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Regulation of serotonin 5-HT_{2C} receptors by chronic ligand exposure

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Abstract

The effect of ligand pretreatment on human 5-hydroxytryptamine$_{2C}$ (5-HT_{2C}) receptors was examined in CHO cells expressing high (CHO-1C7; 67±3 pmol/mg) or low (CHO-1C19; 72±10 fmol/mg) levels of the receptor. Seventy-two hours pretreatment of CHO-1C7 cells with various ligands did not affect receptor expression. Pretreatment with inverse agonists enhanced 5-HT-mediated inositol phosphate accumulation with no change in constitutive receptor activity. The enhanced agonist responsiveness was inversely correlated with the intrinsic activity of the pretreatment ligand. Seventy-two hours of pretreatment with the weak agonist, 5-methoxygramine, caused an elevation in constitutive activity but no alteration in 5-HT-mediated signaling. In CHO-1C19 cells, 24 but not 72 h of pretreatment with the inverse agonist mianserin enhanced 5-HT-mediated signaling, with no effect on basal signaling; pretreatment with 5-methoxygramine had no significant effect. These findings highlight differences in the pattern of chronic regulation of 5HT_{2C} receptor signaling between high and low receptor expression levels in a common cellular background.

Keywords: Constitutive activity, Inverse agonist; Receptor theory; Serotonin receptor; Up-regulation

1. Introduction

Despite some early debate over the physiological significance of constitutive (ligand independent) activation of G protein-coupled receptors (GPCRs; Black and Shankley, 1995; Milligan et al., 1995), recent studies of the phenomenon have indicated that it is not necessarily the exclusive result of artificially high receptor expression levels or designed activating mutations, but rather is a naturally occurring property of some GPCRs, such as the dopamine D_{1B} receptor (Tiberi and Caron, 1994) and the 5-hydroxytryptamine$_{2C}$ (5-HT_{2C}) receptor (Burns et al., 1997; Berg et al., 1999; Herrick-Davis et al., 1999; Niswender et al., 1999; Berg et al., 2001). Moreover, the discovery of human diseases characterized by GPCR constitutive activity (Parma et al., 1993; Shenker et al., 1993) raises the potential of using inverse agonist ligands to silence constitutive GPCR activity as therapeutically preferred medicines over neutral antagonist ligands. What remains unclear however is the long-term effects of prolonged inverse agonist and/or neutral antagonist exposure on GPCR expression and signaling.

Serotonin 5-HT_{2C} receptors display a number of features that make them suitable for addressing questions related to current theories of GPCR signaling and regulation. For instance, this receptor is known to undergo RNA editing in the second intracellular loop, leading to various isoforms that display altered signaling properties (Burns et al., 1997). Interestingly, the non-RNA edited 5-HT_{2C} receptor isoform possesses a significant degree of constitutive activity (Niswender et al., 1999). In addition, the 5-HT_{2C} receptor is able to down-regulate in vivo in response to prolonged (>24 h) exposure to both agonists and antagonists/inverse

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agonists. This antagonist-mediated down-regulation is opposite to that seen for many other GPCRs, which tend to up-regulate after chronic antagonist exposure (Pei et al., 1994; MacEwan and Milligan, 1996; Gether et al., 1997).

Numerous studies have investigated the phenomenon of 5-HT_{2C} receptor down-regulation in both in vivo and in vitro systems. While 5-HT_{2C} receptor down-regulation appears to be a reproducible phenomenon in vivo (Sanders-Bush and Breeding, 1988; Roth and Ciaranello, 1991; Pranzatelli et al., 1993) and can be observed in primary cultures of rat choroid plexus (Barker and Sanders-Bush, 1993), cell lines transfected with 5-HT_{2C} receptors can behave quite differently. For example, down-regulation to antagonist but not agonist pretreatment has been demonstrated in NIH 3T3 cells (Barker et al., 1994), while no down-regulation to 24-h exposure with any $5HT_{2C}$ ligand was evident in transfected CHO-K1 cells (Berg et al., 1999). Similarly, the effect of ligand pretreatment on the signaling capacity of 5-HT_{2C} receptors is another outcome that appears to be highly dependent on cellular background. For example, chronic pretreatment with inverse agonists decreases signaling in vivo (Sanders-Bush and Breeding, 1988), has no effect on signaling in transfected SH-SY5Y neuroblastoma cells (Newton and Elliott, 1997), or increases signaling in transfected CHO cells (Berg et al., 1999).

A study by Berg et al. (1999) undertook a comprehensive investigation of the effects of 24-h pretreatment with inverse agonists on human 5-HT_{2C} receptors, which were expressed in a CHO-K1 cell line at low levels that did not reveal constitutive receptor activity in acute signaling assays. That study found no effect of ligand pretreatment on 5-HT_{2C} receptor density but did reveal a surprising increase in the signaling responsiveness to the agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). The finding suggested that cell-specific differences between CHO cells and neuronal populations of cells that express 5-HT_{2C} receptors likely lead to different outcomes of prolonged ligand exposure on receptor expression and/or signaling. However, the study of Berg et al. (1999) used a 24-h pretreatment time, whereas in vivo down-regulation of neuronal 5-HT_{2C} receptors is only seen after at least 72 h (Sanders-Bush and Breeding, 1988; Pranzatelli et al., 1993). Considering this finding, one of the aims of experiments in this study was to determine whether pretreatment with an inverse agonist for 72 rather than 24 h could induce 5-HT_{2C} receptor down-regulation analogous to that seen in vivo. A further focus of our investigations was to determine potential correlations between specific ligand properties (i.e., affinity, intrinsic activity) and their effects after prolonged exposure on the 5-HT_{2C} receptor expressed in CHO cells. To address these issues, we have used two CHO cell lines expressing the wild-type (non-RNA edited) human 5-HT_{2C} receptor with minimal and high constitutive activity, respectively, allowing the full spectrum of 5-HT_{2C} drug properties to be determined and their impact on chronic receptor regulation to be assessed in parallel.

2. Materials and methods

2.1. Materials

Minimum essential medium alpha (α-MEM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and hygromycin B were obtained from Invitrogen (Mt. Waverly, Victoria, Australia). [^{3}H]mesulergine (73 Ci/mmol) was from NEN Life Sciences Products, (Boston, MA, USA), [^{3}H]myoinositol (110 Ci/mmol) was from Amersham Pharmacia Biotech, and [^{14}C]inositol phosphate from American Radiolabeled Chemicals (ARC, St Louis, MO, USA). All other drugs and chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture

CHO-K1 cells transfected to stably express the non-RNA edited human 5-HT_{2C} receptor at "low" or "high" levels (see Results) were a generous gift from Dr. William Clarke (Department of Pharmacology, University of Texas Health Science Centre, San Antonio, TX, USA). Cells were grown in α-MEM containing 10% v/v heat-inactivated fetal bovine serum and hygromycin B (50 μg/ml) as a selection agent and were maintained in a humidified incubator at 37 °C, 95% O_2. Upon reaching ~80% confluence, cells were harvested using a trypsin/versene solution and used for experiments or passaged. Cells were not used after passage number 25.

