Modifying Ligand-Induced and Constitutive Signaling of the Human 5-HT₄ Receptor

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G protein–coupled receptors (GPCRs) signal through a limited number of G-protein pathways and play crucial roles in many biological processes. Studies of their *in vivo* functions have been hampered by the molecular and functional diversity of GPCRs and the paucity of ligands with specific signaling effects. To better compare the effects of activating different G-protein signaling pathways through ligand-induced or constitutive signaling, we developed a new series of RASSLs (receptors activated solely by synthetic ligands) that activate different G-protein signaling pathways. These RASSLs are based on the human $5\text{-}HT_{4b}$ receptor, a GPCR with high constitutive G_s signaling and strong ligand-induced G-protein activation of the G_s and $G_{s/q}$ pathways. The first receptor in this series, $5\text{-}HT_4\text{-}D^{100}A$ or Rs1 (RASSL serotonin 1), is not activated by its endogenous agonist, serotonin, but is selectively activated by the small synthetic molecules GR113808, GR125487, and RO110-0235. All agonists potently induced G_s signaling, but only a few (e.g., zacopride) also induced signaling via the G_q pathway. Zacopride-induced G_q signaling was enhanced by replacing the C-terminus of Rs1 with the C-terminus of the human $5\text{-}HT_{2C}$ receptor. Additional point mutations ($D^{66}A$ and $D^{66}N$) blocked constitutive G_s signaling and lowered ligand-induced G_q signaling. Replacing the third intracellular loop of Rs1 with that of human $5\text{-}HT_{1A}$ conferred ligand-mediated G_i signaling. This G_i-coupled RASSL, Rs1.3, exhibited no measurable signaling to the G_s or G_q pathway. These findings show that the signaling repertoire of Rs1 can be expanded and controlled by receptor engineering and drug selection.

INTRODUCTION

Heptahelical G protein–coupled receptors (GPCRs) are the largest family of human cell-surface receptors, encompassing more than 340 hormone receptors and 350–460 olfactory receptors [1,2]. They are activated by peptide hormones, odorants, photons, biogenic amines, phospholipids, and many other extracellular signals. Upon activation, GPCRs undergo conformational changes that allow active and reversible signaling through a limited number of G-protein pathways (G_s, G_i, G_q, $G_{12/13}$). These signals mediate a wide variety of physiological responses, including heart rate, chemotaxis, cell proliferation, neurotransmission, and hormonal responses. Owing to their physiological importance, GPCRs are of great medical interest. Indeed, they are targets for at least 40% of modern pharmaceuticals [3].

Although many drugs target GPCRs, studies of GPCR signaling *in vivo* have been hampered by the lack of specific agonists and antagonists for many of the receptors. GPCRs display molecular and functional diversity, such as the type of G-protein signaling pathway associations, different levels of constitutive activity, and different signaling responses due to different ligand-selective conformations [4,5]. This diversity enables the receptors to transmit unique extracellular signals but hampers efforts to sort out the relative contributions of each signaling pathway or the roles of constitutive signaling for each receptor.

To better study the diversity of GPCRs, we developed receptors activated solely by synthetic ligands (RASSLs) by modifying their structures to render them unresponsive to endogenous hormones. Instead, RASSLs are activated by small-molecule drugs [6], allowing them to be used to activate specific G-protein pathways rapidly and reversibly and to mimic the speed, localization, regulation, and amplification of endogenous GPCR signals [7].

Since it is impractical to convert all GPCRs into RASSLs, we and others have focused on representative G_s-, G_i-, and G_q-coupled GPCRs, which stimulate adenylyl cyclase, inhibit adenylyl cyclase, and stimulate phospholipase-C, respectively. Ro1 (RASSL opioid 1), the prototype RASSL based on a G_i-coupled κ-opioid receptor [8], provided a proof of concept for this strategy. Its G_i response to natural ligands is 0.001% of that of the wildtype receptor, but it is potently activated by the synthetic agonist spiradoline [8–10]. Ro1 decreases heart rate in mice [11] and affects taste sensation in the tongue [9]. In addition to serving as powerful tools to dissect the G-protein signaling *in vivo*, RASSLs can yield insights into fundamental aspects of receptor diversity [12]. For instance, constitutive signaling of Ro1 led to cardiomy-

opathy [11], diminished bone formation [13], and induced hydrocephalus [10]. These constitutive signaling phenotypes would have been difficult or impossible to identify by studying endogenous receptors.

Multiple RASSLs have since been made, including a G_s-coupled RASSL based on the melanocortin-4 receptor [14], a G_q-coupled RASSL based on the histamine 1 receptor [15], and a series of RASSLS based on muscarinic receptors [16]. These RASSLs are useful tools; however, it is still advantageous to derive a series of RASSLs with distinct G-protein signaling from the same parental GPCR. It can be difficult to compare the effects of RASSLs based on different parental GPCRs since these RASSLs could have different pharmacokinetics, constitutive activity, desensitization kinetics, and cellular localization.

To better study GPCRs, we built a series of RASSLs based on the human 5-HT$_4$ receptor (Figure 1), which has several advantages over other serotonin receptors. First, its pharmacological properties are well established [17]. Second, its agonists have milder effects (increased gastrokinesis [18], augmented memory acquisition and retention [19], increased chronotropic and inotropic cardiostimulation [20], and enhanced cortisol release [21]) than other serotonergic drugs. Third, it has a large number

of synthetic ligands, which allows us to identify differences in their effects on that receptor. Fourth, a single mutation ($D^{100}A$) in the mouse 5-HT$_4$ receptor dramatically reduced its affinity for serotonin, its endogenous ligand. This mutation also allows synthetic agonists and antagonists for the wildtype receptor to activate the mutant, turning 5-HT$_4$-$D^{100}A$ into a RASSL [22].

Finally, we reasoned that novel receptors coupling to other signaling pathways could be created by making chimeras of the 5-HT$_4$ receptor with other family members. Altering the G-protein selectivity of GPCRs is often difficult because it is based on receptor conformation determined by multiple regions of the receptor [23]. Changing multiple regions involves large internal mutations that often lead to receptor instability. A better strategy for altering G-protein signaling characteristics is to swap domains between structurally similar receptors within the same family. The 5-HT$_4$ receptor belongs to a family of at least 15 receptors, each with different subfamilies that engage different G-protein signaling pathways. The 5-HT$_4$, 5-HT$_6$, and 5-HT$_7$ subfamilies are G_s coupled, the 5-HT$_1$ subfamily is G_i coupled, and the 5-HT$_2$ subfamily is G_q coupled [24]. These characteristics could expedite our efforts to make purely G_s-, G_i-, and G_q-coupled RASSLs.
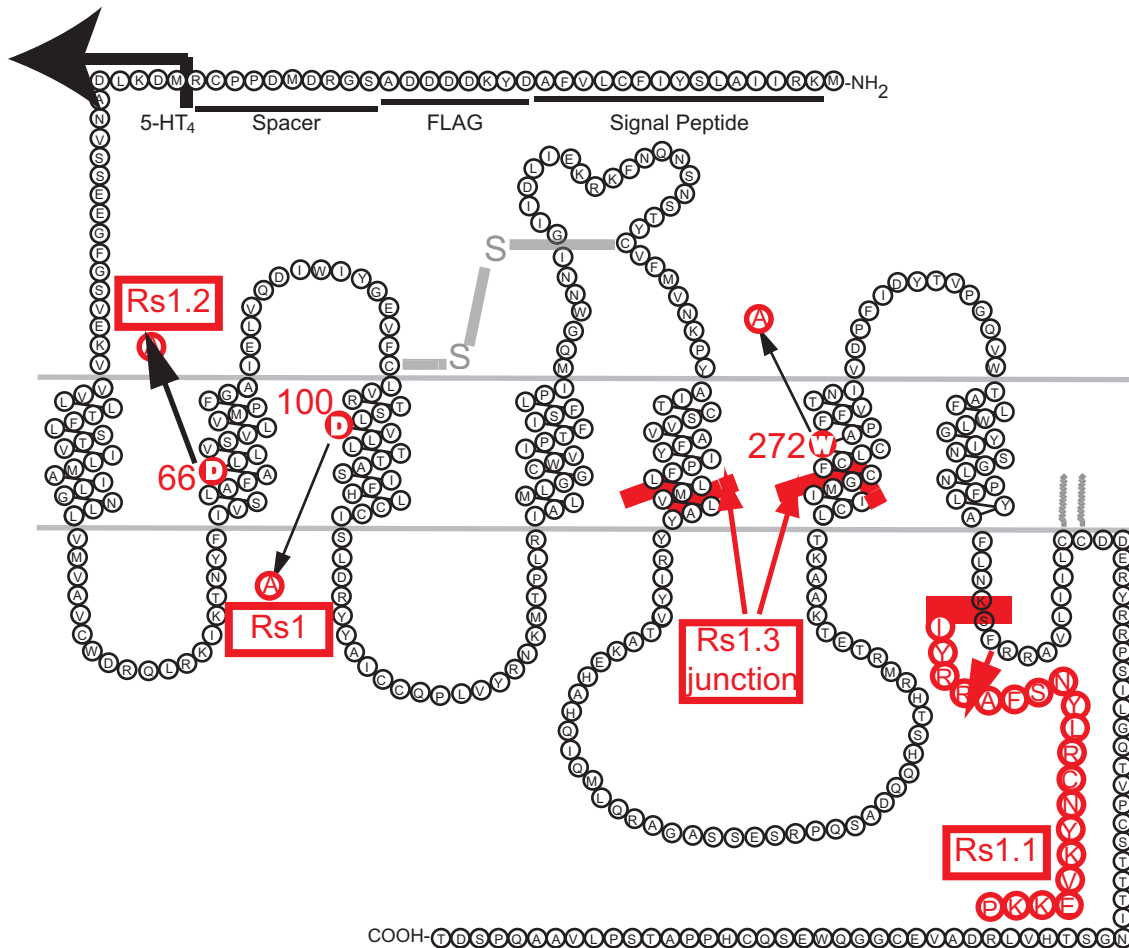
Figure 1. Human 5-HT$_4$-based RASSLs. A signal peptide and the FLAG epitope were added to the N-terminus of the human 5-HT$_4$ receptor. A $D^{100}A$ mutation was introduced by site-directed mutagenesis to create the G_s-coupled RASSL (Rs1). An additional point mutation ($D^{66}A$, $D^{66}N$, or $W^{272}A$) was added to Rs1 to modulate constitutive signaling. Rs1.1 is the Rs1-C-5-HT$_{2C}$ chimera with enhanced G_q signaling. Rs1.2 is Rs1 with an extra $D^{66}A$ mutation that decreased constitutive G_s signaling. Rs1.3 is the Rs1-i3-5-HT$_{1A}$ chimera with G_i signaling. The junctions in Rs1.1 and Rs1.3 indicate ends of domain swapping where the 5-HT$_4$ has been replaced by 5-HT$_{2C}$ or 5-HT$_{1A}$, respectively.
doi:10.1371/journal.pone.0001317.g001

