nature portfolio

Peer Review File

Active Conformations of Neuronal Na+,K+-ATPase isoforms and a Disease-Causing Mutant

Corresponding Author: Professor Poul Nissen

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)
Review of the Manuscript: “Active Conformations of Neuronal Na+,K+-ATPase isoforms and a Disease-Causing Mutant” by Christensen et al.

The authors present a detailed structural study determining four distinct conformations of human alpha-1 and alpha-3 - isoform-specific Na+,K+-ATPases (NKA) under turnover conditions (Na+, ATP and Mg2+). Resolving intermediate states like the pre-phosphorylated Na2E1-ATP, sodium-occluded [Na3]E1-P-ADP, the hitherto unknown intermediate [Na3]E2P, and outward-open E2P offers a valuable, near-complete view of the Na+ transport cycle.
Furthermore, the structural and functional characterization of the Alternating Hemiplegia of Childhood (AHC)-causing alpha-3 Q140L mutant is highly relevant. The proposal that impaired polysaturated phosphatidylethanolamine (PE) binding destabilizes the E2P state, suggesting lipid modification as a potential therapeutic strategy for AHC, is intriguing.
While the technical scope of this work is significant, the novelty of the structural component is somewhat limited by the recent publication of similar structures by Guo et al. (2022) and Nguyen et al. (2022) at Nature Communications.
Additionally, several points regarding the recombinant NKA preparation and functional integrity require clarification and supporting data to fully establish the robustness of the reported structural conclusions.
My specific comments for the authors are as follows:
1. Sample preparation and Quality: The preparation method involves a multi-step in vitro assembly process (purifying FXYD subunit separately, adding it to detergent solubilized alpha-beta, and reconstitution into nanodiscs). Supplementary Figure 2A shows that the majority of the protein, especially alpha-3 eluted at the void volume of the Superdex 200 10/300 column, which often indicates oligomerization and aggregation. Only a minor fraction was collected for downstream analysis. Given this, could the authors confirm why co-expression of all three subunits (alpha, beta and FXYD) was not pursued to ensure proper complex assembly in vivo? More importantly, the authors must provide detailed functional evidence, including but not limited to electrophysiology, phosphorylation, ATPase activity to characterize the specific NKA fraction used for Cryo-EM and ensure it is highly active.
2. Na2E1-ATP conformation: The structure labeled as Na2E1-ATP is classified as inward-open. However, the reported M1/M4 movement (~4Å) is notably smaller than the displacement observed in the fully open state of other P-type ATPases (e.g. Ca2+ ATPase at ~12Å). Moreover, this structure also revealed the binding of two out of three Na+ ions. Based on these observations, the state may be more accurately described as an inward-occluded conformation rather than fully open. Could the authors provide a more detailed structural rationale for classifying this state as ‘inward-open’? Have attempts been made to capture a fully open E1-ATP conformation using a Na+-free buffer?
3. Data Processing and [Na3]E2P Intermediate: The authors utilized an in silico classification approach during Cryo-EM data processing to resolve the various conformational states, including the novel [Na3]E2P intermediate. Given that this intermediate was derived computationally from a heterogenous particle pool, could the authors address the possibility that this method might have artificially separated closely related conformations? What orthogonal validation methods or independent structural evidence can be provided to support the existence and structural uniqueness of [Na3]E2P intermediate?
4. Functional Stoichiometry: Supplementary Table 2 reports very low ouabain binding values (~15.5 and ~8 pmol/mg for alpha-1 and alpha-3, respectively). Calculating the stoichiometry reveals that only a small percentage (~0.14%-0.28%) of the total NKA mass appears capable of ouabain binding. Since ouabain is a high-affinity inhibitor, this metric strongly suggests that only a tiny fraction of the recombinant protein is functionally active. This observation, which echoes the potential issues raised in Comment 1, needs to be reconciled with the functional assays.

5. Q140L Mechanistic Conclusion: The data in Figure 4C shows that the stimulatory effect of PE on the WT's ATPase activity is modest (~1.5-fold). In contrast, the Q140L mutation causes a severe functional defect, reducing the baseline ATPase activity by approximately10-fold. This quantitative difference suggests that impaired PE binding alone may not sufficiently explain the substantial functional impairment caused by the AHC-derived mutation. Consequently, the conclusion (lines 392-397) that impaired protein-lipid interaction is the likely general mechanism in AHC may be overstated and requires tempering or further refinement based on the available functional data.