2.3. Preparation of cell membranes

CHO-1C7 cells were harvested and washed with CHO-HEPES buffer (in mM: NaCl, 110.0; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1.0; glucose, 25.0; HEPES, 20.0; sucrose, 58.4) before centrifuging (400×g, 5 min). The resulting pellet was resuspended in ice-cold low ionic strength HEPES buffer (in mM: HEPES, 50; $MgCl_2$, 2.5; EGTA, 2.0, pH 7.4), homogenized using 3×10 s bursts separated by 30 s on ice with a handheld tissue homogeniser (PT-DA 1205/2EC Polytron Aggregate, Kinematica), and centrifuged (400×g, 10 min). The supernatant was then centrifuged (31,000×g, 30 min, 4 °C) using a Model J2-M1 centrifuge (Beckman, Fullerton, CA USA), and the resulting pellet was resuspended in 3 ml ice-cold low ionic strength HEPES buffer, assayed for protein content using the method of Bradford (1976) with bovine serum albumin as the standard, and frozen at −80 °C until required for experiments. The procedure for membrane preparation from CHO-1C19 cells was identical except that all washing and centrifugation steps were performed on the original pellet rather than the supernatant.

2.4. Saturation binding assays

[^{3}H]mesulergine saturation assays were performed according to a protocol modified from that previously

described by Clarke and coworkers (Berg et al., 1999). Assays were performed in triplicate in a volume of 500 μl low ionic strength HEPES buffer containing 1 mg/ml ascorbic acid, 1 μg of membrane protein from CHO-1C7 cells, or 100 μg of membrane protein from CHO-1C19 cells, and various concentrations of [^{3}H]mesulergine. Two types of saturation binding assays were employed. The first type of assay was a "full" saturation assay, which utilized concentrations of [^{3}H]mesulergine ranging between 0.1 and 20 nM and allowed for an estimation of receptor density and radioligand equilibrium dissociation constant using nonlinear regression (see Data analysis). The second type of assay was a "two-point" saturation binding assay, which used only two concentrations, 0.5 and 5 nM, of [^{3}H]mesulergine and allowed for a calculation of cell surface receptor density only (see Data analysis and Appendix A). In all instances, nonspecific binding was defined as binding in the presence of 1 μM mianserin. Incubation (1 h, 37 °C), in a shaking water bath, was terminated by the addition of 2 ml ice-cold 0.9% w/v NaCl solution followed by rapid filtration using a Brandel cell harvester (Gaithersburg, MD, USA) and washing with 2×2 ml ice-cold 0.9% w/v NaCl solution. Filters (Whatman GF/C) were presoaked in 0.9% w/v NaCl solution containing 1% polyethyleneimine (PEI) at 4 °C for 1 h prior to filtering. Filters were placed in 7-ml scintillation vials (Wallac, Gaithersburg, MD, USA), followed by the addition of 5 ml of scintillation cocktail (Ultima Gold LSC-cocktail, Packard). Vials were then left to stand until the filters became uniformly translucent (at least 2 h) before the radioactivity was determined using a Model 1409 DSA Liquid Scintillation counter (Wallac).

2.5. Competition binding assay

Assays were performed in a volume of 500 μl low ionic strength HEPES buffer containing 1 mg/ml ascorbic acid, 1 nM [^{3}H]mesulergine, and varying concentrations of inhibiting drugs. The reaction was initiated by the addition of 1 μg of membrane protein from CHO-1C7 5-HT$_{2C}$ cells. Experiments were carried out in triplicate at 37 °C for 60 min with nonspecific binding, reaction termination, and radioactivity determination being the same as described above for the saturation binding assays.

2.6. Ligand pretreatment of CHO cells

5-HT$_{2C}$-expressing CHO-K1 cells were passaged as described above, after which a 10×K_I final concentration of different 5-HT$_{2C}$ ligands (see Results) or vehicle equivalent was added to the cell culture medium either 72 or 24 h prior to harvesting the cells for experiments. Cell culture media (together with any treatment ligand) were replaced every 24 h if necessary. For cells that were used in the [^{3}H]inositol phosphate (IP) assay, 0.5 μCi/ml [^{3}H]myoinositol was also added to the flask 24 h prior to harvesting. For each pretreatment experiment in a given passage number, a vehicle-pretreated flask was prepared in parallel.

2.7. [^{3}H] IP accumulation measurements

CHO-1C7 or CHO-1C19 cells were labelled for 24 h with [^{3}H]myoinositol as described above. Cells were then washed with CHO HEPES buffer containing 10 mM LiCl, distributed to assay tubes (~5×10^5 cells/tube) and allowed to incubate for 15 min at 37 °C. Concentration–response curves for the stimulation of IP accumulation by a variety of 5-HT$_{2C}$ ligands were then constructed. The reaction was allowed to proceed for 30 min after the addition of ligand or vehicle control before being stopped with chloroform/methanol (1:2). Tubes were vortexed briefly and placed at 4 °C for 30 min to allow phase separation. Total inositol phosphates were then separated by ion-exchange chromatography on DOWEX AG1-X8 resin, with [^{14}C]inositol-1-phosphate as a recovery standard. The amount of radioactivity in each sample was then determined by liquid scintillation counting on a Tri-Carb 1600 TR Liquid Scintillation Analyzer (Packard Bioscience, Victoria, Australia).

2.8. Data analysis

Complete radioligand saturation binding isotherms were analyzed via nonlinear regression using PRISM 3.02 (Graphpad Software, San Diego, CA, USA) in order to derive individual estimates of B_{max} (total receptor density) and K_D (radioligand–receptor equilibrium dissociation constant). "Two-point" saturation binding experiments were analyzed according to the following equation (see Appendix A):

$$B_{max} = \frac{B1B2([A1] - [A2])}{[A2]B2 - [A2]B1} \quad (1)$$

where [A1] and [A2] denote the low and high concentrations, respectively, of radioligand, and B1 and B2 denote the corresponding counts of specific binding. Competition binding isotherms were analyzed using PRISM according to a one-site empirical mass-action binding model. IC_{50} values were converted to K_I values (competitor–receptor dissociation equilibrium constant) by PRISM.

