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A unique phenotype of 5-HT_{2C}, agonist-induced GTPγ^{35}S binding, transferable to 5-HT_{2A} and 5-HT_{2B}, upon swapping intracellular regions

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1 The human 5-HT_{2C} receptor, when expressed heterologously in various mammalian cell lines (HEK293, SH-EP and NIH-3T3) at various receptor densities (6 to 45 pmol mg^{-1} protein), mediates robust agonist-induced GTPγ^{35}S binding from coupling to G_i subtypes of G proteins, in addition to $G_{q/11}$. Such a phenotype, however, was not seen with the human 5-HT_{2A} and 5-HT_{2B} receptors, indicating their common pathway with 5-HT_{2C} limited to $G_{q/11}$, not including G_i.

2 Because intracellular regions are largely responsible for signalling pathways, we prepared the chimeras of the 5-HT_{2A} and 5-HT_{2B} receptors where the second and third intracellular loops, and the C-terminal region were replaced with the 5-HT_{2C} counterparts.

3 The chimeras showed robust agonist-induced GTPγ^{35}S binding. Relative intrinsic efficacies of agonists from the GTPγ^{35}S binding were nearly identical to the reported values for their parent receptors as measured with Ca^{2+} or [^{3}H]-inositol phosphate accumulation. Also the chimeras displayed the same ligand-binding properties as the parent receptors.

4 We conclude that the phenotype of agonist-induced GTPγ^{35}S binding is unique to 5-HT_{2C} among the 5-HT_2 receptor family, and is transferable to 5-HT_{2A} and 5-HT_{2B}, upon swapping intracellular sequences, without altering their receptor pharmacology.

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Abbreviations: EC_{50}, a half-maximal concentration; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IP, inositol phosphate; NEM, N-ethylmaleimide; K_D, dissociation constant; K_i, inhibition constant; PCR, polymerase chain reaction

Introduction

The family of 5-HT_2 receptors, G protein-coupled receptors with seven transmembrane segments, consists of three subtypes, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. The receptor subtypes are differentially distributed in the brain, with the primary localization of 5-HT_{2A} in cerebral cortex (caudate) (Pazos *et al*., 1987), that of 5-HT_{2B} in hippocampus and midbrain (Flanigan *et al*., 1995), and that of 5-HT_{2C} in choroid plexus (Conn *et al*., 1979). The receptors have been implicated in modulation of feeding behaviour, mood, perception and aggression (Pandey *et al*., 1995; Saxena, 1995; Roth *et al*., 1998). Their ligands, including atypical antipsychotic and hallucinogenic drugs, have been targeted for treatments of anxiety, depression, schizophrenia and obesity (Sanders-Bush & Breeding, 1991; Sandau & Hen, 1994; Dourish, 1995; Tecott *et al*., 1995; Kennett *et al*., 1996; Newton *et al*., 1996). For signal transduction, 5-HT_2 receptor subtypes, when heterologously expressed in mammalian cells (HEK293, CHO, NIH-3T3 and SH-SY5Y cells), have been proposed to share the same signalling pathway, the $G_{q/11}$/phospholipase Cβ/inositol 1,4,5-triphosphate/Ca^{2+} signal (Sanders-Bush & Breeding, 1991; Hartman & Northup, 1996). Recently, we found that the human 5-HT_{2C} receptor, when expressed in HEK293 cells, mediated robust agonist-induced GTPγ^{35}S binding from its additional coupling to G_i subtypes, but not from its coupling to $G_{q/11}$ subtypes due to their slow GTP turnover rates (Alberts *et al*., 1999). The same phenotype was also observed here with the 5-HT_{2C} receptor expressed in different cell lines (human epithelial SH-EP and NIH-3T3) at variable receptor densities, but was not seen with the 5-HT_{2A} and 5-HT_{2B} receptors. We will report here that the 5-HT_{2A} and 5-HT_{2B} chimeras with the second and third intracellular loops and the C-terminus of 5-HT_{2C} display agonist-induced GTPγ^{35}S binding with no detectable change in their receptor pharmacology.

Methods

cDNAs for the human 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2c} receptor were cloned into the PCRscript vector *via* blunt end ligation. The directionality of inserts was determined with polymerase chain reaction (PCR) using primers annealing to the vector and inserts. The 5-HT_{2A} chimera containing the 5-HT_{2C} intracellular regions was prepared using the procedure of gene splicing by overlap extension (Horton *et al*., 1989).

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Briefly, the second intracellular loop (from D172 to I197), the third intracellular loop (L256 to V324) and the C terminal region (F383 to V471) were replaced with the corresponding sequences from the human 5-HT$_{2C}$ receptor (D151-A176, L256-V311 and F371-V458). For each junction of the chimera, we prepared a pair of sense and complementary antisense primers, which cover about 20 nucleotides each of the 5-HT$_{2A}$ and 5-HT$_{2C}$ junctional sequences. PCR was carried out initially using the 5-HT$_{2C}$ cDNA as the template. Then, the 5-HT$_{2A}$ sequences between junctions were added using overlap extension techniques using the junctional PCR products and the 5-HT$_{2A}$ cDNA as the template. Similarly, the 5-HT$_{2B}$ chimera was prepared where the second intracellular loop (from D152 to T177), the third intracellular loop (L239 to V324) and the C terminal region (F383 to V481) were replaced by the corresponding 5-HT$_{2C}$ counterparts (see above). Nucleotide sequences of the chimeras were confirmed with dideoxy sequencing. The cDNA inserts were transferred to a mammalian expression vector, PCI-Neo™ from Promega, and the recombinant vectors were used to transfect human embryonic kidney (HEK)293 cells, human epithelial clonal cells (SH-EP), and NIH-3T3 cells, using Ca^{2+} phosphate precipitation techniques. Transfected cells were selected in the presence of G418 (400 μg ml^{-1}). Membranes from stably transfected cells were prepared by standard procedures including cell homogenization and differential centrifugations as described elsewhere (Alberts *et al*., 1999).

