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The high-energy transition state of the glutamate transporter homologue GltPh

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1st Editorial Decision 17th Jun 2020

Dear Dr Huysmans, dear Dr Boudker,

Thank you again for the submission of your manuscript (EMBOJ-2020-105415) to The EMBO Journal and in addition providing us with a preliminary revision plan. Thank you also for your patience with my response, which got delayed due to detailed discussions in the team regarding your response. As mentioned earlier, your study has been sent to three referees, and we have received reports from all of them, which I enclose below.

The referees acknowledge the potential interest and novelty of your work, although they also express major concerns. While referees #2 and #3 are overall more positive, referee #1 raises major reservations regarding the claims made on differential translocation rates and points to insufficient time resolution as a major technical limitation (ref#1, pts.1,2). Further this referee remains unconvinced as to the predictive value and translational perspective of allosteric modulation (ref#1, pts. 5,6).

We realise that you would - judging from the information provided in the point-by-point letter - be potentially able to address the issues raised by the referees in a revised version of the manuscript.

I judge the comments of the referees to be generally reasonable and can - based on your sensible preliminary response - offer to invite you to revise your manuscript experimentally to address the referees' concerns.

Please feel free to contact me if you have any questions or need further input on the referee comments.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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
Editor
The EMBO Journal

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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:
http://bit.ly/EMBOPressFigurePreparationGuideline

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Method) that follows the model below (see also https://www.embopress.org/page/journal/14602075/authorguide#availabilityofpublishedmaterial). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets (and computer code) produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14602075/authorguide#referencesformat

IMPORTANT: When you send the revision we will require
- 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 (http://emboj.embopress.org/authorguide).
- Expanded View files (replacing Supplementary Information)
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 15th Sep 2020.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In this manuscript Huysmans et al. study the energetics of the major conformational change during glutamate transport, the translocation of the mobile transport domain. The authors initially use bioinformatic approaches together with transporter reconstitution and radioactive tracerflux experiments to identify gain-of-function mutants of the transporter. These gain-of-function mutations - that increase transport rates to up to 10 - 20 times - were then analyzed with a smFRET techniques that groups of Blanchard and Boudker have intensively used in the past.

Measurements of dwell times on inward- and outward-facing conformation as well as of transition frequencies were used for a linear free energy relationship analysis that revealed close structural resemblance of a high-energy transition state with the inward-facing conformation. The authors propose an approach for allosteric modulation of these transporters based on these analyses.

This paper addresses a very important question - the molecular basis of isoform-specific differences in glutamate transporters, with potentially important applications in medicine. However, I cannot see the contribution of the paper to develop novel allosteric modulators of mammalian glutamate transporters, and I have several technical concerns.

1. My major concern is the limited time resolution of the smFRET technique (p. 10 "While the macroscopic rates of the transport domain translocation are slow, translocation of the transport domain is faster than the resolution of our smFRET recordings (10-100 ms)." Faster transitions into intermediate conformation that cannot be resolved might significantly modify the results of the linear free energy relationship analysis. The authors should test possible consequences of this limitation. In single channel recordings of ion channels methods have been developed to correct for such events (Ball et al. (1990). Proc Biol Sci, 242, 61-67; Blatz et al. (1986). Biophys J 49, 967-980). The authors might want to use such approaches to test the validity of their conclusions.

2. With limited time resolution it appears difficult to me to distinguish high transition frequencies from longer residence times. Since some of the mutations are located in HP2 and might affect the likelihood of HP2 closure, this is not a trivial problem. K+ binding was recently shown to stabilize HP2 closure (Kortzak et al. (2019). EMBO J 38, e101468), and the comparison of K+ and Choline+ might provide information about the role of the limited time resolution on correct quantification of dwell times and transitions frequencies.

3. The kinetic heterogeneity is biophysically interesting, but a major limitation for the LFER analysis. The authors state on p. 10 "... we hypothesize that the kinetic heterogeneity exhibited by GltPh is indicative of a highly rugged energy landscape and the existence of long-lived conformations with distinct kinetic properties." Is the LFER analysis still applicable to such energy landscape? In ion channels (Grosman et al. (2000). Nature 403, 773-776), proteins with only one time constants in dwell time analysis were analyzed, and here I find this approach quite logic. I would recommend more discussion of possible limitations of the analysis for the particular GltPh energy landscape.

4. The authors use their analysis "to gain insight into the nature of the most commonly used activation barrier during transport domain motions". For this the authors now choose protein with "typical" behavior. Please provide a statistical analysis for WT and each of the mutants, with dwell times and transitions frequencies for each measurements, and, most importantly, of the identification of the "typical" behavior from these data.

5. The authors state in their abstract that "Based on these analyses, we propose an approach for allosteric modulation of these transporters." Dysfunctional EAATs play a role in certain diseases, and pharmacological EAAT modification might be useful for treating certain acquired diseases. However, stabilizing high-energy transition states is a very generic concept commonly used to increase enzymatic activity, and not a new "approach". Moreover, the new results only allow predictions about the function of allosteric modulators on GltPh; no predictions can be made about the high energy transition states of the EAATs. GltPh is kinetically very different from the EAATs, in which the K+-bound re-translocation represents the rate-limiting step. Most importantly, the transition state for the rate-limiting process in EAATs is unclear; hence application of the concepts developed here on EAATs is speculation. Therefore, I suggest removing this section. The paper

does not provide any new insights into potential modification of EAAT function. No prediction can be made about the high energy transition states of the EAATs.

6. The authors speculate that substrate release instead of translocation becomes rate limiting in the fast mutants (p. 7). This would be an important conclusion, but it is currently not supported by any experimental data. The authors should test this hypothesis experimentally.

Minor points
1. p.3: "These studies suggested that the translocation of the substrate-loaded transport domain from the OFS to the IFS is the rate limited step of the cycle.." Fluorescence spectroscopy and fast substrate application revealed very slow processes associated with Na+ association (Hanelt et al. (2015). J Biol Chem 290, 15962-15972) in GltPh, which are absent in VCF measurements on EAAC1 (Larsson et al. (2004). Proc.Natl.Acad.Sci.U.S.A. 101, 3951-3956). From these findings I would conclude that Na+ association and associated conformational changes are rate-limiting in GltPh, and that evolutionary optimization of these processes accelerated glutamate transport in mammalian transporters. I cannot see how smFRET can disprove such a conclusion, since only dwell times in each of the two states o the transport domain can be assessed. Please discuss this point in more detail.

2. p. 14: ".consistent with the return of the apo transport domain into the OFS being the rate-limiting step of the cycle." In the EAATs, re-translocation is obligatorily K+-bound.