Functional IP accumulation experiments were analyzed by nonlinear regression using the following three parameter Hill equation:

$$Y = E_{min} + \frac{E_{max} - E_{min}}{1 + 10^{(-pEC_{50} - X)}} \quad (2)$$

where Y is the level of response, E_{min} is the bottom plateau, E_{max} is the top plateau, pEC_{50} is the negative base 10 logarithm of the molar concentration of drug required to generate a half-maximal response, and X is the concentration of drug. Ligand response range was calculated by subtracting E_{min} from E_{max}.

Results are expressed as mean±standard error of the mean. Statistical significance was determined by unpaired *t*-test or by one-way analysis of variance (ANOVA), as appropriate. Correlation analysis was performed by calculating the correlation coefficient (r^2). A probability (P) value of 0.05 was taken to indicate statistical significance in all tests.

3. Results

3.1. Radioligand binding

[^{3}H]mesulergine bound in a reversible and saturable manner to the 5-HT$_{2C}$ receptor expressed in CHO-1C7 cell membranes, yielding a monophasic isotherm characterized by a pK_D of 9.05±0.07 and a B_{max} of 67±3 pmol/mg protein (n=3). Similar experiments conducted using CHO-1C19 membranes revealed a pK_D of 8.86±0.28 and a B_{max} of 72±10 fmol/mg protein (n=3). Thus, the two cell lines differed in receptor expression level by almost 1000-fold.

Subsequent radioligand competition binding assays on CHO-1C7 membranes, using a variety of 5-HT$_{2C}$ receptor ligands to displace 1 nM [^{3}H]mesulergine, yielded monophasic isotherms indicative of binding to a single-affinity state of the 5-HT$_{2C}$ receptor (Table 1). These experiments also allowed for the determination of pretreatment concentrations of ligand that would ensure equivalent occupancy of the 5-HT$_{2C}$ receptor.

3.2. Acute effect of 5-HT$_{2C}$ ligands on IP accumulation

To define the functional potency and intrinsic activity of the 5-HT$_{2C}$ ligands under investigation, concentration–response curves for the ability of the ligands to influence the accumulation of [^{3}H]IP in CHO-1C7 cells were generated. The nine ligands tested displayed a broad spectrum of efficacies ranging from the full agonist 5-HT to the full inverse agonists, ritanserin and mianserin (Table 1 and Fig. 1). The compounds lisuride, 5-methoxygramine (5-MXG), and chlorpromazine were closest to being neutral antagonists in this system. For comparison, the functional potency and intrinsic activity of 5-HT, tryptamine, DOI, 5-MXG, and mianserin were also assessed in CHO-1C19 cells. In this cell line, mianserin and 5-MXG behaved as neutral antagonists (Table 2 and Fig. 2).

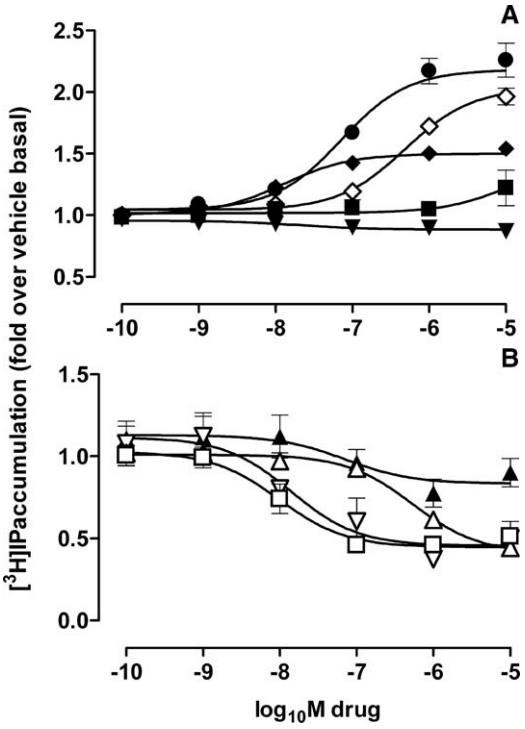

Fig. 1. [^{3}H]Inositol phosphate accumulation responses to various ligands in CHO-1C7 cells. (A) 5-HT (●; n=4), DOI (◆; n=4), tryptamine (◇; n=3), 5-MXG (■; n=3), lisuride (▼; n=3). (B) ritanserin (□; n=3), mianserin (▽; n=3), ketanserin (△; n=3), chlorpromazine (▲; n=3). All points represent the mean±S.E.M. of experiments conducted in triplicate. Error bars that are not shown are contained within the symbol.

3.3. Effects of ligand pretreatment on 5-HT$_{2C}$ receptor expression

Initially, experiments were undertaken to validate that the cell-washing protocol employed was sufficient to remove

Table 1
Functional and binding parameters for 5-HT$_{2C}$ ligands in CHO-1C7 cells

Ligand	Function			Binding	
	E_{max}[a]	pEC$_{50}$[b]	n[c]	pK_I[d]	n[c]
5-HT	2.18±0.13	7.13±0.04	4	6.51±0.11	6
DOI	1.54±0.07	7.91±0.21	4	7.11±0.13	3
Tryptamine	2.07±0.05	6.21±0.03	3	6.23±0.08	3
5-MXG	1.21±0.16	5.04±0.39	3	6.52±0.06	3
Lisuride	0.85±0.02	7.23±0.48	3	7.72±0.11	3
Mianserin	0.41±0.04	7.88±0.06	3	8.02±0.11	3
Ritanserin	0.45±0.03	8.02±0.09	3	9.31±0.22	3
Ketanserin	0.45±0.03	6.31±0.12	3	7.22±0.13	3
Chlorpromazine	0.75±0.05	7.51±0.14	3	7.83±0.11	3

[a] Maximum ligand response (fold over basal).
[b] Negative logarithm of the molar concentration of ligand required to achieve half-maximal response.
[c] Number of experiments.
[d] Negative logarithm of the competitor equilibrium dissociation constant.

Table 2
Functional parameters for 5-HT$_{2C}$ ligands in CHO-1C19 cells

Ligand	E_{max}[a]	pEC$_{50}$[b]	n[c]
5-HT	4.21±0.66	7.30±0.08	5
Tryptamine	3.76±0.26	6.26±0.07	5
DOI	2.95±0.13	6.99±0.07	5
Mianserin	n.d.[d]	n.d.[d]	4
5-MXG	n.d.[d]	n.d.[d]	4

[a] Maximum ligand response (fold over basal).
[b] Negative logarithm of the molar concentration of ligand required to achieve half-maximal response.
[c] Number of experiments.
[d] Not determined.

