Cellular Signalling 63 (2019) 109383

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Bell-shaped agonist activation of 5-HT_{1A} receptor-coupled $G\alpha i_3$ G-proteins: Receptor density-dependent switch in receptor signaling

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ARTICLE INFO

Keywords:
5-HT_{1A} receptor
G-protein coupling
Dimerization
Antibody capture
Biased agonist

ABSTRACT

A previous study observed bell-shaped concentration-response isotherms for activation of $G\alpha_{i3}$ G-protein subunits by high efficacy 5-HT_{1A} receptor agonists in a Chinese hamster ovary (CHO) cell line expressing high levels of these receptors. This suggested that a signaling switch took place in that cell line (from $G\alpha_{i3}$ to activation of other G-proteins) but it was unclear if such effects are observed for 5-HT_{1A} receptors in other cellular environments.

Here, using an antibody capture-based [^{35}S]GTPγS binding assay for $G\alpha_{i3}$ activation, we investigated whether efficacious 5-HT_{1A} receptor agonists (5-HT, F13714, befiradol, NLX-101), prototypical agonists ((+) and (−)8-OH-DPAT), and partial agonist, antagonists, inverse agonists (pindolol, WAY100635, spiperone) produced similar effects on 5 cell lines expressing different levels of human 5-HT_{1A} receptors.

In membranes from cell lines (HeLa, C6-glia and CHO-low) expressing moderate receptor levels (between 1 and 4 pmol/mg of protein), 5-HT, F13714, befiradol and NLX-101 elicited classical sigmoid concentration-response isotherms. In contrast, in cell lines (CHO-high, HEK-293F−) expressing high receptor levels (> 9 pmol/mg) these agonists elicited bell-shaped concentration-response isotherms that peaked at nanomolar-range concentrations and then returned to baseline or below. Spiperone elicited inverse agonist inhibitory sigmoid isotherms in all membrane preparations while WAY100635 was mostly 'silent' for $G\alpha_{i3}$ activation. The other compounds elicited diverse responses in the different cell lines suggesting that other factors, in addition to receptor expression levels, could be influencing $G\alpha_{i3}$ activation.

These data indicate that $G\alpha_{i3}$ G-protein activation by 5-HT_{1A} receptor ligands is highly dependent on receptor expression levels and on cellular background. Moreover, the induction of bell-shape concentration-response isotherms by 5-HT and other high-efficacy agonists is consistent with a switch in signaling to other G-protein-mediated signaling cascades, possibly elicited by receptor conformational changes.

1. Introduction

G-protein-coupled receptors (GPCRs), such as the serotonin 5-HT_{1A} receptor, constitute an important class of therapeutic targets, but much remains to be understood concerning their coupling to heterotrimeric G-protein subtypes and the effects of agonists thereon [1]. 5-HT_{1A} receptors are important targets in the treatment of various central nervous system disorders, including movement disorders [2], depression [3,4], schizophrenia [5], and pain [6], and in vitro studies indicate that they couple mainly to $G\alpha i/o$ protein subtypes, with the following Gα-subtype preference: $G\alpha i_3 > G\alpha i_2 \geq G\alpha i_1 \geq G\alpha o > G\alpha s > G\alpha z$ [7], although cell-type-specific differences were observed in the coupling to second messengers [7].

Such preferential coupling is physiologically relevant, because native rat 5-HT_{1A} receptors couple to different G-proteins in a brain region-dependent manner [8]. Thus, 5-HT_{1A} receptors were found to couple to both $G\alpha i_3$ and Gαo in raphe nuclei, but preferentially to Gαo in hippocampus, and $G\alpha i_3$ in cortex [8], possibly as a result of differential levels of G-protein subtype expression in specific brain regions [9,10]. Such considerations are an important element in the growing understanding of the actions of novel 'biased agonists' at 5-HT_{1A} receptors. Indeed, the recent discovery of compounds that preferentially activate particular G-protein populations, and thereby elicit distinct downstream signaling cascades, has raised the possibility of improved targeting of brain regions involved in therapeutic activities, rather than those that mediate undesirable effects [11,12]. The selective 5-HT_{1A}

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https://doi.org/10.1016/j.cellsig.2019.109383
Received 6 June 2019; Received in revised form 29 July 2019; Accepted 30 July 2019
Available online 31 July 2019

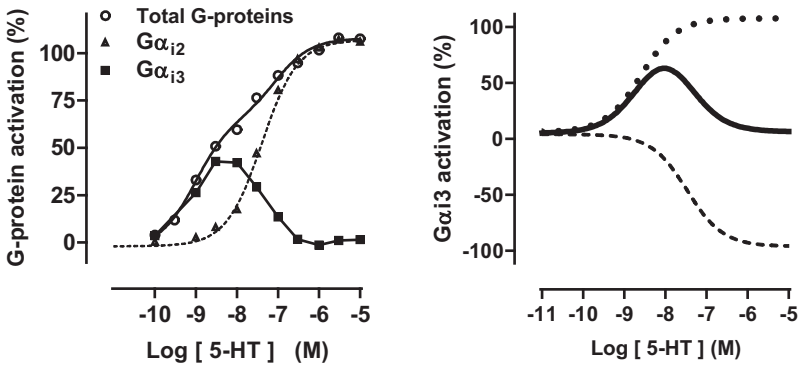

Fig. 1. Effects of 5-HT on G-protein activation in cell lines expressing high levels of 5-HT_{1A} receptors. Left-hand panel: when classical [^{35}S]GTPγS binding experiments are carried out, stimulation curves are biphasic (open circles), consistent with detection of 2 populations of G-proteins [16]. These can be resolved into 2 components corresponding to Gαi_3 activation (black squares) and Gαi_2 activation (triangles). Right-hand panel: Gαi_3 antibody capture technique confirms the bell-shape concentration response effects of 5-HT (solid line). The bell-shape can be modeled mathematically by an algorithm that calculates 2 opposite sigmoid curves: one with a higher potency for the stimulatory phase (dotted line), and one with lower potency for the inhibitory phase (dashed line).

receptor agonist, befiradol (a.k.a. F13640 or NLX-112) preferentially activates Gαo G-proteins and is known to potently activate dorsal raphe nucleus 5-HT_{1A} autoreceptors that are involved in control of motor systems [13]. In contrast, NLX-101 (a.k.a. F15599) preferentially activates other G-proteins (notably Gαi3) and thus exhibits biased agonism at post-synaptic 5-HT_{1A} heteroreceptors, particularly in cortical brain regions controlling mood and cognition [14,15]. Another biased agonist, F13714, also exhibits distinctive G-protein activation patterns in [^{35}S]GTPγS binding experiments on membranes prepared from different brain regions [14].