3. I would like to see the smFRET data at higher time resolution, maybe the authors can show some examples for selected traces in the supplementals.

Referee #2:

General sumary:

This study by Huysmans and colleagues employs single molecule FRET experiments to obtain real-time recordings of transport-associated conformational rearrangements in single protomers of a trimeric glutamate transporter protein. This approach allows measuring single-molecule transition rates for conformational switches between outward facing and inward facing states (OFS and IFS) of a protomer. The analysis identifies the OFS to IFS step as the rate limiting step of the cycle. The authors then introduce perturbations that alter overall cycling rate, such as addition of transported ligands (L-aspartate and Na+) or insertion of point mutations into positions hinted by clever cross-species sequence comparisons, and analyze the effects on individual transition rates. They find that the perturbations predominantly affect the rate of the forward (OFS to IFS) transition. Using linear free energy relationship (LFER) analysis they conclude that the high-energy transition state (TS) that the protomer needs to overcome to complete the rate-limiting isomerization step structurally resembles the IFS conformation. A practical inference of exceptional interest is that small molecules that bind tighter in the IFS conformation are expected to stabilize the TS of the rate-limiting step, and should therefore act as allosteric activators with potential for therapeutic application. The work exposed in this paper is not only a great technical feat, but is also conceptually elegant, and the conclusions are rock solid. Below are a few questions/suggestions the authors might consider to address, to improve clarity in certain places of the manuscript.

Specific comments:

1.) P2, 2nd par: "LFERs correlate... From these correlations, one can infer whether the TS is structurally more similar to the initial or the final conformation."
More precisely, one can infer whether in the TS the perturbed region of the protein resembles its initial or final conformation.

2.) Protein labeling. If I understand the cartoon in Fig. S2B correctly, each trimer was labeled with one donor dye molecule, one acceptor dye molecule, and one biotin-PEG-maleimide anchor. How was this fixed stoichiometry achieved? Or was labeling random, as expected, but this particular population was the only population that generated a FRET signal under TIRF conditions? Please clarify.

3.) P6: "In the presence of 10 mM blocker... we still observed a transition frequency of 0.03 {plus minus} 0.01 s-1... these transitions originated from ~8-17 % of all molecules and constituted an apparent background. When this small sub-population was subtracted... the resulting transition frequency for the WT GltPh... reduced to ~0.01 s-1"
How is this "background" interpreted? What was the rationale for subtracting the rate of the blocker-insensitive subpopulation from the measured rates? If there is a rationale, is it meaningful to measure a rate of 0.01 s-1 over a background of 0.03 {plus minus} 0.01 s-1?

4.) P7: "substrate release can become rate-limiting in more dynamic mutants"
Comparing Figs. S4 and S6, it seems that the dwell time of the IFS state is the same, whether or not substrate is present on the intracellular side. This suggests that the rates of the IFS -> OFS transition for the substrate-bound and the ligand-free protomer (i.e., rates for the two leftward horizontal arrows in Fig. 1B) are very similar. (BTW, such rates are also used in the simulations in Fig. S7B, left panel.) If that is the case, how could substrate dissociation delay the IFS -> OFS transition?
5.) P11, 4th par: "we estimated the rate constants, k(OFS to IFS) and k(IFS to OFS), from the inverse of the mean OFS and IFS lifetimes"
Are the rate constants k(OFS to IFS) and k(IFS to OFS) meant to denote protomer transition rates, as illustrated in Fig. 7B? If so, then the two should be calculated differently from the mean dwell times of the two FRET levels. If I understand correctly, the analysis was done on segments in which transitions occured only between E(FRET) values of ~0.4 and ~0.6, corresponding to OFS/OFS and IFS/OFS arrangements, respectively, of the two FRET labeled subunits. The mean dwell time of the IFS/OFS state is equal to the mean life time of the IFS state of a protomer (the IFS/OFS state is terminated when the protomer which is in the IFS state flips back to OFS): thus, k(IFS to OFS) = 1/tau(IFS/OFS). In contrast, the mean life time of the OFS/OFS state is half that of the OFS state of a protomer (since the OFS/OFS state is terminated by flipping of any one of two protomers): thus, k(OFS to IFS) = 1/2xtau(OFS/OFS). Has this fact been taken into account?

6.) Fig. 7A vs. B: In panel B the rate for the IFS -> OFS transition is slightly larger for the substrate-bound (2 s-1) compared to the unliganded (1.6 s-1) protomer. In contrast, in the black (control) energy diagram in panel A, the barrier is drawn higher for the former step (right side of 2nd barrier) than for the latter (left side of 4th barrier).

Minor:

7.) P2, line 8: "transporters activity" should be "transporter activities"

8.) P3, line 7: "rate-limited" should be "rate-limiting"

9.) P5, line 2: please indicate that the KM refers to that of aspartate (as opposed to Na+)

10.) P5, line 6: "M362A" - in Fig. 1F M362V is shown

11.) P5, line 2 from bottom: "increases transport rate" should be "increases in transport rate"

12.) P11, lines 2 and 3: "the forward" and "the reverse" - delete "the"

13.) P24, line 5: please define "transition frequency". Is this the frequency of transitions either way, or the frequency of cycles?

14.) Fig. 5A-B legend: "lifetimes of... the molecules showing no transitions before photobleaching (black bars)"
The meaning of the black bars is unclear. If there were no transitions, how could dwell times be obtained?

15.) Fig. 5C legend: "molecules falling within 30 % of the most populated bins of the 2D-histograms (yellow to red)"
Maybe identify the corresponding area of the 2-D histogram with a box?

16.) Fig. 7 legend: Please define NAMs (negative allosteric modulators).

17.) Fig. 1F: Please explain abscissa units. What does a negative fold change mean? Does a "-5-fold change" mean a 5-fold decrease?

18.) Fig. S1B: It is a difficult visual task to discern the substrate from the highlighted side chains. Maybe use a single, more contrasting color for the substrate?

19.) Fig. S2C, bottom panel, labels on the right: I don't understand why the label for E(FRET)~0.4 is "OFS/OFS + OFS/IFS". Shouldn't this label be "OFS/OFS", and the label for E(FRET)~0.6 "IFS/OFS + OFS/IFS"? Or did I miss something here?

20.) Fig. S5 legend: "superimposition" should be "superposition" (twice)

21.) Fig. S10: see comments 14.) and 15.)