Overall Recommendation

The manuscript reports compelling structural data and an interesting functional mechanism for an AHC mutant. However, the novelty of the structural findings is somewhat compromised by very recent, parallel publications. Furthermore, the questions surrounding the functional integrity and active stoichiometry of the recombinant enzyme preparation (Comments 1 and 4) introduce significant uncertainty regarding the quality of structural and functional data. Given all reasons above, I don't recommend this manuscript for publication at Nature Communications.

Reviewer #2

(Remarks to the Author)

The manuscript by Christensen et al. entitled "Active conformations of Neuronal Na+,K+ -ATPase isoforms and a Disease-Causing Mutant" reports cryo-EM structures of human α1β1FXYD1 and neuron-specific α3β1FXYD1 isoform complexes under ATPase turn-over conditions, as well as the disease-associated mutant α3(Q104L) β1FXYD1. The authors captured multiple conformational states along the transport cycle, particularly the phosphoenzyme intermediate with sodium-occluded [Na3]E2P state. These structures provide new mechanistic insights into how Na+ ions occupy the three binding sites, especially the low affinity site III, and how the bindings induce conformational changes coupled with phosphorylation. The study also highlights the critical role of the TM1-A-domain linker in mediating the conformational rearrangement during the transition. Furthermore, a specific phospholipid-binding pocket was identified in the Q104L mutant structures, revealing an important regulatory feature of Na+,K+ -ATPase transport activity. The data are solid, and the study makes a significant contribution to understanding the molecular mechanism of Na+,K+ transport.

Major issue:

How did the authors define the different conformational states under turnover conditions? Did they observe clear cryo-EM density for the conserved phosphorylation Asp and for nucleotide density in E1-ATP and E1P-ADP states? The authors should display them in Figure 1 similar as in Supplementary Figure 4 panel B with each assigned state (e.g., close-up density maps around the phosphorylation site and nucleotide-binding pocket).

The overall cryo-EM maps shown in Figure 1A lack sufficient visual detail to evaluate the state assignments. I suggest the authors show the cryo-EM density overlaid with the modelled domains, using the same color scheme for map as for each model and display the nanodisc belt semi-transparent so that internal features are not obscured (also applied for Figure 2A and supplementary figure 3).

The authors compare each turnover state with previously published structures stabilized by inhibitors or nucleotide analogs respectively (PMID: 36075933). It would be helpful to validate the state assignments with structural comparisons for each state.

Minor issues:

Figure 1A: Please indicate each subunit. "[Na3]E1-P-ADP" should be "[Na3]E1P-ADP".

Figure 1B: the residues labels are too small to read clearly and should be enlarged for better visibility. Additionally, the Na+ density at site III appears weaker in the [Na3]E1P-ADP state compared to the [Na3]E2P state. Could this reflect a shift in Na+ occupancy toward the D923 position or indicate a more stable interaction in the latter state?

Supplementary figure 2B: The authors should include the absorbance value on the y-axis.

Supplementary figure 3: Part of the E1-ATP structure and label are missing.

Supplementary figure 4: I would suggest that the authors display a the cutaway view of the electrostatistic surface to illustrate the Na+ entry pathway and clearly label the three distinct binding sites. Please change the label color of the distance markers in Panel B for better visibility and display the corresponding cryo-EM densities for ATP, ADP and D366.

Supplementary figure 8: Please include the local resolution estimation for the final maps for at least one dataset, as well as an assessment of the model-to-map fit quality.

Line 580 and line 583: "pH- 7.4" should be corrected to "pH 7.4"

Line 596 – 597: Why ATP was added during purification before nanodisc reconstitution?

Line 596: "C12E8" should be corrected to "CE".

Line 533: Saposin A : TEV, 1 : 100 (w/w)?

Line 608-609: Dose adding LMNG to Nanodisc samples before grid preparation improve particle orientation distribution or ?

Line 683: "pH 6.45 for 10 on ice" should be corrected to include the proper time unit.

Line 726-727: Why histidine added to the reaction buffer?

Line 727 and 735: "MgCl2 "should be corrected to "MgCl".

Duplicate refs: 16 and 25; 11 and 12.

Reviewer #3

(Remarks to the Author)

The Na^+,K^+-ATPase is essential for maintaining sodium and potassium gradients across cell membranes. Christensen et al. present cryo-EM structures of the human Na^+,K^+-ATPase α1β1FXYD1 and the neuron-specific α3β1FXYD1 isoforms, determined in active, ATP-driven turnover conditions. Through particle classification, the authors identified four sequential structural states along the Na^+ transport cycle. Notably, they resolved the Na_3E2P intermediate, which had not been

structurally observed previously.

