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Identification of psychedelic new psychoactive substances (NPS) showing biased agonism at the 5-HT_{2A}R through simultaneous use of β-arrestin 2 and miniGα_q bioassays

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Abstract

Psychedelic new psychoactive substances (NPS), compounds exerting their main pharmacological effects through the activation of the serotonin 2A receptor (5-HT_{2A}R), continuously comprise a substantial portion of the reported NPS. However, these substances and their exact mechanism of action, differentiating them from non-psychedelic 5-HT_{2A}R agonists, require further characterization. One potentially relevant phenomenon is the occurrence of biased agonism, in which (a) certain signaling pathway(s) is preferentially activated over the other(s). To this end, a new bioassay was developed, monitoring the recruitment of an engineered mini$G\alpha_q$ protein to the activated 5-HT_{2A}R. The setup was designed to be analogous to that of a previously developed bioassay monitoring β-arrestin 2 recruitment through the NanoBiT system, enabling estimation of the potential preference of a substance to trigger recruitment of one protein over the other. This approach yielded several statistically significantly biased agonists within the group of phenylalkylamine psychedelics, more specifically the *N*-benzyl substituted 25H analogues 25H-NBF, 25H-NBMD, 25H-NBOH and 25H-NBOMe. All four compounds show a statistically significant preference towards the recruitment of β-arrestin 2 over mini$G\alpha_q$, as compared to the reference psychedelic substance LSD. We identified markedly different responses for Bromo-DragonFLY in the two bioassays, suggesting biased agonism, though the calculated bias factor equalled out to approximately 0. This demonstrates that the accurate assessment of biased agonism requires both the consideration of the observed trends in addition to the numerical value of the bias factor. A second panel of structural (I-substituted) analogues of the former group of phenylalkylamines showed a similar trend in the ranking order of the bias factors, resulting in one additional compound (25I-NBF) being statistically significantly biased.

Keywords: psychedelics, hallucinogens, biased agonism, G protein-coupled receptor, serotonin 2A receptor

1. Introduction

The serotonin (5-HT) 2A receptor (5-HT_{2A}R) is a G protein-coupled receptor (GPCR) with roles in a wide variety of processes, such as mood, learning, memory, sleep-wake cycles and appetite. The 5-HT_{2A}R receptor is expressed mainly in platelets, the gastro-intestinal tract and the central nervous system, and is involved in several disorders with rather complex etiologies, e.g. depression, eating disorders and schizophrenia.[1, 2] In order to fulfill this multitude of functions, the 5-HT_{2A}R is linked to a variety of downstream signaling pathways via the recruitment of a number of cytosolic proteins, amongst which the canonical $G\alpha_q$ protein and the scaffolding protein β-arrestin 2 (βarr2).[1, 3-6]

Besides exerting physiological and pathological functions, the 5-HT_{2A}R also plays an important role in the mechanism of action of serotonergic psychedelics, substances defined to exert their main pharmacological effects through this receptor.[3] This group of substances can be divided into three structural subclasses: ergolines (with lysergic acid diethylamide (LSD) as prototypical representative), tryptamines (e.g. DMT) and psychedelic phenylalkylamines (such as the naturally occurring mescaline).[7] Apart from the more 'conventional' psychedelics, over the past years a large variety of psychedelic NPS (new psychoactive substances) has been reported: compounds mimicking the effects of e.g. LSD, while being structurally distinct. Psychedelic NPS have been a consistently abundant class within the group of newly detected NPS reported to the United Nations Office on Drugs and Crime (UNODC).[8-11]

The effects sought for by users of psychedelic substances include altered states of consciousness, empathic feelings and mystical experiences. However, their use may also result in severe adverse reactions, amongst which agitation, headaches, convulsions, renal failure, rhabdomyolysis and even death, specifically with the more recent group of NPS.[4, 11, 12] Besides the effects that make these substances attractive to illicit drug users, serotonergic

psychedelics are increasingly recognized for having therapeutically interesting properties. Clinical trials with mainly LSD and psilocybin, in the context of treating addictions and ameliorating distress concerning death, invigorate this hypothesis, and psychedelic substances have been suggested as therapeutically useful in other (psychiatric) conditions as well.[13-15]

Even though the group of psychedelic substances is highly relevant from both a therapeutic and a substance abuse perspective, the precise mechanism of action that distinguishes them from non-psychedelic 5-HT_{2A}R agonists remains elusive.[5, 6, 15] Proposed mechanisms include the activation of distinct signaling events, the occurrence of biased agonism, the binding of these substances to other GPCRs, and the involvement of GPCR dimerization.[4-6, 16] Biased agonism or functional selectivity can be defined as the differential activation of signaling pathways by distinct ligands at the same GPCR. When pathways can be linked to respective effects, this can enable the development of pharmaceuticals with more beneficial therapeutic properties.[17-19] The assessment of this phenomenon is greatly hampered by the difficulty to comparatively interpret the results obtained with distinct assays. More specifically, applied assays can differ in terms of systems used for measuring (readout method, saturability of the system, potential temporal bias, distinct expression levels and the use of native versus modified receptor or transducer constructs), and in cell context (with possibly different expression levels, and co-expression of GPCR isoforms and off-target proteins).[20, 21]

This study is the first to use two distinct yet highly analogous *in vitro* bioassays to systematically investigate the potentially biased agonism of a set of structurally diverse psychedelic NPS, representing both the tryptamine and phenylalkylamine subgroup. To this end, the recruitment of either βarr2 or an engineered mini$G\alpha_q$ protein to the activated 5-HT_{2A}R was monitored.[22] This latter protein consists of the thermodynamically stabilized Ras domain of the $G\alpha_q$ subunit.[23-25] The recruitment of both proteins can be monitored via a luminescent readout, obtained through the NanoBiT® system, in HEK 293T cells transiently transfected with

the concerned constructs. This simultaneous monitoring in highly similar systems, representing signaling events upstream of the signaling cascade, allowed for the assessment of biased agonism of these compounds, via the intrinsic relative activity approach.[18, 19, 26] It is, to the best of our knowledge, the first time a βarr2 recruitment assay is used for the assessment of biased agonism in a large set of psychedelic substances at the 5-HT_{2A}R on a molecular level.

2. Materials and methods

2.1 Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM, supplemented with GlutaMAX®), Hank's Balanced Salt Solution (HBSS), Phusion High Fidelity PCR Master Mix, restriction enzyme EcoRI, Amphotericin B (250 µg/mL) and penicillin/streptomycin (10 000 IU/mL and 10 000 µg/mL) were procured from Thermo Fisher Scientific (Pittsburg, PA, USA). The plasmid containing mini$G\alpha_q$ was a kind gift of Dr. A. Chevigné (Luxembourg Institute of Health). Transfection reagent FuGENE®, thermosensitive alkaline phosphatase (TSAP) and the Nano-Glo Live Cell reagent and LCS Dilution buffer were from Promega (Madison, WI, USA). The analytical standard of LSD (lysergic acid diethylamide), Fetal Bovine Serum (FBS) and poly-D-lysine hydrobromide were purchased from Sigma-Aldrich (Overijse, Belgium). The Human Embryonic Kidney (HEK) 293T cells (passage 20) were kindly gifted by Prof. O. De Wever (Ghent University Hospital, Belgium). The analytical standards of Mescaline HCl (3,4,5-trimethoxy-benzeneethanamine); 2C-H HCl (2,5-dimethoxy-benzeneethanamine); DOH HCl (*DL*-2,5-dimethoxyamphetamine); 25H-NBOH HCl (2-[[[2-(2,5-dimethoxyphenyl)ethyl]amino]methyl]-phenol); 25H-NBOMe HCl (2-(2,5-dimethoxyphenyl)-*N*-(2-methoxybenzyl)ethanamine); 25H-NBF HCl (2-(2,5-dimethoxyphenyl)-*N*-(2-fluorobenzyl)ethanamine); 25H-NBMD HCl (*N*-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(2,5-dimethoxyphenyl)ethanamine); N-Me-2C-H HCl (2,5-Dimethoxy-*N*-methylphenethylamine);

25I-NBOH HCl (2-(((4-iodo-2,5-dimethoxyphenethyl)amino)methyl)phenol); 2C-B-FLY HCl (8-bromo-2,3,6,7-tetrahydro-benzo[1,2-b:4,5-b']difuran-4-ethanamine) and DOI HCl (*DL*-2,5-dimethoxy-4-iodoamphetamine) were bought from Chiron AS (Trondheim, Norway). DIPT HCl (*N*,*N*-Diisopropyltryptamine), DMT hemifumarate (*N*,*N*-Dimethyltryptamine), DPT HCl (*N*,*N*-Dipropyltryptamine), and Bromo-DragonFLY HCl (8-bromo-α-methyl-benzo[1,2-b:4,5-b']difuran-4-ethanamine) and *R*-Bromo-DragonFLY HCl were kindly gifted by Chiron AS. The analytical standards of 25I-NBMD HCl (*N*-(benzo[d][1,3]dioxol-4-ylmethyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine) and 25I-NBF HCl (*N*-(2-fluorobenzyl)-2-(4-iodo-2,5-dimethoxyphenyl)ethanamine) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The standards of 2C-I HCl (4-iodo-2,5-dimethoxy-benzeneethanamine) and 25I-NBOMe HCl (4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine) were kindly gifted by Prof. K. Maudens, who procured them from the Australian Government National Measurement Institute and Cayman Chemical Company, respectively. The structures of all evaluated compounds are given in Figure 1.