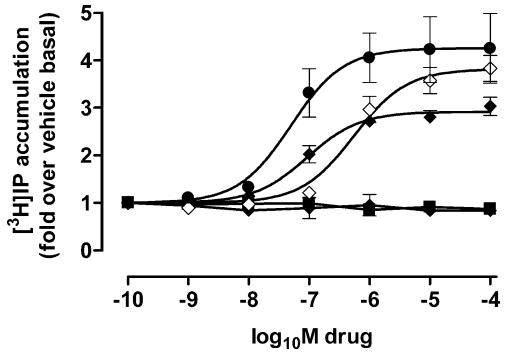

Fig. 2. [^{3}H]Inositol phosphate accumulation responses to various ligands in CHO-1C19 cells. 5-HT (●), tryptamine (◇), DOI (◆), 5-MXG (■), mianserin (▽). Values represent mean±S.E.M. of four to five experiments conducted in triplicate. Error bars not shown are contained within symbol.

the pretreatment ligand after 72 h. [^{3}H]mesulergine full-saturation experiments conducted using membranes prepared from 5-MXG- or mianserin-pretreated CHO-1C7 cells showed no significant change in radioligand pK_D compared to vehicle-treated membranes (vehicle, pK_D=8.93±0.07, n=8; mianserin-pretreated, pK_D=8.62±0.17, n=3; 5-MXG-pretreated, pK_D=8.60±0.14, n=3; ANOVA P>0.05), indicating effective washout of drugs following pretreatment. Furthermore, the same experiments indicated that 72-h pretreatment with either 5-MXG or mianserin had no effect on 5-HT$_{2C}$ receptor-expression levels (data not shown). Subsequent determinations of 5-HT$_{2C}$ receptor density after 72 h of pretreatment utilized a "two-point" saturation binding protocol (see Appendix A).

Six 5-HT$_{2C}$ receptor ligands, chosen for their diversity of intrinsic activities and affinities, had no significant effect on 5-HT$_{2C}$ receptor expression in CHO-1C7 cells after 72 h of pretreatment (Fig. 3).

3.4. Effect of ligand pretreatment on responses to 5-HT

Functional experiments of 5-HT-mediated IP accumulation revealed significant differences in the signaling capacity of ligand-pretreated cells compared to vehicle-pretreated cells. This is in contrast to the lack of effect of chronic ligand pretreatment on 5-HT$_{2C}$ receptor expression. Fig. 4 shows the effect of 5-HT in CHO-1C7 cells exposed to either vehicle mianserin (2×10^{-7} M) or 5-MXG (3×10^{-6} M). Depending on the ligand used for the pretreatment, either the 5-HT response range or the basal constitutive activity of the system was enhanced (Fig. 4, Table 3). Indeed, extending the battery of compounds used in the pretreatment studies revealed a trend for different types of 5-HT$_{2C}$ ligands to differentially affect either of these system response parameters. Specifically, pretreatment of cells with strong inverse agonists, such as mianserin or ritanserin, was characterized by an increase in the range of response to 5-HT with no change in constitutive activity (Table 3 and Fig. 4). In contrast, increased constitutive activity was most pronounced after pretreatment with near-neutral antagonists, such as 5-MXG; this increase in the basal level of [^{3}H]IP accumulation was accompanied by no significant effect on the range of 5-HT-mediated responses (Table 3 and Fig. 4). Importantly, pretreatment of nontransfected CHO cells with 5-MXG for 72 h had no effect on basal IP accumulation (data not shown). This suggests a 5-HT$_{2C}$-mediated effect in CHO-1C7 cells. Pretreatment with the partial agonist DOI had negligible effects on 5-HT responses (Table 3).

The ability of pretreatment ligands to modulate the response range of 5-HT did not correlate with the affinity of the ligand for the 5-HT$_{2C}$ receptor, as determined from competition experiments against [^{3}H]mesulergine (r^2=0.43, P>0.05; data not shown). There was however a significant correlation between the intrinsic activity of ligands in the acute IP accumulation assay and their ability to modulate the response range and pEC$_{50}$ of 5-HT-induced [^{3}H]IP accumulation after pretreatment (r^2=0.72, P=0.03 and r^2=0.66, P<0.05, respectively; Table 3 and Fig. 5). In addition, ligands that were close-to-neutral antagonists in the acute IP accumulation assay (5-MXG, lisuride and chlorpromazine) displayed a tendency to increase the basal activity of CHO-1C7 cells after 72 h of pretreatment (Table 3 and Fig. 5).

To ascertain whether the pretreatment-induced modulation of 5-HT response range and/or increase in basal activity reflected a long-lasting, ligand-induced change in 5-HT$_{2C}$ receptor conformation, 5-HT competition experiments against [^{3}H]mesulergine were carried out in membranes from vehicle-pretreated and ligand-pretreated 5-HT$_{2C}$-expressing CHO-1C7 cells. Neither pretreatment with the full inverse agonist mianserin nor the close-to-neutral antagonist 5-MXG affected the affinity state recognised by 5-HT (Fig. 6).

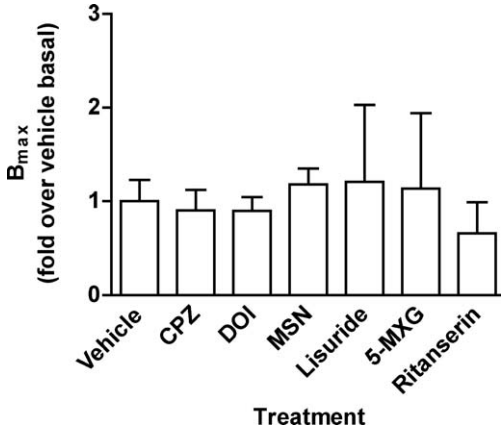

Fig. 3. The effect of ligand pretreatment on 5-HT$_{2C}$ receptor density (B_{max}). CHO-1C7 cells expressing 5-HT$_{2C}$ receptors were pretreated for 72 h with a $10\times K_I$ concentration of ligand prior to extensive washout and membrane preparation (see Materials and methods). Radioligand saturation binding experiments were conducted using two concentrations of [^{3}H]mesulergine (see Appendix A), with nonspecific binding being defined as [^{3}H]mesulergine binding in the presence of 10 μM mianserin. Vehicle (n=7), CPZ (chlorpromazine; n=3), DOI (n=4), MSN (mianserin; n=7), lisuride (n=3), 5-MXG (n=3), ritanserin (n=3). All bars represent mean±S.E.M. of experiments conducted in replicates of six.

