Human members of the solute carrier 1 (SLC1) family of transporters take up excitatory neurotransmitters in the brain and amino acids in peripheral organs. Dysregulation of the function of SLC1 transporters is associated with neurodegenerative disorders and cancer. Here we present crystal structures of a thermostabilized human SLC1 transporter, the excitatory amino acid transporter 1 (EAAT1), with and without allosteric and competitive inhibitors bound. The structures reveal architectural features of the human transporters, such as intra- and extracellular domains that have potential roles in transport function, regulation by lipids and post-translational modifications. The coordination of the allosteric inhibitor in the structures and the change in the transporter dynamics measured by hydrogen–deuterium exchange mass spectrometry reveal a mechanism of inhibition, in which the transporter is locked in the outward-facing states of the transport cycle. Our results provide insights into the molecular mechanisms underlying the function and pharmacology of human SLC1 transporters.

SLC1 transporters constitute a large family of ion-coupled transporters present in all kingdoms of life. There are seven human SLC1 transporters (Extended Data Fig. 1) that have evolved to serve two specialized functions: in the central nervous system, SLC1 excitatory amino acid transporters (EAAT1–EAAT5) take up the neurotransmitter glutamate into the cell. In peripheral organs, EAATs take up glutamate and aspartate, while SLC1 neutral amino acid transporters (ASCT1 and ASCT2) exchange small amino acids between the extra- and intracellular compartments, contributing to the cellular solute homeostasis.

Glutamate is the most important excitatory transmitter in the mammalian brain and it has to be continuously pumped into the cytoplasm to allow for rounds of transmission and prevent cytotoxicity. This essential neurological function is carried out by EAAT1–EAAT5 transporters expressed at the plasma membrane of astrocytes and neurons. In particular, astroglial EAAT1 and EAAT2 orthologues are highly expressed in the hind- and forebrain, respectively, and are responsible for most of the glutamate uptake in the rodent brain. EAATs are powerful molecular pumps that can maintain up to 10^4-fold glutamate gradients by using energy stored in sodium, proton and potassium gradients. Remarkably, their dysregulation has been associated with several neurological diseases, including amyotrophic lateral sclerosis, ataxia, stroke, depression and glioma, making them important drug targets.

ASCTs are structurally related to EAATs, and function as sodium-dependent neutral amino acid exchangers at the plasma membrane. Importantly, ASCT2 is upregulated in several forms of cancer, such as melanoma, lung, prostate and breast cancer, and it is a key drug target in cancer therapy.

Despite the need for small compounds that selectively and allosterically modulate SLC1 human transporters, most of their pharmacology is based on substrate analogues that inhibit transport competitively. The only known selective allosteric modulators of SLC1 transporters are a series of non-competitive EAAT1-selective inhibitors, of which 2-amino-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile (UCPH101) is the best studied. However, its mechanism of action is still poorly understood at the molecular level.

In structural terms, most of our knowledge on the transport mechanism and pharmacology of SLC1 transporters comes from the prokaryotic homologue GltPh that has been crystallized in the main conformational states of the transport cycle, outward-facing states, inward-facing states, as well as in complex with a non-selective and competitive inhibitor of the EAATs. 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 UCPH101. The structures, and supporting functional data, show new architectural features of the EAATs and ASCTs, and unravel the allosteric mechanism of UCPH101-like inhibitors in atomic detail. Taken together, these structural data can prove useful for the design of allosteric compounds with improved selectivity for both EAATs and ASCTs.

**EAAT1 engineering and crystallization**

Purified wild-type EAAT1 lacks transport activity upon reconstitution in synthetic liposomes (Fig. 1a), and was recalcitrant to crystallization. To obtain functional protein suitable for crystallographic studies, we engineered a thermostable EAAT1 (known as EAAT1cryp) that shares an overall ~75% sequence identity with the wild type, and up to ~90% identity at the C-terminal core of the protein (Extended Data Fig. 1; Methods), where the transported substrate and coupled ions are expected to bind. Indeed, purified EAAT1cryp Reconstituted in liposomes showed robust glutamate uptake that depends on opposite gradients of sodium and potassium ions across the bilayer (Fig. 1a and Extended Data Fig. 2a), and was inhibited by the EAAT1-selective compound UCPH101 (half-maximum inhibitory concentration (IC50) 90% identity at the C-terminal core of the protein (Extended Data Fig. 1; Methods), where the transported substrate and coupled ions are expected to bind. Indeed, purified EAAT1cryp Reconstituted in liposomes showed robust glutamate uptake that depends on opposite gradients of sodium and potassium ions across the bilayer (Fig. 1a and Extended Data Fig. 2a), and was inhibited by the EAAT1-selective compound UCPH101 (half-maximum inhibitory concentration (IC50))...
radioactive l-glutamate by purified EAAT1 (grey), EAAT1 cryst (blue), and EAAT1cryst-II (red) reconstituted in liposomes. EAAT1cryst-II was used in the extra- and intra-liposomal solutions (yellow circles). UCPH101 was used in the extra- or intra-liposomal solutions (purple circles). The three Scad forming the TranD (orange) have been highlighted, and several helices and loops in the TranD (orange) have been removed. f, Domain organization diagram of EAAT1cryst monomer.

of 4.5 ± 0.3 μM (mean ± s.e.m.), Hill coefficient 0.92 ± 0.07 (Fig. 1b). These data show that the transport mechanism and pharmacological selectivity are conserved in EAAT1cryst.

