeller-like shape that ensures the trimeric form of 136 the transporter and anchors it to the membrane.

The three TranDs are more peripheral and localize between the blades of the propeller, making protein contacts exclusively with the ScaDs of their own monomer. The TranD-ScaD interface buries  $\sim$ 3,500 Å<sup>2</sup>, including a conserved salt bridge between E256 and K364. This interface is mainly formed by cytoplasmic residues in HP1, TM7, and TM3 (TranD), and TM2, TM4c, and TM5 (ScaD). However, on the extracellular side additional contacts occurred between HP2 and TM4, through residues that are well conserved among human transporters (Extended Data Fig. 5).

Substrate and ion translocation in SLC1 transporters is thought to occur through large rigid-body movements of the TranD, relative to the static ScaD, that move the cargo in an elevator-like fashion across the membrane<sup>23,32</sup>. Thus, during the isomerization to the inward-facing state the TranD-ScaD interface changes drastically on the TranD side, and the novel features observed at this interface in EAAT1<sub>cryst</sub> might influence the distinct TranD dynamics in human SLC1 proteins.

#### **150 Transport domain**

151 One of the most remarkable architectural features of the EAAT1<sub>cryst</sub> TranD is 152 at the TM8 level, in which deletions and insertions compared to GltPh reshape this 153 helix and its interactions with neighboring structural elements important for transport (Fig. 2 and Extended Data Fig. 3). In EAAT1<sub>crvst</sub>, TM8 can be divided into 154 155 extracellular (TM8a), transmembrane (TM8b), and cytoplasmic (TM8c) helices. The 156 loop connecting TM8a and the C-terminal helix of HP2 (HP2b) is six residues shorter 157 in human SLC1 transporters. Consequently, the extracellular ends of TM8a and HP2b 158 are in close proximity and engage in hydrogen bonding and hydrophobic interactions 159 (Fig. 2, upper right inset). HP2 is a key component of the gating machinery that controls the access of substrate and ions to their binding sites in the TranD<sup>25,33-35</sup>, and 160 161 its interactions with TM8b likely play an important role in determining HP2

movements. Notably, several single-cysteine mutations at positions along TM8a in
 EAAT1<sup>36</sup>, and in a rodent EAAT2 ortholog<sup>37</sup> impaired glutamate transport,
 highlighting the significance of this extracellular region for function.

At TM8b level, we found strong electron density for the substrate (L-165 166 aspartate) and one of the sodium-binding sites previously observed in the archeal 167 homologs of SLC1 family (Na2) (Fig. 2), which we modeled with similar coordination than in their structures<sup>25,31</sup>. Remarkably, the carboxylate group of D456 168 169 (TM8b), which coordinates the  $\alpha$ -amino group of the substrate, is also at hydrogen 170 bond distance with the hydroxyl group of S343 (HP1). Moreover, the guanidinium 171 group of R457 (TM8b) engages in hydrogen bonding with HP1 residue G341, and 172 possibly L340 and T342 that point their backbone carbonyl oxygen atoms towards 173 TM8b. Residues S343 and R457 are well conserved in human SLC1 transporters, and substitutions at equivalent positions in EAAT1 (S363 and R477)<sup>38</sup>, and EAAT3 174 (R445)<sup>39</sup> inactivate transport. In addition, the loss-of-function mutation R445W in 175 EAAT3, equivalent to R457 in EAAT1<sub>cryst</sub>, causes human dicarboxylic aminoaciduria 176 due to the lack of aspartate/glutamate reabsorption function in the kidney<sup>12</sup>. Overall, 177 178 the functional studies and our structural data converge to suggest that interactions 179 between conserved human residues at HP1 and TM8b are important to the correct 180 folding and function of the transporters.

On the cytoplasmic side, hydrophilic TM8c extends beyond the membrane plane through a hydrophilic helix (TM8c), and makes contact with residues in TM3 and TM7a (Fig. 2, lower right inset). Notably, EAAT2 deletion mutants in this region have a deleterious effect on transport function and membrane trafficking<sup>40</sup>. Accordingly, a deletion of TM8c beyond E501 in EAAT1 (equivalent to E500 in EAAT2 and E481 in EAAT1<sub>cryst</sub>) decreased glutamate uptake rate by ~2-fold 187 (Extended Data Fig. 2b). The functional data, and the amino acid conservation in
188 TM8c among EAATs, underscore the pivotal role of this structural motif in protein
189 folding and transport kinetics.

190 Scaffold domain

191 The ScaD is less conserved than the TranD in the SLC1 family. In particular, 192 TM4 is highly divergent (Extended Data Fig. 1), and shows several unique 193 architectural features in EAAT1<sub>cryst</sub>. On the extracellular side, TM4a forms inter- and 194 intra-monomeric contacts with TM2 and HP2, respectively. Moreover, an amino acid 195 insertion between TM4b and TM4c (TM4b-c loop) that appeared during the evolution 196 of eukaryotic transporters protrudes into the central vestibule of the EAAT1<sub>crvst</sub> trimer 197 (Fig. 3a and Extended Data Fig. 4). The TM4b-c loop forms the center of the 198 propeller, and makes extensive contacts within and between protomers. Due to the 199 lack of electron density it was not possible to model the outermost residues of the 200 TM4b-c loop (Y200-V210), but they are expected to reach out to the bulk solvent, 201 and expose one of the predicted N-glycosylation sites of the transporter (N204). 202 Notably, despite the lack of sequence identity among human SLC1 transporters, all of 203 them contain predicted N-glycosylation sites in the TM4b-c loop, suggesting a 204 common role of this loop in the posttranslational processing of these proteins.

An additional novel feature of the  $EAAT1_{cryst}$  ScaD architecture is the Nterminal extension of TM1 by an amphipathic helix (TM1a). Positioned nearly parallel to the membrane plane, TM1a forms the tips of the blades in the propeller (Fig. 1c and Fig. 3b). Remarkably, it does not form inter- or intra-monomeric contacts, and its position and amphipathic nature suggest that TM1a somehow interacts with the inner leaflet of the membrane. Indeed, there is a hydrophobic crevice between TM1a and HP1a from the same monomer, where we observe strong 212 non-protein electron density in  $EAAT1_{cryst}$  that likely corresponds to bound detergent 213 or lipid molecules (Fig. 3b). Interestingly, a second hydrophobic crevice is observed 214 between the extracellular part of TM4 and HP2, where there is also strong non-protein 215 electron density (Fig. 3a). A similar crevice was also noted in a substrate-bound 216 structure of GltPh<sup>22</sup>.

The lipidic composition of the bilayer regulates the function of SLC1 transporters<sup>41-45</sup>. Because TM1a-HP1a and TM4-HP2 hydrophobic crevices are at the interface between the TranD and ScaD, where large conformational changes are expected to occur during substrate translocation, they might constitute sites for lipid regulation of transport function.

222 UCPH<sub>101</sub> binding site

The structure of  $EAAT1_{cryst}$  showed strong electron density for UCPH<sub>101</sub> in a hydrophobic pocket facing the inner leaflet of the membrane on the interface between the TranD and ScaD (Fig. 1d,e and Fig. 4a,b). This pocket is formed by residues in TM3, TM7 and TM4c, and extends the TranD-ScaD interface by ~ 500 Å<sup>2</sup>.

The chromene skeleton of UCPH101, the parental group of the UCPH series of 227 228 compounds, is buried deeply in the domain interface, and coordinated by a direct ring-229 stacking interaction with F369 (TM7a), as well as hydrophobic interactions with 230 G120 (TM3), V373 (TM7a) and M231 (TM4c) (Fig. 4b). In addition, the amine group 231 of UCPH<sub>101</sub> forms a hydrogen bond with the main-chain carbonyl of F369, while its 232 carbonitrile group interacts with Y127 (TM3). The methoxy-phenyl and naphthalene 233 groups are more peripheral and partly facing the hydrocarbon core of the membrane. 234 Yet, the former establishes hydrophobic interactions with V124 (TM3), V373 and 235 M231, while the latter is mainly coordinated by F235 (TM4c). The majority of the 236 above-mentioned residues have been reported to be important for the inhibition of an

EAAT1 rodent ortholog by UCPH<sub>101</sub> in cell  $assays^{21}$ . Furthermore, the EAAT1<sub>cryst</sub> double mutant M231I-F235I (EAAT1<sub>cryst-II</sub>) showed >30-fold increase in UCPH<sub>101</sub> IC<sub>50</sub> compared to EAAT1<sub>cryst</sub> in proteo-liposomes (Fig. 1b). Hence, there is an excellent agreement between the crystallographic and functional data.