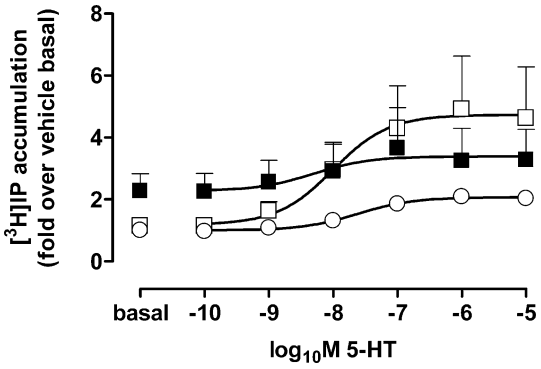

Fig. 4. The effect of pretreatment with selected 5-HT_{2C} ligands on the response to 5-HT in the inositol phosphate accumulation assay in 5-HT_{2C} CHO-1C7 cells. Cells were pretreated with mianserin (2×10^{-7} M) or 5-MXG (3×10^{-6} M) for 72 h prior to extensive washout and determination of 5-HT concentration–response curves (see Materials and methods). Pretreatment ligands were vehicle (○; n=10), ritanserin (□; n=4), 5-MXG (■; n=5). All points represent the mean±S.E.M. of experiments conducted in triplicate. Error bars that are not shown are contained within the symbol.

For comparison to the signaling studies on the CHO-1C17 cells, the low-receptor-expressing CHO-1C19 cells were treated for 24 or 72 h with concentrations corresponding to $10\times K_I$ of the agonist, DOI, the near-neutral antagonist, 5-MXG, the inverse agonist, mianserin, or vehicle control. After 24-h pretreatment and extensive washing to remove residual ligand, 5-HT IP accumulation curves were constructed (Fig. 7). No significant effect was observed on ligand-independent signaling after any of the pretreatments, however a significant increase in 5-HT-mediated IP accumulation, relative to vehicle, was found after mianserin pretreatment (Table 4 and Fig. 7). A similar trend was noted for 5-MXG pretreatment, but this was not statistically significant. 5-HT potency was not significantly altered by pretreatment. Interestingly, when exposure time was extended to 72 h, no difference in 5-HT signaling parameters was observed for any of the pretreatment conditions (Table 4 and Fig. 7).

3.5. Effect of ligand pretreatment on responses to other ligands

Additional experiments were undertaken in CHO-1C7 cells in order to investigate the effect of chronic ligand exposure on the subsequent signaling capacity of another agonist (DOI), an inverse agonist (ritanserin), or a near-neutral antagonist (5-MXG) of the 5-HT_{2C} receptor.

As before, pretreatment of CHO-1C7 cells with 5-MXG caused a significant enhancement of the basal IP accumulation (3.02±0.38, n=11, P<0.01; Table 5 and Fig. 8), whereas pretreatment with either DOI or ritanserin did not affect this parameter. 5-MXG pretreatment also increased the response range of the inverse agonist ritanserin, but its effect on DOI and on itself was only an upward translocation of the curves (Table 5 and Fig. 8). DOI pretreatment had minimal effects on responses to any of the three ligands which is consistent with the lack of effect on 5-HT-mediated signaling (Table 5 and Fig. 8). Ritanserin pretreatment tended to increase the response range to both DOI and 5-MXG (Table 5 and Fig. 8), although this did not reach statistical significance.

4. Discussion

There are two main findings from this study. First, striking differences were noted between the effect of 72-h pretreatment with "neutral" antagonists versus inverse agonists on the signaling capacity of overexpressed recombinant 5-HT_{2C} receptors. Second, the use of a 72-h pretreatment time, which was based on the minimal duration of exposure required to observe 5-HT_{2C} receptor down-regulation in vivo, allowed for a differentiation between chronic ligand effects on the signaling of the receptor at low versus high receptor expression levels, and also suggests that the lack of 5-HT_{2C} receptor down-regulation after prolonged ligand exposure noted in previous studies is due to a characteristic of the CHO host cell rather than the use of an inadequate pretreatment time.

Table 3
The effect of ligand pretreatment on 5-HT-mediated IP accumulation in 5-HT_{2C} CHO-1C7 cells

Pretreatment ligand	pEC_{50}[a]	Basal[b]	E_{max}[c]	Range[d]	n[e]
Vehicle	7.56±0.14	1.00	2.08±0.19	1.09±0.18	10
Chlorpromazine	8.15±0.21	1.92±0.30	4.59±0.66	2.58±0.47	4
DOI	7.25±0.07	1.29±0.31	2.85±0.61	1.56±0.37	4
Mianserin	8.18±0.16	1.53±0.42	5.60±1.60	4.01±1.62[f]	4
Lisuride	7.91±0.03	2.21±0.69	3.72±0.98	1.52±0.29	3
5-MXG	8.08±0.31	2.32±0.72[f]	3.49±1.41	1.16±0.70	4
Ritanserin	8.04±0.09	1.07±0.16	5.55±2.01	4.38±1.88[f]	3

Cells were exposed to a $10\times K_I$ concentration of pretreatment ligand for 72 h followed by extensive washout (see Materials and methods). All values are mean±S.E.M. of the indicated number of experiments conducted in triplicate.

[a] Negative logarithm of the molar concentration of 5-HT required to achieve half-maximal response.
[b] Response in the absence of 5-HT, expressed relative to that of the vehicle-pretreated cells.
[c] Maximum 5-HT response from sigmoidal curve fit.
[d] E_{max} minus E_{min} (minimum ligand response from sigmoidal curve).
[e] Number of experiments conduced in triplicate.
[f] P< 0.05 compared to vehicle-pretreated control.

