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Ligand-directed serotonin 5-HT$_{2C}$ receptor desensitization and sensitization

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Abstract

Exposure of G protein–coupled receptors (GPCRs) to agonists can desensitize receptor signaling and lead to drug tolerance, whereas inverse agonists can sensitize signaling. For example, activation of serotonin 5-HT$_{2C}$ GPCRs is pharmacotherapeutic for obesity, but there is tolerance to the anorectic effect of the only approved 5-HT$_{2C}$ agonist, lorcaserin. We tested the hypothesis that different agonists or inverse agonists differentially desensitize or sensitize, respectively, canonical 5-HT$_{2C}$-mediated activation of phospholipase C (PLC) signaling in vitro. Lorcaserin, which displays potency and efficacy equal to 5-HT, desensitized the 5-HT$_{2C}$ receptor significantly more than 5-HT ($p < 0.05$). Agonist chemotypes such as 2-aminotetralins, with similar potency but lower efficacy than 5-HT, produced little 5-HT$_{2C}$ desensitization. The piperazine agonist 1-(3-chlorophenyl)piperazine (mCPP), with lower potency but similar efficacy as 5-HT, elicited desensitization indistinguishable from 5-HT, while the piperazine agonist aripiprazole, with lower potency and efficacy, did not desensitize 5-HT$_{2C}$-PLC signaling. Several 5-HT$_{2C}$ agonists also were assessed for β-arrestin recruitment—lorcaserin was a 'super-agonist', but a 2-aminotetralin and aripiprazole had nil activity, suggesting they are biased towards 5-HT$_{2C}$-PLC signaling. We observed robust positive correlations between the magnitude of 5-HT$_{2C}$ desensitization and agonist efficacy to stimulate PLC or to recruit β-arrestin. In contrast, different inverse agonists caused different magnitudes of 5-HT$_{2C}$ sensitization that did not correlate with efficacy (or potency) to inhibit constitutive 5-HT$_{2C}$-PLC signaling. Assessment of the 5-HT$_{2C}$-S407A point-mutated receptor indicated this residue's involvement in ligand-dependent desensitization, but we did not observe a role for protein kinase C.These data show that ligand structure uniquely impacts 5-HT$_{2C}$ desensitization and sensitization processes.

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Conflict of interest

The authors declare they have no financial conflicts of interest.

Keywords

5-HT_{2C} receptor; β-arrestin; Aminotetralin; Desensitization; phospholipase C; Sensitization

1. Introduction

The serotonin (5-hydroxytryptamine, 5-HT) 5-HT_{2C} G protein-coupled receptor (GPCR) has pharmacotherapeutic relevance in obesity, substance use disorders, and other neuropsychiatric disorders (Morgan et al., 2012, 2013; Canal et al., 2013a, 2013b, 2014; Higgins et al., 2013; Kasper et al., 2013). The 5-HT_{2C} receptor-preferring agonist lorcaserin is approved for obesity and is being evaluated as a treatment for cocaine addiction (Collins et al., 2017). Lorcaserin, however, requires daily administration for obesity (Arena, 2012; O'Neil et al., 2012; Harvey-Lewis et al., 2016), and a significant number of patients taking lorcaserin develop tolerance to its anorectic effects (Smith et al., 2010), limiting its therapeutic value. 5-HT_{2C} receptor desensitization likely is involved in clinical tolerance that develops after prolonged lorcaserin use.

Canonical 5-HT_{2C} receptor signaling involves agonist binding to cell membrane-embedded receptor, with stabilization of receptor conformation(s) that bind intracellular heterotrimeric G protein containing the $G\alpha_q$ subunit. The $G\alpha_q$ subunit activates by dissociating from the heterotrimer, and then stimulates phospholipase C (PLC). PLC catalyzes hydrolysis of phosphatidylinositol 4,5-bisphosphate to form membrane-bound diacylglycerol (, a protein kinase C (PKC) activator) and intracellular inositol 1,4,5-trisphosphate (IP3, that stimulates calcium release from endoplasmic reticulum). IP3 is degraded to IP2 and IP1; all three are collectively referred to as inositol phosphates (IP).

The 5-HT_{2C} receptor shows agonist–dependent desensitization (Stout et al., 2002)—attenuated signaling after prolonged activation—and like other GPCRs, the extent of 5-HT_{2C} receptor desensitization may depend on the structure of the agonist ligand (Gray and Roth, 2001; Van Oekelen et al., 2003). GPCR desensitization can be mediated by serine/threonine kinases, such as PKC (Benovic et al., 1985), and by G protein-coupled receptor kinases (GRKs) (Pitcher et al., 1998) that phosphorylate intracellular residues, endowing the receptor with high affinity for the polar core of β-arrestin. When bound to a GPCR, β-arrestin, creates steric hindrance, blocking (Kelly et al., 2008) that blocks heterotrimeric G proteins from coupling to the receptor, thus occluding activation of Gα proteins.

Specific mechanisms governing desensitization of 5-HT_{2C} canonical signaling have not been reported, however, some details are known regarding desensitization mechanisms of the 5-HT_{2A} receptor that has ~80% transmembrane sequence homology with the 5-HT_{2C} receptor (Julius et al., 1990). For example, PKC-mediated phosphorylation of serine 421 (S421) at the C-terminus of the 5-HT_{2A} receptor impacts the degree of agonist–induced 5-HT_{2A} receptor desensitization and internalization (Kagaya et al., 1990; Rahman and Neuman, 1993; Bhattacharyya et al., 2002; Gray et al., 2003). The homologue of 5-HT_{2A} receptor residue S421 in the 5-HT_{2C} receptor is S407, suggesting PKC may also be involved in desensitization of the 5-HT_{2C} receptor.

The objectives of this study include reporting information on 5-HT_{2C} receptor desensitization mechanisms and ligand chemical structural properties that impact the magnitude of 5-HT_{2C} receptor desensitization. We also investigated 5-HT_{2C} receptor sensitization—enhanced signaling after prolonged receptor inactivation, i.e., by inverse agonists. A long-term goal of this work is to provide information for rational drug design targeting the 5-HT_{2C} receptor to minimize 5-HT_{2C} receptor desensitization and clinical tolerance.

5-HT_{2C} receptor agonists studied (Fig. 1) included indoleamines (5-HT and Ro 60–0175), piperazine derivatives (1-(3-chlorophenyl) piperazine (mCPP), aripiprazole, and WAY–161503), the benzazepine lorcaserin, the phenethylamine (±) 2,5-dimethoxy-4-iodoamphetamine (DOI) and 2-aminotetralins (2*S*,4*R*)-(−)-*trans*-4-phenyl-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine (4-phenyl-2-dimethylaminotetralin, PAT), (2*S*,4*R*)-(−)-*trans*-4-(3’[*meta*]-bromophenyl)-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine (*meta*-bromo-PAT, MBP). 5-HT_{2C} receptor inverse agonists assessed (Fig. 2) included the diazepine clozapine, the ergoline mesulergine, the benzo-dipyrrole SB 206553, and 2-aminotetralins (2*S*,4*R*)-(−)-*trans*-4-cyclohexyl-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalen-2-amine (4-cyclohexyl-2-dimethylaminotetralin, CAT), (2*S*,4*R*)-(−)-*trans*-4-(3’[*meta*]-trifluoromethylphenyl)-*N*,*N*-dimethyl-1,2,3,4-tetrahydronaphthalene-2-amine (*meta*–trifluoro-PAT, MFP) (Sakhuja et al., 2015; Liu et al., 2017). We assessed both canonical $G\alpha_q$ signaling (via production of IP) and β-arrestin recruitment. We also used site-directed mutagenesis and PKC inhibition studies to interrogate the role of phosphorylation regarding 5-HT_{2C} receptor desensitization.

