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The ion-coupling mechanism of human excitatory amino acid transporters

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **3rd May 2021**

Dear Dr Reyes,

Thank you for the submission of your manuscript (EMBOJ-2021-108341) to The EMBO Journal. Please accept my apologies for the unusual delay with the peer-review of your work due to protracted referee input and detailed discussions in the team. Your manuscript has been sent to two reviewers and we have received reports from both of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In more detail, referee #1 points to concerns on insufficient proof for a functional role of Ca2+ binding on transport substrate function and the level of insights provided into requirement of extra- versus intracellular Ca2+ and its entry paths (ref#1, pt.1). Referee #2 agrees in that the proposed competition between Ca2+ and Na+ needs is not satisfactorily supported at this stage and needs consolidation via numerous experiments (ref#2, pt.1). Further, this reviewer requests refinement of the K+ location annotated to the EAAT structure (ref#2, pt.2).

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I have to emphasize though that given the required additional support for key aspects of the study, we will need a strong revision to move forward towards publication of this article at the EMBO Journal.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

I this context I also want to point to our adjusted GTA We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact us at any time to discuss an adapted revision plan for your manuscript should you need additional time, and also if you see a paper with related content published elsewhere.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).

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Referee #1:

The study provides a comprehensive analysis of the ion coupling mechanism of the glutamate transporter, EAAT1. This topic has been studied for many years initially using electrophysiological and site-directed mutagenesis methods, and in more recent years with structural biology and molecular dynamics simulations. During this time a number of conflicting proposals were put forward. This current study has managed to bring all these studies together and provide a detailed analysis of the process that is consistent with many earlier proposals. It is wonderful to see it all come together in a way that ties it all together.
Whilst the majority of the work is clearly presented and very convincing, the one aspect of the work that is superficially addressed is the proposal that Ca2+ binds to the transporter to presumably modulate the function of the transporter. This is a very novel proposal and needs further clarification. Convincing evidence is provided that Ca2+ binds to an outward facing conformation of the transporter at the Na3 site and that Ca2+ is not transported by EAAT1. But, this implies that Ca2+ is able to influence the function of the transporter. There are a few points that need clarification:
1. If Ca2+ binds to the outward facing conformation of the transporter with a Kd of 2 mM, then you would expect that Ca2+ would impact on the function of the transporter under physiological conditions. This idea should be explored in greater depth. I would like to see the impact of a Ca2+ titration on the rate of uptake of substrate. Presumably, this would be via extracellular Ca2+, but it would be worthwhile testing the effect of Ca2+ in both the intracellular and extracellular solutions.
2. Whilst the above experiment may address the issue as to whether the relevant Ca2+ is intracellular or extracellular, it would be nice to use molecular dynamics simulations to get a better understanding of the entry pathways of Ca2+ into the Na3 site.
3. In the discussion of the potential role of Ca2+, the authors have speculated as to the impact of variations in intracellular Ca2+. If Ca2+ is binding to the outward facing state, then extracellular Ca2+ is more physiologically relevant. This needs to be clarified.
This additional work on the role of Ca2+ suggested may be better suited to a separate study, but I am happy to leave this suggestion to the authors

Referee #2:

Glutamate is the most abundant excitatory neurotransmitter in the CNS and the glutamate transporters (EAATs 1-5) are important in the re-uptake of glutamate from the synaptic cleft. Central to the mechanism of the EAATs is the coupling between Na+, H+ and glutamate as well as K+, which catalyses the transition back to the outward-state after releasing sodium and substrate. The manuscript by the Reyes lab builds upon their previous crystal structure of EAAT1 with a combination of spectroscopy, crystal structures in complex with anomalous scatters mimicking Na+/K+ sites as well as a mutant deficient in proton coupling, a cryo EM structure of EAAT1, transport assays and HDX-MS measurements. By combining these methods they have been able to validate the location of the three Na+ sites, the elevator-structural transitions, as well as the likely proton-coupling site. These are important results, even if they are confirmatory rather than novel. Perhaps most interesting is the position of the counter K+ ion and the competition seen with Ca2+. Overall, while I see the potential scientific impact of this interesting study, it needs some further controls for supporting their main findings.