Here, we describe a new series of RASSLs developed to modify the ligand-induced and constitutive signaling of the human 5-HT$_4$ receptor. These modified GPCRs will help us better study the effect of constitutive G$_s$ signaling and ligand-induced G$_s$, G$_s$/G$_q$, and G$_i$ signaling *in vivo*.

RESULTS

Human 5-HT$_4$ D^{100}A is a G$_s$-coupled RASSL

The D^{100}A mutation in the mouse 5-HT$_4$ receptor converts it into a RASSL [22], but its effects on the human 5-HT$_4$ receptor have not been tested. We now extend these findings to the human 5-HT$_4$ receptor (Figure 1). To determine if antagonists for the wildtype 5-HT$_4$ receptor also activate the human 5-HT$_4$-D^{100}A mutant, we tested a variety of compounds. The mutant receptor was not activated by serotonin (Figures 2, 3A), but it was activated by agonists (cisapride, zacopride), partial agonists (RS23597, RS39604, RS67333), antagonists (GR113808, RO110-0235), and an inverse agonist (GR125487) for the wildtype 5-HT$_4$ receptor (Figure 2). Interestingly, GR125487 showed a specific response, as demonstrated by the steep concentration-response curve (Figure 3D). In addition, GR113808, GR125487, and RO110-0235 potently activated G$_s$ signaling of 5-HT$_4$-D^{100}A without stimulating the wildtype receptor (Figures 3B–E). The mutant receptor was selectively activated by multiple synthetic ligands (GR113808, GR125487, and RO110-0235) but not serotonin. We named it Rs1 (RASSL serotonin 1) (Figure 1).

Rs1 has a high level of constitutive signaling

We next examined the constitutive signaling of Rs1 in more detail. Rs1 showed greater constitutive signaling than the wildtype receptor at all levels of transfection (Figure 4A). Constitutive activity was observable when only 25 ng of receptor cDNA per 5×10^6 HEK293 cells was transfected (Figure 2). The highest level of constitutive activity, achieved with 5.4 μg of receptor cDNA per 5×10^6 HEK293 cells, was 1.5 times greater than that of the wildtype 5-HT$_4$ receptor (49.6 ± 1.25 nM vs. 32.5 ± 4.04 nM, $p < 0.005$) and >10-fold higher than that of the control receptors (the β_2-adrenergic and parathyroid hormone receptors), which have low levels of constitutive signaling (Figure 4A). Despite the high level of constitutive signaling, both the 5-HT$_4$ receptor and Rs1 could still be further activated by zacopride (Figure 4B).

Ligand-specific G$_q$ signaling in Rs1

Before constructing Rs1-5HT$_{2C}$ chimeras to make a G$_q$-signaling RASSL, we assayed inositol phosphate 1 (IP1) accumulation by Rs1 via constitutive or ligand-induced signaling. Rs1 showed no measurable difference in constitutive G$_q$ signaling (Figure 5) as compared to mock-transfected cells in the IP1 and calcium mobilization assays. Upon activation by cisapride, zacopride, RS23597, RS39604, or RS67333, Rs1 showed 2–3.5-fold higher G$_q$ signaling than the wildtype 5-HT$_4$ receptor ($p < 0.005$) (Figure 5). Surprisingly, GR113808, GR125487, and RO110-0235 activated predominately G$_s$ (Figure 2) and little G$_q$ signaling (Figure 5) by Rs1. These are the same ligands used to selectively
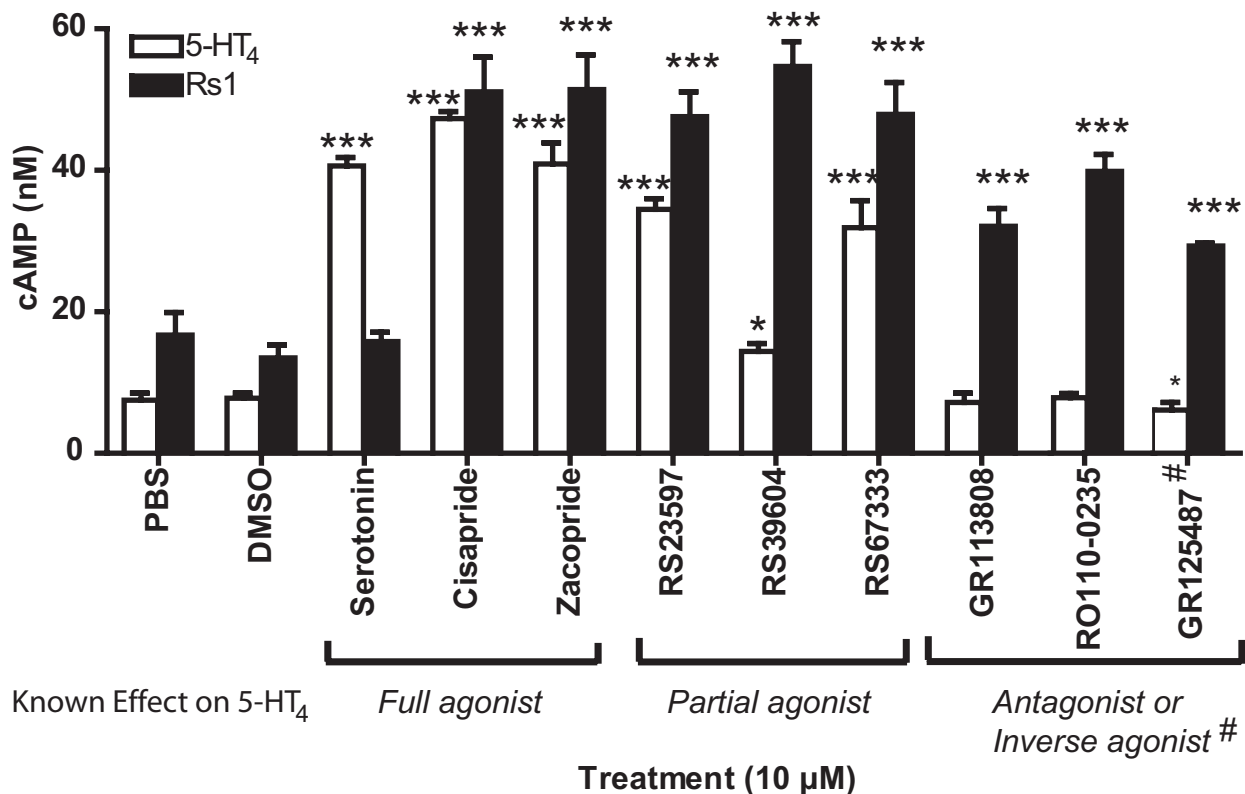
Figure 2. Rs1 is a G$_s$-coupled RASSL. Rs1 was efficiently activated by small compounds known to be full agonists (cisapride, zacopride), partial agonists (RS39604, RS67333, and RS23597), antagonists (GR113808, RO110-0235), or inverse agonists (GR125487) for the wildtype 5-HT$_4$ receptor. It was not activated by its endogenous agonist (serotonin). Values are mean±SD of three independent experiments in which 25 ng of 5-HT$_4$ or Rs1 receptor cDNA was electroporated into 5×10^6 cells. DMSO, dimethyl sulfoxide.
doi:10.1371/journal.pone.0001317.g002