2.2 Cloning of the miniGα_q constructs in the NanoBiT system

Four miniGα_q constructs were generated, N- or C-terminally fused to the NanoBiT subunits (miniGα_q-LgBiT, miniGα_q-SmBiT, LgBiT-miniGα_q and SmBiT-miniGα_q) according to standard cloning procedures, as described previously for the receptor constructs (5-HT_{2A}R-LgBiT and 5-HT_{2A}R-SmBiT).[22] The polymerase chain reaction (PCR) was performed by mixing 2.5 μM of each of the forward and reverse primers (for the former two constructs, the forward primer was ATCCAA*GAATTC*ACCATGATCGAGAAGCAGC and the reverse primer ATCCAA*GAATTC*CCGACGAGGTTGTACTCTCTCAGG, for the latter two ATCCAA*GAATTC*AATGATCGAGAAGCAGC and ATCCAA*GAATTC*TCAGACGAGGTTGTACTCTCTCAGG, respectively; EcoRI restriction sites are underlined) with PCR Mastermix and 100 pg of the template DNA. The three-step

PCR consisted of initial denaturation (98°C, 30 s), denaturation (98°C, 10 s), annealing (66.9°C, 20 s), elongation (72°C, 25 s) and final elongation (72°C, 5 min), of which the three middle steps were repeated 32 times. After purification, the reaction product was digested with the restriction enzyme EcoRI. Three microgram of each of the respective vectors (NB MCS-1 to -4) was digested with the same restriction enzyme, purified, and digested with TSAP. The digested vectors and inserts were ligated using T4 DNA ligase, the products were heat-shock transformed into chemically competent *E.coli*, and plated onto ampicillin-containing agar plates. After growing overnight, colonies containing the aimed construct were selected via PCR, and grown in LB broth. The correctness of the constructs was verified via Sanger sequencing.

2.3 Routine Cell culture

Human embryonic Kidney Cells (HEK 293T) were routinely maintained in DMEM (with GlutaMAX®), supplemented with 10 % heat-inactivated FBS, 100 IU/mL of penicillin, 0.25 µg/mL amphotericin B and 100 µg/mL streptomycin, in a humidified atmosphere of 37 °C and 5 % CO_2. Cells were used until passage 25.

2.4 Transient transfection protocol: selection of the optimal combination and use for compound screening

The βarr2 and mini$G\alpha_q$ recruitment assays in the NanoBiT® system were performed via a transient transfection protocol, as described before.[22, 27] In brief, routinely cultured HEK 293T cells were seeded in 6-well plates, at a density of 500 000 cells per well, and incubated overnight. Subsequently, each well was transfected with 3.3 µg of DNA, consisting of equal quantities of both the receptor and βarr2 or mini$G\alpha_q$ constructs, in a 3:1 FuGENE®:DNA ratio according to the manufacturer's protocol. Twenty-four hours post transfection, the cells were reseeded into poly-D-lysine coated 96-well plates at a density of 50 000 cells per well, and incubated for another 24 h before the readout took place. First, the cells were washed twice with

HBSS, and 100 µL of HBSS was pipetted into each well. Then, 25 µL of the Nano-Glo Live Cell reagent (substrate, diluted 1/20 in Nano-Glo LCS Dilution buffer according to manufacturer's protocol) was added, and the plate was placed in the Tristar^2 LB 942 multimode microplate reader (Berthold Technologies GmbH & Co, Germany). After equilibration of the luminescent signal, the 13.5 x concentrated agonist(s) was (were) added to each well, and the signal was monitored for 2 h.

For the selection of the optimal combination of mini$G\alpha_q$- and 5-HT_{2A}R-constructs in the NanoBiT® system, each of the combinations was transfected and tested at a fixed concentration of 1 µM LSD. In the experiments performed for characterization of the ligand bias of the compounds, all tested compounds were tested at concentrations of (100 µM) – (25 µM) – (10 µM) – 1 µM – 10^{-7} M – 10^{-8} M – 10^{-9} M – 10^{-10} M – 10^{-11} M – (10^{-12} M), in at least three independent experiments, each performed in duplicate. The βarr2 and mini$G\alpha_q$ assays were performed in parallel, using the same dilutions of the compounds for both assays, and LSD was run as a reference agonist on all plates. Appropriate solvent controls were included in all experiments.

2.5 Data processing and statistical analysis

The time-luminescence profiles obtained during the luminescence analysis were corrected for interwell variability and used for the calculation of the area under the curve (AUC), as previously described in more detail.[28] After subtraction of the AUC of the corresponding solvent control, concentration-response curves were generated via GraphPad Prism software (San Diego, CA, USA), using the three-parametric nonlinear regression model. This model implies the use of a Hill slope fixed at 1, which is a requirement to be fulfilled for the implementation of the ligand bias calculation described below.[18, 26] Data points associated with the highest concentration(s) were excluded in case of a reduction of 20 % or more, as

compared to the next (less concentrated) dilution. This 20% cut-off point for exclusion was defined a priori, based on previous experience with multiple receptor systems, and was applied consistently, with as a rationale that (very) high concentrations could potentially lead to cell toxicity or solubility issues, and inclusion of these data points could inadvertently skew the obtained results.[29] For each separate experiment, the data were normalized for the maximal response of the reference agonist LSD, and finally bundled to obtain the total EC_{50} and E_{max} values for each compound.

To quantify the tendency of a compound to induce preferential recruitment of either βarr2 or miniGα_q, the 'intrinsic relative activity' (RA_i) was calculated for each compound in each bioassay.[18, 26] This value defines the ratio of E_{max}/EC_{50} of the tested compound to the E_{max}/EC_{50} ratio of the reference agonist, in this case LSD:

$$RA_{i,\text{ reference agonist}}^{\text{pathway}} = \frac{\frac{E_{\max,i}}{EC_{50,i}}}{\frac{E_{\max,\text{ LSD}}}{EC_{50,\text{LSD}}}} = \frac{EC_{50,\text{ LSD}} \times E_{\max,i}}{E_{\max,\text{LSD}} \times EC_{50,i}}$$

For each individual compound, the obtained RA_i values per pathway were then combined into the bias factor, β_i, using the following formula:[18, 26]

$$\beta_i = \log\left(\frac{RA_{i,\text{ LSD}}^{\beta\text{arr2}}}{RA_{i,\text{ LSD}}^{\text{miniG}\alpha\text{q}}}\right)$$

This formula implies that the bias factor for the reference agonist LSD is equal to 0 (as further discussed in section 3.2). Compounds with a positive value for β have a greater relative tendency towards recruitment of βarr2, while compounds with a negative β tend to favor recruitment of miniGα_q. The statistical significance of the difference of each β from 0 was determined via a Kruskal-Wallis analysis (the non-parametric alternative to one-way ANOVA), with post-hoc Dunn's test in GraphPad Prism. Furthermore, bias plots were generated to

qualitatively assess the extent of ligand bias towards one pathway or the other. The normalized AUC values of the three independent experiments of the miniGα_q bioassay were plotted against those obtained in the βarr2 assay. Curves were fit using the centered second order polynomial (quadratic) fitting, using GraphPad Prism software.[30]

