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Applications of Yeast-Based Signaling Sensor for Characterization of Antagonist and Analysis of Site-Directed Mutants of the Human Serotonin 1A Receptor

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ABSTRACT: The monoamine neurotransmitter serotonin (5-HT) regulates a wide spectrum of human physiology through the 5-HT receptor family. One such receptor, the 5-HT_{1A} receptor (HTR1A), is the most widely studied subtype and represents a significant molecular target in medicinal and therapeutic fields. Yeast-based fluorescent reporter systems have proven to be especially useful for GPCR assays, since detection using a fluorescent reporter considerably simplifies measurement procedures. However, previously reported systems using enhanced green fluorescent protein (EGFP) as the reporter in yeast still showed low signal-to-noise (S/N) ratios, making EGFP difficult to apply as an easily accessible tool. Therefore, we constructed a refined yeast-based GPCR biosensor employing a high-sensitivity strain that incorporated both a Gα-engineered receptor and a fluorescent reporter (*ZsGreen*). As we report here, the refined yeast-based fluorescent biosensor was applied successfully to antagonist characterization and analysis of site-directed mutants of the HTR1A receptor. Pindolol, a known antagonist of HTR1A, specifically inhibited agonist-induced signaling, demonstrating the ease of evaluating inhibition effects using our reporter strain. Characterization of site-specific receptor mutants confirmed the role of specific targeted residues, including the highly conserved DRY motif, in the activation of HTR1A. Thus, our refined yeast biosensor strain, which incorporates a *ZsGreen* reporter and an engineered Gα receptor, is expected to serve as a simple and practical sensing tool for evaluating the ligand candidates and defining residues important to the function of human GPCRs.

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Introduction

Serotonin [5-hydroxytryptamine (5-HT)], a monoamine compound, is an essential neurotransmitter involved in diverse functions in nearly every organ system in the human body (Berger et al., 2009; Roth et al., 2004; Wacker et al., 2013). The activity of 5-HT is mediated through activation of members of a large family of 5-HT receptor proteins that can be grouped into seven subfamilies (5-HT_{1-7}) on the basis of sequence homology and signaling mechanisms (Kroeze et al., 2002). With the exception of the 5-HT_3 receptor, which is a ligand-gated ion channel, family members are heterotrimeric guanine nucleotide binding protein (G-protein)-coupled receptors (GPCRs). GPCRs belong to a large and diverse family of integral membrane proteins that participate in the regulation of many cellular processes and, therefore, represent key targets for pharmacological treatment. The serotonergic system is the target of many prescribed drugs, including atypical antipsychotics, anti-migraine medications, anxiolytics, and antidepressants (Berger et al., 2009).

5-HT also is a well-known mitogen for several types of non-tumoral cells (Nemecek et al., 1986; Sibella-Argüelles, 2001; Takuwa et al., 1989), and this mitogenic activity relates to the function of several known oncogenes (Julius et al., 1989). Depending on tumor type, the antagonists of either 5-HT_2 (Launay et al., 1996) or 5-HT_1 receptors have been found to inhibit 5-HT-induced tumor growth (Seuwen and Pouysségur, 1990). Among subtype 1 receptors, the 5-HT_{1A} receptor (HTR1A) is the most widely studied, and has been shown to be involved in the proliferation of several types of human tumor cells (Cattaneo et al., 1993, 1995; Ishizuka et al., 1992; Launay et al., 1996; Rajamannan et al., 2001). For instance, antagonists of HTR1A and known serotonin-uptake inhibitors such as pindobind,

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fluoxetine, and NAN-190 were reported to inhibit the growth of prostate tumor cell lines (Abdul et al., 1994, 1995; Dizeyi et al., 2004). Thus, HTR1A is a molecular target for cancer therapy. The development of a simple and convenient detection system for assaying HTR1A activity has great potential in the identification of new anti-neoplastic agents.

The eukaryotic unicellular yeast *Saccharomyces cerevisiae* is a useful microbial host organism for studying GPCRs as monomolecular models (Ishii et al., 2010; Minic et al., 2005). Because of their non-competitive and monopolistic G-protein signaling pathway, yeast serve as simplified experimental systems compared to the complicated signaling pathways used by higher eukaryotic cells (Iguchi et al., 2010; Togawa et al., 2010). Upon expression in yeast cells, a variety of human GPCRs have been demonstrated to transduce signals, either through the endogenous yeast Gα-subunit (Gpa1p) or via a yeast-human chimeric G-protein (Brown et al., 2000; Fukuda et al., 2011; Ishii et al., 2014). Yeast cells have been employed successfully for the expression of many types of human GPCRs, and have been adapted (by the addition of enzymatic and growth reporter genes) for use in various applications, including the screening of ligands and of mutagenized receptors (Klco et al., 2006; Minic et al., 2005; Nakamura et al., 2013a; Scarselli et al., 2007).

The recent establishment of fluorescent reporter gene assays for GPCRs provides a convenient assay for measuring receptor activity: the cell culture is simply diluted into buffers and the fluorescence is read using fluorometric instruments (Ishii et al., 2006, 2012a,b). A flow cytometer is a powerful tool, especially for comparative studies, quantitative screening, and cell sorting (Ishii et al., 2008, 2012a,b). However, systems using the enhanced green fluorescent protein-encoding locus (*EGFP*; derived from *Aequorea victoria*) as a reporter gene have exhibited comparatively weak fluorescence (Nakamura et al., 2013b). This property represents an important restriction in applying *EGFP* as a reporter for a wide variety of GPCRs and applications, because the resulting low signal-to-noise (S/N) ratio presents a detection limitation. We recently developed a refined yeast-based fluorescence biosensor by using the tetrameric *Zoanthus* sp. green fluorescent protein (encoded by *ZsGreen*). As reported by Nakamura et al. (2013b), the *ZsGreen* clearly exhibited brighter fluorescence than EGFP when used as a reporter in yeast. Specifically, a previously described EGFP reporter yeast strain was re-engineered by replacing a pair of *EGFP* reporter genes with *ZsGreen* genes, while also replacing the endogenous *GPA1* locus with a chimeric gene coding for a yeast Gpa1-human $G\alpha_{i3}$ protein (Gpa1/$G\alpha_{i3}$ transplant; G_{i3}tp). The resulting yeast strain showed more than 30-fold increased fluorescence than the previous *EGFP* reporter strain, and the improved version was used to detect agonist-promoted signaling by several human GPCRs whose natural ligands were short peptides, such as somatostatin, neurotensin, and angiotensin (Nakamura et al., 2013b, 2014b).

