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Peer Review Information

Journal: Structural and Molecular Biology
Manuscript Title: Structural mechanism of muscle nicotinic receptor desensitization and block by curare
Corresponding author name(s): Dr Ryan Hibbs, Professor Michael Stowell

Reviewer Comments & Decisions:

Decision Letter, initial version, with author responses (blue):

Our ref: NSMB-A45683

January 3rd, 2022

Dear Ryan,

I wish you a Happy New Year! Thank you again for submitting your manuscript "Structural mechanism of muscle nicotinic receptor desensitization and block by curare" (NSMB-A45683) to NSMB. It has now been seen by three expert referees and their comments are below. I hope you will be pleased to see that all three reviewers find that the work is of high interest and quality. Therefore, we'll be happy in principle to publish the paper in Nature Structural & Molecular Biology, pending minor revisions to implement the referees' suggestions and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements, probably only in the first two weeks of the New Year. Please do not upload the final materials and make any revisions until you receive this additional information from us.

To facilitate our work at this stage, we would appreciate if you could send us the main text as a word file. Please make sure to copy the NSMB account (cc'ed above).

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040

Referee expertise:

Reviewer #1: pGLIC function and pharmacology
Reviewer #2: cryo-EM of membrane proteins
Reviewer #3: pGLIC function and pharmacology

Reviewer #1 (Remarks to the Author):

This new study from the Hibbs lab presents for the first-time structures at 2.5 -2.7 Å resolution of the muscle-type nicotinic acetylcholine receptor (nAChR) in the Apo (resting, closed channel, activatable) and desensitized (agonist (carbamylcholine) bound) states and also in the presence of the competitive antagonist d-tubocurarine (dTC), or dTC and carbamylcholine. The structures, determined from single particle cryo-EM data, are of native receptors purified in detergent from Torpedo electric organ and then reincorporated into lipid nanodiscs. This report is a very important extension of their previous determination of the Torpedo nAChR structure in the presence of the peptide neurotoxin α-bungarotoxin. The report, which is presented in a clear and concise manner, provides a wealth of new and surprising structural information that significantly advances our understanding of the structural changes associated with receptor desensitization and the mechanism of inhibition by dTC. The conclusions are well supported by the data presented. The report includes appropriate consideration of the relationship between the new structural data and prior structural, biochemical, and mutational studies. The study is a major contribution to our understanding of the structures of neurotransmitter-gated ion channels in different conformational starts and provides data crucial for future computational studies of the conformational transitions of the muscle nAChR or for the search for novel nAChR therapeutic agents.

The new results that I find most important:

1. The localization of high and low affinity cholesterol sites and of phospholipid. nAChR purified and reconstituted with soy lipids (which lack cholesterol) bind ~5 cholesterol /nAChR, consistent with previous results. With cholesterol supplementation, there are ~25 cholesterol/nAChR, as seen for the Torpedo nAChR in its native membrane. The structure determined in the absence of added Chol identifies cholesterol at 3 subunit interfaces in the inner membrane leaflet, with additional Chol seen after Chol supplementation. It has long been known the Chol is necessary for gating of nAChRs, and these results, defining nAChR residues interacting with Chol are a major advance over previous low-resolution studies from Nigel Unwin identifying Chol/phospholipid/nAChR interactions in native Torpedo membranes.

2. Differences in structure between resting and desensitized states. The asymmetry of the desensitized structure, with substantial differences between the orientation of the M4 helices of the two alpha subunits, is unexpected and contrasts to the symmetric structure of the resting state. The difference in orientation of the M4 helix relative to the M1 and M3 helices identified three Phe, one in each of the helices, that were likely to make important energetic contributions to state transitions and in fact had been identified in previous studies by mutational analyses as determinants of agonist gating (EC50). In this report, replacement of 2 of the residues by Ala is shown to increase the rate of recovery from desensitization, suggesting that these interactions are important determinants of the stability of the desensitized state.

3. Structure of the ion channel in resting vs desensitized state. Though there are no big surprises here, it is wonderful to have the resolution necessary to see waters at the level of the desensitization gate (M2-2) and to clearly determine that the permeability barrier in the resting state occurs at M2-9 and M2-16 (not M2-13). These results allow new interpretations of mechanisms of desensitization and state dependent binding of drugs in the channel based upon based upon previous photoaffinity labeling and mutational studies.

4. dTC stabilizes a desensitized state structure different than that stabilized by Carb. With the dTC -bound structure determined in the presence of 0.5 mM dTC, dTC is shown to bind to 4 sites: the two agonist sites, a site near the extracellular end of the ion channel, and a site near the extracellular end of the M1, M3, and M4 helices of αΥ. While these allosteric sites may be of low affinity for dTC and not necessarily important for the action of dTC itself, the last site defines a novel allosteric binding site and it will be of particular interest in the future to determine whether the αΥ site is possibly a binding site for therapeutically important positive or negative allosteric modulators. While the structure of the ion channel is the same in the presence of Carb or dTC, the asymmetric disposition of the M4 helices in the Carb structure is not seen in the presence of dTC, a difference most likely resulting from the binding of dTC to the αΥ helix bundle site.