2. Materials and methods

2.1. Compounds

All commercial compounds were ≥ 99% pure according to the manufacturer, unless noted. 5-HT hydrochloride was purchased from Alfa Aesar (Ward Hill, MA). DOI hydrochloride (> 98%), mCPP hydrochloride, Ro 60–0175 fumarate, clozapine, SB 206553 hydrochloride hydrate, and chelerythrine chloride (≥ 95%) were purchased from Sigma Aldrich (St. Louis, MO). WAY-161503 hydrochloride and mesulergine hydrochloride were purchased from Tocris Biosciences (Bristol, U.K.). Lorcaserin hydrochloride (> 98%) was purchased from Chem Scene (Monmouth Junction, NJ). [^{3}H]Myo–inositol (specific activity 22.5 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). The 5-HT_{2C} receptor agonists PAT (Booth et al., 2009) and its *meta*–bromophenyl substituted analog, MBP (Canal et al., 2014), as well as, the 5-HT_{2C} receptor inverse agonists MFP (Liu et al., 2017), and CAT (Sakhuja et al., 2015) were synthesized in our laboratory; free bases were converted to hydrochloride salts (Booth et al., 2009). The novel aminotetralin derivatives were > 99% pure, according to high–resolution mass spectrum, ^{1}H and ^{13}C nuclear magnetic resonance, and high performance liquid chromatography data (Sakhuja et al., 2015).

2.2. Human 5-HT_{2C} receptor S407A construct

The human 5-$HT_{2C–INI}$ (wild-type, WT) complementary deoxyribonucleic acid (cDNA) cloned in the pcDNA3.1 + vector was obtained from Missouri S&T cDNA Resource Center

(Rolla, MO). Point mutation of 5-HT_{2C} receptor residue 407 from serine (WT) to alanine was made by polymerase chain reaction using the QuikChange II Site–Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. Mutagenesis and sequencing primers were obtained from Life Technologies (Carlsbad, CA). The primers used to make the 5-$HT_{2C–INI}$ S407A point mutant were 5'–cgccactgctttggctgggagggagct–3' (sense) and 5'–agctccctcccagccaaagcagtggcg–3' (anti-sense). Polymerase chain reaction was performed as previously described, with optimization (Canal et al., 2011). Parental DNA in the reaction mixture was digested using restriction endonuclease *Dpn*I (from diplococcus pneumoniae) at 37 °C for one hour. Digestion mixture (2 μl) was transformed into XL1–Blue supercompetent cells by heat pulse at 42 °C for 45 s. The transformed reaction was incubated in Super Optimal broth with Catabolite repression medium (Sigma–Aldrich) and then plated onto Luria–Bertani agar plates containing 100 μg/ml ampicillin. A single colony was selected for sequencing, and the point mutation was confirmed using the Sanger sequencing method by Genewiz (South Plainfield, NJ).

2.3. Cell culture and transfections

Chinese hamster ovarian K1 (CHO-K1) cells (ATCC, CCL–61) were used for 5-HT_{2C} receptor–$G\alpha_q$–IP assays. They were grown in a humidified incubator at 37 °C with 5% carbon dioxide in Dulbecco's modified Eagle's medium (10–013–CV, Mediatech, Manassas) with 10% fetal bovine serum and 1% penicillin/streptomycin (SV30079.01, Thermo Scientific, Waltham, MA). Cells from pass 4–20 were used in functional assays (counting pass one as first pass from a stock purchased from ATCC). CHO-K1 cells were grown to 70–80% confluency in 10 cm plates, washed with phosphate buffered saline (PBS), and then transiently transfected for 20 h overnight in an incubator with 15 μg 5-$HT_{2C–INI}$ pcDNA (5-HT_{2C} WT or 5-HT_{2C} S407A) and 30 μl Turbofect reagent (Thermo-Fisher), in 6 ml Dulbecco's modified Eagle's medium containing 5% dialyzed fetal bovine serum and 4 ml Opti-MEM. Initial, pilot results from [^{3}H]mesulergine saturation binding experiments indicated that transiently transfected CHO-K1 cells expressed 5-HT_{2C} receptor at a density of 1.5 ± 0.2 pmol/mg protein.

2.4. Desensitization and sensitization of 5-HT_{2C} receptor canonical signaling

To assess 5-HT_{2C} receptor desensitization, or sensitization, we measured activity at the canonical 5-HT_{2C}–PLC/IP signaling pathway. For the control (not desensitized or sensitized) condition, 24 h post-–transfection, medium was removed from 10 cm plates containing cells at ~ 90% confluency and was replaced with 14 ml of inositol–free Dulbecco's modified Eagle's medium containing 5% dialyzed fetal bovine serum. Cells then were detached via scraping, and 2 μCi/ml [^{3}H] Myo–inositol was added. Cells then were mixed by vortexing, and 270 μl of cell suspension per well was seeded into 48–well CellBind® plates (Corning, Lowell, MA) and placed in an incubator at 37 °C with 5% carbon dioxide. After 2–4 h, cells adhered to the plate. Then, 30 μl of inositol–free Dulbecco's modified Eagle's medium vehicle (basal condition) was added under culture conditions for 20 h. For desensitization and sensitization conditions, the same procedure as above was used except that 20–4 h after 5-HT_{2C}–expressing cells adhered to plates, 30 μl agonist or inverse agonist test ligand at a final concentration of 1 μM was added to cells under culture conditions for 20 h. The concentration of test ligands (except aripiprazole) was

> 25–fold higher than EC/IC_{50} values for 5-HT_{2C}–mediated PLC/IP signaling in the control condition, i.e. treatments were at saturating concentrations, curtailing the possibility of differences in desensitization or sensitization based on divergent ligand potencies. Aripiprazole had very low potency (EC_{50} ~ 1 μM; Table 1) as a 5-HT_{2C} partial agonist and it had low solubility in the desensitization assay, thus, it was used at same concentration (1 μM) as for other agonists. At 1 μM, tested ligands do not have relevant affinity for $G\alpha_q$-coupled receptors endogenously expressed in CHO-K1 cells (i.e., muscarinic, purinogenic, thrombin, and calcitonin GPCRs), minimizing the possibility of off target receptor cross-talk in these studies.

To assess the role of PKC in ligand-induced 5-HT_{2C} receptor desensitization, chelerythrine, a selective inhibitor of PKC (K_i = 700 nM), was included at 10 μM (i.e., > 10-times *K*i to ensure interaction with PKC over the course of the assay) (Herbert et al., 1990) (Absolinova et al., 2010).

After 20 h, medium was removed, and then cells were washed with PBS three times for 5 min each. For agonist–induced desensitization, the same agonist (1–10,000 nM in inositol–free Dulbecco's modified Eagle's medium containing 100 mM lithium chloride (LiCl) and 10 μM pargyline) was added to cells to re–stimulate the 5-HT_{2C} receptor; for inverse agonist sensitization, 5-HT (1–10,000 nM in inositol–free Dulbecco's modified Eagle's medium containing 100 mM (LiCl) and 10 μM pargyline) was added to re–stimulate the receptor. Medium was discarded 45 min later, and 400 μl of 50 mM formic acid was added to each well to stop reactions. The acid was neutralized one hour later by addition of 200 μl 150 mM ammonium hydroxide, and the culture plates were frozen at −40 °C overnight. After thawing and centrifugation at 1000*g* for 5 min, supernatant from the cell mixture was added to individual BioRad anion–exchange columns containing AG 1–X8 formate–form resin. After washing each loaded column with 10 ml deionized water, [^{3}H]IP were eluted with 4 ml of 800 mM ammonium formate into vials. An aliquot of 1 ml was mixed vigorously with 10 ml scintillation cocktail (ScintiVerse Cocktail, Fisher), and [^{3}H] scintillations were counted on a PerkinElmer Tri–Carb 2190TR liquid scintillation counter. The data presented are from three or four independent experiments that included quadruplicate samples for each data point.

2.5. 5-HT_{2C} receptor β-arrestin recruitment

The DiscoverX PathHunter β-arrestin kit, containing human osteosarcoma U2OS cells stably expressing the 5-$HT_{2C\text{-}INI}$ receptor, was utilized to quantify agonist–induced β-arrestin recruitment to the 5-HT_{2C} receptor per the manufacturer's protocol with no deviations (DiscoverX, Fremont, CA). Cells were plated at a density of 10,000 cells/well into a 96–well plate using cell plating 19 media (DiscoverX), and treated with agonist for 2 h, followed by a 1 h enzyme complementation incubation at room temperature. Luminescence readings were obtained using a Synergy H1 reader (Biotek, Winooski, VT). The data presented are from three independent experiments that included quadruplicate samples for each data point, except for 5-HT positive control, which included two samples for each data point.