A. cAMP (nM) vs Log [5HT] (M) — ○ 5-HT$_4$, ● Rs1

B. cAMP (nM) vs Log [Zacopride] (M) — ○ 5-HT$_4$, ● Rs1

C. cAMP (nM) vs Log [GR113808] (M) — ○ 5-HT$_4$, ● Rs1

D. cAMP (nM) vs Log [GR125487] (M) — ○ 5-HT$_4$, ● Rs1

E. cAMP (nM) vs Log [RS110-0235] (M) — ○ 5-HT$_4$, ● Rs1

F.

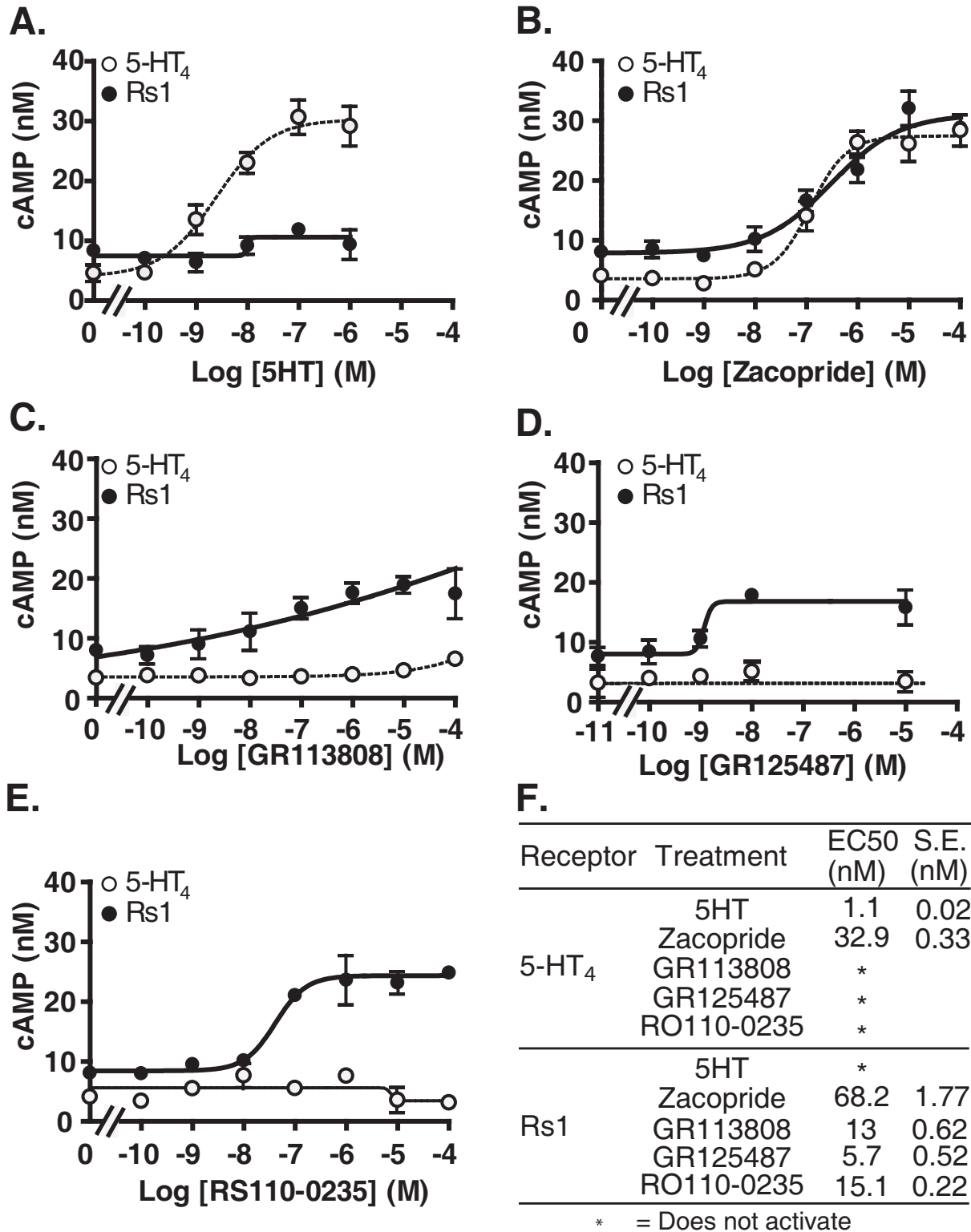| Receptor	Treatment	EC50 (nM)	S.E. (nM)
5-HT$_4$	5HT	1.1	0.02
	Zacopride	32.9	0.33
	GR113808	*	
	GR125487	*	
	RO110-0235	*	
Rs1	5HT	*	
	Zacopride	68.2	1.77
	GR113808	13	0.62
	GR125487	5.7	0.52
	RO110-0235	15.1	0.22

* = Does not activate

Figure 3. Rs1 activation occurs in the nanomolar range. (A–E) Rs1 transfectants were stimulated with increasing amounts of drugs. The D^{100}A mutation in Rs1 makes the receptor insensitive to serotonin. It was efficiently activated by GR113808, GR125487, and RO110-0235, which do not activate the wildtype 5-HT$_4$ receptor. Values are mean±standard deviation of three independent experiments in which 25 ng of 5-HT$_4$ or Rs1 receptor cDNA was electroporated into 5×10^6 HEK293 cells. (F) Best-fit estimate of the half-maximal effective concentration (EC50). Values are mean±SEM of three independent experiments.
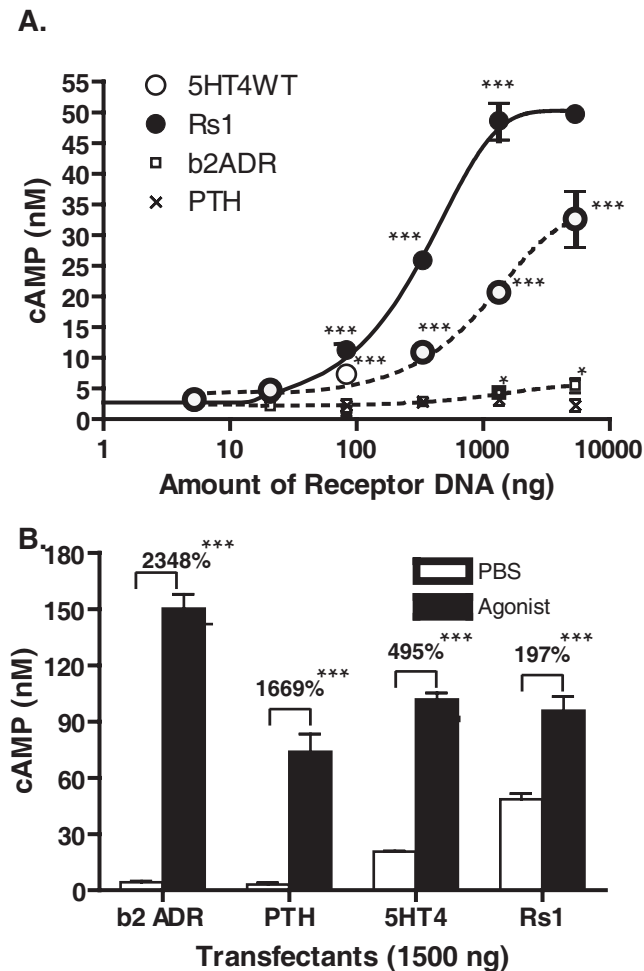doi:10.1371/journal.pone.0001317.g003

A.

