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# Arylpiperazine agonists of the serotonin 5-$HT_{1A}$ receptor preferentially activate cAMP signaling versus recruitment of β-arrestin-2

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ABSTRACT

G protein-coupled receptors (GPCRs) mediate biological signal transduction through complex molecular pathways. Therapeutic effects of GPCR-directed drugs are typically accompanied by unwanted side effects, owing in part to the parallel engagement of multiple signaling mechanisms. The discovery of drugs that are 'functionally selective' towards therapeutic effects, based on their selective control of cellular responses through a given GPCR, is thus a major goal in pharmacology today. In the present study, we show that several arylpiperazine ligands of the serotonin 5-$HT_{1A}$ receptor (5-$HT_{1A}$R) preferentially activate 3′,5′-cyclic adenosine monophosphate (cAMP) signaling versus β-arrestin-2 recruitment. The pharmacology of these compounds is thus qualitatively different from the endogenous agonist serotonin, indicating functional selectivity of 5-$HT_{1A}$R-mediated response pathways. Preliminary evidence suggests that phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) downstream of 5-$HT_{1A}$R is a substrate of functionally selective signaling by partial agonists. We propose that the compounds described in the present study are useful starting points for the development of signaling pathway-selective drugs targeting 5-$HT_{1A}$R.



## 1. Introduction

G protein-coupled receptors (GPCRs) form the largest group of integral membrane proteins in the human genome. Extensive analysis of sequence data and structural motifs has led to the definition of five main GPCR families, each of which comprises numerous subgroups and branches.[1] Members of these branches specialize in the detection of an astonishingly diverse set of stimuli, including peptides, small molecules and even photons.[2] By transferring information encoded in these stimuli from extracellular to intracellular space, GPCRs play central roles in virtually all domains of physiology, including sensory functions such as smell, taste, vision and pain. The canonical pathway of GPCR-mediated signaling involves intracellular coupling to heterotrimeric G proteins and subsequent activation of effector proteins, which in turn control the production or mobilization of second messengers such as 3′,5′-cyclic adenosine monophosphate (cAMP), phosphoinositides and calcium.[3] In addition, G protein-independent signaling pathways have been described in various cells and tissues, typically involving intracellular adaptor proteins such as the β-arrestins.[4] Binding of endogenous molecules or drugs (ligands) to their cognate GPCRs thus causes multifaceted cellular responses, which can be measured as a ligand's 'pluridimensional efficacy' when a given GPCR is interrogated using pharmacological assays.[5] Intriguingly, it is now known that GPCR-mediated responses can be selectively modulated by different ligands acting at a given GPCR. For example, some ligands activate or block all responses mediated by the receptor ('balanced' agonists and antagonists, respectively) while others significantly affect only a subset of the responses and thus qualify as 'biased' ligands. This form of 'functional selectivity' in GPCR-mediated signaling has fundamental implications for the discovery and development of new drugs as well as their clinical use.[6] Perhaps most importantly, it could be exploited to reveal those signaling pathways that underlie the therapeutic efficacy of a given

*Abbreviations:* 5-HT, 5-hydroxytryptamine; cAMP, 3′,5′-cyclic adenosine monophosphate; ERK1/2, extracellular signal-regulated kinases 1/2; GPCR, G protein-coupled receptor; $LogEC_{50}$, logarithm of agonist concentration that induces half-maximal response; $E_{max}$, maximal response induced by an agonist.

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ligand versus those that are associated with its unwanted side effects. Drugs could then be designed to selectively control therapeutic signaling pathways without causing side effects, thus being biased towards clinical efficacy. Given that the GPCR superfamily represents about 15% of the 'druggable genome',[7] and given that GPCRs serve as the main molecular targets for more than 25% of marketed drugs,[8] the development of compounds that act as functionally selective ligands for GPCRs is a promising and active area of research.