3. Results

3.1 Determination of the optimal configuration of GPCR and miniGα_q

For the determination of biased agonism, a bioassay was developed to monitor the recruitment of the engineered miniGα_q protein to the 5-HT$_{2A}$R, highly similar to a previously reported βarr2 bioassay.[22-25] Analogously with this latter assay, the NanoBiT® technology (Nanoluciferase Binary Technology) was employed, a technique specifically designed for the monitoring of protein-protein interactions. To this end, the nanoluciferase enzyme is split into two inactive parts, each of which is fused to one of the potentially interacting proteins, in this case the miniGα_q protein and the 5-HT$_{2A}$R. Upon agonist binding, miniGα_q is recruited to the activated receptor, resulting in functional complementation of the enzyme, which can be picked up by a bioluminescent signal in the presence of the enzyme's substrate.[31] The four possible combinations of receptor and effector constructs were generated, and tested for the optimal sensitivity, as visually depicted in Figure 2A. For each of those combinations, the activation profile of unstimulated cells was compared with that of cells stimulated with 1 µM of the psychedelic agonist LSD. As visualized in Figure 2A, the upper two combinations (5-HT$_{2A}$R-SmBiT with LgBiT-miniGα_q and 5-HT$_{2A}$R-LgBiT with SmBiT-miniGα_q) both showed an increased signal upon stimulation with LSD. The latter combination was selected based on the increase in signal upon agonist stimulation. Furthermore, this option employs the same receptor construct (5-HT$_{2A}$R-LgBiT) as that employed in a previously developed βarr2 bioassay,[22] thereby eliminating an extra source of variability that would be introduced when using different

constructs. In contrast with the successful setup of a HEK 293T system stably expressing 5-HT_{2A}R-LgBiT with SmBiT-βarr2 [32], repeated attempts to generate a similar stable system for 5-HT_{2A}R-LgBiT with SmBiT-mini$G\alpha_q$ were unsuccessful, for unknown reasons. Hence, rather than using stable cell systems for the evaluation of biased agonism (as performed before for cannabinoid, opioid and adenosine receptors), we used transiently transfected cells here.[28, 30, 33]

3.2 LSD as a reference agonist

Figure 2B demonstrates the verification of the concentration-dependence of our new platform. Assessing the potential occurrence of ligand bias for a given compound requires the measurement of both bioassays, and comparison of the results to a reference agonist to eliminate system bias and observational bias while also guaranteeing comparability of the experimental conditions.[19, 21] LSD is a well-characterized psychedelic compound that was previously used as the reference agonist in experiments assessing the activity of compounds at the 5-HT_{2A}R [22, 32]. Figure 2C shows the concentration-response curves obtained using LSD in the bioassays used here. The Figure shows two indistinguishably overlapping curves, with largely overlapping confidence intervals of the EC_{50} values: 5.95 nM (with a 95 % confidence interval of 3.54 – 11.5 nM) for the βarr2 bioassay, and 5.70 nM (2.79 – 12.8 nM) for the newly developed mini$G\alpha_q$ bioassay. Therefore, we implemented LSD as the reference agonist within this set of experiments.

3.3 Analysis of twelve psychedelic substances with divergent structures

We then employed the optimized mini$G\alpha_q$ bioassay simultaneously with the previously developed βarr2 bioassay, in order to determine the potential biased agonism of a set of psychedelic NPS. In the initial approach, a structurally divergent set of compounds was chosen, comprising 3 tryptamines and 9 diversely substituted phenylalkylamine compounds. All

structures are shown in Figure 1. The EC_{50} (as a measure of the potency of that compound in that specific bioassay) and E_{max} (as a measure of efficacy) values were determined, and, together with the values obtained for the reference agonist LSD, this allowed for the calculation of the bias factor, β. A negative β value implies a preference of that compound towards the mini$G\alpha_q$ bioassay, a positive value towards the βarr2 bioassay, and a value (close to) 0 implies a lack of preference towards either, relative to the reference agonist LSD for which β = 0. All values are given in Table 1, and the associated overlay curves, representing the concentration-response curves of the compounds in the respective bioassays, are shown in Figure 3.

Upon implementation of a Kruskal-Wallis analysis with post hoc Dunn's test, several compounds showing statistically significant differences in bias compared to LSD were identified: 25H-NBF (β value of 0.928, p = 0.0023), 25H-NBMD (β value of 0.733, p = 0.0222), 25H-NBOH (β value of 0.750, p = 0.0130) and 25H-NBOMe (β value of 0.710, p = 0.0222).

3.4 Analysis of eight structurally related analogues

Following the identification of several compounds showing a bias statistically significantly different from LSD, a second panel of structurally related substances was assayed to verify whether there was a link between certain structural features and the observed (lack of) biased agonism. Furthermore, as Bromo-DragonFLY represented a peculiar case, with no statistically significant relative bias (β value of 0.033), despite clearly different activation profiles for the mini$G\alpha_q$ and βarr2 bioassay (Figure 3), we also analyzed its structural analogue 2C-B-FLY, as well as the enantiopure *R*-Bromo-DragonFLY. The results of this series of experiments, more specifically the EC_{50} and E_{max} values per compound and per bioassay, and their respective bias factors, are shown in Table 2. The associated overlays of the concentration-response curves obtained in the respective bioassays, are shown in Figure 4.

4. Discussion

Serotonergic psychedelics are defined as substances exerting their pharmacological actions mainly through activation of the 5-HT_{2A}R. On a cellular level, receptor activation by an agonist can lead to the recruitment of a trimeric G protein to the receptor. In the case of the 5-HT_{2A}R, the receptor mainly interacts with the $G\alpha_q$ protein, resulting in activation of phospholipase C (PLC), accumulation of inositol phosphates (IP) and diacylglycerol, and the release of intracellular Ca^{2+}. Other signaling events include phospholipase A (PLA) activity, with the associated arachidonic acid release, and Ca^{2+} mobilization through channels in the cell membrane. In addition to the canonical G protein-related signaling pathways, other proteins can interact with the activated GPCR, such as β-arrestin 2 (βarr2). This latter scaffolding protein can not only influence the internalization and desensitization of the receptor, but can also induce signaling events.[1, 3-6] It remains elusive which (combination) of these effects is responsible for the psychedelic actions of those compounds, and to what extent phenomena such as biased signaling, receptor dimerization and the binding of other receptors are involved in the mechanism of action of those substances.[4-6, 16]

The 5-HT_{2A}R was one of the first GPCRs for which functional selectivity was recognized.[17, 34, 35] The best described phenomenon at this receptor is the selective activation of either the PLC or the PLA pathway. In this context, several agonists with preference towards either pathway have been identified, and along with high-throughput screening methods for bias, several models have been developed to predict signaling properties.[34, 36-40] Additionally, the potential agonist-directed preference towards either $G\alpha_q$ activation or Ca^{2+} mobilization has been explored.[41] Furthermore, biased agonism has been proposed as the mechanism behind the psychedelic nature of some, but not all 5-HT_{2A}R agonists, as differences in transcriptome patterns were uncovered between psychedelic and non-psychedelic substances, through the differential activation of the $G_{i/o}$ protein and the Src pathway.[42, 43] Contrarily, potential preference of βarr recruitment as compared to activation of the G protein pathway has only

recently been explored.[35] To maximally allow comparison between recruitment of either $G\alpha_q$ or βarr2, we developed a bioassay monitoring the recruitment of an engineered mini$G\alpha_q$ protein, in order to complement our previously described βarr2 recruitment assay.[22] The comparison of potency and efficacy data obtained in these two distinct, yet highly analogous recruitment bioassays, each monitoring separate upstream signaling events in the same cell line and through the same technique, enables the estimation of potentially biased agonism within the tested compounds.

Following the selection of the optimal configuration of the 5-HT_{2A}R and the engineered mini$G\alpha_q$ protein in the NanoBiT® system, enabling the most sensitive monitoring, and the verification of the concentration-dependence of this response, the newly developed bioassay was used simultaneously with the previously developed βarr2 bioassay for the assessment of biased agonism.[22] A prerequisite of this type of experiments is the use of a suitable reference agonist, thereby guaranteeing optimal comparability of the experiments and eliminating system and observational bias.[19, 21] Within the employed system, the well-described psychedelic compound LSD was found to show no preference towards either recruitment of mini$G\alpha_q$ or βarr2, reflected by the practically overlapping curves of the two bioassays, and the highly similar EC_{50} values (as illustrated by the overlapping confidence intervals). The obtained EC_{50} values were within the same range as those previously reported with βarr2 bioassays performed in either transiently transfected cells and cells stably expressing the bioassay components.[22, 32] Therefore, LSD was selected as the reference agonist. In literature, LSD has been identified as both unbiased or biased, depending on which pathways were monitored in which receptor subtype. Agonist-directed signaling has been described for LSD at the 5-HT_{2C}R, where PI hydrolysis, PKC translocation and Ca^{2+} were compared between LSD and serotonin.[44] From crystal structure data, the LSD-bound 5-HT_{2B}R showed hallmarks of an apparently βarr-biased state. This was supported by comparing the outcome of a Tango (βarr2 recruitment) assay