B.

Figure 4. Rs1 has significant constitutive activity. (A) Rs1 and the wildtype 5-HT$_4$ receptor both had much higher constitutive activity than the β_2-adrenergic receptor (β_2-ADR) and parathyroid hormone receptor (PTH). ***$p < 0.005$, and * $p < 0.05$ vs. mock-transfected cells (t test). **(B)** Rs1 was still activated when 1.5 µg of receptor cDNA was electroporated into 5×10^6 HEK293 cells. Rs1 only showed a 197% increase over constitutive signaling. 5-HT$_4$ and Rs1 were stimulated with 1 µM of zacopride. β_2-ADR and PTH were stimulated with 1 µM of isoproterenol and human PTH (1-34) peptide, respectively. Activation of any of the four receptors resulted in significantly higher cAMP over the PBS mock treated samples. ***$p < 0.005$ (t test). All experiments were repeated three times. Values are mean±SD.
doi:10.1371/journal.pone.0001317.g004

activate the G$_s$ signaling of Rs1 without activating the G$_s$ signaling of the wildtype 5-HT$_4$ receptor (Figure 2). Therefore, we could use drugs with distinct chemical structures (Figure S1) to activate G$_s$ or G$_s$/G$_q$ signaling of Rs1. The once controversial use of conformation-specific ligands to alter G-protein coupling and other receptor functions has now been demonstrated in several other GPCRs [25–29]. This is the first time that agonist-dependent functional selectivity has been shown in a RASSL.

Replacing the C-terminus of Rs1 with 5-HT$_{2C}$ increases G$_q$ signaling

To make a purely G$_q$ signaling RASSL from Rs1, we exchanged the intracellular loops of Rs1 with those of the G$_q$-coupled human 5-HT$_{2C}$ receptor. By transferring domains at different junctions of intracellular loops, we made 12 different Rs1-5-HT$_{2C}$ chimeras

(Figures S2, S6, S7, S8, S9, S10, S11, S12, S13, S14, S15, S16, S17). To characterize them, we used RS23597 because it activated G$_q$ signaling of Rs1 but not the wildtype 5-HT$_4$ receptor, as measured by calcium mobilization assays (Figure S3C).

Replacing the second (i2) or third intracellular loop (i3) of Rs1 eliminated both G$_s$ and G$_q$ signaling (Figure S3). Only the carboxyl chimera (Rs1-C-5-HT$_{2C}$) showed enhanced G$_q$ signaling in response to cisapride, zacopride, RS23597, RS39604, and RS67333 (Figure 5), showing that the ligand-induced specificity of signaling was preserved. Constitutive and ligand-induced G$_s$ signaling were also largely preserved (Figures S3A, S3B).

These data indicated that i2 and i3 are both necessary for G$_s$-coupling of the 5-HT$_4$ receptor. They also suggested that the C-terminus of 5-HT$_{2C}$ contains a G-protein coupling domain for G$_q$ signaling or that the C-terminus of 5-HT$_4$ receptor promotes G$_q$ signaling. However, we were unable to completely alter the G-protein preference of this receptor for G$_q$ signaling. We amplified the G$_q$ signaling of Rs1 by domain swapping the C-terminus with 5-HT$_{2C}$. None of the other 12 chimeras showed enhanced G$_q$ signaling, even when multiple internal segments were combined. We did not proceed further with these experiments because substitutions of multiple internal domains also decreased cell-surface expression of the receptors (data not shown). Since G$_q$ signaling of Rs1-C-5-HT$_{2C}$ was activated by cisapride, zacopride, and RS23597 but not by serotonin, we named it Rs1.1 (Table 1).

Moreover, when Rs1-C-5-HT$_{2C}$ was activated by cisapride, zacopride, RS23597, RS39604, or RS67333, signaling was 4.5–6.8-fold higher than constitutive signaling ($p < 0.005$). Signaling increased 1.3-fold in response to GR125487 ($p < 0.05$), 1.7-fold in response to GR113808 ($p < 0.05$), and 2.2-fold in response to RO110-0235 ($p < 0.005$). This suggests that the carboxylic tail may play a role in functional selectivity in 5-HT$_4$ or 5-HT$_{2C}$ receptors (Figure S3).

A purely G$_s$ signaling RASSL with low levels of constitutive signaling

Since the high constitutive activity of the 5-HT$_4$-D^{100}A mutant causes significant phenotypes in transgenic mice [30] and could not be controlled by inverse agonists [31], we attempted to lower the Rs1 constitutive activity by making additional point mutations. We focused on the D^{66}N and W^{272}A mutations, which reduce constitutive signaling of the mouse 5-HT$_4$ receptor [31,32]. Rs1-D^{66}A, Rs1-D^{66}N, and Rs1-W^{272}A significantly reduced constitutive signaling (Figure 6A). The cell-surface expression of Rs1-D^{66}A and Rs1-D^{66}N was similar to that of Rs1 (Figure 6B), so the reduction in constitutive signaling was probably not linked to lower cell-surface expression. Surprisingly, the D^{66}A and D^{66}N mutations also abolished zacopride-induced G$_q$ signaling (Figure 6C). Thus, we created two RASSLs exhibiting pure G$_s$ signaling and low constitutive signaling. Unfortunately, the efficacy of the ligand-induced G$_s$ response was also significantly compromised, diminishing the utility of these receptors.