Major points.

1. The analysis and calculation of Na+ binding affinity is assessed by a tryptophan residue, which is located ca. 10 Å from the Na+ sites. The obvious question is whether the change in tryptophan fluorescence faithfully reports on Na+ binding. It is important too:

I) show the raw traces of the tryptophan fluorescence with and without Na+.
ii) the change in Trp fluorescence with a mutant that can no longer bind Na+.
iii) the Trp fluorescence change with L-aspartate after Na+ addition
iv) the Trp fluorescence with Na+ and in the presence of the outward-inhibitor UCPH
v) mutations that abolish Ca2+ binding.
vi) binding affinities for Ca2+ with and without UCPH since the conclusion is that Ca2+ binds on the inside.

These experiments will confirm that the Trp is mostly reporting on Na+ binding and the proposed competition with the Ca2+ site. The outward-inhibitor is a useful control as they should restrict dynamics also contributing to the fluorescent signal.

2. K+ binding catalyses the return step of the transport cycle and, as such, binds on the "inside". However, structural studies have been carried out for the "outward" facing crystal structure and K+ could not be modelled in the inward-facing cryo EM structure. The obvious question is whether the Rb+ signals reports on the "physiological transported K+" site in the trapped "outward" state obtained in the presence of an inhibitor. I think it would be informative to compare the Kd for K+ with and without the UCPH inhibitor to verify if the binding affinities are symmetric. If so, this would give support for the current K+ location in the detergent structure.

Minor points

1. The W287 is not thought to contribute to a change in fluorescence as it is located on the scaffold. However, movement of the transport domain could still change its environment and report a change in fluorescence. Indeed it is positioned similar to the F273W mutant used in the bacterial homologue GltPh to monitor Na+ binding by Trp fluorescence. An appropriate control would be to further mutate this residue to phenylalanine.

2. It was concluded that Na+ binding was not pH dependent as the apparent Kd didnt change from pH 6 to 10, but substrate binding was pH dependent, Fig. 2B. However, the pH dependance of substate binding was measured in the presence of only 0.5 mM NaCl, which is far below the Kd for sodium at 20 mM. As such, can this conclusion really be made from this data?

3. The conclusion that E373 is the proton coupling site seems to have been embellished a little here, since its pretty much the only candidate that could fulfil such a role and the previous studies showing this are clear. Indeed no other residues were experimentally tested here. I agree its an important to validate this, but some re-writing seems required to more faithfully represent the current standing in the field, e.g., it would make sense to point out the repositioning of E406 between the apo and bound structures in the EAAT3 cryo-EM structure (Qiu et al, 2021) and in MD simulations (Kortzak et al., 2019).

Referee #1:

The study provides a comprehensive analysis of the ion coupling mechanism of the glutamate transporter, EAAT1. This topic has been studied for many years initially using electrophysiological and site-directed mutagenesis methods, and in more recent years with structural biology and molecular dynamics simulations. During this time a number of conflicting proposals were put forward. This current study has managed to bring all these studies together and provide a detailed analysis of the process that is consistent with many earlier proposals. It is wonderful to see it all come together in a way that ties it all together.
We thank Referee #1 for pointing out the in-depth and comprehensive nature of our work.

Whilst the majority of the work is clearly presented and very convincing, the one aspect of the work that is superficially addressed is the proposal that Ca2+ binds to the transporter to presumably modulate the function of the transporter. This is a very novel proposal and needs further clarification. Convincing evidence is provided that Ca2+ binds to an outward facing conformation of the transporter at the Na3 site and that Ca2+ is not transported by EAAT1. But, this implies that Ca2+ is able to influence the function of the transporter.
We agree with Referee #1 that the effect of Ca2+ on glutamate transport has not been characterized to its full extent. This will require probing kinetics, as well as steady-state transport varying intra- and extracellular [Ca2+], respectively, and it is out of the scope of the current manuscript, which already constitutes an in-depth functional and structural characterization of ion-coupled transport.

