Gα Protein Signaling Bias at Serotonin 1A Receptor

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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 Gαi/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 Gαi/o/z family members. Our concentration-response analysis of the activation of each Gαi/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 Gαz that was unique among the agonists tested: we found a biased factor of +2.19 when comparing the activation of Gαz versus Gα13 by F-15599, while it was -0.29 for 8-hydroxy-2-(di-n-propylamino)tetrahydroindole. Similarly, vortioxetine showed a biased factor of +1.06 for Gαz versus Gα13, 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 therapeutically relevant cellular outcomes.

SIGNIFICANCE STATEMENT

Serotonin 1a receptor (5-HT1AR) activates several members of the Gαi/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, Gαs-biased compounds activating β2-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)tetrahydroindole; AC, adenylyl cyclase; BRET, bioluminescence resonance energy transfer; CHO, Chinese hamster ovary cells; Emax, 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 Gz proteins that are classified into four subfamilies according to their sequence homology: Gzα, Gzq, Gz12/13, and Gz12/13 (Wett-Church 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; SvoB Strac 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 is expressed in many central and peripheral physiologic functions including sleep, pain, temperature homeostasis, processing of emotions, and response to stress (Bjorvain and Ursin, 1998; Polter and Li, 2010; Garcia-Garcia et al., 2014; Albert and Vahid-Ansari, 2019; Haleem, 2019; Razekarivony et al., 2021; Voronova, 2021; Pohrson 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 exclusively sensitive to pertussis toxin suggesting coupling to Gz12/13 family members with minimal activity toward members of the Gzα, Gzq, and Gz12/13 protein families (Raymond et al., 1999; Kooistra et al., 2021; Pandyszekeres et al., 2022). 5-HT1AR preferentially couples to members of the numerous Gz12/13 and several natural and synthetic agonists are available, this receptor provides an excellent model to explore the potential Gz 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 Gz proteins remains obscure. Here, we investigate the activation bias in response to ten structurally diverse 5-HT1AR agonists by obtaining their unique profiles of Gz protein activation using an optimized cell-based bioiminescence resonance energy transfer (BRET) assay.

Materials and Methods

Cell Cultures and Transfections. Human embryonic kidney 293T cell line (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% CO2. Cells were routinely monitored for possible mycoplasma contamination. Two million cells were seeded in each well of 96-well plates in medium without antibiotics for 4 hours and then transfected with a mixture of 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). G1/1-venus156-230 and G2/2-venus1-152 were generous gifts from Dr. Nevin A. Lambert (Augusta University, Augusta GA). Gz 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-14281), vortioxetine (HY-15414A), sumatriptan succinate (HY-B0121), nicotine (HY-N0049), ibiperone (HY-A0056), aripiprazole (HY-14546), buspirone (HY-B1115), F-15598 (HY-19862), and 8-OH-DPAT (HY-15688). 5-HT hydrochloride was purchased from Tocris (55475/50). 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 MgCl2 and 0.1% glucose), collected in 1.5 ml tubes, and centrifuged for 5 minutes at 500 x 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 G1/1-2-venus (collected using the emission filter 535/530) to the light emitted by masGRK3CT-Nluc (475/530). 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 (Emax). Importantly, we excluded Hill slopes 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 Gz protein bias, we adopted the equation from (Winpenny et al., 2016). Briefly, we first calculated the mean Log(Emax/E50) for each agonist; then, we calculated the ΔLog(Emax/E50) normalized to the reference agonist, in this case 5-HT:

\[ \Delta \text{Log} = \log \left( \frac{E_{\text{max}}}{E_{50}} \right)_{\text{test}} - \log \left( \frac{E_{\text{max}}}{E_{50}} \right)_{\text{reference}} \]  

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

\[ \Delta \Delta \text{Log} = \Delta \log \left( \frac{E_{\text{max}}}{E_{50}} \right)_{\text{pathway 1}} - \Delta \log \left( \frac{E_{\text{max}}}{E_{50}} \right)_{\text{pathway 2}} \]