241 Several mechanistically-relevant observations can be made regarding the UCPH<sub>101</sub> binding pocket in EAAT1<sub>cryst</sub>: i) it is over 15 Å away from the substrate and 242 243 sodium binding sites, suggesting that UCPH<sub>101</sub> does not preclude substrate binding, as 244 expected for a non-competitive allosteric inhibitor; ii) it faces the inner leaflet of the 245 membrane, implying that UCPH<sub>101</sub> accesses its binding site from the lipidic, and not 246 the aqueous phase, when applied extracellularly; iii) it is fully contained in a single 247 subunit, in agreement with the lack of cooperativity observed in proteo-liposome (Fig. 1b), and cell assays<sup>21</sup>; iv) a comparison of the EAAT1-5 sequences suggests that the 248 249 main determinants of UCPH<sub>101</sub> selectivity for EAAT1 are in TM4c, where M231 and 250 F235 are the only coordinating residues that differ between EAAT1 and all other 251 EAATs (Extended Data Fig. 1). Consistently, the equivalent residues in EAAT2 are 252 isoleucine, and the double mutant EAAT1<sub>cryst-II</sub>, containing M231I and F235I, shows a 253 >30-fold increase in the UCPH<sub>101</sub> IC<sub>50</sub> compared to EAAT1<sub>cryst</sub>.

#### 254 UCPH<sub>101</sub>-unbound state

To better understand the conformational changes of the transporter induced by UCPH<sub>101</sub>, we set out to determine the structure of the UCPH<sub>101</sub>-unbound state. As mentioned above, EAAT1<sub>cryst</sub> was refractory to crystallization in the absence of the compound and instead, we solved the structure of EAAT1<sub>cryst-II</sub> UCPH<sub>101</sub>-unbound state. For comparison, we determined the structure of the EAAT1<sub>cryst-II</sub> UCPH<sub>101</sub>bound state, using a large excess of the compound in the crystallization conditions (see Methods).

262 The structure of EAAT1<sub>cryst-II</sub> in the UCPH<sub>101</sub>-bound state is nearly identical to 263 that of the EAAT1<sub>cryst</sub> with the exception of the mutated I231 and I235 side chains, 264 and a  $\sim 2$  Å movement of UCPH<sub>101</sub> methoxy-phenyl and naphthalene groups away 265 from them (Fig. 4c and Extended Data Fig. 6a). Interestingly, the EAAT1<sub>crvst-II</sub> 266 UCPH<sub>101</sub>-unbound structure has an overall similar conformation to the UCPH<sub>101</sub>-267 bound state, but shows notable differences. First, the UCPH<sub>101</sub> binding pocket 268 contains no excess electron density, and the side chain of F369 moves outward by as 269 much as 1.9 Å, partly occupying the volume for UCPH<sub>101</sub> chromene group (Fig. 4c). 270 Second, there is a small rigid-body movement of the entire TranD that is shifted by as 271 much as 0.7 Å, compared to the UCPH<sub>101</sub>-bound structures (Extended Data Fig. 6b). 272 Interestingly, this conformational change shows the EAAT1<sub>cryst</sub> TranD is able to 273 undergo rigid-body movements relative to the ScaD, and highlights the importance of 274 such movements for the function of the human transporters, as it has been shown for the prokaryotic homolog $^{23,24}$ . 275

The structural changes observed in the  $UCPH_{101}$ -unbound structure unambiguously demonstrate that the assigned binding pocket of  $UCPH_{101}$  is correct, and that within the restricted environment of the crystal lattice,  $UCPH_{101}$  induces both local and global conformational changes of the transporter that optimize its coordination in an outward-facing state.

#### 281 Transport domain dynamics

The coordination of  $UCPH_{101}$  in the crystal structures, wedged between the TranD (TM3 and TM7a) and the ScaD (TM4c), as well as the effect of the M231I-F235I mutations on the  $UCPH_{101}$  potency strongly suggest that  $UCPH_{101}$  inhibits transport by trapping the transporter in an outward-facing state. Consistently, the rigid-body movements of the TranD to isomerize into the inward-facing state would separate the coordinating residues in the TranD from those in the ScaD, and disrupt the UCPH<sub>101</sub> coordination. Hence, under equilibrium conditions where the transporters are sampling outward- and inward-facing states, the expected effect of UCPH<sub>101</sub> binding is to shift the equilibrium in favor of the outward-facing state.

To gain insights into the effects of  $UCPH_{101}$  binding to the transporters at equilibrium, we probed the detergent solubilized  $EAAT1_{cryst}$  by hydrogen-deuterium exchange mass spectrometry (HDX-MS). HDX-MS measures the rate of exchange of backbone amide hydrogen atoms that depends on solvent accessibility and hydrogen bonding, and provides valuable information on the dynamics and conformational changes of proteins<sup>46,47</sup>.

We compared the HDX behavior of the  $EAAT1_{cryst}$  in the presence and absence of UCPH<sub>101</sub>. Overall, the deuterium uptake pattern of  $EAAT1_{cryst}$  shows dynamic structural elements in both the TranD and ScaD (Extended Data Fig. 7-9), and reveals the unstructured and solvent-exposed nature of several regions that were not resolved in the crystal structures, including the TM3-TM4a (peptide 153-173) and TM4b-c (peptide 200-208) loops, as well as the N- (peptide 1-28) and C- termini (peptides 490-522) (Extended Data Fig. 10).

304 Binding of UCPH<sub>101</sub> significantly decreased deuterium uptake in several areas 305 of the TranD including its binding pocket (residues 112-123 and 370-374), and the 306 surrounding area (residues 354-369), while it left the uptake in the ScaD unchanged 307 (Fig. 5a). It also decreased deuterium uptake in distant residues (336-349 and 420-308 430) at the tips of HP1 and HP2 involved in substrate coordination and occlusion, 309 suggesting that UCPH<sub>101</sub> induces conformational changes in the transporter upon 310 binding. To gain insights into the nature of those conformational changes, we first 311 compared the TranD areas in which UCPH<sub>101</sub> decreased uptake with those buried at

312 the interface with ScaD in the EAAT1<sub>cryst</sub> structure, and found that they correlate 313 remarkably well (Fig. 5a-c). Second, we built a model of the EAAT1<sub>crvst</sub> inwardfacing state, based on a recently solved structure of GltPh<sup>24</sup>, to assess the changes in 314 315 solvent accessibility in a possible transition between inward- and outward-facing 316 states (Fig. 5c,d). Indeed, the comparison between the structure and the model shows 317 that the UCPH<sub>101</sub>-modified areas detected by HDX-MS transit as rigid bodies from 318 being solvent-exposed, in the inward-facing state, to buried at the TranD-ScaD 319 interface, in the outward-facing state. Such conformational change is expected to 320 decrease the dynamics of alpha helices and/or the solvent accessibility of the loops in 321 the UCPH<sub>101</sub>-modified areas and thus, is consistent with the observed decrease in 322 deuterium uptake. Overall, the HDX-MS and structural analysis support the 323 stabilization of the outward-facing state, at the expense of the inward-facing state(s), 324 induced by UCPH<sub>101</sub>.

#### 325 UCPH<sub>101</sub>- and TBOA<sub>TFB</sub>-bound state

The distant position of  $UCPH_{101}$  from the substrate and the HP2, a structural element that controls extracellular access to the binding site<sup>25</sup>, suggests that the UCPH<sub>101</sub>-bound transporters could undergo the conformational changes required to exchange the substrate with the extracellular solution. To test this, we solved the crystal structure of EAAT1<sub>cryst</sub> in complex with both UCPH<sub>101</sub> and (2*S*,3*S*)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate (TBOA<sub>TFB</sub>), a potent and nonselective TBOA derivative<sup>48</sup>, at 3.7 Å resolution (Extended Data Table 1).

Overall, the UCPH<sub>101</sub>-TBOA<sub>TFB</sub> bound structure is similar to that of the UCPH<sub>101</sub>bound state, with the exception of HP2 that adopts an "open" conformation and packs against the TM4b-c loop, disrupting the coordination of the Na2 (Fig. 6a,b). These conformational changes resemble those previously observed in the structure of the GltPh-TBOA complex, and are in excellent agreement with the proposed competitive
 inhibitory mechanism of TBOA-like compounds<sup>25</sup>.