Referee #3:

This is a very nice manuscript that describes the application of an elegant methods to determine equilibrium transitions in apo and substrate bound GltPh transporters. The results indicate that the transition state occurs late on the translocation pathway and resembles the inward-facing configuration. This is an important finding that adds to our structural picture of how these transporters operate, which is impossible to gain from structural data only. Therefore, these functional data are critical for our understanding of transport mechanism. I have only some minor comments for the authors to address:
1) How do the authors explain the "background" transitions in the presence of the blocker TBOA? Are these artifacts that would occur in the absence of any transporter under observation?
2) The dwell time histograms indicate several kinetic components in the OFS. While this is unlikely

to be caused by lack of saturation of the Asp binding step, is it possible that the Na+ binding sites are not fully saturated? In other words, could Na+ binding and dissociation equilibria contribute to the heterogeneity in kinetics? What happens when the Na+ concentration is lowered?
3) Negative allosteric modulators (NAMs) are shown in Fig. 7C, but not described in detail in the discussion, with respect to their mechanism.
4) The free energy profiles in Fig. 7A and C are not well described in the discussion. What is the significance of the several energy barriers? How was their relative height determined?
5) There are kinetic data that were published previously for mammalian EAATs that may be relevant for the discussion of these results. For example, kinetic heterogeneity was observed in homo-exchange conditions by Watzke et al., JGP 2001, and energy barriers for translocation were determined by Mim et al., Biochemistry, 2007.

Dear Dr Klimmeck,

*Thank you for your email detailing the editorial decision on our submission **EMBOJ-2020-105415**. We are pleased that the Referees find that our paper addresses "**a very important question**" and that our work is an "**important contribution**" based upon an "**elegant**" approach with "**rock solid**" results. We thank Referee #1 in particular for the in-depth critical reading of our manuscript. We address the concerns of all Referees in full below.*

Most significantly, we have added a complete set of Glt_{Ph} dynamics under apo conditions and when bound to Na^+/L-Asp for all mutants at an increased time resolution of 10 ms. These data are entirely in line with our reported findings at 100 ms resolution and are summarized in a new Supplementary Figure 7.

We hope that you will find our revised manuscript suitable for publication in the EMBO Journal.

Best regards,

Olga Boudker
Gerard Huysmans

Referee #1:

In this manuscript, Huysmans et al. study the energetics of the major conformational change during glutamate transport, the translocation of the mobile transport domain. The authors initially use bioinformatic approaches together with transporter reconstitution and radioactive tracerflux experiments to identify gain-of-function mutants of the transporter. These gain-of-function mutations - that increase transport rates to up to 10 - 20 times - were then analyzed with a smFRET techniques that groups of Blanchard and Boudker have intensively used in the past. Measurements of dwell times on inward- and outward-facing conformation as well as of transition frequencies were used for a linear free energy relationship analysis that revealed close structural resemblance of a high-energy transition state with the inward-facing conformation. The authors propose an approach for allosteric modulation of these transporters based on these analyses.

This paper addresses a very important question - the molecular basis of isoform-specific differences in glutamate transporters, with potentially important applications in medicine. However, I cannot see the contribution of the paper to develop novel allosteric modulators of mammalian glutamate transporters, and I have several technical concerns.

1. My major concern is the limited time resolution of the smFRET technique (p. 10 "While the macroscopic rates of the transport domain translocation are slow, translocation of the transport domain is faster than the resolution of our smFRET recordings (10-100 ms)." Faster transitions into intermediate conformation that cannot be resolved might significantly modify the results of the linear free energy relationship analysis. The authors should test possible consequences of this limitation. In single channel recordings of ion channels methods have been developed to correct for such events (Ball et al. (1990). Proc Biol Sci, 242, 61-67; Blatz et al. (1986). Biophys J 49, 967-980). The authors might want to use such approaches to test the validity of their conclusions.

Referee #1 raises two potentially different points here:

1 – My major concern is the limited time resolution of the smFRET technique (p. 10 "While the macroscopic rates of the transport domain translocation are slow, translocation of the transport domain is faster than the resolution of our smFRET recordings (10-100 ms)." Faster transitions into intermediate conformation that cannot be resolved might significantly modify the results of the linear free energy relationship analysis.

We thank the Referee for this insightful point. The Referee raises the concern that during the apparent instantaneous transitions from the OFS to IFS (and back), there might be short-lived intermediates that we cannot observe at the100 or 10 ms time resolution of our experiments. Thus, we may be missing transitions in our recordings, which is indeed likely, and the question is whether and how this affects our findings.

A similar situation was described in detail in recent DNA and protein folding studies using single-molecule techniques (e.g., Chung et al. 2015 Science, Sturzenegger et al. 2018 Nat Comms, Hoffer et al. 2019 PNAS). Like in our studies, folding and unfolding reactions showed slow macroscopic rates with the transition appearing as instantaneous jumps in the single-molecule recordings with ms time resolution. However, when these reactions were probed with different single-molecule techniques achieving µs time resolution, gradual folding was observed. The intermediates rapidly sampled during the transition are part of the transition conformational ensemble and define the shape of the energy barrier, but not its location relative to the terminal states. This reasoning applies to the Glt_{Ph} OFS-to-IFS transitions in the same way as to macromolecular folding processes.

Similarly, intermediates that are in rapid exchange with the OFS or the IFS are parts of the OFS and IFS conformational ensembles, respectively.

We discuss these points and the appropriate references on ***p. 12*** *of the revised manuscript, from "****Correspondingly, intermediate positions of the domain, manifesting in intermediate*** E_{FRET} ***values, are not observed even though they must be traversed on a faster time scale…"***

2 – The authors should test possible consequences of this limitation. In single channel recordings of ion channels methods have been developed to correct for such events (Ball et al. (1990). Proc Biol Sci, 242, 61-67; Blatz et al. (1986). Biophys J 49, 967-980). The authors might want to use such approaches to test the validity of their conclusions.

The Referee is again pointing out that we might be missing transitions at the experimental time resolution employed. This is an important point. Missed transitions may significantly alter the observed dwell times, as well as the estimated rates of specific transitions, which may potentially affect our results and conclusions. Indeed, our dwell-time distributions obtained at 100 msec time resolution do indicate that we might be missing transitions (Figure S3 and S6), and we are grateful to the Referee for prompting us to take this matter into further consideration.