Radioactive ligand binding was measured in membranes expressing recombinant receptors, using filtration techniques as described elsewhere (Alberts *et al*., 1999). Briefly, binding of [^{3}H]-5-HT, [^{3}H]-mesulergine, [^{3}H]-LSD and [^{3}H]-ketanserin was measured in medium that contained (mM): NaCl 100, $MgCl_2$ 2, EDTA 1, HEPES/Tris (pH 7.4) 20, the radioactive ligand at varying concentrations (0.5–20 nM for typical binding profiles), and 5–10 μg membrane protein, in a total volume of 500 μl. Reaction mixtures were incubated at 23°C for 60 min, and rapidly filtered over Whatman GF/B filters under vacuum, which were then washed three times with 4 ml of an ice cold 50 mM Tris/HCl buffer (pH 7.4). Non-specific binding was estimated in the presence of unlabelled clozapine or ketanserin in excess (100 μM). Ligand stock solutions were prepared in 0.1% ascorbic acid. Displacement of [^{3}H]-mesulergine, [^{3}H]-LSD or [^{3}H]-ketanserin at 2 nM by test compounds at various concentrations (competition assay) was carried out in the same manner.

GTPγ^{35}S binding was measured following the procedure reported previously (Alberts *et al*., 1999) in medium that contained (mM): HEPES 25, NaCl 100, EDTA 1, $MgCl_2$ 3, dithiothreitol 0.5, digitonin 0.003%, GTPγ^{35}S ($3-5\times10^5$ c.p.m./assay) 2 nM, and 10 μg membrane protein in a volume of 120 μl. Membranes were preincubated with 100 μM 5′-adenylylimidodiphosphate for 30 min at room temperature, subsequently with 10 μM GDP for 10 min on ice. For some experiments, membranes were first treated with N-ethylmaleimide (NEM) (100 μM) for 30 min, and excess NEM was removed with dithiothreitol. Test ligands were included at 10 μM, unless indicated otherwise.

Reaction mixtures were incubated for 45 min at 30°C, and were filtered over a Whatman GF/B filter under vacuum. Filters were washed three times with 4 ml of an ice-cold buffer that contained (mM): NaCl 100, Tris/HCl 20, pH 8.0, $MgCl_2$ 25. Agonist-induced GTPγ^{35}S binding was obtained by subtracting that observed without agonists. Binding data were analysed using a nonlinear regression method (Sigma Plot), and presented as mean ± s.e.mean. ($n=3$).

Results

We obtained several mammalian cell lines, HEK293-A, SH-EP-A, NIH-3T3 and HEK293-B, stably expressing the human 5-HT$_{2C}$ receptor at various receptor densities; 45 ± 3, 12.4 ± 1, 11 ± 1 and 6.6 ± 0.1 pmol mg^{-1} protein, respectively, as estimated from maximal binding of [^{3}H]-mesulergine (antagonist). The dissociation constant for [^{3}H]-mesulergine was not altered (2–3.8 nM) (Table 1). In all these cell lines, 5-HT concentration-dependently induced GTPγ^{35}S binding (Figure 1), and the magnitude of GTPγ^{35}S binding seems to follow generally that of receptor density; e.g., 724, 437, 282 and 213 fmol mg^{-1} protein for the HEK293-A, SH-EP-A, NIH-3T3 and HEK293-B, respectively (Table 1, Figure 1). As the receptor density was getting less, however, the half maximal concentration for 5-HT (EC_{50}) for GTPγ^{35}S binding seems to be getting greater. For example, the EC_{50} values of 40 and 140 nM for 5-HT were observed for the cell line of the highest (45 pmol mg^{-1} protein) and lowest receptor density (6.6 pmol mg^{-1} protein), respectively. This suggests participation of more receptors in low affinity states in G protein coupling, as receptor density decreases.

To examine the effect of receptor density on agonist efficacy, three agonists of differential efficacies were examined for GTPγ^{35}S binding; a full agonist, Org37684, a partial agonist, quipazine, and a weak partial agonist LSD. Enhancement of GTPγ^{35}S binding by Org37684 (10 μM) ranged from 93 to 101% as normalized to that of 5-HT (10 μM), quipazine from 52 to 67%, and LSD from 23 to 29% in these cell lines, with no appreciable dependence on receptor density (Figure 1). The values were also in good agreement with those reported in the literature as obtained with [^{3}H]-IP accumulation and Ca^{2+} signals under minimal receptor reserves (Newton *et al*., 1996; Porter *et al*., 1999). It appears that the relative ability of agonists to induce GTPγ^{35}S binding is independent of receptor density, in contrast to downstream signals, which are markedly affected by receptor reserves. Also noteworthy is the observation that 5-HT-induced GTPγ^{35}S binding in these cell lines was blocked by NEM, a specific inhibitor of G_i subtypes of G proteins (Winslow *et al*., 1987), and also by mesulergine, a selective 5-HT$_{2C}$ antagonist (Figure 1). This confirms the coupling of 5-HT$_{2C}$ to G_i, in addition to $G_{q/11}$ subtypes (Alberts *et al*., 1999).