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is a hormone and neurotransmitter whose physiological roles include the regulation of mood, cognition, sleep, sex and appetite. Serotonin mediates its effects via a family of receptor proteins, most of which are GPCRs.[9–11] The first member of the 5-HT receptor family to be cloned and extensively characterized was 5-$HT_{1A}$R. 5-$HT_{1A}$Rs are abundantly expressed in the brain, where (among other roles) they act as somatodendritic autoreceptors to regulate the firing activity of 5-HT neurons. The principal mechanism of signal transduction employed by 5-$HT_{1A}$R involves coupling to heterotrimeric $G_{i/o}$ proteins, $G_{i/o}$-mediated inhibition of adenylate cyclase and subsequent reduction of intracellular cAMP. For several ligands of 5-$HT_{1A}$R, functional selectivity has been documented with respect to signaling through specific $G_{i/o}$ protein subtypes (reviewed in Refs. 12,13). However, there are currently no ligands with functional selectivity for $G_{i/o}$-mediated cAMP signaling versus β-arrestin pathways downstream of 5-$HT_{1A}$R. In fact, to the best of our knowledge, there is only one report in the academic literature explicitly showing recruitment of β-arrestin-2 to 5-$HT_{1A}$R, that is, direct physical contact between the two proteins.[14] Given the general therapeutic potential of G protein-biased and β-arrestin-biased signaling, respectively,[4] as well as the therapeutic use of 5-$HT_{1A}$R as a drug target,[15] we sought to identify such functionally selective ligands. To this end, we decided to select a pilot set of high-affinity 5-$HT_{1A}$R ligands belonging to the class of long-chain arylpiperazines previously studied by our group (Fig. 1). This approach was prompted by a recent report which showed how structural manipulation of the long-chain arylpiperazine scaffold of aripiprazole, a partial agonist of the dopamine $D_2$ receptor, produced compounds with functional selectivity for the β-arrestin versus the G protein/cAMP pathway.[16] For the scope of the present experiments, we selected from our chemical library three 1-(2-pyridyl)piperazine derivatives that were known to potently stimulate 5-$HT_{1A}$R-mediated [$^{35}$S]GTPγS binding[17] (compounds **1**–**3**), and three additional 1-(2-pyridyl)piperazine derivatives (compounds **4**–**6**)[17] (Fig. 1) whose ability to activate 5-$HT_{1A}$R was unknown. Due to the presence of the basic nitrogen-containing 1-(2-pyridyl)piperazine system that is responsible for ligand interactions with Asp3.32 of 5-$HT_{1A}$R, the critical site for ligand binding and signal transduction,[18] we expected compounds **4**–**6** to possess agonist activity as well. Thus, for this initial exploration, we have selected close structural analogs characterized by subtle differences in the linker flexibility and terminal fragment structure.

## 2. Materials and methods

### 2.1. Drugs and reagents

All cell culture media and supplements were from Life Technologies (Stockholm, Sweden). Drugs were obtained from Sigma–Aldrich (Stockholm, Sweden; catalog numbers: forskolin, F6886; 5-hydroxytryptamine hydrochloride [5-HT], H9523; (±)-8-hydroxy-2-(dipropylamino)tetralin hydrobromide [(±)-8-OH-DPAT], H8520; pargyline, P8013; theophylline, T1633). A Chinese hamster ovary (CHO) cell line that stably expresses the human 5-$HT_{1A}$ receptor (hereafter referred to as CHO-1A) was kindly provided by Prof. Kelly Berg, University of Texas Health Science Center at San Antonio, USA. Receptor surface expression levels are relatively low in CHO-1A cells, equaling about 140 fmol/mg protein.[19] Cell passage number was <10 for all of the experiments reported here. Antibodies against phosphorylated ERK1/2 and calnexin were from Cell Signaling Technology (Beverly, MA, USA; 9101L, used at 1:500) and Sigma–Aldrich (C4731, used at 1:4000), respectively. The PathHunter eXpress HTR1A CHO-K1 β-Arrestin GPCR Assay was obtained from DiscoveRx (Fremont, CA, USA). Arylpiperazine test compounds were synthesized at the University of Bari and used in the form of hydrochloride salts. Compounds **1** and **6** were racemic mixtures.

### 2.2. Cell culture and drug treatments

CHO-1A cells were grown in culture medium (MEM alpha supplemented with 5% FBS and 50 μg/mL geneticin) at 37 °C in an atmosphere of 5% $CO_2$/95% air. For seeding into 96-well plates and assays of cAMP accumulation, cells were resuspended in culture medium at a concentration of $5 \times 10^4$/mL and 200 μL were dispensed for a final number of $10^4$ cells/well. After 24 h, cells in 96-well plates were rinsed with Hank's balanced salt solution (HBSS) and subsequently switched to 100 μL/well of serum-free medium (DMEM/F-12) for an additional 24 h before treatment. Drugs were prepared at 2× of their final concentration in serum-

