

Selective Positive Allosteric Modulation of β 2AR Using Antibody Fragments Targeting the Third Intracellular Loop

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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)

In this manuscript, the authors present a novel antibody fragment engineering strategy to selectively modulate GPCR signaling through targeting the intrinsically disordered third intracellular loop (ICL3) region. Recognizing that traditional drug discovery approaches face challenges in achieving receptor isoform selectivity due to conserved orthosteric binding sites, and that the conformational heterogeneity of ICL3 limits structure-based small molecule approaches, the researchers repurpose a previously characterized monoclonal antibody (Mab5) that binds to the β -adrenergic receptor ICL3. They engineer two antibody fragments - a single-chain variable fragment (ScFv5) and a single-domain antibody (VhhL5) - and demonstrate that while ScFv5 broadly enhances signaling across all three β AR isoforms, VhhL5 acts as a selective positive allosteric modulator specifically for β 2AR. The authors show that VhhL5 achieves this selectivity by disrupting receptor autoregulation, thereby enhancing receptor-G protein coupling and downstream signaling, providing proof-of-concept for using engineered intrabodies to target disordered GPCR domains for selective receptor modulation. These are fundamental issues that should be addressed to fully substantiate the manuscript's conclusions.

1- The initial experiments are done with a fusion receptor that contains the SPASM module. The SPASM sensor methodology is well-described, but the manuscript would benefit from a rigorous validation that the sensor doesn't alter receptor pharmacology. For example the C-tail of β 2AR is shown to act as an autoinhibitory module for the receptor activation (PMC10085991). It is conceivable that the addition of the SPASM could alter receptor pharmacology.

2- The study employs a short 12-amino acid linker (4xGSG) as the negative control for the ~250-amino acid ScFv5 fusion. This significant size discrepancy is a concern, as the GPCR C-terminus is a known allosteric modulator of receptor function (e.g., as an autoinhibitory module in β 2AR). The addition of a large protein domain like ScFv5, independent of its binding activity, could sterically or allosterically alter receptor pharmacology in ways that the short linker control cannot account for. A more appropriate negative control, such as fusing a size-matched, non-binding ScFv or single-domain antibody, is needed to ensure the observed effects are due to specific ICL3 engagement.

3- The manuscript reports subtle but significant differences in maximal signaling response (e.g., ~20-25% increases). It is well-documented that signaling output (both E_{max} and EC_{50}) is highly dependent on the number of receptors expressed at the cell surface. The current method measures total protein expression, which is insufficient, as a 20% difference in surface localization could fully account for a 20% change in E_{max} . To validate that the observed changes in signaling are due to allosteric modulation and not variations in receptor trafficking or expression, the authors must quantify the surface levels of all tested receptor constructs (wild-type, fusions, and mutants).

4- Kind of related to the previous point, other experiments that compare different mutations should also measure the surface localization of the receptors (For example figure 4).

5- The manuscript would be strengthened by including additional negative controls that test VhhL5 against a structurally and sequentially unrelated GPCR. While the authors argue that targeting variable loops circumvents the promiscuity of conserved orthosteric pockets, it remains possible that binders targeting intrinsically disordered regions could exhibit a different form of promiscuity. Demonstrating a lack of effect on an unrelated receptor would bolster the claim that this strategy can achieve high specificity.

6- The majority of the experiments and characterization in Figures 1-3 were generated using a SPASM sensor system where the antibody fragment is directly fused to the GPCR. This creates an artificial 1:1 stoichiometry and an extremely high local concentration that may not reflect a physiological context. The authors switched to a more relevant co-expression system in Figure 4 to validate the PAM effect. However, the selectivity of VhhL5 should be demonstrated in the co-expression system with β 1AR and β 3AR.

7- The discussion about the interaction mechanism (MoRFs, SLiMs) is highly speculative, particularly given that the alanine scanning mutagenesis failed to identify a clear structural motif responsible for the effect. Furthermore, the discussion does not adequately address the potential differences between the experimental systems used. The tethered SPASM sensor and the membrane-anchored co-expression system provide different levels of accessibility, orientation, and concentration of the VhhL5. The manuscript should acknowledge these differences and discuss how they might influence the results and their interpretation, rather than treating them as equivalent.

8- The proposed model in figure 5 is a bit misleading in my opinion. It implies that the VhhL5 interacts with a short part of ICL3 and pulls it away, while itself is soluble. Just thinking in terms of simple thermodynamics, I cannot imagine how this configuration would be possible.

Minor:

1- SFig. 3a is referenced in the manuscript after all other panels in SFig 3.

2- The principles behind the "SPASM sensor" and the "v- β 2AR-S-pep" luminescence assay are not immediately clear from the text. A brief, description of how each assay works (e.g., "...measured by ratiometric FRET between donor/acceptor fluorophores flanking a flexible linker that reports on proximity...") would greatly improve readability for a non-specialist audience.

Reviewer #2

(Remarks to the Author)

This manuscript explores allosteric modulation of the β 2-adrenergic receptor signaling through engineered antibody fragments. The authors employ a suite of modern tools including: FRET-based biosensors, membrane-tethered constructs, and bioluminescent coupling assays to dissect receptor-G protein interactions and intracellular loop regulation. While the study offers useful tools and a thoughtful framework for targeting GPCR intracellular domains, the conceptual advance is narrow and does not meet the threshold of broad novelty or impact expected for publication in this journal. Nonetheless, the work may be of interest to specialists developing receptor-targeted modulators or intracellular biosensor platforms.

Major comments

1. Limited novelty of VhhL5 as a modulator.

While the study emphasizes the development of VhhL5 as a selective allosteric modulator, it should be noted that ScFv5 is already a functional PAM, albeit without selectivity. Moreover, VhhL5 is not a newly identified nanobody, which diminishes the novelty of the approach. Overall, limited novelty substantively diminishes the impact of the study.

2. -Neglect of β -arrestin pathway modulation and unclear interpretation of arrestin-KO data

The arrestin-KO experiments are not convincingly interpreted. If VhhL5 acts via a β -arrestin-independent mechanism, one would expect stronger signaling in wild-type cells due to β -arrestin-mediated desensitization, yet this enhancement is not observed. More importantly, interrogation of β -arrestin signaling was not assessed for any of the antibodies. Is VhhL5 also a PAM for β -arrestin signaling or is it biased toward G protein signaling?

3. Lack of evidence for VhhL5-induced activation in the absence of agonist

The central mechanistic claim—that VhhL5 enhances signaling by relieving ICL3 autoregulation—is insufficiently supported. The authors do not test whether VhhL5 alone can induce G protein activation in the absence of agonist. This is relevant in light of previous work (e.g., Samama et al., 1993 PMID: 8095262) showing that mutations in ICL3 can induce constitutive receptor activity.

4. Confusing use of Nb6 as a control

" β 2AR-Nb6 fusion has an increased FRET ratio compared to the control but is lower than the β 2AR-VhhL5 fusion (SFig. 3c)." The inclusion of β 2AR-Nb6 fusion as a control (SFig. 3c) is poorly justified. Although Nb6 is a nanobody for KOR, the authors report it exhibits weak binding to β 2AR without explaining the basis or implications of this observation. If the goal is to assess the specificity of VhhL5, a more rigorous approach would involve testing a broader panel of GPCRs using the SPASM system to provide a clearer selectivity profile.

5. Unsupported structural claim regarding G protein coupling

The authors propose that VhhL5 binding disrupts ICL3 autoregulation without impairing G protein coupling, but no direct evidence—such as molecular dynamics simulations or structural analysis—is provided to support this. Additional modeling or biophysical data would be necessary to validate this key mechanistic point.

6. Poor figure annotation and limited quantification rigor

Several figures lack clear labeling, axes definitions, or group annotations, which impairs readability (e.g. the concentration of Iso applied..). In addition, more robust and standardized quantification methods would improve data clarity and allow for more accurate interpretation and comparison across conditions (e.g. “ β 2AR and β 3AR show ~25% increase in E_{max} compared to the ~20% for β 1AR (SFig. 1d,e,g).”..)

Minor issues

- “Lastly, ICL3 presents interfaces that do not occlude effector binding..” I suggest to replace the “effector” with G protein or beta-arrestin to be more specific.
- “ β 2AR and β 3AR show ~25% increase in E_{max} compared to the ~20% for β 1AR (SFig. 1d,e,g).” The figure does not clearly show the percentage to the readers.
- “VhhL5 is unable to modulate ..” This statement is misleading that SFig. 3 e-h does show some effects.

Reviewer #3

(Remarks to the Author)

The authors initially used fusion constructs to directly demonstrate the non-selective signaling promotion of ScFv5 toward β -adrenoceptors, as well as its similar affinity for these receptors.