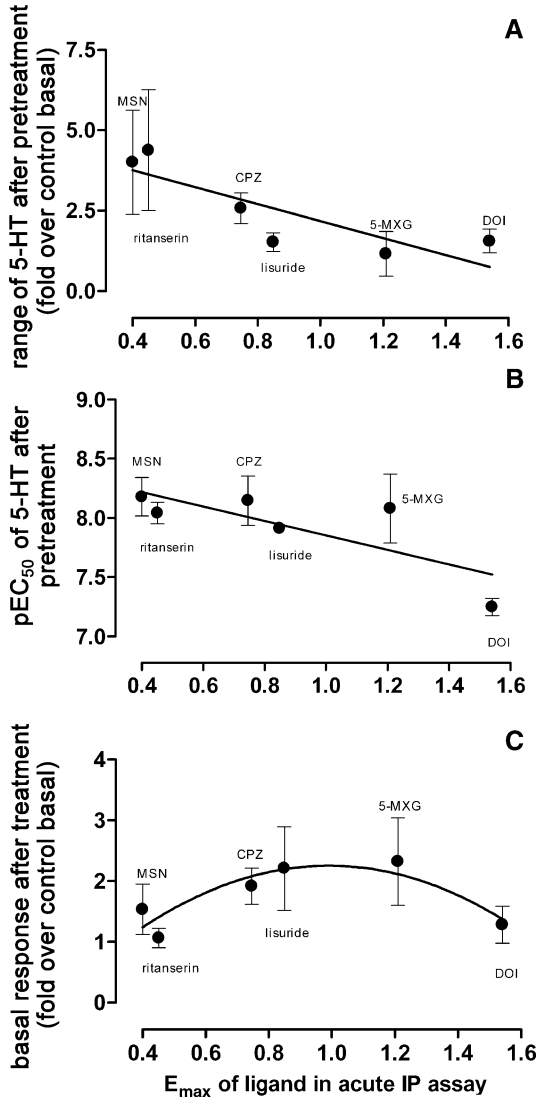

Fig. 5. Correlation analysis between the E_{max} (intrinsic activity) of 5-HT_{2C} ligands in the inositol phosphate accumulation assay and the effect of 72-h ligand pretreatment on 5-HT-mediated signaling in CHO-1C7 cells. The acute maximum ligand effect (E_{max}) was determined for each ligand in the IP accumulation assay (see Table 1). These ligands were then used at a $10\times K_I$ concentration (determined from radioligand competition binding; see Table 1) to treat 5-HT_{2C} CHO-1C7 cells for 72 h prior to extensive washout (see Materials and methods) and determination of 5-HT concentration–response curves. (A) Correlation between acute ligand E_{max} and 5-HT response range after 72-h ligand pretreatment (r^2=0.72; P=0.03). (B) Correlation between acute ligand E_{max} and the pEC_{50} of 5-HT after 72-h ligand pretreatment (r^2=0.66; P<0.05). (C) Relationship between acute ligand E_{max} and the effect on 5-HT_{2C} receptor basal activity after 72-h ligand pretreatment. All panels: MSN (mianserin; n=4), ritanserin (n=3), CPZ (chlorpromazine; n=4), lisuride (n=3), 5-MXG (n=5), DOI (n=4). All points represent the mean±S.E.M. of experiments conducted in triplicate. Error bars that are not shown are contained within the symbol.

The lack of effect of ligand pretreatment on 5-HT_{2C} receptor density in either CHO-1C7 or CHO-1C19 cells is in contrast to in vivo observations with the 5-HT_{2C} receptor, and suggests that it is some cellular characteristic of CHO cells themselves that does not allow down-regulation to

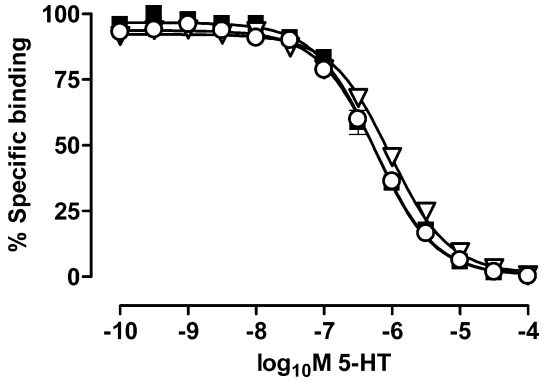

Fig. 6. Competition between [^{3}H]mesulergine and 5-HT at 5-HT_{2C} receptors in ligand-pretreated CHO-1C7 cell membrane homogenates. The affinity of 5-HT for the 5-HT_{2C} receptor was determined after 72-h pretreatment of 5-HT_{2C} CHO-1C7 cells with a $10\times K_I$ concentration of mianserin or 5-MXG followed by extensive washout (see Materials and methods). Control (vehicle pretreatment, ○; n=6), 5-MXG pretreatment (■; n=3) and mianserin pretreatment (▽; n=3). All points represent the mean±S.E.M. of experiments conducted in triplicate. Error bars that are not shown are contained within the symbol. Curves are the best-fit values for a one-site competition binding model (see Data analysis).

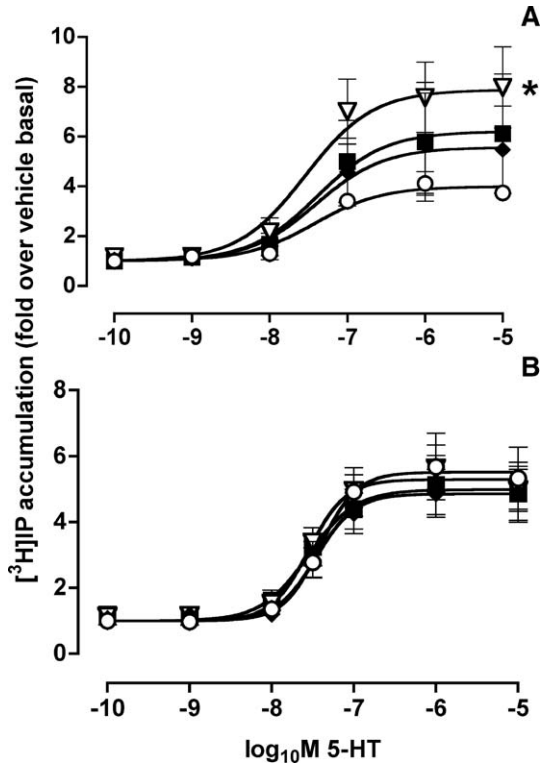

Fig. 7. Effect of 24- and 72-h ligand pretreatment on 5-HT-mediated IP accumulation in CHO-1C19 cells. Cells were treated for 24 (A) or 72 h (B) with ligand at concentrations equal to $10\times K_I$, followed by extensive washout and assay of 5-HT-mediated signaling. Pretreatment conditions: vehicle (○; n=4), DOI (◆; n=4), mianserin (▽; n=4), 5-MXG (■; n=4). Values represent mean±S.E.M. of experiments conducted in triplicate. Error bars not shown are contained within symbol. * P<0.05, Dunnett's post hoc comparison of means.

Table 4
The effect of ligand pretreatment on 5-HT-mediated IP accumulation in 5-HT_{2C} CHO-1C19 cells

Pretreatment ligand	pEC_{50}[a]	E_{max}[b]	n[c]
24 h			
Vehicle	7.39±0.06	3.94±0.38	4
DOI	7.33±0.06	5.47±1.74	4
Mianserin	7.54±0.15	7.70±0.14[d]	4
5-MXG	7.47±0.10	6.12±2.51	4
72 h			
Vehicle	7.31±0.16	5.51±0.97	5
DOI	7.28±0.14	5.12±0.94	5
Miansern	7.48±0.05	5.30±0.67	4
5-MXG	7.49±0.08	5.00±0.86	5

Cells were exposed to a $10\times K_I$ concentration of pretreatment ligand for either 24 or 72 h followed by extensive washout (see Materials and methods). All values are mean±S.E.M. of the indicated number of experiments conducted in triplicate.

[a] Negative logarithm of the molar concentration of 5-HT required to achieve half-maximal response.

[b] Maximum 5-HT response from sigmoidal curve fit.

[c] Number of experiments conduced in triplicate.

[d] $P<0.05$ compared to vehicle-pretreated control.

occur rather than the use of a short pretreatment period or differences in receptor expression levels. Lack of pretreatment-induced down-regulation of 5-HT_{2C} receptors in CHO cells is interesting in and of itself, because it suggests that CHO cells may lack important accessory proteins necessary for the behavior of the receptors in vivo (Sanders-Bush and Breeding, 1988; Roth and Ciaranello, 1991; Pranzatelli et al., 1993) and in some lines that endogenously express the receptor (Barker and Sanders-Bush, 1993).

