

Calcium induced N-terminal gating and pore collapse in connexin-46/50 gap junctions

Corresponding Author: Dr Steve Reichow

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript describes the cryo-EM structures of Cx46/Cx50 gap junction channels in a buffer containing Ca²⁺ ions in lipid nanodiscs. The structures reveal the Ca²⁺ ion binding sites at the extracellular site (EC1 site) and N-terminal site (NT site), which induce the N-terminal rearrangement. These observations implicate how the occluded and gated conformation of the gap junction channel can be mediated by Ca²⁺ ions, demonstrating that the remodeling is consistent with the iris model proposed long ago.

The structural analyses are reliable, and the interpretations are carefully described. My concerns are minor, but the authors should address several points raised below for publication.

1.
In the cryo-EM map of the occluded conformation of Cx46/Cx50, the space on the rotational axis of the pore surrounded by the N-termini is filled with unassigned densities, which are not mentioned in the manuscript. Similar pore densities are found in the map of gated conformation, although it is not well resolved, which is discussed as a "single Ca²⁺ ion could cross-link the NT domains" in Fig. 5. However, the volume of these densities seems too large to be accounted for by a single Ca²⁺ ion. Similarly, the pore densities have been observed in the cryo-EM structures of Cx36 and Cx43 (Lee et al. Nat. Commun. 14, 1347, 2023, Lee et al. Nat. Commun. 14, 931, 2023), which have been implicated as lipids.
In this manuscript, the pore size is discussed based on only the assigned model (Fig. 2c), but it is recommended that the authors describe their additional interpretations about undefined pore densities. This is because the pore density may affect the channel permeability.
2.
For Ca²⁺ binding, the densities for the EC1 site are clearly visible, but those for the NT site are ambiguous. The gated conformation obtained by 3D classification does not have enough densities to assign Ca²⁺, and as mentioned above, densities remain on the axis of the pore surrounded by NT.
Given this, the NT site for Ca²⁺ binding seems relatively speculative.
Although the manuscript notes the NT site as tentative, the discussion about the NT conformation sounds limited to the NT site to readers.
I suggest that the authors either strengthen the NT site with additional validation data, such as functional analysis by mutagenesis, where the amino acids capture Ca²⁺, or weaken the expressions so that the interpretation is not conclusive, like by describing the similar NT conformations found in other connexins even in no Ca²⁺ buffer.
3.
Regarding the EC1 site, the structures show that the Ca²⁺ binding site is different between Cx26 and Cx46/Cx50. The resolution of this study is particularly high and is therefore highly reliable. It would be good to add the authors' interpretation in the discussion section as to whether the difference in the EC1 site between Cx26 and Cx46/Cx50 has biological significance or is simply a coincidence.
4.
The dynamic N-terminal conformational change is very interesting, and this kind of configuration is observed in the cryo-EM structures of Cx43 (PLN: Pore lining N-terminus, GCN: Gate covering N-terminus, Lee et al. 2023). Also, the cryo-EM

structures of Cx43 and Cx36, showing the pore densities similarly, did not contain Ca^{2+} in the buffers. The authors might want to add an interpretation as to whether the NT movement observed in Cx46/Cx50 is Ca^{2+} specific or whether this structural change may generally occur as the opening and closing of connexin channels.

5.

It is worthwhile to consider that the Cx46/Cx50 structures have been observed in the presence of 20 mM Ca^{2+} , a high concentration that is unlikely to occur in vivo. Can the authors mention about this?

6.

The authors mentioned the "iris model" proposed by Zampighi et al. long ago, but the movement summarized in Figs and movies provides the impression of a pseudo-2-fold movement of paired subunits along a diagonal line rather than an iris. Indeed, the crystallographic studies by Oshima et al. (2011) demonstrated an asymmetric NT rearrangement with a 2-fold rotational crystallographic axis, reminiscent of the rearrangement depicted in Fig. 4d in this work. The functional assays by Oh et al. (2000) support individual subunit movement rather than concerted subunit change (= iris model). My understanding is that the "iris model" assumes the concerted and identical rearrangement of the six subunits simultaneously. Given this, the consistency between the NT movement in this text and the "iris model" sounds misleading to me. A modification of the text would be encouraged.

Minor points

1. line 105, "the" is duplicated. iris model is quoted using single quotation marks, but other parts use double quotation marks.
2. line 176, significant -> significant.
3. line 277, transmebrane -> transmembrane,
4. line 278, assoicated -> associated
5. line 289, rearrangments -> rearrangements
6. line 304, ambiguitiy -> ambiguity
7. line 318, dynmaic -> dynamic
8. line 321, hemichannels -> hemichannels

Reviewer #2

(Remarks to the Author)

The manuscript describes the calcium-induced N-terminal gating mechanism in connexin46/50 gap junctions. As Ca^{2+} -induced gating plays a crucial role in protecting healthy cells from cytotoxic signals triggered during ischemic conditions, understanding this mechanism is of significant biological importance. To investigate this process, the authors determined the cryo-EM structures of native Cx46/50 in the presence of Ca^{2+} , which revealed the Ca^{2+} binding sites. Among these, Ca^{2+} was found to bind to the N-terminal (NT) domain, triggering its conformational dynamics. The finding that Ca^{2+} binding induces conformational changes in the NT domain is particularly intriguing and aligns with previous observations that the NT domain of Cx43 and Cx36 gap junctions also undergoes conformational changes upon CHS or lipid binding (PMID: 36805660, PMID: 39455592). This study may provide further evidence that conformational changes or dynamic properties of the NT domain play a key role in regulating connexin gating, although the diversity of gating mechanisms may be subtype-dependent.

In this context, the structural snapshots of Ca^{2+} -induced conformational changes in the NT domain of Cx46/50, along with the accompanying structural dynamics of the overall structure (Fig. 4), are interesting and potentially significant. However, the exact gating mechanism of Cx46/50 remains unclear, and alternative gating mechanisms cannot be entirely ruled out. The authors favor the "iris model" for channel gating, but the current structural data do not fully validate this model. To strengthen their hypothesis, functional studies such as electrophysiological assays, dye-transfer experiments, or molecular dynamics (MD) simulations would be valuable.

It is acknowledged that performing functional assays may pose practical challenges for two main reasons. First, as Cx46/50 was purified from native cells, designing mutants for functional studies may be difficult. However, biochemical studies using ectopic expression could complement structural analysis to further validate the proposed model. Second, the NT domain, which contains the conserved Trp4 residue where Ca^{2+} binds, is essential for proper channel function as it interacts with the hydrophobic core of Cx46/50. Consequently, designing mutations that selectively disrupt Ca^{2+} binding without interfering with native function is highly challenging. Nevertheless, efforts in this direction would significantly strengthen the proposed model.

Another potential gating mechanism involves lipid-mediated occlusion, as suggested by cryo-EM studies of Cx43 and Cx36 from other research groups (PMID: 39455592, PMID: 36805660). While the proposed mechanism in Cx43 and Cx36—where lipid double-layer densities obstruct the channel pore and correlate with NT domain conformational states (PLN and FN states)—has yet to be fully demonstrated (since these observations were made in purified cryo-EM samples rather than in native cell environments, which could be further investigated via cryo-ET), it remains possible that connexins share common gating principles. This notion is supported by the observation that mefloquine, a specific inhibitor of Cx36, also blocks Cx46/50, suggesting a shared inhibition mechanism.