After further investigating the interaction sites of ScFv5 with the ICL3 of β 2AR, they developed a β 2-selective nanobody (vhhL5) by grafting the complementarity-determining regions (CDRs) of ScFv5 onto vhh scaffolds. Using a cAMP assay with fusion constructs, they demonstrated the specific positive allosteric effect of vhhL5 on β 2AR. This effect was also confirmed through co-expression of vhhL5 with β 2AR.

In the third part of the study, the authors explored the mechanisms underlying the positive allosteric effect of vhhL5. They found that vhhL5 enhances receptor signaling by promoting β 2AR coupling to the helix 5 of Gs. Additionally, vhhL5 was shown to relieve the constraints imposed by ICL3, supporting their previous Nature paper on ICL3 autoregulation.

Suggestions:

In Figure 5, the author presents an illustrative model suggesting that VhhL5 relieves spatial constraints in ICL3 to enhance β 2AR signaling. However, this speculation is currently supported only by cell-based assays. It would strengthen the study if the author could include structural data. Incorporating molecular dynamics (MD) simulations to demonstrate the interaction between the nanobody and ICL3 would be a reasonable addition.

A previous study (J. Am. Chem. Soc. 2024, 146, 41, 28527–28537) found that, unlike β 2AR, the ICL3 of β 1AR promotes receptor signaling by facilitating Gs coupling. This direct promotional role of β 1AR ICL3 may influence the selective modulation of β 2AR by VhhL5 and should be discussed.

My conclusion: Overall, I find this paper very interesting. The experimental design is creative, and the results appear solid.

Reviewer #4

(Remarks to the Author)

In this study, the authors employ clever antibody engineering to convert a pharmacologically inactive GPCR targeting Fab into a positive allosteric modulator. Experimental data presented in the manuscript indicate that the resulting antibody fragments exert their signaling effects by modulating the conformation of ICL3 to release its autoinhibitory activity. Like other allosteric sites, ICL3 is less conserved across receptors. Targeting of this region could provide subtype selectivity, which is desirable for therapeutics, but is challenging to achieve with small molecule drugs, due to the conformational flexibility of ICL3. The authors demonstrate that antibodies are well-poised to target ICL3 providing an alternative pharmacological strategy to control GPCR function. There are not many antibodies that functionally modulate GPCR signaling, making this new mechanism of action of interest to the pharmacological community; however, additional experimental support would strengthen the manuscript for publication at Nature Communications.

Major Points

1. Throughout the manuscript, the authors refer to receptor isoforms to describe B1AR, B2AR, B3AR. Subtype or subfamily may be a more appropriate term as the three receptors are produced by different genes. Please review and address accordingly.
2. To make the findings in the paper more significant and accessible to a non-GPCR expert, the authors might consider explicitly mentioning ICL3's role as a regulatory region in the introduction.
3. In the first paragraph of the results section, the authors state that removing the constant region of the Fab fragment would expose the G protein binding site of B2AR. To avoid confusion, I recommend modifying the language to indicate that this was a hypothesis.
4. The manuscript would be strengthened by additional biochemical characterization of ScFv5, VhhL5, VhhH5 compared to

Fab5. A binding experiment demonstrating that unlike Fab5, ScFv5, VhhL5, and possibly VhhH5 facilitate G α s binding would provide mechanistic support for the observed changes in signaling assays. This could be carried out through pull downs with recombinant proteins or perhaps a FRET-based measurement in the presence of excess G α s.

5. Throughout the manuscript, signaling assays are normalized based on total-protein. Are the receptor-ScFv fusions expressed at similar levels on the cell-surface? Inclusion of this additional control would further verify that the observed changes in signaling assays are not simply due to increased trafficking of the receptor-ScFv fusions to the plasma membrane.

6. The disparity between binding strength and increases in cAMP levels for VhhL5 and VhhH5 is interesting, especially as the LC binds primarily to the proximal region of ICL3, whereas the HC is responsible for distal interactions. An additional sentence broadly describing the heavy and light chain interfaces should be included to bring the reader's attention to Fig. 2a as well as a figure illustrating the structure in the supplemental. Alternatively, the Fig. 2e caption could be expanded to highlight this point. Do mutations in the context of the Vhh decrease receptor binding or cAMP production more substantially than mutations in the ScFv?

7. It is difficult to compare Fig. 2d and 2g as the data is normalized in one panel, but not the other. For clarity, I recommend that the authors are consistent in their data presentation in the main text figures.

8. Discrepancies between binding and signaling effects (similar to those observed for VhhH5 and VhhL5 and B2AR mentioned in point 6) are seen for VhhL5 and B1AR, B2AR, and B3AR. While binding does not necessarily impart pharmacological activity, additional mechanistic experiments would further support this observation. Is an increase in cAMP observed with VhhL5 when B2AR ICL3 is swapped into B1AR or B3AR or does replacement of B2AR ICL3 lead to no change in cAMP?

9. A substantial FRET ratio increase is seen with the SPASM sensor for Nb6, which was raised for a different receptor (Supplementary Fig. 3b). I'm not clear on whether the FRET ratio used as a measure of binding strength is truly informative of a true interaction for these constructs. An additional control to mitigate any increases in the FRET ratio due to non-specific binding would be informative and help to clean up the manuscript. Can the signal be quenched through the addition of exogenous nanobody or can a binding interaction be detected outside of the SPASM construct? This point has implications for observations made throughout the manuscript including the mutagenesis of ScFv5 (Fig. 2c) and activity of VhhL5 on other receptors (Fig. 3b).

10. The data presented in Fig. 4 clearly confirms that many of the observations presented in earlier figures is not an artifact of the SPASM setup and really nails home the main point of the paper – modulating ICL3 conformation can allosterically influence receptor signaling. Additional mechanistic data would strengthen the conclusions drawn from these experiments. A radioligand competition binding assay investigating whether VhhL5 enhances the KHi affinity for an agonist in the presence of G α s or the G α s c-terminal peptide following the ternary complex model would greatly complement the data presented in Fig. 4f.

11. Does VhhL5 interact similarly with the QDG(AAA) variant?

12. Prior work from the same group indicates that deletion of ICL3 from B2AR increases non-cognate G protein activity. Does introduction of VhhL5 recapitulate this result? If so, it would further support the nanobodies proposed mode of action and add additional pharmacological capacity to the nanobody.

13. The discussion mentions that nanobodies are generally antagonists or negative allosteric modulators. While this is true, additional nanobody agonists are reported. Please update the text accordingly. Some references include nanobodies for the apelin receptor (Ma et al. Sci. Adv. 2020) and melanocortin-4 (Fontaine et al, Nat. Commun. 2024).

Minor Points

1. Please define SPASM in the main text.

2. Figure panel 1d is missing a key and has a different y-axis label than the other panels.

3. Fig. 4f is not called out in the main text

4. Introducing the fact that much of ICL3 is disordered in the B2AR Fab5 structures earlier in the manuscript would set the reader up to appreciate the extensive analysis of IDRs in the discussion.

5. If the orientation of heavy chain and light chain was investigated in the construction of the ScFv it is worth mentioning in the text.

6. In the nanobody expression and purification section of the methods please correct the size of the centrifugal filter – I think it should be 10 kDa not 10,000 kDa.

(Remarks to the Author)

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

Reviewer #6

(Remarks to the Author)

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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all of my concerns with substantial new experimental data. The revised manuscript is significantly strengthened and I have no further comments.

Reviewer #2

(Remarks to the Author)

The authors have been responsive and addressed all my concerns.

Reviewer #3

(Remarks to the Author)

I am happy with the revised version

Reviewer #4

(Remarks to the Author)

The inclusion of additional controls, experimental data, and the localization of the VhhL5 binding site via both experimental and computational studies addresses many of my concerns regarding the initial manuscript draft. Overall, the authors integrate this new data to propose an interesting mechanistic model for VhhL5. While the manuscript does contain some unusual and thought-provoking observations, appropriate controls are included. A few minor edits could improve the clarity of the manuscript for publication:

1. I appreciate the thought and effort that went into the structural modeling. In particular, the methods are incredibly detailed and clearly walk the reader through the author's rationale. Some of this clarity is lost in the main text and figures. Perhaps Fig. 4c-e would be better suited for supplementary data as they largely support the experimental data in Fig. 4b and observations made from full length ICL3 models in panels f-j. Alternatively, inclusion of visuals, such as those shown in SFig 7d and 7f alongside the principle component analysis in Fig. 4c may help the reader follow along in the main text figures.
2. It is tricky to appreciate the similarities and differences between the starting models in SFig. 8. Coloring different features (ICLs, Ndel1, Ndel2, middle, Cdel1) on the models would be informative.
3. The observation that miniG protein recruitment is modestly attenuated (SFig. 12), but the B2AR-S peptide interaction is increased (representative of coupling) is significant and could be more clearly stated in the text to better support the model presented in Fig. 7. The authors included the following language in their response to the reviewers that nicely and concisely summarizes their findings "VhhL5 binds to the ICL3 N-terminus to release C-terminal regulatory constraints that steer ICL3 away from the G protein binding site and facilitate G protein coupling". To clarify the model, I suggest that this wording be incorporated into the manuscript.

