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Evidence that 5-HT$_{2A}$ receptor signalling efficacy and not biased agonism differentiates serotonergic psychedelic from non-psychedelic drugs

Aurelija Ippolito[1] | Sridhar Vasudevan[1] | Shaun Hurley[2] | Gary Gilmour[2] | Frederick Westhorpe[2] | Grant Churchill[1] | Trevor Sharp[1]

[1]University Department of Pharmacology, Oxford, UK

[2]Compass Pathways plc, London, UK

Correspondence
Aurelija Ippolito and Trevor Sharp, University Department of Pharmacology, Mansfield Road, Oxford, UK.
Email: aurelija.ippolito@univ.ox.ac.uk and trevor.sharp@pharm.ox.ac.uk

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Background and Purpose: Serotonergic psychedelic drugs are under investigation as therapies for various psychiatric disorders, including major depression. Although serotonergic psychedelic drugs are 5-HT$_{2A}$ receptor agonists, some such agonists are not psychedelic, potentially due to differences in 5-HT$_{2A}$ receptor ligand bias or signalling efficacy. Here, we investigated 5-HT$_{2A}$ receptor signalling properties of selected psychedelic and non-psychedelic drugs.

Experimental Approach: G_q-coupled (Ca^{2+} and IP_1) and β-arrestin2 signalling effects of six psychedelic drugs (psilocin, 5-MeO-DMT, LSD, mescaline, 25B-NBOMe and DOI) and three non-psychedelic drugs (lisuride, TBG and IHCH-7079) were characterised using SH-SY5Y cells expressing human 5-HT$_{2A}$ receptors. Ligand bias and signalling efficacy were measured using concentration–responses curves, compared with 5-HT. The generality of findings was tested using rat C6 cells which express endogenous 5-HT$_{2A}$ receptors.

Key Results: In SH-SY5Y cells, all psychedelic drugs were partial agonists at both 5-HT$_{2A}$ receptor signalling pathways and none showed significant ligand bias. In comparison, the non-psychedelic drugs were not distinguishable from psychedelic drugs in terms of ligand bias properties but exhibited the lowest 5-HT$_{2A}$ receptor signalling efficacy of all drugs tested. The latter result was confirmed in C6 cells.

Conclusion and Implications: In summary, all psychedelic drugs tested were unbiased, partial 5-HT$_{2A}$ receptor agonists. Importantly, the non-psychedelic drugs lisuride, TBG and IHCH-7079 were discriminated from psychedelic drugs, not through ligand bias but rather by low efficacy. Therefore, low 5-HT$_{2A}$ receptor signalling efficacy may explain why some 5-HT$_{2A}$ receptor agonists are not psychedelic, although a larger panel of drugs should be tested to confirm this idea.

KEYWORDS
5-HT, 5-HT$_{2A}$ receptor, biased agonism, G_q and β-arrestin2 signalling, psychedelic, serotonin

Abbreviations: 25B-NBOMe, N-(2-methoxybenzyl)-1-(2, 5-dimethoxy-4-bromophenyl)-2-aminoethane; 5-MeO-DMT, 5-methoxy-N,N-dimethyltryptamine; DOI, 2,5-dimethoxy-4-iodo-amphetamine hydrochloride; IHCH-7079, (6bR,10aS)-8-(2-Methoxyphenethyl)-3-methyl-2,3,6b,7,8,9,10,10aoctahydro-1H-pyrido[3′,4′:4,5]pyrrolo[1,2,3-de]quinoxaline; IP_1, inositol monophosphate; TBG, tabernanthalog.

1 | INTRODUCTION

Serotonergic psychedelic drugs are in development for the treatment of various psychiatric disorders, ranging from major depression and anxiety to substance misuse disorder and anorexia (Goodwin et al., 2022). In particular, each of **psilocybin**, 5-methoxy-N,N-dimethyltryptamine (**5-MeO-DMT**) and lysergic acid diethylamide (**LSD**) have progressed to clinical trials of depression and anxiety (Carhart-Harris et al., 2021; Holze et al., 2023; Reckweg et al., 2023). As a result, there is increasing interest in the molecular mechanisms by which such agents induce their psychotropic effects.

The **5-HT$_{2A}$ receptor** is central to the subjective experience elicited by serotonergic psychedelic drugs. Clinical PET imaging studies using the 5-HT$_{2A}$ radioligand, [^{11}C]Cimbi-36, report a strong correlation between 5-HT$_{2A}$ receptor occupancy and psychedelic experience intensity following psilocybin administration (Madsen et al., 2018; Sharp & Barnes, 2020). Moreover, the psychedelic effects of psilocybin and LSD in human volunteers were attenuated by the 5-HT$_{2A}$ receptor antagonist **ketanserin** (Becker et al., 2023; Holze et al., 2021; Valle et al., 2016). In preclinical studies, the 5-HT$_{2A}$ receptor agonist-induced head-twitch response in rodents is widely considered a surrogate marker of psychedelic effects in humans; this response is abolished by 5-HT$_{2A}$ receptor knockout or selective antagonists (González-Maeso et al., 2007; Jennings et al., 2008), and the potency of agonists that induce head-twitches in rodents correlates with hallucinogenic potency in humans (Halberstadt et al., 2020).

An interesting development is reports of putative non-psychedelic 5-HT$_{2A}$ receptor agonists, specifically 5-HT$_{2A}$ receptor agonists that lack the propensity to evoke head-twitches (Cameron et al., 2020; Dong et al., 2021; Dongmei et al., 2022). For example, the 5-HT$_{2A}$ receptor ligands tabernanthalog (**TBG**) and IHCH-7079 did not induce head-twitches in mice, yet evoked 5-HT$_{2A}$ receptor-mediated effects in other in vivo models (Cameron et al., 2020; Dongmei et al., 2022). Although the definition of TBG and IHCH-7079 as non-psychedelic is currently based on head-twitch data in mice, the 5-HT$_{2A}$ receptor agonist **lisuride** fails to evoke head-twitches in mice (Dongmei et al., 2022) and lacks psychedelic effects in humans (Claus et al., 1998; Herrmann et al., 1977; Schmidt et al., 2002). The reason why some 5-HT$_{2A}$ receptor agonists are psychedelic and not others is currently unknown.

A feature of G protein-coupled receptors is their capacity to signal through both G protein-dependent and β-arrestin2-dependent pathways (Kenakin, 1995; Reiter et al., 2012; Urban et al., 2007). The 5-HT$_{2A}$ receptor has been shown to signal via $G_{q/11}$ (to activate **phospholipase C** and increase **inositol trisphosphate** and intracellular Ca^{2+} [Sharp & Barnes, 2020]) as well as β-arrestin2 and other pathways (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003; Xia, Gray, et al., 2003; Xia, Hufeisen, et al., 2003). Divergence in the psychedelic effects of 5-HT$_{2A}$ receptor agonists may be due to preferential signalling through G_q- or β-arrestin2-mediated pathways (biased agonism) (Pottie et al., 2023), but there are discordant findings in the recent literature. For example, whereas IHCH-7079 is claimed to be β-arrestin2-biased (Dongmei et al., 2022), other data suggest G_q bias in non-psychedelic drugs (Kaplan et al., 2022). There are also conflicting reports for lisuride, with evidence that the drug is G_q-biased in some studies (Pogorelov et al., 2023) while having no G_q bias in others (Dongmei et al., 2022). An alternative explanation for non-psychedelic 5-HT$_{2A}$ receptor agonists is partial agonism; that is, agonists with low 5-HT$_{2A}$ receptor efficacy may exhibit a more limited repertoire of behavioural effects. For example, drugs exhibiting weak partial agonism at either the **m-opioid receptor** or the benzodiazepine binding site have been linked to a low propensity to induce respiratory depression or sedation, respectively, versus other behavioural effects (Gillis et al., 2020; Haefely et al., 1990).