Additional complexity in G-protein activation by agonists has been observed using [^{35}S]GTPγS binding assays performed on membranes from a CHO cell line expressing high levels of 5-HT_{1A} receptors. 5-HT yielded 'flat' concentration-response isotherms i.e. with low Hill coefficients (illustrated in Fig. 1, left-hand panel) [16] consistent with the presence of 2 different responses. Moreover, the [^{35}S]GTPγS binding induced by low concentrations of 5-HT was prevented by pre-incubation of membranes with anti-Gαi_3 antibodies or by siRNA knock-down of Gαi_3 expression [16,17]. In contrast, the [^{35}S]GTPγS binding induced by high concentrations of 5-HT was prevented by preincubation of membranes with anti-Gαi_2-specific antibodies (*unpublished observation*) or by siRNA knock-down of Gαi_2 expression [16,17]. These observations indicate that 5-HT elicited coupling of 5-HT_{1A} receptors to different G-protein subtypes, depending on agonist concentration. This was further explored in experiments using an antibody-capture-based [^{35}S]GTPγS binding assay targeting Gαi_3 G-proteins. These experiments yielded bell-shaped concentration-response isotherms with 5-HT [16]. Both the ascending and descending components of the isotherms were prevented by pre-treatment of membranes with pertussis toxin and were also blocked by WAY100635, a selective 5-HT_{1A} receptor antagonist.

The occurrence of bell-shape concentration-response curves for cell signaling responses is a not-unusual, but often poorly understood, phenomenon for a variety of receptor subtypes and accumulating reports indicate that they may be important to drive specific physiological responses (see Discussion section). In the case of 5-HT_{1A} receptors, the data indicate they can efficiently couple to both Gαi_3 and Gαi_2 G-proteins and exhibit a bell-shaped 'switch' in coupling from Gαi_3 to Gαi_2 in an agonist concentration-dependent manner, possibly reflecting specific ligand binding properties at receptor monomers or dimers, as previously suggested [16,18]. However, the studies described above were restricted to recombinant human (h) 5-HT_{1A} receptors expressed in a specific cell type (i.e. CHO cells) and the possibility remained that the G-protein coupling switch was specifically related to this particular cell line. The present study therefore evaluated whether Gαi_3 activation by 5-HT_{1A} receptor agonists exhibited distinct properties in other h5-HT_{1A}-transfected cell lines. In addition to CHO cells expressing high levels of receptor, as used previously [16,17], we tested 4 other different cell lines stably expressing h5-HT_{1A} receptors: HeLa, C6-glial and CHO-low cells expressing moderate amounts of h5-HT_{1A} receptors and HEK-293F cells expressing high amounts of h5-HT_{1A} receptors. The results show that bell-shape concentration response isotherms, likely reflecting a 'switch' from Gαi_3 to Gαi_2 G-protein activation, are observed in the cell lines expressing high levels of h5-HT_{1A} receptors but not in the cell lines expressing moderate levels of h5-HT_{1A} receptors. This suggests that the switch in G-protein coupling, which is most readily observed with high efficacy agonists, depends particularly on receptor expression levels.

2. Materials and methods

2.1. Membrane preparations

Cell lines stably expressing recombinant human 5-HT_{1A} receptors were obtained from different sources as described previously [9]. Briefly, Hela cells were from a previously-characterized HA7 clone [19]; C6-glia, CHO-low and HEK-293F cell lines were prepared in-house [20]. CHO-high cells were obtained commercially from Euroscreen/Perkin Elmer. In all cases, cells were grown to confluence in Dulbecco's Modified Eagle Medium enriched with 10% fetal calf serum, L-glutamine, sodium citrate and antibiotics. Following 2 washes with cold PBS, 20 mM HEPES pH 7.0 containing 10 mM EDTA was applied over the cells which were then scraped from the flask, transferred to a centrifuge tube and homogenized with a polytron. Membranes were washed twice by cycles of 10 min 55,000 *g* centrifugation/resuspension in 20 mM HEPES pH 7.0 containing 0.1 mM EDTA at 4 °C. Final pellets were resuspended HEPES buffer containing 100 mM NaCl, 5 mM $MgCl_2$ and 50 mM GDP.

As reported previously [9], h5-HT_{1A}-transfected cell lines expressed different amounts of receptors, ranging from approximately 1.1–1.3 pmol/mg proteins (C6-glia and HeLa, respectively) to 3.6 pmol/mg proteins (CHO-low), based on saturation binding assays using the antagonist radiotracer, [^{3}H]WAY100635. Membranes from CHO-high and HEK-293F cells expressed much higher levels of h5-HT_{1A} receptors: 9.6 and > 10 pmol receptor/mg protein, respectively (also measured by saturation with [^{3}H]WAY100635, data not shown).

2.2. Antibody capture [^{35}S]GTPγS binding Scintillation Proximity Assay (SPA)

The antibody capture [^{35}S]GTPγS binding assay allows detection of the activation of specific Gα-subtypes on the basis of antibody selectivity. The antibodies used in the present study detected both Gαi_3 and Gαi_1 G-protein subtypes [21]. However, mRNA levels of Gαi1 protein subtype, as measured by RT-PCR, are much lower than those of Gαi_3 mRNA in all cell lines tested [7,9,22] and 5-HT_{1A} receptors couple strongly to Gαi_3 and more poorly to Gαi_1 [7] so the present assay conditions essentially detect Gαi_3 activation.

Assays were performed in 96-well plates using the same buffer (20 mM HEPES buffer containing 100 mM NaCl, 3 mM $MgCl_2$ and 3 μM GDP), and incubation conditions. Cell membranes together with the

tested drugs and [^{35}S]GTPγS (0.2 nM) were incubated for 60 min under constant agitation (600 rpm). Incubation was terminated by adding detergent (Nonidet NP-40) and the plates were further agitated for 30 min before addition of 0.2 mg anti-G$\alpha i_{1/3}$-specific monoclonal antibodies (Biomol) to each well. The primary antibodies were allowed to react for 60 min under agitation before adding 50 ml of the secondary antibodies (anti-mouse coupled to SPA beads, Amersham) that were allowed to react for another 60 min. Plates were then centrifuged at 1000 *g* for 15 min. Radioactivity was immediately measured on a scintillation counter.

2.3. Data analysis

All data are expressed as mean ± s.e.m. of at least three independent determinations, each point performed in duplicate. Concentration-response isotherms were analysed by nonlinear regression, using GraphPad Prism (GraphPad Software Inc., San Diego, CA). To determine pEC_{50} and Emax for sigmoid curves, the value of the minimum and maximum asymptotes was not fixed. For bell-shape concentration-response isotherms, analysis in GraphPad Prism used an equation which models 2 opposite sigmoid curves (Fig. 1B): Y = Gap + Section1 + Section2.

Where:

$$Section1 = Span1/(1 + 10\text{^}(LogEC_{50\_1} - X))$$

$$Section2 = Span2/(1 + 10\text{^}(X - LogEC_{50\_2}))$$

$$Span1 = Emax1 - Emin1$$

$$Span2 = Emax2 - Emin2$$

When using this equation, Hill coefficients of the 2 opposing sigmoid curves were fixed to 1, minima of the 2 curves (Emin1 and Emin2) were fixed to zero, and maximal inhibition of the second phase (Emax2) was also limited to 100% (i.e. full inhibition of basal [^{35}S]GTPγS binding to Gαi_3).

2.4. Compounds

The compounds tested herein were selected based on previous [^{35}S]GTPγS binding studies of 'total' G-protein activation indicating that they had different levels of agonist efficacy at 5-HT_{1A} receptors. 5-HT, F13714, F13640, NLX-101 and (+)8-OH-DPAT have been described as 'full agonists'. (−)8-OH-DPAT and (−)pindolol have been described as partial agonists, whereas spiperone and WAY100635 display antagonist and inverse agonist properties, respectively. In addition, several of the compounds (notably the chemical congeners, F13714, befiradol and NLX-101) exhibit differing in vivo 'biased agonist' properties for a range of neurochemical, behavioral and brain imaging parameters. Whilst potently and efficaciously activating 5-HT_{1A} receptors, they exhibit differing properties for G-protein activation and this translates to distinct brain region targeting in vivo (see Table 1 for summary and references).