Engineering Rs1 for G$_i$ Signaling

To engineer a G$_i$-signaling RASSL based on Rs1, we replaced its intracellular loops with those of 5-HT$_{1A}$, a G$_i$ signaling receptor [33]. Of four Rs1-5HT$_{1A}$ chimeras (Figures 7, S4, S18, S19, S20, S21), only the two containing i2 and i3 from Rs1 were expressed at a level similar to that of Rs1 (Figure S5D). Replacing those loops abolished constitutive and ligand-induced G$_s$ signaling at both low and high levels of receptor cDNA (25 ng and 4.8 µg per 5×10^6 cells) (Figures S5A, S5B). Interestingly, this RASSL showed no evidence of constitutive signaling via the G$_i$ or G$_s$ pathway. These findings
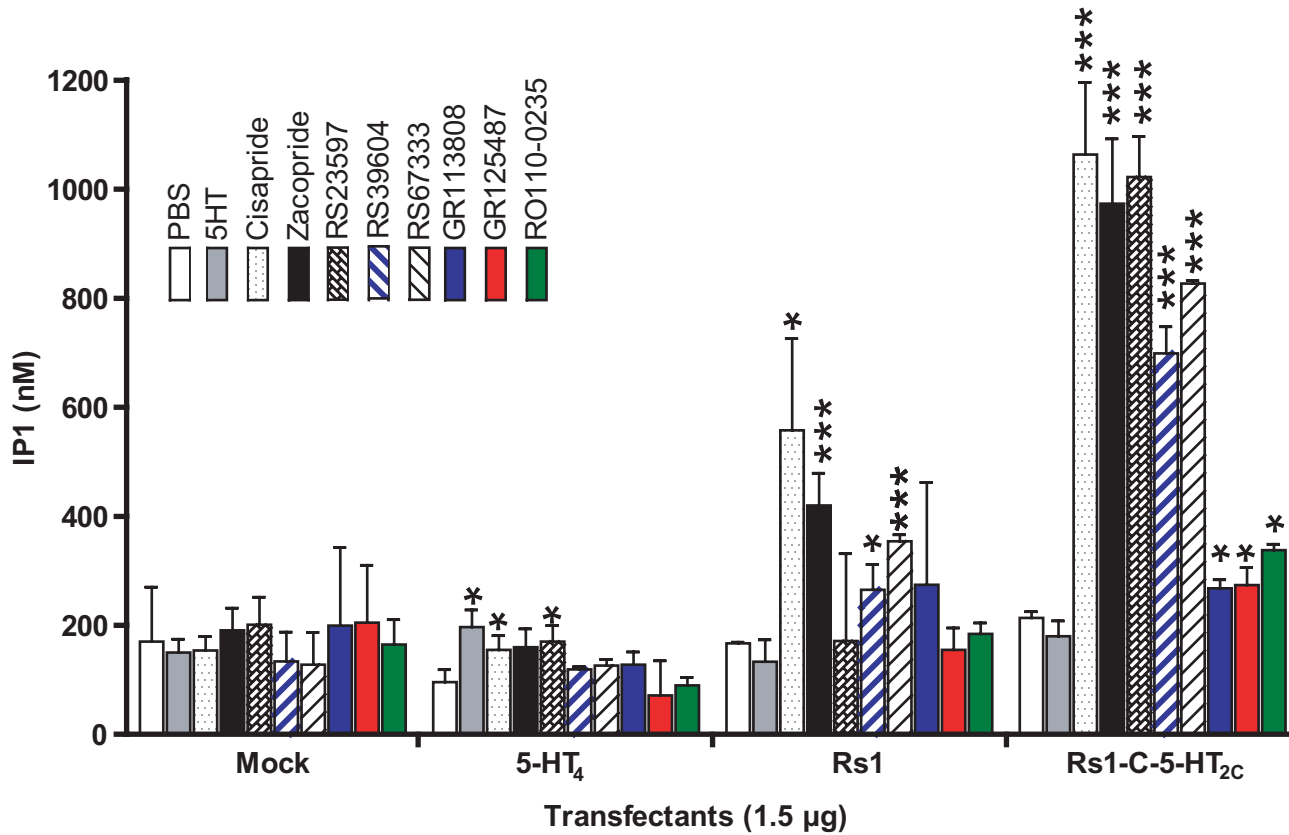
Figure 5. The wildtype 5-HT$_4$ receptor, Rs1, and the Rs1-C-5-HT$_{2C}$ chimera exhibit different G$_q$ signaling properties. G$_q$ signaling was analyzed by measuring the accumulation of inositol 1 phosphate (IP1). The 5-HT$_4$ receptor showed increased G$_q$ signaling when activated by serotonin, cisapride, and zacopride but not by RS39604 or RS67333, relative to mock treatment with HBSS. Rs1 showed significantly higher G$_q$ signaling than the wildtype 5-HT$_4$ receptor when activated by cisapride, zacopride, RS39604, and RS67333 but not by serotonin. G$_q$ signaling of Rs1-C-5HT$_{2C}$ chimera was activated by cisapride, zacopride, RS23597, RS39604, and RS67333 and was minimally activated by RO110-0235. GR113808 and GR125487 did not activate G$_q$ signaling by any of the three receptors. ***$p < 0.005$, * $p < 0.05$ vs. mock-transfected cells (t test). Values are $\pm$SD of three experiments.
doi:10.1371/journal.pone.0001317.g005

strongly imply that both i2 and i3 are required for G$_s$ signaling of Rs1. In addition, activation of the Rs1-i3-5-HT$_{1A}$ chimera with zacopride significantly inhibited cAMP accumulation induced by 10 μM apomorphine (agonist for dopamine 1 receptors) in HEK293 cells co-transfected with 1.5 μg of Rs1 receptor and 0.5 μg of dopamine 1 receptor (per 5×10^6 cells; Figure S5C and Figure S8A). This inhibition was smaller than that of μ-opioid receptor stimulated by [D-Ala2, D-Leu5]-enkephalin (DADLE). Both responses were abolished by 50 nM pertussis toxin, indicating the involvement of G$_i$ signaling (Figure 7B). Unfortunately, the potency (amount of drug needed to reach an effect) of ligand-induced G$_i$ signaling was significantly reduced (Figure 7C). While these results are encouraging, future experiments are needed to determine if low potency may be due to nonspecific effects, or will reproduce in other cell types. Since Rs1-i3-5HT$_{1A}$ exhibited G$_i$ but not G$_s$ signaling, we named it Rs1.3 (Figure 1, Table 1).

DISCUSSION

A new series of RASSLs
We report here a new series of RASSLs to study multiple G-protein signaling pathways. Many GPCRs activate multiple G-protein signaling pathways and exhibit a wide range of constitutive signaling activities. Our new RASSLs will help us better study the effect of stimulating canonical signaling pathways (G$_s$/G$_q$, G$_s$, G$_i$), with different constitutive signaling using a single receptor

backbone and different synthetic agonists. These new RASSLs will also allow a systematic examination of the different functional domains of the 5-HT$_{4B}$ receptor.

These new RASSLs could help us better dissect the physiological significance of constitutive signaling *in vivo*. Constitutive

Table 1. Controlling the G-protein signaling of Rs1

Receptors	Description	Constitutive signalling G$_s$	G- protein signaling		
			G$_i$	G$_s$	G$_q$
5-HT$_4$	5-HT$_4$	++	–	+++	+
Rs1	5-HT$_4$-D^{100}A	+++	–	+++	+
Rs1.1	Rs1-C-5-HT$_{2C}$	+++	N/A	+++	++
Rs1.2	Rs1-D^{66}A	+	N/A	+	–
Rs1.3	Rs1-i3-5-HT$_{1A}$	–	+	–	N/A

Constitutive signaling and ligand-induced signaling of Rs1 were successfully controlled by point mutations, drug choice, and domain swapping. G$_q$ signaling of Rs1 could be activated by zacopride or RS23597 but not GR113808, GR125487 or RO110-0235. The signaling was significantly increased by switching the carboxyl tail (Rs1.1). Attempts to decrease constitutive activity also decreased ligand-induced G$_s$ signaling and abolished G$_q$ signaling (Rs1.2). Replacing i3 of Rs1 with that of 5-HT$_{1A}$ resulted in a G$_i$ coupled RASSL with no G$_{s/q}$ signaling and G$_i$ signaling (Rs1.3).
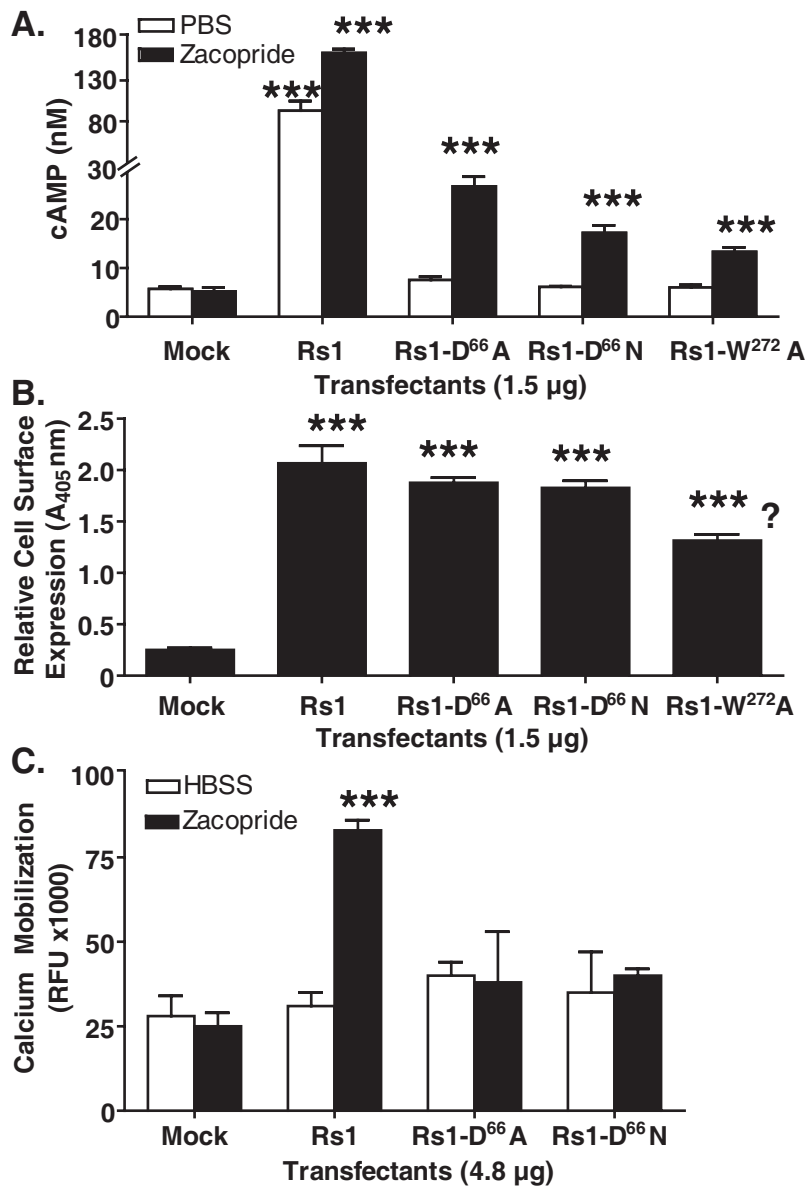doi:10.1371/journal.pone.0001317.t001