In contrast to effects on receptor density, 72-h ligand pretreatment of overexpressed CHO-1C7 cells revealed interesting effects on both agonist-dependent and agonist-independent receptor signaling. In the case of agonist-dependent receptor activity, pretreatment with inverse agonists, such as mianserin and ritanserin, significantly increased the response range of 5-HT with no change in basal activity; a similar finding was made by Berg et al. (1999), who demonstrated a potentiation of the IP accumulation response to DOI after pretreatment for 24 h with the 5-HT_{2C} inverse agonist [5-methyl-1-(3-pyridilcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole; SB 206553]. An additional finding in our study in CHO-1C7 cells is that this potentiation of 5-HT signaling after 72-h pretreatment was inversely correlated with the intrinsic activity of the pretreatment ligand in the acute IP accumulation assay (Fig. 5). In the case of agonist-independent (constitutive) receptor activity, pretreatment with the near-neutral antagonist 5-MXG caused a significant increase in this basal activity, with no subsequent effect on 5-HT response range. Although not significant, there was also a trend for the near-neutral antagonists, lisuride and chlorpromazine, to increase basal IP accumulation.

Given the differential effects in CHO-1C7 cells of pretreatment with inverse agonists, on the one hand, and near-neutral antagonists, on the other, it is likely that different mechanisms underlie the phenomena. Indeed, using CHO-1C19 cells, Berg et al. (1999) demonstrated that the effect of pretreatment with inverse agonists on agonist-dependent signaling was mediated by an up-regulation of $G_{q/11}$ proteins that couple to the 5-HT_{2C} receptor; this is also likely to be the mechanism operative with inverse agonists in our study. However, with respect to our findings with the neutral antagonists, one possible explanation for their effect on constitutive receptor activity

Table 5
The effect of 5-HT_{2C} ligand pretreatment on receptor-mediated [^{3}H]IP accumulation in 5-HT_{2C} CHO-1C7 cells

Test ligand	Pretreatment ligand	pEC_{50}[a]	Basal[b]	E_{max}[c]	Range[d]
DOI	Vehicle (4)	7.32±0.13	1.00±0.00	1.52±0.14	0.49±0.15
	DOI (4)	7.54±0.16	1.48±0.09	2.03±0.24	0.56±0.16
	5-MXG[e] (5)	7.60±1.83	3.13±0.61[f]	3.28±0.41[g]	0.67±0.71
	Ritanserin (3)	7.19±0.32	1.88±0.35	2.59±0.62	1.02±0.41
5-MXG	Vehicle (4)	6.01±0.47	1.00±0.00	1.71±0.15	0.55±0.11
	DOI (3)	n.d.[e]	1.95±0.37	n.d.[e]	n.d.[e]
	5-MXG (3)	5.49±1.26	3.08±1.21	4.11±1.26	0.73±0.33
	Ritanserin (4)	5.40±0.14	1.03±0.34	2.55±0.85	1.41±0.50
Ritanserin	Vehicle (4)	7.76±0.05	1.00±0.00	0.40±0.03	−0.67±0.06
	DOI (4)	7.38±0.02[g]	1.25±0.15	0.50±0.03	−0.82±0.13
	5-MXG (4)	7.72±0.09	2.87±0.45[f]	1.37±0.15[f]	−1.46±0.22
	Ritanserin (3)	7.84±0.06	1.08±0.21	0.50±0.07	−0.51±0.17

Cells were exposed to a $10\times K_I$ concentration of pretreatment ligand for 72 h of pretreatment followed by extensive washout. Concentration–response curves were then generated for each ligand ("test ligand"). All values are mean±S.E.M. of experiments conducted in triplicate. The number of experiments is shown in parentheses.

[a] Negative logarithm of the molar concentration of ligand required to achieve half-maximal response.

[b] Response in the absence of ligand.

[c] Maximum ligand response from sigmoidal curve fit.

[d] E_{max} minus E_{min} (minimum ligand response from sigmoidal curve fit).

[e] Not determined.

[f] $P<0.01$ compared to vehicle-pretreated control.

[g] $P<0.05$ compared to vehicle-pretreated control.

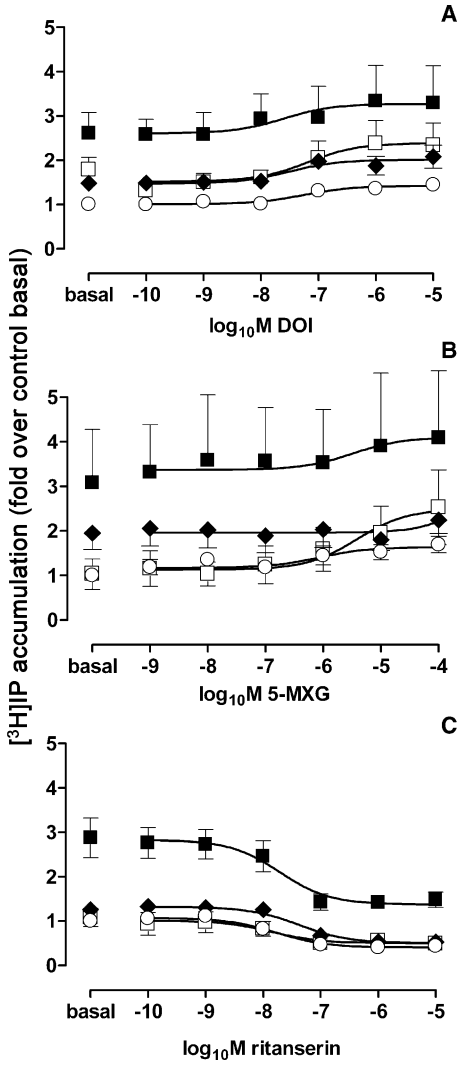

Fig. 8. The effect of 5-HT_{2C} ligand pretreatment on the response to an agonist, neutral antagonist, or inverse agonist in inositol phosphate accumulation assay using 5-HT_{2C} CHO-1C7 cells. Cells were treated for 72 h with a 10×K_I concentration of ligand prior to washout (see Materials and methods) and determination of concentration–response curves to the ligands shown on the horizontal axis of each graph. Pretreatments were vehicle (○; n=4). (A) DOI, (◆; n=4), 5-MXG (■; n=4), ritanserin (□; n=4). (B) DOI, (◆; n=3), 5-MXG (■; n=3), ritanserin (□; n=4). (C) DOI, (◆; n=4), 5-MXG (■; n=4), ritanserin (□; n=3). All points represent the mean±S.E.M. of experiments conducted in triplicate. Error bars that are not shown are contained within the symbol.

is that pretreatment with these agents leads to a sustained redistribution of the inactive receptor state/active receptor state equilibrium in CHO-1C7 cells. This explanation is unlikely however, because 5-HT competition against [^{3}H]mesulergine conducted in membranes from vehicle-treated CHO-1C7 cells or cells pretreated for 72 h with 5-MXG revealed no change in the affinity of 5-HT for the 5-HT_{2C} receptor; an enhancement in affinity would have been expected if pretreatment led to more receptors being in the active state.