DiscoverRX does not provide information on the expression level of 5-HT_{2C} receptor in the assay kit, however, Dr. John Allen (University of Texas Medical Branch–Galveston) kindly

reported to us the cells in the DiscoverRX kit express 5-$HT_{2C\text{-}INI}$ at about 1.2 pmol/mg protein, measured by [^{3}H]mesulergine saturation binding (n = 3). This receptor binding site density closely matched what we observed following transient transfections of CHO-K1 cells.

2.6. Data transformations and statistical analyses

Functional pharmacology data were analyzed using nonlinear regression curve–fitting algorithms in GraphPad Prism, 6.0 for Mac (San Diego, CA), and were fitted using the "log(agonist) vs. response (three parameters)" model. The degree of desensitization or sensitization was defined by the following equation:

$$100 - \frac{\left(DesensitizedE_{max}\right) * 100}{\left(ControlE_{max}\right)} = \%\quad desensitization$$

Calculations of one–way analysis of variance (ANOVA) with Tukey's multiple–comparison *post hoc* tests were performed to compare the effects of ligands on 5-HT_{2C} receptor desensitization or sensitization. Calculations of two–way ANOVA with Sidak's multiple–comparison post hoc test were performed to compare the differential effects of ligand–induced desensitization between WT and S407A 5-HT_{2C} receptor and the effects of PKC inhibition. Pearson correlation coefficients were calculated to determine the relationship between second messenger signaling outputs (IP signaling E_{max}, β-arrestin recruitment E_{max}, IP signaling pEC_{50}, β-arrestin recruitment pEC_{50}) and the degree of receptor desensitization and sensitization. Ligand efficacies and potencies are presented as mean values (± standard error of the mean [S.E.M]), with the adjusted *P* values from aforementioned statistical analyses. Unless otherwise stipulated, *P* values are noted with asterisks in figures and are defined as *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$; and ****, $P < 0.0001$. All asterisks in figures represent differences from vehicle group unless otherwise indicated.

3. Results

3.1. 5-HT_{2C} receptor agonist–dependent activation and β-arrestin recruitment

3.1.1. 5-HT_{2C} receptor agonist effect on IP production after 20 h incubation of cells with vehicle only—The potencies (pEC_{50}) and efficacies (maximal efficacy [E_{max}]) of agonist ligands at the 5-HT_{2C} receptor IP signaling pathway following 20 h of incubation of cells with vehicle only are shown in Table 1. Post–hoc tests revealed that mCPP and aripiprazole were less potent than 5-HT, DOI, Ro 60–0175, WAY–161503, and lorcaserin ($P < 0.05$). The pEC_{50} values of the other 5-HT_{2C} agonists in Table 1 were not statistically different from each other. Regarding efficacy, Ro 60–0175, WAY–161503, lorcaserin, mCPP, and DOI were full agonists, while MBP, PAT, and aripiprazole were partial agonists ($P < 0.05$), relative to 5-HT (Table 1).

3.1.2. 5-HT_{2C} receptor–β-arrestin recruitment—The pEC_{50} and E_{max} values of 5-HT, Ro 60–0175, lorcaserin, MBP, and aripiprazole to recruit β-arrestin to the 5-HT_{2C}

receptor are shown in Table 2. Lorcaserin showed increased E_{max} relative to all other agonists tested ($P < 0.05$). Both MBP and aripiprazole had negligible effects on 5-HT_{2C} receptor β-arrestin recruitment. The effect of Ro 60–0175 was not different from the endogenous ligand, 5-HT.

3.1.3. Agonist-dependent desensitization of 5-HT_{2C} receptor IP production—

Pretreatment of 5-HT_{2C}-expressing CHO-K1 cells for 20 h with 1 μM of test agonists (5-HT, Ro 60–0175, WAY–161503, lorcaserin, mCPP, DOI, MBP, PAT, and aripirazole) did not alter the pEC_{50} to stimulate IP production, ($P < 0.05$), data not shown. In contrast, with the exception of aripiprazole, pretreatment with test agonists resulted in desensitization as measured by a significant reduction in agonist ligand efficacy (E_{max}) ($F_{9,36,} = 63.5$; $P < 0.0001$) (Fig. 3). The 5-HT_{2C} full agonist lorcaserin produced the largest degree of desensitization, with an 85 ± 2.6% reduction in E_{max} after desensitization compared to control conditions. 5-HT preincubation resulted in a 63 ± 3.2% reduction in subsequent E_{max}. The other 5-HT_{2C} full agonists tested (Ro 60–0175, DOI, mCPP, and WAY–161503) desensitized 5-HT_{2C}-mediated IP signaling with a magnitude similar to 5-HT, as evidenced by reductions in E_{max} values from the desensitized condition that were 59 ± 5.4%, 73 ± 4.1%, 69 ± 1.8%, and 79 ± 0.5% (respectively) of the corresponding E_{max} values obtained from control conditions. Preincubation with the partial agonists PAT and MBP resulted in 29 ± 5.0% and 38 ± 4.9% reductions in subsequent E_{max} values, respectively. The rank order for agonist–induced desensitization of 5-HT_{2C} receptor IP signaling was: lorcaserin > WAY-161503 > (±)–DOI > mCPP > 5-HT > Ro 60–0175 > MBP > PAT > aripiprazole.

Pearson correlation analyses assessed the relationship between desensitization and ligand pharmacology at 5-HT_{2C} receptor IP and β-arrestin signaling pathways (Fig. 4). As shown in Fig. 4A and B, there were significant positive correlations between ligand E_{max} values for stimulating 5-HT_{2C} receptor IP (Fig. 4A) or β-arrestin (Fig. 4B) signaling and desensitization of IP signaling ($r = 0.91$, $P < 0.01$ and $r = 0.88$, $P < 0.05$, respectively). In contrast, ligand potency (pEC_{50}) at either pathway did not correlate with desensitization (Fig. 4C and D).

3.2. Inverse agonist–dependent inhibition and sensitization of 5-HT_{2C} receptor IP production

The potency and efficacy of the 5-HT_{2C} receptor inverse agonists following 20 h of vehicle administration are shown in Table 3. Clozapine was the most efficacious, and SB 206553 was the most potent, 5-HT_{2C} inverse agonist, respectively, at reducing basal 5-HT_{2C}-mediated basal IP production. To assess 5-HT_{2C} receptor sensitization, CHO-K1 cells expressing the 5-HT_{2C} receptor were incubated with 1 μM inverse agonists for 20 h. After washing the cells, the efficacy of 5-HT to stimulate 5-HT_{2C}–mediated IP production was assessed. Sensitization was defined as an elevated E_{max} value of 5-HT in the sensitization condition compared to cells without pretreatment of inverse agonists. As shown in Fig. 5, each of the 5-HT_{2C} inverse agonists caused sensitization of the 5-HT_{2C} receptor ($F_{5, 25} = 27.84$; $P < 0.0001$). The efficacy of 5-HT was increased by 69 ± 6.3%, 41 ± 4.7%, 29 ± 3.2%, 27 ± 9.1%, 26 ± 4.0% in cells pretreated with the inverse agonists SB 206553, CAT, clozapine, mesulergine, and MFP, respectively.

Pearson correlation analyses assessed the relationship between sensitization and inverse agonist pharmacology at inhibiting basal 5-HT_{2C}–induced IP production (Fig. 6). As shown in Fig. 6A and B, there were no significant positive correlations between inverse agonist efficacy or potency values for inhibiting 5-HT_{2C} IP production sensitization of IP signaling (r = −0.06 and r = 0.38, respectively).