Notably, EAAT1cryst crystallized in the presence of UCPH101, and we solved its inhibitor-bound structure (see Methods and Extended Data Table 1), but it was refractory to crystallization in the absence of UCPH101. To overcome this, we introduced Met231Ile and Phe235Ile mutations in the inhibitor-binding pocket (Extended Data Fig. 1), and solved the crystal structures of ‘EAAT1cryst-Il’ in both the presence and absence of the inhibitor (Extended Data Table 1). Purified EAAT1cryst-Il also showed robust sodium- and potassium-dependent glutamate uptake, and the UCPH101 IC50 value increased more than 30-fold (131 ± 38 μM, Hill coefficient 0.92 ± 0.0; Fig. 1a, b), as expected owing to the mutations in the inhibitor-binding pocket (see below).

Domain organization

The structure of EAAT1cryst shows a symmetric homotrimer in a substrate-bound and UCPH101-bound outward-facing conformation (Fig. 1c–e), with an overall GltPh-like fold21,22 (Extended Data Fig. 3). Each subunit is composed of two domains: a scaffold domain (Scad), including transmembrane helices TM1–TM2 and TM4–TM5; and a transport domain (TranD), including TM3, TM6–TM8 and re-entrant helical loops 1–2 (HP1–HP2; Fig. 1f). The three Scads form a compact central structure with a propeller-like shape that ensures the trimeric form of the transporter and anchors it to the membrane (Extended Data Fig. 4). The three TranDs are more peripheral and localize between the blades of the propeller, making protein contacts exclusively with the Scads of their own subunit. The TranD–Scad interface buries approximately 3,500 Å², including a conserved salt bridge between Glu256 and Lys364, through interactions of residues in the cytoplasmic parts of HP1, TM7 and TM3 (TranD), and TM2, TM4c and TM5 (Scad). On the extracellular side, additional contacts occurred between residues in HP2 and TM4 that are well conserved among human transporters (Extended Data Fig. 5).

Substrate and ion translocation in SLC1 transporters is thought to occur by large rigid-body movements of the TranD, relative to the static Scad, that move the cargo in an elevator-like fashion across the membrane22,30. Thus, during the isomerization to the inward-facing state, the TranD–Scad interface changes markedly on the TranD side, and the new features observed at this interface in EAAT1cryst might influence the distinct TranD dynamics in human SLC1 proteins.

Transport domain

One of the most remarkable architectural features of the EAAT1cryst TranD is in TM8, where there are deletions and insertions compared to prokaryotic homologues (Fig. 2 and Extended Data Fig. 3). In EAAT1cryst, TM8 can be divided into extracellular (TM8a), transmembrane (TM8b) and cytoplasmic (TM8c) helices. The loop connecting TM8a and HP2 is six residues shorter in human SLC1 transporters than in the prokaryotic counterparts, and brings their extracellular ends in close contact through hydrogen bonding and hydrophobic interactions (Fig. 2). HP2 is a dynamic element that controls the access of substrate and ions to their binding sites in the TranD24,31–33 and its interactions with TM8b probably have important roles in determining HP2 movements. Consistently, single cysteine mutations at positions along TM8a in EAAT1 (ref. 34) and in a rodent EAAT2 orthologue35 impaired glutamate transport, highlighting the importance of this extracellular region for function.

In TM8b, we found strong electron density for the substrate (L-aspartate) and one of the sodium ions (Na2) in similar sites to the structures of prokaryotic homologues24,29 (Fig. 2). Notably, the carbohydrate group of Asp456 (TM8b), which coordinates the α-amino group of the substrate, is also at hydrogen bond distance with the hydroxyl group of Ser343 (HP1). Moreover, the guanidinium group of Arg457 (TM8b) engages in hydrogen bonding with HP1 residue Gly341, and possibly Leu340 and Thr342 that point their backbone carbonyl oxygen atoms towards TM8b. Residues Ser343 and Arg457 are well conserved in human SLC1 transporters, and substitutions at equivalent positions in EAAT1 (Ser363 and Arg477)36 and EAAT3 (Arg445)37 inactivate transport. In addition, the loss-of-function mutation Arg445Trp in EAAT3, equivalent to Arg457 in EAAT1cryst, causes human dicarboxylic aminoaciduria38. Overall, the functional

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**Figure 1 | Function and architecture of EAAT1cryst.** a, b, Uptake of radioactive l-glutamate by purified EAAT1 (grey), EAAT1cryst (blue), and EAAT1cryst-II (red) reconstituted in liposomes. a, Transport was abolished when choline (Ch) was used in the extra- or intra-liposomal solutions (yellow circles). b, UCPH101 inhibits glutamate transport in a concentration-dependent manner. Plots in a and b depict an average of three independent experiments performed with duplicate measurements, and error bars represent s.e.m.

c, d, Structure of EAAT1cryst trimer viewed from the extracellular solution (c) and from the membrane (d), highlighting the Scad (teal) and TranD (orange). e, EAAT1cryst monomer viewed parallel to the membrane. The Scad is represented as solvent-accessible surface (teal), and several helices and loops in the TranD (orange) have been removed. f, Domain organization diagram of EAAT1cryst monomer.

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**Figure 2 | Transport domain.** EAAT1cryst TranD with substrate (spheres) and one sodium ion (purple sphere) bound. Omit F0–F1 densities contoured at 2.3σ (black mesh) show the position of the ligands. TM8a–c (blue) interacts with other regions on the TranD (orange), including HP1 (left inset), HP2 (top right inset), and TM3 and TM(7) (bottom right inset).
Figure 3 | Scaffold domain. a, TM4 (purple) makes extensive contacts with the ScaD of the neighbouring monomer (monomer 2, teal surface), and the TranD (orange) of the same subunit (monomer 1). The TranD of monomer 2, and several transmembrane helices of monomer 1 were removed for clarity of display. $F_o - F_c$ density in the HP2 and TM4 crevice is contoured at 2.7σ (red mesh). b, Two monomers of EAATcryst 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 $F_o - F_c$ density (purple mesh, contoured at 2.7σ).