Overall, the manuscript is well-written, and the proposed Ca^{2+} -induced N-terminal gating and pore collapse mechanism in

Cx46/50 is scientifically intriguing, particularly given the biological relevance of Ca^{2+} -induced gating in gap junction channels. However, additional validation through complementary experimental approaches would be beneficial in excluding alternative gating mechanisms proposed for other connexins. The following points should be considered to further improve the manuscript:

Major points:

- 1) The gating mechanism observed in Cx43 and Cx36, where the channel pore is obstructed by lipid double-layer densities, should be considered. Since Cx46/50 was reconstituted in lipid nanodiscs, similar to Cx43 and Cx36, were similar double-layered densities observed in Ca^{2+} -treated Cx46/50?
- 2) The poor map quality of the NT domain may result from conformational heterogeneity. Performing a protomer-focused 3D classification would be highly beneficial for quantitatively analyzing the conformational diversity of the NT domain. The Ca^{2+} -induced gated states 1 and 2 resemble the GCN state observed in other connexins. Analyzing the positional distribution of the NT domain could provide insights into its structural dynamics. Additionally, the weak density of calcium bound to the NT domain may be due to this structural heterogeneity.
- 3) All structures were solved with D6 symmetry. Solving the Cx46/50 structures in C1 symmetry should be considered, particularly since typical symmetry-induced artifacts are observed in the NT domains of the Ca^{2+} -induced gated states 1 and 2. Additionally, imposing C1 symmetry could further validate the structural features of the NT domain.
- 4) It is tempting to suggest that Ca^{2+} binding to the NT domain of Cx46/50 and the resulting dynamic behavior of the NT domain may share similarities with the mechanism by which mefloquine induces structural changes in the NT domain of Cx36. This potential shared mechanism, in which both processes inhibit connexin function, warrants further consideration.
- 5) The authors identified two distinct calcium-binding sites: the NT domain and the EC1 site. While both sites are described as contributing to channel blocking through different mechanisms, is there evidence of cooperativity between them, or do they independently contribute to inhibition?
- 6) The Ca^{2+} -induced blocking of Cx46/50 could be mediated either through direct Ca^{2+} binding to the channel or through calmodulin binding. Since calmodulin is known to bind to Cx46/50 and inhibit channel function, could its involvement in the observed gating mechanism be considered?

Reviewer #3

(Remarks to the Author)

The manuscript by Flores et al describes the Ca^{2+} -bound states of the lens gap junction channel Cx46/50. The authors show that in the presence of Ca^{2+} the protein can exist in several gated states, and the NT (N-terminal) region plays a key role in this. The manuscript points to a previously suggested iris-like model of connexin channel gating based on their cryo-EM observations.

I think the work is of high quality, the illustrations are of very high quality, and it was a pleasure to read this manuscript. The authors are the top experts in this field and this work is clearly at the forefront methodologically.

Despite this, my main concern is the over-interpretation of the cryo-EM density maps around the NT region. This is really quite a poorly resolved region in the density maps in this study.

The features in this region are ambiguous and noisy in the occluded map, and do not readily allow confined Ca^{2+} ion placement - this would imply that a substantial part of this story is standing on a weak foundation and the manuscript has to be reformulated to address this concern. I am not sure this would necessarily invalidate the claim of a bound Ca^{2+} ion in that position - but in the absence of some orthogonal experiments to confirm this, the proposed binding site for Ca^{2+} needs to be described with a lot of caution.

Occluded state map / model. Fig 1 and ED Fig 3g. It is clear that much of the story is relying on the Ca^{2+} atom bound close to the D3 in the NT region. However, while the map for the NT as presented in ED Fig3g is just about ok for very rough building of the N-terminal polypeptide - it is difficult to correlate the map quality with the figure in ED Fig3c (local resolution). Even if formally the NT regions are estimated to have high local resolution, inspection of all maps reveals that model building in this is tough and placing ions anywhere in this density map is not possible. It is not unreasonable from the chemical environment point of view to place Ca^{2+} ions there: there is a D3 residue there and the acetylated glycine G2, and this should be an excellent place for Ca^{2+} ion coordination. The changes are observed upon addition of Ca^{2+} to the protein, so this is consistent too. But cryo-EM densities and the accompanying models do not provide sufficiently strong evidence for the bound Ca^{2+} ions there (and I think the specific side-chain conformations are also modelled optimistically).

I am struggling to suggest a clear experiment to address this specific point. However the following few come to mind:

1. One could imagine improving the resolution further by collecting a lot more cryo-EM data and isolating more clear density maps corresponding to the NT region. Contingent on map improvement, the authors might be able to boost their claims.
2. Alternatively (or perhaps in addition) - modifying/mutating the G2 and/or D3 residues to see the effect of that on Ca^{2+} binding and on the conformation could be an option. I can imagine these changes might radically affect the NT behaviour, and might bring a set of new problems.
3. Perhaps some MD simulations would be useful to at least some extent validate the proposed Ca^{2+} ion placement / stability at the N-terminus.

I think the authors should come up with an optimal experimental and/or computational approach to support their claims - otherwise they might need to update the manuscript substantially to reflect the accuracy of their cryo-EM reconstructions and the reliability of their models.

I also have a few minor points that could be addressed to improve the manuscript.

- Line 114. Why was 20 mM Ca²⁺ used? Maybe an explanation for this Ca²⁺ concentration could be helpful here for the reader.
- Fig 5, line 833. The authors write that NT "It is proposed that NT pairing may also be supported by inter-subunit Ca²⁺ binding" - maybe this should be phrased more carefully, in light of the main point above. The green dashed circles are used in this figure to highlight some sort of NT arrangements.
- ED Fig 3i. How do the authors distinguish the density that they interpret as a bound Ca²⁺ in the EC1 site from the densities they interpret as ordered water molecules? Is this something that is very clearly evident from the maps? It might be useful to show the maps in these regions at several thresholds.
- While I understand that it is impossible to tell the difference between Cx46 and Cx50, and formally one can model either one of those two connexin proteins into the density - it might be reasonable to present the Table 1 with a single map entry per condition ("Ca²⁺ occluded Cx46/50" and "Ca²⁺ gate 1 Cx46/50"), merging the identical entries from "Magnification" to "Map resolution range (Å)". This would also reduce the number of EMDB entries.
- The manuscript might benefit from some comparisons of the observed connexin conformations with the other connexin structures - in the current manuscript there are only sequence-based comparisons in ED Fig 4. In case some of the proposed modes of gating have parallels with other connexin channels - this might be discussed (e.g., in the gating models or broader implications part of the discussion).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my concerns adequately in their response.
I have no further comment.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

I thank the authors for thoroughly addressing all my concerns. The added amphipol cryo-EM data effectively clarifies the role of lipids, and the MD simulations alongside 3DVA support the revised asymmetric gating model. Removing the ungrounded N-terminal Ca²⁺ ion assignment was appropriate and substantially improves the manuscript's rigor. I have no further concerns and recommend the manuscript for publication.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

I commend the authors on the thorough job they have done on the revised manuscript. All my comments have been addressed well and I think the manuscript is now acceptable for publication.

