nature portfolio

Peer Review File

Molecular insights into the α6β4 nicotinic acetylcholine receptor function and ligand recognition

Corresponding Author: Professor Yan Zhao

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)

The paper by Su et al. reports the high-resolution structure of the α6β4 nicotinic acetylcholine receptor bound to either nicotine or the pre-clinical agonist tebanicline determined by cryo-EM. This receptor is significant because it is thought to be present in sensory neurons in the dorsal root ganglia (DRG) and is associated with pain. Structural studies of this receptor have been limited until now due to the unknown chaperones required for its expression. However, these chaperones were discovered in recent years, allowing the authors to use them to express sufficient amounts of protein for purification and structural determination.

The obtained structure reveals standard features of a nicotinic receptor, including a defined subunit stoichiometry, details of the orthosteric binding pockets, interactions with the two agonists, unique inter-subunit stabilizing interactions proposed to assist in subunit assembly, and details of the ion-conductive pore in the desensitized conformation. Specifically, the structure reveals differences in receptor-ligand interactions between α6β4 and α4β2 that may account for the lower affinity of nicotine for α6β4 compared to α4β2. Additionally, the structure reveals an interaction between the chlorine atom on tebanicline and a main chain carbonyl group on the complementary face of the orthosteric sites, which may contribute to the unusually high affinity of this ligand.

The paper is clearly written and logically organized, largely paralleling previous structural work on α4β2, α3β4, and α1β1γδ receptors. However, it provides a few new insights, including the detailed receptor-ligand interactions that stabilize tebanicline in the orthosteric site and interactions that stabilize nicotine, offering a possible explanation for nicotine's lower affinity for the α6β4 receptor compared to α4β2. Additionally, the analysis of subunit stoichiometry based on inter-subunit contact areas for candidate pentameric combinations of subunits provides a rationale for the observed stoichiometry.

Major Points-

1. References: Many of the cited references are review articles, and in some cases, the review does not address the referenced topic. For instance, reference 12, which is a general reference on molecular counterparts of pain, does not mention α6β4 receptors, the topic of the reference. Similarly, reference 23, which discusses the conserved coupling element across all nicotinic receptors among many other topics, would benefit from citing the original discovery. Additionally, some references are subject to interpretation rather than providing hard evidence. For example, reference 9 shows that knocking out BARP, which affects α6β4 receptor expression in vitro, reduces analgesic efficacy in vivo, but it does not confirm whether this effect is due to reduced α6β4 receptor expression. Reference 16, which is a purely structural paper, is cited to support the order of subunit assembly, but this reference does not provide any experimental evidence on the order of subunit assembly.

2. Tebanicline's Effect: The paper gives the impression that the analgesic effect of tebanicline is due to its action on α6β4 receptors. However, tebanicline is much more potent on α4β2 receptors than α6β4 receptors, suggesting that α4β2 receptors might be a more logical target for analgesia (https://jpet.aspetjournals.org/content/285/2/777.long).

3. Chlorine Atom Interaction: The structure shows an apparently novel interaction between the chlorine atom on tebanicline and a main chain carbonyl group on the complementary face of the orthosteric site. However, a similar interaction between

the chlorine atom of the high-affinity agonist epibatidine and an equivalent main chain carbonyl group was previously observed in the complex with an α7/AChBP chimera (https://www.nature.com/articles/nn.2908).

4. Fig. 9b Interpretation: In Fig. 9b, it is stated that R172 from the principal face of β4, which does not form a ligand binding site, inserts between a cluster of aromatic residues, destabilizing the hydrophobic cavity. However, R172 with its positive charge more likely acts as a pseudo-agonist, stabilizing the surrounding aromatic residues. This observation was originally made for the non-ligand binding site in the structure of the α4β2 receptor (reference 16) and should be credited.

Minor Points-

1. Ligand Identification: In Fig. 3, ABT-594 is labeled as the ligand bound in the orthosteric binding site, but the ligand is actually tebanicline. Most readers may not be familiar with ABT-594.

2. Residue loop location: On line 245, L142 is indicated as being in loop D, but L142 is in loop E.

3. Residue Numbering Consistency: Residue numbering sometimes starts with the first residue of the signal peptide as residue 1, rather than the first residue of the mature protein. For instance, in Fig. 2e, α6β4 and α3β4 are superimposed, but the residue numbering for α6β4 starts with the signal peptide, while that for α3β4 starts with the mature protein. This inconsistency is confusing. Please indicate the residue numbering convention used in the paper, and use the first residue of the mature protein as residue 1, as this will be more familiar to most readers.

4. Grammatical Errors: Numerous grammatical errors throughout the manuscript should be corrected.

Reviewer #2

(Remarks to the Author)
In this work, Su et al. present a structural study on the human nicotinic acetylcholine receptor α6β4. Broadly, nicotinic receptors are categorized into two main classes: muscle-type and neuronal-type, based on their subunit composition and physiological roles. The α6β4 receptor is a neuronal-type receptor. While research in this field has predominantly focused on the two major subtypes, α4β2 and α7, α6-containing receptors, recognized as a minor subtype, also play significant physiological roles. Although the functions of α6β4 subtypes are not yet fully understood, recent studies have revealed the presence of these receptors in several native tissues.

One challenge in studying this receptor subtype has been the difficulty of producing functional receptors recombinantly in mammalian cell lines. In this study, the authors successfully expressed and purified the α6β4 receptor by co-expressing three chaperone proteins—IRE1, SUL2B1, and BARP—ensuring proper receptor function. They solved two cryo-EM structures of the α6β4 receptor bound to nicotine and tabanicline, respectively. These structures provide valuable insights into the ligand-binding modes, the rules of heteropentamer assembly, and the ion permeability properties of this particular receptor subtype.