The other members of the 5-HT$_2$ receptor family, the human 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors, on the other hand, showed no appreciable level of 5-HT-induced GTPγ^{35}S binding, when heterologously expressed in SH-EP at the receptor density of 1.8 ± 0.2 and 8.4 ± 0.2 pmol mg^{-1} protein, respectively (Figure 2). The 5-HT$_{2A}$ receptor density was estimated from [^{3}H]-ketanserin binding and the 5-HT$_{2B}$ receptor density from [^{3}H]-LSD binding. Because intracellular regions are largely responsible for signalling pathways, we prepared here the chimeras of the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor where the second, third intracellular loops and the

Table 1 Comparison of the 5-HT$_{2C}$, 5-HT$_{2A}$, 5-HT$_{2A/2C}$, 5-HT$_{2B}$ and 5-HT$_{2B/2C}$ receptors for their ligand binding and 5-HT-induced GTPγ^{35}S binding, when heterologously expressed in diverse cell lines at various receptor densities

Receptor cell lines	*[^{3}H]-Mesulergine binding* K_D, *nM*	B_{max}*	*GTPγ^{35}S binding* E_{max}†	EC_{50}, *nM*	*Hill*
Human 5-HT$_{2C}$					
HEK293-A	2.0±0.1	45±3	724±36	40±2	0.88±0.04
SH-EP-A	3.8±0.4	12.4±2	437±39	83±8	0.82±0.05
NIH-3T3	3.6±0.5	11.9±0.6	282±19	124±16	0.92±0.09
HEK293-B	2.0±0.2	6.6±0.1	213±8	140±21	0.92±0.1
	[^{3}H]-Ketanserin binding				
Human 5-HT$_{2A}$					
SH-EP	3.4±0.4	1.8±0.2	Not detectable		
Human 5-HT$_{2A/2C}$					
SH-EP	2.7±0.3	6.5±0.5	587±35	680±95	0.93±0.12
	[^{3}H]-LSD binding				
Human 5-HT$_{2B}$					
SH-EP	2.7±0.1	8.4±0.2	Not detectable		
Human 5-HT$_{2B/2C}$					
SH-EP	1.6±0.1	2.8±0.1	193±8	25±3	0.93±0.09

*pmol mg^{-1} protein; †pmol mg^{-1} protein.

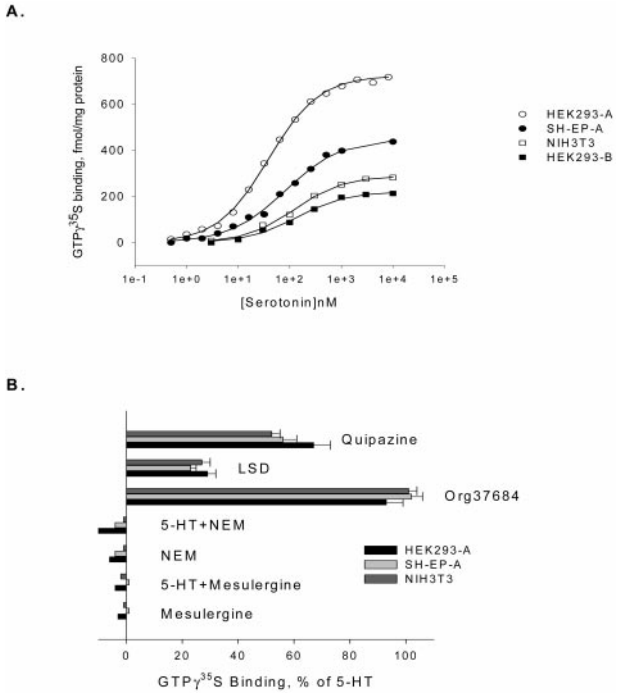

Figure 1 Agonist-induced GTPγ^{35}S binding at the human 5-HT$_{2C}$ receptor heterologously expressed in HEK293, SH-EP and NIH-3T3 cells at various receptor densities. (A) GTPγ^{35}S binding was induced by 5-HT at various concentrations in cell membranes (see Table 1). We obtained 5-HT-induced GTPγ^{35}S binding by subtracting that observed in the absence of agonists. We measured binding at 30°C for 45 min, using filtration techniques, as described in the section of 'Methods'. Solid lines represent the data fitting to the model for a single class of binding sites. The parameters thus obtained are shown in Table 1. (B) 5-HT(1 μM)-induced GTPγ^{35}S binding was blocked by mesulergine (100 μM) in cell membranes from HEK293-A, SH-EP-A and NIH-3T3 cells expressing the human 5-HT$_{2C}$ receptor. The GTPγ^{35}S binding was blocked by treatment with NEM (100 μM), an inhibitor of G_i subtypes of G proteins. Also shown are comparisons of three agonists, Org367684, quipazine and LSD with respect to their ability to enhance GTPγ^{35}S binding in the cell lines.

C-terminal region were replaced with the 5-HT$_{2C}$ counterparts (see the section of 'Methods').

