nature portfolio

Peer Review File

$G\alpha_{o/z}$-biased coupling profile of the dopamine D3 receptor

Corresponding Author: Professor Cesare Orlandi

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper focuses on the G protein coupling specificity of the D2-like dopamine receptors and most specifically the dopamine D3 receptor. They make use of a BRET beta gamma release assay to show that the D3R couples selectively to Galpha o and z whereas the D2R and D4R couples to all Gi/o/z members. They use D2/D3 chimeric receptors to reveal that ICL2 residues are a determinant of this different selectivity as are residues within the C-terminal helix of Galpha o/i G proteins. They make use of the CAMPER mice system to measure the effect of D2R and D3R selective agonists on intracellular cAMP levels in CA1 hippocampal neurons. In general, the experiments are well performed and the data is clear.However, the coupling specificity of the D3R has been studied before. As highlighted by the authors, papers almost 20 years ago described the same specificity for G alpha O and G alpha Z. The efforts to describe the structural basis of this specificity reveal new information around the role of ICL2 using receptor chimeras. The authors rationalise these observations against the emerging structural data but I feel it would have been a nice addition for the authors to take this one step further and experimentally investigate some of the hypotheses generated here. Finally, it's difficult to align the data from the measurement of cAMP in slice with the rest of the paper. As prsented I don't think you can conclude taht the responses driven by the different agonists are simply down to the differential coupling selectivity of the D2 and d3 receptors. Specific comments are as follows:

D3R coupling specificity: The coupling specificity of the D3R for GalphaO and GalphaZ have been investigated and identified before (10.1023/a:1006988603199, 10.1021/acsptsci.3c00339, doi:10.1124/jpet.107.134296). The use of the beta gamma release BRET assay as a measure of G protein activation does indeed have the advantage that the G alpha subunit is unmodified although the beta/gamma subunits are fused to a fluorescent protein. By usign this appraoch the authors ahve confirmed the conclusions of previous papers. The results obtained by Zanetti and colleagues match those obtained by Lane et al in 2008 showing that the D3R selectively couples to G alpha o over G alpha i1-3. The approach by Lane et al used 35S GTPgS binding using the pertussis toxin resistant variants (C to I) of the G protein alpha subunits, and like this current paper showed that this coupling specificity was preserved for all agonists tested. The elevated levels 35SGTPgS binding observed with Gai2 observed in this paper do not, as the authors of the present paper state, reflect receptor mediated basal signalling but rather different levels of G protein expression as can be seen from the various western blots. Consistent with the data from Zanetti et al. agonist stimulated G protein activation was only observed when Galphao was present leading to a consistent conclusion that D3R couples selectively to Galphao without the 'complications' in interpretation suggested by the present authors. Similarly, while the C to I mutation in the alpha helix C-terminus of the G alpha subunits is a modification, the same modification was made to each Gi/o subunit and this did not alter the G protein specificity of either D2R or D3R as compared to the present paper. Similarly, while they used different BRET constructs, the paper by Monnich and coworkers find essentially the same pattern of D3R coupling selectivity. A more useful discussion might be a focus on the other papers which reveal a different pattern of selectivity and the approach taken in these. Are they measuring different aspects of G protein coupling and can this explain the difference? Do we get any insight into the steps of G protein selectivity in this manner? Can the authors rule out the possibility that in a different cellular background (with different accessory proteins) the D3R might show a distinct G protein coupling profile compared to this one obtained in a HEK background?

Structural basis of selectivity: The authors reveal that ICL2 appears to be a determinant of G protein selectivity for D3R, although their data also shows that ICL3 also likely plays some role. The authors then go on the highlight some specific residue differences within ICL2 that might play key roles in this specificity. It's not clear why the authors did not take this further and investigate this with additional mutants. This additional information would strengthen the paper.

While the authors do investigate the determinants of selectivity for Gao over Gai they do not do the same thing for galphaZ, this would be a good addition to the paper with experiments similar to those shown in figure 4. How do the various D2/D3 chimeras couple to Galpha Z?

The slice experiments with CAMPER mice are difficult to align with the previous data in the paper. It is not clear to what extent D2R or D3R are contributing to each response. Suminirole is not very selective so would likely be acting through both receptors at a concentration of 100 nM and the response would likely be a mix of the two receptor signals. ML-417 is D3R-selective but can activate the D2R albeit with low potency. ML-417 in these slice experiments gives a very small signal and causes an increase in cAMP which is difficult to reconcile with the authors results in Figure 3 which shows D3R activation of Gz and Go to cause a decrease in cAMP. Which of Gao/z is contributing to this response in figure 7 and what is the evidence for this? Which G proteins are expressed in these cells? The D2R can also couple efficiently to both Galphao and GalphaZ (and indeed displays preference for them based on agonist potencies) so how can the apparently opposite effects of a D2R-selective and a D3R-selective agonist be reconciled simply through G protein coupling? To me this would argue, that if indeed these are D2 vs D3 signals then there is an additional level of complexity here that the authors have not addressed. I think this needs to be addressed with further experiments. The judicious use of selective D3R and D2R antagonists at appropriate concentrations might be useful here.

The phrase ‘unexpected coupling profile of D3R’ (line 225) is odd. This coupling profile has been seen before as described above and in their paper.

The interpretation of sumanirole having slow off-rate kinetics does not seem correct to me, sumanirole and isoprotenerol are acting at distinct receptors so one would not expect isoprotenerol to compete with or block the action of sumanirole. Rather the sustained effect of sumanirole to negate the effect of isopretenerol reflects the ongoing activation of Gai/o/z subunits.

The authors refer to the G protein coupling selectivity as ‘bias’. Against the background of biased agonism, I think that this is unnecessarily confusing. I think stating it as G protein coupling selectivity is a clearer description of this phenomenon.

Reviewer #2

(Remarks to the Author)
Zanetti et al present an in-depth study around the signaling of the D3 dopamine receptor subtype and its ability to couple to G proteins. Coupling of the D3 has long been more difficult to understand compared to the D2 and D4 subtypes and this manuscript finally sets them side by side in the same selective functional assay. This is an important question, the experiments are well thought out and very complete (including convincing rescue and over-competition experiments) and define the signaling profile of the D3R. I have only minor concerns as outlined below.

Enthusiasm is only slightly tempered (as highlighted in the discussion) that these questions have been previously addressed by others; particularly in the Lane (2008) paper referenced which came to similar conclusions. This paper is however not as complete or clean, and the field was far from settled given the structure data and other conflicting papers.

There are several instances in the literature of evidence that the manuscript data appear to conflict; however, the authors do a solid job of explaining how these other studies may have come to a different conclusion. The most difficult to address being the cryo-EM structure of D3R-Gi complexes. These should be slightly further clarified that in a system of purified proteins without other interactors it may be possible for the two proteins to interact as is seen in the structure.

In Figure 7, treatment with ML417 paradoxically raises the levels of cAMP in this preparation. While the proposed description of the mechanism (AC sensitization by Gbetagamma) is intriguing and I think very interesting in this native system, because this action is so important to the field it would be worth including alternative explanations such as ML417 having some small activity on a different receptor (unlikely given its published selectivity profile) or antagonist response on the D2R (it’s very weak partial agonist response on the D2R could act as a functional antagonist response in an ex vivo prep if there is underlying basal ‘tone’.

It would be intriguing to look at beta-arrestin signaling in your system and with your chimeric receptors to complete the D3R pharmacology profile, but is beyond the scope of this manuscript.

Reviewer #3

(Remarks to the Author)
In this manuscript, Zanetti et al. investigated the G protein subtype preference underlying dopamine D3 receptor activation. It is quite interesting that, within the D2-like family (D2, D3, and D4), only D3 does not couple to Gi1-i3 subtypes. They further looked into the structural mechanism and identified that the unique sequence in ICL2 of D3R may play an important role. They also examined how the unique coupling profile of D3R affects its signaling at the neuronal level. This is an interesting study with information useful for general researchers. I have some questions regarding the interpretation of data and conclusions that I hope will help improve the manuscript.
1. The nanoBRET assay used here fundamentally reports Gβ1γ2 availability to bind the GRK3CT sensor, yet for Gα

subtypes they may have preferred pairs, as shown in the TRUPATH paper (Olsen et al., NCB 2020). This raises the question: Different Gβ and Gγ isoforms can alter heterotrimer assembly, basal dissociation, and effector engagement. It is therefore unclear whether the observed coupling hierarchy would remain unchanged if a different Gβγ pair were used. For example, in the TRUPATH paper, they show that Gi2 prefers the Gβ3γ8 pair; can they compare this with Gβ1γ2?
2. In Figure 3D, it is unclear why Gi2 at high concentration doesn't affect GoA signaling at D2R. In Figure 2, D2-GoA vs. d2-Gi2, quinpirole behaves very differently and is much weaker at Gi2. It would be expected to see a BRET ratio change if Gi2 dominates the Gβ1γ2 coupling and signaling with high Gi2 present. Basically, the Gi2 should act as an antagonist against GoA as their potency and efficacy are very different.
3. Figure 6 proposes that steric clashes and/or electrostatic repulsion (e.g., involving D350 in Gαi2 and T142 in D3R ICL2) explain D3R's inability to productively engage Gi proteins. While the structural rationale is reasonable, this mechanistic interpretation would be strengthened by targeted point-mutagenesis experiments.
3. Although sumanirole and ML417 show distinct receptor preferences in nanoBRET assays in HEK293 cells, it remains unclear whether the single concentration used in CA1 slices (100 nM) lies within a selective window in this physiological preparation; higher concentrations can be tested. Regarding ML417's unique effect, the authors showed that ML417 is very selective between D2 and D3. Are there any published data or can they confirm ML417 does not hit other Gs-coupled receptors and contribute to the increase in cAMP levels, such as closely related D1R and D5R (both Gs coupled)?
4. In Figure 7D, if ML417's effect is through hijacking the Gβγ, then they can do the same experiment but using forskolin to directly activate the ACs, and see if they can see the cAMP inhibition by ML417 in neurons.

Minor comments:
What are the values in Figure 1C, Emax (e.g., 0, 0.03,… 0.15) as they seem not match the deltaBRET ratio in Figure 1A?
Adding an annotation within the panel to indicate which radar plots correspond to each receptor would help prevent confusion.
Lines 140-141 may need rewording as ML-417 activates Gz at D3R.
Standardize naming throughout (e.g., consistently use GoA or GαoA rather than mixing GoA/GαoA).
It is helpful to provide tables summarizing values (e.g., pEC50 and Emax for D2R/D3R/D4R across Gi/o/z subtypes).
Please add a color key bar for the electrostatic surface rendering and clarify in the legend what the colors represent in Fig. 6A.

Reviewer #4

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

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have addressed my previously indicated concerns and I feel this revised version adds significant clarity. The experiments are well thought out and described. The impact of the findings are only slightly tempered by prior data. The reason for the published structural data of D3 binding to Gi, but not well signaling, remains a bit unclear. I suggest publication of the manuscript.

Reviewer #3

(Remarks to the Author)
The authors have addressed my questions.

Reviewer #4

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

Reviewer #1 (Remarks to the Author):

The paper focuses on the G protein coupling specificity of the D2-like dopamine receptors and most specifically the dopamine D3 receptor. They make use of a BRET beta gamma release assay to show that the D3R couples selectively to Galpha o and z whereas the D2R and D4R couples to all Gi/o/z members. They use D2/D3 chimeric receptors to reveal that ICL2 residues are a determinant of this different selectivity as are residues within the C-terminal helix of Galpha o/i G proteins. They make use of the CAMPER mice system to measure the effect of D2R and D3R selective agonists on intracellular cAMP levels in CA1 hippocampal neurons. In general, the experiments are well performed and the data is clear. However, the coupling specificity of the D3R has been studied before. As highlighted by the authors, papers almost 20 years ago described the same specificity for G alpha O and G alpha Z. The efforts to describe the structural basis of this specificity reveal new information around the role of ICL2 using receptor chimeras. The authors rationalise these observations against the emerging structural data but I feel it would have been a nice addition for the authors to take this one step further and experimentally investigate some of the hypotheses generated here. Finally, it's difficult to align the data from the measurement of cAMP in slice with the rest of the paper. As presented I don't think you can conclude that the responses driven by the different agonists are simply down to the differential coupling selectivity of the D2 and D3 receptors. Specific comments are as follows:

D3R coupling specificity: The coupling specificity of the D3R for GalphaO and GalphaZ have been investigated and identified before (10.1023/a:1006988603199, 10.1021/acsptsci.3c00339, doi:10.1124/jpet.107.134296). The use of the beta gamma release BRET assay as a measure of G protein activation does indeed have the advantage that the G alpha subunit is unmodified although the beta/gamma subunits are fused to a fluorescent protein. By usign this appraoch the authors ahve confirmed the conclusions of previous papers. The results obtained by Zanetti and colleagues match those obtained by Lane et al in 2008 showing that the D3R selectively couples to G alpha o over G alpha i1-3. The approach by Lane et al used 35S GTPgS binding using the pertussis toxin resistant variants (C to I) of the G protein alpha subunits, and like this current paper showed that this coupling specificity was preserved for all agonists tested. The elevated levels 35SGTPgS binding observed with Gai2 observed in this paper do not, as the authors of the present paper state, reflect receptor mediated basal signalling but rather different levels of G protein expression as can be seen from the various western blots. Consistent with the data from Zanetti et al. agonist stimulated G protein activation was only observed when Galphao was present leading to a consistent conclusion that D3R couples selectively to Galphao without the 'complications' in interpretation suggested by the present authors. Similarly, while the C to I mutation in the alpha helix C-terminus of the G alpha subunits is a modification, the same modification was made to each Gi/o subunit and this did not alter the G protein specificity of either D2R or D3R as compared to the present paper. Similarly, while they used different BRET constructs, the paper by Monnich and coworkers find essentially the same pattern of D3R coupling selectivity. A more useful discussion might be a focus on the other papers which reveal a different pattern of selectivity and the approach taken in these. Are they measuring different aspects of G protein coupling and can this explain the difference? Do we get any insight into the steps of G protein selectivity in this manner?