There are a few points that need clarification:

1. If Ca2+ binds to the outward facing conformation of the transporter with a Kd of 2 mM, then you would expect that Ca2+ would impact on the function of the transporter under physiological conditions. This idea should be explored in greater depth. I would like to see the impact of a Ca2+ titration on the rate of uptake of substrate. Presumably, this would be via extracellular Ca2+, but it would be worthwhile testing the effect of Ca2+ in both the intracellular and extracellular solutions.
Purified $EAAT1_{CRYST}$ in detergent solutions is at equilibrium between outward- and inward-facing states (Canul-Tec et al., Nature 2017), hence the Ca2+ binding parameters that we determined reflect, in principle, binding to both states. Upon suggestion of Referee #2 (Major poin 1), during revision we have performed binding experiments in the presence of UCPH101, an allosteric inhibitor that traps the transporters in outward-facing states. We did not observe significant changes in Na+, Na+/transmitter, or Ca2+ binding parameters, respectively, suggesting that ligands bind similarly to outward- and inward-facing states. These results are expected, as the tranD moves across the membrane as nearly rigid-body exposing the same binding sites to opposite sides of the membrane. Moreover, “symmetry” in ligand binding affinity to outward- and inward-facing states has been observed in prokaryotic homolog GltPh (Reyes et al., Nat. Struct. Mol. Biol. 2013). Hence, we conclude that Ca2+ is able to bind to outward- and inward-facing states, respectively, with apparent KD values ~ 2 mM.
Regarding Ca2+ effect on transmitter transport, as stated above, we strongly believe that it deserves a separate study involving time-resolved techniques like patch-clamp electrophysiology. However, to shed some light on this problem, during revision we performed two types of experiments:
i) In cells expressing EAAT1CRYST, we measured steady-state uptake at saturating (60 uM) and sub-saturating (20 uM) glutamate concentrations, respectively, in the presence of 5 mM

extracellular Ca2+, or after substituting Ca2+ for Mg2+ (a divalent cation that does not compete with Na+ or yields Trp fluorescence changes in purified EAAT1CRYST; not shown). Under these conditions, we did not observe significant differences between Ca2+ and Mg2+ buffers. These results are somehow expected, as extracellular Ca2+ binding is too weak to out-compete 3Na+/1H+/1transmitter coupled binding. However, our uptake assay is not able to resolve potential Ca2+ effects on EAAT1 kinetics.
ii) We also compared EAAT1CRYST-mediated uptake in liposomes loaded with K+ and different Ca2+ concentrations. Interestingly, we observed an inhibitory effect of Ca2+ with IC50 ~ 5 mM. This effect could be due to facilitation of transmitter re-binding, as Ca2+ and transmitter are thermodynamically coupled, supporting the idea that under physiological conditions with cytoplasmic [glutamate] in the mM range, Ca2+/transmitter coupled binding could possibly affect transport rate.
In summary, although additional experimental work is needed to determine if Ca2+ plays modulatory role(s) in EAAT-mediated glutamate transport at the synapse, we think that is important to make the readers aware of this possibility. In the revised manuscript, we have edited the section on "Ca2+ binding at Na3" to show and discuss results on Ca2+ binding in the presence of UCPH101 and to mutant $D400_{380}N$ at Na3, respectively (revised Fig. 5B), as well as the effect of Ca2+ in cells and liposomes (revised Fig. EV5).

2. Whilst the above experiment may address the issue as to whether the relevant Ca2+ is intracellular or extracellular, it would be nice to use molecular dynamics simulations to get a better understanding of the entry pathways of Ca2+ into the Na3 site.
We agree with Referee #1 that MD simulations and other experiments will be important to characterize the role of Ca2+ on glutamate transport, but they are outside the scope of this work.