Together with previous structural studies on receptors α4β2, α3β4, and α7, this work deepens our understanding of the shared and distinct features within the nAChR family. However, the functional studies in this paper are relatively weak, leaving the critical questions regarding the α6-containing receptor unaddressed. I believe the impact of this study could be greatly enhanced by incorporating additional functional experiments and providing a more comprehensive comparison with other subtypes. This would help uncover the unique mechanisms of this specific receptor subtype, distinguishing it from other receptors in the family.

Below are specific comments for the authors to consider.
1. The authors report that co-expression of the α6β4 receptor with protein chaperones yielded a pentameric receptor with a 2α6:3β4 stoichiometry. Then the authors constructed theoretical pentamer models to explore the exclusion of other possible assembly stoichiometries, such as the 5β4 and 1α6:4β4 assemblies. However, the manuscript lacks a clear description of the methods used to build these models, leaving it unclear whether the comparisons made between these theoretical models are applicable to real receptor assembly or physiologically relevant. In reality, the formation of these aberrant stoichiometries would likely involve compensatory rearrangements to prevent clashes or gaps in the structure.

Additionally, the manuscript does not address why the most plausible stoichiometry, 3α6:2β4, was not considered in the analysis. Could the authors provide an explanation for this omission?

2. In line 120, the authors predict that E185 plays a critical role in the assembly of the α6β4 receptor, noting that this residue is not conserved across other nicotinic receptor subtypes. However, did the authors perform any mutagenesis experiments to validate this hypothesis? It would be interesting for the authors to further investigate, from a structural perspective, why the α6β4 receptor is difficult to produce in a functional form.

3. The authors hypothesize that the α6β4 receptor and the α7 receptor may have distinct coupling mechanisms due to the presence of V76 in the coupling region of the α6β4 receptor, whereas the corresponding residue in the α7 receptor is K45, which is known to be critical for receptor gating. To gain a comprehensive understanding of the gating mechanism in the α6β4 receptor, the authors should conduct functional experiments to validate this hypothesis.

4. Two important references are notably absent from the work and should be cited and discussed:

• Morales-Perez, C., Noviello, C., & Hibbs, R. (2016). X-ray structure of the human α4β2 nicotinic receptor. Nature, 538, 411–415. This paper presents the first structure of the human α4β2 receptor.

• Maldifassi, M. C. et al. (2023). Human α6β4 Nicotinic Acetylcholine Receptor: Heterologous Expression and Agonist Behavior Provide Insights into the Immediate Binding Site. Molecular Pharmacology, 103(6), 339-347. This paper provides a comprehensive biochemical study of the human α6β4 receptor.

Minors:
1. Line 208, “Bothe” I think it is a typo.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript by Su, et al. adequately addresses some of the issues raised in the initial review. However, several minor issues remain as follows.
1. Citing original rather than review articles was requested. Furthermore, some citations again focused on a topic different from that in the text.
a. References 1-3 are cited for the first biochemically isolated neurotransmitter receptor. While reference 1 is an appropriate review for this topic, references 2 and 3, although mentioning this, are on different topics. Please cite work directly addressing the text assertion.
b. Reference 5 is cited for the Cys-loop receptor superfamily, but this reference is on accessory proteins.
c. Reference 6 is cited for the many possible subunit combinations in neuronal nicotinic receptors, but this reference is again on accessory proteins.
d. Reference 7 is cited for the regional distribution of alpha6* receptors, but it is a review of the challenges in studying alpha6* receptors. A reference therein by Le Novere, Zoli, and Changeux (1996) contains the actual data on alpha6* regional distribution.
2. My original report questioned whether Tebanicline’s analgesic effect was due to action on α6β4 rather than α4β2 receptors, the latter of which bind the drug with higher affinity. The author’s response is puzzling in two ways. First, in their response they refer to Tebanicline as inhibiting the receptors, whereas the authors and others clearly show it activates them. Second, the observation that Tebanicline’s effect was greater in BARP knock-out animals, which show reduced α6β4 surface expression in some neurons, does not necessarily mean the effect is mediated solely through these receptors; a much more comprehensive assessment of nAChR subtype expression across many neuronal types would be required to draw this conclusion. In sum, although there is evidence that an action on α6β4 receptors may contribute to Tebanicline’s analgesic effect, it should be acknowledged that the drug may also act on α4β2 receptors, which show even higher affinity.
3. On lines 202-206 and 265, the contribution of L121 to ligand binding is demonstrated, but they do not reference the original work showing L121 and equivalent residues in complementary subunits contribute to ligand binding (https://pubmed.ncbi.nlm.nih.gov/9295287/).
4. On line 222 the phrase “was observed in the structures of a7 nicotinic receptors34 “ should read: “ was observed in the structure of a soluble a7AChBP chimeric ligand binding domain34”.
5. On line 262 of Discussion, where a conserved triad of electrostatic interactions is mentioned, the corresponding residues are unclear since several distinct electrostatic interactions are described. Please indicate which residues are being referred to.
6. There are several grammatical errors: lines 90, 92,120, 136, 140-141, 142, 150, 196, 259.

Reviewer #2

(Remarks to the Author)
The reviewer's rebuttal and revised manuscript addresses my comments. I recommend publication.

Point-by-point response for

Molecular insights into the α6β4 nicotinic acetylcholine receptor function and ligand recognition

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TO REVIEWER REPORT #1

The paper by Su et al. reports the high-resolution structure of the α6β4 nicotinic acetylcholine receptor bound to either nicotine or the pre-clinical agonist tebanicline determined by cryo-EM. This receptor is significant because it is thought to be present in sensory neurons in the dorsal root ganglia (DRG) and is associated with pain. Structural studies of this receptor have been limited until now due to the unknown chaperones required for its expression. However, these chaperones were discovered in recent years, allowing the authors to use them to express sufficient amounts of protein for purification and structural determination.

