nature portfolio

Peer Review File

Molecular basis of human taurine transporter uptake and inhibition

Corresponding Author: Dr Kaige Yan

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript describes eleven cryo-EM structures of human taurine transporter (TauT), which has mutations in intracellular loops to form a Fab complex with or without four substrates/six inhibitors, at a resolution of 2.9–3.4 Å. The structures with Tau and its analogues have been verified through binding and uptake experiments using TauT mutants. The comparison with GAT-1 illustrates TauT-specific interactions in substrate-amino residue and Na+ recognition. These findings are important for understanding the molecular function and transport mechanism of TauT. However, the current manuscript lacks a thorough introduction of the research background, making it unclear how these findings relate to drug discovery as claimed by the authors. Furthermore, it is necessary to clearly demonstrate the significance of elucidating the inhibitory mechanism of several molecules that interact with TauT more weakly compared to the best substrate taurine (Tau).

1. The relevance of this research to drug discovery for neurological diseases is unclear. In the Abstract, line 25. "It is a pharmacological target for various neuropsychiatric diseases" is not clearly described in the Introduction. On the other hand, the Discussion seems to focus solely on the various cancer diseases (line 448). The authors should re-write the introduction.
a) Lines 104-105: There is a question whether the problem of "no TauT-targeting drugs have been investigated in clinical trials" can be resolved by determining the structure of TauT. Please clearly demonstrate the usefulness of TauT inhibitors in treating neurodegenerative diseases.
b) Lines 83-103: Although the sentences describe the utilities and characteristics of various TauT inhibitors, most citated studies examined only the inhibitory effects on TauT transport or showed negative pharmacological effects (Ref-37 shows that inhibitors decrease in brain taurine levels). Please cite the literature that examined therapeutic or beneficial pharmacological effects.
c)Furthermore, several references in this section are not properly cited, making it impossible to determine their validity. Please review the entire text and cite appropriately. This reviewer will state only those noticed below.

There is an error in the citation of Ref-34, which describes "Effect of nipecotic acid on GABA release from locust synaptosomes" not the vertebrate nervous system as described in line 92.

Ref-35: If the sentence "I4AA has shown potential in interacting with TauT in BRB cells, playing a crucial role in the prevention of retinal diseases" means that I4AA plays an important role in preventing retinal diseases, this explanation may be inappropriate as I4AA has no such pharmacological effect.

Ref-36: "The longer carbon chain length of 5AVA may lead to a unique bent configuration, enhancing its interaction with TauT" should be confirmed. This paper shows that IC50 values for beta-alanine, GABA, and 5AVA were 55.5, 1014, and 1420 microM, respectively, indicating that the longer the carbon chain, the weaker the interaction with TauT.

"TauT-targeting compounds" in lines 84-85 include 5-aminovaleric acid and homotaurine. Is this correct? Do these exert their pharmacological effects by targeting TauT? Ref-33 shows that homotaurine inhibited TauT function with IC50 value of 3.4 mM, indicating a very low affinity.

2) Please consider the relationship between the N-terminus segment (residues 38-46) and transport function. Although lines 327-331 indicate the interactions between Arg41 and Asp416 in TM8/Tyr315 in TM6, and between Trp44 and Phe50 in TM1a/Tyr315 in TM6, these interactions were not inspected. Do these mutations affect transport activity?

3) Please clarify that GES is a TauT inhibitor, not the substrate, to consider the relationship between inhibitor properties and conformation status. The authors argue that GES is the only inhibitor that showed an occluded conformation among the inhibitors tested, while other inhibitors show an inward-open conformation. This study also shows that all tested substrates stabilize TauT in an intermediate conformation or an occluded conformation (high affinity ligands such as Tau and beta-Ala seem to show an occluded conformation), suggesting GES as a substrate of TauT.

Minors:
Line 171: "Extended Data Figs. 2l and 3" should be Extended Data Figs.2 and 3.
Line 710: Concentrations of compounds used in Extended Data Fig.1 are missing.
Line 838: The order of TauT(apo), TauT(Tau), and TauT(GABA) should be same as those in Fig.3a.

Reviewer #2

(Remarks to the Author)
The manuscript by Du, Cheng, Xie and colleagues is a very dense and comprehensive manuscript covering a lot of different aspect related not only to the taurine transporter but also to the closer members of the immediate protein family. The manuscript is well written (sometimes maybe a “the” ect. Is missing; the text should undergo thorough proof reading.).
I can only congratulate the authors on a job nicely done. There are only minor things that I would want to have mended.

The start of the results section should be indicated – by a sub-heading or at least by a page break.

Line 243: “These findings provide helpful information to increase our understanding of the substrate specificity of GABA subfamily.”
This sentence is not really written well: What shall GABA subfamily mean? This needs to be specified. Also, the sentence is kind of a concluding statement for a paragraph or section of the paper. However, there is another concluding paragraph following thereafter (lines 246-249). The authors should decide which one to keep and also, they should add another sentence which leads the reader to the next section. Otherwise, it is difficult to follow.

Line 346: “dose-dependently“ – the authors mean “concentration-dependently”.

The only thing that “bothers” me a bit is the distinction between substrates, substrate analogues and inhibitors – how can the authors distinguish among those? Sometimes, it is not really easy nor obviously overt which compound belongs to what category.
Maybe the authors want to comment on this – and also include a sentence to clarify this to the reader of the article.

Reviewer #3

(Remarks to the Author)
The manuscript “Molecular basis of human taurine transporter uptake and inhibition” presents and describes 11 cryo-EM structures of the human taurine transporter (TauT, SLC6A6). The structures are presented in the apo form, bound to taurine, b-alanine, guanidinoacetate, γ-aminobutyric acid (GABA), nipecotic acid, imidazole-4-acetic acid, piperidine-4-sulfonic acid, guanidinoethyl sulfonate, 5-aminovaleric acid and homotaurine. The cryo-EM structures are supported with functional experiements to determine the binding site of these ligands. The maps are of good quality but the work presented requires revision to improve clarity.
Some of the models and maps require correction, reprocessing, etc (see below).

A general comment for future journal submissions, I strongly recommend to match the name of the map with the model as the random job numbers on the map files are not helpful for locating the corresponding coordinate files.

Few major problems regarding the models and maps:

1- P4S-bound model:
If the corresponding map is “cryosparc_P41_J5913_006_volume_map_sharp.mrc”, the density for the P4S is ambigous and does not follow the shape or pose of the compound. Extra steps of processing, masking and local refinement, cleaning the particle stack might be helpful to improve the density. But at this stage, the map does not support modeling P4S.
(Is it a lose binder? Is it partial occupancy?)
Further, the density presented in Figure 1 is not reflected in the cryo-EM map. The sigma level perhaps is not selected at the level the noise is not present. Please clarify this part.

2- I4AA-bound model:
Assuming the corresponding map is: cryosparc_P41_J6512_006_volume_map_sharp.mrc
The density quality around residues 47 – 58 is rather poor, consider removing the region.
The density for the ligand is “very poor, it does not allow unambigous modeling of the compound” and tehrefore any discussions based on this model and P4S-bound model should be revisited once the density is imporved (I suggest more extensive processing, as suggested above) after ensuring the correct pose of the compound is modeled in the density.

3- 5AVA mode, shouldn't the N04 be positioned differently? There is apparently a water molecule (?) density hydrogen bonding with the nitrogen atom, please check that.

4- Model refinement statistics: clash scores are high. Please improve the model to fit the accepted standards in the field.
Please improve the models to not have any ramachandran outliers (disallowed % should be zero)
Please include CaBLAM values (should be below 1% to be acceptable).

Please note that the updated coordinates should be re-deposited into PDB and the updated validation reports should be presented.

Below please find some more comments that will hopefully help the authors to improve their manuscript.

Noteable issues:

On maps and models:
The Fab has not been modelled into any of the structures presented despite a good density for them. The authors should model the Fab into each of the structures presented.
The authors have modelled in N-acetyl glucosamine into the taurine, b-ala and apo structures, however the density is not rich in this area. The authors should consider removing them from the models.
The density for residues 180-188 in the TauTapo, TauTTau, TauTb-ala, TauTGAA, TauTGABA as well as inhibitor-bound states does not seem strong enough to model.
Similarly in all models and maps, if at the sigma level that the noise from the detergent micelle is removed, the density for certain regoins of the model does not support modeling, please remove the residues.
Modeling should be done a bit more carefully, another example is residues 418–439 in NPE-bound model, where information on a some of the residues is missing. In this case I suggest not to remove the residues, rather set the occupancy of the flexible side chains to zero.

TauTb -ala model:
Density for Asp401 side chain is lacking. Additionally, the density for sodium in the Na2 is not strong and is currently modelled in only 1.3 Å away from Asp401. Could the authors comment on this?

TauTGAA model:
The density for residues 47 to 56 does not seem rich enough to model these residues in.

GES-bound model:
There probably is a water-mediated interaction with N135. Please investigate the the local density.

In the legend of any figure that shows density, sigma level and the program that estimates it, should be reported.

The shortening of taurine to Tau should be avoided if possible to avoid confusion with tau proteins. However, the shortening of taurine transporter to TauT is common in literature and appropriate.

Lines 46-48: It is not clear what the authors are trying to communicate with the last line '…albeit with relatively lower affinity in the nervous system.' Is this to say that the affinity for taurine is dependent on where the receptor is expressed?

Lines 104 – 105: Despite the promise of these compounds, no TauT-targeting drugs have been investigated in clinical trials. – Please elaborate at what stage of trials and "why" the compounds failed.

Lines 158 – 192 Architecture of TauT – the language is too general and not clear which part corresponds to which model, e.g., lines 187 – 188 refers to modeling residues 38 – 47 of the N terminus, however, this section is not modeled in all the maps (missing in NPE, P4S, etc). Please be more specific.

Lines 205-207: The author's speculate that 'Phe300 may serve as a key structural element for Tau recognition', by comparing the Tau-binding site in hTauT and the GABA binding site in hGAT1. Phe300 (hTauT numbering) is conserved in these two transporters and appear to occupy the same space so it is not clear how the authors came to this conclusion. In Zhu et al. (2023), the amino group of GABA interact with the hydroxyl Tyr60, which is not coserved in hTauT. The authors should clarify this point.

