nature portfolio

Peer Review File

A neurodevelopmental disorder mutation locks G proteins in the transitory pre-activated state

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This manuscript by Knight, Dohlman and an all-star team of collaborators describes the phenotype of a disease-causing mutation (K46E) in the Gα subunit of the G protein Go (Goα). The team demonstrates, in compelling fashion, that this mutation creates a stable form of Goα that is locked in the nucleotide-free (apo) state, resulting in a Gα subunit that remains bound to both the GPCR and the Gβγ subunit. This Goα-K46E protein exhibits a "dominant-negative" phenotype that prevents release of Gβγ and activation of linked downstream signaling events thus causing severe neurological deficits. The authors demonstrate that this K46 residue is preserved in all Ga subunits and is critical for nucleotide binding/exchange and G protein activation. The team also presents the atomic structure of this complex (hDRD2:Goa(K46E):β1γ2 bound to dopamine). These findings are entirely novel and important on several levels. The findings: 1) identify the mechanism-of-action of a disease-causing mutation thus allowing for the theoretical possibility of a therapeutic intervention; 2) fill an important hole in our mechanistic understanding for the basis of GPCR-G protein activation, a first view of the first committed step of G protein activation; 3) provide the first Cryo-EM structure of the nucleotide-free "apo" state of a Ga subunit, providing atomic detail of this mechanism; and 4) describe an important new tool (induced mutation at pinK-E) for studying GPCR-G protein coupling and high-throughput ligand screening. Overall, these findings are highly significant. The study is a beautiful "tour-de-force" characterization of a disease-causing human mutation that identifies the underlying molecular mechanism of the disease at the atomic, protein and cellular level with broad implications for medicine, cell biology and pharmacology. Below are my mostly minor critiques.

Critiques for attention:

1.In my opinion, the title is confusing and does not convey the focus and take home message of the paper. The current title is: "A neurodevelopmental deficit that locks G proteins in the transitory pre-activated state." This is unclear to me as a reader. A more accurate title that conveys the findings would be something like the following: "A mutation in the human GNAO gene underlying a neurological disorder locks G proteins in the transitory pre-activated state." This suggested title is just an example, but I believe a better title can and should be provided.

2. Abstract: The actual Cryo-EM structure reported in the study (human DRD2:Goα(K46E):β1γ2 bound to dopamine) should be mentioned/clarified in the abstract.

3. The original/earlier characterization of this K46E mutation is unclear. It seems the first report of this variant/mutation is found in Ref 34, and references therein. On page 3, the authors write: "Following a recent comprehensive screen of mutations associated with neurological disorders in humans, we identified a substitution (GaoK46E) that seemed likely to prevent guanine nucleotide

binding , and thereby impose the first transformation state of the activation process (Figure 1A) (22,23). Refs 22 is Slepak et al (1993) JBC, and Ref 23 is Aponovich et (1998) JBC. While relevant, they do not describe the recent screen of mutations associated with neurological disorders and do not cite Ref 34.

On page 4, the authors write: “Following a comprehensive screen of Gao mutations associated with a neurological disorder in humans, we identified a subset of mutations that suppress Gbg dissociation in cells. Because these mutants inhibit signaling in the presence of wildtype Ga, they may be regarded as dominant negative mutants (25,26). Of the 55 variants tested, the best performing mutant had a charge substitution at a conserved lysine that bridges the b and g phosphates of GTP. Replacement of the lysine with a negatively charged glutamate (Gao K46E) is likely to be incompatible with GDP or GTP binding, due to charge-charge repulsion, postulated that Gao K46E persists in the nucleotide-free state and, consequently, remains associated with Gbg and receptor. energy transfer (BRET) to monitor Ga-Rluc8 dissociation from Gbg-GFP2 following GPCR activation (Figure 2A) (27). Again, the recent screen of mutations was not shown or cited. Refs 25 is: Herskowitz et al (1987) Nature; Ref 26 is: Barren et al (2007) J. Neurosci Res; Refs 27 is: Olsen et al (2020) Nat Chem Biol. While relevant, none of these describe the recent screen of mutations associated with neurological disorders no was Ref 34 cited.

In short, this screening data, if previously reported, should be cited, or if this is the first report, presented in the Supplemental data. Or if the results are being prepared for another manuscript, say that in the text and show minimal supporting data in Supplemental.

4. Ref 34 is incomplete in the bibliography.

5. Figure 5 and supplemental show Cryo-EM structure of the D2R-Goα-K26E-Gβγ complex. The authors say in the abstract and text that this complex can be easily purified but the purified proteins used for the complex structure are not shown and should be shown as gels in Supplemental. Obviously the complex was isolated resulting in a structure, but seeing the purity of the starting material is helpful.

6. The Figure legends for all figures (including Supplemental) need to be improved and made more clear to the reader. For example, the legend for Figure 2 reads. “pinKE mutations prevent Gαβγ dissociation”. This legend is incomplete and should read something along the lines of Figure 2: Ga subunits expressing the pinKE mutation prevent Gβγ dissociation”. Likewise, Figure 3 legend reads: “inhibition of effector function”. This should read: Figure 3: “Placing the pinK-E mutation into Ga subunits prevents Gβγ-directed regulation of effector function”. These are just two examples and alternative titles can be conjured. All Figure legends, including supplemental, need a second look and should be modified and improved as needed.

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8. In Fig 4E, it would be helpful to connect the dots in the Rmax data since this a concentration response curve with calculated Kd.

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Reviewer #2 (Remarks to the Author):

In this study the authors describe a new variant of the Go subunit, p.K46E, which is associated to neurodevelopmental disorder and locks in a genetically dominant fashion the G protein in a transitory pre-activation state by permanently binding to the receptor and Beta-Gamma subunits, preventing the interaction with the effectors. The authors functionally characterize this mutant via several assays including nucleotide binding, catalytic activity, subunit association and ion channel function, which they complemented with MD simulations of the WT and mutant Go subunit in the APO and GTP bound states. Notably, they also show that this variant can be purified and structurally solved in complex with agonist-bound receptors.

This is a sound study of highly relevance for the field, as it contributes to shed further light on the first steps of G protein activation, it lays the foundation for new tools to ease the determination of receptor-G protein complex structures and ligand type discrimination, as well as it provides a mechanistic understanding of the functional consequences of a variant associated to a severe neurological disorder.

There are however a few points that the authors might want to consider to improve the manuscript:

• The authors should discuss if and how this study relates to the recent work by the Lambert group (https://www.nature.com/articles/s41589-022-01231-z) referred G protein variants (Gi1 4A) that are

able to bypass intermediate-state complexes by adopting conformations similar to receptor-bound G proteins independently of nucleotide release. The Go K46E and the Gi1 4A variants appear to bias G protein's activation at different points of the transition path, but they both somehow favor the formation of a receptor-G protein complex. The Gi1 4A variant does so by bypassing intermediate states and reducing coupling selectivity requirements. Is the Go K46E variant expected to work in a similar or different way?

• It would be useful to carry out a few more structural comparisons: first, it would be interesting to compare the Go conformation of the DRD2-Go K46E complex with other available Go complexes. The analysis should focus on the conformational changes observed for the Ras domain. Any difference? Are these (if any) limited to the P-loop region or do they propagate elsewhere? Then possibly focus on the whole receptor complex. In the absence of a DRD2-Go WT complex, one could perhaps consider the DRD2-Gi1 complexes (e.g. 8IRS). How does the receptor-G protein interface look like? Do they have a similar docking mode? How about the DRD2 conformations? Any difference that might be linked to the binding of the Go mutant and stabilization of the agonist-bound receptor?

• It would be helpful to make a 3D cartoon representation by aligning the Ras domain of Galpha subunits of the DRD2-Galphao p.K46E, NTR1-Gi1 and B2AR-Gs complexes to highlight the different AHD roto-translations observed. This supplementary figure should be referenced in the corresponding results describing the AHD alternative poses observed.

• Regarding MD simulations: please provide more information about which rotamer was used to model the Go mutants. Clearly, there should be a strong dependence of the simulation results on the input coordinates. Along this line, any specific reason why to start the simulations from active conformations? Please provide RMSD plots in the SI to check for trajectory convergence of the different simulations.

• Do the conformers of the Go K46E APO MD trajectory visit the side chains conformations and interactions, involving K46E and more in general the P-loop residues, observed in the DRD2-Go K46E experimental structure?

• Figure S2 doesn't provide any detail regarding the contact analysis. It would be interesting to provide the contact statistics both for the GTP and APO forms. Ribbon representations are very difficult to read. Please use cartoons instead. Either energy minimize or refine with Rosetta FastRelax to improve geometry of representative structures for better rendering.

Minor points:

• A curiosity: Is it possible to model the Mg cofactor in the catalytic pocket? Any density for it?

• In the latest paragraph of the Results the authors comment: "Superimposition with the same region in the Gi1 structure, along with electrostatic analysis [….]". However no figure or table is referenced here. Are the authors referring to the 1GIA panel shown between panel 5D and 5E? Please specify. Also, it will be good to quantify via RMSD the "slight arrangement of the aforementioned loop".

• In the “Inhibition of effector function” section: please correct “an measure”

Reviewer #3 (Remarks to the Author):

Knight and colleagues perform an extensive series of experiments to characterize a disease-associated Go mutant that introduces a charge switch in the guanine nucleotide binding site. While many experiments have been performed the data don’t fully support the main claim that the disease-causing phenotype of the protein is a persistent nucleotide-free state that acts as a dominant negative by binding and sequestering activated receptors. One major problem is that there is little direct evidence that receptor-pinKE Go complexes are highly stable in the presence of nucleotide, and there were missed opportunities to demonstrate this. Instead, there is direct evidence that pinKE mutants do bind GDP at concentrations present in cells, and that some residual exchange and activation is present (Fig. S1). Overall, it appears that this is a mutant that binds nucleotides poorly and is unstable but survives to some extent because of the charge switching. The broader practical or conceptual significance of the findings is also a bit unclear, as structural characterization of the nucleotide-free intermediate is performed regularly with wild-type proteins using the same procedures used here, and it is not surprising that this mutation would negatively affect nucleotide binding.

Specific comments:

Purification of complexes for structural characterization was done in the presence of apyrase and/or the absence of GDP, therefore it is not surprising that the resulting complex is nucleotide-free. If the authors are confident in their model it is not clear why apyrase was present during purification, and the manuscript would have been much stronger if purification had been carried out in the presence of GDP. Apart from radioligand binding experiments where receptors and G proteins are artificially tethered there is no direct evidence that activated receptors and pinKE G proteins stay intact as a complex in the presence of nucleotides. I’m not suggesting the authors determine new structures, but showing size-exclusion chromatography with and without physiological GDP would strengthen the manuscript considerably. BRET experiments that directly monitor receptor-G protein association in the presence and absence of nucleotides would also help considerably and would seem within reach for this group.

TRUPATH data shown in Figure 2 don’t include GoA and GoB, which is odd considering the human mutation is in Go. Perhaps this is because the defect with Go was only partial, as shown in Fig. S1. This is especially true for D2R, where the defect is really quite modest. On page 4 the effect of pinKE

on the TRUPATH responses are first described as “diminished” and then later as showing that betagamma release was “blocked”. Neither of these descriptions match the data.

The TRUPATH results in Figure 2 labeled as net BRET appear to be ligand-induced changes (deltaBRET). For the basal BRET ratios given as bar graphs it is not clear if this is raw or net BRET, and the difference is critical for assessing how well each alpha subunit interacts with betagamma in the first place. The very low values in Fig. S1C suggest these are net BRET, in which case they indicate that some subunits (Gi3) associate with betagamma very poorly to begin with, despite being expressed better that others (GoA).

How do the authors explain the sometimes dramatic decrease in basal BRET in the TRUPATH assay with pinKE? If all Rluc-tagged alpha subunits associate with betagamma the only possible explanation is a difference in basal conformation. Or do some pinKE fail to associate with betagamma, perhaps after misfolding, lowering BRET?

The authors claim (pg. 4) that their TRUPATH results “demonstrate that Lys 46 is needed for proper Gbetagamma dissociation”. They go on to say that TRUPATH “provides a direct and quantitative readout of Galpha Gbg association”. Since TRUPATH, like any BRET assay, can report either proximity/dissociation or orientation these statements are simply incorrect.

On page 4 the authors postulate that pinKE mutants will fail to dissociate from receptors as well as betagamma, but then say they will test this by looking at free betagamma release with GRKct. This is a non-sequitur that doesn’t test receptor-G protein association at all.

The betagamma release experiments in Fig. 1B,C are shown only as deltaBRET, which obscures the baseline. This is a critical parameter in these experiments as it demonstrates proper Galpha expression, folding, and association with betagamma. These results need to be plotted as net BRET.

The proposed dominant negative effect shown in Fig. 1C is only valid if it is also shown that wt GoA is expressed at the same levels in the presence and absence of the pinKE mutant. There is a difference between a dominant negative mechanism and simply rendering part of the G proteins available inactive.

The Go trace in Fig. 2 B is identical to the Go + Go trace in Fig. 2C, and these are said to be representative after activation of MOR.

The exact same traces are reused in Fig. S1E in the same way, although here they are said to be due to activation of D2R. Indeed, all of the traces said to be D2R in S1 are identical to traces said to be MOR in Fig. 2.

Figure S1E also shows receptor-G protein interaction data, specified as D2R, that appears identical to data shown in Fig. 2, receptor unspecified.

The authors claim that their GRK experiments show that the Go pinKE mutant forms a non-productive complex with betagamma. How can this be reconciled with the substantial TRUPATH response in Fig. S1?