Reviewer #5

(Remarks to the Author)

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Reviewer #6

(Remarks to the Author)

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Reviewer #1 (Remarks to the Author):

In this manuscript, the authors present a novel antibody fragment engineering strategy to selectively modulate GPCR signaling through targeting the intrinsically disordered third intracellular loop (ICL3) region. Recognizing that traditional drug discovery approaches face challenges in achieving receptor isoform selectivity due to conserved orthosteric binding sites, and that the conformational heterogeneity of ICL3 limits structure-based small molecule approaches, the researchers repurpose a previously characterized monoclonal antibody (Mab5) that binds to the β -adrenergic receptor ICL3. They engineer two antibody fragments - a single-chain variable fragment (ScFv5) and a single-domain antibody (VhhL5) - and demonstrate that while ScFv5 broadly enhances signaling across all three β AR isoforms, VhhL5 acts as a selective positive allosteric modulator specifically for β 2AR. The authors show that VhhL5 achieves this selectivity by disrupting receptor autoregulation, thereby enhancing receptor-G protein coupling and downstream signaling, providing proof-of-concept for using engineered intrabodies to target disordered GPCR domains for selective receptor modulation. These are fundamental issues that should be addressed to fully substantiate the manuscript's conclusions.

We thank the reviewer for recognizing the novelty and extent of our efforts.

We appreciate the in-depth review and have provided substantial new experimental data to address their concerns. A summary of new figures that address major concerns is listed below.

New SFig. 1: *C-tail fusion does not alter receptor function* – Identical Emax for C-tail tethered fluorescent constructs compared to unlabeled (dark) receptor

New SFig. 10: *Matched cell surface expression levels* - Fusion of or co-transfection with VhhL5 does not alter β 2AR's surface expression.

New SFig. 11a: *Selectivity of VhhL5* - Co-transfection of VhhL5 does not modulate cAMP Emax for two related β xAR subtypes (β 1AR, β 3AR), one unrelated Gs coupled receptor (D1R), and one promiscuous (Gs/Gq) receptor (PTH1R).

New Fig. 4: *VhhL5 binding site* – VhhL5 binds to the ICL3 N-terminus through multiple dynamic inter-molecular contacts.

1- The initial experiments are done with a fusion receptor that contains the SPASM module. The SPASM sensor methodology is well-described, but the manuscript would benefit from a rigorous validation that the sensor doesn't alter receptor pharmacology. For example the C-tail of β 2AR is shown to act as an autoinhibitory module for the receptor activation (PMC10085991). It is conceivable that the addition of the SPASM could alter receptor pharmacology.

We appreciate the reviewer highlighting potential differences in receptor's pharmacology upon C-tail tethering. We have included a **new SFig. 1** demonstrating that under matched surface expression, maximal cAMP response (Emax) to isoproterenol is identical with or without C-terminal fusion. We do observe a subtle increase in the EC₅₀ of the tethered construct. Nonetheless, our primary observation for allosteric modulation through antibody fragments involves a change in Emax, with no observed effects on EC₅₀ for the targeted receptor.

SFig. 1 is included in the resubmission with the following sentence added to the Results: "The SPASM sensor involves a C-terminal fusion to the receptor, which has been shown to be important for regulating β 2AR activation⁹. We found that at matching surface expression (SFig. 1a,b), C-terminal fusion to β 2AR does not influence signaling Emax (SFig. 1c,d) but modestly increases EC₅₀ (SFig. 1c,e)."

2- The study employs a short 12-amino acid linker (4xGSG) as the negative control for the ~250-amino acid ScFv5 fusion. This significant size discrepancy is a concern, as the GPCR C-terminus is a known

allosteric modulator of receptor function(e.g., as an autoinhibitory module in β_2 AR). The addition of a large protein domain like ScFv5, independent of its binding activity, could sterically or allosterically alter receptor pharmacology in ways that the short linker control cannot account for. A more appropriate negative control, such as fusing a size-matched, non-binding ScFv or single-domain antibody, is needed to ensure the observed effects are due to specific ICL3 engagement.

We agree with the reviewer that the size discrepancy of the ScFv5 and additional moieties could alter the receptor's pharmacology. To address this concern, we provide data on a non-specific ScFv (ScFvG6) that shows substantially lower interaction with the receptor, compared to ScFv5 and no modulation of signaling (SFig. 2). For the VhhL5, we now include Vhhv3.1, a derivative of VhhL5 with altered CDR sequences. Vhhv3.1 serves as a negative control that shows no measurable interaction (FRET) and does not modulate β_2 AR signaling (SFig. 5; S.Table 1). Further, while a previously reported nanobody, Nb6 (binder of Kappa opioid receptor's ICL3) shows a significant interaction with β_2 AR, it does not modulate cAMP signaling (SFig. 5).

A description of these controls is included in the Results:

(ScFvG6) - "The observed response is unique to ScFv5, as fusion of a previously reported ScFv (G6) that binds vascular endothelial growth factor has a reduced interaction with β_2 AR, and no effect on β_2 AR signaling (SFig. 2d,e,f)²⁶."

(Nb6) - "To test the specificity of β_2 AR's response to VhhL5, we tested a previously reported single-domain antibody (Nb6) that binds the ICL3 of Kappa Opioid Receptor (KOR)²⁸. KOR and β_2 AR ICL3s do not share a conserved sequence (SFig. 5d). β_2 AR-Nb6 fusion has an increased FRET ratio compared to the control, but is lower than the β_2 AR-VhhL5 fusion (SFig. 5e). Furthermore, the cAMP response elicited by VhhL5 appears to be specific to this antibody fragment, as Nb6 fusion has no effect on β_2 AR signaling (SFig. 3f-h)."

(Vhhv3.1) - "To optimize VhhL5 for recombinant expression and purification, we derived Vhhv3.1 that contains sub-regions of CDRs 1 and 3 of the Fab5 light chain that are in proximity to the ICL3 (PDB ID: 2R4S). While soluble purification was observed for Vhhv3.1 (SFig. 6b), it did not interact with β_2 AR (SFig. 6c) nor did it modulate cAMP signaling (SFig. 6d) in the context of a SPASM sensor. Thus, Vhhv3.1 was used as a negative control in further experimentation throughout this study."

3- The manuscript reports subtle but significant differences in maximal signaling response (e.g., ~20-25% increases). It is well-documented that signaling output (both E_{max} and EC_{50}) is highly dependent on the number of receptors expressed at the cell surface. The current method measures total protein expression, which is insufficient, as a 20% difference in surface localization could fully account for a 20% change in E_{max} . To validate that the observed changes in signaling are due to allosteric modulation and not variations in receptor trafficking or expression, the authors must quantify the surface levels of all tested receptor constructs (wild-type, fusions, and mutants).

We thank the reviewer for highlighting the possibility that the modulation observed could be derived from changes in the surface expression of the receptor upon antibody fragment inclusion. To address this, we have included surface expression data for β_2 AR SPASM sensor fusions with and without the VhhL5, and for the un-tether configuration with co-expression of VhhL5 (**new SFig. 10**). We observed no differences in surface expression in the presence of VhhL5 for either of the receptor configurations used in this study.

This observation is now noted in the Results: "The observed allosteric modulation of β_2 AR by VhhL5 is not influenced by changes in receptor surface expression levels (SFig. 10)."

4- Kind of related to the previous point, other experiments that compare different mutations should also

measure the surface localization of the receptors (For example figure 4).

As noted for earlier comments, we have provided sample data for each of the expression formats to demonstrate that changes in cell surface expression do not contribute to observed modulation of cAMP signaling.

5- The manuscript would be strengthened by including additional negative controls that test VhhL5 against a structurally and sequentially unrelated GPCR. While the authors argue that targeting variable loops circumvents the promiscuity of conserved orthosteric pockets, it remains possible that binders targeting intrinsically disordered regions could exhibit a different form of promiscuity. Demonstrating a lack of effect on an unrelated receptor would bolster the claim that this strategy can achieve high specificity.

We thank the reviewer for this suggestion. We have tested the un-tethered configuration (receptor-mCit + VhhL5-mCer) for class A dopamine 1 receptor (D₁R) and class B parathyroid 1 receptor (PTH₁R) (**new SFig. 11a**). We observe no significant modulation of the maximal cAMP response of either of these two receptors upon co-expression of VhhL5.

We have included these controls in the Results: “Further, VhhL5 exhibits β_2 AR selectivity, with no observable modulation at β_x AR subtypes (β_1 AR and β_3 AR), and two unrelated GPCRs (PTH₁R and D₁R) (SFig. 11a).”