(Remarks on code availability)

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We thank the reviewers for their careful and constructive assessment of our manuscript. We apologize for the extended time required to complete this revision. The reviewers raised important concerns regarding interpretation of the N-terminal cryo-EM density, the proposed Ca^{2+} -binding sites, conformational heterogeneity, the potential contribution of lipids, and the relationship of our findings to other connexin-gating mechanisms. Addressing these points required substantial re-analysis and additional experimental and computational work, and the manuscript has been extensively revised as a result.

Most importantly, we have removed the previously proposed NT Ca^{2+} -binding site from the atomic models and from our interpretation of the cryo-EM maps. The EC1 site is now the only Ca^{2+} -binding site assigned directly from experimental density. We also performed new long-timescale MD simulations of Cx46 and Cx50 with multiple replicates to assess other potential Ca^{2+} interaction sites, and we collected a new cryo-EM dataset of Ca^{2+} -treated Cx46/50 reconstituted in amphipol to test the potential role of lipids in mediating gating. Together, these studies support a revised model in which distributed, transient Ca^{2+} interactions bias the NT toward a heterogeneous ensemble of displaced conformations, while lipids may stabilize or enrich more ordered gated states.

The revised manuscript also includes expanded comparisons with the proposed Cx26 Ca^{2+} gating model, the low-pH-gated Cx46/50 and with structural and functional studies of other connexins. These additions have shifted the central interpretation toward an ensemble mechanism involving heterogeneous NT remodeling, asymmetric pore collapse, and a shared NT–TM2 mechanical pathway that responds to diverse physiological signals. The manuscript has been substantially reorganized and now includes new Figures 1–7, new supplementary analyses, and extensively revised Results and Discussion sections. We believe these changes have made the work considerably stronger, more rigorous, and more appropriately cautious in its structural interpretation. We respond to each comment in detail below.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript describes the cryo-EM structures of Cx46/Cx50 gap junction channels in a buffer containing Ca^{2+} ions in lipid nanodiscs. The structures reveal the Ca^{2+} ion binding sites at the extracellular site (EC1 site) and N-terminal site (NT site), which induce the N-terminal rearrangement. These observations implicate how the occluded and gated conformation of the gap junction channel can be mediated by Ca^{2+} ions, demonstrating that the remodeling is consistent with the iris model proposed long ago.

The structural analyses are reliable, and the interpretations are carefully described. My concerns are minor, but the authors should address several points raised below for publication.

1. In the cryo-EM map of the occluded conformation of Cx46/Cx50, the space on the rotational axis of the pore surrounded by the N-termini is filled with unassigned densities, which are not mentioned in the manuscript. Similar pore densities are found in the map of gated conformation, although it is not well resolved, which is discussed as a “single Ca^{2+} ion could cross-link the NT domains” in Fig. 5. However, the volume of these densities seems too large to be accounted for by a single Ca^{2+} ion.

Similarly, the pore densities have been observed in the cryo-EM structures of Cx36 and Cx43 (Lee et al. Nat. Commun. 14, 1347, 2023, Lee et al. Nat. Commun. 14, 931, 2023), which have been implicated as lipids. In this manuscript, the pore size is discussed based on only the assigned model (Fig. 2c), but it is recommended that the authors describe their additional interpretations about undefined pore densities. This is because the pore density may affect the channel permeability.

Response: We agree and have substantially expanded our discussion of the unassigned pore densities in the revised manuscript. The revised text now distinguishes between the two Ca^{2+} -bound conformations. In the Ca^{2+} -bound destabilized open-state (formerly termed the “occluded” state), additional high-frequency density surrounding the NT domains is present, likely due to unresolved conformational heterogeneity. This interpretation has been clarified in the Supplementary Figure 3 legend.

In contrast, the Ca^{2+} -bound gated state contains more substantial pore-lining densities beneath the displaced NT domains. As the reviewer noted, these resemble the pore-associated densities reported for Cx36 and Cx43, as well as the pore-bound lipids observed in our recently reported low-pH-gated Cx46/50 structures (Jarodsky et al. 2026). Although these densities are less well resolved than in our low-pH gated structures and therefore cannot be assigned with confidence, we now discuss the possibility that these features may represent pore-associated lipids that stabilize the gated conformation.

To directly investigate this possibility, we collected and analyzed a new cryo-EM dataset of Cx46/50 reconstituted in amphipol under identical Ca^{2+} conditions. In the absence of a surrounding lipid bilayer, Ca^{2+} still induces substantial NT destabilization and delocalization, demonstrating that lipids are not required to initiate NT remodeling. However, the more ordered gated conformations and associated pore densities observed in nanodiscs are largely absent in amphipol, consistent with a role for lipids in stabilizing or enriching specific Ca^{2+} -gated conformations rather than triggering channel closure. These findings are presented in the new Results section, " Ca^{2+} gating shares structural features with lipid-stabilized low-pH gating" and accompanying Figure 2.

The Supplementary Figure 3 legend has also been revised to clarify the interpretation of the remaining unassigned density:

g–h, Cx46 and Cx50 models fit to NT domain density for the destabilized open-state (residues 2–19) and gated state (residues 7–19), respectively. Sidechains for residues 7–17 of gated state 1 were truncated beyond C β due to limited local resolution. Note that both maps contain additional unassigned densities in the pore region near the NT domains, attributed to unresolved heterogeneity. In the case of the Ca^{2+} -bound gated state, pore-lining densities beneath the NT may further represent pore-bound lipids (see Fig. 2).

2. For Ca^{2+} binding, the densities for the EC1 site are clearly visible, but those for the NT site are ambiguous.

The gated conformation obtained by 3D classification does not have enough densities to assign Ca^{2+} , and as mentioned above, densities remain on the axis of the pore surrounded by NT. Given this, the NT site for Ca^{2+} binding seems relatively speculative.

Although the manuscript notes the NT site as tentative, the discussion about the NT conformation sounds limited to the NT site to readers.

I suggest that the authors either strengthen the NT site with additional validation data, such as functional analysis by mutagenesis, where the amino acids capture Ca^{2+} , or weaken the expressions so that the interpretation is not conclusive, like by describing the similar NT conformations found in other connexins even in no Ca^{2+} buffer.

Response: We agree that the original assignment of an NT-bound Ca^{2+} ion was not sufficiently supported by the cryo-EM density. We have therefore removed this ion from the atomic models and eliminated the corresponding interpretation from the manuscript. The EC1 site is now the only Ca^{2+} -binding site assigned directly from the cryo-EM maps.