On page 4 the GRKct method is said to measure the ability of a mutant to inhibit receptor activation. This isn't even close to what this assay measures.

The basal BRET ratios in Fig. S1C suggest that the pinKE Go mutants associate to some extent with betagamma without activation of a receptor. This directly contradicts the model the authors introduce on pages 5-6, that the pinKE Go only sequesters betagamma in complex with an activated receptor. This model seems to be introduced to explain why the pinKE mutant did not sequester betagamma away from GIRK channels, but it seems more likely that the failure of the pinKE to inhibit betagamma-medated GIRK activity has something to do with how the experiment was done, since wt Go also didn't sequester betagamma well; there is no significant difference between wt and pinKE in Fig. 3B,C.

Shortly thereafter the authors say that when D2 is expressed both pinKE and RC mutants reduce the effect of potassium (i.e. sequester betagamma) compared to wt Go, but why should wt fail to sequester betagamma completely in this case? Wild-type alpha also sequesters betagamma.

Another inconsistency appears in this experiment when the RC mutant apparently supports agonist-induced GIRK signals slightly better than wt. If this mutant can't release betagamma how does this work, and how can it be reconciled with the BRET experiments?

While the thermostability measurements do not show a difference between GTPgS and GDP for the pinKE mutant the meaning of this is not clear for this mutant. In contrast they do show that the pinKE mutant does bind GDP within the physiological concentration range. Therefore, this mutant is not likely to be "locked" in the nucleotide-free state in cells. Similarly, BODIPY-GTP does produce a signal with pinKE at 50 nM, as does GTPgS at 1 micromolar. This may be a mutant that folds poorly and binds guanine nucleotides poorly, but these data suggest there is some residual binding.

The bilayer interferometry experiments also directly contradict the model where activated receptor is necessary for pinKE to bind betagamma.

The radioligand binding experiments do suggest that, when fused to a receptor, the pinKE mutant survives and folds well enough to maintain high affinity agonist binding in the presence of nucleotide. This is some of the clearest data in the manuscript, but in this case one wonders if this represents the receptor chaperoning an unstable heterotrimer, which would not happen with normal receptor:G protein stoichiometry and without the tether. Could this perhaps help explain some of the unusual GIRK results with and without receptors?

Reviewer #4 (Remarks to the Author):

The authors reported two cryoEM structures of D2R-GαoK46E in the presence and absence of a stabilizing fab. In my opinion, the location of AHD domain is very interesting especially that it is determined without any stabilizing Fab. However, the discussion of this structure is very limited in the paper. It is well known that the AHD domain has a variable position relative to the Ras domain. Could the authors speculate why the specific mutation would result in a relatively stable Ras domain opening? Maybe via its interface with Gbg? I would recommend show the Gbg interface in the figure. The comparisons with other G protein complex structures are only described in text without any figures or supplementary figures. When compared that with the b2-adrenergic receptor-Gs protein complex crystal structure, the author doesn't mention that that structure has a nanobody binding to the Ga protein in-between the AHD and Ras domain which biases the Ras domain position relative to AHD domain. There are so many G protein complex structures there and it is not clear how the authors that the NTSR1-G protein complex is more similar to their structure. I think a more comprehensive comparison with existing relevant structures are necessary.

The AHD density in the structure in without scFv16 is not very clear which doesn't allow confident modeling. This is probably due to the dynamic nature of the AHD domain to the Ras Domain. While I understood that the local resolution map showing the poor resolution in the Figure S3, I would recommend that the authors describe that too in the text. Right now, it reads like only the AHD density in the structure with svFv16 is poorly ordered. In Page7, last sentence "The EM density maps were sufficiently clear to place the receptor, the three G protein subunits, scFv16, and the bound ligand", it is not accurate because of the AHD domain of Ga domain.

I would suggest that the authors reconsider the color choices to improve clarity of the Figure 5D.

a) Gao is blended in with Gb. I would suggest use less bright colors or colors with more contrast from Ga;

b) Please consider use two different colors (or two different shades of cyan) for the AHD and Ras Domain for Ga domain so that the rotation angle would be more obvious.

c) The distance label is very hard to see. Please consider use a dark color for the line and get rid of the distance label. Instead, explain in the caption that the lines represent distances < 4 Å.

Reviewer #5 (Remarks to the Author):

The manuscript, entitled "A neurodevelopmental deficit that locks G proteins in the transitory pre-activated state by Knight et al. represents a very interesting study on the functional consequences of two mutations in the Gαo-gene on the balance of the G protein cycle. The most interesting finding is a charge-exchanging mutation that is demonstrated in the current manuscript to prevent binding of nucleotides to the G protein, without disturbing the ability to bind Gβγ. Furthermore, this K46E mutation induces a high affinity state of the receptor, stabilizing the ternary complex and thereby working as a dominant negative form. The mechanistic explanation how this mutation is causing dysfunction of the G protein is worked out with great care, including structural analysis.

The study is carried out with great expertise and elegant experiments and even solves the structure of the receptor bound G protein complex my means of Cryo EM. The findings are quite clear and convincing and have implications far beyond providing a better understanding of the molecular mechanism of a rare disease-inducing mutation. The mutant G protein will most likely also be a valuable tool for studying G protein signaling, function and receptor selectivity. The methods are described very well.

There are some issues that need to be addressed:

1) There is one issue that is apparent in the experimental results, however it is not mentioned and not discussed in the manuscript: In Figure 2A and also in the supplemental Fig 1 not only the agonist induced signal is severely affected by the 46KE mutation but also the basal BRET signal between Gα and Gβγ. I'm missing a discussion about this observation. In light of the capability of Gao 46KE to still bind Gβγ, we would not expect a decrease in the basal BRET signal, unless there is a much-reduced expression of Gβγ or a diminished capability of Gβγ binding to Gα46KE or the conformation of the Heterotrimer is different, leading to a change in resonance energy transfer. Each of the possibilities could be addressed experimentally. In the latter case it seems to be feasible and interesting to compare the basal BRET between wt Gα and Gα46KE in permeabilized cells treated with apyrase in the presence of an receptor + agonist. If the main mechanism of this mutation is to prevent nucleotide binding we should expect to observe similar BRET signals. This issue is important to mechanistically link the observed cellular phenotype exclusively to the proposed mechanism and if so, would strengthen the argumentation of the authors.

2) The title of the paper as well as a statement in the discussion: “Finally, our findings establish a potential mechanistic basis for human disease. The Gao K46E and Gao R209C mutations have been implicated in a pathogenic condition characterized by seizures, movement disorders, intellectual disability and developmental delay 33,36,53-56 “ strongly implicates, that the 46KE Mutation has been described as a mutant that is responsible for a severe neurological disease. I screened the literature provided by the authors carefully and also literature beyond the cited ones, however I could not find the evidence the authors are suggesting. Of course, there is plenty of evidence for the R209C mutant to be implicated in pathogenic conditions. I probably have overlooked it, but if not, I would feel misled by the title and the discussion. It would be helpful for the reviewer (and also for other readers) to more specifically cite the literature in this context. Furthermore, please provide the literature with the first description of the disease-associated mutation. If there would be evidence for the K46E mutation to occur in patients, it would be highly interesting to look for differences in the phenotype also in comparison to the R209C Mutation that might be attributable to the differential functional consequences of these two mutations.

Minor comments:

A decrease in the BRET signal between Gα and Gβγ clearly correlates with activation and might indicate full dissociation, however unless proven in the same experimental setting it should not automatically interpreted as such. Reasoning behind this is, that both the dipole angle of the fluorophore and the distance between the donor and acceptor influence the BRET amplitude. Also, the BRET signal goes not down to zero with full G protein activation. For all four subfamilies of G proteins there is evidence for uncomplete dissociation in the literature.

REVIEWER COMMENTS

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This manuscript by Knight, Dohlman and an all-star team of collaborators describes the phenotype of a disease-causing mutation (K46E) in the Gα subunit of the G protein Go (Goα). The team demonstrates, in compelling fashion, that this mutation creates a stable form of Goα that is locked in the nucleotide-free (apo) state, resulting in a Gα subunit that remains bound to both the GPCR and the Gβγ subunit. This Goα-K46E protein exhibits a "dominant-negative" phenotype that prevents release of Gβγ and activation of linked downstream signaling events thus causing severe neurological deficits. The authors demonstrate that this K46 residue is preserved in all Gα subunits and is critical for nucleotide binding/exchange and G protein activation. The team also presents the atomic structure of this complex (hDRD2:Goa(K46E):β1γ2 bound to dopamine). These findings are entirely novel and important on several levels. The findings: 1) identify the mechanism-of-action of a disease-causing mutation thus allowing for the theoretical possibility of a therapeutic intervention; 2) fill an important hole in our mechanistic understanding for the basis of GPCR-G protein activation, a first view of the first committed step of G protein activation; 3) provide the first Cryo-EM structure of the nucleotide-free "apo" state of a Gα subunit, providing atomic detail of this mechanism; and 4) describe an important new tool (induced mutation at pinK-E) for studying GPCR-G protein coupling and high-throughput ligand screening. Overall, these findings are highly significant. The study is a beautiful "tour-de-force" characterization of a disease-causing human mutation that identifies the underlying molecular mechanism of the disease at the atomic, protein and cellular level with broad implications for medicine, cell biology and pharmacology. Below are my mostly minor critiques.

We are grateful to the reviewer for their time, effort, and enthusiastic evaluation of our manuscript

Critiques for attention:

1.In my opinion, the title is confusing and does not convey the focus and take home message of the paper. The current title is: "A neurodevelopmental deficit that locks G proteins in the transitory pre-activated state." This is unclear to me as a reader. A more accurate title that conveys the findings would be something like the following: "A mutation in the human GNAO gene underlying a neurological disorder locks G proteins in the transitory pre-activated state." This suggested title is just an example, but I believe a better title can and should be provided.

At the reviewer's suggestion, the title has been changed to: "*A neurodevelopmental disorder mutation locks G proteins in the transitory pre-activated state"*

2. Abstract: The actual Cryo-EM structure reported in the study (human DRD2:Goα(K46E):β1γ2 bound to dopamine) should be mentioned/clarified in the abstract.

At the reviewer's suggestion, we have added new text to the abstract: "*We demonstrate further that the nucleotide-free G protein can be easily purified in complex with agonist-bound receptors and use cryo-electron microscopy to determine the structure of* $G\alpha_o^{K46E}$ *in complex with Gβγ and the dopamine D2 receptor."*

3. The original/earlier characterization of this K46E mutation is unclear. It seems the first report of this variant/mutation is found in Ref 34, and references therein. On page 3, the authors write:

“Following a recent comprehensive screen of mutations associated with neurological disorders in humans, we identified a substitution (GαoK46E) that seemed likely to prevent guanine nucleotide binding , and thereby impose the first transformation state of the activation process (Figure 1A) (22,23). Refs 22 is Slepak et al (1993) JBC, and Ref 23 is Aponovich et (1998) JBC. While relevant, they do not describe the recent screen of mutations associated with neurological disorders and do not cite Ref 34.

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In short, this screening data, if previously reported, should be cited, or if this is the first report, presented in the Supplemental data. Or if the results are being prepared for another manuscript, say that in the text and show minimal supporting data in Supplemental.

We have added a citation to our newly-published screening paper, as well as a sentence describing the known properties of the pinKE mutant, as follows:

“Following a recent comprehensive screen of mutations associated with neurological disorders in humans, we identified a substitution ($G\alpha_o^{K46E}$) that seemed likely to prevent guanine nucleotide binding, and thereby impose the first transformation state of the activation process (Figure 1A). Of the 55 mutations tested, $G\alpha_o^{K46E}$ most strongly suppressed signaling in cells.”

4. Ref 34 is incomplete in the bibliography.

This is a bug in EndNote, which we have manually corrected.

5. Figure 5 and supplemental show Cryo-EM structure of the D2R-Goα-K26E-Gβγ complex. The authors say in the abstract and text that this complex can be easily purified but the purified proteins used for the complex structure are not shown and should be shown as gels in Supplemental. Obviously the complex was isolated resulting in a structure, but seeing the purity of the starting material is helpful.

At the reviewer’s suggestion we have added Figure S3 showing a Coomassie stained gel of the purified complex following size exclusion chromatography, performed in the absence and presence of apyrase.

6. The Figure legends for all figures (including Supplemental) need to be improved and made more clear to the reader. For example, the legend for Figure 2 reads. “pinKE mutations prevent

Gαβγ dissociation". This legend is incomplete and should read something along the lines of Figure 2: Gα subunits expressing the pinKE mutation prevent Gβγ dissociation". Likewise, Figure 3 legend reads: "inhibition of effector function". This should read: Figure 3: "Placing the pinK-E mutation into Gα subunits prevents Gβγ-directed regulation of effector function". These are just two examples and alternative titles can be conjured. All Figure legends, including supplemental, need a second look and should be modified and improved as needed.

At the reviewer's suggestion, the figure legends have been revised and clarified.

7. All BRET figures fail to indicate what is being measured in the actual figure Y-axis. It is helpful to the reader to know what is being measured as BRET. If measuring Gβγ dissociation, label the y axis as: Gβγ dissociation (ΔBRET). Same for the Normalize fluorescence in Fig 4.

At the reviewer's suggestion, the terms netBRET, delta-BRET, maximum amplitude, normalized fluorescence, and Rmax are now defined in the legends.

8. In Fig 4E, it would be helpful to connect the dots in the Rmax data since this a concentration response curve with calculated Kd.

Done.