6- The majority of the experiments and characterization in Figures 1-3 were generated using a SPASM sensor system where the antibody fragment is directly fused to the GPCR. This creates an artificial 1:1 stoichiometry and an extremely high local concentration that may not reflect a physiological context. The authors switched to a more relevant co-expression system in Figure 4 to validate the PAM effect. However, the selectivity of VhhL5 should be demonstrated in the co-expression system with β_1 AR and β_3 AR.

We agree with the need for these additional experiments. We now included data on the effect of VhhL5 on the maximal cAMP response of β_1 AR and β_3 AR. We observe no significant increase in the maximal cAMP response (**new SFig. 11a**), demonstrating that VhhL5 exhibits selectivity in both the SPASM sensor and un-tethered systems.

We have included these controls in the Results: “Further, VhhL5 exhibits β_2 AR selectivity, with no observable modulation at β_x AR subtypes (β_1 AR and β_3 AR), and two unrelated GPCRs (PTH₁R and D₁R) (SFig. 11a).”

7- The discussion about the interaction mechanism (MoRFs, SLiMs) is highly speculative, particularly given that the alanine scanning mutagenesis failed to identify a clear structural motif responsible for the effect....

In our initial submission, we had not defined a potential receptor interface for the antibody fragments. In this resubmission, we used scanning deletion mutagenesis to highlight the N-terminus of ICL3 as the putative binding site for VhhL5 (**new Fig. 4a,b**). Our computational analyses (**new Fig. 4c-j**), combined with site-directed mutagenesis of antibody CDRs (Fig. 2c,d) suggest a dynamically distributed structural interface. Our discussion now centers on these new insights and acknowledges the complexity of the interaction beyond established mechanisms (MoRFs, SLiMs).

See discussion new para 4: “We propose a model wherein antibody binding to ICL3 releases distal spatial constraints to facilitate receptor activation and enhanced G protein coupling....”

Discussion revised para 5: “Thus far, bimolecular interactions with IDRs have focused on short linear

motifs (SLiMs)”

...Furthermore, the discussion does not adequately address the potential differences between the experimental systems used. The tethered SPASM sensor and the membrane-anchored co-expression system provide different levels of accessibility, orientation, and concentration of the VhhL5. The manuscript should acknowledge these differences and discuss how they might influence the results and their interpretation, rather than treating them as equivalent.

We acknowledge that all configurations used in this manuscript may provide different accessibility, orientation, and concentrations of VhhL5. All statistical comparisons, FRET and cAMP, are performed between matched configurations (SPASM, membrane-tethered) to account for these differences.

The potential differences between the two systems are included in the Results Excerpt – ‘The SPASM fusion and membrane tethered system may provide different accessibility, orientation, and concentration of VhhL5.’

We now include this in the revised Methods section on Statistical Analysis: “To address potential differences in the levels of accessibility, orientation, and concentration on the modulation of antibody fragments, all statistical comparisons for FRET and cAMP are performed using matched ER/K linked or membrane-tethered configurations.”

8- The proposed model in figure 5 is a bit misleading in my opinion. It implies that the VhhL5 interacts with a short part of ICL3 and pulls it away, while itself is soluble. Just thinking in terms of simple thermodynamics, I cannot imagine how this configuration would be possible.

We apologize for this confusion. Our new scanning deletion mutagenesis data demonstrate that the N-terminus of ICL3 as the primary interaction site (**new Fig. 4a**). Further, the site-directed mutagenesis of VhhL5 (Fig. 2), combined with molecular dynamics simulations (**new Fig. 4**) suggest a dynamic interaction through multiple contacts. With this new information, we now have revised the model figure (**new Fig. 7**) to demonstrate the site-of-action (N-terminal region), while acknowledging the dynamic nature of the interaction (shaded region). Further, in response to the comments from reviewer 2, we have now uncovered a suppressive effect of VhhL5 on receptor internalization that correlates with suppressed pERK1/2.

Minor:

1- SFig. 3a is referenced in the manuscript after all other panels in SFig 3.

We thank the reviewer for pointing out the placement of this data. We have modified the figure to place panel (a) at the end of the supplemental figure. We have revised and modified the manuscript accordingly.

2- The principles behind the "SPASM sensor" and the "v-β2AR-S-pep" luminescence assay are not immediately clear from the text. A brief, description of how each assay works (e.g., "...measured by ratiometric FRET between donor/acceptor fluorophores flanking a flexible linker that reports on proximity...") would greatly improve readability for a non-specialist audience.

We thank the reviewer for this recommendation. We have now added a description of these assays in the Results section:

Excerpt from the results: “We used a linker topology termed systematic protein affinity strength modulation (SPASM) sensor which enables high local concentrations (~ 1-10μM) of a bimolecular interacting pair with 1:1 stoichiometry²³. The GPCR SPASM sensor consists of a single polypeptide chain, (N-to-C terminus): GPCR - SPASM module - antibody fragment²⁴. The SPASM module consists of FRET donor/acceptor pair

(mCerulean/mCitrine) flanking an α -helical ER/K linker²⁵. The unique biophysical properties of the ER/K α -helix enable ratiometric FRET measurements of the relative interaction strengths between the proteins flanking the SPASM module²³.”

Reviewer #2 (Remarks to the Author):

This manuscript explores allosteric modulation of the β_2 -adrenergic receptor signaling through engineered antibody fragments. The authors employ a suite of modern tools including: FRET-based biosensors, membrane-tethered constructs, and bioluminescent coupling assays to dissect receptor-G protein interactions and intracellular loop regulation. While the study offers useful tools and a thoughtful framework for targeting GPCR intracellular domains, the conceptual advance is narrow and does not meet the threshold of broad novelty or impact expected for publication in this journal. Nonetheless, the work may be of interest to specialists developing receptor-targeted modulators or intracellular biosensor platforms.

We thank the reviewer for this summary evaluation. We disagree with the claim of limited novelty, especially as we demonstrate, to our knowledge the first instance of positive allosteric modulation of GPCRs using an antibody fragment that selectively targets the sequence divergent intrinsically disordered loop region (ICL3). We have now provided substantial additional data to address the major concerns related to β -arrestin functional pathways (**new Fig. 6**) and the combined use of computational and experimental approaches to identify the ICL3 N-terminus as the likely VhhL5 binding site (**new Fig. 4**).

Major comments

1. Limited novelty of VhhL5 as a modulator.

While the study emphasizes the development of VhhL5 as a selective allosteric modulator, it should be noted that ScFv5 is already a functional PAM, albeit without selectivity. Moreover, VhhL5 is not a newly identified nanobody, which diminishes the novelty of the approach. Overall, limited novelty substantively diminishes the impact of the study.

We respectfully disagree with this reviewer comment. ScFv5 and VhhL5 were both newly developed for this study. While they both share CDRs with a previously reported Fab5 monoclonal antibody fragment (Rasmussen et al., Nature, 2007), multiple experimental iterations were necessary to successfully transpose CDR sequences to create functional antibody fragments. Further, as discussed in the Results, Fab5 was previously suggested to hinder β_2 AR-Gs coupling given its spatial overlap with the G protein binding cavity. Our approach to reduce antibody size to minimize steric hinderance and consequent positive allosteric modulation through ICL3 is a novel concept that is the focus of this study.

2. -Neglect of β -arrestin pathway modulation and unclear interpretation of arrestin-KO data

The arrestin-KO experiments are not convincingly interpreted. If VhhL5 acts via a β -arrestin-independent mechanism, one would expect stronger signaling in wild-type cells due to β -arrestin-mediated desensitization, yet this enhancement is not observed. More importantly, interrogation of β -arrestin signaling was not assessed for any of the antibodies. Is VhhL5 also a PAM for β -arrestin signaling or is it biased toward G protein signaling?

We thank the reviewer for this insightful suggestion. We now include data demonstrating that receptor internalization and pERK signaling are suppressed by VhhL5, both of which are associated with the β -arrestin pathway. These effects are unique to VhhL5, as a related antibody fragment, Vhhv3.1 does not influence cAMP signaling (**new SFig. 6c,d**) and has no effect on β_2 AR internalization (**new SFig. 14a,b**). Given our observation that receptor internalization blockade through β -arrestin KO (**new SFig. 14c,d**) only

partially suppresses VhhL5 modulation of cAMP signaling – (26% for *barr1/2* KO [Fig. 6f] compared to 48% for WT [Fig. 5b]), we conclude that the PAM activity of VhhL5 is derived from the combined effects of VhhL5 on both G protein and β -arrestin pathways (**new Fig. 7**). The suppression of pERK signaling demonstrates that VhhL5 is a G protein-biased allosteric modulator. We have now included this new experimental data (**new Fig. 6a-d**), a new Results section, and a new Discussion paragraph highlighting the influence of VhhL5 on β_2 AR internalization and G protein biased signaling.