To investigate how Ca^{2+} might nevertheless promote NT remodeling, we performed new long-timescale MD simulations of Cx46 and Cx50. Importantly, these simulations are presented as an independent analysis rather than validation of the previously assigned density. The simulations reproduce Ca^{2+} coordination at the experimentally resolved EC1 site and identify several additional, transient interaction regions distributed along the pore, including acidic residues within the NT domain. Inspection of individual trajectories further reveals inter-subunit coordination geometries that are associated with weakening of the hydrophobic NT–TM1/TM2 anchoring interactions and, in some cases, more pronounced NT displacement.

We have revised the manuscript throughout to make clear that these NT interactions are transient, low-affinity, and inferred from MD rather than directly resolved by cryo-EM. We also explicitly qualify the trajectory-level observations as mechanistic hypotheses derived from a limited number of simulations. The revised

interpretation is therefore not that Ca^{2+} binds to a single defined NT site, but that distributed and dynamically exchanging Ca^{2+} interactions may collectively destabilize NT anchoring and bias the channel toward the ensemble of displaced conformations observed experimentally. These results are presented in the new Results sections on pages 8-10 and in new Figures 5 and 6.

3. Regarding the EC1 site, the structures show that the Ca^{2+} binding site is different between Cx26 and Cx46/Cx50. The resolution of this study is particularly high and is therefore highly reliable. It would be good to add the authors' interpretation in the discussion section as to whether the difference in the EC1 site between Cx26 and Cx46/Cx50 has biological significance or is simply a coincidence.

Response: We agree that the original manuscript did not sufficiently address the relationship between the Cx46/50 EC1 site and the previously proposed Cx26 mechanism. We have now added a detailed comparison in the revised Results section *“Comparison with the proposed Cx26 Ca^{2+} -gating mechanism”* (pages 10–11), new Figure 7, and new Supplementary Figure 10.

This analysis shows that the Ca^{2+} -coordination geometry proposed for Cx26 is not reproduced in Cx46/50. In Cx46/50, the corresponding TM1/EC1 residues instead form a stable network of direct and water-mediated interactions that is preserved across the apo, destabilized open, and gated states, whereas Ca^{2+} is resolved at a distinct EC1 site coordinated primarily by D51 and E62 from adjacent subunits. We also compare the accompanying electrostatic and conformational consequences of Ca^{2+} binding, including the absence of resolved NT domains in the Cx26 crystal structures and the pronounced NT remodeling observed here.

We have been cautious not to assign a specific biological function to this difference without direct comparative functional data. However, the revised Discussion now considers it in the context of isoform-specific sequence and structural features, particularly the limited conservation of E62 across the connexin family. We therefore interpret the distinct sites as potentially reflecting isoform-dependent modes of Ca^{2+} recognition rather than a simple discrepancy between structures. At the same time, both models share a common consequence (Ca^{2+} -dependent remodeling of the pore electrostatic environment), while the heterogeneous nature of the induced NT conformations resolved in our study may reconcile why the NT of Cx26 was not resolved in the previous crystallographic study. The revised manuscript develops this comparison in the section *“Reconciling prior Ca^{2+} -gating models across connexins”*.

4. The dynamic N-terminal conformational change is very interesting, and this kind of configuration is observed in the cryo-EM structures of Cx43 (PLN: Pore lining N-terminus, GCN: Gate covering N-terminus, Lee et al. 2023). Also, the cryo-EM structures of Cx43 and Cx36, showing the pore densities similarly, did not contain Ca^{2+} in the buffers. The authors might want to add an interpretation as to whether the NT movement observed in Cx46/Cx50 is Ca^{2+} specific or whether this structural change may generally occur as the opening and closing of connexin channels.

Response: We agree. The revised manuscript now distinguishes between the Ca^{2+} dependence of the gating stimulus and the broader structural mechanism of NT-mediated closure. Our data do not suggest that NT movement is unique to Ca^{2+} . Rather, Ca^{2+} appears to bias Cx46/50 toward a conformational pathway that is also accessed by other gating stimuli.

The most direct comparison is with our recently described low-pH-gated Cx46/50 structures. As shown in new Figure 2 and the accompanying Results section, *“ Ca^{2+} gating shares structural features with lipid-stabilized low-pH gating”* both conditions produce closely related destabilized-open and gated NT conformations, together with coordinated inward movement of TM2 about the conserved proline kink. The upstream triggers differ, but the resulting structural transitions are remarkably similar.

We have broadened this interpretation in the revised Discussion under the new heading *“Distinct gating stimuli converge on a shared NT–TM2 mechanical axis”*. There, we compare our findings with structural and functional observations from other connexins. These comparisons support the interpretation that the NT–TM2 axis represents a conserved gating module that can be engaged by Ca^{2+} , low pH, CO_2 , voltage, and

potentially other regulatory inputs. Thus, we now interpret the NT conformational changes observed here not as a Ca^{2+} -specific structural state, but as a more general connexin gating transition whose occupancy is promoted by Ca^{2+} in Cx46/50.

5. It is worthwhile to consider that the Cx46/Cx50 structures have been observed in the presence of 20 mM Ca^{2+} , a high concentration that is unlikely to occur in vivo. Can the authors mention about this?

Response: Agreed. We now explicitly acknowledge that 20 mM Ca^{2+} is a non-physiological, saturating condition and clarify both why it was used and how the resulting structures and simulations should be interpreted.

In the Results, we added the rationale for using elevated Ca^{2+} during cryo-EM sample preparation:

Nanodisc-embedded channels were exchanged into buffer containing 20 mM Ca^{2+} and inspected by negative stain electron microscopy to confirm sample homogeneity and nanodisc incorporation (Supplementary Fig. 1). The high Ca^{2+} conditions were selected to promote occupancy of low-affinity sites and to support the relatively high protein concentrations required for cryo-EM analysis.

We also added a more extensive discussion of this limitation and its physiological implications:

Notably, the MD simulations were initiated from the destabilized open-state and, like the cryo-EM experiments, were performed under saturating Ca^{2+} (20 mM) to enhance sampling of transient coordination events. Consequently, the simulations do not capture the altered coordination environment that likely emerges following NT disengagement and formation of the fully gated conformations, where additional Ca^{2+} interactions and lipid-mediated stabilization may further reshape the energetic landscape. Accordingly, the shallow free-energy minima and nanosecond Ca^{2+} dwell times observed here likely reflect the intrinsic Ca^{2+} -responsive landscape of channels under saturating conditions, and may more closely resemble the millimolar Ca^{2+} regulation reported for connexin hemichannels. By contrast, the markedly greater Ca^{2+} sensitivity of gap junction uncoupling in cells may arise from conformational-state-dependent Ca^{2+} engagement, lipid-mediated stabilization of displaced NT conformations, cooperative behavior within junctional plaques, and additional cellular regulatory mechanisms, including interactions with auxiliary proteins such as calmodulin⁵². Together, these findings support a model in which distributed, low-affinity Ca^{2+} interactions progressively destabilize NT anchoring, biasing the channel toward pore-occluding conformations while providing multiple opportunities for physiological regulation to tune the gating response.

This revised text makes clear that the experiments were designed to define the intrinsic structural response of Cx46/50 under saturating Ca^{2+} , rather than to reproduce the apparent Ca^{2+} sensitivity of gap junction uncoupling in cells.