9. In many places, the authors have incomplete sentences that end with a reference number. For example, on page 17, the sentence: "Bacterial expression was used for production of recombinant full length human Gαo, as described previously for Gαi 69, and as modified by 20." The end of the sentence with ref 20 needs to written to complete the sentence. Similarly, on page 19, the sentence: "GTP binding and hydrolysis was determined as described previously 41 and as modified in 74." The end of the sentence with ref 74 needs to be rewritten to complete the sentence. There are other examples throughout the text. The authors should carefully re-read the entire text and correct these with complete sentences.

Done.

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This is a sound study of highly relevance for the field, as it contributes to shed further light on the first steps of G protein activation, it lays the foundation for new tools to ease the determination of receptor-G protein complex structures and ligand type discrimination, as well as it provides a mechanistic understanding of the functional consequences of a variant associated to a severe neurological disorder.

We are grateful to the reviewer for their time, effort, and enthusiastic evaluation of our manuscript

• The authors should discuss if and how this study relates to the recent work by the Lambert group (https://www.nature.com/articles/s41589-022-01231-z) referred G protein variants (Gi1 4A) that are able to bypass intermediate-state complexes by adopting conformations similar to receptor-bound G proteins independently of nucleotide release. The Go K46E and the Gi1 4A variants appear to bias G protein's activation at different points of the transition path, but they both somehow favor the formation of a receptor-G protein complex. The Gi1 4A variant does so by bypassing intermediate states and reducing coupling selectivity requirements. Is the Go K46E variant expected to work in a similar or different way?

The structure of the Gαo-4A mutant shows that the α5 helix is detached from the Ras domain even in GDP-bound trimer state. The authors conclude that the 4A mutant bypasses the intermediate state of the α5 helix. To investigate whether G_O K46E mutant has a similar behavior, we performed MD simulations on the wildtype-G_O trimer with GDP bound and also on the Gαo-K46E mutant trimer (without GDP, since this mutant does not bind to nucleotides). Within the short time scale of the MD simulations we only expect to see the early events of weakening of α5 helix interaction with the Ras domain. We performed 5 runs of MD simulations, each 1.5 μs long. This totaled to 7.5 μs trajectory for each system. We then calculated the interaction energy of the α5 residues with the residues in the interface with the Ras domain. We replaced Figure S2A with a new figure that shows the time series of the moving average of these interaction energies (averaged over the 5 simulations for each time point). The total interaction energy of the α5 helix with the Ras domain in $G\alpha_o^{K46E}$ is weaker than in the wildtype. Additionally, the interaction energies for $G\alpha_o^{K46E}$ show more fluctuations than in the wildtype. These data suggest similar mechanisms for $G\alpha_o^{K46E}$ and Gαi-4A. ~~Of course the MD simulations do not capture the dislodging of the H% helix.~~

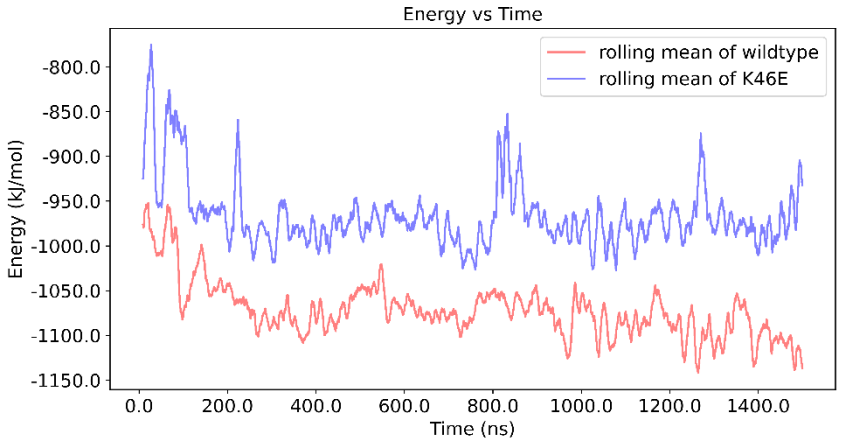
At the reviewer's suggestion, we have added the following text highlighting the Gi14A findings:

"Lambert and colleagues described two types of Gα mutants, both of which bind stably with agonist-occupied receptor but fail to release GDP. One strategy involved inserting four amino acids and extending the α5 helix, mimicking the translation that normally occurs during receptor engagement. Under these circumstances receptor-G protein subtype selectivity was diminished."

And

"We then examined the contact frequency (percentage of MD snapshots that make contact with GTP) of various residues in the nucleotide binding site (Supplementary Figure S2). This analysis revealed that residues Glu-43, Ser-44, Gly-45, Lys-46, Lys-181 and Thr-182 weaken their contact frequency by more than 30% in the $G\alpha_o^{K46E}$ mutant as compared to wildtype $G\alpha_o$. The total interaction energy of the α5 helix with the Ras domain is weaker in $G\alpha_o^{K46E}$ than in wildtype $G\alpha_o$. Similarly, the structure of the previously described GDP-bound $G\alpha_o$-4A mutant shows that the α5 helix is detached from the Ras domain [18]. These data suggest that the K46E and -4A mutants have similar effects on the α5 helix. In summary, our MDS analysis reveals how the Lys-46-Glu substitution affects the structure and dynamics of $G\alpha_o$, and provides a rationale for the observed reduction in nucleotide binding affinity."

New Figure S2A



• It would be useful to carry out a few more structural comparisons: first, it would be interesting to compare the Go conformation of the DRD2-Go K46E complex with other available Go complexes. The analysis should focus on the conformational changes observed for the Ras domain. Any difference? Are these (if any) limited to the P-loop region or do they propagate elsewhere? Then possibly focus on the whole receptor complex. In the absence of a DRD2-Go WT complex, one could perhaps consider the DRD2-Gi1 complexes (e.g. 8IRS). How does the receptor-G protein interface look like? Do they have a similar docking mode? How about the DRD2 conformations? Any difference that might be linked to the binding of the Go mutant and stabilization of the agonist-bound receptor?

We thank the reviewer for their comments and suggestions. We have now compared the D2R-GoA^{K46E} complex to the D2R-Gi1 complex (PDB ID: 8IRS) and have added a new figure (Figure 6) to the main text. In addition, we compared buried surface area (bsa) and contacts not only of the bound ligands (dopamine) but also the G protein interface, and summarized these findings in new supplementary tables as well as new text, as follows:

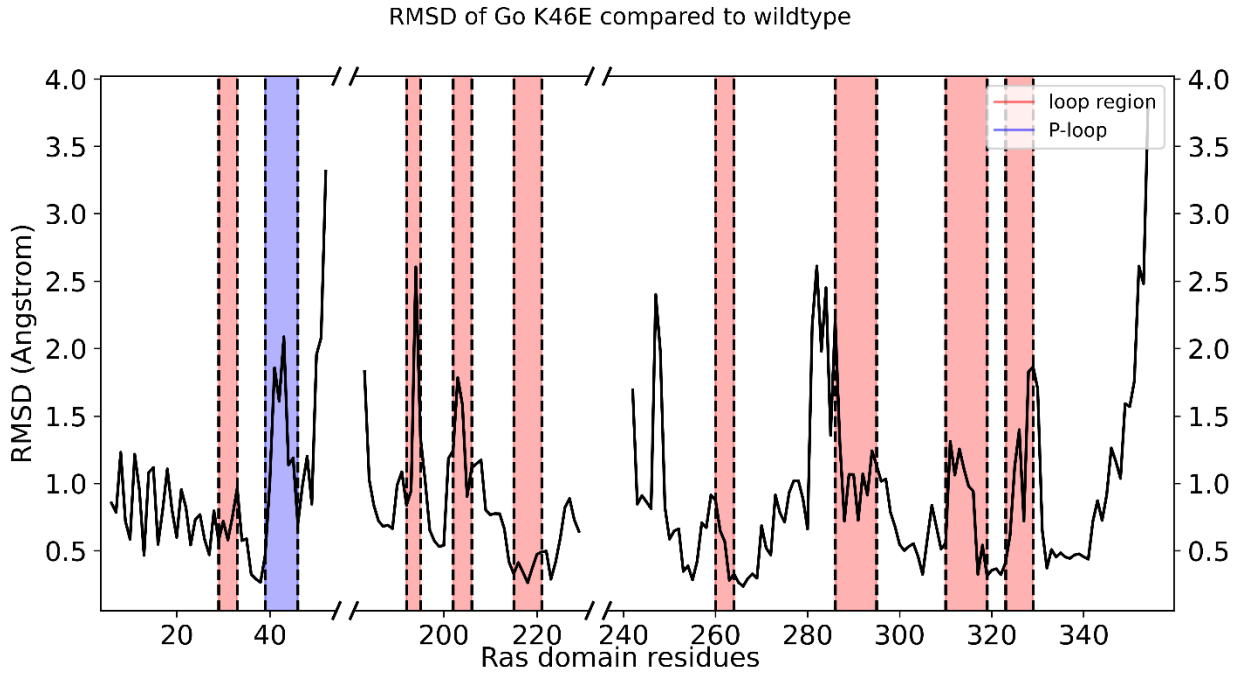
"To date, there exist three active state structures of D2R in complex with a G protein (PDB ID: 6VMS, 7JVR, and 8IRS). However, these structures are in complex with G_{i1}, not G_o, and bound with the agonists rotigotine or bromocriptine, not dopamine. Superposition of the receptors from our D2R-G_o^{K46E} structure (without scFv16) and from D2R-G_{i1} (PDB ID: 8IRS) reveals an RMSD of 1.50 Å of all aligned α carbons (Figure 6A). Most of the divergence between the two structures is at the extracellular ends of the transmembrane (TM) domains and in the extracellular loops. In comparison to D2R-G_{i1}, TM5 and TM6 in the D2R-G_o^{K46E} structure are more ordered, have more a helical content and are extended by 3 and 5 residues, respectively. In contrast, the orthosteric binding site residues superpose well in the two structures. The bound

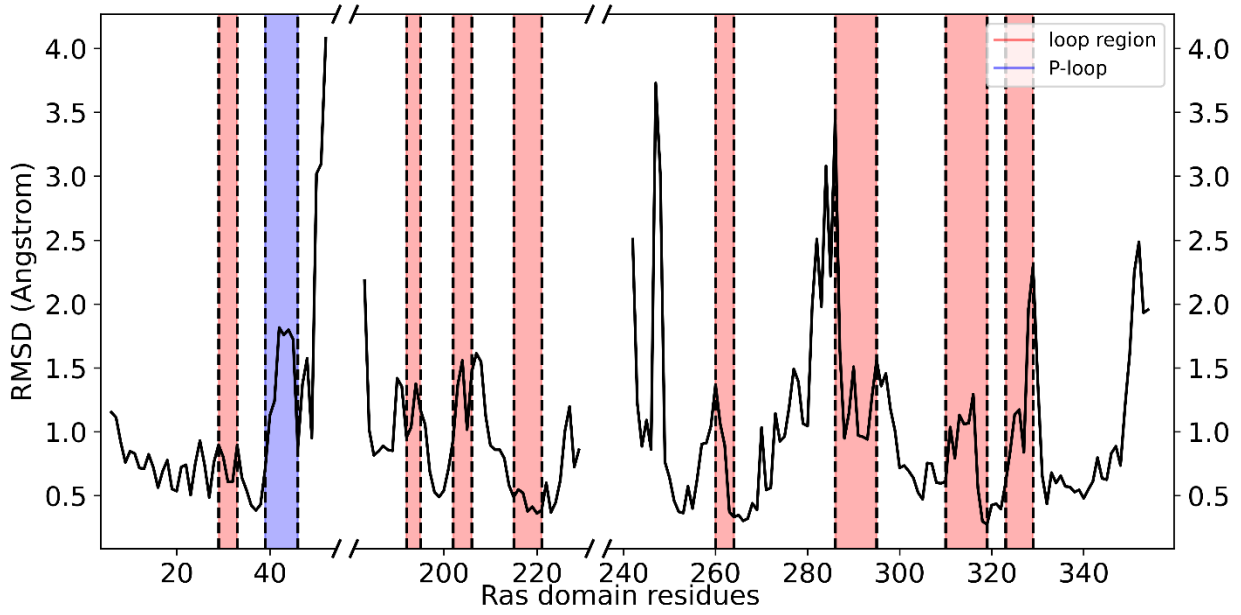
ligands occupy a similar area and engage overlapping residues, even though rotigotine is larger than dopamine and thus occupies more buried surface area (bsa) (272 Å^2 versus 131 Å^2, Figure 6B, Table S2). Furthermore, a comparison of our dopamine-bound D2R complex with the dopamine-bound D1R structure (PDB ID: 7F1O) revealed that the binding-site residues are conserved in these receptor subtypes (Table S2).