versus that of a Ca^{2+} mobilization assay, applied at the 5-HT_{2B}R.[45, 46] However, LSD appeared relatively unbiased at the 5-HT_{1B}R, when comparing the outcomes of a Tango versus a cAMP inhibition assay.[46] Importantly, the comparative outcome of bioassays that monitor receptor activation at different levels of signaling may strongly be influenced by a 'plateauing' effect in bioassays that monitor more downstream effects, as recently elegantly demonstrated by Gillis and Gondin and colleagues for the mu opioid receptor.[47] This may yield a somewhat distorted view on biased agonism. As a consequence, at present it is not entirely clear to what extent LSD can effectively be considered as βarr-biased at the 5-HT_{2A}R. A more detailed description of the available information on the βarr2 pathway for psychedelic substances is provided further in the discussion section. Irrespective of the discussion to what extent LSD is to be considered βarr-biased, it may serve as a reference psychedelic, to which (the bias of) other psychedelics can be compared. Furthermore, several signaling properties at the 5-HT_{2A}R have been comparatively explored for LSD: $G\alpha_q$ activation versus Ca^{2+} mobilization, PLC versus PLA activation, and the induction of distinct transcriptome fingerprints.[37, 41-43] The comparison of those results to ours is not straightforward, as different comparator molecules and signaling pathways were used. From the above, it is clear that statements on ligand bias should always include referral to the utilized reference agonist(s), the assessed pathways and cell types. Although we employed bioassays that are maximally similar, there are still some variables that may need to be taken into account for the interpretation of the results. E.g., the βarr2 and mini$G\alpha_q$ fusion proteins with SmBiT may intrinsically have different turnover times and/or may differ in strength and/or duration in terms of coupling to the receptor. As more stable complexes may show higher apparent efficacies, this is an important consideration that should be kept in mind.[48] Moreover, results are not only influenced by the assay system per se, but also by the cellular context. As the βarr2 and mini$G\alpha_q$ bioassays utilized here both

employ overexpressed assay components, one should be cautious when attempting to extrapolate these findings to native expression systems.[20]

The highly analogous bioassays were used to analyze a first panel of structurally diverse psychedelic compounds, consisting of three tryptamines and nine phenylalkylamines. All substances were identified as agonists for both the recruitment of miniGα_q and βarr2, covering a broad range of both potency (EC_{50}) and efficacy (E_{max}) values, as can be derived from Table 1. The Table also provides the calculated bias factors (β), with the respective p values obtained from the Kruskal-Wallis analysis. A visual representation of the β values is given in Figure 5. From this set of twelve compounds, four psychedelics were identified as statistically significantly biased towards the recruitment of βarr2, relative to the reference agonist LSD: 25H-NBF (β value of 0.928, p = 0.0023), 25H-NBMD (β value of 0.733, p = 0.0222), 25H-NBOH (β value of 0.750, p = 0.0130) and 25H-NBOMe (β value of 0.710, p = 0.0222). As this value is logarithmic, a β value of 1 is to be interpreted as a tenfold preference of the agonist towards the recruitment of βarr2, over miniGα_q, relative to LSD. All calculated β values were positive, ranging from 0.009 for mescaline, which is essentially unbiased, to 0.928 for 25H-NBF. The latter suggests that this ligand induces a receptor conformation preferentially resulting in recruitment of βarr2 over miniGα_q. Within this subset of psychedelic substances, the highest bias factors can be linked to the *N*-benzyl substituted subgroup of phenylalkylamines. While four statistically significantly biased phenylalkylamines were identified, no statistically significantly biased tryptamines were discovered, despite relatively high bias factors (0.33 – 0.57).

When looking closely into the results obtained with the second panel, containing selected structural analogues of the phenylalkylamines in the first test panel, several trends can be observed in Table 2 and Figures 4 and 5. Both the structurally related analogue 2C-B-FLY of Bromo-DragonFLY, and its enantiopure isomer *R*-Bromo-DragonFLY, show similar profiles,

with markedly higher efficacies for the miniGα_q bioassay than for the βarr2 recruitment assay, and potencies within the same order of magnitude. In literature, it has been reported that the *R*-enantiomer of Bromo-DragonFLY is mainly responsible for the activity of the racemic mixture.[49] In line with this, we found that the *R*-enantiomer is capable of inducing the recruitment of βarr2 and miniGα_q. Analogously to 2C-H and DOH, 2C-I and DOI show no statistically significant preference towards either pathway compared to LSD, with overlapping curves for 2C-I and an increased E_{max} for the miniGα_q over the βarr2 bioassay for DOI. Within the category of *N*-benzyl substituted psychedelics (-NBF, -NBMD, -NBOH and -NBOMe), the trend in bias factor reveals that, within both the group of 25I-substances and that of 25H-substances, the -NBF has the highest bias factor, and the -NBOMe has the lowest, resulting in 25I-NBF being statistically significantly biased towards the βarr2 recruitment assay compared to LSD. While for 25I-NBF, 25I-NBMD and 25I-NBOH, the βarr2 curves are higher and more leftward shifted than those of miniGα_q, this is not the case for 25I-NBOMe, exemplifying how a relatively small difference in structure can influence the bias profile of a compound.[45, 50, 51] Whether the tethering of the methoxy groups on the phenyl moiety of the phenethylamine core (as in (*R*)-Bromo-DragonFLY and 2C-B-FLY), or the substitution of that core with an ortho-substituted *N*-benzyl group is determinative for a certain bias or particular activation profiles, has to be confirmed with more structural analogues. Additionally, the recent elucidation of the agonist-bound structure of the 5-HT_{2A}R with an engineered Gα_q protein will presumably shed more light on the structural features required for activation of one pathway or the other.[52] Although Kim et al. did not specifically determine ligand bias, the agonist-bound 5-HT_{2A}R structure shows a differential binding mode of 25CN-NBOH as compared to LSD, which could potentially result in distinct signaling properties. Interestingly, a BRET analysis with βarr2 and an engineered Gα_q protein revealed a higher efficacy for 25CN-NBOH in the βarr2 assay, when compared to serotonin as a reference.[52]

As seen in Figure 5 and Tables 1 and 2, all calculated bias factors (β) are (at least slightly) positive, indicating a stronger preference towards the recruitment of βarr2 over miniGα_q, compared to the reference psychedelic substance LSD. Important to note is the fact that, within the test group, no "perfectly biased" substances were identified, only activating one of the two bioassays.[21] First described to interact with the 5-HT_{2A}R over 20 years ago, the exact functions of βarr2 at this receptor still remain largely elusive.[53] On the one hand, 5-HT_{2A}R structural data indicate psychedelic substances to show a preference towards agonist-induced receptor desensitization,[54] with LSD claimed to be biased towards βarr2 recruitment over nonpsychedelic lysergamides (as discussed above).[45] Quantitative phosphoproteomics revealed different receptor phosphorylation patterns between psychedelic and non-psychedelic substances.[55] On the other hand, knockout mice and cell lines suggest divergent functions for this protein in the response to psychedelic and non-psychedelic ligands: the behavioral head twitch response is abrogated in βarr1/2 knockout mice in response to non-psychedelic substances, but not in response to DOI and *N*-methyltryptamines. In this context, it has been hypothesized that the recruitment of βarr may result in differential consequences for non-psychedelic versus psychedelic substances.[56-59] Based on the calculated bias factors, the present data show a (slight) preference of the tested substances towards the recruitment of βarr2 over that of miniGα_q compared to LSD, without enabling a distinction between the function of βarr2 in signaling, desensitization, internalization, or a combination of these. Further complicating the interpretation of bias factors, is the lack of knowledge about which extent of bias is required to result in a differential response *in vivo*. It must therefore be noted that the statistically significant differences in bias factor cannot easily be translated to (relevant) differences in biological effects. The latter requires empirical determination with in vivo experiments.[60]