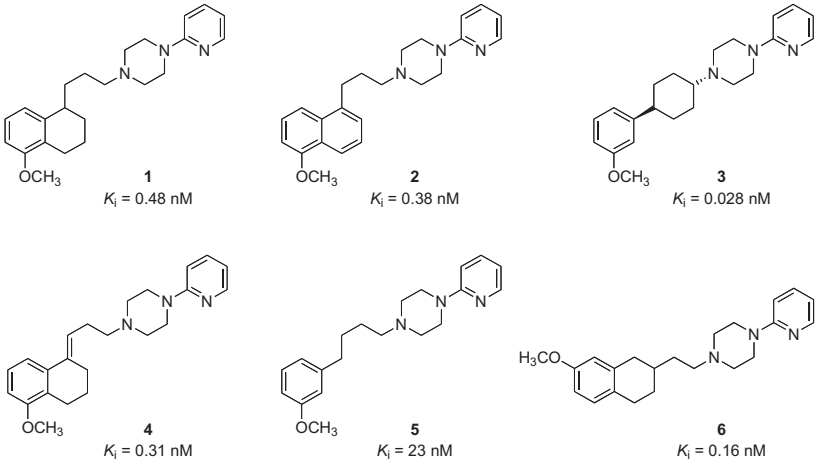


**Figure 1.** Long-chain arylpiperazine ligands with high affinity for 5-$HT_{1A}$R. See Refs. 17,38 for details.

free medium containing 5 μM pargyline and 2.5 mM theophylline. Stock solutions of 5-HT and (±)-8-OH-DPAT (1 mM in water) were freshly prepared from powder on the day of each experiment, while the test compounds and forskolin were prepared from refrigerated or frozen stocks in DMSO or water, respectively. For each treatment condition, 100 μL of 2× drug solution were dispensed per well. To measure their impact on forskolin-stimulated cAMP accumulation, test compounds were co-incubated with 1 μM forskolin for 15 min. Untreated wells received 100 μL of serum-free medium containing pargyline and theophylline, and 1% DMSO was used as a vehicle control (DMSO concentration was 0.1% at 10 μM of the test compounds). Plates were incubated at 37 °C and 5% $CO_2$/95% air. Medium was removed from all wells before addition of 100 μL lysis buffer (1% Triton X-100 in 0.1 M HCl) and incubation at room temperature for 15 min. Lysates were collected in polypropylene tubes and stored at −20 °C until analysis.

For phosphorylation assays, CHO-1A cells ($2 \times 10^5$ per well) were seeded into 12-well plates and grown in culture medium (see above) for 24 h. Cells were then switched to serum-free medium (DMEM/F-12) and grown for an additional 24 h before treatment. Agonists were diluted to their final concentration (1 μM) in serum-free medium containing 5 μM pargyline and 2.5 mM theophylline. After addition of agonists, CHO-1A cells were incubated at 37 °C and 5% $CO_2$/95% air for five minutes. This treatment time frame is based on previous experiments in CHO cell lines expressing low surface levels of human 5-$HT_{1A}$R (≈50 fmol receptor per mg protein), which showed that agonist-induced phosphorylation of ERK1/2 is maximal after five minutes.[20] Treatment was stopped by rinsing the cells with ice-cold PBS, adding 100 μL of protein extraction buffer (150 mM NaCl, 25 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1% SDS; containing 1× Halt protease and phosphatase inhibitor cocktail, Thermo Scientific, Göteborg Sweden) per well and then quickly placing the 12-well plates on dry ice. Cell lysates were thawed and harvested after keeping the plates at −20 °C over night.

### 2.3. Measurement of cAMP signaling

Concentrations of cAMP in cell lysates from 96-well plates (see above) were determined using a commercial ELISA (cyclic AMP Complete ELISA Kit, KA0320; Abnova, Taiwan) according to the manufacturer's instructions for acetylated samples.

### 2.4. Measurement of β-arrestin-2 recruitment

Recruitment of β-arrestin-2 to 5-$HT_{1A}$R was measured using a commercially available kit (PathHunter eXpress HTR1A CHO-K1 β-Arrestin GPCR Assay, DiscoveRx 93-0696E2CP0M) according to the manufacturer's instructions. PathHunter eXpress CHO-K1 cells were thawed, resuspended in cell plating reagent, seeded into 96-well plates ($10^4$ cells per well) and incubated for 24–48 h at 37 °C and 5% $CO_2$. Dilution series of each test compound (from 10 mM stocks in methanol) were prepared at 11× of the final concentration (0.1–100 μM) and dispensed in volumes of 10 μL per well, after which cells were incubated for 90 min at 37 °C. Following addition of PathHunter® detection reagents (55 μL per well), 96-well plates were incubated for another 60 min at room temperature. Finally, luminescence signals from the assay were captured using a bench top plate reader (VICTOR3, Perkin Elmer, Waltham, MA, USA).

### 2.5. Measurement of ERK1/2 phosphorylation

Cellular lysates were briefly sonicated (to reduce sample viscosity), heated to 95 °C for ten minutes (to denature proteins) and centrifuged (to remove cellular debris). Cleared lysates were then mixed with loading buffer to obtain final concentrations of 50 mM Tris, 8% glycerol, 1.6% SDS, 0.008% bromophenol blue and 4% β-mercaptoethanol in each sample. Proteins were separated by SDS–PAGE using 4–12% Bis–Tris gels and MOPS SDS running buffer (NuPAGE, Life Technologies), followed by transfer to PVDF membranes (Immobilon-P, 0.45 μm pore size; Millipore IPVH00010; Stockholm, Sweden) and parallel immunodetection of phosphorylated ERK1/2 and calnexin. Specific protein bands were visualized and their densitometric intensity was measured using ImageJ software (http://rsbweb.nih.gov/ij/). To control for protein loading and transfer, resulting numerical values for phosphorylated ERK1/2 were divided by the values for calnexin. Note that calnexin was used instead of total ERK1/2 in order to avoid stripping and reprobing of membranes, which had not worked sufficiently well in extensive preliminary experiments. Finally, values from untreated cells were subtracted from all other experimental groups, and values after treatment with 5-HT were defined as 100% within each experiment. Results from three experiments, each carried out in triplicate, were pooled for statistical analysis.