The authors also determined the cryo-EM structure of a disease-associated mutant, α3-Q140L, linked to Alternating Hemiplegia of Childhood (AHC). They showed that the Q140L mutation disrupts a specific lipid-binding pocket, revealing a lipid-dependent regulatory mechanism for Na^+,K^+-ATPase activity. Overall, this is a solid and well-performed study, with high-quality cryo-EM data. I therefore support its publication at Nature Communications.

I have one minor suggestion:
To convince the readers that the models are accurately built for the entire complex, the authors should include figures showing the cryo-EM densities in transparent together with the fitted models for all four states from each dataset in Figures 1–3. In the zoomed-in panels (for example, Figure 1B), the Na^+ and lipid densities should also be displayed along with densities of the surrounding transmembrane regions.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided a comprehensive and convincing revision that thoroughly addresses my previous concerns. In particular, the revised manuscript successfully clarifies the unique value of studying the enzyme under active turnover conditions. The additional evidence provided for the [Na3]E2P intermediate is robust and offers a crucial, previously unobserved view of this transition.

In addition, the authors have effectively addressed queries regarding the monomeric protein yield and functional stoichiometry. I also appreciate the refined discussion of the AHC-associated mutation, which now provides a more dual-mechanism involving both E2 state destabilization and lipid interaction.

In summary, the authors have demonstrated significant scientific rigor in this revision, and I am pleased to recommend the manuscript for publication.

Reviewer #2

(Remarks to the Author)
The authors have addressed all my concerns, and I have no further comments.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed my concerns.

Response to reviewers

We thank the three reviewers for insightful comments and suggestions. Below are our responses to each point raised.

Reviewer #1 (Remarks to the Author):

Review of the Manuscript: “Active Conformations of Neuronal Na+,K+-ATPase isoforms and a Disease-Causing Mutant” by Christensen et al.

The authors present a detailed structural study determining four distinct conformations of human alpha-1 and alpha-3 -isoform-specific Na+,K+-ATPases (NKA) under turnover conditions (Na+, ATP and Mg2+). Resolving intermediate states like the pre-phosphorylated Na2E1-ATP, sodium-occluded [Na3]E1-P-ADP, the hitherto unknown intermediate [Na3]E2P, and outward-open E2P offers a valuable, near-complete view of the Na+ transport cycle. Furthermore, the structural and functional characterization of the Alternating Hemiplegia of Childhood (AHC)-causing alpha-3 Q140L mutant is highly relevant. The proposal that impaired polysaturated phosphatidylethanolamine (PE) binding destabilizes the E2P state, suggesting lipid modification as a potential therapeutic strategy for AHC, is intriguing. While the technical scope of this work is significant, the novelty of the structural component is somewhat limited by the recent publication of similar structures by Guo et al. (2022) and Nguyen et al. (2022) at Nature Communications.

- We do indeed acknowledge that Guo et al. (2022) and Nguyen et al. (2022) presented cryo-EM studies of of Na,K-ATPase, and we cite and discuss these papers. The key message of the two papers relates primarily to models for the gating mechanism of cation binding and occlusion. The studies used classical inhibitors and inactivating point mutations, but reached conflicting conclusions on a so-called ‘sliding-door’ versus a ‘hinged-door’ movement of M1 in the occlusion mechanism.
  We present a wider mechanistic study based on higher resolution cryo-EM structures and fluorescence analysis of E1/E2 conformational equilibria. Our cryo-EM samples are full-length, wild-type human Na,K-ATPase isoforms under active turn-over conditions (i.e. not influenced by mutations nor inhibitors), thus representing the full cycle.
  Our studies yield novel, important insights into both general and isoform-specific mechanisms. These include not least the novel and crucial Na+-transport intermediate [Na3]E2P, providing critical information of a sequential E1P-E2P transition mechanism, and important information to the general field of P-type ATPases. Furthermore, we provide convincing explanations for i) the physiologically important property of a lower apparent Na+ affinity of a3 vs. a1, ii) evidence for the specific protein-lipid interactions of relevance to physiological conditions, and iii) suggestions for a potential rescue of AHC mutations of putative clinical importance. For the specific point of Na+ binding, our study clearly supports a ‘sliding-door’ mechanism, in agreement with Guo et al. (2022) (as noted in our manuscript, lines 118-124) and earlier studies of Ca2+-ATPase (e.g. Winther et al. 2013). Furthermore, we investigate isoform-specific properties of the neuronal a3 compared to the ubiquitous a1 isoform, the E1P-to-E2P transition with a novel [Na3]E2P state, and effects of the AHC-causing mutant Q140L of a3. Thus, our studies resolve a long-standing question concerning structural intermediates in the active Na+ transport cycle and provide entirely new information on isoform-specific properties, and a potential role of specific lipid-protein interactions in neurological disease