Currently, there are few systematic comparisons of 5-HT$_{2A}$ receptor ligand bias or signalling efficacy of psychedelic versus non-psychedelic drugs. Moreover, many claims of drugs having 5-HT$_{2A}$ receptor ligand bias are often not supported by quantitative measurements of bias such as ΔΔlog (E_{max}/EC_{50}) analysis (Kenakin, 2017). To fill this gap here we compared, side-by-side, 5-HT$_{2A}$ receptor ligand bias and signalling efficacy for a panel of psychedelic and non-psychedelic drugs using a fully quantitative approach. The psychedelic drugs selected were the tryptamines psilocin (active psilocybin metabolite) and 5-MeO-DMT, the ergoline LSD, and the phenethylamines **mescaline**, 25B-NBOMe and **DOI** (see Section 2 for chemical structures). These agents were compared with the non-psychedelic drugs TBG, IHCH-7079 and lisuride. Drugs were selected to be chemically diverse, since receptor stabilisation in a particular state may determine ligand bias (Dongmei et al., 2022; Kim et al., 2020; McCorvy et al., 2018; Wacker et al., 2013). Experiments first investigated

What is already known

- Serotonergic psychedelic drugs are under investigation as therapies for various psychiatric disorders, including major depression.
- Serotonergic psychedelic drugs are 5-HT$_{2A}$ receptor agonists, but some such agonists are not psychedelic.

What does this study add

- Non-psychedelic drugs could be discriminated from psychedelic drugs by low 5-HT$_{2A}$ receptor signalling efficacy.
- Non-psychedelic drugs could not be discriminated from psychedelic drugs by 5-HT$_{2A}$ receptor biased signalling.

What is the clinical significance

- This study aids the discovery of nonpsychedelic 5-HT$_{2A}$ receptor agonists with potential clinical advantages over their psychedelic comparators.

5-HT_{2A} receptor-mediated G_q or β-arrestin2 signalling properties in a cell line expressing recombinant human 5-HT_{2A} receptors and, then, key findings were confirmed using a cell line expressing endogenous rat 5-HT_{2A} receptors.

2 | METHODS

2.1 | Cell culture

SH-SY5Y neuroblastoma cells (RRID:CVCL_0019) transfected with the human 5-HT_{2A} receptor (Newton et al., 1996) were maintained in culture medium: Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM Glutamax, 10% (v/v) foetal bovine serum (FBS), 100 IU μg^{-1} ml^{-1} penicillin/streptomycin and 480-$\mu g\ ml^{-1}$ G418 (to maintain transfection selection pressure). C6 glioma cells that endogenously express the rat 5-HT_{2A} receptor (Meller, Harrison, Elliott, & Sharp, 2002; Meller, Harrison, & Sharp, 2002) (ATCC CCL-107, RRID:CVCL_0194) were maintained in Ham's F12 nutrient mix containing 2 mM Glutamax, 10% (v/v) FBS and 100 IU μg^{-1} ml^{-1} penicillin/streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.2 | Assay of agonist-evoked cytosolic Ca^{2+}

Cells were plated in 96-well black/clear bottom plates at a density of 40,000 (SH-SY5Y) or 60,000 (C6) cells/well, 48 h (SH-SY5Y) or 24 h (C6) before the day of experiment. The 10% FBS culture medium was replaced with culture medium containing 10% dialysed FBS, to avoid potential receptor desensitisation by serotonin (5-hydroxytryptamine; **5-HT**) in the FBS.

On the day of experiment the culture medium was aspirated, and cells were washed twice with 200 µl Hanks' balanced salt solution (HBSS) containing calcium (HBSS-Ca^{2+}). Next, 100 µl of assay buffer containing 4 µM Fluo-4-AM (Life Technologies), 0.02% Pluronic F127 and 2.5 mM probenecid in HBSS-Ca^{2+} was added to each well, and the plate was incubated at room temperature (RT) for 1 h to allow dye loading, followed by 37°C for 30 min to allow for intracellular esterase action. The assay buffer was aspirated, and cells were washed twice with HBSS-Ca^{2+} before addition of 90 µl/well HBSS-Ca^{2+}. Cells were allowed to equilibrate for 15 min at RT before fluorescence recordings at the same temperature.

Baseline fluorescence was measured on a plate reader (BMG Optima) from the plate bottom at 480/520 nm excitation/emission every 3 s for 30 s prior to addition of one of 10 µl agonist, 10 µl agonist/agonist combination or 10 µl agonist plus 10 µl antagonist MDL-100,907 (also named **volinanserin**) 15 min before agonist addition, after which fluorescence was recorded for a further 3 min (a time frame which allowed for the agonist-induced response to reach a maximum). In each well, the final concentration of DMSO was 0.1% (v/v). A raw trace demonstrating an agonist-evoked Ca^{2+} response over time in SH-SY5Y cells is shown in Figure S1A.

In some experiments, Schild plots were constructed to confirm that drugs considered to be partial agonists demonstrated the expected antagonist properties when in combination with a full agonist. In each experiment, a concentration-response for 5-HT (10 µM–0.1 nM) was run both in the absence and then the presence of increasing concentrations of the partial agonist. The partial agonist and 5-HT were added simultaneously, because preincubation of cells with a partial agonist could lead to receptor desensitisation (Lewis et al., 2023).

2.3 | Assay of agonist-evoked inositol monophosphate (IP_1) accumulation

For **IP_1** assays, SH-SY5Y cells were plated in 384-well white low-volume plates 24 h before the day of experiment at a density of 20,000 cells/well. As above, the 10% FBS-supplemented culture medium was replaced with 10% dialysed FBS. On the day of experiment, the medium was aspirated and 10 µl of buffer containing LiCl and 4 µl of agonist/antagonist were added to each well. The plate was incubated at 37°C for 1 h before addition to each well of 3 µl IP_1-d2 and anti-IP_1-d2 dissolved in lysis buffer (CisBio HTRF IP-One G_q Kit, RRID:AB_2904131) and then incubated further at RT for 1 h. A calibration curve was run prior to commencing experiments (according to manufacturer's instructions).

Fluorescence was measured on a plate reader (Tecan Infinite F1200) from the top of the plate at 620/340 nm and 665/340 nm excitation/emission. The final concentration of DMSO in each well was 0.1% (v/v).

2.4 | Assay of agonist-evoked β-arrestin2 recruitment

β-arrestin2 recruitment was measured using the genetically encoded borealis arrestin biosensor packaged in a mammalian baculovirus vector (Montana Molecular). The fluorescent biosensor undergoes a change in conformation upon recruitment to the receptor, leading to a reduction in fluorescence (Hoare et al., 2020).

SH-SY5Y cells were plated in 96-well black/clear-bottom plates at a density of 40,000 cells/well 48 h before the day of experiment. The 10% FBS-supplemented culture medium was replaced with 10% dialysed FBS. The following day, to each well was added 50 µl of a transfection mix containing 8-µl β-arrestin2 sensor, 15 µl human-5-HT_{2A} receptor, 3 µl GPCR kinase 2, 3-µl **GPCR kinase 3** (all packaged in Mammalian Baculovirus vectors, Montana Molecular), 0.6 µl sodium butyrate and 21.4 µl media. The transfection mix was aspirated, and cells washed twice with DPBS containing calcium (DPBS-Ca^{2+}) before addition of 100 µl DPBS-Ca^{2+} to each well. The cells were allowed to equilibrate for 30 min at RT.

Baseline fluorescence was measured on a plate reader (BMG Omega) from the bottom of the plate at 485/520 excitation/emission every 75 s for 5 min prior to addition of 50 µl agonist, after which

fluorescence was recorded for a further 30 min. The final concentration of DMSO in each well was at 0.1% (v/v). A raw trace demonstrating agonist-evoked β-arrestin2 recruitment over time in SH-SY5Y cells is shown in Figure S1B.