To Clarify:

dTC sites: Is the αϒ helix bundle site occupied by dTC in both the dTC structure and the Carb-dTC structure? Does dTC not bind to the αδ site? If not, why not, since the M4 helix is already "detached" in that site?

Minor comments:
(i) Line 259. Rather than ask the reader to go to the Methods, perhaps state simply here that the structure is determined "in the presence of 500 uM dTC, a concentration 25-fold higher than necessary to occupy both agonist sites" (rather than "at higher concentrations").

(ii) line 586. "as previously described" What reference??

Response to Reviewer #1
To Clarify: dTC sites: Is the αϒ helix bundle site occupied by dTC in both the dTC structure and the Carb-dTC structure? Does dTC not bind to the αδ site? If not, why not, since the M4 helix is already "detached" in that site?
Our response: We could only observe density for d-tubo at α/γ helix bundle site in the pure d-tubo bound condition, where we used the higher concentration of d-tubo. One of the possible reasons for occupying one site, and not the other, is that the M4 helix at the α/δ site is already detached and filled with lipids after d-tubo occupies the two orthosteric site. Further, superposition of d-tubo from the αγ site on the αδ site shows that d-tubo would clash with the M4 helix at the α/δ site. Thus, while M4 is indeed detached in both sites, the M4 opens to a lesser degree at the αδ site.
Minor comments:
(i) Line 259. Rather than ask the reader to go to the Methods, perhaps state simply here that the structure is determined "in the presence of 500 uM dTC, a concentration 25-fold higher than necessary to occupy both agonist sites" (rather than "at higher concentrations").
Our response: This is a great suggestion. We added to the main text the following statement: "The structure with only the antagonist bound was determined in the presence of 500 µM *d*-tubo, a concentration 50-fold higher than the necessary to occupy both agonist sites."

(ii) line 586. "as previously described" What reference??
Our response: Thank you for catching this error in omitting the reference. We now include two references here, in the Methods section:
9. Rahman, M. M. et al. Structure of the Native Muscle-type Nicotinic Receptor and Inhibition by Snake Venom Toxins. Neuron 106, 952-962 e955, doi:10.1016/j.neuron.2020.03.012 (2020).

10. Rahman, M. M., Worrell, B. T., Stowell, M. H. B. & Hibbs, R. E. in Methods in enzymology Vol. 653 (eds Daniel L. Minor & Henry M. Colecraft) 189-206 (Academic Press, 2021).

Reviewer #2 (Remarks to the Author):

This study presents cryo-EM structures of muscle-type nicotinic acetylcholine receptor from Torpedo in its apo-form resting state, the agonist carbachol-bound desensitized state, the antagonist d-tubocurarine (tubo)-bound desensitized-like state, and the carbachol/d-tubo-bound state at high resolution (2.5-3.2 Å). This study reveals an unusual asymmetric conformational changes between the resting and desensitized states, which could not find in the neuronal nAChRs. Interestingly, the antagonist d-tubo is not only bound in the two classical orthosteric sites, but also in two allosteric sites in the transmembrane domain. The antagonism mode of d-tubo is a new one, and it is different from the classical antagonists, such as alpha-BgTX. The allosteric sites of d-tubo may provide clues to develop new blockers which are selective for muscle receptors. Overall, this study is an important work and provide novel insights into the gating of muscle-type nicotinic receptor. I support the acceptance of this manuscript.

Minor comments,
1. Line 99: "Cholesterol quantification estimated that 4-5 cholesterol molecules per receptor remained bound through purification in the presence of soy lipids alone (Fig. 1b), …". This sentence describes the third column "Apo" in Fig. 1b.
I wonder what does the last column "soy polar lipid" in Fig. 1b mean. Or what is the sample preparation process of this one?

2. Line 213: "…nicotinic receptor, WT and mutants, expressed in HEK cells (Figs. 4e-g)." Since there is only one double mutant F233A+F414A in Figs. 4e-g, it is better to describe it as "…nicotinic receptor, WT and the mutant F233A+F414A, …".

Response to Reviewer #2
Minor comments:
1. Line 99: "Cholesterol quantification estimated that 4-5 cholesterol molecules per receptor remained bound through purification in the presence of soy lipids alone (Fig. 1b), …". This sentence describes the third column "Apo" in Fig. 1b.
I wonder what does the last column "soy polar lipid" in Fig. 1b mean. Or what is the sample preparation process of this one?
Our response: The "soy polar lipids" condition was used as a control, because there is no cholesterol present in this lipid mixture. The sample preparation is as described in the "Lipid Assays" section in Methods. We have added explanatory detail to the beginning of this methods section to make it more clear how to prepare the samples.