339 In the substrate-binding site, we observed excess electron density for the bulky 340 TBOA<sub>TFB</sub> (Fig. 6c), but due to lack of resolution, we were not able to unambiguously 341 orient the compound. In order to fit the TBOA<sub>TFB</sub> molecule into the density, we 342 initially positioned the TBOA moiety of TBOA<sub>TFB</sub> using the TBOA-bound GltPh 343 structure as a guide. In this position, it remained stable during several successive 344 cycles of refinement that yielded a reasonable fit into the electron density. The 345 additional benzoylamino and trifluoromethyl groups of  $TBOA_{TFB}$  localized in a 346 hydrophobic cavity mainly formed by residues in HP1b and TM7a, and possibly by 347 residues in TM2 and TM4c. Interestingly, the interactions of these groups with the 348 transporter could explain the  $\sim$ 1500-fold increase in inhibitory potency of TBOA<sub>TFB</sub>, 349 compared to TBOA, that has been observed in EAAT1 transport assays<sup>48</sup>.

Indeed, we also observed  $UCPH_{101}$  bound in this structure with an identical coordination than in the substrate-bound state. Therefore, despite the lower resolution of the TBOA<sub>TFB</sub> bound structure, it shows that  $UCPH_{101}$  binding at its allosteric site does not preclude the movements of HP2 involved in substrate and sodium binding from the extracellular solution.

#### 355 Inhibitory mechanisms of EAAT1

The structures of  $EAAT1_{cryst}$  reveal new architectural features of human SLC1 transporters, and the first non-competitive inhibitory mechanism of this family of proteins in molecular detail. UCPH<sub>101</sub> is an EAAT1-selective inhibitor with a bipartite coordination by residues in both the TranD and ScaD, and the downward rigid-body movements of the TranD during transport disrupt such coordination. This implies that upon binding, UCPH<sub>101</sub> "glues" the TranD to the ScaD in the outward-facing states, and precludes the translocation reaction of the transport cycle, but not the substratebinding/unbinding reactions from the extracellular solution (Fig. 7).

The inhibitory mechanism of UCPH<sub>101</sub> contrasts with that of substrate-analog competitive inhibitors like TBOA. The binding pocket of TBOA-like compounds overlap to some extent with that of the substrate<sup>25</sup>, and some of these molecules can bind the transporter from both the extra- and intracellular aqueous solutions<sup>49,50</sup>. Therefore, they inhibit transport by precluding substrate binding on either side of the membrane. Moreover, TBOA-like compounds are not selective among glutamate transporters due to the high amino acid conservation in the substrate-binding sites.

The mechanistic differences observed in  $UCPH_{101}$  over other known inhibitors make it an extremely valuable pharmacological tool, to isolate and study the conformational changes that EAAT1 undergoes upon substrate and ion binding. Remarkably, the  $UCPH_{101}$  allosteric binding site observed in EAAT1<sub>cryst</sub> highlights a cavity that can facilitate the design of selective compounds for other human SLC1 transporters, and possibly the long-sought positive modulators of glutamate uptake.

377

#### 379 **METHODS**

#### 380 **Construct optimization**

We used fluorescence-detection size-exclusion chromatography (FSEC)<sup>51</sup> to 381 382 screen solubilization conditions and EAAT1 variants fused to enhanced green 383 fluorescent protein (eGFP). EAAT1 N-terminal fusions solubilized in dodecanoyl 384 sucrose (DDS, Anatrace) were found to have good solubility and mono-dispersity by 385 FSEC in clear lysates. However, EAAT1 looses its transport activity and 386 chromatographic monodispersity upon purification. To increase its stability, we used consensus mutagenesis<sup>26</sup>, and screened EAAT1 variants with different consensus 387 388 mutations in the predicted transmembrane helices by FSEC. The apparent melting 389 temperature (Tm) of the most stable EAAT1 construct was >20 °C over that of the 390 wild-type EAAT1, but the mutated transporter was still refractory to crystallization. 391 We reasoned that the extracellular region between TM3-4c could interfere with 392 crystallization, and changed it for the shorter TM3-4c sequence from ASCT2 393 (Extended Data Fig.1). In addition, we mutated the two predicted N-glycosylation 394 sites of the transporter (N155T and N204T mutations) to further improve 395 crystallizability.

#### 396 Expression and purification

All constructs were introduced into pcDNA3.1(+) (Invitrogen) with Nterminal Strep-tag II affinity tag followed by eGFP and PreScission protease cleavage site, and expressed in HEK293F cells (ATCC, mycoplasma test negative) grown in Excell293 medium (Sigma) and supplemented with 4mM L-glutamine (Sigma) and  $5\mu$ g/ml Phenol red (Sigma-Aldrich) to densities of 2.5 x 10<sup>6</sup> cells ml<sup>-1</sup>. Cells were transiently transfected in Freestyle293 medium (Invitrogen) using poly-ethylenimine (PEI) (Tebu-bio) at a density of 2.5 x 10<sup>6</sup> cells ml<sup>-1</sup>, diluted with an equivalent volume 404 of Excell293 6 hours post-transfection, and treated with 2.2 mM valproic acid
405 (Sigma) 12 hours after dilution of the cultures. Cells were collected at ~48 h post406 transfection.

407 Initial screens of constructs and detergent solubilization buffers were done in 408 small-scale (5-10 ml), and cells were collected, mechanically disrupted with a 409 douncer and solubilized in 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl buffer 410 supplemented with 1 mM L-asp, 1 mM EDTA, 1 mM Phenylmethylsulfonyl fluoride 411 (PMSF), 1 mM Tris(2-carboxyethyl)phosphine (TCEP), 1:200 (v/v) dilution of 412 mammalian protease inhibitor cocktail (Sigma), 10 % glycerol, 2% detergent and 413 0.4% cholesterol hemisuccinate (CHS) (Anatrace). After 1-hour incubation at 4 °C, 414 clear lysates were obtained by ultracentrifugation (247,000 g for 45 min). A high-415 throughput auto-sampler was used to inject the lysates in a SRT SEC-500 column 416 (Sepax Technologies) equilibrated in 50 mM HEPES/Tris-base, pH 7.4, 200 mM 417 NaCl buffer supplemented with 1 mM L-asp, 1 mM (TCEP), 5 % glycerol, 3 x CMC 418 detergent, and  $\sim 0.01\%$  CHS, in line with fluorescence detection (Photon technology 419 international) for FSEC analysis.

420 Large-scale expression was done in 2-4 l cultures with cells collected in 50 mM HEPES/Tris-base, pH 7.4, 50 mM NaCl buffer supplemented with 1 mM L-asp, 421 422 1 mM EDTA, 1 mM PMSF, 1 mM TCEP, and 1:200 (v/v) dilution of mammalian 423 protease inhibitor cocktail (Sigma), and disrupted in an cell homogenizer 424 (EmulsiFlex-C5, Avestin) after 3 runs at 15,000 Psi. The resulting homogenate was 425 clarified by centrifugation (4,500 g, 0.5 h) and the crude membranes were collected 426 by ultracentrifugation (186,000 g for 1.5 h). Membranes were washed once with the 427 above-mentioned buffer and finally homogenized with a douncer in a buffer 428 containing 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM

429 EDTA, 1 mM TCEP, and 10% Glycerol, snap-frozen in liquid  $N_2$  and stored at -80°C 430 at 0.5 g of membranes ml<sup>-1</sup>.

431 Membrane solubilization was done by thawing out and supplementing the 432 membrane homogenate with 2% DDS, 0.4% CHS, and 25 µM UCPH<sub>101</sub> (Abcam). 433 After 1-hour incubation, the insoluble material was removed by ultracentrifugation 434 (186,000g for 1 h), and Strep-Tactin sepharose resin (GE Healthcare) was added to 435 the supernatant and rotated for 2 h. Resin was washed with 25 column volumes of 50 436 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM TCEP, 5% 437 Glycerol, 0.05% DDS, 0.01% CHS and 25  $\mu$ M UCPH<sub>101</sub>, and the protein was eluted 438 with the same buffer supplemented with 2.5 mM D-desthiobiotin.