F13714 fumarate, befiradol (aka NLX-112 or F13640) fumarate, NLX-101 (aka F15599) fumarate and WAY100635 dihydrochloride were synthesized at the Centre de Recherche Pierre Fabre (Castres, France). 5-Hydroxytryptamine (5-HT) creatinine sulphate, (±)-8-OH-DPAT bromohydrate, (−)8-OH-DPAT bromohydrate, (−)pindolol base and spiperone hydrochloride, were purchased from Sigma-RBI (St. Quentin Fallavier, France). Drugs were dissolved in distilled water or 10% DMSO at 10^{-3} M, and dilutions were prepared in the appropriate assay buffer.

Table 1
Binding affinity and agonist properties on the compounds tested. pKi values were determined previously by competition binding on cell membranes from CHO-high cells.

Compound	pKi	Agonist / antagonist properties	References
5-HT	9.21	Endogenous neurotransmitter; full agonist	[16]
F13714	10.40	Selective 5-HT_{1A} biased agonist; in vivo 5-HT_{1A} autoreceptor activator	[14,24]
Befiradol [a]	9.49	Selective 5-HT_{1A} biased agonist; preferential Gαo activator; in vivo 5-HT_{1A} autoreceptor bias	[9,25]
NLX-101 [b]	8.57	Selective 5-HT_{1A} biased agonist; preferential Gαi activator; in vivo cortical 5-HT_{1A} bias	[14]
(+)8-OH-DPAT	9.33	Full agonist, also activates 5-HT7 receptors at higher doses	[14,50]
(−)8-OH-DPAT	9.19	Partial agonist	[16,50]
(−)Pindolol	8.19	Partial agonist; in vivo 5-HT_{1A} autoreceptor bias. Also blocks beta-adrenergic receptors.	[16,51]
WAY100635	9.25	Antagonist	[16,23]
Spiperone	7.00	Inverse agonist. Also blocks D_2 and 5-HT_{2A} receptors	[16,23]

[a] Befiradol is also known as NLX-112 or F13640.
[b] NLX-101 is also known as F15599.

3. Results

3.1. 5-HT and biased agonists

In the 3 cell lines expressing modest levels of h5-HT_{1A} receptors (HeLa, C6-glia and CHO-low), 5-HT and the high efficacy biased agonists (F13714, befiradol and NLX-101) elicited sigmoid concentration-response curves reflecting robust [^{35}S]GTPγS binding to Gαi_3 (Fig. 2). The maximal effects (Emax values) of the biased agonists were generally similar to those of 5-HT (i.e. full agonism, Table 2). For comparison, maximal stimulation (relative to basal binding = 100%) elicited by 5-HT was 161 ± 9% for HeLa cells, 141 ± 14% for C6-glial cells and 146 ± 15% for CHO-low cells (n = at least 3 in all cases). In contrast to Emax values, the potency of the agonists (pEC_{50} values) varied: F13714 was the most potent, NLX-101 was the least potent and befiradol showed intermediated potency.

In membranes from cell lines expressing higher levels of h5-HT_{1A} receptors (CHO-high and HEK-293F−), 5-HT and the biased agonists produced bell-shaped concentration-response curves which rise to a peak and then decrease back to basal levels (for 5-HT) or descend below basal levels in the case of F13714, befiradol and NLX-101 (Fig. 2). The bell-shape concentration-response isotherms could be modeled by nonlinear regression into a stimulatory effect followed by an inhibitory effect at higher agonist concentrations (Fig. 1B; Tables 3 and 4). Differences were observed in the effects of the biased agonists. F13714 showed comparatively minor Gαi_3 stimulation in membranes from CHO-high and HEK-293F cells (Fig. 2). In contrast, NLX-101 produced a more pronounced 'bell-shape' in CHO-high and HEK-293F cells. The profile of befiradol was intermediate.

3.2. (+) and (−) 8-OH-DPAT

In the 3 cell lines expressing modest levels of h5-HT_{1A} receptors (HeLa, C6-glia and CHO-low), (+)8-OH-DPAT elicited sigmoid stimulation curves with maximal effects approaching those of 5-HT (see Table 2 and Fig. 3). Although (−)8-OH-DPAT also elicited sigmoid concentration-response isotherms in these same cell lines, they reached a plateau at submaximal [^{35}S]GTPγS binding to Gαi_3.

In membranes from cell lines expressing higher levels of h5-HT_{1A} receptors, (+)8-OH-DPAT induced a bell-shaped isotherm in CHO-high cell membranes but produced a predominantly inhibitory response in