Results: See new section titled “*VhhL5 suppress β_2 AR internalization*”

Discussion excerpt: “Our data suggests that suppression of receptor internalization as a parallel mechanism for the PAM activity of VhhL5 (Fig. 6). In addition to augmenting cAMP signaling at the plasma membrane, blocking receptor internalization diminishes pERK signaling, a hallmark on endomembrane signaling in GPCRs. Furthermore, knockout of β -arrestin1/2, central regulators of β_2 AR internalization that also contribute to pERK signaling at endomembrane compartments, only partially abolished cAMP augmentation by VhhL5 (Fig. 6f). These data support the role of VhhL5 as a partially G protein signaling biased modulator (Fig. 7). The precise role of VhhL5 in suppressing the β -arrestin pathway is unclear, although recent studies have highlighted synergies between receptor ICL3 and C-tail that comprise their respective binding sites⁹.”

3. Lack of evidence for VhhL5-induced activation in the absence of agonist

The central mechanistic claim—that VhhL5 enhances signaling by relieving ICL3 autoregulation—is insufficiently supported. The authors do not test whether VhhL5 alone can induce G protein activation in the absence of agonist. This is relevant in light of previous work (e.g., Samama et al., 1993 PMID: 8095262) showing that mutations in ICL3 can induce constitutive receptor activity.

We now present data demonstrating no measurable basal activity of β_2 AR in the presence of VhhL5 (**new SFig. 11b**). Basal effects of VhhL5 were measured relative to a previously reported single-point mutation, L272A, that renders the receptor constitutively active (in the absence of ligand). While our assay does detect significant constitutive activity for the β_2 AR-L272A mutant, no measurable effects are observed in the presence or absence of VhhL5. Note – these assays were performed in the presence of IBMX to suppress phosphodiesterase activity. We have now included this observation in the Results.

Results excerpt – “VhhL5 does not significantly activate the receptor in the absence of agonist, suggesting the requirement for an agonist for its allosteric activity (SFig. 11b).”

4. Confusing use of Nb6 as a control

“ β_2 AR-Nb6 fusion has an increased FRET ratio compared to the control but is lower than the β_2 AR-VhhL5 fusion (SFig. 3c).” The inclusion of β_2 AR-Nb6 fusion as a control (SFig. 3c) is poorly justified. Although Nb6 is a nanobody for KOR, the authors report it exhibits weak binding to β_2 AR without explaining the basis or implications of this observation. If the goal is to assess the specificity of VhhL5, a more rigorous approach would involve testing a broader panel of GPCRs using the SPASM system to provide a clearer selectivity profile.

As mentioned in our original submission, Nb6 was selected to highlight the distinct KOR and β_2 AR ICL3 sequences. In the resubmission, we have further clarified that the KOR residues that interface with Nb6 in high-resolution structures are distinct from β_2 AR sequence in this region. However, we recognize that while Nb6 does not modulate β_2 AR cAMP signaling (SFig. 5f-h), it does have moderate interactions as observed in FRET measurements (SFig. 5e). Inspired by the reviewer comment, we now include data on an additional single domain antibody, Vhhv3.1 that was derived during our iterations to enhance VhhL5 recombinant protein expression. Vhhv3.1 has a modified amino acid sequence (S.Table 1), and neither demonstrates significant interaction (FRET) (**new SFig. 6c**) nor modulation of cAMP signaling (**new SFig. 6d**). We have

included this information in the Results.

Results Nb6 rationale – “KOR and β_2 AR ICL3s do not share a conserved sequence (SFig. 5d). β_2 AR-Nb6 fusion has an increased FRET ratio compared to the control, but is lower than the β_2 AR-VhhL5 fusion (SFig. 5e). Furthermore, the cAMP response elicited by VhhL5 appears to be specific to this antibody fragment, as Nb6 fusion has no effect on β_2 AR signaling (SFig. 3f-h).”

Results Vhhv3.1 data – “To optimize VhhL5 for recombinant expression and purification, we derived Vhhv3.1 that contains sub-regions of CDRs 1 and 3 of the Fab5 light chain that are in proximity to the ICL3 (PDB ID: 2R4S). While soluble purification was observed for Vhhv3.1 (SFig. 6b), it did not interact with β_2 AR (SFig. 6c) nor did it modulate cAMP signaling (SFig. 6d) in the context of a SPASM sensor. Thus, Vhhv3.1 was used as a negative control in further experimentation throughout this study.”

5. Unsupported structural claim regarding G protein coupling

The authors propose that VhhL5 binding disrupts ICL3 autoregulation without impairing G protein coupling, but no direct evidence—such as molecular dynamics simulations or structural analysis—is provided to support this. Additional modeling or biophysical data would be necessary to validate this key mechanistic point.

We now include experimental data for miniGs recruitment to β_2 AR measured using an established BRET assay (**new SFig. 12a**). VhhL5 has a modest effect on miniGs recruitment to the receptor (**new SFig. 12b,d**). In contrast, both luminescence (SFig. 12f,g) and FRET (**new Fig. 5c,d**) demonstrate enhanced β_2 AR-S peptide interactions consistent with enhanced G protein activation.

In our original submission, we had not identified the VhhL5 interaction site on the receptor. We now provide data from molecular dynamics (MD) simulations combined with scanning deletion mutagenesis to establish the N-terminus of ICL3 as the VhhL5- β_2 AR interaction interface (**new Fig. 4**). We have previously shown that mutagenesis of the ICL3 C-terminus [QDG(AAA)] releases receptor autoregulation to enhance G protein activation and cAMP signaling (*Sadler et al., Nature*, 23). Further, VhhL5 binding does not augment cAMP signaling in the QDG(AAA) mutant (Fig. 5e,f). Together, these data support our model, wherein VhhL5 binding to the β_2 AR N-terminus alters the ICL3 conformation ensemble to displace C-terminal (QDG) constraints to release ICL3 autoregulation. Displacing ICL3 to ‘open’ conformational states (*Sadler et al., Nature*, 23) enhances S peptide access to the β_2 AR cytosolic cavity augmenting G protein activation and consequent downstream cAMP signaling (**new Fig. 7**).

Results excerpt: “To determine whether VhhL5 can facilitate the release of ICL3 autoregulation in the presence of agonist, we assessed G protein recruitment (miniGs) and coupling ($\alpha 5$ helix of $G_{\alpha s}$ – S peptide) (Fig 5c, SFig. 12a). A modest but significant decrease (7%) was observed for miniGs recruitment quantified using a bioluminescence resonance energy transfer (BRET) assay in the presence of VhhL5 (SFig. 12b,d). This decrease was not observed when miniGs recruitment was challenged with Vhhv3.1 (SFig. 12c,e). In contrast, the β_2 AR-S peptide interaction measured using both luminescence (SFig. 12f) and FRET-based assays (Fig. 5c) show significant increases at saturating agonist concentration (SFig. 12g, Fig. 5d), suggesting enhanced G protein activation³¹. This is consistent with the observed increase in downstream cAMP signaling mediated by VhhL5 (Fig. 3c, Fig. 5b).

ICL3 occlusion of the G protein binding site is dependent on residues in the C-terminal region of ICL3 forming spatial distance constraints with other regions of the receptor⁶. Mutagenesis of spatially constrained residues ²⁵⁰QDG to AAA enhances cAMP signaling (Fig. 5e-f), consistent with previous observations⁶. VhhL5 is unable to further augment cAMP signaling downstream of the QDG(AAA) mutant (Fig. 5f). This effect is also observed in the context of ScFv5 (SFig. 13a-d). We previously reported that GPCRs signal through non-cognate G proteins in the absence of ICL3⁶. VhhL5 has no measurable effect on G_q mediated IP_x accumulation downstream of β_2 AR (SFig. 13e). This is consistent with our finding that

the releasing autoregulatory constraints in β_2 AR ICL3 alone are not sufficient to enhance non-cognate G protein coupling⁶. Together, these observations suggest that β_2 AR ICL3 binders augment G_s coupling and subsequent cAMP signaling by releasing autoregulatory interactions in ICL3.”

6. Poor figure annotation and limited quantification rigor

Several figures lack clear labeling, axes definitions, or group annotations, which impairs readability (e.g. the concentration of Iso applied..). In addition, more robust and standardized quantification methods would improve data clarity and allow for more accurate interpretation and comparison across conditions (e.g. “ β_2 AR and β_3 AR show ~25% increase in E_{max} compared to the ~20% for β_1 AR (SFig. 1d,e,g).”..)

We thank the reviewer for the insight to improve the readability of the figures and quantification rigor. We have addressed this issue by modifying the figure panels to ensure consistent axis labeling and have included the represented value for the E_{max} to all dose responses.

Minor issues

- “Lastly, ICL3 presents interfaces that do not occlude effector binding..” I suggest to replace the “effector” with G protein or beta-arrestin to be more specific.