Figures 3 and 5 reveal a remarkable observation for Bromo-DragonFLY, described in literature as extremely potent, showing a markedly different activation profile in the two bioassays: the compound reaches an E_{max} of 287 % in the miniGα_q recruitment assay, whereas 'only' 122 % was reached in the βarr2 bioassay. In a previous study, which assessed βarr2 recruitment via a cell line stably expressing the constituents of this bioassay, this compound did not differ markedly from other tested compounds.[7, 32, 61] Our new findings provide a reasonable explanation for the discrepancy between our previous findings and those in literature, and invigorate the need for implementing different bioassays in the functional characterization of substances. Moreover, the case of Bromo-DragonFLY also nicely illustrates an inherent limitation of the commonly employed 'relative activity' approach. The overlay of the miniGα_q and βarr2 concentration-response curves (shown in Figure 3) shows two clearly distinct curves, with largely different E_{max} values. On the other hand, the EC_{50} values obtained from the two bioassays are similar (3.32 nM in the miniGα_q bioassay, versus 1.53 nM in the βarr2 bioassay), with overlapping 95 % confidence intervals. However, because the ratio between the EC_{50} values of the two bioassays is the inverse of that of the E_{max} values, the bias factor equals out to 0.033, in stark contrast with the qualitative interpretation of the results. The important message that should be derived from this is that biased agonism cannot -and should not- be summarized by solely the calculated bias factor β. A more visual representation of the bias factor is the bias plot, as shown in Figure 6. The graph for Bromo-DragonFLY shows a curve which is bent towards the axis on which the percentage of miniGα_q activation is placed. While not giving a quantitative value, the concomitant use of this approach can help to prevent overlooking exceptional cases, such as this compound.[18, 19, 21, 26]

Figure 6 therefore shows the individual bias plots of each of the psychedelics included in the test panel. Roughly three types of plots can be observed: (i) the compounds showing a visually clear preference towards the recruitment of miniGα_q ((*R*)-Bromo-DragonFLY, 2C-B-FLY and

mescaline); (ii) the compounds showing a qualitative preference towards the recruitment of βarr2 (25H/I-NBF, 25H/I-NBMD, 25H/I-NBOH, 25H/I-NBOMe and N-Me-2C-H); and (iii) the more ambiguous compounds, of which the curve starts at one side of that of the reference agonist, crosses the curve of the reference agonist and stops at the other side, or where the curve coincides with that of the reference agonist. Given the positive β factors calculated for all tested psychedelics, indicating a (slight) relative preference towards the recruitment of βarr2, the occurrence of this first group is remarkable, and emphasizes the necessity of also including a qualitative assessment of the potentially biased agonism, as outlined above. Importantly, for all substances for which the bias factor indicated a statistically significant relative bias towards the recruitment of βarr2, the bias plot supports this finding.

Another qualitative method to visually estimate the occurrence of biased agonism between two bioassays, is the depiction of the E_{max} values of each of the tested substances in each of the assays, and evaluation of the change in ranking order. Similarly to the bias plot, this representation takes into account the efficacies of the agonists, without making a priori assumptions on the data, as was the case for the quantitative approach, where a Hill slope of 1 in the nonlinear regression analysis is assumed.[21] This 'change in ranking' approach was applied on the obtained data, and the results are shown in Figure 7, allowing for the rapid identification of qualitatively biased substances. In Figure 7, these substances can be recognized via lines crossing each other, as an indication of a different relative ranking of the efficacies in the two bioassays, indicative of the occurrence of bias. Here, the Figure shows several crossing lines, and outspoken differences in both directions, as indicated by the color code (a red color for compounds that differ 9-10 ranks between the two assays, blue for 7-8 ranks difference, green for 5 or 6, and black for a difference of less than five places in the ranking order of the two assays). Employing similar bioassays with different receptor systems (cannabinoid receptor CB1 and the mu opioid receptor), the rank order of the relative efficacies remained largely the

same between the two bioassays, with less crossing lines in this graphical presentation.[30, 33] Here, however, we observed remarkable differences in ranking (substances shifting 9 or 10 places) for 25H-NBF, 25I-NBF, 25I-NBMD and DIPT, with 25H-NBF and 25I-NBF coming forward as strongly biased towards βarr2 recruitment via all three assessment methods. Also for the other *N*-benzyl substituted phenethylamines, except for 25I-NBOMe, this graph shows rather large differences in ranking order between the two employed assays, consistent with their qualitative (and quantitative) preference towards the βarr2 assay (Figures 5 and 6). Despite the large difference in E_{max} values between the two employed bioassays, the ranking order of (*R*)-Bromo-DragonFLY changes less. In short, even upon applying two maximally similar bioassays, the use of different methodologies to assess biased agonism yields a distinct view on what compounds can be classified as biased towards one pathway or the other, emphasizing the need of applying different methods to obtain an estimate of bias.

In conclusion, we report on the successful development of a bioassay that monitors the recruitment of an engineered mini$G\alpha_q$ protein to the activated 5-HT_{2A}R. Following selection of the optimal configuration of this assay and the selection of LSD as a reference psychedelic substance, the optimized bioassay was used together with a previously developed analogous βarr2 bioassay for the assessment of biased agonism. The use of two highly analogous bioassays, monitoring recruitment upstream in the signaling pathway in a similar cell environment, and using the same technology, allows for an optimally comparative assessment. A panel of 3 tryptamines and 9 diversely substituted phenylalkylamines was subjected to analysis, and bias factors were calculated through the relative activity approach. Employing this method, several agonists statistically significantly biased relative to LSD, were identified within the group of phenylalkylamine psychedelics, more specifically the *N*-benzyl substituted 25H analogues 25H-NBF, 25H-NBMD, 25H-NBOH and 25H-NBOMe. Several trends were observed when testing a panel of 8 additional structural phenylalkylamine analogues. Although

no stringent conclusions could be drawn as to which structural features are responsible for the observed effects, *N*-benzylsubstituted substances and compounds in which the methoxy groups on the phenylalkyl moiety are tethered appear to be initiating particular signaling patterns. Overall, all bias factors were positive, indicative of a preference (either statistically significant or not) towards the recruitment of βarr2 over miniGα_q relative to the reference compound, LSD. Comparison of the calculated bias factors with the qualitative assessment of the overlay curves and respective bias plots of the compound responses, shows apparent contrasts in conclusions that can be drawn, thereby hinting at the necessity of combining different approaches in conclusion making.

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Conflict of interest

The authors declare that they have no conflict of interest.

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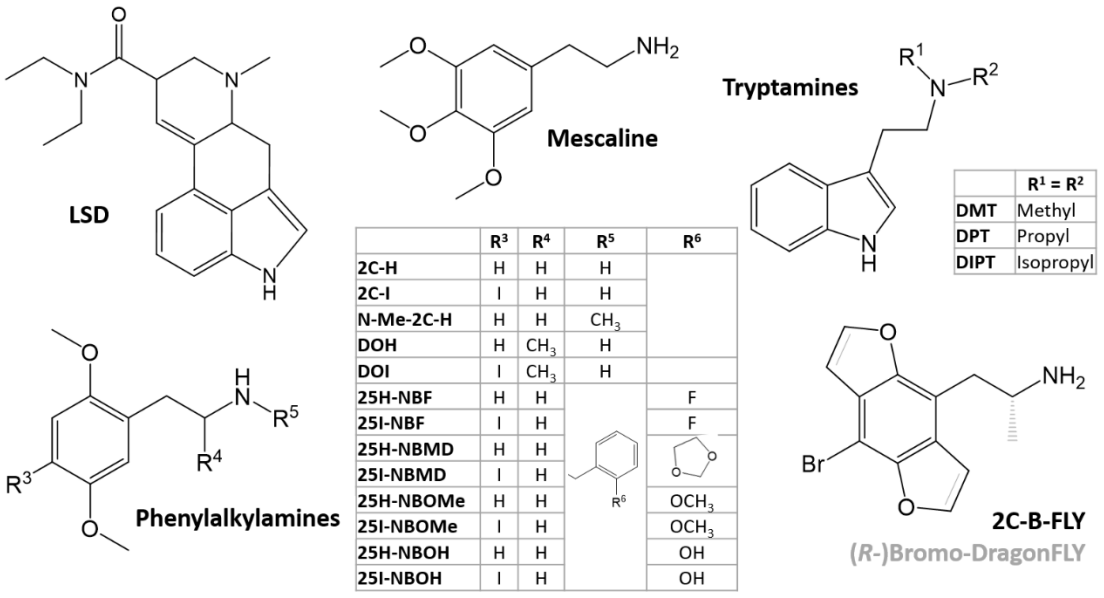
FIGURES



	R³	R⁴	R⁵	R⁶
2C-H	H	H	H	
2C-I	I	H	H	
N-Me-2C-H	H	H	CH_3	
DOH	H	CH_3	H	
DOI	I	CH_3	H	
25H-NBF	H	H		F
25I-NBF	I	H		F
25H-NBMD	H	H		
25I-NBMD	I	H		
25H-NBOMe	H	H		OCH_3
25I-NBOMe	I	H		OCH_3
25H-NBOH	H	H		OH
25I-NBOH	I	H		OH

	R¹ = R²
DMT	Methyl
DPT	Propyl
DIPT	Isopropyl

Figure 1: Structures of the compounds used for the determination of potential ligand bias: LSD, mescaline, phenylalkylamines and tryptamines, with the respective R-groups in the table next to the basic structure, and 2C-B-FLY and (*R*)-Bromo-DragonFLY, where the additional features of the latter compound are shown in grey: the methyl group and the aromatic core, whereas 2C-B-FLY has a non-aromatic heterocyclic structure.