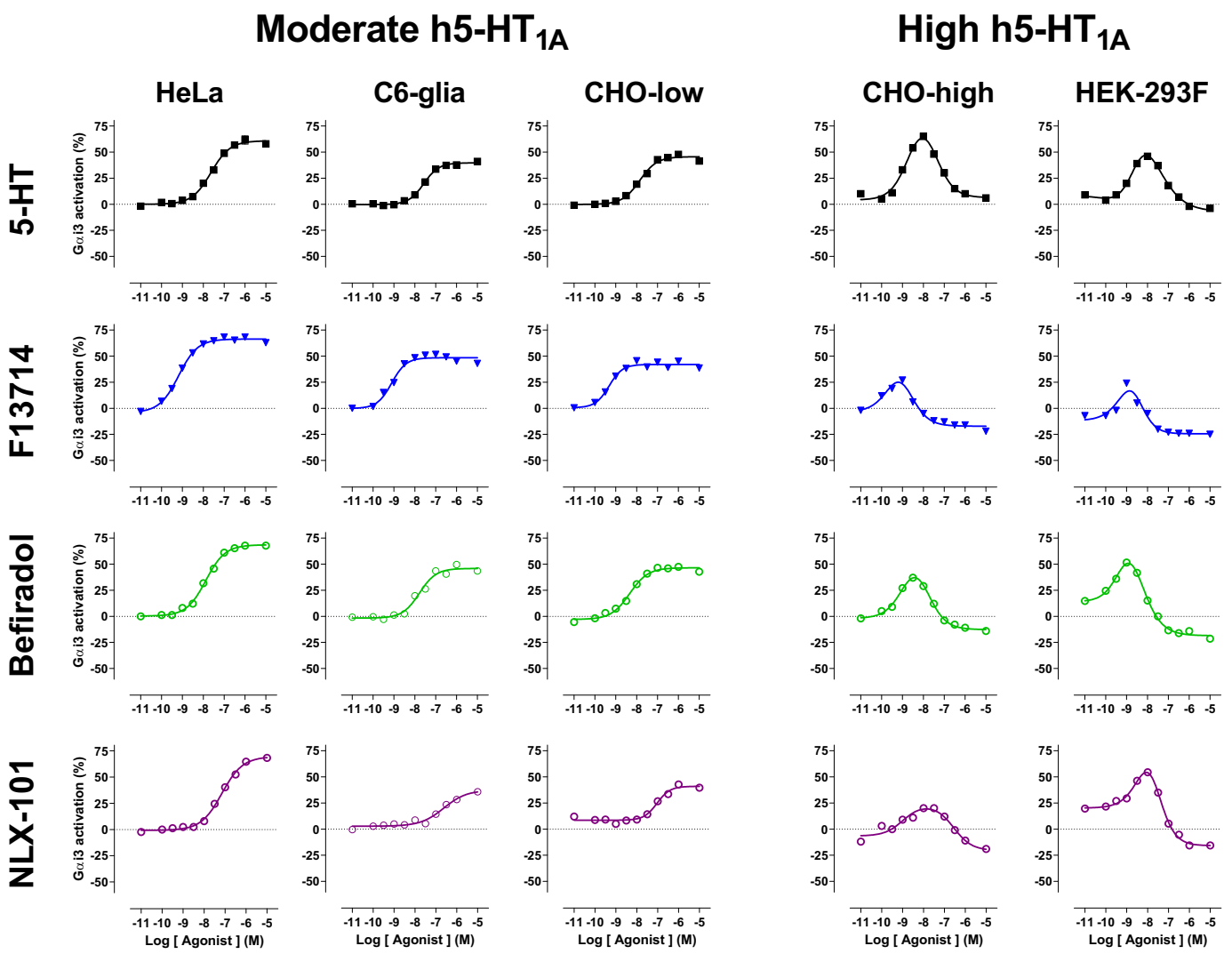

Fig. 2. Effects of 5-HT and of the biased agonists, F13714, befiradol and NLX-101, on $G\alpha i_3$ G-protein activation detected by an immunocapture / [^{35}S]GTPγS binding assay in cell lines expressing moderate (HeLa, C6-glial, CHO-low) or high (CHO-high, HEK-293−) levels of h5-HT$_{1A}$ receptors. Points are averages of at least 3 experiments and are expressed as % of basal [^{35}S]GTPγS binding.

HEK-293F cell membranes. (−)8-OH-DPAT also induced a slightly bell-shaped isotherm in CHO-high cell membranes with a modest inhibitory component but produced a sigmoid stimulation curve in HEK-293F cell membranes (Fig. 3).

3.3. Partial agonist, antagonist and inverse agonist

In 2 of the cell lines expressing modest levels of h5-HT$_{1A}$ receptors (HeLa, C6-glia), (−)pindolol produced no effects on [^{35}S]GTPγS binding to $G\alpha i_3$ (Fig. 4, Tables 2–4). In contrast, on CHO-low-h5-HT$_{1A}$ cell membranes, (−)pindolol concentration-dependently inhibited

Table 2
Analysis of $G\alpha i_3$ activation in cell lines expressing moderate levels of h5-HT$_{1A}$ receptors: HeLa, C6-Glial and CHO-low.

$G\alpha i_3$ activation by was determined by an immunocapture-based [^{35}S]GTPγS binding assay. Data are presented as average ± s.e.m. of at least 3 independent determinations. All concentration-response curves were sigmoid. Maximal effects (Emax) are expressed as % relative to a saturating concentration of 5-HT (10 μM).

	HeLa-h5-HT$_{1A}$			C6-Glial-h5-HT$_{1A}$			CHO-low-h5-HT$_{1A}$		
Compound	Emax	pEC$_{50}$	nH	Emax	pEC$_{50}$	nH	Emax	pEC$_{50}$	nH
5-HT	100	7.61 ± 0.03	0.94 ± 0.03	100	7.52 ± 0.08	1.21 ± 0.19	100	7.76 ± 0.05	1.05 ± 0.07
F13714	104 ± 3	9.13 ± 0.08	1.06 ± 0.07	118 ± 10	9.09 ± 0.22	1.71 ± 0.35	99 ± 9	9.10 ± 0.29	1.79 ± 0.24
Befiradol	112 ± 5	7.87 ± 0.04	0.94 ± 0.05	115 ± 15	7.80 ± 0.11	1.08 ± 0.13	101 ± 6	8.32 ± 0.12	1.04 ± 0.07
NLX-101	113 ± 7	7.17 ± 0.26	1.06 ± 0.13	87 ± 27	6.70 ± 0.03	1.14 ± 0.41	87 ± 10	6.87 ± 0.25	1.56 ± 0.22
(+)8-OH-DPAT	87 ± 4	7.80 ± 0.06	1.07 ± 0.17	101 ± 19	7.70 ± 0.12	0.91 ± 0.13	91 ± 7	7.63 ± 0.15	1.01 ± 0.18
(−)8-OH-DPAT	53 ± 3	7.86 ± 0.08	1.28 ± 0.18	27 ± 5	7.82 ± 0.24	1.73 ± 0.26	49 ± 8	8.09 ± 0.19	1.11 ± 0.23
(−)Pindolol	Inactive			Inactive			−69 ± 24	7.02 ± 0.07	1.05 ± 0.16
WAY100635	Inactive			Inactive			Inactive		
Spiperone	−49 ± 9	6.92 ± 0.19	0.76 ± 0.18	−46 ± 4	7.59 ± 0.21	1.51 ± 0.34	−78 ± 18	7.11 ± 0.12	1.11 ± 0.11

Table 3
Effects of h5-HT_{1A} receptor ligands on Gαi_3 activation in a cell line expressing high levels of h5-HT_{1A} receptors: CHO-high.

Gαi_3 activation by was determined by an immunocapture-based [^{35}S]GTPγS binding assay. Data are presented as mean ± s.e.m. of at least 3 independent determinations. For bell-shape curves, two Emax and pEC_{50} values were determined (for the ascending and the descending phases of the curves). For sigmoid curves, a single Emax and pEC_{50} value was determined. Emax values are expressed as % of basal [^{35}S]GTPγS binding. Hill coefficients (nH) were fixed to 1 for bell-shape curves. The Δ pEC_{50} is the separation between pEC_{50_1} and pEC_{50_2}, also shown as fold-difference.

Compound	Curve shape	Emax1	Emax2	pEC_{50_1}	nH_1	pEC_{50_2}	nH_2	Δ pEC_{50} (*fold difference*)
5-HT	Bell-shape	95 ± 8	−87 ± 6	8.74 ± 0.05	1.00	7.31 ± 0.06	1.00	1.43 (*27*)
F13714	Bell-shape	65 ± 24	−78 ± 15	9.17 ± 0.36	1.00	8.00 ± 0.49	1.00	1.17 (*15*)
Befiradol	Bell-shape	84 ± 12	95 ± 3	8.84 ± 0.13	1.00	7.88 ± 0.09	1.00	0.96 (*9*)
NLX-101	Bell-shape	131 ± 75	45 ± 13	10.17 ± 0.88	1.00	6.85 ± 0.16	1.00	3.32 (*2090*)
(+)8-OH-DPAT	Bell-shape	73 ± 20	−83 ± 10	8.97 ± 0.23	1.00	7.65 ± 0.11	1.00	1.32 (*21*)
(−)8-OH-DPAT	Bell-shape	52 ± 23	−40 ± 32	8.34 ± 0.08	1.00	7.53 ± 0.48	1.00	0.81 (*6*)
(−)Pindolol	Sigmoid	65 ± 8		8.00 ± 0.13	1.00 ± 0.22			
WAY100635	Inactive	0						
Spiperone	Sigmoid		−45 ± 7			7.23 ± 0.12	−0.95 ± 0.07	

[^{35}S]GTPγS binding. WAY100635 did not elicit measurable effects on [^{35}S]GTPγS binding to membranes of any of these 3 cell lines, whereas spiperone produced inhibition in all cases.