The obtained structure reveals standard features of a nicotinic receptor, including a defined subunit stoichiometry, details of the orthosteric binding pockets, interactions with the two agonists, unique inter-subunit stabilizing interactions proposed to assist in subunit assembly, and details of the ion-conductive pore in the desensitized conformation. Specifically, the structure reveals differences in receptor-ligand interactions between α6β4 and α4β2 that may account for the lower affinity of nicotine for α6β4 compared to α4β2. Additionally, the structure reveals an interaction between the chlorine atom on tebanicline and a main chain carbonyl group on the complementary face of the orthosteric sites, which may contribute to the unusually high affinity of this ligand.

The paper is clearly written and logically organized, largely paralleling previous structural work on α4β2, α3β4, and α1β1γδ receptors. However, it provides a few new insights, including the detailed receptor-ligand interactions that stabilize tebanicline in the orthosteric site and interactions that stabilize nicotine, offering a possible explanation for nicotine's lower affinity for the α6β4 receptor compared to α4β2. Additionally, the analysis of subunit stoichiometry based on inter-subunit contact areas for candidate pentameric combinations of subunits provides a rationale for the observed stoichiometry.

Reply: We appreciate the reviewer's positive comments on our manuscript and are grateful for his/her feedback, which has significantly improved our work.

Major Point 1

References: Many of the cited references are review articles, and in some cases, the review does not address the referenced topic. For instance, reference 12, which is a general reference on molecular counterparts of pain, does not mention α6β4 receptors, the topic of the reference. Similarly, reference 23, which discusses the conserved coupling element across all nicotinic receptors among many other topics, would benefit from citing the original discovery. Additionally, some references are subject to interpretation rather than providing hard evidence. For example, reference 9 shows that knocking out BARP, which affects α6β4 receptor expression in vitro, reduces analgesic efficacy in vivo, but it does not confirm whether this effect is due to reduced α6β4 receptor expression. Reference 16, which is a purely structural paper, is cited to support the order of subunit assembly, but this reference does not provide any experimental evidence on the order of subunit assembly.

Reply: We thank the reviewer for raising this important issue. We agree that we should cite the original discovery. Accordingly, we have cited the literature that provides solid evidence for our statements where the reviewer pointed out. Additionally, we have reviewed the entire manuscript to ensure that we do not solely cite review articles.

For the reference 16, although the authors suggested a sequence for subunit assembly during receptor assembly, they do not provide the biochemical evidence to support this claim. Furthermore, we reviewed the literature and found no evidence to corroborate this assertion. As a result, we have removed this description of subunit assembly in the revised version of our manuscript.

Major Point 2

Tebanicline's Effect: The paper gives the impression that the analgesic effect of tebanicline is due to its action on α6β4 receptors. However, tebanicline is much more potent on α4β2 receptors than α6β4 receptors, suggesting that α4β2 receptors might be a more logical target for analgesia (https://jpet.aspetjournals.org/content/285/2/777.long).

Reply: We appreciate this comment and for bringing this literature to our attention. Tebanicline has been demonstrated to exert an antinociceptive effect in rodent models of neuropathic pain following systemic administration[1-4]. According to the literature the reviewer mentioned[3], tebanicline is indeed more potent in inhibiting α4β2 receptors than α6β4 receptors. However, the analgesic effect of tebanicline was decreased in animals with reduced α6β4 surface expression[4]. These findings suggest that inhibiting α6β4 receptors significantly contributes to the analgesic effect of tebanicline.

Reference

[1] Decker, M. W. et al. Antinociceptive effects of the novel neuronal nicotinic acetylcholine receptor agonist, ABT-594, in mice. Eur J Pharmacol 346, 23-33, doi:10.1016/s0014-2999(98)00042-9 (1998).
[2] Bannon, A. W. et al. ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective antinociceptive agent acting via neuronal nicotinic acetylcholine receptors: II. In vivo characterization. J Pharmacol Exp Ther 285, 787-794 (1998).
[3] Donnelly-Roberts, D. L. et al. ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine]: a novel, orally effective analgesic acting via neuronal nicotinic acetylcholine receptors: I. In vitro characterization. J Pharmacol Exp Ther 285, 777-786 (1998).
[4] Knowland, D. et al. Functional α6β4 acetylcholine receptor expression enables pharmacological testing of nicotinic agonists with analgesic properties. J Clin Invest 130, 6158-6170, doi:10.1172/jci140311 (2020).

Major Point 3

Chlorine Atom Interaction: The structure shows an apparently novel interaction between the chlorine atom on tebanicline and a main chain carbonyl group on the complementary face of the orthosteric site. However, a similar interaction between the chlorine atom of the high-affinity agonist epibatidine and an equivalent main chain carbonyl group was previously observed in the complex with an α7/AChBP chimera (https://www.nature.com/articles/nn.2908).

Reply: We appreciate the reviewer's comments and for bringing this literature to our attention. Our structure

reveals a halogen bond between the chlorine atom on tebanicline and the carbonyl oxygen of N132 (now updated to N111 in the revised manuscript) in the β4 subunit, further confirmed by molecular dynamics simulations. Inspired by the reviewer's comments, we compared our structure with one determined in the literature and found that this bond is conserved[1]. Consequently, we have included this structural comparison as Figure 1* and added a brief discussion in the revised manuscript. Starting from lines 220-222, it reads: "Similar interactions between the chlorine atom and the carbonyl group on the complementary face of the orthosteric binding site was observed in the structures of α7/AChBP chimera receptors[34](Figure 3d).".