We have now measured a complete set of dynamics for all mutants at 10 ms time resolution. These data have a disadvantage that they contain more background transitions due to the reduced signal-to-noise, whereby some of the noise is misinterpreted as transitions by trace idealization. These are well-known tradeoffs in all efforts to achieve quantitative analyses of dwell time distributions. However, qualitatively, the dwell-time distributions do not reveal evidence of additional dynamic modes with shorter time constants. Furthermore, we find an expected large percentage of non-dynamic trajectories. Finally, the population distributions match those obtained at 100 ms time resolution. These data suggest that we are not missing a

*large number of transitions at 100 ms time resolution. We have included these data in a new figure (**Supplementary Figure 7**) and we modified on **p. 9** from **"Recordings performed with 10 ms time …"***

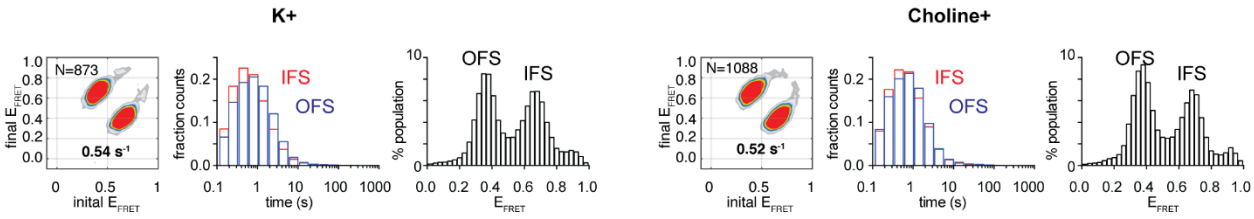
*We also recalculated the time constants used in the linear LFER analysis using correction factors according to Blatz and Magleby (1986). In most cases, the rate constants change less than 10 %, with the most substantial effects on the rates of the OFS to IFS transition of the apo transporter. Following these analyses, we replotted the 2D histograms in **Figure 5** of the main text and in **Supplementary Figures 11 -13** of the revised Supplemental Data. We also included the reference in the main manuscript on **p. 9-10** ("**To account for missed transitions we mathematically corrected the estimated FRET-state lifetimes following established procedures (Blatz & Magleby, 1986).** ") and **p. 11**: **("…each Glt_{Ph} transporter with intrinsically homogeneous dynamics can be described by a pair of characteristic OFS and IFS lifetimes that we mathematically corrected for the missed transitions (Blatz & Magleby, 1986).")***

*Using these corrected rate constants, we recalculated the LFERs. Overall, correcting the rate constants did not change the outcome. M362V GltPh was the only mutant for which energy differences changed so that Leffler slopes increased to 1.5. Therefore, we added this mutant to the same panel as all other HP2 mutants that provide evidence for transition state stabilization by HP2 mutations (**Figure 6** of the main text).*

2. With limited time resolution it appears difficult to me to distinguish high transition frequencies from longer residence times. Since some of the mutations are located in HP2 and might affect the likelihood of HP2 closure, this is not a trivial problem. K+ binding was recently shown to stabilize HP2 closure (Kortzak et al. (2019). EMBO J 38, e101468), and the comparison of K+ and Choline+ might provide information about the role of the limited time resolution on correct quantification of dwell times and transitions frequencies.

This comment is of a similar nature to the prior comment. The Referee rightly points out that there might be fast transitions out of the OFS as the transporter attempts to cross the transition barrier that our finite time resolution misses. As described above, we do not appear to appreciably miss bona fide transitions to the IFS. However, a multitude of much more transient barrier crossing attempts likely exist. These would be treated in our analysis as part of the OFS ensemble (even if they involve a considerable transport domain movement).

*We share the Referee's views that HP2 conformations are critical determinants of the rates of the OFS to IFS transitions, as highlighted in the Discussion (p. 15-16). We have modified the appropriate sentence on **p. 15** to include the suggested reference: **"Collectively, these data suggest that the conformationally flexible HP2 (…) serves as a master-regulator of substrate binding, translocation, release, and recycling of the apo transporter (Kortzak, Alleva et al., 2019)."** However, we find that population histograms and dwell-time distributions in the presence of either Choline+ or K+ are indistinguishable from each other (we measure these routinely, and an example is shown in **Rebuttal Figure 1**). Thus, K+ ions alter neither the dynamics nor state populations of the transporter in our experiments, consistent with our observations that HP2 closing does not seem to be a rate-limiting factor in either apo or Na/Asp-bound states.*



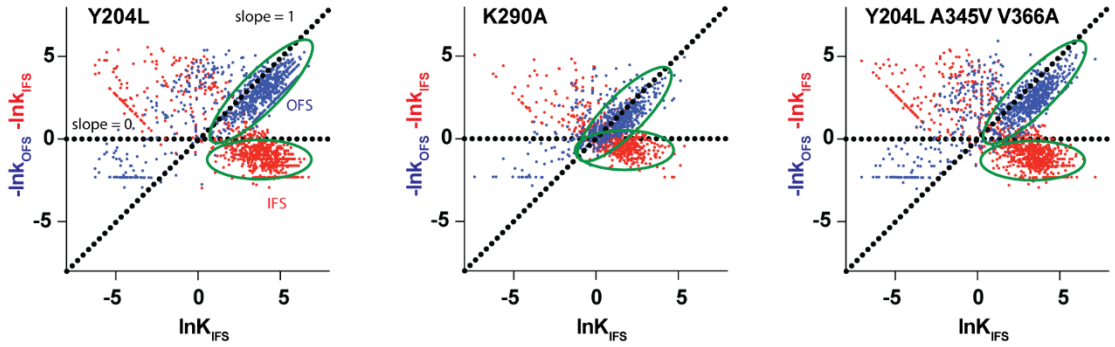
Rebuttal Figure 1: Transport domain dynamics of apo Glt_{Ph} in dodecylmaltoside*. For each condition, K+ (**Left**) and Choline+ (**Right**), the following plots are shown: Left, transition density plots. Shown on the panels are the number of trajectories analyzed and the population-wide mean transition frequency calculated as the ratio of the total number of transitions and the total trajectory lifetime. Middle, dwell-time distributions of the OFS in blue and the IFS in red. Right, time-averaged population distributions of E_{FRET}.*

3. The kinetic heterogeneity is biophysically interesting, but a major limitation for the LFER analysis. The authors state on p. 10 "... we hypothesize that the kinetic heterogeneity exhibited by GltPh is indicative of a highly rugged energy landscape and the existence of long-lived conformations with distinct kinetic properties." Is the LFER analysis still applicable to such energy landscape? In ion channels (Grosman et al. (2000). Nature 403, 773-776), proteins with only one time constants in dwell time analysis were analyzed, and here I find this approach quite logic. I would recommend more discussion of possible limitations of the analysis for the particular GltPh energy landscape.

