Serotonin G Protein-Coupled Receptor-Based Biosensing Modalities in Yeast

Bettina Lengger, Emma E. Hoch-Schneider, Christina N. Jensen, Tadas Jakočiūnas, Anja A. Petersen, Thomas M. Frimurer, Emil D. Jensen, and Michael K. Jensen*

Cite This: *ACS Sens.* 2022, 7, 1323−1335

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Serotonin is a key neurotransmitter involved in numerous physiological processes and serves as an important precursor for manufacturing bioactive indoleamines and alkaloids used in the treatment of human pathologies. In humans, serotonin sensing and signaling can occur by 12 G protein-coupled receptors (GPCRs) coupled to Gα proteins. In yeast, human serotonin GPCRs coupled to Gα proteins have previously been shown to function as whole-cell biosensors of serotonin. However, systematic characterization of serotonin biosensing modalities between variant serotonin GPCRs and application thereof for high-resolution serotonin quantification is still awaiting. To systematically assess GPCR signaling in response to serotonin, we characterized reporter gene expression at two different pHs of a 144-sized library encoding all 12 human serotonin GPCRs in combination with 12 different Gα proteins engineered in yeast. From this screen, we observed changes in the biosensor sensitivities of >4 orders of magnitude. Furthermore, adopting optimal biosensing designs and pH conditions enabled high-resolution high-performance liquid chromatography-validated sensing of serotonin produced in yeast. Lastly, we used the yeast platform to characterize 19 serotonin GPCR polymorphisms found in human populations. While major differences in signaling were observed among the individual polymorphisms when studied in yeast, a cross-comparison of selected variants in mammalian cells showed both similar and disparate results. Taken together, our study highlights serotonin biosensing modalities of relevance to both biotechnological and potential human health applications.

KEYWORDS: GPCR, serotonin, 5-HT receptor, polymorphism, biosensor, yeast
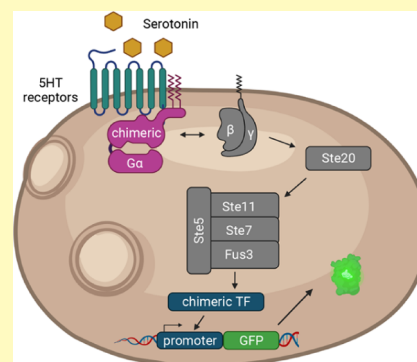
Serotonin is a monoamine neurotransmitter largely confined to the digestive and central nervous systems of humans and implicated in a plethora of biological functions in humans, including functions in mood, feelings, eating, and sleeping.[1] In humans, a total of 13 serotonin receptor genes and 1 pseudogene are found and encode for a total of 12 serotonin G protein-coupled receptors (GPCRs) and 1 ionotropic channel, together mediating serotonin signaling.[2] Collectively, GPCRs are seven-transmembrane proteins, which allow cells to respond to extracellular stimuli by coupling the binding of a ligand to the activation of intracellular signaling pathways.[3] The intracellular signaling through GPCRs occurs *via* a ligand-mediated conformational change, with the GPCRs serving as guanine-exchange factors to activate heterotrimeric guanine nucleotide-binding proteins (G proteins), consisting of the three subunits Gα, Gβ, and Gγ.[4] Binding of a ligand to the GPCR promotes a conformational change in the receptor, which in turn activates the GPCR-bound Gα subunit of the G protein. The exchange of Gα-bound GTP to GDP promotes the dissociation of the G protein from the GPCR and the separation of the Gα subunit from the Gβγ dimer.[5] Following dissociation, the subunits relay intracellular signaling to ultimately effectuate an adequate transcriptional reprograming

in response to the extracellular milieu.[6] While these modules constitute the core of GPCR signaling, a dearth of knowledge of how the approx. 800 GPCRs encoded in the human genome couple through 16 different Gα subunits challenges our understanding of GPCR signaling,[7] notwithstanding the structure−affinity relationship between the great diversity of ligands and the GPCRs which have evolved to respond to them, including light, hormones, and small molecules, such as serotonin.[8,9]