2.5 | Materials

Psilocin (4-hydroxy-N, N-dimethyltryptamine; supplied by Compass Pathways), 5-MeO-DMT (5-methoxy-*N*,*N*-tryptamine; Cambridge Bioscience), DOI (2,5-dimethoxy-4-iodo-amphetamine hydrochloride; Cambridge Bioscience), mescaline (3,4,5-trimethoxyphenethylamine; Cambridge Bioscience), LSD (lysergic acid diethylamide; Chiron), 25B-NBOMe (N-(2-methoxybenzyl)-1-(2, 5-dimethoxy-4-bromophenyl)-2-aminoethane hydrochloride; Chiron), lisuride maleate (Bio-Techne), TBG (tabernanthalog; supplied by Compass Pathways), IHCH-7079 ((6bR,10aS)-8-(2-methoxyphenethyl)-3-methyl-2,3,6b,7,8,9,10,10aoctahydro-1H-pyrido[3′,4′:4,5]pyrrolo[1,2,3-de]quinoxaline; Axon Medchem) and volinanserin (MDL-100,907; Bio-Techne) were dissolved in DMSO, and 5-HT hydrochloride (Enzo Life Sciences) was dissolved in deionised water, to obtain 10 mM stock solutions. On the day of experiment, working solutions were obtained by diluting drugs in HBSS-Ca^{2+} (Ca^{2+}/IP_1 assays) or DPBS-Ca^{2+} (β-arrestin2 assays).

2.6 | Data processing and statistical analysis

Statistical analyses were performed using GraphPad Prism software. For Ca^{2+} assays, baseline fluorescence was averaged and maximum fluorescence reached (a single value for the peak response) was expressed as a % of baseline (corrected for vehicle addition) (Figure S1A). Peak response rather than area under the curve was taken but these measures both yield similar results (Charlton & Vauquelin, 2010). For IP_1 assays, data were converted to emission at 665/620 values, which were then converted to intracellular IP_1 concentrations using the IP_1 calibration curve. Data were normalised to the mean of the vehicle controls included within each assay. Although it appears on a small number of concentration–response curves that the lowest agonist concentrations generated a signal, this effect was within the standard error of the mean of the controls and not statistically significant. For β-arrestin2 assays, fluorescence was averaged over the first 5 min and then measurements after agonist addition over the remaining 30 min were normalised to baseline. Steady states were then calculated using the 'baseline then rise to steady state time course' curve fit on GraphPad Prism, and this was used as an endpoint measurement of cumulative response, that is, accumulation of β-arrestin recruited to the receptor and leading to a plateau in fluorescence change (Figure S1B).

Concentration–response curves were generated using the variable Hill slope function of GraphPad Prism, and then, Hill slopes were assessed for significant deviation from unity using the extra sum-of-squares *F*-test (Gillis et al., 2020). For the vast majority of curves, the null hypothesis was accepted (i.e., the Hill slope did not significantly differ from unity) and concentration–response curves were generated by GraphPad Prism. This used the non-linear regression function 'log[agonist] versus response (three parameter)' and the curve fit parameters 'potency, bottom plateau and efficacy (top)'. For the few concentration–response curves with Hill slopes that significantly differed from unity, curves were generated using the non-linear regression function of GraphPad Prism, 'log[agonist] versus response (variable slope)' curve fit'. The efficacy for each 5-HT_{2A} receptor agonist was normalised to the response to 10 μM 5-HT ($E_{max} = 1$), and each point was expressed as mean ± SEM value of five independent experiments carried out in duplicate.

Schild plots were constructed by plotting log[dose ratio − 1] versus [partial agonist] and using the 'straight line' curve fit on GraphPad Prism. The extra sum-of-squares *F*-test was used to ascertain whether the Schild plot slope significantly differed from one. The plot was interpolated to obtain a pA_2 value (*x*-intercept).

Ligand bias was quantified using the method of Kenakin (2017) which measures bias in comparison to 5-HT since this is an endogenous, balanced agonist of the 5-HT_{2A} receptor. Specifically, log (E_{max}/EC_{50}) values were calculated for each agonist in each pathway using E_{max} and EC_{50} values derived by averaging these parameters across replicates for each assay, and then compared with the reference agonist 5-HT by calculation of Δlog (E_{max}/EC_{50}) (log $(E_{max}/EC_{50})_{agonist}$ − log $(E_{max}/EC_{50})_{5\text{-}HT}$). Finally, ΔΔlog ($E_{max}/EC_{50}$) values were calculated (Δlog $(E_{max}/EC_{50})_{pathway\ 1}$ − Δlog $(E_{max}/EC_{50})_{pathway\ 2}$) and used to assess significance of either bias between two signalling pathways or the same signalling pathway between two cell lines. A one-way ANOVA followed by a post hoc Dunnett's test (which corrects for multiple comparisons) was performed on ΔΔlog (E_{max}/EC_{50}) values to ascertain the statistical significance of bias between two signalling pathways. *P*-values less than 0.05 were considered statistically significant.

Log (E_{max}/EC_{50}) values were used rather than log(τ/K_A) values, because the vast majority of Hill slopes were not significantly different from unity. In cases where the Hill slope does not significantly differ from unity, log (E_{max}/EC_{50}) and log(τ/K_A) values can be used interchangeably (Kenakin, 2017; Winpenny et al., 2016) because they give similar results (Kenakin, 2017; Winpenny et al., 2016).

2.7 | Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023).

3 | RESULTS

3.1 | Effect of 5-HT_{2A} receptor agonists on cytosolic Ca^{2+} in SH-SY5Y cells

Initial experiments determined the effect of psychedelic and non-psychedelic 5-HT_{2A} receptor agonists on G_q signalling activity via

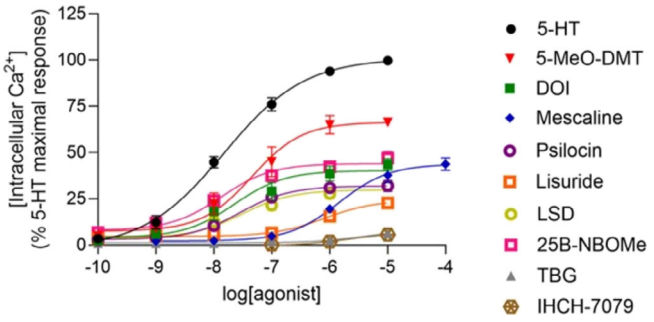

FIGURE 1 Effect of psychedelic and non-psychedelic drugs on cytosolic Ca^{2+} in SH-SY5Y cells expressing the human 5-HT_{2A} receptor. Each point is the mean ± SEM value of five independent experiments performed in duplicates. Responses are relative to 10 μM 5-HT.

measurement of cytosolic Ca^{2+} in SH-SY5Y cells expressing recombinant human 5-HT_{2A} receptors. The psychedelic drugs elicited concentration-dependent increases in Ca^{2+} (Figure 1) and showed variable potencies, with 25B-NBOMe and DOI being the most potent and mescaline being around two orders of magnitude less potent than other drugs (Figure 1 and Table 1). All psychedelic drugs had lower efficacy than 5-HT (Figure 1), with LSD and psilocin being the least efficacious (E_{max} values 0.31 and 0.34, respectively; Table 1).

The three non-psychedelic drugs, namely lisuride, TBG and IHCH-7079, also evoked concentration-related Ca^{2+} responses. These drugs displayed the lowest efficacy of all drugs tested, with TBG and IHCH-7079 producing marginal responses of 0.086 and 0.074 at 10 μM, respectively, whereas lisuride displayed low efficacy of 0.25 and a potency similar to mescaline (Figure 1 and Table 1). The Ca^{2+} responses to both non-psychedelic and psychedelic drugs (10 μM) were blocked by the selective 5-HT_{2A} receptor antagonist volinanserin (1 μM), confirming the role of this receptor in G_q signalling activity (Figure S2A).

