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Gα Protein Signaling Bias at Serotonin 1A Receptor[S]

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ABSTRACT

Serotonin 1A receptor (5-HT1AR) is a clinically relevant target because of its involvement in several central and peripheral functions, including sleep, temperature homeostasis, processing of emotions, and response to stress. As a G protein coupled receptor (GPCR) activating numerous $G\alpha_{i/o/z}$ family members, 5-HT1AR can potentially modulate multiple intracellular signaling pathways in response to different therapeutics. Here, we applied a cell-based bioluminescence resonance energy transfer assay to quantify how ten structurally diverse 5-HT1AR agonists exert biased signaling by differentially stimulating $G\alpha_{i/o/z}$ family members. Our concentration-response analysis of the activation of each $G\alpha_{i/o/z}$ protein revealed unique potency and efficacy profiles of selected agonists when compared with the reference 5-hydroxytryptamine, serotonin. Overall, our analysis of signaling bias identified groups of ligands sharing comparable G protein activation selectivity and also drugs with unique selectivity profiles. We observed, for example, a strong bias of F-15599 toward the activation of $G\alpha_{i3}$ that was unique among the agonists tested: we found a biased factor of +2.19 when comparing the activation of $G\alpha_{i3}$ versus $G\alpha_{i2}$ by F-15599, while it was -0.29 for 8-hydroxy-2-(di-n-propylamino) tetralin. Similarly, vortioxetine showed a biased factor of +1.06 for $G\alpha_z$ versus $G\alpha_{oA}$, while it was -1.38 for vilazodone. Considering that alternative signaling pathways are regulated downstream of each Gα protein, our data suggest that the unique pharmacological properties of the tested agonists could result in multiple unrelated cellular outcomes. Further investigation is needed to reveal how this type of ligand bias could affect cellular responses and to illuminate the molecular mechanisms underlying therapeutic profile and side effects of each drug.

SIGNIFICANCE STATEMENT

Serotonin 1a receptor (5-HT1AR) activates several members of the $G_{i/o/z}$ protein family. Here, we examined ten structurally diverse and clinically relevant agonists acting on 5-HT1AR and identified distinctive bias patterns among G proteins. Considering the diversity of their intracellular effectors and signaling properties, this data reveal novel mechanisms underlying both therapeutic and undesirable effects.

Introduction

G protein coupled receptors (GPCRs) are the largest group of membrane receptors in mammals. About 35% of approved drugs regulate GPCRs; thus, they are a vital target for drug discovery (Campbell and Smrcka, 2018; Sriram and Insel, 2018). GPCRs are seven transmembrane proteins that couple and transduce signals by activating heterotrimeric G proteins and β-arrestins which further regulate various downstream signaling pathways. The activation of intracellular effectors by GPCR ligands is sometimes thought to be balanced, despite the fact that a limited number of studies explored this issue. However, biased ligands with the ability to preferentially stimulate a signaling pathway over another one have also been identified (Urban et al., 2007; Kenakin, 2019). The first evidence of signaling bias emerged from the observation that individual serotonergic receptors could control distinct intracellular pathways (Roth and Chuang, 1987). Later, drug discovery efforts suggested that G protein biased agonists targeting μ-opioid receptors could maintain analgesic effects while reducing adverse outcomes (Raehal et al., 2005; DeWire et al., 2013; Schmid et al., 2017). On the contrary, β-arrestin biased agonists targeting angiotensin II type-1 receptors have been proposed to reduce blood pressure and increase cardiac performance (Violin et al., 2010). More recently, $G\alpha_s$-biased compounds activating β_2-adrenergic receptors with no apparent involvement of β-arrestin-mediated signaling have been identified as potential candidates for treating asthma (Kim et al., 2021). As a consequence, the functional selectivity between Gα proteins and β-arrestins has become a classic example of clinically relevant ligand bias (Gurevich and Gurevich, 2020). However, ligand bias producing selective activation of Gα protein subtypes is an event that has been rarely investigated (Von Moo et al., 2022; Voss et al., 2022). It has been demonstrated that a biased agonist acting on adenosine 1 receptor and selective to GoB exhibit less cardiorespiratory depression compared

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; serotonin, 5-HT1AR, serotonin 1A receptor; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino) tetralin; AC, adenylyl cyclase; BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary cells; E_{max}, maximal efficacy; GPCR, G protein coupled receptor; GRK3CT, G protein-coupled receptor kinase 3 C-terminus; pEC50, negative logarithm of the half maximal effective concentration.

with non-biased G protein agonists (Wall et al., 2022). As a result, it might be possible to understand the molecular process underlying drug efficacy and potency by looking into biased G protein signaling pathways. In mammals, 16 genes encode for Gα proteins that are classified into four subfamilies according to their sequence homology: $G\alpha_s$, $G\alpha_q$, $G\alpha_{12/13}$, and $G\alpha_{i/o/z}$ (Wettschureck and Offermanns, 2005). Given that GPCR-G protein coupling can be selective toward a subset of G proteins, different ligands acting on the same receptor could potentially control alternative downstream signaling cascades (Berg et al., 1998; Zheng et al., 2010; Fleetwood et al., 2021; Wright and Bouvier, 2021; Kim et al., 2022).

