nature portfolio

Peer Review File

Functional and structural basis of a negative allostery within GABAB hetero-tetramers

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript “Functional and structural basis of a negative allostery within GABAB hetero-tetramers” describes the investigation of the structural and functional organization of GABAB receptors, a class C GPCR critically involved in inhibitory neurotransmission and implicated in diverse neurological conditions. While GABAB receptors are known to function as obligatory GB1–GB2 heterodimers, emerging evidence suggests they can also form higher-order oligomers with potential roles in signaling integration and disease. Using subunit-specific nanobodies, the authors provide direct evidence for the existence of GABAB hetero-tetramers in neurons and demonstrate that their function can be selectively disrupted by disease-associated mutations, underscoring their physiological significance. Functional assays reveal distinct pharmacological profiles between heterodimers and tetramers, with agonists showing reduced efficacy in tetramers, while positive allosteric modulators remain effective. Structural determination by cryo-EM resolves the GABAB hetero-tetramer, revealing an asymmetric extracellular domain arrangement that enforces negative allosteric control, preventing simultaneous activation of both heterodimers. The work provides new insights into GPCR oligomerization, with important implications for therapeutic strategies targeting GABAB receptors.
Overall, I find this study interesting, original and of sufficient conceptual novelty to justify publication in Nature communications. I have a couple of comments that should be addressed during revision:

1) Detection and identity of GABAB oligomers: The authors use the two GB1-recognizing nanobodies Nb-4C10rFc and Nb-4C10mFc to detect GABAB heterotetramers. However, to my understanding, this nanobody combination should also detect GB1 homodimers. How do the authors ensure that their PLA signals specifically and exclusively stem from GABAB heterotetramers?
Along this line, the authors use the two nanobodies Nb-4C10rFc (against GB1) and Nb-5E9mFc (against GB2) to detect GABAB heterodimers. However, this nanobody combination would not be able to distinguish between ‘isolated’ GABAB heterodimers (as the authors claim) and heterodimeric units within a GABAB heterotetramer. A revised manuscript should provide more data to strengthen the authors’ conclusion on which oligomers they actually detect.

2) Expression levels of heterodimers and heterotetramers: For all pharmacological experiments involving receptor mutants (Figs. 2, 4, and 5; Extended Data Fig. 3), the authors need to control cell surface expression levels of the various heterodimer/heterotetramer receptor assemblies. Without equal expression levels of the various oligomers, direct functional comparisons of potencies and efficacies cannot be precisely determined. In addition, quantifying the ligand effects at the various oligomers and mutants using state-of-the-art efficacy measures such as those derived from the operational model, would be more quantitative and deliver statistically testable parameters for ligand efficacies.

Minor:
a) Ligand concentrations in Figure legends: In some Figure legends, ligand concentrations are given as x mM, whereas in the text it says x microM.

b) Figure legend: Extended Data Fig. 1: There is an error in this sentence: “ASA: The ER retention signal RSR in the C-terminal coiled-coil domain of GB1 was mutated to RSR that facilitate the cell surface expression.”

c) Figure 4d: In the manuscript text it says (lines 264-266): “We found that the maximal Ca2+ release response induced by GABA increased by 30% for GABAB receptor formed with GB1A397N/N399T and GB2 and by 80% for GABAB receptor formed with GB1E414N and GB2, compared to the wild type GABAB receptor”. The data displayed in Figure 4d are the other way around: 30% increase for GB1E414N and 80% increase for GB1A397N/N399T.

Reviewer #2

(Remarks to the Author)
Review of “Functional and structural basis of a negative allostery within GABAb hetero-tetramers” by Shen et al.

The association of individual molecules of GPCRs with other copies of the same receptor, other membrane resident proteins, or molecules of distinct GPCRs has been an intense area of study for multiple decades. Evidence has continued to mount that GPCR oligomer assemblies behave differently than GPCRs evaluated in unassembled form. Studies of such assemblies are further complicated in the study of class C GPCRs which form constitutive dimers (either homomers or heteromers), which is required for function. The heterodimeric GPCR GABAb consists of GB1 and GB2 building blocks, which play separate roles in the mechanism of activation. To further complicate matters, evidence (including past work from these authors) suggests that GABAb undergoes further assembly to form heterotetramers (dimer of heterodimers); however, such higher order assemblies have proven challenging to fully characterize using more traditional biochemical methods. In this manuscript the authors describe the development of new tools (nanobodies) and the application of new pharmacological (BRET), biochemical, and structural analyses (CryoEM) to dissect GABAb assemblies.

I am enthusiastic about the work overall. The new data generated in these studies is quite interesting and provocative. Providing strong evidence that GPCRs impart allosteric effects on either other via discrete higher order assemblies would be beneficial for pharmacological studies and drug development. The data presented here offer new angles to understand mechanisms by which GABAb can form heterotetrameric assemblies and how such assemblies might differ from the more conventional heterodimers. These data will be of interest to the broader GPCR pharmacological community. However, several aspects of the experimental design and the assumptions that underly interpretation of these data should be addressed before acceptance for publication.
Major Comments:
*For the proximity ligation assays it is unclear to me how the authors account for the bivalent nature of the nanobody-Fc constructs (such as 4F10-Fc) used for receptor binding. Fc constructs are dimeric (as it seems are the reagents used to detect 4F10-Fc). Using a dimeric binding construct (and a dimeric detection reagent) would seem to be a good way to artificially induce dimerization of the target being studies. The authors should consider whether they could perform a similar assay using monomeric nanobody detection reagents. It would also be good to include a nanobody detection reagent that binds to some other target on the cell surface and show that less PLA signal is observed (when mixed with 4F10-Fc).
*For this same PLA experiment, how do the authors rule out that any of the signal attributed to tetramerization results from GB1-GB1 homodimerization? Design of a probe to measure this species would help strengthen evidence.
*For experiments measuring heterodimer and heterotetramer signaling with CODA-RET the authors use LgBit and HiBit tagged receptors to allow measurement of activation (Gi recruitment) only at receptor pairs. This is an interesting experimental design, but it also likely affects the oligomeric propensities of the receptors under study (as shown later when attempting to purify hetero-tetramers). Since HiBit has high affinity for LgBit it is hard to discern whether the complexes formed would apply to endogenous receptors or if this is an artefact of introduction of the complementary tags. Could the authors use SmBit (a lower affinity peptide that binds LgBit) instead of HiBit to reduce the contribution of tag-mediated assembly has on the pharmacological responses observed?
*When performing BRET assays using CODA-RET the authors report BRET ratios for assessing receptor activation. To make these measurements the authors also must record bulk luminescence values (from LgBit + HiBit), which would report on the total amount of nLuc complex formed in a given cell population. This would seem to be valuable information for understanding how much heterodimer vs heterotetramer is formed under analogous conditions. Could the authors report nLuc luminescence in a meaningful format to provide insight into this topic?
*It seems intuitive that higher older oligomer assembly (such as tetramerization) would be highly dependent on the level of a given protein at the cell surface. This type of consideration would seem to be particularly important for receptor mutants (like data in Figure 2) which might have altered stability (or expression levels). Can any of the altered behavior of any receptor variants be simply explained by alterations in levels of cell surface protein? This could be measured by flow cytometry staining and would seem to be most important for assessment of pathogenic receptor mutants.
*It seems possible that the LgBit-HiBit interaction could play a role in dictating the geometry observed in the interaction between the two subunits in the heterotetramer structure. Are there observations that would argue against this? If not, I suggest devising alternative mechanisms for promoting assembly to see if this changes the heterotetramer geometry observed.
*The observation that transplanting the extended loop found in Drosophila loop 3 into human receptor affects calcium signaling is interesting. It seems like this effect should be tested in more direct measurements of heterotetramerization (PLA or CODA-RET).
*For the data shown in Figure 5d, the observation that heterotetramer efficacy was decreased by 50% when a GB1 subunit that does not bind ligand is introduced was interesting. I think that performing a similar measurement for heterodimer signaling would also be useful.
*For the data shown in Figure 6c, it seems there is no negative control where dimerization between two Cys containing constructs is not observed. This control is needed to say whether the assembly mechanism described here is needed for covalent heterotetramer formation (or if simply overexpressing two Cys-containing constructs in the same cell is enough to

observe these effects)
*I did not find sequence listings for any of the receptor construct designs or the nanobodies (and nanobody constructs) identified in this study. This data is essential for others to be able to build on these findings. If there are intellectual property considerations restricting sharing of sequence information, then reference must be made to the patent disclosures so relevant information can be obtained once possible.
Minor Comments:
*It looks like the binding experiments to determine nanobody affinities (Extended Fig 1 and Extended Table 1) were only performed once. These should be repeated at least once more to provide error bars on the measurements and give a sense of whether different nanobodies and constructs actually have statistically distinguishable binding.
*Nanobody binding constants are listed as Kd values, but it seems these numbers were derived from flow cytometry staining experiments. Such values are better described as EC50s (and not Kds).
*In the caption for Ext Figure 1b the concentration of nanobody is listed as 1 mM. This seems high, perhaps double check?
*In Extended Data Fig. 1 could the authors define “RSR”?
*On lines 162-164 the authors describe the GB2KKXX receptor, but it is not clear to me when it is applied (in this portion of the text) or how it functions. Based on Ext Data Fig 1 it looks like wild type hGB1a (used as GB1) has an ER retention signal. How can this be combined with GB2KKXX and result in receptor at the cell surface? I am likely just missing some important details, but it would be helpful of the authors could be more explicit about which GB1/GB2 variants are used in what context.
*For Figure 1e, could the authors add some additional text to the figure legend more clearly explaining what the bar graphs in the inset represent? Same request for Figure 2c-d.
*Starting at line 188 the authors begin discussion of experiments using baclofen. It seems that baclofen was not used in Figure 1 experiments. Could the authors add text explaining this choice (and whether they might have missed anything by not using baclofen in Fig 1)?
*Could the authors comment on why they think they don’t see any signal for Nb-4C10-Bi in the CryoEM structure? Is it even important for inducing heterotetramerization?
*When assessing the functionality of constructs used for structural studies the authors use a calcium mobilization assay. Could the authors comment on why this wasn’t used on constructs described earlier in the paper? It might be good to do a few spot checks to see if calcium mobilization correlates with the G protein assays described earlier in the manuscript.
*In lines 291-294 the authors write about whether one or both VFTs can bind to ligand. How was this assessed? By modelling? Some additional context would be helpful.
*This might be addressed in other forms, but it would be useful to provide exact product numbers for some of the unusual reagents (PIA probes, etc).
*A few small spelling/grammar suggestions:
Title: suggest removing word “a”
Line 47: “evidences” should be “evidence” and “oligomer” should be “oligomers”
Line 530 “Thers”—unsure what this is supposed to say