Additionally, several points regarding the recombinant NKA preparation and functional integrity require clarification and supporting data to fully establish the robustness of the

reported structural conclusions.
My specific comments for the authors are as follows:
1. Sample preparation and Quality: The preparation method involves a multi-step in vitro assembly process (purifying FXYD subunit separately, adding it to detergent solubilized alpha-beta, and reconstitution into nanodiscs). Supplementary Figure 2A shows that the majority of the protein, especially alpha-3 eluted at the void volume of the Superdex 200 10/300 column, which often indicates oligomerization and aggregation. Only a minor fraction was collected for downstream analysis. Given this, could the authors confirm why co-expression of all three subunits (alpha, beta and FXYD) was not pursued to ensure proper complex assembly in vivo?

- It is common to observe a distribution in size-exclusion chromatography of samples from nanodisc reconstitution of membrane proteins, and we have no concerns with the quality of the purified material used here. Generally, lipid nanodiscs would be considered preferred membrane protein sample presentation modes over detergent micelles.
  A well-documented P. pastoris expression is used. It is likely that a mammalian expression system can provide a co-expressed complex[1], but then contamination of all endogenous subunits must be avoided and controlled for. This is not relevant here for the P. pastoris system, which is therefore highly preferred.

  Previous studies have shown that co-expression of FXYD1 with alpha + beta (a/b) subunits in P. pastoris has greater variability than reconstitution (pubmed 16608841), while, on the other hand, reconstitution of a/b with separately expressed FXYD1 turned out to be an efficient and reproducible tool for the generation of stable and functional a/b/FXYD complexes (pubmed 21228272, 26429909, 21449573). The structures we present here reveal a single FXYD1 molecule bound near the aTM9 segment, i.e. in the same position as FXYD2 in native renal Na,K-ATPase, demonstrating a fully integral and correct assembly of the a/b/FXYD1 complex.

  The size exclusion chromatograms for the nanodisc reconstituted complexes indeed show majority of the protein eluting at a larger size than the monomeric complexes applied to grids. Purifying the Na,K-ATPase in detergent on the other hand provides a primarily monomeric elution profile, and the oligomeric assemblies (dimers and larger) thus result from the SapA-nanodisc reconstitution and are not due to mis-assembly of a/b/FXYD. Numerous efforts to optimise the reconstitution, and yield of the monomeric fraction have been conducted. In brief, testing lipids, detergents, protein:lipid:saposin ratios, direct reconstitution from membranes, and variation in temperature and timing have not led to a predominantly monomeric profile. The oligomeric peak contained soluble and highly active Na,K-ATPase (probed by the Baginsky ATPase activity assay), but was not suited for single-particle cryo-EM due to a highly heterogeneous (but not denatured, misfolded or misassembled) nature of the particles. The monomeric fraction also shows proper ATPase activity, and for the EM-studies we aimed for and present here, it was far superior compared to detergent samples. Hence, we used the small fraction of monomeric ATPase in SapA nanodisc to obtain the best results.

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[1] *Concerning purity and activity of Na,K-ATPase preparations applied to cryo-EM grids, it is interesting to compare our data to those reported in Nguyen et al. 2022 (Guo et al. report no functional data). Nguyen et al. described HEK293 expression and purification of a3b1FXYD6. The partially purified soluble protein in LMNG applied to the Sephadex column has a specific binding capacity of 0.9 nmoles/mg. Compared to about 6.5 nmoles/mg for pure Na,K-ATPase, the preparation is about 15% pure. Also, the specific Na,K-ATPase activity of 254 umole/mg/hr is about half that of a3b1FXYD1 used in the current work (range 400-600 umoles/mg/hr). Judging by SDS gels the purity of protein applied to the grids in our study is higher in the present work than in Nguyen et al. (compare Figs 2S and 1S in Nguyen et al.). Yet convincing structural data on the different conformations, stabilized by BeFx, ADP-AlFx, and MgFx were obtained in Nguyen et al., and we infer that the absolute activity of the protein applied to grids is less important than proof of the validity of the structural data obtained by reference to appropriate controls or previously published structures.*

- In addition to the measured ATPase activity of nanodisc reconstituted Na,K-ATPase (approx.12 µmol P_i/mg/min), the different structures/conformations show a functional protein as these depend on the specific ligand combinations present, Na/Mg with ATP or Na/Mg/K with ATP (corresponding to Na-ATPase and Na,K-ATPase reaction conditions, respectively). Furthermore In the Na/Mg/ATP condition, three of the four conformations detected (Na2E1, [Na3]E1P-ADP and E2P) are similar to structures determined previously of inhibitor-stabilized native shark/pig Na,K-ATPase and, in effect, serve as controls for also the new features, in particular the [Na3]E2P intermediate in the Na,K-ATPase reaction.