In membranes from cell lines expressing higher levels of h5-HT_{1A} receptors, (−)pindolol robustly stimulated [^{35}S]GTPγS binding to membranes of CHO-high cells but produced a slight inhibition in membranes from HEK-293F cells. WAY100635 produced no effect in CHO-high cell membranes but inhibited [^{35}S]GTPγS binding to membranes of HEK-293F cells. Spiperone showed inverse agonist properties (Fig. 4, Tables 2–4).

4. Discussion

The main findings of the present study are as follows.

Firstly, in three cell lines expressing moderate levels of 5-HT_{1A} receptors (Hela, C6-glial and CHO-low) 5-HT and three highly efficacious agonists (F13714, befiradol and NLX-101), exhibited sigmoid concentration-response effects for activation of Gαi_3 G-proteins (Fig. 2). In each case, the maximal effects of the synthetic agonists resembled that of 5-HT, i.e. they acted essentially as 'full agonists' (Emax values close to 100%, relative to 5-HT, Table 2), as has been previously reported for these compounds **[9,14]**. However, the potency of the agonists (pEC_{50} values) varied widely with F13714 being the most potent and NLX-101 the least potent, in accordance with the order of affinity of the compounds (Table 1). These data show that the agonists are capable of achieving maximal level of Gαi_3 stimulation in the 3 cell lines expressing moderate levels of h5-HT_{1A} receptors.

Secondly, 5-HT and these same highly efficacious agonists exhibited bell-shaped concentration-response effects for activation of Gαi_3 G-proteins in membranes of two other cell lines expressing high levels of 5-HT_{1A} receptors (CHO-high and HEK-293F−). Thus, 5-HT produced a symmetrical-looking concentration-response curve reaching a maximal stimulation at about 10 nM (Fig. 2). This is similar to our previous observation **[16]** and also similar to the EC_{50} value for Gαi_3 activation determined for the cell lines expressing moderate levels of h5-HT_{1A} receptors (Table 2). It is therefore tempting to suggest that bell-shape concentration-response isotherms occur under conditions where high receptor expression levels are combined with half-maximal stimulation of the receptor by 5-HT, thus triggering a switch in G-protein coupling. In any case, Gαi_3 G-protein activation is not detectable at saturating concentrations of 5-HT, indicating that, under such conditions, receptor signaling is mediated by other G-protein subtypes. As concerns the effects of the biased agonists, it is noticeable that the shape of the bell-shape concentration-response isotherms differs from one compound to another. Maximal Gαi_3 activation by F13714 is modest and there is a small separation between the pEC_{50} values of the stimulatory phase and the inhibitory phase (Δ pEC_{50} value, Tables 3 and 4). In contrast, NLX-101 has a wider separation in pEC_{50} values, suggesting that the switch in G-protein signaling of 5-HT_{1A} receptors from Gαi_3 to another G-protein occurs less readily with this agonist. This suggests that NLX-101 preferentially activates Gαi_3 G-proteins whereas F13714 may preferentially activate Gαi_2. It should be borne in mind that these biased agonists exhibit divergent properties in a range of electrophysiological, neurochemical, behavioral and brain imaging studies **[12]**. F13714 and befiradol more prominently activate 5-HT_{1A} autoreceptors whereas NLX-101 preferentially activates cortical 5-HT_{1A} heteroreceptors. The present observations suggest that the capacity to induce a signaling switch from Gαi_3 to Gαi_2 may contribute to the differing in vivo profiles of the compounds. It is also noteworthy that high concentrations of F13714, befiradol and NLX-101 all decrease Gαi_3 activation below

Table 4
Effects of h5-HT_{1A} receptor ligands on Gαi_3 activation in a cell line expressing high levels of h5-HT_{1A} receptors: HEK-293F−.

Gαi_3 activation by was determined by an immunocapture-based [^{35}S]GTPγS binding assay. Data are presented as mean ± s.e.m. of at least 3 independent determinations. For bell-shape curves, two Emax and pEC_{50} values were determined (for the ascending and the descending phases of the curves). For sigmoid curves, a single Emax and pEC_{50} value was determined. Emax values are expressed as % of basal [^{35}S]GTPγS binding. Hill coefficients (nH) were fixed to 1 for bell-shape curves. The Δ pEC_{50} is the separation between pEC_{50_1} and pEC_{50_2}, also shown as fold-difference.

Compound	Curve shape	Emax1	Emax2	pEC_{50_1}	nH_1	pEC_{50_2}	nH_2	Δ pEC_{50} (*fold difference*)
Serotonin	Bell-shape	79 ± 13	−85 ± 13	8.53 ± 0.07	1.00 [a]	7.41 ± 0.06	1.00	1.12 (*13*)
F13714	Bell-shape	57 ± 39	−83 ± 24	9.42 ± 0.24	1.00 [a]	8.20 ± 0.24	1.00	1.22 (*17*)
Befiradol	Bell-shape	59 ± 19	−79 ± 21	8.93 ± 0.50	1.00 [a]	8.05 ± 0.07	1.00	0.88 (*8*)
NLX-101	Bell-shape	57 ± 14	−93 ± 7	8.75 ± 0.13	1.00 [a]	7.35 ± 0.09	1.00	1.40 (*25*)
(+)8-OH-DPAT	Bell-shape	20 ± 13	−44 ± 6	8.27 ± 0.25	1.00 [a]	7.00 ± 0.29	1.00	1.27 (*19*)
(−)8-OH-DPAT	Sigmoid	19 ± 7		9.55 ± 0.27			1.24 ± 0.32	
(−)Pindolol	Sigmoid		−7 ± 1	6.98 ± 0.10			1.01 ± 0.18	
WAY100635	Sigmoid		−20 ± 3	7.49 ± 0.12			2.13 ± 0.03	
Spiperone	Sigmoid		−50 ± 2	6.97 ± 0.06			0.96 ± 0.12	