439 The eluted eGFP-transporter fusion was concentrated to 1-2 mg ml<sup>-1</sup> using 100-kDa cutoff membranes (Millipore), and digested with His-tagged PreScission 440 441 protease overnight at 4 °C. The protease was removed by reverse Ni-NTA (Qiagen) 442 affinity chromatography, and the flow through containing the transporter was 443 concentrated to 500 µl, ultra-centrifuged (86,900 g, 20 min), and applied to a 444 Superose 6 10/300 gel filtration column (GE Healthcare) equilibrated with 50 mM 445 HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 1 mM TCEP, 5% Glycerol, 446 0.25% decanoyl sucrose (DS, Sigma), 0.05% CHS and 100 µM UCPH<sub>101</sub>. To obtain 447 the UCPH<sub>101</sub> unbound structure, the protocol was identical, but the allosteric inhibitor 448 was omitted from all buffers. To obtain the UCPH<sub>101</sub>-TBOA<sub>TFB</sub> bound structure, the 449 protein sample was supplemented with 3 mM TBOA<sub>TFB</sub> (Tocris) before the injection 450 in the gel filtration column equilibrated with 50 mM HEPES/Tris-base, pH 7.4, 200 451 mM NaCl, 1 mM TCEP, 5% Glycerol, 0.25% decanoyl sucrose (DS, Sigma), 0.05% 452 CHS, 300 µM TBOA<sub>TFB</sub> and 100 µM UCPH<sub>101</sub>.

453 Protein samples after the solubilization step were kept on ice or at 4 °C at all times.

#### 454 Crystallization and structure determination

Purified protein was concentrated to 3.5-4.0 mg ml<sup>-1</sup> and 1 mM UCPH<sub>101</sub> was 455 456 added in experiments with the inhibitor-bound transporters. Initial vapor diffusion 457 crystallization screens were done by mixing 300 nl of protein and reservoir solution in 458 sitting drops, dispensed by a Mosquito robot (TTP labtech) in 96-well Greiner plates. 459 The purified transporters form three-dimensional crystals in several conditions 460 containing low molecular weight polyethylene glycols. The best-diffracting crystals 461 were obtained after manual optimization using 1.6  $\mu$ l hanging drops at 4 °C, obtained 462 by mixing equal volumes of protein supplemented with 0.2% n-Octyl-b-D-463 glucopyranoside (BOG, Anatrace) and 0.04% CHS, and reservoir solutions containing 464 100 mM Tris, pH 8.2, 50 mM CaCl<sub>2</sub>, 50 mM BaCl<sub>2</sub>, and 28-30% PEG 400. Crystals 465 appeared after 24-48 h and reached their maximum size after a week. Crystals were 466 flash-frozen in liquid nitrogen before X-ray diffraction data collection without any 467 further cryo protection.

468 X-ray diffraction data were collected at beamlines PROXIMA-1 at the 469 SOLEIL synchrotron (St Aubin, France) and at beamlines at the European 470 Synchrotron Radiation Facility (Grenoble, France). In general, 2-3 data sets from 471 single crystals were collected, and indexed, integrated, scaled and merged using XDS package<sup>52</sup>. Due to the anisotropic nature of the diffraction data, the DEBYE and 472 473 STARANISO programs were applied to scale it using the STARANISO server 474 (http://staraniso.globalphasing.org/). The software performs an anisotropic cut-off of 475 merged intensity data with a Bayesian estimation of the structure amplitudes, and 476 applies an anisotropic correction to the data. Table 1 shows the refinement statistics 477 for the full sets of reflections truncated at the best high-resolution along h, k or l axis, values given by AIMLESS<sup>53</sup>, before the anisotropic corrections computed by the 478

479 STARANISO software. The corrected anisotropic amplitudes were then used for molecular replacement in PHASER<sup>54</sup>, using the scaffold and transport domains of 480 481 GltPh (PDB code 2NWL) as independent search models. The initial electron density 482 maps were clearly interpretable, and the final model was obtained through rounds of manual building in COOT<sup>55</sup> and refinement in Buster<sup>56</sup>, until reaching good 483 484 crystallographic statistics and stereochemistry (Extended Data Table 1). The model 485 contains one EAAT1<sub>cryst</sub> monomer per asymmetric unit and most of the EAAT1<sub>cryst</sub> 486 polypeptide (residues 37-487), with the exception of some residues in the 487 extracellular loops between TM3-4a, TM4b-4c, TM5-6 and TM7b-HP2a. Sequence 488 assignment was aided by anomalous difference Fourier maps from diffraction data 489 collected with low energy X-rays (1.77 Å) to highlight the sulfur atoms of methionine 490 and cysteine residues (Extended Data Fig. 11). EAAT1<sub>crvst-II</sub>, as well as the 491 EAAT1<sub>crvst</sub> UCPH<sub>101</sub>-TBOA<sub>TFB</sub> bound structures were solved by the same approach 492 above mentioned, but using the EAAT1<sub>cryst</sub> TranD and ScaD as independent search 493 models for molecular replacement.

The stereochemical properties of the final models were analyzed with the Molprobity server (http://molprobity.biochem.duke.edu/). At least 95% of the residues in all models are in the Ramachandran favored region. Protein interfaces were analyzed with the PISA server (http://www.ebi.ac.uk/msd-srv/prot\_int/pistart.html). Structural alignments were done with Superpose in the CCP4 suite. All structural figures were prepared with PyMOL Molecualr Graphics System, Schrodinger, LLC.

500 **Radioactive substrate transport assays** 

501 Unilamellar liposomes were made at 9:1 molar ratio of 1-palmitoyl-2-oleoyl-502 sn-glycero-3-phosphocholine (Avanti Polar Lipids) and CHS, in a buffer containing 503 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl and 1 mM L-asp. The transporters

were purified as described above, but excluding the reverse chromatography step after
protease cleavage, and using a Superose 6 10/300 column equilibrated with 50 mM
HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM L-asp, 0.5 mM TCEP, 0.0632%
DDS, 0.01264% CHS, and 5% glycerol.

508 To reconstitute the protein, liposomes were first mixed with DDS at a 1:2 509 (w/w) lipid-to-detergent ratio for 1 h, and then the purified transporters were added at 510 a 1:40 (w/w) protein-to-lipid ratio. Detergent removal was done at 4° C using SM-2 biobeads (BioRad) at 100 mg ml<sup>-1</sup>. The internal solution of the liposomes was 511 512 exchanged using 10 freeze-thaw cycles in the appropriate buffer. After extrusion 513 through 400-nm polycarbonate membranes (Avanti Polar Lipids), the 514 proteoliposomes were concentrated by ultracentrifugation (150,000 g for 30 min at  $4^{\circ}$ C) and resuspended at 20 mg of lipids ml<sup>-1</sup>, for immediate use. 515

516 Substrate transport was assayed at 37°C. The uptake reaction was initiated by 517 diluting the proteo-liposomes 10-fold into a buffer containing 50 mM HEPES/Trisbase, pH 7.4, 200 mM NaCl, 50 µM L-glutamate, and 5 µM [<sup>14</sup>C]-L-glutamate 518 519 (PerkinElmer), and 2.5% glycerol. After 30 min, 200-µl aliquots were diluted 5-fold 520 into ice-cold quench buffer (50 mM HEPES/Tris-base, pH 7.4, 200 mM ChCl, and 521 2.5% glycerol), followed by immediate filtration and wash on nitrocellulose 0.22-µm 522 filters (Millipore). Radioactivity was quantified by liquid scintillation using a Tri-523 Carb 3110TR counter (PerkinElmer). For the UCPH<sub>101</sub> titrations, proteo-liposomes 524 were both pre-incubated for 20 min at room temperature, and assayed in the presence 525 of  $UCPH_{101}$ . Background radioactivity was estimated from protein-free liposomes, 526 and subtracted from the uptake data. Data was fitted to a Hill equation of the form:

527  $F=F_{\infty} + \Delta F_{\infty}/(1+(IC_{50}/[UCPH_{101}])^n)$ 

528 Where  $F_{\infty}$  is the final level of inhibition,  $\Delta F_{\infty}$  is the final amplitude of the UCPH<sub>101</sub> 529 effect, and n is the Hill coefficient.

To titrate the rate of L-glutamate transport by  $EAAT1_{cryst}$ , proteo-liposomes were assayed in the presence of 0, 5, 50 or 200  $\mu$ M L-glutamate supplemented with 1, 5, 5, or 5  $\mu$ M [<sup>14</sup>C]-L-glutamate, respectively. At each substrate concentration, the initial rate of transport was calculated by a linear fit to 120 s and 180 s uptake measurements with origin fixed at zero. Background radioactivity was estimated from protein-free liposomes, and subtracted from the uptake data.