3. In the discussion of the potential role of Ca2+, the authors have speculated as to the impact of variations in intracellular Ca2+. If Ca2+ is binding to the outward facing state, then extracellular Ca2+ is more physiologically relevant. This needs to be clarified.
We regret the lack of clarity in this regard. EAAT1CRYST Ca2+/Ba2+ bound structure is in an outward facing state because of both crystal-contacts and the presence of allosteric inhibitor UCPH101 that stabilizes such states.
As explained above, and in the revised manuscript (Results section: Ca2+ binding to Na3), in a cellular context with ~120 Na+ outside, Ca2+ binding is too weak to out-compete 3Na+/1H+/1transmitter coupled binding. However, it seems more likely that due to Ca2+/transmitter thermodynamic coupling, Ca2+ could aid cytoplasmic glutamate (at high [glutamate]) to re-bind.

This additional work on the role of Ca2+ suggested may be better suited to a separate study, but I am happy to leave this suggestion to the authors
We appreciate the suggestion by Referee #1 to leave the additional work related to the role of Ca2+ to a separate study.

Referee #2:

Glutamate is the most abundant excitatory neurotransmitter in the CNS and the glutamate transporters (EAATs 1-5) are important in the re-uptake of glutamate from the synaptic cleft. Central to the mechanism of the EAATs is the coupling between Na+, H+ and glutamate as well as K+, which catalyses the transition back to the outward-state after releasing sodium

and substrate. The manuscript by the Reyes lab builds upon their previous crystal structure of EAAT1 with a combination of spectroscopy, crystal structures in complex with anomalous scatters mimicking Na+/K+ sites as well as a mutant deficient in proton coupling, a cryo EM structure of EAAT1, transport assays and HDX-MS measurements. By combining these methods they have been able to validate the location of the three Na+ sites, the elevator-structural transitions, as well as the likely proton-coupling site. These are important results, even if they are confirmatory rather than novel. Perhaps most interesting is the position of the counter K+ ion and the competition seen with Ca2+. Overall, while I see the potential scientific impact of this interesting study, it needs some further controls for supporting their main findings.

We thank Referee #2 for highlighting the impact of our work, and for suggesting important controls to strengthen our findings. During revision, we carried out those controls, that include probing ligands binding in the presence of UCPH101, a mutant at Na3 site that impairs both Na+ and Ca2+ binding, as well as Phe mutations of Trp residues. The results are described below and included in the revised manuscript

Major points.

1. The analysis and calculation of Na+ binding affinity is assessed by a tryptophan residue, which is located ca. 10 Å from the Na+ sites. The obvious question is whether the change in tryptophan fluorescence faithfully reports on Na+ binding. It is important to:

I) show the raw traces of the tryptophan fluorescence with and without Na+.

Revised Fig. 1A shows tryptophan-fluorescence changes induced by Na+ (blue) and K+ (black), respectively. Na+ induces robust fluorescence changes that enable titrations and quantification of apparent KD values. In contrast, K+ induces minor fluorescence changes, precluding such measurements, and showing that Trp-fluorescence reports on Na+-binding.

ii) the change in Trp fluorescence with a mutant that can no longer bind Na+.