Lines 233 – 234, Interesting choice to mutate Glu and Leu to Cys. Are there previous studies suggesting such replacement? Please elaborate on the choice. Please also elaborate on the effects of mutations in increasing the activity of the transporter.

Lines 718 -721: In the methods it states that 'For the inhibition assay, the cells were incubated in PBS containing 0.5 μCi/mL [3H]taurine and different concentrations of P4S (MedChemExpress), I4AA (MedChemExpress), GES (MedChemExpress), 5AVA (MedChemExpress), nipecotic acid (MedChemExpress) or Htau (MedChemExpress)'. Please state the concentrations used and the rational behind the specific concentrations.

Lines 199-200: The wording makes the description of the interactions ambiguous as to whether it applies to the substrate or Na+ ion in Na1. Reword for clarification.
Lines 240-243: This sentence requires rewording to make it clear what the authors are trying to communicate. Is it that the

mutations showed a significant decrease in transport activity? Further explanation as to why there is a decrease in transport activity for taurine, GABA and GAA were observed considering the mutants were based on residues in GABA transporter proteins would be beneficial.
Line 246: 'exhibited varying impacts' is a vague sentence. It would be helpful to be more detailed.
Lines 267-273: In describing the interactions, the authors should be specific as to whether the interaction is with the backbone or the amino acid residue side chain. This would also then be beneficial for understanding the results of the mutagenesis.
Lines 297-299: The sentence 'The TauTTau and TauTGABA….. (Extended Fig. 7).' is unclear. It should be reworded to make it unambiguous as to which structures were superimposed and the corresponding RMSD values.
Line 305: The authors state Gly56 undergoes noteable conformational changes. It is not clear what is meant by this considering there is no side chain. Is it that the residue is moved?

Figure 1. The densities presented in panel c are difficult to see. It would be beneficial to make them more obvious by making them darker or less transparent.

Figure 2. It would be beneficial to label the distances of the interactions shown in each panel. Change the figure title from '..analogues binding of TauT' to '…analogues binding to TauT'.
Line 825: Change 'of the Taurine' to 'of taurine'

Figure 4. Consider using a lighter shade of brown for the 2D structure of the compound in panel D. It is challenging to see the black font.

Figure 5. It is not clear from the schmetic or the legened as to what the red lines from the different ligand mean? Is it the step it blocks?

Extended figure 1: In panel b) it would be clearer to replace the label 'WT', 'EM' and 'GFP' with taurine to make it consistent with the other labels. You have already indicated which data refers to TauTWT and TauTEM. In the legend also indicate that the GFP expressing control is shown in green. Be more precise with the line 'Data were normalized to TauTWT' by stating 'Data were normalized to taurine uptake by TauTWT'
In the legend of c) indicate the number of replicates.
In panel d) it is not clear which part of the chromatogram the fractions correspond to. Indicate on the chromatogram the fractions collected.

Extended data figure 2. The paneles are too small to recognise any features, an option could be to make it into e.g., two figures at least, with substarte-bound states and inhibitor-bound states. Also not clear why only TM1, TM3, TM6 and TM8 are shown out of the 12 TMs.

Extended data figure 4. The figure legend does not make it clear which panel is for substrate site mutations and which are for ion binding site mutations. There is a lack of detail for these experiments. Were these conducted at a single dose and compared to WT? How was it determined that the mutants expressed and that the mutations decreased transport due to disrupting protein-ligand interactions and not a lack of protein expression? A full dose-response of these experiments would be more informative.

Extended data figure 6. The same comment as extended data fig. 4 in relation to the set up of the experiment. Additionally, statistics were performed on the data presented in Extended data fig. 4 but not for the mutants presented in Extended data fig. 6.

Extended data figure 8. It would be better to have error bars presented in both directions. It would also be beneficial to indicate the number of replicates.

Extended data Figure 10, Line 1005: Change 'TauTNipecotic acid' to 'TauTnipecotic acid' for consistency. Also change this in Supplementary figure 10 so it is consistent throughout the paper

Minor ponits:
Lines 29 – 31: The sentence is not clear – It this modofication correct?
The structures TauT bound to substrate (taurine) and substrate analogues (β-alanine, guanidinoacetate, and γ-aminobutyric acid), are captured in distinct conformations.
Line 88 – 90: The sentence is not clear… "The high molecule flexibility"??
Line 57: Remove the qualitative assessmment 'groundbreaking'
Line 101: The line begins with 'As promising drugs…' but only one drug is then stated in this sentence (5AVA). Change this to read 'As a promising drug candidate'
Line 117: Change 'These structures reveal atomic details of substrate, substrate analogues and their associated ions recognition' to 'These structures reveal atomic details of substrate, substrate analogue and ion recognition'
Line 118: Change 'enhance our capacity' to 'enhance capacity'
Line 138: Remove 'definitively'
Line 149: Remove the repeated mention of 'finally'

Line 172-173: Add a reference to support the statement that '..are proposed to alter surface trafficking, expression, and stabilit of TauT'
Line 175: replace the comma with and such that it reads '…positioned at the membrane's midpoint and is found to be…'
Line 200: Add taurine to limit ambiguity such that it reads 'The remaining oxygen atoms of taurine engage in..'
Line 213: Change 'alanine substitutions including Phe58…' to 'alanine substitutions of Phe58…'
Line 215: replace 'binding to Tau' to 'binding of taurine'
Line 234: Replace 'boost' with 'increase'
Line 268: change 'surrounding' to 'surrounded'
Line 281: Change 'experiences prominent displacement' to 'is prominently displaced'
Line 316-343: In the description of the extracellular gate, the salt bridge between Arg66 and Asp459 is not described. This salt bridge is well described in other SLC6 members and should be discussed here.
Line 340: Change 'Taut's cryo-EM structures' to 'the cryo-EM structures of TauT'
Line 358: Change 'sites' to 'site'
Line 364: Change 'them' to 'compounds' to remove ambiguity
Line 365: Change 'of other' to 'of the other'
Line 409: Add 'the' so it reads for the substrate binding site'
Line 628: Change 'for enhancing' to 'to enhance'
Line 629: Report the g value rather than the rpm value
Line 630, 640, 644, 656: Change 'TauTEM' to 'TauTEM' to be consisten with the introduction
Line 652: Change 'through' to 'by'
Line 660: Change 'experiment' to 'experiments'
Line 664: Change 'state' to 'states' in both instances
Line 670: Change 'application in grids' to 'application to grids'
Line 671: Change 'using' to 'use'
Line 672: Repeated use of 'were'
Line 676: Report what value the energy filter was set to.
Line 688: Change 'worse' to 'with a larger value'
Line 694: Delete 'generate'
Line 697: Change 'in the CryoSPARC' to 'in CryoSPARC'

Reviewer #4

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

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately addressed my concerns and improved the manuscript.

Reviewer #2

(Remarks to the Author)
The authors have responded to my comments and requests in an adequate manner. I can see that my points were only minor in nature but the authors have incorporated all suggestions and overall, the manuscript shaped well during this process of revision.

Reviewer #3

(Remarks to the Author)
Thanks to the authors, the revised version of the manuscript is highly improved. The quality of cryo-EM densities (in the very few problematic data sets) have also been improved by additional collection and processing. However, the quality of the cryo-EM models still does not meet the accepted criteria in the field. The main emphasis and majority of the paper is on the structural studies, therefore it is critical that the models are error free.
The resolution of cryo-EM maps are reasonably good and it should be possible to get the CaBLAM values below 1%. There are also still a few Ramachandran outliers in the models that should be corrected.
I can recommend using ISOLDE (https://tristanic.github.io/isolde/) and coot in parallel (real space refinement in coot, reducing the refinement weight value) and use the input model's restraints in Phenix refinement for the final refinement stage. This should help the authors to improve the model values.

Other than the general quality of the models that need to be improved, the beta-Ala pose needs be revisited, it appears the amine group direction is not correct (please check more carefully the cryo-EM density).

Reviewer #4

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

Version 2:

Reviewer comments:

Reviewer #3

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

Reviewer #4

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

Response to reviewer's comments

We deeply appreciate the insightful and constructive comments provided by the reviewers and editor. We have made point-by-point responses as below. To ensure clarity, the reviewer's criticisms and/or suggestions are shown in dark red, whereas our responses are shown in dark blue. The text in the manuscript has also been revised accordingly and is highlighted with a yellow background. We hope that our responses can address the reviewers' concerns and our revised manuscript is suitable to be published in *Nature Communications*.

We have modified our formatting in our revised manuscript to comply with the formatting instructions of *Nature Communications*.

Reviewer #1 (Remarks to the Author):

This manuscript describes eleven cryo-EM structures of human taurine transporter (TauT), which has mutations in intracellular loops to form a Fab complex with or without four substrates/six inhibitors, at a resolution of 2.9−3.4 Å. The structures with Tau and its analogues have been verified through binding and uptake experiments using TauT mutants. The comparison with GAT-1 illustrates TauT-specific interactions in substrate-amino residue and Na^+ recognition. These findings are important for understanding the molecular function and transport mechanism of TauT. However, the current manuscript lacks a thorough introduction of the research background, making it unclear how these findings relate to drug discovery as claimed by the authors.

Response: We thank the reviewer for the comment. We have revised the research background as shown in the responses below.

Furthermore, it is necessary to clearly demonstrate the significance of elucidating the inhibitory mechanism of several molecules that interact with TauT more weakly compared to the best substrate taurine (Tau).

Response: We thank the reviewer for the thoughtful suggestion. You have raised an important point regarding the significance of elucidating the inhibitory mechanisms of molecules that interact with TauT more weakly compared to the best substrate taurine. While some inhibitors of TauT have been reported, there remains limited information about the detailed mechanisms of these inhibitors, especially those with weaker binding affinities.

We fully agree that understanding the inhibitory mechanisms of these weaker binders is crucial for several reasons. Firstly, it can provide insights into the structural and functional features that determine the specificity and potency of TauT inhibitors.

Secondly, elucidating the mechanisms of weaker inhibitors can help identify potential off-target effects and guide the design of more selective and high-potency inhibitors. This is particularly important given the complex nature of TauT and its interactions with various substrates and inhibitors.