Reviewer #3

(Remarks to the Author)
GABAB, as a class C GPCR, is known to function as heterodimers. Whether higher-order oligomers exist and their physiological relevance is not clear. In this manuscript, Shen et al. employed multiple approaches to study the role of GABAB hetero-tetramers in cellular signaling. To do so, they first developed nanobodies that could recognize different subunits (GB1 and GB2, for example). Using the nanobody-based PLA assays, they could detect oligomerizations in both overexpressed HEK cells and isolated neurons. Both hetero-dimers and hetero-tetramers can signal through G proteins but with different potency or efficacy. Some clinically relevant mutations may function via the change of hetero-tetramer stability or formation. A cryo-EM structure of GABAB Hetero-tetramers has been determined and displays different symmetry compared to the hetero-dimer structure. This manuscript provides evidence from different aspects to support that GABAB hetero-tetramers may exist physiologically. The other evidence that these tetramers play important roles in GABAB’s function needs more data.
1. A few major questions related to the physiological relevance of hetero-tetramers. What is the percentage of GABAB hetero-tetramers vs hetero-dimers in neurons and what determines this ratio? Figure 1b quantified both hetero-dimers and tetramers on cell membranes, do they represent all populations of GABAB in cells or neurons? Is the tetramer formation inducible upon condition changes or receptor activation?
2. The two nanobodies are useful tools in this study. In Figure 1a & 1b, will the binding of the nanobody affect another binding? If yes, this will affect the quantitation of dimers or tetramers in cells. One experiment that can be done is to measure the binding affinity of Nb5E9 after long incubation of GABAB with Nb-4C10.
3. Line 162-164: Additionally, we introduced an ER retention signal at the C-terminal end of GB2 (GB2KKXX) to regulate its trafficking to the cell membrane, which occurs only upon interaction with GB1. This sentence and the assay are unclear. GB2KKXX can retain GB2 in the ER membrane, why does interaction with GB1 then help its trafficking to the cell membrane? Particularly, the CODA-RET assay will measure bioluminescence from the cell membrane, ERs, and Golgi. Line 173 says 100 μM, in the Figure 1e legend it was 100 mM.
4. When examining the clinical variants, the assay was specifically designed for measuring dimers or tetramers. Those mutations may still affect signaling. A regular BRET including GB2-Nluc and Venus-miniGi1 can be performed to see if the mutants affect G protein signaling compared to WT.
5. Another major concern is the use of NanoBit and Nanobody in determining the hetero-tetramer structures. Although they were essential for successful structure determination, they weaken the physiological relevance of the hetero-tetramers. Particularly their sample preparation without NanoBit or Nanobody didn’t allow them to get enough hetero-tetramers, which

indicates the hetero-tetramers are a very small population compared to hetero-dimers.
6. Line 255-269. Here the authors tried to introduce glycosylation sites to prevent dimerization or oligomerization. Why did it not work? Was the presence of glycosylation confirmed by mass spec or other approaches? Glycosylation can significantly increase the size of the proteins by introducing a large sugar chain, which is sufficient to disrupt the interface.
Minor points
Line 101 says the HEK293T cells expressing the receptors were injected as antigens? Is this true as I see from the Methods, it was cell membrane extraction.
Is the VFT of GB2 very different from GB1? So Nb-5E9 only binds to GB2's VFT?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised version of the manuscript "*Functional and structural basis of a negative allostery within GABAB heterotetramers*" has improved over its original version. I find the new data, clarifications and new discussions overall convincing.

There are two (minor) remaining issues that I feel need to be addressed:

1) *GB1-homodimers*: I find the authors' argumentation overall plausible. Nevertheless, I believe it would strengthen the author's explanation if they provided some experimental data to back-up their key arguments.
2) *Quantification of ligand efficacies*: I maintain that the functional effects of the disease-relevant mutations (e.g. on ligand efficacy) should be quantified using state-of-the-art pharmacological methods. For instance, the operational model yields a parameter, i.e. $\log(\tau/K_A)$ - a surrogate for ligand efficacy, that allows to quantify and statistically test differences in ligand efficacies across oligomers and disease-relevant mutations. Comparing TOP values, as done in the current version of the manuscript, is insufficiently quantitative.

Reviewer #2

(Remarks to the Author)
The authors have nicely addressed my questions and comments. I have two minor requests for clarification/follow up:
*I don't see any mention of the relevant patent describing nanobodies (CN120024449A (State: disclosure)) described in either the 'competing interests' or the nanobody methods section. Perhaps it is included somewhere else, but I cannot find it.
*For new data shown in Extended Data Fig 2d, do all conditions use HEK293 cells transfected to express both mGluR2 and gabaB? Or are different transfection conditions used for the different analyses? This should be clarified in the figure legend.

Reviewer #3

(Remarks to the Author)
The authors have addressed my questions. I recommend the publication of the manuscript.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am convinced by the additional work and clarifications that the authors have incorporated into this revised version of the manuscript. The revisions have substantially again strengthened the paper, and the authors now present a clear, compelling, and well-supported case for their conclusions. The experimental data are interpreted with appropriate rigor, now fully warranting publication in Nature Communications.

Particularly commendable is the authors' excellent integration of their findings within the existing literature. This careful and comprehensive contextualization greatly enhances its accessibility to non-specialists seeking to understand GABAB receptor biology.

Overall, this is a high-quality and mature piece of work, and I congratulate the authors on a thoughtful and thorough revision.

Point-by-point answers to reviewers' comments

Reviewer #1 (Remarks to the Author):

The manuscript "Functional and structural basis of a negative allostery within $GABA_B$ hetero-tetramers"; describes the investigation of the structural and functional organization of $GABA_B$ receptors, a class C GPCR critically involved in inhibitory neurotransmission and implicated in diverse neurological conditions. While $GABA_B$ receptors are known to function as obligatory GB1, GB2 heterodimers, emerging evidence suggests they can also form higher-order oligomers with potential roles in signaling integration and disease. Using subunit-specific nanobodies, the authors provide direct evidence for the existence of $GABA_B$ hetero-tetramers in neurons and demonstrate that their function can be selectively disrupted by disease-associated mutations, underscoring their physiological significance. Functional assays reveal distinct pharmacological profiles between heterodimers and tetramers, with agonists showing reduced efficacy in tetramers, while positive allosteric modulators remain effective. Structural determination by cryo-EM resolves the $GABA_B$ hetero-tetramer, revealing an asymmetric extracellular domain arrangement that enforces negative allosteric control, preventing simultaneous activation of both heterodimers. The work provides new insights into GPCR oligomerization, with important implications for therapeutic strategies targeting $GABA_B$ receptors.
Overall, I find this study interesting, original and of sufficient conceptual novelty to justify publication in Nature communications. I have a couple of comments that should be addressed during revision:

We wish to thank the referee for his/her very positive general comment. As detailed below, we did our best to clarify all the issues that have been raised.

1) Detection and identity of $GABA_B$ oligomers: The authors use the two GB1-recognizing nanobodies Nb-4C10rFc and Nb-4C10mFc to detect $GABA_B$ heterotetramers. However, to my understanding, this nanobody combination should also detect GB1 homodimers. How do the authors ensure that their PLA signals specifically and exclusively stem from $GABA_B$ heterotetramers?

It is true that the nanobodies used in the PLA assay allow the detection of GB1 proximity. Accordingly, this approach cannot exclude that part of the signal measured can be due to the presence of GB1 homodimers at the cell surface. However, such homodimers likely represent a very minor fraction of the signal. Indeed, GB1 contains an ER retention sequence that prevents it from reaching the trans-golgi network, thanks to its recognition by the ER gatekeeper PRAF2 (Doly et al., 2015). GB1 surface expression is also controlled at a later step by msec7-ARF acting at a second retention site (a di Leucine motif) within its C-terminal tail (Restituito et al., Mol Cell Neurosci 2004). These retention systems are prevented by the GB1-GB2 coiled coil interaction, allowing GB1 to reach the cell surface only when associated with GB2 (Couve et al., JBC 1998; Margeta-Mitrovic et al., Neuron 2000). Moreover, in the absence of GB2, GB1 is rapidly degraded, leading to a very low amount of GB1 subunits (Gassmann et al., J Neurosci 2004), further indicating that GB1 homodimers, if any, represents a very small fraction of the GB1 subunits at the cell surface. One could propose a possible dissociation of GB1-GB2 heterodimers while at the cell surface, enabling the formation of GB1 homodimers.

Again, such a possibility is very unlikely due to the strong association between these two subunits brought by the coiled coil interaction between their C-terminal intracellular domains (Kammerer et al., Bioch 1999; Calver et al., J Neurosci 2001). Even the tetramers were shown to be very stable and not capable of exchanging subunits (Comps-Agrar et al., FASEB J 2012).

The possible existence of GB1 homodimers is now indicated in our manuscript (Page 15, Line 369-383), with enough explanation to convince the reader that they very likely represent a very minor proportion of the $GABA_B$ receptors at the cells surface.

Along this line, the authors use the two nanobodies Nb-4C10rFc (against GB1) and Nb-5E9mFc (against GB2) to detect $GABA_B$ heterodimers. However, this nanobody combination would not be able to distinguish between $GABA_B$ heterodimers (as the authors claim) and heterodimeric units within a $GABA_B$ heterotetramer. A revised manuscript should provide more data to strengthen the authors'conclusion on which oligomers they actually detect.

Yes, the referee is perfectly right, the nanobodies combination detect any GB1-GB2 heterodimers, whether strict dimers, or being part of larger complexes. This is now clearly stated in our manuscript (Page 15, Line 384-397). Although we could not specifically detect strict GB1-GB2 heterodimers, such a determination is not necessary for our conclusion, what being important is the detection of the proximity between these heterodimers.

2) Expression levels of heterodimers and heterotetramers: For all pharmacological experiments involving receptor mutants (Figs. 2, 4, and 5; Extended Data Fig. 3), the authors need to control cell surface expression levels of the various heterodimer/heterotetramer receptor assemblies. Without equal expression levels of the various oligomers, direct functional comparisons of potencies and efficacies cannot be precisely determined.

We thank the reviewer for the valuable comments. Indeed, we already systematically measured cell-surface expression levels of the $GABA_B$ receptor mutants in the cells used for the functional assays shown in Figs. 2, 4, and 5; Extended Data Fig. 3. These data are shown below (**Figure R1**). Note that most receptors are expressed at a similar level as the wild type, but a few (E368D and E414N) show a two-fold lower expression level. Such lower expression has to be taken into account in interpreting the functional data, but clearly, a two-fold lower expression of the E368D mutant cannot per se explain a 2-log shift, in baclofen potency on the heterodimer assay, nor a total loss of function in the tetramer assay. Regarding the disruption of the negative cooperativity within the tetramer, one mutant (A397N/N399T) displays a similar expression as the WT, and this mutation results in an increase of 80% of the maximal effect. The lower effect observed with the E414N mutant compared with $GB1^{A397N/N399T}$ is likely explained by its 2-fold lower expression level relative to WT. This is now described and discussed in our revised manuscript (Page 11, Line 271-273).