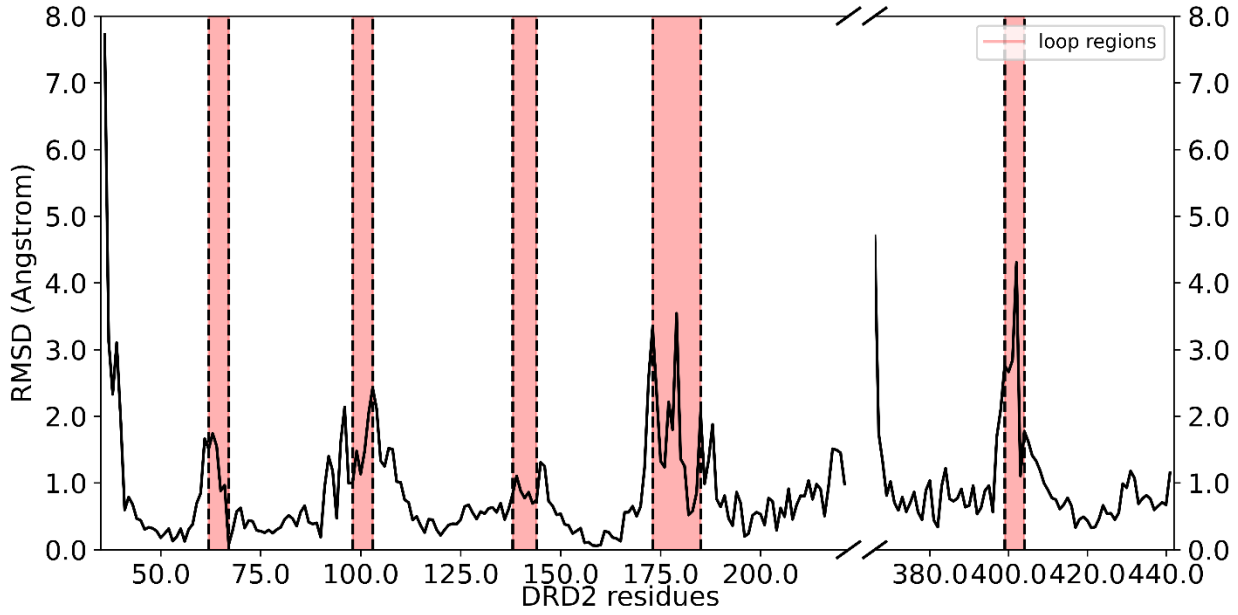
Comparison of the intracellular interface of D2R-G_o^{K46E} and D2R-G_{i1} revealed an approximate 0.5 Å off-center shift of the two G protein heterotrimer complexes, a difference that reverberates through the complex (Figure 6A). For the D2R-G_o^{K46E} complex, $G\alpha_o$ contributes 12 sidechain residues and 915 Å^2 of bsa to the interface; the C-terminal α-helix alone contributes 9 out of 12 residues and 773 Å^2 of the total bsa (Table S3). For the D2R-G_{i1} complex, $G\alpha_{i1}$ contributes 16 sidechain residues and 940 Å^2 of bsa to the interface; the C-terminal α-helix contributes 12 out of 16 residues and 808 Å^2 of the total bsa (Table S4). Conversely, the D2R receptor contributes 16 and 19 sidechain residues to the G_o and G_{i1} protein interfaces, respectively, 14 of which are shared between the two structures (Figure 6C).

Activation motif changes in GPCRs signify a transition from the inactive to active state, and vice versa. Rearrangement of the phenylalanine in the PIF connector region, rotation of the tyrosine in the tyrosine toggle switch (NPxxY), and disruption of an ionic-lock in the E/DRY motif have all been reported to be important for receptor activation, but can be receptor- and ligand-specific [51]. The PIF connector motif is likely to have an important role in connecting the agonist binding pocket to downstream conformational rearrangements required for receptor activation [17]. The NPxxY and E/DRY motifs have been proposed as stabilizing elements of an active conformation [52,53]. These motifs are superimposed in the activated D2R-G_o^{K46E} and D2R-G_{i1} structures, and far less so when compared to the inactive D2R receptor structure (PDB ID: 6CM4) [54] (Figure 6D). Thus, structurally speaking, the single substitution in G_{oA}^{K46E} has no apparent negative impact on ligand binding or activation of D2R. We conclude that the pinKE mutation locks the receptor in the agonist-bound, transitory pre-activated state."

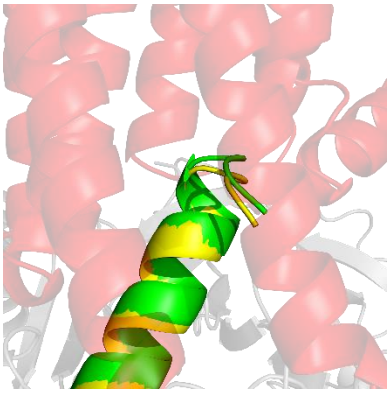
In addition, we have compared our D2R-G_o^{K46E} complex structure with 5 GPCR-G_o complexes (PDB IDs: 7D77, 7TS0, 7W2Z, 7EJ8, 8DZQ). We selected those structures because they have better than 3Å resolution and only one gap in the Ras domain. We calculated the root mean square deviation of every residue in the D2R-G_o^{K46E} and GPCR-G_o complexes. The average RMSD over the 5 pairs are plotted in the figure panels below. We also plotted the average RMSD variation for every residue among the 5 GPCR-G_o complexes. As seen in these plots the level of variations in RMSD of the loop region, the P-loop region (residues 40-45) and the spatially close HG region (residues 272-286) among the different Gαo-wildtype complexes are the same as that from the D2R-G_o^{K46E} structure. Therefore, we conclude that the D2R-G_o^{K46E} structure is not appreciably different from that of the other GPCR-G_o complexes.

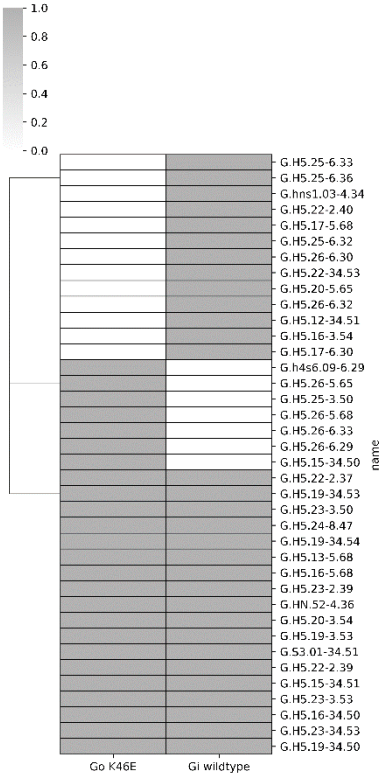

RMSD of Go K46E compared to wildtype
RMSD (Angstrom)
Ras domain residues
loop region
P-loop
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
20
40
200
220
240
260
280
300
320
340


RMSD of Ras domain of WT Go protein
RMSD (Angstrom)
Ras domain residues
loop region
P-loop
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
20
40
200
220
240
260
280
300
320
340



We also compared the orientation of the α5 helix in D2R-G_{i1} (PDB ID: 8IRS) with our D2R-G_o^{K46E} structure. As shown in the figure panels below, there is little difference between the α5 helix of G_i wildtype (green) and G_o^{K46E} mutant (yellow), with an overall backbone RMSD of 0.23Å. Although the spatial positioning of the α5 helices of the two structures are very similar, the residue contacts between the α5 helix helix and receptor are slightly different as shown in the contact map. This difference could stem from the fact that there are three residue differences in the α5 helix of $Gα_o$ and that of $Gα_{i1}$. Therefore, we conclude that the G_o^{K46E} mutant structure shares a high degree of structural similarity to the $Gα_{i1}$ wildtype structure.





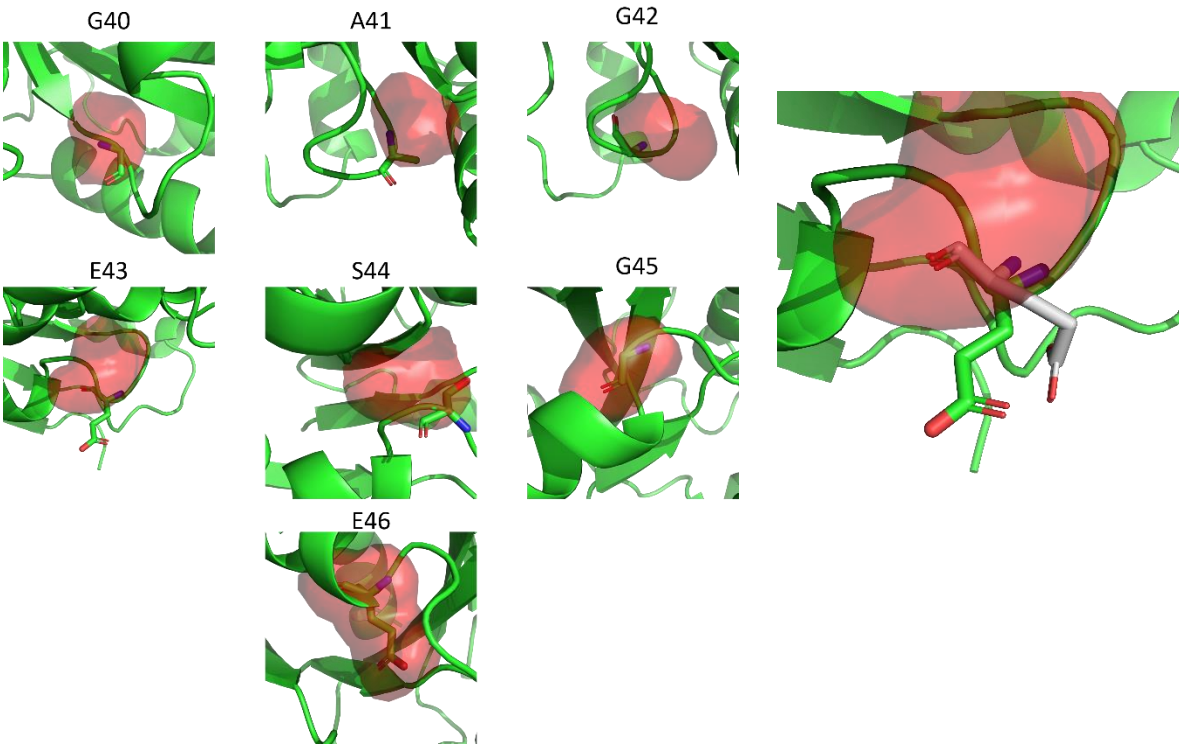
• It would be helpful to make a 3D cartoon representation by aligning the Ras domain of Galpha subunits of the DRD2-Galphao p.K46E, NTR1-Gi1 and B2AR-Gs complexes to highlight the different AHD roto-translations observed. This supplementary figure should be referenced in the corresponding results describing the AHD alternative poses observed.

To show alternative AHD conformations in these poses we added a new figure (Supplemental Figure S6) where we have overlaid NTR1-G_{i1}, B2AR-G_s and our solved D2R-G_o^{K46E} structures .

• Regarding MD simulations: please provide more information about which rotamer was used to model the Go mutants. Clearly, there should be a strong dependence of the simulation results on the input coordinates. Along this line, any specific reason why to start the simulations from active conformations? Please provide RMSD plots in the SI to check for trajectory convergence of the different simulations.

We used the rotamers of the Gαo wildtype structure to generate the K46E mutant starting structure for both the monomer and the trimer simulations. The starting structure of the K46E mutant had the same rotamers as the wildtype. This was done to see if α5 helix weakens its interaction with Ras domain in the K46E mutant compared to wildtype G_O. We also performed MD simulations on the active state G_O monomer to compare the results with the thermostability and nucleotide binding assays, which were performed with the monomeric G_O proteins. We have now provided the RMSD versus time plots for all simulations in Supplemental Figure S2B.

• Do the conformers of the Go K46E APO MD trajectory visit the side chains conformations and interactions, involving K46E and more in general the P-loop residues, observed in the DRD2-Go K46E experimental structure?

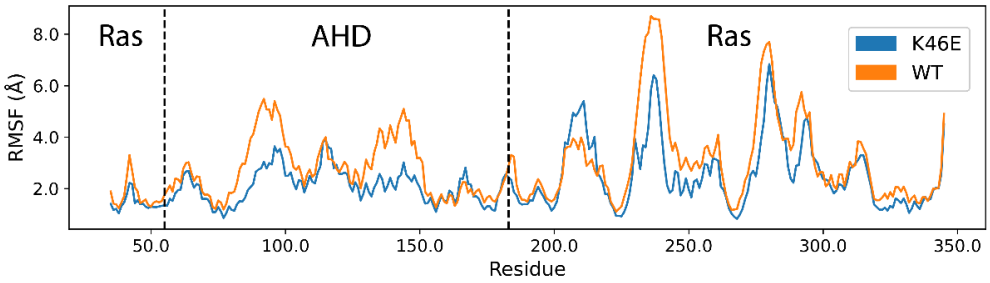


The starting conformations of the Gαo-K46E mutant trimer simulations were generated using the wildtype Gαo structure as template. To understand if the sidechain conformation of P-loop residues moved from the wildtype conformations to those conformations in the D2R-Gαo-K46E mutant crystal structure, we calculated the spatial density of the side chain atoms of the residues in the P-loop. As shown in red spheres, the side chain conformations of all the residues (except for E43) in the P-loop are indeed sampled by the MD simulation trajectories starting from wildtype Gαo side chain conformations.

• Figure S2 doesn't provide any detail regarding the contact analysis. It would be interesting to provide the contact statistics both for the GTP and APO forms. Ribbon representations are very difficult to read. Please use cartoons instead. Either energy minimize or refine with Rosetta FastRelax to improve geometry of representative structures for better rendering.

We have substituted the line representation of the structures (formerly Figure S2A) with a quantitative RMSF plot (new Figure S2B). This plot shows that the flexibility of the wildtype residues (as reflected by RMSF) are higher than those in the K46E mutant (for all but residues 206-216). This provides a quantitative view of the differences in the flexibility of K46E and wildtype Gαo.

New Figure S2B



Minor points:

• A curiosity: Is it possible to model the Mg cofactor in the catalytic pocket? Any density for it?

Although the cryoEM maps are at decent overall resolutions, they do not show density for magnesium.

• In the latest paragraph of the Results the authors comment: "Superimposition with the same region in the Gi1 structure, along with electrostatic analysis [….]". However no figure or table is referenced here. Are the authors referring to the 1GIA panel shown between panel 5D and 5E? Please specify. Also, it will be good to quantify via RMSD the "slight arrangement of the aforementioned loop".