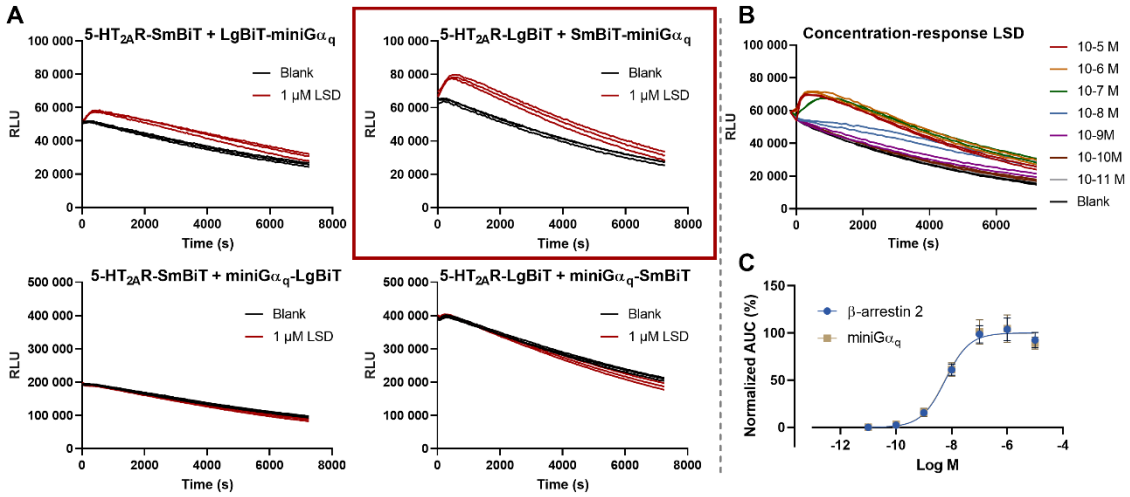


Figure 2: (A) Selection of the optimal configuration of 5-HT$_{2A}$R and miniGα_q constructs in the NanoBiT® system. Activation profiles of each of the four possible combinations are shown, with black lines representing the solvent controls ('Blank') and red curves the agonist-stimulated cells ('1 µM LSD'). Triplicates of one representative experiment are shown, and the selected combination is emphasized in a red frame. RLU: Relative Light Units (B) Corrected activation profiles obtained by testing different concentrations of LSD in the miniGα_q bioassay. Concentration-dependence of the monitored signals can be observed. (C) Overlay of the concentration-response curves of LSD in both the miniGα_q and β-arrestin 2 bioassays.

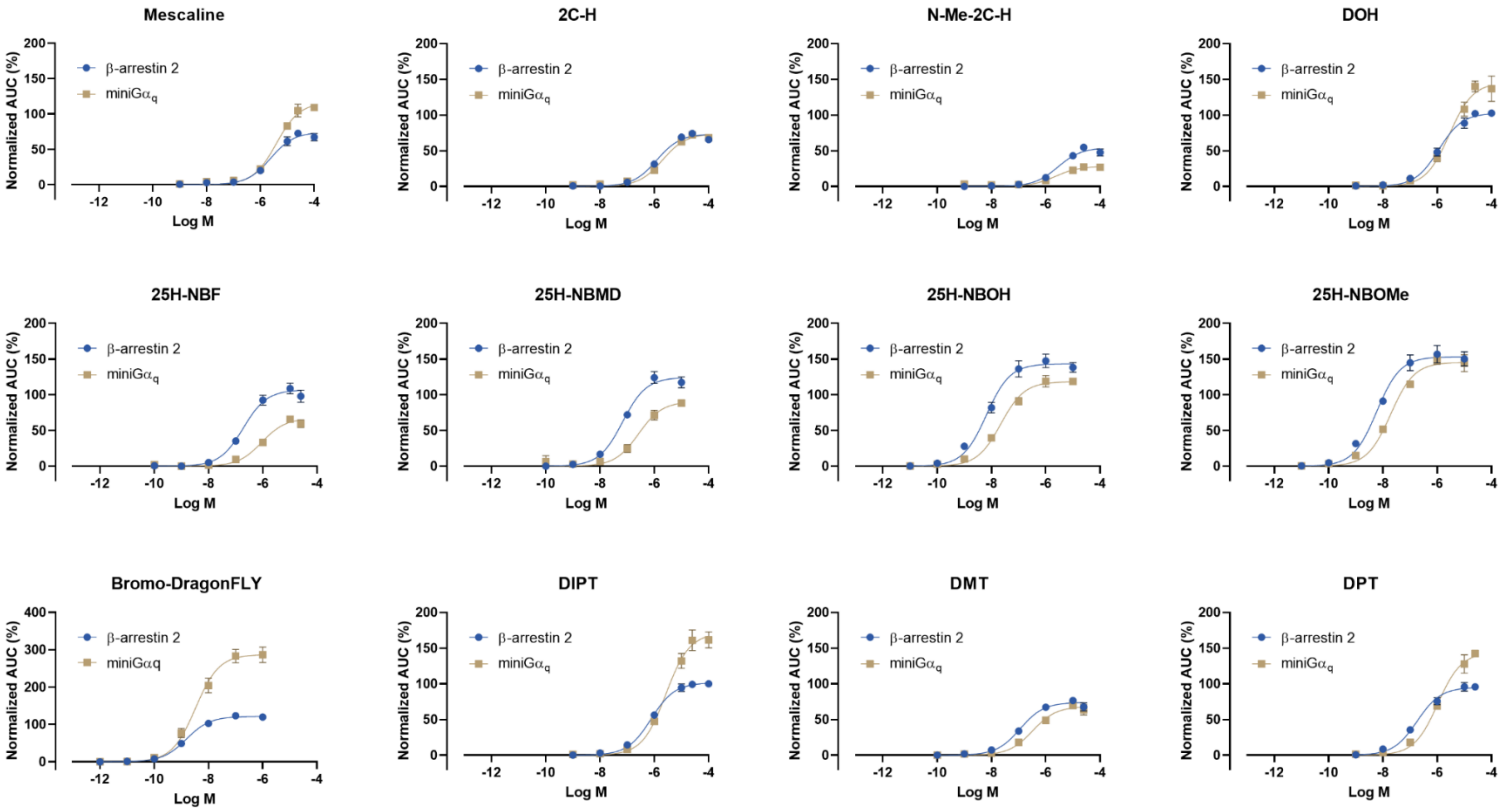


Figure 3: Overlay figures of the obtained concentration-response curves of each compound, in both of the employed bioassays. The blue dots represent the curves obtained with the β-arrestin 2 bioassay, the beige squares represent those obtained with the miniGα_q bioassay. Note the different y-axis-scale used for Bromo-DragonFLY. Each data point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean).