3.2 | Effect of 5-HT_{2A} receptor agonists on IP_1 accumulation in SH-SY5Y cells

Next, the effect of the psychedelic and non-psychedelic drugs on G_q signalling activity in the SH-SY5Y cells was determined upstream of the Ca^{2+} response by measuring the accumulation of intracellular IP_1. As with Ca^{2+}, all psychedelic drugs elicited concentration-dependent increases in IP_1 (Figure 2). The rank order of potency in the IP_1 assay varied across the psychedelic drugs and was similar to that observed in the Ca^{2+} assay, with 25B-NBOMe and LSD being the most potent and mescaline again being the least potent (Figure 2 and Table 1). All the psychedelic drugs were less efficacious than 5-HT (E_{max} values ranging from 0.59 to 0.75; Table 1) except DOI, which was equally efficacious.

For the non-psychedelic drugs, as observed in the Ca^{2+} assay, both lisuride and TBG elicited concentration-related increases in IP_1 accumulation. Importantly, these drugs displayed the lowest efficacy

TABLE 1 Signalling parameters of psychedelic and non-psychedelic drugs at the human 5-HT_{2A} receptor.

	Ca^{2+}		IP_1		β-arrestin2				
Drug	E_{max}	pEC_{50}	E_{max}	pEC_{50}	E_{max}	pEC_{50}	Ca^{2+} Δlog (E_{max}/EC_{50})	IP_1 Δlog (E_{max}/EC_{50})	β-arrestin2 Δlog (E_{max}/EC_{50})
5-HT	1.00 ± 0.030	7.83 ± 0.092	1.00 ± 0.041	7.15 ± 0.11	1.00 ± 0.022	7.34 ± 0.063	0.00 ± 0.13	0.00 ± 0.13	0.00 ± 0.091
5-MeO-DMT	0.66 ± 0.035	7.34 ± 0.17	0.70 ± 0.050	8.18 ± 0.27	0.71 ± 0.035	7.75 ± 0.16	−0.68 ± 0.20	0.87 ± 0.19	0.26 ± 0.17
DOI	0.40 ± 0.025	7.64 ± 0.22	1.02 ± 0.041	7.13 ± 0.11	0.80 ± 0.012	8.14 ± 0.045	−0.60 ± 0.24	−0.011 ± 0.13	0.70 ± 0.079
Mescaline	0.43 ± 0.020	5.84 ± 0.12	0.70 ± 0.059	5.67 ± 0.21	0.64 ± 0.034	5.76 ± 0.13	−2.37 ± 0.15	−1.63 ± 0.16	−1.77 ± 0.15
Psilocin	0.32 ± 0.013	7.55 ± 0.14	0.59 ± 0.038	7.81 ± 0.30	0.39 ± 0.032	7.37 ± 0.20	−0.79 ± 0.17	0.43 ± 0.21	−0.38 ± 0.21
Lisuride	0.24 ± 0.015	6.10 ± 0.14	0.46 ± 0.025	8.97 ± 0.24	0.22 ± 0.018	7.46 ± 0.30	−2.37 ± 0.17	1.48 ± 0.17	−0.54 ± 0.31
LSD	0.30 ± 0.016	7.51 ± 0.19	0.75 ± 0.038	8.93 ± 0.21	0.60 ± 0.021	8.64 ± 0.14	−0.86 ± 0.22	1.66 ± 0.16	1.08 ± 0.15
25B-NBOMe	0.44 ± 0.018	7.87 ± 0.16	0.68 ± 0.036	8.92 ± 0.23	1.05 ± 0.031	8.27 ± 0.10	−0.33 ± 0.18	1.60 ± 0.17	0.95 ± 0.12
TBG	0.081 ± 0.020	5.43 ± 0.39[a]	0.46 ± 0.036	5.34 ± 0.19	0.22 ± 0.022	5.74 ± 0.33	−3.50 ± 0.41	−2.15 ± 0.15	−2.20 ± 0.34
IHCH-7079	0.069 ± 0.026	5.65 ± 0.54[a]	Inverse agonist	7.07 ± 0.19	0.35 ± 0.020	7.38 ± 0.17	−3.35 ± 0.55	Inverse agonist	−0.41 ± 0.19

Note: Data were derived from concentration–response curves for Ca^{2+}, IP_1 and β-arrestin2 readouts in SH-SY5Y cells expressing the human 5-HT_{2A} receptor. E_{max}, pEC_{50} and Δlog (E_{max}/EC_{50}) values are the mean ± SEM values of five independent experiments performed in duplicates. E_{max} and Δlog (E_{max}/EC_{50}) values are relative to 10 μM 5-HT. Non-psychedelic drugs in italics.

[a]pEC_{50} value is an estimate due to low E_{max}.

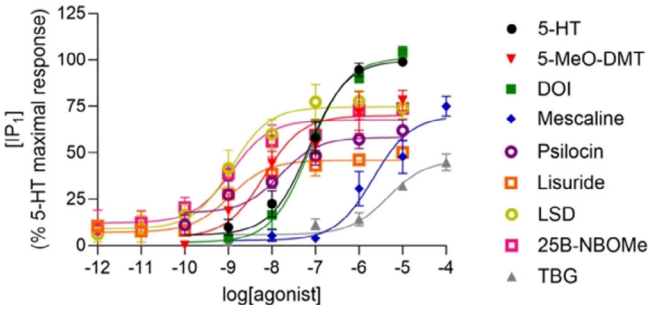

FIGURE 2 Effect of psychedelic and non-psychedelic drugs on IP_1 accumulation in SH-SY5Y cells expressing the human 5-HT_{2A} receptor. Each point is the mean ± SEM value of five independent experiments performed in duplicates. Responses are relative to 10 μM 5-HT.

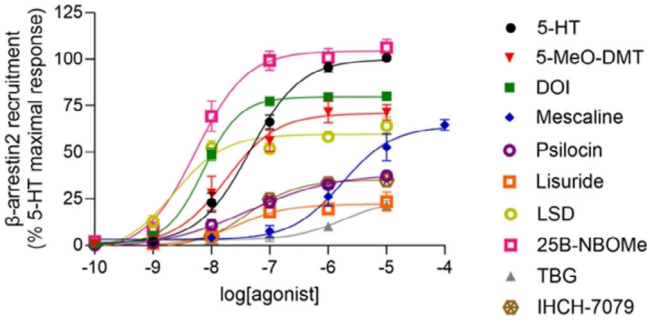

FIGURE 3 Effect of psychedelic and non-psychedelic drugs on β-arrestin2 recruitment in SH-SY5Y cells expressing the human 5-HT_{2A} receptor. Each point is the mean ± SEM value of five independent experiments performed in duplicates. Responses are relative to 10 μM 5-HT.

of all drugs tested (E_{max} value, 0.46; Figure 2 and Table 1). Interestingly, IHCH-7079 elicited a concentration-dependent decrease in IP_1 (Figure S3), suggesting that this drug acted as a fairly potent (pEC_{50} 7.07) inverse agonist in this assay (note constitutive activity is not visible in the Ca^{2+} assay).

3.3 | Effect of 5-HT_{2A} receptor agonists on β-arrestin2 recruitment in SH-SY5Y cells

Next, experiments examined the effects of psychedelic and non-psychedelic drugs on 5-HT_{2A} receptor signalling via β-arrestin2 in the SH-SY5Y cells. As with the assays of G_q signalling, all drugs tested elicited concentration-dependent increases in β-arrestin2 recruitment (Figure 3). Moreover, as with the Ca^{2+} and IP_1 assays, 25B-NBOMe and LSD were the most potent psychedelic drugs tested, and mescaline was once more the least potent (Figure 3 and Table 1). As observed in the Ca^{2+} and IP_1 assays, in the β-arrestin2 assay the psychedelic drugs were less efficacious than 5-HT (E_{max} values ranging from 0.39 to 0.80; Table 1), except for 25B-NBOMe which showed equal efficacy to 5-HT (Figure 3 and Table 1).

For the non-psychedelic drugs, each of lisuride, TBG and IHCH-7079 elicited concentration-related increases in β-arrestin2 recruitment (Figure 3 and Table 1). Moreover, as with the other assays, the non-psychedelic drugs again all demonstrated the lowest efficacy (E_{max} values 0.22–0.35), although that of IHCH-7079 was close to that of psilocin (Table 1). The β-arrestin2 response to both psychedelic and non-psychedelic drugs (10 μM) was abolished by pretreatment with volinanserin (1 μM), also confirming that the β-arrestin2 signalling was 5-HT_{2A} receptor-mediated (Figure S2B).