### 2.6. Data analysis

Data analysis was carried out using GraphPad Prism 5.04 (GraphPad Software, La Jolla, CA). Results from concentration–response assays for cAMP signaling and β-arrestin-2 recruitment were normalized to the maximal effect of 5-HT (100%) and subsequently analyzed by nonlinear regression. Several of the test compounds displayed complex, non-sigmoidal, approximately bell-shaped concentration–response curves, particularly in the cAMP assay. Data for these compounds were analyzed by fitting a model based on the Gaussian distribution, as described in detail by Christopoulos et al. (see Eqs. 4 and 5 in Ref. 21). Standard concentration–response functions (sigmoidal models) were used for analysis of the remaining compounds as appropriate. Where possible, estimates of potency ($LogEC_{50}$) and maximal agonist effect ($E_{max}$) were derived as best-fit parameters from these curve fitting procedures. Data from ERK1/2 phosphorylation experiments were analyzed by one-way ANOVA with Dunnett's post-test to compare agonist-treated groups with the untreated control group.

## 3. Results

### 3.1. Concentration–response curves for cAMP signaling and β-arrestin-2 recruitment

Several of the compounds used in the present study are potent and efficacious agonists in terms of G protein activation via 5-$HT_{1A}$R, as previously determined by [$^{35}$S]GTPγS binding (see Ref. 17). To determine their potency and maximal effects in terms of downstream signaling, we measured compound-induced inhibition of forskolin-stimulated cAMP accumulation in a Chinese hamster ovary cell line stably expressing human 5-$HT_{1A}$R (hereafter referred to as CHO-1A). In every experiment, 5-HT was included as a reference (full agonist) and experimental results for all test compounds were expressed as percentages of the maximal agonist effect ($E_{max}$) induced by 5-HT. For comparative purposes, the established 5-$HT_{1A}$R agonist (±)-8-OH-DPAT was also included. Note that this racemic compound is a partial agonist of 5-$HT_{1A}$R, while its enantiomer (+)-8-OH-DPAT is a full agonist.[22] In our cAMP signaling assays, the arylpiperazine compounds displayed unusual concentration–response behavior (Fig. 2). While the data for 5-HT and (±)-8-OH-DPAT were in line with the sigmoidal concentration–response curve typically observed for agonists, the response to several test compounds showed a peak at submaximal concentrations and a dip at the highest concentrations tested. Data for