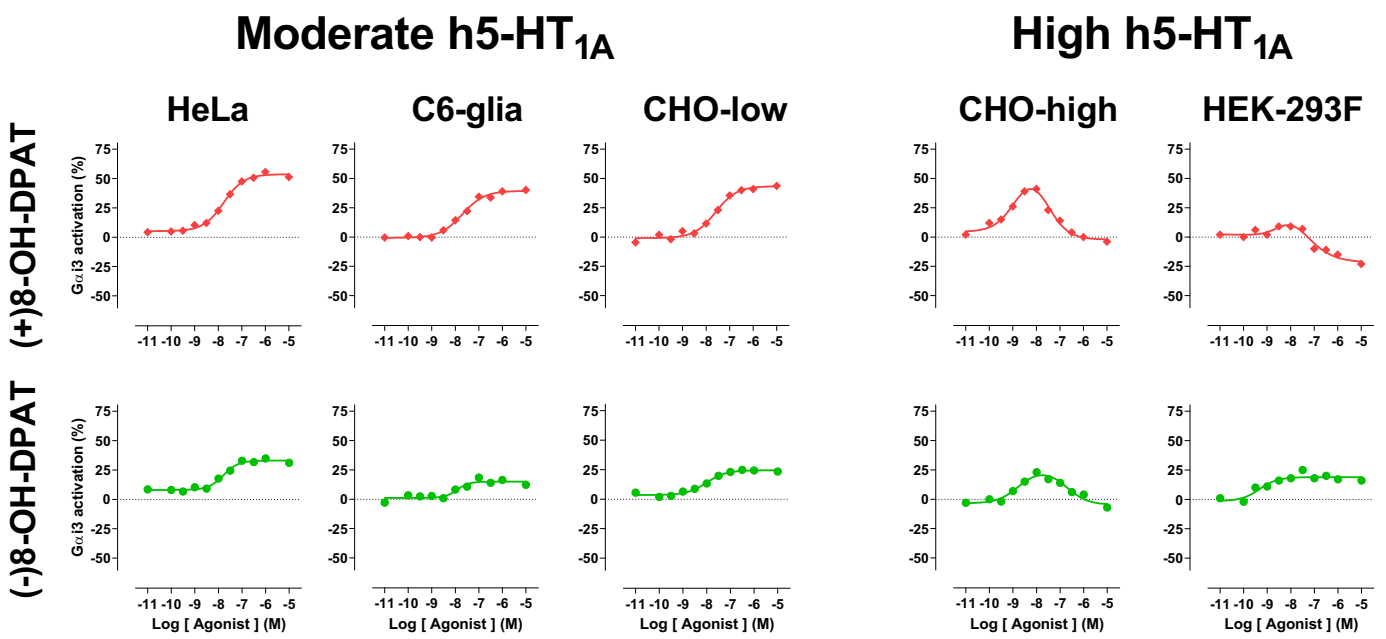

Fig. 3. Effects of (+) and (−)8-OH-DPAT on Gαi$_3$ G-protein activation detected by an immunocapture / [^{35}S]GTPγS binding assay in cell lines expressing moderate (HeLa, C6-glial, CHO-low) or high (CHO-high, HEK-293F−) levels of h5-HT$_{1A}$ receptors. Points are averages of at least 3 experiments and are expressed as % of basal [^{35}S]GTPγS binding.

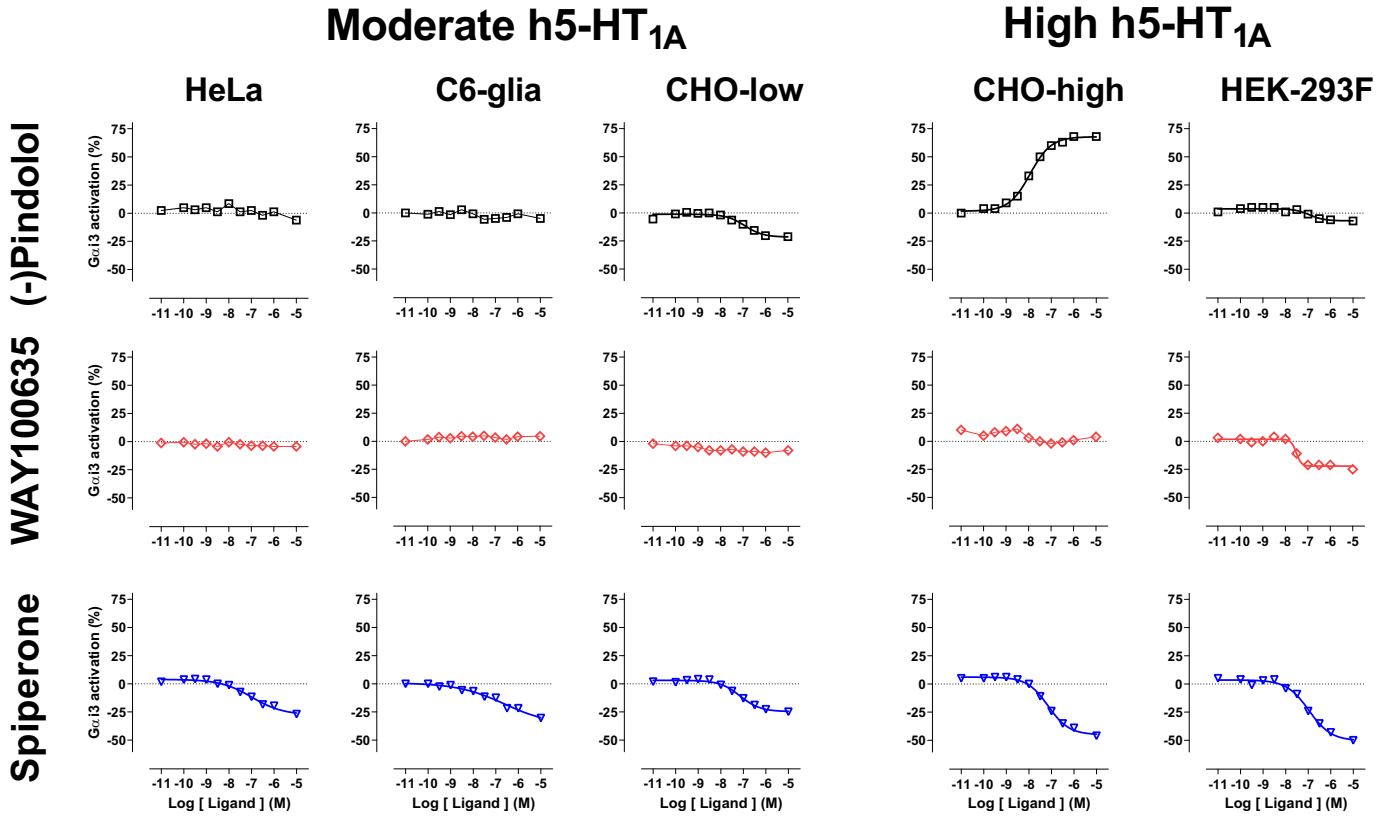

Fig. 4. Effects of the partial agonists, (−)pindolol, the antagonist, WAY100635, and the inverse agonist, spiperone, on Gαi$_3$ G-protein activation detected by an immunocapture / [^{35}S]GTPγS binding assay in cell lines expressing moderate (HeLa, C6-glial, CHO-low) or high (CHO-high, HEK-293F−) levels of h5-HT$_{1A}$ receptors. Points are averages of at least 3 experiments and are expressed as % of basal [^{35}S]GTPγS binding.

basal levels, thus acting as pseudo-inverse agonists. This may reflect induction of a h5-HT$_{1A}$ receptor confirmation that is resistant to Gαi$_3$ coupling and therefore suppresses endogenous Gαi$_3$ constitutive activation.

Thirdly, the other compounds tested, with lower levels of agonist efficacy (partial agonists, antagonist or inverse agonist) exhibited

complex patterns of response in the different cell membranes. Both (+) and (−)8-OH-DPAT induced bell-shaped Gαi$_3$ activation in CHO-high cell membranes but their effects in HEK-293F cell membranes were divergent (Fig. 3). Spiperone elicited inhibition of Gαi$_3$ activation in all 5 cell lines (including both CHO cell lines), but the 'neutral antagonist' WAY100635 [23], which had previously shown partial agonist effects for Gαi$_3$ activation in CHO-high cell membranes [16], exhibited inverse agonism in HEK-293F cell membranes (Fig. 4). Finally, (−)pindolol exhibited sigmoid, but opposite, responses in CHO-low and CHO-high cell membranes (inverse agonism and agonism, respectively) and little effect in the other cell membranes. Thus, although it seems clear that some kind of conformational change occurs when high concentrations of 5-HT (or other high-efficacy agonists) interact with highly-expressed 5-HT$_{1A}$ receptors, the disparate effects of the partial agonists / antagonists suggest that additional mechanisms may intervene in h5-HT$_{1A}$ receptor-mediated G-protein activation. This may be due to cell-type-specific conditions, such as differing levels of constitutive activity, which can influence both the amplitude and the direction of response for partial agonists, as observed previously for activation of Gαi$_3$ by (−)pindolol at h5-HT$_{1A}$ receptors [16].

