nature portfolio

Peer Review File

Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1

Corresponding Author: Dr Jing-Xiang Wu

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 several structures of the betaine/GABA transporter in different conformations.

The cryoEM data appears to be well processed and the map resolution is reasonable for the resolution that has been reported.

I have a few issues that I would like to see the authors address in a revised manuscript:

There is very limited functional characterization of the transporter based on the structural analysis, I only see a few mutants and uptake experiments that were conducted. I would like to see more functional analysis of the protein based on the structures.

The modeling of Na3 seems to be precarious at best. The density is very weak and without any functional experiments to support its placement, thus I would not model this or make it to be a major conclusion of the manuscript.

The BPDBPA binding site is very interesting but looking closely at the modeling of the inhibitor, I can imagine that if it were rotated by 180 degrees it might also fit the density well and make similarly reasonable interactions. Can the authors perform such an analysis? It would be useful to make sure that the reported pose is the correct pose.

Finally the writing of the manuscript is fairly rough in places, if the authors could improve sentence structure and phrasing that would improve the quality of the manuscript.

Reviewer #2

(Remarks to the Author)
The manuscript by Zhou and co-workers on Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1 provides an excellent description of the structure of the BGT-1 transporter bound to various drugs. The BGT-1 transporter is a member of the GABA transporter sub-family of SLC6 transporters of which the structures of a number of the transporters have been determined over the last 15-20 years.
The novelty of this work lies in the description of potential drug binding sites because inhibitors of BGT-1 transporters have the potential to be developed into anti-epileptic drugs. Structures are presented for BGT-1 in the apo state and with the substrates GABA and betaine, the competitive inhibitor ATPCA and the allosteric inhibitor BPDBA.
Issues of concern:
1.The determination of the structures of BGT-1 appear to be expertly carried out and all the expected features of a SLC6 transporter are confirmed, including a descriptions of the substrate and competitive inhibitor site. The description of the allosteric inhibitor binding site is novel and has the potential to provide important leads in drug discovery.
The allosteric inhibitor binding site for BPDBA is novel with an unusual location – being below the substrate binding site, which raises an import question that has not been addressed – namely how does the inhibitor access its binding site? The BPDBA is a very hydrophobic molecule and it is conceivable that it crosses the cell membrane and accesses the site from the intracellular side of the transporter. If this work is to be of value for the drug discovery field then this mechanism of access needs to be established. This could be achieved by a combination of molecular dynamics simulations. First, to establish

whether the molecule preferentially enters cell membranes. Second, this could be followed up with course grain molecular dynamics simulations followed up with atomic level molecular dynamics, and I would recommend that this be done. Alternatively, if the drug accesses the site via the external vestibule and substrate binding site, then this too could be addressed by MD simulations. It would also be useful to follow up with a more detailed analysis of the kinetics of drug action in a cellular system to confirm the mechanism of drug access. Without this mechanism of access data, the information that can be gained from a description of the allosteric site will be limited.
2. There is also a suggestion that the BGT-1 transporter contains 3 Na+ binding sites, which is unusual for SLC6 transporters. The only transporter in the SLC6 family where this has been definitively demonstrated is GlyT2 and the published evidence for 3 Na+ ions for BGT-1 is at best cursory (Matskevitch et al., JBC, 1999) and needs to be done using more definitive methodology as used for GlyT2 (Roux and Supplisson, Neuron, 2000). If the authors want to make the claim that there are 3 Na+ for BGT-1 then they need to back this up with convincing experimental evidence. The description of the 3rd Na+ binding site would also benefit from MD analysis to demonstrate that the binding of Na+ is possible at this site.

Reviewer #3

(Remarks to the Author)
Reviewer comments

The authors of the manuscript “Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1” presents models of hBGT1 in apo state as well as bound to the two substrates GABA and betaine, the substrate-like inhibitor ATPCA and the allosteric inhibitor BPDBA. The BPDBA structure is intriguing as it identifies a novel allosteric pocket in the SLC6 subfamily, and will surely be of great interest to the field, but unfortunately as the currently manuscript stands, it lacks important functional validation. For the manuscript to be suitable for publication in Nature Communications a number of concerns should be addressed.

General comments
The source of compounds, in particular the non-commercially available ligands ATPCA and BDPBA, is absent. Analytical assessment including chemical identity and purity of the compounds as well as basic pharmacological assessment is indispensable before publication. A Materials section needs to be added to the Methods section describing this fully and documentation included. With regards to the structural work, various refinement is needed. Also, a major concern is the low extent of functional studies to validate the structural work. All over, This challenges what firm conclusions can be drawn. A specific list of items are given below which, for clarity, is divided between structural biology and pharmacology.

Xinjing Chen is mentioned in the “Author contributions” but is not listed as an author (or in the acknowledgements?

Major comments
Structural biology: The authors are overall commended for their structural work, particularly for the use of wildtype human BGT1 without any fiducial markers.
• The unsharpened maps should be provided for evaluation.
• Please report the CaBLAM values of all models in the extended data table 1 (should be below 1% to be acceptable)
• The reported Clash scores are high (extended data table 1), please work on improving the models
• Remove the NAGs from the models, there is no density to support modelling glycosylation
• There are several Ramachandran outliers in all the models, please correct them.

Specific comments to individual structures
Apo structures:
Apo-occluded map is extremely noisy, please revisit the processing and refinement.

GABA-bound BGT1:
There is no signal for Na ion at the Na1 site to be modelled in GABA-bound BGT1. Rotamers of residues S327 and S330 coordinating Cl should be corrected.

Betaine-bound BGT1
Should be “re-processed” as the map is showing strong artefacts. It is not possible to comment on this model and map at its current situation.

ATPCA-bound BGT1
At r.m.s.d. level of 4.5 where the noise from the micelle is removed, there is no density for water molecule or Na1, please either improve the density or remove.
Is D394 coordinating Na2? Please correct the rotamer
There is no signal for a Na ion or the water molecule at the proposed Na3 site. Any known or observed uncertainties should be laid forward.

Revisit the residue rotamers for proper Cl coordination

BPDBA-bound BGT1
Density around the compound is ambiguous and noisy. General modelling is also poor. Can the compound be modelled in

two different poses? The rotamers of residues S294 and S327 and N58 coordinating Cl ion should be corrected.

Furthermore, there are several comments to the figures – see list below.

Pharmacology
Improved pharmacological assessment and data analysis is warranted.
• There is no validation of the functionality of the cryo-EM construct. In case the data in Figure 2 is from the GFP-MBP tagged hBGT1, this should be stated clearly and compared to a non-tagged BGT1 wt construct. In relation to this, there is a complete lack of error estimations (± s.e.m. or CI) throughout the manuscript, making judgement of the claims like "similar to previously reported" very difficult.
• No pharmacological assessment of ATPCA is shown. This should be included.
• It sounds implausible that the transporter adopts an occluded conformation prior to ATPCA binding, since both extra- and intracellular pathway to the ATPCA binding site are occluded? It sounds more likely that this is just one of the conformations that the transporter naturally resides in, in the absence of ligand. Some comment on this should be included in the manuscript.
• For the substrate binding site a limited mutagenesis study of the E52 residue is performed but only a single concentration of d6-GABA (2 µM) is used. Given previous studies on this residue (PMID: 32747622) it would be relevant to obtain potencies (i.e. provide full curves) for at least GABA as this was reported to be potentiated by the E52Y mutant. Couldn't the reduced uptake simply be a result of reduced expression? Care should be taken not to overinterpret the data.
• The binding site of BPDBA is indeed very interesting, but it would be much more convincing with point mutations of interactions with the phenyl ring / π-cage (which should not be involved in substrate binding/transport) alike what was performed with E52A/Y.
• There is a complete lack of both experimental and discussion about how BPDBA reaches its binding site. Is it through the transport cycle (binding from outside) in a process similar to that proposed for GAT1 and tiagabine or is it binding from the cytosolic side? In Extended Fig. 8 it is indicated that BDBPA is going through the membrane but this is not commented on in the text. This aspect should be discussed in the text, and experiments to support the hypothesis provided.
• The authors suggest that the previous reported lowering of Km by BPDBA could be due to "locking" the transporter in a binding capable, but transport-incapable state. This could relatively easy be tested experimentally by preincubation with BPDBA and then measuring GABA binding.
• A major follow-up study was conducted on BDPBA and 71 analogues (PMID: 28991462) following the cited Kragholm paper, but seems to have been neglected by the authors. In this paper a prediction for an allosteric site was actually proposed which is vastly different from the one presented here. It would be interesting to compare and provide the needed validation. Can the previous site be refuted?
• A lot of effort is put into comparing the BGT1 structure to GAT1, especially in relation to the E52 and Q299 residues. Why was GAT3 not chosen since this is more close to BGT1 in overall sequence identity?

Perspectivation
• It is unusual for a SLC6 transporter to have two endogenous substrates (betaine and GABA). Have you considered the physiological relevance of this?
• Given the identification of a novel allosteric pocket would this potentially enable structure-based design of PAMs?

Minor comments

Comments related to figures:
- Provide n's for all pharmacology data
- Fig. 1c can Na and Cl atoms be made more clearly visible?
- Fig. 2c shouldn't GABA be depicted as a twitter ion?
- Fig 3 Please show with interactions with dashed lines
- Fig. 4b a hydrogen is missing in the BPDBA structure (the amide NH)
- Extended Fig. 1c Is this conducted with purified protein? Reference is missing in the main text
- Extended Data Fig. 1c "toatal" should be "total".
- Extended data Fig. 2 please show the representative good 2D classes. It is not possible to distinguish the particles in the representative micrograph
- Extended data Fig 3 Processing and refinement need to be revisited as artefacts are reflected in GSFSC graphs
- Extended Data Fig. 8c supported by relevant text, this figure would be valuable as a main figure.

