

Reversible lipid mediated pH-gating of connexin-46/50 by cryo-EM

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Please find the attached doc file.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

(Remarks on code availability)

The manuscript by Jarodsky et al is a very interesting article describing the difference in structures of the connexin46/50 gap junction at high and low pH. In a lipid environment (nanodiscs made of DMPC, rather than the native environment) the change of pH causes the infiltration of lipids into the pore and causes apparent closure of the channels caused by an upward movement of the N-terminal helix. Surprisingly, this is reversible.

The study is interesting. The possible involvement of lipids in the regulation of connexins and other large pore channels appears to be a recurring theme in the recent structural studies that have been carried out. The link between pH and lipids has not been observed before. Within the limits of the experimentation (using nanodiscs with DMPC rather than native lipids) the experiments seem well done with extensive classification of cryo-EM data sets.

There are a few points that need to be addressed:

The high pH buffer is HEPES. However, instead of using a similar buffer such as MES, (that could be used at pH 5.8), the authors use a completely different buffer by swapping to succinate. It is therefore possible that the change in buffering system affects the structure as well as, or even instead of, the pH? Given that they see the same conformation in amphipols using "Low pH buffer" as they see in HEPES, this is probably not an issue, but I note in their methods that the "Low pH buffer" is not explicitly defined when discussing the amphipols experiments. The buffer needs to be defined and some

comment should be made regarding the differing buffering systems.

They discuss the possibility of His95 and His17 being important for pH sensitivity and gating. As they note in the results section, the literature surrounding this is not clear cut. However, the discussion is written in such a way that leads the reader to believe that the structures reported here explain how the residues contribute to pH sensitivity. This is slightly misleading.

There is only minimal comparison with other connexin structures. While I wouldn't expect a detailed comparison of all the different structures, the movement of TM2 around the proline seems similar to that reported for Cx26 (Brotherton, et al Structure, 2022, eLife, 2024) and movements of TM2 are also mentioned for Cx43 (Lee et al, Nat Comm, 2023).

In Figure 1b, a "pore plug" is mentioned at low threshold levels. Is this perhaps an artefact of the symmetry?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Attached.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
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(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed the reviewers comments.

(Remarks on code availability)

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Point-by-point Response

We thank the reviewers for their thoughtful and constructive feedback, which has significantly improved the clarity and quality of our manuscript. In response, we have revised the text, figures, and methods, as well as additional new data to address all major concerns.

Notably, we have further refined our gated-state structure and now present a more conservative model that better reflects the local resolution limitations within the NT gating domain. We have also expanded structural comparisons to other connexin isoforms and gating mechanisms, clarified key interpretations and methodological details, and strengthened our discussion of pH- and lipid-mediated gating. In addition, we now explicitly acknowledge key limitations of the current study. A detailed, point-by-point response to each reviewer comment is provided below.

Reviewers #1/2:

The authors have used sophisticated cryo-EM techniques to investigate the reversible, lipid-mediated pH-gating of connexin-46/50. While they have attempted several approaches to resolve various states, there are several critical and major concerns that need to be addressed. In general, to support most of their conclusions, they need to improve the quality of the maps in certain important regions, which are central to the discussion in this manuscript. Many statements are overly speculative and not supported by map densities. The authors either need to collect more data and classify structural heterogeneity more effectively or moderate their claims accordingly.

Major Concerns:

1. MSP Belt Tightness & Nanodisc Stability

- The MSP belt appears too tight, which may influence conformational changes in the N-terminal (NT) domain, particularly at low pH, where it is proposed to be stabilized by lipids.
- Nanodiscs do not behave well at low pH, and MSP stability decreases under such conditions, potentially affecting lipid entry. As a result, the lipid density observed near the NT may not be physiologically relevant.

Response: We thank the reviewers for raising these concerns regarding the use of nanodiscs and the potential influence of MSP belt tightness and pH-dependent instability on our structural observations, which we address below. As for the overarching concern, that our *in vitro* nanodisc systems may not fully replicate the physiological membrane environments, we certainly agree and we explicitly acknowledge this limitation in the manuscript,

pg. 12:

However, it is important to note that a direct role of lipids in gap junction gating remains to be validated through cellular functional studies.

pg. 15

Moreover, as noted above, the physiological relevance of lipid-mediated gating remains to be established in cellular systems, including investigations into potential lipid specificity and defining the translocation pathway(s) into and out of the pore.

Regarding MSP belt tightness: While the reviewers don't provide their rationale for making this assertion, we respectfully note that the use of MSP1E1 in our system resulted in highly homogeneous populations of gap junction channels in the open state under neutral pH conditions. Notably, our structures of Cx46/50 in MSP1E1 nanodiscs remain, to our knowledge, the only system to provide such a well-defined and consistent open-state conformation of the NT-domain for gap junction channels under non-gating conditions. Furthermore, our 3D variability analysis (3DVA) demonstrates that even under these conditions conformational flexibility persists, especially in TM2 and other TMs, indicating that intrinsic dynamics are preserved and not overly constrained by the nanodisc scaffold. In fact, we have unpublished data showing that nanodiscs using MSP1E1 can contain more than one Cx46/50 channel, and still preserve the channels in a stabilized open-state.

Regarding MSP behavior at low pH: we understand the concern that potential destabilization of the MSP belt could, in principle, influence lipid redistribution. However, several lines of evidence strongly argue against this, or for it being a dominant contributor to the observed lipid infiltration or NT gating:

1. **Reversibility:** A key finding of our study is the reversible nature of the lipid-dependent gating transition. Upon restoring samples from low pH to neutral pH, >96% of channels return to the open conformation, with concomitant removal of pore-bound lipids (a key result and aspect of rigor that has been missing from other gap junction structural studies that observe lipids inside the channel pore). This reversibility strongly supports a non-artifactual mechanism and preserved integrity of our nanodisc system.
2. **Detergent Controls:** In parallel experiments using amphipol-reconstituted channels, we show that low pH alone was insufficient to induce pore closure, strongly supporting our conclusion that lipids are required for this gating transition.
3. **Stability at Low pH:** Although not shown in the original manuscript, we have additional data demonstrating that Cx46/50 nanodiscs reconstituted with MSP1E1 and DMPC are stable at pH 5.8 for extended periods (≥ 1 week at 4°C), as assessed by negative stain EM (image now included in our revised Extended Data Fig. 1). These findings further support the stability and suitability of our system under the experimental conditions used.
4. We would further note that this same MSP system has recently been used by our lab to capture a distinct gating mechanism in Cx46/50 in response to Ca^{2+} , where NT movement is similarly induced but without clear evidence of lipid infiltration (Flores et al. BioRxiv 2025).