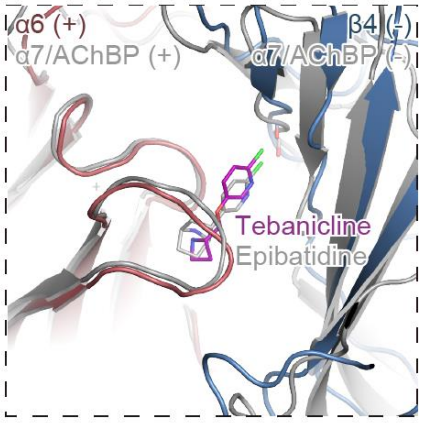

Figure 1*. The ligand binding pockets of α6β4 and α7/AChBP chimera structures (corresponding to Fig 3d). The structures of α6β4 and α7/AChBP are shown as cartoon. The of α6 and β4 subunits are colored in burgundy and blue, respectively, while the structure of α7/AChBP is colored in gray. Tebanicline and epibatidine molecules are shown as purple and gray sticks, respectively.

Reference

[1] Li, S. X. et al. Ligand-binding domain of an α7-nicotinic receptor chimera and its complex with agonist. Nat Neurosci 14, 1253-1259, doi:10.1038/nn.2908 (2011).

Major Point 4

Fig. 9b Interpretation: In Fig. 9b, it is stated that R172 from the principal face of β4, which does not form a ligand binding site, inserts between a cluster of aromatic residues, destabilizing the hydrophobic cavity. However, R172 with its positive charge more likely acts as a pseudo-agonist, stabilizing the surrounding aromatic residues. This observation was originally made for the non-ligand binding site in the structure of the α4β2 receptor (reference 16) and should be credited.

Reply: We appreciate the reviewer's comments and insights. Following reviewer's comment, we have checked structure of α4β2 receptor in its desensitized state and found that R149 (equivalent to R172 in α6β4, which has been updated to R151 in our revised manuscript) forms cation-π interactions with aromatic residues Y95 and Y196[1]. These interactions likely allow R149 to function as a pseudo-agonist, stabilizing the surrounding aromatic residues (Figure 2*c). However, in the non-ligand binding site of our α6β4 structure, the interaction between R172 (now updated to R151 in the revised manuscript) and these aromaric residues were absent, leading to a reorganization of cluster of aromatic residues (Figure 2*d, e). A similar phenomena was also presented in the non-ligand binding site of the α3β4 structures[2] (Figure 2*f). Therefore, we hypothesize that R172 (now updated

to R151 in the revised manuscript) and its equivalent residues play a non-equivalent role in receptors with distinct subunit compositions.

In our revised manuscript, we have incorporated related structural analysis and included a brief discussion in the revised manuscript. In lines 188-194, it reads “In the α4β2 structure, a previous study mentioned that R149 (equivalent to R151 in α6β4) forms cation-π interactions with surrounding aromatic residues (Supplementary Fig. 9c), functioning as a pseudo-agonist[26]. However, in the non-ligand binding site of α6β4 structure, the interactions between R151 and these aromatic residues were absent (Supplementary Fig. 9d, e). Similar phenomena were also observed in the α3β4 structure[27](Supplementary Fig. 9f). Therefore, we hypothesize that R151 and its equivalent residues play a non-equivalent role in receptors with distinct subunit compositions.”.

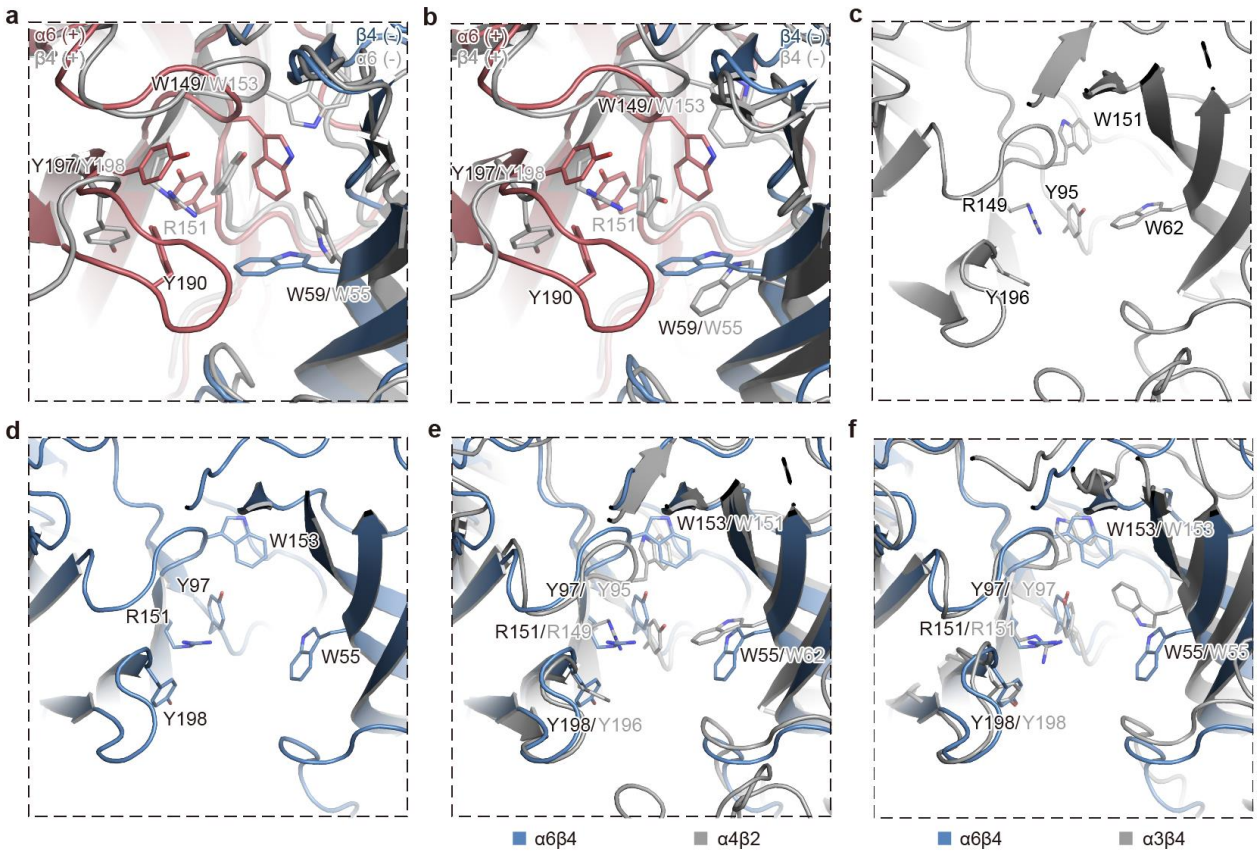

Figure 2*. The non-ligand binding sites between different nicotinic receptors (corresponding to Supplementary Fig 9).