6. The authors mentioned the “iris model” proposed by Zampighi et al. long ago, but the movement summarized in Figs and movies provides the impression of a pseudo-2-fold movement of paired subunits along a diagonal line rather than an iris. Indeed, the crystallographic studies by Oshima et al. (2011) demonstrated an asymmetric NT rearrangement with a 2-fold rotational crystallographic axis, reminiscent of the rearrangement depicted in Fig. 4d in this work. The functional assays by Oh et al. (2000) support individual subunit movement rather than concerted subunit change (= iris model). My understanding is that the “iris model” assumes the concerted and identical rearrangement of the six subunits simultaneously. Given this, the consistency between the NT movement in this text and the “iris model” sounds misleading to me. A modification of the text would be encouraged.

Response: We agree that our original comparison to the iris model was too direct. Our intent was to emphasize that Ca^{2+} gating involves coordinated subunit rearrangements that narrow and reshape the pore, not to imply the concerted, sixfold-symmetric motion proposed in the classical iris model. We have revised the manuscript throughout to dampen this direct comparison and make this distinction more clear.

We also appreciate the reviewer highlighting the work of Oshima et al. The asymmetric, approximately twofold arrangement observed for the gating-biased Cx26 M34A mutant is indeed more closely related to the channel stretching, pore collapse, and paired NT configurations observed in our 3D variability analysis. Together with functional evidence supporting non-concerted subunit transitions, these comparisons reinforce our interpretation that connexin gating proceeds through heterogeneous and asymmetric rearrangements rather than a uniform iris-like constriction.

We now discuss this more carefully in the revised Discussion under “Reconciling prior Ca^{2+} -gating models across connexins”:

Early electron crystallography studies of native gap junctions gave rise to the iris model, in which coordinated subunit rearrangements constrict the pore²⁷⁻²⁹. Although our structures reveal a considerably more complex and heterogeneous process than originally envisioned, they support the central premise that collective subunit motions contribute to gating. Rather than producing a simple symmetric constriction, these rearrangements reshape the pore through asymmetric stretching and collapse while simultaneously creating the geometric conditions that permit NT displacement and steric occlusion. A low-resolution structure of the gating-biased Cx26M34A mutant¹⁵, which displayed an oblique pore geometry together with central NT-associated densities, is likewise consistent with this revised view. Thus, pore constriction and NT-mediated occlusion may be viewed as complementary features of the same gating mechanism.

Minor points

1. line 105, “the” is duplicated. iris model is quoted using single quotation marks, but other parts use double quotation marks.
2. line 176, significant -> significant.
3. line 277, transmebrane -> transmembrane,
4. line 278, assoicated -> associated
5. line 289, rearrangments -> rearrangements
6. line 304, ambiguity -> ambiguity
7. line 318, dynmaic -> dynamic
8. line 321, hemichannels -> hemichannels

Response: Thank you – these mistakes have been corrected.

Reviewer #2 (Remarks to the Author):

The manuscript describes the calcium-induced N-terminal gating mechanism in connexin46/50 gap junctions. As Ca^{2+} -induced gating plays a crucial role in protecting healthy cells from cytotoxic signals triggered during ischemic conditions, understanding this mechanism is of significant biological importance. To investigate this process, the authors determined the cryo-EM structures of native Cx46/50 in the presence of Ca^{2+} , which revealed the Ca^{2+} binding sites. Among these, Ca^{2+} was found to bind to the N-terminal (NT) domain, triggering its conformational dynamics. The finding that Ca^{2+} binding induces conformational changes in the NT domain is particularly intriguing and aligns with previous observations that the NT domain of Cx43 and Cx36 gap junctions also undergoes conformational changes upon CHS or lipid binding (PMID: 36805660, PMID: 39455592). This study may provide further evidence that conformational changes or dynamic properties of the NT domain play a key role in regulating connexin gating, although the diversity of gating mechanisms may be subtype-dependent.

In this context, the structural snapshots of Ca^{2+} -induced conformational changes in the NT domain of Cx46/50, along with the accompanying structural dynamics of the overall structure (Fig. 4), are interesting and potentially significant. However, the exact gating mechanism of Cx46/50 remains unclear, and alternative gating mechanisms cannot be entirely ruled out. The authors favor the “iris model” for channel gating, but the current structural data do not fully validate this model. To strengthen their hypothesis, functional studies such as electrophysiological assays, dye-transfer experiments, or molecular dynamics (MD) simulations would be valuable.

It is acknowledged that performing functional assays may pose practical challenges for two main reasons. First, as Cx46/50 was purified from native cells, designing mutants for functional studies may be difficult. However, biochemical studies using ectopic expression could complement structural analysis to further validate the proposed model. Second, the NT domain, which contains the conserved Trp4 residue where Ca^{2+} binds, is essential for proper channel function as it interacts with the hydrophobic core of Cx46/50. Consequently, designing mutations that selectively disrupt Ca^{2+} binding without interfering with native function is highly challenging. Nevertheless, efforts in this direction would significantly strengthen the proposed model.

Another potential gating mechanism involves lipid-mediated occlusion, as suggested by cryo-EM studies of Cx43 and Cx36 from other research groups (PMID: 39455592, PMID: 36805660). While the proposed mechanism in Cx43 and Cx36—where lipid double-layer densities obstruct the channel pore and correlate with NT domain conformational states (PLN and FN states)—has yet to be fully demonstrated (since these observations were made in purified cryo-EM samples rather than in native cell environments, which could be further investigated via cryo-ET), it remains possible that connexins share common gating principles. This notion is supported by the observation that mefloquine, a specific inhibitor of Cx36, also blocks Cx46/50, suggesting a shared inhibition mechanism.

Overall, the manuscript is well-written, and the proposed Ca^{2+} -induced N-terminal gating and pore collapse mechanism in Cx46/50 is scientifically intriguing, particularly given the biological relevance of Ca^{2+} -induced gating in gap junction channels. However, additional validation through complementary experimental approaches would be beneficial in excluding alternative gating mechanisms proposed for other connexins.

Response: We thank the reviewer for their careful assessment and poignant critiques. We believe these concerns are all addressed in our point-by-point responses below.

The following points should be considered to further improve the manuscript:

Major points:

1) The gating mechanism observed in Cx43 and Cx36, where the channel pore is obstructed by lipid double-layer densities, should be considered. Since Cx46/50 was reconstituted in lipid nanodiscs, similar to Cx43 and Cx36, were similar double-layered densities observed in Ca^{2+} -treated Cx46/50?

Response: We do not observe the well-defined lipid double-layer reported in the pores of Cx43 and Cx36. However, the Ca^{2+} -bound gated Cx46/50 reconstructions contain unassigned pore-lining densities beneath the displaced NT domains that could represent more heterogeneous or partially occupied lipids. In the revised manuscript, we compare these features with the more clearly resolved pore-associated lipids in our recently reported low-pH-gated Cx46/50 structures. Although the densities under Ca^{2+} conditions are less well defined and cannot be assigned confidently, their similar location suggested that lipids might contribute to stabilization of the gated state.