Scaffold domain

The ScaD contains highly divergent regions, especially in TM4 (Extended Data Fig. 1), in which EAATcryst shows several unique architectural features. On the extracellular side, TM4a forms inter- and intra-subunit contacts with TM2 and HP2, respectively. Moreover, an amino acid insertion between TM4b and TM4c (TM4b–c loop) that appeared during the evolution of eukaryotic transporters protrudes into the central vestibule of the EAATcryst trimer (Fig. 3a and Extended Data Fig. 4). The TM4b–c loop forms the centre of the propeller, and makes extensive contacts within and between protomers. Although we could not model the outermost residues in the TM4b–c loop (Tyr200–Val210), they are expected to reach out to the bulk solvent exposing an N-glycosylation site (Asn204). Notably, all human SLC1 transporters contain predicted N-glycosylation sites in the TM4b–c loop, suggesting a conserved role of this loop in the post-translational processing of these proteins.

Figure 4 | UCPH101-binding site. a, Lateral view of EAAT1cryst monomer showing UCPH101 bound (pink) between the TranD (orange) and ScaD (teal). b, c, UCPH101 coordination and $F_o - F_c$ densities contoured at 2.0σ (blue mesh) in EAAT1cryst (b) and EAAT1cryst-II (c), respectively.

Studies and our structural data converge to suggest that interactions between conserved human residues at HP1 and TM8b are important to the correct folding and function of the transporters. On the cytoplasmic side, TM8 extends beyond the membrane through a hydrophilic helix (TM8c) that makes contact with TM3 and TM7a (Fig. 2). EAAT2 deletion mutants in this region have a deleterious effect on transport function and membrane trafficking. Accordingly, an EAAT1 deletion in TM8c decreased the rate of glutamate uptake by approximately 2-fold (Extended Data Fig. 2b). Indeed, the functional data and the TM8c amino acid conservation among EAATs underscore the pivotal role of this structural motif in protein folding and transport kinetics.

UCPH101-binding site

The structure of EAAT1cryst showed strong electron density for UCPH101 in a hydrophobic pocket between TM3, TM7 and TM4c at the TranD–ScaD interface, where the compound extends the interface by around 500 Å² (Fig. 1d, e and 4a, b). The chromene skeleton, the parental group of the UCPH series of compounds, is buried deeply in the domains interface, and coordinated by a direct ring-stacking interaction with Phe369 (TM7a), as well as hydrophobic interactions with Gly120 (TM3), Val373 (TM7a) and Met231 (TM4c) (Fig. 4b). In addition, the amine group of UCPH101 forms a hydrogen bond with the main-chain carbonyl of Phe369, while its carbonitrile group interacts with Tyr127 (TM3). The methoxy-phenyl and naphthalene groups appear partially facing the hydrocarbon core of the membrane. Yet, the former establishes hydrophobic interactions with Val124 (TM3), Val373 and Met231, whereas the latter is mainly coordinated by Phe235 (TM4c). Most of these residues are important for UCPH101 transport inhibition of an EAAT1 rodent orthologue. Furthermore, the Met231Ile/Phe235Ile double mutant (EAAT1cryst-II) showed a more than 30-fold increase in the UCPH101 IC50 value compared to EAAT1cryst in proteo-liposomes (Fig. 1b). Hence, there is excellent agreement between the crystallographic and functional data.

Several mechanistically relevant observations can be made about the UCPH101 binding pocket in EAAT1cryst: (1) it is more than 15 Å away from the substrate- and sodium-binding sites, suggesting that UCPH101 does not preclude extracellular substrate binding; (2) it faces the inner leaflet of the membrane, indicating that UCPH101 accesses its binding site from the lipid, and not the aqueous phase, when applied extracellularly; (3) it is fully contained in a single subunit, in agreement with the lack of cooperativity observed in proteo-liposome
(Fig. 1b) and cell assays\(^{20}\); and (4) a comparison of the EAAT1–EAAT5 sequences suggests that the main determinants of UCPH101 selectivity for EAAT1 are in TM4c, where Met231 and Phe235 are the only coordinating residues that differ between EAAT1 and all other EAATs (Extended Data Fig. 1). Consistently, the double-mutant EAAT1\textsuperscript{cryst-II} containing EAAT2 residues equivalent to Met231 and Phe235 shows a large increase in the IC\textsubscript{50} value.

**UCPH101–bound state**

To understand better the conformational changes of the transporter induced by UCPH101, we set out to determine the structure of the EAAT1\textsuperscript{cryst} UCPH101–bound state, but it was refractory to crystallization. Instead, we solved the structure of the EAAT1\textsuperscript{cryst-II} UCPH101–unbound state and the EAAT1\textsuperscript{cryst-II} UCPH101–bound state for comparison, using an excess of the compound in the crystallization conditions (see Methods).

The EAAT1\textsuperscript{cryst-II} UCPH101–bound state is nearly identical to that of the EAAT1\textsuperscript{cryst} with the exception of the Ile231 and Ile235 side chains, and a roughly 2 Å movement of UCPH101, methoxy-phenyl and naphthalene groups away from them (Fig. 4c and Extended Data Fig. 6a). However, the EAAT1\textsuperscript{cryst-II} UCPH101–unbound structure shows notable differences with the bound state. First, there is no excess electron density for UCPH101, and the side chain of Phe369 moves outward by as much as 1.9 Å, partly occupying the volume of the UCPH101 chromene group (Fig. 4c). Second, there is a small rigid-body movement of the entire TranD that shifts by as much as 0.7 Å, compared to the UCPH101–bound structures (Extended Data Fig. 6b). This conformational change shows the EAAT1\textsuperscript{cryst} TranD is able to move as rigid bodies relative to the ScaD, and highlights the importance of such movements for the function of the human transporters, as it has been shown for GltPh\textsuperscript{22,23}.