a. Comparison of nicotine binding pockets in α6-β4 and β4-α6 interfaces. The key residues are shown as sticks. **b.** Comparison of nicotine binding pockets in α6-β4 and β4-β4 interfaces. The key residues are shown as sticks. The nicotine is shown as sticks and spheres. **c.** The non-ligand binding site of α4β2 receptor. The key residues for interaction are shown as sticks. **d.** The non-ligand binding site of α6β4 receptor. The key residues are shown as sticks. **e.** Comparison of non-ligand binding site of α6β4 and α4β2. The key residues are shown as sticks. The α6β4 and α4β2 structures are colored in blue and gray, respectively. **f.** Comparison of non-ligand binding site of α6β4 and α3β4. The key residues are shown as sticks. The α6β4 and α3β4 structures are colored in blue and gray, respectively.

Reference

[1] Morales-Perez, C. L., Noviello, C. M. & Hibbs, R. E. X-ray structure of the human α4β2 nicotinic receptor.

Nature **538**, 411-415, doi:10.1038/nature19785 (2016).
[2] Gharpure, A. *et al.* Agonist Selectivity and Ion Permeation in the α3β4 Ganglionic Nicotinic Receptor. *Neuron* **104**, 501-511.e506, doi:10.1016/j.neuron.2019.07.030 (2019).

Minor Point 1

Ligand Identification: In Fig. 3, ABT-594 is labeled as the ligand bound in the orthosteric binding site, but the ligand is actually tebanicline. Most readers may not be familiar with ABT-594.

Reply: We appreciate the reviewer for pointing out this typo. We have replaced ABT-594 with tebanicline in Figure 3 and have thoroughly reviewed the entire manuscript to ensure that no similar issues remain.

Minor Point 2

Residue loop location: On line 245, L142 is indicated as being in loop D, but L142 is in loop E.

Reply: Thank you for pointing out this typo. We have carefully checked the position of residue L142 (now updated to L121 in the revised manuscript) and found that it is located in loop E. We have corrected this in the revised manuscript.

Minor Point 3

Residue Numbering Consistency: Residue numbering sometimes starts with the first residue of the signal peptide as residue 1, rather than the first residue of the mature protein. For instance, in Fig. 2e, α6β4 and α3β4 are superimposed, but the residue numbering for α6β4 starts with the signal peptide, while that for α3β4 starts with the mature protein. This inconsistency is confusing. Please indicate the residue numbering convention used in the paper, and use the first residue of the mature protein as residue 1, as this will be more familiar to most readers.

Reply: Thank you for pointing out this issue. We have thoroughly examined the text and figures to ensure that the first residue of the mature protein is designated as residue 1.

Minor Point 4

Grammatical Errors: Numerous grammatical errors throughout the manuscript should be corrected.

Reply: Thank you for raising this issue. We have thoroughly reviewed the entire document and made extensive corrections through a language polishing service.

TO REVIEWER REPORT #2

In this work, Su et al. present a structural study on the human nicotinic acetylcholine receptor α6β4. Broadly, nicotinic receptors are categorized into two main classes: muscle-type and neuronal-type, based on their subunit composition and physiological roles. The α6β4 receptor is a neuronal-type receptor. While research in this field

has predominantly focused on the two major subtypes, α4β2 and α7, α6-containing receptors, recognized as a minor subtype, also play significant physiological roles. Although the functions of α6β4 subtypes are not yet fully understood, recent studies have revealed the presence of these receptors in several native tissues.

One challenge in studying this receptor subtype has been the difficulty of producing functional receptors recombinantly in mammalian cell lines. In this study, the authors successfully expressed and purified the α6β4 receptor by co-expressing three chaperone proteins—IRE1, SUL2B1, and BARP—ensuring proper receptor function. They solved two cryo-EM structures of the α6β4 receptor bound to nicotine and tabanicline, respectively. These structures provide valuable insights into the ligand-binding modes, the rules of heteropentamer assembly, and the ion permeability properties of this particular receptor subtype.

Together with previous structural studies on receptors α4β2, α3β4, and α7, this work deepens our understanding of the shared and distinct features within the nAChR family. However, the functional studies in this paper are relatively weak, leaving the critical questions regarding the α6-containing receptor unaddressed. I believe the impact of this study could be greatly enhanced by incorporating additional functional experiments and providing a more comprehensive comparison with other subtypes. This would help uncover the unique mechanisms of this specific receptor subtype, distinguishing it from other receptors in the family.

Reply: We appreciate the reviewer's positive comments on our manuscript and thank you for the time and effort invested in reviewing to improve our work.

Major Point 1

The authors report that co-expression of the α6β4 receptor with protein chaperones yielded a pentameric receptor with a 2α6:3β4 stoichiometry. Then the authors constructed theoretical pentamer models to explore the exclusion of other possible assembly stoichiometries, such as the 5β4 and 1α6:4β4 assemblies. However, the manuscript lacks a clear description of the methods used to build these models, leaving it unclear whether the comparisons made between these theoretical models are applicable to real receptor assembly or physiologically relevant. In reality, the formation of these aberrant stoichiometries would likely involve compensatory rearrangements to prevent clashes or gaps in the structure.