More importantly, the authors must provide detailed functional evidence, including but not limited to electrophysiology, phosphorylation, ATPase activity to characterize the specific NKA fraction used for Cryo-EM and ensure it is highly active.

We do indeed show functional data for the cryo-EM sample, and it is fully active. All WT samples used for cryo-EM showed clear Na+,K+,Mg2+ dependent ATPase activity. The ATPase assay is a golden standard for P-type ATPases. The Spec. Act. of Saposin samples was 12µmol/mg/min for a1b1FXYD1, 6.7 for a3b1FXYD1 and 2.5 for a1Q140Lb1FXYD1, with >95% ouabain sensitive activity for wt samples and >90% for mutant. Activities have been included in the manuscript. Those activities are about half of detergent solubilised samples shown in Fig 3C. The difference is caused in part by an over-estimation of protein concentration due to an unknown amount of SapA molecules in the nanodisc, and potentially also a tight nature of the disc that may slow activity to some extend compared to lipid-detergent micelles. Still, we consider the activity level in lipid nanodiscs consistent with a fully active enzyme.
Electrophysiological measurements of samples in Saposin A nanodiscs are not possible, as such experiments require compartmentalization or large lipid-bilayers, or deposition to solid-support membranes. Due to the nature of nanodisc reconstituted protein, with particles held together by a scaffold protein, such electrophysiological studies are implausible.
In addition, the different cryo-EM structures/conformations are determined from samples flash-cooled at active ATPase turnover conditions, and we present multiple conformations (including phosphoenzyme intermediates) involved in ion-binding/release, ATP-binding, Na,K-ATPase phosphorylation, and nucleotide release, covering a near complete view of the Na-transport cycle.

2. Na2E1-ATP conformation: The structure labeled as Na2E1-ATP is classified as inward-open. However, the reported M1/ M4 movement (~4Å) is notably smaller than the displacement observed in the fully open state of other P-type ATPases (e.g. Ca2+ ATPase at ~12Å).

The approx. 4Å movement of M1 and the ‘sliding-door’ mechanism of the cytoplasmic gate is in agreement with Guo et al (2022) (reported 4.6Å shift of M1). While the approx. 4-5Å movement of M1 is smaller than the estimated 12Å of SERCA, the opening is significant and sufficient for gating the solvent accessible pathway from the cytoplasm to the Na^+ binding sites. See sup. Fig 5A and Guo et al (2022). The Na,K-ATPase and Ca2+ ATPases are different proteins and although they share the same ‘sliding-door’ mechanism, the extend of the M1 movement differs as also previously described in other publications such as Guo et al (2022). Apparently, Ca2+-ATPases and Na,K-ATPases are a bit different here, but qualitatively they are doing the same thing.

Moreover, this structure also revealed the binding of two out of three Na+ ions. Based on these observations, the state may be more accurately described as an inward-occluded conformation rather than fully open. Could the authors provide a more detailed structural rationale for classifying this state as ‘inward-open’?

> An occluded state with 2 Na ions would not be expected to progress through the cycle, unless a proton for example is bound to the third ion binding site. While not all structures had local resolutions high enough to identify three bound sodium ions in the occluded E1P state (but e.g. wild-type a3 and a1 do in the Na-ATPase cycle), the states were assigned based on the structure of the TM-domain and comparison to structures of known conformations stabilised with metal fluorides or nucleotide analogues. An open E1 and occluded E1 state can be distinguished by the relative position of M1, which slides towards the cytoplasmic domains in the E1-ATP -> [Na3]E1P-ADP transition. In addition to the position of M1, the ‘inward open’ (non-phosphorylated) and ‘Na-occluded’ (phosphorylated) states can be distinguished by the positioning of cytoplasmic domains and nucleotide, which are in a tight arrangement in the Na-occluded [Na3]E1P-ADP compared to E1-ATP (inward-open). Figures of the cytoplasmic domains near the phosphorylation sites have now been included in the main figures 1 and 2 for further clarification.

Have attempts been made to capture a fully open E1-ATP conformation using a Na+-free buffer?

> Great suggestion, this was considered but was not done for this study, which has focused on turn-over conditions.

3. Data Processing and [Na3]E2P Intermediate: The authors utilized an in silico classification approach during Cryo-EM data processing to resolve the various conformational states, including the novel [Na3]E2P intermediate. Given that this intermediate was derived computationally from a heterogenous particle pool, could the authors address the possibility that this method might have artificially separated closely related conformations?