In the present study, we generated a fluorescent biosensor able to detect signaling by human HTR1A, an amine receptor, in response to the natural agonist 5-HT. This biosensor used living yeast cells harboring *ZsGreen* reporter genes and a $G_{i3}tp$ gene. While the previous *EGFP*-containing versions of HTR1A-expressing yeast strains produced low levels of green fluorescence in response to 5-HT exposure, the current *ZsGreen*-containing HTR1A-expressing strains displayed higher levels of fluorescence, with signal exhibiting dose-dependence in response to various 5-HT concentrations. We additionally demonstrated that our yeast biosensor strain permitted characterization of an antagonist and analysis of site-directed mutants of the human HTR1A receptor.

Materials and Methods

Plasmid Construction

All plasmids used in this study are listed in Table I. All primers used for plasmid construction are listed in Supplementary Table SI.

A DNA fragment encoding the human HTR1A protein was PCR-amplified from pPR3-HTR1A (Nakamura et al., 2013a) using

Table I. Yeast strains and plasmids used in this study.

Strain or plasmid	Specific features	Source
Strain		
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann et al. (1998)
IMFD-70	BY4741 *sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::P_{FIG1}-EGFP far1Δ*	Togawa et al. (2010)
IMFD-72	BY4741 *sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::EGFP his3Δ::P_{FIG1}-EGFP far1Δ gpa1Δ::$G_{i3}tp$*	Ishii et al. (2012a)
IMFD-70ZsD	BY4741 *sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::ZsGreen his3Δ::P_{FIG1}-ZsGreen far1Δ*	Nakamura et al. (2013b)
IMFD-72ZsD	BY4741 *sst2Δ::AUR1-C ste2Δ::LEU2 fig1Δ::ZsGreen his3Δ::P_{FIG1}-ZsGreen far1Δ gpa1Δ::$G_{i3}tp$*	Nakamura et al. (2013b)
Plasmid		
pGK421	Yeast expression vector containing *PGK1* promoter, *PGK1* terminator, 2 μ origin, and *MET15* marker	Togawa et al. (2010)
pGK421-HTR1A	Human HTR1A receptor expression in pGK421	This study
pGK421-HTR1A-D82N	Human HTR1A receptor (D82N) mutant expression in pGK421	This study
pGK421-HTR1A-D116N	Human HTR1A receptor (D116N) mutant expression in pGK421	This study
pGK421-HTR1A-C109A	Human HTR1A receptor (C109A) mutant expression in pGK421	This study
pGK421-HTR1A-C187A	Human HTR1A receptor (C187A) mutant expression in pGK421	This study
pGK421-HTR1A-S199A	Human HTR1A receptor (S199A) mutant expression in pGK421	This study
pGK421-HTR1A-T200A	Human HTR1A receptor (T200A) mutant expression in pGK421	This study
pGK421-HTR1A-N386V	Human HTR1A receptor (N386V) mutant expression in pGK421	This study
pGK421-HTR1A-D133A	Human HTR1A receptor (D133A) mutant expression in pGK421	This study
pGK421-HTR1A-R134A	Human HTR1A receptor (R134A) mutant expression in pGK421	This study
pGK421-HTR1A-Y135A	Human HTR1A receptor (Y135A) mutant expression in pGK421	This study

primers #1 and #2. The resulting amplicon was digested with *Nhe*I + *Bgl*II, and then ligated between the *PGK1* promoter (P_{PGK1}) and the *PGK1* terminator (T_{PGK1}) in a similarly digested pGK421 (Togawa et al., 2010), yielding the plasmid pGK421-HTR1A.

The plasmids for expressing mutated HTR1A proteins were constructed as follows. To introduce point mutations, gene fragments encoding the upstream and downstream parts of mutated HTR1A were respectively PCR-amplified from pGK421-HTR1A using primers #1 + #3 and #4 + #2 (for D82N); #1 + #5 and #6 + #2 (for D116N); #1 + #7 and #8 + #2 (for C109A); #1 + #9 and #10 + #2 (for C187A); #1 + #11 and #12 + #2 (for S199A); #1 + #13 and #14 + #2 (for T200A); #1 + #15 and #16 + #2 (for N386V); #1 + #17 and #18 + #2 (for D133A); #1 + #19 and #20 + #2 (for R134A); #1 + #21 and #22 + #2 (for Y135A). These amplified fragments then were used as the templates for overlap PCR with primers #1 and #2. The resulting mutated *HTR1A* amplicons were digested with *Nhe*I + *Bgl*II and ligated into similarly digested pGK421, resulting in plasmids designated pGK421-HTR1A-D82N, -D116N, -C109A, -C187A, -S199A, -T200A, -N386V, -D133A, -R134A, or -Y135A, respectively.

Yeast Strains and Media

All yeast strains used in this study are listed in Table I. Transformation with plasmids was performed using the lithium acetate method (Gietz et al., 1992).

Synthetic dextrose (SD) medium contained 6.7 g/L yeast nitrogen base without amino acids (YNB) (BD-Diagnostic Systems, Sparks, MD) and 20 g/L glucose. For SDM71 media, SD medium was adjusted to pH 7.1 with 200 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) (Nacalai Tesque, Kyoto, Japan). YPD medium contained 10 g/L yeast extract (Nacalai Tesque), 20 g/L peptone (BD-Diagnostic Systems), and 20 g/L glucose. Amino acids and nucleotides (20 mg/L histidine, 60 mg/L leucine, and 20 mg/L uracil) were supplemented into SD medium to compensate for relevant auxotrophies. For solid medium, agar was added at 20 g/L.