Additionally, the manuscript does not address why the most plausible stoichiometry, 3α6:2β4, was not considered in the analysis. Could the authors provide an explanation for this omission?

Reply: We appreciate the reviewer's comment and fully agree that providing a clear description of the methods used to build these models is essential. To clarify, based on the α6β4 receptor structure obtained in our work, we generate theoretical pentameric models, including the 5β4 and 1α6:4β4 assemblies. To build the 1α6:4β4 receptor model, the α6 (4) subunit and β4 (5) subunit are removed from our experimental α6β4 structure, leaving one α6 subunit (α6 (1)) and two β4 subunits (β4 (2) and β4 (3)) as the initial model. Subsequently, the β4 subunits were added sequentially using a pairwise alignment approach. Specifically, during the addition of the fourth β4 subunit, the β4(3) subunit from the initial model was used as a structural reference and aligned with the β4(2) subunit of the incoming two-subunit (Figure 3*). The fifth β4 subunit was then added in a similar manner, resulting in a model consisting of four β4 subunits and one α6 subunit. The 5β4 model was constructed using a similar stepwise alignment process. After building these models, energy minimization was performed to optimize their structures (Figure 3*). These results support our hypothesis that the 2α6:3β4 form is more stable than the 5β4 and 1α6:4β4 forms under our experimental conditions. A similar modeling approach has been

successfully applied in the analysis of α4β2 receptors[1].

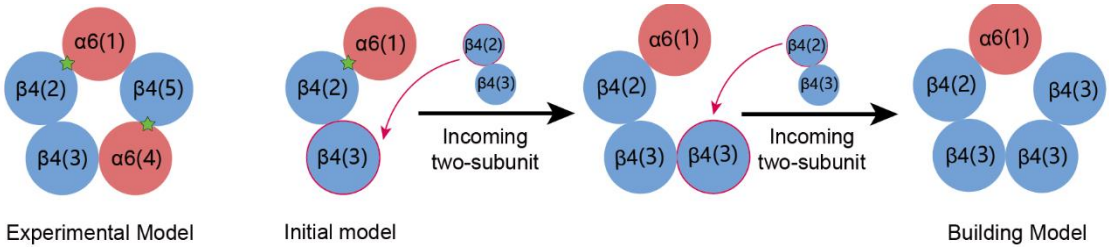

Figure 3*. The flowchart of generating theoretical pentameric models (corresponding to Supplementary Fig 8a). To build the 1α6:4β4 receptor model, the α6 (4) subunit and β4 (5) subunit are removed from our experimental α6β4 structure, leaving one α6 subunit (α6 (1)) and two β4 subunits (β4 (2) and β4 (3)) as the initial model. Subsequently, the β4 subunits were added sequentially using a pairwise alignment approach. Specifically, during the addition of the fourth β4 subunit, the β4(3) subunit from the initial model was used as a structural reference and aligned with the β4(2) subunit of the incoming two-subunit. The fifth β4 subunit was then added in a similar manner, resulting in a model consisting of four β4 subunits and one α6 subunit. The 5β4 model was constructed using a similar stepwise alignment process. After building these models, energy minimization was performed to optimize their structures.

In our revised manuscript, we have incorporated the method of building these models. In lines 149-153, it reads "To explain why other theoretically possible subunit stoichiometries were not presented, generate theoretical pentameric models, including the 5β4 and 1α6:4β4 assemblies through aligning the subunits pairwise based on the α6β4 receptor structure obtained in our experiment. These models were then optimized using energy minimization (Supplementary Fig. 8a)." Additionally, the specific step-by-step method for building these models is provided in the legend of Supplementary Fig. 8a.

Regarding the omission of the 3α6:2β4 stoichiometry, while we could theoretically generate this model using a similar method, we lack experimental data for the α6-α6 subunit interface. Consequently, we are unable to compare the α6-α6 interface in the generated model with experimental results, and thus cannot draw accurate conclusions. For this reason, the 3α6:2β4 model is not discussed in the manuscript.

Reference

[1] Walsh, R. M., Jr. *et al.* Structural principles of distinct assemblies of the human α4β2 nicotinic receptor. *Nature* **557**, 261-265, doi:10.1038/s41586-018-0081-7 (2018).

Major Point 2

In line 120, the authors predict that E185 plays a critical role in the assembly of the α6β4 receptor, noting that this residue is not conserved across other nicotinic receptor subtypes. However, did the authors perform any mutagenesis experiments to validate this hypothesis? It would be interesting for the authors to further investigate, from a structural perspective, why the α6β4 receptor is difficult to produce in a functional form.

Reply: We thank the reviewer for this comment and fully agree that more functional characterization is necessary. Following the reviewer's suggestion, we replaced E185 (now updated to E155 in the revised manuscript) with either a smaller side chain (E155A) or larger side chains (E155W and E155F), disrupting the electrostatic interactions involving residue E155 or creating steric hindrance at this position. Two-electrode

voltage clamp experiments revealed that these three mutations significantly reduced current amplitudes (Figure 4*), despite the same amount of RNA being injected for each mutant into oocytes. This finding supports the important role of residue E155 in the α6β4 receptor assembly.

In our revised manuscript, we have included these data in Supplementary Figure 6 and added a brief discussion. In lines 120-124, it reads, “To investigated the functional role in α6β4 receptor, we evaluated the peak currents for WT α6β4 receptor and some mutants, including E155A, E155W, and E155F. Two-electrode voltage clamp experiments revealed that these three mutations significantly reduced current amplitudes (Supplementary Fig. 6e).”.