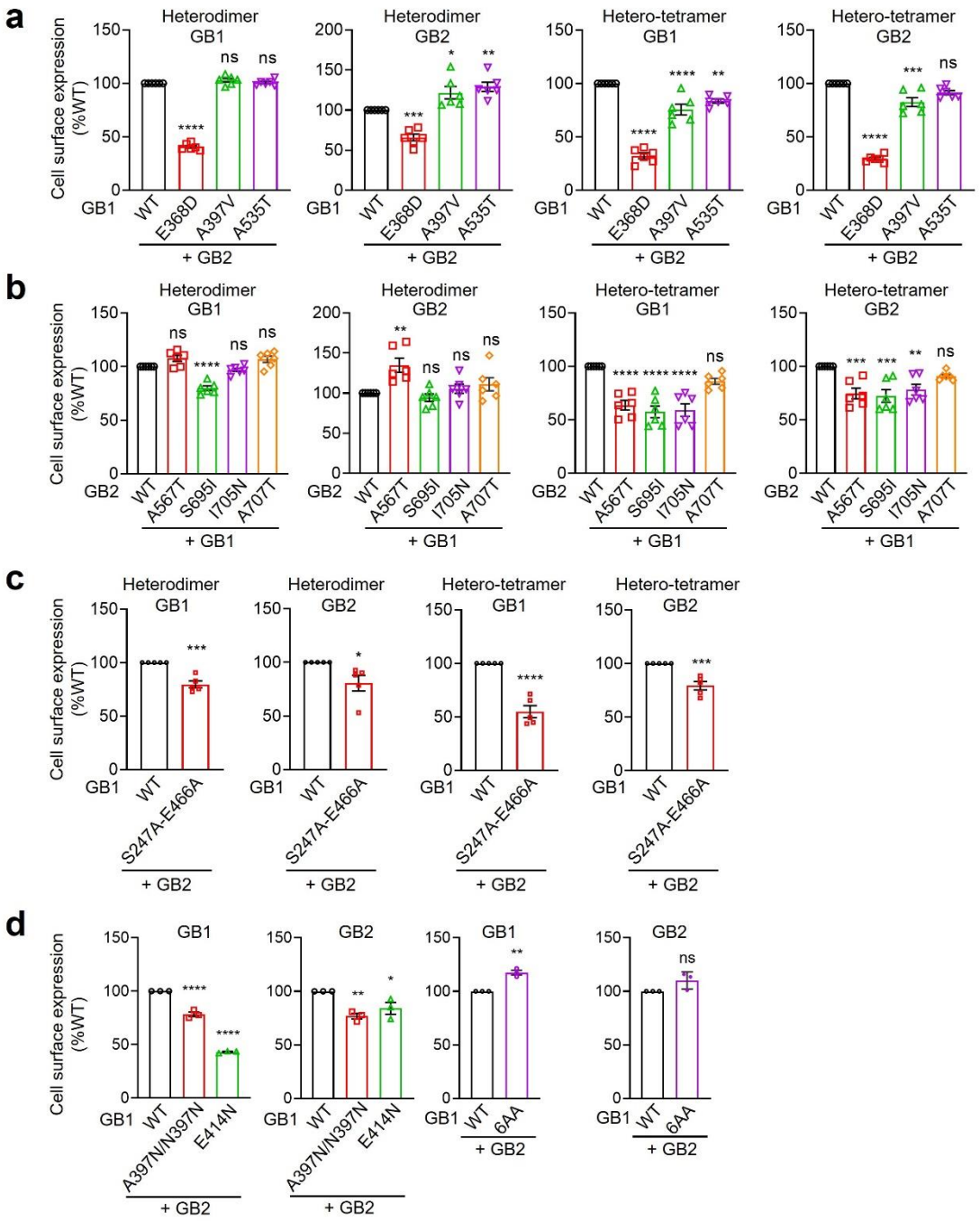


Figure R1. Expression levels of GABA$_B$ heterodimers and heterotetramers. a-d, Expression of the indicated receptors in Figs. 2, 4, 5 and Extended Data Fig. 5. Data are present as mean ± s.e.m. from at least three independent experiments. Data are analyzed using one-way analysis of variance (ANOVA) with a Dunnett's post-hoc multiple comparison test or unpaired t-test (two-tailed) to determine significance (compared with WT group). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and not significant (ns).

These data are presented in the new **Extended Data Fig. 4c, Extended Data Fig. 5e, Extended Data Fig. 9a, e and Extended Data Fig. 11b** of the revised manuscript.

In addition, quantifying the ligand effects at the various oligomers and mutants using state-of-the-art efficacy

measures such as those derived from the operational model, would be more quantitative and deliver statistically testable parameters for ligand efficacies.

We thank the reviewer for the valuable suggestion. We have reanalyzed the data using the estimated TOP values of the Prism fits. These data have been updated in the inserted panels in **Figure 1, Figure 2, Figure 5, Figure 1, Extended Data Fig. 5, Extended Data Fig. 12**.

Minor:

a) Ligand concentrations in Figure legends: In some Figure legends, ligand concentrations are given as x mM, whereas in the text it says x microM.

We thank the reviewer for pointing it out. We have corrected the mM into μM in the revised manuscript.

b) Figure legend: Extended Data Fig. 1: There is an error in this sentence: ASA: The ER retention signal RSR in the C-terminal coiled-coil domain of GB1 was mutated to RSR that facilitate the cell surface expression.;

We thank the reviewer for pointing it out. We have corrected sentence as "The ER retention signal RSR (Arg-Ser-Arg) at the C-terminal end of the coiled-coil domain of GB1 was mutated to ASA (Ala-Ser-Ala) to prevent ER retention, allowing cell surface expression of the constructs.".

c) Figure 4d: In the manuscript text it says (lines 264-266): We found that the maximal Ca^{2+} release response induced by GABA increased by 30% for $GABA_B$ receptor formed with GB1A397N/N399T and GB2 and by 80% for $GABA_B$ receptor formed with GB1E414N and GB2, compared to the wild type $GABA_B$ receptor. The data displayed in Figure 4d are the other way around: 30% increase for GB1E414N and 80% increase for GB1A397N/N399T.

We have corrected this sentence as follow: "Ca^{2+} release response induced by GABA increased by 80% for $GABA_B$ receptor formed with $GB1^{A397N/N399T}$ and GB2 and by 30% for $GABA_B$ receptor formed with $GB1^{E414N}$ and GB2". Furthermore, we added a sentence (Page 11, Line 271-273) to explain that the lower effect observed with the E414N mutant compared with $GB1^{A397N/N399T}$ is likely related to its lower expression, as indicated in our answer to the first point above.

Reviewer #2 (Remarks to the Author):

Review of "Functional and structural basis of a negative allostery within GABAb hetero-tetramers" by Shen et al.

The association of individual molecules of GPCRs with other copies of the same receptor, other membrane resident proteins, or molecules of distinct GPCRs has been an intense area of study for multiple decades. Evidence has continued to mount that GPCR oligomer assemblies behave differently than GPCRs evaluated in

unassembled form. Studies of such assemblies are further complicated in the study of class C GPCRs which form constitutive dimers (either homomers or heteromers), which is required for function. The heterodimeric GPCR GABAb consists of GB1 and GB2 building blocks, which play separate roles in the mechanism of activation. To further complicate matters, evidence (including past work from these authors) suggests that GABAb undergoes further assembly to form heterotetramers (dimer of heterodimers); however, such higher order assemblies have proven challenging to fully characterize using more traditional biochemical methods. In this manuscript the authors describe the development of new tools (nanobodies) and the application of new pharmacological (BRET), biochemical, and structural analyses (CryoEM) to dissect GABAb assemblies.

I am enthusiastic about the work overall. The new data generated in these studies is quite interesting and provocative. Providing strong evidence that GPCRs impart allosteric effects on either other via discrete higher order assemblies would be beneficial for pharmacological studies and drug development. The data presented here offer new angles to understand mechanisms by which GABAb can form heterotetrameric assemblies and how such assemblies might differ from the more conventional heterodimers. These data will be of interest to the broader GPCR pharmacological community. However, several aspects of the experimental design and the assumptions that underly interpretation of these data should be addressed before acceptance for publication.

We wish to thank the referee for his/her very positive general comment. As detailed bellow, we did our best to satisfy his/her specific comments.

Major Comments:

1) For the proximity ligation assays it is unclear to me how the authors account for the bivalent nature of the nanobody-Fc constructs (such as 4F10-Fc) used for receptor binding. Fc constructs are dimeric (as it seems are the reagents used to detect 4F10-Fc). Using a dimeric binding construct (and a dimeric detection reagent) would seem to be a good way to artificially induce dimerization of the target being studies. The authors should consider whether they could perform a similar assay using monomeric nanobody detection reagents. It would also be good to include a nanobody detection reagent that binds to some other target on the cell surface and show that less PLA signal is observed (when mixed with 4F10-Fc).

We thank the reviewer for this comment. As the cells were fixed with 4% formaldehyde before incubation with 4F10-Fc followed by PLA detection, the dimeric or tetrameric state should be unaffected by the nanobody-Fc. This have now been explained in our revised manuscript.

We thank the referee for proposing another negative control, measuring PLA dots between two cell surface receptors not interacting. For this, we used mGlu2 as we do have high affinity nanobodies specific of this receptor (Scholler et al., Nat Comun 2017; Oosterlaken et al., Nature 2025), and $GABA_B$ is known not to interact with mGluRs (Rives et al., EMBO J 2009). As shown in **Figure R2**, the expression of the mGlu2 homodimer was validated by quantifying PLA-dots obtained with the mGlu2 nanobodies, and were found to represent 138% those obtained with the GB1-GB2 nanobodies, suggesting a higher expression level of the mGlu2 homodimers

than $GABA_B$ heterodimers. In contrast, the mGlu2-GB1 number of PLA-dots per μm^2 represent about 21% of the values measured with GB1-GB2, offering a good estimate of PLA signals resulting from a random proximity between two non-interacting cell surface proteins expressed at this level.

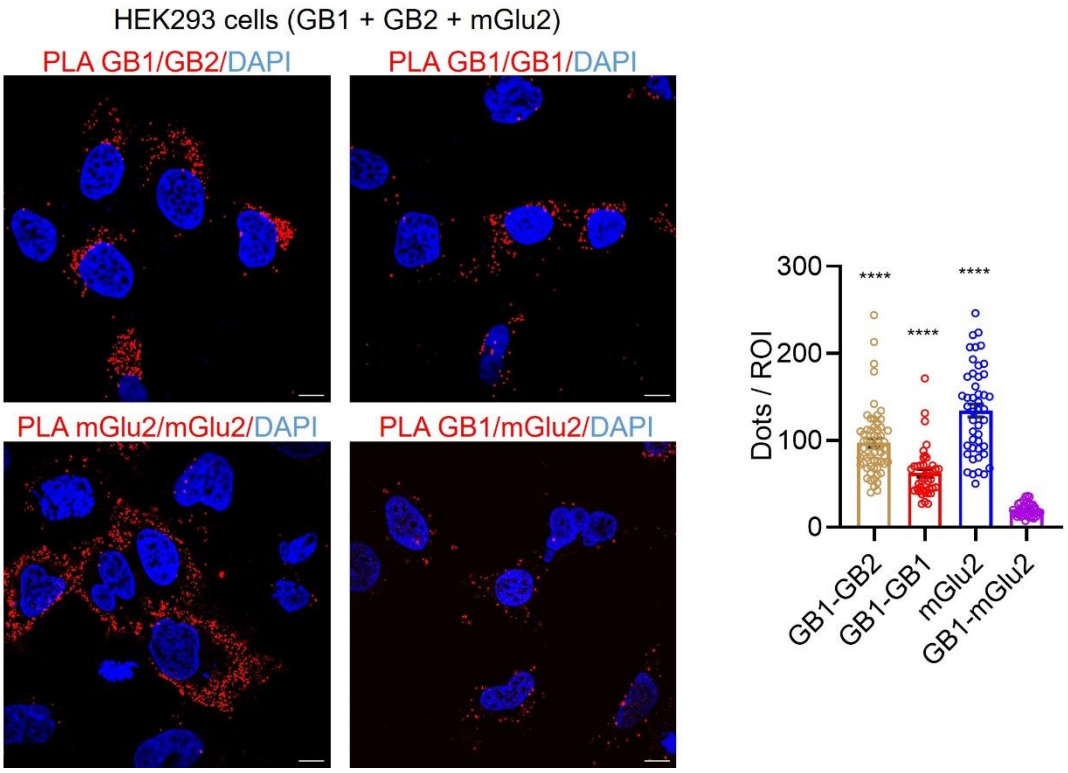


Figure R2. **Detection of $GABA_B$ hetero-tetramer expression.** Representative images of PLA experiments performed with Nb-4C10^{rFc} and Nb-5E9^{mFc} (anti-GB1/GB2), or Nb-4C10^{rFc} and Nb-4C10^{mFc} (anti-GB1/GB1), or DN1^{rFc} and DN1^{mFc} (anti-mGlu2), or Nb-4C10^{rFc} and DN1^{mFc} (anti-GB1/mGlu2) in HEK293 cells expressing indicated proteins. PLA signal is shown in red dots, cell nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI) in blue. Scale bar: 10 µm. Data are representative of three independent experiments performed on different cultures. The histograms show mean ± s.e.m. of PLA dot number per region of interest from 37 to 61 cells. Data are analyzed using one-way analysis of variance (ANOVA) with a Dunnett 's post-hoc multiple comparison test to determine significance (compared with GB1-mGlu2 group). ****$P < 0.0001$.

These data are presented in the new **Extended Data Fig. 2** of the revised manuscript.

2) For this same PLA experiment, how do the authors rule out that any of the signal attributed to tetramerization results from GB1-GB1 homodimerization? Design of a probe to measure this species would help strengthen evidence.