HTR1A Signaling Assay

HTR1A signaling assays followed previously described procedures (Nakamura et al., 2013b, 2014a) except for changes in the ligand. In brief, to assay signal activation from human HTR1A, the yeast strains transformed with the wild-type or mutated HTR1A expression plasmids were grown in SD medium (supplemented as needed) at 30 °C overnight; the cells then were inoculated into 5 mL of the respective fresh SD medium to give an initial optical density of 0.03 at 600 nm ($OD_{600} = 0.03$). The cells were incubated at 30 °C on a rotary shaker at 150 rpm for up to 18 h, then harvested, washed, and resuspended in water to yield an $OD_{600} = 10$. The resulting yeast cell suspensions were added (at 10 μL/well; $OD_{600} = 1$) to the wells of 96-well cluster dishes containing fresh SDM71 medium (80 μL/well) supplemented with 10× concentrated 5-HT (Nacalai Tesque) (10 μL/well) at various concentrations or with distilled water (without 5-HT; control). For antagonist assays, 100 μM or 1 mM 5-HT (80 μL/well) was alternatively supplemented with 10× concentrated pindolol (Tocris

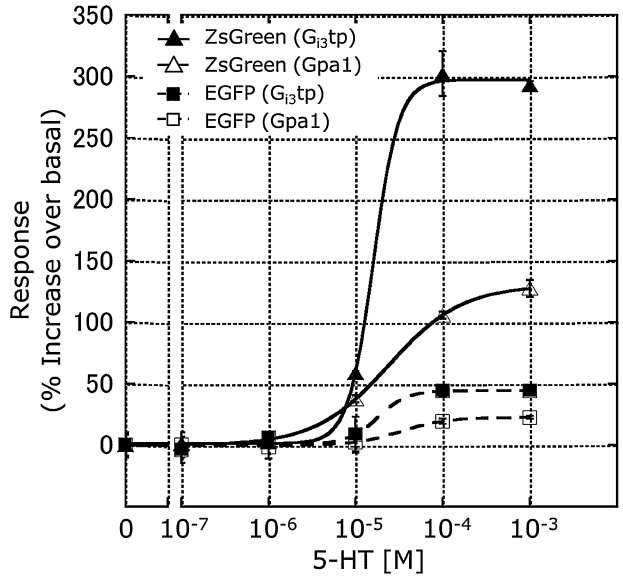

Figure 1. Dose–response curves for 5-HT-specific HTR1A signaling activities of recombinant yeast cells. Yeast strains IMFD-70 (EGFP (Gpa1)), IMFD-72 (EGFP (G_{i3}tp)), IMFD-70ZsD (*ZsGreen* (Gpa1)), and IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) were transformed with pGK421-HTR1A. All transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT at various concentrations. The GFP fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

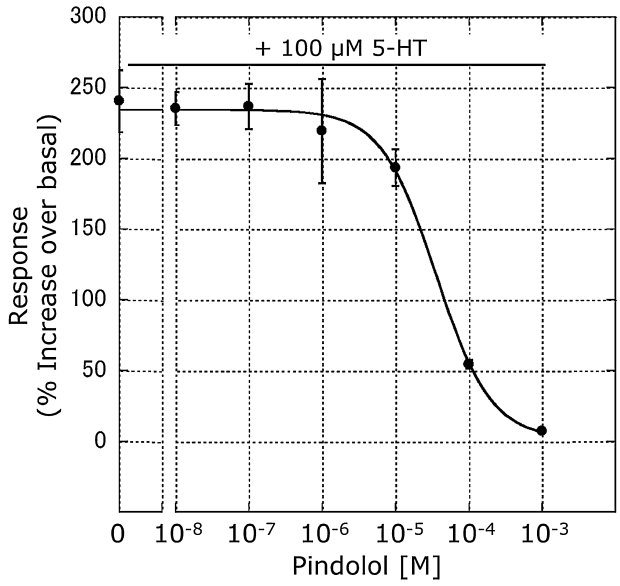

Figure 2. Inhibition by pindolol of 5-HT (100 μM)-mediated functional responses of HTR1A. The dose–response relationship to pindolol was determined using recombinant yeast strain IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) transformed with pGK421-HTR1A. The transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT (100 μM) and pindolol at various concentrations. The *ZsGreen* fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

Bioscience, Bristol, United Kingdom) (10 μL/well) at various concentrations or with distilled water (without pindolol; control). The plates were incubated at 30 °C with shaking (150 rpm) for 4 h. After incubation, the cultures were diluted with 1 mL of sheath fluid, and fluorescence was analyzed by flow cytometry (see below). Half-maximal effective concentration (EC_{50}) values and half-maximal inhibitory concentration (IC_{50}) values were determined using KaleidaGraph4.0 to fit the data to a dose-response logistic model.