We plan to address this gap by resolving the high-resolution structures of TauT in complex with

these reported inhibitors, combined with functional analysis. This endeavor may largely contribute to the development of high-potency and selective TauT inhibitors, ultimately enhancing our understanding of TauT's role in biological processes and potential therapeutic applications.

1. The relevance of this research to drug discovery for neurological diseases is unclear. In the Abstract, line 25. "It is a pharmacological target for various neuropsychiatric diseases" is not clearly described in the Introduction. On the other hand, the discussion seems to focus solely on the various cancer diseases (line 448). The authors should re-write the introduction.

Response: We thank the reviewer for the insightful comment. In response, we have carefully revised the manuscript to better emphasize the significance of our research. Specifically, we have rewritten a new sentence in lines 22-23 of the Abstract. Additionally, we have thoroughly revised the Introduction (lines 78-98) and the Discussion section (line 425) to clearly elucidate the relevance of our research to related physiological health and cancers. We believe these revisions significantly enhance the clarity and impact of our work.

a) Lines 104-105: There is a question whether the problem of "no TauT-targeting drugs have been investigated in clinical trials" can be resolved by determining the structure of TauT. Please clearly demonstrate the usefulness of TauT inhibitors in treating neurodegenerative diseases.

Response: We sincerely apologize for any confusion caused to the reviewer and would like to clarify clearly. The determination of the structure of TauT is not sufficient to address the problem of "no TauT-targeting drugs have been investigated in clinical trials". The structure of TauT may provide theoretical guidance for the molecule design targeting TauT.

We apologize for having misunderstood the relationship between TauT and neurodegenerative diseases. Literature indicates that TauT is predominantly linked to retinal health and a range of cancers, with scant information available regarding its connection to neurodegenerative diseases. To date, TauT inhibitors may not be inadequate for the treatment of neurodegenerative conditions.

b) Lines 83-103: Although the sentences describe the utilities and characteristics of various TauT inhibitors, most citated studies examined only the inhibitory effects on TauT transport or showed negative pharmacological effects (Ref-37 shows that inhibitors decrease in brain taurine levels). Please cite the literature that examined therapeutic or beneficial pharmacological effects.

Response: We thank the reviewer for the insightful comment. We apologize for not clearly clarifying the therapeutic potential of TauT inhibitors in this section. Upon further review of the literature, we found that while most studies have focused on the inhibitory effects of TauT inhibitors on taurine transport or their negative pharmacological effects, there is emerging evidence suggesting potential therapeutic applications.

For instance, elevated expression levels of TauT have been observed in various cancers, such as gastric and colorectal cancer, and are associated with poor prognosis and advanced tumor stages. Inhibition of taurine transport by targeting TauT has been suggested as a promising strategy for

anticancer therapy. Specifically, guanidinoethyl sulfonate (GES), a substrate-mimetic inhibitor of TauT, has been shown to effectively reduce taurine levels in the brain and plasma, and may have unexplored antitumor properties. Additionally, recent studies have indicated that inhibiting TauT could restore antitumor immunity, which is impaired by the exhaustion of environmental taurine depleted by tumor cells.

We have rewritten the sentences in lines 78-93 and revised several references in this part to better reflect the current understanding of the therapeutic potential of TauT inhibitors. We believe these revisions provide a clearer picture of the potential benefits of targeting TauT for therapeutic purposes.

c) Furthermore, several references in this section are not properly cited, making it impossible to determine their validity. Please review the entire text and cite appropriately. This reviewer will state only those noticed below.

Response: We thank the reviewer for pointing it out. We deeply apologize for not properly citing the references in this section and bringing trouble to the reviewer's understanding. We have reviewed the entire text and cited the references appropriately.

There is an error in the citation of Ref-34, which describes "Effect of nipecotic acid on GABA release from locust synaptosomes" not the vertebrate nervous system as described in line 92.

Response: We are grateful to the reviewer for their suggestion and we sincerely apologize for the error in our original citation within this sentence. Upon reviewing the reference, we have made the necessary correction and now cited the correct reference in line 84. The revised reference describes the effect of nipecotic acid on the vertebrate nervous system.

Ref-35: If the sentence "I4AA has shown potential in interacting with TauT in BRB cells, playing a crucial role in the prevention of retinal diseases" means that I4AA plays an important role in preventing retinal diseases, this explanation may be inappropriate as I4AA has no such pharmacological effect.

Response: We apologize for our over-interpretation of the literature. The inhibitor I4AA does interact with TauT in blood-retinal barrier (BRB) cells, but it does not have a pharmacological effect on retinal diseases. We previously speculated that it might play a role in preventing retinal diseases, which was not accurate. We have revised the description in lines 84-85 to clarify this point.

Ref-36: "The longer carbon chain length of 5AVA may lead to a unique bent configuration, enhancing its interaction with TauT" should be confirmed. This paper shows that IC50 values for beta-alanine, GABA, and 5AVA were 55.5, 1014, and 1420 microM, respectively, indicating that the longer the carbon chain, the weaker the interaction with TauT.

Response: Thank you for pointing this out. We deeply apologize for the misunderstanding of Ref-36. The literature indicates that 5AVA contains a C4 linker between the ionic groups, whereas GABA has a C3 linker. Despite the longer carbon chain in 5AVA, it exhibits comparable affinity to TauT as

GABA, which has a shorter C3 linker. This suggests that the C4 linker in 5AVA may adopt a unique bent conformation to interact with TauT, rather than simply correlating longer chain length with weaker interaction. We have revised the sentence in lines 88-89 to accurately reflect this understanding.

"TauT-targeting compounds" in lines 84-85 include 5-aminovaleric acid and homotaurine. Is this correct? Do these exert their pharmacological effects by targeting TauT? Ref-33 shows that homotaurine inhibited TauT function with IC50 value of 3.4 mM, indicating a very low affinity.

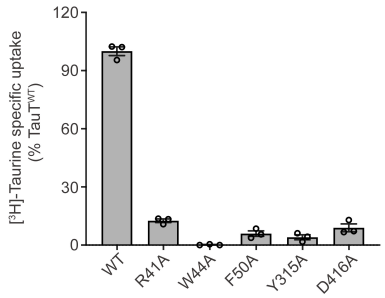
Response: Thank you for pointing this out. We deeply apologize for the inaccurate description. Both 5-aminovaleric acid (5AVA) and homotaurine are indeed inhibitors of TauT. However, it is not clearly established in the literature that their pharmacological effects are exerted specifically through targeting TauT. Additionally, while homotaurine has been explored for its potential therapeutic effects in conditions such as Alzheimer's disease, these effects are likely mediated through other mechanisms, such as its interaction with $GABA_A$ receptors, rather than through its inhibitory action on TauT.

Homotaurine, which is structurally similar to taurine with a C3-linker between the sulfonate group and amino group, has been shown to inhibit TauT function in a concentration-dependent manner, with an IC_{50} value of 3.4 mM. This relatively high IC_{50} value indicates that homotaurine has a low affinity for TauT.

We have revised the description in lines 78-83 to accurately reflect these points.

2. Please consider the relationship between the N-terminus segment (residues 38-46) and transport function. Although lines 327-331 indicate the interactions between Arg41 and Asp416 in TM8/Tyr315 in TM6, and between Trp44 and Phe50 in TM1a/Tyr315 in TM6, these interactions were not inspected. Do these mutations affect transport activity?

Response: We thank the reviewer for the comment. To inspect these interactions in N-terminus segment, we mutated residues Arg41, Trp44, Phe50, Tyr315 and Asp416 to alanine. The generated mutants were further examined to assess their influence on the transport activity of taurine (Authors' Response Fig. 1). All of these mutations exhibit significantly decrease in the transport activity of taurine.



Authors' Response Fig. 1 Transport activity of $TauT^{WT}$ and its mutants. Biochemical validation of the N-terminal interaction residues using [^{3}H]taurine uptake assay. The transport activity of each

mutant is normalized to that of $TauT^{WT}$. Assays were conducted with three independent biological replicates (n=3). Data are mean ± s.e.m.

3. Please clarify that GES is a TauT inhibitor, not the substrate, to consider the relationship between inhibitor properties and conformation status. The authors argue that GES is the only inhibitor that showed an occluded conformation among the inhibitors tested, while other inhibitors show an inward-open conformation. This study also shows that all tested substrates stabilize TauT in an intermediate conformation or an occluded conformation (high affinity ligands such as Tau and beta-Ala seem to show an occluded conformation), suggesting GES as a substrate of TauT.

Response: Thank you for your insightful comment. We appreciate your attention to the clarification of GES as a TauT inhibitor. As indicated in Ref-33 and Ref-36, GES (guanidinoethyl sulfonate) is indeed an inhibitor of TauT, not a substrate. We have revised the description in lines 86-87 to accurately reflect this categorization.

While GES-bound TauT was captured in an occluded conformation, similar to the taurine-bound structure, other inhibitors-bound TauT were captured in an inward-open conformation. This difference in conformational status does not necessarily imply that GES acts as a substrate. Instead, it may reflect the unique binding mode and structural characteristics of GES. For instance, GES has a more complex structure compared to other inhibitors, with its guanidine group occupying a similar site to the amino group of taurine and its sulfate group overlapping with the sulfate group of taurine. This binding mode, along with the external factors during sample preparation, may contribute to the observed occluded conformation.

Minors:
Line 171: "Extended Data Figs. 2l and 3" should be Extended Data Figs.2 and 3.

Response: We thank the reviewer for the suggestion. We have changed the description to Supplementary Figs. 2h and 4 in lines 151-152.

Line 710: Concentrations of compounds used in Extended Data Fig.1 are missing.

Response: We thank the reviewer for pointing it out. We have added the concentrations of compounds used in Extended Data Fig. 1 in lines 600-602.

Line 838: The order of TauT(apo), TauT(Tau), and TauT(GABA) should be same as those in Fig.3a.

Response: We thank the reviewer for the suggestion. We have adjusted the order to match the order in Figure 3a (line 829).

Reviewer #2 (Remarks to the Author):
The manuscript by Du, Cheng, Xie and colleagues is a very dense and comprehensive manuscript covering a lot of different aspect related not only to the taurine transporter but also to the closer members of the immediate protein family. The manuscript is well written (sometimes maybe a “the”

ect. Is missing; the text should undergo thorough proof reading.).