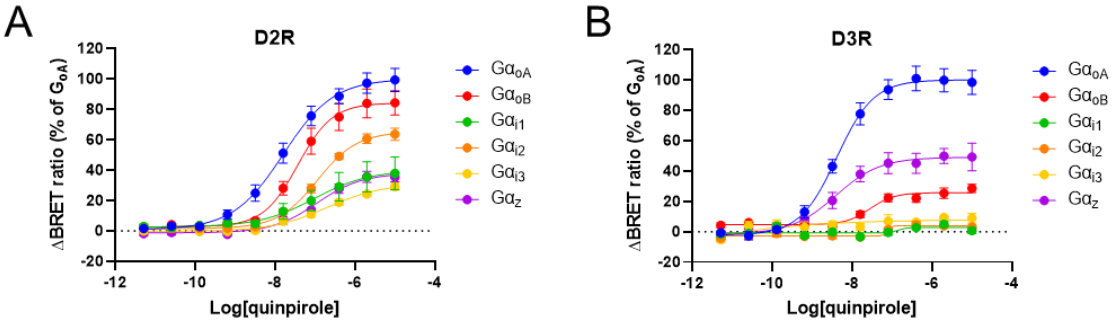
We appreciate Reviewer 1's thorough analysis of our manuscript in the context of the existing literature. In response to their suggestions, we have revised the discussion to reduce emphasis on studies reporting conclusions similar to ours and instead placed greater focus on the studies describing a distinct coupling profile for D3R.

The idea that different assays capture distinct aspects of G protein coupling is thought-provoking. TRUPATH and related approaches detect an increase in the distance between Gα and Gβγ subunits upon GPCR activation, whereas our G protein nanoBRET assay measures the release of Gβγ, enabling its interaction with GRK3ct-Nluc at the membrane. One possible explanation is that, in the presence of $G\alpha_i$, D3R fails to efficiently release Gβγ, resulting in no detectable signal in our nanoBRET assay, while still undergoing a conformational change sufficient to reduce the BRET signal in TRUPATH. Although intriguing, this hypothesis remains speculative, as such behavior has not been previously reported and is not supported by existing data on D3R. Indeed, different groups using TRUPATH and similar BRET-based configurations have reported inconsistent findings.

Can the authors rule out the possibility that in a different cellular background (with different accessory proteins) the D3R might show a distinct G protein coupling profile compared to this one obtained in a HEK background?

The effect of the cellular background on the interpretation of the coupling results is a very important point. In the revised manuscript, we addressed this issue by obtaining a concentration-response curve to quinpirole applied to Chinese Hamster Ovary (CHO) cells transfected with D2R or D3R and each $G_{i/o/z}$ protein. Similar to our observations in HEK293 cells, we detected activation of all six $G\alpha_{i/o/z}$ proteins by D2R, while D3R only activated $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$ (new **Supplementary Figure 3**).

Supplementary Figure 3

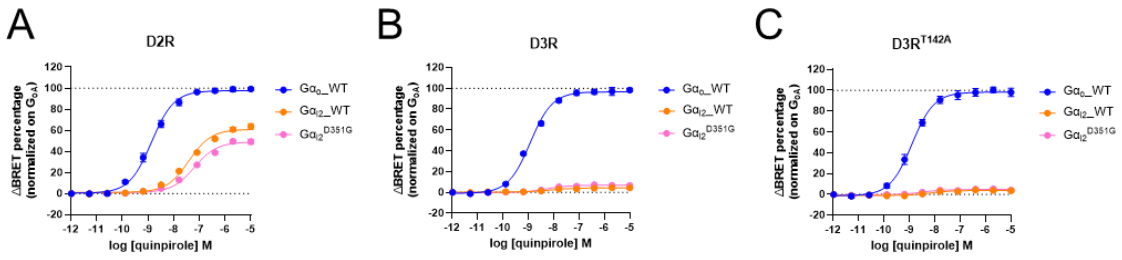


Supplementary Figure 3. D2R and D3R response to quinpirole in CHO cells. Concentration–response curves were obtained using the G protein nanoBRET assay in CHO cells transfected with D2R (**A**) or D3R (**B**) and indicated Gα proteins. Cells were stimulated with increasing concentrations of quinpirole. Data are shown as mean ± SEM of values normalized to the $G\alpha_{oA}$ signal. N=5 independent replicates.

Structural basis of selectivity: The authors reveal that ICL2 appears to be a determinant of G protein selectivity for D3R, although their data also shows that ICL3 also likely plays some role. The authors then go on the highlight some specific residue differences within ICL2 that might play key roles in this specificity. It's not clear why the authors did not take this further and investigate this with additional mutants. This additional information would strengthen the paper.

We appreciate the Reviewer's suggestion, and we have now included the proposed experiment. We undertook site-directed mutagenesis to generate mammalian expression clones encoding point-mutated versions of D3R ($D3R^{T142A}$) and $G\alpha_{i2}$ ($G\alpha_{i2}^{D351G}$). These mutants were evaluated using the G protein nanoBRET assay (new **Supplementary Figure 4**). Introduction of a glycine at position 351 of $G\alpha_{i2}$, the residue that is found at the corresponding position in $G\alpha_{o}$, resulted in no change in signal upon D2R activation (new **Supplementary Figure 4A**), or D3R activation (new **Supplementary Figure 4B**). Likewise, the $D3R^{T142A}$ mutant failed to produce D3R-mediated activation of $G\alpha_{i2}$ (new **Supplementary Figure 4C**). Together, these results indicate that the receptor:G protein interaction interface requires contributions from multiple sites to support stable complex formation and to promote GDP-to-GTP exchange on $G\alpha_{i2}$ and heterotrimer dissociation efficiently. These results are described in the revised manuscript (**lines 233-242**).

Supplementary Figure 4



Supplementary Figure 4. Mutating $D3R^{T142A}$ and $G\alpha_{i2}^{D351G}$ has no effect on G-protein coupling. (**A-C**) Concentration-response curves measured using the G protein nanoBRET assay for D2R (**A**), D3R (**B**) and $D3R^{T142A}$ (**C**) co-transfected with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{i2}^{D351G}$. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=4 independent replicates.

While the authors do investigate the determinants of selectivity for Gao over Gai they do not do the same thing for galphaZ, this would be a good addition to the paper with experiments similar to those shown in figure 4. How do the various D2/D3 chimeras couple to Galpha Z?

We believe the Reviewer is referring to Figure 5, where we tested D2R and D3R chimeras (ICL2 and ICL3 swaps) with $G\alpha_{o}$ and $G\alpha_{i2}$, but not with $G\alpha_{z}$. We now provide additional experiments evaluating the coupling of each receptor and $G\alpha_{z}$ (new **Figure 5B**). Our results demonstrate that the coupling to $G\alpha_{z}$ is not altered by swapping the ICL2 and ICL3 between D2R and D3R.

Figure 5

B

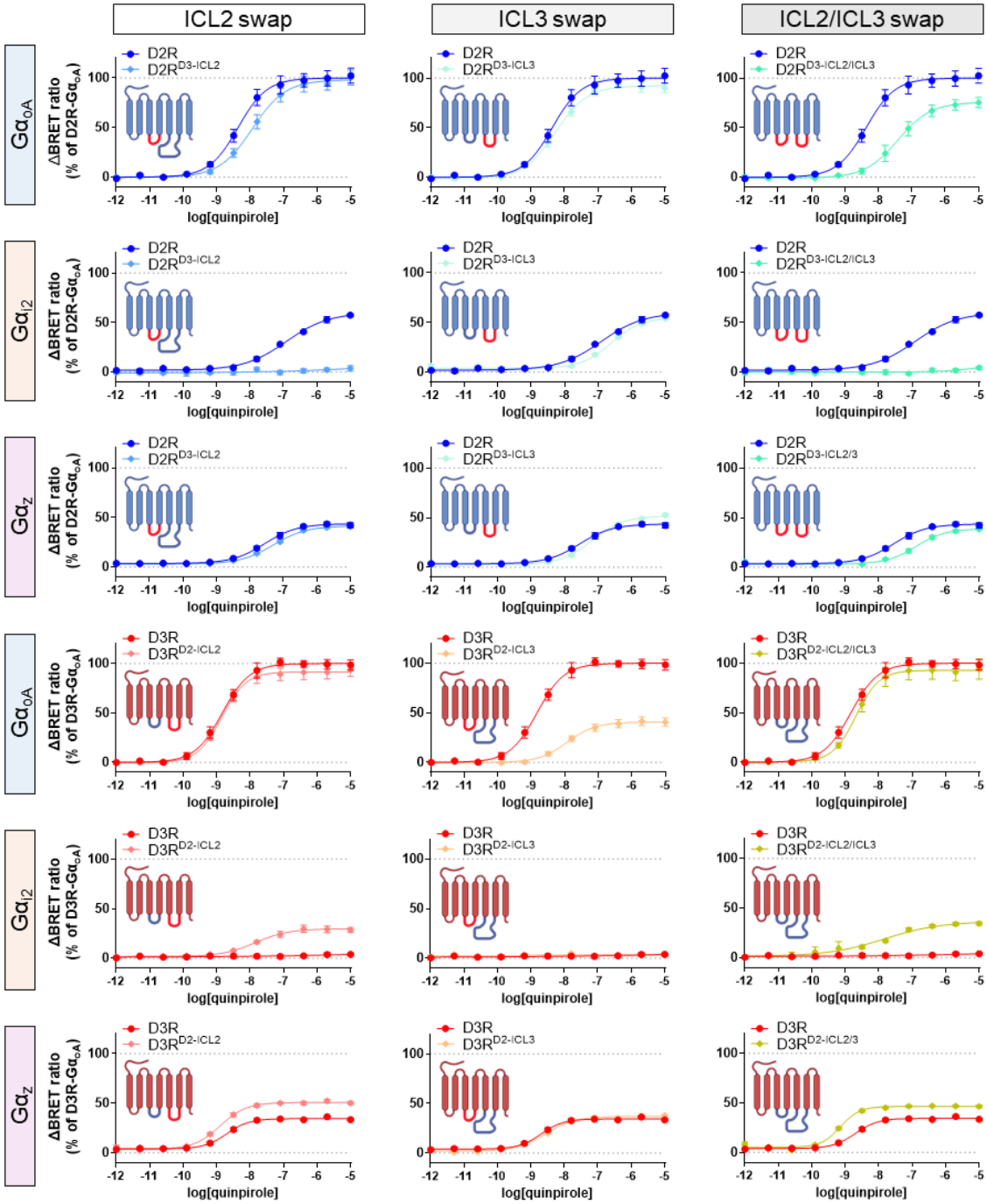


Figure 5.

(**B**) Concentration–response curves obtained using the G protein nanoBRET assay for GαoA and $G\alpha_{i2}$ activation by wild-type and loop-swapped D2R and D3R constructs. Schematic representations of the used chimeric receptor constructs in which ICL2 and/or ICL3 of D2R and D3R were swapped are inserted in each graph. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Reference curves for wild-type D2R and D3R with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{z}$ are reproduced multiple times to facilitate comparisons. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=5 independent replicates.