> This was addressed extensively during processing. We concluded that merging conformations would not have resolved the complex in its entirety and the good fit and refinement of an atomic model with proper stereochemistry would not be possible. For instance, merging the occluded [Na3]E1P-ADP (providing transmembrane density) with outward open E2P states (providing density for cytoplasmic domains) would have caused conflicting orientations of the β-subunit and α-transmembrane domain. Thus, density would have been distorted or likely have been averaged out.
> The confidence in our map and thus the structure of the [Na3]E2P intermediate conformation, can also be described statistically from the b-factor values of the map extracted from cryoSPARC (See table 1). The b-factor in cryo-EM, also known as the atomic displacement parameter, models the observed spread of the map density by atomic movement to account for flexibility or uncertainty. A high b-factor (above 150 for resolutions below 4Å) is an indication for uncertainty and a high spread in map density, which could be caused for example by inappropriately merging two or more conformation into a single map (high map density spread). The global b-factors obtained for the different conformations, including the [Na3]E2P intermediate, remain near or below 100 (see table 1), which is generally accepted as good and sufficient for reliable modelbuilding. Additionally, for each dataset we observe similar b-factor values for the novel [Na3]E2P as for the other, well-known conformations extracted, indicating

comparable confidence in the map features of the novel conformations and those previously described by crystallography with the use of inhibitors.

What orthogonal validation methods or independent structural evidence can be provided to support the existence and structural uniqueness of [Na3]E2P intermediate?

- This is discussed in the paper (solution scattering, MD, mutants & occlusion assays, fluorescence measurements and kinetic considerations, and also the comparison to the bacterial Ca2+-ATPase LMCA1 stabilized with a 4-Ala insert (Basse Hansen et al. 2025 40016426).

4. Functional Stoichiometry: Supplementary Table 2 reports very low ouabain binding values (~15.5 and ~8 pmol/mg for a1 and a3, respectively). Calculating the stoichiometry reveals that only a small percentage (~0.14%-0.28%) of the total NKA mass appears capable of ouabain binding. Since ouabain is a high-affinity inhibitor, this metric strongly suggests that only a tiny fraction of the recombinant protein is functionally active. This observation, which echoes the potential issues raised in Comment 1, needs to be reconciled with the functional assays.

For clarification, the ouabain-binding method is used to determine the amount of active Na,K-ATPase in a **crude** yeast membrane and is given as pmol Na,K-ATPase sites per mg of **total** protein. The value is in line with previous measures of Na,K-ATPase isoforms expressed in yeast membranes (e.g. pubmed 11546674).
The values of ouabain binding and Na,K-ATPase activities in Table 2S are within the normal range for crude *P.pastoris* membrane preparations (which can vary by 2-3 -fold) (see PMID 15708860; 20388710;23430748). The derived turn-over number of, for example, a1b1FXYD1 (8496 min^{-1}) shows that the Na,K-ATPase is fully active and compatible with the ouabain binding. Using the values presented in Table S2 the specific turnover can be calculated by dividing the Na,K-ATPase activity by the ouabain binding capacity:

$$Turnover = \frac{0.132\mu mol \cdot mg^{-1} \cdot min^{-1}}{15.5pmol \cdot mg^{-1}} = 8496min^{-1}$$

This value is quite similar to that of purified kidney Na,K-ATPase (PMID 4276443) or purified a1b1FXYD1 expressed in P. pastoris (see PMID 15708860;23430748). Thus, one can assume that the calculated turn-over numbers for a3b1FXYD1 and other complexes based on Na,K-ATPase activity and ouabain binding in the yeast membranes are reliable.

5. Q140L Mechanistic Conclusion: The data in Figure 4C shows that the stimulatory effect of PE on the WT's ATPase activity is modest (~1.5-fold). In contrast, the Q140L mutation causes a severe functional defect, reducing the baseline ATPase activity by approximately10-fold. This quantitative difference suggests that impaired PE binding alone may not sufficiently explain the substantial functional impairment caused by the AHC-derived mutation. Consequently, the conclusion (lines 392-397) that impaired protein-lipid interaction is the likely general mechanism in AHC may be overstated and requires tempering or further refinement based on the available functional data.

We agree that the inhibitory effect of the Q140L mutation on Na,K-ATPase activity represents the contribution of two aspects of the mechanism , namely (1) by destabilization of E2 or stabilization of E1, as inferred in Fig. 3 D and 4 A+B, and (2) by

interference with binding of the 18:0,22:6PE, as inferred from Fig. 3C and Fig. 4C+D. The stimulation of Na,K-ATPase activity by 18:0,22:6PE binding (seen also previously with the purified protein) is a valid, physiological effect. We have modified the text to clarify these points. It remains to be seen whether the proposed strategy to increase the degree of unsaturation of brain PE in vivo in order to stimulate Na,K-ATPase activity is a feasible approach, but such studies go far beyond the scope of the work presented here.