Response: Thank you for your positive feedback and for pointing out the need for additional proofreading. We have carefully reviewed the manuscript and made corrections to ensure clarity and grammatical accuracy. We have added missing articles such as "the" in lines 51, 112, 293, 310, 426, and other relevant locations. We appreciate your attention to detail and have taken steps to enhance the overall quality of the manuscript.

I can only congratulate the authors on a job nicely done. There are only minor things that I would want to have mended. The start of the results section should be indicated – by a sub-heading or at least by a page break.

Response: We thank the reviewer for the suggestion. We have added a sub-heading in the start of the results section in line 100.

Line 243: "These findings provide helpful information to increase our understanding of the substrate specificity of GABA subfamily." This sentence is not really written well: What shall GABA subfamily mean? This needs to be specified. Also, the sentence is kind of a concluding statement for a paragraph or section of the paper. However, there is another concluding paragraph following thereafter (lines 246-249). The authors should decide which one to keep and also, they should add another sentence which leads the reader to the next section. Otherwise, it is difficult to follow.

Response: We apologize for the confusion caused by the unclear wording. We appreciate the reviewer's suggestion to clarify the terminology. The term "GABA subfamily" refers to one of the subgroups within the solute carrier 6 (SLC6) family, which includes TauT, GAT1, GAT2, GAT3, BGT1, and CT1. We have revised the sentence in line 222 to clearly specify the GABA subfamily.

Additionally, we have reviewed the structure of the paragraph and deleted the redundant concluding paragraph to avoid repetition and improve clarity.

To better guide the reader to the next section, we have added a transitional sentence: "The substrate analogues β-alanine, GABA, and GAA demonstrate potent inhibitory effects on TauT. Determining the structures of TauT in complex with these ligands will further advance our understanding of TauT." This sentence now appears in lines 222-225, providing a smooth transition to the subsequent discussion.

Line 346: "dose-dependently"– the authors mean "concentration-dependently".

Response: We thank the reviewer for the suggestion. We have changed the "dose-dependently" to "concentration-dependently" in lines 326-327.

The only thing that "bothers" me a bit is the distinction between substrates, substrate analogues and inhibitors – how can the authors distinguish among those? Sometimes, it is not really easy nor obviously overt which compound belongs to what category. Maybe the authors want to comment

on this – and also include a sentence to clarify this to the reader of the article.

Response: We sincerely thank the reviewer for raising this important and insightful point. We agree that distinguishing between substrates, substrate analogues, and inhibitors can sometimes be challenging and not always straightforward. To address this, we have provided a detailed explanation of our classification criteria.

Firstly, based on well-established evidence from multiple studies, taurine is recognized as the primary transport substrate of TauT, and thus we categorize it as a substrate. For other compounds, we classify common amino acids (such as β-alanine) and substrates of other members of the GABA subfamily (for example, GABA for GAT1-3 and GAA for CT1) as substrate analogues. These compounds share structural similarities with taurine and are transported by related transporters within the same family. The remaining compounds, which are uncommon amino acids or ligands without specific transport proteins, are classified as inhibitors.

We apologize for not having clearly clarified this distinction in the article. Following your recommendation, we have added a sentence in the Introduction section (lines 78-83) to elucidate our classification criteria and provide clarity for the reader. This addition will help to better guide the reader in understanding how we categorize these compounds in our study.

Reviewer #3 (Remarks to the Author):

The manuscript "Molecular basis of human taurine transporter uptake and inhibition" presents and describes 11 cryo-EM structures of the human taurine transporter (TauT, SLC6A6). The structures are presented in the apo form, bound to taurine, β-alanine, guanidinoacetate, γ-aminobutyric acid (GABA), nipecotic acid, imidazole-4-acetic acid, piperidine-4-sulfonic acid, guanidinoethyl sulfonate, 5-aminovaleric acid and homotaurine. The cryo-EM structures are supported with functional experiements to determine the binding site of these ligands. The maps are of good quality but the work presented requires revision to improve clarity. Some of the models and maps require correction, reprocessing, etc (see below).

A general comment for future journal submissions, I strongly recommend to match the name of the map with the model as the random job numbers on the map files are not helpful for locating the corresponding coordinate files.

Response: Thank you for your valuable feedback. We sincerely apologize for the oversight in not naming the map files appropriately, which may have caused confusion in locating the corresponding coordinate files. We have now corrected this by ensuring that the names of the maps are matched with their respective coordinate files. We appreciate your understanding and look forward to your continued guidance.

Few major problems regarding the models and maps:

1-P4S-bound model:

If the corresponding map is "cryosparc_P41_J5913_006_volume_map_sharp.mrc", the density for the P4S is ambiguous and does not follow the shape or pose of the compound. Extra steps of

processing, masking and local refinement, cleaning the particle stack might be helpful to improve the density. But at this stage, the map does not support modeling P4S. (Is it a lose binder? Is it partial occupancy?) Further, the density presented in Figure 1 is not reflected in the cryo-EM map. The sigma level perhaps is not selected at the level the noise is not present. Please clarify this part.

Response: Thank you very much for your insightful comments and suggestions. We have taken the following steps to address the issues raised:

1. Improving the Density Map Quality

We have collected an additional 5,665 images and combined them with the previous cryo-EM images for further processing. This has led to an improved resolution of the map to 3.12 Å. Additionally, we have employed extra processing steps, including local refinement, to enhance the density map quality. These steps have significantly improved the clarity and accuracy of the density map.

2. Addressing the Density Ambiguity for P4S

We have carefully re-evaluated the density map for the P4S region. The new map now supports the modeling of P4S, and the density is consistent with the shape and pose of the compound. We have ensured that the density map accurately reflects the structure of $TauT^{P4S}$.

3. Updating Figure 1

We apologize for the previous discrepancy between the density presented in Figure 1 and the cryo-EM map. After improving the resolution and refining the density map, we have updated Figure 1 to accurately reflect the density at the sigma level where noise is not present.

4. Clarifying the Density Representation

We have carefully selected the sigma level to ensure that the noise is minimized while still clearly representing the density features. The new map and corresponding figures now provide a clear and accurate representation of the density.

We have also considered the possibility of partial occupancy or weak binding for P4S, and our improved map now provides a clearer basis for further analysis.

We appreciate your detailed feedback and believe that these improvements significantly enhance the quality and clarity of our work. We look forward to your continued guidance.

2- I4AA-bound model:

Assuming the corresponding map is: cryosparc_P41_J6512_006_volume_map_sharp.mrc

The density quality around residues 47–58 is rather poor, consider removing the region.

The density for the ligand is "very poor, it does not allow unambiguous modeling of the compound" and therefore any discussions based on this model and P4S-bound model should be revisited once the density is imporved (I suggest more extensive processing, as suggested above) after ensuring the correct pose of the compound is modeled in the density.

Response: We appreciate your constructive feedback and suggestions. We have taken the following steps to address the issues raised:

1. Improving Density Quality

We have collected an additional 5,319 images and combined them with the previous cryo-EM images for further processing. This effort has led to an improved resolution of the map to 2.92 Å. The density quality around residues 47–58 has been enhanced, and we have retained the residues by

setting the occupancy of the flexible side chains to zero to better reflect their dynamic nature.

2. Enhancing Ligand Density

We apologize for the previous poor density for the ligand I4AA, which did not allow unambiguous modeling of the compound. After improving the resolution and refining the density map, the new map now supports the modeling of I4AA at a sigma level where noise is minimized. We have carefully revisited the discussions based on I4AA and the P4S-bound model and have revised the related sections in lines 345-347, 349-351, 356-360, 377-382, 388-394, 462-464, and 466-469.

3. Processing and Refinement Steps

To further enhance the density quality, we have employed local refinement, as suggested. This step has improved the clarity and accuracy of the density map, allowing for more reliable modeling of the compound.

We believe these improvements significantly enhance the quality and reliability of our work.

3- 5AVA mode, shouldn't the N04 be positioned differently? There is apparently a water molecule (?) density hydrogen bonding with the nitrogen atom, please check that.

Response: We thank the reviewer for pointing it out. We have checked the local density and indeed found a water molecule density. We have repositioned the N04 of 5AVA to hydrogen bond with the water molecule. Additionally, we have revised the discussion related to 5AVA in our manuscript in lines 356, 389-390, 462-464 and 468-469.

4- Model refinement statistics: clash scores are high. Please improve the model to fit the accepted standards in the field. Please improve the models to not have any ramachandran outliers (disallowed % should be zero). Please include CaBLAM values (should be below 1% to be acceptable).

Response: We thank you for your valuable suggestions regarding the model refinement statistics. We have taken the following steps to address the issues raised:

1. Improving Model Refinement Statistics

We have carefully refined our model to meet the accepted standards in the field. The clash scores have been significantly reduced, and the Ramachandran outliers have been eliminated, bringing the disallowed percentage to zero.

2. CaBLAM Values

We deeply apologize that despite our best efforts, the CaBLAM values have not been brought below 1%. It is worth noting that achieving CaBLAM values below 1% can be challenging, even in high-resolution models. For instance, in recent studies, it was observed that many refined models, even those with high-resolution maps (2.8-3 Å), still had CaBLAM outliers (***Tan, Jiaxin et al. (2024); Yan, Rui et al. (2024); Hu, Tuo et al. (2024); Li, Yue et al. (2024); Ji, Wenming et al. (2024)***). This suggests that our current CaBLAM values are not uncommon in the field, especially considering the resolution of our map.

We have detailed the improved model refinement statistics in Authors' Response Fig. 2. We believe these improvements significantly enhance the quality and reliability of our work. We look forward to your continued guidance and feedback.

	#1 TauT^{Tau} (EMDB: 61970) (PDB: 9K1B)	#2 TauT^{Apo} (EMDB: 61943) (PDB: 9K0C)	#3 TauT$^{\beta\text{-}Ala}$ (EMDB: 61948) (PDB: 9K0N)	#4 TauT^{GAA} (EMDB: 61949) (PDB: 9K0O)	#5 TauT^{GABA} (EMDB: 61974) (PDB: 9K1F)	#6 TauT^{P4S} (EMDB: 61975) (PDB: 9K1H)	#7 TauT^{HAA} (EMDB: 61976) (PDB: 9K1I)	#8 TauT^{GES} (EMDB: 61983) (PDB: 9K1X)	#9 TauT^{SAVA} (EMDB: 61983) (PDB: 9K1X)	#10 TauT^{Hist} (EMDB: 61987) (PDB: 9K21)	#11 TauT$^{Thioacetic\ acid}$ (EMDB: 61985) (PDB: 9K1Z)
Clash score	6.52	6.05	4.55	6.09	4.90	6.12	5.93	7.14	5.77	6.19	6.97
Disallowed (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CaBLAM values	3.88	3.93	2.41	3.29	3.39	2.85	3.40	2.41	2.04	2.85	2.30

Authors' Response Fig. 2 Cryo-EM validation statistics including the clash score, ramachandran outliers and CaBLAM values.