The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) plays essential roles in the central and peripheral nervous systems (Mann, 1999; Nichols and Nichols, 2008; Švob Štrac et al., 2016; Sharp and Barnes, 2020). Among the GPCRs endogenously activated by 5-HT, 5-HT1A receptor (5-HT1AR) is widely expressed and it has been implicated in many central and peripheral physiologic functions including sleep, pain, temperature homeostasis, processing of emotions, and response to stress (Bjorvatn and Ursin, 1998; Polter and Li, 2010; Garcia-Garcia et al., 2014; Albert and Vahid-Ansari, 2019; Haleem, 2019; Razakarivony et al., 2021; Voronova, 2021; Pehrson et al., 2022). In the brain, 5-HT1AR acts as either a somatodendritic autoreceptor to control activity-dependent 5-HT release (Sprouse and Aghajanian, 1987) or as a postsynaptic heteroreceptor to reduce neuronal excitability and firing rates (Riad et al., 2000; Garcia-Garcia et al., 2014). Previous studies have shown that signaling cascades activated by 5-HT1AR are exquisitely sensitive to pertussis toxin suggesting coupling to $G\alpha_{i/o/z}$ family members with minimal activity toward members of the $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12/13}$ protein families (Raymond et al., 1999; Kooistra et al., 2021; Pandy-Szekeres et al., 2022). Since 5-HT1AR preferentially couples to members of the numerous $G\alpha_{i/o/z}$ and several natural and synthetic agonists are available, this receptor provides an excellent model to explore the potential Gα protein bias. Furthermore, many approved drugs acting on 5-HT1AR elicit different therapeutic effects and adverse responses (Celada et al., 2013). Nevertheless, the question of whether these diverse outcomes depend on the biased activation of Gα proteins remains obscure. Here, we investigate the activation bias in response to ten structurally diverse 5-HT1AR agonists by obtaining their unique profiles of Gα protein activation using an optimized cell-based bioluminescence resonance energy transfer (BRET) assay.

Materials and Methods

Cell Cultures and Transfections. Human embryonic kidney 293T cell line cells (RRID:CVCL_1926) were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Gibco, 10567-014) supplemented with 10% fetal bovine serum (Biowest, S1520), non-essential amino acids (Gibco, 11140-050), penicillin 100 units/ml and streptomycin 100 μg/ml (Gibco, 15140-122), and amphotericin B 250 μg/ml (ThermoFisher, 15290-018) at 37°C and 5% CO_2. Cells were routinely monitored for possible mycoplasma contamination. Two million cells were seeded in each well of 6-well plates in medium without antibiotics for 4 hours and then transfected with a 1:3 ratio of DNA plasmid (2.5 μg) and polyethylenimine (7.5 μl) (VWR, AAA43896). The optimal Gα:Gβγ transfection ratio identified for $G\alpha_{oA}$, $G\alpha_{oB}$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_z$ were 4:1. The empty vector pcDNA3.1 was used to normalize the ratio of transfected plasmids. Transiently transfected cells were incubated for 16 hours before being tested.

DNA Plasmids and Chemicals. The plasmid encoding human 5-HT1AR was obtained from the cDNA Resource Center (www.cdna.org) (HTR01A0000). $G\beta 1\text{-venus}^{156\text{-}239}$ and $G\gamma 2\text{-venus}^{1\text{-}155}$ were generous gifts from Dr. Nevin A. Lambert (Augusta University, Augusta GA). Gα proteins and masGRK3CT-Nluc constructs were generous gifts from Dr. Kirill A. Martemyanov (UF Scripps Biomedical Research, Jupiter, FL). The following 5-HT1AR agonists were purchased from MedChemExpress: vilazodone (HY-14261), vortioxetine (HY-15414A), sumatriptan succinate (HY-B0121), nuciferine (HY-N0049), flibanserin (HY-A0095), aripiprazole (HY-14546), buspirone (HY-B1115), F-15599 (HY-19863), and 8-OH-DPAT (HY-15688). 5-HT hydrochloride was purchased from Tocris (3547/50). All chemicals were resuspended according to manufacturers' instructions, aliquoted, and stored at -20°C until use.

G Protein NanoBRET Assay. The day after transfection, cells were briefly washed with phosphate buffered saline, resuspended in BRET buffer (phosphate buffered saline supplemented with 0.5 mM $MgCl_2$ and 0.1% glucose), collected in 1.5 ml tubes, and centrifuged for 5 minutes at 500 × g. Pelleted cells were resuspended in 500 μl of BRET buffer and 25 μl of cells were plated in 96-well white microplates (Greiner Bio-One). The nanoluc substrate furimazine (N1120) was purchased from Promega and used according to the manufacturer's instructions. BRET measurements were obtained using a POLARstar Omega microplate reader (BMG Labtech). All measurements were performed at room temperature and BRET signal was determined by calculating the ratio of the light emitted by $G\beta 1\gamma 2\text{-venus}$ (collected using the emission filter 535/30) to the light emitted by masGRK3CT-Nluc (475/30). In kinetics assays, the baseline value (basal BRET ratio) was averaged from recordings of the five seconds before agonist injection. In concentration-response experiments, 30 μl of cells per well were plated and mixed with the nanoluc substrate furimazine. Initial readings were performed to establish basal BRET ratio and then agonists at 12 concentrations were added. BRET signal was recorded for 3 minutes. ΔBRET ratios were obtained by subtracting the basal BRET ratio from the maximal amplitude measured.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism version 9 software (RRID:SCR_002798). Sample size was not predetermined; however, each experiment was performed at least five times before statistical analysis was done. Concentration-response curves were fitted to a sigmoidal four-parameter logistic function (variable slope analysis) to quantify agonist potencies (pEC50) and maximal responses (E_{max}). Importantly, we excluded Hill slope values that did not lie between 0.7 and 1.4 (Winpenny et al., 2016). At least five independent biologic replicates were used for each experiment. To obtain the Gα protein bias, we adopted the equation from (Winpenny et al., 2016). Briefly, we first calculated the mean Log(E_{max}/EC50) for each agonist; then, we calculated the ΔLog(E_{max}/EC50) normalized to the reference agonist, in this case 5-HT:

$$\Delta\mathrm{Log} = \log\left(\frac{\mathrm{Emax}}{\mathrm{EC50}}\ test\right) - \log\left(\frac{Emax}{EC50}\ reference\right) \quad (1)$$

Finally, we calculated the bias factor (ΔΔLog) between different Gα proteins using the following equation:

$$\Delta\Delta\mathrm{Log} = \Delta\log\left(\frac{\mathrm{Emax}}{\mathrm{EC50}}\ pathway\ 1\right) - \Delta\log\left(\frac{Emax}{EC50}\ pathway\ 2\right) \quad (2)$$

All of our calculations error were associated with 95% confidence intervals (CI).