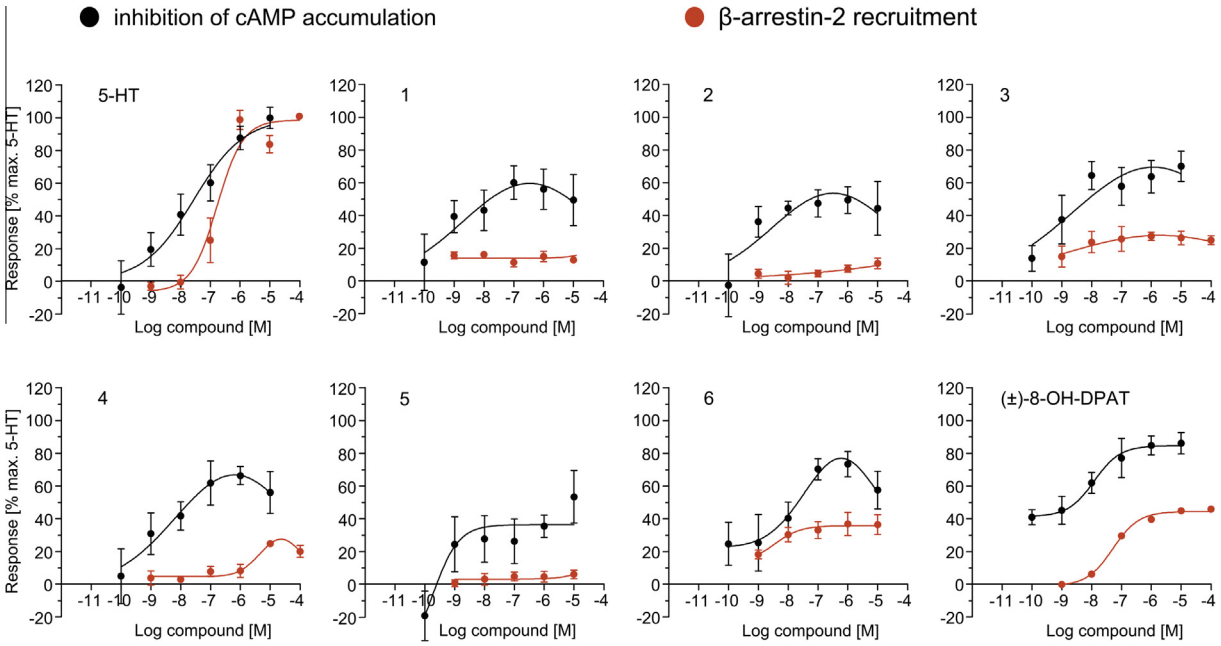


**Figure 2.** Concentration–response curves for 5-$HT_{1A}$R-mediated cAMP signaling and β-arrestin-2 recruitment. Activity towards the cAMP pathway (black symbols) was assessed by stimulating CHO-1A cells with 1 μM forskolin and measuring compound-mediated inhibition of forskolin-stimulated cAMP accumulation after 15 min of incubation. Cellular cAMP concentration after stimulation with 1 μM forskolin in the absence of compounds was defined as 0% (baseline) and the maximal effect of 5-HT (reference, full agonist) was defined as 100%. Agonist effects of the test compounds were expressed as percentages on this scale. Activity in terms of β-arrestin-2 recruitment (red symbols) was assessed in an analogous manner, with the maximal effect of 5-HT set to 100%, but here the baseline was defined by the signal in untreated cells and was subtracted from all other values. Curves represent pooled results from 3 to 4 independent experiments, each carried out in duplicate, with each assay plate containing 5-HT. Activity of (±)-8-OH-DPAT towards β-arrestin-2 recruitment was measured in two experiments. Therefore, corresponding data are shown without error bars. Data for all other compounds in both signaling assays are shown as means ± SEM.

these compounds were thus best approximated by a bell-shaped fit based on the Gaussian distribution (see Ref. 21 for details).

Compound **5** showed variable responses and was poorly described by either sigmoidal or bell-shaped fits. The concentration–response curve for compound **3** did not show a dip at the highest concentration, but overall the alternative fit yielded better parameter estimates and was therefore applied. Note that bell-shaped concentration–response curves have previously been observed for several ligands of 5-$HT_{1A}$R[23] and even 5-HT itself.[24] Possible mechanisms include activation of inhibitory and stimulatory G proteins, agonist-dependent switching between G protein subtypes, and time-dependent changes in coupling between receptor and G proteins.[23,24] The concentration–response curve for (±)-8-OH-DPAT indicated sizeable activity towards cAMP signaling even at the lowest concentration (Fig. 2). The reason for this is not known.

Where possible, estimates of potency ($LogEC_{50}$; logarithm of agonist concentration that induces a half-maximal response) and maximal agonist effect ($E_{max}$) were derived from curve fitting and summarized in Table 1. These estimates indicate that all six arylpiperazine ligands are partial agonists of cAMP signaling via 5-$HT_{1A}$R, with apparent $E_{max}$ values ranging from 53.4% to 73.5%. (±)-8-OH-DPAT behaved as a near-full agonist with an apparent $E_{max}$ of 84.8%.

In terms of β-arrestin-2 recruitment, 5-HT and (±)-8-OH-DPAT behaved as full and partial agonists, respectively. Concentration–response behavior of the arylpiperazine ligands was again mostly non-sigmoidal (Fig. 2), yielding very weak to moderate partial agonism in terms of apparent $E_{max}$ values (range: 5.9–35.9%; see Table 1). Most compounds showed both lower potency and weaker maximal effects in terms of β-arrestin-2 recruitment compared to cAMP signaling. Exceptions are compounds **3** and **6**, which were more potent in terms of β-arrestin-2 recruitment. Potency estimates for β-arrestin-2 recruitment could not be computed for **1**, **2** and **5**, the three compounds showing lowest maximal effect in this assay.

**Table 1**
Potency ($LogEC_{50}$) and maximal agonist effects ($E_{max}$) of 5-$HT_{1A}$R-mediated cAMP signaling and β-arrestin-2 recruitment

| Compound | Inhibition of cAMP accumulation | | β-Arrestin-2 recruitment | |
|---|---|---|---|---|
| | $LogEC_{50}$ | $E_{max}$ | $LogEC_{50}$ | $E_{max}$ |
| 5-HT | −7.56 ± 0.25 | 100 | −6.77 ± 0.16 | 100 |
| **1** | −9.11 ± 0.63 | 59.6 ± 8.6 | n.c. | 16.2 ± 1.2[a] |
| **2** | −8.90 ± 0.60 | 53.6 ± 8.3 | n.c. | 10.7 ± 3.1[a] |
| **3** | −9.10 ± 0.52 | 69.5 ± 6.6 | −9.49 ± 1.12 | 27.9 ± 3.1 |
| **4** | −8.53 ± 0.44 | 66.9 ± 8.2 | −5.47 ± 0.27 | 22.8 ± 4.1 |
| **5** | n.c. | 53.4 ± 16.1[a] | n.c. | 5.9 ± 2.7[a] |
| **6** | −7.68 ± 0.54 | 73.5 ± 7.7[a] | −8.45 ± 0.80 | 35.9 ± 3 |
| (±)-8-OH-DPAT | −7.92 ± 0.35 | 84.8 ± 4.8 | −7.29 ± 0.23 | 44.5 ± 2.9 |

$E_{max}$ values (expressed as percentages of the maximal effect of 5-HT) for the test compounds were obtained by curve fitting (see Section 2) as well as from simple plots of the concentration–response data shown in Figure 2.