Please note that the updated coordinates should be re-deposited into PDB and the updated validation reports should be presented.

Response: Thank you to the reviewer for the reminder. We have re-deposited the updated coordinates into PDB and we have presented the updated validation reports.

Below please find some more comments that will hopefully help the authors to improve their manuscript.
Notable issues:
On maps and models:
The Fab has not been modelled into any of the structures presented despite a good density for them. The authors should model the Fab into each of the structures presented. The authors have modelled in N-acetyl glucosamine into the taurine, β-Ala and apo structures, however the density is not rich in this area. The authors should consider removing them from the models.

Response: We thank the reviewer for the suggestion. We have modelled the Fab into each of the structures of TauT and we have removed the previously modelled N-acetyl glucosamine from the models of taurine-bound, beta-alanine-bound and apo structures. We have re-deposited the updated coordinates into PDB and we have presented the updated validation reports.

The density for residues 180-188 in the TauT^{apo}, TauT^{Tau}, TauT$^{\beta\text{-}Ala}$, TauT^{GAA}, TauT^{GABA} as well as inhibitor-bound states does not seem strong enough to model. Similarly in all models and maps, if at the sigma level that the noise from the detergent micelle is removed, the density for certain regions of the model does not support modeling, please remove the residues.

Response: We thank the reviewer for the suggestion. We have removed the residues 180-188 in each of the structures of TauT. At the sigma level that the noise from the detergent micelle is removed, we have set the occupancy of the flexible side chains of residues 418-439 to zero in the NPE-bound model. As for the GAA-bound model, we have removed residues 47-54. Additionally, we have checked all models and maps and set the occupancy of the poor density side chains to zero.

Modeling should be done a bit more carefully, another example is residues 418–439 in NPE-bound model, where information on a some of the residues is missing. In this case I suggest not to remove the residues, rather set the occupancy of the flexible side chains to zero.

Response: We thank the reviewer for the comment. We have set the occupancy of the flexible side chains of residues 418-439 to zero in the NPE-bound model.

TauT$^{\beta\text{-Ala}}$ model:

Density for Asp401 side chain is lacking. Additionally, the density for sodium in the Na2 is not strong and is currently modelled in only 1.3 Å away from Asp401. Could the authors comment on this?

Response: We apologize for the poor density of sodium in the Na2 site and the residue Asp401 in the model of TauT$^{\beta\text{-Ala}}$ previously. We collected an additional 2,051 images and conducted image processing combined with previous cryo-EM images. The resolution of the map of TauT$^{\beta\text{-Ala}}$ was improved to 3.21 Å. The density quality of residue Asp401 and sodium in the Na2 site has been improved slightly. The map now supports the modelling of residue Asp401 and sodium in the Na2 site better.

TauT^{GAA} model:

The density for residues 47 to 56 does not seem rich enough to model these residues in.

Response: We thank the reviewer for the suggestion. We have removed residues 47 to 54 in the TauT^{GAA} model.

GES-bound model:

There probably is a water-mediated interaction with N135. Please investigate the local density.

Response: We thank the reviewer for the comment. We have investigated the local density and indeed found a water molecule density. Additionally, we have revised the interaction mode of GES in our manuscript in lines 357-358.

In the legend of any figure that shows density, sigma level and the program that estimates it, should be reported.

Response: We thank the reviewer for pointing it out. We have reported the sigma level and the program that estimates it in the legends of Figure 1, Figure 3, Supplementary Fig. 2, and Supplementary Fig. 8 (previous Extended Data Fig. 7).

The shortening of taurine to Tau should be avoided if possible to avoid confusion with tau proteins. However, the shortening of taurine transporter to TauT is common in literature and appropriate.

Response: We thank the reviewer for the thoughtful suggestion. We have checked the full manuscript and replaced “Tau” with “taurine”.

Lines 46-48: It is not clear what the authors are trying to communicate with the last line ‘…albeit with relatively lower affinity in the nervous system.’ Is this to say that the affinity for taurine is dependent on where the receptor is expressed?

Response: We apologize for our false description and for bringing confusion to the reviewer. Taurine is a weak agonist for both the $GABA_A$ receptor and the glycine receptor, so the correct meaning is that taurine has a lower affinity with these two receptors. We have revised the description in line 43.

Lines 104–105: Despite the promise of these compounds, no TauT-targeting drugs have been investigated in clinical trials. – Please elaborate at what stage of trials and "why" the compounds failed.

Response: We thank the reviewer for the suggestion. Through literature review, we found no clinical reports related to these small molecule compounds interacting with TauT. We hope that by obtaining the high-resolution structures of TauT in complex with these small molecule compounds, we can lay a theoretical foundation for the design of small molecule drugs targeting TauT in the future, and accelerate the future relevant clinical drug research.

Lines 158–192: Architecture of TauT–the language is too general and not clear which part corresponds to which model, e.g., lines 187–188 refers to modeling residues 38–47 of the N terminus, however, this section is not modeled in all the maps (missing in NPE, P4S, etc). Please be more specific.

Response: We apologize for not stating clearly and specifically. We have revisited the paragraphs on the "Architecture of TauT" and clarified which part of this paragraphs corresponds to which model more specifically. We have revised related sentences in lines 139-140, 150 and 165.

Lines 205-207: The author's speculate that 'Phe300 may serve as a key structural element for Tau recognition', by comparing the Tau-binding site in hTauT and the GABA binding site in hGAT1. Phe300 (hTauT numbering) is conserved in these two transporters and appear to occupy the same space so it is not clear how the authors came to this conclusion. In Zhu et al. (2023), the amino group of GABA interact with the hydroxyl Tyr60, which is not coserved in hTauT. The authors should clarify this point.

Response: We apologize to the reviewer for the confusion caused by our incorrect conclusion. We had naively assumed that the different interaction mode of the amino group between TauT and GAT1 indicated that Phe300 was critical for taurine recognition in TauT, which was an incorrect assumption. Replacing Phe300 with alanine abolished the transport activity of TauT, emphasizing the critical role of Phe300 in taurine transport. We have removed this incorrect conclusion from our manuscript. The revised text lies on lines 181-183.

In Zhu et al. (2023), the amino group of substrate GABA interacts with the hydroxyl group of Tyr60. Mutation of Y60A reduces the activity substantially, while Y60F does not affect transport activity, indicating a minor role for the hydroxyl group of Tyr60 in substrate recognition. Residue Tyr60 in GAT1 corresponds to Gly57 in TauT. In our manuscript, mutation of G57A slightly reduces the transport activity of TauT but still retains some transport activity. Based on this, we speculate that Gly57 plays a minor role in taurine recognition.

Lines 233–234: Interesting choice to mutate Glu and Leu to Cys. Are there previous studies suggesting such replacement? Please elaborate on the choice. Please also elaborate on the effects of mutations in increasing the activity of the transporter.

Response: We thank the reviewer for the thoughtful comment. Our choice to mutate Glu to Cys was based on a previous related study of ***Yahara, Tohru et al. (2014)***. In this study, researcher mutated the residue Glu406 in *rat* TauT to corresponding residue Cys in several GATs. Multiple alignment analysis revealed that residue Glu406 is conserved between human, mouse, rat, dog and monkey orthologs of TauT. In addition, in the study of ***Colas, Claire et al. (2020)***, researchers claimed that residue Cys144 is unique to CT1 within the SLC6 family and is suspected to be involved in the substrate specificity of CT1. Based on these two studies, we chose to mutate Leu to Cys in our research.

The molecules GAA and GABA possess longer carbon linkers than the molecule taurine. Mutations of residues Glu and Leu to Cys lead to a reduction in the side chain bulkiness of these residues, which results in the substrate binding cavity becoming larger. The larger volume of the substrate binding cavity may better accommodate the longer carbon linkers of GAA and GABA. The mutation E406C results in an increase in the transport activity for GABA, leading us to speculate that Glu406 is involved in the substrate specificity of TauT. The residue Leu134 in TauT corresponds to Cys144 in CT1, which is unique to CT1. The mutation L134C exhibits an increase in the transport activity for GAA, leading us to assume that Leu134 plays an important role in the substrate specificity between taurine and GAA.

Lines 718-721: In the methods it states that 'For the inhibition assay, the cells were incubated in PBS containing 0.5 μCi/mL [^{3}H]taurine and different concentrations of P4S (MedChemExpress), I4AA (MedChemExpress), GES (MedChemExpress), 5AVA (MedChemExpress), nipecotic acid (MedChemExpress) or Htau (MedChemExpress)'. Please state the concentrations used and the rational behind the specific concentrations.

Response: We apologize to the reviewer for not previously stating the concentrations used. We have now added the concentrations used in lines 600-602. For the inhibition assay, we chose specific concentrations for different inhibitors based on the study by ***Stary D and Bajda M. (2023)***.

Lines 199-200: The wording makes the description of the interactions ambiguous as to whether it applies to the substrate or Na^+ ion in Na1. Reword for clarification.

Response: We thank the reviewer for pointing it out. We have reworded the clarification to make the description of interactions unambiguously in lines 176-178.

Lines 240-243: This sentence requires rewording to make it clear what the authors are trying to communicate. Is it that the mutations showed a significant decrease in transport activity? Further explanation as to why there is a decrease in transport activity for taurine, GABA and GAA were observed considering the mutants were based on residues in GABA transporter proteins would be

beneficial.

Response: We apologize for not clearly explaining and bringing confusion to the reviewer. Mutations of residues Gly57, Phe58 and Leu306 showed a significant decrease in transport activity of taurine, GAA and GABA. We have explained our speculation for the reduction in the transport activity and we have rewritten the sentences in lines 216-220.