*We agree with the Referee that the kinetic heterogeneity is an "inconvenient truth" even as this phenomenon appears widespread in nature, including in ion channels (e.g. Auerbach & Lingle (1986) J Physiol). When we examine individual molecules, the majority show single-exponential OFS and IFS survival plots, and, therefore, undergo two-state transitions. These molecules meet the criteria to perform LFER analysis so long as both fast and slow dynamic modes encounter the same principle barrier. The 2D histograms in Figure 5 of the manuscript show that, in most mutants, the mean IFS lifetime distributions vary comparatively little, while the OFS lifetimes vary over several orders of magnitude. Qualitatively, this observation suggests that heterogeneity is mostly due to differences in the OFS to IFS transition rates and, therefore, variable energy differences between the OFS and the transition state (TS). In contrast, the energy differences between the TS and the IFS remains similar, producing little differences in kinetic constants. These considerations were included in the first paragraph on **p. 12** from **"Already a qualitative comparison…".***

*We do not yet know what the molecular basis of the observed kinetic differences between the molecules may be. Generally, one can consider these unknown differences as structural perturbations and conduct an LFER analysis on each mutant plotting kinetic constants obtained for each molecule as a function of the equilibrium constant. Examples of such analyses are shown in **Rebuttal Figure 2**. From the graphs, it is evident that while there is heterogeneity, a majority of the molecules (between ~80 and 90 %) fall along the same trend lines with Leffler coefficients close to 1 and 0 for the OFS-to-IFS and IFS-to-OFS transitions, respectively. Thus, for the majority of molecules, the energies of the IFS and the TS are affected similarly, suggesting that the TS and IFS must be structurally similar. These analyses further suggest that ~ 80-90 % of molecules cross the same TS barrier.*

*We do not feel comfortable introducing this type of analysis in the paper because of its intrinsic complexity. However, we added a comment on the possibility of distinct TSs on **p. 11 starting with "Other dynamic modes…".***



Rebuttal Figure 2: Leffler plots constructed using transition rate constants estimated for each molecule for the following variants: Y204L (left), K290A (middle), and Y204L/A345V/V366A (right) mutants. *Transition rates from the OFS to IFS are in blue, and those from the IFS to the OFS are in red. Black dotted lines through the origin have slopes of 0 and 1 to guide the eye. Green ellipsoids highlight the dominant fraction of molecules with OFS and IFS along the same trendline with slopes close to 1 and 0, respectively.*

4. The authors use their analysis "to gain insight into the nature of the most commonly used activation barrier during transport domain motions". For this the authors now choose protein with "typical" behavior. Please provide a statistical analysis for WT and each of the mutants, with dwell times and transitions frequencies for each measurements, and, most importantly, of the identification of the "typical" behavior from these data.

This question pertains to the data presented in Figure 5A and B, which are pooled data from three or more experimental repeats and are for illustration purposes only. Each repeat was analyzed independently, and the mean lifetimes of molecules in the top 30 % of highest density histogram bins were averaged. The errors in Figure 5C represent the S.E.M. over the independent repeats, as indicated in the figure legend. We now include 2D histograms for each independent repeat in a new ***Supplementary Figure 11*** *and we highlight the molecules that were selected to calculate the typical lifetimes with a box. We provide the same information for the measurements under apo conditions in the new* ***Supplementary Figure 13****.*

5. The authors state in their abstract that "Based on these analyses, we propose an approach for allosteric modulation of these transporters." Dysfunctional EAATs play a role in certain diseases, and pharmacological EAAT modification might be useful for treating certain acquired diseases. However, stabilizing high-energy transition states is a very generic concept commonly used to increase enzymatic activity, and not a new "approach". Moreover, the new results only allow predictions about the function of allosteric modulators on GltPh; no predictions can be made about the high energy transition states of the EAATs. GltPh is kinetically very different from the EAATs, in which the K+-bound re-translocation represents the rate-limiting step. Most importantly, the transition state for the rate-limiting process in EAATs is unclear; hence application of the concepts developed here on EAATs is speculation. Therefore, I suggest removing this section. The paper does not provide any new insights into potential modification of EAAT function. No prediction can be made about the high energy transition states of the EAATs.

We agree with the Referee that our work does not provide direct insight into the design of EAAT activators and we acknowledge that re-translocation of the K+-bound transporter domain is rate-

*limiting in EAATs, as we have noted in the penultimate paragraph of our Discussion (**p.16-17**). We also agree that transition-state stabilization is not new in itself. However, turning this concept into practice to develop allosteric modulators is challenging even for enzymes (e.g., Hammam et al (2017) and Zorba et al (2019), referenced at the end of the Discussion).*

*Our work provides a step-by-step "recipe" on how to locate the transition state for a particular transporter. Applying this approach to relevant human proteins will reveal whether knowledge of the transition state structure is pharmacologically actionable. Such a conceptual advance is new to the best of our knowledge. We have rewritten the appropriate paragraphs of the Discussion to state this more clearly on **p.16-17**.*

6. The authors speculate that substrate release instead of translocation becomes rate limiting in the fast mutants (p. 7). This would be an important conclusion, but it is currently not supported by any experimental data. The authors should test this hypothesis experimentally.

*We speculate that release slows transport in K290A and Y204L/K290A Glt_{Ph} because their translocation dynamics are increased significantly more than the uptake rates. Thus, we reason that either substrate binding or release become rate-limiting in these mutants, occurring on tens of seconds time scale. It is unlikely that binding is that slow, considering that these mutants show nM substrate affinity similar to the WT in 200 mM NaCl used in our experiments. Moreover, Hanelt et al. showed that Na+ binding occurs with a kinetic rate of 5 s^{-1} M^{-1}. Most importantly, we observe many more long-OFS dwell in the asymmetric transport conditions (with Na+/Asp in the external liposome solution and K+ in the internal solution) than in symmetric apo conditions (with K+ in both external and internal solutions). If binding occurred on a tens of seconds time scale, we would have observed many apo protomers escaping back into the IFS before binding substrate, increasing the number of shorter dwells. Consistently, the dwell-time distributions under transport conditions and in symmetric substrate-saturating conditions (with both external and internal solutions containing 200 mM Na+/100 µM L-Asp) were similar. We have added these reasonings on **p. 8** of the revised manuscript starting with: **"It is, in principle, possible that Na^+ and L-Asp binding to the OFS is rate-limiting in the dynamic mutants…"***

*Direct measurement of substrate release from the IFS is possible by cross-linking Glt_{Ph} mutants in the IFS and introducing a Trp in TM1 as a reporter (Oh and Boudker, 2018). Qualitatively, these measurements indicate that substrate release from WT Glt_{Ph} is slow, occurring on tens of seconds time scale, as previously reported (**Rebuttal Figure 3,** and Oh and Boudker, 2018). In these experiments, HP2 mutants with reduced substrate affinity show significantly faster release. We are reluctant to include these data in the manuscript because deriving accurate rate constants, particularly if there are multiple kinetic components, is challenging.*

Figure for referees removed

Minor points
1. p.3: "These studies suggested that the translocation of the substrate-loaded transport domain from the OFS to the IFS is the rate limited step of the cycle.." Fluorescence spectroscopy and fast substrate application revealed very slow processes associated with Na+ association (Hanelt et al. (2015). J Biol Chem 290, 15962-15972) in GltPh, which are absent in VCF measurements on EAAC1 (Larsson et al. (2004). Proc.Natl.Acad.Sci.U.S.A. 101, 3951-3956). From these findings I would conclude that Na+ association and associated conformational changes are rate-limiting in GltPh, and that evolutionary optimization of these processes accelerated glutamate transport in mammalian transporters. I cannot see how smFRET can disprove such a conclusion, since only dwell times in each of the two states o the transport domain can be assessed. Please discuss this point in more detail.