Taken together, we believe the combined biochemical, structural, and control data, and orthogonal gating studies all support the validity of our conclusions that pH-gating of Cx46/50 is reversibly facilitated by lipids when using the selected MSP-nanodisc system. Nevertheless, we have revised the text to more clearly acknowledge the limitations regarding physiological relevance.

2. NT Helix Density & Pore Radius Calculation

- Despite the overall high resolution, the NT region exhibits weak density, indicating flexibility and structural heterogeneity.
- Discussions regarding NT interactions lack strong map support.
- The weak density in this region also calls into question the accuracy of pore radius calculations, which may lead to erroneous interpretations.

Response: We appreciate the reviewers' careful assessment of the NT domain and agree that this region remains structurally heterogeneous, limiting our initial interpretations. As part of our revised analysis, we undertook more extensive particle classification routines to further enrich for the fully gated conformation (see response at the end, for details), yet the NT-domain continued to exhibit limited resolvability for many sidechain features, consistent with their intrinsic flexibility and/or positional variability under gating conditions.

To better reflect the limitations in map quality within the NT, we have revised our structural models of the gated state by pruning unresolved NT sidechains to their C β atoms, consistent with the lack of resolvable sidechain features in this region. Residues 2-3 also remain unresolved and are not modeled, consistent with our original models. These modeling decisions are now explicitly stated in the revised Methods section, Main text and related Figure legends.

In response to the reviewers' valid concern regarding interpretation of NT interactions, we have removed prior descriptions of inter-subunit NT interactions from both the main text and Fig. 1, as we agree that the available map density does not support such specific conclusions.

Regarding the pore radius calculations: we also agree with this concern. As such, the previous pore profile analyses (Fig. 1i and Fig. 7b) have been replaced with revised panels (new Fig. 1i and Fig. 7b) that are meant to illustrate the displacement of the NT helices from the open-state conformation, measured as the change in

C α position. We believe this more conservative and structurally grounded analysis more accurately reflects the intended purpose of this analysis for comparing the observed conformational changes of the NT-domain.

3. Lipid Density & Model Reliability

- The authors claim to have built 400 lipid molecules, but most of the observed density corresponds only to the acyl chains, making this an overstatement.
- The modelled head groups lack clear supporting density, making inferred interactions unreliable.
- Weak lipid density at the pore suggests low occupancy. Further classification or additional data collection may reveal particle classes without lipids, which would impact the proposed model.

Response: We thank the reviewers for raising these points, addressed below.

Clarification of number of lipids. We agree, our initial wording may have unintendedly implied the extent to which full lipid molecules were resolved. As revised, we now explicitly state that the majority of lipid densities correspond primarily to the acyl chain regions, with a limited subset displaying resolved lipid headgroups. Our updated description on pg. 4 now reads:

Over 400 lipid densities (mostly single acyl chains of DMPC) and >900 water molecules were also resolved, with extracellular leaflet lipid chains exhibiting long-range hexagonal packing, consistent with previous observations⁴¹.

Regarding lipid headgroups/interactions. As part of our revised analysis, we performed more focused particle classification to enrich for channels with gated subunit conformations (see concluding response, below). This led to modest improvements in map quality for the subset of lipid headgroups that were resolved. We have accordingly updated our main text and Figures to reflect these new map densities, and we address the remaining limitations with revised descriptions of lipid interactions to emphasize proximity to protein residues, rather than definitive atomic distances. For example, we now state that certain lipid headgroups are “located in close proximity to” or “adjacent to” protein residues, rather than asserting precise distances.

Pore-bound lipid densities. Even after further refinement and improvement of the pore-bound lipid densities, we agree that they remain weaker than expected for a well-defined/stabilized interaction. However, we interpret this not as an indication of low lipid occupancy, but rather as a reflection of conformational heterogeneity in lipid binding within the pore.

This interpretation is supported by multiple observations:

1. No lipid-free gated state detected: Across all classifications and datasets, we do not observe a gated NT conformation in the absence of pore-bound lipid density (even at neutral pH, the rare gated-state that was classified showed pore-bound lipid densities).
2. Amphipol control: In amphipol-reconstituted samples, where lipid entry into the pore is excluded, low pH alone does not induce the NT gating transition – highlighting the necessity of lipids for the observed conformational change.
3. Energetic considerations: The gated NT conformation exposes hydrophobic surfaces on both the NT and TM1/2 lining the pore lumen, which would be energetically unfavorable in the absence of lipid occupation. Thus, it is unlikely that the observed conformation could be stabilized without the presence of lipids – or some other molecule/driving force to support this state.

We have updated our discussion of these results and interpretations – see for example, pg. 5:

Although the local resolution of these lipids is somewhat limited, likely reflecting positional and/or conformational heterogeneity, substantial regions were sufficiently resolved to permit interpretation for atomic modeling.

4. Plug-Like Density & Symmetry Artifacts

- The central plug-like density, interpreted as pore occlusion, was identified using D6 symmetry. However, symmetry axes often exhibit artificial densities that may not reflect the true protein structure. The authors should verify whether this density persists in symmetry-free reconstructions.

Response: We agree. Notably, in our initial analysis, the plug-like density within the pore was observed in both D6 and C1 reconstructions, leading us to consider it a potentially relevant feature. However, following the reviewers' suggestion and as part of our revised analysis, we performed additional particle classification to further enrich for gated-state channels, and re-refined both C1 and D6 maps using these more focused particle sets. Both the C1 and D6 maps are now prominently displayed in Fig. 1. Notably, in the resulting maps, the central plug-like density is no longer observed in either symmetry condition, indicating that this feature was either a reconstruction artifact or reflected a component of heterogeneity that had been removed upon further classification. We have therefore removed all references to the plug-like density and its potential interpretation from the revised manuscript, including associated figure panels and discussion.