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
5-HT1AR Uniquely Activates Heterotrimeric G<sub>i/o/z</sub> 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 G<sub>z</sub> protein family (G<sub>x<sub>o</sub></sub>, G<sub>x<sub>a</sub></sub>, G<sub>x<sub>c</sub></sub>, G<sub>x<sub>15</sub></sub>, and G<sub>x<sub>13</sub></sub>) 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, G<sub>z</sub> dissociates from G<sub>β</sub><sub>γ</sub>-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<sub>x<sub>o</sub></sub>, while we did not observe any activation of G<sub>x<sub>a</sub></sub>, G<sub>x<sub>c</sub></sub>, G<sub>x<sub>15</sub></sub>, and G<sub>x<sub>13</sub></sub> (Fig. 1B). With the goal of measuring the activation of each member of the G<sub>i/o/z</sub> family, we then optimized the stoichiometry of each expressed G<sub>z</sub> and G<sub>β</sub><sub>γ</sub>-venus by titrating the amount of transfected G<sub>z</sub> subunits against a constant amount of transfected G<sub>β</sub><sub>γ</sub>-venus. As expected, by increasing the amount of transfected G<sub>z</sub>, we observed a reduction in the basal activity; in fact, suboptimal expression of G<sub>z</sub> allows free G<sub>β</sub><sub>γ</sub>-venus to interact with GRK3CT-Nluc, increasing the BRET signal detected in the absence of GPCR stimulation. Therefore, optimal G<sub>x</sub>:G<sub>β</sub><sub>γ</sub> ratios were selected based on low basal BRET ratio and high maximal amplitude for each G<sub>i/o/z</sub> protein (Supplemental Fig. 1).

G<sub>z</sub> 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<sub>x<sub>o</sub></sub> over G<sub>x<sub>a</sub></sub> and G<sub>x<sub>c</sub></sub> (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<sub>i/o/z</sub> protein family. Then, we quantitatively measured G 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<sub>x<sub>a</sub></sub> showed the highest amplitude which represents the highest efficacy measured as ΔBRET ratio followed by G<sub>x<sub>13</sub></sub>, G<sub>x<sub>15</sub></sub>, G<sub>x<sub>o</sub></sub>, and G<sub>x<sub>c</sub></sub> (Fig. 2A). For the purpose of evaluating potency and efficacy, we conducted concentration-response studies for each G<sub>z</sub> protein in response to increasing concentrations of 5-HT ranging from 2 µM to 100 µM (Fig. 2B). Our analysis of potency and efficacy, measured as maximal ΔBRET ratio, suggest a preferential activation of G<sub>x<sub>13</sub></sub> over G<sub>x<sub>15</sub></sub> (Fig. 2, C and D; Supplemental Table 1). However, caveats due to possible differences in individual G<sub>z</sub> protein properties, such as protein expression levels, endogenous expression of specific regulatory proteins (i.e., RGS proteins), or efficiency in terms of releasing G<sub>β</sub><sub>γ</sub>-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 G<sub>z</sub> proteins produced the highest ΔBRET ratio within one minute, with the exception of G<sub>x<sub>15</sub></sub> which still reached its highest amplitude within three minutes (Fig. 2, E and F; Supplemental Fig. 2).

Structurally Diverse 5-HT1AR Agonists Produce Different G<sub>z</sub> Protein Coupling Profiles. We hypothesized that structurally diverse 5-HT1AR agonists could elicit a ligand bias at the G<sub>z</sub> 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 G<sub>β</sub><sub>1/2</sub>-venus without 5-HT1AR. As expected, while we could measure in real time the activation of individual G<sub>i/o/z</sub> protein by 5-HT1AR (Supplemental Fig. 4A), we did not observe any signal in mock-transfected cells (Supplemental Fig. 4B).}

![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 G proteins, this system was developed by fusing the donor luciferase with an effector of G<sub>β</sub><sub>γ</sub> (masGRK3CT-ntlc), and a split acceptor, venus, fused with G<sub>β/1</sub> and G<sub>β/2</sub> subunits, allowing the use of wild type G<sub>z</sub> subunits. (B) Representative traces obtained by applying 100 µM 5-HT on HEK293 cells transfected with 5-HT1AR and each one of G<sub>x</sub>, G<sub>x<sub>a</sub></sub>, G<sub>x<sub>c</sub></sub>, G<sub>x<sub>15</sub></sub>, and G<sub>x<sub>13</sub></sub> 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.](image-url)
coef- cacy and potency (Fig. 3, B and C), we evaluated a transduction
5-HT1AR. A formal analysis of signaling bias revealed the ex-
istence of two groups of 5-HT1AR agonists. After examining the
very high concentrations (Fig. 3