A plot of the E_{max} values for all the drugs in the different assays (SH-SY5Y cells) revealed the consistently low efficacy of the non-psychedelic versus psychedelic drugs (Figure S4).

3.4 | Ligand bias properties of psychedelic and non-psychedelic agents

With agonist activity data generated from the IP_1 and β-arrestin2 assays, ligand bias in 5-HT_{2A} receptor-mediated signalling was assessed. To cancel out system-specific differences such as downstream signalling amplification for each assay, agonist potencies and maximal efficacies were converted to Δlog (E_{max}/EC_{50}) values with 5-HT being the reference agonist. A scatter plot of Δlog (E_{max}/EC_{50}) values then allowed a comparison of the activity of each agonist in these two pathways (Kenakin, 2017) (Figure 4). In this plot, agonists falling close to the line of unity (i.e., possessing similar Δlog (E_{max}/EC_{50}) values in each assay) have low bias, whereas agonists that deviate from the line of unity (i.e., possessing different Δlog (E_{max}/EC_{50}) values in each assay) display bias.

Generally, drugs with high IP_1 signalling activity also had high β-arrestin2 signalling activity (Figure 4). For the psychedelic drugs, although there were minor deviations from the line of unity, none of drugs showed a statistically significant bias relative to 5-HT (ΔΔlog

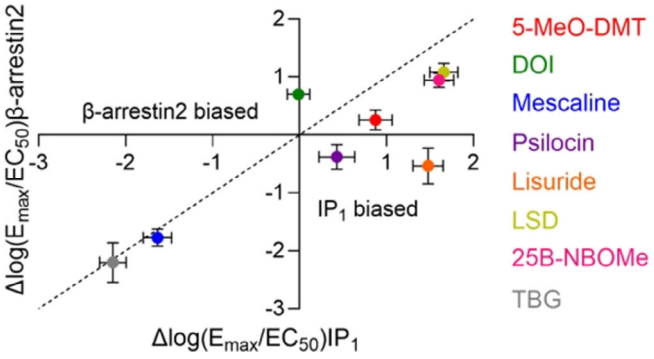

FIGURE 4 Scatter plot comparing the activity (Δlog (E_{max}/EC_{50}) values) of psychedelic and non-psychedelic drugs on 5-HT_{2A} receptor-mediated IP_1 and β-arrestin2 signalling pathways in SH-SY5Y cells. In this plot, the more positive the *x* or *y* value, the greater activity in a particular pathway. The further a drug deviates from the line of unity, the more biased the agonist. Each point represents a mean ± SEM value from five independent experiments performed in duplicates.

TABLE 2 5-HT_{2A} receptor signalling bias (ΔΔlog (E_{max}/EC_{50} values) of psychedelic and non-psychedelic drugs.

Drug	IP_1 vs. β-arrestin ΔΔlog (E_{max}/EC_{50})	Ca^{2+} vs. IP_1 ΔΔlog (E_{max}/EC_{50})	Ca^{2+} vs. β-arrestin ΔΔlog (E_{max}/EC_{50})	SH-SY5Y vs. C6 ΔΔlog (E_{max}/EC_{50})
5-HT	0.00 ± 0.16	0.00 ± 0.19	0.00 ± 0.16	0.00 ± 0.25
5-MeO-DMT	0.62 ± 0.26	−1.56 ± 0.27**	−0.94 ± 0.26	0.46 ± 0.27
DOI	−0.71 ± 0.15	−0.59 ± 0.27	−1.30 ± 0.25	0.072 ± 0.33
Mescaline	0.14 ± 0.22	−0.73 ± 0.22	−0.59 ± 0.21	−0.83 ± 0.24
Psilocin	0.81 ± 0.30	−1.22 ± 0.27*	−0.41 ± 0.27	0.41 ± 0.29
Lisuride	2.01 ± 0.35****	−3.84 ± 0.24****	−1.83 ± 0.35**	0.22 ± 0.38
LSD	0.58 ± 0.22	−2.52 ± 0.27****	−1.94 ± 0.26**	0.26 ± 0.28
25B-NBOMe	0.66 ± 0.21	−1.94 ± 0.25***	−1.28 ± 0.22	−0.34 ± 0.28
TBG	0.058 ± 0.38	−1.36 ± 0.44**	−1.30 ± 0.53	−1.24 ± 0.81
IHCH-7079	n.d.	n.d.	−2.94 ± 0.58****	−0.56 ± 1.49

Note: Data were derived from concentration–response curves for Ca^{2+}, IP_1 and β-arrestin2 readouts in and SH-SY5Y and C6 cells expressing the human and rat 5-HT_{2A} receptor, respectively. ΔΔlog (E_{max}/EC_{50}) values are the mean ± SEM values of five independent experiments performed in duplicates. Non-psychedelic drugs in italics.

Abbreviation: n.d., not determined due to inverse agonist action in IP_1 assay.

*P < 0.05.**P < 0.01.***P < 0.001.****P < 0.0001 (one-way ANOVA and followed by a post hoc Dunnett's multiple comparisons test).

(E_{max}/EC_{50}) values, P > 0.05 post hoc Dunnett's test following one-way ANOVA; Table 2). In the case of the non-psychedelic drugs, lisuride showed a statistically significant (P < 0.0001) bias towards IP_1 versus β-arrestin2 signalling (Figure 4 and Table 2), whereas TBG showed no ligand bias. In contrast, IHCH-7079 showed clear evidence of β-arrestin2 bias although a ΔΔlog (E_{max}/EC_{50}) value could not be calculated (i.e., the drug exhibited agonist activity in the β-arrestin2 assay but inverse agonist activity in the IP_1 assay). Thus, a comparison of agonist activity at IP_1 versus β-arrestin2 signalling failed to distinguish between psychedelic and non-psychedelic 5-HT_{2A} receptor agonists. It is noteworthy that 5-HT showed equal signalling activity in IP_1 and β-arrestin2 assays (a balanced agonist) since a comparison of signalling efficacy in the IP_1 assay with signalling efficacy in the β-arrestin2 assay at each concentration of 5-HT yielded a plot with a slope of unity (Figure S5; Kolb et al., 2022). It is also noteworthy that there was no clear pattern between ligand bias and chemical structure.

As with the plot of IP_1 versus β-arrestin2 signalling, a scatter plot of Δlog (E_{max}/EC_{50}) values to compare agonist activity in the Ca^{2+} and β-arrestin2 signalling pathways did not discriminate between the psychedelic and non-psychedelic agonists (Figure S6A and Table 2). Finally, a scatter plot of Δlog (E_{max}/EC_{50}) values obtained from the Ca^{2+} and IP_1 assays allowed comparison of agonist activity of what should be the same G_q signalling pathway. This plot also did not distinguish between psychedelic and non-psychedelic drugs. However, the plot revealed that relative to 5-HT, the majority of drugs (aside from DOI and mescaline) exhibited a bias towards activity in the IP_1 assay compared with the Ca^{2+} assay, with the ergolines, LSD and lisuride showing the greatest bias of all agonists tested (Figure S6B and Table 2).

Overall, the different scatter plots revealed, importantly, that ligand bias pattern did not distinguish between psychedelic and non-psychedelic agents.

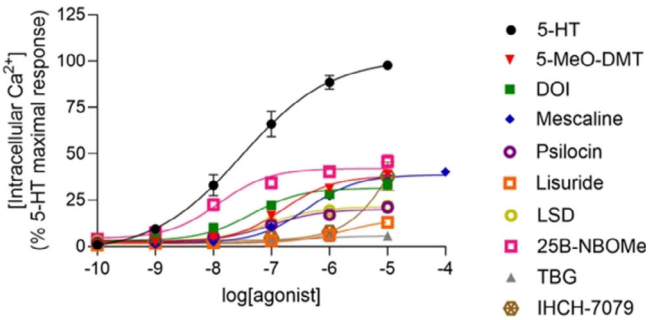

FIGURE 5 Effect of psychedelic and non-psychedelic drugs on cytosolic Ca^{2+} in C6 cells expressing the rat 5-HT_{2A} receptor. Each point is the mean ± SEM value of five independent experiments performed in duplicates. Responses are relative to 10 μM 5-HT.