It is likely that the effects of ligand pretreatment on 5-HT_{2C} signaling, discussed in the preceding paragraphs, reflect multiple (pleiotropic) coupling modes of the receptor when it is overexpressed, as a different pattern of behaviors was noted in the lower-expressing CHO-1C19 cell line. Intriguingly, enhanced IP accumulation to 5-HT following mianserin pretreatment was lost at 72 h in CHO-1C19, but not CHO-1C7 cells. This finding suggests a biphasic regulation of 5-HT_{2C} signaling by the cellular environment (i.e., increased signaling followed by reduced signaling) that is lost when the receptor is overexpressed (i.e., increased signaling remains unaltered), perhaps due to a saturation of intracellular regulatory proteins. Functional assays examining the effect of prolonged ligand exposure on 5-HT_{2C} receptors in native tissue are scarce; it may well be that an acute, inverse agonist-mediated, up-regulation in receptor signaling is also observed within 24 h in vivo but is then "reset" to lower levels as the cellular environment adapts.

Traditional two-state theory implies that the unliganded, active form of the receptor, denoted R*, signals in an identical manner to agonist bound form, AR*. However, the results obtained with 5-MXG in the CHO-1C7 cells suggest that this assumption may not accurately reflect the reality of GPCR signaling. In explaining the effect of 5-MXG, it is necessary to assume that the unliganded form of the receptor signals in a manner different to that of the agonist driven conformation. Just as different agonists can drive conformations of GPCRs that differ in their ability to couple to specific effectors (Kenakin, 1997), it is reasonable to assume that unliganded conformations of receptors also possess effector-coupling preferences, distinct from that of agonist-bound forms. Thus, one way in which 5-MXG could selectively enhance constitutive activity without affecting agonist mediating signaling is by up-regulating a cellular signaling cascade sensitive to R* but not AR*. Similarly, selective up-regulation of specific members of the $G_{q/11}$ protein family, which may couple preferentially to the agonist-bound conformation of the 5-HT_{2C} receptor but not the unliganded receptor, may be a mechanism by which inverse agonists can potentiate agonist-mediated signaling without affecting constitutive basal activity.

In recent years there has been much debate on the therapeutic significance of neutral antagonists versus inverse agonists in a physiological setting (Black and Shankley, 1995; Milligan et al., 1995). It may be argued that in the majority of cases involving blockade of GPCRs by therapeutic drugs, there is little reason to assume that an inverse agonist would have any particular advantage over a neutral antagonist. Our study shows that for the 5-HT_{2C} receptor at least, inverse agonists and neutral antagonists can have profoundly different outcomes on receptor signaling after chronic exposure. That is, even after removal of the ligand, the system remains altered. This occurs, although the acute effects would be indistinguishable in most physiological settings. A better understanding of chronic drug effects would broaden the range of pharmacological manipulation of GPCRs. The advantages would become

increasingly important as we learn more about aberrant GPCR signaling in various disease states.

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Appendix A. Theoretical rationale and validation of the "two-point" saturation binding assay

The most common method used to obtain an approximate estimate of maximal receptor density without performing a complete saturation binding experiment is to use a single, "maximal" concentration of radioligand, with the tacit assumption that this is sufficient to provide near-saturating levels of receptor occupancy under all experimental conditions studied. However, this approach suffers from two main drawbacks. First, it cannot guarantee an unambiguous measure of maximal receptor density if an experimental treatment has caused a significant alteration of receptor density. Second, the high concentrations of radioligand routinely required for this approach can limit its practical application if cost becomes a factor. An alternative method that can provide accurate measures of maximal receptor density under varying conditions of receptor expression, while utilizing relatively low concentrations of radioligand, is the "two-point" saturation assay. The theoretical basis for this approach is as follows.

Assuming standard mass-action kinetics, the specific binding, B, of a particular concentration of radioligand $[A]$ to a single class of receptor follows the following hyperbolic relationship:

$$B = \frac{B_{\max}[A]}{[A] + K_D} \tag{A1}$$

where $B_{\max}$ represents the maximal (asymptotic) density of receptor binding sites, and K_D denotes the equilibrium dissociation constant of the radioligand. This equation can be rearranged as shown below:

$$K_D = \frac{B_{\max}[A] - B[A]}{B} \tag{A2}$$

Because K_D is a constant specific to each radioligand–receptor pair, the relationship between two different levels of specific binding ($B1$ and $B2$) corresponding to two different concentrations of radioligand ($[A1]$ and $[A2]$) can be shown to be

$$\frac{B_{\max}[\mathrm{A1}] - \mathrm{B1}[\mathrm{A1}]}{\mathrm{B1}} = \frac{B_{\max}[\mathrm{A2}] - \mathrm{B2}[\mathrm{A2}]}{\mathrm{B2}} \tag{A3}$$

which, on simplification yields the following equation:

$$B_{\max} = \frac{\mathrm{B1B2}([\mathrm{A1}] - [\mathrm{A2}])}{[\mathrm{A1}]\mathrm{B2} - [\mathrm{A2}]\mathrm{B1}} \tag{A4}$$

This approach has been used previously for the calculation of $B_{\max}$ values in assays of radioligand binding to muscarinic acetylcholine receptors (Lazareno and Birdsall, 1995; Christopoulos, 2000). It should be noted that this method assumes that the K_D value between different receptor preparations is unaltered by any experimental treatment. In our experience, the method allows for calculation of reliable estimates of the $B_{\max}$, relative to estimates obtained from complete saturation binding curve analysis, provided that the experimental error is ≤10% CV (A. Christopoulos, unpublished). Shown in Table 6 is a comparison of the $B_{\max}$ results obtained for [^{3}H]mesulergine in this study from three separate "full" (eight-point) or two-point (0.5 an 5 nM) saturation binding assays, where it can be seen that a very good agreement is obtained between the two methods of analysis.

Table 6
Comparison of $B_{\max}$ values using two different methods

	$B_{\max}$ (pmol/mg protein)	
	Full saturation[a]	Two-point saturation[b]
Experiment 1	73	70
Experiment 2	93	98
Experiment 3	195	207

[a] Estimated via nonlinear regression.
[b] Calculated via Eq. (A4).

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