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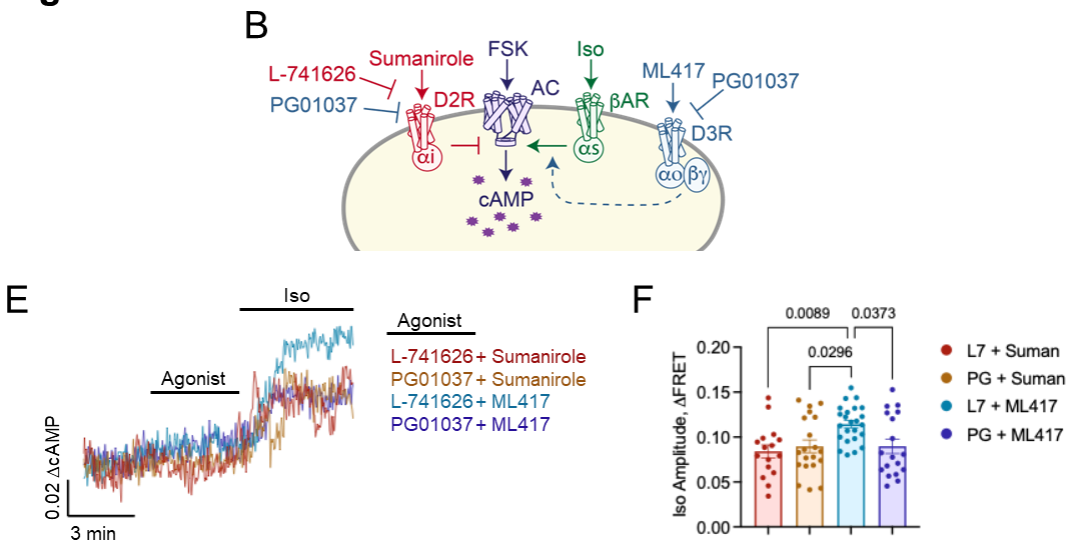
We appreciate this insightful perspective. Upon testing various antagonists, we found L-741626 exhibited selectivity toward D2R over D3R. Unfortunately, we were not able to validate a D3R selective antagonist, relative to D2R, in our hands (new **Supplementary Figure 5**). We found PG01037 was slightly more potent at D3R but nonetheless strongly inhibited both D2R and D3R. The brain slice cAMP imaging experiments were repeated with co-application of these antagonists (new **Figure 7 E-F**). We found both L-741626 (10 μM) and PG01037 (10 μM) blocked Sumanirole-

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

mediated cAMP inhibition, suggesting Sumanirole operates through D2R-$G\alpha_i$ in CA1 neurons. Moreover, Iso-mediated cAMP synthesis was not impacted by either antagonist. We next observed that L-741626 had no effect on ML417-mediated sensitization of Iso-induced cAMP production. On the other hand, PG01037 blocked the ML417's sensitization of cAMP following Iso application. We interpret the results such that PG01037 blocks ML417 on D3R. Meanwhile, L-741626 blocks D2R, which is not robustly activated by ML417 at 100 nM in CA1 neurons. These data support the notion that ML417 acts through D3R-$G\alpha_o$-$G\beta\gamma$ to adjust cAMP in CA1 neurons.

The phrase 'unexpected coupling profile of D3R' (line 225) is odd. This coupling profile has been seen before as described above and in their paper.

We adjusted the wording and changed "unexpected" to "unusual" to highlight how this coupling profile is unique.

The interpretation of sumanirole having slow off-rate kinetics does not seem correct to me, sumanirole and isoprotenerol are acting at distinct receptors so one would not expect isoprotenerol to compete with or block the action of sumanirole. Rather the sustained effect of sumanirole to negate the effect of isoprotenerol reflects the ongoing activation of Gai/o/z subunits.

We appreciate the Reviewer's comment, and we removed the reference to the possible slow off-rate kinetics of sumanirole from the text.

The authors refer to the G protein coupling selectivity as 'bias'. Against the background of biased agonism, I think that this is unnecessarily confusing. I think stating it as G protein coupling selectivity is a clearer description of this phenomenon.

We agree with the Reviewer that traditionally the term 'bias' has been applied to studies aiming at discerning the preferential activation of β-arrestin vs G proteins. However, the use of 'bias' has recently been extended to include the entirety of the GPCR signaling transducers as outlined here:

A community Biased Signaling Atlas (PMID: 36973443)

"*For GPCRs, biased signaling has classically been studied for the four G protein families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) and the arrestin family (Fig. 1). It additionally could extend to GPCR kinases (GRKs), which are also transducers — that is, receptor-binding effector proteins that phosphorylate the C termini of activated receptors to promote arrestin recruitment. Recently, however,* ***the scope of the term has been greatly expanded to include bias among any of the 27 specific transducer subtypes — 16 G proteins (with separately signaling α and βγ subunits), 7 GRKs and 4 arrestin proteins — which can differ in their functional outcome****."*

Community guidelines for GPCR ligand bias: IUPHAR review 32 (PMID: 35106752)

" '*Biased signalling' is the ligand-dependent activation of certain pathways over others, and can lead to a 'functionally selective' response. 'Biased signalling' became generally accepted after evidence*

accumulated that the rank order of ligands by potency could be different for different pathways engaged by a single receptor (Roth & Chuang, 1987; Spengler et al., 1993) or inversion of the ligand modality (Azzi et al., 2003; Baker, Hall, & Hill, 2003). ***The most frequently studied pathway-bias has been that between G proteins and arrestins, while more recent studies have compared G protein families and even subtypes belonging to the same G protein family****."*

According to these guidelines, we refer to the ability of the Dopamine D3 receptor to selectively engage $G_{o/z}$ but not G_i as signaling bias.

Reviewer #2 (Remarks to the Author):

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Enthusiasm is only slightly tempered (as highlighted in the discussion) that these questions have been previously addressed by others; particularly in the Lane (2008) paper referenced which came to similar conclusions. This paper is however not as complete or clean, and the field was far from settled given the structure data and other conflicting papers.

We agree with the Reviewer and acknowledge the seminal findings reported by Lane et al. (2008). Given the substantial body of literature published since 2008 that has contributed to ongoing confusion regarding the G protein coupling profile of D3R, we believe that our study provides the conclusive evidence necessary to resolve this debate.

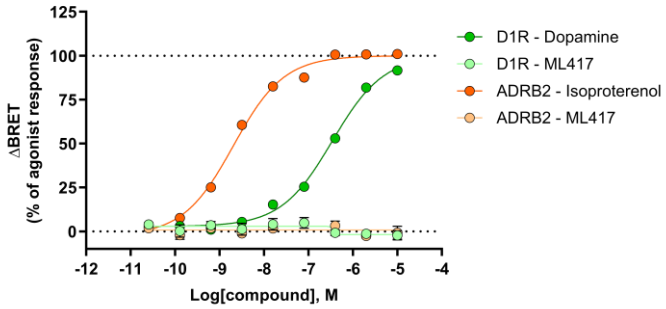
There are several instances in the literature of evidence that the manuscript data appear to conflict; however, the authors do a solid job of explaining how these other studies may have come to a different conclusion. The most difficult to address being the cryo-EM structure of D3R-Gi complexes. These should be slightly further clarified that in a system of purified proteins without other interactors it may be possible for the two proteins to interact as is seen in the structure.

We thank the Reviewer for their suggestion. We agree that interactions observed in a purified protein system may not be observed in the presence of other interactors. Additionally, purification of complexes from insect cells and stabilization using antibody fragments might facilitate the generation of stable complexes that would otherwise not be observed in mammalian systems. We have now clarified this better in the revised manuscript (**lines 226-232**).

In Figure 7, treatment with ML417 paradoxically raises the levels of cAMP in this preparation. While the proposed description of the mechanism (AC sensitization by Gbetagamma) is intriguing and I think very interesting in this native system, because this action is so important to the field it would be worth including alternative explanations such as ML417 having some small activity on a different receptor (unlikely given its published selectivity profile) or antagonist response on the D2R (it's very weak partial agonist response on the D2R could act as a functional antagonist response in an ex vivo prep if there is underlying basal 'tone'.

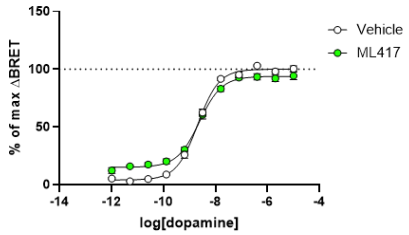
ML417 was originally discovered and pharmacologically characterized in the laboratory of Dr. David R. Sibley at the NINDS (PMID: 32342685). That study reported a comprehensive functional profiling of ML417 across 168 known GPCRs, including the Gs-coupled dopamine D1R and D5R, and demonstrated a high degree of selectivity for D3R. This reference is now included in our manuscript. In addition, to independently confirm selectivity, we tested ML417 for activity at other monoaminergic

receptors, specifically dopamine D1R and the β2-adrenergic receptor (ADRB2). As shown below, ML417 failed to activate either receptor even at high concentrations, further supporting the strong D3R selectivity reported in the literature.



Following the Reviewer's suggestion, we now included a comment on ML417 possibly acting as a weak partial agonist at D2R to reduce the tonic inhibition of adenylyl cyclase (**lines 277-279**). However, we obtained a concentration-response of dopamine at D2R in the presence of 100 nM ML417, the same concentration used in the experiments reported in Figure 7 (new **Supplementary Figure 6**). We found that, again, ML417 acts as a partial D2R agonist at this concentration but fails to inhibit dopamine stimulation of D2R. Therefore, we believe this explanation of the results in **Figure 7** is unlikely.

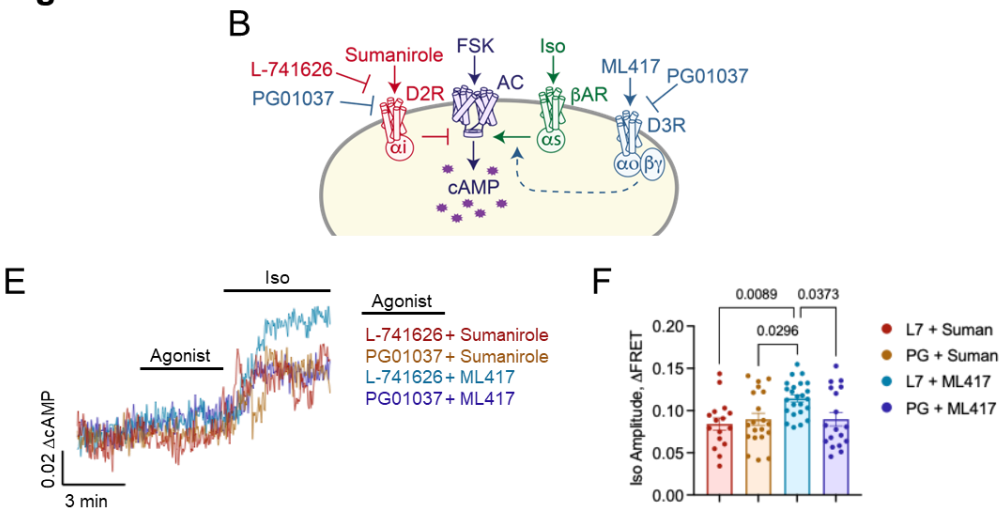
Supplementary Figure 6



Supplementary Figure 6. Weak partial agonism of ML417 at the D2R does not inhibit dopamine responses. Concentration-response curves measured using the G protein nanoBRET assay for D2R in wild-type HEK293T cells co-transfected with $G\alpha_{oA}$. Increasing concentrations of dopamine were applied in the presence of vehicle (white) or 100 nM ML417 (green). Data shown as mean ± SEM. N=5 independent replicates.

Finally, we have now performed experiments with an antagonist selective for D2R and also an antagonist targeting both D2R and D3R. Unfortunately, we were not able to validate a D3R selective antagonist (new **Supplementary Figure 5**). The D2R antagonist (L-741626) had no effect on ML417's impact on cAMP signaling, whereas the D2R/D3R antagonist (PG01037) indeed blunted ML417's cAMP sensitization (new **Figure 7E-F**). These data provide evidence that ML417 does not strongly signal through D2R in CA1 neurons, but rather the observed effects are driven through the D3R.

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

It would be intriguing to look at beta-arrestin signaling in your system and with your chimeric receptors to complete the D3R pharmacology profile, but is beyond the scope of this manuscript.

In the revised manuscript, we introduced an analysis of β-arrestin2 recruitment to D2R, D3R, and D4R measured using a NanoBiT complementation assay following stimulation with quinpirole and dopamine (new **Figure 3D-E**). The experimental methods and corresponding results are now fully described in the revised manuscript. While these experiments completed the pharmacological characterization of D2R, D3R, and D4R, they did not reveal a D3R-specific response that would justify further investigation using the chimeric receptors.