3.5 | Effect of 5-HT_{2A} receptor agonists on cytosolic Ca^{2+} in C6 cells

Results obtained from the SH-SY5Y cells suggested that psychedelic drugs could not be distinguished from non-psychedelic drugs on the basis of ligand bias. Rather, the data suggested that the non-psychedelic drugs consistently had very low efficacy at 5-HT_{2A} receptors compared with the psychedelic drugs. To test this latter observation further, the effect of all drugs on G_q activity was examined in a different cell model, specifically C6 glioma cells endogenously expressing the rat 5-HT_{2A} receptor.

As observed in the Ca^{2+} assay using SH-SY5Y cells, all psychedelic drugs caused a concentration-dependent increase in cytosolic Ca^{2+} in C6 cells and with a lower efficacy than 5-HT (Figure 5 and Table 3). The relative potency of the psychedelic drugs was similar to that observed in SH-SY5Y cells, with 25B-NBOMe and LSD being the most potent and mescaline being the least potent (Figure 5 and

TABLE 3 Potency and efficacy of psychedelic and non-psychedelic drugs at the rat 5-HT_{2A} receptor.

Drug	E_{max}	pEC_{50}	$\Delta\log(E_{max}/EC_{50})$
5-HT	1.00 ± 0.058	7.51 ± 0.15	0.00 ± 0.21
5-MeO-DMT	0.38 ± 0.015	6.79 ± 0.10	−1.14 ± 0.18
DOI	0.31 ± 0.016	7.35 ± 0.16	−0.67 ± 0.22
Mescaline	0.38 ± 0.013	6.39 ± 0.10	−1.54 ± 0.18
Psilocin	0.20 ± 0.012	7.01 ± 0.23	−1.20 ± 0.23
Lisuride	0.15 ± 0.025	5.76 ± 0.29	−2.58 ± 0.34
LSD	0.21 ± 0.0082	7.09 ± 0.12	−1.12 ± 0.19
25B-NBOMe	0.42 ± 0.018	7.90 ± 0.15	0.013 ± 0.21
TBG	0.057 ± 0.11	6.48 ± 0.68[b]	−2.27 ± 0.69
IHCH-7079	1.09 ± 2.27[a]	4.68 ± 1.37	−2.79 ± 1.38

Note: Data were derived from concentration–response curves for Ca^{2+} readout in C6 cells. E_{max}, pEC_{50} and $\Delta\log(E_{max}/EC_{50})$ measurements are the mean ± SEM values of five independent experiments performed in duplicates. E_{max} values are relative to 10 μM 5-HT. Non-psychedelic drugs in italics.

[a]E_{max} value derived from incomplete concentration–response curve.

[b]pEC_{50} value is an estimate due to low E_{max}.

Table 3). Importantly, and also in keeping with results from the SH-SY5Y cells, the non-psychedelic drugs lisuride and TBG had the lowest efficacy of all drugs tested in the C6 cells (Figure 5 and Table 3). Surprisingly, IHCH-7079 appeared to have relatively high efficacy in the C6 cells (Figure 5). However, follow-up experiments with volinanserin revealed nonselectivity in the actions of IHCH-7079 in the C6 cells. Thus, whereas the Ca^{2+} responses to 10 μM of the psychedelic drugs as well as lisuride and TBG were abolished by 1 μM volinanserin (Figure S7B), the Ca^{2+} response to either 10 or 100 μM IHCH-7079 was not completely blocked by 1 μM volinanserin (Figure S7C). Taking the latter result into account, IHCH-7079 also had the expected low efficacy in the C6 cell model.

A $\Delta\log(E_{max}/EC_{50})$ scatter plot of Ca^{2+} responses for the C6 and SH-SY5Y cells emphasised the similarity in agonist activity in the two cell lines expressing the rat and human 5-HT_{2A} receptors, respectively (Figure S7A). None of the agonists were significantly biased towards Ca^{2+} signalling in one cell line over another ($\Delta\Delta\log(E_{max}/EC_{50})$ values, $P > 0.05$ post hoc Dunnett's test following one-way ANOVA; Table 2). Therefore, on the basis of the drugs tested here, the rat and human 5-HT_{2A} receptors have similar pharmacology despite having only 87% sequence homology (Barnes et al., 2021).

3.6 | Schild analysis of Ca^{2+} response in C6 cells to non-psychedelic and psychedelic 5-HT_{2A} receptor agonists

Finally, given the very low 5-HT_{2A} receptor signalling efficacy of each of lisuride, TBG and IHCH-7079, a Schild analysis was carried out using the C6 cells to test for evidence that these drugs also clearly exhibited antagonist properties as expected of drugs with weak partial agonist properties. Thus, Schild plots revealed that in the presence of 5-HT, each of lisuride, TBG and IHCH-7079 increased the response of low 5-HT concentrations and caused a parallel, rightward shift of the response of 5-HT at high concentrations (Figure S8 and Table 4). Schild plots for the psychedelic agonists also confirmed the partial agonist properties of these drugs (Table 4). Note that in some cases, statistical analysis revealed that the Schild plot slopes were significantly less than 1, resulting in a potential overestimate of the pA_2 values.

TABLE 4 Schild plot slopes, linear regression and pA_2 measurements for psychedelic and non-psychedelic drugs calculated from Ca^{2+} responses in the presence of 5-HT in C6 cells expressing rat 5-HT_{2A} receptors.

Drug	Schild plot slope	Linear regression R^2	pA_2
5-MeO-DMT	1.00	0.982	6.31
DOI	0.362	0.832	9.37[a]
Mescaline	0.927	0.883	6.76
Psilocin	1.33	0.969	6.66
Lisuride	0.314	0.850	9.734[a]
LSD	0.900	0.993	6.68
25B-NBOMe	0.474	0.756	8.53[a]
TBG	0.302	0.758	9.03[a]
IHCH-7079	1.88	0.881	7.40

Note: Non-psychedelic drugs in italics.

[a]pA_2 value calculated despite a Schild plot slope being statistically significantly different from 1.

Overall, as observed in the SH-SY5Y cells, the data from the C6 cells confirmed that the non-psychedelic drugs lisuride, TBG and IHCH-7079 were very low efficacy 5-HT_{2A} receptor agonists compared with the psychedelic drugs.

4 | DISCUSSION

It is unknown why some 5-HT_{2A} receptor agonists are psychedelic and others are not, with both biased agonism and partial agonism being plausible explanations (see Section 1). Although it is currently unclear whether the subjective effects of psychedelic drugs are necessary for their therapeutic effects, 5-HT_{2A} receptor agonists without psychedelic effects are of clinical interest given the potential practical and safety issues associated with the use of psychedelic drugs. The present study characterised the 5-HT_{2A} receptor signalling properties of a panel of psychedelic drugs (psilocin, 5-MeO-DMT, LSD, mescaline, 25B-NBOMe and DOI) and non-psychedelic drugs (lisuride, TBG and IHCH-7079), with a focus on G_q- versus β-arrestin2-coupled pathways. We report two key findings: (i) The psychedelic drugs were 5-HT_{2A} receptor agonists in models of both G_q and β-arrestin2 signalling and were typically unbiased relative to 5-HT and with lower efficacy than 5-HT, and (ii) the non-psychedelic drugs lisuride, TBG and IHCH-7079 were indistinguishable from psychedelic drugs in terms of their ligand bias properties but exhibited the lowest 5-HT_{2A} receptor

signalling efficacy of all drugs tested. Finally, it is noteworthy that the two ergolines tested, lisuride and LSD, showed greater signalling activity in the IP_1 compared with Ca^{2+} assay.