Referee 1 correctly points out that Na+ binding is on the timescale of ~1 sec at 200 mM NaCl (Hanelt et al., 2015) in the absence of L-Asp. This is still very fast compared to the translocation of the substrate loaded transport domain in the WT protein. However, it approaches the translocation rate in our fastest mutant and may become a factor. We now comment on this on ***p.8 "Notably, in our fastest Y204L/K290A/A345V/V366A mutant, achieving turnovers of 1 s, all rate constants become similar, and substrate binding might also impact the transport rate."***

Further, we agree with the Referee that multiple steps of the transport cycle were likely optimized through evolution, not limited to translocation dynamics. In this context, it is striking that HP2 mutations identified through bioinformatics, as well as humanizing R276S/M395R mutations, affect both translocation rates and binding. We comment on this in the first paragraph of the revised discussion on ***p. 15****. "Thus, mutations in the transport domain can optimize multiple steps of the transport cycle at once, perhaps mimicking the natural evolutionary process."*

2. p. 14: ".consistent with the return of the apo transport domain into the OFS being the rate-limiting step of the cycle." In the EAATs, re-translocation is obligatorily K+-bound.

The Referee is correct, we have incorporated this change on ***p.16*** *in the paragraph starting at: "****PAMs of human EAATs****…"*

3. I would like to see the smFRET data at higher time resolution, maybe the authors can show some examples for selected traces in the supplementals.

Example traces were included in panel A of the new ***Supplementary Figure 7****.*

Referee #2:

General sumary:

This study by Huysmans and colleagues employs single molecule FRET experiments to obtain real-time recordings of transport-associated conformational rearrangements in single protomers of a trimeric glutamate transporter protein. This approach allows measuring single-molecule transition rates for conformational switches between outward facing and inward facing states (OFS and IFS) of a protomer. The analysis identifies the OFS to IFS step as the rate limiting step of the cycle. The authors then introduce perturbations that alter overall cycling rate, such

as addition of transported ligands (L-aspartate and Na+) or insertion of point mutations into positions hinted by clever cross-species sequence comparisons, and analyze the effects on individual transition rates. They find that the perturbations predominantly affect the rate of the forward (OFS to IFS) transition. Using linear free energy relationship (LFER) analysis they conclude that the high-energy transition state (TS) that the protomer needs to overcome to complete the rate-limiting isomerization step structurally resembles the IFS conformation. A practical inference of exceptional interest is that small molecules that bind tighter in the IFS conformation are expected to stabilize the TS of the rate-limiting step, and should therefore act as allosteric activators with potential for therapeutic application. The work exposed in this paper is not only a great technical feat, but is also conceptually elegant, and the conclusions are rock solid. Below are a few questions/suggestions the authors might consider to address, to improve clarity in certain places of the manuscript.

Specific comments:

1.) P2, 2nd par: "LFERs correlate... From these correlations, one can infer whether the TS is structurally more similar to the initial or the final conformation."
More precisely, one can infer whether in the TS the perturbed region of the protein resembles its initial or final conformation.

We agree and have rewritten this sentence at the bottom of ***p.2*** *as "****From these correlations, one can infer whether the perturbed region of the protein resembles the initial or the final conformation in the TS****".*

2.) Protein labeling. If I understand the cartoon in Fig. S2B correctly, each trimer was labeled with one donor dye molecule, one acceptor dye molecule, and one biotin-PEG-maleimide anchor. How was this fixed stoichiometry achieved? Or was labeling random, as expected, but this particular population was the only population that generated a FRET signal under TIRF conditions? Please clarify.

The labeling is random. However, only the molecules carrying three distinct labels pass data selection. Surface immobilization via a biotin-streptavidin bridge ascertains that at least one transport domain is labeled with biotin-PEG11. Selection of trajectories with one catastrophic photobleaching event assures the analysis of only those GltPh transporters that contain one donor and one acceptor fluorophore. We clarify this point in revised Methods.

3.) P6: "In the presence of 10 mM blocker... we still observed a transition frequency of 0.03 {plus minus} 0.01 s-1... these transitions originated from ~8-17 % of all molecules and constituted an apparent background. When this small sub-population was subtracted... the resulting transition frequency for the WT GltPh... reduced to ~0.01 s-1". How is this "background" interpreted? What was the rationale for subtracting the rate of the blocker-insensitive subpopulation from the measured rates? If there is a rationale, is it meaningful to measure a rate of 0.01 s-1 over a background of 0.03 {plus minus} 0.01 s-1?

The Referee correctly points out that WT protein dynamics are close to background. Background likely stems from non-functional misfolded molecules that do not bind TBOA. Their presence puts an uncertainty on the "real" frequency of WT GltPh dynamics and the reported value of 0.01 s^{-1} *should be taken with a grain of salt. However, we recently measured single-molecule transport activity of GltPh under equivalent conditions, which produced similar mean*

*value of ca 0.01 s^{-1} (Ciftci et al (2020) Sci Adv, eaaz1949). We made a note of this in the revised manuscript on **p.7**.*

*We have recently measured single-molecule transport for several Glt_{Ph} mutants used in this study. These experiments show a convincing correlation between mean dynamics and transport rates without uncertainties associated with bulk transport of radiolabeled L-Asp (**Rebuttal Figure 4**).*

Figure for referees removed

4.) P7: "substrate release can become rate-limiting in more dynamic mutants"
Comparing Figs. S4 and S6, it seems that the dwell time of the IFS state is the same, whether or not substrate is present on the intracellular side. This suggests that the rates of the IFS -> OFS transition for the substrate-bound and the ligand-free protomer (i.e., rates for the two leftward horizontal arrows in Fig. 1B) are very similar. (BTW, such rates are also used in the simulations in Fig. S7B, left panel.) If that is the case, how could substrate dissociation delay the IFS -> OFS transition?