Figure 3

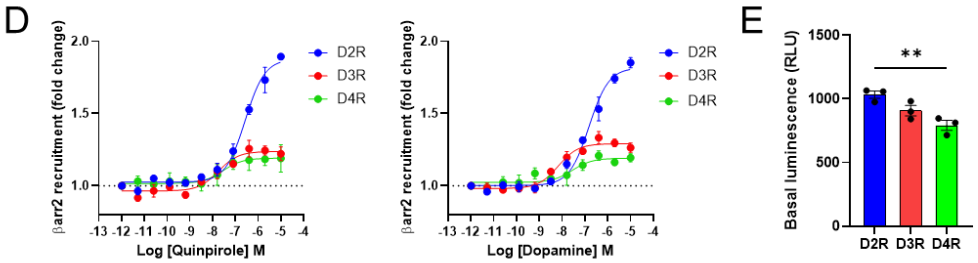


Figure 3.
(**D**) Concentration-response curves illustrating β-arrestin2 recruitment to D2R, D3R, and D4R following stimulation with quinpirole and dopamine, normalized as fold change, with response observed at the lowest concentration of ligand treated as 1. (**E**). Basal luminescence observed following co-transfection of receptor-SmBiT and βarr2-LgBiT. Data are shown as mean ± SEM. N=3 independent replicates. **P < 0.01, determined by one-way ANOVA and Tukey post hoc test.

Reviewer #3 (Remarks to the Author):

In this manuscript, Zanetti et al. investigated the G protein subtype preference underlying dopamine D3 receptor activation. It is quite interesting that, within the D2-like family (D2, D3, and D4), only D3 does not couple to Gi1-i3 subtypes. They further looked into the structural mechanism and identified that the unique sequence in ICL2 of D3R may play an important role. They also examined how the unique coupling profile of D3R affects its signaling at the neuronal level. This is an interesting study with information useful for general researchers. I have some questions regarding the interpretation of data and conclusions that I hope will help improve the manuscript.

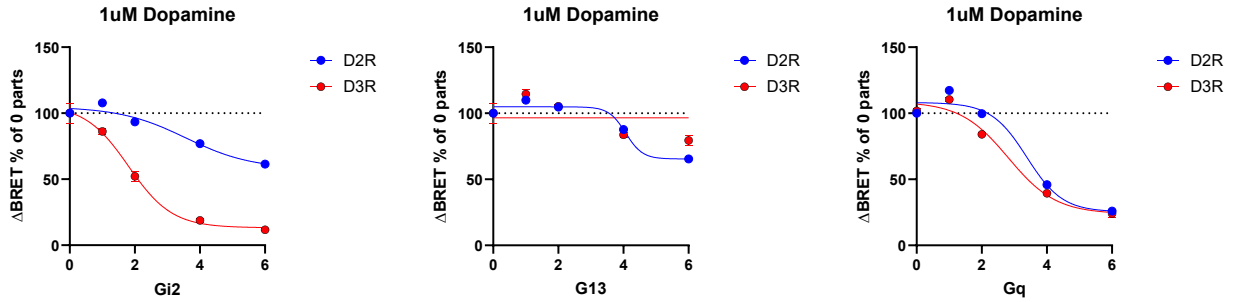
1. The nanoBRET assay used here fundamentally reports Gβ1γ2 availability to bind the GRK3CT sensor, yet for Gα subtypes they may have preferred pairs, as shown in the TRUPATH paper (Olsen et al., NCB 2020). This raises the question: Different Gβ and Gγ isoforms can alter heterotrimer assembly, basal dissociation, and effector engagement. It is therefore unclear whether the observed coupling hierarchy would remain unchanged if a different Gβγ pair were used. For example, in the TRUPATH paper, they show that Gi2 prefers the Gβ3γ8 pair; can they compare this with Gβ1γ2?

We thank the Reviewer for their observations on the possible role of Gβγ in defining the readout of GPCR cell-based assays. In designing the TRUPATH systems, Dr. Bryan Roth and colleagues observed that a better signal was obtained by pairing defined Gβγ heterodimers with individual Gα proteins. We agree that, in our BRET assay, the maximal effect measured may be influenced by the use of a specific Gβγ pair (in our case, $G\beta_1\gamma_2$). In support of our conclusions:

1. We believe that being consistent in the use of the same Gβγ pair enhances our ability to detect meaningful differences. We can clearly observe the signal generated by D2R and D4R, using $G\alpha_{i2}\beta_1\gamma_2$, suggesting that the heterotrimer dissociation is not inhibited in this combination. This has been extensively observed for a large number of receptors and Gα proteins by us (e.g., PMID: 40944639, 40568800, 40563241, 37567783, 33784795) and others (e.g., PMID: 37742189, 33667409, 26628681, 38412860, 19258039).
2. TRUPATH experiments performed in the Roth lab using the $G\beta_3\gamma_8$ combination also showed a lack of coupling to D3R (see figure 3.4 of Olsen, R. H. J. *Development of Next-generation G Protein Biosensors for Deconvoluting 529 the Complexity of GPCR Signaling*, The University of North Carolina at Chapel Hill, 530 (2019)).
3. In "*Diversity of the Gβγ complexes defines spatial and temporal bias of GPCR signaling*" by Masuho et al. (PMID: 33667409), the same BRET-based assay we used in our paper we applied to explore D2R activation across all possible Gβγ combinations. As shown in Figure 1E of that study, altering the Gβγ pairing produced only minor differences in signal amplitude in the G protein nanoBRET assay, consistent with the approach used in our work.

2. In Figure 3D, it is unclear why Gi2 at high concentration doesn't affect GoA signaling at D2R. In Figure 2, D2-GoA vs. d2-Gi2, quinpirole behaves very differently and is much weaker at Gi2. It would be expected to see a BRET ratio change if Gi2 dominates the Gβ1γ2 coupling and signaling with high Gi2 present. Basically, the Gi2 should act as an antagonist against GoA as their potency and efficacy are very different.

This is a particularly interesting and important observation, and one that also initially surprised us. The original experiment was performed in the background of global G protein KO cells. We speculated that this could affect the overall signaling measured and not be fully comparable to what was reported in **Figure 2**, where the background was wild-type HEK293T cells. We now performed additional experiments in the wild-type HEK293T cells (see below). We also included additional controls in which we co-transfected $G\alpha_{13}$ or $G\alpha_{q}$, Gα subunits that are not activated by either D2R or D3R. We expected that increasing the expression of a non-activatable Gα protein ($G\alpha_{i2}$ for D3R or $G\alpha_{13}$/$G\alpha_{q}$ for both D2R and D3R) would lead to a signal that originates exclusively from the 1:12 fraction of transfected $G\alpha_{o}$. However, the results of this set of experiments indicate that even the presence of a high amount of non-activatable Gα protein does not completely block the signal obtained by transfecting 1 part of the activatable $G\alpha_{o}$.



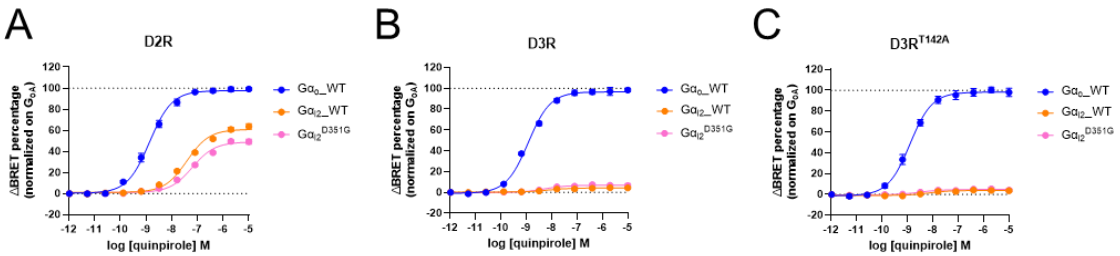
Based on our current data, we can only speculate on the molecular mechanisms underlying this phenomenon that could depend on possible roles of RGS proteins, possible unproductive interactions of receptors with G proteins (see PMID: 32817560), or variation in expression levels between Gα proteins. We acknowledge that this conceptual framework would require additional experimental validation, which we believe falls beyond the scope of the current manuscript. Considering that, at this stage, the results are difficult to interpret and do not contribute to the central message of the manuscript, we chose to remove this dataset (original **Figure 3D** and original **Supplementary Figure 3**) and prevent potential confusion.

3. Figure 6 proposes that steric clashes and/or electrostatic repulsion (e.g., involving D350 in Gαi2 and T142 in D3R ICL2) explain D3R's inability to productively engage Gi proteins. While the structural rationale is reasonable, this mechanistic interpretation would be strengthened by targeted point-mutagenesis experiments.

We appreciate the Reviewer's suggestion, and we have now included the proposed experiments in the revised manuscript. We undertook site-directed mutagenesis to generate mammalian expression clones encoding point-mutated versions of D3R ($D3R^{T142A}$) and $G\alpha_{i2}$ ($G\alpha_{i2}^{D351G}$). These mutants were evaluated using the G protein nanoBRET assay (new **Supplementary Figure 4**). Introduction of a glycine at position 351 of $G\alpha_{i2}$, the residue that is found at the corresponding position in $G\alpha_{o}$, resulted in no change in signal upon D2R activation (new **Supplementary Figure 4A**) or D3R activation (new **Supplementary Figure 4B**). Likewise, the $D3R^{T142A}$ mutant failed to produce D3R-mediated activation of $G\alpha_{i2}$ (new **Supplementary Figure 4C**). Together, these results indicate that the receptor:G protein interaction interface requires contributions from multiple sites to support stable

complex formation and to promote GDP-to-GTP exchange on $G\alpha_{i2}$ and heterotrimer dissociation efficiently. These results are described in the revised manuscript (**lines 233-242**).

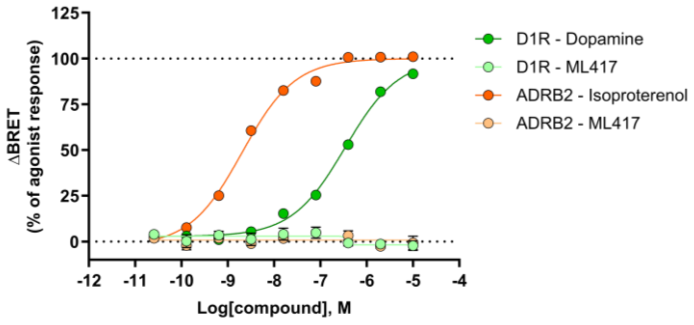
Supplementary Figure 4



Supplementary Figure 4. Mutating $D3R^{T142A}$ and $G\alpha_{i2}^{D351G}$ has no effect on G-protein coupling. (**A-C**) Concentration-response curves measured using the G protein nanoBRET assay for D2R (**A**), D3R (**B**) and $D3R^{T142A}$ (**C**) co-transfected with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{i2}^{D351G}$. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=4 independent replicates.

4. Although sumanirole and ML417 show distinct receptor preferences in nanoBRET assays in HEK293 cells, it remains unclear whether the single concentration used in CA1 slices (100 nM) lies within a selective window in this physiological preparation; higher concentrations can be tested. Regarding ML417's unique effect, the authors showed that ML417 is very selective between D2 and D3. Are there any published data or can they confirm ML417 does not hit other Gs-coupled receptors and contribute to the increase in cAMP levels, such as closely related D1R and D5R (both Gs coupled)?

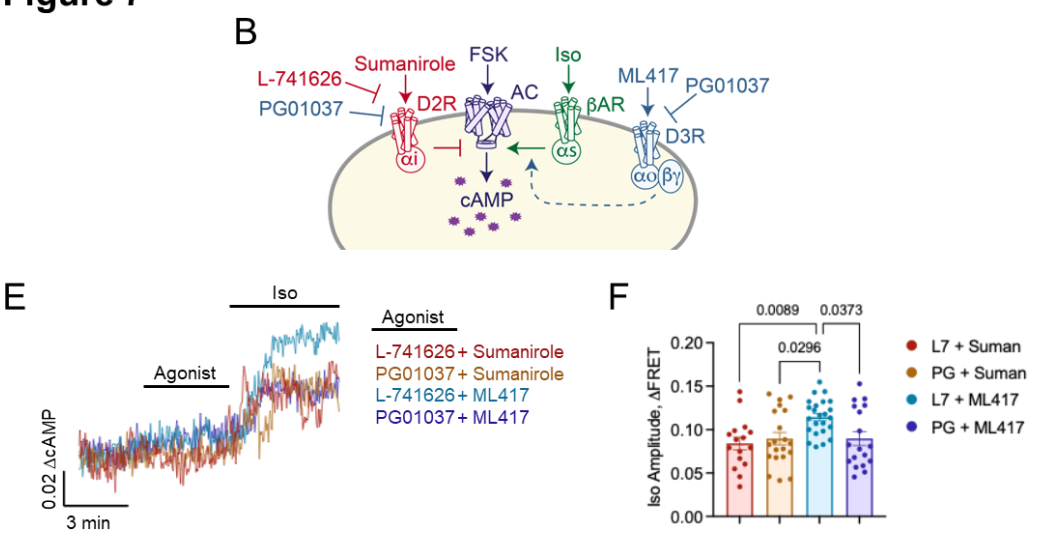
For the analysis of ML417 effects in brain slices, we used a concentration of 100 nM that was selected based on our concentration–response curves, indicating that this concentration selectively activates D3R without engaging D2R (less than 7% of dopamine-mediated activation). ML417 was originally discovered and pharmacologically characterized in the laboratory of Dr. David R. Sibley at the NINDS (PMID: 32342685). That study reported a comprehensive functional profiling of ML417 across 168 known GPCRs, including the Gs-coupled dopamine D1R and D5R, and demonstrated a high degree of selectivity for D3R. This reference is now included in our manuscript. In addition, to independently confirm selectivity, we tested ML417 for activity at other monoaminergic receptors, specifically dopamine D1R and the β2-adrenergic receptor (ADRB2). As shown below, ML417 failed to activate either receptor even at high concentrations, further supporting the strong D3R selectivity reported in the literature.