Comments related to text:
- Abstract – add that no fiducial markers were used in the structural work
- Line 41 Sentence says "Recent studies", but only two studies are mentioned and one of them is from 2013 so not really recent. Please adapt wording.
- Line 53-55 BGT1 is reported to mainly regulate extrasynaptic GABA levels, presumably more than synaptic levels mentioned here. Should be included and referenced accordingly.
- Line 68 "growing" interest? It seems that not much has happened in recent years so this appears as an overstatement. Please adapt
- Line 79 along the same lines, compounds may have therapeutic potential. Maybe more safe to say 'as lead structures'?
- Line 87-88 "reveal … conformational transitions along the transport cycle." Is a little overstated. Please tone this down as only "half" the transitions of the cycle is presented.
- Line 98-99 "hBGT1 exhibited favourable biochemical properties when solubilized in lauryl maltose neopentyl glycol (LMNG) supplemented with cholesteryl hemisuccinate (CHS)". Please better explain what "favourable biochemical

properties" means.
- Line 106 Resolution for apo structures does not correlate with what is reported elsewhere in the manuscript.
- Line 124 was part of EL2 also not modelled? Indicated by the small dashed line in Fig. 1?
- Line 130 No error estimation is presented in the text or figure legend. Please provide ± or confidence interval.
- Line 159 Based on distance between the carbonyl oxygen and the hydrogen of the quaternary ammonium group, one should be able to determine if it can be classified as either an electrostatic interaction or a salt bridge. A salt bridge is also an electrostatic interaction.
- Line 179-183 Selection of hGlyT1, hTauT, and hNET for comparison should be explained better. Why were these transporters best for comparison of substrate binding site? Also, mentioning GlyT2 here seems unnecessary.
- Line 182 It says that ATPCA is an inhibitor. Please correct.
- Remove 's' in Discussion headline
- Line 190-195 Without better explanation of why this is important, it just seems like a lot of words (and a figure) to say NET binds noradrenaline differently.
- Line 200 Please include error parameters.
- Line 229 When stating that BGT1's Km for GABA is comparable to previously reported (26µM), you cannot simultaneously state that that of ATPCA (21 µM) is lower than GABA. Either your presented Km for GABA of 53 µM is higher than previous or GABA and ATPCA are the same.
- Line 291-292 "…preventing its transition to the inward-facing conformation and impairing the substrate binding." This sounds like the substrate binding occurs at the inward facing conformation.
- Line 299-300 "Despite high sequence conservation between GAT1-3 and BGT1, their responses to BPDBA differ significantly". Please provide more detail for this statement.
- Line 308-309 "These features likely explain the lack of BPDBA inhibitory activity against GAT1 (Extended Data Fig. 7a, b)". The figure shows structure, nothing about activity.
- Line 343-345 The area of the putative sodium 3 site in the local resolution maps on Extended data Fig. 3c looks to be among the best resolved, yet it is stated that there are limitations local resolution at this specific spot. Please clarify and provide zoom in view of the local resolution as well as overlay of cryo-EM map to show the position.
- Line 362-363 There is no figure showing any movement of EL4?
- Line 393-395 It appears implausible that the transporter adopts an occluded conformation prior to ATPCA binding, since both extra- and intracellular pathway to the ATPCA binding site are occluded? Isn't it much more likely that this is just one of the conformations that the transporter naturally resides in, in the absence of a ligand.
- Line 494 + 496 2 million cells per well in both 6-well and 96-well plate. That must be a mistake?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I have no further concerns, I thank the authors for responding carefully to each of my comments.

Reviewer #2

(Remarks to the Author)
The revised version of this manuscript is significantly improved compared to the original submission with the inclusion of important preliminary MD analysis and some attempts to better characterize the functional properties of the transporter. However, there still remains an issue that requires further work.
1. The functional characterization of the transporter has been done in an usual way by relying on liquid chromatography and mass spec to track the uptake of labelled GABA and betaine instead of the more traditional radiolabelled uptake methods or electrophysiological methods. The results obtained in this study are significantly different to previous values reported for uptake and inhibition. For example the IC50 for ATPCA inhibition of GABA uptake is 260 uM compared to previousl;y published values of 2.5 uM (ref 23) and whilst small variations do occur between different assays - the difference here (2 orders of magnitude) requires further investigation and clarification. Isuggest that similar uptake assays to that used in previous studies be used to allow direct comparison with previous work.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily responded to most comments and the manuscript has vastly improved. Nicely done! Only two things remain:

1) There is a poor/discontinuous density for BPDBA – Fig. 4b - this may be due to the flexibility of the ring but should be duly addressed.

2) In the pharmacological analysis of ATPCA (Fig. 2b) it is highly surprising that the Ki is so high (almost 10x right-shifted) compared to previous reports, whereas GABA is more close to the expected. What is the reason for this? How may this influence the results and interpretation?

Reviewer #4

(Remarks to the Author)

General comments: the study “Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1” by Zhuo et al. resolves a number of structures with kinetic measurements to elucidate the molecular basis for hGBT1 transporters. The authors made a significant effort to address key concerns from reviewers such as many molecular dynamic simulations aimed to understand the dynamics and binding of BPDBA.

The study solved structures for basically four substates, betaine, GABA, ATPCA and BPDBA. It turns out the results of BPDBA seem the most interesting, but the introduction sounds weak and not focused on BPDBA.

The title was about substrate recognition, but the Discussion section, in my opinion, does not elaborate and further discuss the implications of the substrate recognition mechanism. I am still not sure what the key take-away messages for the substrate recognition mechanisms are. The mechanisms may be different when ATPCA or BPDBA is present. Of course, the hydrogen networks, binding moieties are discussed in the results, but it might be good to summarize the results in Discussion.

In Figure 5, a schematic for the reaction coordinate used in simulations may be extremely helpful.

Why was the measurement of ATPCA uptake not reported in K_m value for comparison with GABA and betaine? I am not sure how the binding affinity of ATPCA, GABA and betaine can be compared?

One reason that the investigation of ATPCA was performed is “ATPCA, a bioisosteric analog developed to mimic the amino group of β-alanine or GABA, was originally reported to act both as a selective inhibitor and a transportable substrate of human betaine/GABA transporter 1”, I think it would be more convincing if the authors elaborate a bit more in this paragraph about the chemistry relevance of ATPCA in comparison with betaine.

There is a room for improvement in figure 7 summarizing the results. I think since the structures of all substrate-bound GPT1 are solved, I think RMSD, labels of occluded, inward-facing structures should be more relevant than those of ATPCA-bound or BPDBA bounds.

Since this study is overwhelmed with a number of substrate-bound structures for a substrate recognition mechanism, it might help readers if there is a table summarizing RMSD and key features of those structures.

Overall, I think one more revision would help to make this study clearer and more valuable to the field.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In the previous review of this manuscript, I pointed out the two orders of magnitude difference between the IC50 value for ATPCA reported in this paper compared to previous papers. The revised manuscript has ignored this comment and even tried to play down the significance of an IC50 value. The small change in IC50/Km values that are generated when using the Cheng Prusoff equation do not in any way provide a reasonable explanation for the differences. An IC50 of 238 uM is still ~ 2 orders of magnitude different and cannot be explained by different experimental conditions. I recommend that the experiments be conducted in a way that allow direct comparison with previous results so that we can be confident in the results presented. If the authors do not wish to do radiolabelled uptake assays, they could also do two electrode voltage clamp recordings from Xenopus laevis oocytes to get direct meeasures of transport as outlined in: Matskevitch et al., (1999) Journal of Biological Chemistry 274, 16709-16716 Functional Characterization of the Betaine/γ-Aminobutyric Acid Transporter BGT-1 Expressed in Xenopus Oocytes.

Reviewer #3

(Remarks to the Author)
The authors have responded carefully to remaining comments. I have no further comments.

Reviewer #4

(Remarks to the Author)
The revision has addressed my concerns, and improved the clarity.

Version 3:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
I am happy with the additions to the manuscript

Point-to-point response to reviewer comments

We sincerely thank the editors and reviewers for their careful evaluation of our manuscript and for the insightful and constructive comments. These suggestions have been invaluable for improving the clarity, rigor, and overall quality of the work. In response, we have revised the manuscript extensively, incorporating additional analyses and new data where appropriate, and have clarified the scope and interpretation of our conclusions. All comments raised by the reviewers have been carefully considered and addressed in a point-by-point manner below.

Reviewer #1 (Remarks to the Author):

This manuscript describes several structures of the betaine/GABA transporter in different conformations.

The cryoEM data appears to be well processed and the map resolution is reasonable for the resolution that has been reported.

Response: We thank the reviewer for the careful evaluation of our manuscript and for the constructive comments. We have addressed each point as detailed below.

I have a few issues that I would like to see the authors address in a revised manuscript:

Comment 1:

There is very limited functional characterization of the transporter based on the structural analysis, I only see a few mutants and uptake experiments that were conducted. I would like to see more functional analysis of the protein based on the structures.

Response: We thank the reviewer for this important comment regarding functional validation.

In the revised manuscript, we have expanded the functional characterization of hBGT1 in a structure-guided manner to provide more direct experimental support for the structural observations.

First, we quantified apparent transport kinetics for endogenous substrate betaine under identical experimental conditions (Fig. 2a). These analyses yielded apparent K_m and V_{max} values.

Second, we extended the structure-guided mutational analysis of residues implicated in substrate recognition by introducing additional mutations at key positions within the substrate-binding pocket (Fig. 2e). In particular, we performed full kinetic characterization of

the E52 mutant, determining complete apparent K_m and V_{max} curves for GABA uptake, which highlights the central role of this residue in coordinating the cationic moieties of transported substrates (Fig. 2f).

Third, we supplemented the functional analysis of the substrate-like inhibitor ATPCA by quantifying its inhibitory potency on GABA uptake, determining IC_{50} values and deriving an apparent K_i under defined conditions (Fig. 3a).

Fourth, to evaluate the functional consequences of allosteric inhibition, we performed GABA transport kinetics for wild-type hBGT1 in the presence of BPDBA (Extended Data Fig. 7a). These measurements reveal a predominant reduction in apparent transport turnover with minimal effects on the apparent Km for GABA, consistent with an allosteric mode of inhibition.

Finally, we functionally tested residues identified from the BPDBA-bound structure by mutational analysis. Mutation of C402, a residue involved in stabilizing interactions with BPDBA, led to a modest increase in the BPDBA IC_{50}, indicating that this residue contributes to BPDBA inhibition and providing functional support for the structurally defined allosteric binding pocket (Fig. 4e).

Together, these complementary uptake, kinetic, and mutational analyses were designed to validate and contextualize the structural findings rather than to provide an exhaustive pharmacological characterization of all transport properties of hBGT1. We have clarified this scope explicitly in the revised manuscript.

Comment2:

The modeling of Na3 seems to be precarious at best. The density is very weak and without any functional experiments to support its placement, thus I would not model this or make it to be a major conclusion of the manuscript.

Response: We thank the reviewer for this comment and agree that the available data do not support confident modeling of an additional sodium-binding site at this position.

Upon re-evaluation of the cryo-EM maps, we consider the density in this region to be insufficient for assigning a defined Na^+ ion, particularly in the absence of supporting functional evidence. Accordingly, we have removed any modeling, description, or interpretation of a Na3 site from the Results and no longer present it as a defined ion-binding position in the manuscript.

The region is now mentioned only briefly in the Discussion, where it is explicitly framed as a highly tentative and speculative observation discussed in the broader context of recent structural and functional studies on GlyT2 within the SLC6 family (Lines 521-530). We

explicitly note the limitations of the current data and refrain from drawing any functional conclusions regarding a Na3 site in hBGT1.

Comment3:

The BPDBPA binding site is very interesting but looking closely at the modeling of the inhibitor, I can imagine that if it were rotated by 180 degrees it might also fit the density well and make similarly reasonable interactions. Can the authors perform such an analysis? It would be useful to make sure that the reported pose is the correct pose.

Response: We thank the reviewer for this insightful suggestion regarding the binding pose of BPDBA.

We agree that in earlier iterations of the cryo-EM map, local density quality in the BPDBA-binding region was limited and could, in principle, allow ambiguity in ligand orientation. To address this concern, we performed additional data reprocessing and refinement, resulting in a substantially improved map with enhanced local resolution and reduced noise in the inhibitor-binding pocket. BPDBA was subsequently re-modeled into this refined density.