Rstudio software was used to visualize the results as a cluster dendrogram. Data were grouped into different clusters and the "complete" method was used for hierarchical clustering using distance matrix (Euclidean) dendrogram on R-studio (R Core Team, 2013).

All the data are reported as mean ± S.D. One-way ANOVA was applied to determine statistical differences and P values were reported as follows in the figure: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. Post

hoc tests used and number of replicates are indicated in figure legends. Given the exploratory character of the study, calculated *P* values cannot be interpreted as hypothesis-testing but only as descriptive.

Results

5-HT1AR Uniquely Activates Heterotrimeric $G_{i/o/z}$ Family Members. To confirm the reported G protein coupling profile of 5-HT1AR, we expressed 5-HT1AR in HEK293 cells together with a representative member of each Gα protein family ($G\alpha_o$, $G\alpha_s$, $G\alpha_q$, $G\alpha_{15}$, and $G\alpha_{13}$) and we used a G protein nanoBRET assay to measure the coupling efficiency to each G protein in response to 5-HT stimulation (Fig. 1A). Briefly, after activation of the receptor with 5-HT, Gα dissociates from Gβγ-venus that is now free to associate with the BRET donor G protein-coupled receptor kinase 3 C-terminus (GRK3CT)-Nluc and generate a BRET signal (Hollins et al., 2009; Masuho et al., 2015). As expected, 5-HT1AR efficiently activated $G\alpha_o$, while we did not observe any activation of $G\alpha_s$, $G\alpha_q$, $G\alpha_{15}$, and $G\alpha_{13}$ (Fig. 1B). With the goal of measuring the activation of each member of the $G\alpha_{i/o/z}$ family, we then optimized the stoichiometry of each expressed Gα and Gβγ-venus by titrating the amount of Gα subunits against a constant amount of transfected Gβγ-venus. As expected, by increasing the amount of transfected Gα, we observed a reduction in the basal activity; in fact, suboptimal expression of Gα allows free Gβγ-venus to interact with GRK3CT-Nluc, increasing the BRET signal detected in the absence of GPCR stimulation. Therefore, optimal Gα:Gβγ ratios were selected based on low basal BRET ratio and high maximal amplitude for each $G\alpha_{i/o/z}$ protein (Supplemental Fig. 1).

Gα Protein Coupling Profile of 5-HT1AR Stimulated with the Endogenous Agonist 5-HT. According to experimental data available on the GPCR database (https://gproteindb.org/), 5-HT1AR favors $G\alpha_o$ over $G\alpha_i$ and $G\alpha_z$ (Kooistra et al., 2021; Pandy-Szekeres et al., 2022). To confirm this ranking order, we applied 100 μM of 5-HT on HEK293 cells transiently transfected with 5-HT1AR and each member of the $G\alpha_{i/o/z}$ protein family. Then, we quantitatively measured Gα protein activation via the same BRET assay described above as it allows to monitor G protein activation in real time (Hollins et al., 2009; Masuho et al., 2015). We observed that within sixty seconds, $G\alpha_{oA}$ showed the highest amplitude which represents the highest efficacy measured as ΔBRET ratio followed by $G\alpha_{oB}$, $G\alpha_{i2}$, $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_z$ (Fig. 2A). For the purpose of evaluating potency and efficacy, we conducted concentration-response studies for each Gα protein in response to increasing concentrations of 5-HT ranging from 2 pM to 100 μM (Fig. 2B). Our analysis of potency and efficacy, measured as maximal ΔBRET ratio, suggest a preferential activation of $G\alpha_o$ over $G\alpha_{i/z}$ (Fig. 2, C and D; Supplemental Table 1). However, caveats due to possible differences in individual Gα protein properties, such as protein expression levels, endogenous expression of specific regulatory proteins (i.e., RGS proteins), or efficiency in terms of releasing Gβγ-venus, do not allow a direct comparison. Later on, we will use the physiologic ligand 5-HT as a reference to compare potency and efficacy of 5-HT1AR agonists. Taking into consideration that time to reach an equilibrium could affect maximal amplitude measurements, especially at low concentration of agonist, we analyzed each condition over three minutes. We observed that the majority of Gα proteins produced the highest ΔBRET ratio within one minute, with the exception of $G\alpha_z$, which still reached its highest amplitude within three minutes (Fig. 2, E and F; Supplemental Fig. 2).

Structurally Diverse 5-TH1AR Agonists Produce Different Gα Protein Coupling Profiles. We hypothesized that structurally diverse 5-HT1AR agonists could elicit a ligand bias at the Gα protein level. To test this hypothesis, we examined nine agonists, including clinically approved antidepressant agents and we compared them with the endogenous ligand 5-HT as a reference (Supplemental Fig. 3). Five of these compounds are reported to act as partial agonists on 5-HT1AR while 8-OH-DPAT and sumatriptan are reported to act as full agonists (Alexander et al., 2021). In addition, we included in our study a highly selective 5-HT1AR full agonist, F-15599 (also known as NLX-101) for which biased activity has been reported both in vitro and in vivo (Newman-Tancredi et al., 2009; Vidal et al., 2018; Depoortere et al., 2021). To ensure that the tested agonists do not activate any endogenous receptor, we transfected cells with pcDNA3.1 along with masGRK3CT-Nluc and Gβ1γ2-venus without 5-HT1AR. As expected, while we could measure in real time the activation of individual $G_{i/o/z}$ protein by 5-HT1AR (Supplemental Fig. 4A), we did not observe any signal in mock-transfected cells (Supplemental