3.3. Agonist–dependent desensitization of S407A 5-HT_{2C} receptor IP production

The 5-HT_{2C} receptor serine residue S407, at the distal end of the intracellular C terminus, is in a position homologous to 5-HT_{2A} receptor residue S421 that is involved in agonist–induced desensitization, putatively, via a phosphorylation event (Gray et al., 2003). To test for a functional role of S407 in 5-HT_{2C}–IP desensitization, residue S407 was mutated to alanine (S407A), precluding its phosphorylation. 5-HT_{2C}–IP functional assays then were performed, as above. Agonist ligands assessed were 5-HT, mCPP, RO 60–0175, DOI, lorcaserin, and MBP. The potency (but not efficacy—see below) of only mCPP at the S407A 5-HT_{2C} receptor was significantly reduced compared to WT 5-HT_{2C} receptor; pEC_{50} = 8.15 ± 0.11 and 7.41 ± 0.21 M, respectively ($P < 0.05$).

Similar to results for the WT 5-HT_{2C} receptor, all agonists caused desensitization (lowered E_{max}) of S407A 5-HT_{2C}–IP signaling, however, desensitization caused by 5-HT, DOI, and lorcaserin was blunted at S407A relative to the WT receptor (Fig. 7). The E_{max} of 5-HT, DOI, and lorcaserin at S407A was reduced by 38 ± 4.1%, 38 ± 6.0%, and 54 ± 5.2%, respectively, in the desensitization conditions, magnitudes significantly lower compared to desensitization observed at WT 5-HT_{2C} ($P < 0.001$). The E_{max} for Ro 60–0175, mCPP, and MBP at S407A was reduced by 71 ± 3.8%, 55 ± 7.0%, and 36 ± 8.0%, respectively, in the desensitization conditions—magnitudes not different from desensitization observed at WT 5-HT_{2C}.

3.4. Effect of PKC inhibition on 5-HT-induced 5-HT_{2C} receptor desensitization

PKC is a downstream effector of the 5-HT2C receptor. We hypothesized that PKC phosphorylates activated 5-HT_{2C} receptors contributing to desensitization via a negative feedback mechanism. To assess this hypothesis, 5-HT_{2C} receptor–expressing cells were treated with 10 μM chelerythrine, a selective, cell–permeable PKC inhibitor, 10 min prior to performing the receptor desensitization protocol. In the absence of chelerythrine, 5-HT-induced desensitization resulted in the reduction of 5-HT efficacy to stimulate IP production by 45 ± 3.0% compared to 5-HT_{2C} receptor–expressing cells that were not pretreated with 5-HT. When cells were treated with 10 μM chelerythrine (which has no effect on 5-HT-induced IP production in non-desensitized cells, data not shown), 10 min prior to 5-HT-induced desensitization, the efficacy of 5-HT at desensitized receptors was reduced by 52 ± 4.8%, an effect not statistically different than control conditions (n = 3) (Fig. 8).

4. Discussion

We assessed several structural classes of 5-HT_{2C} receptor ligands and observed that desensitization and sensitization of 5-HT_{2C} receptor canonical signaling is dependent on ligand pharmacology and structure. For example, ligands with partial agonism, including,

the arylpiperazine aripiprazole and the phenylaminotetralins PAT and MBP cause nil or low desensitization, while full agonists such as indole 5-HT and benzazepine lorcaserin cause substantially more 5-HT_{2C} receptor desensitization. We observed a significant positive correlation between agonist *efficacy* and desensitization magnitude at both canonical 5-HT_{2C}–IP signaling as well as 5-HT_{2C}–mediated β-arrestin signaling pathways, suggesting agonist efficacy impacts 5-HT_{2C} receptor desensitization. In contrast, agonist *potency* did not correlate with desensitization. DOI and MBP, for example, showed similar potencies as 5-HT_{2C} agonists, but DOI caused more robust desensitization than MBP. Regarding 5-HT_{2C} receptor sensitization, neither inverse agonist efficacy nor potency correlated with the magnitude of sensitization.

The magnitude of 5-HT_{2C} receptor signaling desensitization was different for agonists of different structural classes. Desensitization was greatest after prolonged stimulation with the benzazepine lorcaserin. Surprisingly, lorcaserin desensitized the 5-HT_{2C} receptor more robustly than the endogenous indole 5-HT, despite each having similar maximal efficacies (E_{max}) and potencies (pEC_{50}) at the 5-HT_{2C} IP signaling pathway. Lorcaserin also desensitized the 5-HT_{2C} receptor more than the indole Ro 60–0175, the phenethylamine DOI, the aminotetralins MBP and PAT, the piperazines mCPP, WAY-161503, and the arylpiperazine aripiprazole, as shown in Fig. 9.

Lorcaserin was a “super agonist” at recruiting β-arrestin to the 5-HT_{2C} receptor, with an efficacy significantly greater than the 5-HT_{2C} full agonists 5-HT and Ro 60–0175. The extremely high efficacy of lorcaserin to recruit β-arrestin may account for it’s remarkable desensitization of 5-HT_{2C} IP signaling that is more robust than any other agonist tested (Roth et al., 2017; Wacker et al., 2017; Rajagopal and Shenoy, 2018). In contrast, MBP and aripiprazole showed very low to nil efficacy to recruit β–arrestin, and they produced low to nil 5-HT_{2C} receptor desensitization effects. These data suggest MBP and aripiprazole are biased toward PLC/IP signaling, or that a certain degree of canonical $G\alpha_q$ activation is required to recruit β-arrestin to 5-HT_{2C}, which subsequently causes receptor desensitization. MBP and aripiprazole are partial agonists of the 5-HT_{2C}–$G\alpha_q$–IP pathway, and a recent report concludes that G protein activation is required for β-arrestin recruitment (Grundmann et al., 2018). MBP- and aripiprazole-mediated partial activation of 5-HT_{2C} and $G\alpha_q$ may be insufficient to recruit β-arrestin, therefore preventing 5-HT_{2C}–$G\alpha_q$ desensitization.

Inverse agonist ligand structure also appears to impact 5-HT_{2C} receptor sensitization. Preincubation of the 5-HT_{2C} receptor with the benzo-dipyrrole inverse agonist SB 206553 sensitized the 5-HT_{2C} receptor to activation by 5-HT (i.e., increased E_{max} of 5-HT) to an extent that was larger than all other inverse agonists, including the aminotetralins MFP and CAT, the ergoline mesulergine, and the benzazepine clozapine. Interestingly, SB 206553 and MFP have about equal 5-HT_{2C} receptor inverse agonist potency and efficacy, however, SB 206553 sensitized the 5-HT_{2C} receptor to a greater extent than MFP.

The 5-HT_{2C} receptor sensitization we observed after 20 h of inverse agonist exposure likely is due to an increase of functional receptor at the cell surface, which has been shown in 5-HT_{2C} expressing cells treated for 18 h with 1 μM SB 206553 (Chanrion et al., 2008). During optimization, we observed that pretreatment with SB 206553 for 8 h or less does not

sensitize the 5-HT_{2C} receptor (data not shown). Similarly, others have shown that a 30 min SB 206553 pretreatment is insufficient to sensitize 5-HT_{2C} receptors (Schlag et al., 2004). These data suggest that new mRNA translation, post-translational modifications, trafficking and insertion into the membrane may underlie 5-HT_{2C} sensitization after inverse agonist treatment.

Like other GPCRs, ligand-induced 5-HT_{2C} receptor desensitization and sensitization is likely a consequence of unique interactions between ligand and receptor that impact G-protein coupling, receptor phosphorylation, and β-arrestin binding (Liggett, 2011; Nobles et al., 2011; Rajagopal and Shenoy, 2018). We observed that the structure of the agonist ligand also impacts involvement of 5-HT_{2C} receptor residue S407 (homologue of 5-HT_{2A} receptor S421) in the desensitization process. For example, desensitization of the S407A point-mutated 5-HT_{2C} receptor by lorcaserin, 5-HT, and DOI was less than for the WT 5-HT_{2C} receptor. Meanwhile, desensitization by MBP, mCPP, and Ro 60–0175 was the about the same for the WT and S407A 5-HT_{2C} receptor. These results suggest that structurally-different agonists may stabilize different active receptor conformations, altering spatial orientation of 5-HT_{2C} receptor serine residues and perhaps impacting access of serine/threonine kinases.