The structural changes observed in the UCPH101–unbound structure unambiguously demonstrate that the assigned binding pocket of UCPH101 is correct, and that within the restricted environment of the crystal lattice, UCPH101 induces both local and global conformational changes of the transporter that optimize its coordination in an outward-facing state.

**Transport domain dynamics**

The coordination of UCPH101 in the crystal structures, wedged in the TranD–ScaD interface, as well as the effect of the Met231Ile–Phe235Ile mutations on its potency strongly suggest that UCPH101 inhibits transport by trapping the transporter in an outward-facing state. Consistently, the rigid-body movements of the TranD to isomerize the transporter into the inward-facing state would separate the coordinating residues in the TranD from those in the ScaD, and disrupt the UCPH101 coordination. Hence, under equilibrium conditions in which the transporters are sampling outward- and inward-facing states, the expected effect of UCPH101 binding is to shift the equilibrium in favour of the outward-facing state.

To gain insights into the effects of UCPH101 binding to the transporters at equilibrium, we probed the detergent-solubilized EAAT1\textsuperscript{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\textsuperscript{45,46}.

We compared the HDX behaviour of the EAAT1\textsuperscript{cryst} in the presence and absence of UCPH101. Overall, the deuterium uptake pattern of EAAT1\textsuperscript{cryst} shows dynamic structural elements in both the TranD and ScaD (Extended Data Figs 7, 8), 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 (peptides 490–522) termini (Extended Data Fig. 9).

Binding of UCPH101 markedly decreased deuterium uptake in several areas of the TranD including its binding pocket (residues 112–123 and 370–374) and the surrounding area (residues 354–369), whereas it left the uptake in the ScaD unchanged (Fig. 5a). It also decreased deuterium uptake in distant residues (336–349 and 420–430) at the tips of HP1 and HP2 involved in substrate coordination and occlusion, suggesting that UCPH101 induces conformational changes in the transporter upon binding. To gain insights into the nature of those conformational changes, we first compared the TranD areas in which UCPH101 decreased uptake with those buried at the interface with ScaD in the EAAT1\textsuperscript{cryst} structure, and found that they correlate remarkably well (Fig. 5a–c). Second, we built a model of the EAAT1\textsuperscript{cryst} inward-facing state, based on a recently solved structure of GltPh\textsuperscript{23}, to assess the changes in solvent accessibility in a possible transition between the inward- and outward-facing states (Fig. 5c, d). The comparison between the structure and the model shows that the bound outward-facing state, are coloured dark blue for comparison with a. c, d, Comparison between EAAT1\textsuperscript{cryst} structure in the UCPH101–bound outward-facing state (c) and a model of an inward-facing state based on the GltPh \textsuperscript{unlocked} state (PDB code 4X2S) (d), in which the TranD (coloured as in a) undergoes a large rigid body movement towards the cytoplasm.
UCPH101-modified areas detected by HDX-MS transit as rigid bodies from being solvent-exposed, in the inward-facing state, to buried at the TranD–ScaD interface, in the outward-facing state. Such conformational change is expected to decrease the dynamics of α-helices and/or the solvent accessibility of the loops in the UCPH101-modified areas, and is thus consistent with the observed decrease in deuterium uptake. Overall, the HDX-MS and structural analysis support the stabilization of the outward-facing state at the expense of the inward-facing state(s), induced by UCPH101.

**UCPH101- and TBOATFB-bound state**

The distant position of UCPH101 from the substrate and HP2, a structural element that controls extracellular access to the substrate-binding site24, suggests that the UCPH101-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 EAAT1crys in complex with both UCPH101 and (2S,3S)-3-[4-(trifluoromethyl)benzoylamino]benzyl oxy]aspartate (TBOATFB), a potent and non-selective TBOA derivative24, at 3.7 Å resolution (Extended Data Table 1).

Overall, the UCPH101–TBOATFB-bound structure is similar to that of the UCPH101-bound state, with the exception that HP2 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 compounds24.

In the substrate-binding site, we observed excess electron density for the bulky TBOATFB (Fig. 6c), but owing to the lack of resolution, we were not able to unambiguously orient the compound. Hence, we initially positioned the TBOA moiety of TBOATFB using the TBOA-bound GltPh structure as a guide. After crystallographic refinement, the additional benzamino and trifluoromethyl groups of TBOATFB in EAAT1crys localized in a hydrophobic cavity mainly formed by residues in HP1b and TM7a, and possibly by residues in TM2 and TM4c, probably explaining the observed 1,500-fold increase in EAAT1 inhibitory potency compared with TBOA27.

In the TBOATFB-bound structure, UCPH101 is bound with an identical coordination to the substrate-bound state. Therefore, the EAAT1crys structures show that UCPH101 binding at its allosteric site does not preclude the movements of HP2 involved in substrate and sodium binding from the extracellular solution.

**Inhibitory mechanisms of EAAT1**

The structural and functional EAAT1crys data reveal architectural features of human SLC1 transporters, and a molecular mechanism of allosteric inhibition. The binding of UCPH101 ‘glues’ the TranD to the ScaD in the outward-facing states, and precludes substrate translocation but not binding from the extracellular solution (Fig. 7). Non-conserved residues in the ScaD determined UCPH101 selectivity for EAAT1. This inhibitory mechanism contrasts with that of substrate-analogue inhibitors such as TBOA. The binding pocket of TBOA-like compounds overlaps with the conserved substrate-binding site24, and explains the lack of selectivity among glutamate transporters and the inhibition of substrate binding observed on either side of the membrane48,49.