In the updated structure, the overall position of BPDBA undergoes only minor adjustment relative to earlier models, while its orientation is preserved: the dichlorophenyl ring is directed toward the protein core, and the benzyl group extends toward the membrane-proximal cavity. Importantly, the density corresponding to the dichlorophenyl ring is now well resolved, with distinct features attributable to both chlorine substituents, providing direct, orientation-specific density support for the reported pose (Fig. 4b).

We additionally tested an alternative model in which BPDBA was rotated by 180 degrees within the pocket. This orientation resulted in pronounced steric clashes with residues in IL2 and failed to account for the observed density, indicating that it does not represent a physically plausible binding mode.

Finally, to evaluate the stability of BPDBA within the intracellular pocket, we next performed unbiased conventional MD simulations starting from the BPDBA-bound structure (Fig. 5c). Consistent with these structural observations, molecular dynamics simulations initiated from the experimentally observed pose showed stable retention of BPDBA within the pocket, whereas alternative orientations were not supported (Fig. 5).

Together, the improved cryo-EM density, steric constraints imposed by the pocket architecture, and independent MD analyses collectively support the binding pose reported in the manuscript.

Comment4:

Finally the writing of the manuscript is fairly rough in places, if the authors could improve

sentence structure and phrasing that would improve the quality of the manuscript.

Response: We thank the reviewer for this critical feedback regarding the clarity and phrasing of the manuscript. We have carefully revised the manuscript to improve clarity, sentence structure, and overall readability throughout the text.

Reviewer #2

The manuscript by Zhou and co-workers on Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1 provides an excellent description of the structure of the BGT-1 transporter bound to various drugs. The BGT-1 transporter is a member of the GABA transporter sub-family of SLC6 transporters of which the structures of a number of the transporters have been determined over the last 15-20 years.

The novelty of this work lies in the description of potential drug binding sites because inhibitors of BGT-1 transporters have the potential to be developed into anti-epileptic drugs. Structures are presented for BGT-1 in the apo state and with the substrates GABA and betaine, the competitive inhibitor ATPCA and the allosteric inhibitor BPDBA.

Response: We thank the reviewer for the careful reading of our manuscript and for the positive and constructive comments. We address the specific points raised by the reviewer below.

Issues of concern:

Issue 1:

The determination of the structures of BGT-1 appear to be expertly carried out and all the expected features of a SLC6 transporter are confirmed, including a descriptions of the substrate and competitive inhibitor site. The description of the allosteric inhibitor binding site is novel and has the potential to provide important leads in drug discovery.

The allosteric inhibitor binding site for BPDBA is novel with an unusual location – being below the substrate binding site, which raises an import question that has not been addressed – namely how does the inhibitor access its binding site? The BPDBA is a very hydrophobic molecule and it is conceivable that it crosses the cell membrane and accesses the site from the intracellular side of the transporter. If this work is to be of value for the drug discovery field then this mechanism of access needs to be established. This could be achieved by a combination of molecular dynamics simulations. First, to establish whether the molecule preferentially enters cell membranes. Second, this could be followed up with course grain molecular dynamics simulations followed up with atomic level molecular dynamics, and I would recommend that this be done. Alternatively, if the drug accesses the site via the

external vestibule and substrate binding site, then this too could be addressed by MD simulations. It would also be useful to follow up with a more detailed analysis of the kinetics of drug action in a cellular system to confirm the mechanism of drug access. Without this mechanism of access data, the information that can be gained from a description of the allosteric site will be limited.

Response: We thank the reviewer for raising this important question regarding how BPDBA accesses its intracellular allosteric binding site.

To address this point, we performed a series of molecular dynamics simulations to assess BPDBA membrane association, potential access pathways, and binding stability (Fig. 5; Extended Data Fig. 8). First, in unbiased simulations with multiple BPDBA molecules initially placed in the extracellular solvent, analysis of ligand trajectories revealed a reproducible redistribution of BPDBA positions toward the membrane along the bilayer normal, resulting in enrichment near the membrane interface rather than isotropic diffusion in bulk solvent, consistent with its hydrophobic nature (Fig. 5a, b).

Second, starting from the BPDBA-bound cryo-EM structure, conventional MD simulations showed stable retention of BPDBA within the intracellular pocket while allowing local rearrangements of the ligand and surrounding residues (Fig. 5c, d; Extended Data Fig. 8a). To explore feasible exit or access routes, we further applied enhanced-sampling simulations, including well-tempered metadynamics, together with independent steered MD trajectories. Across these simulations, BPDBA reproducibly sampled a lateral, membrane-facing pathway near the TM4–TM5-TM8–IL2 region, rather than routes involving passage through the extracellular vestibule and the central substrate-binding site (Fig. 5e–i; Extended Data Fig. 8b–c). We note that under the conditions sampled, the simulations did not provide support for direct entry from bulk cytosol through the intracellular gate.

In parallel, cell-based uptake kinetics indicate that BPDBA predominantly reduces the maximal transport rate of hBGT1 with minimal changes in the apparent Km for GABA, consistent with an allosteric mode of inhibition (Extended Data Fig. 7a). While these measurements do not directly resolve the temporal sequence of inhibitor access, they are compatible with a membrane-mediated mechanism in which BPDBA is captured from a membrane-proximal environment and guided into the intracellular pocket via a lateral opening.

Together, these simulation results and functional data support a model in which BPDBA exits the bulk solvent and becomes enriched within a membrane-proximal environment surrounding the transporter, from which it can access the intracellular allosteric pocket through a lateral, membrane-facing opening. We have revised the Results and Discussion to describe these findings and to clarify their implications for structure-guided inhibitor design.

Issue 2:

There is also a suggestion that the BGT-1 transporter contains 3 Na+ binding sites, which is unusual for SLC6 transporters. The only transporter in the SLC6 family where this has been definitively demonstrated is GlyT2 and the published evidence for 3 Na+ ions for BGT-1 is at best cursory (Matskevitch et al., JBC, 1999) and needs to be done using more definitive methodology as used for GlyT2 (Roux and Supplisson, Neuron, 2000). If the authors want to make the claim that there are 3 Na+ for BGT-1 then they need to back this up with convincing experimental evidence. The description of the 3rd Na+ binding site would also benefit from MD analysis to demonstrate that the binding of Na+ is possible at this site.

Response: We thank the reviewer for this comment regarding the possibility of a third Na^+ binding site in hBGT1.

We fully agree that establishing the existence and functional relevance of a third Na^+ binding site would require rigorous experimental validation, comparable to the approaches previously applied to GlyT2, including definitive functional and/or electrophysiological measurements. In the present study, we do not seek to make a claim that hBGT1 operates with a three-Na^+ transport stoichiometry.

To avoid overinterpretation, we have removed any description or modeling of a Na3 site from the Results and do not present it as an established structural or functional feature of hBGT1. The region is now mentioned only briefly in the Discussion, where it is explicitly framed as a highly speculative observation discussed in the context of recent structural and functional studies on GlyT2 within the SLC6 family (Lines 521-530). We emphasize the limited quality of the cryo-EM density in this region and the absence of supporting functional evidence, and we refrain from drawing any conclusions regarding Na^+ stoichiometry or mechanistic roles in hBGT1.

We note the reviewer's suggestion that molecular dynamics simulations could be used to assess the feasibility of Na^+ binding at a putative third site. However, given that MD simulations alone cannot establish a bona fide Na^+ binding site or transport stoichiometry in the absence of rigorous functional validation, we chose not to pursue additional MD analysis of Na^+ binding at this site and do not advance any claims regarding a third Na^+ in hBGT1.

Reviewer #3

The authors of the manuscript "Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1" presents models of hBGT1 in apo state as well as bound to the two substrates GABA and betaine, the substrate-like inhibitor ATPCA and the allosteric inhibitor BPDBA. The BPDBA structure is intriguing as it identifies a novel allosteric pocket

in the SLC6 subfamily, and will surely be of great interest to the field, but unfortunately as the currently manuscript stands, it lacks important functional validation. For the manuscript to be suitable for publication in Nature Communications a number of concerns should be addressed.

Response: We thank the reviewer for the thorough evaluation of our manuscript and for recognizing the potential interest of the BPDBA-bound structure and the identification of a novel allosteric pocket within the SLC6 family. We appreciate the constructive comments and address the specific concerns raised below.

General comments:

The source of compounds, in particular the non-commercially available ligands ATPCA and BDPBA, is absent. Analytical assessment including chemical identity and purity of the compounds as well as basic pharmacological assessment is indispensable before publication. A Materials section needs to be added to the Methods section describing this fully and documentation included. With regards to the structural work, various refinement is needed. Also, a major concern is the low extent of functional studies to validate the structural work. All over, This challenges what firm conclusions can be drawn. A specific list of items are given below which, for clarity, is divided between structural biology and pharmacology.

Xinjing Chen is mentioned in the "Author contributions" but is not listed as an author (or in the acknowledgements?

Response: We thank the reviewer for raising these important points regarding compound documentation, structural refinement, functional validation, and authorship clarity.

In the revised manuscript, we have added a dedicated subsection in the Methods describing the source and supplier-reported chemical identity and purity of all compounds used in this study, including unlabeled ligands (GABA, betaine, ATPCA and BPDBA) as well as the stable isotope–labeled tracers/standards used for uptake quantification (Lines 621-627). The relevant information provided by the commercial supplier is now included to ensure transparency and reproducibility.

With respect to the structural work, we have revisited data processing and model refinement for all cryo-EM datasets (Extended Data Fig. 3). Atomic models were further refined to improve geometry and overall model quality, with updated validation statistics reported in the revised manuscript (Extended Data Table. 1). In addition, unsharpened maps have been made available as part of the revision materials to facilitate assessment of the underlying density.

To better contextualize the structural observations, we have expanded the set of structure-guided functional measurements, including additional uptake analyses, transport kinetics, and quantitative assessment of inhibitor effects (Figs. 2-4; Extended Data Fig. 7a). These

experiments are intended to support and interpret the structural findings within the scope of the present study, rather than to provide a comprehensive pharmacological characterization.

Finally, we have addressed the authorship-related inconsistency noted by the reviewer. Xinjing Chen is no longer listed under Author Contributions and is now appropriately acknowledged for her contributions during early-stage construct cloning and screening.

Major comments

Structural biology:

The authors are overall commended for their structural work, particularly for the use of wildtype human BGT1 without any fiducial markers.

• The unsharpened maps should be provided for evaluation.

• Please report the CaBLAM values of all models in the extended data table 1 (should be below 1% to be acceptable)

• The reported Clash scores are high (extended data table 1), please work on improving the models

• Remove the NAGs from the models, there is no density to support modelling glycosylation

• There are several Ramachandran outliers in all the models, please correct them.

Response:

We thank the reviewer for the positive assessment of our structural work and for the constructive suggestions aimed at further improving model quality and validation.

- Provide unsharpened maps: In the revised submission, unsharpened cryo-EM maps for all structures have been made available to facilitate independent evaluation of the underlying density.

- Report CaBLAM Statistics: We have also updated Extended Data Table 1 to include CaBLAM statistics for all atomic models, which are now within acceptable ranges in all cases.