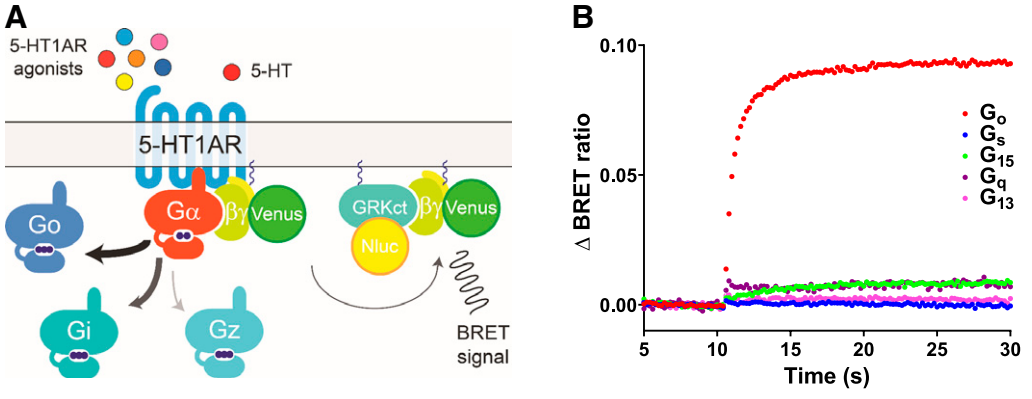

Fig. 1. 5-HT1AR stimulation in a BRET-based assay. (A) Cartoon illustration of cell-based BRET assay. GPCR stimulation triggers the dissociation of heterotrimeric G proteins leading to changes in the BRET signal that represents an index of GPCR activation. To limit artifacts due to the modification of Gα proteins, this system was developed by fusing the donor luciferase with an effector of Gβγ (masGRK3-CT-nluc), and a split acceptor, venus, fused with Gβ1 and Gγ2 subunits, allowing the use of wild type Gα subunits. (B) Representative traces obtained by applying 100 μM 5-HT on HEK293 cells transfected with 5-HT1AR and each one of G_s, G_q, G_{13} G_{15}, and G_o proteins. Recording started 10 seconds before agonist application to obtain a basal BRET signal. ΔBRET ratio was calculated by subtraction of the basal BRET at each time point.

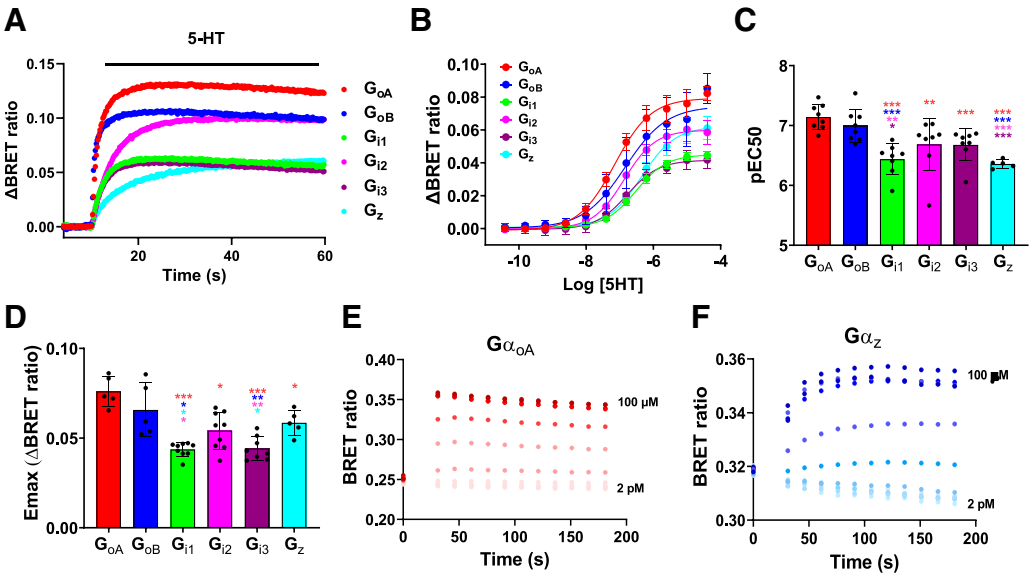

Fig. 2. Pharmacological characterization of 5-HT1AR. (A) Representative ΔBRET ratio responses after application of 100 μM 5-HT for each G$\alpha_{i/o/z}$ family member. Basal BRET ratio was measured for 10 seconds before agonist application. (B) Concentration-response curves of different Gα proteins after 5-HT application. Each data point represents the mean ± S.D. (n = 5 independent replicates). (C) pEC50 obtained from concentration-response curves for each Gα protein. Each bar graph represents the mean ± S.D. (n = 5 independent replicates). (D) Efficacy (E_{max}) reported as quantified ΔBRET ratio for different Gα proteins. Each bra graph represents the mean ± S.D. (n = 5 independent replicates). (E-F) Representative curves showing the concentration-dependent increase in ΔBRET ratio over time for Gα_o and Gα_z recorded over three minutes. Increasing 5-HT concentrations are represented by darker colors ranging from 2 pM (lighter) to 100 μM (darker). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ determined by one-way ANOVA and Tukey post hoc test (n = 5). Color code: red * indicates comparison with G_{oA}; dark blue * indicates comparison with G_{oB}; pink * indicates comparison with G_{i2}; purple * indicates comparison with G_{i3}; cyan * indicates comparison with G_z.