The unique inhibitory mechanism of UCPH101 makes it an extremely valuable pharmacological tool to study the conformational changes that EAAT1 undergoes upon substrate and ion binding. Remarkably, the UCPH101 allosteric binding site 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.

**Figure 6 | UCPH101–TBOATFB-bound EAAT1crys structure.** a, The movement of HP2 (purple) partly exposes the substrate-binding pocket to the solvent and shows a molecule of TBOATFB (purple sticks) bound to it. UCPH101 is also observed in this structure (pink sticks). b, The tip of HP2 moves as much as 9.5 Å in the UCPH101–TBOATFB-bound (purple) compared to the UCPH101-bound (orange) structures, placing the carbonyl oxygen of Ala420 away from Na2 (green sphere). c, Omit map F0 – F1 density for the TBOATFB molecule is contoured at 2.3σ (black mesh), and some of the residues at Van der Waals or hydrogen-bond distance from the compound are represented as sticks.

**Figure 7 | EAAT1 inhibitory mechanisms.** Schematic representation of TBOA-like competitive and UCPH101-like allosteric inhibition. Competitive inhibitors occupy the substrate-binding pocket and preclude substrate binding. UCPH101-like compounds bind at the TranD–ScaD interface and block the movement of the TranD (orange) relative to the ScaD (teal).
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METHODS

No statistical methods were used to predetermine sample size. Experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Construction optimization. We used fluorescence-detection size-exclusion chromatography (FSEC) to screen solubilization conditions and EAAT1 variants fused to enhanced green fluorescent protein (eGFP). EAAT1 N-terminal fusions solubilized in dodecanoyl succine (DDS, Anatscare) were found to have good solubility and membrane retention by FSEC in clear lysates. However, EAAT1 loses its transport activity and chromatographic monodispersity upon purification. To increase its stability, we used consensus mutagenesis16, and screened EAAT1 variants with different consensus mutations in the predicted transmembrane helices by FSEC. The apparent melting temperature (Tm) of the most stable EAAT1 construct was more than 20°C higher than that of the wild-type EAAT1, but the mutated transporter was still refractory to crystallization. We reasoned that the divergent extracellular region between TM3–4 could interfere with crystallization, and changed it for the equivalent region in ASC167, the shortest one among human SLC1 members (Extended Data Fig. 1). In addition, we mutated the two predicted N-glycosylation sites of the transporter (Asn155Thr and Asn204Thr mutations) to improve crystallizability further.

Expression and purification. All constructs were introduced into pcDNA3.1 (+) (Invitrogen) with N-terminal 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 3 mM TBOATFB (Tocris) before the UCPH101 unbound structure, the protocol was identical, but the allosteric inhibitor HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM TCEP, 5% glycerol, 0.25% decanol succine (Sigma), 0.05% CHS, 300 µM TBOATFB, and 100 µM UCPH101. Protein samples after the solubilization step were kept on ice or at 4°C at all times.

Crystallization and structure determination. Purified protein was concentrated to 3.5–4.0 mg ml−1 and 1 mM UCPH101 was added in experiments with the inhibitor-bound transporters. Initial vapour diffusion crystallization screens were performed using a robot (Greiner) and reservoir solution in sitting drops, dispensed by a mosquito robot (TTP labtech) in 96-well Greiner plates. The purified transporters form three-dimensional crystals in several conditions containing low molecular mass polyelectrolyte glycols. The best-diffacting crystals were obtained after manual optimization using 1.6 µl hanging drops at 4°C, obtained by mixing equal volumes of protein supplemented with 0.2% n-octyl-β-d-glucopyranoside (BOG, Anatscare) and 0.04% CHS, and reservoir solutions containing 100 mM Tris, pH 8.2, 50 mM CaCl2, 50 mM BaCl2 and 28–30% PEG 400. Crystals appeared after 43–48 h and reached their maximum size after 1 week. Crystals were flash-frozen in liquid nitrogen before X-ray diffraction data collection without any further cryo protection.

X-ray diffraction data were collected at beamline PROXIMA-1 at the SOLEIL synchrotron (St Aubin, France), and at beamlines ID23-1 and ID23-2, as well as ID29 and ID30B at the European Synchrotron Radiation Facility (Grenoble, France). In general, 2–3 datasets from single crystals were collected, and indexed, integrated, scaled and merged using the XDS package22. Owing to the anisotropic nature of the diffraction data, the DEBYE and STARANISO programs were applied using the STARANISO server (http://staraniso.globalphasing.org). The software performs an anisotropic cut-off of merged intensity data, a Bayesian estimation of the structure amplitudes, and applies an anisotropic correction to the data. Extended Data Table 1 shows the refinement statistics for the full sets of reflections truncated at the best high-resolution along the h, k or l axis, values given by AIMLESS23, before the anisotropic corrections computed by the STARANISO software. The corrected anisotropic amplitudes were then used for molecular replacement in PHASER24, using the scaffold and transport domains of GltPh (PDB code 2NW1) as independent search models. The initial electron density maps were clearly interpretable, and the final model was obtained through rounds of manual building in COOT25 and refinement in BUSTER26, until reaching good crystallographic statistics and stereochemistry (Extended Data Table 1). The model contains one EAAT1cryst monomer per asymmetric unit and most of the EAAT1cryst polypeptide (residues 37–487), with the exception of some residues in the extracellular loops between TM3–4a, TM4b–4c, TM5–6 and TM7b–HP2a. The stereochemical properties of the final models were analysed with the Molprobity server (http://molprobity.biochem.duke.edu/). At least 95% of the residues in all models are in the Ramachandran favoured region. Protein interfaces were analysed 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. Molecular Graphics System, Schrodinger, LLC.

