Impact of pathogenic mutations of the GLUT1 glucose transporter on channel dynamics using ConsDYN enhanced sampling [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Background: The solute carrier (SLC) family of membrane proteins is a large class of transporters for many small molecules that are vital for the cell. Several pathogenic mutations are reported in the glucose transporter subfamily SLC2, causing Glut1-deficiency syndrome (GLUT1DS1, GLUT1DS2), epilepsy (EIG2) and cryohydrocytosis with neurological defects (Dystonia-9). Understanding the link between these mutations and transporter dynamics is crucial to elucidate their role in the dysfunction of the underlying transport mechanism.

Methods: Predictions from SIFT and PolyPhen provided an impression of the impact upon mutation in the highly conserved RXGRR motifs, but no clear differentiation could be made by these methods between pathogenic and non-pathogenic mutations. Therefore, to identify the molecular effects on the transporter function, insight from molecular dynamic simulations is required. We studied a variety of pathogenic and non-pathogenic mutations, using a newly developed coarse-grained simulation approach ‘ConsDYN’, which allows the sampling of both inward-open and outward-occluded states. To guarantee the sampling of large conformational changes, we only include conserved restraints of the elastic network introduced upon coarse-graining, which showed similar reference distances between the two conformational states (≤1 Å difference).

Results: We capture the ‘conserved dynamics’ between both states using ConsDYN. Simultaneously, it allowed us to considerably lower
the computational costs of our study. This approach is sufficiently sensitive to capture the effect of different mutations, and our results clearly indicate that the pathogenic mutation in GLUT1, G91D, situated at the highly conserved RXGRR motif between helices 2 and 3, has a strong impact on channel function, as it blocks the protein from sampling both conformational states.

**Conclusions:** Using our approach, we can explain the pathogenicity of the mutation G91D when we observe the configurations of the transmembrane helices, suggesting that their relative position is crucial for the correct functioning of the GLUT1 protein.

**Keywords**
GLUT1 glucose transporter deficiency syndrome, Human glucose transporters, SLC transporter family, transport mechanism, molecular dynamics simulation, Martini force field, coarse-grained simulations, enhanced sampling method
Introduction
The solute carrier (SLC) transporter superfamily is known to play a key role in the transport of small molecules. The superfamily comprises 52 families, and at least 386 different transporter genes have so far been identified in humans (Hediger et al., 2013; Higuchi et al., 2018). This family of membrane proteins is a large class of transporters for many small molecules such as glucose that are vital for the cell, and can be found in all kingdoms of life. Of particular interest are the glucose transporters SLC2A1 from the SLC2 subfamily; GLUT1 mutations are associated with GLUT1 deficiency syndrome (GLUT1DS1 and GLUT1DS2), and some forms of spasticity (Dystonia-9) and epilepsy (EIG2) (Klepper et al., 2016; Mongin et al., 2016). Shedding light on the molecular mechanism of the channel transport function, can enable us to understand the difference between pathogenic and benign mutations that have been observed in human subjects. Glucose transporter GLUT1 is built of 12 transmembrane helices (TMs) and exhibits a two-fold symmetry plane joining the two times six TM helices over a bridging helix on the cytoplasmic side of the membrane (see Figure 1A). Throughout the SLC transporters, a highly conserved RXGRR-motif is found between TM2 and TM3 and between TM8 and TM9 at the intracellular side of the corresponding loops (Pao et al., 1998; Sato & Mueckler, 1999). Several mutations at these anchor points are known to be disease-related, such as G91D and R92W which are known to cause GLUT1DS1 (Klepper et al., 2001; Klepper & Voit, 2002), whereas R93W is associated with GLUT1DS2 (Joshi et al., 2008).

Here, we investigate the effect of different mutations on the dynamic of the human GLUT1 protein. As the dynamic response upon mutation may depend on the conformational state, we aimed to simulate both the outward-occluded state (O₀) and inward-open state (Iₒ). For GLUT1, however, only the Iₒ state is available; therefore, simulations of GLUT3, which is evolutionarily quite close to GLUT1, in the O₀ state were analysed. GLUT3 is also suspected to be associated with neurological disorders such as Alzheimer (An et al., 2018; Gu et al., 2018; Simpson et al., 1994; Szablewski, 2017).