536 For the cell-based transport uptake, cells were collected 36 h post-transfection, and washed three times and resuspended at a density of 50 x  $10^6$  cells ml<sup>-1</sup> in 11 mM 537 HEPES/Tris-base, pH 7.4, 140 mM ChCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM 538 539 MgCl<sub>2</sub>, and 10 mM D-glucose, for immediate use. The uptake assay was performed 540 similarly to the one described for the proteo-liposomes, but using a reaction buffer 541 containing 11 mM HEPES/Tris-base, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 10 mM D-glucose, 50  $\mu M$  L-glutamate, and 5  $\mu M$  [ $^{14}C$ ]-L-542 543 glutamate, and 0.8-µm nitrocellulose filters. Background radioactivity was estimated 544 from cells transfected with empty vector, and subtracted from the uptake data.

#### 545 Hydrogen-deuterium exchange mass spectrometry

HDX-MS experiments were performed with transporters purified as described
in the proteo-liposome section, and using a Superose 6 5/150 gel filtration column
equilibrated with 50 mM HEPES/Tris-base, 200 mM NaCl, pH7.4, 1 mM L-asp, 0.5
mM TCEP, 0.0632% DDS, 0.01264% CHS, and 5% glycerol.

550 The purified EAAT1<sub>cryst</sub> was incubated in ice for 30 min with 2.2% DMSO at 551 a monomer concentration of 5.2  $\mu$ M, in the presence and absence of 102  $\mu$ M 552 UCPH<sub>101</sub>, respectively. Prior to labeling, 10  $\mu$ L of the unbound and UCPH<sub>101</sub>-bound

553 EAAT1<sub>cryst</sub> solution was equilibrated for 10 min at room temperature. Deuterium 554 exchange was initiated by adding 40 µL of D<sub>2</sub>O buffer (50 mM HEPES, pD 7.3, 200 555 mM NaCl, 1 mM L-asp, 5% glycerol, 0.0632% DDS, 0.01264% CHS, 0.5 mM 556 TCEP) supplemented or not with 101.2  $\mu$ M UCPH<sub>101</sub>. Assuming that the K<sub>D</sub> and IC<sub>50</sub> 557 values of UCPH<sub>101</sub> are similar,  $\sim 96\%$  of the transporters would remain bound during 558 deuterium labeling. Aliquots of 10.4 pmols of protein were removed at defined 559 deuterium exchange time points (from 10 sec to 60 min) and quenched upon mixing 560 with an ice-cold acidic solution (0.75% formic acid, 5% glycerol) to decrease the pH 561 to 2.6. Quenched samples were immediately snap-frozen in liquid N2 and stored at -562 80°C until analysis.

563 Prior to mass analysis, quenched samples were rapidly thawed and 564 immediately injected into a cooled nanoACQUITY UPLC HDX system (Waters 565 corp.) maintained at 0°C. 8.6-pmol protein samples were on-line digested for 2 min at 566 20 °C using an in-house packed immobilized pepsin cartridge (2.0 x 20 mm, 66 µL 567 bed volume). The resulting peptides were trapped and desalted onto a C18 Trap 568 column (VanGuard BEH 1.7 µm, 2.1 x 5 mm, Waters corp.) at a flow rate of 100 569 µL/min of 0.15% formic acid, and then separated in 10 min by a linear gradient of acetonitrile from 5 to 40% at 40 µL/min using an ACQUITY UPLC<sup>TM</sup> BEH C18 570 571 analytical column (1.7 µm, 1 x 100 mm, Waters corp.). After each run, the pepsin 572 cartridge was manually cleaned with two consecutive washes of 1% formic acid, 5% 573 acetonitrile, 1.5 M guanidinium chloride, pH 2.5. Blank injections were performed 574 between each run to confirm the absence of carry-over.

575 Mass spectra were acquired in resolution and positive mode on a Synapt G2-Si 576 HDMS mass spectrometer (Waters corp.) equipped with a standard electrospray 577 ionization source, as described previously<sup>57</sup>. Peptides were identified from

undeuterated protein samples acquired in MS<sup>E</sup> mode by database searching in 578 579 ProteinLynX Global Server 3.0 (Waters corp.). Each fragmentation spectrum was 580 manually inspected for assignment validation. Deuterium uptake values were 581 calculated for each peptide using DynamX 3.0 (Waters corp.). Only one unique 582 charge state was considered per peptide and no adjustment was made for back-583 exchange. HDX-MS results are reported as relative deuterium uptake values expressed in mass unit or fractional exchange<sup>57</sup>. A statistical analysis was performed 584 with MEMHDX<sup>58</sup> using a False Discovery Rate of 1%. 585

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#### 784 Author contribution

J.C.C.-T., and R.A. optimized and performed protein expression, purification and crystallization, and R.A. performed molecular biology; J.C.C.-T., R.A., and N.R. collected crystallographic data, and J.C.C.-T., P.L., and N.R. analyzed diffraction data and structures; E.C. and R.A performed and analyzed uptake experiments; E.C. prepared protein samples for HDX-MS; S.B. collected and analyzed HDX-MS data with help from E.C.; All authors contributed to the experimental design of the project and manuscript preparation. N.R. conceived and supervised the project.

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Author Information Coordinates and structure factors for  $EAAT1_{cryst}$ -UCPH<sub>101</sub>, EAAT1cryst-UCPH<sub>101</sub>-TBOA<sub>TFB</sub>,  $EAAT1_{cryst-II}$ -UCPH<sub>101</sub>, and  $EAAT1_{cryst-II}$  crystal structures have been deposited in the Protein Data Bank with accession codes 5LLM, 5MJU, 5LM4, 5LLU, respectively. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to N.R. (nreyes@pasteur.fr)



802 Figure 1 | Function and architecture of EAAT1<sub>cryst</sub>. a-b, Uptake of radioactive L-803 glutamate by purified EAAT1 (grey), EAAT1<sub>cryst</sub> (blue), and EAAT1<sub>cryst-II</sub> (red) 804 reconstituted in liposomes. Transport was abolished when choline ( $Ch^+$ ) was used as 805 the main cation in the extra- or intra-liposomal solutions (yellow circles) (a). UCPH<sub>101</sub> inhibits glutamate transport in a concentration dependent manner (b). Plots 806 807 depict an average of three independent experiments performed with duplicate 808 measurements, and error bars represent s.e.m. c-d, Structure of EAAT1<sub>crvst</sub> trimer 809 viewed from the extracellular solution (c) and from the membrane (d), with the ScaD 810 (teal) and TranD (orange) as surfaces, and UPCH<sub>101</sub> bound between them (white 811 spheres). e, EAAT1<sub>cryst</sub> monomer viewed parallel to the membrane. The ScaD domain 812 is represented as surface (teal), and several helices and loops in the TranD (orange) 813 have been removed form clarity of display. f, Domain organization diagram of 814 EAAT1<sub>cryst</sub> monomer.



**Figure 2** | **Transport domain.** EAAT1<sub>cryst</sub> transport domain viewed from the 823 membrane with substrate (spheres) and one sodium ion (purple spheres) bound. Omit 824 Fo-Fc densities contoured at 2.3  $\sigma$  (black mesh) show the position of the ligands. 825 TM8a-c (blue) interacts with other regions on the TranD (orange), including HP1 (left

826 inset), HP2 (upper right inset), and TM3 and TM7 (lower right inset).





Figure 3 | Scaffold domain. a, TM4 (purple) makes extensive contacts with the ScaD of the neighboring monomer (monomer 2, teal surface), and the TranD (orange) of its own polypeptide (monomer 1). The TranD of monomer 2, and several TMs of monomer 1 were removed for clarity of display. Fo-Fc density in the HP2-TM4 crevice is contoured at  $2.7\sigma$  (red mesh). **b**, Two monomers of EAAT<sub>crvst</sub> show TM1a laying parallel to the membrane. The third monomer is not shown. TM1 (teal) and HP1a (dark blue) form a hydrophobic crevice containing non-protein Fo-Fc density (purple mesh, contour  $2.7\sigma$ ).



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**Figure 4** | **UCPH**<sub>101</sub> **binding site. a**, Lateral view of EAAT1<sub>cryst</sub> monomer from the membrane showing UCPH<sub>101</sub> bound (pink) between the TranD (orange) and ScaD (teal). **b-c**, UCPH<sub>101</sub> (pink) coordination and Fo-Fc densities contoured at 2.0 $\sigma$  (blue mesh) in EAAT1<sub>cryst</sub> (**b**) and EAAT1<sub>cryst-II</sub> (**c**), respectively. Side chains of residues in TM3, TM4c, and TM7 involved in coordination are shown. F369 side chain moves outward in the EAAT1<sub>cryst-II</sub> unbound state (grey) (**c**).