*We agree that the text was unclear. The Referee is correct, the transitions themselves are not delayed. Instead, the slow release must result in the transporter making multiple transitions before releasing the substrate. To clarify this, we have added a sentence on **p. 8**: "**We speculate that substrate release from the IFS becomes rate-limiting in these dynamic mutants with the transport domain visiting IFS multiple times before releasing substrate** (**Figure 1B**)."*

5.) P11, 4th par: "we estimated the rate constants, k(OFS to IFS) and k(IFS to OFS), from the inverse of the mean OFS and IFS lifetimes"
Are the rate constants k(OFS to IFS) and k(IFS to OFS) meant to denote protomer transition rates, as illustrated in Fig. 7B? If so, then the two should be calculated differently from the mean dwell times of the two FRET levels. If I understand correctly, the analysis was done on segments in which transitions occured only between E(FRET) values of ~0.4 and ~0.6, corresponding to OFS/OFS and IFS/OFS arrangements, respectively, of the two FRET labeled subunits. The mean dwell time of the IFS/OFS state is equal to the mean life time of the IFS state of a protomer (the IFS/OFS state is terminated when the protomer which is in the IFS state flips back to OFS): thus, k(IFS to OFS) = 1/tau(IFS/OFS). In contrast, the mean life time of the OFS/OFS state is half that of the OFS state of a protomer (since the OFS/OFS state is

terminated by flipping of any one of two protomers): thus, k(OFS to IFS) = 1/2xtau(OFS/OFS). Has this fact been taken into account?

We thank the Referee for pointing out the lack of clarity in the text. All transitions are included, but the overwhelming majority of these are between E_{FRET} of 0.4 to 0.6. Only one protomer is moving in these traces, and it is always the same protomer. We know this because elevator transition is associated with translocation and rotation of the transport domain. Thus, the movement of one or the other protomer is distinguishable. Consequently, the mean dwell times directly correlate to the flipping of one distinct protomer. The probability of two protomers moving at the same time is low, particularly in less dynamic transporters. We clarify this on ***p. 7*** *of the revised manuscript: "****Typically, only one protomer at a time displays recurring transitions between…"*** *and provided references to our previous work, where we considered the issue in more detail.*

6.) Fig. 7A vs. B: In panel B the rate for the IFS -> OFS transition is slightly larger for the substrate-bound (2 s-1) compared to the unliganded (1.6 s-1) protomer. In contrast, in the black (control) energy diagram in panel A, the barrier is drawn higher for the former step (right side of 2nd barrier) than for the latter (left side of 4th barrier).

We have modified the energy diagrams to reflect the relative differences better.

Minor: We thank the Referee for the careful reading and have modified these

7.) P2, line 8: "transporters activity" should be "transporter activities"

8.) P3, line 7: "rate-limited" should be "rate-limiting"

9.) P5, line 2: please indicate that the KM refers to that of aspartate (as opposed to Na+)

10.) P5, line 6: "M362A" - in Fig. 1F M362V is shown

11.) P5, line 2 from bottom: "increases transport rate" should be "increases in transport rate"

12.) P11, lines 2 and 3: "the forward" and "the reverse" - delete "the"

13.) P24, line 5: please define "transition frequency". Is this the frequency of transitions either way, or the frequency of cycles?

Done 7-13.

14.) Fig. 5A-B legend: "lifetimes of... the molecules showing no transitions before photobleaching (black bars)"
The meaning of the black bars is unclear. If there were no transitions, how could dwell times be obtained?

*For molecules without transitions, the time of photobleaching was used to create the black histograms. This is now clarified in the legend as follows: "****and the photobleaching time of molecules showing no transitions (black bars)****"*

15.) Fig. 5C legend: "molecules falling within 30 % of the most populated bins of the 2D-

histograms (yellow to red)"
Maybe identify the corresponding area of the 2-D histogram with a box?

We thank the Referee for this suggestion, and we use a box to highlight these bins in ***Supplementary Figures 11 and 13*** *that show the independent repeats.*

16.) Fig. 7 legend: Please define NAMs (negative allosteric modulators).
Done

17.) Fig. 1F: Please explain abscissa units. What does a negative fold change mean? Does a "-5-fold change" mean a 5-fold decrease?

*The Referee is correct, and we have clarified this in the Figure legend: "****(F) Fold increase (positive values) or decrease (negative values)…****"*

18.) Fig. S1B: It is a difficult visual task to discern the substrate from the highlighted side chains. Maybe use a single, more contrasting color for the substrate?

We have tried a number of alternative representations but unfortunately could not find a particularly satisfying representation.

19.) Fig. S2C, bottom panel, labels on the right: I don't understand why the label for E(FRET)~0.4 is "OFS/OFS + OFS/IFS". Shouldn't this label be "OFS/OFS", and the label for E(FRET)~0.6 "IFS/OFS + OFS/IFS"? Or did I miss something here?

As isomerization of one protomer includes a rotation in addition to the translational movement, only the flipping of one protomer gives the distinct E_{FRET} *change. Consequently, flipping of the other will not change the* E_{FRET}*. This is illustrated in Supplementary Figure 2A. We now highlight this in the legend to this figure: "****Note that in the latter case, only isomerization of one distinct protomer gives rise to a measurable distance change.****"*

20.) Fig. S5 legend: "superimposition" should be "superposition" (twice)

21.) Fig. S10: see comments 14.) and 15.)

Done 20 - 21

Referee #3:

This is a very nice manuscript that describes the application of an elegant methods to determine equilibrium transitions in apo and substrate bound GltPh transporters. The results indicate that the transition state occurs late on the translocation pathway and resembles the inward-facing configuration. This is an important finding that adds to our structural picture of how these transporters operate, which is impossible to gain from structural data only. Therefore, these functional data are critical for our understanding of transport mechanism.

We thank this Referee for the positive assessment of the work.

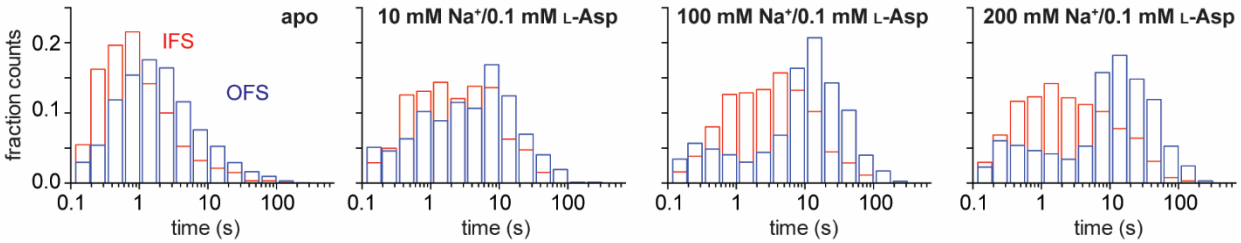
I have only some minor comments for the authors to address:
1) How do the authors explain the "background" transitions in the presence of the blocker

TBOA? Are these artifacts that would occur in the absence of any transporter under observation?