Fig. 4B). Similarly, we confirmed activation of each Gα subunit when using high concentrations of each selected agonist in our optimized G protein nanoBRET assay (Supplemental Fig. 4, C–K). Next, we generated concentration-response curves, and we calculated efficacy (E_{max}) and pEC50 for individual agonists with each of the six Gα proteins (Fig. 3A; Supplemental Fig. 5; Table 1). We first observed a wide range of efficacy values, ranging between 0–180% of 5-HT response. As expected, some drugs performed as partial agonists while others can be considered as full agonists. Interestingly, some agonists showed super agonistic properties for certain G proteins compared with the reference ligand. For instance, flibanserin, F-15599, 8-OH-DPAT, and vilazodone showed Gα_{oB} maximum response ranging from 125% to 180% when compared with the endogenous ligand 5-HT. Overall, the application of six independent readouts, one for each Gα protein, revealed a multifaceted response otherwise difficult to appreciate. This data established that the most potent agonists other than 5-HT were vilazodone, F-15599, flibanserin, buspirone, and 8-OH-DPAT across Gα proteins. Interestingly, we also found that some agonists were not able to activate specific Gα proteins: for example, aripiprazole could not activate Gα_{i1} while Gα_{i3} was only activated at very high concentrations (over 100 μM). Meanwhile, sumatriptan activates Gα_o but it only showed activation to G_i at extremely high concentrations (Fig. 3**;** Supplemental Fig. 5).

Ligand-Dependent Gα Protein Signaling Bias at 5-HT1AR. A formal analysis of signaling bias revealed the existence of two groups of 5-HT1AR agonists. After examining the signaling properties of the different ligands and calculating efficacy and potency (Fig. 3, B and C), we evaluated a transduction coefficient as Log(E_{max}/EC50) for each agonist (Fig. 3D). To comprehensively analyze similarities and differences among Gα protein activation patterns elicited by each agonist we applied a cluster analysis (Fig. 3D). Accordingly, our data revealed two groups of agonists: 1) 5-HT, F-15599, vilazodone, buspirone, 8-OH-DPAT, and flibanserin; and 2) vortioxetine, sumatriptan, nuciferine, and aripiprazole. Finally, with a Hill slope proximal to 1, we evaluated the bias factor, expressed as a ΔΔLog(E_{max}/EC50), produced by each agonist across Gα proteins after normalization to the reference 5-HT (Fig. 4). Agonists that did not reach the plateau for certain G proteins were excluded from our bias calculations.

Using these data, we generated paired comparisons that suggest biased activation of a test Gα protein over a reference Gα (positive values), or the opposite (negative values). This set of data revealed for example that vilazodone, 8-OH-DPAT, nuciferine, sumatriptan, and aripiprazole have a bias toward G$\alpha_{oA/B}$ over Gα_{i1-3} and Gα_z, while vortioxetine prefers G$\alpha_{i/z}$ activation over Gα_o. On the contrary, F-15599 and flibanserin reveal mixed activation between G_o and $G_{i/z}$. Both agonists show preference activation toward G_{oB}, G_{i1}, and G_{i3} over G_{oA}, G_{i2}, and G_z (Fig. 4). Collectively, we found that individual agonists showed unique Gα protein activation profiles.

Discussion

In the last two decades, a great deal of effort has been put forward to understand ligand bias and functional selectivity between Gα proteins and β-arrestins (Rankovic et al., 2016; Smith et al., 2018; Wisler et al., 2018; Eiger et al., 2022). In fact, ligands that show biased properties hold the promise to be more effective and safer therapeutics (Gurevich and Gurevich, 2020). However, a limited number of studies investigated signaling bias within Gα proteins (Masuho et al., 2015; Von Moo et al., 2022; Voss et al., 2022). In this study, using the physiologic ligand 5-HT as a reference, we showed that