Flow Cytometric Analysis

Flow cytometry measurements of green fluorescence followed the previously described procedure (Ishii et al., 2012b). In

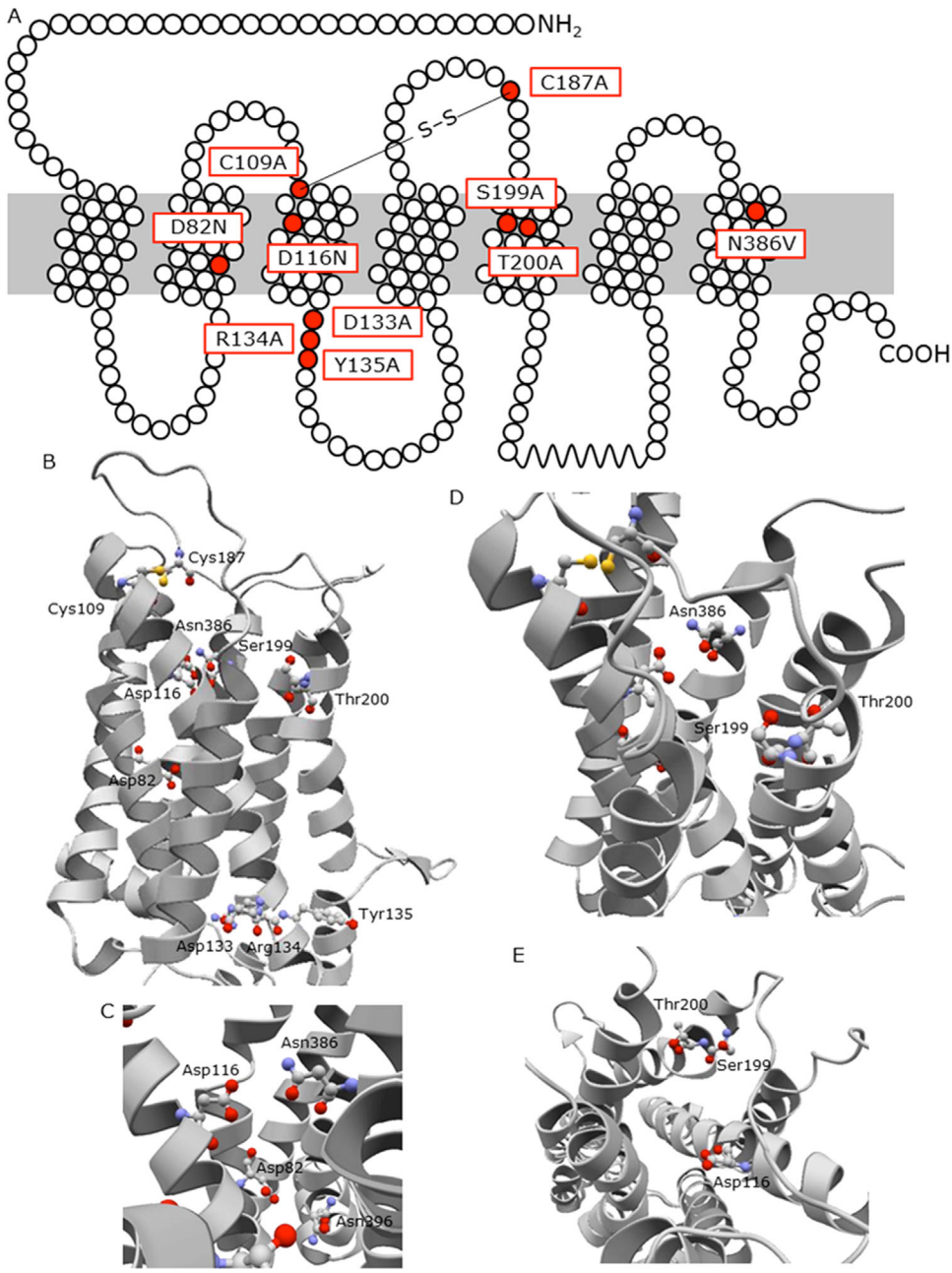

Figure 3. (**A**) Schematic illustration of the human HTR1A receptor showing the positions of the analyzed mutations. Each circle represents an amino acid residue. The positions of amino acids mutated in this study are indicated as red circles. (**B**) The predicted structure of HTR1A. SWISS-MODEL server and structural information of human HTR1B were used to obtain the modeling structures of HTR1A. (**C**) Close-up views of predicted structures of HTR1A in proximity of Asp82 and Asp116. (**D**) Close-up views of predicted structures of HTR1A in proximity of Ser199, Thr200, and Asn386. (**E**) Close-up views of predicted structures of HTR1A in proximity of a 5-HT binding site. (**B**–**E**) The colors are coded as a carbon (gray), a nitrogen (blue), an oxygen (red), and a sulfur (yellow). Figures were created using PDBjViewer version 4.4 (Kinoshita and Nakamura, 2004).

brief, fluorescent cells were detected using a BD FACSCanto II flow cytometer equipped with a 488-nm blue laser (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); the data were analyzed using BD FACSDiva software (v5.0; Becton, Dickinson and Company). The GFP fluorescence signal was collected through a 530/30 nm band-pass filter and the GFP-A mean of 10,000 cells was defined as 'green fluorescence intensity'.

Data Analysis

Data represent mean ± standard deviation for $n = 3$ (representing three independent transformants). The response was expressed as the percentage increase in the presence of a test reagent at the indicated concentrations over the respective basal (unstimulated) mean of fluorescence intensity value.