Line 264: 'exhibited varying impacts' is a vague sentence. It would be helpful to be more detailed.

Response: We thank the reviewer for the thoughtful comment. We have revised the description more detailed in lines 239-240.

Lines 267-273: In describing the interactions, the authors should be specific as to whether the interaction is with the backbone or the amino acid residue side chain. This would also then be beneficial for understanding the results of the mutagenesis.

Response: We thank the reviewer for the insightful suggestion. We have revised the description of the interactions in lines 247-251.

Lines 297-299: The sentence 'The $TauT^{Tau}$ and $TauT^{GABA}$….. (Extended Fig. 7).' is unclear. It should be reworded to make it unambiguous as to which structures were superimposed and the corresponding RMSD values.

Response: We thank the reviewer for the suggestion. We have reworded the statement to make it unambiguous in lines 276-279.

Line 305: The authors state Gly56 undergoes notable conformational changes. It is not clear what is meant by this considering there is no side chain. Is it that the residue is moved?

Response: We apologize to the reviewer for our ambiguous statement. Residue Gly56 undergoes a change in position rather than conformation. We have revised the clarification in lines 284-285.

Figure 1. The densities presented in panel c are difficult to see. It would be beneficial to make them more obvious by making them darker or less transparent.

Response: We thank the reviewer for the suggestion. We have reduced the transparency of the densities presented in panel c of Figure 1 to make them more obvious.

Figure 2. It would be beneficial to label the distances of the interactions shown in each panel. Change the figure title from '..analogues binding of TauT' to '…analogues binding to TauT'.

Response: We thank the reviewer for the comment. We have labelled the distances of the interactions shown in each panel of Figure 2. In addition, we have changed the figure title in line 820.

Line 825: Change ‘of the Taurine’ to ‘of taurine’

Response: We thank the reviewer for the suggestion. We have changed ‘of the Taurine’ to ‘of taurine’ in lines 820-821.

Figure 4. Consider using a lighter shade of brown for the 2D structure of the compound in panel D. It is challenging to see the black font.

Response: We apologize for not clearly displaying the 2D structure of the compound 5AVA. We have now used a lighter shade of brown to make it more visible.

Figure 5. It is not clear from the schematic or the legend as to what the red lines from the different ligand mean? Is it the step it blocks?

Response: We apologize for not clarifying clearly and causing confusion to the reviewer. The red lines mean that the small molecules block TauT in the corresponding conformation. We have stated this in the legend of Figure 5 in lines 866-867.

Extended figure 1: In panel b) it would be clearer to replace the label ‘WT’, ‘EM’ and ‘GFP’ with taurine to make it consistent with the other labels. You have already indicated which data refers to $TauT^{WT}$ and $TauT^{EM}$. In the legend also indicate that the GFP expressing control is shown in green. Be more precise with the line ‘Data were normalized to $TauT^{WT}$’ by stating ‘Data were normalized to taurine uptake by $TauT^{WT}$’
In the legend of c) indicate the number of replicates.
In panel d) it is not clear which part of the chromatogram the fractions correspond to. Indicate on the chromatogram the fractions collected.

Response: We thank the reviewer for the insightful suggestions. In panel b), we have replaced the label ‘WT’, ‘EM’ and ‘GFP’ with taurine. Following your recommendation, we have indicated in the legend in line 10 of new Supplementary Fig. 1 (corresponding to previous Extended Data Fig. 1) that the GFP-expressing control is shown in green, and we have made the statement more precise in line 11 of new Supplementary Fig. 1.

In the legend of c), we have indicated the number of replicates in line 15 of new Supplementary Fig. 1. In panel d), e) and f), we have indicated the fractions collected on the chromatogram, and we have stated this in the legend in lines 20-21 of new Supplementary Fig. 1.

Extended data figure 2. The panels are too small to recognize any features, an option could be to make it into e.g., two figures at least, with substrate-bound states and inhibitor-bound states. Also not clear why only TM1, TM3, TM6 and TM8 are shown out of the 12 TMs.

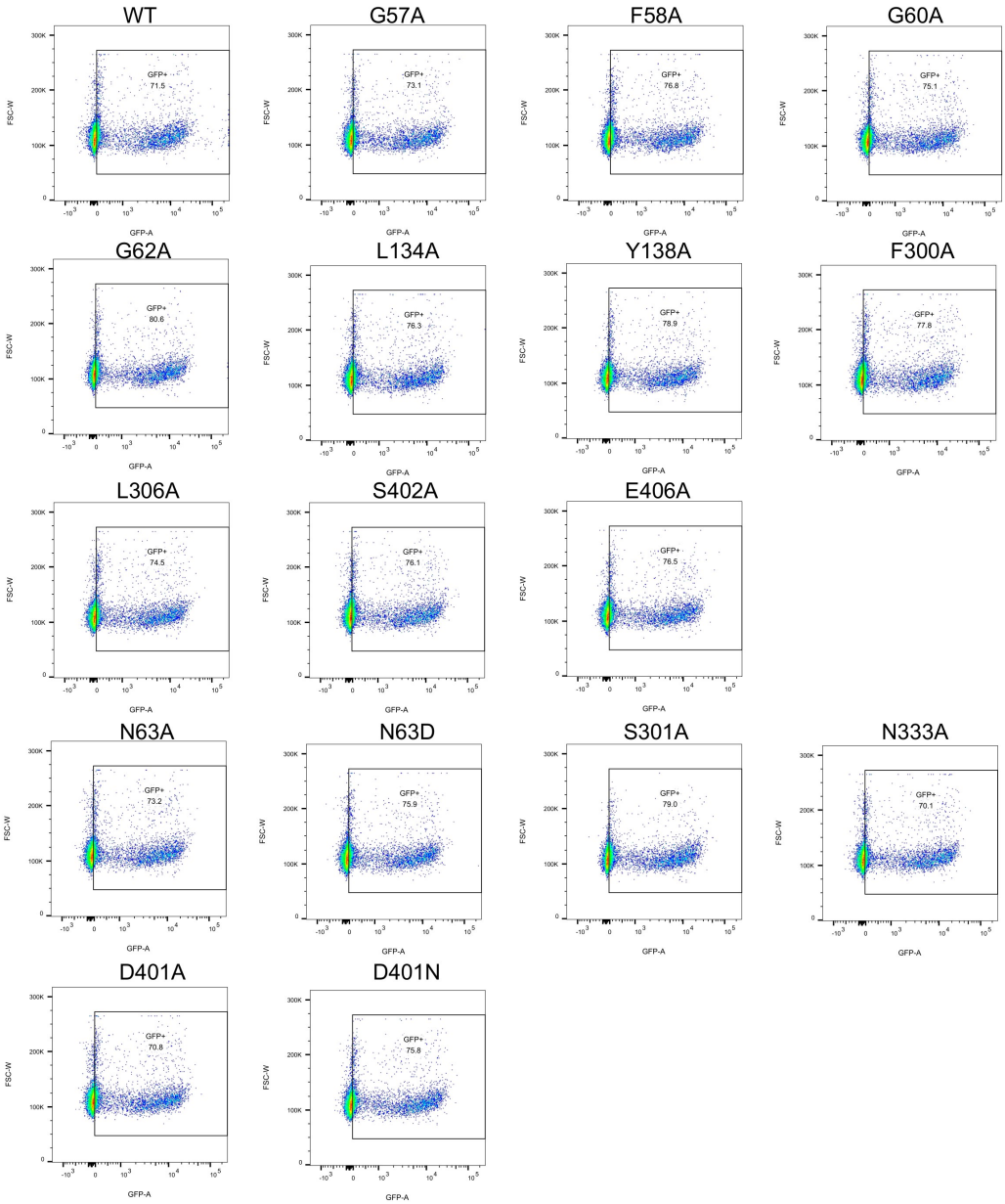
Response: We thank the reviewer for the suggestion. We have divided the figures into two new figures: one showing the densities of transmembrane helices (new Supplementary Fig. 2) and the other showing the eleven structures of TauT (new Supplementary Fig. 3).

We apologize for not previously displaying all 12 transmembrane helices. Since the overall structure of all models is similar, the main difference is reflected in TM1. We have chosen to shown all 12 transmembrane helices of the TauT$^{\beta\text{-Ala}}$ structure, as well as the density of TM1 in the inward-open and intermediate conformations (new Supplementary Fig. 2).

Extended data figure 4. The figure legend does not make it clear which panel is for substrate site mutations and which are for ion binding site mutations. There is a lack of detail for these experiments. Were these conducted at a single dose and compared to WT? How was it determined that the mutants expressed and that the mutations decreased transport due to disrupting protein-ligand interactions and not a lack of protein expression? A full dose-response of these experiments would be more informative.

Response: We apologize for not describing clearly in the legend and lacking the details for these experiments. We have revised the legend to make it clearer which panel is for substrate site mutations and which is for ion binding site mutations in line 61 of new Supplementary Fig. 5 (corresponding to previous Extended Data Fig. 4). Additionally, we have rewritten the method of transport assay and described the process of data normalization in lines 594-598 in our manuscript.

We appreciate the reviewer for pointing out the necessity of verifying the expression of the mutants. Cells were harvested 48 hours after being transfected with the plasmids of wild-type or mutants of TauT. And we have analyzed the positive rate of GFP by flow cytometry (Authors' Response Fig. 3). The results showed that the mutants of TauT exhibited nearly identical expression levels compared with wild-type TauT.