SH-EP cell lines stably expressing the chimeras were obtained under G-418 selection, and their ligand binding properties were characterized. The 5-HT$_{2A/2C}$ chimera bound [^{3}H]-ketanserin with a K_D of 2.9±0.5 nM, similar to that (3.4±0.4 nM) for the wild type 5-HT$_{2A}$, and maximal binding of 6.5±0.5 pmol mg^{-1} protein. Also the 5-HT$_{2B/2C}$ chimera bound [^{3}H]-LSD with a K_D of 1.6±0.1 nM (*cf.* 2.7±0.1 nM for the wild type 5-HT$_{2B}$) and maximal binding of 2.8±0.1 pmol mg^{-1} protein. Furthermore, binding affinities of 15 serotonergic ligands were evaluated, using competition experiments with [^{3}H]-ketanserin for the 5-HT$_{2A}$ receptor and the 5-HT$_{2A/2C}$ chimera, and with [^{3}H]-LSD for the 5-HT$_{2B}$ receptor and the 5-HT$_{2B/2C}$ chimera. These values were compared to those for 5-HT$_{2C}$ as obtained from competition experiments with [^{3}H]-mesulergine (Table 2). All displacement data fitted to one-site binding model. Even for agonists, two-site binding model was avoided here, because the receptor population in high affinity states for agonists is small (<10%) and poorly resolved, in part due to high receptor density and in part due to small affinity differences in low and high affinity states; e.g., the 5-HT K_i ratio for low to high affinity sites being less than 10 in the 5-HT$_{2B}$ receptor and 5-HT$_{2B/2C}$ chimera.

A plot of pK$_i$ values of test ligands between 5-HT$_{2A}$ and 5-HT$_{2A/2C}$ showed a high correlation coefficient (r^2) of 0.98 with a slope of 1 (linear regression analysis) (Figure 3, Table 2), but they were quite different from those of 5-HT$_{2C}$. For example, spiperone bound 5-HT$_{2A}$ and 5-HT$_{2A/2C}$ with a K_i value of 0.8 and 0.6 nM, respectively, but bound 5-HT$_{2C}$ with a K_i value of 1047 nM. Also metergoline, clozapine, ketanserin and LSD bound 5-HT$_{2A}$ and 5-HT$_{2A/2C}$ with 10–20 fold higher affinities than those for 5-HT$_{2C}$, but mesulergine with a 30 fold lower affinity.

Similarly, the pK$_i$ values of test ligands between the 5-HT$_{2B}$ receptor and the 5-HT$_{2B/2C}$ chimeras also showed a high correlation coefficient of 0.99, with a slope of 1 (Figure

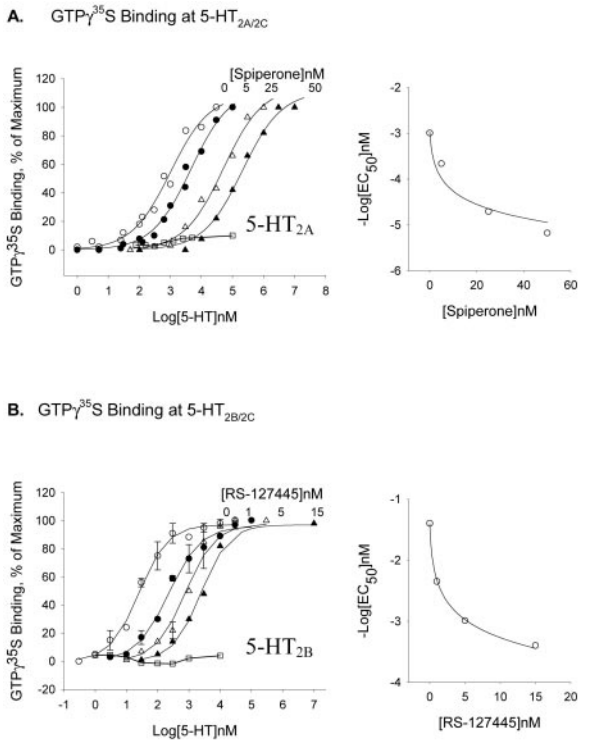

Figure 2 5-HT-induced GTPγ^{35}S binding in SH-EP cell membranes expressing the 5-HT$_{2A/2C}$ and 5-HT$_{2B/2C}$ chimeras in the presence or absence of selective antagonists. (A) GTPγ^{35}S binding was measured as a function of 5-HT concentrations in the presence of spiperone (antagonist) at 0, 5, 25 and 50 nM. Amounts of 5-HT-dependent GTPγ^{35}S binding, as obtained by subtracting that observed without the agonist, were normalized to the level observed with 5-HT at a saturating concentration (10 μM). Spiperone by itself showed no appreciable effects on GTPγ^{35}S binding, but shifted 5-HT dose-response profiles to the right. Spiperone-induced shifts of the EC_{50} value for 5-HT were plotted as a function of metergoline concentrations, and the solid line represents a non-linear regression fit to the equation of competitive interaction, $-\log[\text{5-HT } EC_{50}] = -\log([\text{Spiperone}] + K_D) - \log C$ (C = constant) (Lew & Angus, 1995). (B) GTPγ^{35}S binding was measured as a function of 5-HT concentrations in the presence of RS-127445 (antagonist) at 0, 1, 5 and 15 nM. Amounts of 5-HT-dependent GTPγ^{35}S binding, as obtained by subtracting that observed without the agonist, were normalized to the level observed with 5-HT at a saturating concentration (10 μM). RS-127445 by itself showed no appreciable effects on GTPγ^{35}S binding, but shifted 5-HT dose-response profiles to the right. RS-127445-induced shifts of the EC_{50} value for 5-HT were plotted as a function of RS-127445 concentrations, and the solid line represents a non-linear regression fit to the equation of competitive interaction, $-\log[\text{5-HT } EC_{50}] = -\log([\text{RS-127445}] + K_D) - \log C$ (C = constant) (Lew & Angus, 1995).