5. In Figure 7D, if ML417's effect is through hijacking the Gβγ, then they can do the same experiment but using forskolin to directly activate the ACs, and see if they can see the cAMP inhibition by ML417 in neurons.

We have repeated similar experiments, but with the inclusion of antagonists to select for D2R or D2R/D3R. Please see also responses to Reviewers #1 and #2. Briefly, we were unable to validate antagonists selective for D3R in our hands (new **Supplementary Figure 5**). Utilization of the selective D2R antagonist (L-741626) blocked Sumanirole inhibition of cAMP but had no effect on ML417. The D2R/D3R antagonist (PG01037) blocked both the Sumanirole and ML417 effects on cAMP (new **Figure 7E-F**). These data support the concept that ML417 acts through D3R-Gα_o-Gβγ to adjust cAMP in CA1 neurons. An important feature of the CAMPER sensor is the capability to detect bidirectional cAMP changes from baseline (*i.e.*, Gα_i-mediated inhibition), which was observed in the Sumanirole treatment in the original data. ML417 did not reduce baseline cAMP in these experiments, suggesting its primary mode of action is not through Gα_i.

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

Minor comments:

What are the values in Figure 1C, Emax (e.g., 0, 0.03,… 0.15) as they seem not match the deltaBRET ratio in Figure 1A? Adding an annotation within the panel to indicate which radar plots correspond to each receptor would help prevent confusion.

We apologize, as the representative kinetics trace reporting Gα_o activation in response to D3R stimulation in **Figure 1A** was indeed not representative of the signal we normally obtain in G protein

nanoBRET. We now include a representative kinetics trace that aligns with the results quantified in **Figures 1B** and **1C**. Additionally, receptors analyzed in **Figure 1C** are now indicated as suggested.

Lines 140-141 may need rewording as ML417 activates Gz at D3R.

We apologize for the confusion. The point we wanted to make is that ML417 activates D3R but not D2R, except for $G\alpha_o$, but only at higher concentrations. We added “by D2R” at the end of the sentence to improve clarity.

Standardize naming throughout (e.g., consistently use GoA or GαoA rather than mixing GoA/GαoA).

We normally use $G\alpha_{oA}$ when referring to the Gα subunit, and G_{oA} if we are referring to the heterotrimeric G protein. We understand that this is unnecessarily confusing, and we have now simplified this by using Gα throughout the manuscript, including the title.

It is helpful to provide tables summarizing values (e.g., pEC50 and Emax for D2R/D3R/D4R across Gi/o/z subtypes).

Tables summarizing pEC_{50} and E_{max} values are now provided (**Supplementary Data 1-3**).

Please add a color key bar for the electrostatic surface rendering and clarify in the legend what the colors represent in Fig. 6A.

We thank the Reviewer for their suggestion. We have now clarified in the legend what the colors of the electrostatic surface rendering represent and also included a scale bar in the revised manuscript.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

We asked other Reviewer to check the rebuttal and they are happy with the revisions made.

Reviewer comment:

I read in detail the critiques from reviewer 1 and the rebuttal. It would be my opinion that they fully addressed the relatively minor critiques and added additional experiments and editing to clarify findings. I would stand by my other comments that this could now be suitable for publication and I believe they thoroughly addressed reviewer #1 as well.

Reviewer #2 (Remarks to the Author):

The authors have addressed my previously indicated concerns and I feel this revised version adds significant clarity. The experiments are well thought out and described. The impact of the findings are only slightly tempered by prior data. The reason for the published structural data of D3 binding to Gi, but not well signaling, remains a bit unclear. I suggest publication of the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have addressed my questions.

Reviewer #4 (Remarks to the Author):

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

We thank all the Reviewers for their constructive feedback on our work that led to an improved final manuscript.

Reviewer #1 (Remarks to the Author):

The paper focuses on the G protein coupling specificity of the D2-like dopamine receptors and most specifically the dopamine D3 receptor. They make use of a BRET beta gamma release assay to show that the D3R couples selectively to Galpha o and z whereas the D2R and D4R couples to all Gi/o/z members. They use D2/D3 chimeric receptors to reveal that ICL2 residues are a determinant of this different selectivity as are residues within the C-terminal helix of Galpha o/i G proteins. They make use of the CAMPER mice system to measure the effect of D2R and D3R selective agonists on intracellular cAMP levels in CA1 hippocampal neurons. In general, the experiments are well performed and the data is clear. However, the coupling specificity of the D3R has been studied before. As highlighted by the authors, papers almost 20 years ago described the same specificity for G alpha O and G alpha Z. The efforts to describe the structural basis of this specificity reveal new information around the role of ICL2 using receptor chimeras. The authors rationalise these observations against the emerging structural data but I feel it would have been a nice addition for the authors to take this one step further and experimentally investigate some of the hypotheses generated here. Finally, it's difficult to align the data from the measurement of cAMP in slice with the rest of the paper. As presented I don't think you can conclude that the responses driven by the different agonists are simply down to the differential coupling selectivity of the D2 and D3 receptors. Specific comments are as follows:

D3R coupling specificity: The coupling specificity of the D3R for GalphaO and GalphaZ have been investigated and identified before (10.1023/a:1006988603199, 10.1021/acsptsci.3c00339, doi:10.1124/jpet.107.134296). The use of the beta gamma release BRET assay as a measure of G protein activation does indeed have the advantage that the G alpha subunit is unmodified although the beta/gamma subunits are fused to a fluorescent protein. By usign this appraoch the authors ahve confirmed the conclusions of previous papers. The results obtained by Zanetti and colleagues match those obtained by Lane et al in 2008 showing that the D3R selectively couples to G alpha o over G alpha i1-3. The approach by Lane et al used 35S GTPgS binding using the pertussis toxin resistant variants (C to I) of the G protein alpha subunits, and like this current paper showed that this coupling specificity was preserved for all agonists tested. The elevated levels 35SGTPgS binding observed with Gai2 observed in this paper do not, as the authors of the present paper state, reflect receptor mediated basal signalling but rather different levels of G protein expression as can be seen from the various western blots. Consistent with the data from Zanetti et al. agonist stimulated G protein activation was only observed when Galphao was present leading to a consistent conclusion that D3R couples selectively to Galphao without the 'complications' in interpretation suggested by the present authors. Similarly, while the C to I mutation in the alpha helix C-terminus of the G alpha subunits is a modification, the same modification was made to each Gi/o subunit and this did not alter the G protein specificity of either D2R or D3R as compared to the present paper. Similarly, while they used different BRET constructs, the paper by Monnich and coworkers find essentially the same pattern of D3R coupling selectivity. A more useful discussion might be a focus on the other papers which reveal a different pattern of selectivity and the approach taken in these. Are they measuring different aspects of G protein coupling and can this explain the difference? Do we get any insight into the steps of G protein selectivity in this manner?

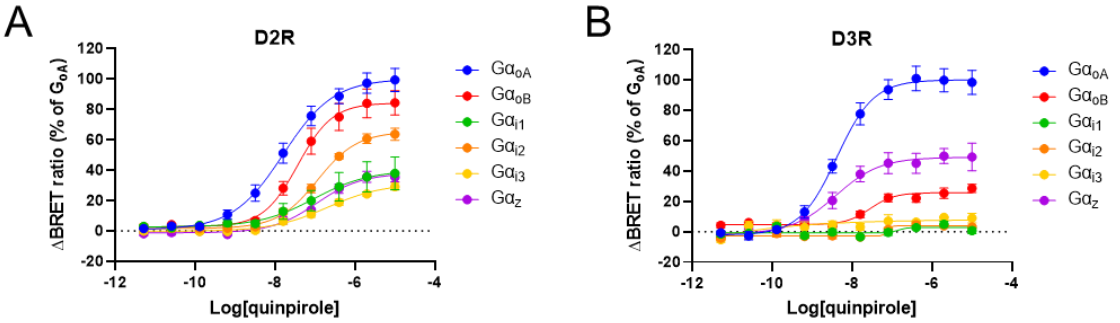
We appreciate Reviewer 1's thorough analysis of our manuscript in the context of the existing literature. In response to their suggestions, we have revised the discussion to reduce emphasis on studies reporting conclusions similar to ours and instead placed greater focus on the studies describing a distinct coupling profile for D3R.

The idea that different assays capture distinct aspects of G protein coupling is thought-provoking. TRUPATH and related approaches detect an increase in the distance between Gα and Gβγ subunits upon GPCR activation, whereas our G protein nanoBRET assay measures the release of Gβγ, enabling its interaction with GRK3ct-Nluc at the membrane. One possible explanation is that, in the presence of $G\alpha_i$, D3R fails to efficiently release Gβγ, resulting in no detectable signal in our nanoBRET assay, while still undergoing a conformational change sufficient to reduce the BRET signal in TRUPATH. Although intriguing, this hypothesis remains speculative, as such behavior has not been previously reported and is not supported by existing data on D3R. Indeed, different groups using TRUPATH and similar BRET-based configurations have reported inconsistent findings.

Can the authors rule out the possibility that in a different cellular background (with different accessory proteins) the D3R might show a distinct G protein coupling profile compared to this one obtained in a HEK background?

The effect of the cellular background on the interpretation of the coupling results is a very important point. In the revised manuscript, we addressed this issue by obtaining a concentration-response curve to quinpirole applied to Chinese Hamster Ovary (CHO) cells transfected with D2R or D3R and each $G_{i/o/z}$ protein. Similar to our observations in HEK293 cells, we detected activation of all six $G\alpha_{i/o/z}$ proteins by D2R, while D3R only activated $G\alpha_{oA}$, $G\alpha_{oB}$, and $G\alpha_z$ (new **Supplementary Figure 3**).

Supplementary Figure 3

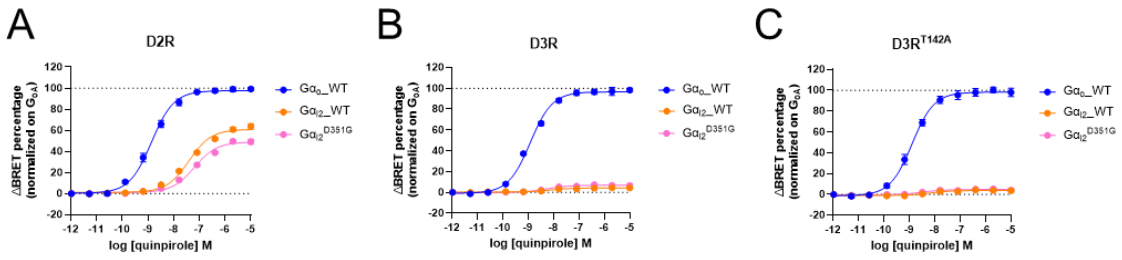


Supplementary Figure 3. D2R and D3R response to quinpirole in CHO cells. Concentration–response curves were obtained using the G protein nanoBRET assay in CHO cells transfected with D2R (**A**) or D3R (**B**) and indicated Gα proteins. Cells were stimulated with increasing concentrations of quinpirole. Data are shown as mean ± SEM of values normalized to the $G\alpha_{oA}$ signal. N=5 independent replicates.

Structural basis of selectivity: The authors reveal that ICL2 appears to be a determinant of G protein selectivity for D3R, although their data also shows that ICL3 also likely plays some role. The authors then go on the highlight some specific residue differences within ICL2 that might play key roles in this specificity. It's not clear why the authors did not take this further and investigate this with additional mutants. This additional information would strengthen the paper.