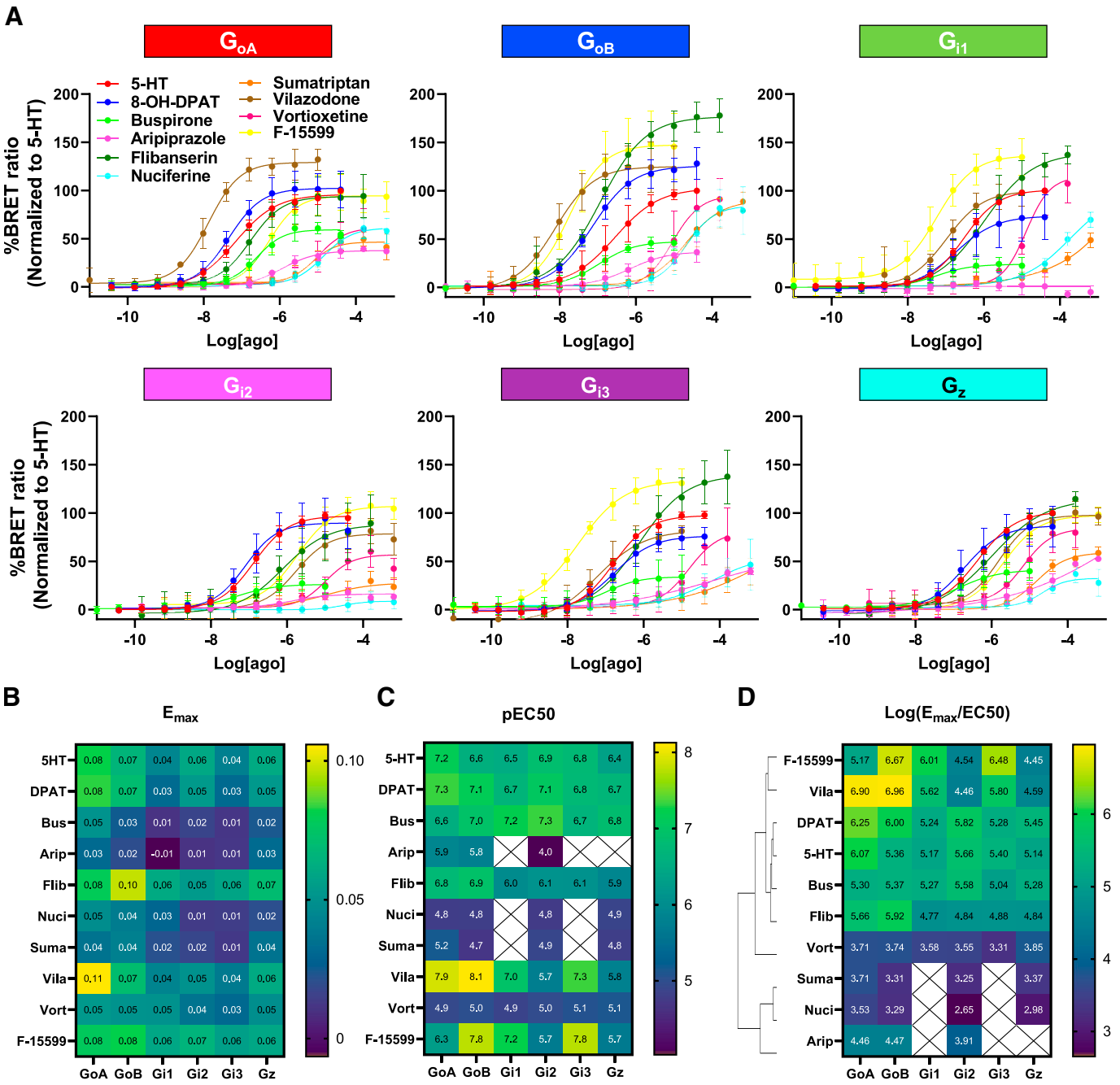

Fig. 3. Concentration-response curves and activation heatmaps for each Gα protein obtained with different 5-HT1AR agonists. (A) Full concentration-response curves for each of the ten indicated 5-HT1AR agonists with each of the six $G\alpha_{i/o/z}$ proteins. Each data point represents the mean ± S.D. (n = 5–10 independent replicates). (B) Efficacy values reported as maximal ΔBRET ratio (E_{max}) for each 5-HT1AR agonist using the activation of the six Gα proteins as readout. Colors represents the mean E_{max} value according to the color-coded scale reported to the right (n = 5–10 independent replicates). (C) Potency reported as pEC50 values for each agonist/Gα protein pair. Colors represents the mean pEC50 value according to the color-coded scale reported to the right (n = 5–10 independent replicates). (D) Both values were used to calculate the transduction coefficient as Log(E_{max}/EC50) and to cluster the ten agonists according to their profile of G protein activation. Colors represent the Log(E_{max}/EC50) value according to the color-coded scale reported to the right.

the action of structurally diverse 5-TH1AR agonists can be biased toward different $G\alpha_{i/o/z}$ family members. From the concentration-response curves obtained for ten 5-HT1AR agonists we were able to estimate EC50 and E_{max} for each one of six activated Gα proteins. Analyzing these data, we discovered that some 5-HT1AR ligands perform as super, full, or partial agonists when compared with the endogenous ligand 5-HT in their ability to trigger the release of specific Gα subunits. A striking example is the selective 5-HT1AR agonist F-15599 that acts as a super agonist in the activation of $G\alpha_{i1}$, as a full agonist in the activation of $G\alpha_{oA}$, $G\alpha_{i2}$, $G\alpha_{i3}$, and $G\alpha_{z}$, while it is a partial agonist in the activation of $G\alpha_{oB}$.

From these data, we calculated a bias factor that was later normalized over 5-HT to compare the relative activation of

TABLE 1
Efficacy and potency values of indicated 5-HT1AR agonists acting on each $G\alpha_{i/o/z}$ protein. Each value represents the mean ± S.D. of 5–10 independent replicates

Compound	Gα protein	Emax ± S.D.	pEC50 ± S.D.	Compound	Gα protein	Emax ± S.D.	pEC50 ± S.D.
	GoA	0.082 ± 0.003	7.20 ± 0.15		GoA	0.048 ± 0.010	4.78 ± 0.18
	GoB	0.066 ± 0.015	6.59 ± 0.46		GoB	0.040 ± 0.007	4.82 ± 0.39
5-HT	Gi1	0.045 ± 0.002	6.52 ± 0.07	Nuciferine	Gi1	0.030 ± 0.003	
	Gi2	0.060 ± 0.007	6.86 ± 0.09		Gi2	0.013 ± 0.009	4.75 ± 1.15
	Gi3	0.042 ± 0.005	6.78 ± 0.06		Gi3	0.014 ± 0.009	
	Gz	0.061 ± 0.006	6.36 ± 0.06		Gz	0.020 ± 0.002	4.90 ± 0.66
	GoA	0.084 ± 0.020	7.33 ± 0.20		GoA	0.036 ± 0.006	5.17 ± 0.20
	GoB	0.070 ± 0.008	7.14 ± 0.06		GoB	0.042 ± 0.006	4.72 ± 0.18
8-OH-DPAT	Gi1	0.032 ± 0.002	6.68 ± 0.18	Sumatriptan	Gi1	0.021 ± 0.004	
	Gi2	0.054 ± 0.016	7.05 ± 0.18		Gi2	0.018 ± 0.005	4.92 ± 0.63
	Gi3	0.032 ± 0.003	6.77 ± 0.12		Gi3	0.013 ± 0.007	
	Gz	0.053 ± 0.015	6.70 ± 0.19		Gz	0.036 ± 0.004	4.81 ± 0.08
	GoA	0.050 ± 0.006	6.57 ± 0.10		GoA	0.106 ± 0.019	7.88 ± 0.15
	GoB	0.025 ± 0.004	6.97 ± 0.09		GoB	0.071 ± 0.010	8.13 ± 0.14
Buspirone	Gi1	0.011 ± 0.003	7.20 ± 0.20	Vilazodone	Gi1	0.043 ± 0.004	6.97 ± 0.08
	Gi2	0.017 ± 0.005	7.33 ± 0.19		Gi2	0.045 ± 0.004	5.75 ± 0.12
	Gi3	0.012 ± 0.007	6.66 ± 0.87		Gi3	0.039 ± 0.006	7.34 ± 0.19
	Gz	0.024 ± 0.006	6.85 ± 0.18		Gz	0.061 ± 0.005	5.81 ± 0.13
	GoA	0.032 ± 0.009	5.88 ± 0.21		GoA	0.053 ± 0.018	4.94 ± 0.18
	GoB	0.020 ± 0.005	5.76 ± 0.28		GoB	0.050 ± 0.010	5.02 ± 0.22
Aripiprazole	Gi1			Vortioxetine	Gi1	0.049 ± 0.009	4.86 ± 0.14
	Gi2	0.011 ± 0.003	4.03 ± 0.82		Gi2	0.037 ± 0.008	4.98 ± 0.30
	Gi3	0.013 ± 0.001			Gi3	0.031 ± 0.013	5.08 ± 1.02
	Gz	0.020 ± 0.002			Gz	0.048 ± 0.009	5.14 ± 0.20
	GoA	0.078 ± 0.019	6.77 ± 0.14		GoA	0.077 ± 0.014	6.27 ± 0.11
	GoB	0.097 ± 0.008	6.93 ± 0.12		GoB	0.081 ± 0.017	7.77 ± 0.21
Flibanserin	Gi1	0.061 ± 0.003	5.96 ± 0.38	F-15599	Gi1	0.060 ± 0.008	7.24 ± 0.14
	Gi2	0.054 ± 0.013	6.13 ± 0.23		Gi2	0.066 ± 0.003	5.71 ± 0.10
	Gi3	0.059 ± 0.010	6.08 ± 0.19		Gi3	0.056 ± 0.002	7.76 ± 0.11
	Gz	0.070 ± 0.008	5.91 ± 0.32		Gz	0.060 ± 0.004	5.67 ± 0.13