We thank the reviewer for pointing out this generalization. It has been modified to ‘G protein binding’.

- “ β_2 AR and β_3 AR show ~25% increase in E_{max} compared to the ~20% for β_1 AR (SFig. 1d,e,g).” The figure does not clearly show the percentage to the readers.

We thank the reviewer for pointing out this opportunity for clarification. The relative percentages for the E_{max} have been identified on the right side of the maximum response of each dose response curve.

- “VhhL5 is unable to modulate ..” This statement is misleading that SFig. 3 e-h does show some effects.

We thank the reviewer for pointing out this overstating language. We have modified the language and included information stating that VhhL5 has a modest but significant effect into the EC_{50} of β_1 AR.

Reviewer #3 (Remarks to the Author):

The authors initially used fusion constructs to directly demonstrate the non-selective signaling promotion of ScFv5 toward β -adrenoceptors, as well as its similar affinity for these receptors.

After further investigating the interaction sites of ScFv5 with the ICL3 of β_2 AR, they developed a β_2 -selective nanobody (vhhL5) by grafting the complementarity-determining regions (CDRs) of ScFv5 onto vhh scaffolds. Using a cAMP assay with fusion constructs, they demonstrated the specific positive allosteric effect of vhhL5 on β_2 AR. This effect was also confirmed through co-expression of vhhL5 with β_2 AR.

In the third part of the study, the authors explored the mechanisms underlying the positive allosteric effect of vhhL5. They found that vhhL5 enhances receptor signaling by promoting β_2 AR coupling to the helix 5 of G_s . Additionally, vhhL5 was shown to relieve the constraints imposed by ICL3, supporting their previous Nature paper on ICL3 autoregulation.

We thank the reviewer for this detailed summary evaluation.

Suggestions:

In Figure 5, the author presents an illustrative model suggesting that VhhL5 relieves spatial constraints in ICL3 to enhance β_2 AR signaling. However, this speculation is currently supported only by cell-based assays. It would strengthen the study if the author could include structural data. Incorporating molecular

dynamics (MD) simulations to demonstrate the interaction between the nanobody and ICL3 would be a reasonable addition.

We appreciate the need for further structural characterization. In the original manuscript submission, we had not identified the VhhL5 - β_2 AR binding interface. To address the binding site, we performed scanning deletion mutagenesis through the ICL3 (**new Fig. 4a-b**). We observed that deletions encompassing the ICL3 N-terminus (Ndel1 and Ndel2) significantly decrease the β_2 AR – VhhL5 interaction strength as measured using a SPASM sensor. To gain further structural insight, we utilized a combination of AlphaFold3, previously reported all-atom molecular dynamics (MD) simulations, and coarse-grained simulations (**new data Fig. 4c-j**). The collective modeling and simulations support the ICL3's N-terminus as the β_2 AR-VhhL5 binding interface and reveal potential binding poses (**new SFig. 8**). These structural data suggest that VhhL5 binding to the ICL3 N-terminus allosterically modulates the ICL3 conformational landscape to release spatial constraints at the C-terminus (QDG; Fig. 5f), which in turn release autoregulation and facilitate receptor activation (model figure – **new Fig. 7**). Our model figure also incorporates new mechanistic insight related to VhhL5 suppressing receptor trafficking (**new Fig. 6**), a parallel mechanism that enhances G protein signaling.

These data are summarized in a New Results section titled 'Structural Models of β_2 AR bound to VhhL5'

A previous study (J. Am. Chem. Soc. 2024, 146, 41, 28527–28537) found that, unlike β_2 AR, the ICL3 of β_1 AR promotes receptor signaling by facilitating Gs coupling. This direct promotional role of β_1 AR ICL3 may influence the selective modulation of β_2 AR by VhhL5 and should be discussed.

We thank the reviewer for this suggestion. We have now highlighted these observations in the introduction and discussion sections.

Excerpt from Introduction: “The third intracellular loop (ICL3) is one such loop region with demonstrated roles in GPCR function, including modulating receptor-G protein selectivity^{5,6}, receptor autoregulation^{6,7}, G protein engagement⁸, and interacting with other cytosolic regions within the receptor⁹. These features of ICL3 make it a potential target for allosteric modulators^{6,7,10,11}”

Excerpts from Discussion: “Our findings suggest that VhhL5 interactions with the β_2 AR ICL3 N-terminus allosterically disrupt autoregulatory constraints at the ICL3 C-terminus (Fig. 5f) to facilitate receptor activation and enhance G protein coupling (Fig. 5d)^{6,8,31}. ”

“While our FRET sensor measurements show VhhL5 interactions with all three β_x AR subtypes (Fig. 3b), the lack of measurable signaling effects on β_1 and β_3 (Fig. 3d,e; SFig. 11a) suggest the sequence composition of the β_2 AR ICL3 is important for selective allosteric modulation by VhhL5. Specificity to the β_2 AR ICL3 is potentially rendered through a combination of unique molecular contacts within of the VhhL5 interaction site, allosteric communication within the ICL3 region, or subtype-specific structural roles of ICL3⁸. ”

My conclusion: Overall, I find this paper very interesting. The experimental design is creative, and the results appear solid.

We thank the reviewer for this summary evaluation and hope we have adequately addressed their comments.

Reviewer #4 (Remarks to the Author):

In this study, the authors employ clever antibody engineering to convert a pharmacologically inactive

GPCR targeting Fab into a positive allosteric modulator. Experimental data presented in the manuscript indicate that the resulting antibody fragments exert their signaling effects by modulating the conformation of ICL3 to release its autoinhibitory activity. Like other allosteric sites, ICL3 is less conserved across receptors. Targeting of this region could provide subtype selectivity, which is desirable for therapeutics, but is challenging to achieve with small molecule drugs, due to the conformational flexibility of ICL3. The authors demonstrate that antibodies are well-poised to target ICL3 providing an alternative pharmacological strategy to control GPCR function. There are not many antibodies that functionally modulate GPCR signaling, making this new mechanism of action of interest to the pharmacological community; however, additional experimental support would strengthen the manuscript for publication at Nature Communications.

We thank the reviewer for recognizing the significance and extent of our efforts. We appreciate the in-depth review and have provided substantial new experimental data to address their concerns.

Major Points

1. Throughout the manuscript, the authors refer to receptor isoforms to describe B1AR, B2AR, B3AR. Subtype or subfamily may be a more appropriate term as the three receptors are produced by different genes. Please review and address accordingly.

We appreciate this suggestion. We have modified the manuscript to refer the beta-adrenergic receptors as subtypes instead of previously used of isoforms.

2. To make the findings in the paper more significant and accessible to a non-GPCR expert, the authors might consider explicitly mentioning ICL3's role as a regulatory region in the introduction.

We thank the reviewer for this suggestion. This submission now provides additional information and references about the role of ICL3 as a regulatory region in the introduction.

Excerpt from introduction:

“The third intracellular loop (ICL3) is one such loop region with demonstrated roles in GPCR function, including modulating receptor-G protein selectivity^{5,6}, receptor autoregulation^{6,7}, G protein engagement⁸, and interacting with other cytosolic regions within the receptor⁹. These features of ICL3 make it a potential target for allosteric modulators^{6,7,10,11}.”

3. In the first paragraph of the results section, the authors state that removing the constant region of the Fab fragment would expose the G protein binding site of B2AR. To avoid confusion, I recommend modifying the language to indicate that this was a hypothesis.

We agree with this comment. We have modified the language to reframe this as a hypothesis that is supported by our data.

Excerpt from results:

“We hypothesized that in the absence of the constant regions of the Fab fragment, interactions between the variable chains of Fab5 and β_2 AR will not hinder G protein accessibility to its binding site within the receptor.”

4. The manuscript would be strengthened by additional biochemical characterization of ScFv5, VhhL5, VhhH5 compared to Fab5. A binding experiment demonstrating that unlike Fab5, ScFv5, VhhL5, and possibly VhhH5 facilitate G α s binding would provide mechanistic support for the observed changes in signaling assays. This could be carried out through pull downs with recombinant proteins or perhaps a

FRET-based measurement in the presence of excess G α s.

We acknowledge the need for further mechanistic insights. In our original submission, we demonstrated an overlap between VhhL5 and the release of ICL3 autoregulation through an established mutagenesis strategy [QDG(AAA) mutant in the ICL3 C-terminus]. Using VhhL5 as an antibody prototype, we now provide data on (1) antibody specificity; (2) site of binding; (3) overlap with G protein binding site; and (4) structural basis for G protein activation. (1) We demonstrate that our observed allosteric modulation is unique to VhhL5 using a distinct antibody fragment, Vhhv3.1 which was a byproduct of our antibody sequence optimization strategy to enhance recombinant protein expression (**new SFig. 6c,d**). (2) Using scanning deletion mutagenesis combined with MD simulations we now demonstrate that VhhL5 binds the ICL3 N-terminus through a distributed dynamic binding interface (**new Fig. 4**). (3) Using a BRET miniGs recruitment assay, we find that VhhL5 has only a modest effect (7%) on occluding the G protein binding cavity (**new SFig. 12b**). (4) Lastly, we observe stronger receptor-S peptide interactions (G α s α 5 helix) in the presence of VhhL5 (**new Fig. 5c,d**; **SFig. 12f,g**) supporting a mechanism for enhanced G protein activation. Together, these new data support a mechanistic model wherein VhhL5 binds to the ICL3 N-terminus to release C-terminal regulatory constraints that steer ICL3 away from the G protein binding site and facilitate G protein coupling (**new Fig. 7**).