5. Heterogeneous Structural States & Data Processing

- The manuscript discusses multiple states leading up to the "Low pH Induced an Ensemble of Gated and Open States" but later concludes that these states are mixtures of different conformations.
- Since the earlier structures likely represent averaged density maps, their significance is diminished.
- The study should focus on asymmetric subunit conformations rather than enforcing D6 symmetry after identifying asymmetric particles.
- Structural heterogeneity is still evident, as indicated by standard deviations. The authors must precisely isolate symmetric or asymmetric but homogeneous particles for refinement to ensure the final maps represent distinct states accurately.

Response: We thank the reviewers for raising this point. Indeed, the structural heterogeneity (particularly at low pH) is a key feature of this system and we agree that this complicates interpretation of symmetry-imposed reconstructions. In response, we have revised the manuscript to better delineate the distinct insights gained from both symmetric and asymmetric processing strategies.

The D6-symmetrized reconstructions were critical for defining the high-resolution features of the most primary conformational states, specifically the stabilized-open, destabilized-open and gated-state configurations. These idealized models allowed us to identify key structural rearrangements, including pore-lining lipids and the more nuanced changes in the NT and TM2 for the destabilized-open state, which we were not able to resolve at lower resolutions achieved by symmetry-expansion and focused refinement methods performed on single subunits. Importantly, by leveraging D6-symmetry, this approach also enabled us to detect rare gated-state and destabilized-open state populations under neutral pH conditions (transitions that were also not evident in our asymmetric processing approach).

However, we fully acknowledge that these D6-refined structures do not represent the dominant conformational state of channels under low pH conditions. Our subunit-focused classification and symmetry expansion analyses revealed that most channels exist in asymmetric, heterogeneous ensembles consisting of both open and gated subunits. This ensemble-level heterogeneity was essential for demonstrating that under cryo-EM conditions gating in Cx46/50 is not a fully concerted or cooperative process.

Moreover, we would like to emphasize that this level of conformational heterogeneity, and the use of both symmetric and asymmetric processing approaches, reflects an ongoing challenge in the field of gap junction structural biology. Similar degrees of structural heterogeneity have been reported in other connexin structures, including Cx26, Cx32, Cx36 and Cx43, where authors similarly employed symmetry-imposed reconstructions to resolve high-resolution states, alongside C1 or subunit-level analyses to reveal asymmetric features. Our approach is therefore consistent with established methodological practices used to address the inherent complexity of these high-order oligomeric membrane proteins.

We have updated the manuscript to:

- Clearly state that D6 reconstructions represent idealized endpoints rather than the dominant ensemble.
- Clarify how each analytical approach (D6 vs. C1) contributes complementary insights.
- Reference comparable examples in the field where similar methodological strategies have been employed.

This ensemble-level heterogeneity also highlights an important methodological point. It is important to emphasize that the D6- (or C6-) symmetrized cryo-EM reconstructions presented in this work do not represent the dominant conformations present under low pH conditions. Rather, these idealized reconstructions serve as high-resolution endpoints that highlight key structural rearrangements, including lipid interactions and NT/TM2 conformational changes, which were otherwise obscured at the lower resolutions obtained through our focused subunit-level analyses. Consistent with this, our subunit-level classification approaches revealed that the majority of channels adopt heterogeneous assemblies composed of both open and gated subunits. Together, these complementary strategies provide a more complete view of the gating process, where D6/C6-symmetrized reconstructions define high-resolution detail, while subunit-based analyses capture ensemble-level heterogeneity. Similar methodological strategies have been employed in recent studies of other connexins (e.g., Cx26, Cx32, Cx36, Cx43)^{53-56,67}, underscoring both the complexity of gap junction structural characterization and the value of integrating symmetric and asymmetric approaches.

We appreciate the reviewers' constructive critique, which has helped us clarify the interpretation and limitations of our data and to place our work in the broader context of current methodological approaches to connexin structural studies.

6. Neutral pH Data Processing

- What happens when the neutral pH dataset is processed separately?
- Would this yield a neutral pH open state? Why was it combined with the low pH dataset?

Response: We thank the reviewers for the opportunity to clarify these points regarding our data processing strategy.

The neutral pH datasets (collections #3 and #6) were indeed processed independently prior to employing our mixed-dataset approach for classification of rare-states, as described in our Methods section. When analyzed separately (e.g., using heterogeneous refinement, 3DVA, or symmetry expansion and signal subtraction followed by focused 3D classification) we consistently observed only the primary conformation of the stabilized open-state (see Fig. 3, Extended Data Fig. 8, Extended Data Fig. 9, and Supplemental Movie 1). These findings are consistent with our previously published structural characterization of Cx46/50 in MSP1E1 nanodiscs under similar neutral pH conditions (Flores et al., 2020).

To enhance our ability to identify and resolve minor states that we hypothesized may be present, but only poorly sampled under these conditions, we implemented a combined dataset processing strategy. Specifically, we pooled particles from both neutral and low pH datasets to leverage the dominant state captured under each condition (i.e., open at neutral pH and gated at low pH). This approach allowed us to leverage the major population from one condition to help isolate the minor population from the other. Notably, this strategy was successful and enabled us to extract a rare population of lipid-mediated gated-state particles present under neutral pH conditions, which we were unable to resolve using conventional approaches on the neutral dataset alone.

We hope this explanation resolves any confusion. We have further clarified this rationale and strategy in the revised text on pg. 9.

Rare states detected at neutral pH

Previously, under neutral pH cryo-EM conditions, we have only detected the stabilized open-state of Cx46/50^{40,41}. Consistent with this, heterogenous refinement and 3DVA performed on neutral pH nanodisc datasets (Collections #3 and #6), as well as the amphipol low-pH dataset (Collection #2), revealed exclusively open-state channels with minimal NT heterogeneity, with variability limited to a slight “wobble” of TM2 (Supplemental Movie 1, Extended Data Fig. 8 & 9).