Revised Fig. 1B shows that mutation $D400_{380}N$ at Na3 greatly impairs Na binding, further re-assuring that fluorescence signals report on Na+ binding to conserved sites observed in the structure.

iii) the Trp fluorescence change with L-aspartate after Na+ addition

Revised Fig. 1C shows fluorescence changes of Asp titrations in the presence (blue) and absence (black) of Na+, respectively. In the former, but not in the latter, we observed significant fluorescence changes, as expected for Na+/transmitter coupled binding.

iv) the Trp fluorescence with Na+ and in the presence of the outward-inhibitor UCPH

Revised Fig. 1B and 1D show that saturating concentrations of $UCPH_{101}$ inhibitor do not affect significantly Na+ (red circles) or Asp KD (red circle) values, respectively, suggesting that outward- and inward-facing apo transporters bind Na+, and Na+/transmitter with similar affinities, as it has been reported in prokaryotic homolog GltPh (Reyes et al., Nat. Struct. Mol. Biol. 2013). These results further indicate that tranD movements associated to membrane translocation do not contribute greatly to steady-state fluorescence signal.

v) mutations that abolish Ca2+ binding.

Revised Fig. 4B shows that mutation $D400_{380}N$ at Na3 greatly impairs Ca2+ binding, supporting Ca2+ occupancy of this site, as well as competition with Na+.

vi) binding affinities for Ca2+ with and without UCPH since the conclusion is that Ca2+ binds on the inside.

We regret the lack of clarity regarding Ca2+ binding sidedness. The following is our response to Referee #1, who also raised concern about this issue:

Purified $EAAT1_{CRYST}$ in detergent solutions is at equilibrium between outward- and inward-facing states (Canul-Tec et al., Nature 2017), hence the Ca2+ binding parameters that we determined reflect, in principle, binding to both states. Upon suggestion of Referee #2 (Major poin 1), during revision we have performed binding experiments in the presence of UCPH101, an allosteric inhibitor that traps the transporters in outward-facing states. We did not observe significant changes in Na+, Na+/transmitter, or Ca2+ binding parameters, respectively, suggesting that ligands bind similarly to outward- and inward-facing states. These results are expected, as the tranD moves across the membrane as nearly rigid-body exposing the same binding sites to opposite sides of the membrane. Moreover, "symmetry" in ligand binding affinity to outward- and inward-facing states has been observed in prokaryotic homolog GltPh (Reyes et al., Nat. Struct. Mol. Biol. 2013). Hence, we conclude that Ca2+ is able to bind to outward- and inward-facing states, respectively, with apparent KD values ~ 2 mM.

Regarding Ca2+ effect on transmitter transport, as stated above, we strongly believe that it deserves a separate study involving time-resolved techniques like patch-clamp electrophysiology. However, to shed some light on this problem, during revision we performed two types of experiments:

i) In cells expressing EAAT1CRYST, we measured steady-state uptake at saturating (60 uM) and sub-saturating (20 uM) glutamate concentrations, respectively, in the presence of 5 mM extracellular Ca2+, or after substituting Ca2+ for Mg2+ (a divalent cation that does not compete with Na+ or yields Trp fluorescence changes in purified EAAT1CRYST; not shown). Under these conditions, we did not observe significant differences between Ca2+ and Mg2+ buffers. These results are somehow expected, as extracellular Ca2+ binding is too weak to out-compete 3Na+/1H+/1transmitter coupled binding. However, our uptake assay is not able to resolve potential Ca2+ effects on EAAT1 kinetics.

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In summary, although additional experimental work is needed to determine if Ca2+ plays modulatory role(s) in EAAT-mediated glutamate transport at the synapse, we think that is important to make the readers aware of this possibility. In the revised manuscript, we have edited the section on "Ca2+ binding at Na3" to show and discuss results on Ca2+ binding in the presence of UCPH101 and to mutant $D400_{380}N$ at Na3, respectively (revised Fig. 5B), as well as the effect of Ca2+ in cells and liposomes (revised Fig. EV5).

These experiments will confirm that the Trp is mostly reporting on Na+ binding and the proposed competition with the Ca2+ site. The outward-inhibitor is a useful control as they should restrict dynamics also contributing to the fluorescent signal.

We thank Referee #2 for suggesting these important controls to confirm that Trp fluorescence signals arise from changes in tranD dynamics upon Na+ and Ca2+ binding involving Na3, as opposed to tranD rigid-body movements, and that ligands bind outward- and inward-facing states with similar apparent affinities in detergent solutions.