For more than 3 decades, yeast has served as a platform for studying human GPCRs[10,11] with great potential in both medical and biotechnological application areas.[8] The vast majority of GPCR studies in yeast are based on the mating pathway naturally activated by pheromones through the yeast mating factor GPCRs Ste2/3.[12] Upon ligand activation, successful coupling of a heterologous GPCR with the yeast

mating pathway can occur through coupling to the yeast Gα protein Gpa1, which subsequently activates the mitogen-activated protein (MAP) kinase cascade consisting of Ste5/Ste7/Ste11, ultimately resulting in the activation of hundreds of pheromone-responsive genes.[8] While a few studies demonstrated the coupling of human GPCRs to Gpa1,[10,13,14] our ability to couple exogenous GPCRs to the mating pathway was greatly facilitated by the development of chimeric Gα proteins, consisting of Gpa1 with the final five amino acids swapped with those of a Gα protein known to interact with a given GPCR.[11,15−17] Likewise, the knockout of SST2, a negative regulator of GPA1, and FAR1, an inducer of cell cycle arrest during mating, has been a key step to increase heterologous GPCR signaling in yeast, as demonstrated previously,[18,19] ultimately enabling the development of whole-cell biosensors based on >50 GPCRs.[8,16,20]

For serotonin GPCRs, 3 out of the 12 human serotonin GPCRs have successfully coupled to the yeast mating pathway, namely, 5-HT1A,[11] 5-HT1D,[11,15] and the 5-HT4 receptors.[14,16,20,21] Likewise, in yeast, 10 mutants of the 5-HT1A receptor have been engineered to elucidate polymorphisms impacting receptor activation,[15] while Kapolka et al. recently reported the coupling of 5-HT4 with all 10 chimeric Gα protein variants.[20] Importantly, while yeast only provides a minimal platform for studying heterologous GPCR signaling through its mating pathway, physiologically relevant pharmacological properties and receptor specificity for the Gα chimera have shown to be consistent with the EC$_{50}$ values and cognate mammalian Gα protein coupling, respectively.[11,15] Likewise, for biotechnological purposes, microbial production in yeast of a range of GPCR agonists with clinical applications can offer a solution for supply chain stability and scalability of production.[22,23] However, optimizing heterologous biosynthetic pathways for human bioactives in yeast using metabolic engineering is often a tedious endeavor, involving complex engineering to create optimal pathway designs for fermentation-based manufacturing of such bioactives. Here, GPCR-based serotonin biosensors have shown promising results with a 5-HT1A-based sensor coupled to the Gpa1/Gαi3 chimera, resulting in a sensor with 300% increase over basal fluorescence after activation with serotonin.[15] Using a 5-HT4-based sensor, Ehrenworth et al. demonstrated that yeast-produced serotonin could be detected with a 2-fold change.[14] Still, while serotonin receptors have been characterized in yeast, no systematic approach has been performed to study the Gα protein coupling of all human serotonin receptors, and the use of current best-performing serotonin GPCRs for biotechnological purposes suffers from low dynamic output ranges and lack of established high-resolution workflows.[14]

In this study, we describe the systematic characterization of human serotonin GPCR-mediated biosensing modalities in yeast. Specifically, we characterize signaling in 144 different engineered yeast strains expressing all 12 human serotonin receptors in combination with 12 different Gα protein designs at two different pHs and furthermore present the characterization of 19 serotonin GPCR polymorphisms mined from the 1000 Genome Project.[24,25] From this, we report serotonin dose−response curves for >30 biosensing designs and apply an optimized and high-performance liquid chromatography (HPLC)-validated biosensing workflow for high-resolution screening of a yeast strain library engineered to produce serotonin. Collectively, these results provide a new biosensing

resource-based chimeric Gα coupling of the human serotonin GPCRs.