We appreciate the Reviewer's suggestion, and we have now included the proposed experiment. We undertook site-directed mutagenesis to generate mammalian expression clones encoding point-mutated versions of D3R ($D3R^{T142A}$) and $G\alpha_{i2}$ ($G\alpha_{i2}^{D351G}$). These mutants were evaluated using the G protein nanoBRET assay (new **Supplementary Figure 4**). Introduction of a glycine at position 351 of $G\alpha_{i2}$, the residue that is found at the corresponding position in $G\alpha_o$, resulted in no change in signal upon D2R activation (new **Supplementary Figure 4A**), or D3R activation (new **Supplementary Figure 4B**). Likewise, the $D3R^{T142A}$ mutant failed to produce D3R-mediated activation of $G\alpha_{i2}$ (new **Supplementary Figure 4C**). Together, these results indicate that the receptor:G protein interaction interface requires contributions from multiple sites to support stable complex formation and to promote GDP-to-GTP exchange on $G\alpha_{i2}$ and heterotrimer dissociation efficiently. These results are described in the revised manuscript (**lines 233-242**).

Supplementary Figure 4



Supplementary Figure 4. Mutating $D3R^{T142A}$ and $G\alpha_{i2}^{D351G}$ has no effect on G-protein coupling. (**A-C**) Concentration-response curves measured using the G protein nanoBRET assay for D2R (**A**), D3R (**B**) and $D3R^{T142A}$ (**C**) co-transfected with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{i2}^{D351G}$. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=4 independent replicates.

While the authors do investigate the determinants of selectivity for Gao over Gai they do not do the same thing for galphaZ, this would be a good addition to the paper with experiments similar to those shown in figure 4. How do the various D2/D3 chimeras couple to Galpha Z?

We believe the Reviewer is referring to Figure 5, where we tested D2R and D3R chimeras (ICL2 and ICL3 swaps) with $G\alpha_o$ and $G\alpha_{i2}$, but not with $G\alpha_z$. We now provide additional experiments evaluating the coupling of each receptor and $G\alpha_z$ (new **Figure 5B**). Our results demonstrate that the coupling to $G\alpha_z$ is not altered by swapping the ICL2 and ICL3 between D2R and D3R.

Figure 5

B

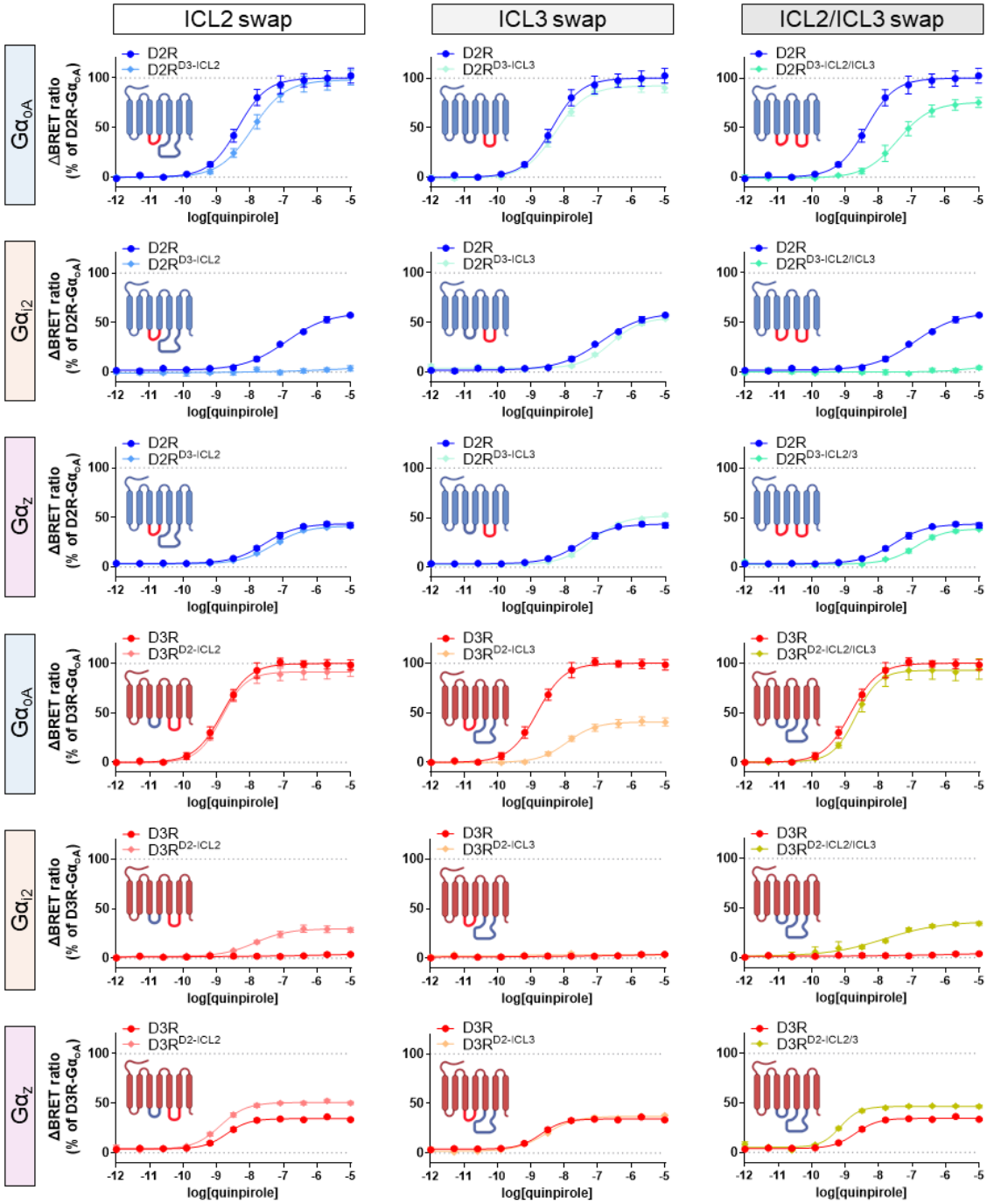


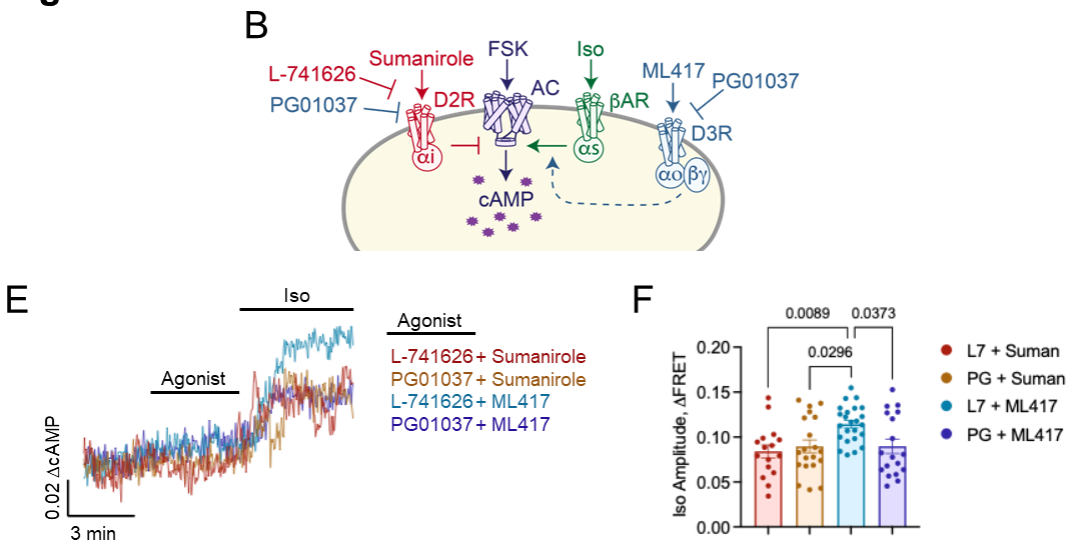
Figure 5.

(**B**) Concentration–response curves obtained using the G protein nanoBRET assay for GαoA and $G\alpha_{i2}$ activation by wild-type and loop-swapped D2R and D3R constructs. Schematic representations of the used chimeric receptor constructs in which ICL2 and/or ICL3 of D2R and D3R were swapped are inserted in each graph. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Reference curves for wild-type D2R and D3R with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{z}$ are reproduced multiple times to facilitate comparisons. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=5 independent replicates.

The slice experiments with CAMPER mice are difficult to align with the previous data in the paper. It is not clear to what extent D2R or D3R are contributing to each response. Suminirole is not very selective so would likely be acting through both receptors at a concentration of 100 nM and the response would likely be a mix of the two receptor signals. ML-417 is D3R-selective but can activate the D2R albeit with low potency. ML-417 in these slice experiments gives a very small signal and causes an increase in cAMP which is difficult to reconcile with the authors results in Figure 3 which shows D3R activation of Gz and Go to cause a decrease in cAMP. Which of Gao/z is contributing to this response in figure 7 and what is the evidence for this? Which G proteins are expressed in these cells? The D2R can also couple efficiently to both Galphao and GalphaZ (and indeed displays preference for them based on agonist potencies) so how can the apparently opposite effects of a D2R-selective and a D3R-selective agonist be reconciled simply through G protein coupling? To me this would argue, that if indeed these are D2 vs D3 signals then there is an additional level of complexity here that the authors have not addressed. I think this needs to be addressed with further experiments. The judicious use of selective D3R and D2R antagonists at appropriate concentrations might be useful here.

We appreciate this insightful perspective. Upon testing various antagonists, we found L-741626 exhibited selectivity toward D2R over D3R. Unfortunately, we were not able to validate a D3R selective antagonist, relative to D2R, in our hands (new **Supplementary Figure 5**). We found PG01037 was slightly more potent at D3R but nonetheless strongly inhibited both D2R and D3R. The brain slice cAMP imaging experiments were repeated with co-application of these antagonists (new **Figure 7 E-F**). We found both L-741626 (10 μM) and PG01037 (10 μM) blocked Sumanirole-

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

mediated cAMP inhibition, suggesting Sumanirole operates through D2R-$G\alpha_i$ in CA1 neurons. Moreover, Iso-mediated cAMP synthesis was not impacted by either antagonist. We next observed that L-741626 had no effect on ML417-mediated sensitization of Iso-induced cAMP production. On the other hand, PG01037 blocked the ML417's sensitization of cAMP following Iso application. We interpret the results such that PG01037 blocks ML417 on D3R. Meanwhile, L-741626 blocks D2R, which is not robustly activated by ML417 at 100 nM in CA1 neurons. These data support the notion that ML417 acts through D3R-$G\alpha_o$-$G\beta\gamma$ to adjust cAMP in CA1 neurons.

The phrase 'unexpected coupling profile of D3R' (line 225) is odd. This coupling profile has been seen before as described above and in their paper.

We adjusted the wording and changed "unexpected" to "unusual" to highlight how this coupling profile is unique.

The interpretation of sumanirole having slow off-rate kinetics does not seem correct to me, sumanirole and isoprotenerol are acting at distinct receptors so one would not expect isoprotenerol to compete with or block the action of sumanirole. Rather the sustained effect of sumanirole to negate the effect of isoprotenerol reflects the ongoing activation of Gai/o/z subunits.

We appreciate the Reviewer's comment, and we removed the reference to the possible slow off-rate kinetics of sumanirole from the text.

The authors refer to the G protein coupling selectivity as 'bias'. Against the background of biased agonism, I think that this is unnecessarily confusing. I think stating it as G protein coupling selectivity is a clearer description of this phenomenon.

We agree with the Reviewer that traditionally the term 'bias' has been applied to studies aiming at discerning the preferential activation of β-arrestin vs G proteins. However, the use of 'bias' has recently been extended to include the entirety of the GPCR signaling transducers as outlined here:

A community Biased Signaling Atlas (PMID: 36973443)

"*For GPCRs, biased signaling has classically been studied for the four G protein families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) and the arrestin family (Fig. 1). It additionally could extend to GPCR kinases (GRKs), which are also transducers — that is, receptor-binding effector proteins that phosphorylate the C termini of activated receptors to promote arrestin recruitment. Recently, however,* ***the scope of the term has been greatly expanded to include bias among any of the 27 specific transducer subtypes — 16 G proteins (with separately signaling α and βγ subunits), 7 GRKs and 4 arrestin proteins — which can differ in their functional outcome****."*

Community guidelines for GPCR ligand bias: IUPHAR review 32 (PMID: 35106752)

" '*Biased signalling' is the ligand-dependent activation of certain pathways over others, and can lead to a 'functionally selective' response. 'Biased signalling' became generally accepted after evidence*

accumulated that the rank order of ligands by potency could be different for different pathways engaged by a single receptor (Roth & Chuang, 1987; Spengler et al., 1993) or inversion of the ligand modality (Azzi et al., 2003; Baker, Hall, & Hill, 2003). ***The most frequently studied pathway-bias has been that between G proteins and arrestins, while more recent studies have compared G protein families and even subtypes belonging to the same G protein family****."*

According to these guidelines, we refer to the ability of the Dopamine D3 receptor to selectively engage $G_{o/z}$ but not G_i as signaling bias.