We thank the reviewer for their comment and apologize for the lack of detail in describing the comparison between the two structures. We have revised Figure 5 and the paragraph, as follows:

"Superposition with the same region in the $G\alpha_{i1}$ structure, along with electrostatic analysis, revealed that the K46E substitution causes a slight rearrangement of the aforementioned loop (rmsd of 1.094 Å and 0.598 Å when comparing $G\alpha_{i1}$-GTP (PDB ID: 1GIA) with D2R-G_o^{K46E} and

D2R-G_o^{K46E} + scFv16, respectively*) and is shifted toward the front of the pocket along with a charge reversal to a negatively charged pocket (Figures 5D and 5E).”*

• In the “Inhibition of effector function” section: please correct “an measure”

Corrected

Reviewer #3 (Remarks to the Author):

Knight and colleagues perform an extensive series of experiments to characterize a disease-associated Go mutant that introduces a charge switch in the guanine nucleotide binding site. While many experiments have been performed the data don't fully support the main claim that the disease-causing phenotype of the protein is a persistent nucleotide-free state that acts as a dominant negative by binding and sequestering activated receptors. One major problem is that there is little direct evidence that receptor-pinKE Go complexes are highly stable in the presence of nucleotide, and there were missed opportunities to demonstrate this. Instead, there is direct evidence that pinKE mutants do bind GDP at concentrations present in cells, and that some residual exchange and activation is present (Fig. S1). Overall, it appears that this is a mutant that binds nucleotides poorly and is unstable but survives to some extent because of the charge switching. The broader practical or conceptual significance of the findings is also a bit unclear, as structural characterization of the nucleotide-free intermediate is performed regularly with wild-type proteins using the same procedures used here, and it is not surprising that this mutation would negatively affect nucleotide binding.

We appreciate the feedback and welcome the opportunity to elaborate on why the findings are significant and surprising. This paper demonstrates that the purified Gαo mutant has a 1000-fold reduction in affinity for GDP, yet binds normally to Gβγ. The mutant is sufficiently stable to co-purify with an agonist-bound GPCR and Gβγ and to inhibit GPCR signaling by a variety of measures and in a variety of cell systems, including yeast, oocytes and human cells.

Specific comments:

Purification of complexes for structural characterization was done in the presence of apyrase and/or the absence of GDP, therefore it is not surprising that the resulting complex is nucleotide-free. If the authors are confident in their model it is not clear why apyrase was present during purification, and the manuscript would have been much stronger if purification had been carried out in the presence of GDP. Apart from radioligand binding experiments where receptors and G proteins are artificially tethered there is no direct evidence that activated receptors and pinKE G proteins stay intact as a complex in the presence of nucleotides. I'm not suggesting the authors determine new structures, but showing size-exclusion chromatography with and without physiological GDP would strengthen the manuscript considerably. BRET experiments that directly monitor receptor-G protein association in the presence and absence of nucleotides would also help considerably and would seem within reach for this group.

Thank you for the suggestion, which was also made by reviewer #1. We now provide size-exclusion chromatography data, with and without apyrase, demonstrating the stability of the complex during purification (new Figure S3). In addition, we provide a Coomassie stained gel documenting the purity of the complex.

Unfortunately our repeated attempts to perform BRET in permeabilized cells were unsuccessful, showing no effect of apyrase even for the wildtype control.

TRUPATH data shown in Figure 2 don't include GoA and GoB, which is odd considering the human mutation is in Go. Perhaps this is because the defect with Go was only partial, as shown in Fig. S1. This is especially true for D2R, where the defect is really quite modest. On page 4 the effect of pinKE on the TRUPATH responses are first described as "diminished" and then later as showing that betagamma release was "blocked". Neither of these descriptions match the data.

The reviewer raises several valid points. We chose to present the data for GoA and GoB in the supplemental figures to highlight the data with most striking effects and to illustrate that the effect of the pinKE substitution was conserved across Gα families.

The modest effect for D2R, as illustrated in Figure S1D, was obtained using a sub-optimal pairing of TRUPATH components for GoA and GoB (Gβ1γ2 as opposed to the optimal Gβ3γ8 pairing described in the original TRUPATH paper). We elected to show these data because Gβ1γ2 were used for structural determination in this manuscript and because they still show a diminished, albeit modest, response relative to wildtype. The consequence of suboptimal transducer pairings can be further appreciated by the reduced span (50-60% reduction) between MOR with optimal pairings (Figure S1A, span~0.2 and ~0.3 for wildtype GoA and GoB, respectively) and D2R with suboptimal pairings (Figure S1D, span~0.1 for both wildtype GoA and GoB). As such, we believe the effect of the pinKE mutant illustrated in Figure S1D may only appear modest relative to a diminished BRET response of wildtype Gα.

We agree that the language used to describe the results is inconsistent and have modified the text accordingly. However, the TRUPATH responses for all optimal Gα/β/γ combinations containing the pinKE mutant are diminished. We have rephrased the text accordingly.

The TRUPATH results in Figure 2 labeled as net BRET appear to be ligand-induced changes (deltaBRET). For the basal BRET ratios given as bar graphs it is not clear if this is raw or net BRET, and the difference is critical for assessing how well each alpha subunit interacts with betagamma in the first place. The very low values in Fig. S1C suggest these are net BRET, in which case they indicate that some subunits (Gi3) associate with betagamma very poorly to begin with, despite being expressed better that others (GoA).

How do the authors explain the sometimes dramatic decrease in basal BRET in the TRUPATH assay with pinKE? If all Rluc-tagged alpha subunits associate with betagamma the only possible explanation is a difference in basal conformation. Or do some pinKE fail to associate with betagamma, perhaps after misfolding, lowering BRET?

The reviewer is correct that Gβγ appears to associate less well with several mutant Gα-Rluc constructs as compared with wildtype Gα-Rluc. The basal BRET ratios in Figure 2 and Figure S1C are raw BRET ratios from cells treated with vehicle (“basal”) and are presented to illustrate changes in mutant Gα-Rluc association with Gβγ. In cases where the reduction is modest (e.g. Gαi1 and Gαi2), we believe the ratiometric nature of the BRET method still provides meaningful results. We infer that the decrease in basal BRET ratio may be due to poor expression, misfolding, or an altered orientation of Rluc/GFP2. As such, we have added an additional statement in the manuscript to better emphasize these potential explanations:

“*Notably, a subset of pinKE mutants displayed significantly reduced luminescence, basal BRET ratios, or both, indicating poor expression or association, respectively (Supplemental Figures S1C and S1D)*” *While* $G\alpha_{oA}$ *appeared to express more poorly than the other subtypes tested (Supplementary Figure S1C), it bound particularly well to Gβγ under basal conditions (Supplementary Figure S1D). Together, these data suggest that Lys46 is needed for proper Gβγ dissociation.”*

The authors claim (pg. 4) that their TRUPATH results “demonstrate that Lys 46 is needed for proper Gbetagamma dissociation”. They go on to say that TRUPATH “provides a direct and

quantitative readout of Gα-Gβγ association". Since TRUPATH, like any BRET assay, can report either proximity/dissociation or orientation these statements are simply incorrect.

On page 4 the authors postulate that pinKE mutants will fail to dissociate from receptors as well as betagamma, but then say they will test this by looking at free betagamma release with GRKct. This is a non-sequitur that doesn't test receptor-G protein association at all.

At the suggestion of the reviewer, we have added new text as follows:

"The BRET method used above provides a direct and quantitative readout of distance between donor and acceptor, in this case bound to Gα and Gβγ~~of Gα-Gβγ association~~, and therefore is well suited for comparison of the pinKE substitutions in multiple Gα subtypes... While the diminished BRET signal can reflect diminished proximity, it is also dependent on the dipole angle of the fluorophores within each Gα subtype. In addition, it is possible that some G proteins do not fully dissociate following receptor activation. As an additional test of our hypothesis, ~~To test this~~ we used an alternative BRET method…"

The betagamma release experiments in Fig. 1B,C are shown only as deltaBRET, which obscures the baseline. This is a critical parameter in these experiments as it demonstrates proper Galpha expression, folding, and association with betagamma. These results need to be plotted as net BRET.

In our experience the baseline BRET values show substantial fluctuations between independent experiments and require normalization for reporting and analysis in a meaningful way. In contrast, changes in BRET induced by ligand application are stable and report the behavior of the system that we desire to analyze. We routinely perform control experiments to ensure proper behavior of components of the system that include looking at changes in BRET upon omission of Galpha and reversal of agonist induced response back to baseline upon application of antagonists. It is customary in the field to report BRET responses as deltaBRET to facilitate the analysis of their kinetics which is difficult to do if netBRET are presented instead. Thus, it is our preference to plot these data as deltaBRET, consistent with the format used in many of our previous publications.

The proposed dominant negative effect shown in Fig. 1C is only valid if it is also shown that wt GoA is expressed at the same levels in the presence and absence of the pinKE mutant. There is a difference between a dominant negative mechanism and simply rendering part of the G proteins available inactive.

Our use of the term "dominant negative" is consistent with the original 1987 *Nature* article by Ira Herskowitz. The abstract is copied here:

"*Molecular biologists are increasingly faced with the problem of assigning a function to genes that have been cloned. A new approach to this problem involves the manipulation of the cloned gene to create what are known as 'dominant negative' mutations. These encode mutant polypeptides that when overexpressed disrupt the activity of the wild-type gene. There are many precedents for this kind of behaviour in the literature-some oncogenes might be examples of naturally occurring dominant negative mutations.*"

The Go trace in Fig. 2 B is identical to the Go + Go trace in Fig. 2C, and these are said to be representative after activation of MOR.

The exact same traces are reused in Fig. S1E in the same way, although here they are said to be due to activation of D2R. Indeed, all of the traces said to be D2R in S1 are identical to traces said to be MOR in Fig. 2.

Figure S1E also shows receptor-G protein interaction data, specified as D2R, that appears identical to data shown in Fig. 2, receptor unspecified.

We apologize for the error. Figure 2B/C are correct and are representative of activation by dopamine binding to D2R. Figure S1E has been replaced with the intended data panel, which is representative of activation by DAMGO binding to MOR.

The authors claim that their GRK experiments show that the Go pinKE mutant forms a non-productive complex with betagamma. How can this be reconciled with the substantial TRUPATH response in Fig. S1?

On page 4 the GRKct method is said to measure the ability of a mutant to inhibit receptor activation. This isn't even close to what this assay measures.

The nano-luciferase complementation assay measures association between receptor and Gβγ. Compared to wildtype, the pinKE mutant exhibits dramatically increased receptor association, and also prominent dominant negative activity in the GRK assay, indicating the mutant binds GPCRs and blocks receptor activation. The TRUPATH and GRK BRET experiments largely agree but in this case the difference could be attributed to the different Gβγ pairings (Gγ8 vs Gγ2). Differences could also result from the use of modified Gα in TRUPATH versus unmodified Gα in the GRK experiments. These data, together with the protein binding and GIRK activity data, indicate that the mutation impedes Gβγ release following receptor activation.

The basal BRET ratios in Fig. S1C suggest that the pinKE Go mutants associate to some extent with betagamma without activation of a receptor. This directly contradicts the model the authors introduce on pages 5-6, that the pinKE Go only sequesters betagamma in complex with an activated receptor. This model seems to be introduced to explain why the pinKE mutant did not sequester betagamma away from GIRK channels, but it seems more likely that the failure of the pinKE to inhibit betagamma-medated GIRK activity has something to do with how the experiment was done, since wt Go also didn't sequester betagamma well; there is no significant difference between wt and pinKE in Fig. 3B,C.

Shortly thereafter the authors say that when D2 is expressed both pinKE and RC mutants reduce the effect of potassium (i.e. sequester betagamma) compared to wt Go, but why should wt fail to sequester betagamma completely in this case? Wild-type alpha also sequesters betagamma.

We make a distinction between binding and sequestration. Our data indicates that pinKE forms a stable and nonproductive complex with agonist-bound receptor (and Gβγ). Whereas purified pinKE binds normally to Gβγ (e.g. by BLI) is less able to release Gβγ (e.g. by BRET, GIRK, and cryo-EM) upon receptor activation, as follows:

"Together, these data support the conclusion that both $G\alpha_o^{K46E}$ and $G\alpha_o^{R209C}$ mutants sequester Gβγ, but $G\alpha_o^{K46E}$ does so only in the presence of activated receptor. Stated differently, triadRC acts as a Gβγ-specific dominant negative mutant and pinKE is a receptor-specific dominant negative mutant."

Another inconsistency appears in this experiment when the RC mutant apparently supports agonist-induced GIRK signals slightly better than wt. If this mutant can't release betagamma how does this work, and how can it be reconciled with the BRET experiments?

The oocyte data (with D2R) show no difference between the WT (black) and RC (blue) responses, while the HEK293 data (with MOR) show significantly less responses between the WT (black) and RC (blue) responses. We interpret these results to indicate that endogenous Gαi proteins in the two cell systems couple to different extents to the D2R versus MOR receptors giving us the signals we see in the RC cases. In contrast, the BRET signal comes only from the labeled Gα and Gβγ proteins and not the endogenous G proteins, that's why there is no signal in the RC case.

While the thermostability measurements do not show a difference between GTPgS and GDP for the pinKE mutant the meaning of this is not clear for this mutant. In contrast they do show that the pinKE mutant does bind GDP within the physiological concentration range. Therefore, this mutant is not likely to be "locked" in the nucleotide-free state in cells. Similarly, BODIPY-GTP does produce a signal with pinKE at 50 nM, as does GTPgS at 1 micromolar. This may be a mutant that folds poorly and binds guanine nucleotides poorly, but these data suggest there is some residual binding.