Figure 6. Abolishing the constitutive activity of Rs1 eliminates ligand-induced G_s and G_q signaling. (A) The $D^{66}A$, $D^{66}N$, and $W^{272}A$ mutations each decreased the constitutive signaling of Rs1, as shown by cAMP HTRF HiRange assay. **(B)** Cell-surface expression levels of Rs1-$D^{66}A$, Rs1-$D^{66}N$, and Rs1 were similar and higher than that of Rs1-$W^{272}A$. *** $p < 0.05$ vs. Rs1 (*t* test). **(C)** The $D^{66}A$ or $D^{66}N$ mutations also abolished G_q signaling of Rs1, as shown by HTRF IP1 assay.
doi:10.1371/journal.pone.0001317.g006

signaling is crucial for many physiological processes and many diseases. Up to 40% of all GPCRs [34], including the 5-HT$_4$ receptor [35], show significant constitutive activity, and Rs1 could be a good model for these receptors. Like the mouse 5-HT$_4$-$D^{100}A$ receptor [22], Rs1 had a higher level of constitutive signaling than the wildtype 5-HT$_4$ receptor. Using the tetracycline transactivator (Tet) system, we have already made an Rs1 transgenic mouse in which Rs1 expression is driven by the osteoblast-specific Col1α-1 2.3-kb promoter fragment. These mice exhibited dramatically increased bone formation [30]. These and other findings strongly suggest that constitutive signaling can drive potent phenotypic changes *in vivo*. We found only constitutive G_s signaling in Rs1 but did not observe any constitutive G_q signaling. It would be interesting to generate new or modify existing RASSLs with increased G_i or G_q constitutive signaling in the future to examine

the effect of both agonist-mediated and constitutive signaling in various physiological processes. Since signaling would only be dependent on expression rather than on circulating hormones, these RASSLs have an intrinsic advantage for studying constitutive signaling when combined with a Tet system.

In addition, the 5-HT$_4$ RASSLs have a large set of agonists with different activities that could be useful in future studies. Many of the Rs1 agonists used in this study activated Rs1 with an EC$_{50}$ in the nanomolar range, allowing to us activate Rs1 effectively. Having an abundant selection of ligands for Rs1 helped us find strong evidence of ligand-induced functional selectivity on Rs1. Having a greater variety of drugs to choose from could be valuable for *in vivo* studies. For instance, we could use GR125487 to activate Rs1 while suppressing the basal activity of wildtype 5-HT$_4$. Alternatively, we could potentially study Rs1 constitutive signaling by using drugs such
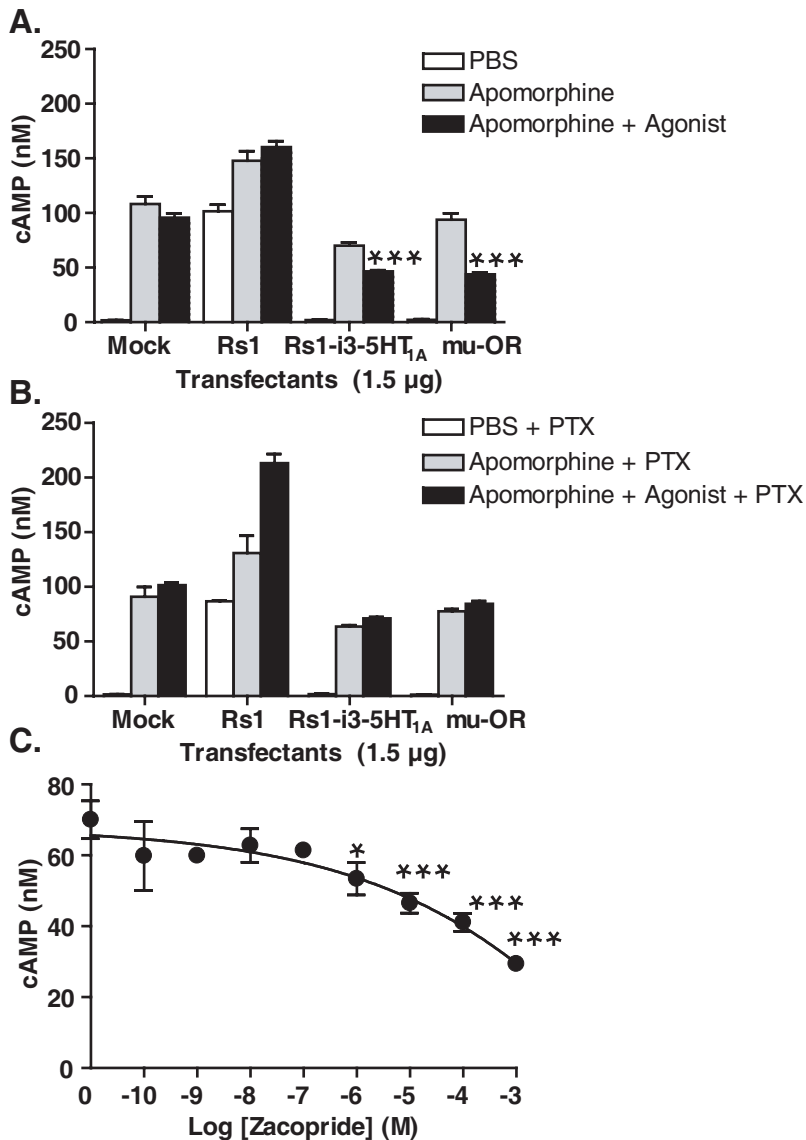
Figure 7. Replacing i3 of Rs1 with that of 5-HT$_{1A}$ results in a receptor, Rs1-i3-5-HT$_{1A}$, with weak G$_i$ signaling. (A) Rs1-i3-5-HT$_{1A}$ chimera decreased cAMP accumulation. It also showed little constitutive G$_s$ signaling, in contrast to Rs1. All HEK293 transfectants were electroporated with 0.6 μg of human dopamine 1 receptor and 1.5 μg of Rs1, Rs1-i3-5-HT$_{1A}$ chimera, or human mu-opioid receptor. The transfectants were stimulated with 10 μM apomorphine (agonist for the dopamine 1 receptor) to increase basal cAMP level in order to observe G$_i$ signaling. Rs1 and Rs1-i3-5-HT$_{1A}$ were then stimulated with 10 μM zacopride. The mu-opioid receptor was stimulated with 10 μM DADLE. ***p<0.005, * p<0.05 vs. apomorphine (t test). **(B)** Treatment with pertussis toxin (PTX) abolished the decreased cAMP accumulation of Rs1-i3-5HT$_{1A}$ and mu-opioid receptor, indicating that the decreased cAMP accumulation seen in panel (A) was due to G$_i$ signaling. The results are representative of three independent experiments. Values are mean±SD. **(C)** Rs1-i3-5HT$_{1A}$ required a large amount of zacopride for maximal G$_i$ response. The data are representative of two independent experiments. ***p<0.005, *p<0.05 (t test), zacopride vs. no treatement.
doi:10.1371/journal.pone.0001317.g007

as RO116-0086 or RO116-1148 [31] to decrease constitutive signaling of the wildtype 5-HT$_4$ receptor but not of Rs1.

Finally, this new series of RASSLs may make it possible to perform *in vivo* studies in which Rs1 is activated with minimal side effects. Since knockout of the 5-HT$_4$ receptor does not cause overt side effects [36], treatment with GR113808, GR125487, and R0110-0235 (antagonists and inverse agonists for the wildtype receptor) may have minimal side effects as well. This eliminates the need to knock out the endogenous 5-HT$_4$ receptors for most *in vivo* experiments [10]. Since the same agonists can be used to activate all of the RASSLs within this series and therefore+ engage

different G-protein signaling pathways (Table 1), we can more easily compare the effect of activating the G$_s$, G$_{s/q}$, or G$_i$ pathway.