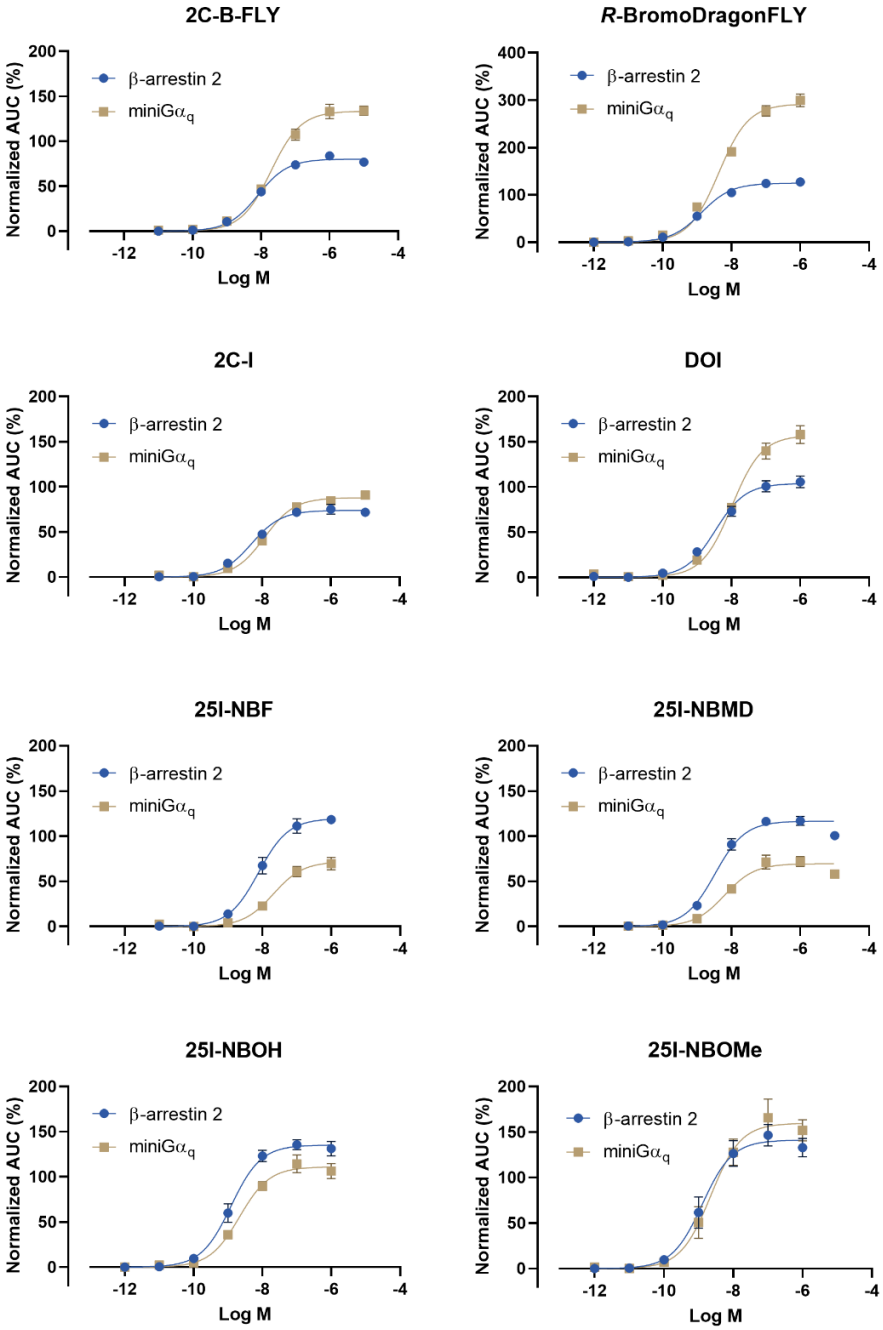


Figure 4: Overlay figures of the obtained concentration-response curves of the additional compounds, in both of the employed bioassays. The blue dots represent the curves obtained with the β-arrestin 2 bioassay, the beige squares represent those obtained with the miniGα_q bioassay. Note the different y-axis-scale used for *R*-Bromo-DragonFLY. Each data point represents the mean of three independent experiments, each performed in duplicate ± SEM (standard error of the mean).

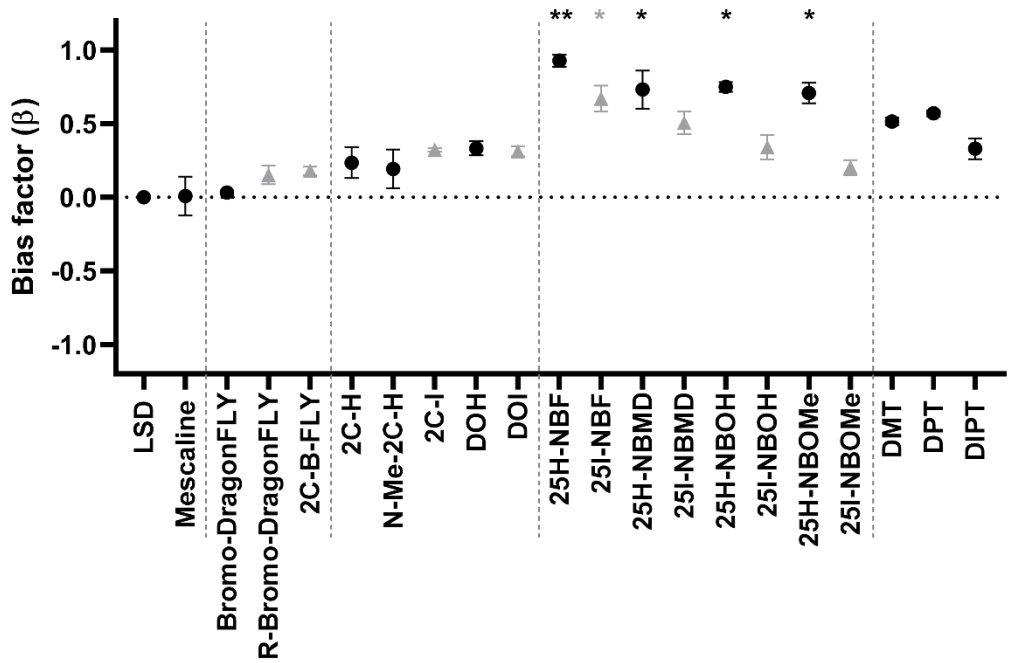


Figure 5: Visual representation of the calculated bias factors (β) for each of the tested compounds, calculated from three independent experiments. The β of reference agonist LSD is 0, a positive value implies a preference towards βarr2 recruitment and a negative value towards miniGα_q recruitment. Black circles represent substances included in the first tested group, grey triangles their respective structural analogues from the second test panel, both ± SEM (standard error of the mean). *: $P < 0.05$; **: $P < 0.01$.

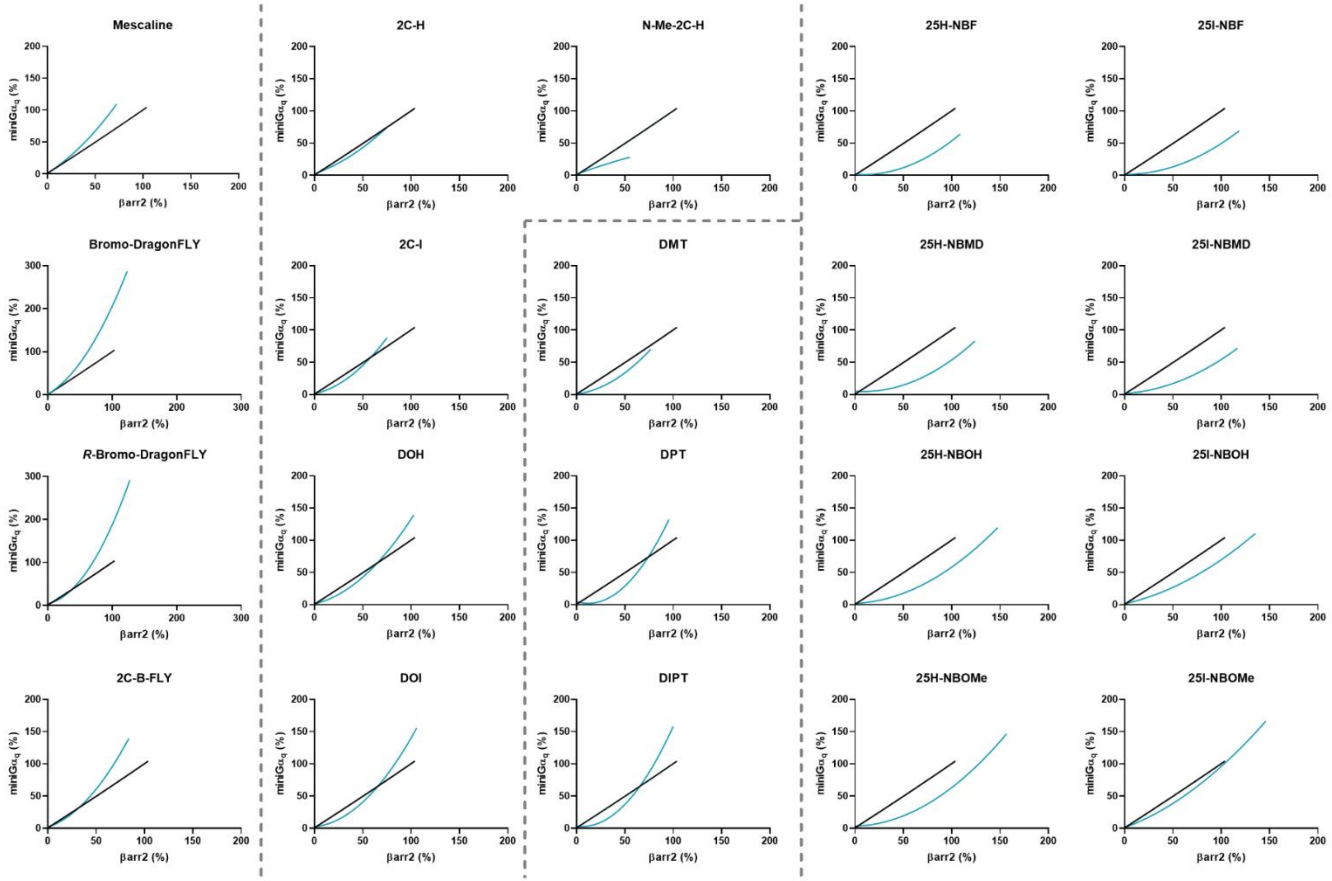


Figure 6: Individual bias plots of each of the substances assessed in the two bioassays, with the same subdivision as used in Figure 5. Each point represents the effect of one concentration of an agonist, visualizing the respective responses in each of the bioassays, where the x-axis shows the

percentage activation of the β-arrestin 2 bioassay and the y-axis that of the miniGα_q bioassay. The black line visualizes reference agonist LSD, the blue line is that of the tested substance. Curves were fit using a centered second order (quadratic) polynomial function.