[a] Where curve fitting yielded ambiguous parameter estimates, values drawn from plots of the data are listed in the table. $LogEC_{50}$ values were derived by curve fitting of the concentration–response data shown in Figure 2. All values are presented ±SEM. n.c., not computable.

### 3.2. Partial agonists of 5-$HT_{1A}$R favor cAMP signaling over β-arrestin-2 recruitment

To establish whether any of the tested ligands qualify as functionally selective agonists, we attempted to analyze our data using two recently published methods.[25,26] However, fitting the proposed models to our data yielded ambiguous parameter estimates, most likely due to the unusual concentration–response curves described above, such that we were unable to carry out a rigorous

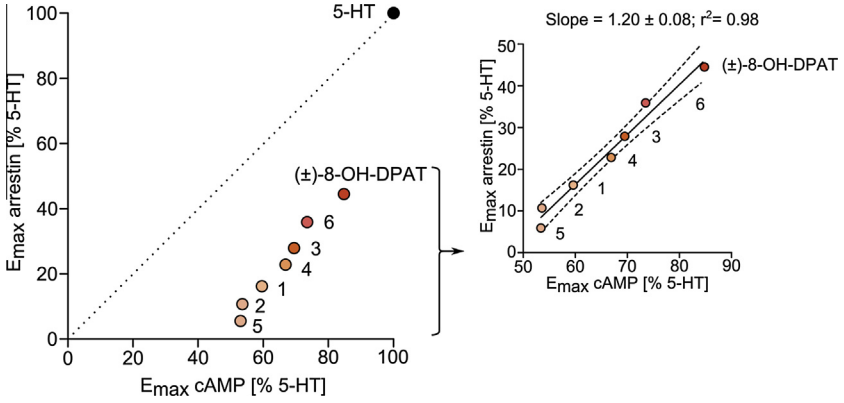


**Figure 3.** Correlation plots of agonist effects in terms of cAMP signaling and β-arrestin-2 recruitment. The full agonist 5-HT is 'balanced' by definition, that is, has equal maximal effects of 100% at both cellular response pathways. Balanced partial agonists should also have equal maximal effects at both pathways, thus following a linear correlation (see dotted line in left panel). However, the compounds tested in the present study display a sizable preference for the cAMP pathway, which is apparent as a deviation from the linear correlation defined by the reference agonist 5-HT. A separate plot of their agonist effects indicates a close correlation between $E_{max}$ cAMP and $E_{max}$ arrestin, with a slope of 1.20 ± 0.08 (95% CI: 0.98–1.42; $r^2$ = 0.98; see dotted lines in right panel). See Figure 2 and Table 1 for the underlying data.

quantitative analysis and statistical evaluation. We therefore employed a simple and conceptually straightforward method, which identifies functional selectivity of a group of agonists from a correlation plot of maximal effects across two cellular response pathways.[27,28] If a given compound has no preference for either of the pathways, that is, the relative maximal agonist effect (expressed as % 5-HT) in pathway 1 is the same as in pathway 2, the compound is considered 'balanced'. The distribution of effects for a series of balanced partial agonists thus follows a straight line, the upper boundary of which is defined by the full agonist 5-HT (which is balanced by definition, that is, its maximal effect is defined as 100% at both pathways). Correlation plots of our data indicate that all test compounds display functional selectivity, in that there is a sizable preference for cAMP signaling over β-arrestin-2 recruitment (Fig. 3, left panel). In a separate plot for these compounds, we found a close linear correlation between their $E_{max}$ cAMP and $E_{max}$ arrestin (slope = 1.20 ± 0.08; 95% CI: 0.98–1.42; $r^2$ = 0.98; see right panel in Fig. 3).

### 3.3. Differential stimulation of ERK1/2 phosphorylation by two arylpiperazine ligands of 5-$HT_{1A}$R

Compounds **2** and **3** showed similar activity towards cAMP signaling ($E_{max}$ 53.6% vs 69.5%) but a sizeable difference in terms of β-arrestin-2 recruitment ($E_{max}$ 10.7% vs 27.9%), raising the possibility that additional cellular responses are differentially affected by these two compounds. To test this, we focused on phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2; also known as p44/42 MAPK). These kinases have previously been used as readouts for functional selectivity at 5-HT receptors including 5-$HT_{1A}$R (see Ref. 13 for review) and represent a common node within β-arrestin-dependent signaling pathways.[4] Exposure of CHO-1A cells to 1 μM 5-HT for five minutes caused a 6.4-fold increase in phosphorylation of ERK1/2 compared to basal values. The relative agonist effect of 1 μM **3** was 30.4% (different from basal; one-way ANOVA with Dunnett's post-test, $^{***}p$ <0.001), whereas compound **2** failed to stimulate phosphorylation (Fig. 4). Given that **2** and **3** are nearly equiactive towards cAMP at a concentration of 1 μM, this result supports the notion that the two compounds are functionally selective agonists of 5-$HT_{1A}$R.