We calculated ef-
from 125% to 180% when compared with the endogenous li-
dern the physiologic ligand 5-HT as a reference, we showed that
be more effective and safer therapeutics (Gurevich and Gure-
last two decades, a great deal of effort has been put
found that individual ago-
cluster analysis (Fig. 3D). Accordingly, our data revealed two
groups of agonists: 1) 5-HT, F-15599, vilazodone, buspirone,
8-OH-DPAT, and fibanserin; and 2) vortioxetine, sumatriptan,
nuciferine, and aripiprazole. Finally, with a Hill slope proximal
to 1, we evaluated the bias factor, expressed as a ΔΔLog(Emax/
EC50), produced by each agonist across Gz proteins after nor-
malization 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 Gz protein over a reference
Gz (positive values), or the opposite (negative values). This
set of data revealed for example that vilazodone, 8-OH-DPAT,
and fibanserin have a bias toward Gz2, while vortioxetine prefers Gz2
activation over Gz3. On the contrary, F-15599 and fibanserin
reveal mixed activation between Gz and Gz. Both agonists
show preference activation toward Gz2,2 and Gz2, and Gz (Fig. 4).
Collectively, we found that individual ago-
nists showed unique Gz 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 Gz 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 Gure-
vich, 2020). However, a limited number of studies investi-
gated signaling bias within Gz 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. 2. Pharmacological characterization of 5-HT1AR. (A) Representative ΔBRET ratio responses after application of 100 μM 5-HT for each Gz].

We 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 consid-
ered as full agonists. Interestingly, some agonists showed super
agonistic properties for certain G proteins compared with the reference
lignand. For instance, fentanyl, F-15599, 8-OH-D-
PAT, and vilazodone showed Gz1 maximum response ranging
from 125% to 180% when compared with the endogenous li-
gand 5-HT. Overall, the application of six independent read-
outs, one for each Gz 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, fibanserin, buspirone, and 8-OH-DPAT across Gz proteins.
Interestingly, we also found that some agonists were
not able to activate specific Gz proteins: for example, aripipra-
zole could not activate Gz3, while Gz3 was only activated at
very high concentrations (over 100 μM). Meanwhile, sumatrip-
tan activates Gz0 but it only showed activation to G1 at ex-
tremely high concentrations (Fig. 3; Supplemental Fig. 5).

Ligand-Dependent Gz Protein Signaling Bias at
5-HT1AR. A formal analysis of signaling bias revealed the ex-
istence of two groups of 5-HT1AR agonists. After examining the
signaling properties of the different ligands and calculating effi-
cacy and potency (Fig. 3, B and C), we evaluated a transduction
coefficient as Log(Emax/EC50) for each agonist (Fig. 3D). To com-
prehensively analyze similarities and differences among Gz
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 fibanserin; and 2) vortioxetine, sumatriptan,
nuciferine, and aripiprazole. Finally, with a Hill slope proximal
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Using these data, we generated paired comparisons that
suggest biased activation of a test Gz protein over a reference
Gz (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
Gz2,2 over Gz1,3 and Gz2, while vortioxetine prefers Gz2,2 ac-
tivation over Gz3. On the contrary, F-15599 and fibanserin
reveal mixed activation between Gz and Gz. Both agonists
show preference activation toward Gz2,2 and Gz2,2 and Gz (Fig. 4).
Collectively, we found that individual ago-
nists showed unique Gz protein activation profiles.
the action of structurally diverse 5-HT1AR agonists can be biased toward different Gαi/o/z family members. From the concentration-response curves obtained for ten 5-HT1AR agonists we were able to estimate EC50 and Emax 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 Gαi1, as a full agonist in the activation of GαoA, Gαi2, Gαi3, and Gαz, while it is a partial agonist in the activation of GαoB.

From these data, we calculated a bias factor that was later normalized over 5-HT to compare the relative activation of...
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 G 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; 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 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; 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 proteins could mediate selective activation of signaling pathways in the brain, which might explain the preference of 5-HT1AR agonists in particular brain regions (Valdizán et al., 2010; Newman-Tancredi et al., 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.

### TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Gα protein</th>
<th>Emax ± S.D.</th>
<th>pEC50 ± S.D.</th>
<th>Compound</th>
<th>Gα protein</th>
<th>Emax ± S.D.</th>
<th>pEC50 ± S.D.</th>
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<tr>
<td>GoA</td>
<td>0.082 ± 0.003</td>
<td>7.20 ± 0.15</td>
<td>4.78 ± 0.18</td>
<td>GoA</td>
<td>0.048 ± 0.010</td>
<td>4.82 ± 0.39</td>
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<tr>
<td>GoB</td>
<td>0.061 ± 0.015</td>
<td>6.59 ± 0.46</td>
<td>4.90 ± 0.66</td>
<td>GoA</td>
<td>0.040 ± 0.007</td>
<td>5.17 ± 0.20</td>
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<td>5-HT</td>
<td>0.045 ± 0.002</td>
<td>6.52 ± 0.07</td>
<td>4.75 ± 1.15</td>
<td>GoA</td>
<td>0.030 ± 0.003</td>
<td>5.72 ± 1.80</td>
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</tr>
<tr>
<td>Gi1</td>
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<td>6.86 ± 0.09</td>
<td>4.81 ± 0.08</td>
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<td>4.92 ± 0.63</td>
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<tr>
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<td>4.98 ± 0.18</td>
<td>Gi2</td>
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<td>4.90 ± 0.66</td>
<td></td>
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<tr>
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<td>6.36 ± 0.06</td>
<td>5.75 ± 0.12</td>
<td>Gz</td>
<td>0.036 ± 0.006</td>
<td>5.17 ± 0.20</td>
<td></td>
</tr>
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<td>GoA</td>
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<td>7.35 ± 0.20</td>
<td>5.72 ± 0.14</td>
<td>GoA</td>
<td>0.106 ± 0.019</td>
<td>7.88 ± 0.15</td>
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<tr>
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<td>0.070 ± 0.008</td>
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<td>5.81 ± 0.13</td>
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<td>0.071 ± 0.010</td>
<td>8.13 ± 0.14</td>
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</tr>
<tr>
<td>8-OH-DPAT</td>
<td>0.032 ± 0.002</td>
<td>6.68 ± 0.18</td>
<td>5.81 ± 0.13</td>
<td>GoA</td>
<td>0.016 ± 0.009</td>
<td>4.98 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>Gi1</td>
<td>0.054 ± 0.016</td>
<td>7.05 ± 0.18</td>
<td>5.91 ± 0.32</td>
<td>GoA</td>
<td>0.040 ± 0.017</td>
<td>6.97 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Gi2</td>
<td>0.032 ± 0.003</td>
<td>6.77 ± 0.12</td>
<td>6.76 ± 0.18</td>
<td>Gi2</td>
<td>0.045 ± 0.004</td>
<td>5.75 ± 0.12</td>
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</tr>
<tr>
<td>Gi3</td>
<td>0.053 ± 0.015</td>
<td>6.70 ± 0.19</td>
<td>6.85 ± 0.18</td>
<td>Gi3</td>
<td>0.039 ± 0.006</td>
<td>7.34 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>GoA</td>
<td>0.050 ± 0.006</td>
<td>6.67 ± 0.10</td>
<td>6.85 ± 0.18</td>
<td>Gi3</td>
<td>0.061 ± 0.005</td>
<td>5.81 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>GoB</td>
<td>0.025 ± 0.004</td>
<td>6.97 ± 0.09</td>
<td>7.05 ± 0.18</td>
<td>GoB</td>
<td>0.053 ± 0.018</td>
<td>4.94 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>Buspirone</td>
<td>0.011 ± 0.003</td>
<td>7.20 ± 0.20</td>
<td>7.05 ± 0.18</td>
<td>GoB</td>
<td>0.050 ± 0.010</td>
<td>5.02 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Gz</td>
<td>0.024 ± 0.006</td>
<td>6.85 ± 0.18</td>
<td>7.14 ± 0.12</td>
<td>Gz</td>
<td>0.053 ± 0.018</td>
<td>4.94 ± 0.18</td>
<td></td>
</tr>
<tr>
<td>GoA</td>
<td>0.032 ± 0.009</td>
<td>5.88 ± 0.21</td>
<td>7.14 ± 0.12</td>
<td>GoA</td>
<td>0.053 ± 0.018</td>
<td>4.94 ± 0.18</td>
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</tr>
<tr>
<td>GoB</td>
<td>0.020 ± 0.005</td>
<td>5.76 ± 0.28</td>
<td>7.14 ± 0.12</td>
<td>GoB</td>
<td>0.050 ± 0.010</td>
<td>5.02 ± 0.22</td>
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</tr>
<tr>