3), but were quite different from those for 5-HT$_{2C}$. RS-127445, a 5-HT$_{2B}$-selective antagonist (Bonhaus *et al*., 1999), for example, bound 5-HT$_{2B}$ and 5-HT$_{2B/2C}$ with a K_i value of 0.14 and 0.18 nM, respectively, but bound 5-HT$_{2C}$ with a K_i of 405 nM. Also clozapine, 5-HT, LSD, quipazine and 5-CT showed 7–20 fold higher affinities for 5-HT$_{2B}$ and 5-HT$_{2B/2C}$ than those for 5-HT$_{2C}$. It should be noted that the pK_i values reported here for the wild types of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ are in good agreement with those reported in the literature (Newton *et al*., 1996; Sleight *et al*., 1996; Roth *et al*., 1998). It appears that the substitution of the intracellular regions of 5-HT$_{2A}$ or 5-HT$_{2B}$ with the corresponding 5-HT$_{2C}$ regions had no appreciable effects on their ligand binding properties. This is consistent with the conventional view that ligand binding pockets of G protein coupled receptors are primarily contributed by transmembrane segments and extracellular loops, but not by intracellular regions.

Functionally, the 5-HT$_{2A/2C}$ and the 5-HT$_{2B/2C}$ chimeras showed robust 5-HT-induced GTPγ^{35}S binding, unlike their parent receptors (Table 2). For the 5-HT$_{2A/2C}$ chimera, 5-HT dose-dependently enhanced GTPγ^{35}S binding with an EC_{50} of 787 ± 95 nM and maximal binding of 587 fmol mg^{-1} protein. Spiperone, a selective antagonist for the 5-HT$_{2A}$ receptor, did not appreciably affect the basal binding, but concentration-dependently shifted the 5-HT dose-response profile to the right. The 5-HT EC_{50} value increased from 787 to 4616, 42523 and 164376 nM in the presence of spiperone at 5, 25 and 50 nM, respectively (Figure 2). The data fitted to the equation for competitive interactions, $-\log[\text{5-HT } EC_{50}] = -\log([\text{spiperone}] + K_D) - \log C$ (Lew & Angus, 1995), where K_D is the dissociation constant for spiperone and C is the ratio of $K_D/EC_{50\text{-control}}$. Non-linear regression analysis (Figure 2) yielded the K_D of 0.5 nM for spiperone, which is close to its K_i value (0.6 nM) from equilibrium binding experiments, and the C value of 5.1×10^{-4} as predicted.

At the 5-HT$_{2B/2C}$ chimera, 5-HT also concentration-dependently enhanced GTPγ^{35}S binding with an EC_{50} of 25 ± 3 nM and maximal increase of 193 fmol mg^{-1} protein (Figure 2). RS-127445, a selective antagonist for the 5-HT$_{2B}$ receptor, did not appreciably affect the basal binding, but concentration-dependently shifted the 5-HT concentration-response profile to the right. The 5-HT EC_{50} value increased from 25 to 223, 974 and 2495 nM in the presence of RS-127445 at 1, 5 and 15 nM, respectively (Figure 2). The data fitted to the same equation (Lew & Angus, 1995) and non-linear regression analysis (Figure 2) yielded the K_D of 0.14 nM for RS-127445, which is close to its K_i value (0.18 ± 0.02 nM) from equilibrium binding experiments, and the C value of 3.1×10^{-4} also being close to that predicted. Also noteworthy is our observation that other antagonists, ketanserin, clozapine, and mesulergine, hardly affected the basal GTPγ^{35}S binding at the both chimeras (data not shown).

We also examined 10 serotonergic agonists for their ability to induce GTPγ^{35}S binding at 5-HT$_{2A/2C}$, 5-HT$_{2C}$ and 5-HT$_{2B/2C}$; 2-Me-5HT, 5-CT, α-Me-5-HT, LSD, Org37684, mCPP, TFMPP, quipazine, DOI and DOB (Table 3). Their responses, after normalization to that of 5-HT (10 μM), were compared to their relative efficacy for 5-HT$_{2A}$ and 5-HT$_{2B}$ as reported in the literature (parenthesis) with intracellular Ca^{2+} signals (Porter *et al*., 1999). The order of the relative efficacy at 5-HT$_{2A/2C}$ from GTPγ^{35}S binding was α-Me-5-HT > DOB > 5-CT > Quipazine > 2-Me-5-HT > Org37684 > DOI > LSD > mCPP > TFMPP, and was nearly identical to that reported with 5-HT$_{2A}$ in the literature (Porter *et al*., 1999). A plot of these values at 5-HT$_{2A/2C}$ vs those at 5-HT$_{2A}$ showed a correlation coefficient of 0.91 with a slope of 1 (data not shown). Only the values for DOI and LSD showed over 10% variations from their reported values (47 vs 61% for DOI and 27 vs 44% for LSD). Moreover, the 5-HT$_{2A/2C}$, although containing intracellular parts of 5-HT$_{2C}$, displayed agonist efficacies quite different from those at 5-HT$_{2C}$. For example, Org37684, DOI and mCPP were moderate or weak partial

Table 2 Comparison of ligand binding affinities to the human 5-$HT_{2A/2C}$, 5-HT_{2A}, 5-$HT_{2B/2C}$ and 5-HT_{2C} receptors