- Reduction of High Clash Scores: To address concerns regarding model geometry and stereochemical quality, we revisited model building using a combination of manual corrections and iterative refinement procedures. This comprehensive re-evaluation resulted in a substantial overall improvement in model quality, including reduced clashscores and improved backbone geometry (Extended Data Table. 1).

- Removal of Unsupported N-linked Glycans (NAGs): Given the absence of well-defined density supporting glycosylation, all N-linked glycans (NAGs) have been removed from the final models.

- Correction of Ramachandran Outliers: All previously identified Ramachandran outliers have been corrected through manual rebuilding and subsequent refinement. The updated models now have 0% outliers, with >97% of residues in the favored regions. These improved statistics are included in Extended Data Table 1.

As a result of these revisions, the stereochemical parameters of all models, including Ramachandran and CaBLAM statistics, show clear improvement (Extended Data Table. 1). In addition, overall model–map agreement has improved, as reflected by enhanced real-space fit metrics, and is now consistent with the resolution and quality of the cryo-EM maps.

Specific comments to individual structures

Apo structures:

Apo-occluded map is extremely noisy, please revisit the processing and refinement.

Response: We thank the reviewer for this comment. We have revisited the apo-occluded dataset and re-evaluated the reconstruction to improve map interpretability. Both unmasked and loosely masked reconstructions show reduced noise compared to the previous version. Based on the updated map, the corresponding atomic model was re-examined and refined to ensure consistency with the improved density.

GABA-bound BGT1:

There is no signal for Na ion at the Na1 site to be modelled in GABA-bound BGT1. Rotamers of residues S327 and S330 coordinating Cl should be corrected.

Response: We thank the reviewer for pointing this out. We have revisited the GABA-bound dataset and re-evaluated the cryo-EM map in this region. In the updated reconstruction, the density at the Na1 site is more clearly defined and is consistent with the presence of a Na^+ ion. Accordingly, the Na1 site has been re-assessed and modeled with this improved density in mind.

In addition, the rotamers of residues S327 and S330 coordinating the Cl^- ion have been corrected. Together, these adjustments lead to improved agreement between the atomic model and the experimental density, as well as overall improvements in model geometry.

Betaine-bound BGT1

Should be “re-processed” as the map is showing strong artefacts. It is not possible to comment on this model and map at its current situation.

Response: We thank the reviewer for this comment. The betaine-bound dataset has been reprocessed to address the observed map artefacts. The updated reconstruction shows reduced artefacts and improved overall map interpretability compared to the previous version. Based

on the revised map, the corresponding atomic model was re-evaluated, rebuilt where appropriate, and refined to ensure consistency with the improved density. As a result, the revised betaine-bound structure now shows improved agreement between the atomic model and the cryo-EM map, as well as overall enhanced model quality.

ATPCA-bound BGT1

At r.m.s.d. level of 4.5 where the noise from the micelle is removed, there is no density for water molecule or Na1, please either improve the density or remove.

Is D394 coordinating Na2? Please correct the rotamer

There is no signal for a Na ion or the water molecule at the proposed Na3 site. Any known or observed uncertainties should be laid forward.

Revisit the residue rotamers for proper Cl coordination.

Response: We thank the reviewer for these detailed comments. We have revisited the ATPCA-bound dataset and re-evaluated the corresponding cryo-EM map.

In the updated reconstruction, the density at the Na1 site is consistent with modeling of a Na^+ ion at r.m.s.d. levels where micelle-related noise is reduced, whereas the previously modeled water molecule is not supported by the density and has therefore been removed.

Density consistent with Na^+ coordination at the Na2 site is observed in the revised map, and the rotamer of D394 has been corrected accordingly.

With respect to the previously proposed Na3 region, no convincing density supporting placement of a Na^+ ion or associated water molecule is observed in the revised map. To avoid overinterpretation, these species have been removed from the atomic model, and no conclusions are drawn regarding the presence or functional relevance of a third Na^+ binding site in the ATPCA-bound structure.

Residue rotamers involved in Cl^- coordination have also been revisited and adjusted where necessary to ensure proper geometry and consistency with the experimental density.

BPDBA-bound BGT1

Density around the compound is ambiguous and noisy. General modelling is also poor. Can the compound be modelled in two different poses? The rotamers of residues S294 and S327 and N58 coordinating Cl ion should be corrected.

Response: We thank the reviewer for this comment. We have revisited the BPDBA-bound dataset and re-evaluated the corresponding cryo-EM map and atomic model. As part of this process, overall model quality and geometry were carefully reassessed and improved. Following reprocessing and refinement, the local density in the BPDBA-binding region is

improved, allowing a more reliable assessment of ligand placement and surrounding side-chain conformations.

In the revised structure, BPDBA adopts an orientation consistent with our previous model, with the dichlorophenyl ring positioned toward the protein interior and the benzyl moiety extending toward the membrane-proximal cavity. The density for the dichlorophenyl group is better defined in the updated map, supporting this orientation within the limits of the local resolution.

We also evaluated an alternative model in which BPDBA was rotated by 180° within the pocket. This orientation is incompatible with the observed density and results in steric clashes with residues in the IL2 region, and was therefore not considered a plausible binding mode.

In addition, molecular dynamics simulations initiated from the experimentally observed pose show stable retention of BPDBA within the pocket, providing independent support for the modeled orientation (Fig. 5). Finally, the rotamers of residues S294, S327, and N58 involved in Cl^- coordination have been revisited and corrected to ensure proper geometry and consistency with the experimental density.

Pharmacology

Improved pharmacological assessment and data analysis is warranted.

• There is no validation of the functionality of the cryo-EM construct. In case the data in Figure 2 is from the GFP-MBP tagged hBGT1, this should be stated clearly and compared to a non-tagged BGT1 wt construct. In relation to this, there is a complete lack of error estimations (± s.e.m. or CI) throughout the manuscript, making judgement of the claims like "similar to previously reported" very difficult.

Response: We thank the reviewer for this comment. In the revised manuscript, we have added explicit validation of the functionality of the cryo-EM construct. To assess whether the N-terminal GFP–MBP fusion affects transporter function, we directly compared tagged and untagged wild-type hBGT1 under identical experimental conditions. As described in the Results (Extended Data Fig. 1c), the two constructs exhibit comparable expression levels and GABA uptake activities, indicating that the fusion tag does not substantially perturb hBGT1 function.

We have also revised the manuscript to provide quantitative uncertainty information for all functional measurements. The number of independent experiments (n) is now indicated for each dataset, and data are reported with appropriate measures of variability, including mean ± s.e.m. for uptake and kinetic analyses and mean ± s.d. for bar plots, as specified in the figure legends. These revisions allow the functional data to be evaluated based on internal consistency and experimental variability, without relying on direct comparisons to previously

reported values.

• No pharmacological assessment of ATPCA is shown. This should be included.

Response: We thank the reviewer for this comment. In the revised manuscript, we now include a functional assessment of ATPCA using cell-based GABA-d_6 uptake assays. Under the experimental conditions used in this study, ATPCA inhibits hBGT1-mediated GABA-d_6 uptake with an apparent IC_{50} of 260 µM. An estimated apparent K_i of 238 µM was derived assuming a competitive mode of inhibition (Fig. 3a). These measurements provide a quantitative functional characterization of ATPCA that complements the structural analysis presented in the manuscript.

• It sounds implausible that the transporter adopts an occluded conformation prior to ATPCA binding, since both extra- and intracellular pathway to the ATPCA binding site are occluded? It sounds more likely that this is just one of the conformations that the transporter naturally resides in, in the absence of ligand. Some comment on this should be included in the manuscript.

Response: We thank the reviewer for this insightful comment and agree with the underlying interpretation. We have revised the manuscript to clarify that the occluded-like conformation observed in the ATPCA-bound structure is not interpreted as being induced prior to ligand binding. Instead, ATPCA is now described as stabilizing a substrate-like occluded conformation that belongs to the conformational ensemble sampled during substrate engagement by hBGT1 (Lines 485-499).

As a substrate-like inhibitor, ATPCA adopts interactions that are closely related to those of endogenous substrates and stabilizes an occluded arrangement that is similar, but not identical, to the occluded conformations observed with GABA or betaine. This interpretation is consistent with a conformational selection framework acting within substrate-bound states, rather than a ligand-induced conformational transition.

• For the substrate binding site a limited mutagenesis study of the E52 residue is performed but only a single concentration of d6-GABA (2 µM) is used. Given previous studies on this residue (PMID: 32747622) it would be relevant to obtain potencies (i.e. provide full curves) for at least GABA as this was reported to be potentiated by the E52Y mutant. Couldn't the reduced uptake simply be a result of reduced expression? Care should be taken not to overinterpret the data.

Response: We thank the reviewer for this important comment. In the revised manuscript, we have expanded the functional analysis of E52 mutants by performing full concentration–response measurements for GABA uptake rather than relying on a single substrate concentration.

Kinetic analysis reveals that mutation of E52 results in a measurable increase in the apparent Km together with a reduction in Vmax, providing a quantitative description of the altered transport behavior (Fig. 2f). These changes are consistent with a direct effect of the E52 mutations on substrate recognition and transport efficiency and place the reduced uptake observed in the initial single-point assay into a kinetic framework.

Based on these full kinetic measurements, we have revised the text to reflect these findings with appropriate caution and without overinterpretation of single-concentration uptake data.

• The binding site of BPDBA is indeed very interesting, but it would be much more convincing with point mutations of interactions with the phenyl ring / π-cage (which should not be involved in substrate binding/transport) alike what was performed with E52A/Y.

Response: We thank the reviewer for this constructive suggestion. We agree that mutational analysis of residues that are not directly involved in substrate binding or transport provides an effective strategy to functionally validate a distinct allosteric binding site.

In the revised manuscript, we have added structure-guided functional validation by targeting C402, a residue located outside the central substrate-binding pocket that participates in stabilizing BPDBA within the halogen clamp subsite (Fig. 4d). Mutation of C402 resulted in modest shifts in BPDBA potency, with approximately twofold and threefold increases in IC_{50} for C402S and C402A, respectively (Fig. 4e).

The limited magnitude of these effects is consistent with a multivalent mode of BPDBA recognition, in which individual peripheral interactions contribute additively to ligand stabilization rather than serving as dominant determinants. Within the scope of the present study, these results provide functional support for a BPDBA-specific allosteric pocket.

• There is a complete lack of both experimental and discussion about how BPDBA reaches its binding site. Is it through the transport cycle (binding from outside) in a process similar to that proposed for GAT1 and tiagabine or is it binding from the cytosolic side? In Extended Fig. 8 it is indicated that BDBPA is going through the membrane but this is not commented on in the text. This aspect should be discussed in the text, and experiments to support the hypothesis provided.

Response: We thank the reviewer for raising this important point. We agree that how BPDBA reaches its intracellular binding pocket is central to understanding the mechanism of allosteric inhibition.

In the revised manuscript, we have expanded the Discussion to explicitly address potential access routes (Lines 556-560). Based on the structural location of the BPDBA-binding pocket and the physicochemical properties of the compound, we discuss three plausible access routes: (i) entry through the canonical extracellular vestibule along the substrate translocation

pathway; (ii) access from the cytosolic side through the intracellular gate; and (iii) entry via a lateral, membrane-proximal route at the protein–lipid interface.