While these new RASSLs may be useful for studying G protein signaling *in vivo*, they have several limitations. Despite significant efforts to screen all available antagonists, we could not identify inverse agonists that would lower constitutive signaling by Rs1. Fortunately, we can use conditional expression systems such as the tetracycline transactivator system to control Rs1 expression and constitutive activation. Although we were successful in finding mutations that reduced Rs1 constitutive signaling these same mutations adversely affected the agonist mediated signaling (Rs1.2), suggesting that the

$D^{66}N$ and $W^{272}A$ mutations are critical for all aspects of G_s signaling. Other mutations could be explored that may provide a more optimal reduction in G_s basal activity without affecting ligand-induced receptor activation. In addition, the relatively small G_i signaling of Rs1.3 can only be activated by a relatively high concentration of zacopride, thus limiting its usefulness *in vivo*.

Insights into G-protein signaling by Rs1

Our study yielded insights into the G-protein selectivity and functional selectivity (differential effects of ligands on the same receptor) of Rs1. Of the 12 Rs1-5-HT$_{2C}$ chimeras that are expressed on the cell surface, none of the i2 or i3 chimeras showed any G_s or G_q signaling. Evidently, these intracellular loops of Rs1 are crucial for signaling via those pathways. The importance of i2 and i3 for G_s signaling of Rs1 is further supported by the lack of G_s signaling by the Rs1-i2-5-HT$_{1A}$ and Rs1-i3-5-HT$_{1A}$ chimeras. This is the first study showing the importance of i2 and i3 in both G_s and G_q signaling of the human 5-HT$_4$-$D^{100}A$ receptor.

We also found that i3 domain swapping abolished all G_s signaling and enabled Rs1 to stimulate G_i signaling of 5-HT$_{1A}$. The role of i2 and i3 in the G_i signaling of 5-HT$_{1A}$ receptor has been extensively reported. The entire N-terminus [37] and C-terminus of i2 [38] of 5-HT$_{1A}$ are thought to be sufficient to support G-protein coupling, but not signaling. On the other hand, the N-terminus [39] and C-terminus of i3 of 5-HT$_{1A}$ [40,41] seem to be essential for the G_i signaling of 5-HT$_{1A}$. In fact, replacing the N-terminus of the i3 of the α_2-adrenergic receptor with that of 5-HT$_{1A}$ resulted in a chimera that signals like a 5-HT$_{1A}$ receptor when stimulated by a α_2-adrenergic receptor agonist [42]. Since Rs1-5HT$_{1A}$ chimeras with multiple internal domains replaced are not significantly expressed on the cell surface (data not shown), it may be difficult to further improve the potency of the Rs1.3 using our current approach. We hypothesize that replacing the N- and C-terminal portions of i2 and i3 instead of the whole i2 and i3 loops may increase the potency of Rs1.

We also showed that various drugs can differentially activate G-protein signaling of Rs1. Functional selectivity has been reported for many receptors. It led to divergent fates of internalization for the dopamine D_1 receptor [25], various binding specificities for gonadotropin-releasing hormone receptors, and different levels of activation of G proteins for the β_2-adrenergic [26], mu-opioid [27], dopamine D_2 [28], and human 5-HT$_{2A}$ [29] receptors.

The indoleamine derivatives GR113808, GR125487, and RO110-0235 did not fully activate G_q signaling of Rs1, Rs1.1, or the 5-HT$_4$ receptor. On the other hand, the benzamide derivatives cisapride, zacopride, RS23597, RS39604, and RS67333 activated the G_q signaling of Rs1. These findings may reflect distinct conformational changes caused by indoleamine and benzamide derivatives.

The possibility of functional selectivity is further supported by the results obtained with Rs1-C-5HT$_{2c}$ and Rs1 point mutants (Rs1-$D^{66}A$ and Rs1-$D^{66}N$). The $D^{100}A$ mutation and replacement of the C-terminus amplified G_q signaling by the 5-HT$_4$ receptor. The addition of $D^{66}A$ and $D^{66}N$ abolished G_q signaling. Since $D^{100}A$ is located in the binding pocket of the 5-HT$_4$ receptor, this mutation in Rs1 may have changed the configuration of the binding pocket, making the receptor more susceptible to G_q activation by zacopride and RS23597. This response was even more pronounced when the $D^{100}A$ mutation was combined with domain swapping of the C-terminus with that of 5-HT$_{2C}$. Thus, it is reasonable to hypothesize that these changes modified the ligand-selective receptor conformation [5], changing the receptor susceptibility to functional selectivity.

Conclusions

Our studies with Rs1 provide a proof-of-concept for making a series of RASSLs with different signaling properties. Recently, Armbruster *et al.* made a series of RASSLs based on the muscarinic M3 and M4 receptors, which have low constitutive activity. These RASSLs each couple different G-protein signaling pathways and can be activated by clozapine-N-oxide, an inert ligand with high bioavailability [16]. These RASSLs nicely complement our Rs1 RASSLs with varying constitutive activity. In addition, we predict that some RASSLs with the same canonical G-protein signaling (G_s, G_i, or G_q) will have different *in vivo* phenotypes due to noncanonical signaling. This growing collection of RASSLs will greatly facilitate our efforts to understand the physiological significance of the inherent signaling diversity of GPCRs.

An ideal series of RASSLs would have receptors with different combinations of low and high basal signaling, with robust ligand-induced effects for each major pathway, and potent inverse agonists. Although we have not achieved this goal with the Rs1 series, we are hopeful that it can be achieved with other receptors in the future. Indeed, RASSLs based on the muscarinic receptors [16] show great promise, as there are naturally occurring, or published mutants of the muscarinic reports that activate each of the major G protein signaling pathways.

MATERIALS AND METHODS

Constructing human 5-HT$_4$ mutant cDNA and Rs1-5-HT$_{1A}$, and Rs1-5-HT$_{2C}$ chimeras

The human 5-HT$_4$ receptor cDNA (a gift from Dr. Bryan Roth, University of North Carolina) was used in all experiments. To improve expression and allow detection of the receptor, we added a signal peptide from influenza hemagglutinin [43] and a FLAG epitope (DYKDDDDA) at the N-terminus. 5-HT$_4$ was then subcloned by PCR; the primers, ATCGATCGgcggccgcGTGAG-CAAGGGCGAGGAGCTGTTC and ATCGATCG gcggccgc-CTAAGTGTCACTGGGCTGAGCAGCC, were inserted into the Not1 restriction site (gcggccgc) of the pUNIV-5-HT$_{2C}$-INI plasmid to replace the 5-HT$_{2C}$-INI (a gift from Dr. Bryan Roth) in frame with the signal peptide and the FLAG epitope. The receptor was then mutated ($D^{100}A$) with a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primer GTCTT-GTTCGGACATCTCTG**gcc**GT CCTGCTCACAACGGCAT-CG (Figures 1, S2). The following mutant sense primers were used: 5-HT$_4$-$D^{66}A$, TTCATTGTATCTCTTGCTTTTGCG**gca**CT-GCT GGTTTCGGTGCTGG-GATG; 5-HT$_4$-$D^{66}N$, TTCA-TTGTATCTCTTGCTTTTGCG**aac**C TGCTGGTTTCGGT-GCT-GGTGATG; and 5-HT$_4$-$W^{272}A$, GTTGCTTCTGCC-TCTGCT GGGCGCCA**gcc**TTTGTCA-CCAATATTGTGG. The sense primer used to replace the carboxyl chimera for Rs1.1 (Table 1) was AGTTACTCTTCC**gcg**GCCGCGAATT-CAG TGGATCCACTAGTAAC. The Rs1-5-HT$_{1A}$ and Rs1-5-HT$_{2C}$ chimeras were made by PCR fusion. The mutations are indicated by boldface, lower-case letters.

HEK293 maintenance and electroporation

Early-passage (≤ 20) HEK293 cells were maintained in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with sodium pyruvate (Invitrogen) and 10% Fetalplex (Gemini Bio-Products, West Sacramento, CA). Receptors were electroporated into HEK293 cells as described [44]. The electroporated cells were reconstituted into a suspension using DMEM with 10% heat-inactivated, dialyzed fetal bovine serum (Thermo-Fisher Scientific,

Logan, UT). The transfection efficiency was monitored by flow cytometry, and the cell-surface expression of the receptor was determined by FLAG ELISA (enzyme-linked immunosorbent assay) the next day.