These data are summarized in a New Results sections titled ‘Structural Models of β_2 AR bound to VhhL5’, and ‘VhhL5 enhances β_2 AR-Gs coupling by disrupting spatial distance constraints in ICL3’

5. Throughout the manuscript, signaling assays are normalized based on total-protein. Are the receptor-ScFv fusions expressed at similar levels on the cell-surface? Inclusion of this additional control would further verify that the observed changes in signaling assays are not simply due to increased trafficking of the receptor-ScFv fusions to the plasma membrane.

We acknowledge this potential confound in our data interpretation. To address this question, we have included surface expression data (**new SFig. 10**) showcasing that inclusion of VhhL5 does not alter surface expression (anti-HA-HRP) for similar levels of total receptor protein (mCitrine levels - fused to the receptor).

6. The disparity between binding strength and increases in cAMP levels for VhhL5 and VhhH5 is interesting, especially as the LC binds primarily to the proximal region of ICL3, whereas the HC is responsible for distal interactions. An additional sentence broadly describing the heavy and light chain interfaces should be included to bring the reader’s attention to Fig. 2a as well as a figure illustrating the structure in the supplemental. Alternatively, the Fig. 2e caption could be expanded to highlight this point. Do mutations in the context of the Vhh decrease receptor binding or cAMP production more substantially than mutations in the ScFv?

1) We appreciate this suggestion. We have included the following sentence to highlight the interaction interfaces of Vhh fragments captured in the high-resolution structure.

Results excerpt - “Based on a 4 Å cut off, the light chain of Fab5 interacts with the most N-terminal region of the ICL3 while the heavy chain interface involves both N- and C- terminal residues of ICL3 that are resolved in the β_2 AR-Fab5 complex structure (PDB: 2R4S).”

The crystal structure representation (PDB 2R4S) is notated as **SFig. 3** illustrates the interaction interface of the heavy and light chain of Fab5 and ICL3.

2) We agree that there is a lack of correlation between interaction strength and maximal cAMP responses. Specifically, VhhL5 has a stronger interaction compared to VhhH5 but both exhibit similar maximal cAMP.

In contrast, our control antibody Nb6 does have a moderate interaction with β_2 AR but does not modulate cAMP signaling. We have now included an additional control, Vhhv3.1 that neither interacts nor modulates cAMP signaling. These data illustrate that interaction alone appears insufficient for disrupting ICL3 autoregulation and consequently positive allosteric modulation of receptor activity. We have now included the following sentences in the discussion to highlight this observation.

Discussion excerpt – “Our data highlights the complexity of this interaction. First, receptor-Vhh binding, assessed by FRET measurements, does not correlate with modulation of downstream signaling. We observe this phenomenon across different Vhh fused to the same GPCR (Fig. 2, SFig. 5, SFig. 6), as well as different GPCRs fused to the same Vhh (Fig. 3). Furthermore, our Vhh optimization (SFig. 6) and computational interface characterization (Fig. 9) highlight the importance of a distributed interaction across all three CDRs. While the VhhL5 binding site on the β_2 AR ICL3 that we identified is consistent with the structural interface of the light chain of Fab5, structural data would point to an interaction centered on CDR1 and CDR3 contacts (SFig. 3). Our modeling data is consistent with the importance of CDR1 and CDR3 (Fig. 4), indicating CDR2 as a potential site for future optimization of efficacy, affinity and selectivity. Our study provides foundational insights into how Vhh can modulate GPCR signaling through the ICL3 region. Future structural work will delineate the complex modes of interaction that make modulation through ICL3 possible.”

7. It is difficult to compare Fig. 2d and 2g as the data is normalized in one panel, but not the other. For clarity, I recommend that the authors are consistent in their data presentation in the main text figures.

We appreciate this recommendation. We have addressed this issue by normalizing all the data to the experimental control of each specific biological replicate. The figures have been modified accordingly (Fig. 2d,g).

8. Discrepancies between binding and signaling effects (similar to those observed for VhhH5 and VhhL5 and B2AR mentioned in point 6) are seen for VhhL5 and B1AR, B2AR, and B3AR. While binding does not necessarily impart pharmacological activity, additional mechanistic experiments would further support this observation. Is an increase in cAMP observed with VhhL5 when B2AR ICL3 is swapped into B1AR or B3AR or does replacement of B2AR ICL3 lead to no change in cAMP?

The reviewer comments highlight the need to further understand the underlying mechanism of action of allosteric modulation. In the revision, we have taken a first step by demonstrating that the ICL3 N-terminus is essential for VhhL5 binding, consistent with the published crystal structure (**new Fig. 4**). Further, our computational analyses reveal that VhhL5 binds through a distributed dynamic interaction that expands the insights provided by the high-resolution structural snap-shot. In contrast, data in our original submission highlights the overlap of antibody mechanism with the release of autoregulatory constraints at the ICL3 C-terminus [QDG(AAA)]. Hence, we conclude that Vhh binds through a dynamic interface that modulates the ICL3 conformational ensemble to disrupt distal spatial distance constraints.

While the reviewer suggestion of swapping ICL3s is intriguing, it is outside the scope of this study. We have previously used insertions into PTH1R, a receptor with a short (8 aa) ICL3, to demonstrate the autonomous ability of this disordered region in gating G protein coupling. However, the suggested receptor isoforms have relatively long loop regions with distinct amino acid compositions that likely have both intra-ICL3 and ICL3-receptor (TM/C-tail/ICL2) interactions that would influence our interpretation of any measurements. As noted in response to (6), further structural characterization of the allosteric mechanism of antibody fragments is necessary and will form the focus of future studies.

9. A substantial FRET ratio increase is seen with the SPASM sensor for Nb6, which was raised for a different receptor (Supplementary Fig. 3b). I'm not clear on whether the FRET ratio used as a measure of

binding strength is truly informative of a true interaction for these constructs. An additional control to mitigate any increases in the FRET ratio due to non-specific binding would be informative and help to clean up the manuscript. Can the signal be quenched through the addition of exogenous nanobody or can a binding interaction be detected outside of the SPASM construct? This point has implications for observations made throughout the manuscript including the mutagenesis of ScFv5 (Fig. 2c) and activity of VhhL5 on other receptors (Fig. 3b).

We thank the reviewer for suggesting the need for additional controls. In the resubmission, we have incorporated an additional control Vhhv3.1 which exhibits no measurable interaction (FRET) or cAMP modulation through β_2 AR (**new SFig. 6c,d**). Additionally, we have included experimental data performing scanning deletion mutagenesis of β_2 AR ICL3, which quenched the FRET ratio when most N-terminal regions were depleted (**new Fig. 4a-b**). These data support our interpretation of FRET as a surrogate for the strength of the receptor-Vhh interaction.

10. The data presented in Fig. 4 clearly confirms that many of the observations presented in earlier figures is not an artifact of the SPASM setup and really nails home the main point of the paper – modulating ICL3 conformation can allosterically influence receptor signaling. Additional mechanistic data would strengthen the conclusions drawn from these experiments. A radioligand competition binding assay investigating whether VhhL5 enhances the KHi affinity for an agonist in the presence of G α s or the G α s c-terminal peptide following the ternary complex model would greatly complement the data presented in Fig. 4f.

We thank the reviewer for this suggestion. Our prior studies (Sadler et al, 2023) show that ICL3 deletion does not significantly alter the K_d of ligand binding, suggesting limited allostery between this loop and the ligand binding site. This hypothesis is also supported by data in our original submission, demonstrating that VhhL5 does not affect EC₅₀ of cAMP signaling (SFig. 5a, SFig. 9c). Hence, we chose not to pursue this line of enquiry in our manuscript. We do agree with the need for further mechanistic insight and have focused our efforts in the revision on characterizing G protein binding and G protein activation. We find that VhhL5 does not alter miniGs binding (**new SFig. 12b,d**), consistent with ICL3 not affecting ternary complex formation. Nonetheless, VhhL5 does augment access to the G protein binding cavity, as measured by an enhanced interaction with the G α s C-terminus (**new Fig. 5c,d**). Lastly, in our revision we show that VhhL5 has no effect on the basal activity of β_2 AR (**new SFig. 11b**). Together, these data support our model of VhhL5 releasing ligand-dependent gating of ICL3 (**new Fig. 7**), thereby enhancing G protein coupling and augmenting the maximal cAMP response.