Methods
Starting structures
The crystal structures of both Iₒ and O₀ states are available in the PDB; 5EQI for GLUT1 Iₒ state (Deng et al., 2015), and 4ZW9 for GLUT3 O₀ state (Kapoor et al., 2016); these were used as starting points for the simulations. Based on the reported pathogenic mutations of GLUT1 in the conserved RXGRR motif region, we searched for additional mutations at the corresponding positions of GLUT3 and included them in our study. The selected mutations are situated in the conserved RXGRR-motif distal to the channel. An overview of all mutations for GLUT1 and GLUT3 studied in this work is provided in Table 1. Due to the high sequence identity (~70%) between the proteins, we intentionally did not build a homology model for one or the other protein. This is justified, as our main aim is to characterize the global opening and closing mechanism rather than to look into atomistic details such as protonation states. Moreover, we now avoid additional uncertainties about details of the structure as would be inevitably introduced during the homology building process. Our composite scheme using coarse-grained molecular dynamics with a conserved elastic network ConsDYN is explained in the approach below and further details are supplied in the supporting methods, available in the deposited code (Feenstra, 2019b).

Molecular dynamics simulations
In this study, we employ molecular dynamics (MD) simulations using the GROMACS 4.0.5 programme package (Hess et al., 2008). For efficiency reason, we investigated the applicability of the MARTINI coarse-grained (CG) force field (Arnarez et al., 2015; de Jong et al., 2013; Hsu et al., 2017; Monticelli et al., 2008; Periole et al., 2009), which is about 500-fold faster than the full-atomistic GROMOS (May et al., 2014). In addition, we modified the elastic network that is used

![Figure 1: GLUT1 structural overview. (A) Pipe representation of the inward-open (Iₒ) conformation (PDB-ID: 5EQI) of GLUT1 situated in the lipid bilayer. Note that the protein structure has a two-fold rotational symmetry and the two conserved RXGRR-motifs are located at the junctions of the transmembrane (TM) helices 2 and 3 and TM8-TM9. The red arrows symbolize the inside and outside distances. Note that we number the helices starting from TM1 at the beginning of the N-terminus of the transporter (dark blue in the pipe representation). (B) Pipe representation of the Iₒ conformation (PDB-ID: 5EQI) of GLUT1 viewing on the outward facing part of the channel inside the periplasm. The four main mutation sites, G91, R92, R333, and R334, are highlighted in magenta. (C) Definition of the order parameters to follow the motion of the helices over the ConsDYN simulations.](image-url)
in MARTINI based on our starting conformations, including only elastic network constraints that differed less than 1Å between the I₀ and O₀ states. This allows transition between both states, while maintaining protein structure stability. This composite scheme will henceforward be referred to as ConsDYN (CON-Served DYNamics). The detailed computational set-up is provided in the Supporting Methods (Feenstra, 2019b).

Analysis of channel dynamics

Essential dynamics analysis was performed on the GLUT1 and GLUT3 simulations using built-in analysis tools on GROMACS. To allow this comparison between these two homologous proteins, and allow for focusing on overall motions of the channel region, we selected the structurally conserved helical segments, as summarized in Supporting Table S2, available as extended data (Feenstra, 2019a). Then, the covariance and eigenvalue calculation was performed on the ensemble of both wild-type systems, using the full atomistic (AT), coarse-grained (CG) and ConsDYN simulations.

To analyse the channel dynamics from the ConsDYN, we defined several order parameters as previously proposed by Nagarathinam et al. (2018), by measuring distances between adjacent TM helices, at the intracellular (in)- and extracellular (out)sides of the protein (see Figure 1B, C). For each of the ring of six central helices that make up the channel, TM2, TM1, TM5, TM8, TM7, TM11 (and back to TM2), we defined an ‘inside’ and ‘outside’ segment of ten residues (See Figure 1 and extended data, Supporting Figure S3 (Feenstra, 2019a)). Comparing the distances of the mutations to both wild types allows us to capture abnormal behaviour and identify the mutations that have the highest impact on the opening and closing mechanism (see Results and Discussion).