Compounds	*5-HT2A/2C* K_i, *nM*	*5-HT2A* K_i, *nM*	*5-HT2C* K_i, *nM*	*5-HT2B/2C* K_i, *nM*	*5-HT2B* K_i, *nM*
Spiperone	0.6±0.05	0.8±0.06	1047	704±39	482±38
Metergoline	0.14±0.01	0.17±0.03	3.1±0.7	1.7±0.1	0.8±0.1
Clozapine	4.9±0.2	3.5±0.6	38±2	7.6±1	6.7±0.9
Mesulergine	33±2	39±4	1.1±0.2	3.6±0.2	3.5±0.2
Ketanserin	2.7±0.3	2.7±0.2	48	90±5	93±6
1-NP	5.2±0.2	5.7±0.1	3.7±0.2	3.8±0.8	3.1±0.2
RS-127445	418±38	422±31	405±38	0.18±0.02	0.14±0.03
5-HT	656±51	506±42	142±4	15±2.5	21±2
LSD	1.9±0.2	2.9±0.2	20±2	2.1±0.1	1.6±0.1
DOI	40±4	34±3	85±6	32±3	25±3
mCPP	174±10	176±11	298±21	78±10	61±5
Org37684	361±23	320±18	181±9	116±5	109±7
Quipazine	1810±76	1643±67	2686±249	164±16	175±21
TFMPP	146±12	174±16	211±9	213±16	229±18
5-CT	1717±164	1868±67	2455±249	92±12	91±5

Competition binding experiments aginst [^{3}H]-ketanserin (2 nM) for 5-HT_{2A} and 5-$HT_{2A/2C}$, [^{3}H]-LSD for 5-HT_{2B} and 5-$HT_{2B/2C}$, and [^{3}H]-mesulgerine for 5-HT_{2C} were carried with test ligands at various concentrations in membranes from SH-EP cells or HEK293 cells stably expressing indicated receptors. IC_{50} values were obtained from dose-response profiles for test ligands at six different concentrations. Test ligand concentrations were continuously adjusted until IC_{50} values fell in the middle range of dose-response profiles. IC_{50} values converted to K_i values using Cheng–Prusoff equation.

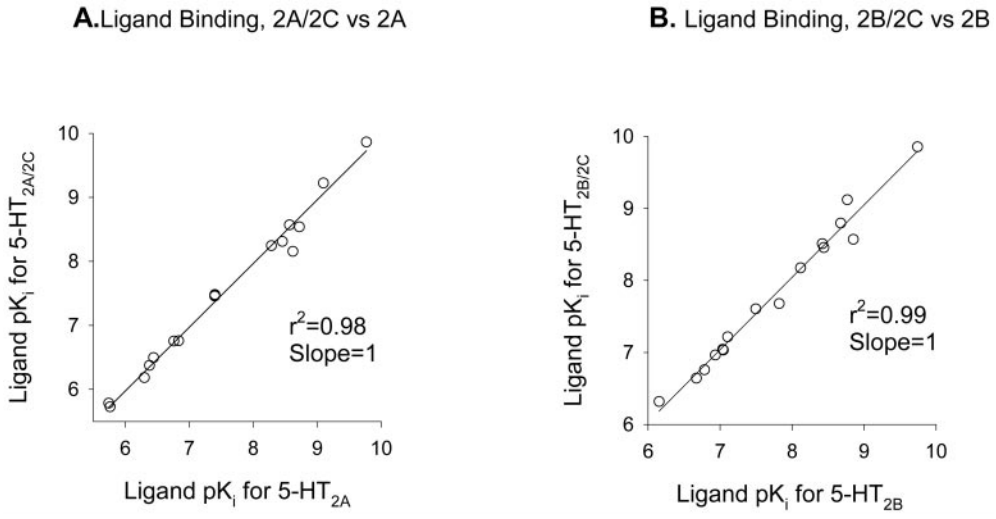

Figure 3 Comparison of ligand binding properties between 5-$HT_{2A/2C}$ and 5-HT_{2A}, and between 5-$HT_{2B/2C}$ and 5-HT_{2B}. (A) The plot shows the correlation of the pK_i values of 15 test ligands (Table 2) from competition experiments using [^{3}H]-ketanserin (2 nM) in membranes for 5-HT_{2A} and 5-$HT_{2A/2C}$ expressed in SH-EP cells. The solid line represents linear regression analysis, with a correlation coefficient (r^2) of 0.98 with a slope of 1. (B) A similar plot showing the correlation of the pK_i values from 5-HT_{2B} and 5-$HT_{2B/2C}$ receptors expressed in SH-EP cells; r^2 of 0.99 with a slope of 1. [^{3}H]-LSD was used for competition experiments.

agonists at 5-$HT_{2A/2C}$ (40 to 20% of 5-HT), but full agonists at 5-HT_{2C} (over 80% of 5-HT).

With the 5-$HT_{2B/2C}$ chimera, the order of the relative efficacy from GTPγ[35]S binding was 5-CT> DOB> 2-Me-5HT>α-Me-5-HT> DOI> LSD> TFMPP> Org37684> mCPP> Quipazine, and was very close to that reported earlier for 5-HT_{2B} (Porter *et al*., 1999). A plot for the values at 5-$HT_{2B/2C}$ vs those at 5-HT_{2B} showed a correlation coefficient of 0.93 with a slope of 0.93 from linear regression analysis (data not shown). Only the values for α-Me-5-HT and DOI showed over 10% variations from their reported values (64 vs 77% for α-Me-5-HT and 52 vs 65% for DOI). Again, the 5-$HT_{2B/2C}$ chimera, although containing intracellular parts of 5-HT2C, displayed agonist efficacies quite different from those at 5-HT_{2C}. For example, Org37684 and mCPP are weak partial agonists at the 5-$HT_{2B/2C}$ chimera, but full agonists at the 5-HT_{2C} receptor.