<td>Aripiprazole</td>
<td>0.011 ± 0.003</td>
<td>4.03 ± 0.82</td>
<td>7.05 ± 0.18</td>
<td>Aripiprazole</td>
<td>0.013 ± 0.001</td>
<td>4.98 ± 0.30</td>
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<tr>
<td>Gi2</td>
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<td>4.03 ± 0.82</td>
<td>7.05 ± 0.18</td>
<td>Gi2</td>
<td>0.037 ± 0.008</td>
<td>4.98 ± 0.30</td>
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</tr>
<tr>
<td>Gz</td>
<td>0.020 ± 0.002</td>
<td>4.03 ± 0.82</td>
<td>7.05 ± 0.18</td>
<td>Gz</td>
<td>0.031 ± 0.013</td>
<td>5.08 ± 1.02</td>
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</tr>
<tr>
<td>GoA</td>
<td>0.078 ± 0.019</td>
<td>6.77 ± 0.14</td>
<td>7.05 ± 0.18</td>
<td>GoA</td>
<td>0.048 ± 0.009</td>
<td>5.14 ± 0.20</td>
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</tr>
<tr>
<td>GoB</td>
<td>0.097 ± 0.008</td>
<td>6.93 ± 0.12</td>
<td>7.05 ± 0.18</td>
<td>GoB</td>
<td>0.077 ± 0.014</td>
<td>6.27 ± 0.11</td>
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<tr>
<td>Flibanserin</td>
<td>0.061 ± 0.003</td>
<td>5.96 ± 0.38</td>
<td>7.24 ± 0.14</td>
<td>F-15599</td>
<td>0.060 ± 0.008</td>
<td>7.24 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Gi1</td>
<td>0.054 ± 0.013</td>
<td>6.13 ± 0.23</td>
<td>7.24 ± 0.14</td>
<td>Gi1</td>
<td>0.060 ± 0.008</td>
<td>7.24 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Gi3</td>
<td>0.059 ± 0.010</td>
<td>6.08 ± 0.19</td>
<td>7.24 ± 0.14</td>
<td>Gi3</td>
<td>0.056 ± 0.002</td>
<td>7.76 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>GoA</td>
<td>0.070 ± 0.008</td>
<td>5.91 ± 0.32</td>
<td>7.24 ± 0.14</td>
<td>GoA</td>
<td>0.060 ± 0.004</td>
<td>5.67 ± 0.13</td>
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The table indicates that the preferential coupling of 5-HT1AR with Gα proteins in vivo data revealed that treating rats with pertussis toxin, which inactivate Na+/H+ exchangers while Gαi1, Gαi2, and Gαi3 cannot (Garnovskaya et al., 1997). In another study using antisense approach, knock down of Gα proteins in CHO cells, it was demonstrated a preferential coupling of 5-HT1AR with Gαi2 rather than Gαi3 in suppressing cAMP levels (Rauly-Lestienne et al., 2011). Additionally, in vivo data revealed that treating rats with pertussis toxin, which inactivate all Gα family members with the exception of Gαi2, followed by treatments with 5-HT1AR agonist 8-OH-DPAT, elevated adrenocorticotrophic hormone and oxytocin levels, while an antisense-induced decrease in hypothalamic Gα2 levels dramatically inhibited oxytocin and adrenocorticotrophic 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 Gα 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.
is biased to Gα11 and Gα13, while buspirone is biased to Gαi and Gαi2, 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 Gβγ proteins released by Gαi preferentially activate GIRK channels compared with Gαi (Zhang et al., 2002; Sadja and Reuveny, 2009; Anderson et al., 2020). As a consequence, drugs that are biased to Gαi 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 Gαo compared with Gαi. 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

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**Fig. 4.** Heatmaps of the bias factor for structurally diverse agonists with different Gα proteins subtypes. For each agonist, the Emax/EC50 values were normalized to the Emax/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) filbanserin (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).
van den Buuse M, Martin S, Holgate J, Matthaei K, and Hendry I (2007) Mice deficient in the alpha subunit of Gα(z) show changes in pre-pulse inhibition, anxiety and responses to 5-HT(1A) receptor stimulation, which are strongly dependent on the genetic background. Psychopharmacology (Berl) 195:273–283.

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