Results and discussion

Prediction of mutation impact

For each of the mutants of GLUT1 and GLUT3 considered here, Table 1 lists the predicted impact upon mutation obtained from SIFT (Sim et al., 2012) and PolyPhen-2 (Adzhubei et al., 2010). Most mutations are classified as likely pathogenic by both methods, with the exception of GLUT1 R93Q, and GLUT3 R91C and R91H. However, these methods are trained on the dbSNP database which also includes these known mutations, so this should be no surprise. Moreover, these predictions do not

<table>
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<th>Protein</th>
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<th>Clinical significance*</th>
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<tbody>
<tr>
<td>GLUT1</td>
<td>G91D</td>
<td>(Klepper et al., 2001; Klepper &amp; Voit, 2002) GLUT1DS1 omim:606777 affect protein function probably damaging</td>
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<tr>
<td></td>
<td>R93W</td>
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<tr>
<td></td>
<td>R333W</td>
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<tr>
<td></td>
<td>R334Q</td>
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<tr>
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<td></td>
<td>R331W</td>
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* References given to literature describing clinical appearance, OMIM entries, and dbSNP entries given, if available.
allow us to gain any insights into the mechanism by which these mutations may affect transporter function.

**Verification of constraining approach**

Firstly, we want to verify if our conservation-based constraining approach for coarse-grained MD simulations (ConsDYN) is able to sample both I₀ and O₀ states. We performed essential dynamics (ED) analysis (Amadei et al., 1993; Van Aalten et al., 1997) to compare AT, traditional CG MARTINI, and ConsDYN simulations, as described in the Methods. Figure 2 shows 2D plots of the first two (largest) ED eigenvectors, representing the extracted correlated motions over the ensemble of our simulations. The sampling of the different states, inward-open and outward-occluded, in AT simulation hardly converges. The regular CG simulations already sample more intermediate conformations, but there is no overlap. The ConsDYN simulations, on the other hand, sample many states intermediate to inside-open and outside-open starting states, compared to other simulations. This means our goal of improved sampling of large conformational transitions has been attained.

**Probing conformational changes**

To probe for the degree of the conformational changes during the simulations of the wild types and the mutants in more detail than done with the ED analysis, two distances were used to describe the opening and closing of the periplasmic and cytoplasmic sides of the transporter. Nagarathinam et al. (2018) studied a bacterial homolog of GLUT1 and GLUT3, and analysed the movement between TM5 and TM8. In the extended data, Figure S4, we can see that the distributions obtained from our ConsDYN simulations, resemble those reported by Nagarathinam et al. (2018), providing an independent validation that our ConsDYN approach is able to sample biologically relevant conformational states for large scale motions, such as those involved in the glucose transporter mechanism. Nevertheless, there are differences between the distance distributions in our work and that of Nagarathinam et al. (2018), which is not surprising when comparing human glucose transporters with a bacterial multidrug transporter (see also extended data, Figure S4 and Table S3 (Feenstra, 2019a)).

Therefore, in addition to the TM5-TM8 distances, we extended the analysis to other helices along and across the channel rim that make up for the entire SLC channel architecture, allowing us to monitor changes in their position (see Figure 1B, C). For each of these order parameters, we calculate the distance at the inside and outside of the protein with respect to the membrane. Using this analysis, we can immediately observe the changes occurring between the inward-open and outward-occluded states. We see several distances changing significantly during this process: TM1/TM2(in), TM1/TM5(out), TM1/TM7(out), and TM5/TM11(in) are all closing, while TM1/TM2(out), TM1/TM5(in), TM1/TM7(in), TM2/TM11(in), TM2/TM8(out), and TM5/TM11(out) are opening (see extended data, Table S3 and Figure S4 for more details (Feenstra, 2019a)); these motions are also schematically summarised in extended data, Figure S3 (Feenstra, 2019a). Table 2 summarises the overlap between the observed distribution of distances between the wild type and each of the mutants for TM5 and TM11 that exhibited the strongest