Molecular modeling and mutagenesis studies reported in the literature provide support that structurally-different agonists interact with the same GPCR differently and result in different pharmacological potency and efficacy. For example, 5-HT, DOI, lorcaserin, mCPP, Ro 60–0175 and WAY-161503 (all shown here to be high efficacy agonists at the canonical 5-HT_{2C} receptor signaling pathway) contain one or more chemical moieties capable of forming hydrogen or halogen bonding interactions. The 5-OH moiety of 5-HT is proposed to hydrogen bond with 5-HT_{2C} receptor residues D3.32, S3.36, and Y7.43 (Bray and Goddard, 2008; Canal et al., 2011; Liu et al., 2017), putatively, stabilizing a 5-HT_{2C} receptor conformation that leads to robust activation. Experimental results supporting this proposal include that tryptamine (same structure as 5-HT except without the 5-OH moiety) is a 5-HT_{2C} agonist with reduced affinity and lower functional potency and efficacy than serotonin (Porter et al., 1999; Canal et al., 2011). Analogously, the aminotetralins PAT and MBP lack a hydrogen bond donor in a position that is analogous to the 5-OH moiety of 5-HT, perhaps, explaining why these compounds are partial agonists at the 5-HT_{2C} receptor (Table 1). Thus, it appears that agonist chemical structure impacts efficacy to, in turn, impact 5-HT_{2C} receptor desensitization.

Our data are consistent with previous observations that PKC is not involved in short-term (< 1 h) 5-HT-induced desensitization of the 5-HT_{2C} receptor (Berg et al., 2001). It was previously shown that PKC plays a significant role only in long-term (> 24 h of agonist pretreatment) 5-HT_{2C} receptor desensitization processes, whereas, GRK, may mediate short-term 5-HT_{2C} receptor desensitization (< 10 min). In contrast, for the 5-HT_{2A} receptor, there is a significant role for PKC in short–term 5-HT-induced desensitization (Anji et al., 2001; Bhattacharyya et al., 2002), indicating unique signaling regulatory mechanisms for the highly homologous 5-HT_{2A} and 5-HT_{2C} receptors.

5. Conclusions

In summary, our studies suggest that ligand chemical structure impacts 5-HT_{2C} receptor desensitization and sensitization. There is a robust, positive correlation between the efficacy of 5-HT_{2C} receptor agonist–elicited IP production and β-arrestin recruitment and the magnitude of 5-HT_{2C} receptor desensitization. Accordingly, agonist structure, efficacy to activate G proteins, and efficacy to recruit β-arrestin should be considered when designing 5-HT_{2C} receptor agonists to possess low desensitization liability, i.e. to avoid tolerance to clinical effects. Conversely, drug design involving 5-HT_{2C} receptor inverse agonism should take into account the phenomenon of 5-HT_{2C} receptor sensitization that can result in elevated endogenous 5-HT_{2C} receptor signaling after discontinuation of the inverse agonist treatment. Results here indicated that the magnitude of 5-HT_{2C} receptor signaling desensitization or resensitization was different for agonists or inverse agonists of different structural classes, suggesting, structure—activity relationships to inform drug design targeting the 5-HT_{2C} (and closely-related) GPCRs could be unique for each medicinal chemical scaffold.

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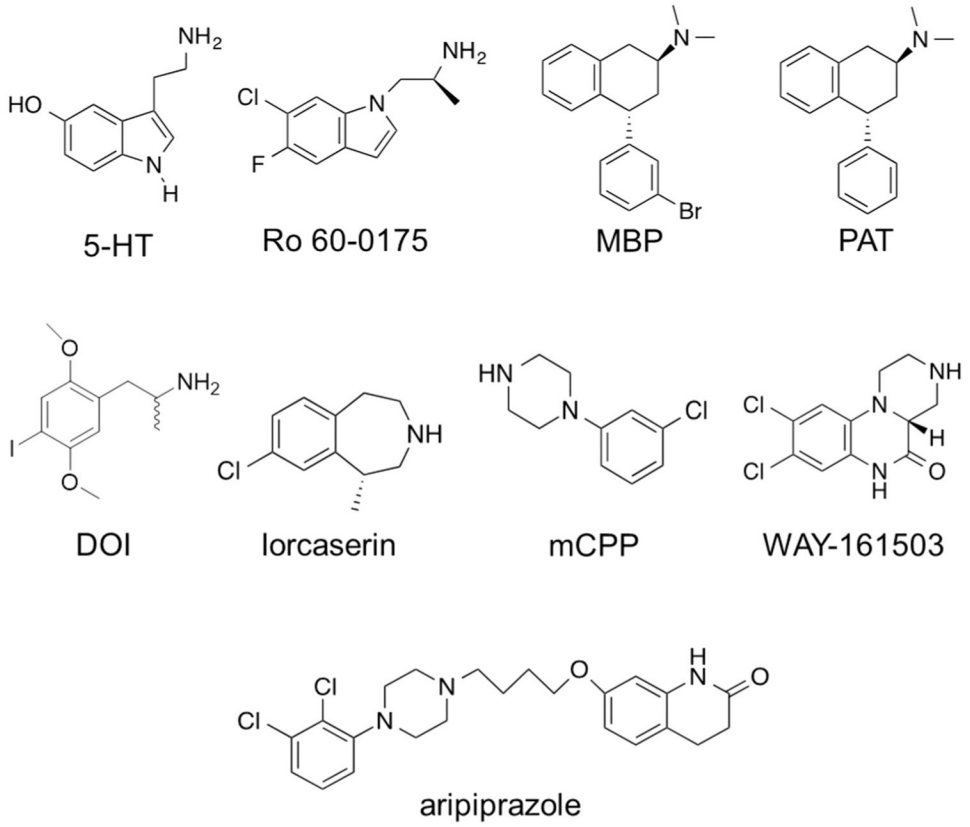

Fig. 1.
5-HT_{2C} receptor agonists tested for desensitization.

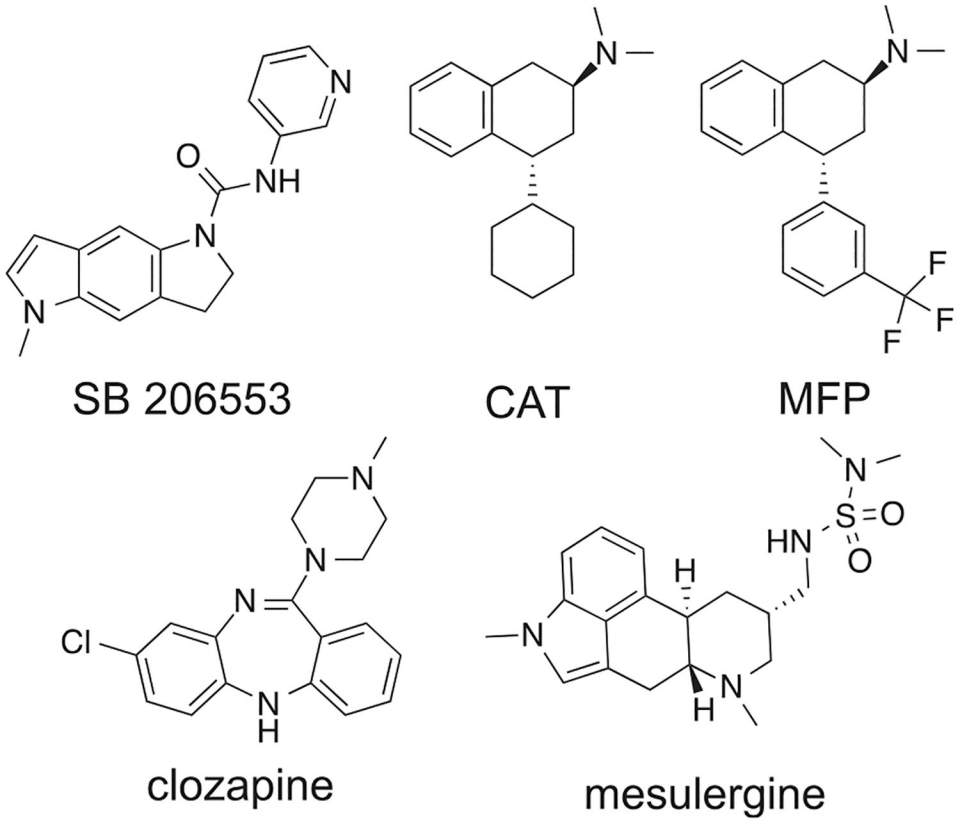

Fig. 2.
5-HT_{2C} receptor inverse agonists tested for sensitization.