2. K+ binding catalyses the return step of the transport cycle and, as such, binds on the "inside". However, structural studies have been carried out for the "outward" facing crystal structure and K+ could not be modelled in the inward-facing cryo EM structure. The obvious question is whether the Rb+ signals reports on the "physiological transported K+" site in the trapped "outward" state obtained in the presence of an inhibitor. I think it would be informative to compare the Kd for K+ with and without the UCPH inhibitor to verify if the binding affinities are symmetric. If so, this would give support for the current K+ location in the detergent structure.

As it occurs with Na+/H+/transmitter binding, K+ binding to transporters is not a specific feature of outward- or inward-facing states, and to the best of our knowledge, there is no experimental evidence suggesting that K+ binds different sites from inside and outside, respectively. In contrast, K+ binding to a single site from either outside or inside is expected because the tranD moves nearly as a rigid-body to opposite sides of the membrane.

Under physiological ionic gradients, K+ binds on the inside simply because cytoplasmic [K+] >> [Na+]. Indeed, it is well established that when ionic gradients are experimentally inverted, the transport cycle works in reverse mode, and external K+ binds on the outside and is transported into the cytoplasm. Indeed, early electrophysiological determination of K+ transport stoichiometry by the Attwell (Nature 1988; this reference has now been added to the manuscript), and Kavanaugh (Nature 1996) labs was done by titrating the effect of external (not cytoplasmic) K+ on transport. Hence, K+ binds both outward- and inward-facing states, and the two K+-bound states are physiologically relevant.

Regarding Rb+ binding, we demonstrate in the manuscript that Rb+ is a functional K+ analog, as it is counter-transported in liposome uptake assays (revised Fig. 4A). Hence, we don’t see strong reasons to believe that Rb+ interacts differently than K+ with the transporter. Regretfully, as shown in revised Fig. 1A, K+ does not yield robust fluorescence changes to enable titrations and KD determination. We agree with Referee #2 that verifying similar K+ affinities in the presence and absence of UCPH101 could provide some support to the location of the K+ binding site observed in the structure. However, differential K+ affinities under those conditions would not directly argue against a single K+ site to bind the ion from outside and inside, respectively.

(Additional comment via email exchange with Editor):

Referee #2: Basically the authors are saying that they cannot measure K+ binding by tryptophan fluorescence, but they can measure Na+. Its a little surprising since the location of the proposed K+ position is close to the Na+ binding sites from what I can recall. If the other controls for the Trp fluorescence assay that I suggested work out (as expected), this information will provide some further confidence in the assay. Based on this data one can then speculate why K+ is not able to to be detected by the Trp binding assay. Regarding the impact of the paper I think that if they cannot validate the binding mode for K+ (which is thought to be specific to the inward and not the outward-facing state) then they will need to tone down the K+ section and/or add a caveat to mention that it possible K+ may bind differently in the inward-facing conformation.

Indeed, Na+ (as well as Ca2+ and transmitter), but not K+, induce robust Trp-fluorescence changes (revised Fig. 1). Although, we do not understand the structural details underlying Trp-fluorescence changes in EAAT1CRYST, individual Phe mutations of W267 and W473 (suggested by Referee #2, see below Minor point #1) show that both Trp residues are required to probe Na+ and transmitter binding with this assay, and suggest that Trp fluorescence changes arise from short- and long-range effects. Moreover, binding experiments in the presence and absence of UCPH101 (also suggested by Referee #2) suggest that elevator-like

movements of the tranD do not contribute to Trp fluorescence. As for the lack of fluorescence signal associated to K+ binding, we can only speculate that tranD dynamics around the two Trp are not very different between Apo and K+ bound states, as opposed to Apo and Na+/transmitter bound states. Consistently, changes in HDX profiles between K+-bound and Na+/transmitter bound states show differential protein dynamics under the two conditions.
In the revised manuscript, we have extensively edited the section on Trp-fluorescence binding assay, and show lack of fluorescence signal associated to K+, as well as describe the effect of Phe mutations. However, considering that: K+ binding is not specific of inward-facing states; tranD tranlocates ligands binding sites as a rigid-body; there is no experimental evidence suggesting that K+ binds different sites in outward and inward-facing states, respectively; the Rb+-bound structure represents the first structural determination of a counter-transported ion binding site; we kindly disagree to "tone-down" the K+ section or suggest that K+ may bind differently from the inside.