857 Figure 5 | Transport domain dynamics. a, UCPH<sub>101</sub> decreased deuterium uptake in 858 several regions (black) of the TranD at the interface with the ScaD. The alpha carbon 859 atoms of the limiting residues for these regions are shown as back spheres. Unaffected regions of the TranD (orange) or those not detected during HDX-MS 860 861 analysis (grey) are also shown (sequence coverage ~77%). b, Residues of the 862 EAAT1<sub>cryst</sub> TranD buried at the interface with the ScaD, in the UPCH<sub>101</sub>-bound 863 outward-facing state, are colored dark blue for comparison with (a). c-d, Comparison 864 of  $EAAT1_{cryst}$  structure in the UPCH<sub>101</sub>-bound outward-facing state (c) with a model 865 of an inward-facing state based on the GltPh "unlocked" state (PDB 4X2S) (d). The 866 ScaD domain (pseudo-transparent surface cyan) is in identical positions in the 867 structure and the model, while the TranD (color code as in a) undergoes a large rigid 868 body movement towards the cytoplasm. 869



881 Figure 6 | UCPH<sub>101</sub>-TBOA<sub>TFB</sub> bound EAAT1<sub>cryst</sub> structure. a, The TranD and 882 ScaD of the EAAT1<sub>cryst</sub> monomer are represented in orange and cyan, respectively. 883 The movement of HP2 (purple) partly exposes the substrate-binding pocket to the 884 solvent and shows a molecule of TBOA<sub>TFB</sub> (purple sticks) bound to it. UCPH<sub>101</sub> is 885 also observed in this structure (pink sticks). b, The tip of HP2 moves as much as 9.5 886 Å in the UCPH<sub>101</sub>-TBOA<sub>TFB</sub>- (purple), compared to the UCPH<sub>101</sub>-substrate bound 887 (orange) structures, moving the carbonyl oxygen of A420 away from Na2 (green 888 sphere). c, Omit map Fo-Fc density for the TBOA<sub>TFB</sub> molecule is contoured at  $2.3\sigma$ 889 (black mesh), and some of the residues at Van deer Waals or H-bond distance from 890 the compound are represented as sticks.



900Figure 7 | EAAT1 inhibitory mechanisms. Schematic representation of the901molecular mechanisms of action of TBOA-like competitive and UCPH101-like902allosteric inhibitors in EAAT1. Competitive inhibitors, bind to the substrate-binding903pocket in the TranD and preclude substrate (black ellipse) binding from both sides of904the membrane. UCPH101-like compounds bind at the TranD-ScaD interface and block905the movement of the TranD (orange) relative to the ScaD (teal), but allow substrate906binding from the extracellular solution.

#### 908 Extended Data Table 1. Data collection and refinement statistics.

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	EAAT1 <sub>cryst</sub>	EAAT1 <sub>cryst-II</sub>	EAAT1 <sub>cryst-II</sub>	EAAT1 <sub>cryst</sub>
	UCPH <sub>101</sub>	UCPH <sub>101</sub>		$UCPH_{101}$ and
	bound	bound		TBOA <sub>TFB</sub>
Data and a strant				bound
Data collection*	DC	DC	DC	DC
Space group	P03	P03	P03	P63
Cell dimensions	122 27 00 07	100.11.00.00	122 22 00 57	124 22 00 01
a=b, c(A)	123.27,89.87	123.11, 89.62	123.32, 89.57	124.33, 90.81
$\alpha = \beta, \gamma$ (°)	90.0, 120.0	90.0, 120.0	90.0, 120.0	90.0, 120.0
Wavelength Decolution (Å)	0.979 45 00 <b>3 35</b> (2 24	0.970 45 02 <b>2 1</b> (2 10	1.009 45 07 <b>3 33</b> (3 41	0.9// 46.21. <b>2.71</b> (2.01
Resolution (A)	45.89 <b>-3.25</b> (3.34- 3.25)	45.82 <b>-3.1</b> (3.18- 3.1)	45.87 <b>-3.32</b> (3.41- 3.32)	46.31 <b>-3.71</b> (3.81- 3.71)
Anisotropy direction <sup>\$</sup>				
Resolution where				
$CC_{1/2} > 0.3$				
Overall (Å)	3.37	3.1	3.32	3.71
along h, k axis (A)	3.75	3.68	3.85	4.35
along l axis (A)	3.25	3.1	3.32	3.71
Measured reflections	333978 (24261)	290672 (21906)	326273 (21829)	141904 (11176)
Unique reflections	12338 (902)	14115 (1 032)	11556 (834)	8570 (628)
Completeness (%)	100 (100)	99.9 (99.9)	100 (100)	99.9 (100.0)
Mn (I) half-set	0.99 (0.22)	1 (0.43)	0.99 (0.31)	0.99 (0.373)
correlation		150(00)	10 (0 7)	121(00)
$l/\sigma(l)$	11.7 (0.7)	15.8 (0.8)	13(0.7)	12.1 (0.9)
R <sub>merge</sub>	0.20 (6.90)	0.1(6./1)	0.15 (8.16)	0.14(3.71)
Redundancy	27.1 (26.9)	20.6 (21.2)	28.2 (26.2)	16.3 (14.3)
Structure				
Definement				
Resolution cut-off $(\text{Å})$	45 80- <b>2 25</b>	45 80- <b>3 10</b>	20 00- <b>2 22</b>	25 00- <b>3 71</b>
No. of Work / Tost	43.09- <b>3.23</b> 0801 /475	10725/528	0251/445	23.00 <b>-3.71</b> 6860/684
reflections	JUJ1/4/J	107237320	7231/443	0000/004
$R_{\rm cryst}$ (%) / $R_{\rm free}$ (%)	21.9/24.1	21.7/25.9	20.9/25.3	22.7/25.4
No. of protein atoms	3002	2960	2995	3008
No. of heteroatoms	42	42	10	62
<i>B</i> factors (Å) <sup>2</sup>				
Protein	129.5	111.8	137.0	135.5
Heteroatoms	107.3	99.5	125.6	132.7
R.m.s. deviations				
from ideal				
Bond lengths (A)	0.009	0.01	0.009	0.009
Bond angles (°)	1.06	1.12	1.05	1.03

910 One crystal was used to collect diffraction datasets for each structure, except in the EAAT1<sub>cryst</sub>

911 UCPH<sub>101</sub> and TFB-TBOA bound structure, where datasets from three crystals were merged.

5% of reflections were used for calculation of  $R_{\text{free}}$ .

912 913 \*Values in parentheses are for the highest-resolution shell.

914 <sup>\$</sup>The anisotropy directions where computed with AIMLESS.