It should be mentioned that the present study did not carry out antagonist experiments (e.g. with WAY100635) to verify that all the effects of the compounds are abolished by a 5-HT$_{1A}$ receptor antagonist. Such experiments were done previously for 5-HT using the same G-protein targeting technique in CHO-5-HT$_{1A}$-high cells [14], and antagonist experiments were also done using classical [^{35}S]GTPγS binding on HeLa and C6-glial cell lines [20]. The likelihood that other receptor subtypes may influence the present data is therefore low, notably in the case of the biased agonists, F13714, befiradol and NLX-101, which are highly selective for 5-HT$_{1A}$ [14,24,25]. However, in the case of pindolol and 8-OH-DPAT, which are only partially-selective for 5-HT$_{1A}$ receptors, the possibility of an interaction with endogenous receptors cannot be formally excluded.

Taken together, the above considerations suggests that there may be several factors involved in determining the profile of Gαi$_3$ G-protein activation but, at least for high efficacy agonists, the results clearly point to receptor expression level as being a key parameter driving Gαi$_3$ coupling to h5-HT$_{1A}$ receptors and the bell-shaped switch in G-protein activation response (see additional discussion below).

4.1. Previous reports of bell-shape isotherms

Bell-shape concentration-response isotherms have been described in other functional assays performed on recombinant cells expressing other GPCR. For example, stimulation of muscarinic M$_2$ receptors expressed in CHO cells by high efficacy agonists led to inhibition of forskolin-stimulated cAMP production at low agonist concentrations followed by a return to basal forskolin-stimulated cAMP at higher agonist concentrations [26,27]. This inverted bell-shape (or U-shape) concentration-response phenomenon was influenced by M$_2$ receptor expression levels and the descending phase was sensitive to pertussis toxin [28], while the ascending phase was prevented by siRNA targeting Gα$_s$ subunits [26]. Similarly, agonists elicited U-shape concentration-response isotherms for inhibition of forskolin-stimulated cAMP production in CHO cells expressing high levels of porcine α$_{2A}$ adrenoceptors, the descending phase being blocked by pertussis toxin, while the ascending phase was absent in cell lines expressing low hα$_{2A}$ receptor levels [29]. In addition, bell-shape concentration-response isotherms have been reported for [^{35}S]GTPγS binding to membranes of CHO cells expressing high levels of adenosine A$_1$ receptors [30,31] or μ-opioid receptors [32] and for β2 adrenoceptor signaling via Gαs and adenylyl cyclase in a human breast cell line [33].

Thus, bell-shape concentration-response isotherms are observed with different GPCRs and are generally associated with high levels of receptor expression in heterologous systems, but have also been detected in vivo. For example, in cardiac tissue from transgenic animals overexpressing α$_2$ adrenoceptors [34], the full agonists isoproterenol and epinephrine led to Gαs-mediated positive inotropic response on isolated left atrium at low agonist concentrations, and, at higher agonist concentrations, to a PTX-sensitive Gαi-mediated negative inotropic response. This switch in α$_2$ adrenoceptor coupling from Gαs to Gαi proteins was not detected with the partial agonist norepinephrine. Interestingly, a similar switch in isoproterenol-mediated α$_2$ adrenoceptor signaling from Gαs to Gαi has also been demonstrated in cultured peritoneal macrophage cells originating from non-transgenic animals [35], and this signaling switch, which was prevented by PTX pretreatment, was a necessary step for induction of immune response to phorbol myristate acetate [35]. A study of human primary umbilical vascular cells also reported bell-shaped concentration-response curves associated with specific G-protein subtypes and leading to differential signaling outputs (cAMP, cGMP, pERK1/2) [36]. Taken together, these observations indicate that although bell-shape concentration-response isotherms are more often described for high-expressing heterologous systems, they are also seen in physiological tissues and may have functional and/or pathological consequences.

4.2. Mechanisms underlying bell-shape concentration-response effects

Although the present data, together with the above-mentioned literature, point to high receptor expression levels as being a key factor in detection of bell-shape responses, the exact mechanisms underlying such responses are a matter of discussion. One group [27,28] suggested that U-shaped concentration-response in forskolin-stimulated cAMP production was due to a progressive saturation of muscarinic M$_2$ receptor coupling to a "preferred Gα protein" (Gαi), followed by progressive receptor coupling to a "second preferred Gα protein" (Gαs), thus resulting in neutralization of the inhibitory response mediated by Gαi by the opposite effect of stimulatory Gαs. A similar idea was described for adenosine A1-induced cAMP accumulation in CHO cells. Low concentrations of agonists induced inhibition via a pertussis toxin-sensitive Gαi mechanism, but high concentrations produced stimulation attributed to A1-Gαs coupling [37]. In the case of 5-HT$_{1A}$ receptors, such a pattern of response may correspond to activation of different G-proteins by agonists under low receptor expression conditions i.e. sigmoid curves with a maximal plateau of activation for Gαi$_3$ with additional G-protein activation via a "second preferred G-protein", such as Gαi$_2$ [7,38].

However, in the present case of a bell-shape pattern of Gαi$_3$ activation, a successive cumulative coupling to a "preferred G-protein" and then to a "second preferred G-protein" cannot account for the observations – a different mechanism must be involved. Indeed, under high receptor expression conditions, saturation of h5-HT$_{1A}$-mediated Gαi$_3$ signaling does not reach a plateau at high agonist concentrations but returns to basal levels, indicating a loss of Gαi$_3$ activation and a switch in h5-HT$_{1A}$ receptor signaling toward a different G-protein, in a manner that prevents Gαi$_3$ activation. Given that the bell-shape response is agonist concentration-dependent, these observations argue strongly in favor of direct receptor-receptor interaction, potentially involving formation of 5-HT$_{1A}$ homodimers (or homo-oligomers). Indeed, like other GPCRs, 5-HT$_{1A}$ receptors have been shown to dimerize (and heterodimerize) in vitro. 5-HT$_{1A}$ homodimers may be formed constitutively and be the predominant conformation of receptors expressed at the cell surface, especially at high receptor expression levels, thus influencing G-protein signaling [39–43]. Moreover, 5-HT$_{1A}$ dimerization may be enhanced by certain agonists such as 8-OH-DPAT, while the inverse agonist, methysergide, was found to reduce dimer formation potentially via a mechanism involving Gαs subunits [44].