4.1 | 5-HT_{2A} receptor ligand bias did not discriminate between psychedelic and non-psychedelic drugs

One explanation for why some 5-HT_{2A} receptor agonists are psychedelic and not others is biased agonism, that is, preference for one 5-HT_{2A} receptor signalling pathway versus another. Here, all the psychedelic and non-psychedelic drugs tested activated both 5-HT_{2A} receptor-mediated G_q and β-arrestin2 signalling (with the exception of IHCH-7079, which behaved as an inverse agonist in the IP_1 assay—see below). Although lisuride and IHCH-7079 displayed preference for one signalling pathway versus another, ligand bias (relative to 5-HT) did not discriminate between psychedelic and non-psychedelic drugs. Thus, none of the psychedelic drugs showed significant G_q versus β-arrestin2-biased signalling. Moreover, of the non-psychedelic drugs tested, whereas lisuride was biased towards G_q signalling, IHCH-7079 was biased towards β-arrestin2 signalling, and TBG showed no ligand bias.

As far as we are aware, this is the first report of 5-HT_{2A} receptor ligand bias for TBG. The finding that the drug does not exhibit a preference for G_q and β-arrestin2 signalling suggests that bias does not explain its non-psychedelic behavioural profile in mice (Cameron et al., 2020). Our observation that IHCH-7079 has a preference for the β-arrestin2 pathway agrees with the claims of a recent study (Dongmei et al., 2022) but, in support, we now provide quantitative evidence of β-arrestin2-biased signalling. More specifically, IHCH-7079 was a weak agonist for β-arrestin2 pathway but interestingly displayed inverse agonist properties in the G_q signalling assay (IP_1 accumulation), such that a fully quantitative measure of bias was not possible. Presumably, IHCH-7079 behaves as an inverse agonist and agonist for G_q and β-arrestin signalling, respectively, because it stabilises the 5-HT_{2A} receptor in a state that favours the latter pathway. Our finding that lisuride shows G_q-biased signalling is in accord with a previous study using in vivo models of 5-HT_{2A} receptor signalling (Pogorelov et al., 2023). Other studies have assessed G_q and β-arrestin2 signalling of lisuride (measured by BRET-based G_q and β-arrestin2 recruitment) in HEK293T cells transfected with human 5-HT_{2A} receptor (Dongmei et al., 2022; Wallach et al., 2023), but again, we provide ligand bias quantification for this drug.

Our principal model of G_q and β-arrestin2-biased signalling used the human 5-HT_{2A} receptor and measurement of IP_1 accumulation and β-arrestin2 recruitment combined with a scatter plot of Δlog (E_{max}/EC_{50}) values for each agonist in each signalling pathway combined with statistical analysis of ΔΔlog (E_{max}/EC_{50}) values (Kenakin, 2017). A strength of this model is that the use of a reference agonist (here 5-HT) accounts for assays with potential differences in receptor reserves and signal amplification. The current study also generated a scatter plot of Δlog (E_{max}/EC_{50}) values for Ca^{2+} versus β-arrestin2 signalling and corresponding ΔΔlog (E_{max}/EC_{50}) values, and this also did not discriminate between the psychedelic and non-psychedelic drugs. However, it should be noted that the latter plot is limited to the extent that Ca^{2+} was measured under non-equilibrium conditions, whereas β-arrestin2 measurements were performed at equilibrium (see below).

There are currently few quantitative studies of 5-HT_{2A} receptor biased signalling of psychedelic versus non-psychedelic drugs. Our data showing that psilocin and other psychedelic drugs lack ligand bias are in accord with a very recent study also reporting that psilocin, LSD, DOI and 5-MeO-DMT have similar activity at human 5-HT_{2A} receptor-mediated G_q and β-arrestin2 signalling (Wallach et al., 2023), although ligand bias was not quantified using Δlog (E_{max}/EC_{50}) values. The current study focused on G_q and β-arrestin2 signalling because these are the best-characterised 5-HT_{2A} receptor-mediated pathways. Nevertheless, it is possible that psychedelic and non-psychedelic drugs might be discriminated by 5-HT_{2A} receptor signalling via other pathways. For example, there is evidence of 5-HT_{2A} receptor coupling to the $G_{12/13}$ protein (Kurrasch-Orbaugh et al., 2003; Qu et al., 2005), and previous studies have shown that the G_i inhibitor pertussis toxin reduces LSD- but not lisuride-stimulated gene expression changes (González-Maeso et al., 2007). In spite of this evidence, there is a lack of agreement regarding the ability of the 5-HT_{2A} receptor to couple to the G_i protein (Gaitonde et al., 2024; Kim et al., 2020). There is also evidence that the 5-HT_{2A} receptor has the capacity to signal via the arachidonic acid pathway (downstream of the PLA_2 pathway) (Berg et al., 1998). However, it is reported that DOI, LSD and lisuride all showed signalling preference for arachidonic acid versus G_q signalling, indicating that bias at this particular 5-HT_{2A} receptor signalling pathway does not discriminate between psychedelic and non-psychedelic agonists (Berg et al., 1998). Although 5-HT_{2A} receptor signalling pathways other than those involving G_q and β-arrestin2 are yet to be fully defined, the current data cannot be extrapolated to such pathways.

4.2 | Non-psychedelic drugs had low efficacy 5-HT_{2A} receptor signalling compared with psychedelic drugs

A general feature of the drugs tested here is that they had lower efficacy compared with 5-HT in both G_q and β-arrestin2 assays and thereby were partial 5-HT_{2A} receptor agonists. This finding was robust and consistent across two cell systems (human neuroblastoma SH-SY5Y, rat C6 glioma). Interestingly, the non-psychedelic drugs lisuride, TBG and IHCH-7079 displayed the lowest efficacies (at concentrations at which signalling was demonstrated to be 5-HT_{2A}-mediated) of all agonists tested in both cell systems. This finding accords with a recent study reporting that compared with psychedelic drugs, lisuride, IHCH-7079 and IHCH-7086 (another putative non-psychedelic) exhibited low efficacy in both G_q and β-arrestin2 signalling pathways mediated by human 5-HT_{2A} receptors (Dongmei et al., 2022).

Similarly, other recent papers report that the putative non-psychedelic drugs Ariadne (Cunningham et al., 2023) and **2-Br-LSD** (Lewis et al., 2023), as well as lisuride (Glatfelter et al., 2024), show low efficacy in human 5-HT_{2A} receptor-coupled G_q and β-arrestin2 signalling pathways compared with the psychedelic drugs **DOM** and LSD. These findings taken together with the current data suggest that a feature of non-psychedelic drugs that differentiates them from psychedelic drugs is their lower efficacy at 5-HT_{2A} receptors.

It was recently concluded that a certain level of efficacy is required for a 5-HT_{2A} receptor agonist to elicit a head-twitch response in mice (Wallach et al., 2023). Specifically, of 14 phenethylamines tested, those with the lowest 5-HT_{2A} receptor signalling efficacy lacked the propensity to evoke head-twitches. On the other hand, other studies suggest that low efficacy 5-HT_{2A} receptor agonists are still able to evoke antidepressant-like effects. For example, lisuride, TBG and IHCH-7086 were all reported to have such effects in mouse behavioural models as well as measurements of plasticity (Cameron et al., 2020; Dongmei et al., 2022; Lewis et al., 2023). It is, however, currently unknown whether these potential therapeutic properties of low efficacy 5-HT_{2A} receptor agonists translate to humans or indeed whether non-psychedelic drugs such as TBG and IHCH-7079 lack the ability to evoke subjective effects in humans as predicted by mouse head-twitch data. The 5-HT_{2A} receptor agonists, Ariadne and 2-Br-LSD, which are ineffective in the head-twitch model, are reported to elicit some psychoactive effects when administered at high doses to humans (Cunningham et al., 2023; Lewis et al., 2023). This outcome raises the possibility that there may be a continuum in psychedelic effects across the spectrum of 5-HT_{2A} receptor agonists rather than a clear segregation of psychedelic versus non-psychedelic propensities.