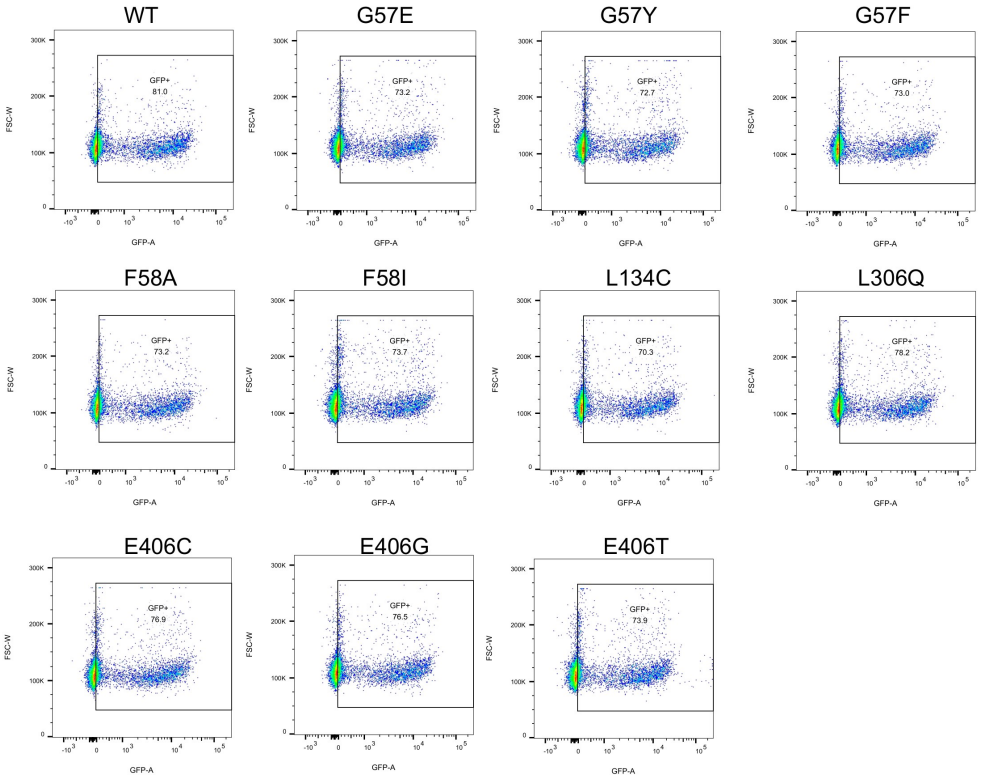
Authors' Response Fig. 3 Flow cytometry dot plot of GFP-TauT or its mutants, expression in HEK293T cells.

Extended data figure 6. The same comment as extended data fig. 4 in relation to the set up of the experiment. Additionally, statistics were performed on the data presented in Extended data fig. 4 but not for the mutants presented in Extended data fig. 6.

Response: We thank the reviewer for the suggestion. We have rewritten the method of transport assay and described the process of data normalization in lines 594-598. Additionally, we have now performed statistical analysis on the data presented in new Supplementary Fig. 7 (corresponding to previous Extended Data Fig. 6).

We appreciate the reviewer for pointing out the necessity of verifying the expression of the mutants. Cells were harvested 48 hours after being transfected with the plasmids of wild-type or mutants of

TauT. And we have analyzed the positive rate of GFP by flow cytometry (Authors' Response Fig. 4). The results showed that the mutants of TauT exhibited nearly identical expression levels compared with wild-type TauT.



Authors' Response Fig. 4 Flow cytometry dot plot of GFP-TauT or its mutants, expression in HEK293T cells.

Extended data figure 8. It would be better to have error bars presented in both directions. It would also be beneficial to indicate the number of replicates.

Response: We thank the reviewer for the suggestion and have shown the error bars in both directions in new Supplementary Fig. 9 (corresponding to previous Extended Data Fig. 8). Additionally, we have indicated the number of replicates in the legend in lines 118-119 of new Supplementary Fig. 9.

Extended data Figure 10, Line 1005: Change 'TauT$^{\text{Nipecotic acid}}$' to 'TauT$^{\text{nipecotic acid}}$' for consistency. Also change this in Supplementary figure 10 so it is consistent throughout the paper.

Response: We thank the reviewer for the suggestion. We have ensured the consistency of description of TauT$^{\text{Nipecotic acid}}$ in new Supplementary Fig. 11 (corresponding to previous Extended Data Fig. 10). Additionally, we have reviewed the entire manuscript to ensure consistency.

Minor ponits:
Lines 29–31: The sentence is not clear – Is this modofication correct?

The structures TauT bound to substrate (taurine) and substrate analogues (β-alanine, guanidinoacetate, and γ-aminobutyric acid), are captured in distinct conformations.

Response: We thank the reviewer for the suggestion. We have rewritten the sentence in lines 26-28.

Line 88–90: The sentence is not clear… "The high molecule flexibility"??

Response: We apologize for not describing clearly. Based on the suggestions from reviewer#1, we have made most changes to the Introduction section, and in the process we have removed this unclear sentence.

Line 57: Remove the qualitative assessment 'groundbreaking'.

Response: We thank the reviewer for the suggestion. We have removed 'groundbreaking' in the sentence in line 52.

Line 101: The line begins with 'As promising drugs…' but only one drug is then stated in this sentence (5AVA). Change this to read 'As a promising drug candidate'.

Response: We thank the reviewer for pointing it out. We have made most changes to the Introduction section, and in the process we have removed this unclear sentence.

Line 117: Change 'These structures reveal atomic details of substrate, substrate analogues and their associated ions recognition' to 'These structures reveal atomic details of substrate, substrate analogue and ion recognition'.

Response: We thank the reviewer for the suggestion. We have rewritten the sentence in lines 96-98.

Line 118: Change 'enhance our capacity' to 'enhance capacity'

Response: We thank the reviewer for the suggestion. We have rewritten the sentence in lines 96-98.

Line 138: Remove 'definitively'

Response: We thank the reviewer for the suggestion. We have removed 'definitively' in line 118.

Line 149: Remove the repeated mention of 'finally'

Response: We thank the reviewer for the suggestion. We have removed the repeated mention of 'finally' in line 130.

Line 172-173: Add a reference to support the statement that '..are proposed to alter surface trafficking, expression, and stabilit of TauT'

Response: We thank the reviewer for the comment. The densities of the N-acetyl glucosamine in the $TauT^{Apo}$, $TauT^{Tau}$ and $TauT^{\beta\text{-}Ala}$ are not rich. Based on the suggestion from the reviewer, we have removed them from the models.

Line 175: replace the comma with and such that it reads '…positioned at the membrane's midpoint and is found to be…'

Response: We thank the reviewer for the suggestion. Following your recommendation, we have replaced the comma with and in line 152.

Line 200: Add taurine to limit ambiguity such that it reads 'The remaining oxygen atoms of taurine engage in..'

Response: We thank the reviewer for the suggestion. We have added taurine to in line 178.

Line 213: Change 'alanine substitutions including Phe58…' to 'alanine substitutions of Phe58…'

Response: We thank the reviewer for the suggestion. We have changed 'including' to 'of' in line 189.

Line 215: replace 'binding to Tau' to 'binding of taurine'

Response: We thank the reviewer for the suggestion. We have replaced 'binding to Tau' to 'binding of taurine' in line 191.

Line 234: replace 'boost' with 'increase'

Response: We thank the reviewer for the comment. We have replaced 'boost' with 'increase' in line 210.

Line 268: change 'surrounding' to 'surrounded'

Response: We thank the reviewer for the comment. Following your recommendation, we have changed 'surrounding' to 'surrounded' in line 245.

Line 281: Change 'experiences prominent displacement' to 'is prominently displaced'

Response: We thank the reviewer for the suggestion. We have changed 'experiences prominent displacement' to 'is prominently displaced' in line 260.

Line 316-343: In the description of the extracellular gate, the salt bridge between Arg66 and Asp459 is not described. This salt bridge is well described in other SLC6 members and should be discussed here.

Response: We apologize for not having described the interaction between Arg66 and Asp459 in our previous description of the extracellular gate. We have now described related interactions in lines 305-306.

Line 340: Change ‘TauT’s cryo-EM structures’ to ‘the cryo-EM structures of TauT’

Response: We thank the reviewer for the suggestion. We have changed ‘TauT’s cryo-EM structures’ to ‘the cryo-EM structures of TauT’ in lines 321-322.

Line 358: Change ‘sites’ to ‘site’

Response: We thank the reviewer for the suggestion. We have changed ‘sites’ to ‘site’ in line 338.

Line 364: Change ‘them’ to ‘compounds’ to remove ambiguity

Response: We thank the reviewer for the comment. We have changed ‘them’ to ‘compounds’ to remove ambiguity in line 344.

Line 365: Change ‘of other’ to ‘of the other’

Response: We thank the reviewer for the suggestion. We have changed ‘of other’ to ‘of the other’ in line 345.

Line 409: Add ‘the’ so it reads for ‘the substrate binding site’

Response: We thank the reviewer for the suggestion. Following your recommendation, we have added ‘the’ in line 385.

Line 628: Change ‘for enhancing’ to ‘to enhance’

Response: We thank the reviewer for the suggestion. Following your recommendation, we have changed ‘for enhancing’ to ‘to enhance’ in line 504.

Line 629: Report the g value rather than the rpm value

Response: We thank the reviewer for the suggestion. We have reported the g value in line 505.

Line 630, 640, 644, 656: Change ‘$TauT_{EM}$’ to ‘$TauT^{EM}$’ to be consistent with the introduction

Response: We thank the reviewer for the comment. We have changed ‘$TauT_{EM}$’ to ‘$TauT^{EM}$’ in lines 506, 516, 520 and 532.

Line 652: Change ‘through’ to ‘by’

Response: We thank the reviewer for the comment. We have changed ‘through’ to ‘by’ in line 528.

Line 660: Change ‘experiment’ to ‘experiments’

Response: We thank the reviewer for the comment. We have changed ‘experiment’ to ‘experiments’ in line 536.

Line 664: Change ‘state’ to ‘states’ in both instances

Response: We thank the reviewer for the comment. We have changed ‘state’ to ‘states’ in both instances in line 540.

Line 670: Change ‘application in grids’ to ‘application to grids’

Response: We thank the reviewer for the comment. We have changed ‘application in grids’ to ‘application to grids’ in line 546.

Line 671: Change ‘using’ to ‘use’

Response: We thank the reviewer for the suggestion. We have changed ‘using’ to ‘use’ in line 547.

Line 672: Repeated use of ‘were’

Response: We thank the reviewer for pointing it out. We have revised the statement in line 548.

Line 676: Report what value the energy filter was set to.

Response: We thank the reviewer for pointing it out. Following your recommendation, we have reported the value the energy filter was set to in lines 551-552.

Line 688: Change ‘worse’ to ‘with a larger value’

Response: We thank the reviewer for the suggestion. We have changed ‘worse’ to ‘with a larger value’ in lines 564-565.