2. Line 213: "…nicotinic receptor, WT and mutants, expressed in HEK cells (Figs. 4e-g)." Since there is only one double mutant F233A+F414A in Figs. 4e-g, it is better to describe it as "…nicotinic receptor, WT and the mutant F233A+F414A, …".
Our response: Thank you for this helpful suggestion. We have modified the text accordingly. The sentence now reads:
"As the *Torpedo* muscle-type receptor does not express well in HEK cells, and solution exchange in oocytes is slow, we conducted these experiments by patching the αβγδ mouse muscle nicotinic receptor, WT and the mutant F233A + F414A, expressed in HEK cells (Figs. 4e-g)."

Reviewer #3 (Remarks to the Author):

Summary of results:

The manuscript describes the Torpedo nicotinic acetylcholine receptor that is a close relative to the muscle nAChR in the apo state as well as agonist and antagonist bound.

Originality and significance:
The novelty of the present study is the detailed analysis of ASYMMETRIC conformational changes between the different states. The detailed insights provided into the conformational change pathways based on the respective subunit, its relation to the agonist binding site and the extent of conformational change. Overall, the study is beautifully conducted, the results are intuitively presented and allow the reader to immerse and understand in a structural context how these receptors move during conformational transitions.

Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions:
All written sections, images and videos are very clear, the introduction and conclusion are supported by the experimental approach and results.

I have very little if anything to criticize here which made this review difficult when it should not be.

Detailed summary:
Cholesterol
Initially, the authors determined two cryo-EM structures in soybean lipid nanodiscs with and without additional cholesterol. Cholesterol has been shown to modulate channel activity and interestingly in Torpedo membranes a high cholesterol amount has been observed.

The receptor structure is identical in both cholesterol conditions. In the low cholesterol conditions high affinity binding sites in the inner membrane leaflet in close proximity to the MX helix are observed, whereas in high cholesterol conditions mimicking those of Torpedo membranes low affinity additional sites are identified.

Carbachol
The acetylcholine analogous carbachol binds at the two orthosteric agonist binding sites between alpha and delta/gamma subunits that contract upon agonist binding with the quaternary ammonium unsurprisingly engaging in a cation-pi interaction. The description of the carbachol induced conformational changes in the ECDs and TMDs is intuitive.

Gates
In the resting state there are two barrier points towards hydrated permeant ions observed, 9' and 16'. In the desensitized state the pore is funnel shaped. The desensitization gate is located towards the cytosolic mouth of the pore at the 2' level. The location of this gate varies in the channel superfamily.

Tubocurarine
Interestingly a structure of the antagonist with AChBP has been deposited to the protein data bank in 2011 but never published.
Binding sites 1 and 2 are the orthosteric sites in which the toxin binds in different orientations. Additionally at high concentrations the toxin also binds between 16' and 20' at the extracellular end of the TM domain pore and functions as a pore blocker, and at the extracellular end of the alpha-gamma M4 helical interface with other transmembrane helices, stabilizing a conformation similar to alpha-delta with carbachol/desensitized conformation.

Suggested improvements/questions:
Questions:
- For the toxin Torpedo structures published in Neuron, particle alignment was facilitated by the presence of a toxin that bound to the ECD. How was particle alignment possible here?
-
Minor points:
- Some of the videos seem to be excessively long with 30 sec of the same rocking motion while others seem short with 3 seconds.

Response to Reviewer #3
Suggested improvements/questions:
Questions:
- For the toxin Torpedo structures published in Neuron, particle alignment was facilitated by the presence

of a toxin that bound to the ECD. How was particle alignment possible here?

Our response: Great question. In other heteromeric receptors in the superfamily, including other nicotinic receptors, Fab fragments, a toxin molecule or other fiduciary markers have been essential in particle alignment, and we expected this to hold true for the Torpedo receptor. However, we found that particle alignment proceeded smoothly in all cases for this receptor. We suspect that the large asymmetric features from the C-termini and F-loops of the γ and δ subunits provide well ordered 'handles' for particle alignment. Large and ordered C-termini like in the Torpedo receptor have not been observed, yet, in other heteromeric pGLIC. We have added a sentence related to particle alignment to the Methods:
"The potential problem of pseudo-symmetry in particle alignment turned out to not present a challenge, likely due to the large and well-ordered C-termini and F-loops visible at low resolution in the γ and δ subunits."

Minor points:
- Some of the videos seem to be excessively long with 30 sec of the same rocking motion while others seem short with 3 seconds.
Our response: We have shortened the 30 second videos to 5 seconds.

Final Decision Letter:

4th Feb 2022

Dear Ryan,

We are now happy to accept your revised paper "Structural mechanism of muscle nicotinic receptor desensitization and block by curare" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

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Kind regards,
Florian

Florian Ullrich, Ph.D.
Associate Editor
Nature Structural & Molecular Biology
ORCID 0000-0002-1153-2040