We have clarified this above in our response to Referee 2, point 3

2) The dwell time histograms indicate several kinetic components in the OFS. While this is unlikely to be caused by lack of saturation of the Asp binding step, is it possible that the Na+ binding sites are not fully saturated? In other words, could Na+ binding and dissociation equilibria contribute to the heterogeneity in kinetics? What happens when the Na+ concentration is lowered?

*The Referee raises an interesting point. Do the long dwell times arise from empty Na^+-sites? The last Na^+ to bind stabilizes HP2 closure and incomplete saturation of that site might delay the OFS to IFS transition. If so, lowering the Na^+ concentration might favor slower dynamic modes. The apparent measured Na^+ affinity in the presence of 1 mM L-Asp is below 1 mM (Reyes, NSMB 2013). Thus, 200 mM Na^+ used in our experiments is highly saturating. Nevertheless, we measured the dynamics in the presence of 1 mM L-Asp and variable Na^+ concentrations (**Rebuttal Figure 5**). We observed indistinguishable dwell-time distributions at saturating 100 and 200 mM Na+. Only when we reduced Na+ concentration 10 mM, we observed increased dynamics suggesting the presence of apo protein. We note in the revised manuscript (**p. 11**): **"It is also unlikely to arise from incompletely saturated Na^+ sites because we used ion concentrations in excess of 200-fold over the K_D values…"***



Rebuttal Figure 5: *OFS (blue) and IFS (red) dwell-time distributions of WT Glt_{Ph} at different Na+-concentrations (as indicated) in dodecylmaltoside.*

3) Negative allosteric modulators (NAMs) are shown in Fig. 7C, but not described in detail in the discussion, with respect to their mechanism.

*We have modified the Discussion to include the effect of NAMs on **p. 16** as follows: **"In contrast, molecules that bind tighter to the OFS would increase the height of the barrier and serve as negative allosteric modulators (NAMs) (Figure 7C).**"*

4) The free energy profiles in Fig. 7A and C are not well described in the discussion. What is the significance of the several energy barriers? How was their relative height determined?

We have modified panel A of Figure 7 to clarify the processes associated with each energy barrier. We note that the heights of the barriers are not drawn to scale with the rate constants as given in panel B. This is clarified in the revised figure legend.

5) There are kinetic data that were published previously for mammalian EAATs that may be relevant for the discussion of these results. For example, kinetic heterogeneity was observed in

homo-exchange conditions by Watzke et al., JGP 2001, and energy barriers for translocation were determined by Mim et al., Biochemistry, 2007.

We thank the Referee for pointing out these studies. We now include Mim et al in our Discussion on ***p.17*** *to compare the rate-limiting energy barriers of the transport cycle in mammalian glutamate transporters with those in* Glt_{Ph}*. Watzke et al report double-exponential relaxation currents of EAAC1 assigned to closing of the extracellular gate and domain translocation or opening of the intracellular gate. These currents occur on a much faster timescale than our measurements here, as is expected for mammalian transporter. We feel that without comparative smFRET dynamics of mammalian glutamate transporters, it is speculative at best to correlate these with dynamic heterogeneity of the elevator movement in* Glt_{Ph}*.*

1st Revision - Editorial Decision 28th Aug 2020

Dear Dr Huysmans,

This is to share enclosed the mentioned formatting edits and comments by our production team, which we kindly ask you to consider in your final revised manuscript version leaving changes in track mode.

Thank you in advance for your cooperation.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Editor
The EMBO Journal

Dear Dr Huysmans, dear Dr Boudker,

Thank you for submitting your amended manuscript (EMBOJ-2020-105415R) to The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor remaining issues related to formatting and data representation as detailed below which need to be addressed at re-submission.

Further, I will share additional changes and comments from our production team during the next days to be considered.

Please contact me at any time if you have further questions.

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Again, please contact me at any time if you need any additional help.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Introduce ORCID IDs for all corresponding authors (O.B.) via our online manuscript system. Please see below for additional information.

>> Recheck callouts for figures 5C which needs to be introduced before 6A in the main text.

>> Please add a separate 'Statistical analysis' section in the material & methods part of the manuscript.

>> Rename the current 'Supplementary Information' to 'Appendix' and add a ToC on the first page. Change the nomenclature of supplementary figures and tables to 'Appendix Figure S1, S2...' and 'Appendix Table S1, S2...' and adjust the callouts in the main text and legends accordingly.

****

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Referee #1:

The authors have addressed all my concerns, and I have no further criticisms. I recommend acceptance of this interesting and well performed paper.

Referee #2:

This study convincingly demonstrates that in a trimeric glutamate transporter protein the step from the outward-facing to the inward-facing state is the rate limiting step of the cycle. Furthermore, the high-energy transition state that the protomer needs to overcome to complete the rate-limiting isomerization step structurally resembles the inward-facing state.

In this revised manuscript the authors have addressed all my concerns raised by the original version. I have no further requests.

Referee #3:

This is a comprehensive revision of the original version of the manuscript. I have no further concerns and recommend publication as is.

Dear Dr. Klimmeck,

Thank you for your email from August 28 and the decision to accept our manuscript EMBOJ-2020-105415R for publication. We have addressed all your queries and those from the production team in the revised manuscript text (with track changes). We also submitted a revised Appendix and changed all callouts.

We hope that the formatted revision is now suitable for publication in the EMBO Journal.

Best regards,

Olga Boudker
Gerard Huysmans

2nd Revision - Editorial Decision 18th Sep 2020

Dear Dr Huysmans, dear Dr Boudker,

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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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.

A- Figures

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.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- 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.

2. Captions

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.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- 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.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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.

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Our sample size largely exceeds the accepted number (> 100 molecules) to provide population-wide averages.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	All samples were included and analyzed in the same rigorous way. Acquired traces were selected for analysis using the following criteria: a single catastrophic photobleaching event, ascertaining GltPh labeling with a single LD55P-MAL and LD655-MAL label; over 10:1 signal-to-background noise ratio; over 5:1 signal-to-signal noise ratio; FRET lifetime of at least 5 s; a maximum of 1 donor blink per trajectory.
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4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	yes

Is there an estimate of variation within each group of data?	yes
Is the variance similar between the groups that are being statistically compared?	yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

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11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The protein structure has been submitted to the PDB (accession code 6V8G)
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