The referee is right. This point has also been raised by the first referee. See our answer to his/her first point above. In summary, although we cannot exclude this possibility, a number of previously reported data indicate a very low amount (if any) of GB1 homodimers at the cell surface. Moreover, we don't see how one could discriminate a strict GB1 homodimer, from a dimeric GB1 within a $GABA_B$ tetramer. This point is now clearly

discussed in our revised manuscript (Page 15, Line 369-397).

3) For experiments measuring heterodimer and heterotetramer signaling with CODA-RET the authors use LgBit and HiBit tagged receptors to allow measurement of activation (Gi recruitment) only at receptor pairs. This is an interesting experimental design, but it also likely affects the oligomeric propensities of the receptors under study (as shown later when attempting to purify hetero-tetramers). Since HiBit has high affinity for LgBit it is hard to discern whether the complexes formed would apply to endogenous receptors or if this is an artefact of introduction of the complementary tags. Could the authors use SmBit (a lower affinity peptide that binds LgBit) instead of HiBit to reduce the contribution of tag-mediated assembly has on the pharmacological responses observed?

This is indeed an interesting point. Our goal was to get information on the functional properties of the $GABA_B$ receptor tetramers, versus the heterodimers. When analyzing the heterodimer, as the later is strongly stabilized by the coiled coil domain, the high affinity interaction between LgBiT and HiBiT will not change much the interaction between the subunits within these already stable dimers. In that situation the number of tetramers, influencing the functional assay are kept to their natural level based on the surface density of the $GABA_B$ heterodimers. These can eventually be disrupted as illustrated in our previous studies (Maurel et al., Nat Methods 2008; Comps-Agrar et al., EMBO J 2011). For the analysis of the functional properties of the tetramer, one need to have the highest proportion possible of tetramers in order to have a good signal, mainly generated by these complexes, with as less as possible contamination by strict heterodimeric responses. This is the reason why we choose to use the high affinity component of the NanoBiT system. This allowed us to characterize the pharmacological and functional properties of the tetramers selectively. This is of course, unrelated to what could occur in native cells where a mixture of the strict heterodimers and tetramers likely exists, making then the functional responses difficult to interpret. We have here to admit that our data are more qualitative than quantitative, as the responses measured here in the so called “heterodimer assay” are likely contaminated by some generated by natural tetramers. This is well illustrated by the increase in signaling observed with the mutant expected to prevent the interaction between the GB1 VFTs within tetramer (Fig. 4).

4) When performing BRET assays using CODA-RET the authors report BRET ratios for assessing receptor activation. To make these measurements the authors also must record bulk luminescence values (from LgBit + HiBit), which would report on the total amount of nLuc complex formed in a given cell population. This would seem to be valuable information for understanding how much heterodimer vs heterotetramer is formed under analogous conditions. Could the authors report nLuc luminescence in a meaningful format to provide insight into this topic?

We thank the reviewer for raising this important point. Obviously, these data were recorded for any experiments shown in our original manuscript, and are now included in the revised version. As shown in **Figure R3**, the luminescence signal showed no significant difference between $GABA_B$ WT and mutants.

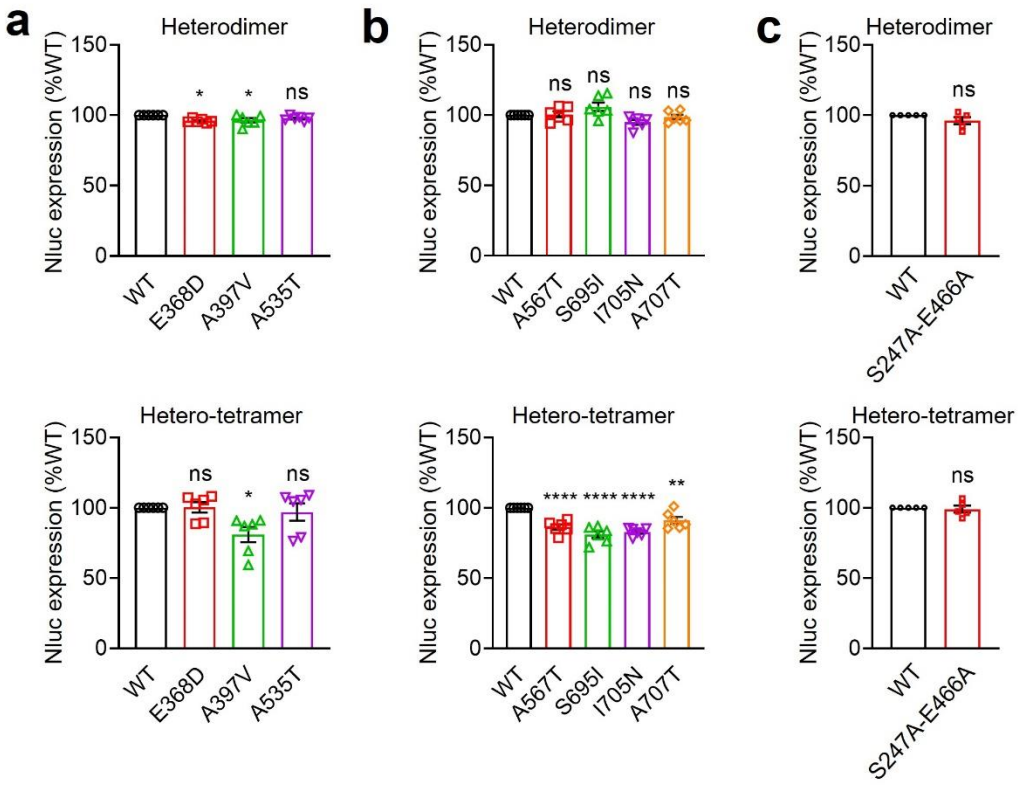


Figure R3. Nluc expression levels of $GABA_B$ heterodimers and heterotetramers. a-c, Total luminescence of indicated recombinant Nluc in both $GABA_B$ heterodimers or hetero-tetramers. Data are present as mean ± s.e.m. from at least three independent experiments, and were analyzed using one-way analysis of variance (ANOVA) with a Dunnett's post-hoc multiple comparison test or unpaired t-test (two-tailed) to determine significance (compared with WT group). ****$P < 0.0001$, **$P < 0.01$, *$P < 0.05$ and not significant (ns).

These data are presented in the new **Extended Data Figure 3d, Extended Data Figure 5f and Extended Data Figure 11c** of the revised manuscript.

We also performed a dedicated experiment in which we compared the nLuc signals obtained using the GB1-GB2 constructs (heterodimers), with those obtained using the GB1-GB1 constructs (tetramers). We also performed, in the same cells, an ELISA after cell permeabilization to estimate the total amount of $GABA_B$ receptors, taking advantage of the HA-tagged GB1 subunits. Under such condition, we measured 5.46 ± 0.17 luminescence unit per Elisa-signal unit for the heterodimer, and 3.67 ± 0.12 for the tetramer (**Table R1**). Assuming there will be half of the Nluc formed if all heterodimers were assembled into tetramers, the value obtained for the tetramers if higher than expected. This can be due to the different constructs used, and the likely existence of some GB1 homodimers in the intracellular compartments. Whatever, these data suggest that the HiBiT-LgBiT system, as expected and discussed in this rebuttal, very likely stabilizes the tetramers, increasing the proportion of $GABA_B$ receptors in a tetrameric form.

	Nluc expression	GB1 total expression	Normalization (Nluc/GB1)	Normalization (mean ± s.e.m)	Heterodimer vs. Heterotetramer
Heterodimer	1419910	295990	4.80	5.46 ± 0.17	0.67
	1427106	277720	5.14		
	1437310	262900	5.47		
	1451246	252320	5.75		
	1460473	255470	5.72		
	1497626	254800	5.88		
Heterotetramer	1100823	353050	3.12	3.67 ± 0.12	
	1129390	309550	3.65		
	1121223	301380	3.72		
	1130176	285980	3.95		
	1160330	295170	3.93		
	1140993	309470	3.67		

Table R1. The ratio of $GABA_B$ heterodimer vs hetero-tetramer determined by Nluc expression and GB1 total expression. Cells expressing NanoBiT-tagged $GABA_B$ receptor complexes were analyzed to compare luminescence signals (Nluc) between heterodimeric and tetrameric forms. Total receptor expression was quantified by ELISA after cell permeabilization using an antibody against the HA-tagged GB1 subunit. The relative Nluc-to-GB1 expression ratios reflect differences in receptor assembly states, distinguishing heterodimeric from heterotetrameric forms. Data shown are representative of three independent experiments.

These data are presented in the new **Extended Table 2** of the revised manuscript.

5) It seems intuitive that higher older oligomer assembly (such as tetramerization) would be highly dependent on the level of a given protein at the cell surface. This type of consideration would seem to be particularly important for receptor mutants (like data in Figure 2) which might have altered stability (or expression levels). Can any of the altered behavior of any receptor variants be simply explained by alterations in levels of cell surface protein? This could be measured by flow cytometry staining and would seem to be most important for assessment of pathogenic receptor mutants.

This point has also been raised by the first referee. See our answer to his/her second point above (**Figure R1**). To summarize: most mutants show very similar expression level as the WT, but two of them are expressed at a 2-fold lower level, providing explanation for their peculiar functional response, but in perfect line with our conclusion.

6) It seems possible that the LgBit-HiBit interaction could play a role in dictating the geometry observed in the interaction between the two subunits in the heterotetramer structure. Are there observations that would argue against this? If not, I suggest devising alternative mechanisms for promoting assembly to see if this changes the heterotetramer geometry observed.

Three of our previous studies support the cryoEM structure observed of the $GABA_B$ tetramer, and the specific

organization of the subunits. In a first study (Maurel et al., Nat Meth 2008), we revealed a similar, slightly lower FRET signal between GB1 and GB1, to that measured between GB1 and GB2, for the same amount of receptors at the cell surface. In contrast, a much lower FRET was observed between GB2 and GB2. Based on these data, taking into account the Ro value of the FRET pair used, we proposed a tetramer assembly with a close contact between GB1 subunits from two $GABA_B$ heterodimers, and the GB2 subunits on the side, too distant to generate high FRET. In a second study, we examined the possibility to cross-link 7TM domains of GB1 with either GB1 or GB2 after inserting Cys at different positions in the TMs (Xue et al., Nat Commun 2019), in either an inactive or active state. The data obtained lead to a model that is close, though more compact to that observed in the cryoEM structure of the tetramer. At least, the contact zone between each of the 4 subunits proposed, are in line with those observed in the structure. In the third study (Comps-Agrar et al., EMBO J 2009, confirmed in Stewart et al., Neuropharmacology 2018), we identified a site in the GB1 VFT as possibly being the interacting site at the level in the $GABA_B$ tetramer. Indeed, mutations at this site resulted in a decrease in FRET between the GB1 VFTs, and most importantly, in the negative allosteric control of one heterodimer by the other. This site corresponds perfectly to that identified in the cryoEM structure for one GB1 subunit. We however proposed a symmetric association, while the structure revealed an asymmetric GB1-GB1 association. This later finding nicely explains that a single binding site was accessible per tetramer (Stewart et al., 2018)

As these studies were all performed without the LgBiT-HiBiT system, this is consistent with this system not influencing the general organization of the tetramer.

7) The observation that transplanting the extended loop found in Drosophila loop 3 into human receptor affects calcium signaling is interesting. It seems like this effect should be tested in more direct measurements of heterotetramerization (PLA or CODA-RET).

We previously reported that steric hindrance at the level of the GB1-VFT interaction between the heterodimers did prevent the negative allostery between the heterodimers (leading to higher signaling efficacy, and increasing the number of accessible binding sites: Stewart et al., Neuropharmacology 2018), but did not fully prevent tetramer formation. As discuss in this previous study, we assume the tetramers can still form through interactions at the level of the 7TM domains, consistent with what is observed in our cryoEM structure. We cannot exclude that the drosophila specific loop does the same. Whatever, we did examine the effect of this loop on the PLA signals measured between GB1 and GB1, and observed a lower number of dots/μm^2 (**Figure R4**). A similar value was obtained with the DGB1 constructs in which we added the sushi domains to allow the generation of PLA signal with our tools, indicating that the addition of the drosophila loop in the human receptor results in the same PLA signal as that measured with the drosophila receptors. Taken into account the background signal measured between GB1 and mGlu2, the introduction of the drosophila loop led to a two-fold decrease in the PLA signal, indicating a lower proportion of tetramers.