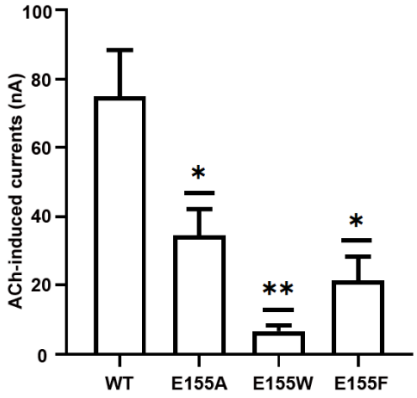

Figure 4*. ACh-induced currents of wild-type α6β4 receptor and its mutants in two-electrode voltage clamp experiment (corresponding to Supplementary Fig 6e). Data represent the mean ± S.E.M. from three separate experiments. The ACh-induced currents of E155A, E155W and E155F are significantly lower than that of wild-type α6β4 receptor. $P < 0.05$ for all mutants, unpaired t-test.

Major Point 3

The authors hypothesize that the α6β4 receptor and the α7 receptor may have distinct coupling mechanisms due to the presence of V76 in the coupling region of the α6β4 receptor, whereas the corresponding residue in the α7 receptor is K45, which is known to be critical for receptor gating. To gain a comprehensive understanding of the gating mechanism in the α6β4 receptor, the authors should conduct functional experiments to validate this hypothesis.

Reply: We thank the reviewer for this comment and agree with reviewer it would be wonderful to include functional date to support our hypothesis. The residue V76 (now updated to V46 in the revised manuscript) is not conserved among nAChRs, and the equivalent residue of V46 in most other nAChR subunits is lysine. Therefore, we engineered a V46K mutation and studied the effect of this mutation on agonist-induced desensitization of α6β4 receptor using two-electrode voltage clamp experiments.

Notably, two-electrode voltage clamp experiments revealed that the rate of desensitization of V76K mutation was significantly faster than that of WT receptor (Figure 5*). Structural comparisons of desensitized α6β4 and α7 receptors showed that in the α7 receptor, K45-equivalent residue of V46 in α6, forms an inter-subunit electrostatic interaction with E172 in the desensitization state, potentially stabilizing the receptor in this state more rapidly. In contrast, this interaction is absent in α6β4 receptor structure, which may explain its slower

desensitization rate compared to α7.

In our revised manuscript, we have included these data in Supplementary Figure 7 and add a brief discussion. In lines 136-144, it reads “To investigated the functional role of V46 in α6β4 receptor, we generated a V46K$^{\alpha 6}$ mutant. Two-electrode voltage clamp experiments revealed that the rate of desensitization of V46K mutation was significantly faster than that of WT receptor (Supplementary Fig. 7d). Structural comparisons between the desensitization states of α6β4 and α7 receptors provided additional insights. In the α7 receptor, K45-equivalent residue of V46 in α6, forms an inter-subunit electrostatic interaction with E172 in the desensitization state[25] (Supplementary Fig. 7c), potentially stabilizing the receptor in this state more rapidly. In contrast, this interaction is absent in α6β4 receptor structure, which may explain its slower desensitization rate compared to α7.”. Additionally, in the method study, we have added a method for desensitization study. In lines 401-405, it reads “To characterize desensitization, currents were evoked by 1 mM ACh at 1 min intervals with ND96 washout, and the time constant was measured by fitting the current decays with two-phase exponential association equation using Origin 9.0 software (OriginLab Corporation, Northampton, MA). All values were normalized to those produced by ACh for each individual cell.”

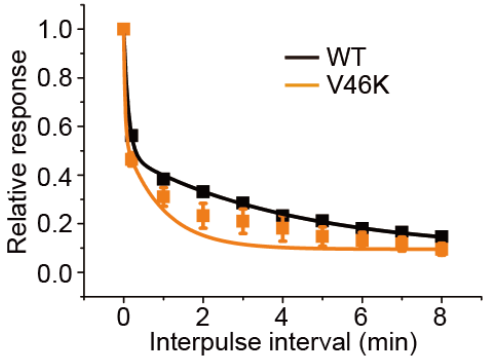

Figure 5*. The desensitization rates of wild-type α6β4 receptor and its mutant in two-electrode voltage clamp experiment (corresponding to Supplementary Fig 7d). Time course of ACh response for wild-type α6β4 receptor and its mutant. Data represent the mean ± S.E.M. from three separate experiments. The black and orange curves represent α6β4^{WT} receptor and its mutant (α6β4^{V46K}), respectively. α6β4^{WT}: Tau values (fast) = 2.92, Tau values (slow) = 0.08; α6β4^{V46K}: Tau values (fast) = 0.67, Tau values (slow) = 0.02.

Major Point 4

Two important references are notably absent from the work and should be cited and discussed:

• Morales-Perez, C., Noviello, C., & Hibbs, R. (2016). X-ray structure of the human α4β2 nicotinic receptor. Nature, 538, 411–415. This paper presents the first structure of the human α4β2 receptor.

• Maldifassi, M. C. et al. (2023). Human α6β4 Nicotinic Acetylcholine Receptor: Heterologous Expression and Agonist Behavior Provide Insights into the Immediate Binding Site. Molecular Pharmacology, 103(6), 339-347. This paper provides a comprehensive biochemical study of the human α6β4 receptor.

Reply: We greatly appreciate the reviewer for bringing these two important references to our attention. We agree that both papers are highly relevant to our study and will significantly enhance the context of our work.

In our revised manuscript, we have included the following citations in the revised manuscript and discuss them.

In lines 144-147, it reads “Additionally, two intra-subunit electrostatic interactions occur in α6 between E45, D138 and R208 (Supplementary Fig. 7e), with this triad of electrostatic interactions serving as a conserved coupling element among most nAChRs[25-29]”.

In lines 176-177, it reads “Previous research also demonstrated that the W149 is essential for ligand binding[30].”

In lines 188-190, it reads “In the α4β2 structure, a previous study mentioned that R149 (equivalent to R151 in α6β4) forms cation-π interactions with surrounding aromatic residues (Supplementary Fig. 9c), functioning as a pseudo-agonist[26]”.