		() 1a		1b		σ		2a		
	10	20	30	40	50	60	70	80	90	100
EAAT1cryst	20 OGVSKRTLLAKI	KVONITKEDVKSF	LRRNALLLIT	LAVILGVVLG	FLLRPY	-PLSPREVKY	FAFPGELLMR	MLKMLILPLI	VSSLITGLAS	LDAKASGRLGMRAV
EAAT1	20 QGVSKRTLLAK	KVQNITKEDVKSY	LFRNAFVLLT	TAVIVGTILG	FTLRPY	-RMSYREVKY	FSFPGELLMR	MLQMLVLPLI	ISSLVTGMAA	LDSKASGKMGMRAV
EAAT2	28PK	RHLGLRLCD-K	LGKNLLLTLTV	FGVILGAVCG	GLLRLZ	SPIHPDVVML	IAFPGDILMR	MIRMLILPLI	ISSLITCLSC	LDAKASGRLGTRAM
EAAT3	7ĸ	GC <mark>E</mark> -WKRFI	LENNWVLLSTV	AAVVLGITTG	VLVREH	ISNLSTLERFY	FAFPGEILMR	MLKLIILPLI	IS <mark>SMIT</mark> GVAA	LDSNVSGKIGLRAV
EAAT4	28 ESLQQRALRTRI	RLOTMTLEHVLRF	LRRNAFILLT	/SAVVIGVSLA	FALRPY	I–Q <mark>L</mark> TYRQIKY	FS FPGELLMRI	MLQMLVLPLI	V9 <mark>5LVTGM</mark> AS	LONKATGRMGMRAA
EAAT5	6ILA	RGRDVC	-RRNGLLILSV	LSVIVGCLLG	FFLRTF	R-RLSPQEISY	FQFPGELLMRI	MLKMMILPLV	VSSLMSGLAS	LDAKTSSRLGVLTV
ASCT1	16 AGPAAGPG-AP	STA-AGRARRCAGE	LRRQALVLLT	/SGVLAGAGLG	AALRGI	L-S <mark>L</mark> SRTQVTY	LAFPGEMLLR	MLRMIILPLV	VCSLVSGAAS	LDASCLGRLGGIAV
ASCT2	31 QGAAAG-GYC-0	SRDQVRRC	LRANLLVLLT	VAVVAGVALG	LGVSGAGGAI	L-ALGPERLSA	FVFPGELLLR	LLRMITLPLV	VCSLIGGAAS	LDPGALGRLGAWAL
	) 3				() 4a		4b )			
	12	0 130	140	150	160	170	180	190	200	210
EAAT1cryst	125 VYYMSTTILAV	LGIILVLIIHPGA	ASAAITA-SVO	AAGSAENAPS	KEVLDSFLDI	LARNIFPSNLV	SAAFRSYSTT	YEERTITG		
EAAT1	125 VYYMITTIIAV	IGIIIVIIIHPGK	STKENMI	REGKIVR	VTAADAFLDI	LIRNMFPPNLV	EACFROFETN	YEKRSFKV		PIQAN-ETLV
EAAT2	122 VYYMSTTIIAAV	LGVILVLAIHPGN	PKLKKQIG	PGKKNDE	VSSLDAFLDI	LIRNLFPENLV	ACFOOIQTV	TKKVL-VAP	)	PPDEE-A
EAAT3	96 VYYFCTTLIAV	ILGIVLVVSIKPGV	POKVGEIZ	RTGSTPE	VSTVDAMLDI	LIRNMFP <mark>E</mark> NLV	ACFOOYNTK	REEVKP	·	PSDPE-M
EAAT4	133 VYYMVTTIIAV	FIGILMVTIIHPGK)	GSKEGL	REGRIET	IPTADAFMDI	LIRNMFP <mark>P</mark> NLV	EACFROFRTO	STRVVTRIM	WRTENGSEPG	ASMPPPFSV-ENGT
EAAT5	94 AYYLWTTFMAV	IVGIFMVSIIHPGS	AAQKETTE	QSGK-PI	MSSADALLDI	LIRNMFP <mark>A</mark> NLV	EATFKQYRTK	TTPVVK-	S-P-	KVAPEEAPPR
ASCT1	119AYFGLTTLSAS	ALAVALAFIIKPGS	GAQTLQSSDLG	LEDSGPPPVP	KETVDSFLDI	LARNLFPSNLV	VAAFRTYATD	YK-VV	TQN-S	
ASCT2	133 LFFLVTTLLAS	ALGVGLALALQPGA	ASAAINA-SVO	AAGSAENAPS	KEVLDSFLDI	LARNIFPSNLV	SAAFRSYSTT	YEERNIT		
	Δ	1								
				σ	4c			5a		
	23	0 240	250	260	270	280	290	300	310	320
EAAT1crvst	208		RV-KVPVGO-	EVEGMNILGL	VVFSIVFGIZ	ALGENGEOGOL		ATMKLVAT IM	WYAPLGILFT	TAGKIVEMEDLEVI
EAAT1	211 GAVINN	/SEAMETLTRI	TEE-LVPVPG-	SVNGVNALGU	VVFSMCFGF	TGNMKEOGOA	REFEDSINE	ATMRLVAVTM	WYAPVGILFT	TAGKIVEMEDMGVT
EAAT2	206NATSAV	SLINETVIEVPEE	FKM-VIKKGLE	FROGMNVLGL	IGFFIAFG17	MGKMGDOAKL	WDEFNILNE	IVMKLVIMIM	WYSPLGIACI	ICGKIIAIKDLEVV
EAAT3	178NMTEES	TAVMTTAISKN-K	FKE-YKIVGM-	YSDGINVLGL	IVFCLVFGL	IGKMÆKGQI	LVDFFNALSD	ATMKIVQIIM	CYMPLGILFI	IAGKIIEVEDWEIF
EAAT4	235-SFLEN	TRALGTLOEML-S	FEE-TVPVPG-	-SANGINALGL	VVFSVAFGL	/IGGMKHK <mark>G</mark> RV	LRDFFDSLNE	AIMRLVGIII	WYAPVGILFI	IAGKILEMEDMAVL
EAAT5	181 RILIYGVQEEN	SHVONFALDLTPP	PEVVYKSEPG-	TSDGMNVLGI	VFFSATMGIN	ALGRMGDS <mark>G</mark> AP	LVSFCQCLNE:	SV <mark>M</mark> KI VAVAV	WYFPFGIVFI	IAGKILEMDDPRAV
ASCT1	203	SSGN-V	THE-KIPIGT-	EIEGMNILGL	VLFALVLGV#	ALKKLGSE <mark>G</mark> ED	LIRFFNSLNE	ATMVLVSWIM	IWYVPVGIMFI	VGS <mark>KIVEMKD</mark> IIVL
ASCT2	215	G	rrv-kvpvgQ-	-EVEGMNILGL	VVFAIVFGVA	ALRKLGPE <mark>G</mark> EL	LIRFFNSFNE	ATMVLVSWIM	WYAPVGIMFI	VAGKIVEMEDVGLL
				그	88					
	0	6		HP1a	– ĭ	() HP1b		7a		7b
	34	0 350	360	370	380	390	400	410	420	430
EAAT1cryst	291 GGOLGMYMVTV	VGLVIHGLIVLPL	IYFLITRKNP	VFIAGILOAL	ITALGTSSS	SATLP ITFKCL	EENNGVDKRI	TREVLEVGAT	INMOGTALYE	AVAAIFIAQVNNYE
EAAT1	311 GGOLAMYTVTV.	VGLLIHAVIVLPL	LYFLVTRKNP	WFIGGLLOAL	ITALGTSSS	SATLP ITFKCL	EENINGVDKRV	TREVLEVGAT	INMIGTALYE	ALAAIFIAQVNNFE
EAAT2	310 AROLGMYMVTV	IGLIHGGIFLPL	I YFVVTRKNP	SFFAGIFOAW	ITALGTASS/	AGTLPVTFRCL	EENLGIDKRV	TRFVLPVGAT	INMOGTALYE	AVAAIFIAOMNGVV
EAAT3	280-RKLGLYMATV	TGLAIHSIVILPL	IYFIVVRKNP	RFAMGMAQAL	LTALMISSS	SATLPVTFRCA	EENNQVDKRI	TRFVLPVGAT	INMOGTALYE	AVAAVFIAQINDLD
EAAT4	336GGQLGMYTLTV	IVGLELHAGIVLPL	IYFLVTHRNPE	PFIGCMLQAL	ITAMGTSSSS	SATLP ITFRCL	EEGLGVDRRI'	TREVLPVGAT	VNMDGTALYE	ALAAIFIAQVNNYE
EAAT5	290 GKKLGFYSVTV	CGLVLHGLFILPL	LYFFITKKNPI	IVFIRGILQAL	LIALATSSS	SATLP ITFKCL	LENNHIDRRI	ARFVLPVGAT	INMOGTALYE	AVAAIFIAQVNNYE
ASCT1	291 VTSLGKYIFAS	IL <mark>GHVIH</mark> GGIVLPL	IYFVFTRKNPE	RELLGLLAPE	ATAFATC <mark>SS</mark> S	SATLPSMMKCI	EENNGVDKRI:	SRFILPIGAT	VNMDGAAIFQ	CVAAVFIAQLNNVE
ASCT2	299FARLGKYILCC	LIGHAIHGLLVLPL	IYFLFTRKNPY	R <mark>FLWGIVTPL</mark>	ATAFGTSSS	BATLPLMMKCV	EENNGVAKHI	SRFILPIGAT	VNMDGAALFO	CVAAVFIAQLSQQS
								ΔΔ		
	() HP	2a (	) HP2b	) () 8a		8b		) () 8c		
	45	460	470	480	490	500	510	520	530	
EAAT1cryst	401 LDFGOIITISI	TATAASIGAAGIPO	AGLVTMVIVL	AVGLPTDDIT	LIIAVDWLLI	RFRTMVNVLG	DALGAGIVEH	-LSRKELE-K	ODAELG	
EAAT1	421 INFGOIITISI	TATAASIGAAGIPO	AGLVTMVIVL	SVGLPTDDIT	LIIAVDWFLI	RLRTTTNVLG	DSLGAGIVEH	-LSRHELK-N	RDVENG	
EAAT2	420 LDGGQIVTVSL	TATLASVGAASIPS	AGLVTMLLIL	AVGLPTEDIS	LLVAVDWLLI	ORMRTSVNVVG	DSFGAGIVYH	-LSKSELD-T	IDSOHR	
EAAT3	389LGIGQIITISI	TATSASIGAAGVPO	AGLVTMVIVLS	BAVGLPAEDVT	LIIAVDWLLI	DRFRTMVNVLG	DAFGTGIVEK	-LSKKELE-Q	MDVSSE	
EAAT4	446 LNLGQITTISI	TATAASVGAAGIPO	AGLVTMVIVLI	SVGLPTEDIT	LIIAVDWFLI	DRLRTMINVLG	DSIGAAVIEH	-LSORELE-L	QEAELT	
EAAT5	400 LDFGQIITISI	TATAASIGAAGIPO	AGLVTMVIVLI	SVGLPTDDIT	LIIAVDWALI	DRFRTMINVLG	DALAAGIMAH	-ICRKDFARD	TGTEKL	
ASCT1	401 INAGOIFTILV	TATASSVGAAGVPA	GGVLTIATILE	AIGLPTHDLP	LILAVIMIVI	ORTTTVVNVEG	DALGAGILHH	-LNQKATK-K	GEQELA	
ASCT2	409 LDFVKIITILV	TATASSVGAAGIPA	GCVLTLAIIL	AVNLPVDHIS	LILAVDWLVI	ORSCTVLNVEG	DALGAGLION	YVDRTESR-S	TEPELI	