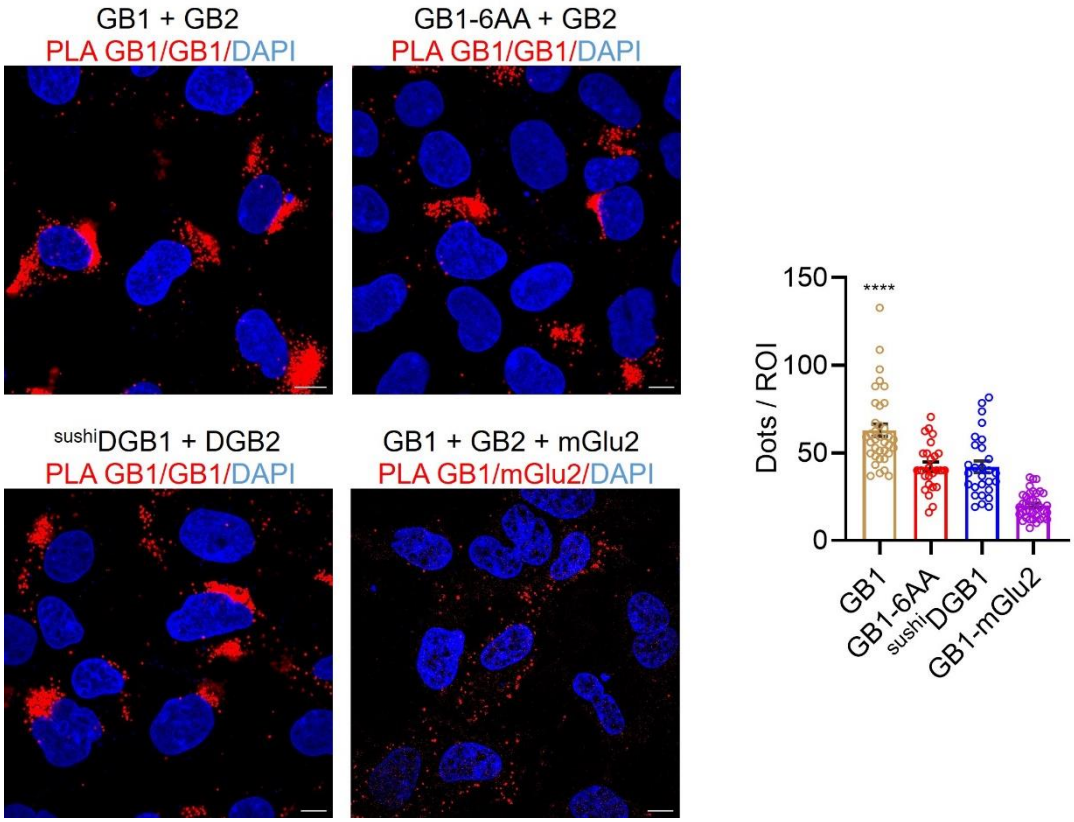


Figure R4. Drosophila loop reduces GB1 tetramerization. Representative images of PLA experiments performed with Nb-4C10^{rFc} and 4C10^{mFc} (anti-GB1^{WT}/GB1^{WT}), or Nb-4C10^{rFc} and Nb-4C10^{mFc} (anti-GB1^{6AA}/GB1^{6AA}), or Nb-4C10^{rFc} and Nb-4C10^{mFc} (anti-^{sushi}DGB1/^{sushi}DGB1), or Nb-4C10^{rFc} and DN1^{mFc} (anti-GB1/mGlu2) in HEK293 cells expressing indicated proteins. PLA signal is shown in red dots, cell nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) in blue. Scale bar: 10 µm. Data are representative of three independent experiments performed on different cultures. The histograms show mean ± s.e.m. of PLA dot number per region of interest from 37 to 61 cells. Data are analyzed using one-way analysis of variance (ANOVA) with a Dunnett 's post-hoc multiple comparison test to determine significance (compared with GB1-6AA group). ****P < 0.0001.

These data are presented in the new **Extended Data Figure 9d** of the revised manuscript.

8) For the data shown in Figure 5d, the observation that heterotetramer efficacy was decreased by 50% when a GB1 subunit that does not bind ligand is introduced was interesting. I think that performing a similar measurement for heterodimer signaling would also be useful.

We thank the reviewer for this suggestion. We have measured G protein activation in the $GABA_B$ heterodimer with the mutation of S247A-E466A in the GB1 subunit. As shown in **Figure R5**, and as expected by the mutation of the unique GABA binding site in this heterodimer, this mutant totally lost the ability to be activated by GABA. This is consistent with a previous report (Comps-Agrar, L. et al. EMBO J 2011).

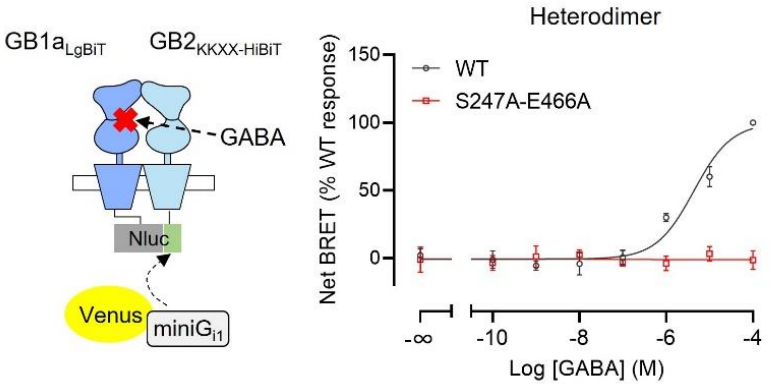


Figure R5. GABA-induced G proteins in $GABA_B$ heterodimer WT and mutant with S247A-E466A in GB1 subunit. The effect of GABA on the G protein recruitment to $GABA_B$ heterodimer WT or S247A-E466A mutant. Data are presented as mean ± s.e.m. from five independent biological replicates each performed in triplicates.

These data are presented in the new **Extended Data Figure 11a** of the revised manuscript.

9) For the data shown in Figure 6c, it seems there is no negative control where dimerization between two Cys containing constructs is not observed. This control is needed to say whether the assembly mechanism described here is needed for covalent heterotetramer formation (or if simply overexpressing two Cys-containing constructs in the same cell is enough to observe these effects)

To address the concern regarding a negative control in Figure 6c, we also generated four additional mutant constructs, none of these alternative mutants led to crosslinked oligomers (**Figure R5**). These results demonstrate that crosslinking of heterodimers is not simply driven by the presence of cysteines. Rather, covalent crosslinking occurs specifically due to the spatial proximity of residues (GB1-K590$^{1.38}$C and GB2-A617$^{4.52}$C, or GB1-D831^{TM7}C and GB2-A617$^{4.52}$C), supporting the idea that the observed heterotetramer formation reflects a defined assembly mechanism rather than non-specific interactions resulting from the overexpression of two cysteine-containing constructs in the same cell.

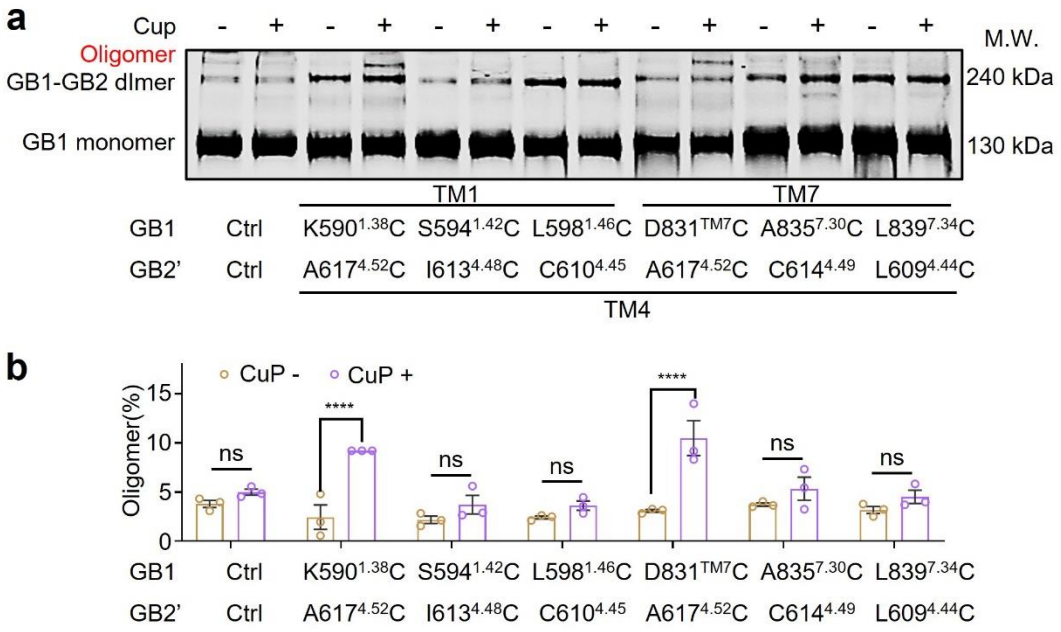


Figure R5. Crosslinking of cell surface GB2 and GB1' subunits between the indicated residues mutated into cysteine, without (CuP-) or with CuP (CuP+). a, Represent blots of the crosslinking experiment. **b,** Percentage of hetero-tetramer are presented as mean ± s.e.m. from three independent biological replicates, and analyzed using unpaired t-test (two-tailed) to determine significance (compared with CuP-). ****P < 0.0001 and not significant (ns).

These data are presented in the new **Extended Data Figure 13** of the revised manuscript.

10) I did not find sequence listings for any of the receptor construct designs or the nanobodies (and nanobody constructs) identified in this study. This data is essential for others to be able to build on these findings. If there are intellectual property considerations restricting sharing of sequence information, then reference must be made to the patent disclosures so relevant information can be obtained once possible.

The amino acid and nucleic acid sequences encoding NB-4C10 and NB-5E9 have been included in Chinese patent CN120024449A (State: disclosure). They will then be accessible to everyone for basic research as soon as the patent is published.

Minor Comments:

a) It looks like the binding experiments to determine nanobody affinities (Extended Fig 1 and Extended Table 1) were only performed once. These should be repeated at least once more to provide error bars on the measurements and give a sense of whether different nanobodies and constructs actually have statistically distinguishable binding.

There experiments have been performed three times. We have clarified it in the figure legend of Extended Data Fig. 1 and added n numbers in Extended Table 1.

b) Nanobody binding constants are listed as Kd values, but it seems these numbers were derived from flow cytometry staining experiments. Such values are better described as EC50s (and not Kds).

Our nanobodies specifically target GB1 or GB2 subunit, demonstrating a strict stoichiometric ratio of 1:1 (nanobody:receptor). Based on this, we measured the binding affinity using flow cytometry staining experiments. We then measured the mean staining value obtained through the fluorescence signal detected by the flow cytometer, and expressed these values as a function of the concentrations of nanobodies used for the staining. The saturation curves were fitted using GraphPad Prism software by the nonlinear dose-response method to determine the affinity of the nanobodies, which was represented by the equilibrium dissociation constant (K_D) between the nanobody and receptor, using a one-site specific binding equation:

$$Y = B_{max} \times X/(KD + X),$$

where X is the concentration of nanobodies, Y is the response to the specific binding, B_{max} is the maximum specific binding in the same units as Y, and KD is the equilibrium dissociation constant in the same units as X. Saturation curves obtained were already shown in **Extended data Fig. 1**.

c) In the caption for Ext Figure 1b the concentration of nanobody is listed as 1 mM. This seems high, perhaps double check?