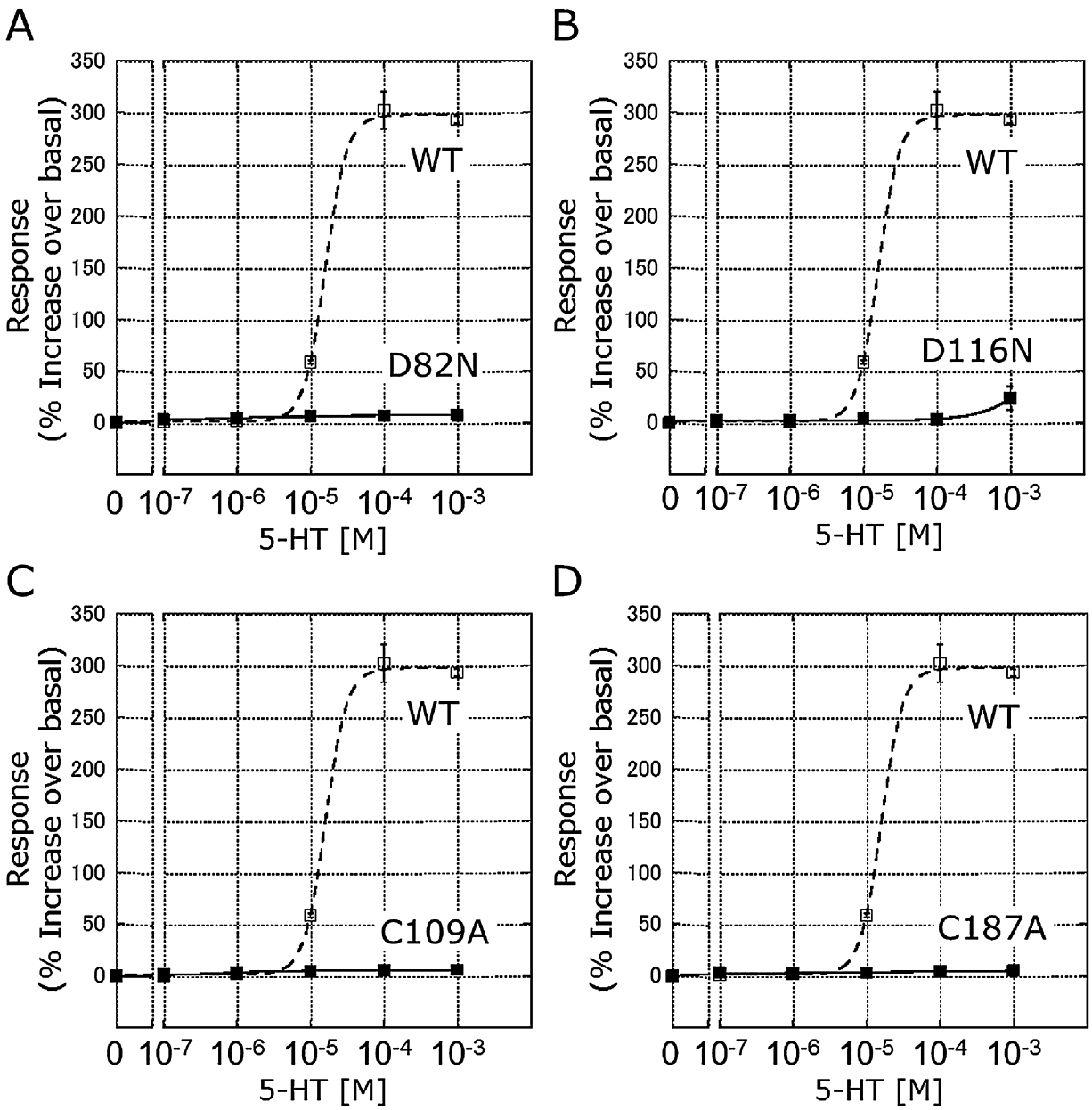

Figure 4. Ligand response of wild-type (WT) and mutant (D82N, D116N, C109A, C187A) HTR1A receptors. The dose–response relationship to 5-HT was determined using recombinant yeast strain IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) expressing WT (open squares, □) or mutant (filled squares, ■) HTR1A receptor. Dose–response curves are shown for (**A**) D82N mutant, (**B**) D116N mutant, (**C**) C109A mutant, and (**D**) C187A mutant. The transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT at various concentrations. The *ZsGreen* fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

Modeling of HTR1A Structure

The modeling structure of human HTR1A was obtained from SWISS-MODEL server (http://swissmodel.expasy.org/) using the structure of human HTR1B as a template (4IAR, http://www.rcsb.org/pdb/explore/explore.do?structureId=4IAR) (Wang et al., 2013). Figures were created using PDBjViewer version 4.4 (Kinoshita and Nakamura, 2004).

Western Blot Analysis

Western blot analysis basically followed previously described procedures (Ishii et al., 2012b) with some modifications. Collected cells were suspended in 10 mM Tris-HCl (pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to give an OD_{600} of 100, and 200 μL of cell suspension was disrupted using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) with 0.5 mm glass beads. Cell lysates were centrifuged at 1,000×*g* for 5 min and the pellet was then washed three times with 10 mM Tris-HCl containing 1 mM PMSF. The pellet was resuspended in 200 μL of SDS solubilization buffer (50 mM Tris-HCl [pH 7.8], 2% SDS [w/v], 100 mM ethylene diamine tetraacetic acid [EDTA], 40 mM 2-mercaptoethanol [2-ME]), and the suspension was boiled at 95 °C for 5 min and then centrifuged at 10,000×*g* for 5 min. The supernatant was collected and diluted with an equivalent volume of 2× sample buffer (25 mM Tris-HCl [pH 6.8], 4% SDS [w/v], 20% glycerol [w/v], 10% 2-ME [v/v], 0.1 mg/mL bromophenol blue [BPB]). Twenty microliters of each sample was loaded onto a 12.5% polyacrylamide gel and proteins were separated by electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane (Immobilon-FL; Millipore, Billerica, MA) by electroblotting. Western blots were performed as follows: rabbit anti-HTR1A antibody (GeneTex, Irvine, CA) for the wild-type or mutated HTR1A was primarily used at dilutions of 1:500 in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween-20 [v/v]). Anti-rabbit secondary antibody conjugated with alkaline phosphatase (Promega, Madison, WI) was used at dilutions of 1:5,000 in TBST. Chemiluminescent visualization was performed with Amersham CDP-Star Detection Reagent (GE Healthcare, Buckinghamshire, United Kingdom) and the signal was detected using a lumino-image analyzer LAS-4000 system (GE Healthcare).

Results and Discussion

Comparison of Agonistic Sensitivity Between HTR1A-Expressing Yeast Strains With Different Reporters and G-Proteins

The first aim of this study was to generate a fluorescence-based yeast biosensor for monitoring 5-HT-promoted signaling via human HTR1A, an amine receptor. The second aim was to assess the utility of the biosensor for characterization of antagonists and analysis of site-directed mutant proteins. To monitor HTR1A signaling, we utilized the previously constructed yeast strain IMFD-70 (Togawa et al., 2010), which contains two *EGFP* reporter genes, and the recently engineered yeast strain IMFD-70ZsD (Nakamura et al., 2013b), which contains two *ZsGreen* reporter genes (Table I). For each of these reporter genes, expression was controlled by the signal responsive *FIG1* promoter, allowing the detection of agonist-stimulated signal (Togawa et al., 2010).