Overall Recommendation
The manuscript reports compelling structural data and an interesting functional mechanism for an AHC mutant. However, the novelty of the structural findings is somewhat compromised by very recent, parallel publications. Furthermore, the questions surrounding the functional integrity and active stoichiometry of the recombinant enzyme preparation (Comments 1 and 4) introduce significant uncertainty regarding the quality of structural and functional data. Given all reasons above, I don't recommend this manuscript for publication at Nature Communications.

In short, we are of the opinion that we have responded fully to the reviewer's questions and requested clarifications, and that we have made appropriate changes to the text and figures where needed. The findings are novel, providing important insights into structure and function of Na,K-ATPase in general, the specific properties of neuronal a3 isoform of Na,K-ATPase compared to the ubiquitous a1 isoform, and of the disease-causing a3-Q140L mutant. The studies are based on functional and well-characterized samples obtained from well-documented preparations (as detailed above and see also the foonote 1 referring to the papers by Guo et al. and Nguyen et al.). Furthermore the strategy of determining the cryo-EM structures in active turn-over conditions may have implications for other ion pumps and transporter proteins

Reviewer #2 (Remarks to the Author):

The manuscript by Christensen et al. entitled "Active conformations of Neuronal Na+,K+ -ATPase isoforms and a Disease-Causing Mutant" reports cryo-EM structures of human α1β1FXYD1 and neuron-specific α3β1FXYD1 isoform complexes under ATPase turn-over conditions, as well as the disease-associated mutant α3(Q104L) β1FXYD1. The authors captured multiple conformational states along the transport cycle, particularly the phosphoenzyme intermediate with sodium-occluded [Na3]E2P state. These structures provide new mechanistic insights into how Na+ ions occupy the three binding sites, especially the low affinity site III, and how the bindings induce conformational changes coupled with phosphorylation. The study also highlights the critical role of the TM1-A-domain linker in mediating the conformational rearrangement during the transition. Furthermore, a specific phospholipid-binding pocket was identified in the Q104L mutant structures, revealing an important regulatory feature of Na+,K+ -ATPase transport activity. The data are solid, and the study makes a significant contribution to understanding the molecular mechanism of Na+,K+ transport.
Major issue:
How did the authors define the different conformational states under turnover conditions? Did they observe clear cryo-EM density for the conserved phosphorylation Asp and for nucleotide density in E1-ATP and E1P-ADP states?

The conformations were initially classified by comparison to literature and published structures of known conformations trapped by inhibitors.

In addition, the cryo-EM data of e.g. a3 wt structures show clear density in the different conformations for both nucleotides (ATP and ADP) and bound ions, and for phosphorylation of the conserved Asp. Phosphorylation of Asp is unfortunately difficult to observe for some structures with low local resolution in the cytoplasmic domain (especially for the Q140L mutant). The conformational state of these structures was inferred by comparison of the TM and cytoplasmic domains to the higher resolution structures for wild-type presented in this study and previously published structures.

The authors should display them in Figure 1 similar as in Supplementary Figure 4 panel B with each assigned state (e.g., close-up density maps around the phosphorylation site and nucleotide-binding pocket).

Close views of the nucleotide binding sites/phosphorylation have been included in the main figures, Fig. 1C and Fig 2B

The overall cryo-EM maps shown in Figure 1A lack sufficient visual detail to evaluate the state assignments. I suggest the authors show the cryo-EM density overlaid with the modelled domains, using the same color scheme for map as for each model and display the nanodisc belt semi-transparent so that internal features are not obscured (also applied for Figure 2A and supplementary figure 3).

- An additional figure has been prepared showing a map to model fit with and without disc for the 4 alpha 3 models shown in Figures 1 and 3. Local resolutions have been included for further clarity.

The authors compare each turnover state with previously published structures stabilized by inhibitors or nucleotide analogs respectively (PMID: 36075933). It would be helpful to validate the state assignments with structural comparisons for each state.

- An additional figure has been prepared.

Minor issues:
Figure 1A: Please indicate each subunit. “[Na3]E1-P-ADP” should be “[Na3]E1P-ADP”.

- We will adhere to this nomenclature.