Line 694: Delete ‘generate’

Response: We thank the reviewer for the suggestion. We have deleted ‘generate’ in line 571.

Line 697: Change ‘in the CryoSPARC’ to ‘in CryoSPARC’

Response: We thank the reviewer for the suggestion. We have changed ‘in the CryoSPARC’ to ‘in CryoSPARC’ in line 574.

Reviewer #4 (Remarks to the Author):

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

Response: We thank the reviewer ***#4*** for the suggestions.

Response to reviewer's comments

We deeply appreciate the insightful and constructive comments provided by the reviewers and editor. We have made point-by-point responses as below. To ensure clarity, the reviewer's criticisms and/or suggestions are shown in dark red, whereas our responses are shown in dark blue. The text in the manuscript has also been revised accordingly and is highlighted with a yellow background. We hope that our responses can address the reviewers' concerns and our revised manuscript is suitable to be published in *Nature Communications*.

Reviewer #1 (Remarks to the Author):

The authors have adequately addressed my concerns and improved the manuscript.

Response: We thank the reviewer for the comment.

Reviewer #2 (Remarks to the Author):

The authors have responded to my comments and requests in an adequate manner. I can see that my points were only minor in nature but the authors have incorporated all suggestions and overall, the manuscript shaped well during this process of revision.

Response: Thank you for your positive feedback.

Reviewer #3 (Remarks to the Author):

Thanks to the authors, the revised version of the manuscript is highly improved. The quality of cryo-EM densities (in the very few problematic data sets) have also been improved by additional collection and processing. However, the quality of the cryo-EM models still does not meet the accepted criteria in the field. The main emphasis and majority of the paper is on the structural studies, therefore it is critical that the models are error free. The resolution of cryo-EM maps are reasonably good and it should be possible to get the CaBLAM values below 1%.

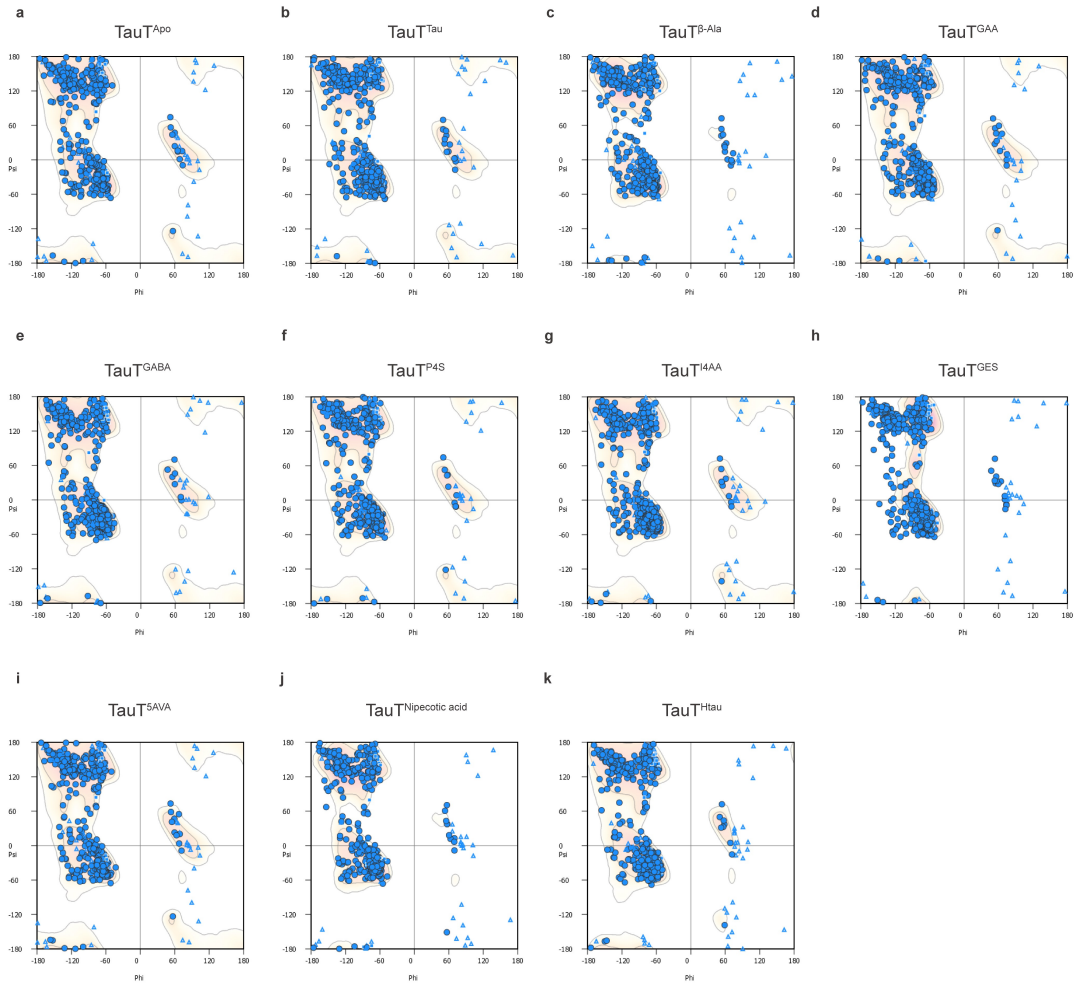
Response: We thank you for your valuable suggestions regarding the model refinement statistics. Following your suggestion, we have adjusted the models in coot by reducing the refinement weight value and used ISOLDE to further refine models in parallel. Additionally, we have used Phenix refinement for the final refinement stage. Following these steps, we have improved the model values and the CaBLAM values have been brought to around 1%. The detailed model refinement statistics are shown in Authors' Response Fig. 1. In addition, we have described these refinement steps in the Methods section in lines 577-583.

	#1 TauT^{Tm}	#2 TauT^{Apo}	#3 Tau$T^{\beta\text{-}Ala}$	#4 TauT^{GAA}	#5 TauT^{GABA}	#6 TauT^{PNS}	#7 TauT^{HAA}	#8 TauT^{GES}	#9 TauT^{SAVA}	#10 TauT^{Hist}	#11 Tau$T^{Quisconic\ acid}$
	(EMDB: 61970)	(EMDB: 61943)	(EMDB: 61948)	(EMDB: 61949)	(EMDB: 61974)	(EMDB: 61975)	(EMDB: 61976)	(EMDB: 61983)	(EMDB: 61983)	(EMDB: 61987)	(EMDB: 61985)
	(PDB: 9K1B)	(PDB: 9K0C)	(PDB: 9K0N)	(PDB: 9K0O)	(PDB: 9K1F)	(PDB: 9K1H)	(PDB: 9K1I)	(PDB: 9K1X)	(PDB: 9K1V)	(PDB: 9K21)	(PDB: 9K1Z)
Favored	97.09	97.86	97.35	97.02	97.72	97.18	97.32	97.18	97.99	97.32	97.72
Disallowed (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CaBLAM values	1.07	0.81	0.94	1.10	0.68	0.95	0.95	1.08	0.95	0.68	1.08

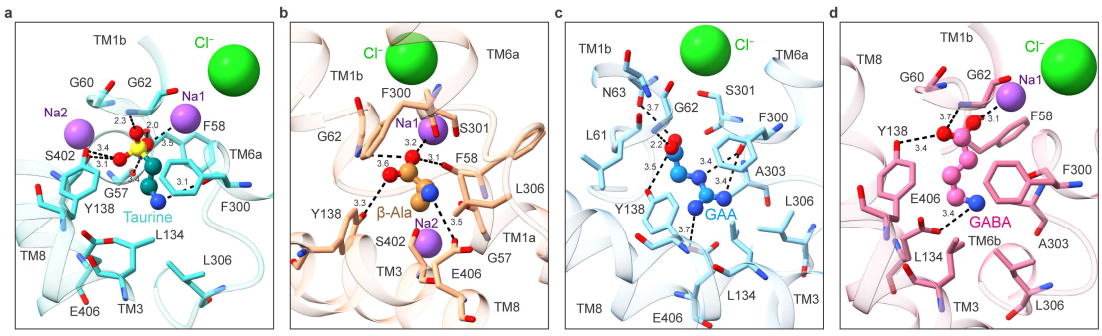
Authors' Response Fig. 1 Cryo-EM validation statistics including the Ramachandran plots and CaBLAM values.

There are also still a few Ramachandran outliers in the models that should be corrected. I can recommend using ISOLDE (https://tristanic.github.io/isolde/) and coot in parallel (real space refinement in coot, reducing the refinement weight value) and use the input model's restraints in Phenix refinement for the final refinement stage. This should help the authors to improve the model values.

Response: We thank you for your valuable suggestions regarding the model refinement statistics. Following your suggestion, we have adjusted the value of Ramachandran outliers to zero. The corresponding Ramachandran plots are shown in Authors' Response Fig. 2. Additionally, we have revised the distances labelled in Authors' Response Fig. 3.



Authors' Response Fig. 2 Ramachandran plots of structures of TauT.



Authors' Response Fig. 3 Taurine and taurine analogues binding to TauT. a-d, Interactions of taurine (a), taurine analogues: β-Alanine (b), guanidinoacetate (c), and γ-aminobutyric acid (d) with residues in the central binding site of TauT. Interaction distances are represented as dashed lines with values representing angstroms.

Other than the general quality of the models that need to be improved, the beta-Ala pose needs be revisited, it appears the amino group direction is not correct (please check more carefully the cryo-EM density).

Response: We apologize for the discrepancy between the beta-Ala pose and the cryo-EM density. We have revisited the amino group direction of beta-Ala, making it better match the density.

Reviewer #4 (Remarks to the Author):

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

Response: We thank the reviewer ***#4*** for the suggestions.

Response to reviewer's comments

We deeply appreciate the insightful and constructive comments provided by the reviewers and editor. We have made point-by-point responses as below. To ensure clarity, the reviewer's criticisms and/or suggestions are shown in dark red, whereas our responses are shown in dark blue. The text in the manuscript has also been revised accordingly and is highlighted with a yellow background. We hope that our responses can address the reviewers' concerns and our revised manuscript is suitable to be published in *Nature Communications*.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed my concerns. I have no further comments.

Response: Thank you for your positive feedback.

Reviewer #4 (Remarks to the Author):

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

Response: Thank you for your positive feedback.