■ EXPERIMENTAL SECTION

Cultivation of Bacteria and Yeast. The chemically competent *Escherichia coli* DH5α strain was used for plasmid propagation and cloning. *E. coli* strains were grown in 2xYT media supplemented with 100 μg/mL ampicillin at 37 °C and 250 rpm. Yeast was grown in a synthetic complete-dropout medium, made with 6.7 g/L yeast nitrogen base without amino acids (Sigma), 1.4 g/L yeast synthetic dropout medium supplements (Sigma) lacking uracil, histidine, leucine, and tryptophan, supplemented with 2% w/v glucose. Histidine, uracil, leucine, and tryptophan were added as needed for auxotrophic selection. Yeast in preculture tubes was grown at 30 °C and 250 rpm, while incubation in 96-well deep plates took place at 30 °C and 300 rpm.

GPCR and 5-HT4b Variant Sourcing. The protein sequences of GPCRs were selected on uniprot.com and translated into nucleic acid sequence using the EMBOSS Backtranseq tool[9,26] and ordered as biobricks *via* Twist Bioscience, except for gBL10, which was taken from the NCBI database entry NM_000870.6. The complete list of all synthetic genes used can be found in Supporting Information Table S6.

For human 5-HT4b variants, the identified transcript for human 5HT4b (ENST00000377888) was identified on Ensembl through the International Genome Sample Resource database to find information on global population variants and distribution.[24,25] Single amino acid variants and their population frequency were identified through the Ensembl genome browser using the Haplosaurus tool for the previously specified transcript ID.[25] Due to the codon-optimization of the 5HT4b receptor, variants were designed with the amino acid variation of interest irrespective of base-pair changes. Of the 20 5-HT4b single amino acid variants listed on the Haplosaurus protein-haplotype browser on Ensembl, 19 were tested in *Saccharomyces cerevisiae* due to a cloning issue of one of the variants.

Plasmid Construction and Transformations in *E. coli*. All plasmids in this study were cloned using uracil specific excision reagent (USER) cloning (New England Biolabs) and the EasyClone method.[27] Genetic parts for assembly into plasmids and USER vector plasmids were amplified using PhusionU polymerase (Thermo Fisher Scientific). Plasmids containing the GPCRs had a Kozak sequence (AAAACA) in front of the start codon of the receptor. Synthetic genes were ordered from TWIST Bioscience, and custom oligos were ordered from IDT or used from previous publications.[28] The complete list of all gBlocks and plasmids and yeast strains can be found in Supporting Information Tables S6−S8. The plasmids were transformed into the chemically competent DH5α strain by heat-shocking for 45 s at 42 °C and recovered on Luria−Bertani plates supplemented with 100 μg/mL ampicillin.

Construction of Yeast Strains. For plasmid-based GPCR expression, library strains were constructed by transforming plasmids containing the respective serotonin GPCR under a CCW12 promoter and a HIS3 marker. For yeast transformations, plasmids and linear DNA parts for integration were transformed into yeast using the lithium acetate/single-stranded carrier DNA/PEG method.[29] The plasmids were transformed into yeast strains yWS2261−yWS2272, representing optimized sensor strains with different Gα protein backgrounds.[16] The transformed yeast cells were selected on SC-HIS plates.

For integration of the GPCRs into the yeast genome, plasmids with overlap to the genomic sites as described by Jessop-Fabre et al. were engineered to contain the respective genetic sequence to be integrated [serotonin GPCR, 5-HT4(b) variant].[27] The plasmids were NotI-digested for 4 h, the NotI enzyme was heat-inactivated, and linear fragments were integrated into yeast genomic sites with the help of a Cas9 plasmid and a gRNA plasmid targeting the respective integration site.[27]