In this context, the bell-shape agonist concentration-response isotherms of Gαi$_3$ activation in membranes of cells expressing high levels of h5-HT$_{1A}$ receptors could be related to a conformational change, hypothetically resulting from receptor dimerization and/or occupancy

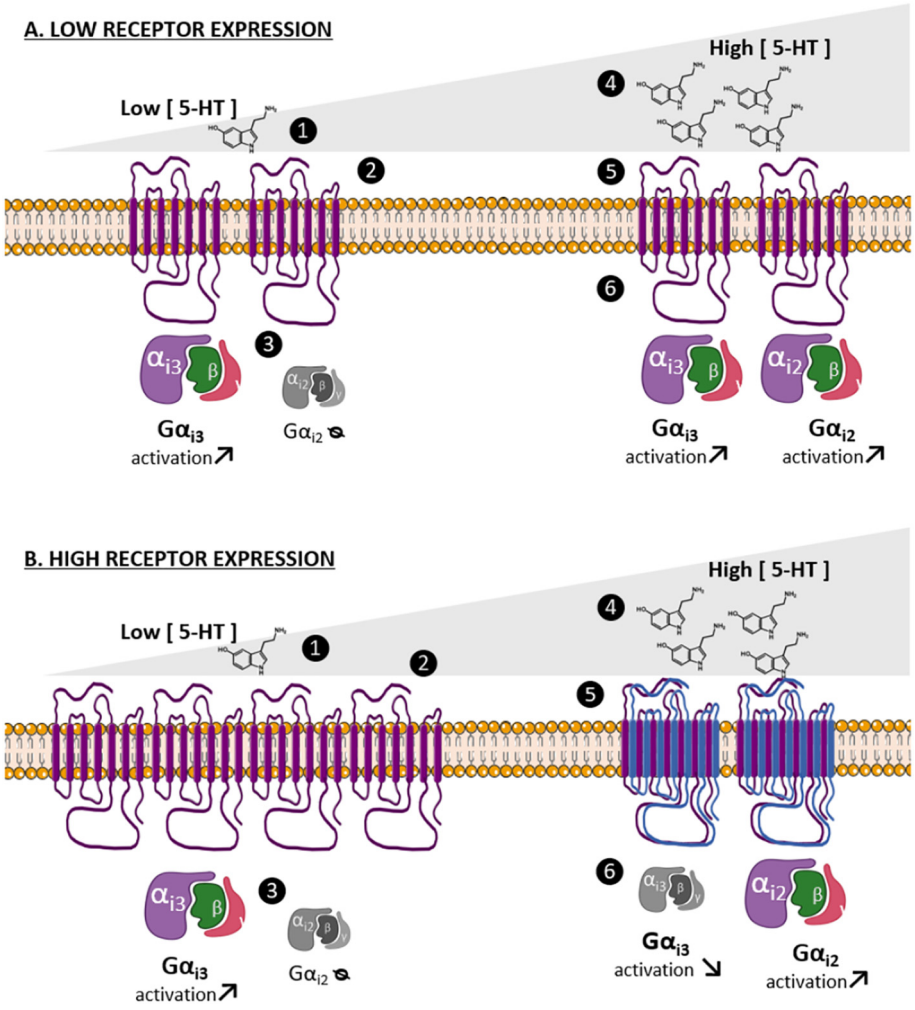

Fig. 5. Proposed mechanism of agonist-induced 5-HT_{1A} receptor G-protein coupling switch.
A. Low receptor expression: when low concentrations of 5-HT are present (1), 5-HT_{1A} receptors present in a conformation (2) which favors activation of $G\alpha i_3$ G-proteins (3). When high concentrations of 5-HT are present (4), 5-HT_{1A} receptors (5) additionally activate other G-proteins (notably $G\alpha i_2$) in addition to $G\alpha i_3$ (6).
B. High receptor expression: when low concentrations of 5-HT are present (1), 5-HT_{1A} receptors present in a conformation (e.g. monomers (2)) which favors activation of $G\alpha i_3$ G-proteins (3). In contrast, when high concentrations of 5-HT are present (4), 5-HT_{1A} receptors present in a conformation (e.g. homodimers (5)) which preferentially activates other G-proteins (notably $G\alpha i_2$) and suppresses activation of $G\alpha i_3$ (6).

of both h5-HT_{1A} receptor protomers, thus eliciting a "switch" in signaling (see proposed mechanism in Fig. 5). This is suggested by the observation that peak $G\alpha i_3$ activation in CHO-high or HEK-293F cell membranes occurs at agonist concentrations that are similar to the EC_{50} values for $G\alpha i_3$ activation in the other cell lines (Fig. 2), possibly corresponding to half-maximal receptor occupancy. As discussed in the Introduction, a switch in G-protein coupling of h5-HT_{1A} is likely to be toward $G\alpha i_2$, a G-protein which appears particularly efficient at activating $ERK_{1/2}$ phosphorylation [45,46] and that, like $G\alpha i_3$, is highly expressed (at least at the mRNA level) in C6-glial, CHO and HEK cells [9], although immunoblot experiments have shown that HeLa more abundantly express $G\alpha i_3$ than $G\alpha i_2$ [38]. Receptor dimerization has been found to be important for $ERK_{1/2}$ phosphorylation for other GPCRs. In the case of muscarinic M_3 receptor dimers, activation of a single protomer was sufficient to activate G-protein signaling, but activation of both receptor protomers was necessary for induction of $ERK_{1/2}$ phosphorylation [47]. It is interesting that befiradol and F13714 (which elicit efficient switches in coupling over a narrow concentration range - see ΔpEC_{50} data in Table 3) potently elicit ERK phosphorylation in rat dorsal raphe, whereas NLX-101 (which less readily induces a coupling switch from $G\alpha i_3$ to $G\alpha i_2$, Table 3) elicits markedly less potent and less efficacious ERK phosphorylation effects in this brain region [14,48,49]. In contrast, NLX-101 was relatively potent for eliciting ERK phosphorylation in frontal cortex, possibly due to its biased agonist activation of specific G-protein subtypes and intracellular cascades therein [14]. Overall, although receptor dimerization is an attractive hypothesis to account for the present bell-shaped activation patterns of $G\alpha i_3$ activation, the present study does not formally address this issue and direct experimental verification is necessary to confirm this interpretation.

5. Conclusions

GPCR-mediated G-protein signaling switches have been described in a variety of heterologous expression systems and the present study on h5-HT_{1A} receptors supports the interpretation that high levels of receptor expression are a key factor in determining the capacity of high efficacy agonists to elicit such as switch. Thus, 5-HT, as well as F13714, befiradol and NLX-101, induce a switch in G-protein signaling in membranes from high-expressing cell lines but not in membranes from cell lines expressing moderate levels of h5-HT_{1A} receptors. Signaling switches have also been described for other receptors in native tissue or cells, so it is possible that this phenomenon may be of physiological and/or pathological significance for responses influenced by 5-HT_{1A} receptor activation, such as control of mood, cognition and movement. Moreover, in view of the recent discovery of 5-HT_{1A} receptor 'biased agonists', the present data suggest that novel compounds could be identified that induce/stabilize receptor conformations that preferentially activate specific G-protein signaling cascades in brain regions expressing different densities of 5-HT_{1A} receptors. Finally, the present study indicates that care should be taken to carry out detailed agonist concentration-response experiments when investigating 5-HT_{1A} receptor-mediated cellular activation in tissues expressing high levels of this receptor.

Acknowledgements

The experiments were funded by Pierre Fabre Laboratories. All the authors, apart from MAV, were employees of Pierre Fabre Laboratories at the time of the experiments.

Competing interests

ANT and MAV are employees and stockholders of Neurolixis Inc.
AMO, FL and JCM are employees of Pierre Fabre Laboratories.
ANT and DC were previously employees of Pierre Fabre Laboratories.

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