Drugs

5-HT, isoproterenol, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). Cisapride, zacopride, GR113808, GR1254875, RS23597-190 HCl, RS39604 HCl, and RS67333 HCl were from Tocris (Bristol, UK). Human parathyroid hormone peptide (amino acids 1–34) was from Bachem Biosciences (King of Prussia, PA). RO110-0235 was generously donated by Renee Martin (Roche, Palo Alto, CA).

Measuring cell-surface expression by FLAG ELISA

Cell-surface receptor expression was measured with a FLAG ELISA as described [45]. Cells seeded in poly-D-lysine-coated 96-well plates were fixed with 100 µl of 4% paraformaldehyde for 10 min at room temperature, washed, and stained with 100 µl of staining buffer (DMEM, 10% FBS, and 1 mM $CaCl_2$) containing anti-FLAG M1 antibody (1:1000; Sigma-Aldrich) for 1 h at 25°C. The samples were washed three times with wash buffer (PBS and 1 mM $CaCl_2$) and stained with 100 µl of staining buffer with rat anti-mouse IgG antibody conjugated with horseradish peroxidase (1:1000; Bio-Rad Laboratories, Hercules, CA). After 30 min, the samples were washed with wash buffer, placed on a rocker for 10 min, and washed again. This process was repeated two more times. Then, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate (200 µl; Sigma-Aldrich) was added to the samples. After rocking for 30 min, 200 µl of the substrate was transferred to new 96-well plates, and optical density was measured with Victor 3 (PerkinElmer, Waltham, MA) at 405 nm. All samples contain three replicates, and all experiments were repeated at least three times.

Measurement of cAMP production in intact cells

To improve assay consistency and minimize pipetting error in the 384-well plates, we modified the high-range HTRF assay (CisBio International, Bagnols-sur-Cèze, France) by seeding, stimulating, and lysing the cells in 96-well plates and using the lysate instead of live cells to determine cAMP production. The remainder of the analysis was performed according to the manufacturer's instructions

G$_i$ Assay

G$_i$ signaling was examined in cells transfected with 1.5 µg of receptor cDNA, 0.6 µg of human dopamine 1 receptor cDNA, and pcDNA3 (up to 6 µg). The co-transfectants were stimulated first with 100 µl of KRBG buffer containing IBMX for 10 min at room temperature and then with 50 µl of PBS containing 10 µM apomorphine (agonist for the dopamine 1 receptor) and 10 µM zacopride for 10 min at 37°C. The cells were lysed in 50 µl of lysis buffer, and 5 µl of lysate was used in the HiRange HTRF assay.

Fluorometric imaging plate reader assay to measure calcium mobilization

To measure calcium mobilization, 4.8 µg of receptor cDNA, 0.6 µg of DsRed plasmid, and 0.6 µg of human bombesin receptor cDNA were electroporated into 5×10^6 HEK293 cells as above [44]. Hank's balanced salt solution (10 ml) with 20 mM HEPES, 0.25 mM probenecid acid (Sigma-Aldrich), and 2% pluronic acid (Sigma-

Aldrich) was added to each bottle of Calcium 4 (Molecular Devices, Sunnyvale, CA), and 100 µl of the resulting solution was added to each well for 1 h at 37°C before measurement. Assays were performed with a FLEX Station (Molecular Devices), with excitation of 485 nm, emission of 525 nm, and cut-off of 515 nm, as recommended by the manufacturer.

Determination of IP1 production in intact cells

A modified version of the IP1 protocol was used (CisBio International). HEK293 cells were washed once with calcium-free PBS and dissociated from flasks with cell dissociation buffer (Invitrogen). Cells (5×10^6) were electroporated as described above. Then, 10^5 cells were placed in DMEM supplemented with 10% decomplemented, dialyzed against FBS, and seeded onto 96-well plates coated with poly-D-lysine. The next day, the cells were stimulated with agonists in 50 µl of 1× stimulation buffer for 30 min at 37°C and lysed for 10 min with 9 µl of lysis/detection buffer. Then, 14 µl of lysate was added to 384-well plates and subjected to High-Range HTRF assay as described above, except that 3 µl of cAMP-d2 and anti-cAMP-cryptate solution were added to each well.

Data analysis

cAMP and IP1 values were analyzed with GraphPad Prism 4 (GraphPad Software, San Diego, CA). Calcium mobilization results were analyzed with SoftMax Pro v5 (Molecular Devices). Statistical significance was determined with paired Student's t tests.

SUPPORTING INFORMATION

Figure S1   Chemical structures of the compounds used in the study. All the chemicals used in the experimental are shown.
Found at: doi:10.1371/journal.pone.0001317.s001 (1.92 MB EPS)

Figure S2   Rs1-5-HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s002 (12.17 MB EPS)

Figure S3   The second and third intracellular loops (i2 and i3) of Rs1 are crucial for Gs and Gq signaling. (A, B) Rs1-5-HT2C chimeras with swaps of i2 and i3 could no longer process Gs signals, at either 25 ng or 4.8 µg of receptor cDNA per 5×106 HEK293 cells. (C) Gq signaling of Rs1 was abolished when i2 and i3 of Rs1 were replaced with those of 5-HT2C. The Gq signaling was measured by calcium mobilization assay. (D) Only chimeras with a single domain swap were expressed on the cell surface. The results represent three independent experiments. All figures were representative of three independent experiments.
Found at: doi:10.1371/journal.pone.0001317.s003 (2.03 MB EPS)

Figure S4   Rs1-5-HT1A chimeras. All modifications were made on Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s004 (3.82 MB EPS)

Figure S5   Replacing the second or third intracellular loop (i2 or i3) of Rs1 with 5-HT1A alters G protein signaling. (A, B) Rs1-5-HT1A chimeras with swaps of the second (i2) and third intracellular (i3) loops no longer signal via the Gs pathway, regardless of whether cells were transfected with 25 ng or 4.8 µg of receptor cDNA per 5×106 HEK293 cells. This suggests that the second and third intracellular loops are crucial for acute Gs

signaling of Rs1. ***p<0.001 vs. mock transfected (t test). (C) Replacing i3 of Rs1 resulted in a Gi signaling receptor. All HEK293 transfectants were electroporated with 0.6 µg of the human dopamine 1 receptor and 1.5 µg of Rs1, Rs1-5HT1A chimeras, or the mu-opioid receptor. Rs1 and Rs1-5HT1a chimeras were treated with 10 µM apomorphine (an agonist for dopamine 1 receptor) or with 10 µM apomorphine and 10 µM zacopride. Transfectants with 0.6 µg of the human dopamine 1 receptor and 1.5 µg of the mu-opioid receptor served as positive controls. 10 µM DADLE was used in place of zacopride to stimulate mu-opioid receptors. ***p<0.005, *p<0.05 vs. apomorphine (t test). (D) Rs1, (i1, i2 and i30 were expressed on the cell surface. Cell-surface expression and calcium mobilization of the chimeras were examined at 4.8 µg of receptor DNA per 5×10^6 HEK293 cells. The results are representative of three independent experiments. Values are mean±SD. ***p<0.001 vs. mock transfected (t test).
Found at: doi:10.1371/journal.pone.0001317.s005 (1.67 MB EPS)

Figure S6 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s006 (2.98 MB EPS)

Figure S7 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s007 (2.96 MB EPS)

Figure S8 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s008 (3.00 MB EPS)

Figure S9 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s009 (2.99 MB EPS)

Figure S10 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s010 (2.99 MB EPS)

Figure S11 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s011 (2.99 MB EPS)

Figure S12 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s012 (3.00 MB EPS)

Figure S13 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s013 (2.99 MB EPS)

Figure S14 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s014 (3.00 MB DOC)

Figure S15 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s015 (2.99 MB EPS)

Figure S16 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s016 (3.00 MB EPS)

Figure S17 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s017 (2.96 MB EPS)

Figure S18 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s018 (3.00 MB EPS)

Figure S19 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s019 (2.96 MB DOC)

Figure S20 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s020 (3.40 MB EPS)

Figure S21 High-resolution representation of Rs1-5HT1A and Rs1-5HT2C chimeras. All modifications made to Rs1 (Figure 1). Red lines indicate the junctions of chimeras. The amino acids exchanged are shown by the amino acid alignment.
Found at: doi:10.1371/journal.pone.0001317.s021 (3.03 MB EPS)

Author Contributions

Conceived and designed the experiments: BC WC JN TN LP SC EH. Performed the experiments: WC. Analyzed the data: BC WC JN TN LP SC EH. Contributed reagents/materials/analysis tools: BC. Wrote the paper: WC.

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