The serotonin production strains were constructed as described previously.[30] The plasmid pCfB9221 containing enzymes HsDDC
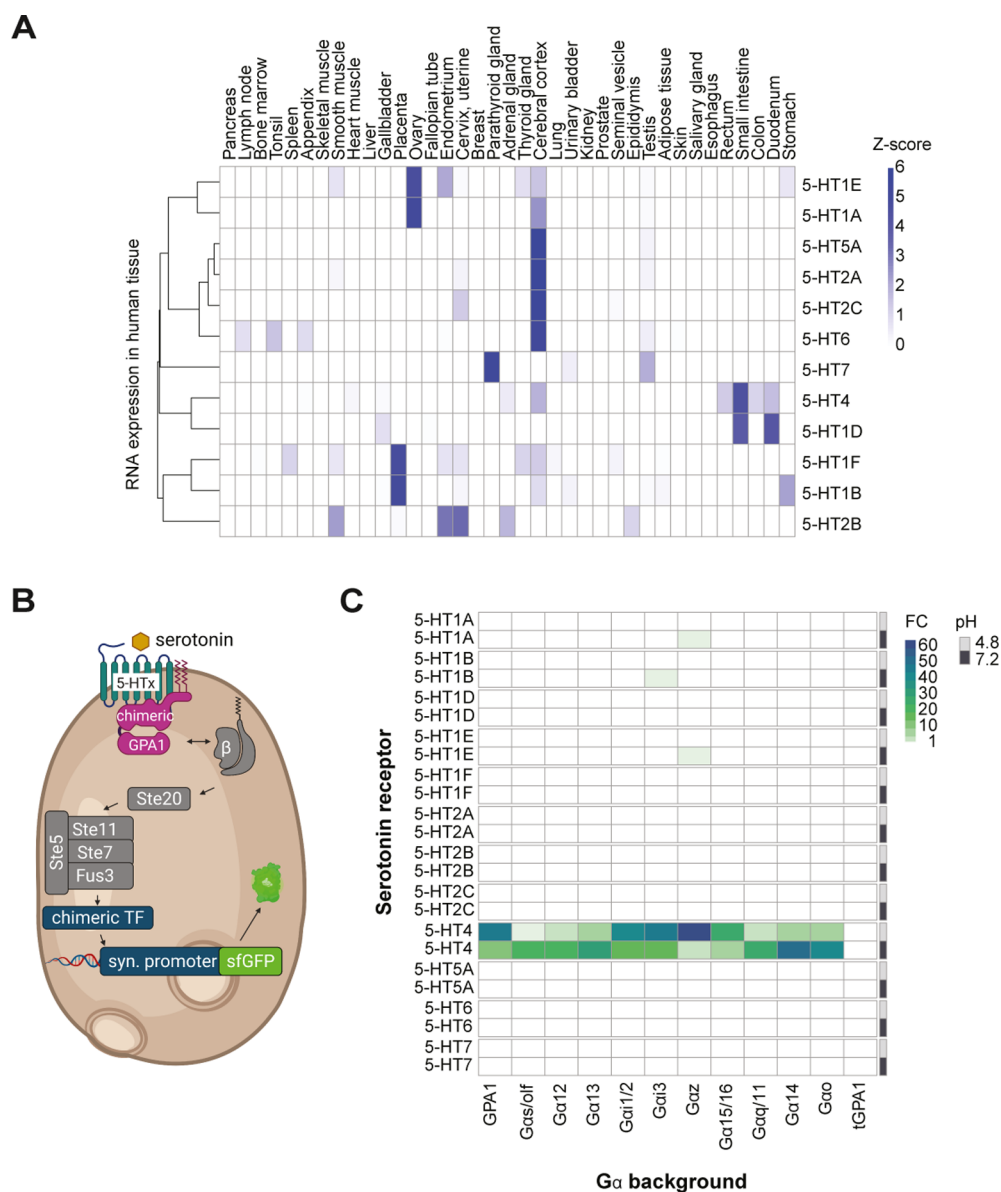
Figure 1. Exploring serotonin GPCR functionality in yeast. (A) Heat map of transcripts of serotonin GPCR expression in human tissues and organs from Human Protein Atlas.[39] Color key indicates relative expression levels normalized by row. (B) Schematic of the engineered yeast mating pathway,[16] coupled to human serotonin GPCRs. Serotonin binds to the 5-HT class of GPCRs; the associated engineered Gpa1-based chimeric $G\alpha$ protein dissociates into one $G\alpha$ subunit and a $G\beta\gamma$ dimer to induce the MAP-kinase cascade (Ste5/Ste7/Ste11), which in turn activates a chimeric transcription factor (chimeric TF), binding to a synthetic promoter to enable expression of superfolder green fluorescent protein (sfGFP). Created with BioRender.com. (C) Heat map of 12 serotonin GPCRs expressed from centromeric plasmids in 12 different $G\alpha$ background yeast strains. Fold change shown in color (FC) represents the ratio of fluorescence between the induced (100 μM serotonin) and uninduced state (0 μM serotonin). FC values represent the average of triplicate median values sampled by flow cytometry with 10,000 events analyzed for each triplicate in both induced and noninduced conditions. Note that breaks in the color range for 1C are not equidistant for the lower end of the scale to allow for representation of the lower-induced variants.