In addition, all of these engineered yeast strains harbored *ste2*Δ, *sst2*Δ, and *far1*Δ alleles, rendering the cells deficient in (respectively) the yeast single GPCR; the yeast principal negative regulator of G-protein signaling (RGS); and the yeast G1-cyclin-dependent kinase inhibitor, which induces G1 arrest in response to signaling (Ishii et al., 2008, 2012b; Togawa et al., 2010). These deletions allow (respectively) expression of human GPCRs in the absence of native (yeast) receptor; hypersensitive agonist stimulation even at low ligand concentrations; and continued

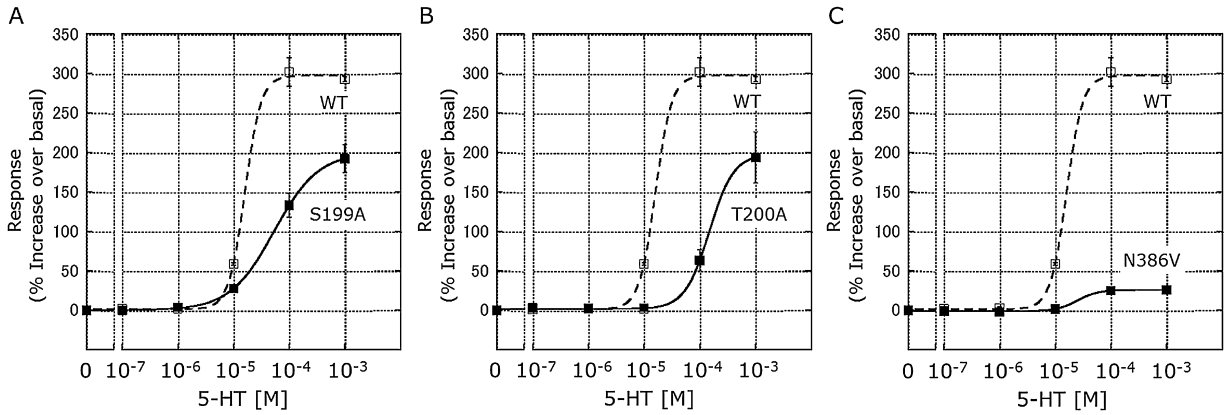

Figure 5. Ligand response of wild-type (WT) and mutant (S199A, T200A, N386V) HTR1A receptors. The dose–response relationship to 5-HT was determined using recombinant yeast strain IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) expressing WT (open squares, □) or mutant (filled squares, ■) HTR1A receptors. Dose–response curves are shown for (**A**) S199A mutant, (**B**) T200A mutant, and (**C**) N386V mutant. The transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT at various concentrations. The *ZsGreen* fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

growth (lack of growth arrest) and plasmid retention despite receptor activation.

The human HTR1A receptor is known to transduce signals in yeast cells through the endogenous yeast Gα-subunit (Gpa1p) (Brown et al., 2000). A yeast-human chimeric G-protein, in which the carboxyl-terminal 5 amino acid residues of Gpa1p (KIGII) are replaced with the equivalent residues from human $G\alpha_{i3}$ (ECGLY) (Gpa1/$G\alpha_{i3}$ transplant, G_{i3}tp), also has been shown to improve signal transmission via $G\alpha_i$-specific HTR1A in yeast (Brown et al., 2000).

To test the effects of the replacement of fluorescent reporter gene and introduction of the yeast-human chimeric G-protein for signaling in yeast cells expressing the human HTR1A receptor, we compared the fluorescence of four types of yeast cells that express the combination of either *ZsGreen* or *EGFP* reporter gene and either the yeast endogenous G-protein (Gpa1p) or the yeast-human chimeric G-protein (G_{i3}tp). The human HTR1A receptor was expressed via a multicopy episomal plasmid under the control of the constitutive *PGK1* promoter (pGK421-HTR1A) (Table I). Yeast strains IMFD-70 (EGFP, Gpa1p), IMFD-72 (EGFP, G_{i3}tp), IMFD-70ZsD (*ZsGreen*, Gpa1p), and IMFD-72ZsD (*ZsGreen*, G_{i3}tp) (Table I) (all harboring pGK421-HTR1A) were constructed and subjected to signaling assays. As shown in Figure 1, 5-HT induced dose-dependent increases in fluorescence responses in all four strains. The half-maximal effective concentration (EC_{50}) values were 33 μM (IMFD-70), 18 μM (IMFD-72), 24 μM (IMFD-70ZsD), and 16 μM (IMFD-72ZsD). These results indicated that the sensitivity of HTR1A for 5-HT was increased in by replacement of the endogenous Gα with the yeast-human chimeric Gα (G_{i3}tp). Since the EC_{50} value of HTR1A signaling for 5-HT has been reported as 1.3–3.2 μM in mammalian cells (Fargin et al., 1989; Knight et al., 2003; Raymond et al., 1992), the sensitivity of engineered yeast cells was comparable to that of mammalian cells. Upon the addition of agonist, yeast strains IMFD-70 and IMFD-72 showed modest responses with the maximal percentage increase over the basal value (%E_{max}) (23%, IMFD-70; 44%, IMFD-72) (Fig. 1). In contrast, the %E_{max} values of strains IMFD-70ZsD and IMFD-72ZsD were 127% and 302%, respectively (Fig. 1). Because the IMFD-72ZsD (*ZsGreen*, G_{i3}tp) strain expressing HTR1A provided the highest fluorescence response, further studies were conducted using the yeast strain IMFD-72ZsD.