To test this possibility, we collected a new cryo-EM dataset of Cx46/50 reconstituted in amphipol and exposed to the same Ca^{2+} conditions. Ca^{2+} still produced substantial NT destabilization and delocalization in the absence of a lipid bilayer, indicating that lipids are not required to initiate NT remodeling. However, the more ordered gated NT conformations and associated pore-lining densities observed in nanodiscs were largely absent in amphipol. These findings support a model in which Ca^{2+} is sufficient to destabilize the open-state NT, whereas pore-associated lipids may stabilize or enrich particular conformations within the broader gated ensemble.

These new data and comparisons are presented in Figure 2 and the revised Results section, “ *Ca^{2+} gating shares structural features with lipid-stabilized low-pH gating*” (pages 6-7). We have also expanded the Discussion under “*Distinct gating stimuli converge on a shared NT–TM2 mechanical axis*” (page 12) to place these findings in the broader context of other connexin structures and functional studies. Rather than proposing that all connexins gate through an identical lipid-filled pore state, we now emphasize that different gating stimuli and isoforms may converge on a shared NT–TM2 remodeling pathway, with lipids contributing to different degrees to the stabilization of pore-occluding conformations.

2) The poor map quality of the NT domain may result from conformational heterogeneity. Performing a protomer-focused 3D classification would be highly beneficial for quantitatively analyzing the conformational diversity of the NT domain. The Ca^{2+} -induced gated states 1 and 2 resemble the GCN state observed in other connexins. Analyzing the positional distribution of the NT domain could provide insights into its structural dynamics. Additionally, the weak density of calcium bound to the NT domain may be due to this structural heterogeneity.

Response: We agree that the poor map quality of the NT domain for the gated-states primarily reflects substantial conformational heterogeneity. We attempted protomer-focused classification using an approach similar to that applied in our recent low-pH-gating study, but this did not yield stable, interpretable NT classes for the Ca^{2+} -bound dataset. We therefore analyzed the conformational landscape using 3D variability analysis (3DVA) of a symmetry-expanded particle stack without imposing symmetry on the resulting PC motions.

This analysis revealed a continuum of asymmetric NT configurations rather than a set of discrete states. Individual channels contain different combinations of open-like and gated NT domains, frequently involving neighboring subunits or paired NT interactions across the pore – consistent with a broad and highly heterogeneous ensemble. Although we were unable to resolve the dataset into sufficiently robust protomer classes for a quantitative occupancy analysis, the 3DVA analysis directly demonstrates that the poor NT densities in the symmetrized maps arises from heterogeneous and non-concerted NT rearrangements. These results are presented in Figure 3, Supplemental Figure 7, and the corresponding Results section.

We also agree that this heterogeneity precludes confident assignment of Ca^{2+} at the NT in the destabilized open-state (formerly “occluded state”) cryo-EM map. Upon re-analysis, we concluded that the original NT Ca^{2+} site was not sufficiently supported and have removed the ion from the atomic models and all associated cryo-EM interpretations. The EC1 site is now the only Ca^{2+} site assigned directly from experimental density.

To explore how Ca^{2+} might nevertheless contribute to NT destabilization, we performed new long-timescale MD simulations. These simulations are presented as an independent mechanistic analysis rather than validation of the removed cryo-EM assignment. They reproduce Ca^{2+} coordination at the EC1 site and identify additional transient interaction regions along the pore, including inter- and intra-subunit interactions involving acidic NT residues. In several trajectories, these interactions coincide with weakening of the NT–TM1/TM2 anchoring contacts and more pronounced NT displacement. We have carefully qualified these observations as qualitative and derived from a limited number of trajectories. The revised interpretation is therefore not that Ca^{2+} occupies a single defined NT site, but that distributed, transient Ca^{2+} interactions may bias the NT toward the heterogeneous displaced and potentially paired NT-NT conformations observed experimentally.

3) All structures were solved with D6 symmetry. Solving the Cx46/50 structures in C1 symmetry should be considered, particularly since typical symmetry-induced artifacts are observed in the NT domains of the Ca^{2+} -induced gated states 1 and 2. Additionally, imposing C1 symmetry could further validate the structural features of the NT domain.

Response: We agree that the D6-symmetrized reconstructions provide an averaged representation of the NT domains and can introduce symmetry-related features that do not capture the underlying asymmetric conformations. Indeed, we have performed C1 refinements and classifications. However, these did not yield an improved or interpretable NT reconstruction, likely because no single asymmetric state dominates the dataset.

As described in our response to the preceding comment, we therefore used 3DVA of a symmetry-expanded particle stack to characterize the asymmetric conformational landscape directly. This analysis revealed distinct NT conformations within the same channel, together with approximately twofold stretching and collapse of the channel scaffold (see Figure 3). We now make clear that the D6 maps are used for high-resolution analysis of the channel scaffold and visualization of averaged NT states, whereas the asymmetric 3DVA provides the more representative description of NT heterogeneity.

The revised Results conclude:

“Together, these analyses indicate that Ca²⁺ gating is mediated by a continuum of asymmetric NT configurations coupled to coordinated remodeling of the channel scaffold, rather than by a simple transition between open and closed states.”

This ensemble-view of the gating mechanism is also described in our revised Discussion section (page 11).

4) It is tempting to suggest that Ca²⁺ binding to the NT domain of Cx46/50 and the resulting dynamic behavior of the NT domain may share similarities with the mechanism by which mefloquine induces structural changes in the NT domain of Cx36. This potential shared mechanism, in which both processes inhibit connexin function, warrants further consideration.

Response: We agree that this is an interesting potential comparison. Both Ca²⁺-dependent gating of Cx46/50 and mefloquine inhibition of Cx36 involve remodeling of the NT domain and loss of channel function, raising the possibility that distinct regulatory inputs can converge on a common NT-centered gating machinery.

In the revised manuscript, we have focused the detailed structural comparisons on systems that are most directly related to the present mechanism, including lipid-mediated low-pH gating of Cx46/50 and the previously proposed Ca²⁺-gating mechanism of Cx26. We also broaden the Discussion to propose that the NT–TM2 axis functions as a conserved mechanical module engaged by multiple physiological stimuli.

We have not developed a more direct mechanistic equivalence with mefloquine inhibition because the initiating interaction and structural pathway may differ between pharmacological inhibition from those produced by physiological gating stimuli. Nonetheless, we now acknowledge that pharmacological ligands may also exploit this shared NT-centered conformational landscape in the Discussion, under *“Broader implications”*.

5) The authors identified two distinct calcium-binding sites: the NT domain and the EC1 site. While both sites are described as contributing to channel blocking through different mechanisms, is there evidence of cooperativity between them, or do they independently contribute to inhibition?

Response: This is an important question. However, following re-analysis, the revised manuscript no longer identifies two discrete Ca²⁺-binding sites. The EC1 site is the only site assigned directly from the cryo-EM density; the previously proposed NT site has been removed from the models and interpretation because it was not sufficiently supported by the map quality.

The new MD simulations identify several transient Ca²⁺ interaction regions along the pore, including acidic residues within the NT domain, but they do not provide clear evidence for cooperativity between the EC1 site and these NT interactions. Indeed, the EC1 coordination environment remains largely unchanged across the cryo-EM gating ensemble, and how occupancy at this site is coupled to NT remodeling remains unresolved.