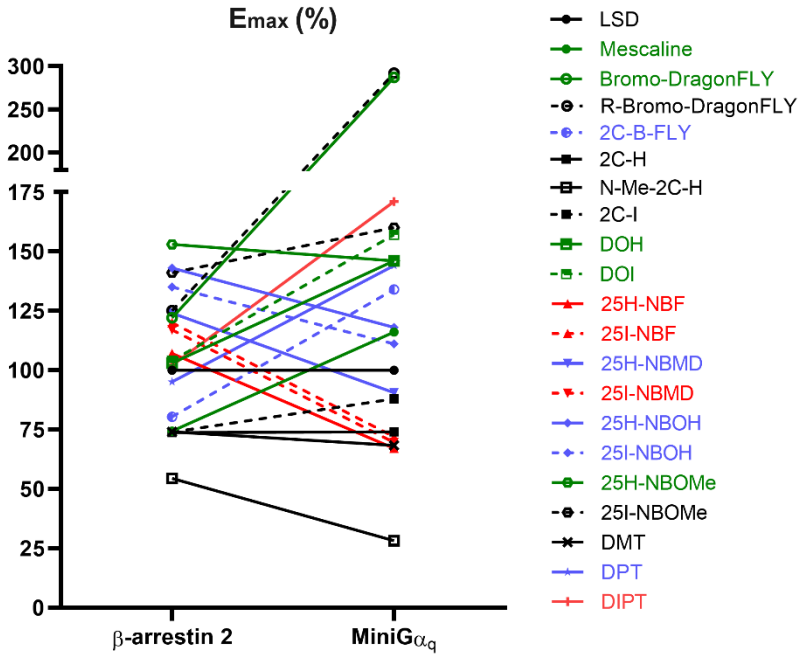


Figure 7: Visual representation of the qualitative comparison of the efficacies of each of the individual test substances in the two bioassays. The substances from the original panel are depicted by full lines, those of the second panel by dashed lines. Differences in ranking order of the E_{max} values between the two bioassays are indicative of a qualitative preference towards either, and the colors visualize this difference: red stands for a difference of 9-10 ranks between the two bioassays, blue for 7-8, green for 5 or 6, and black for less than 5.

TABLES

Table 1: Overview of the obtained potency (EC_{50}) and efficacy (E_{max}) parameters in both of the employed bioassays. These values, together with those of the reference agonist LSD, allowed for the calculation of the bias factor β, indicating a preference towards the βarr2 bioassay (positive value) or the miniGα_q bioassay (negative value). The bias factors that are statistically significantly different from 0 (with a P value <0.05) are marked in bold. E_{max} values are normalized to the maximal response of the reference agonist LSD. CI: 95 % confidence interval.

	βarr2		**MiniGα_q**			
Compound	EC_{50} (nM) (CI)	E_{max} (%) (CI)	EC_{50} (nM) (CI)	E_{max} (%) (CI)	β	P
LSD	5.95 (3.54 - 11.5)	100 (91.3 - 109)	5.70 (2.79 - 12.8)	100 (88.6 - 111)	0	N.A.
Mescaline	2372 (1574 - 3594)	74.3 (68.3 - 88.8)	3906 (2828 - 5357)	116 (109 - 125)	0.009	> 0.99
Bromo-dragonFLY	1.53 (1.11 - 2.11)	122 (115 - 128)	3.32 (2.10 - 5.17)	287 (267 - 308)	0.033	> 0.99
2C-H	1220 (865 - 1711)	73.7 (69.1 - 78.5)	1992 (1304 - 3062)	74 (68.1 - 80.3)	0.235	> 0.99
N-Me-2C-H	2773 (1704 - 4510)	54.4 (49.1 - 60.3)	2249 (1026 - 5037)	28.2 (24.2 - 33.0)	0.194	> 0.99
DOH	1132 (787 - 1624)	103 (96.2 - 110)	2793 (1773 - 4457)	146 (133 - 160)	0.334	> 0.99
25H-NBF	190 (130 - 277)	107 (99.9 - 114)	911 (593 - 1369)	67.1 (61.4 - 73.1)	**0.928**	**0.0023**
25H-NBMD	68 (46.1 - 98.0)	124 (116 - 133)	261 (140 - 490)	90.5 (79.5 - 103)	**0.733**	**0.022**
25H-NBOH	6.50 (4.19 - 9.76)	143 (135 - 152)	22.1 (15.5 - 31.7)	118 (112 - 125)	**0.750**	**0.013**
25H-NBOMe	5.91 (3.79 - 8.91)	153 (144 - 163)	19.3 (13.5 - 27.8)	146 (138 - 154)	**0.710**	**0.022**
DMT	114 (82.8 - 155)	74 (70.2 - 78)	328 (201 - 530)	68.3 (62.8 - 74.1)	0.516	0.24
DPT	181 (124 - 266)	95.1 (89.3 - 101)	1051 (686 - 1596)	144 (132 - 157)	0.570	0.075
DIPT	759 (585 - 976)	102 (97.1 - 106)	2659 (1850 - 3872)	171 (158 - 186)	0.330	> 0.99

Table 2: Overview of the obtained potency (EC_{50}) and efficacy (E_{max}) parameters in both the employed bioassays. These values, together with those of the reference agonist LSD, allowed for the calculation of the bias factor β, indicating a preference towards the βarr2 bioassay (positive value) or the miniGα_q bioassay (negative value). The bias factors that are statistically significantly different from 0 (with a P value <0.05) are marked in bold. E_{max} values are normalized to the maximal response of reference agonist LSD. CI: 95 % confidence interval.

	βarr2		**MiniGα_q**			
Compound	EC_{50} (nM) (CI)	E_{max} (%) (CI)	EC_{50} (nM) (CI)	E_{max} (%) (CI)	β	P
LSD	5.95 (3.54 - 11.5)	100 (91.3 - 109)	5.70 (2.79 - 12.8)	99.8 (88.6 - 111)	0	N.A.
2C-B-FLY	8.07 (6.12 - 10.5)	80.3 (77.0 - 83.6)	19.4 (14.1 - 26.7)	134 (127 - 140)	0.180	> 0.99
***R*-Bromo-DragonFLY**	1.33 (1.02 - 1.74)	125 (120 - 130)	4.24 (3.04 - 5.80)	292 (278 - 307)	0.152	> 0.99
2C-I	4.90 (3.29 - 7.13)	73.9 (69.9 - 77.9)	11.7 (8.95 - 15.3)	87.9 (84.2 - 91.6)	0.323	> 0.99
DOI	3.39 (2.97 - 4.97)	104 (97.8 - 110)	10.2 (7.39 - 13.9)	157 (148 - 167)	0.313	> 0.99
25I-NBF	7.70 (5.32 - 11.0)	120 (112 - 128)	20.6 (12.3 - 34.9)	71.9 (64.6 - 79.8)	**0.671**	**0.026**
25I-NBMD	3.31 (2.40 - 4.56)	117 (111 - 123)	6.32 (3.46 - 11.0)	69.6 (63.2 - 76.2)	0.506	0.19
25I-NBOH	1.22 (0.878 - 1.69)	135 (128 - 142)	2.15 (1.41 - 3.27)	111 (104 - 119)	0.341	> 0.99
25I-NBOMe	1.27 (0.708 - 2.27)	141 (128 - 154)	2.23 (1.15 - 4.32)	160 (142 - 177)	0.202	> 0.99