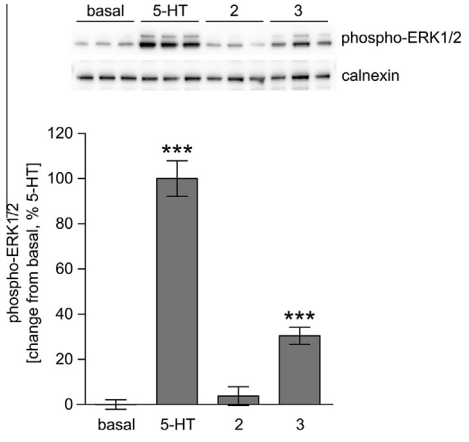


**Figure 4.** Agonist-induced phosphorylation of ERK1/2 downstream of 5-$HT_{1A}$R. CHO-1A cells were treated for five minutes with 1 μM 5-HT, **2** or **3**. Phosphorylated ERK1/2 (phospho-ERK1/2) was detected by Western blot and quantified using densitometry. Calnexin was detected in parallel to control for equal protein loading and transfer. Three experiments were carried out (representative example shown here), with each of the four conditions assayed in triplicate (see top panel in the figure), and baseline-subtracted results were expressed as percentages relative to 5-HT (±SEM). Data were analyzed by one-way ANOVA with Dunnett's post-test (comparison of the three agonists versus basal; $^{***}p$ <0.001). Compound **3** is a partial agonist for ERK1/2 phosphorylation, achieving 30.4% of the effect induced by 5-HT. In contrast, compound **2** fails to induce phosphorylation compared to basal values.

## 4. Discussion

Recent years have seen a surge of publications reporting non-classical effects of GPCR ligands, such as patterns of agonist activity that are inconsistent with established definitions of efficacy. These non-classical behaviors have been described by a variety of terms including 'functional selectivity' and 'biased agonism',[29] referring (for example) to agonist-specific control of individual signaling pathways coupled to a given GPCR. Two important potential applications emerge from the recognition of this 'new pharmacology'. First, signaling pathway-selective ligands could be used as tools to define those GPCR-mediated cellular responses that underlie therapeutic efficacy versus drug side effects. Second, drugs could subsequently be designed to selectively affect therapeutic signaling pathways without causing side effects, thus being biased towards clinical efficacy. Recent results from a small trial in humans with a biased agonist of the μ receptor, a GPCR targeted by opioid compounds, support this latter notion.[30] In the present study, we attempted to identify ligands of the serotonin 5-$HT_{1A}$ receptor (5-$HT_{1A}$R) that enable selective control of its response pathways. This receptor is an important pharmacotherapeutic target for disorders of the nervous system, including anxiety,

depression and pain.[15] Our experiments were focused on cAMP signaling and β-arrestin-2 recruitment downstream of 5-$HT_{1A}$R, employing a small series of long-chain arylpiperazine ligands as test compounds. These compounds were chosen because long-chain arylpiperazine ligands of the dopamine $D_2$ receptor were recently shown to display functional selectivity towards β-arrestin recruitment versus cAMP signaling.[16] In all experiments, the test compounds were directly compared with the endogenous agonist 5-HT.

Relative to 5-HT, our results suggest that all tested compounds favor cAMP signaling over β-arrestin-2 recruitment downstream of 5-$HT_{1A}$R. Thus, it can be argued that the tested compounds display functional selectivity relative to the endogenous agonist. We emphasize that, in the context of our results, functional selectivity is used as 'a general term to describe all possible instances of ligands differentially influencing receptor behavior, including differences in pathway engagement'.[31] In this sense, biased agonism or 'ligand-biased signaling' is a subset of functional selectivity, the bona fide demonstration of which requires rigorous quantitative methods.[6,31,32] Generally speaking, there are several possible explanations for the apparent difference in pathway engagement reported here, that is, the fact that the arylpiperazine compounds displayed a sizeable preference for cAMP signaling versus β-arrestin-2 recruitment. One explanation would be that receptor-effector coupling efficiency is simply much higher between 5-$HT_{1A}$R and cAMP signaling, such that all compounds would appear more potent and efficacious towards this pathway. Similarly, because one of the studied pathways is subject to amplification (cAMP; measurement of intracellular second messenger) while the other is not (β-arrestin-2; recombinant assay system based on enzyme complementation), apparent potency and maximal effects could be confounded, leading to erroneous conclusions regarding compound activity.[32] Indeed, 5-HT and (±)-8-OH-DPAT are more potent in terms of cAMP signaling in our experiments, suggesting that receptor–effector coupling efficiency might be higher for this pathway. However, the difference in apparent potency between the two pathways is less than 10-fold for each compound (Table 1). Further, both in terms of potency and maximal agonist effect, the behavior of the compounds 'does not 'track' with the variation of the physiologically relevant (i.e., endogenous) agonist',[31] supporting the notion that the tested arylpiperazines are functionally selective for cAMP signaling relative to 5-HT.