In lines 195-197, it reads “Nicotine, the agonist, activates various nAChRs, such as α6β4, α4β2 and α3β4. However, its affinity for α6β4 is similar to α3β4, while is 20-100 times lower than α4β2. Therefore, we compared the nicotine-bound structure of α6β4 with those of α4β2 and α3β4, respectively[26,27]”.

Minor Point

Line 208, “Bothe” I think it is a typo.

Reply: We appreciate your pointing out this typo. It should be 'both,' and we have corrected it in the revised manuscript.

Point-by-point response for

Molecular insights into the α6β4 nicotinic acetylcholine receptor function and ligand recognition

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TO REVIEWER REPORT #1

Minor Point 1

Citing original rather than review articles was requested. Furthermore, some citations again focused on a topic different from that in the text.
a. References 1-3 are cited for the first biochemically isolated neurotransmitter receptor. While reference 1 is an appropriate review for this topic, references 2 and 3, although mentioning this, are on different topics. Please cite work directly addressing the text assertion.
b. Reference 5 is cited for the Cys-loop receptor superfamily, but this reference is on accessory proteins.
c. Reference 6 is cited for the many possible subunit combinations in neuronal nicotinic receptors, but this reference is again on accessory proteins.
d. Reference 7 is cited for the regional distribution of alpha6 receptors, but it is a review of the challenges in studying alpha6* receptors. A reference therein by Le Novere, Zoli, and Changeux (1996) contains the actual data on alpha6* regional distribution.*

Reply: We appreciate the reviewer's careful evaluation of our citations and the recommendation to cite original research articles rather than review papers. To address these concerns, we have updated our references accordingly, as reflected in the revised manuscript.

Minor Point 2

My original report questioned whether Tebanicline's analgesic effect was due to action on α6β4 rather than α4β2 receptors, the latter of which bind the drug with higher affinity. The author's response is puzzling in two ways. First, in their response they refer to Tebanicline as inhibiting the receptors, whereas the authors and others clearly show it activates them. Second, the observation that Tebanicline's effect was greater in BARP knock-out animals, which show reduced α6β4 surface expression in some neurons, does not necessarily mean the effect is mediated solely through these receptors; a much more comprehensive assessment of nAChR subtype expression across many neuronal types would be required to draw this conclusion. In sum, although there is evidence that an action on α6β4 receptors may contribute to Tebanicline's analgesic effect, it should be acknowledged that the drug may also act on α4β2 receptors, which show even higher affinity.

Reply: We thank the reviewer for highlighting these important issues. Tenanicline activates the α6β4 receptor and serves as its agonist. The reference to inhibition in our previous response was a typographical error. We agree with the reviewer's viewpoint that tebanicline also act on α4β2 receptors, which exhibit higher affinity. Our perspective is that tebanicline could alleviate pain by activating the α6β4 receptor, as evidenced by the reduced analgesic effect observed in animal models with decreased α6β4 surface expression[1].

Reference

1 Knowland, D. *et al.* Functional α6β4 acetylcholine receptor expression enables pharmacological testing

of nicotinic agonists with analgesic properties. *J Clin Invest* **130**, 6158-6170, doi:10.1172/jci140311 (2020).

Minor Point 3

On lines 202-206 and 265, the contribution of L121 to ligand binding is demonstrated, but they do not reference the original work showing L121 and equivalent residues in complementary subunits contribute to ligand binding (https://pubmed.ncbi.nlm.nih.gov/9295287/).

Reply: We thank the reviewer for raising this important issue. We agree that we should cite the original work is necessary and we cited this paper in our revised manuscript. In lines 205-206, it reads "A similar difference was also observed between α4β2 and α3β4 structures[2]. In line 267-268, it reads "the residue L121 in loop E is also essential for ligand binding, similar to its function in α4β2 structure[2]."

Reference

2 Gharpure, A. *et al.* Agonist Selectivity and Ion Permeation in the α3β4 Ganglionic Nicotinic Receptor. *Neuron* **104**, 501-511.e506, doi:10.1016/j.neuron.2019.07.030 (2019).

Minor Point 4

On line 222 the phrase "was observed in the structures of a7 nicotinic receptors34 "should read: " was observed in the structure of a soluble a7AChBP chimeric ligand binding domain34".

Reply: Thank you for pointing out this issue. We have revised it in our revised manuscript. In lines 222-224, it reads "Similar interactions between the chlorine atom and the carbonyl group on the complementary face of the orthosteric binding site was observed in the structures of a soluble α7-AChBP nicotinic receptors[3] (Figure 3d)."

Reference

3 Li, S. X. *et al.* Ligand-binding domain of an α7-nicotinic receptor chimera and its complex with agonist. *Nat Neurosci* **14**, 1253-1259, doi:10.1038/nn.2908 (2011).

Minor Point 5

On line 262 of Discussion, where a conserved triad of electrostatic interactions is mentioned, the corresponding residues are unclear since several distinct electrostatic interactions are described. Please indicate which residues are being referred to.

Reply: Thank you for your valuable suggestion. We have revised the manuscript to specify the exact residues involved in the conserved trial of electrostatic interactions. In lines 264-265, it reads "We hypothesize that the conserved triad of electrostatic interactions, including $E155^{\alpha6}$-$R83^{\beta4}$ and $D152^{\alpha6}$-$R83^{\beta4}$, is pivotal in the subunit assembly of α6β4."

Minor Point 6

There are several grammatical errors: lines 90, 92,120, 136, 140-141, 142, 150, 196, 259.

Reply: Thank you for pointing out the grammatical errors. We have carefully reviewed the indicated lines and corrected the grammar and wording accordingly to improve clarity and readability. Please find the revised text in the updated manuscript. We appreciate your thorough review.