However, we hypothesized that this approach may have lacked the sensitivity to detect rare conformational states that could still be present at neutral pH. To enhance our ability to resolve potential low-abundance states, we combined particles from both neutral and low pH nanodisc datasets

(Collections #3, #4 and #5, #6), thereby increasing representation of various conformational states in the pool of particles. We then reanalyzed the combined particle set using 3DVA, leveraging the well-sampled conformational states from the low pH particles to guide the detection of rare, structurally similar states within the neutral pH subset. This strategy successfully resolved all three states (stabilized open, destabilized open, and gated) allowing for their classification and independent reconstruction from particles derived exclusively from the neutral pH dataset (Fig. 5a; see Methods).

7. Resolution Claims & Model Interpretation

- Line 133: The claim that heteromeric/-typic assembly patterns of Cx46/50 were indistinguishable is questionable.
- At 2.2 Å resolution, individual residues should be distinguishable. If they are not, the reported resolution may be overestimated or your maps are still averaged density.

Response: We appreciate the reviewers' comments and welcome the opportunity to clarify these points.

At the global resolution of 2.2 Å (now 2.3 Å in our revised gated-state map), most side chains (particularly in well-ordered regions) are clearly resolved, as shown in Extended Data Fig. 5. At positions conserved between Cx46 and Cx50, side chains are well defined. In contrast, density is weaker or ambiguous at isoform-divergent positions, consistent with averaging across two nearly identical sequences rather than a limitation of global map quality.

Our statement that heteromeric/-typic assemblies are “indistinguishable” refers not to resolution, but to the intrinsic limitations of current cryo-EM classification methods when applied to systems with high sequence identity and compositional heterogeneity. Although our prior cross-linking mass spectrometry confirmed the presence of heteromeric Cx46/50 channels (Myers et al., Nature 2018), isoform-specific features remain too subtle and spatially dispersed to support reliable classification. Cx46 and Cx50 share over 80% sequence identity across structured regions, and the few differing residues are often solvent-exposed (i.e., conformationally dynamic), and sparsely distributed – further reducing the signal available to discriminate subunit identity.

This challenge is further compounded by the combinatorially large number of possible subunit arrangements in a dodecameric assembly. Similar issues have been reported for co-expressed Cx26 and Cx32, where high-resolution AFM and Fab-labeling (Naulin et al., J. Biol. Chem. 2021) demonstrated largely stochastic subunit incorporation, underscoring the broader challenge of resolving compositional heterogeneity in such systems by cryo-EM.

Notably, these limitations persist even at higher resolution. In our prior study using datasets refined to 1.9 Å (Flores et al., Nat. Commun. 2020), we observed similar ambiguity at isoform-divergent positions, and provide detailed map-to-model comparisons to illustrate these limitations. The challenges/limitations of this system have also been described in detail in our inaugural structural characterization (see Myers et al. Nature 2018).

We are currently developing Fab-based fiducial labeling strategies to enable isoform-specific assignment in future cryo-EM studies, though these efforts are still in early stages and beyond the scope of the current work.

Given our previous detailed analysis and description of these limitations with native Cx46/50 channels, we do not feel the need to delve into such detail in this current work. We have revised the manuscript text to clarify that the inability to distinguish heteromeric/-typic assemblies arises from both the high structural similarity of the isoforms and the methodological limitations of cryo-EM, rather than an overstatement of resolution. We also display map-to-model quality at these sites of sequence similarity and difference in Extended Data Fig. 5.

pg. 4

Heteromeric/-typic assembly patterns of Cx46 and Cx50 could not be distinguished from the cryo-EM data, consistent with our prior studies^{40,41}, reflecting the >80% sequence identity across structured regions of the two isoforms and methodological limitations of current cryo-EM classification methods.

Minor Points:

- The methods section should clearly explain how asymmetric maps were generated.
[Done](#)
- Lines 40–43: The statement is unclear and should be reworded for clarity.
[Done](#)
- Line 1162: Specify which signal was subtracted.
[Done](#)
- Lines 965–967: The observed density alone is insufficient to confirm occlusion.
[Removed](#)
- Line 290: Indicate the corresponding supplementary movie number.
[Done](#)
- Line 555: Clarify whether affinity purification or ion exchange chromatography was used.
[Done](#)
- Figure 6: Adjust the lipid color scheme for better visibility.
[Done](#)

In summary, the authors need to improve map quality, particularly in critical regions, to support their conclusions. Additional data collection and classification could resolve heterogeneity and strengthen structural interpretations.

[Response:](#) We appreciate the reviewers' overarching concern and agree that addressing structural heterogeneity is essential for supporting the mechanistic conclusions of our study. As noted, the degree of conformational variability in our data is both a challenge and, in our view, an informative feature of the system, shedding light on the ensemble-based nature of pH- and lipid-mediated gating in connexin channels.

In response to the reviewers' recommendation, we have implemented additional data processing strategies and expanded our classification efforts beyond what was included in the original submission. Specifically, to improve the refinement of the fully gated state, we applied a revised workflow that builds on our subunit-level classification approach. This included a substantial increase in data usage: instead of a 100k particle subset, we now applied symmetry expansion, signal subtraction, and 3D classification across the full low-pH dataset (476,719 particles, symmetry-expanded to over 5 million subunits).

We then filtered for channels containing ≥ 10 gated subunits (empirically determined to be optimal for maximizing particle count while limiting contamination by ungated subunits), yielding a set of 106,741 channels, which we refined to generate a more homogenous representation of the fully gated state. While this approach did not significantly improve the resolvability of NT sidechains, it did enhance the definition of pore-bound lipid headgroup density – an important feature of our lipid-mediated gating model. This revised refinement also led to the disappearance of the previously reported plug-like density at the symmetry axis (as described in responses to Points #4 and #5 above), further improving the interpretability of this conformation. This updated approach is detailed in the revised Methods section, and associated Extended Data Figure 3.