Minor points

1. The W287 is not thought to contribute to a change in fluorescence as it is located on the scaffold. However, movement of the transport domain could still change its environment and report a change in fluorescence. Indeed it is positioned similar to the F273W mutant used in the bacterial homologue GltPh to monitor Na+ binding by Trp fluorescence. An appropriate control would be to further mutate this residue to phenylalanine.
In order to gain insight on the contribution of Trp residues to the fluorescence signal, and as suggested by Referee #2 (Minor point 1, see below), we probed individual Phe mutants. As expected, W473F mutation in the transport domain abolished Na+ induced fluorescence changes, consistent with W473F being the main fluorescence reporter. Unexpectedly, W267F mutation in the scaffold domain significantly decreased Trp fluorescence changes suggesting that W267 is required to preserve changes in tranD dynamics upon binding, or that it senses binding through long-range conformational changes, or both. In the revised manuscript, we have extensively edited this section, and removed the discussion associated to distances between Trp residues and substrate binding site.

2. It was concluded that Na+ binding was not pH dependent as the apparent Kd didnt change from pH 6 to 10, but substrate binding was pH dependent, Fig. 2B. However, the pH dependance of substate binding was measured in the presence of only 0.5 mM NaCl, which is far below the Kd for sodium at 20 mM. As such, can this conclusion really be made from this data?
In our view, this conclusion can only be made at low Na+ concentrations. Both Na+ and protons lead to formation of substrate-occluded states. Promoting such states with high Na+ would mask the coupling efficiency of the proton, and vice versa the coupling efficiency of Na+ is lower at acidic pH. In the absence of substrate, Na+ Kd is ~20 mM, but due to their thermodynamic coupling in the presence of transmitter Na+ Kd decreases. This has been shown in prokaryotic homologs (Reyes et al., Nat. Struct. Mol. Biol. 2013).
In other words, the thermodynamic coupling efficiency of one ion to the substrate is a function of other coupled ions, and is maximal at lower concentrations of the latter.

3. The conclusion that E373 is the proton coupling site seems to have been embellished a little here, since its pretty much the only candidate that could fulfil such a role and the previous studies showing this are clear. Indeed no other residues were experimentally tested here. I agree its an important to validate this, but some re-writing seems required to more faithfully represent the current standing in the field, e.g., it would make sense to point out the

repositioning of E406 between the apo and bound structures in the EAAT3 cryo-EM structure (Qiu et al, 2021) and in MD simulations (Kortzak et al., 2019).

We would like to insist that there are other residues that could contribute to proton-coupling in the tranD beyond E406, like Y405. In our work, we discarded other candidates based on a comprehensive alignment of EAAT and ASCT mammalian orthologs (revised Fig EV1), lack of proton effect on Na binding (revised Fig. 3A), as well as mutagenesis (Y405F) (revised Fig. 3C). In addition, we do cite and discuss previous work on proton-coupling, not only related to EAAT3 cryo-EM structure (Qiu et al, 2021) and MD simulations (Heinzelmann and Kuyucak, 2014), but more importantly related to the seminal work by the Grewer laboratory (Grewer et al., JBC 2003) that pointed to equivalent E406 residue as the main proton acceptor in transport (see line 182 in revised manuscript "Our results agree well with early studies of rodent ortholog EAAC1 showing that equivalent mutation to $E406_{386}Q$ (E373Q) impairs pH-dependence of apparent glutamate-binding (Grewer et al., 2003), as well as with molecular dynamic simulations of transmitter binding (Heinzelmann and Kuyucak, 2014), and support the role of E406 carboxylate as the main proton acceptor in the transport cycle".