With 5-HT_{2C}, the relative efficacies of test agonists from GTPγ[35]S binding were also similar to those reported earlier for 5-HT_{2C}, using FLIPR-Ca^{2+} imaging (Porter *et al*., 1999), except for a few compounds (Table 1). For instance, the value for Org37684 was 94% from the current GTPγ[35]S binding assay and 55–76% from Ca imaging (Porter *et al*., 1999; Vickers *et al*., 2001). Such differences need to be clarified with further study, but could not be attributed to receptor density of cell lines we used here. First of all, we have shown above that the relative efficacy of Org37684 from GTPγ[35]S binding was not dependent on receptor density in

Table 3 Relative efficacy of serotonergic agonists for the 5-HT$_{2A/2C}$, 5-HT$_{2B/2C}$ and 5-HT$_{2C}$ receptors as measured with GTPγ^{35}S binding in comparison with the corresponding values (parenthesis) in the literature for 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ as measured with Ca^{2+} signals in the presence of minimal receptor reserves (Porter *et al*., 1999)

	5-HT$_{2A/2C}$ (5-HT$_{2A}$)		*5-HT$_{2B/2C}$ (5-HT$_{2B}$)*		*5-HT$_{2C}$*	
	Relative GTPγ^{35}S binding, % of 5-HT					
Compounds	*%*		*%*		*%*	
α-Me-5-HT	95±6	(97)	5-CT	92±3 (90)	Org37684	94±5 (55–76)
DOB	81±4	(74)	DOB	77±2 (69)	α-Me-5-HT	90±3 (92)
5-CT	71±2	(65)	2-Me-5-HT	74±3 (72)	5-CT	84±4 (85)
Quipazine	68±3	(62)	α-Me-5-HT	64±2 (77)	mCPP	80±7 (65)
2-Me-5-HT	56±5	(55)	DOI	52±3 (65)	DOI	76±2 (57)
Org37684	48±4	(45)	LSD	45±10 (51)	2-Me-5-HT	69±5 (70)
DOI	47±5	(61)	TFMPP	33±4 (34)	DOB	64±3 (65)
LSD	27±7	(44)	Org37684	29±2 (34)	Quipazine	52±2 (57)
mCPP	18±2	(22)	mCPP	22±3 (24)	TFMPP	45±2 (54)
TFMPP	17±2	(20)	Quipazine	17±5 (17)	LSD	20±2 (29)

GTPγ^{35}S binding was measured in isolated membranes from cells expressing indicated chimeras, in the presence or absence of test ligands at 10 μM. The net increase was normalized to the maximal effect of 5-HT at 10 μM. These values represent the mean±s.e. ($n=3$).

our cell lines (Figure 1). Also no appreciable discrepancy was noted for other agonists (DOI, 2-Me-5-HT and DOB) that showed the efficacy value from 50–70% in both assays. The same was for a partial agonist, LSD, with its values in the range of 20% in both assays. If receptor reserve were to contribute to agonist efficacy, one would expect more pronounced differences in efficacy of partial than full agonists. Moreover, receptor density seems not to be the major determinant in target G protein couplings, because the CHO cell line showing agonist-induced GTPγ^{35}S binding to G$\alpha_{q/11}$ and Gα_i (Cussac *et al*., 2002) was of higher receptor density (20 pmol mg^{-1}) than the most cell lines we examined here. For example, we observed here GTPγ^{35}S binding to only Gα_i as receptor density varied from 2 to 45 pmol mg^{-1} protein.

Discussion

The human 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors show nearly 80% homology at transmembrane segments, but only 50% homology in their overall primary sequences, largely due to divergences in intracellular regions (only 30% homology). Despite such divergences in the regions that play critical roles in signal transduction, the members of 5-HT$_2$ receptor family have been proposed to share the same signalling pathway, $G_{q/11}$/phospholipase Cβ/IP$_3$/Ca^{2+} signals (Sanders-Bush & Breeding, 1991). In this study, we found, however, that only 5-HT$_{2C}$, but not 5-HT$_{2A}$ and 5-HT$_{2B}$, when heterologously expressed in HEK293 or SH-EP cells, displayed robust agonist-induced GTPγ^{35}S binding from its additional coupling to NEM-sensitive G_i, not from its coupling to $G_{q/11}$. The unique coupling of 5-HT$_{2C}$ to G_i subtypes was further strengthened by our current observation that the 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors, when intracellular regions (second, third and C terminal regions) were replaced with the 5-HT$_{2C}$ counterparts, gained the phenotype of robust agonist-induced GTPγ^{35}S binding.