In this context, the molecular dynamics analyses performed in this study are compatible with route (iii) (Fig. 5; Extended Data Fig. 8). In unbiased simulations, BPDBA molecules exhibit a redistribution toward membrane-proximal regions (Fig. 5a, b), and enhanced-sampling simulations starting from the bound state consistently sample a lateral, membrane-facing pathway connecting the binding pocket with the surrounding lipid environment (Fig. 5e-h). These observations correspond to the trajectories illustrated in Extended Data Fig. 8 and are now explicitly described and discussed in the main text.

While additional experimental approaches would be required to definitively establish the dominant access route in vivo, such analyses lie beyond the scope of this structure-focused study. Accordingly, we present the lateral, membrane-associated pathway as a mechanistically plausible model supported by structural context and MD simulations, rather than as a definitive transport mechanism.

• The authors suggest that the previous reported lowering of Km by BPDBA could be due to "locking" the transporter in a binding capable, but transport-incapable state. This could relatively easy be tested experimentally by preincubation with BPDBA and then measuring GABA binding.

Response: We thank the reviewer for this insightful comment. We agree that a model in which BPDBA "locks" the transporter in a binding-competent but transport-incompetent state could, in principle, be examined using substrate-binding assays performed in the presence of BPDBA.

In the revised manuscript, however, we no longer advance this mechanistic interpretation. To directly assess the effect of BPDBA under our experimental conditions, we performed additional transport kinetics measurements for hBGT1 in the presence of BPDBA (Extended Data Fig. 7a). In contrast to previous reports, BPDBA did not reduce the apparent Km for GABA, whereas a pronounced reduction in Vmax was observed, consistent with an allosteric inhibitory effect under the conditions tested.

Based on these results, we have withdrawn the proposed explanation linking BPDBA-mediated inhibition to a decrease in Km or to stabilization of a substrate-binding–competent, transport-incompetent "locked" state. Accordingly, this hypothesis is no longer discussed in the manuscript, and we do not pursue substrate-binding assays in the present study.

• A major follow-up study was conducted on BDPBA and 71 analogues (PMID: 28991462) following the cited Kragholm paper, but seems to have been neglected by the authors. In this paper a prediction for an allosteric site was actually proposed which is vastly different from

the one presented here. It would be interesting to compare and provide the needed validation. Can the previous site be refuted?

Response: We thank the reviewer for drawing attention to this important follow-up study examining BPDBA and its analogues. We agree that this work provides valuable pharmacological insight and warrants explicit discussion.

In the revised manuscript, we now discuss this study in detail and place it in the context of the structural framework presented here (Lines 531-555). The previous work combined extensive structure–activity relationship analysis with docking and homology modeling based on outward-facing transporter conformations, leading to the proposal of an interaction region within the extracellular vestibule (often referred to as the S2 site). These analyses were necessarily inference-based, given the lack of experimental transporter structures available at the time, and provided an important functional perspective on BPDBA selectivity.

In contrast, the present study is based on experimentally determined cryo-EM structures of hBGT1 and directly visualizes BPDBA bound within a previously uncharacterized intracellular cavity adjacent to the intracellular gate. Rather than refuting the previously proposed site, we interpret the apparent discrepancy as arising from differences in transporter conformational state and methodological resolution. The intracellular pocket observed here is not accessible in outward-facing models and therefore could not have been identified by earlier docking approaches.

We further note that prior SAR and chimera data are compatible with a model in which determinants of BPDBA recognition and access are distributed across multiple regions of the transporter. Within this framework, earlier studies may capture chemical features important for ligand selectivity or access under outward-facing conditions, whereas the cryo-EM structures define a stable inhibitory binding mode in the conformational states resolved in this work. We have revised the Discussion to explicitly compare these models and to clarify how the present structural data refine, rather than contradict, previous pharmacological interpretations.

• A lot of effort is put into comparing the BGT1 structure to GAT1, especially in relation to the E52 and Q299 residues. Why was GAT3 not chosen since this is more close to BGT1 in overall sequence identity?

Response: We thank the reviewer for this thoughtful question. We agree that GAT3 is more closely related to BGT1 in overall sequence identity and represents an important member of the SLC6 GABA transporter subfamily. However, our comparison strategy in this study was guided primarily by the availability of structurally and functionally comparable reference states rather than by overall sequence similarity alone.

At the time of our analysis, available GAT3 structures were predominantly resolved in inward-facing conformations, including apo or inhibitor-bound states, and lacked high-resolution substrate-bound or occluded-like conformations suitable for direct comparison with the substrate-bound states of hBGT1 presented here. More recent GAT3 structures with GABA bound likewise adopt inward-facing conformations and remain limited in resolution, which constrains state-matched analysis of substrate recognition mechanisms.

By contrast, GAT1 has been structurally characterized in multiple functional states, including well-resolved substrate-bound conformations, providing a more appropriate framework for assessing how specific residues—such as the positions corresponding to hBGT1 E52 and Q299—contribute to differences in substrate handling. In this context, GAT1 serves as a structurally informative comparator for dissecting mechanistic features of substrate recognition, rather than as a proxy for overall sequence relatedness. We have clarified this rationale in the revised manuscript (Lines 156-161).

Perspectivation

• It is unusual for a SLC6 transporter to have two endogenous substrates (betaine and GABA). Have you considered the physiological relevance of this?

Response: We thank the reviewer for raising this point. We agree that recognition of two endogenous substrates by an SLC6 transporter is relatively uncommon and warrants consideration of physiological context.

Available evidence suggests that hBGT1 functions primarily outside classical synaptic GABA clearance and is instead associated with regulation of extrasynaptic GABA levels and cellular responses to osmotic stress, conditions under which betaine serves as a compatible osmolyte (Lines 51-58). Within this framework, the ability of hBGT1 to accommodate both substrates likely reflects context-dependent functionality rather than dual high-capacity transport under the same physiological conditions.

We have added a brief discussion to clarify this point while deliberately avoiding further speculation beyond the scope of the present structural and functional data (Lines 514-517).

• Given the identification of a novel allosteric pocket would this potentially enable structure-based design of PAMs?

Response: We thank the reviewer for this forward-looking question. In principle, identification of a previously unrecognized allosteric pocket provides a structural framework that could inform future structure-based ligand design efforts.

In the present study, however, our data establish the existence, location, and inhibitory engagement of this pocket by BPDBA. We do not address whether alternative ligands could

act as positive allosteric modulators (PAMs), nor do our structures demonstrate stabilization of transport-competent states through binding at this site. Accordingly, we refrain from drawing conclusions regarding the feasibility of PAM development.

We note more generally that the intracellular, membrane-accessible nature of this pocket and its apparent subtype selectivity identify it as a structurally tractable allosteric site. This observation highlights conceptual opportunities for future exploration of allosteric modulation, including—but not limited to—PAMs, while remaining beyond the scope of the current work. The Discussion has been revised accordingly to reflect this perspective without overinterpretation (Lines 603-606).

Minor comments

Comments related to figures:

- Provide n's for all pharmacology data

Response: The number of independent biological replicates (n) is now clearly stated in all relevant figure legends (Figs. 2, 3, 4, Extended Data Fig. 1,7).

- Fig. 1c ◊ can Na and Cl atoms be made more clearly visible?

Response: The atomic representations of Na^+ (purple spheres) and Cl^- (green spheres) have been enlarged and their labels made more prominent for clarity.

- Fig. 2c ◊ shouldn't GABA be depicted as a twitter ion?

Response: GABA is now correctly depicted in its zwitterionic form (charged ammonium and carboxylate groups).

- Fig 3 ◊ Please show with interactions with dashed lines

Response: All interaction diagrams (Figs. 3c, 4c, etc.) now use dashed lines to clearly indicate hydrogen bonds and halogen bonds.

- Fig. 4b ◊ a hydrogen is missing in the BPDBA structure (the amide NH)

Response: The chemical structure of BPDBA has been corrected to include the missing hydrogen.

- Extended Fig. 1c ◊ Is this conducted with purified protein? Reference is missing in the main text

Response: Extended Data Fig. 1c (now Extended Data Fig. 1d) presents a cell-based GABA-d_6 uptake assay conducted in HEK293T cells expressing hBGT1, with nonspecific

uptake determined using an empty-vector control. The resulting specific transport activity (total minus nonspecific) is presented in the main figures (Fig. 2a). This description has been added to the revised figure legend, and the figure is appropriately referenced in the main text (Lines 138-139).

- Extended Data Fig. 1c ◊ "toatal" should be "total".

Response: The typo ("toatal") has been corrected to "total" in the revised version of the relevant figure (now Extended Data Fig. 1d).

- Extended data Fig. 2 ◊ please show the representative good 2D classes. It is not possible to distinguish the particles in the representative micrograph

Response: The figure has been updated to include panels showing clear, representative 2D class averages alongside the raw micrograph.

- Extended data Fig 3 ◊ Processing and refinement need to be revisited as artefacts are reflected in GSFSC graphs

Response: All cryo-EM data processing workflows have been revisited. The updated Gold-Standard FSC curves in the revised figure now show improved resolution estimates and reduced indications of processing artifacts.

- Extended Data Fig. 8c ◊ supported by relevant text, this figure would be valuable as a main figure.

Response: Promotion to main figure: As suggested, this panel (illustrating the membrane-mediated access pathway for BPDBA) has been promoted to main Fig. 7. Supporting text describing this mechanistic model has been added to the Results section (Lines 432-435).

Comments related to text:
- Abstract – add that no fiducial markers were used in the structural work
Response: We have revised the Abstract to explicitly state that all structures were determined by single-particle cryo-EM without the use of fiducial markers.

- Line 41 ◊ Sentence says "Recent studies", but only two studies are mentioned and one of them is from 2013 so not really recent. Please adapt wording.
Response: We agree with the reviewer. The wording has been revised from "Recent studies" to "Several studies" to accurately reflect the scope and timing of the cited literature.

- Line 53-55 ◊ BGT1 is reported to mainly regulate extrasynaptic GABA levels, presumably more than synaptic levels mentioned here. Should be included and referenced accordingly.

Response: We appreciate the reviewer's clarification on this important point. We have revised this section to clarify that hBGT1 is primarily associated with regulation of extrasynaptic GABA rather than fast synaptic clearance and have updated the references accordingly.

- Line 68 ◊ "growing" interest? It seems that not much has happened in recent years so this appears as an overstatement. Please adapt
Response: We agree that this wording was an overstatement and have revised the text to remove the implication of a recent increase in research activity.

- Line 79 ◊ along the same lines, compounds may have therapeutic potential. Maybe more safe to say 'as lead structures'?
Response: We have revised the wording to describe the compounds as lead structures rather than implying direct therapeutic potential.

- Line 87-88 ◊ "reveal … conformational transitions along the transport cycle." Is a little overstated. Please tone this down as only "half" the transitions of the cycle is presented.
Response: We agree with the reviewer and have toned down the wording to avoid implying that the full transport cycle is captured.