11. Does VhhL5 interact similarly with the QDG(AAA) variant?

VhhL5 does interact similarly to QDG(AAA) variant. However, new scanning deletion mutagenesis data show that the regions important for the interaction are contained in the most N-terminal region of the ICL3 and depletion of the regions containing the QDG sequence did not significantly quench interaction strength between β_2 AR and VhhL5 (**new Fig. 4**).

12. Prior work from the same group indicates that deletion of ICL3 from B2AR increases non-cognate G protein activity. Does introduction of VhhL5 recapitulate this result? If so, it would further support the nanobodies proposed mode of action and add additional pharmacological capacity to the nanobody.

We thank the reviewer for this insight. To address this question, we performed IP₁ measurements in the absence/presence of VhhL5. The introduction of VhhL5 does not measurably increase signaling through G α_q (non-cognate G protein for β_2 AR) as shown in **new SFig. 13e**. This result is consistent with our previous finding that mutating autoregulatory residues in β_2 AR ICL3 (QDG-AAA) are not sufficient to increase non-cognate signaling (Sadler et al, Nature, 2023, Extended Data Figure 8g).

Results excerpt – “We previously reported that GPCRs signal through non-cognate G proteins in the absence of ICL3⁶. VhhL5 has no measurable effect on G_q mediated IPx accumulation downstream of β_2 AR (SFig. 13e). This is consistent with our finding that the releasing autoregulatory constraints in β_2 AR ICL3 alone are not sufficient to enhance non-cognate G protein coupling⁶.”

13. The discussion mentions that nanobodies are generally antagonists or negative allosteric modulators. While this is true, additional nanobody agonists are reported. Please update the text accordingly. Some references include nanobodies for the apelin receptor (Ma et al. Sci. Adv. 2020) and melanocortin-4 (Fontaine et al, Nat. Commun. 2024).

We thank the reviewer for this comment. We modified the language used in the discussion of this re-submission to specify that the single domain antibody fragments tend to be antagonist or negative allosteric modulators when they interface either ICL3 or intracellular domains of GPCRs and included additional examples.

Excerpt from discussion:

“Vhh are demonstrated to mediate pharmacological inhibition or activation of GPCRs^{36–39}. However, Vhh that target intracellular domains described in the literature thus far predominantly function as either antagonists or negative allosteric modulators¹⁹. A notable exception is an intrabody PAM of the 5HT2A receptor that lacks a characterized binding site³⁸.”

Minor Points

1. Please define SPASM in the main text.

We thank this reviewer for this suggestion. We have included the definition, layout and functionality of the SPASM sensor in the result section.

Results excerpt: “We used a linker topology termed systematic protein affinity strength modulation (SPASM) sensor which enables high local concentrations (~ 1 -10 μ M) of a bimolecular interacting pair with 1:1 stoichiometry²³. The GPCR SPASM sensor consists of a single polypeptide chain, (N-to-C terminus): GPCR - SPASM module - antibody fragment²⁴. The SPASM module consists of FRET donor/acceptor pair (mCerulean/mCitrine) flanking an α -helical ER/K linker²⁵. The unique biophysical properties of the ER/K α -helix enable ratiometric FRET measurements of the relative interaction strengths between the proteins flanking the SPASM module²³. The SPASM sensor involves a C-terminal fusion to the receptor⁹, which does not alter receptors surface expression (SFig. 1a,b) or signaling E_{max} (SFig. 1c,d) but modestly increases EC₅₀ (SFig. 1c,e).”

2. Figure panel 1d is missing a key and has a different y-axis label than the other panels.

We thank the reviewer for pointing out this labeling error. We have modified the figure to match the axis labels, and it now contains all keys.

3. Fig. 4f is not called out in the main text

We thank the reviewer for catching the absence of panel 4F in the main text. We have modified the figures and made sure all panels are called out in the main text.

4. Introducing the fact that much of ICL3 is disordered in the B2AR Fab5 structures earlier in the manuscript would set the reader up to appreciate the extensive analysis of IDRs in the discussion.

We thank the reviewer for this suggestion. We have included the following text in the introduction:

“The third intracellular loop (ICL3) is one such loop region with demonstrated roles in GPCR function, including modulating receptor-G protein selectivity^{5,6}, receptor autoregulation^{6,7}, G protein engagement⁸, and interacting with other cytosolic regions within the receptor⁹. These features of ICL3 make it a potential target for allosteric modulators^{6,7,10,11}. However, the inherent conformational heterogeneity of ICL3 limits the use of structure-based small-molecule discovery approaches to target this region.”

5. If the orientation of heavy chain and light chain was investigated in the construction of the ScFv it is worth mentioning in the text.

We have now included description of the construction of ScFv5 in the method section. We used a previously reported design strategy for transitioning from antibody fragments (Fab) to single-chain variable fragments (ScFvs) (Barbosa de Aguiar, *Scientific reports*, 2021). There were no systematic attempts to vary orientation of heavy and light chain.

Excerpt from methods: “The ScFv5 sequence was derived from the heavy and light chain variable domain of Mab5, monoclonal antibody used to resolve the structure of β_2 AR. ScFv5 sequence is as follows (N-to-C terminus): 113 amino acid of Mab5 heavy chain, 5x GGGGS linker, 107 amino acids of Mab5 light chain (sequence in S.Table 1)⁴⁶”

6. In the nanobody expression and purification section of the methods please correct the size of the centrifugal filter – I think it should be 10 kDa not 10,000 kDa.

We thank the reviewer for pointing out this error. It is now listed as 10kDa.

Reviewer #5 (Remarks to the Author):

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

We appreciate any feedback embedded into the previous reviewers' comments provided by this reviewer.

Reviewer #6 (Remarks to the Author):

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

We appreciate any feedback embedded into the previous reviewers' comments provided by this reviewer.

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my concerns with substantial new experimental data. The revised manuscript is significantly strengthened and I have no further comments.

Reviewer #2 (Remarks to the Author):

The authors have been responsive and addressed all my concerns.

Reviewer #3 (Remarks to the Author):

I am happy with the revised version

Reviewer #4 (Remarks to the Author):

The inclusion of additional controls, experimental data, and the localization of the VhhL5 binding site via both experimental and computational studies addresses many of my concerns regarding the initial manuscript draft. Overall, the authors integrate this new data to propose an interesting mechanistic model for VhhL5. While the manuscript does contain some unusual and thought-provoking observations, appropriate controls are included. A few minor edits could improve the clarity of the manuscript for publication:

1. I appreciate the thought and effort that went into the structural modeling. In particular, the methods are incredibly detailed and clearly walk the reader through the author's rationale. Some of this clarity is lost in the main text and figures. Perhaps Fig. 4c-e would be better suited for supplementary data as they largely support the experimental data in Fig. 4b and observations made from full length ICL3 models in panels f-j. Alternatively, inclusion of visuals, such as those shown in SFig 7d and 7f alongside the principle component analysis in Fig. 4c may help the reader follow along in the main text figures.

We agree with the reviewer that Fig. 4c-e belongs in the supplementary material and as suggested by the reviewer, we have moved **Fig. 4c-e** into **SFig. 7a,c,d**, where they interleave with other visuals that enable interpretation of the data.

2. It is tricky to appreciate the similarities and differences between the starting models in SFig. 8. Coloring different features (ICLs, Ndel1, Ndel2, middle, Cdel1) on the models would be informative.

We agree with the reviewer that coloring of the features in the models in **SFig. 8** would be informative. As suggested, we have modified colors in structural models to match the histogram of sum of the contact frequencies. The colors were selected to match the four ICL3 deletions (Ndel1, Ndel2, Middel, and Cdel1) represented in **Fig. 4**.

3. The observation that miniG protein recruitment is modestly attenuated (SFig. 12), but the B2AR-S peptide interaction is increased (representative of coupling) is significant and could be more clearly stated in the text to better support the model presented in Fig. 7. The authors included the following language in their response to the reviewers that nicely and concisely summarizes their findings “VhhL5 binds to the ICL3 N-terminus to release C-terminal regulatory constraints that steer ICL3 away from the G protein binding site and facilitate G protein coupling”. To clarify the model, I suggest that this wording be incorporated into the manuscript.

We appreciate the reviewer’s observation. As suggested, we have incorporated the following language into the result section summarizing the model figure **Fig. 7**.

Excerpt from the result section: “Together, our findings suggest that VhhL5 binds to the ICL3 N-terminus to release C-terminal regulatory constraints that steer ICL3 away from the G protein binding site and facilitate G protein coupling in addition to hindering receptor trafficking (Fig. 7).”

Reviewer #5 (Remarks to the Author):

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

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