Antagonist Inhibition Assay for Human HTR1A Using IMFD-72ZsD Yeast Strain

To test whether the IMFD-72ZsD background was appropriate for detecting antagonist inhibition of HTR1A, strain IMFD-72ZsD harboring pGK421-HTR1A was grown in the presence of 100 μM 5-HT in combination with various concentrations of pindolol, a known HTR1A antagonist. Fluorescence intensities of the yeast cells then were measured by flow cytometry (Fig. 2). Decreased fluorescence was observed with increasing concentration of pindolol; exposure to 100 μM pindolol decreased the fluorescence response to near basal levels (i.e., those seen in the absence of 5-HT). The half-maximal inhibitory concentration (IC_{50}) value was 36 μM. Repeating of the experiment at a 10-fold higher concentration of agonist (in medium containing 1 mM 5-HT and various concentrations of pindolol) yielded similar results (IC_{50} = 0.13 mM, Supplementary Fig. S1). These results indicated that our IMFD-72ZsD-based system would be appropriate for antagonist inhibition assays of the human HTR1A receptor.

Analysis of Site-Directed Mutants of Human HTR1A Using IMFD-72ZsD Yeast Strain

To test whether IMFD-72ZsD was appropriate for analysis of site-directed mutants of HTR1A, plasmids expressing distinct mutants of HTR1A were constructed (Table I). The 10 individual residues targeted within the human HTR1A receptor are shown in Figure 3A

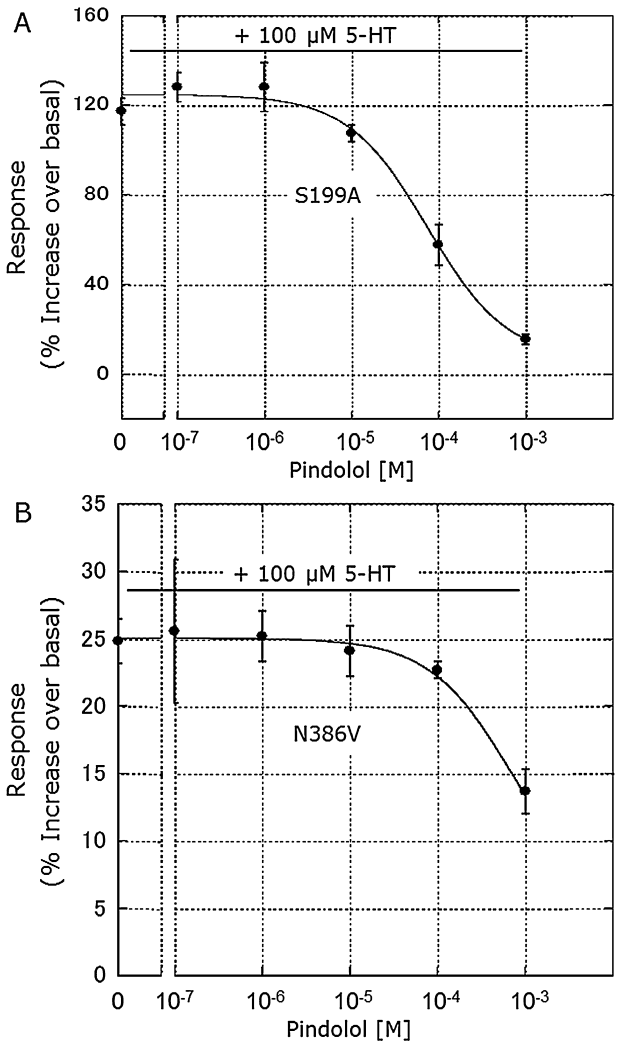

Figure 6. Inhibition by pindolol of 5-HT (100 μM)-mediated functional responses in mutant HTR1A receptors. The dose–response relationship to pindolol was determined using recombinant yeast strain IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) expressing HTR1A mutants. Dose–response curves are shown for (**A**) S199A mutant and (**B**) N386V mutant. The transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT (100 μM) and pindolol at various concentrations. The *ZsGreen* fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

and B. Initially, the previously reported HTR1A mutants (D82N, D116N, C109A, C187A, S199A, T200A, and N386V) were evaluated (Figs. 4 and 5). The expression levels of these mutant receptors were moderately equivalent to the wild-type HTR1A receptor (Supplementary Fig. S2).