the six Gα proteins by each agonist (Fig. 4). For each agonist analyzed we generated 15 direct comparisons highlighting their specific preference toward the activation of individual G protein subtypes. This is extremely relevant because even Gα proteins sharing high degree of homology can activate unique intracellular effectors generating distinct cellular responses. Indeed, studies on individual Gα protein knock out animals frequently revealed a lack of compensating mechanisms, supporting this notion (Jiang et al., 2001; Leck et al., 2004; van den Buuse et al., 2007; Jiang and Bajpayee, 2009; Muntean et al., 2021). More specifically, several studies on 5-HT1AR implicate the activation of such alternative signaling cascades both in vitro and in vivo. For instance, stimulation of 5-HT1AR in rat pituitary GH4C1 cells showed that $G\alpha_o$, but not $G\alpha_i$, inhibits calcium channels without affecting adenylyl cyclase (AC) function (Liu et al., 1994, 1999). On the other hand, 5-HT1AR activation in Chinese hamster ovary cells (CHO) transfected cells showed that $G\alpha_{i2}$ and $G\alpha_{i3}$ can activate Na^+/H^+ exchangers while $G\alpha_{i1}$, $G\alpha_o$, and $G\alpha_z$ cannot (Garnovskaya et al., 1997). In another study using antisense knock down of Gα proteins in CHO cells, it was demonstrated a preferential coupling of 5-HT1AR with $G\alpha_{i3}$ rather than $G\alpha_{i2}$ in suppressing cAMP levels (Rauly-Lestienne et al., 2011). Additionally, in vivo data revealed that treating rats with pertussis toxin, which inactivate all $G\alpha_{i/o}$ family members with the exception of $G\alpha_z$, followed by treatments with 5-HT1AR agonist 8-OH-DPAT, elevated adrenocorticotropic hormone and oxytocin levels compared with control rats, while an antisense-induced decrease in hypothalamic $G\alpha_z$ levels dramatically inhibited oxytocin and adrenocorticotropic hormone responses to 8-OH-DPAT (Serres et al., 2000). Finally, studies on G protein control over individual AC isoforms established unique patterns of inhibition by each $G\alpha_{i/o/z}$ family member (Sadana and Dessauer, 2009; Ostrom et al., 2022). Altogether, the activation of unique effectors downstream of highly similar Gα proteins could generate signaling bias explaining distinctive cellular responses.

Region- and cell-specific patterns of expression of Gα protein subunits may also lead to differential cellular responses induced by activation of the same receptor. In this context, it has been shown that in the brain, 5-HT1A exists as an autoreceptor in serotonergic neurons of the raphe nuclei and as a heteroreceptor in cortical and hippocampal regions (Altieri et al., 2013; You et al., 2016). In ex vivo experiments, 8-OH-DPAT failed to inhibit forskolin-induced cAMP accumulation in serotonergic neurons in the dorsal raphe nucleus, while buspirone effectively showed a concentration-dependent inhibition of cAMP accumulation (Valdizan et al., 2010) confirming previous reports (Clarke et al., 1996; Johnson et al., 1997). Importantly, while buspirone acts as a full agonist on autoreceptors inhibiting the synthesis of 5-HT and neuronal firing, it behaves as a partial agonist at postsynaptic receptors in cortex and hippocampus (Shireen and Haleem, 2005). On the contrary, it was shown that the compound F-15599 preferentially acts on postsynaptic receptors located in the frontal cortex over somatodendritic 5-HT1A autoreceptors (Newman-Tancredi et al., 2009; Lladó-Pelfort et al., 2010). Slope values of concentration-responses curves differed between frontal cortex and raphe nuclei suggesting an underlying mechanism that relies on the preferential activation of selected Gα proteins in different brain regions (Valdizán et al., 2010; Newman-Tancredi et al., 2022). In this context, our data showed that F-15599