We also note that despite these advances, the subunit-level classification approach did not resolve the nuanced difference between the stabilized- and destabilized open-states. For this reason, we have retained the original models for the open and destabilized-open states, which were derived using 3DVA-based classification.

We hope the reviewers recognizes our continued and rigorous efforts to resolve the substantial intrinsic heterogeneity of Cx46/50 at low pH. We believe our combined use of high-resolution symmetry-imposed models

and subunit-level ensemble analysis represents the most comprehensive and robust depiction of the gating transitions accessible from these datasets.

Reviewer #3:

The manuscript by Jarodsky et al is a very interesting article describing the difference in structures of the connexin46/50 gap junction at high and low pH. In a lipid environment (nanodiscs made of DMPC, rather than the native environment) the change of pH causes the infiltration of lipids into the pore and causes apparent closure of the channels caused by an upward movement of the N-terminal helix. Surprisingly, this is reversible.

The study is interesting. The possible involvement of lipids in the regulation of connexins and other large pore channels appears to be a recurring theme in the recent structural studies that have been carried out. The link between pH and lipids has not been observed before. Within the limits of the experimentation (using nanodiscs with DMPC rather than native lipids) the experiments seem well done with extensive classification of cryo-EM data sets.

There are a few points that need to be addressed:

1. The high pH buffer is HEPES. However, instead of using a similar buffer such as MES, (that could be used at pH 5.8), the authors use a completely different buffer by swapping to succinate. It is therefore possible that the change in buffering system affects the structure as well as, or even instead of, the pH? Given that they see the same conformation in amphipols using “Low pH buffer” as they see in HEPES, this is probably not an issue, but I note in their methods that the “Low pH buffer” is not explicitly defined when discussing the amphipols experiments. The buffer needs to be defined and some comment should be made regarding the differing buffering systems.

Response: We thank the reviewer for this insightful comment. Indeed, MES would have provided a more chemically similar buffering system to HEPES and could have minimized the possibility of buffer-specific effects in our experimental design. However, as the reviewer also notes, the lack of gating transition of Cx46/50 in amphipol under low pH conditions using the same succinate-based buffer system strongly suggests that the observed gating transition is driven by pH and not an artifact of the buffer composition.

We have revised the Methods section to explicitly define the low pH buffer used in our amphipol experiments. Additionally, we now include a brief comment in the manuscript noting that, although a different buffer system was used at low pH, the absence of gating transitions for Cx46/50 in amphipols under these same low-pH conditions supports the conclusion that the observed conformational changes are pH-dependent rather than buffer-specific.

pg. 19

We note, that although a different buffer system was used at low pH (succinate versus HEPES), the absence of gating transitions for Cx46/50 in amphipols under these same low-pH conditions (described below) supports the conclusions made in this work that the observed conformational changes are pH-dependent rather than buffer-specific.

2. They discuss the possibility of His95 and His17 being important for pH sensitivity and gating. As they note in the results section, the literature surrounding this is not clear cut. However, the discussion is written in such a way that leads the reader to believe that the structures reported here explain how the residues contribute to pH sensitivity. This is slightly misleading.

Response: We agree, our original discussion may have inadvertently implied a more definitive role for His95 and His17 in pH sensing than is currently supported by the structural data or literature. We have revised the relevant section of the Discussion to clarify that while these residues are candidates for contributing to pH sensitivity, their roles remain unresolved and require further experimental validation. We now emphasize that our structures provide a framework to guide future studies but do not yet establish a direct mechanistic role for these histidines in gating.

pg. 15

While these structural interactions support our proposed gating mechanism and underscore the potential importance of conserved histidine residues in pH-sensing, the functional roles of these histidine residues remain unresolved and further studies are needed to validate the specific protonation events involved and their mechanistic consequences. This includes exploring the possible contribution of non-histidine residues, such as N-terminal acidic residues^{68,69}.

3. There is only minimal comparison with other connexin structures. While I wouldn't expect a detailed comparison of all the different structures, the movement of TM 2 around the proline seems similar to that reported for Cx26 (Brotherton, et al Structure, 2022, eLife, 2024) and movements of TM 2 are also mentioned for Cx43 (Lee et al, Nat Comm, 2023).

Response: We appreciate this suggestion and have expanded our comparative analysis in our revised Results subsection, "*Comparison to other connexin structures.*" In this section, in addition to comparing NT conformation and lipid binding sites, we include both structural and quantitative assessments of TM2 orientation, highlighting conserved movement around the hinge-proline (P88 in Cx46/50), which aligns with prior observations in Cx26 and Cx43 (Brotherton et al., 2022, eLife, 2024; Lee et al., 2023).

pg. 11

Correlated with NT displacement, distinct conformational changes in TM2 were also observed. Compared to the stabilized open-state of Cx46/50, all other models display a more bent conformation localized to the cytoplasmic region of TM2 beyond the conserved proline kink (P88 in Cx46/50; P87 in Cx26 and Cx43) (Fig. 7a). When compared to this region of TM2 in the pH-gated state of Cx46/50, Cx43 at pH 8.0 showed the smallest deviation (Ca r.m.s.d. = 1.7 Å), followed by Cx26 treated with CO₂ (Ca r.m.s.d. = 4.4 Å), while Cx26 at low pH exhibited the largest deviation (Ca r.m.s.d. = 7.2 Å). These observations support a likely conserved mechanistic feature, where a hinge-like motion of TM2 is coupled to the conformational state of the NT.

We also discuss this NT–TM2 coupling in our discussion, where we further note that the role of the conserved proline in voltage gating was originally proposed in Cx26 and 32 (Suchyna et al., 1993; Ri et al., 1999), and our recent cryo-EM study of Cx46/50 under Ca²⁺-gating conditions (Flores et al., 2025) reveals a similar displacement of TM2. These comparisons reinforce a broader model in which NT-TM2 coupling serves as a conserved mechanism for connexin gating across different isoforms and stimuli.

pg. 13

Our findings, together with prior cryo-EM studies, highlight a consistent pattern of coordinated movement between TM2 and the NT, facilitated by a conserved proline kink (P88 in Cx46/50) that serves as a hinge element^{49,53-55,62}. This hinge confers structural flexibility of TM2, allowing it to accommodate NT rearrangements during pH-dependent gating transitions (Fig. 8a,b). Notably, missense mutations at this site in Cx50 have been linked to congenital cataracts^{63,64}, underscoring the physiological relevance. Mutagenesis studies at the homologous proline site in Cx26 and Cx32 previously implicated this site as a transduction element in voltage-gating^{65,66}. Consistently, TM2 displacement originating from P88 is also observed in our recent cryo-EM structure of Cx46/50 in the presence of Ca²⁺⁶². Collectively, these observations support a conserved mechanism of NT-TM2 coupling via the P88 hinge that modulates connexin gating under diverse physiological conditions.