Radioactive substrate transport assays. Unilamellar liposomes were made at 9:1 molar ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and CHS, in a buffer containing 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl and 1 mM t-Asp. The transporters were purified as described above, but instead of being resuspended in 2% detergent, liposomes were treated with 2.2 mM valproic acid (Sigma) 12 h after diluting the cultures. The eluted eGFP-transporter fusion was concentrated to 1–2 mg ml−1 using 100-kDa cutoff membranes (Millipore), and digested with His-tagged PreScission protease overnight at 4°C. The protease was removed by reverse Ni-NTA (Qiagen) affinity chromatography, and the flow through containing the transporter was concentrated to 500 µl, ultra-centrifuged (86,900g, 20 min), and applied to a Superose 12 (10/300) gel filtration column (GE Healthcare) equilibrated with 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM t-Asp, 1 mM TCEP, 5% glycerol, 0.05% DDS, 0.01% CHS and 25 µM UCPH101. To obtain the UCPH101 unbound structure, the protocol was identical, but the allosteric inhibitor was omitted from all buffers. To obtain the UCPH101–TBOATFB-bound structure, the protein sample was supplemented with 3 mM TBOATFB (Tocris) before the injection in the gel filtration column equilibrated with 50 mM HEPES/Tris-base, pH 7.4, 200 mM NaCl, 1 mM TCEP, 5% glycerol, 0.25% decanol succine (Sigma), 0.05% CHS, 300 µM TBOATFB and 100 µM UCPH101. The stereochemical properties of the final models were analysed with the Molprobity server (http://molprobity.biochem.duke.edu/). At least 95% of the residues in all models are in the Ramachandran favoured region. Protein interfaces were analysed with the PISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html). Functional assays were done with Superpose in the CCP4 suite. All structural figures were prepared with PyMOL. Molecular Graphics System, Schrodinger, LLC.

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into ice-cold quench buffer (50 mM HEPES/Tris-base, pH 7.4, 200 mM ChCl, and 2.5% glycerol), followed by immediate filtration and wash on nitrocellulose 0.22 μm filters (Millipore). Radioactivity was quantified by liquid scintillation using a Tri-Carb 3110TR counter (PerkinElmer). For the UCPH101 titrations, proteo-liposomes were both pre-incubated for 20 min at room temperature, and assayed in the presence of UCPH101. Background radioactivity was estimated from protein-free liposomes, and subtracted from the uptake data. Data was fitted to a Hill equation of the form:

\[ F = F_{\infty} + \Delta F_{\infty} / (1 + (IC_{50}/[\text{UCPH101}])^n) \]

in which \( F_{\infty} \) is the final level of inhibition, \( \Delta F_{\infty} \) is the final amplitude of the UCPH101 effect, and \( n \) is the Hill coefficient.

To titrate the rate of l-glutamate transport by EAAT1cryst, proteo-liposomes were assayed in the presence of 0, 5, 50 or 200 μM UCPH101 -glutamate substituted with 1, 5, 5, or 5 μM \(^{14}\text{C}\)-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.

For the cell-based transport uptake, cells were collected 36 h after transfection, and washed three times and resuspended at a density of 50 × 10^6 cells ml⁻¹ in 11 mM HEPES/Tris-base, pH 7.4, 140 mM ChCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM d-glucose, for immediate use. The uptake assay was performed similarly to the one described for the proteo-liposomes, but using a reaction buffer containing 11 mM HEPES/Tris-base, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 10 mM d-glucose, 50 μM l-glutamate, and 5 μM \(^{14}\text{C}\)-l-glutamate, and 0.8-μm nitrocellulose filters. Background radioactivity was estimated from cells transfected with empty vector, and subtracted from the uptake data.

**Hydrogen–deuterium exchange mass spectrometry.** HDMS 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, pH 7.4, 1 mM l-Asp, 0.5 mM TCEP, 0.0632% DDS, 0.01264% CHS and 5% glycerol.

The purified EAAT1cryst was incubated in ice for 30 min with 2.2% DMSO at a monomer concentration of 5.2 μM, in the presence and absence of 102 μM UCPH101, respectively. Before labelling, 10 μl of the unbound and UCPH101-bound EAAT1cryst solution was equilibrated for 10 min at room temperature. Deuterium exchange was initiated by adding 40 μl of D₂O buffer (50 mM HEPES, pH 7.3, 200 mM NaCl, 1 mM l-Asp, 3% glycerol, 0.0632% DDS, 0.01264% CHS, 0.5 mM TCEP) supplemented or not with 102 μM UCPH101. Assuming that the dissociation constant (\( K_d \)) and IC₅₀ values of UCPH101 are similar, around 96% of the transporters would remain bound during deuterium labelling. Aliquots of 10.4 pmol of protein were removed at defined deuterium exchange time points (from 10 s to 60 min) and quenched upon mixing with an ice-cold acidic solution (0.75% formic acid, 5% glycerol) to decrease the pH to 2.6. Quenched samples were immediately snap-frozen in liquid N₂ and stored at −80°C until analysis.