We demonstrated that the purified pinKE mutant has a 1000-fold reduction in affinity for GDP yet binds with wildtype affinity to Gβγ; we have now replaced the term "locked" to reflect that the mutant binds poorly to guanine nucleotides.

The bilayer interferometry experiments also directly contradict the model where activated receptor is necessary for pinKE to bind betagamma.

The radioligand binding experiments do suggest that, when fused to a receptor, the pinKE mutant survives and folds well enough to maintain high affinity agonist binding in the presence of nucleotide. This is some of the clearest data in the manuscript, but in this case one wonders if this represents the receptor chaperoning an unstable heterotrimer, which would not happen with normal receptor:G protein stoichiometry and without the tether. Could this perhaps help explain some of the unusual GIRK results with and without receptors?

Our data indicates that pinKE forms a stable and nonproductive complex with agonist-bound receptor and, consequently, with Gβγ. Whereas purified pinKE is stable and binds normally to Gβγ (e.g. by BLI) is less able to release Gβγ (e.g. by BRET, GIRK, and co-purification) upon receptor activation. We have clarified the text that pinKE forms a nonproductive complex with these proteins and avoid any suggestion that receptor is required for Gβγ binding to occur. We have clarified further that this unproductive complex makes Gβγ unavailable to wildtype endogenous Gα subunits.

Reviewer #4 (Remarks to the Author):

The authors reported two cryoEM structures of D2R-GαoK46E in the presence and absence of a stabilizing fab. In my opinion, the location of AHD domain is very interesting especially that it is determined without any stabilizing Fab. However, the discussion of this structure is very limited in the paper. It is well known that the AHD domain has a variable position relative to the Ras domain. Could the authors speculate why the specific mutation would result in a relatively stable Ras domain opening? Maybe via its interface with Gbg? I would recommend show the Gbg interface in the figure. The comparisons with other G protein complex structures are only described in text without any figures or supplementary figures. When compared that with the b2-adrenergic receptor-Gs protein complex crystal structure, the author doesn't mention that that structure has a nanobody binding to the Gα protein in-between the AHD and Ras domain which biases the Ras domain position relative to AHD domain. There are so many G protein complex structures there and it is not clear how the authors that the NTSR1-G protein complex is more similar to their structure. I think a more comprehensive comparison with existing relevant structures are necessary.

We thank the reviewer for their comments and suggestions. We have now compared the D2R-GoA^{K46E} complex to the D2R-Gi1 complex (8IRS) (new Figure 6). For this, we compared buried surface area (bsa) and contacts not only of the bound ligands (dopamine) but also the G protein interface. We have also created supplementary tables with these data and summarized these findings with the following text to the manuscript:

"To date, there exist three active state structures of D2R in complex with a G protein (PDB ID: 6VMS, 7JVR, and 8IRS). However, these structures are in complex with G_{i1}, not G_o, and bound with the agonists rotigotine or bromocriptine, not dopamine. Superposition of the receptors from our D2R-G_o^{K46E} structure (without scFv16) and from D2R-G_{i1} (PDB ID: 8IRS) reveals an RMSD of 1.50 Å of all aligned α carbons (Figure 6A). Most of the divergence between the two structures is at the extracellular ends of the transmembrane (TM) domains and in the extracellular loops. In comparison to D2R-G_{i1}, TM5 and TM6 in the D2R-G_o^{K46E} structure are more ordered, have more a helical content and are extended by 3 and 5 residues, respectively. In contrast, the orthosteric binding site residues superpose well in the two structures. The bound ligands occupy a similar area and engage overlapping residues, even though rotigotine is larger than dopamine and thus occupies more buried surface area (bsa) (272 Å^2 versus 131 Å^2, Figure 6B, Table S2). Furthermore, a comparison of our dopamine-bound D2R complex with the dopamine-bound D1R structure (PDB ID: 7F1O) revealed that the binding-site residues are conserved in these receptor subtypes (Table S2).

Comparison of the intracellular interface of D2R-G_o^{K46E} and D2R-G_{i1} revealed an approximate 0.5 Å off-center shift of the two G protein heterotrimer complexes, a difference that reverberates through the complex (Figure 6A). For the D2R-G_o^{K46E} complex, $G\alpha_o$ contributes 12 sidechain residues and 915 Å^2 of bsa to the interface; the C-terminal α-helix alone contributes 9 out of 12 residues and 773 Å^2 of the total bsa (Table S3). For the D2R-G_{i1} complex, $G\alpha_{i1}$ contributes 16 sidechain residues and 940 Å^2 of bsa to the interface; the C-terminal α-helix contributes 12 out of 16 residues and 808 Å^2 of the total bsa (Table S4). Conversely, the D2R receptor contributes 16 and 19 sidechain residues to the G_o and G_{i1} protein interfaces, respectively, 14 of which are shared between the two structures (Figure 6C).

Activation motif changes in GPCRs signify a transition from the inactive to active state, and vice versa. Rearrangement of the phenylalanine in the PIF connector region, rotation of the tyrosine in the tyrosine toggle switch (NPxxY), and disruption of an ionic-lock in the E/DRY motif have all

been reported to be important for receptor activation, but can be receptor- and ligand-specific [51]. The PIF connector motif is likely to have an important role in connecting the agonist binding pocket to downstream conformational rearrangements required for receptor activation [17]. The NPxxY and E/DRY motifs have been proposed as stabilizing elements of an active conformation [52,53]. These motifs are superimposed in the activated D2R-G_o^{K46E} and D2R-G_{i1} structures, and far less so when compared to the inactive D2R receptor structure (PDB ID: 6CM4) [54] (Figure 6D). Thus, structurally speaking, the single substitution in G_{oA}^{K46E} has no apparent negative impact on ligand binding or activation of D2R. We conclude that the pinKE mutation locks the receptor in the agonist-bound, transitory pre-activated state."

And

"In contrast, in the β_2-adrenergic receptor-G_s protein complex crystal structure (PDB ID: 3SN6), the AHD is positioned further from the Ras-like domain, possibly due to the presence of a stabilizing nanobody at the domain interface;"

The AHD density in the structure in without scFv16 is not very clear which doesn't allow confident modeling. This is probably due to the dynamic nature of the AHD domain to the Ras Domain. While I understood that the local resolution map showing the poor resolution in the Figure S3, I would recommend that the authors describe that too in the text. Right now, it reads like only the AHD density in the structure with svFv16 is poorly ordered. In Page7, last sentence "The EM density maps were sufficiently clear to place the receptor, the three G protein subunits, scFv16, and the bound ligand", it is not accurate because of the AHD domain of Gα domain.

We thank the reviewer for their comment and agree with their observation. We have deleted the sentence from the manuscript.

I would suggest that the authors reconsider the color choices to improve clarity of the Figure 5D.
a) Gαo is blended in with Gb. I would suggest use less bright colors or colors with more contrast from Gα;
b) Please consider use two different colors (or two different shades of cyan) for the AHD and Ras Domain for Gα domain so that the rotation angle would be more obvious.
c) The distance label is very hard to see. Please consider use a dark color for the line and get rid of the distance label. Instead, explain in the caption that the lines represent distances < 4 Å.

We thank the reviewer for their suggestions, we have amended the figure as suggested in comment (c). For the AHD, it is now enclosed with an outlined red circle to draw attention to the AHD. We have also contrasted the lettering of the components to allow for better visualization.

Reviewer #5 (Remarks to the Author):

The manuscript, entitled “A neurodevelopmental deficit that locks G proteins in the transitory pre-activated state by Knight et al. represents a very interesting study on the functional consequences of two mutations in the Gαo-gene on the balance of the G protein cycle. The most interesting finding is a charge-exchanging mutation that is demonstrated in the current manuscript to prevent binding of nucleotides to the G protein, without disturbing the ability to bind Gβγ. Furthermore, this K46E mutation induces a high affinity state of the receptor, stabilizing the ternary complex and thereby working as a dominant negative form. The mechanistic explanation how this mutation is causing dysfunction of the G protein is worked out with great care, including structural analysis.

The study is carried out with great expertise and elegant experiments and even solves the structure of the receptor bound G protein complex my means of Cryo EM. The findings are quite clear and convincing and have implications far beyond providing a better understanding of the molecular mechanism of a rare disease-inducing mutation. The mutant G protein will most likely also be a valuable tool for studying G protein signaling, function and receptor selectivity. The methods are described very well.

We are grateful to the reviewer for their time, effort, and enthusiastic evaluation of our manuscript

There are some issues that need to be addressed:

1) There is one issue that is apparent in the experimental results, however it is not mentioned and not discussed in the manuscript: In Figure 2A and also in the supplemental Fig 1 not only the agonist induced signal is severely affected by the 46KE mutation but also the basal BRET signal between Gα and Gβγ. I’m missing a discussion about this observation. In light of the capability of Gαo 46KE to still bind Gβγ, we would not expect a decrease in the basal BRET signal, unless there is a much-reduced expression of Gβγ or a diminished capability of Gβγ binding to Gα46KE or the conformation of the Heterotrimer is different, leading to a change in resonance energy transfer. Each of the possibilities could be addressed experimentally. In the latter case it seems to be feasible and interesting to compare the basal BRET between wt Gα and Gα46KE in permeabilized cells treated with apyrase in the presence of an receptor + agonist. If the main mechanism of this mutation is to prevent nucleotide binding we should expect to observe similar BRET signals. This issue is important to mechanistically link the observed cellular phenotype exclusively to the proposed mechanism and if so, would strengthen the argumentation of the authors.

The reviewer is correct that Gβγ appears to associate less well with several mutant Gα-Rluc constructs as compared with wildtype Gα-Rluc. The basal BRET ratios in Figure 2 and Figure S1C are raw BRET ratios from cells treated with vehicle (“basal”) and are presented to illustrate changes in mutant Gα-Rluc association with Gβγ. In cases where the reduction is modest (e.g. Gαi1 and Gαi2), we believe the ratiometric nature of the BRET method still provides meaningful results. We infer that the decrease in basal BRET ratio may be due to poor expression, misfolding, or an altered orientation of Rluc/GFP2. As such, we have added an additional statement in the manuscript to better emphasize these potential explanations:

“*Notably, a subset of pinKE mutants displayed significantly reduced luminescence, basal BRET ratios, or both, indicating poor expression or association, respectively (Supplemental Figures S1C and S1D)*” *While* $G\alpha_{oA}$ *appeared to express more poorly than the other subtypes tested*

(Supplementary Figure S1C), it bound particularly well to Gβγ under basal conditions (Supplementary Figure S1D). Together, these data suggest that Lys46 is needed for proper Gβγ dissociation."

Unfortunately our repeated attempts to perform BRET in permeabilized cells were unsuccessful, showing no effect of apyrase even for the wildtype control. As an alternative, and as suggested by reviewers #1 and 3, we now provide size-exclusion chromatography data with and without apyrase, demonstrating the stability of the complex during purification (new Figure S3). In addition, we provide a Coomassie stained gel of the purified complex, demonstrating the purity of the materials.

2) The title of the paper as well as a statement in the discussion: "Finally, our findings establish a potential mechanistic basis for human disease. The Gαo K46E and Gαo R209C mutations have been implicated in a pathogenic condition characterized by seizures, movement disorders, intellectual disability and developmental delay 33,36,53-56 " strongly implicates, that the 46KE Mutation has been described as a mutant that is responsible for a severe neurological disease. I screened the literature provided by the authors carefully and also literature beyond the cited ones, however I could not find the evidence the authors are suggesting. Of course, there is plenty of evidence for the R209C mutant to be implicated in pathogenic conditions. I probably have overlooked it, but if not, I would feel misled by the title and the discussion. It would be helpful for the reviewer (and also for other readers) to more specifically cite the literature in this context. Furthermore, please provide the literature with the first description of the disease-associated mutation. If there would be evidence for the K46E mutation to occur in patients, it would be highly interesting to look for differences in the phenotype also in comparison to the R209C Mutation that might be attributable to the differential functional consequences of these two mutations.

We have added a citation to the newly-published comprehensive screening paper, as well as a sentence describing the known properties of the pinKE mutant, as follows:

"*pinKE was one of 13 mutations, all located near the nucleotide-binding pocket, all of which suppress Gβγ signaling*."
In a screen of 55 Ga_o mutations, each associated with a neurological disorder in humans [25], we identified a subset of 29 mutations that suppress Gbg dissociation in cells. Because these

We have also added text describing the phenotype of the R209C and K46E mutant patients, as follows:

"*Finally, our findings establish a potential mechanistic basis for human disease. The* $Gα_o^{K46E}$ *and* $Gα_o^{R209C}$ *mutations have been implicated in a pathogenic condition characterized by seizures, movement disorders, intellectual disability and developmental delay. One individual with the K54E mutation exhibited focal, tonic, spasm, and tonic spasm seizures beginning 6 hours after birth. That individual died in her sleep at 23 months of age. Individuals with the R209C mutation are far less likely to have seizures but do exhibit involuntary movement, difficulty speaking, as well as intellectual and developmental delay."*

Minor comments:
A decrease in the BRET signal between Gα and Gβγ clearly correlates with activation and might indicate full dissociation, however unless proven in the same experimental setting it should not automatically interpreted as such. Reasoning behind this is, that both the dipole angle of the fluorophore and the distance between the donor and acceptor influence the BRET amplitude.

Also, the BRET signal goes not down to zero with full G protein activation. For all four subfamilies of G proteins there is evidence for uncomplete dissociation in the literature.