4. In Figure 1b, a "pore plug" is mentioned at low threshold levels. Is this perhaps an artefact of the symmetry?

Response. We agree (see also our response to Reviewers 1/2 on this topic). We thank both sets of reviewers for prompting this clarification.

Notably, in our initial analysis, the plug-like density within the pore was observed in both D6 and C1 reconstructions, leading us to consider it a potentially relevant feature. However, following the reviewers' suggestion and as part of our revised analysis, we performed additional particle classification to enrich for fully

gated channels, and re-refined both C1 and D6 maps using these focused particle sets. Both C1 and D6 maps are now prominently displayed in Fig. 1. In the resulting maps, the central plug-like density is no longer observed in either symmetry condition, indicating that this feature was either a reconstruction artifact or reflected a component of heterogeneity that had been removed upon further classification. We have therefore removed all references to the plug-like density and its potential interpretation from the revised manuscript, including associated figure panels and discussion.

Point-by-Point Response

Reviewer #3 confirmed all their concerns were fully resolved and now supports publication of the revised manuscript.

Reviewer #1/2 provided additional comments, which we address in our point-by-point response below. In doing so, we highlight clear examples of fabricated references used by these Reviewers to question the novelty and significance of our findings. This behavior constitutes a serious breach of scientific integrity and substantially undermines the credibility of the reviewers' entire evaluation, which as we detail below, is problematic throughout.

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Reviewer Report

Summary

Jarodsky *et al.* investigate the mechanism of pH-dependent gating of connexin-46/50 using cryo-electron microscopy and complementary biochemical analyses. While the topic is timely and of general interest, the manuscript lacks conceptual novelty, methodological rigor, and a coherent mechanistic narrative. Several conclusions are speculative and not sufficiently supported by the data presented.

Major Comments

1. Inconsistencies between hypothesis

The authors initially identify connexin-46/50 as forming homomeric gap junctions and present maps and models generated using D6 symmetry. They later combine datasets and identify asymmetric channel conformations, concluding that the presence of fully symmetric gated gap junctions are rare. However, Figures 6c and 6d indicate that only about 1.7% of the particles adopt a symmetric gated conformation, while the majority are asymmetric.

In the first part of the manuscript, the discussion focuses solely on the D6-symmetry imposed maps, which are later revealed to represent a mixture of symmetric and asymmetric conformations. Therefore, drawing mechanistic conclusions solely from the D6-symmetry imposed "gated" state is misleading. The manuscript should clearly distinguish whether the hemichannels or the complete gap junctions exhibit symmetric or asymmetric conformations under neutral and acidic conditions.

Separate analyses of the symmetric and asymmetric maps are necessary to achieve a more accurate and detailed mechanistic understanding. Furthermore, reporting the distinct asymmetric states (such as two-, three-, or four-gated conformations) would greatly enhance the novelty and mechanistic clarity of the study.

Response: This concern was already raised by the reviewers in their first round of comments. We responded to it directly and in detail in our original point-by-point rebuttal, and we made extensive revisions to the manuscript to address it.

We believe these efforts have fully addressed this concern. For completeness and transparency, we refer the editorial team and reviewers to our initial response, included as an **Appendix** below.

2. Figures and data presentation

The figures do not effectively support the stated conclusions. In several panels, the superposition of two structures is indistinguishable due to similar color schemes, key residues discussed in the text are missing from the figures, and the transparent surface rendering gives a misleading impression of lipid density by exaggerating weak features. Overall, the visual presentation suffers from inadequate color contrast, missing labels, and unconvincing densities, which significantly reduce the clarity and impact of the structural claims.

Response: We disagree:

- **Structural Superpositions & Contrast:** The structures being compared are clearly distinguished using high-contrast representations (e.g., white versus dark gray – see Fig. 1d, Fig. 4d).
- **Residue Labeling:** All residues and protein domains discussed in the main text are appropriately labeled in the corresponding figures.
- **Lipid Density Representation:** Contrary to the reviewers' statement, lipid densities are shown in full opacity in all main figures. Additionally, model-to-density fitting for these key lipids is provided in Extended Data Fig. 5, supporting our interpretations.

3. Lack of a coherent narrative or conclusion

The manuscript reads as a collection of loosely connected observations rather than a cohesive mechanistic story. The discussion reiterates prior literature but does not integrate the new results into a unified model. There is **no clear mechanism** explaining how pH modulates gating through structural transitions.

Additionally, connexin-43 (Cx43) structures (e.g., Lee *et al.*, *Nat. commun.*, 2023; PMID: 36805660) have demonstrated multiple hemichannel conformations and well-defined lipid densities that are consistently positioned along the transmembrane helices. In contrast, the lipids modeled in the present work appear in opposite orientations, with no supporting hypothesis or discussion about **lipid entry, exit, or rearrangement during conformational change**. This omission represents a major conceptual gap, as the supposed lipid-mediated gating cannot be reconciled with the structural evidence or prior connexin studies.

Response: The claims of omission and that our manuscript lacks mechanistic detail are completely unfounded. We provide extensive structural evidence and interpretation regarding lipid orientation, our proposed mechanism of proton-mediated lipid entry/exit, and detail lipid rearrangements involved in the gating transition. Critically, we demonstrate reversibility of the lipid-mediated gating in response to pH changes, a central and novel finding that underscores the physiological relevance of the mechanism. To our knowledge, this study provides the first high-resolution structural characterization of a pH-dependent conformational change in any connexin isoform. Thus, our work offers a substantial mechanistic and conceptual advance in the field of gap junction biology.