Generally, marginal interactions of $G_{q/11}$ and GTPγ^{35}S *in vitro* have been attributed to slow GTP turnover rates of $G_{q/11}$ in isolated states (Smrcka *et al*., 1991). Nevertheless, several studies have reported interactions of $G_{q/11}$ with GTPγ^{35}S *in vitro*, seemingly in cell line and/or receptor isoform dependent manners. As noted above, one isoform of the human 5-HT$_{2C}$ receptor, the fully RNA-edited VSV, when heterologously expressed in CHO cells, reportedly mediates agonist-induced GTPγ^{35}S binding to G$\alpha_{q/11}$ and Gα_i (Cussac *et al*., 2002). On the other hand, a differentially RNA-edited 5-HT$_{2C}$ isoform, the ISV (also used in the current study), when expressed in HEK293 cells, mediated GTPγ^{35}S binding to only Gα_i, but not to G$\alpha_{q/11}$. This was evidenced by the complete disappearance of 5-HT-induced GTPγ^{35}S upon treatment of HEK cells with Pertussis toxin and also upon treatment of membranes with NEM (100 μM), a sulfhydryl agent selectively modifying Gα_i (Alberts *et al*., 1999). With rat 5-HT$_{2A}$ receptors, similar cell line-specific GTPγS sensitivity has been reported. Agonist-induced high affinity sites on the rat 5-HT$_{2A}$ receptor were reduced by GTPγS, when heterologously expressed in mouse NIH-3T3 cells, but not when expressed in HEK293 cells (Szele & Pritchett, 1993). Also it has been recently proposed that 5-HT$_{2A}$ mediates 5-HT-induced GTPγ^{35}S binding in rat coronal brain sections, judging from marked inhibition of the GTPγ^{35}S binding by MDL 100105, a racemic form of MDL 100907, a 5-HT$_{2A}$-selective antagonist (Adlersberg *et al*., 2000). This proposal of the direct 5-HT$_{2A}$ involvement in GTPγ^{35}S binding, among many 5-HT receptors in the brain, however, needs to be strengthened by the demonstration of the insensitivity of the GTPγ^{35}S binding to antagonists selective for other 5-HT receptors, and the identification of target Gα proteins.

In any event, such phenotypic differences could be attributed to several factors. (1) Individual cell lines possess structurally restrained G protein coupled receptor complexes that contain cell-specific effectors, G proteins, and accessory proteins, often leading to different phenotypes (Alberts *et al*., 2000a). (2) In the case of human 5-HT$_{2C}$ receptors, RNA-editing seems to alter target G proteins, such as the coupling of non-edited isoforms to G_{13}, but not extensively edited isoforms (Price *et al*., 2001). It seems to be useful in the future to examine various RNA-edited 5-HT$_{2C}$ isoforms for their ability to couple to Gα_i. (3) In the case of 5-HT$_{2A}$, considerable sequence divergences exist between the rat and the human receptors, particularly in the C terminal region,

and could contribute to phenotypic differences. (4) Receptor over expression could be another factor potentially affecting G protein couplings, but not in this case involving 5-HT$_{2C}$. Again the 5-HT$_{2C}$ receptor (VSV) in CHO cells (Cussac *et al*., 2002) that has been reported to mediate interactions of GTPγ^{35}S with $G_{q/11}$ *in vitro* was also highly over-expressed with a receptor density of 20 pmol mg^{-1} protein, which was similar to or greater than that of most HEK293, NIH-3T3 and SH-EP cell lines used in the current study.

It is also noteworthy that 5-HT EC_{50} values in GTPγ^{35}S binding at 5-HT$_{2C}$ in this study were greater than the 5-HT binding constant for high affinity sites (3 nM from [^{3}H]-5-HT binding), but less than that for low affinity sites (142 nM from competition experiments). Similar trends have been observed with diverse catecholamine receptors heterologously expressed in various cell lines at low to moderate receptor density. With human D2long receptors expressed in SH-SY5Y cells at a density of 3 pmol mg^{-1} protein, agonist EC_{50} values in GTPγ^{35}S binding were 20–70 fold greater than their binding constants for high affinity sites, but not exceeding those for their low affinity sites (Alberts *et al*., 2000a). With 5-HT$_{1B}$, 5-HT$_{1D}$ and 5-HT$_{1F}$ expressed in HEK293 cells at receptor density of 0.97, 0.75 and 0.32 pmol mg^{-1} protein, respectively, 5-HT EC_{50} values were 37, 17 and 29 fold greater, respectively, than their binding constants for high affinity sites as measured with [^{3}H]-5-HT (Alberts *et al*., 2000b). At present, precise kinetic analyses are not available for non-hydrolysable GTPγ^{35}S binding to target G proteins during a 40 min incubation period, but we may speculate some factors contributing to this phenotype.

First of all, in the presence of a relatively high level of GTPγ^{35}S at 2 nM here (*cf*., 0.05 or 0.2 nM in the studies by Cussac *et al*. (2002) and Adlersberg *et al*. (2000), respectively), maximal GTPγ^{35}S binding may be largely limited by the availability of endogenous target G-proteins. Also one may assume that a single receptor in structurally restrained G protein coupling complexes may mediate GTPγ^{35}S binding to more than one $G\alpha_i$ protein during the 40 min-incubation period, and may alternate between high and low affinity states. Under such dynamic situations, it is reasonable to observe agonist EC_{50} values to be intermediate between those for high and low affinity states.

In this study, we also established that 5-HT$_{2A/2C}$ and 5-HT$_{2B/2C}$ chimeras retained the same pharmacology as their parent receptors (Porter *et al*., 1999; Newton *et al*., 1996), and could serve as their surrogates. The advantages of using these chimeras over their parent receptors are their robust functional signals as 5-HT$_{2C}$, and convenient and accurate determinations of ligand intrinsic efficacy, using agonist-induced GTPγ^{35}S binding in isolated membranes. In summary, the phenotype of agonist-induced GTPγ^{35}S binding is unique to 5-HT$_{2C}$ among the 5-HT$_2$ receptor family, and is transferable to 5-HT$_{2A}$ and 5-HT$_{2B}$, upon swapping intracellular sequences, without altering their receptor pharmacology.

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