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**Figure 2.** Two-dimensional essential dynamics plot of the simulations. Note that the time-scale of the full-atomistic simulations only samples conformations around the I₀ and O₀ states, while the ConsDYN samples a large number of conformations between both states. AT, full-atomistic; CG, coarse-grained; ConsDYN, conserved CG; I₀, inward open state (PDB-ID: 5EQI); O₀, outward occluded state (PDB-ID: 4ZW9).
Table 2. Overview of the changes in inside and outside distances between transmembrane helix 5 (TM5) and TM11, as quantified using the overlap in distributions of the wild type and mutant (small value is large change), and the shift of the peak location (including the direction; positive is to larger distances). The pathogenic mutations are underlined. All the large shifts (above 0.5) and small overlaps (below 0.6) are set to bold. For a visual aid, the shifts are set in italic.

<table>
<thead>
<tr>
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<th>GLUT3</th>
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<th>R331W</th>
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effects and conformational changes during the simulations. The direction of change is quantified by the difference in the position (‘shift’) of the maximum of the distribution; negative being a ‘closing’ motion, and positive ‘opening’ (the complete table of the distributions for all order parameters are available in the extended data, Table S3 (Feenstra, 2019a)). The corresponding conformational distributions from the ConsDYN simulations, calculated as a function of the inner and outer distances between TM5 and TM7 are given in Figure 3.

Impact of mutations on dynamics

Not all mutations have a high impact on the overall dynamics (extended data, Table S3 and Figure S4 (Feenstra, 2019a)). However, in GLUT1, the reported pathogenic mutation G91D has a profound effect on the dynamics of the protein (i.e., a low overlap and large shift, see Table 2). Also when we consider the distance of TM5-TM11, as shown in Figure 3, the strongest effect is observed for the pathogenic G91D mutant: its distribution varies strongest from the wild types, and hardly visits the inward-open and outward-occluded states. Furthermore, Figure 3 shows that for the pathogenic R92W and R333W mutations, only one state or small parts from both can be accessed. For the benign mutant R93Q, in contrast, it can be seen that both states, inward-open and outward-occluded, are sampled thoroughly during the simulations. Assuming that the relative distance between the two helices is crucial for the correct functioning, this strongly suggests that the pathogenic mutations directly affect the opening and closing mechanism of the GLUT1 transporter.

Mutations in GLUT3 show similar behaviour in TM dynamics compared to those in GLUT1. Here, two mutants with strong abnormal behaviour can be identified: G89V and R91H (Table 2; extended data Figure S4 (Feenstra, 2019a)) shows the corresponding distance distribution plots). Additionally, similar to the observations for pathogenic mutations on GLUT1, these mutations no longer sample intermediate states associated with the transport function, unlike the wild type and many of the other mutations. This strongly suggests that the corresponding mutations between GLUT1 and GLUT3 also have the same direct blocking effects on the opening and closing mechanism of the GLUT3 transporter. However, it should be noted that we cannot make any conclusions about the clinical significance of these GLUT3 mutants, as none have been reported to be pathogenic.

Conclusion

Using extensive ConsDYN simulations of GLUT1 and GLUT3 wild type and several clinically relevant mutations, we provide an effective way to study dynamic effects of mutations on the molecular mechanism of human glutamate transporter proteins. Without using full-atomistic details, we were able to get insight into the opening and closing mechanisms, which may account for the (dys)function of the SLC family caused by pathogenic mutations around the conserved RXGRR-motif. Through these mutations (especially G91D, R92W and R333W in GLUT1), the distances between TM5 and TM11, across the rim of the transporter channel structure, are affected the strongest and can be used as order parameters to elucidate abnormal behaviour in the dynamics of the transporter opening and closing mechanism. Comparing atomistic (AT), coarse-grained
Figure 3. Distance plots of the inner and outer distances along the order parameter TM5-TM11 over the complete time span of the simulation (see Figure 1C). Colour code: wild types I in purple (PDB-ID: 5EQI), O in green (PDB-ID: 4ZW9), mutants in orange. Pathogenic mutants are highlighted in red. It should be noted that in contrast to the benign R93Q mutant, the pathogenic mutants do not sample the I and O states during the simulation, which strongly indicates that the mutation blocks the proper opening and closing mechanism.