Before mass analysis, quenched samples were rapidly thawed and immediately injected into a cooled nanoACQUITY UPLC HDX system (Waters corp.) maintained at 0°C. Protein samples (8.6 pmol) were online digested for 2 min at 20°C using an in-house packed immobilized pepsin cartridge (2.0 × 20 mm, 66 μl bed volume). The resulting peptides were trapped and desalted onto a C18 Trap column (VanGuard BEH 1.7 μm, 2.1 × 5 mm, Waters corp.) at a flow rate of 100 μl min⁻¹ 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⁻¹ using an ACQUITY UPLC BEH C18 analytical column (1.7 μm, 1 × 100 mm, Waters corp.). After each run, the pepin cartridge was manually cleaned with two consecutive washes of 1% formic acid, 5% acetonitrile, 1.5 M guanidinium chloride, pH 2.5. Blank injections were performed between each run to confirm the absence of carry-over.

Mass spectra were acquired in resolution and positive mode on a Synapt G2-Si HDMS mass spectrometer (Waters corp.) equipped with a standard electrospray ionization source, as described previously. Peptides were identified from undeuterated protein samples acquired in MS² mode by database searching in ProteinLynX Global Server 3.0 (Waters corp.). Each fragmentation spectrum was manually inspected for assignment validation. Deuterium uptake values were calculated for each peptide using DynamX 3.0 (Waters corp.). Only one unique charge state was considered per peptide and no adjustment was made for back-exchange. HDMS results are reported as relative deuterium uptake values expressed in mass unit or fractional exchange. A statistical analysis was performed with MEMHDX using a False Discovery Rate of 1%. Data availability. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with accession codes SLM (EAAT1cryst-UCPH101), 5MJU (EAAT1cryst-UCPH101-TBOA-7FB), 5LM4 (EAAT1cryst-II-UCPH101) and SLLU (EAAT1cryst-I2). All other data are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Alignment of human SLC1 transporters.
Amino acid sequences of EAAT1–EAAT5, ASCT1–ASCT2 and EAAT1cryt are compared. The boundaries of the α-helices (cylinders) in the TranD (orange) and ScaD (teal) seen in the EAAT1cryt structure are shown. To confer crystallizability, the region between TM3 and TM4c (arrows) from ASCT2 was transferred to a thermally stabilized EAAT1. To improve crystal formation in the absence of UPCH101, Met231Ile and Phe235Ile mutations (circles) were introduced to generate EAAT1cryt-II. These substitutions are found in EAAT2. Other residues involved in UPCH101 coordination are more conserved (triangles). Sequences were aligned with Jalview59.

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Extended Data Figure 2 | EAAT1\textsubscript{cryst} and EAAT1 glutamate uptake.

(a) Initial rates of L-glutamate uptake from purified EAAT1\textsubscript{cryst} reconstituted in liposomes. The solid line is the fit of a Michaelis–Menten equation to the data with $K_m = 21 \pm 10 \mu\text{M}$ and $V_{\text{max}} = 13 \pm 1 \text{ pmol } \mu\text{g}^{-1} \text{ protein min}^{-1}$. The graph is the mean of three independent experiments, and error bars represent s.e.m.

(b) L-glutamate uptake was measured in HEK293F cells expressing wild-type EAAT1 (black circles) and a truncated mutant beyond Glu501 (red symbols). The initial rate of uptake decreased by approximately 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 $0.16 \pm 0.03 \text{ min}^{-1}$ and $0.08 \pm 0.03 \text{ min}^{-1}$ for EAAT1 and the truncated mutant, respectively. The graphs are means of four independent experiments performed in duplicate. Error bars represent the s.e.m.
Extended Data Figure 3 | EAAT1\textsubscript{cryst} and GltPh structural comparison. 

\(a\), \(b\), EAAT1\textsubscript{cryst} aligns to a monomer of GltPh (PDB code 2NWL), with a 
\(C_\alpha\) root mean squared deviation (r.m.s.d.) value of 1.4 Å. The ScaDs (EAAT1\textsubscript{cryst} teal, and GltPh purple; \(a\)), and TranDs (EAAT1\textsubscript{cryst} 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) buries 3,000 Å² in the trimerization interface through extensive contacts with the two other subunits (teal and purple surfaces), including six intermolecular salt bridges (shown as green sticks for the monomer in black). The surface area buried at the trimeric interface in the other two monomers is coloured light pink. Only residues that contribute ≥10 Å² of buried surface area are highlighted.
Extended Data Figure 5 | TranD–ScaD interface. a, b, EAAT1 crys monomer viewed from the membrane (solid black line). Residues in the TranD (coloured black) bury 1,760 Å² 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.
Extended Data Figure 6 | Superposition of EAAT1<sub>cryst</sub> and EAAT1<sub>cryst-II</sub> structures. a, b, The transport domains of EAAT1<sub>cryst</sub> (teal) and EAAT1<sub>cryst-II</sub> (pink) UCPH<sub>101</sub>-bound structures superimpose accurately after aligning their scaffold domains (a). The overall C<sub>α</sub> r.m.s.d. value was 0.3 Å. However, the same alignment done with EAAT1<sub>cryst-II</sub> UCPH<sub>101</sub>-bound and-unbound structures shows a small but global movement of the transport domain (b), with a small increase in the overall C<sub>α</sub> r.m.s.d. of 0.1 Å. c, d, Anomalous difference Fourier maps contoured at 2.8σ (pink mesh), from data collected at low energy X-rays (1.77 Å), show the correct sequence registry in both the TranD (orange, a) and the ScaD (teal, b).
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>cryst</sub> sequence corresponds to a unique peptide. The 57 peptides coloured blue were further selected for HDX-MS data extraction and analysis. The two additional N-terminal residues (that is, Gly and Pro) that remain after protein purification are also shown. The transmembrane helices of the TranD (orange) and the ScaD (cyan) are indicated above the sequence.
Extended Data Figure 8 | UCPH101 effect on the local hydrogen exchange behaviour of EAAT1cryst. a, HDX profiles of EAAT1cryst (see Methods) in the apo unbound (top) and UCPH101-bound (bottom) state. The relative fractional uptake determined for each peptide and at each time point is plotted as a function of peptide position. The black to red lines correspond to data acquired from 10 s up to 1 h, respectively.