- Line 98-99 ◊ "hBGT1 exhibited favourable biochemical properties when solubilized in lauryl maltose neopentyl glycol (LMNG) supplemented with cholesteryl hemisuccinate (CHS)". Please better explain what "favourable biochemical properties" means.
Response: We have revised the text to clarify what is meant by "favourable biochemical properties" by explicitly describing the solubility, monodispersity, and stability of hBGT1 in LMNG/CHS (Lines 101-103).

- Line 106 ◊ Resolution for apo structures does not correlate with what is reported elsewhere in the manuscript.
Response: We thank the reviewer for their careful reading and for identifying this inconsistency. The reported resolutions for the apo structures have been corrected throughout the manuscript and are now consistent with the values presented in the corresponding methods section, figure legends, and Extended Data Table 1. This revision ensures the accurate and transparent reporting of our structural data.

- Line 124 ◊ was part of EL2 also not modelled? Indicated by the small dashed line in Fig. 1?
Response: Yes, a segment within EL2 could not be reliably modeled due to limited local density and is now indicated by the dashed line in Fig. 1. This limitation is now explicitly noted in the text.

- Line 130 ◊ No error estimation is presented in the text or figure legend. Please provide ± or confidence interval.
Response: We thank the reviewer for highlighting the need for comprehensive error reporting. In response, we have systematically reviewed and updated all quantitative functional data throughout the manuscript.

In the text, fitted parameters such as K_m, V_{max}, IC_{50}, and apparent K_i are now reported with their corresponding 95% confidence intervals.
In the figures, graphical data (e.g., uptake curves, bar graphs) are presented with appropriate error bars (mean ± s.e.m.), and the number of independent biological replicates (n) is explicitly stated in each figure legend.
These revisions ensure that all quantitative claims are presented with transparent measures of variability and reproducibility, aligning with standard practices in pharmacological and biochemical reporting.

- Line 159 ◊ Based on distance between the carbonyl oxygen and the hydrogen of the quaternary ammonium group, one should be able to determine if it can be classified as either an electrostatic interaction or a salt bridge. A salt bridge is also an electrostatic interaction.
Response: We thank the reviewer for this clarification. We agree that salt bridges are a subset of electrostatic interactions and have revised the text to describe this interaction simply as an electrostatic interaction, avoiding overclassification.

- Line 179-183 ◊ Selection of hGlyT1, hTauT, and hNET for comparison should be explained better. Why were these transporters best for comparison of substrate binding site? Also, mentioning GlyT2 here seems unnecessary.
Response: We have revised the text to clarify our rationale for selecting hGlyT1, hTauT, and hNET as structural comparators. These transporters were chosen because they represent distinct SLC6 subfamilies with published high-resolution structures in substrate-bound states, enabling a systematic analysis of how conserved architecture adapts to chemically diverse substrates (glycine, taurine, and monoamines, respectively). This comparative approach allows us to contextualize BGT1's substrate recognition within the broader SLC6 framework. In line with the reviewer's suggestion, the mention of GlyT2 in this specific context has been removed.

- Line 182 ◊ It says that ATPCA is an inhibitor. Please correct.
Response: We have standardized the terminology throughout the manuscript and now consistently refer to ATPCA as a substrate-like inhibitor, clarifying at first mention that it is transportable.

- Remove ‘s’ in Discussion headline
Response: We have corrected the section heading from “Discussions” to “Discussion”.

- Line 190-195 ◊ Without better explanation of why this is important, it just seems like a lot of words (and a figure) to say NET binds noradrenaline differently.
Response: We thank the reviewer for this comment. We have revised this section to clarify the rationale for including NET in the comparison by explicitly explaining how differences in ligand orientation and pocket electrostatics across SLC6 transporters help rationalize substrate selectivity and recognition in hBGT1.

- Line 200 ◊ Please include error parameters.

Response: Appropriate error parameters and sample sizes are now reported in the corresponding figure legend, with the number of independent experiments (n) clearly indicated.

- Line 229 ◊ When stating that BGT1's Km for GABA is comparable to previously reported (26µM), you cannot simultaneously state that that of ATPCA (21 µM) is lower than GABA. Either your presented Km for GABA of 53 µM is higher than previous or GABA and ATPCA are the same.
Response: We have revised this section to reflect our data. We have revised the wording to avoid implying that our structural observations directly explain lower K_m or IC_{50} values, and now frame these interactions as contributing to ATPCA binding and potency in line with previous studies.

- Line 291-292 ◊ "…preventing its transition to the inward-facing conformation and impairing the substrate binding." This sounds like the substrate binding occurs at the inward facing conformation.
Response: We have revised this statement to clarify that substrate binding does not occur in the inward-facing conformation and that inhibition refers to impaired progression through subsequent steps of the transport cycle.

- Line 299-300 ◊ "Despite high sequence conservation between GAT1-3 and BGT1, their Responses to BPDBA differ significantly". Please provide more detail for this statement.
Response: We thank the reviewer for this comment. We have revised the text to clarify how the responses of GAT1–3 and BGT1 to BPDBA differ, specifying that BPDBA selectively inhibits hBGT1 but shows little to no inhibitory activity toward GAT1–3, and linking this difference to variations in the architecture of the BPDBA-binding pocket.

- Line 308-309 ◊ "These features likely explain the lack of BPDBA inhibitory activity against GAT1 (Extended Data Fig. 7a, b)". The figure shows structure, nothing about activity.
Response: We thank the reviewer for pointing this out. We agree that Extended Data Fig. 7a, b shows structural comparisons rather than functional measurements. We have therefore revised the wording to clarify that these structural features provide a rationale for BPDBA selectivity, without implying that the figure itself reports inhibitory activity.

- Line 343-345 ◊ The area of the putative sodium 3 site in the local resolution maps on Extended data Fig. 3c looks to be among the best resolved, yet it is stated that there are limitations local resolution at this specific spot. Please clarify and provide zoom in view of the local resolution as well as overlay of cryo-EM map to show the position.
Response: We thank the reviewer for their careful observation regarding the local resolution in the region around the putative Na3 site. We have carefully re-evaluated the density in the region corresponding to the putative Na3 site. While the local resolution around this area is reasonably high, the density itself is weak and lacks the distinct spherical features and coordination geometry required to confidently assign a sodium ion or water molecule. To prevent overinterpretation, we have taken the following actions:

Density and Modeling: In the reprocessed maps, the density in this region is weak and lacks the distinct spherical features or coordination geometry required to confidently assign a sodium ion or water molecule. The putative Na3 site has been removed from all atomic models and is no longer presented as a defined feature in the Results.
Interpretation: To avoid overinterpretation, this region is now mentioned only briefly in the Discussion. We note its topological correspondence to the functionally validated Na3 site in GlyT2 but explicitly state that our cryo-EM data do not provide sufficient evidence to define it as a sodium-binding site in hBGT1.
These revisions prevent speculative claims and align our conclusions with the resolution and interpretability of the experimental density.

- Line 362-363 ◊ There is no figure showing any movement of EL4?
Response: We agree that no figure demonstrates EL4 movement and have revised the text to avoid implying dynamic motion, describing only the observed positioning in the resolved structures.

- Line 393-395 ◊ It appears implausible that the transporter adopts an occluded conformation prior to ATPCA binding, since both extra- and intracellular pathway to the ATPCA binding site are occluded? Isn't it much more likely that this is just one of the conformations that the transporter naturally resides in, in the absence of a ligand.
Response: We have revised this section to clarify that the ATPCA-bound state is discussed in comparison with occluded-like conformations stabilized by the endogenous substrates GABA and betaine, rather than as a prerequisite or uniquely induced state. The manuscript now treats this conformation as part of the substrate-bound ensemble, without implying adoption prior to ligand binding.

- Line 494 + 496 ◊ 2 million cells per well in both 6-well and 96-well plate. That must be a mistake?
Response: We have corrected the description of cell seeding conditions to remove ambiguity between 6-well and 96-well formats, and the revised Methods now clearly state the respective cell densities and volumes used.

Point-to-point response to reviewer comments

We sincerely thank the editor and the reviewers for their continued careful evaluation of our manuscript and for their constructive and thoughtful comments on the revised version. We are grateful for the opportunity to further improve the clarity and presentation of our work. In response to the remaining concerns, we have carefully revised the manuscript, refined the discussion where appropriate, and clarified specific methodological and interpretational aspects. All comments have been addressed in detail in a point-by-point manner below.

Reviewer #1 (Remarks to the Author):

I have no further concerns, I thank the authors for responding carefully to each of my comments.

Response: We sincerely thank the reviewer for the positive assessment and for the constructive feedback provided throughout the review process. We greatly appreciate the reviewer's careful evaluation of our work.

Reviewer #2 (Remarks to the Author):

The revised version of this manuscript is significantly improved compared to the original submission with the inclusion of important preliminary MD analysis and some attempts to better characterize the functional properties of the transporter.

Response: We sincerely thank the reviewer for the positive assessment of the revised manuscript and for recognizing the improvements made, including the additional MD analyses and the expanded functional characterization. We appreciate the reviewer's continued constructive evaluation of our work.

However, there still remains an issue that requires further work.

1. The functional characterization of the transporter has been done in an usual way by relying on liquid chromatography and mass spec to track the uptake of labelled GABA and betaine instead of the more traditional radiolabeled uptake methods or electrophysiological methods. The results obtained in this study are significantly different to previous values reported for uptake and inhibition. For example the IC50 for ATPCA inhibition of GABA uptake is 260 uM compared to previously published values of 2.5 uM (ref 23) and whilst small variations do occur between different

assays - the difference here (2 orders of magnitude) requires further investigation and clarification. I suggest that similar uptake assays to that used in previous studies be used to allow direct comparison with previous work.

Response: We thank the reviewer for raising this important concern regarding the apparent discrepancy in ATPCA potency compared to previous reports. We have carefully examined the original studies cited (ref. 23 and related work), in which ATPCA was evaluated using radiolabeled uptake assays under initial-rate conditions with ~30 nM or 100 nM substrate concentration. In those studies, ATPCA inhibition of [^{3}H]GABA uptake yielded an IC_{50} value of ~2.5 µM.

In contrast, in the present study uptake was quantified by LC–MS using a substantially higher substrate concentration ([GABA-d_6] = 5 µM). For a transportable substrate analog such as ATPCA, the apparent IC_{50} is expected to scale with substrate concentration according to the Cheng–Prusoff relationship:

$$IC_{50} = K_i(1 + \frac{[S]}{K_m})$$

Accordingly, increasing the substrate concentration from the nanomolar range to micromolar levels is expected to produce a rightward shift in apparent IC_{50} values. Importantly, conversion of IC_{50} to K_i requires an accurate K_m measured under the same assay configuration. Because K_m can vary across expression systems, substrate concentrations, incubation times, and readout methods (radiolabeled initial-rate uptake versus LC–MS-based quantification), the derived K_i values in the present study should be considered apparent parameters specific to the experimental conditions.