The simulations do reveal recurring inter-subunit Ca²⁺ coordination involving neighboring NT domains, as well as interactions bridging proximal and distal regions of the NT. These events are associated with weakening of the NT–TM1/TM2 anchoring contacts and, in some trajectories, more pronounced NT displacement. This is consistent with the paired NT configurations observed by 3D variability analysis and raises the possibility that transient Ca²⁺ bridging contributes collectively to NT destabilization. However, we have avoided describing these observations as evidence of cooperativity, which would require direct functional or energetic measurements. Future mutational and functional studies will be needed to determine whether the EC1 site is mechanistically coupled to the NT and whether the distributed NT interactions act independently or cooperatively.

We clarify this interpretation in the revised Discussion:

“Inspection of individual trajectories further suggests how distributed Ca²⁺ engagement may couple to NT remodeling. Inter-subunit coordination involving distal NT residues at Site 4 is frequently associated with weakening of the hydrophobic anchoring interactions that stabilize the open-state NT, whereas coordination bridging the proximal residue D3 (Site 3) with distal NT residues (Site 4) is more consistently associated with pronounced NT displacement. Although these observations are qualitative and derive

from a limited number of simulations, they provide a plausible molecular explanation for the paired NT configurations observed in the cryo-EM reconstructions and suggest how transient Ca²⁺ coordination could bias the NT toward progressively more displaced conformations.”

6) The Ca²⁺-induced blocking of Cx46/50 could be mediated either through direct Ca²⁺ binding to the channel or through calmodulin binding. Since calmodulin is known to bind to Cx46/50 and inhibit channel function, could its involvement in the observed gating mechanism be considered?

Response: Our purified Cx46/50 preparation does not contain detectable calmodulin or other cytosolic regulatory proteins (see Supplemental Fig. 1). The purification includes extensive stripping of lens fiber-cell membranes before detergent solubilization, followed by ion-exchange and size-exclusion chromatography. Thus, the Ca²⁺-dependent conformational changes observed in the cryo-EM experiments are concluded to arise from direct effects of Ca²⁺ on the purified channel and do not require calmodulin.

This does not exclude an additional role for calmodulin in cells. Calmodulin may provide a separate or complementary layer of regulation that contributes to the apparent Ca²⁺ sensitivity of gap-junction uncoupling under physiological conditions. We now acknowledge this possibility in the revised Discussion:

“By contrast, the markedly greater Ca²⁺ sensitivity of gap junction uncoupling in cells may arise from conformational-state-dependent Ca²⁺ engagement, lipid-mediated stabilization of displaced NT conformations, cooperative behavior within junctional plaques, and additional cellular regulatory mechanisms, including interactions with auxiliary proteins such as calmodulin⁵².”

Reviewer #3 (Remarks to the Author):

The manuscript by Flores et al describes the Ca²⁺-bound states of the lens gap junction channel Cx46/50. The authors show that in the presence of Ca²⁺ the protein can exist in several gated states, and the NT (N-terminal) region plays a key role in this. The manuscript points to a previously suggested iris-like model of connexin channel gating based on their cryo-EM observations.

I think the work is of high quality, the illustrations are of very high quality, and it was a pleasure to read this manuscript. The authors are the top experts in this field and this work is clearly at the forefront methodologically.

Despite this, my main concern is the over-interpretation of the cryo-EM density maps around the NT region. This is really quite a poorly resolved region in the density maps in this study.

The features in this region are ambiguous and noisy in the occluded map, and do not readily allow confined Ca²⁺ ion placement - this would imply that a substantial part of this story is standing on a weak foundation and the manuscript has to be reformulated to address this concern. I am not sure this would necessarily invalidate the claim of a bound Ca²⁺ ion in that position - but in the absence of some orthogonal experiments to confirm this, the proposed binding site for Ca²⁺ needs to be described with a lot of caution.

Occluded state map / model. Fig 1 and ED Fig 3g. It is clear that much of the story is relying on the Ca²⁺ atom bound close to the D3 in the NT region. However, while the map for the NT as presented in ED Fig3g is just about ok for very rough building of the N-terminal polypeptide - it is difficult to correlate the map quality with the figure in ED Fig3c (local resolution). Even if formally the NT regions are estimated to have high local resolution, inspection of all maps reveals that model building in this is tough and placing ions anywhere in this density map is not possible. It is not unreasonable from the chemical environment point of view to place Ca²⁺ ions there: there is a D3 residue there and the acetylated glycine G2, and this should be an excellent place for Ca²⁺ ion coordination. The changes are observed upon addition of Ca²⁺ to the protein, so this is consistent too. But cryo-EM densities and the accompanying models do not provide sufficiently strong evidence for the bound Ca²⁺ ions there (and I think the specific side-chain conformations are also modelled optimistically).

I am struggling to suggest a clear experiment to address this specific point. However the following few come to mind:

1. One could imagine improving the resolution further by collecting a lot more cryo-EM data and isolating more clear density maps corresponding to the NT region. Contingent on map improvement, the authors might be able to boost their claims.
2. Alternatively (or perhaps in addition) - modifying/mutating the G2 and/or D3 residues to see the effect of that on Ca²⁺ binding and on the conformation could be an option. I can imagine these changes might radically affect the NT behaviour, and might bring a set of new problems.
3. Perhaps some MD simulations would be useful to at least some extent validate the proposed Ca²⁺ ion placement / stability at the N-terminus.

I think the authors should come up with an optimal experimental and/or computational approach to support their claims - otherwise they might need to update the manuscript substantially to reflect the accuracy of their cryo-EM reconstructions and the reliability of their models.

Response: We agree with the reviewer's central concern, and appreciate the thoughtful suggestions. Upon re-examination of the maps and models, we concluded that the density near the NT does not support confident placement of a Ca²⁺ ion or detailed interpretation of the associated side-chain conformations. We have therefore removed the proposed NT Ca²⁺ site from the atomic models and eliminated all statements that treat it as a cryo-EM-resolved binding site. The EC1 site is now the only Ca²⁺ site assigned directly from experimental density.

We considered mutational analysis of G2 and D3, as suggested. However, these residues also contribute directly to stabilization of the open-state NT and to the established voltage-sensing mechanism of connexin channels. Their substitution would therefore be expected to alter basal NT conformation and/or channel function independently of Ca²⁺ coordination, making the resulting phenotype difficult to interpret specifically in terms of the proposed site. Rather than using such experiments to support an assignment that is not justified by the maps, we chose to reformulate the manuscript around what can be established more directly – that is Ca²⁺ induces destabilization and dissociation of the NT domain.

To investigate how Ca²⁺ may nevertheless promote NT destabilization, we performed new long-timescale MD simulations of Cx46 and Cx50. These simulations are presented as an independent mechanistic analysis, not as validation of the removed cryo-EM assignment. They reproduce Ca²⁺ coordination at the experimentally resolved EC1 site and identify several additional, transient Ca²⁺ interaction regions distributed along the pore. These include acidic residues within both proximal and distal regions of the NT. In individual trajectories, inter- and intra-subunit coordination involving these residues is associated with weakening of the hydrophobic NT–TM1/TM2 anchoring contacts and, in some cases, more pronounced NT displacement.