At the suggestion of the reviewer, we have added new text as follows:

"*While the diminished BRET signal can reflect diminished proximity, it is also dependent on the dipole angle of the fluorophores within each Gα subtype. In addition, it is possible that some G proteins do not fully dissociate following receptor activation. As an additional test of our hypothesis,* ~~*To test this*~~ *we used an alternative BRET method...*"

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have have improved the manuscript and satisfactorily addressed each of my concerns. Overall, these findings are highly significant. The study is a beautifully executed characterization of a disease-causing human mutation. The study identifies the underlying molecular mechanism of this neurodevelopmental disorder at the atomic, protein and cellular level and the findings have broad implications for medicine, cell biology and pharmacology.

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed all the raised points. I recommend the publication of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have made few substantive changes to the manuscript. Most importantly, there is still no compelling direct evidence that pinKE G proteins bind persistently to active receptors in the presence of physiological concentrations of nucleotides, which is central to the authors' model. Also contrary to the model, the authors show that the pinKE mutant binds GDP across the physiological concentration range. Overall the results are consistent with a mutant that folds poorly, expresses poorly, melts at low temperatures and unsurprisingly functions poorly. Structural characterization of this mutant is not particularly enlightening given the large number of nucleotide-free G protein structures already available.

The authors did not perform size exclusion chromatography as suggested, although they seem to imply that they did. They compare SEC in the absence and presence of apyrase and claim in the methods that the absence of apyrase "represents" physiological concentrations of ATP/GTP. Since no ATP or GTP was added through multiple steps of purification they have no idea how much residual nucleotide is present, if any. It is hard to imagine physiological concentrations of ATP/GTP appearing after 25 column volume washes with nucleotide-free buffer. A known concentration of

nucleotide should have been added. Direct evidence that active receptors and pinKE heterotrimers are stable complexes in the presence of nucleotides is still missing.

The authors claim to show that purified pinKE shows a "1000-fold reduction in affinity for GDP", yet they show nothing of the sort, and indeed they never measure GDP affinity. Figure 4B shows that it takes 1000 times as much GDP to produce a TM for the pinKE that matches the wt, but this does not (!) demonstrate a 1000-fold reduction in affinity, because the TM ranges are dramatically different to begin with. The same figure clearly shows that the pinKE mutant binds to GDP across the range from 10 to 1000 micromolar (i.e. close to the physiological range), similar to what they show for the wild-type. Figure 4C shows the mutant binds to 50 nM GTP. This refutes one of the main claims of the manuscript.

Figure 2A- deltaBRET is still labeled as netBRET. Basal is still not clarified as net or raw BRET ratios.

The authors insist on showing no TRUPATH data for Go in the main figures so as to "highlight the data with the most striking effects". This doesn't seem like a great reason to hide Go in the supplement since the human mutation is in Go. They suggest the modest negative effect of the mutation is due to a suboptimal pairing of TRUPATH components for GoA and GoB. It is difficult to imagine how a suboptimal pairing of components allows a defective G protein to respond better to a receptor. Would a more optimal pairing show no response, and if so, how would this be explained?

The authors do not address the expression of wild-type Go in their "dominant negative" BRET experiments, as suggested. One cannot conclude from the experiment shown in Figure 2C that the presence of pinKE heterotrimers interferes with the activation of wt heterotrimers, unless it can be shown that the same number of wt heterotrimers with labeled betagamma is present in the three conditions. Interference with the wild-type gene is only possible if the wild-type gene product is present in the assay.

The statements "The BRET method used above provides a direct and quantitative readout of distance between donor and acceptor" and "it is also dependent on the dipole angle of the fluorophores within each Galpha subtype" are contradictory.

In the same section the authors still refer to a betagamma release assay as being able to "measure the ability of the mutant to inhibit receptor activation". Again, this assay does not report receptor activation. This comment was ignored.

Instead of showing baseline net BRET in Figure 2B-C as requested the authors argue that independent experiments “require normalization”, that it is customary to show only delta BRET, and that this is “consistent with the format used in many of our previous publications”. This response dismisses the concern raised and is simply wrong. The Martemyanov group has a long history of assessing G protein mutants using this assay and, in every instance I’m aware of, has taken care to include baseline BRET ratios precisely because they provide an indication of how well each mutant interacts with betagamma. Some relevant examples are Figure 2 in PMID: 23222958, Figure 2 in PMID: 26810727, Figure 2 in PMID: 28087732, Figure 1 in PMID: 37548038, and Figure 4 in PMID: 37548038.

The inconsistencies between the authors’ model and the oocyte data persist. Most clearly, neither wt Go nor the KE mutant reverse the increase in GIRK current produced by expressing betagamma-only the RC mutant does so (Fig. 3B-C). Why, if the KE binds normally to betagamma as claimed? The ability of the RC mutant to support receptor-mediated GIRK currents is handwaved away as a difference in endogenous G protein background.

Reviewer #4 (Remarks to the Author):

The authors have sufficiently addressed my comments.

Reviewer #5 (Remarks to the Author):

The authors have addressed all my concerns. It is a bit unfortunate that the BRET experiments in permeabilized cells did not work, however, the inclusion of the stability measurements with purified proteins helps in this context.

Reviewer #3 (Remarks to the Author):

The authors have made few substantive changes to the manuscript. Most importantly, there is still no compelling direct evidence that pinKE G proteins bind persistently to active receptors in the presence of physiological concentrations of nucleotides, which is central to the authors' model. Also contrary to the model, the authors show that the pinKE mutant binds GDP across the physiological concentration range. Overall the results are consistent with a mutant that folds poorly, expresses poorly, melts at low temperatures and unsurprisingly functions poorly.

We respectfully disagree with the reviewer's interpretation of our data. The four BRET methods (Fig. 2) and the radioligand binding assays (Figs. 5A-C) show increased agonist-receptor and receptor-G protein association for pinKE relative to WT, as would be expected for a nucleotide-free G protein. Importantly, all of these experiments were done in cells where there are physiological concentrations of nucleotide. Additionally, using purified proteins, we show negligible binding of pinKE to BODIPY-GTPγS (Fig. 4C) and [^{35}S]GTPγS (Fig. 4D). Nevertheless, this mutant has wildtype binding affinity to Gβγ, indicating that it is properly folded (Fig. 4E).

To improve clarity we have modified a sentence in the abstract as follows:

~~"Using measures of nucleotide binding, catalytic activity, subunit association, and ion channel function, we show that Gα$_o^{K46E}$ binds stably~~ Whereas purified Gα$_o^{K46E}$ binds poorly to guanine nucleotides it retains wild-type affinity for the Gβγ subunits. In cells containing physiological concentrations of nucleotide, Gα$_o^{K46E}$ forms a stable complex with receptors and Gβγ, impedes effector activation, and does so in a genetically dominant manner."

Structural characterization of this mutant is not particularly enlightening given the large number of nucleotide-free G protein structures already available.

Our structure of the D2 dopamine receptor and pinKE is of interest to the larger GPCR and GNAO1 disorder communities because i) there are no other structures of the dopamine receptor with Gα$_o$, representing the most abundant G protein in the brain, ii) there are very few structures that show the complete G protein α subunit or that are solved in the absence of stabilizing nanobodies, iii) the pinKE mutation is responsible for a rare neurological disorder, iv) the mutation is at a lysine residue that is conserved in all large and small GTPase proteins.

The authors did not perform size exclusion chromatography as suggested, although they seem to imply that they did. They compare SEC in the absence and presence of apyrase and claim in the methods that the absence of apyrase "represents" physiological concentrations of ATP/GTP. Since no ATP or GTP was added through multiple steps of purification they have no idea how much residual nucleotide is present, if any. It is hard to imagine physiological concentrations of ATP/GTP appearing after 25 column volume washes with nucleotide-free buffer. A known concentration of nucleotide should have been added. Direct evidence that active receptors and pinKE heterotrimers are stable complexes in the presence of nucleotides is still missing.

This receptor-G protein complex is formed through co-expression, in insect cells, of the receptor and all three G protein subunits. These cells contain physiological concentrations of nucleotide. The presence of these nucleotides prevents receptor-G protein association in cells and in subsequent steps of protein purification. In contrast, the pinKE mutant forms a stable complex with the D2 receptor in cells, and remains associated in the absence or presence of nucleotides (in the presence or absence of added apyrase). The complex remains stable even after 25 column washes with nucleotide-free buffer. Since the complex was purified in the presence of nucleotides, we see no point in readdition of nucleotides after removing them. This provides strong direct evidence that pinKE complexes form a stable complex in cells, in the presence of physiological concentrations of nucleotides.

The authors claim to show that purified pinKE shows a "1000-fold reduction in affinity for GDP", yet they show nothing of the sort, and indeed they never measure GDP affinity. Figure 4B shows that it takes 1000 times as much GDP to produce a TM for the pinKE that matches the wt, but this does not (!) demonstrate a 1000-fold reduction in affinity, because the TM ranges are dramatically different to begin with. The same figure clearly shows that the pinKE mutant binds to GDP across the range from 10 to 1000 micromolar (i.e. close to the physiological range), similar to what they show for the wild-type.

We (PMID: 23954348) and others (PMID: 26258638) have used Tm and Tm changes as a reliable proxy for apo, GDP bound, and GTPγS bound states. The Tm ranges are different because WT binds nucleotide (has a high melting temperature) and KE does not (has a lower melting temperature). The Tm's can be made equal, but only by adding >1000-fold higher concentration of GDP to the mutant. Our interpretation is that a reduction in Tm is due to a reduction in nucleotide binding as stated in the manuscript:

"As shown in Figure 4A, whereas the wildtype protein was more stable when bound to GTPγS than GDP, GαoR209C showed no stabilization in the presence of GTPγS. The GαoK46E variant had a substantially lower Tm, and likewise showed no differences when comparing GTPγS and GDP. Only at 1000-fold higher concentrations of GDP did we observe a Tm close to that of the wildtype protein (Figure 4B)."

Figure 4C shows the mutant binds to 50 nM GTP. This refutes one of the main claims of the manuscript.

Figure 4C clearly shows that WT (black trace) binds properly to GTP while the mutant (red trace) does not.

Figure 2A- deltaBRET is still labeled as netBRET. Basal is still not clarified as net or raw BRET ratios.

We use these terms interchangeably. We have now replaced all instances of netBRET with deltaBRET.

The authors insist on showing no TRUPATH data for Go in the main figures so as to "highlight the data with the most striking effects". This doesn't seem like a great reason to hide Go in the supplement since the human mutation is in Go. They suggest the modest negative effect of the mutation is due to a suboptimal pairing of TRUPATH components for GoA and GoB. It is difficult to imagine how a suboptimal pairing of

components allows a defective G protein to respond better to a receptor. Would a more optimal pairing show no response, and if so, how would this be explained?

The data are presented in a transparent manner. The purpose of the experiment is to determine if the importance of the pinK residue is conserved in multiple different G protein subtypes. We do not know why some G proteins have a stronger signal than others. Given that there are multiple possible pairings of G protein subunit subtypes (16 Gα, 4 Gβ, 12 Gγ) it is reasonable to suggest that different pairings will have effects of different magnitude.

The authors do not address the expression of wild-type Go in their “dominant negative” BRET experiments, as suggested. One cannot conclude from the experiment shown in Figure 2C that the presence of pinKE heterotrimers interferes with the activation of wt heterotrimers, unless it can be shown that the same number of wt heterotrimers with labeled betagamma is present in the three conditions. Interference with the wild-type gene is only possible if the wild-type gene product is present in the assay.

We performed this experiment by co-transfecting equivalent amounts of mutant and wildtype, and confirmed equal expression by western blotting. Thus, the pinKE mutant interferes with activation of wild-type heterotrimers, and does so in a dominant negative manner. We have modified the figure legend to clarify that wild-type is also present in these assays.

The statements “The BRET method used above provides a direct and quantitative readout of distance between donor and acceptor” and “it is also dependent on the dipole angle of the fluorophores within each Galpha subtype” are contradictory.

Taken in context (“While the diminished BRET signal can reflect diminished proximity, it is also dependent on the dipole angle of the fluorophores within each Gα subtype.”) we believe the statements are clear and accurate.

In the same section the authors still refer to a betagamma release assay as being able to “measure the ability of the mutant to inhibit receptor activation”. Again, this assay does not report receptor activation. This comment was ignored.

Receptor-mediated activation is required to release Gβγ. The assay measures binding of free Gβγ to GRK3ct. Thus, the BRET signal is a direct consequence of receptor activation.

To improve clarity, we have substituted “receptor activation” with “receptor-mediated activation.”

Instead of showing baseline net BRET in Figure 2B-C as requested the authors argue that independent experiments “require normalization”, that it is customary to show only delta BRET, and that this is “consistent with the format used in many of our previous publications”. This response dismisses the concern raised and is simply wrong. The Martemyanov group has a long history of assessing G protein mutants using this assay and, in every instance I’m aware of, has taken care to include baseline BRET ratios precisely because they provide an indication of how well each mutant interacts with betagamma. Some relevant examples are Figure 2 in PMID: 23222958, Figure 2 in PMID: 26810727, Figure 2 in PMID: 28087732, Figure 1 in PMID: 37548038, and Figure 4 in PMID: 37548038.

The raw BRET traces and baseline BRET ratios are provided in Figure S1E.

The inconsistencies between the authors' model and the oocyte data persist. Most clearly, neither wt Go nor the KE mutant reverse the increase in GIRK current produced by expressing betagamma- only the RC mutant does so (Fig. 3B-C). Why, if the KE binds normally to betagamma as claimed? The ability of the RC mutant to support receptor-mediated GIRK currents is handwaved away as a difference in endogenous G protein background.

These data fully support our conclusions that pinKE binds poorly to nucleotide and, consequently, forms a non-productive complex with activated receptors.