Thank you for pointing this typo, the concentration was indeed 1 µM. This has been corrected in the revised manuscript, and we carefully checked other similar mistakes in the text and figure legends.

d) In Extended Data Fig. 1 could the authors define RSR?

RSR is now presented as the ER retention motif, and is further indicated as being the Arg-Ser-Arg sequence. It has been modified in Extended Data Fig. 1 to be more clearly.

e) On lines 162-164 the authors describe the $GB2_{KKXX}$ receptor, but it is not clear to me when it is applied (in this portion of the text) or how it functions. Based on Ext Data Fig 1 it looks like wild type hGB1a (used as GB1) has an ER retention signal. How can this be combined with $GB2_{KKXX}$ and result in receptor at the cell surface? I am likely just missing some important details, but it would be helpful of the authors could be more explicit about which GB1/GB2 variants are used in what context.

The following sentence: "Additionally, we introduced an ER retention signal at the C-terminal end of GB2 ($GB2_{KKXX}$) to regulate its trafficking to the cell membrane, which occurs only upon interaction with GB1" has been replaced by:

"In order to perfectly control the surface expression of both GB1 and GB2 subunits, an ER retention motif KKXX was added at the C-terminal end of the GB2 subunit (see Brock et al., JBC 2007). As such, neither GB1,

nor $GB2_{KKXX}$ could reach the cell surface alone. With these subunits, the coiled coil interaction between the C terminal tails of GB1 and GB2 being able to mask both the natural and introduced ER retention motifs of GB1 and GB2, respectively, only the GB1-GB2 heterodimers could reach the cell surface." (Page 7, Line 165-170)

f) For Figure 1e, could the authors add some additional text to the figure legend more clearly explaining what the bar graphs in the inset represent? Same request for Figure 2c-d.

We thank the reviewer for the comment. We have added the description in the Y axis. According to the point #2 of referee #1, the estimated TOP values have been taken from the prism fits for **Figure 2c-d**.

g) Starting at line 188 the authors begin discussion of experiments using baclofen. It seems that baclofen was not used in Figure 1 experiments. Could the authors add text explaining this choice (and whether they might have missed anything by not using baclofen in Fig 1)?

We thank the reviewer for pointing this mistake. Indeed, experiments use to generate Fig. 1e were performed with both GABA and baclofen. In the first figure we choose to present the data with GABA, as it is the endogenous ligand for this receptor. The data obtained with baclofen were shown in Extended data Fig. 4a, b. We now corrected the main text to mention correctly the ligand used: GABA for **Figure 1e**, and baclofen for **Extended Fig 4a, b**. We think it was important to analyze the effect of baclofen on the disease-associated mutation analyses, as it is an approved drug, allowing us to directly assess how these mutations influence responses to a clinically relevant ligand.

h) Could the authors comment on why they think they don't see any signal for Nb-4C10-Bi in the CryoEM structure? Is it even important for inducing heterotetramerization?

We thank the referee for pointing out this point, as we should have explained it in our original manuscript. This is now clarified in the revised version (Page 10, Line 241-243). Indeed, during the characterization of Nb-4C10, we found it binds to the extracellular domain of GB1. Further investigation revealed that it binds to GB1a but not to GB1b, indicating that the pair of sushi domains unique to GB1a are essential for Nb-4C10 binding. In our previous cryo-EM structures of the $GABA_B$ dimer in different states, published in Cell Research (2020) and in Nature (2021), we did not observe density for the sushi domains, indicating that they are highly flexible. We then speculate that the position of Nb-4C10 bound to this flexible domain is very different from particles to particles preventing us from detecting clear density in the position where this nanobody is supposed to be. Although we haven't visualized the density for Nb-4C10 in the cryo-EM structure, we have found that incorporating a sufficiently long linker to connect two 4C10 molecules, forming Nb-4C10-Bi, allows it to bind to two GB1a subunits. This approach aids in the enrichment of heterotetramers during protein purification, thereby increasing the yield of the tetrameric form.

i) When assessing the functionality of constructs used for structural studies the authors use a calcium

mobilization assay. Could the authors comment on why this wasn't used on constructs described earlier in the paper? It might be good to do a few spot checks to see if calcium mobilization correlates with the G protein assays described earlier in the manuscript.

In the functional and pharmacological analysis of the WT and mutant $GABA_B$ receptors in cells, we wanted to discriminate between the activity of heterodimers and tetramers. This is the reason why we used the BRET approach associated with the HiBiT-LgBiT reconstitution approach of the Nluc, as illustrated in **Figure 1**. In **Figure 4** we however used a Ca^{2+} assay (with co-expression of a chimeric Gqi protein) as we wanted to see the change in agonist efficacy resulting from a decreased proportion of tetramers.

j) In lines 291-294 the authors write about whether one or both VFTs can bind to ligand. How was this assessed? By modelling? Some additional context would be helpful.

Previous reports have definitively shown that the VFT binding domain of Class C GPCRs, including that of GB1, maintain an open conformation in the apo state (Mao et al., Cell Res 2020 and Hamidreza Shaye., Nature 2020). Agonist binding induces a closed conformation. To assess the ligand-binding capacity of each VFT domain within our $GABA_B$ tetramer, we performed structural modeling. Specifically, we individually aligned the closed conformation of the GB1 VFT domain (derived from the $GABA_B$ dimer bound to baclofen) to either the GB1 or GB1' subunit within our tetrameric structure. These modeling studies revealed that each GB1 VFT domain could independently adopt a closed conformation without steric clashes, suggesting that each GB1 subunit in the $GABA_B$ tetramer possesses the potential to bind ligand. However, when we attempted to simultaneously model two closed GB1 VFT domains onto the two GB1/GB1' subunits within the tetramer, we observed steric hindrance. This indicates that while both GB1 subunits can potentially bind ligand, only one subunit can undergo VFT domain closure at a given time due to these steric constraints. This is now better explained in our revised manuscript (Page 12, Line 303-312).

k) This might be addressed in other forms, but it would be useful to provide exact product numbers for some of the unusual reagents (PLA probes, etc).

We now provide the number in the revised manuscript. (Sigma, Cat. No. DUO92101).

l) A few small spelling/grammar suggestions:
Title: suggest removing word "a";

The revised title is now: "Functional and structural basis of negative allostery within $GABA_B$ hetero-tetramers".

Line 47: evidences should be evidence, and oligomer should be oligomers;

These mistakes have been corrected.

Line 530 Thers; unsure what this is supposed to say

"Thers" has been replaced by "The".

Reviewer #3 (Remarks to the Author):

$GABA_B$, as a class C GPCR, is known to function as heterodimers. Whether higher-order oligomers exist and their physiological relevance is not clear. In this manuscript, Shen et al. employed multiple approaches to study the role of $GABA_B$ hetero-tetramers in cellular signaling. To do so, they first developed nanobodies that could recognize different subunits (GB1 and GB2, for example). Using the nanobody-based PLA assays, they could detect oligomerizations in both overexpressed HEK cells and isolated neurons. Both hetero-dimers and hetero-tetramers can signal through G proteins but with different potency or efficacy. Some clinically relevant mutations may function via the change of hetero-tetramer stability or formation. A cryo-EM structure of $GABA_B$ Hetero-tetramers has been determined and displays different symmetry compared to the hetero-dimer structure. This manuscript provides evidence from different aspects to support that $GABA_B$ hetero-tetramers may exist physiologically. The other evidence that these tetramers play important roles in GABAB's function needs more data.

1. A few major questions related to the physiological relevance of hetero-tetramers. What is the percentage of $GABA_B$ hetero-tetramers vs hetero-dimers in neurons and what determines this ratio? Figure 1b quantified both hetero-dimers and tetramers on cell membranes, do they represent all populations of $GABA_B$ in cells or neurons? Is the tetramer formation inducible upon condition changes or receptor activation?

The referee raised here an important question to which it is difficult to answer with high precision. When studied in transfected cells using fluorescence photobleaching step analysis, $GABA_B$ receptor tetramers can already be detected, representing 30% of the population, at density as low as 0.15 particules/μm^2 (Calebiro et al., PNAS 2013). When the density is increased, more tetramers, and more larger oligomers were observed, with almost no strict dimers detected at densities higher than as 0.4 particules/μm^2. When analyzed using inter-subunit FRET, we found that the GB1-GB1 FRET, was directly proportional to the expression level, and representing more than 80% of the signal obtained between GB1 and GB2, for a receptor density between 0.1 and 3.0 pmol/mg of proteins (Maurel et al., Nat Meth 2008), then in the range of the endogenous $GABA_B$ receptor density in the brain (0.5 pmol/mg) (Bishoff et al., J Comp Neurol 1999), or in cultured cortical neurons (1.5 pmol/mg) (Comps-Agrar et al., EMBO J 2011). We also previously reported that the GB1-GB1 FRET signal resulting from tetramer formation is not sensitive to agonists (Maurel et al. Nat Meth 2008). Such data already suggest that $GABA_B$ oligomers do not represent a minor proportion of receptors in neurons, especially considering the receptor density is high in specific sub-compartments. However, one cannot exclude the possibility that scaffolding proteins or other $GABA_B$ receptor partners may prevent the formation of, or stabilize such tetramers.

Another important information is that, at least in transfected cells, $GABA_B$ heterodimers are not exchangeable between oligomers (Comps-Agrar et al, FASEB J 2012). Indeed, the data reveal that in the cell plasma membrane the oligomers are quite stable. This suggests that, once formed, they may not be dissociated easily, and may in contrast accumulate.

In our study, we found that the PLA signal (Dots/ROI) measured between GB1 and GB2 is about twice as big

as that measured between GB1 and GB1, both in transfected cells and in cultured neurons. According to the high proximity between heterodimers within a tetramer, we can assume that a single dot will be generated by tetramer when using the heterodimer detection tools. Accordingly, the number of dots detected with the GB1-GB2 detector, very likely represent the total number of strict heterodimers plus the tetramers. In contrast, the dots generated by the GB1-GB1 PLA tools, will only reveal tetramers. Such observations suggest that about half of the $GABA_B$ complexes are tetramers, the other half being strict heterodimers. However, more work is needed to validate the linearity of the PLA signal under our condition, as a function of the protein expression, to make sure such data can be used as a quantification method. Moreover, the GB1-GB1 PLA signal may also account for some heterodimers in close enough proximity, but not assembled into tetramers, as this is allowed by the size of the a nanobody + secondary antibody.

In conclusion, although we cannot provide a good estimate of the proportion of tetramers, our and previous data suggest that they do represent a significant fraction of the receptors. The present discussion is rapidly mentioned in our revised manuscript in the discussion (Page 15, Line 384-397).

2. The two nanobodies are useful tools in this study. In Figure 1a & 1b, will the binding of the nanobody affect another binding? If yes, this will affect the quantitation of dimers or tetramers in cells. One experiment that can be done is to measure the binding affinity of Nb5E9 after long incubation of $GABA_B$ with Nb-4C10.

We thank the reviewer for this important point. To address this, we performed competitive binding assays using HEK293 cells expressing human $GABA_B$ receptors. Cells were incubated with 10 µM Alexa Fluor 647-conjugated nanobody, followed by increasing concentrations of the corresponding unlabeled nanobody (**Figure R8**). We observed that competition occurred in 2 hours after the addition of the competing nanobody only within identical nanobody pairs (Nb-4C10 competed with Nb-$4C10^{AF647}$ **(Figure R8a)**, and Nb-5E9 competed with Nb-$5E9^{AF647}$ **(Figure R8b)**), indicating that Nb-4C10 and Nb-5E9 bind independently to distinct, non-overlapping epitopes. Therefore, the binding of one nanobody does not interfere with the other, ensuring that our dual labeling strategy allows accurate quantification of dimers and tetramers in cells.