We have carefully limited the interpretation of these results. The revised manuscript no longer proposes a single, well-defined NT Ca²⁺-binding site. Instead, it presents a model in which dynamically exchanging, low-affinity Ca²⁺ interactions may collectively bias the NT toward the heterogeneous displaced conformations observed by cryo-EM. We also explicitly state that the trajectory-level observations are qualitative and derive from a limited number of simulations. These new analyses are presented in Figures 5 and 6 and the corresponding revised Results and Discussion sections.

I also have a few minor points that could be addressed to improve the manuscript.

- Line 114. Why was 20 mM Ca²⁺ used? Maybe an explanation for this Ca²⁺ concentration could be helpful here for the reader.

Response: Agreed. We have added a brief explanation at the point where the 20 mM Ca²⁺ condition is first introduced:

“Nanodisc-embedded channels were exchanged into buffer containing 20 mM Ca^{2+} and inspected by negative stain electron microscopy to confirm sample homogeneity and nanodisc incorporation (Supplementary Fig. 1). The high Ca^{2+} conditions were selected to promote occupancy of low-affinity sites and to support the relatively high protein concentrations required for cryo-EM analysis.”

We also expanded the Discussion to clarify that 20 mM Ca^{2+} represents a non-physiological, saturating condition and to explain how the cryo-EM and MD results should be interpreted in that context:

“Notably, the MD simulations were initiated from the destabilized open-state and, like the cryo-EM experiments, were performed under saturating Ca^{2+} (20 mM) to enhance sampling of transient coordination events. Consequently, the simulations do not capture the altered coordination environment that likely emerges following NT disengagement and formation of the fully gated conformations, where additional Ca^{2+} interactions and lipid-mediated stabilization may further reshape the energetic landscape. Accordingly, the shallow free-energy minima and nanosecond Ca^{2+} dwell times observed here likely reflect the intrinsic Ca^{2+} -responsive landscape of channels under saturating conditions, and may more closely resemble the millimolar Ca^{2+} regulation reported for connexin hemichannels. By contrast, the markedly greater Ca^{2+} sensitivity of gap junction uncoupling in cells may arise from conformational-state-dependent Ca^{2+} engagement, lipid-mediated stabilization of displaced NT conformations, cooperative behavior within junctional plaques, and additional cellular regulatory mechanisms, including interactions with auxiliary proteins such as calmodulin⁵². Together, these findings support a model in which distributed, low-affinity Ca^{2+} interactions progressively destabilize NT anchoring, biasing the channel toward pore-occluding conformations while providing multiple opportunities for physiological regulation to tune the gating response.”

- Fig 5, line 833. The authors write that NT “It is proposed that NT pairing may also be supported by inter-subunit Ca^{2+} binding” - maybe this should be phrased more carefully, in light of the main point above. The green dashed circles are used in this figure to highlight some sort of NT arrangements.

Response: Agreed. We have removed the original Figure 5 and the associated statement because the symmetrized representation did not adequately capture the heterogeneous NT configurations revealed by the revised analysis. The green dashed circles and the proposed assignment of specific NT pairs are therefore no longer included.

The new MD simulations do identify transient inter-subunit Ca^{2+} coordination involving NT residues, providing a possible molecular basis for some of the paired NT configurations observed by cryo-EM. However, we now present this only as a qualitative mechanistic hypothesis and do not infer Ca^{2+} -mediated pairing directly from the cryo-EM density. The revised Discussion states:

“Inspection of individual trajectories further suggests how distributed Ca^{2+} engagement may couple to NT remodeling. Inter-subunit coordination involving distal NT residues at Site 4 is frequently associated with weakening of the hydrophobic anchoring interactions that stabilize the open-state NT, whereas coordination bridging the proximal residue D3 (Site 3) with distal NT residues (Site 4) is more consistently associated with pronounced NT displacement. Although these observations are qualitative and derive from a limited number of simulations, they provide a plausible molecular explanation for the paired NT configurations observed in the cryo-EM reconstructions and suggest how transient Ca^{2+} coordination could bias the NT toward progressively more displaced conformations.”

- ED Fig 3i. How do the authors distinguish the density that they interpret as a bound Ca^{2+} in the EC1 site from the densities they interpret as ordered water molecules? Is this something that is very clearly evident from the maps? It might be useful to show the maps in these regions at several thresholds.

Response: Agreed. We now provide a direct comparison of the EC1 density in the Ca^{2+} -bound destabilized open-state, Ca^{2+} -bound gated state, and apo-state maps in new Figure 4. In the Ca^{2+} -bound maps, the spherical density assigned to Ca^{2+} is substantially stronger than the surrounding ordered-water densities, whereas no corresponding feature is present in the apo-state map. This is consistent with the greater electron scattering

expected for Ca^{2+} relative to oxygen and with the local coordination environment formed by E62 and D51 from adjacent subunits.

Finally, the same coordination region is independently recovered in the MD simulations, where Ca^{2+} preferentially samples interactions with E62 and D51. Taken together, the map intensity, absence of the feature in the apo state, favorable coordination chemistry, and independent MD support provide the basis for assigning this density as Ca^{2+} . These analyses are now presented in new Figures 4 and 5 and described in the corresponding Results sections.

- While I understand that it is impossible to tell the difference between Cx46 and Cx50, and formally one can model either one of those two connexin proteins into the density - it might be reasonable to present the Table 1 with a single map entry per condition (" Ca^{2+} occluded Cx46/50" and " Ca^{2+} gate 1 Cx46/50"), merging the identical entries from "Magnification" to "Map resolution range (Å)". This would also reduce the number of EMDB entries.

Response: Agreed - thank you for this suggestion. We have revised Table 1 accordingly.

- The manuscript might benefit from some comparisons of the observed connexin conformations with the other connexin structures - in the current manuscript there are only sequence-based comparisons in ED Fig 4. In case some of the proposed modes of gating have parallels with other connexin channels - this might be discussed (e.g., in the gating models or broader implications part of the discussion).

Response: Agreed. We have substantially expanded the structural comparisons in the revised manuscript. The most direct comparison is now presented in new Figure 2 and the accompanying Results section, " *Ca^{2+} gating shares structural features with lipid-stabilized low-pH gating*" (pages 6–7). Structural overlays show that Ca^{2+} and low pH produce closely related destabilized-open and gated NT conformations, together with coordinated inward movement of TM2 about the conserved proline kink.

We have also broadened the Discussion to compare these findings with structural and functional observations from other connexin isoforms. In the new section "*Distinct gating stimuli converge on a shared NT–TM2 mechanical axis*" (page 12), we discuss evidence from structural and functional studies on other connexins supporting a conserved coupling between NT rearrangement and TM2 motion under diverse physiological gating conditions. These comparisons support the interpretation that different stimuli and isoforms can converge on a shared mechanical pathway, even when the upstream trigger or degree of lipid involvement differs.