Regarding repositioning of E406, we have edited the revised manuscript (line 389) to emphasize on previous reports: "The close proximity of conserved $R479_{459}$ to K_{CT} argues that electrostatic shielding of its sidechain is required for K^+ occlusion and translocation, as suggested by reported apo EAAT3 structure (Qiu et al., 2021) and MD simulations (Kortzak et al., 2019), and hydration of the tranD core is likely key to this process."

Finally, we would like to highlight that in our work, we present the first measurements of H+-coupling efficiency showing that at least ~60% of the H+ binding energy is coupled to substrate binding, and not to other conformational changes of the transport cycle, and that E406Q mutant disrupts this coupling. We also provide structural explanations on how E406 could contribute to stabilize transmitter occlusion through H-bonding with HP2, as well as how E406 contributes to the K+ mechanism by shielding K_{CT} from neighboring R479. These conclusions could not be resolved in previous reports, among other things because the position of the K+ binding site was not determined experimentally using structural-biology techniques.

Dear Dr. Reyes,

Thank you for submitting your revised manuscript (EMBOJ-2021-108341R) to The EMBO Journal, as well as your patience with our response. Your amended study was sent back to the reviewers for re-evaluation, and we have received comments from both of them, which I enclose below.

The referees stated that their issues have been comprehensively resolved and they are now broadly in favour of publication, pending minor revision.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining minor issue stated by referee #2 carefully by adjusting the text and data where appropriate. Further, we need you to consider a number of points related to formatting and data deposition as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Introduce a separate 'Conflict of Interest' section and move it before the references.

>> Release privacy from the PDB/EMD datasets and provide the data processing script as publically accessible information. Add http links on the datasets to the data availability section.

>> Update the bioRxiv citation (Punjabi et al., 2019) as journal publication in the reference list.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 28th Dec 2021.

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Referee #1:

The revised manuscript is significantly improved and I have no major concerns. Whilst the study of Na+/K+/H+ and substrate interactions is very thorough, the main message of the paper will be - that we should also consider Ca2+. This will certainly prompt further investigation because at this point it is a tantalising addition to the subject but not fully explored.

Referee #2:

Overall, I think the authors have done a excellent job and addressing the main technical concerns I had in the previous submission. Importantly, the additional controls showing that Trp fluoresce is reporting on Na+/transmitter binding, with no response to either K+ or L-Asp titrations in the absence of Na+. I only have one further comment below.

Minor points:

Lines 94 to 98 it is written:
"contains two tryptophan residues, W287 in the scaD, and W473 in the tranD. Individual phenylalanine mutants W287F and W473F decreased (delta F/F0 ~5%), and abolished, respectively, Na+/transmitter induced fluorescence signal."

However, I couldn't seem to find the data for the W287F and W473F controls in any of the figures? Please include them if they are not there.

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We agree with Referee #1, and as mention in the revised manuscript, understanding the role of extra and/or intracellular Ca2+ in transmitter transport requires further experimentation.

Referee #2:

Overall, I think the authors have done a excellent job and addressing the main technical concerns I had in the previous submission. Importantly, the additional controls showing that Trp fluoresce is reporting on Na+/transmitter binding, with no response to either K+ or L-Asp titrations in the absence of Na+. I only have one further comment below.

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However, I couldn't seem to find the data for the W287F and W473F controls in any of the figures? Please include them if they are not there.

The results of Trp to Phe mutations are now included in Supp. Fig. 1.

Dear Dr Reyes,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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Corresponding Author Name: Nicolas Reyes
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
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- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
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- definitions of statistical methods and measures:
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  - are tests one-sided or two-sided?
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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