b, The fractional uptake difference plot was generated by subtracting the deuterium uptake values in the UCPH101-unbound from those in the bound state. Negative uptake difference indicates an UCPH101-induced decrease in amide hydrogen exchange. Each dot corresponds to an average of three independent HDX-MS experiments. The four regions (labelled 1–4) that show a statistically significant modification (Wald test; $P < 0.01$) of deuterium uptake upon binding of UCPH101 are highlighted in grey.
Extended Data Figure 9 | HDX-MS results mapped on the crystal structure of ScaD and TranD of EAAT1_cry in the unbound and UCPH_{101}-bound state. The colour code at the bottom shows the average relative fractional uptake measured in both domains after 10 s (top), 10 min (middle) and 1 h (bottom) labelling. Missing regions in the crystal structure are represented by dashed lines. Peptides that show a statistically significant (Wald test; \( P < 0.01 \)) modification of deuterium uptake upon UCPH_{101} binding are labelled. Uncovered regions are coloured light blue.
## Extended Data Table 1 | Data collection and refinement statistics

|                      | EAAT1<sub>cryst</sub>  | EAAT1<sub>cryst</sub>-II | EAAT1<sub>cryst</sub>-II | EAAT1<sub>cryst</sub> UCPH<sub>101</sub> and TBOA<sub>TCH</sub> Bound |
|----------------------|------------------------|---------------------------|---------------------------|-------------------------------------------------|
| **Space group**      | *P6<sub>3</sub>*       | *P6<sub>3</sub>*          | *P6<sub>3</sub>*          | *P6<sub>3</sub>*                                   |
| **Cell dimensions**  |                        |                           |                           |                                                 |
| α=b, c (Å)           | 123.27, 89.87          | 123.11, 89.62             | 123.32, 89.57             | 124.33, 90.81                                     |
| α=β, γ (°)           | 90.0, 120.0            | 90.0, 120.0               | 90.0, 120.0               | 90.0, 120.0                                      |
| Wavelength           | 0.979                  | 0.976                     | 1.009                     | 0.977                                           |
| Resolution (Å)       | 45.89-3.25 (3.34-3.25) | 45.82-3.1 (3.18-3.32)     | 45.87-3.32 (3.41-3.32)    | 46.31-3.71 (3.81-3.71)                           |
| **Anisotropy direction** |                        |                            |                           |                                                 |
| Resolution where CC<sub>1/2</sub> ≥0.3 |                        |                            |                           |                                                 |
| Overall (Å)          | 3.37                   | 3.10                      | 3.32                      | 3.71                                            |
| along h, k axis (Å)  | 3.75                   | 3.68                      | 3.85                      | 4.35                                            |
| along l axis (Å)     | **3.25**               | **3.10**                  | **3.32**                  | **3.71**                                         |
| Measured reflections | 333978 (24261)         | 290672 (21906)            | 326273 (21829)            | 141904 (11144)                                   |
| Unique reflections   | 12338 (902)            | 14115 (1032)              | 11556 (834)               | 8570 (628)                                       |
| Completeness (%)     | 100 (100)              | 99.9 (99.9)               | 100 (100)                 | 99.9 (100.0)                                     |
| CC<sub>1/2</sub>     | 0.99 (0.22)            | 1 (0.43)                  | 0.99 (0.31)               | 0.99 (0.373)                                     |
| I/σ(I)               | 11.7 (0.7)             | 15.8 (0.8)                | 13.0 (0.7)                | 12.1 (0.9)                                       |
| R<sub>merge</sub>    | 0.19 (6.8)             | 0.10 (6.72)               | 0.15 (8.03)               | 0.14 (3.78)                                      |
| Redundancy           | 27.1 (26.9)            | 20.6 (21.2)               | 28.2 (26.2)               | 16.6 (17.7)                                      |

### Structure determination

**Refinement**

| Resolution cut-off (Å) | 34.50-3.25 | 45.80-3.10 | 20.00-3.32 | 25.00-3.71 |
|------------------------|------------|------------|------------|------------|
| No. of Work / Test reflections | 9888 / 474 | 10725 / 528 | 9251 / 445 | 6860 / 684 |
| R<sub>cryst</sub> (%) / R<sub>free</sub> (%) | 21.9 / 24.0 | 21.7 / 25.9 | 20.9 / 25.3 | 22.7 / 25.4 |
| No. of protein atoms   | 3018       | 2960       | 2995       | 3008       |
| No. of heteroatoms     | 42         | 42         | 10         | 62         |
| B factors (Å<sup>2</sup>) |            |            |            |            |
| Protein                | 130.5      | 111.8      | 137.0      | 135.5      |
| Heteroatoms            | 112.9      | 99.5       | 125.6      | 132.7      |

R.m.s. deviations from ideal

| Bond lengths (Å) | 0.009 | 0.01  | 0.009 | 0.009 |
| Bond angles (°)  | 1.06  | 1.12  | 1.05  | 1.03  |

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One crystal was used to collect diffraction datasets for each structure, except in the EAAT1<sub>cryst</sub> UCPH<sub>101</sub> and TBOA<sub>TCH</sub> bound structure, in which datasets from three crystals were merged. 5% of reflections were used for calculation of R<sub>free</sub> values, except for the EAAT1<sub>cryst</sub> UCPH<sub>101</sub>-TBOA<sub>TCH</sub> bound structure in which 10% was used owing to the lower number of total reflections. *Values in parentheses are for the highest-resolution shell. **The anisotropy directions where computed with AIMLESS.