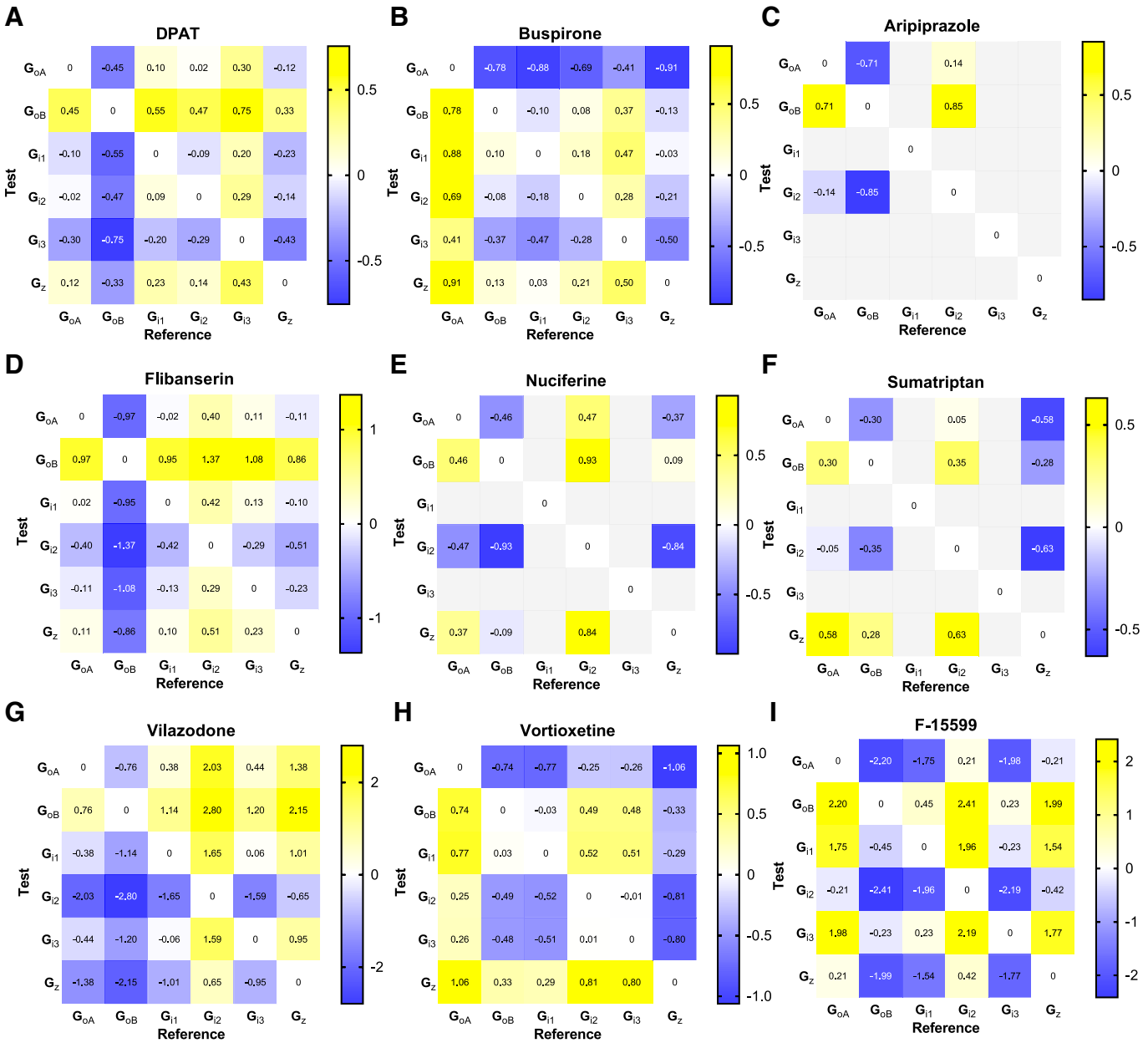

Fig. 4. Heatmaps of the bias factor for structurally diverse agonists with different Gα proteins subtypes. For each agonist, the E_{max}/EC50 values were normalized to the E_{max}/EC50 value of the reference ligand 5-HT. Positive values indicate a bias toward the tested Gα protein, while negative values represent a bias toward the reference Gα protein. Each panel represent an individual agonist: (A) 8-OH-DPAT (B) buspirone (C) aripiprazole (D) flibanserin (E) nuciferine (F) sumatriptan (G) vilazodone (H) vortioxetine, and (I) F-15599. Color-coded scale reported to the right of each panel (n = 5–10 independent replicates).

is biased to $G\alpha_{oB}$, $G\alpha_{i1,}$ and $G\alpha_{i3}$, while buspirone is biased to $G\alpha_{o}$ and $G\alpha_{i2}$ suggesting that these two agonists may mediate distinctive effects at presynaptic and postsynaptic sites because of their intrinsic Gα protein bias. Altogether, these results suggest the existence of a Gα protein-dependent ligand bias at 5-HT1A auto- and hetero-receptors in native brain tissue.

Agonist bias toward selected G protein subfamilies can result in distinguished and unique cellular responses involved in the therapeutic outcome as well as side effects. For instance, it was demonstrated that $G\beta\gamma$ proteins released by $G\alpha_{o}$ preferentially activate GIRK channels compared with $G\alpha_{i}$ (Zhang et al., 2002; Sadja and Reuveny, 2009; Anderson et al., 2020). As a consequence, drugs that are biased to $G\alpha_{o}$ could impact the activation of these channels, which could lead to unique physiologic or pathologic responses. Our data shows that aripiprazole and sumatriptan activation of 5-HT1AR is biased toward $G\alpha_{o}$ compared with $G\alpha_{i}$. This may result in unique properties of these drugs, including adverse effects that involve the activation GIRK channels.

Overall, a full understanding of the molecular implications of ligand bias toward Gα protein subtypes will require further investigations at multiple receptors. This information will possibly address conflicting observations obtained both in vitro and

in vivo and will guide drug development toward safer and more effective therapeutics.

Data Availability

All processed data that support the findings of this study are available within the paper and its Supplemental Material. Raw data are available from the corresponding author upon request.

Authorship Contributions

Participated in research design: Alabdali, Franchini, Orlandi.

Conducted experiments: Alabdali, Franchini.

Contributed new reagents or analytic tools: Alabdali, Franchini.

Performed data analysis: Alabdali.

Wrote or contributed to the writing of the manuscript: Alabdali, Orlandi.

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