The previous report suggested that the D82N and D116N individual substitutions yielded 60 to 100-fold decreases in receptor affinity for 5-HT (Ho et al., 1992). As shown in Figure 4A and B (respectively), the D82N and D116N mutants expressed in yeast were also severely compromised for the ability to promote 5-HT-specific signaling. It has been, respectively, considered that Asp82 is adjacent in space to Asn396 and important for a proper function of the receptor (Sylte et al., 2001), and that the negative charge of the Asp116 residue apparently acts as a counterion for the positive charge of the protonated amine of 5-HT and other HTR1A ligands (Ho et al., 1992; Kuipers et al., 1994; Sylte et al., 1996) (Fig. 3C). Our data obtained using yeast were considerably consistent with the data shown in these previous reports. Similarly, the Cys109 and Cys187 residues are known to be crucial for 5-HT-induced signaling, representing the two halves of a disulfide bridge critical for 5-HT binding to the receptor (Harikumar et al., 2000; Pucadyil et al., 2005; Raymond et al., 1999) (Fig. 3B). As expected, the C109A or C187A mutations effectively abolished HTR1A signaling (Fig. 4C and D, respectively). Many other GPCRs also form disulfide bridges between Cys of the third transmembrane helix (TM 3) and the second extracellular loop, and the structure would be important for ligand-dependent activation (Dohlman et al., 1990; Karnik et al., 2003; Klco et al., 2005). Additionally, the S199A and T200A substitutions were shown to be detected a significant reduction of agonist binding affinities (Ho et al., 1992). Yeast strains expressing HTR1A-S199A and T200A retained partial activities (Fig. 5A and B). The $\%E_{max}$ values for these mutants exhibited 192% and 194% increases, and the EC_{50} values were roughly 50 and 150 μM, respectively. These results were also consistent with the previous reports, which have suggested that the 5-HT binding site is located between TM 3 (with Asp116 as the key recognition site) and TM 5 (providing Ser199 and Thr200 to interact with the 5-OH group of serotonin) (Jacoby et al., 1999; Klabunde and Hessler, 2002) (Fig. 3D and E). In contrast, N386V has been reported to present similar affinity for 5-HT with wild-type receptor while reducing the affinity for the antagonist pindolol (Guan et al., 1992). Indeed, EC_{50} value of the N386V mutant was almost the same as that of wild-type HTR1A (16 μM, wild-type; 25 μM, N386V), although the yeast strain expressing N386V significantly decreased the $\%E_{max}$ value (26%) (Fig. 5C and Supplementary Fig. S3). This result implies that the N386V substitution alters HTR1A signaling activity with only modest changes in affinity for 5-HT. The side chain of Asn386 would be important for active-state conformational change but not for binding of 5-HT (Fig. 3D). Additionally, antagonist inhibition assays were performed for strains expressing the N386V and S199A mutants, using pindolol in the presence of 100 μM 5-HT. Whereas the wild-type and S199A HTR1A strains displayed similar patterns of signal inhibition by pindolol, N386V exhibited only mild inhibition even in the presence of 100 μM pindolol (Figs. 2 and 6). The IC_{50} values were 36 μM (wild-type), 74 μM (S199A), and 710 μM (N386V). These results demonstrated that the IMFD-72ZsD yeast strain reproduced the properties of human HTR1A mutants determined in mammalian cell culture, suggesting that the IMFD-72ZsD background was appropriate for analysis of site-directed receptor mutants.

To extend our mutant analysis, we examined the signaling activities of previously unreported HTR1A mutants (D133A, R134A, or Y135A) using the IMFD-72ZsD yeast strain. The DRY motif, which is highly conserved in the second intracellular loop of many GPCRs (Rovati et al., 2007; Savarese and Fraser, 1992), was selected as the mutation target, because the DRY motif is generally considered to function as a switch for the activation of G-proteins in response to agonist binding (Fig. 3B). The expression levels of these

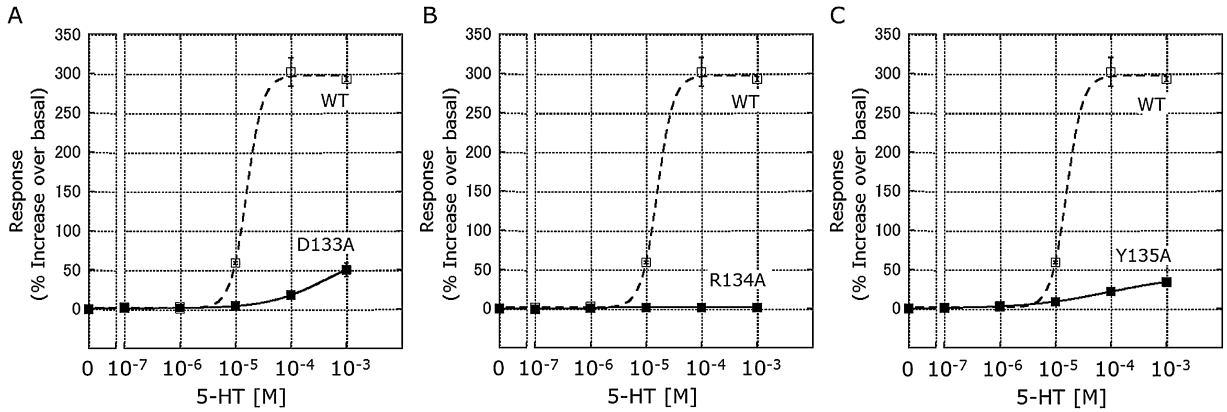

Figure 7. Ligand response of wild-type (WT) and mutant (D133A, R134A, Y135A) HTR1A receptors. The dose–response relationship to 5-HT was determined using recombinant yeast strain IMFD-72ZsD (*ZsGreen* (G_{i3}tp)) expressing WT (open squares, □) or mutant (filled squares, ■) HTR1A receptor. Dose–response curves are shown for (**A**) D133A mutant, (**B**) R134A mutant, and (**C**) Y135A mutant. The transformants were grown in SD selective medium for 18 h. The cells then were incubated for another 4 h in pH-adjusted SD selective medium supplemented with 5-HT at various concentrations. The *ZsGreen* fluorescence of 10,000 cells was measured by flow cytometry. Each data point represents the mean ± standard deviation of three independent transformants.

mutant receptors were almost equivalent to the wild-type HTR1A receptor (Supplementary Fig. S2). As shown in Figure 7, the D133A, R134A, and Y135A single mutants were severely compromised for signal activation in response to 5-HT. These results showed that the IMFD-72ZsD yeast strain was appropriate for use in screening to identify amino acid residues critical for the signaling activity of HTR1A. Because yeast *S. cerevisiae* can functionally express a variety of human GPCRs (but not all GPCRs) (Ishii et al., 2010; Minic et al., 2005), our system would be applicable for assaying other human GPCRs.

Conclusion

In the present study, we constructed a yeast strain (IMFD-72ZsD) that expresses the human HTR1A receptor. This strain incorporates a highly sensitive fluorescent reporter (*ZsGreen*) along with a chimeric Gα protein, yielding increased fluorescence intensity and increased response to 5-HT-specific HTR1A signaling compared to the previous version of this strain. This improvement permitted successful application of the biosensor strain for characterization of an antagonist and analysis of site-directed mutants of the human HTR1A receptor. This yeast biosensor is expected to facilitate research in the development of HTR1A-specific anti-cancer drugs, and also may be of value as a tool for the characterization of other human GPCRs.

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