Reviewer #2 (Remarks to the Author):

Zanetti et al present an in-depth study around the signaling of the D3 dopamine receptor subtype and its ability to couple to G proteins. Coupling of the D3 has long been more difficult to understand compared to the D2 and D4 subtypes and this manuscript finally sets them side by side in the same selective functional assay. This is an important question, the experiments are well thought out and very complete (including convincing rescue and over-competition experiments) and define the signaling profile of the D3R. I have only minor concerns as outlined below.

Enthusiasm is only slightly tempered (as highlighted in the discussion) that these questions have been previously addressed by others; particularly in the Lane (2008) paper referenced which came to similar conclusions. This paper is however not as complete or clean, and the field was far from settled given the structure data and other conflicting papers.

We agree with the Reviewer and acknowledge the seminal findings reported by Lane et al. (2008). Given the substantial body of literature published since 2008 that has contributed to ongoing confusion regarding the G protein coupling profile of D3R, we believe that our study provides the conclusive evidence necessary to resolve this debate.

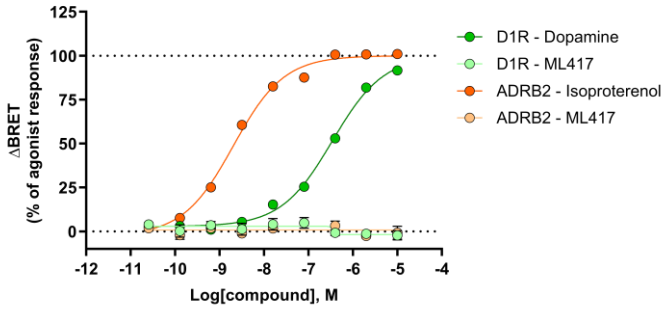
There are several instances in the literature of evidence that the manuscript data appear to conflict; however, the authors do a solid job of explaining how these other studies may have come to a different conclusion. The most difficult to address being the cryo-EM structure of D3R-Gi complexes. These should be slightly further clarified that in a system of purified proteins without other interactors it may be possible for the two proteins to interact as is seen in the structure.

We thank the Reviewer for their suggestion. We agree that interactions observed in a purified protein system may not be observed in the presence of other interactors. Additionally, purification of complexes from insect cells and stabilization using antibody fragments might facilitate the generation of stable complexes that would otherwise not be observed in mammalian systems. We have now clarified this better in the revised manuscript (**lines 226-232**).

In Figure 7, treatment with ML417 paradoxically raises the levels of cAMP in this preparation. While the proposed description of the mechanism (AC sensitization by Gbetagamma) is intriguing and I think very interesting in this native system, because this action is so important to the field it would be worth including alternative explanations such as ML417 having some small activity on a different receptor (unlikely given its published selectivity profile) or antagonist response on the D2R (it's very weak partial agonist response on the D2R could act as a functional antagonist response in an ex vivo prep if there is underlying basal 'tone'.

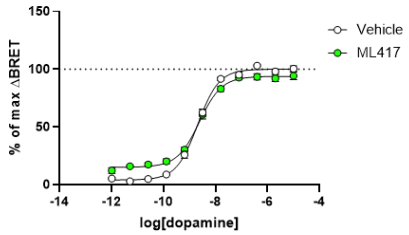
ML417 was originally discovered and pharmacologically characterized in the laboratory of Dr. David R. Sibley at the NINDS (PMID: 32342685). That study reported a comprehensive functional profiling of ML417 across 168 known GPCRs, including the Gs-coupled dopamine D1R and D5R, and demonstrated a high degree of selectivity for D3R. This reference is now included in our manuscript. In addition, to independently confirm selectivity, we tested ML417 for activity at other monoaminergic

receptors, specifically dopamine D1R and the β2-adrenergic receptor (ADRB2). As shown below, ML417 failed to activate either receptor even at high concentrations, further supporting the strong D3R selectivity reported in the literature.



Following the Reviewer's suggestion, we now included a comment on ML417 possibly acting as a weak partial agonist at D2R to reduce the tonic inhibition of adenylyl cyclase (**lines 277-279**). However, we obtained a concentration-response of dopamine at D2R in the presence of 100 nM ML417, the same concentration used in the experiments reported in Figure 7 (new **Supplementary Figure 6**). We found that, again, ML417 acts as a partial D2R agonist at this concentration but fails to inhibit dopamine stimulation of D2R. Therefore, we believe this explanation of the results in **Figure 7** is unlikely.

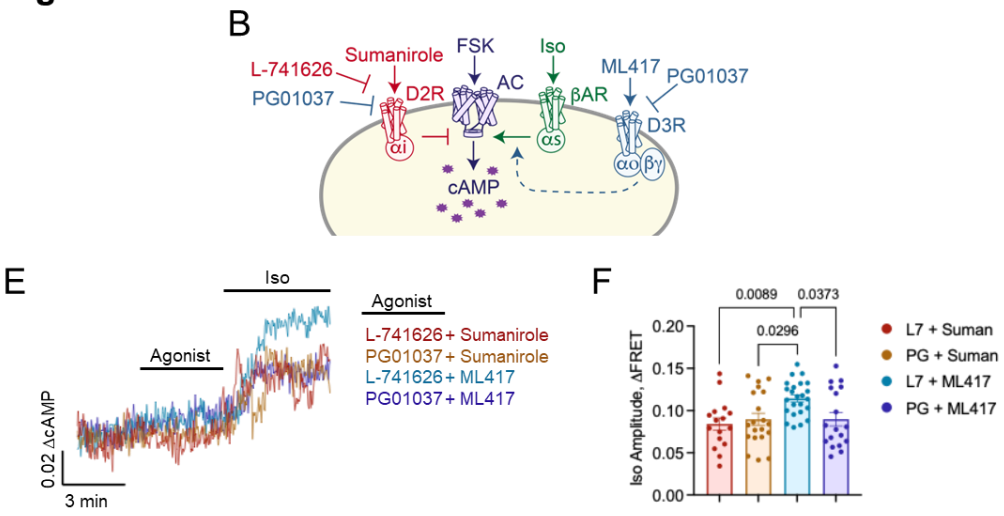
Supplementary Figure 6



Supplementary Figure 6. Weak partial agonism of ML417 at the D2R does not inhibit dopamine responses. Concentration-response curves measured using the G protein nanoBRET assay for D2R in wild-type HEK293T cells co-transfected with $G\alpha_{oA}$. Increasing concentrations of dopamine were applied in the presence of vehicle (white) or 100 nM ML417 (green). Data shown as mean ± SEM. N=5 independent replicates.

Finally, we have now performed experiments with an antagonist selective for D2R and also an antagonist targeting both D2R and D3R. Unfortunately, we were not able to validate a D3R selective antagonist (new **Supplementary Figure 5**). The D2R antagonist (L-741626) had no effect on ML417's impact on cAMP signaling, whereas the D2R/D3R antagonist (PG01037) indeed blunted ML417's cAMP sensitization (new **Figure 7E-F**). These data provide evidence that ML417 does not strongly signal through D2R in CA1 neurons, but rather the observed effects are driven through the D3R.

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

It would be intriguing to look at beta-arrestin signaling in your system and with your chimeric receptors to complete the D3R pharmacology profile, but is beyond the scope of this manuscript.

In the revised manuscript, we introduced an analysis of β-arrestin2 recruitment to D2R, D3R, and D4R measured using a NanoBiT complementation assay following stimulation with quinpirole and dopamine (new **Figure 3D-E**). The experimental methods and corresponding results are now fully described in the revised manuscript. While these experiments completed the pharmacological characterization of D2R, D3R, and D4R, they did not reveal a D3R-specific response that would justify further investigation using the chimeric receptors.

Figure 3

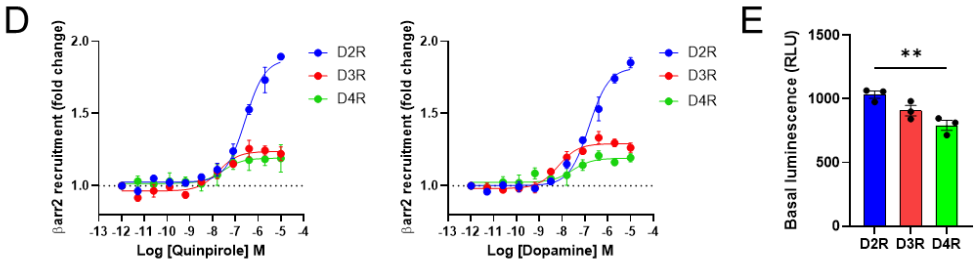


Figure 3.
(**D**) Concentration-response curves illustrating β-arrestin2 recruitment to D2R, D3R, and D4R following stimulation with quinpirole and dopamine, normalized as fold change, with response observed at the lowest concentration of ligand treated as 1. (**E**). Basal luminescence observed following co-transfection of receptor-SmBiT and βarr2-LgBiT. Data are shown as mean ± SEM. N=3 independent replicates. **P < 0.01, determined by one-way ANOVA and Tukey post hoc test.

Reviewer #3 (Remarks to the Author):

In this manuscript, Zanetti et al. investigated the G protein subtype preference underlying dopamine D3 receptor activation. It is quite interesting that, within the D2-like family (D2, D3, and D4), only D3 does not couple to Gi1-i3 subtypes. They further looked into the structural mechanism and identified that the unique sequence in ICL2 of D3R may play an important role. They also examined how the unique coupling profile of D3R affects its signaling at the neuronal level. This is an interesting study with information useful for general researchers. I have some questions regarding the interpretation of data and conclusions that I hope will help improve the manuscript.

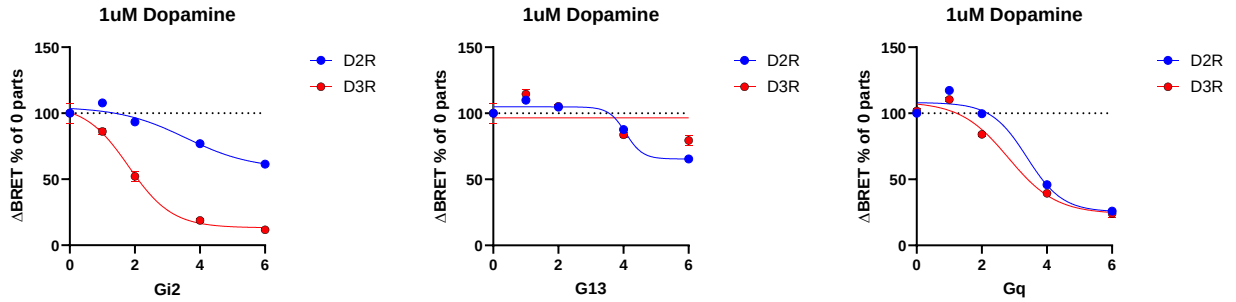
1. The nanoBRET assay used here fundamentally reports Gβ1γ2 availability to bind the GRK3CT sensor, yet for Gα subtypes they may have preferred pairs, as shown in the TRUPATH paper (Olsen et al., NCB 2020). This raises the question: Different Gβ and Gγ isoforms can alter heterotrimer assembly, basal dissociation, and effector engagement. It is therefore unclear whether the observed coupling hierarchy would remain unchanged if a different Gβγ pair were used. For example, in the TRUPATH paper, they show that Gi2 prefers the Gβ3γ8 pair; can they compare this with Gβ1γ2?

We thank the Reviewer for their observations on the possible role of Gβγ in defining the readout of GPCR cell-based assays. In designing the TRUPATH systems, Dr. Bryan Roth and colleagues observed that a better signal was obtained by pairing defined Gβγ heterodimers with individual Gα proteins. We agree that, in our BRET assay, the maximal effect measured may be influenced by the use of a specific Gβγ pair (in our case, $G\beta_1\gamma_2$). In support of our conclusions:

1. We believe that being consistent in the use of the same Gβγ pair enhances our ability to detect meaningful differences. We can clearly observe the signal generated by D2R and D4R, using $G\alpha_{i2}\beta_1\gamma_2$, suggesting that the heterotrimer dissociation is not inhibited in this combination. This has been extensively observed for a large number of receptors and Gα proteins by us (e.g., PMID: 40944639, 40568800, 40563241, 37567783, 33784795) and others (e.g., PMID: 37742189, 33667409, 26628681, 38412860, 19258039).
2. TRUPATH experiments performed in the Roth lab using the $G\beta_3\gamma_8$ combination also showed a lack of coupling to D3R (see figure 3.4 of Olsen, R. H. J. *Development of Next-generation G Protein Biosensors for Deconvoluting 529 the Complexity of GPCR Signaling*, The University of North Carolina at Chapel Hill, 530 (2019)).
3. In "*Diversity of the Gβγ complexes defines spatial and temporal bias of GPCR signaling*" by Masuho et al. (PMID: 33667409), the same BRET-based assay we used in our paper we applied to explore D2R activation across all possible Gβγ combinations. As shown in Figure 1E of that study, altering the Gβγ pairing produced only minor differences in signal amplitude in the G protein nanoBRET assay, consistent with the approach used in our work.