916 Extended Data Figure 1 | Alignment of human SLC1 transporters. Amino acid 917 sequences of EAAT1-5, ASCT1-2 and EAAT1<sub>cryst</sub> are compared. The boundaries of 918 the  $\alpha$ -helices (cylinders) in the TranD (orange) and the ScaD (teal) seen in the 919 EAAT1<sub>crvst</sub> structure are shown. In order to confer crystallizability, the region 920 between TM3 and TM4c (arrows) from ASCT2 was transferred to a thermally 921 stabilized EAAT1. To further improve crystal formation in the absence of UPCH<sub>101</sub>, 922 mutations M231I and F235I (circles) were introduced to generate EAAT1<sub>cryst-II</sub>. These substitutions are found in EAAT2. Other residues involved in UPCH<sub>101</sub> coordination 923 are more conserved (triangles). Sequences were aligned with Jalview<sup>59</sup>. 924



**Extended Data Figure 2** | **EAAT1**<sub>cryst</sub> and **EAAT1 glutamate uptake. a**, Initial rates of L-glutamate uptake from purified EAAT1<sub>cryst</sub> reconstituted in liposomes. The solid line is the fit of a Michaelis-Menten equation to the data with  $K_M=21 \pm 10 \mu M$  and  $V_{max}= 13 \pm 1 \text{ pmol } \mu g^{-1}$  protein min<sup>-1</sup>. The graph is the mean of three independent experiments, and error bars represent s.e.m.. **b**, L-glutamate uptake was measured in HEK293 cells expressing WT EAAT1 (black circles) and a truncated mutant beyond E501 (red symbols). The initial rate of uptake decreased by ~2-fold in the EAAT1 truncated mutant. Data were normalized to the asymptotic level of glutamate uptake based on a monoexponential function. The rates obtained from the fits were 0.16 ± 0.03 and 0.08 ± 0.03 min<sup>-1</sup> for EAAT1 and the truncated mutant, respectively. The graphs are means of 4 independent experiments done in duplicates. Error bars represent the s.e.m..

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947 Extended Data Figure 3 | EAAT1<sub>cryst</sub> and GltPh structural comparison. a-b

948 EAAT1<sub>cryst</sub> aligns to a monomer of GltPh (PDB 2NWL) with α-carbon r.m.s.d. of 1.4

949 Å. The ScaDs (EAAT1<sub>cryst</sub> teal, and GltPh purple,  $\mathbf{a}$ ), and TranDs (EAAT1<sub>cryst</sub> orange

and GltPh, purple, **b**) are shown separately for clarity of display.



Extended Data Figure 4 | EAAT1<sub>cryst</sub> trimeric interface. a-b, Interface of three ScaDs of the EAAT1<sub>cryst</sub> UCPH<sub>101</sub>-bound structure viewed from the extracellular side (a) and from the membrane (b). The TranDs are not shown. The ScaD of one monomer (black) makes extensive contacts with the two other subunits (teal and purple surfaces). The surface area in monomers 2 and 3 buried by monomer 1, is colored in light pink. Only residues that contribute  $\geq 10 \text{ Å}^2$  of buried surface area are highlighted. Side chains of residues that form intermolecular salt bridges are colored in green (sticks).



Extended Data Figure 5 | TranD-ScaD interface. a-b, EAAT1<sub>cryst</sub> monomer viewed from the membrane (solid black line). Residues in the TranD (colored black) bury 1,760  $Å^2$  at the interface with the ScaD (a). This interface extends to the extracellular side of the transporter through interactions between HP2-TM4 (sticks and pseudo-transparent spheres) (b). c, Cytoplasmic view of the monomer displaying the salt bridge between TM7 and TM5. 

a

b



998 Extended Data Figure 6 | Superposition of  $EAAT1_{cryst}$  and  $EAAT1_{cryst-II}$ 999 structures. a-b, The transport domains of  $EAAT1_{cryst}$  (teal) and  $EAAT1_{cryst-II}$  (pink) 1000 UCPH<sub>101</sub>-bound structures superimpose accurately after aligning their scaffold 1001 domains (a). The overall  $\alpha$ -carbon r.m.s.d. was 0.3 Å. However, the same alignment 1002 done with  $EAAT1_{cryst-II}$  UCPH<sub>101</sub>-bound and -unbound structures shows a small but 1003 global movement of the transport domain (b), with a small increase in the overall  $\alpha$ -1004 carbon r.m.s.d. of 0.1 Å.



Extended Data Figure 7 | Peptide coverage map of EAAT1<sub>cryst</sub>. A total of 111 peptides covering 76.3% of the EAAT1<sub>cryst</sub> sequence were identified by data independent MS/MS acquisition after 2 min digestion with immobilized pepsin. Each bar below the EAAT1<sub>crvst</sub> sequence corresponds to a unique peptide. The 57 peptides colored in blue were further selected for HDX-MS data extraction and analysis. The two additional N-terminal residues (i.e., GP) that remain after protein purification are also shown. The transmembrane helices (TM) of the TranD (orange) and the ScaD (cyan) are indicated above the sequence.



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1021 Extended Data Figure 8 | UCPH<sub>101</sub> effect on the local hydrogen exchange 1022 behavior of EAAT1<sub>crvst</sub>. a, HDX profiles of EAAT1<sub>crvst</sub> (see Methods) in the apo 1023 (upper panel) and UCPH<sub>101</sub>-bound state (middle panel). The relative fractional uptake 1024 determined for each peptide and at each time point is plotted as a function of peptide 1025 position. The black to red lines correspond to data acquired from 10 sec up to 1h, 1026 respectively. **b**, The fractional uptake difference plot was generated by subtracting the 1027 deuterium uptake values in the UCPH<sub>101</sub>-unbound from those in the bound state. 1028 Negative uptake difference indicates  $UCPH_{101}$ -induced decrease in amide hydrogen 1029 exchange. Each dot corresponds to an average of three independent HDX-MS 1030 experiments. The four regions (labeled 1 to 4) showing a statistically significant 1031 modification (p < 0.01) of deuterium uptake upon binding of UCPH<sub>101</sub> are highlighted 1032 in grey. 1033



1012 00. 1043 Da.



 $\begin{array}{c} 1044 \\ 1045 \end{array}$ 

1047 Extended Data Figure 10 | HDX-MS results mapped on the crystal structure of 1048 ScaD and TranD of EAAT1<sub>cryst</sub> in the unbound and UCPH<sub>101</sub>-bound state. The 1049 color code at the bottom shows the average relative fractional uptake measured in 1050 both domains after 10 sec (upper panel), 10 min (middle panel) and 1h (lower panel) labeling. Missing regions in the crystal structure are represented by dashed lines. 1051 1052 Peptides showing a statistically significant (p < 0.01) modification of deuterium 1053 uptake upon UCPH<sub>101</sub> binding are labeled. Uncovered regions are colored in light 1054 blue. 1055



1067Extended Data Figure 11 | Sulfur anomalous difference Fourier maps. Anomalous1068difference Fourier maps contoured at the  $2.8\sigma$  (pink mesh), from data collected at low1069energy X-rays (1.77 Å), show the correct sequence registry in both the TranD (orange,1070a) and the ScaD (teal, b).1071