Methods for identification of functionally selective compounds that are based on comparisons of maximal agonist effects, such as the correlation plot we employed (Fig. 3), have been described as 'prone to errors in interpretation in the setting of receptor reserve'.[32] Similar to the issue of coupling efficiency mentioned above, receptor reserve could theoretically account for the apparent differences in signaling pathway engagement and thus confound our results. We think that this is likely not the case, given that the CHO-1A cell line that we used for assays of cAMP signaling expresses relatively low levels of 5-$HT_{1A}$R, namely 140 fmol/mg protein.[19] In cell lines expressing 45 or 500 fmol/mg and in rat hippocampal membranes expressing 233 fmol/mg of 5-$HT_{1A}$R, there is no receptor reserve for 5-$HT_{1A}$R-mediated inhibition of cAMP production.[33,34] Taken together, data from systems in which expression levels of 5-$HT_{1A}$R are very similar to ours suggest that the observed preference of arylpiperazine agonists for cAMP signaling does not arise simply due to nonphysiological overexpression of 5-$HT_{1A}$R or a large receptor reserve. Finally, given that the efficiency of receptor–effector coupling and the impact of receptor reserve on measures of agonist activity downstream of 5-$HT_{1A}$R is strongly dependent on cell type (see Ref. 35 and references therein), it is important to emphasize that the cellular background (CHO-K1) is the same for the two assays that we employed in the present experiments.

Our results show that a partial agonist of 5-$HT_{1A}$R-mediated β-arrestin-2 recruitment, such as **3**, can act as a partial agonist of ERK1/2 activation. In contrast, a compound with little activity towards β-arrestin-2 recruitment, such as compound **2**, fails to stimulate ERK1/2 activation despite potent partial agonism towards cAMP signaling. Regardless of the exact mechanisms, this suggests that two compounds whose $E_{max}$ is similar for cAMP signaling but different for β-arrestin-2 recruitment (see Table 1) can differentially affect the activity of downstream signaling molecules. Thus, even if the observed functional selectivity were merely based on differential coupling efficiencies of the two measured responses (cAMP > arrestin), one could use the two compounds to selectively activate one (cAMP with compound **2**) or both signaling pathways (cAMP and ERK1/2 with compound **3**) downstream of 5-$HT_{1A}$R. This would provide a pharmacological approach through which the contribution of each signaling pathway to corresponding cellular phenotypes could be characterized.

Previous work has identified functionally selective agonists of 5-$HT_{1A}$R (reviewed in Refs. 12,13). In a study comparing the novel agonist F15599 and its congener F13714 with 5-HT and (+)-8-OH-DPAT, Newman-Tancredi and co-workers interrogated four effector pathways (receptor internalization, G protein activation, ERK1/2 phosphorylation and inhibition of cAMP accumulation) in order to detect functional selectivity.[36] The same group also reported that the arylpiperazine derivative aripiprazole shows higher potency towards receptor internalization than G protein activation, suggesting potential bias for this compound.[37] However, to the best of our knowledge, our present experiments provide the first direct evidence for preferential activation of cAMP signaling versus β-arrestin-2 recruitment downstream of 5-$HT_{1A}$R. Importantly, as mentioned above, compound **2** appears to have a strong pathway preference, being a potent partial agonist of cAMP but nearly silent in terms of β-arrestin-2 recruitment and ERK1/2 phosphorylation.

Due to the limited number of compounds studied, conclusions regarding structure–activity relationships are preliminary. Nonetheless, the strong pathway preference shown by the naphthalenyl derivative **2**, compared to the tetrahydronaphthalenyl analogs **1** and **4**, suggests that manipulation of the terminal fragment of these arylpiperazine derivatives might furnish pathway-selective compounds that favor cAMP over β-arrestin-2. Future experiments to determine whether the strong preference for cAMP signaling versus recruitment of β-arrestin-2 displayed by **2** is shared by newly designed arylpiperazine compounds, as well as established arylpiperazine drugs such as buspirone, ipsapirone and gepirone, are warranted. Further, we will analyze the actions of these compounds across a broader spectrum of 5-$HT_{1A}$R-mediated responses (G protein coupling, cAMP signaling, β-arrestin-2 recruitment, ERK1/2 phosphorylation, receptor internalization).

## 5. Conclusion

Our present study is the first to suggest that long-chain arylpiperazines can act as functionally selective agonists of 5-$HT_{1A}$R in terms of cAMP signaling versus β-arrestin-2 recruitment. When compared with the endogenous agonist 5-HT, most of our test compounds display a sizable preference for cAMP both in terms of potency and maximal agonist effect. Additional experiments are needed to clarify the mechanisms by which this pathway preference operates. Nonetheless, it should be possible to synthesize arylpiperazine agonists of 5-$HT_{1A}$R that are devoid of activity towards β-arrestin-mediated cellular responses. Such pathway-specific agonists would be useful probes to further elucidate 5-$HT_{1A}$R-mediated signaling and its physiological consequences.

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