2. In Figure 3D, it is unclear why Gi2 at high concentration doesn't affect GoA signaling at D2R. In Figure 2, D2-GoA vs. d2-Gi2, quinpirole behaves very differently and is much weaker at Gi2. It would be expected to see a BRET ratio change if Gi2 dominates the Gβ1γ2 coupling and signaling with high Gi2 present. Basically, the Gi2 should act as an antagonist against GoA as their potency and efficacy are very different.

This is a particularly interesting and important observation, and one that also initially surprised us. The original experiment was performed in the background of global G protein KO cells. We speculated that this could affect the overall signaling measured and not be fully comparable to what was reported in **Figure 2**, where the background was wild-type HEK293T cells. We now performed additional experiments in the wild-type HEK293T cells (see below). We also included additional controls in which we co-transfected $G\alpha_{13}$ or $G\alpha_{q}$, Gα subunits that are not activated by either D2R or D3R. We expected that increasing the expression of a non-activatable Gα protein ($G\alpha_{i2}$ for D3R or $G\alpha_{13}$/$G\alpha_{q}$ for both D2R and D3R) would lead to a signal that originates exclusively from the 1:12 fraction of transfected $G\alpha_{o}$. However, the results of this set of experiments indicate that even the presence of a high amount of non-activatable Gα protein does not completely block the signal obtained by transfecting 1 part of the activatable $G\alpha_{o}$.



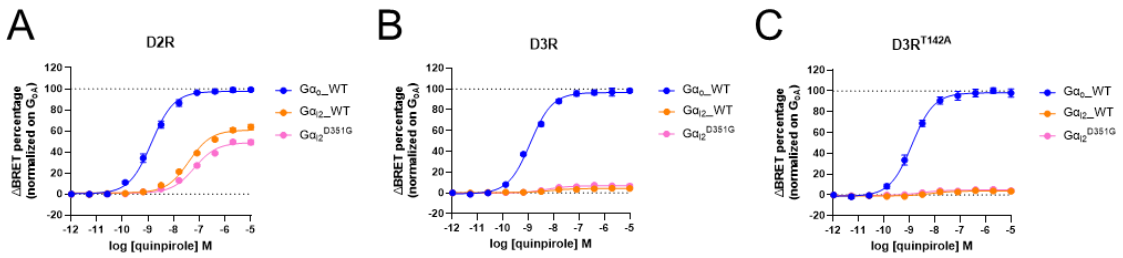
Based on our current data, we can only speculate on the molecular mechanisms underlying this phenomenon that could depend on possible roles of RGS proteins, possible unproductive interactions of receptors with G proteins (see PMID: 32817560), or variation in expression levels between Gα proteins. We acknowledge that this conceptual framework would require additional experimental validation, which we believe falls beyond the scope of the current manuscript. Considering that, at this stage, the results are difficult to interpret and do not contribute to the central message of the manuscript, we chose to remove this dataset (original **Figure 3D** and original **Supplementary Figure 3**) and prevent potential confusion.

3. Figure 6 proposes that steric clashes and/or electrostatic repulsion (e.g., involving D350 in Gαi2 and T142 in D3R ICL2) explain D3R's inability to productively engage Gi proteins. While the structural rationale is reasonable, this mechanistic interpretation would be strengthened by targeted point-mutagenesis experiments.

We appreciate the Reviewer's suggestion, and we have now included the proposed experiments in the revised manuscript. We undertook site-directed mutagenesis to generate mammalian expression clones encoding point-mutated versions of D3R ($D3R^{T142A}$) and $G\alpha_{i2}$ ($G\alpha_{i2}^{D351G}$). These mutants were evaluated using the G protein nanoBRET assay (new **Supplementary Figure 4**). Introduction of a glycine at position 351 of $G\alpha_{i2}$, the residue that is found at the corresponding position in $G\alpha_{o}$, resulted in no change in signal upon D2R activation (new **Supplementary Figure 4A**) or D3R activation (new **Supplementary Figure 4B**). Likewise, the $D3R^{T142A}$ mutant failed to produce D3R-mediated activation of $G\alpha_{i2}$ (new **Supplementary Figure 4C**). Together, these results indicate that the receptor:G protein interaction interface requires contributions from multiple sites to support stable

complex formation and to promote GDP-to-GTP exchange on $G\alpha_{i2}$ and heterotrimer dissociation efficiently. These results are described in the revised manuscript (**lines 233-242**).

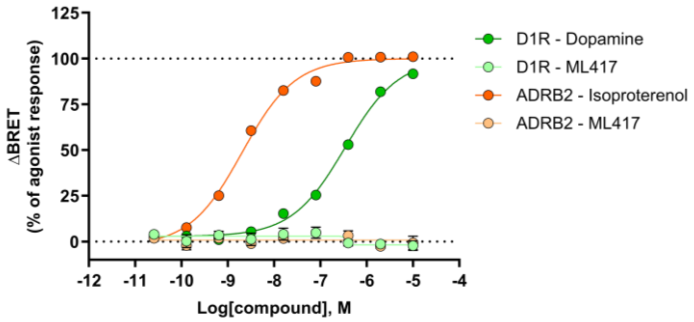
Supplementary Figure 4



Supplementary Figure 4. Mutating $D3R^{T142A}$ and $G\alpha_{i2}^{D351G}$ has no effect on G-protein coupling. (**A-C**) Concentration-response curves measured using the G protein nanoBRET assay for D2R (**A**), D3R (**B**) and $D3R^{T142A}$ (**C**) co-transfected with $G\alpha_{oA}$, $G\alpha_{i2}$, and $G\alpha_{i2}^{D351G}$. All experiments were conducted in wild-type HEK293T cells stimulated with increasing concentrations of quinpirole. Data were normalized to the signal obtained with $G\alpha_{oA}$ and shown as mean ± SEM. N=4 independent replicates.

4. Although sumanirole and ML417 show distinct receptor preferences in nanoBRET assays in HEK293 cells, it remains unclear whether the single concentration used in CA1 slices (100 nM) lies within a selective window in this physiological preparation; higher concentrations can be tested. Regarding ML417's unique effect, the authors showed that ML417 is very selective between D2 and D3. Are there any published data or can they confirm ML417 does not hit other Gs-coupled receptors and contribute to the increase in cAMP levels, such as closely related D1R and D5R (both Gs coupled)?

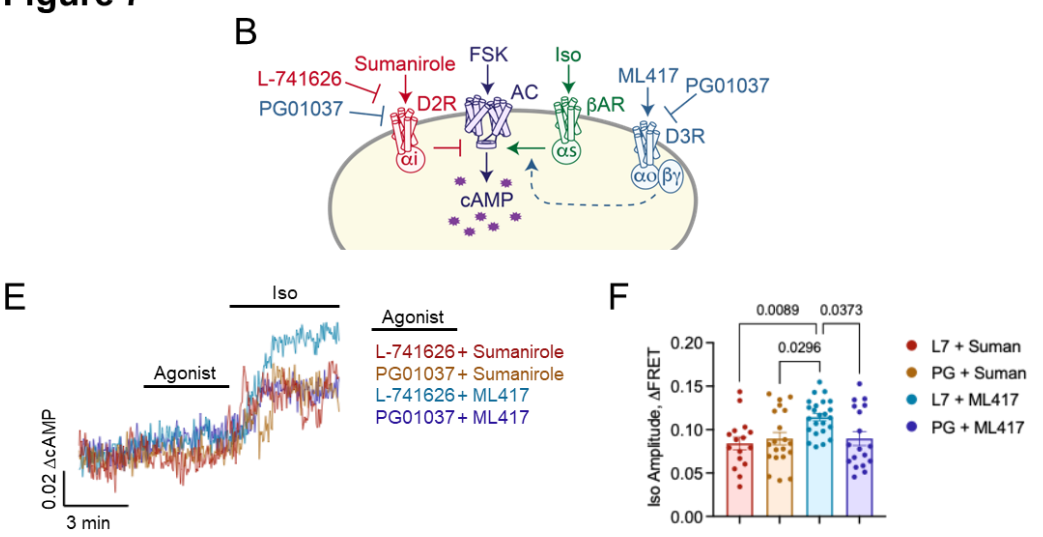
For the analysis of ML417 effects in brain slices, we used a concentration of 100 nM that was selected based on our concentration–response curves, indicating that this concentration selectively activates D3R without engaging D2R (less than 7% of dopamine-mediated activation). ML417 was originally discovered and pharmacologically characterized in the laboratory of Dr. David R. Sibley at the NINDS (PMID: 32342685). That study reported a comprehensive functional profiling of ML417 across 168 known GPCRs, including the Gs-coupled dopamine D1R and D5R, and demonstrated a high degree of selectivity for D3R. This reference is now included in our manuscript. In addition, to independently confirm selectivity, we tested ML417 for activity at other monoaminergic receptors, specifically dopamine D1R and the β2-adrenergic receptor (ADRB2). As shown below, ML417 failed to activate either receptor even at high concentrations, further supporting the strong D3R selectivity reported in the literature.



5. In Figure 7D, if ML417's effect is through hijacking the Gβγ, then they can do the same experiment but using forskolin to directly activate the ACs, and see if they can see the cAMP inhibition by ML417 in neurons.

We have repeated similar experiments, but with the inclusion of antagonists to select for D2R or D2R/D3R. Please see also responses to Reviewers #1 and #2. Briefly, we were unable to validate antagonists selective for D3R in our hands (new **Supplementary Figure 5**). Utilization of the selective D2R antagonist (L-741626) blocked Sumanirole inhibition of cAMP but had no effect on ML417. The D2R/D3R antagonist (PG01037) blocked both the Sumanirole and ML417 effects on cAMP (new **Figure 7E-F**). These data support the concept that ML417 acts through D3R-$G\alpha_o$-Gβγ to adjust cAMP in CA1 neurons. An important feature of the CAMPER sensor is the capability to detect bidirectional cAMP changes from baseline (*i.e.*, $G\alpha_i$-mediated inhibition), which was observed in the Sumanirole treatment in the original data. ML417 did not reduce baseline cAMP in these experiments, suggesting its primary mode of action is not through $G\alpha_i$.

Figure 7



(**B**) Schematic illustration of D2R and D3R signal decoding in dorsal hippocampal CA1 neurons. (**E**) Average trace of cAMP responses to stimulation of: L-741626 (10 mM) with Suminarole (100 nM; n=16 neurons/4 mice), PG01037 (10 mM) with Suminarole (100 nM; n=20 neurons/4 mice), L-741626 (10 mM) with ML417 (100 nM; n=24 neurons/5 mice), or PG01037 (10 mM) with ML417 (100 nM; n=18 neurons/4 mice). Slices were then stimulated with Isoproterenol (Iso; 100 nM). (**F**) Maximum Iso induced cAMP amplitude. One-way ANOVA was performed with Dunnett's multiple comparisons test. F=4.781, R2=0.1624, F (DFn, DFd)=1.603 (3, 74). Data shown as mean ± SEM where each dot represents an individual neuron. Exact P values are depicted on the graphs.

Minor comments:

What are the values in Figure 1C, Emax (e.g., 0, 0.03,… 0.15) as they seem not match the deltaBRET ratio in Figure 1A? Adding an annotation within the panel to indicate which radar plots correspond to each receptor would help prevent confusion.

We apologize, as the representative kinetics trace reporting $G\alpha_o$ activation in response to D3R stimulation in **Figure 1A** was indeed not representative of the signal we normally obtain in G protein

nanoBRET. We now include a representative kinetics trace that aligns with the results quantified in **Figures 1B** and **1C**. Additionally, receptors analyzed in **Figure 1C** are now indicated as suggested.

Lines 140-141 may need rewording as ML417 activates Gz at D3R.

We apologize for the confusion. The point we wanted to make is that ML417 activates D3R but not D2R, except for $G\alpha_o$, but only at higher concentrations. We added “by D2R” at the end of the sentence to improve clarity.

Standardize naming throughout (e.g., consistently use GoA or GαoA rather than mixing GoA/GαoA).

We normally use $G\alpha_{oA}$ when referring to the Gα subunit, and G_{oA} if we are referring to the heterotrimeric G protein. We understand that this is unnecessarily confusing, and we have now simplified this by using Gα throughout the manuscript, including the title.

It is helpful to provide tables summarizing values (e.g., pEC50 and Emax for D2R/D3R/D4R across Gi/o/z subtypes).

Tables summarizing pEC_{50} and E_{max} values are now provided (**Supplementary Data 1-3**).

Please add a color key bar for the electrostatic surface rendering and clarify in the legend what the colors represent in Fig. 6A.

We thank the Reviewer for their suggestion. We have now clarified in the legend what the colors of the electrostatic surface rendering represent and also included a scale bar in the revised manuscript.