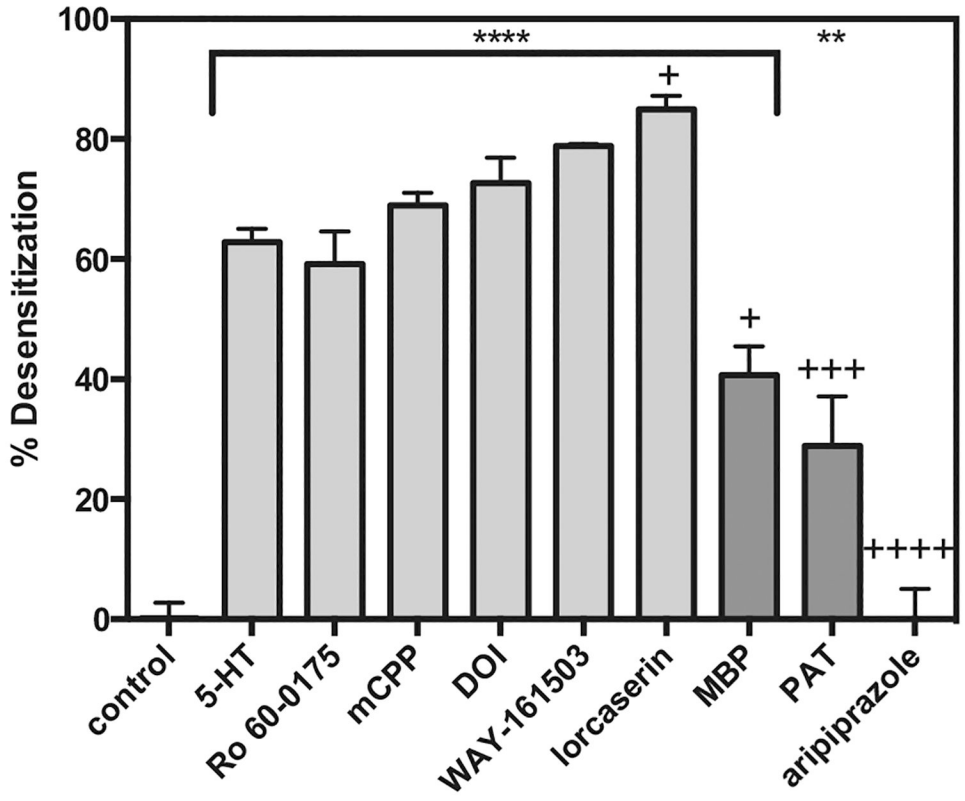

Fig. 3. Agonist–induced desensitization of the 5-HT$_{2C}$ receptor IP pathway.

Cells expressing the 5-HT$_{2C}$ receptor were treated with 1 μM of agonist ligand for 20 h, washed, then re–stimulated with the same agonist (1–10,000 nM). The E_{max} for each agonist at the desensitized 5-HT$_{2C}$ receptor was compared to the E_{max} of the agonist at the non–desensitized 5-HT$_{2C}$ receptor (control condition), and reported as percent desensitization. n = 3 or 4; ** = $P < 0.01$ compared to control; **** = $P < 0.0001$ compared to control; $^{+}$ = $P < 0.05$ compared to 5-HT–induced desensitization; $^{+++}$ = $P < 0.001$ compared to 5-HT–induced desensitization; $^{++++}$ = $P < 0.0001$ compared to 5-HT–induced desensitization, via one-way ANOVA.

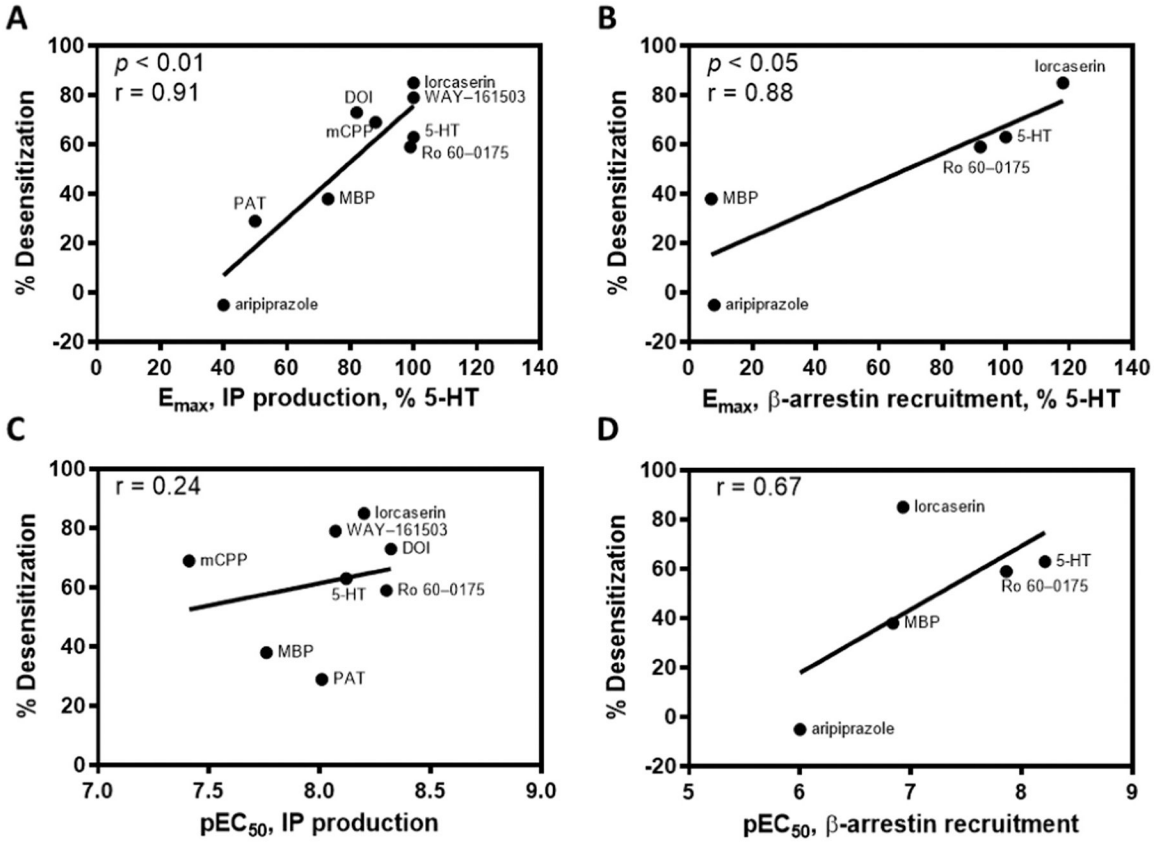

Fig. 4.

5-HT_{2C} receptor agonist efficacy—but not potency—at either IP or β-arrestin pathways significantly correlates with 5-HT_{2C} agonist-induced receptor desensitization of the IP pathway. A. IP production E_{max}; B. β-arrestin recruitment E_{max}; C. IP production pEC_{50}; D. β-arrestin recruitment pEC_{50}.