We encourage editorial team and the reviewers to revisit the relevant sections of the manuscript, which directly address the concerns raised.

4. Lack of novelty and conceptual advance

The manuscript reiterates mechanisms already reported for connexin-46/50 and connexin-26 (e.g., Flores *et al.*, 2020; Brotherton *et al.*, 2022; Meigh *et al.*, 2013). The central premise of lipid-mediated gating under acidic conditions has been previously proposed and partially resolved at comparable resolution in other connexin studies (Lee *et al.*, *Nat. commun.*, 2023). The current study does not significantly extend that understanding—either mechanistically or structurally—and thus offers limited conceptual advancement.

Comparative context:

Similar pH-dependent gating transitions have been described in multiple connexins. Connexin-26 exhibits proton-induced N-terminal helix rearrangements leading to a closed conformation (Brotherton *et al.*, 2022, *Nat. Commun.*), while connexin-43 and connexin-32 show analogous low-pH gating transitions modulated by protonation of cytoplasmic residues (Meigh *et al.*, 2013, *J. Biol. Chem.*; Nielsen *et al.*, 2017, *eLife*). The “neutral destabilized open” and “acidic gated” states proposed here are thus not unique to connexin-46/50 and were previously observed in both homomeric and heteromeric gap junctions. Consequently, the mechanistic insight presented does not constitute a substantive advance over these earlier studies and largely restates already known features of connexin gating.

References:

- Flores, J. E. *et al.* Structural basis of pH gating in connexin-46/50 gap junction channels. *Nat. Commun.* **11**, 505 (2020).
- Brotherton, D. H. *et al.* Proton-dependent conformational rearrangements underlie connexin-26 gating. *Nat. Commun.* **13**, 2750 (2022).
- Meigh, L. *et al.* pH-dependent modulation of connexin-32 and connexin-43 by intracellular protons. *J. Biol. Chem.* **288**, 22758–22767 (2013).
- Nielsen, M. S. *et al.* Structural basis of pH gating and regulation in connexins. *eLife* **6**, e20213 (2017).
- Myers, J. B. *et al.* Structural basis of lipid-mediated gating and multiple conformational states of connexin-43 hemichannels. *Science* **381**, 512–518 (2023).

Response: The assertions made in this critique are demonstrably false and appear to be constructed around the titles of fabricated references (presumably hallucinated by a generative AI tool). The titles cited in support of the reviewers' arguments (highlighted above) do not exist in the scientific literature and are deeply misleading.

Strikingly, several of these fictitious citations list members of our own research group as first authors (e.g., Flores, JE; Myers, JB), despite the fact that no such studies have been written, submitted, or published by our lab. One of the citations, attributed to Flores *et al.*, even appears to be a fabrication of the title of our current manuscript, and used as the basis for arguing a lack of novelty.

This egregious misconduct constitutes a fundamental breach of scholarly integrity and severely compromises the validity and motivation of the reviewers' arguments.

APPENDIX

Here, we provide our initial response to these Reviewers concerns regarding the presentation, analysis, and interpretation of structural heterogeneity, which we believe has already been fully and faithfully addressed in our revised manuscript.

5. Heterogeneous Structural States & Data Processing

- The manuscript discusses multiple states leading up to the "Low pH Induced an Ensemble of Gated and Open States" but later concludes that these states are mixtures of different conformations.
- Since the earlier structures likely represent averaged density maps, their significance is diminished.
- The study should focus on asymmetric subunit conformations rather than enforcing D6 symmetry after identifying asymmetric particles.
- Structural heterogeneity is still evident, as indicated by standard deviations. The authors must precisely isolate symmetric or asymmetric but homogeneous particles for refinement to ensure the final maps represent distinct states accurately.

Response: We thank the reviewers for raising this point. Indeed, the structural heterogeneity (particularly at low pH) is a key feature of this system and we agree that this complicates interpretation of symmetry-imposed reconstructions. In response, we have revised the manuscript to better delineate the distinct insights gained from both symmetric and asymmetric processing strategies.

The D6-symmetrized reconstructions were critical for defining the high-resolution features of the most primary conformational states, specifically the stabilized-open, destabilized-open and gated-state configurations. These idealized models allowed us to identify key structural rearrangements, including pore-lining lipids and the more nuanced changes in the NT and TM2 for the destabilized-open state, which we were not able to resolve at lower resolutions achieved by symmetry-expansion and focused refinement methods performed on single subunits. Importantly, by leveraging D6-symmetry, this approach also enabled us to detect rare gated-state and destabilized-open state populations under neutral pH conditions (transitions that were also not evident in our asymmetric processing approach).

However, we fully acknowledge that these D6-refined structures do not represent the dominant conformational state of channels under low pH conditions. Our subunit-focused classification and symmetry expansion analyses revealed that most channels exist in asymmetric, heterogeneous ensembles consisting of both open and gated subunits. This ensemble-level heterogeneity was essential for demonstrating that under cryo-EM conditions gating in Cx46/50 is not a fully concerted or cooperative process.

Moreover, we would like to emphasize that this level of conformational heterogeneity, and the use of both symmetric and asymmetric processing approaches, reflects an ongoing challenge in the field of gap junction structural biology. Similar degrees of structural heterogeneity have been reported in other connexin structures, including Cx26, Cx32, Cx36 and Cx43, where authors similarly employed symmetry-imposed reconstructions to resolve high-resolution states, alongside C1 or subunit-level analyses to reveal asymmetric features. Our approach is therefore consistent with established methodological practices used to address the inherent complexity of these high-order oligomeric membrane proteins.