In addition, the assay formats differ in several respects beyond substrate concentration, including (i) transient versus stable expression systems, (ii) differences in uptake duration and accumulation windows, and (iii) distinct detection methodologies. These experimental variables can influence apparent potency estimates, particularly for transportable substrate analog such as ATPCA, which may affect the transport cycle beyond simple occupancy of the central binding site.

Importantly, the structural conclusions of this study do not rely on the absolute potency of ATPCA but on its inhibitory effect observed in uptake assays and on the well-resolved canonical binding pose in the S1 site. We have revised the main text [Results section (Lines 232-237), Methods section (Lines 712-715) and Fig. 3a legend] to explicitly state that the IC_{50} and K_i values reported here represent assay-dependent apparent parameters and should not be directly compared with radiolabeled uptake studies performed under substantially different substrate concentrations and

experimental configurations.

While we appreciate the reviewer's suggestion to perform radiolabeled uptake measurements for direct cross-assay comparison, accurate determination of intrinsic kinetic parameters for ATPCA would require a dedicated initial-rate experimental design distinct from the LC–MS–based accumulation framework employed in this study. Such measurements would involve specialized radiolabeled substrates and assay configurations optimized specifically for ATPCA transport kinetics. And our limited access to radioligands precludes this approach at present.

Given that the primary objective of the present work is to establish structural and mechanistic insights under a unified experimental system, we focused on maintaining internal consistency across ligands within the same LC–MS–based assay platform. Within this controlled framework, the comparative inhibitory behavior of ATPCA is reproducible and sufficient to support the structural interpretations presented. We have clarified the assay-dependent nature of the apparent kinetic parameters in the revised manuscript to avoid potential cross-assay overinterpretation (Lines 232-237).

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily responded to most comments and the manuscript has vastly improved. Nicely done! Only two things remain:

Response: We sincerely thank the reviewer for the continued thoughtful and detailed evaluation of our work. We especially appreciate the reviewer's careful reading and constructive suggestions throughout the review process, which have substantially strengthened the clarity and rigor of the manuscript. We address the remaining points in detail below.

1) There is a poor/discontinuous density for BPDBA – Fig. 4b - this may be due to the flexibility of the ring but should be duly addressed.

Response: We thank the reviewer for pointing out the locally weaker/discontinuous density for BPDBA in Fig. 4b. We agree that the density corresponding to the linker region between the piperidine nitrogen and the benzyl substituent is less well defined, becoming continuous only at lower contour levels.

This feature is most consistent with conformational heterogeneity or rotational flexibility of the N–CH_2–aryl linker within the lateral pocket. In contrast, the electron density corresponding to the core scaffold of BPDBA—including the dichlorobenzamide moiety and the central framework—is well resolved and

reproducible across refinements, supporting stable accommodation of the ligand within the allosteric site. Notably, the density corresponding to the terminal phenyl ring itself remains reasonably well defined.

Importantly, ligand binding is further supported by the coherent conformational rearrangements observed in TM1a, TM6b and IL2 in the BPDBA-bound structure, which are absent in substrate-bound states. The protein density surrounding the ligand is continuous and of comparable quality to the rest of the map, indicating that the localized weak density reflects flexibility of the linker region rather than absence of ligand occupancy.

Consistent with this interpretation, MD simulations show that while the BPDBA core remains stably positioned in the lateral pocket, the piperidine–benzyl linker samples multiple conformations, which would be expected to manifest as attenuated or discontinuous density in the averaged cryo-EM reconstruction.

To facilitate evaluation, we have added an enlarged view of the BPDBA density contoured at 3.3σ and 6σ in Extended Data Fig. 7h, allowing visual assessment of the local density features. We have also clarified in the main text that the weaker density is confined to the linker region and is consistent with local conformational flexibility (Lines 291–294).

2) In the pharmacological analysis of ATPCA (Fig. 2b) it is highly surprising that the Ki is so high (almost 10x right-shifted) compared to previous reports, whereas GABA is more close to the expected. What is the reason for this? How may this influence the results and interpretation?

Response: We thank the reviewer for highlighting this important observation. We agree that the apparent K_i value for ATPCA determined in our assay is right-shifted compared to previously reported radiolabeled uptake measurements, whereas the GABA value remains closer to literature estimates.

Several factors likely contribute to this difference. ATPCA is a transportable substrate analog rather than a simple equilibrium binding inhibitor, and its apparent potency can therefore depend strongly on assay configuration. In the present study, uptake was quantified by LC–MS at micromolar substrate concentrations ([GABA-d_6] = 5 µM) under accumulation conditions, whereas prior reports determined ATPCA potency using radiolabeled initial-rate uptake at nanomolar substrate levels. Under such conditions, apparent IC_{50} values are expected to vary with substrate concentration according to the Cheng–Prusoff relationship:

$$IC_{50} = K_i(1 + \frac{[S]}{K_m})$$

Thus, increasing substrate concentration from nanomolar to micromolar levels is expected to produce a rightward shift in apparent IC_{50} values and corresponding K_i estimates when calculated using assay-specific K_m values. Moreover, as ATPCA is transportable, uptake measurements may reflect effects on multiple steps of the transport cycle beyond simple occupancy of a binding site, further contributing to assay-dependent differences.

These observations support that the observed right shift reflects differences in assay configuration rather than altered transport behavior. Importantly, this shift does not affect the structural or mechanistic conclusions of the present study, which rely on the inhibitory effect observed in uptake assays and on the resolved canonical binding pose of ATPCA in the S1 site, independent of the absolute magnitude of the apparent K_i value. We have clarified in the revised manuscript that the ATPCA K_i reported here represents an assay-dependent apparent parameter [Results section (Lines 232-237), Methods section (Lines 712-715) and Fig. 3a legend].

Reviewer #4 (Remarks to the Author):

General comments: the study “Substrate recognition and allosteric inhibition of human betaine/GABA transporter 1” by Zhuo et al. resolves a number of structures with kinetic measurements to elucidate the molecular basis for hGBT1 transporters. The authors made a significant effort to address key concerns from reviewers such as many molecular dynamic simulations aimed to understand the dynamics and binding of BPDBA.

Response: We sincerely thank the reviewer for the thoughtful and constructive evaluation of our study. We greatly appreciate the recognition of the structural and kinetic efforts, as well as the additional molecular dynamics analyses incorporated in the revised manuscript. The reviewer’s comments have been particularly helpful in improving the clarity and overall presentation of the mechanistic framework of substrate recognition and allosteric inhibition. We have carefully revised the manuscript to address the points raised, and provide detailed responses below.

The study solved structures for basically four substates, betaine, GABA, ATPCA and BPDBA. It turns out the results of BPDBA seem the most interesting, but the introduction sounds weak and not focused on BPDBA.

Response: We thank the reviewer for this insightful comment. We agree that the

BPDBA-bound structure provides particularly novel mechanistic insight into the allosteric inhibition of hBGT1. In the revised manuscript, we have refined the Introduction to better reflect the central importance of BPDBA in the conceptual framework of this study.

Specifically, we now (i) clarify the limited structural understanding of allosteric regulation in BGT1 and related SLC6 transporters, (ii) introduce the possibility of membrane-accessible regulatory pockets as a mechanistic question motivating this work, and (iii) explicitly outline how structural characterization of BPDBA extends beyond canonical substrate recognition and reveals a distinct mode of transporter modulation.

All related changes have been highlighted in the revised manuscript for clarity. These revisions align the narrative focus of the Introduction more closely with the mechanistic advances presented in the Results and Discussion.

The title was about substrate recognition, but the Discussion section, in my opinion, does not elaborate and further discuss the implications of the substrate recognition mechanism. I am still not sure what the key take-away messages for the substrate recognition mechanisms are. The mechanisms may be different when ATPCA or BPDBA is present. Of course, the hydrogen networks, binding moieties are discussed in the results, but it might be good to summarize the results in Discussion.

Response: We thank the reviewer for this thoughtful suggestion. We agree that, in the original version, the Discussion did not sufficiently distill the key mechanistic implications of substrate recognition into a clear conceptual framework.

In the revised manuscript, we have added a concise summary paragraph in the Discussion to explicitly synthesize the principal features of substrate recognition in hBGT1(Lines 539-546). Specifically, we clarify (i) the conserved electrostatic core that defines canonical S1 recognition, centered on G57, Y133, the Na1 ion, E52 and Q299; (ii) how subtle differences in pocket geometry contribute to selectivity between GABA and betaine; and (iii) how the substrate-like analog ATPCA engages the same S1 recognition architecture while introducing a limited additional stabilizing contact (e.g., with C399) without redefining the fundamental recognition principle.

We further distinguish canonical S1 substrate recognition from allosteric modulation by BPDBA, which binds to a buried intracellular pocket rather than the S1 site and influences transport by stabilizing a distinct conformational state. These additions aim to clarify the key take-away messages regarding substrate recognition while maintaining the overall structure of the Discussion.

We believe these revisions improve the conceptual clarity of the manuscript and better align the Discussion with the scope indicated by the title.

In Figure 5, a schematic for the reaction coordinate used in simulations may be extremely helpful.

Response: We thank the reviewer for this helpful suggestion. To improve clarity, we have added a schematic illustration of the collective variables used in the molecular dynamics simulations to Fig. 5e, together with a corresponding description in the figure legend.

The schematic visualizes the definition of the reaction coordinates employed in our metadynamics analysis, including the distance-based collective variable (d) describing ligand–pocket separation and the lateral displacement variable (r_gate) along the membrane–protein interface. This addition provides an intuitive graphical summary of the simulation framework and complements the detailed methodological description already presented in the Methods section. No changes were made to the simulation setup or analysis procedures.

Why was the measurement of ATPCA uptake not reported in K_m value for comparison with GABA and betaine? I am not sure how the binding affinity of ATPCA, GABA and betaine can be compared?

Response: We thank the reviewer for raising this important point.

A direct determination of ATPCA K_m was not included in the present study because our primary objective was to characterize the structural basis of substrate recognition and inhibition under a unified experimental framework, rather than to comprehensively re-establish full kinetic parameters for each ligand using ligand-specific assay optimization.

ATPCA is a transportable substrate analog whose transport properties are strongly dependent on assay configuration, including substrate concentration and incubation time. Extraction of an intrinsic K_m for ATPCA would require a dedicated initial-rate design optimized specifically for ATPCA transport. In contrast, our LC–MS–based uptake measurements were configured to maintain consistent conditions across ligands, allowing internal comparison of transport behavior within a single expression system and detection platform.

Accordingly, ATPCA was evaluated using the same LC–MS–based assay configuration employed for GABA and betaine measurements. While absolute kinetic parameters (K_m or K_i) obtained using different assay paradigms (e.g., radiolabeled initial-rate measurements versus LC–MS accumulation assays) are not directly

comparable, the internally consistent framework used here enables reliable comparison of apparent transport behavior among the tested ligands within this study.

We have clarified this point in the revised manuscript to avoid potential overinterpretation of cross-assay kinetic comparisons (Lines 232-237).

One reason that the investigation of ATPCA was performed is "ATPCA, a bioisosteric analog developed to mimic the amino group of β-alanine or GABA, was originally reported to act both as a selective inhibitor and a transportable substrate of human betaine/GABA transporter 1", I think it would be more convincing if the authors elaborate a bit more in this paragraph about the chemistry relevance of ATPCA in comparison with betaine.