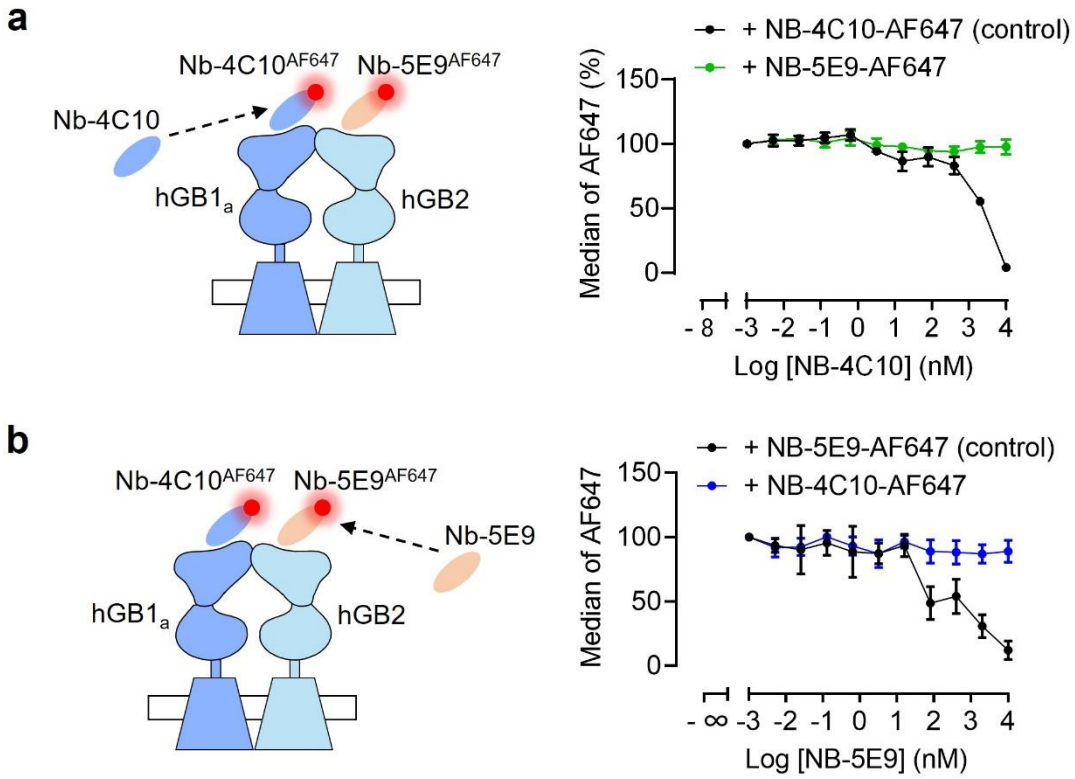


Figure R8. Competitive binding assay of nanobodies to human $GABA_B$ receptor. a, b, HEK293 cells overexpressing human $GABA_B$ receptor were incubated with 10 μM Alexa Fluor 647-conjugated nanobody, followed by the addition of increasing concentrations of unlabeled nanobody for 2 h to allow competitive binding. The percentage of positive events was used as the statistical parameter to characterize their competitive binding to the human $GABA_B$ receptor. Data are presented as mean ± s.e.m. from three independent biological replicates.

These data are presented in the new **Extended Data Fig. 2a, b** of the revised manuscript.

3. Line 162-164: Additionally, we introduced an ER retention signal at the C-terminal end of GB2 ($GB2_{KKXX}$) to regulate its trafficking to the cell membrane, which occurs only upon interaction with GB1. This sentence and the assay are unclear. $GB2_{KKXX}$ can retain GB2 in the ER membrane, why does interaction with GB1 then help its trafficking to the cell membrane? Particularly, the CODA-RET assay will measure bioluminescence from the cell membrane, ERs, and Golgi.

The use of the quality control system, including the addition of KKXX in the GB1 subunit has been clarified in our answer to minor point #e of the referee #2 above.

It is true that CODA-RET assay will measure bioluminescence from all the cell compartments. However, as GABA is known not to penetrate into the cell, only the cell surface receptors will be activated, and then generate a BRET signal. Accordingly, the intracellular nLuc will influence the Venus/nLuc signal calculated. However, we expressed our data as the netBRET (BRET signal with agonist minus the basal BRET signal), and then

always normalize to a positive control. Accordingly, this should not influence much the data presented.

Line 173 says 100 µM, in the Figure 1e legend it was 100 mM.

The mistake. In the figure legend has been corrected (100 µM).

4. When examining the clinical variants, the assay was specifically designed for measuring dimers or tetramers. Those mutations may still affect signaling. A regular BRET including GB2-Nluc and Venus-miniGi1 can be performed to see if the mutants affect G protein signaling compared to WT.

We thank the reviewer for this suggestion.

To evaluate the effects of clinical variants on overall G protein signaling, we performed conventional BRET using GB2-Nluc and Venus-miniGi1. The results were consistent with those obtained from NanoBiT dimer assays (**Fig. 2**). GABA-induced G protein coupling was more profoundly reduced in the $GABA_B$ hetero-tetramers carrying the E368D, A397V, or A535T mutations compared with the corresponding heterodimers. Similarly, rac-BHFF showed only slight or no reduction in efficacy at heterodimers for E368D and A397V (38%, respectively) and no effect for A397V or A535T (**Figure R7**). These findings indicate that these mutations still impact overall receptor signaling, particularly in the tetrameric context.

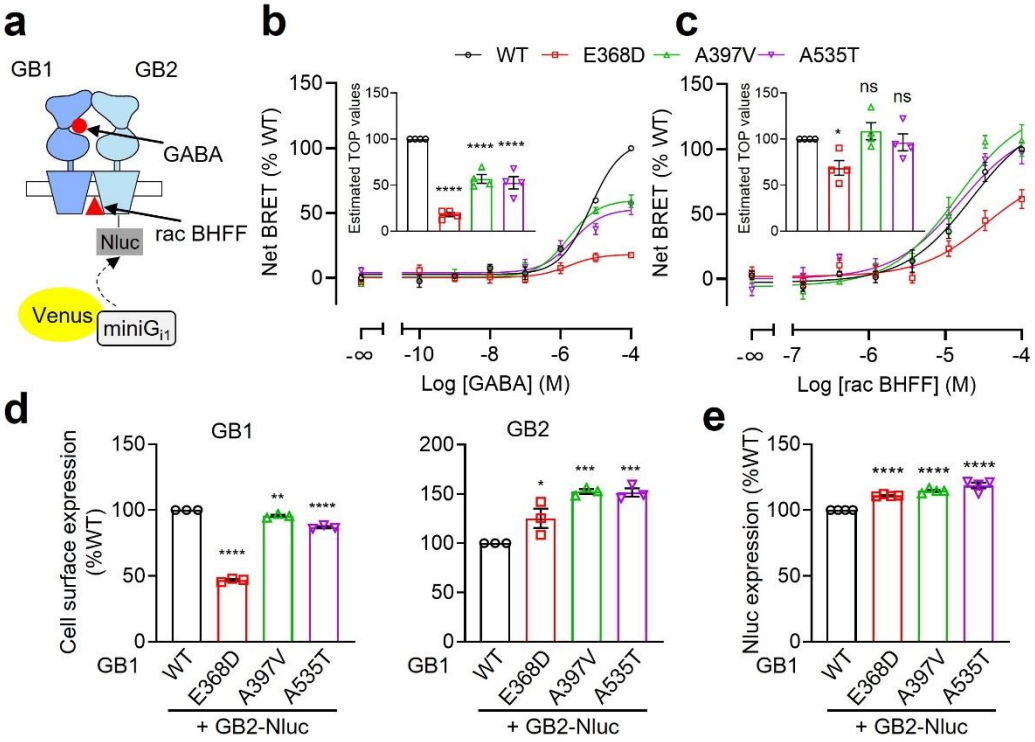


Figure R8. a, Schematic representation of the G protein recruitment to human $GABA_B$ heterodimer by regular BRET using GB2-Nluc and Venus-miniGi1. **b, c,** Effects of GABA (left panel) and rac-BHFF (right panel) on G protein recruitment to the $GABA_B$ heterodimer of WT or indicated mutants (with E368D, A397V, A535T in

GB1, respectively), measured by regular BRET using GB2-Nluc and Venus-miniGi1. The inserted graph represent the estimated TOP values of GABA and rac BHFF on G protein recruitment to $GABA_B$ heterodimer WT or indicated mutants. **d,** Expression of the indicated GB1 WT or indicated mutants (with E368D, A397V, A535T in GB1, respectively) and GB2 receptors. **e,** Total luminescence of indicated recombinant Nluc in both $GABA_B$ heterodimers or hetero-tetramers. Data are presented as mean ± s.e.m. from at four biological independent experiments each performed in triplicates, and analyzed using one-way ANOVA with a Dunnett's post-hoc multiple comparison test to determine significance (compared with WT). ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and not significant (ns).

These data are presented in the new **Extended Data Figure 4e-i** of the revised manuscript

5. Another major concern is the use of NanoBit and Nanobody in determining the hetero-tetramer structures. Although they were essential for successful structure determination, they weaken the physiological relevance of the hetero-tetramers. Particularly their sample preparation without NanoBit or Nanobody didn't allow them to get enough hetero-tetramers, which indicates the hetero-tetramers are a very small population compared to hetero-dimers.

When preparing membrane proteins, they are solubilized using a combination of detergents. These were optimized to get a maximal extraction of the proteins, but not to ensure the preservation of any membrane complexes. For example, the preparation of $GABA_B$ dimers deleted of the coiled-coil domain, easily lead to the isolation of isolated subunits. Obviously, this does not mean the GB1-GB2 dimers are not stable in the plasma membrane, even in the absence of the coiled coil. In cryoEM experiments one want to have the vast majority of the particles corresponding to the same protein complex. Indeed, even like this, the complexity of the system is such that sometimes only 25% of the particles can be used to generate a precise 3D model. Then, if the complexity is increased with additional $GABA_B$ receptor stoichiometry, the situation may be too complex to solve a structure. This is why we used two tricks to stabilize the $GABA_B$ tetramer: i) the LgBiT-HiBiT fused to the C-terminal ends of the GB1 subunits, and also our dimerized nanobody Nb-4C10^{Bi} that bind the sushi domains of both GB1a subunits, and then stabilize the tetramer on the extracellular side. Note that tricks to stabilize specific protein complexes are always used to solve them. With the structure of the complex solved, one has access to important information to test if such a complex exists in real life, through mutations expected to affect the complex, as is being done commonly. We think our previous data, as well as those mutations reported here, plus the crosslinking experiments based on our solved structure, validate our structural data. Moreover, the asymmetric organization of the tetramer provides a clear explanation as why only one binding site is available at a time, as our previous data (Stewart et al., Neuropharmacology 2018).

Additional arguments illustrating the absence of effect of the Hi-Bit-LgBit system, and the presence of the bivalent nanobodies on the geometry of the $GABA_B$ tetramer can also be found in our answer to the point #6 of the second referee.

6. Line 255-269. Here the authors tried to introduce glycosylation sites to prevent dimerization or

oligomerization. Why did it not work? Was the presence of glycosylation confirmed by mass spec or other approaches? Glycosylation can significantly increase the size of the proteins by introducing a large sugar chain, which is sufficient to disrupt the interface.

We thank the reviewer for this suggestion. To assess whether the introduced NXS/T mutations successfully resulted in glycosylation, we compared the electrophoretic mobility of GB1 carrying these mutations with that of wild-type GB1. The NXS/T mutant GB1 exhibited a noticeably higher molecular weight band than wild-type GB1, consistent with the addition of sugar moieties (**Figure R8**). Functionally, introduction of these glycosylation sites led to a marked enhancement of $GABA_B$ receptor activity (**Fig. 4d)**. This effect is due to steric hindrance at tetrameric interaction interfaces introduced by the added sugar chains, which reduces the negative allosteric influence of tetramer formation and thereby restores receptor function. However, we think, in agreement with our previous data (Stewart et al., Neuropharmacology 2018) that the interactions at the level of the TM domains help maintaining the $GABA_B$ tetramer assembly, even though the asymmetric organization of the GB1 VFTs responsible for the negative coupling is prevented.