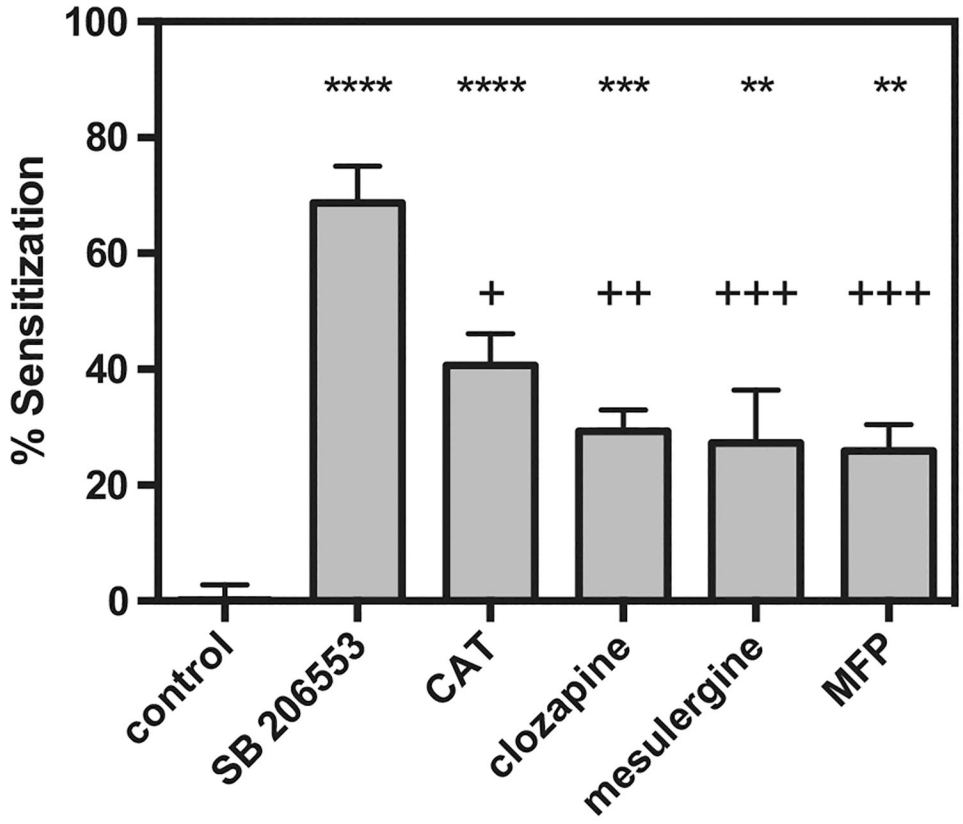

Fig. 5. Inverse agonist sensitization of the 5-HT$_{2C}$ receptor.

Cells expressing the 5-HT$_{2C}$ receptor were treated with 1 μM inverse agonist for 20 h, washed, then re–stimulated with 5-HT (1–10,000 nM). n = 3; ** = $P < 0.01$ compared to control; *** = $P < 0.001$ compared to control; ****= $P < 0.0001$ compared to control; $^{+}$ = $P < 0.05$ compared to SB 206553–induced sensitization; $^{++}$ = $P < 0.01$ compared to SB 206553–induced sensitization; $^{+++}$ = $P < 0.001$ compared to SB 206553–induced sensitization, via one-way ANOVA.

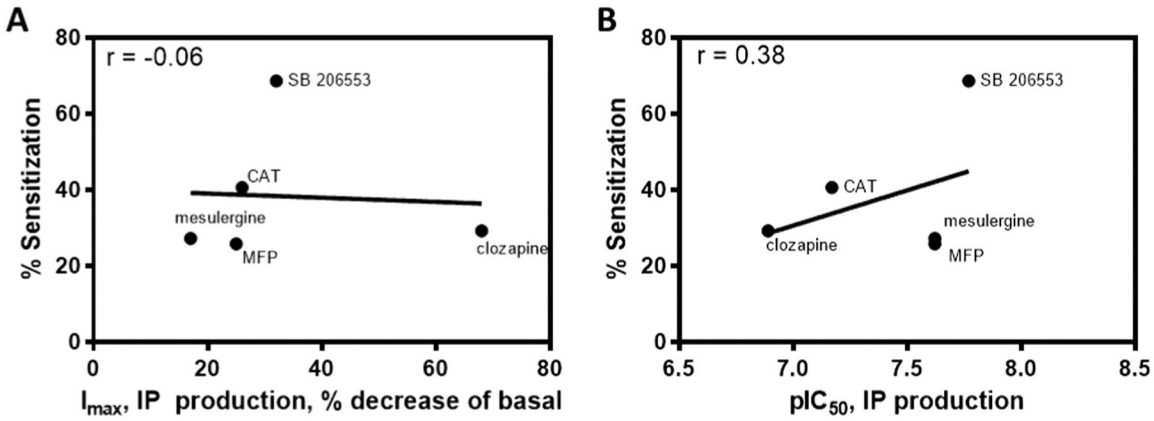

Fig. 6.

Neither 5-HT_{2C} receptor inverse agonist efficacy nor potency at the IP pathway correlates with 5-HT_{2C} receptor sensitization of the IP pathway. A. IP reduction, I_{max} (decrease from basal); B. IP production, pIC_{50}.

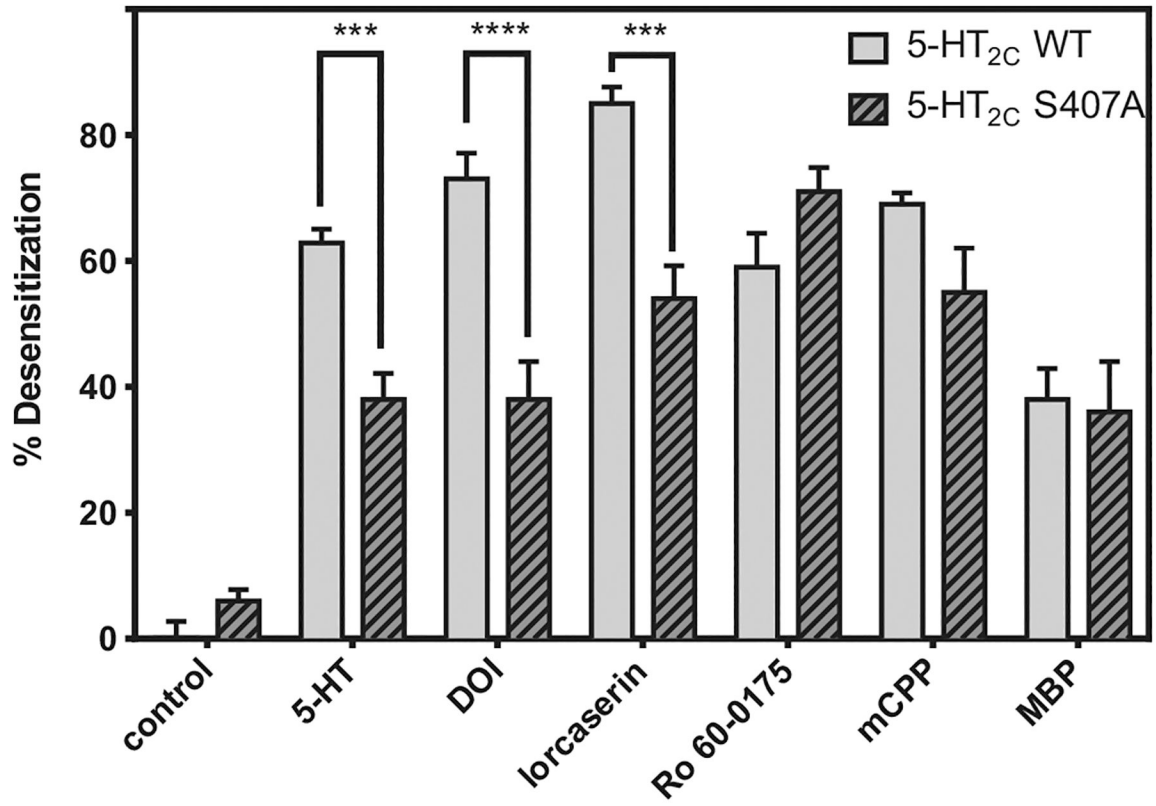

Fig. 7. Agonist–induced desensitization of the S407A 5-HT$_{2C}$ receptor.