MARTINI (CG), and ConsDYN simulations, our work shows that our CG ConsDyn simulations are sufficiently accurate to sample between the conformational states and capture the effect of the mutations on the dynamic and function of these transporter proteins.

Data availability
Underlying data
Crystal structures for GLUT1 I state (Deng et al., 2015) and for GLUT3 O state (Kapoor et al., 2016) were obtained from the Protein Data Bank, under accession numbers 5EQI and 4ZW9, respectively.

Extended data
Open Science Framework: ConsDYN. https://doi.org/10.17605/OSF.IO/F82H5 (Feenstra, 2019a).

The following extended data are available:
- Data.tgz. data files accompanying analyses performed in this study.
- Table S1. Summary of the molecular composition of simulated systems.
- Table S2. Structurally conserved helical segments between 4ZW9 and 5EQI.
- Table S3. Wild-type and Mutant simulations compared by Overlap and shift between TM helix distance distributions.
- Figure S1. Sequence alignment between E. coli multidrug transporter MDFA, and human glucose transporters GLUT1 and GLUT3.
• Figure S2. Pipe representation of the inward-open conformation of the channel.

• Figure S3. Schematic view of the observed pore mechanism going from the inward-open state to the outward-occluded state.

• Figure S4. Distribution of inside and outside helix distances for all examined mutants in GLUT1 and GLUT3.

Extended data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Software availability

Scripts used to setup and analyze the ConsDYN simulations available from:
https://github.com/ibivu/ConsDYN.

Archived source code at time of publication: https://doi.org/10.5281/zenodo.2591477 (Feenstra, 2019b).

License: GNU General Public License 3.0.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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PubMed Abstract | Publisher Full Text

PubMed Abstract | Publisher Full Text | Free Full Text

PubMed Abstract | Publisher Full Text

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Version 1

Reviewer Report 08 May 2019

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Jocelyne Vreede
Van ’t Hoff Institute for Molecular Sciences, University of Amsterdam, Amsterdam, The Netherlands

The paper reports a molecular dynamics simulation study of the glucose transporter GLUT1 using all-atom and coarse grained force fields, in combination with a conserved elastic network. Using essential dynamics analysis and comparison of various distances the authors compared the dynamics of the wild type protein to pathogenic variants in the outward occluded and inward open states. This procedure enabled the prediction of the effect of mutations on the dynamics of GLUT1 and GLUT3.

The manuscript requires a few clarifications to improve my understanding:

- As I am not very familiar with the mechanism of glucose transporters, I would like a few sentences describing how these proteins work, thus giving more context to the different conformational states $O_o$ and $I_o$. Also in the conclusion, a bit more context as to how the states interconvert and the impact of the mutations on these transitions would aid my understanding tremendously.

- The manuscript reports results on mutations at positions 92, 93, 333 and 334, which are highlighted in Fig. 1. Only mutations 92 and 93 are discussed in the introduction. For more context, the mutations at 333 and 334 should be discussed in the introduction as well.

- What motivates the cut-off of 1 angstrom for including constraints in the elastic network? Would 1.5 angstrom or 2 angstrom work as well?

- As essential dynamics analysis is performed on both all-atom and coarse grained simulations, I assume only C-alpha positions are included. Is this assumption correct?

- What do the two eigenvectors shown in Fig. 2 mean? My interpretation is that EV1 is the transition from the $O_o$ to the $I_o$ state, and that EV2 is the transition from the all-atom to coarse grained-constrained description. If this interpretation is correct, would the
conclusion be correct that the dynamics sampled in the different force fields overlap?

- The $O_0$ and $I_0$ states as sampled with the consdyn and the AT approach seem quite different in Fig. 2. What could be the explanation for this difference?

- Snapshots of the conformations at the maxima of the probability histograms would help my understanding of the differences as introduced by the mutations.

- I do not understand how the overlap in the distributions in Table 2 is computed.