Against the idea that low efficacy accounts for the non-psychedelic properties of certain 5-HT_{2A} receptor agonists is the fact that, here, measures of efficacy are set against the most efficacious agonist 5-HT, which might be considered as non-psychedelic. However, hallucinations are a clinical feature of the 5-HT syndrome in humans, and the 5-HT precursor **5-hydroxytryptophan** is well known to elicit head-twitches in mice (Badar, 2024; Goodwin & Green, 1985). Although these are characteristic responses to high 5-HT availability, 5-HT itself will activate multiple interacting, and sometimes opposing, 5-HT receptors (see later). Furthermore, a recent report suggested that 5-HT may need to access intracellular 5-HT_{2A} receptors to elicit psychedelic effects (Vargas et al., 2023), in which case, high 5-HT levels are likely required to penetrate the cell.

4.3 | 5-HT_{2A} receptor ligand bias of ergolines

Surprisingly, our data revealed that the two ergolines LSD and lisuride exhibited the greatest bias of all agonists in the IP_1 versus Ca^{2+} assays, which should be measuring the same G_q signalling pathway. These data suggest that, at least in the case of these ergolines, the measurement of ligand bias may be influenced by assay format.

Both our IP_1 and β-arrestin2 assays were run under conditions in which agonists had likely reached equilibrium with the receptor and therefore likely provided an accurate measure of ligand bias. On the other hand, the Ca^{2+} assay readout was obtained immediately after agonist addition when the drug and receptor were likely under non-equilibrium conditions. Comparison of agonist activity under equilibrium and non-equilibrium conditions potentially leads to inaccurate measures of ligand bias because kinetic differences in agonist on- and off-rates, as well as receptor residency times, can influence measures of agonist efficacy and potency (Finlay et al., 2020; Kenakin, 1984).

Our finding that lisuride and LSD had increased 5-HT_{2A} receptor signalling in the IP_1 versus Ca^{2+} assay agrees with previous studies of ergoline actions at the **5-HT_{2B} receptor** (Bdioui et al., 2018; Unett et al., 2013; Wacker et al., 2017) (a structural 5-HT_{2A} receptor homologue). Interestingly, it is reported that LSD has a unique 5-HT_{2B} receptor binding mode in which a molecular 'lid' hinders drug on- and off-rates and prolongs residency times (Kim et al., 2020; McCorvy et al., 2018; Wacker et al., 2017). Presumably, this also applies to lisuride. Thus, the Ca^{2+} assay may underestimate the activity of LSD and lisuride due to the drugs not having reached equilibrium, leading to an apparent IP_1 versus Ca^{2+} bias. On the other hand, our finding that lisuride shows G_q-biased signalling using the IP_1 and β-arrestin2 assays may be a more accurate finding because both assay conditions were at equilibrium. Overall, our findings with LSD and lisuride support the contention that the kinetics and equilibrium state of signalling assays is an important consideration when measuring ligand bias parameters.

4.4 | Role of 5-HT_{2A} receptor signalling pathways in mediating behavioural effects

Some critical level of 5-HT_{2A} receptor signalling efficacy is likely required to elicit psychedelic effects in that, as noted above, low efficacy 5-HT_{2A} receptor agonists lack an ability to elicit head-twitches in mice and are therefore predicted to have a low psychedelic propensity in humans. In the current study, there was no clear pattern of 5-HT_{2A} receptor ligand bias for the psychedelic and non-psychedelic drugs tested to inform on the likely pathways mediating the psychedelic effects. Moreover, the literature is currently unclear on this point. Some evidence suggests a role for G_q signalling in the head-twitch response; for example, inhibitors of **inositol monophosphatase**, a key enzyme in the G_q signalling pathway, reduced head-twitches induced by DOI and psilocin (Antoniadou et al., 2018). Also, G_q but not β-arrestin2 signalling efficacy was correlated with a propensity to evoke head-twitches (Wallach et al., 2023). However, data generated using β-arrestin2 knockout mice are inconsistent. Although one study reported that LSD-induced head-twitches were attenuated in this mouse (Rodriguiz et al., 2021), other studies found that DOI-induced head-twitches were not (de la Fuente Revenga et al., 2022; Schmid et al., 2008).

There are similarly conflicting findings regarding the 5-HT_{2A} receptor signalling pathways that mediate different behavioural and neuroplastic effects. Thus, inositol monophosphatase inhibitors were

reported to reduce DOI-evoked expression of markers of neural plasticity (Antoniadou et al., 2018), suggesting a role for G_q signalling. Accordingly, a phospholipase C inhibitor prevented the increase in plasticity genes in cultured mouse cortical neurons exposed to LSD and lisuride (González-Maeso et al., 2007). On the other hand, another study reported that several β-arrestin2-biased 5-HT_{2A} receptor agonists showed antidepressant-like activity in tail suspension and forced swim tests in mice (Dongmei et al., 2022). A further complication is recent evidence that some behavioural and neuroplastic effects of psychedelic drugs are not mediated by 5-HT_{2A} receptors alone but that the neurotrophic factor receptor **TrkB** also may play a role (Moliner et al., 2023).

A final point is that whilst the signalling pathways underlying the behavioural effects of 5-HT_{2A} receptor agonists are currently uncertain, other factors add further uncertainty. In particular, most (if not all) psychedelic and non-psychedelic drugs are not selective 5-HT_{2A} receptor agonists and exhibit affinity for other 5-HT receptors and receptors for other neurotransmitters. Moreover, different 5-HT receptors can interact to influence 5-HT_{2A} receptor function at a behavioural level. For example, there is evidence that agonist activity at **5-HT_{1A}** receptors opposes 5-HT_{2A} receptor function, such that drugs with high 5-HT_{1A} versus 5-HT_{2A} receptor activity lack efficacy in the head-twitch model (Warren et al., 2024). Thus, the polypharmacology of 5-HT_{2A} receptor agonists likely plays on the behavioural effects of these drugs.

5 | CONCLUSIONS

The present study reports that the six psychedelic drugs tested were not biased towards either 5-HT_{2A} receptor-mediated G_q or β-arrestin2 signalling pathways. Similarly, three non-psychedelic 5-HT_{2A} receptor agonists, lisuride, TBG and IHCH-7079 also did not demonstrate a consistent bias. Rather, a feature of the latter drugs was their low 5-HT_{2A} receptor efficacy in both G_q and β-arrestin2 signalling pathways. This finding is in accordance with other studies reporting low efficacy of specific non-psychedelic 5-HT_{2A} receptor agonists. Although a quantitative, side-by-side comparison of the 5-HT_{2A} receptor ligand bias of larger panel of psychedelic and non-psychedelic drugs is warranted, our findings support the contention that low efficacy rather than ligand bias accounts for why some 5-HT_{2A} receptor agonists are non-psychedelic.

AUTHOR CONTRIBUTIONS

Conceptualisation: Aurelija Ippolito, Trevor Sharp, Grant Churchill. *Data curation*, *Formal analysis*, *Investigation*: Aurelija Ippolito. *Methodology*: Aurelija Ippolito, Sridhar Vasudevan, Frederick Westhorpe, Grant Churchill, Trevor Sharp. *Writing—original draft*: Aurelija Ippolito, Trevor Sharp. *Review and editing*: Trevor Sharp, Shaun Hurley, Gary Gilmour, Frederick Westhorpe, Grant Churchill. *Supervision*: Trevor Sharp, Grant Churchill, Gary Gilmour, Shaun Hurley. *Resources*: Shaun Hurley, Gary Gilmour, Frederick Westhorpe. *Funding acquisition*: Trevor Sharp.

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CONFLICT OF INTEREST STATEMENT

G.G., F.W. and S.H. are all employees of Compass Pathways plc.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

ORCID

Trevor Sharp https://orcid.org/0000-0001-7434-9713

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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