Cells expressing the WT 5-HT$_{2C}$ (solid bars) or S407A 5-HT$_{2C}$ (striped bars) receptor were treated with 1 μM of agonist ligand for 20 h, washed, then re–stimulated with the same agonist (1–10,000 nM). The E_{max} for each agonist at the desensitized 5-HT$_{2C}$ receptor was compared to the E_{max} of the agonist at the non–desensitized 5-HT$_{2C}$ receptor (control condition), and reported as percent desensitization. n = 3 or 4; *** = $P < 0.001$ compared to 5-HT$_{2C}$ WT desensitization, **** = $P < 0.0001$ compared to 5-HT$_{2C}$ WT desensitization, via two-way ANOVA.

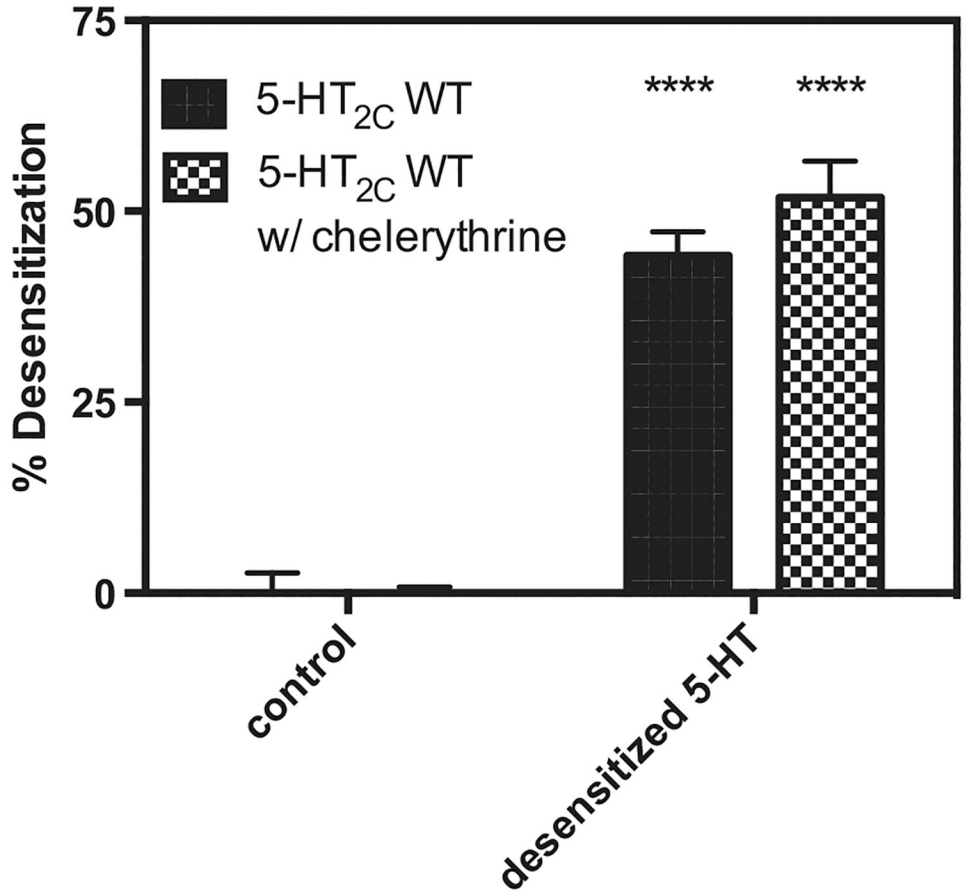

Fig. 8. The PKC inhibitor chelerythrine did not affect 5-HT–induced 5-HT$_{2C}$ receptor desensitization.

Cells expressing the 5-HT$_{2C}$ receptor were treated with 10 μM of 5-HT and vehicle (solid bars) or 10 μM chelerythrine (checkered bars) for 1 h, washed, then re–stimulated with 5-HT (1–10,000 nM). n = 3; **** = $P < 0.0001$ different than 5-HT$_{2C}$ WT control, via two-way ANOVA.

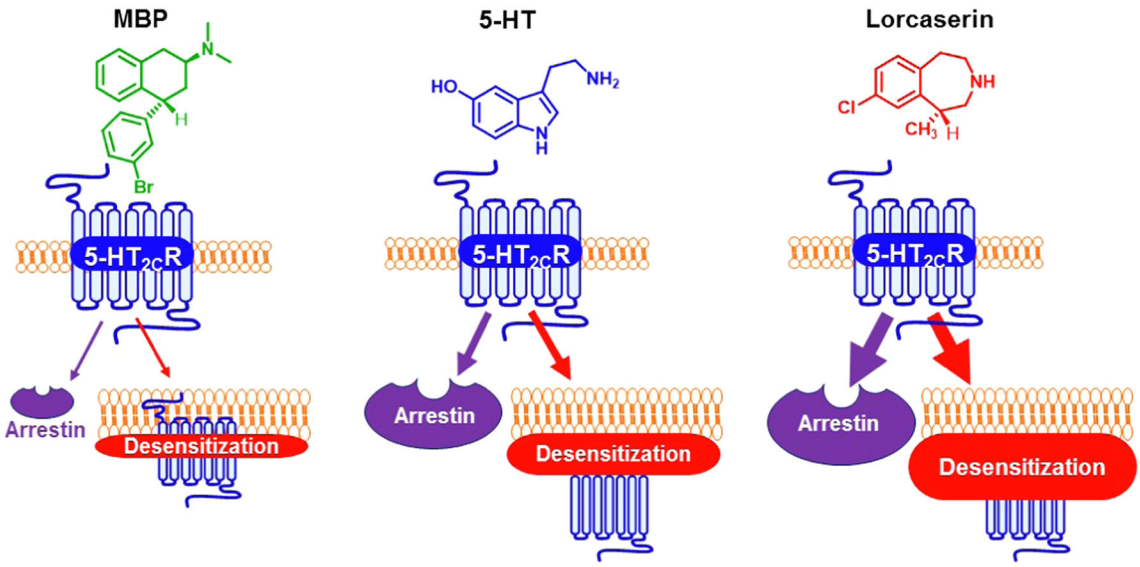

Fig. 9.

Schematic representation of 5-HT_{2C} receptor desensitization by MBP, 5-HT, and Lorcaserin.

Table 1

5-HT_{2C} agonists pEC_{50} and E_{max} values, relative to 5-HT: Inositol phosphate production.

Agonist	pEC_{50}	E_{max} (% 5-HT)
5-HT	8.12 ± 0.10	100 ± 6
WAY-161503	8.07 ± 0.08	100 ± 2
lorcaserin	8.20 ± 0.15	100 ± 1
Ro 60–0175	8.30 ± 0.05	99 ± 6
mCPP	7.41 ± 0.21[a]	88 ± 10
DOI	8.32 ± 0.10	82 ± 4
MBP	7.76 ± 0.04	73 ± 4[a]
PAT	8.01 ± 0.14	50 ± 1[a]
aripiprazole	6.28 ± 0.30[a]	40 ± 4[a]

n = 3 or 4.

[a] = $P < 0.05$ different from 5-HT via one-way ANOVA.

Table 2

A5-HT_{2C} agonists pEC_{50} and E_{max} values, relative to 5-HT: β-arrestin recruitment.

Agonist	pEC_{50}	E_{max} (% 5-HT)
lorcaserin	6.93 ± 0.04[a]	118 ± 5[a]
5-HT	8.21 ± 0.08	100 ± 6
Ro 60–0175	7.86 ± 0.11	92 ± 4
MBP	6.84 ± 0.10[a]	7 ± 4[a]
aripiprazole	< 6[a]	8 ± 1[a]

n = 3.

[a] = $P < 0.05$ different from 5-HT via one-way ANOVA.

Table 3

5-HT_{2C} inverse agonists pIC_{50} and I_{max} values: Inositol phosphate reduction.

Inverse Agonist	pIC_{50}	I_{max} (% decrease from basal)
clozapine	6.89 ± 0.10	68 ± 4
SB 206553	7.77 ± 0.02	32 ± 5
CAT	7.17 ± 0.21	26 ± 4
MFP	7.62 ± 0.10	25 ± 3
mesulergine	7.62 ± 0.13	17 ± 2

n = 3.