We have updated the manuscript to:

- Clearly state that D6 reconstructions represent idealized endpoints rather than the dominant ensemble.
- Clarify how each analytical approach (D6 vs. C1) contributes complementary insights.
- Reference comparable examples in the field where similar methodological strategies have been employed.

pg. 13-14

This ensemble-level heterogeneity also highlights an important methodological point. It is important to emphasize that the D6- (or C6-) symmetrized cryo-EM reconstructions presented in this work do not represent the dominant conformations present under low pH conditions. Rather, these idealized

reconstructions serve as high-resolution endpoints that highlight key structural rearrangements, including lipid interactions and NT/TM2 conformational changes, which were otherwise obscured at the lower resolutions obtained through our focused subunit-level analyses. Consistent with this, our subunit-level classification approaches revealed that the majority of channels adopt heterogeneous assemblies composed of both open and gated subunits. Together, these complementary strategies provide a more complete view of the gating process, where D6/C6-symmetrized reconstructions define high-resolution detail, while subunit-based analyses capture ensemble-level heterogeneity. Similar methodological strategies have been employed in recent studies of other connexins (e.g., Cx26, Cx32, Cx36, Cx43)^{53-56,67}, underscoring both the complexity of gap junction structural characterization and the value of integrating symmetric and asymmetric approaches.

We appreciate the reviewers' constructive critique, which has helped us clarify the interpretation and limitations of our data and to place our work in the broader context of current methodological approaches to connexin structural studies.

[...]

In summary, the authors need to improve map quality, particularly in critical regions, to support their conclusions. Additional data collection and classification could resolve heterogeneity and strengthen structural interpretations.

Response: We appreciate the reviewers' overarching concern and agree that addressing structural heterogeneity is essential for supporting the mechanistic conclusions of our study. As noted, the degree of conformational variability in our data is both a challenge and, in our view, an informative feature of the system, shedding light on the ensemble-based nature of pH- and lipid-mediated gating in connexin channels.

In response to the reviewers' recommendation, we have implemented additional data processing strategies and expanded our classification efforts beyond what was included in the original submission. Specifically, to improve the refinement of the fully gated state, we applied a revised workflow that builds on our subunit-level classification approach. This included a substantial increase in data usage: instead of a 100k particle subset, we now applied symmetry expansion, signal subtraction, and 3D classification across the full low-pH dataset (476,719 particles, symmetry-expanded to over 5 million subunits).

We then filtered for channels containing ≥ 10 gated subunits (empirically determined to be optimal for maximizing particle count while limiting contamination by ungated subunits), yielding a set of 106,741 channels, which we refined to generate a more homogenous representation of the fully gated state. While this approach did not significantly improve the resolvability of NT sidechains, it did enhance the definition of pore-bound lipid headgroup density – an important feature of our lipid-mediated gating model. This revised refinement also led to the disappearance of the previously reported plug-like density at the symmetry axis (as described in responses to Points #4 and #5 above), further improving the interpretability of this conformation. This updated approach is detailed in the revised Methods section, and associated Extended Data Figure 3.

We also note that despite these advances, the subunit-level classification approach did not resolve the nuanced difference between the stabilized- and destabilized open-states. For this reason, we have retained the original models for the open and destabilized-open states, which were derived using 3DVA-based classification.

We hope the reviewers recognizes our continued and rigorous efforts to resolve the substantial intrinsic heterogeneity of Cx46/50 at low pH. We believe our combined use of high-resolution symmetry-imposed models and subunit-level ensemble analysis represents the most comprehensive and robust depiction of the gating transitions accessible from these datasets.

The authors have used sophisticated cryo-EM techniques to investigate the reversible, lipid-mediated pH-gating of connexin-46/50. While they have attempted several approaches to resolve various states, there are several critical and major concerns that need to be addressed. In general, to support most of their conclusions, they need to improve the quality of the maps in certain important regions, which are central to the discussion in this manuscript. Many statements are overly speculative and not supported by map densities. The authors either need to collect more data and classify structural heterogeneity more effectively or moderate their claims accordingly.

Major Concerns:

1. MSP Belt Tightness & Nanodisc Stability

- The MSP belt appears too tight, which may influence conformational changes in the N-terminal (NT) domain, particularly at low pH, where it is proposed to be stabilized by lipids.
- Nanodiscs do not behave well at low pH, and MSP stability decreases under such conditions, potentially affecting lipid entry. As a result, the lipid density observed near the NT may not be physiologically relevant.

2. NT Helix Density & Pore Radius Calculation

- Despite the overall high resolution, the NT region exhibits weak density, indicating flexibility and structural heterogeneity.
- Discussions regarding NT interactions lack strong map support.
- The weak density in this region also calls into question the accuracy of pore radius calculations, which may lead to erroneous interpretations.

3. Lipid Density & Model Reliability

- The authors claim to have built 400 lipid molecules, but most of the observed density corresponds only to the acyl chains, making this an overstatement.
- The modelled head groups lack clear supporting density, making inferred interactions unreliable.
- Weak lipid density at the pore suggests low occupancy. Further classification or additional data collection may reveal particle classes without lipids, which would impact the proposed model.

4. Plug-Like Density & Symmetry Artifacts

- The central plug-like density, interpreted as pore occlusion, was identified using D6 symmetry. However, symmetry axes often exhibit artificial densities that may not reflect the true protein structure. The authors should verify whether this density persists in symmetry-free reconstructions.

5. Heterogeneous Structural States & Data Processing

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- The study should focus on asymmetric subunit conformations rather than enforcing D6 symmetry after identifying asymmetric particles.
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6. Neutral pH Data Processing

- What happens when the neutral pH dataset is processed separately?
- Would this yield a neutral pH open state? Why was it combined with the low pH dataset?

7. Resolution Claims & Model Interpretation

- Line 133: The claim that heteromeric/-typic assembly patterns of Cx46/50 were indistinguishable is questionable.
- At 2.2 Å resolution, individual residues should be distinguishable. If they are not, the reported resolution may be overestimated or your maps are still averaged density.

Minor Points:

- The methods section should clearly explain how asymmetric maps were generated.
- Lines 40–43: The statement is unclear and should be reworded for clarity.
- Line 1162: Specify which signal was subtracted.
- Lines 965–967: The observed density alone is insufficient to confirm occlusion.
- Line 290: Indicate the corresponding supplementary movie number.
- Line 555: Clarify whether affinity purification or ion exchange chromatography was used.
- Figure 6: Adjust the lipid color scheme for better visibility.

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