- What is the unit of the shift in Table 2 and of the distances in Fig. 3?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Molecular simulation of proteins and DNA

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
Lucie Delemotte
Department of Applied Physics, Science for Life Laboratory (SciLifeLab), KTH Royal Institute of Technology, Stockholm, Sweden

This paper describes a computational study of the effect of mutations on GLUT transporter dynamics. The work consists of atomistic and coarse grained simulations, including some using a protocol called ConsDYN which imposes constraints on distances conserved across conformational transitions. The study identifies that several pathogenic and other mutations modify the structural ensemble visited by the protein.

While the topic is important and computational methods are well-suited to answer the question, I have reservations about the study design and the conclusions reached:

1. A pathogenic mutation modifies the function of the protein such that cellular and organism function are altered. Glucose transporters carry out their function, i.e. importing sugars, via an alternating access cycle in which the transporter transits between outward-open and inward-open states via occluded states; whereas sugar binding from the extracellular medium promotes a transition to the inward facing state, sugar release to the inside intracellular medium promotes a return to the outward facing state. Other than the intrinsic dynamics of interconversion between states, the fact that the sugar modifies the stability of states and the kinetics of interconversion is key for function. Thus pathogenicity of a mutation could be due to many factors: among others, sugar binding, unbinding, (de)stabilization of one or more states on the functional cycle, modification of the rate of conversion between states, in the presence and/or absence of sugar. In this paper, the authors investigate the effect of mutations on the dynamics of interconversion between states. I believe the assumption should be spelled out more clearly, and the omission of all other possible effects on the sugar transport cycle should be explained.

Relatedly, were the simulations performed in the presence or absence of sugar? Comparing both cases could lead to increased insights.

2. Why was the analysis limited to outward occluded and inward open states, when high-resolution structures of other states are available? If only two states should be considered, why not consider the inward open and outward open since they are the two end-points of the transport cycle?

3. The ConsDYN method can be an interesting way to promote conversion between states using coarse grained simulations. However, whereas the stated aim on p5 is “to check that ConsDYN is able to sample both I₀ and O₀ states”, Figure 2 reveals that ConsDYN simulations only sample intermediate states, instead of bridging between states. I would thus disagree with the conclusion according to which the method allows to capture the “conserved dynamics” and to “sample between the conformational states”.

Would lowering the force constant of the constraints imposed lead to further exploration of the landscape?
The method also seems to have a serious conceptual drawback, in that it assumes that when switching from a state to another, common contacts are conserved, and additional ones are formed in either states. This does not seem to be a general feature of conformational changes in biological molecules and should be discussed.

4. Table 2 reports changes in distance between helices in the presence and absence of mutations, including pathogenic ones. Whereas the pathogenic G91D seems to cause major changes to the dynamics of the transporter, the other pathogenic mutations only alter some of the distance distributions. It does not appear that applying this methodology and measuring the difference in distance distributions as is done in Table 2 allows to predict pathogenicity. I thus disagree with the conclusions: “the distances between TM5 and TM11 can be used as order parameters to elucidate abnormal behavior in the dynamics of the transporter” and “ConsDYN simulations capture the effect of the mutations on the dynamic and function of the transporter proteins”.

Minor comments:
1. The authors refer to a “channel” in the title and later in the manuscript. Do they mean the transporter lumen? In an alternating access mechanism, a channel is never observed.
2. p5: The difference between Nagarathinam et al. (2018) and this work is ascribed to differences in the transporter protein studied (bacterial vs human) but the authors cannot rule out that the differences can come from differences in the sampling protocol.
3. Were the CG or AA simulations of I_o and O_o analyzed in Figure 3, Table 2 and SI figures?
4. p6: “outer distances between TM5 and TM7 are given in Figure 3” should be replaced by “outer distances between TM5 and TM11 are given in Figure 3”.

Is the work clearly and accurately presented and does it cite the current literature? Yes
Is the study design appropriate and is the work technically sound? Partly
Are sufficient details of methods and analysis provided to allow replication by others? Yes
If applicable, is the statistical analysis and its interpretation appropriate? Yes
Are all the source data underlying the results available to ensure full reproducibility? Yes
Are the conclusions drawn adequately supported by the results? No
**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Membrane proteins, ion channels, molecular dynamics simulations, enhanced sampling

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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