Figure 1B: the residues labels are too small to read clearly and should be enlarged for better visibility. Additionally, the Na+ density at site III appears weaker in the [Na3]E1P-ADP state compared to the [Na3]E2P state. Could this reflect a shift in Na+ occupancy toward the D923 position or indicate a more stable interaction in the latter state?

- The size of text in all figures has been adjusted to at least 8.
  We would be hesitant to compare the suggested relative density intensities/distribution, as such interpretations would be speculative and potentially dominated by slight

differences in local resolution, atomic displacement or flexibility. At first glance, we would not assume that Na+ site III forms a more stable interaction in a transition state (perhaps even to the contrary), but it is an interesting question to follow up on in later studies.

Supplementary figure 2B: The authors should include the absorbance value on the y-axis.

- Absorbance values have been included. The previous version showed normalised values. There was a mistake in the chromatogram of a1b1FXYD1 which became apparent when showing absolute absorbance values. Hence, the shape of the trace slightly changed, although not changing results or conclusions in any way.

Supplementary figure 3: Part of the E1-ATP structure and label are missing.

- We apologize for the mistake. This has been corrected.

Supplementary figure 4: I would suggest that the authors display a the cutaway view of the electrostatistic surface to illustrate the Na+ entry pathway and clearly label the three distinct binding sites. Please change the label color of the distance markers in Panel B for better visibility and display the corresponding cryo-EM densities for ATP, ADP and D366.
Supplementary figure 8: Please include the local resolution estimation for the final maps for at least one dataset, as well as an assessment of the model-to-map fit quality.

- The figures in panel B were moved to the main figures for better assessment of the confirmation. Please note that the distance indications were removed to declutter the figures. Local resolution plots for the main dataset have been included as suppl. figure 10.

Line 580 and line 583: “pH- 7.4” should be corrected to “pH 7.4”

- Corrected.

Line 596 – 597: Why ATP was added during purification before nanodisc reconstitution?

- We have observed the nanodisc to be quite tight when reconstituting inhibitor-stabilised conformations. We added ATP to allow the sample to turn over and move during Nanodisc reconstitution - to loosen the disc, so to say. We later observed that the enzyme is active in the disc but kept the procedure for consistency.

Line 596: “C12E8” should be corrected to “CE”.

- C12E8 is widely used and accepted, and we prefer to stick to this nomenclature.

Line 533: Saposin A : TEV, 1 : 100 (w/w)?

- Thanks for the careful read. This was corrected.

Line 608-609: Dose adding LMNG to Nanodisc samples before grid preparation improve particle orientation distribution or ?

- Including LMNG/detergent before grid preparation generates thinner and more uniform ice for the subsequent cryo-EM data collection.

Line 683: “pH 6.45 for 10 on ice” should be corrected to include the proper time unit.

- ”10’” was changed to “10 min”.

Line 726-727: Why histidine added to the reaction buffer?

- Histidine acts as buffer for pH adjustments. It has historically been used as buffer for P-type ATPases since it is inert to Na,K-ATPase unlike Na+/K+ containing buffers. Furthermore, phosphate buffers that are often used for enzyme activity assays are not suited here since phosphate would interfere, and Tris does not buffer well at the pH optimum.

Line 727 and 735: “MgCl2 “should be corrected to “MgCl”.
Duplicate refs: 16 and 25; 11 and 12.

- Both have been corrected from “MgCl2” to “$MgCl_2$”.

Reviewer #3 (Remarks to the Author):

The Na^+,K^+-ATPase is essential for maintaining sodium and potassium gradients across cell membranes. Christensen et al. present cryo-EM structures of the human Na^+,K^+-ATPase α1β1FXYD1 and the neuron-specific α3β1FXYD1 isoforms, determined in active, ATP-driven turnover conditions. Through particle classification, the authors identified four sequential structural states along the Na^+ transport cycle. Notably, they resolved the Na□E2P intermediate, which had not been structurally observed previously.

The authors also determined the cryo-EM structure of a disease-associated mutant, α3-Q140L, linked to Alternating Hemiplegia of Childhood (AHC). They showed that the Q140L mutation disrupts a specific lipid-binding pocket, revealing a lipid-dependent regulatory mechanism for Na^+,K^+-ATPase activity. Overall, this is a solid and well-performed study, with high-quality cryo-EM data. I therefore support its publication at Nature Communications.

I have one minor suggestion:
To convince the readers that the models are accurately built for the entire complex, the authors should include figures showing the cryo-EM densities in transparent together with the fitted models for all four states from each dataset in Figures 1–3. In the zoomed-in panels (for example, Figure 1B), the Na^+ and lipid densities should also be displayed along with densities of the surrounding transmembrane regions.

- Figures showing map to model fit and local resolution have been included.