The reviewer states accurately that RC, but not KE or WT, suppresses the Gβγ-induced current (Fig. 3B-C). This is because RC forms a nonproductive complex with Gβγ, while KE (which binds normally to Gβγ) and WT do not. In contrast, both mutants suppress receptor-induced current (Fig. 3E), and do so by different mechanisms. KE sequesters activated receptor, in competition with WT, and prevents the release of Gβγ. RC sequesters Gβγ but not receptor; thus RC supports some receptor-mediated signaling through WT endogenous G protein. Again, KE sequesters receptor at physiological concentrations of nucleotide.

Thus, the data support our model that RC is a Gβγ dominant negative mutant while KE is a receptor dominant negative mutant.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed any and all remaining concerns. This is a beautiful story that uncovers important new understanding of G protein signaling, in particular previously unappreciated roles for Go and its contribution to this specific neurological disease.

Reviewer #3 (Remarks to the Author):

Again the manuscript is largely unchanged. The data in clearly contradict the claim that the pinKE mutant fails to bind nucleotide. The claim that there is a highly stable receptor pinKE complex in the presence of physiological nucleotides is still largely unsupported.

There is no compelling evidence that the effects seen in cellular assays are due to stable receptor G protein complexes. The BRET assays used do not look at receptor-G protein association even though such assays do exist, and the current results could be explained just as well by a G protein that fails to release nucleotide or betagamma.

The authors claim that nucleotides present in insect cells prevents receptor G protein association throughout protein purification. This is an astonishing claim. The first step of purification is separating membranes containing receptors and G proteins from cytosolic contents, replacing the latter with nucleotide-free buffer and agonist. What prevents complexes from forming at this step? To simply repeat that the absence of apyrase equals the presence of nucleotides is nonsense. Why not do 25 column washes with a known concentration of nucleotides if the complex is so stable in the presence of nucleotides? Why use nucleotide-free buffer at all? Why not compare how the purification of wt and pinKE differ when nucleotides are present at known concentrations?

The authors simplistically equate Tm with nucleotide binding, but they can have no idea what causes the difference in melting temperature ranges of the wt and pinKE proteins, as many different defects could affect Tm. They don't show us that Tm is equal for wt and pinKE when nucleotides are absent, as their simple interpretation would demand. More importantly, their data clearly show that there is a greater than 10 degree thermal shift in the pinKE as [GDP] increases across the 1-1000 micromolar range, which includes the likely physiological value. This flatly contradicts the statement on line 287 that the pinKE mutant "fails to bind nucleotide entirely". The authors' response is simply nonresponsive and a repetition of what they want to conclude. Of course Tm can be used to indicate GDP binding or measure GDP affinity (as PMID 26258638 did but the authors did

not), the problem here is that Tm measurements in 4B indicate GDP binding in a concentration range that the authors do not wish to acknowledge.

The same is true for 4C. No, the responses aren't equal, but neither of them is zero, and the concentration of nucleotide is only 50 nM.

If the purpose of the TRUPATH experiment is to "determine if the importance of the pinK residue is conserved in multiple different G protein subtypes" why not include the founding subtype, Go? If transparency is the goal why do all of the figures emphasize Go except for this one? If the authors are confident in their results, what harm could it do to add Go to the Figure?

The authors now claim to have confirmed equivalent expression of wt Go in their "dominant negative" experiments by western blotting. There are no western blots in the manuscript, and it is not at all clear how western blotting would distinguish wt from pinKE Go without a specific antibody or epitope.

The oocyte data are a simple failure of a classic experiment that the authors can't explain. For many betagamma effectors a constitutive signal is produced by overexpressing betagamma, and this is reversed by coexpressing alpha since the alpha and effector binding sites generally overlap. This is certainly true for GIRK channels, and also for the GRK betagamma assay the authors now show us in S1E. To explain their oocyte data, which differ completely from their BRET data, the authors invent the concept of a "nonproductive complex with betagamma" that is made by RC but not KE or wt. It's not clear what this could mean when receptors aren't being activated, and in any case, it doesn't explain how binding of wt or KE to betagamma at the GIRK-binding interface could possibly fail to interfere with basal activation of GIRK channels.

Reviewer #5 (Remarks to the Author):

The manuscript has further improved in clarity.

Again it is a bit sad, that the experiments directly addressing receptor G protein complex formation did not work. This could have solved most of the controversy with reviewer 3

In respect to the oocyte data I do see the discrepancy between Figure 3 C and Figure 3F: In the presence of the D2R basal currents are much reduced for pinKE, in the absence of a receptor they are not. Could this mean that the receptor is needed for pinKE to stabilize the Ga Gbg interaction? The reduced BRET values between pinKE and Gbg in the absence of agonist actually go in the same direction. Is this something the authors want to discuss?

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed any and all remaining concerns. This is a beautiful story that uncovers important new understanding of G protein signaling, in particular previously unappreciated roles for Go and its contribution to this specific neurological disease.

Reviewer #3 (Remarks to the Author):

Again the manuscript is largely unchanged. The data in clearly contradict the claim that the pinKE mutant fails to bind nucleotide. The claim that there is a highly stable receptor pinKE complex in the presence of physiological nucleotides is still largely unsupported.

There is no compelling evidence that the effects seen in cellular assays are due to stable receptor G protein complexes. The BRET assays used do not look at receptor-G protein association even though such assays do exist, and the current results could be explained just as well by a G protein that fails to release nucleotide or betagamma.

We respectfully disagree with this reviewer. The binding data in Figs. 4C, 4D and 4E show that the pinKE mutant binds poorly to nucleotide yet binds normally to Gβγ. The BRET assay in Fig. 2D measures association of receptor with the G protein heterotrimer in cells. The radioligand binding data in Fig. 5C show that the mutant stabilizes high affinity agonist binding. The cryo-EM data in Fig. 5 shows the G protein releases nucleotide.

The authors claim that nucleotides present in insect cells prevents receptor G protein association throughout protein purification. This is an astonishing claim. The first step of purification is separating membranes containing receptors and G proteins from cytosolic contents, replacing the latter with nucleotide-free buffer and agonist. What prevents complexes from forming at this step? To simply repeat that the absence of apyrase equals the presence of nucleotides is nonsense. Why not do 25 column washes with a known concentration of nucleotides if the complex is so stable in the presence of nucleotides? Why use nucleotide-free buffer at all? Why not compare how the purification of wt and pinKE differ when nucleotides are present at known concentrations?

The reviewer is correct that the first step of purification is to separate membranes, containing receptors and G proteins, from cytosolic contents. Since the cytosol contains nucleotide, the complex will normally fall apart during this step and remain separated during the subsequent dilution and wash steps; apyrase is normally included during lysis to remove nucleotides and preserve the complex, but is not needed in this case.

The authors simplistically equate Tm with nucleotide binding, but they can have no idea what causes the difference in melting temperature ranges of the wt and pinKE proteins, as many different defects could affect Tm. They don't show us that Tm is equal for wt and pinKE when nucleotides are absent, as their simple interpretation would demand.

We agree that the pinKE mutation could affect Tm, and we were appropriately cautious in our interpretation of the data. Unfortunately, the experiment suggested by the reviewer is not feasible because nucleotide-free WT is stable only when bound to receptor, which would itself

affect Tm. While we do not equate Tm with binding, it might have been reasonable to do so, as dictated by the thermodynamics, since the Tm values for WT and pinKE converge with increasing GDP concentrations (Fig. 4B). Thus one might infer that the Tm values for mutant and WT would be the same at GDP concentrations sufficient to saturate both proteins.

More importantly, their data clearly show that there is a greater than 10 degree thermal shift in the pinKE as [GDP] increases across the 1-1000 micromolar range, which includes the likely physiological value. This flatly contradicts the statement on line 287 that the pinKE mutant "fails to bind nucleotide entirely". The authors' response is simply nonresponsive and a repetition of what they want to conclude. Of course Tm can be used to indicate GDP binding or measure GDP affinity (as PMID 26258638 did but the authors did not), the problem here is that Tm measurements in 4B indicate GDP binding in a concentration range that the authors do not wish to acknowledge.

We agree with the reviewer and have revised the text as follows: "$G\alpha_o^{K46E}$ binds poorly to guanine nucleotides, $G\alpha_o^{R209C}$ binds to nucleotides but fails to release Gβγ."

The same is true for 4C. No, the responses aren't equal, but neither of them is zero, and the concentration of nucleotide is only 50 nM.

If the purpose of the TRUPATH experiment is to "determine if the importance of the pinK residue is conserved in multiple different G protein subtypes" why not include the founding subtype, Go? If transparency is the goal why do all of the figures emphasize Go except for this one? If the authors are confident in their results, what harm could it do to add Go to the Figure?

Please see our previous response to this request.

The authors now claim to have confirmed equivalent expression of wt Go in their "dominant negative" experiments by western blotting. There are no western blots in the manuscript, and it is not at all clear how western blotting would distinguish wt from pinKE Go without a specific antibody or epitope.

We routinely perform western blots to validate plasmids and protocols; accordingly, the statement about western blots has been moved to the methods section. At the reviewer's request we now append the pertinent data, done three times in side-by-side experiments, documenting that WT is expressed at least as well as pinKE.

The oocyte data are a simple failure of a classic experiment that the authors can't explain. For many betagamma effectors a constitutive signal is produced by overexpressing betagamma, and this is reversed by coexpressing alpha since the alpha and effector binding sites generally overlap. This is certainly true for GIRK channels, and also for the GRK betagamma assay the authors now show us in S1E. To explain their oocyte data, which differ completely from their BRET data, the authors invent the concept of a "nonproductive complex with betagamma" that is made by RC but not KE or wt. It's not clear what this could mean when receptors aren't being activated, and in any case, it doesn't explain how binding of wt or KE to betagamma at the GIRK-binding interface could possibly fail to interfere with basal activation of GIRK channels.

See below

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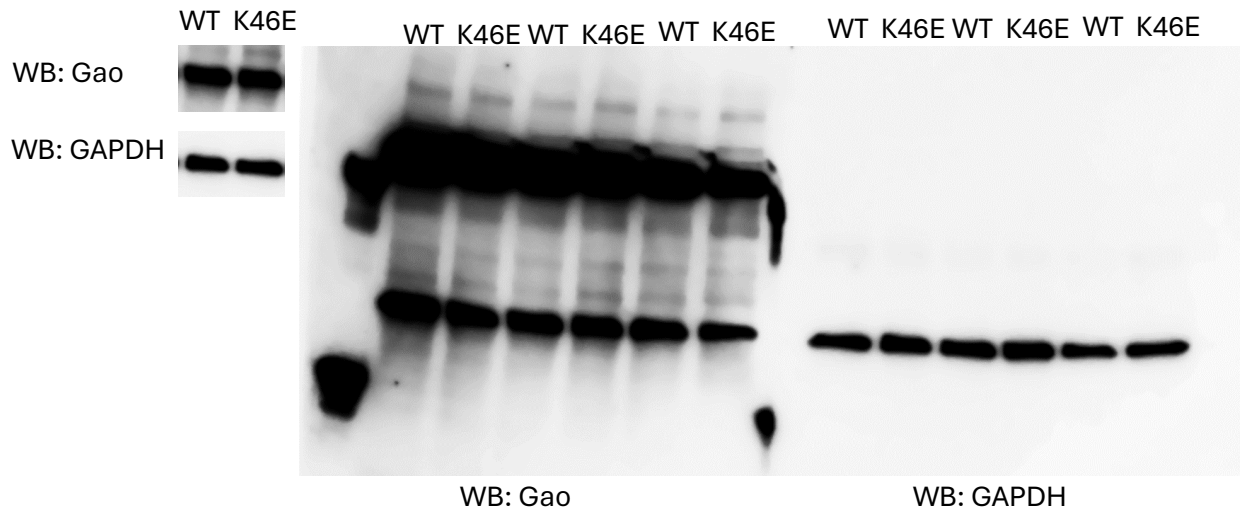
We agree with the interpretation of the two reviewers and have added the comment to the text (underlined) as follows: “These data support the conclusion that both $G\alpha_o^{K46E}$ and $G\alpha_o^{R209C}$ mutants sequester Gβγ, but $G\alpha_o^{K46E}$ does so only in the presence of activated receptor. Accordingly, differences in Fig. 3C and 3F could be due to stabilization by receptor of the G protein heterotrimer. Stated differently, triadRC acts as a Gβγ-specific dominant negative mutant and pinKE is a receptor-specific dominant negative mutant.”



						Gao						Gao/GAPDH				
				177.8073		1	2	3	Average	P value		1	2	3	Average	P value
Gao	WT	43.99	211.01	1.186734		1.186734	0.929692	0.883574	1	0.265375		1.187915	0.840662	0.987134	1.005237	0.143608
Gao	K46E	66.644	188.356	1.059326		1.059326	0.951164	0.651177	0.887222			0.983947	0.808995	0.708942	0.833961	
Gao	WT	89.694	165.306	0.929692												
Gao	K46E	85.876	169.124	0.951164												
Gao	WT	97.894	157.106	0.883574												
Gao	K46E	139.216	115.784	0.651177												
						GAPDH										
				88.13967		1	2	3	Average	P value						
GAPDH	WT	166.948	88.052	0.999005		0.999005	1.105904	0.895091	1	0.078						
GAPDH	K46E	160.108	94.892	1.076609		1.076609	1.175736	0.918519	1.056955							
GAPDH	WT	157.526	97.474	1.105904												
GAPDH	K46E	151.371	103.629	1.175736												
GAPDH	WT	176.107	78.893	0.895091												
GAPDH	K46E	174.042	80.958	0.918519												