Response: We thank the reviewer for this helpful suggestion. To better clarify the chemical rationale for investigating ATPCA, we have revised the corresponding paragraph in the Results section to explicitly contrast ATPCA with betaine (Lines 227-231). In particular, we now note that, unlike betaine—which contains a permanently charged trimethylammonium group—ATPCA retains a substrate-like backbone while introducing a constrained cationic moiety capable of directional hydrogen bonding. This clarification highlights the relevance of ATPCA for probing how hBGT1 accommodates variations in head-group chemistry within the S1 site.

These additions strengthen the chemical context of ATPCA without altering the overall interpretation of our structural and functional data.

There is a room for improvement in figure 7 summarizing the results. I think since the structures of all substrate-bound BGT1 are solved, I think RMSD, labels of occluded, inward-facing structures should be more relevant than those of ATPCA-bound or BPDBA bounds.

Response: We thank the reviewer for this constructive suggestion. We agree that a summary figure should highlight conformational states and structural relationships, rather than primarily emphasizing ligand identity.

In the revised manuscript, Fig. 7 has been updated to clearly define the major conformational states (outward-facing, occluded, and inward-facing) and to present ATPCA- and BPDBA-bound states within this conformational framework, rather than as independent categories. This revision places the emphasis on the transport-related conformational transitions that underlie substrate recognition and inhibition.

To further enhance structural clarity, we have also added explicit Cα RMSD annotations to the conformational comparisons in Fig. 6 and introduced a new summary table (Table 1) compiling RMSD values and key conformational and

recognition features across all resolved states.

Together, these revisions provide a clearer structural context for interpreting ligand-dependent effects and strengthen the mechanistic coherence of the summary model.

Since this study is overwhelmed with a number of substrate-bound structures for a substrate recognition mechanism, it might help readers if there is a table summarizing RMSD and key features of those structures.

Response: We thank the reviewer for this constructive suggestion. To facilitate comparison among the resolved substrate- and inhibitor-bound structures, we have added a summary table (Table 1) that compiles key structural features across the different conformational states. This table includes the assigned conformational state, RMSD values relative to a reference structure, and major mechanistic features such as S1 site occupancy, TM1a orientation, and intracellular pocket engagement.

This consolidated overview is intended to provide readers with a clear and concise comparison of the structural states without duplicating the detailed analyses presented in the main figures. We believe the inclusion of Table 1 improves the accessibility and interpretability of the structural framework described in this study.

Overall, I think one more revision would help to make this study clearer and more valuable to the field.

Response: We sincerely thank the reviewer for this constructive overall assessment. We have carefully revised the manuscript to improve its clarity and presentation in response to the reviewer's comments. We believe that the revised version addresses the concerns raised and enhances the overall readability and impact of the study.

Point-to-point response to reviewer comments

Reviewer #2 (Remarks to the Author):

In the previous review of this manuscript, I pointed out the two orders of magnitude difference between the IC50 value for ATPCA reported in this paper compared to previous papers. The revised manuscript has ignored this comment and even tried to play down the significance of an IC50 value. The small change in IC50/Km values that are generated when using the Cheng Prusoff equation do not in any way provide a reasonable explanation for the differences. An IC50 of 238 uM is still ~ 2 orders of magnitude different and cannot be explained by different experimental conditions. I recommend that the experiments be conducted in a way that allow direct comparison with previous results so that we can be confident in the results presented. If the authors do not wish to do radiolabelled uptake assays, they could also do two electrode voltage clamp recordings from Xenopus laevis oocytes to get direct meeasures of transport as outlined in: Matskevitch et al., (1999) Journal of Biological Chemistry 274, 16709-16716 Functional Characterization of the Betaine/γ-Aminobutyric Acid Transporter BGT-1 Expressed in Xenopus Oocytes.

Response: We sincerely thank the reviewer for raising this concern again. After carefully revisiting both our previous response and the reviewer's comment, we realized that our earlier reply did not adequately convey how seriously we took this issue. We sincerely apologize for that. We did not intend to overlook this concern, nor to downplay the significance of the discrepancy in the ATPCA-related data. On the contrary, we fully understand why the reviewer remained unsatisfied, because an approximately two-orders-of-magnitude difference from the previously reported IC_{50} value is indeed substantial and deserves a much more careful and direct response.

This comment prompted us to reflect more deeply on both our experimental design and the way we interpreted our results. We fully agree that, when a measured pharmacological parameter differs so markedly from the literature, it is not sufficient to attribute the discrepancy only in general terms to assay conditions. Instead, the source of the discrepancy needs to be examined more carefully, the limitations of the assay system need to be defined more clearly, and more direct functional evidence should be provided wherever possible. With this in mind, we made every effort within the scope of this revision to carry out additional experiments that could more directly clarify the functional properties of ATPCA and strengthen the overall interpretation of our study.

First, we repeated the ATPCA inhibition experiment under a lower GABA concentration. The purpose of this experiment was to directly test whether the

apparent IC_{50} measured in our LC–MS-based uptake system depends on substrate concentration and assay context. When we used 2.5 µM GABA instead of 5 µM, the apparent IC_{50} shifted from approximately 260 µM to approximately 230 µM. Although this numerical change was modest and does not reconcile our value with the previously reported result, it confirms that the apparent IC_{50} in our assay is condition-dependent. Rather than overinterpreting this result, we therefore moved the IC_{50} data to the Extended Data and removed the corresponding K_i discussion, because we do not consider further derivation of K_i from this assay-dependent value to be sufficiently reliable.

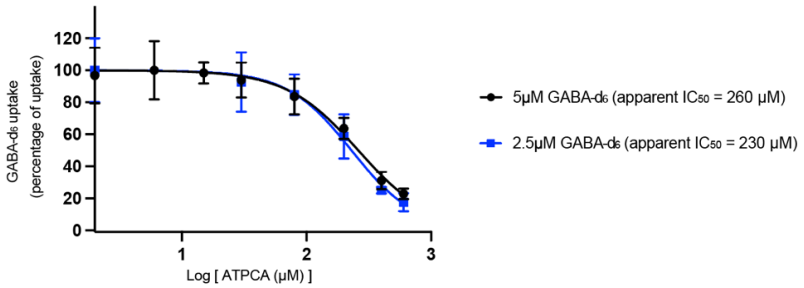

Figure 1 | ATPCA inhibition of GABA uptake under two substrate concentrations (corresponding to Extended Data Fig. 7a)

Second, we directly measured ATPCA uptake by LC–MS and determined its apparent kinetic parameters. We consider this to be a particularly important addition, because it allowed us to characterize ATPCA more directly within our own system as a ligand with substrate-like transport properties, rather than discussing it only indirectly through inhibition data. As shown in the revised manuscript, ATPCA was transported by hBGT1 with an apparent K_m of 57.7 µM, which is in a similar range to the apparent K_m measured for GABA under our assay conditions. These data provide a more direct measure of transport in our system and support our conclusion that ATPCA has transport-related properties toward hBGT1.

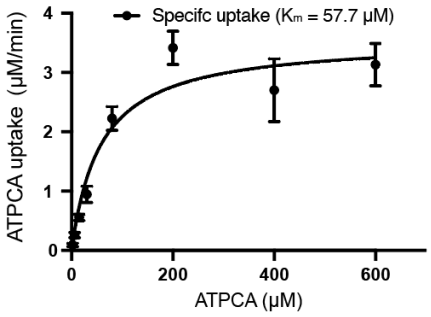

Figure 2 | Transport kinetics of ATPCA by wild-type hBGT1 (corresponding to Fig. 3a)

Third, we added whole-cell patch-clamp recordings in BGT1-expressing HEK293T cells as an independent functional assay. ATPCA, GABA, and betaine all elicited concentration-dependent inward currents, with apparent EC_{50} values of 3.95 µM, 20.3 µM, and 284 µM, respectively. These electrophysiological data independently support that ATPCA is functionally recognized by hBGT1 and provide a direct readout complementary to the LC–MS uptake measurements. Together with the uptake kinetics, these results substantially strengthen the functional characterization of ATPCA in the revised manuscript.

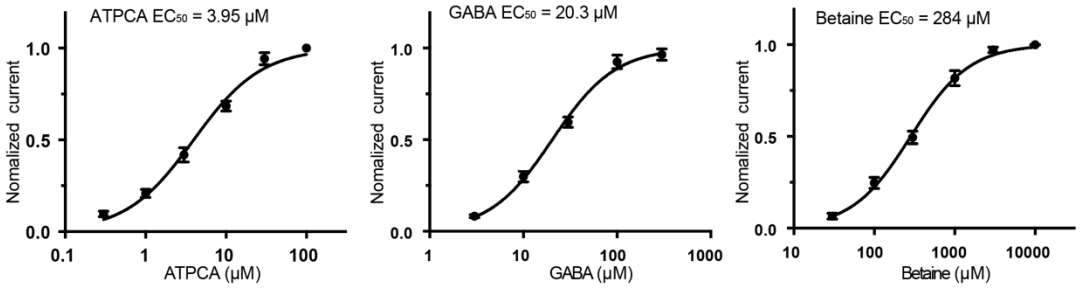

Figure 3 | Concentration–response curves of ATPCA-, GABA- and betaine-activated BGT1 currents in HEK293T cells (corresponding to Fig. 3b)

Accordingly, we have revised the ATPCA-related Results section in the manuscript to incorporate these new findings and to clarify the corresponding interpretation (Lines 232-245). We have also expanded the Methods section to include the procedures for ATPCA uptake measurements and whole-cell patch-clamp recordings (Lines 747-755, 758-767). In addition, we revised Fig. 3 and added a new Extended Data Fig. 7, together with the corresponding figure legends, to present the newly generated transport, electrophysiological, and inhibition data.

At the same time, we would like to state clearly that we agree with the reviewer's point. Radiotracer uptake assays and two-electrode voltage clamp in Xenopus laevis oocytes are highly valuable approaches for direct comparison with previous studies. We carefully considered adding these experiments during revision. However, neither platform is currently established in our laboratory or in the collaborating laboratories that were realistically accessible to us within the revision period, and setting them up would have required substantial additional time and technical preparation beyond what was feasible in this round of revision. We therefore chose to devote our full effort to additional experiments that we could complete rigorously and to a high standard.

Importantly, the purpose of these additional experiments was not to present our assay system as a substitute for the classical pharmacological methods used for precise

kinetic comparison, nor simply to reproduce a previously reported value for its own sake. Rather, our goal was to clarify, within the framework of our own study, how hBGT1 recognizes ATPCA and how ATPCA functionally interacts with this transporter, because this is the central mechanistic question that our work seeks to address.

We hope that the additions made in this revision—including the low-GABA ATPCA inhibition experiment, direct ATPCA uptake kinetics, and whole-cell patch-clamp recordings—demonstrate that we have taken this concern seriously and responded with substantial new functional evidence. We are grateful to the reviewer for raising this important concern, as it prompted us to strengthen both the experiments and the interpretation of ATPCA in the revised manuscript.