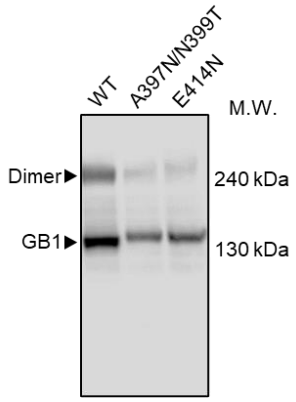


Figure R8. Western blot analysis of the indicated GB1 NXS/T mutants comparison to the wild-type construct.

Minor points

Line 101 says the HEK293T cells expressing the receptors were injected as antigens? Is this true as I see from the Methods, it was cell membrane extraction.

We used HEK293T cell membrane extraction overexpressing GB1 and GB2 as antigens for llama immunization. We corrected in the revised manuscript.

Is the VFT of GB2 very different from GB1? So Nb-5E9 only binds to GB2's VFT?

The amino acid sequence identities between the VFT of GB1 and GB2 are 31.4%. Our data shows that NB-5E9 specifically target the VFT of GB2, not VFT of GB1 (**Extended data Figure 1b**).

Reviewer #1 (Remarks to the Author):

The revised version of the manuscript "*Functional and structural basis of a negative allostery within GABAB heterotetramers*" has improved over its original version. I find the new data, clarifications and new discussions overall convincing.

We wish to thank the referee for his/her positive feedback

There are two (minor) remaining issues that I feel need to be addressed:

1) *GB1-homodimers*: I find the authors' argumentation overall plausible. Nevertheless, I believe it would strengthen the author's explanation if they provided some experimental data to back-up their key arguments.

We understand that the referee would be better convinced that what we observed with the PLA assay using nanobodies targeting the GB1 subunit only, corresponds to $GABA_B$ tetramers, rather than just strict GB1 homodimers. We agree that it would be fantastic if one could find a way to discriminate between these two possible species of $GABA_B$ complexes. However, we did not find a way to discriminate strict GB1 homodimers from associated GB1s within $GABA_B$ tetramers, as is both cases, two GB1 subunits are associated.

We would also like to mention our previous arguments. One could quantify GB1-GB1 complexes in the absence of GB2, such that these would only represent GB1 homodimers. However, the drastic reduction in GB1 in the absence of GB2, as well illustrated in the GB2 KO animals (Gassmann et al., J Neurosci 2004), prevents any comparison with the wild-type animals.
It may also be possible that GB1-GB2 heterodimers, the formation of which is needed for GB1 to be targeted to the cell surface, can dissociate, allowing the isolated GB1 subunits to assemble into homodimers. However, such a possibility is very unlikely due to the strong association between these two subunits brought by the coiled coil interaction between their C-terminal intracellular domains (Kammerer et al., Bioch 1999; Calver et al., J Neurosci 2001). Even the tetramers were shown to be very stable and not capable of exchanging subunits (Comps-Agrar et al., FASEB J 2012).
The fact that GB1 is targeted to the cell surface only when associated with GB2 is well documented, and the molecular partners involved have been identified (see the references in our previous rebuttal).
Our view is that most arguments we used to justify that most GB1-GB1 complexes result from the formation of $GABA_B$ tetramers, rather than homodimers, are well supported by previous data, all published. We did not find any additional data we could provide, not yet reported in the literature, to further support our view.

However, it may be possible to discriminate between the strict GB1 homodimers and the associated GB1 subunits within the $GABA_B$ tetramer, as they likely do not interact via the same interface. Indeed, the cryoEM structure of the GB1 strict dimer (Papasergi-Scott et al., Nature 2020), reveals an interface identical to that involved in the GB1-GB2 interaction, then, totally different from the GB1-GB1 interface within the tetramer. This is expected as the GB1 interface contacting GB2 is already occupied in the tetramer. This is consistent with the data obtained in transfected cells where mutations affecting the GB1-GB1 interface in the tetramer affects the tetramer function, but not that of the heterodimer, as do the 7TM portion of GB1 used as a competitor (Maurel et al., 2008; Comps-Agrar et al. 2011, Stewart et al., 2018). Applying this knowledge to discriminate native GB1 complexes may then be possible, but out of the scope of the present study.

Probably the best argument we have is our estimate of a high proportion of $GABA_B$ heterodimers being associated into tetramers, even in primary neurons, then more than what one would expect for strict GB1 homodimers, as based on the published arguments provided here and previously.

We are sorry not being able to provide further experimental support of this proposal. However, all the data reported in the present and previous papers we and other published nicely fit and can be explained by the tetramer structure we report here: the association via GB1 with GB2 on the side (Maurel et al., Nat Meth 2008; Calebiro et al., PNAS 2013), the negative allostery leading to a single heterodimer within such a complex being able to signal (Maurel 2008, Comps-Agrar 2011…), the GB1 interacting interfaces (Comps-Agrar 2011; Xue et al., Nat Commun 2021), the binding of a single ligand per tetramer (Stewart et al. 2018), the co-IP experiments (Schwenk et al., Nature 2010), the absence of subunit exchange (Comps-Agrar 2012)… such a coherence cannot be just an artifact, as it has to be the result of evolution. This is probably the best argument in favor of the validity of our model.

To further clarify this issue, in addition to the specific paragraph added in the discussion in the previous version, we now added:

- Introduction, when mentioning the $GABA_B$ receptor is a heterodimer: "*stabilized by a coiled-coil interaction between their intracellular tails (Couve et al., JBC 1998; Margeta-Mitrovic et al., Neuron 2000)." (Page 3, Line 62)*
- Results, in the chapter "Expression and activation of $GABA_B$ hetero-tetramers": " *As discussed later, GB1 strict homodimers are not expected to represent a large fraction of the GB1 subunits at the cell surface due to their stable association with GB2 (Kammerer et al., Bioch 1999; Calver et al., J Neurosci 2001), and their retention in the ER in the absence of GB2 (Couve et al., JBC 1998; Margeta-Mitrovic et al., Neuron 2000)*." *(Page 6, Line 139-142)*

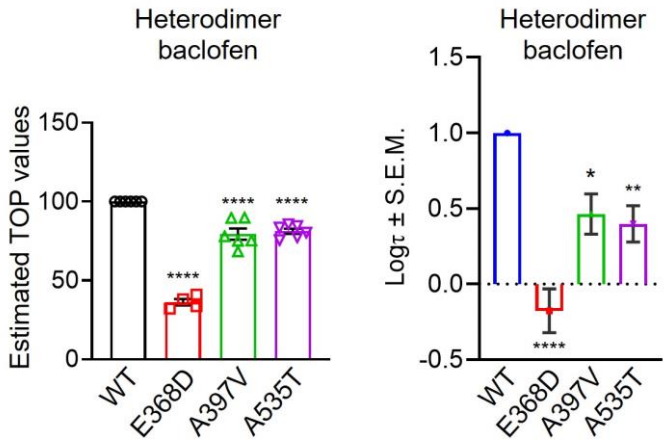
2) *Quantification of ligand efficacies*: I maintain that the functional effects of the disease-relevant mutations (e.g. on ligand efficacy) should be quantified using state-of-the-art pharmacological methods. For instance, the operational model yields a parameter, i.e. $\log(\tau/K_A)$ - a surrogate for ligand efficacy, that allows to quantify and statistically test differences in ligand efficacies across oligomers and disease-relevant mutations. Comparing TOP values, as done in the current version of the manuscript, is insufficiently quantitative.

The referee is right, the maximal response measured, represented by the TOP values from the fitting done using Prism, is not directly related to the maximal receptor efficacy. The use of the operational model can indeed take this into account. Not being so familiar with the operational model for our data interpretation, I did work on this model to figure out the best parameter to represent receptor efficacy. The τ value being the ratio between the total number of activatable receptors (Rtot), over the K_E value, $\log(\tau)$ appears to be a good parameter representing receptor efficacy for constant amounts of receptors. As the K_A is related to ligand affinity on the receptor (it is the concentration of ligand leading to 50% receptor occupancy), the ratio τ/K_A, and it log, did not appear in our hands to reflect ligand efficacy, and this was confirmed by our calculations (see below).
Accordingly, we did calculate the $\log(\tau)$ for our critical experiments using the heterodimer signaling or the tetramer signaling assays for $GABA_B$ WT and mutants stimulated by either the agonists GABA and baclofen, as well as using the PAM BHFF.

An example is given below:

		Heterodimer				Hetero-tetramer			
		n	logτ ± S.E.M.	logKA ± S.E.M.	logτ/KA	n	logτ ± S.E.M.	logKA ± S.E.M.	logτ/KA
Baclofen	GB1-WT	6	1.000 ± 0.000	-4.203 ± 0.124	5.203 ± 0.124	6	1.000 ± 0.000	-4.274 ± 0.203	5.274 ± 0.203
	GB1-E368D	6	-0.177 ± 0.145	-3.642 ± 0.914	3.465 ± 0.317	6	-	-	-
	GB1-A397V	6	0.464 ± 0.133	-4.423 ± 0.181	4.887 ± 0.132	6	-0.826 ± 0.204	-5.136 ± 0.288	4.310 ± 0.342
	GB1-A535T	6	0.398 ± 0.120	-4.606 ± 0.166	5.004 ± 0.134	6	-0.402 ± 0.176	-4.848 ± 0.256	4.447 ± 0.287
rac BHFF	GB1-WT	6	1.000 ± 0.000	-3.955 ± 0.060	4.955 ± 0.060	6	1.000 ± 0.000	-3.967 ± 0.025	4.967 ± 0.000
	GB1-E368D	6	0.410 ± 0.094	-4.343 ± 0.156	4.753 ± 0.087	6	-0.393 ± 0.045	-5.659 ± 0.172	5.266 ± 0.170
	GB1-A397V	6	0.431 ± 0.079	-4.710 ± 0.126	5.140 ± 0.078	6	0.100 ± 0.017	-5.222 ± 0.068	5.322 ± 0.061
	GB1-A535T	6	0.708 ± 0.147	-4.302 ± 0.182	5.010 ± 0.066	6	-0.002 ± 0.019	-5.245 ± 0.076	5.243 ± 0.072

These data illustrate the relationship between log(τ) and the receptor efficacy, assuming all receptors are expressed at the same level, which is not the case for the E368D mutant that shows a two-fold lower expression.
This is also illustrated by the comparison of the Top and log(τ) values determined, as illustrated below for the analysis of the baclofen effect on the WT and $GABA_B$ receptor mutants using the heterodimer assay:



Overall, the analysis made using log(τ) did not change our conclusions. As such, we decided not to change our main figures, as the inserts showing the means of the TOP values will be understandable by most non specialists, but we added our log(τ) analyses in the extended data (extended data Fig. 4 and 6) *(Page 45, Line 1028-1031 and Page 49, Line 1074-1076)*. Indeed, for an accurate use of the operational model, one would need an analysis of the saturation condition for the signaling assay (as measured using various receptor expression levels, as in our case, the receptors carry the system involved in the signal detection). Without such an analysis, the determination of τ may not be optimal.

Reviewer #2 (Remarks to the Author):

The authors have nicely addressed my questions and comments. I have two minor requests for clarification/follow up:
*I don't see any mention of the relevant patent describing nanobodies (CN120024449A (State: disclosure)) described in either the 'competing interests' or the nanobody methods section. Perhaps it is included somewhere else, but I cannot find it.

We thank the reviewer for pointing it out. The required statement has now been included in the revised manuscript under the “Competing Interests” section. *(Page 30, Line 886-888)*

*For new data shown in Extended Data Fig 2d, do all conditions use HEK293 cells transfected to express both mGluR2 and gabaB? Or are different transfection conditions used for the different analyses? This should be clarified in the figure legend.

We thank the reviewer for raising this important point. The necessary clarification has now been added to the legend of Extended Data Fig. 2c in the revised manuscript. *(Page 43, Line 1014)*

Reviewer #3 (Remarks to the Author):

The authors have addressed my questions. I recommend the publication of the manuscript.