and SmTPH was previously constructed and integrated into XI-3.[30] Cofactor enzymes RnPTS and RnSPR, as well as PaPCBD1 and RnDHPR, were cloned on two plasmids and integrated into EasyClone sites X-4 and XII-4, respectively. Additionally, plasmid p2772, constructed previously,[30] containing SmTPH with overlap to TY2 sites, was integrated in the yeast TY2 sites.

The complete list of all yeast strains constructed can be found in Supporting Information Table S9.

Biosensor Assay. Yeast strains were freshly plated and grown on SC plates with respective auxotrophy if required. On day 1, a single colony of a sensing strain was inoculated in SC media with the respective auxotrophy if needed and grown for 24 h. On day 2, the culture was diluted 1:100 in SC media with or without auxotrophy and grown for 16 h. On day 3, the culture was diluted 1:50 in SC media with or without auxotrophy and grown for 2 h. In 96-well flat-bottom plates, 20 μL of the ligand (Sigma-Aldrich: serotonin hydrochloride H9523-100MG, L-tryptophan T0254-1G, and 5-hydroxy-L-tryptophan H9772-1G), dissolved in either Milli-Q (MQ) water or spent media, and 20 μL of the ligand was added to 180 μL of the sensing strain culture except for the experiment in Figure 3D, where additionally ratios of 50:150 and 100:100 μL of the ligand/ biosensor were used. Note that different serotonin concentrations of 10,000, 4000, and 2000 μM were added at 10, 25, or 50% volume, resulting in 1000 μM in-plate concentration in the highest dilution step. For Figure 3F, instead of the ligand, the supernatant of serotonin producing strains was used. The 96-well plate was covered with a PCR

foil and incubated for another 4 h at 30 °C and 300 rpm. The plates were chilled at 4 °C until flow cytometry analysis. The pH of the SC media ranged between 4.7 and 5.3 during all experiments apart from the pH experiment (Figure 3C).

Flow Cytometry Analysis. Flow cytometry analysis was performed on the Miltenyi MACSQuant VYB, using medium mixing and fast running mode. 10,000 events were recorded for each well analyzed, unless otherwise specified. The cells were gated for singlets in the exponential phase, and 10,000 events within the singlet gate were recorded for each well analyzed, apart from Figure 3C, where 6500 events were recorded, and Figure 3F, where 5000 events were analyzed.

Serotonin Production in Yeast. Serotonin-producing yeast strains were inoculated from a single colony and grown for 16 h in SC-URA media (pH 4.9). The cultures were diluted 1:50 and grown for 72 h in SC-URA media in 96-well deep culture plates. The supernatants were harvested by spinning at 4000 rpm for 5 min. The supernatants were carefully transferred into the 96-well plates, covered with aluminum foil, and stored at −80 °C until flow cytometry or HPLC analysis.

HPLC Analysis of Serotonin Production Strains. Analysis of serotonin was done on the Thermo Scientific UltiMate 3000. Solvent A was 0.05% acetic acid, and solvent B was acetonitrile. The column used was Agilent Zorbax C18 4.6 × 100 mm 3σl5-Micron with a Phenomenex AFO-8497 filter (Supporting Information Table S12). The HPLC values were determined according to two standard curves between 0 and 100 μM serotonin hydrochloride in MQ water, one run before and one after the samples, using the Chromeleon Chromatography Data System (CDS) software.

Data Analysis. Data analysis was performed in the R programing language using RStudio, with customized R scripts, making use of the tidyverse, flowCore, and pheatmap packages.[31−35]

For dose−response curves with flow cytometry data and the heat map in Figure 1C, data were obtained in triplicate and the median fluorescence values of each triplicate (consisting of minimum 5000 events, as described in the Biosensor Assay section) were calculated, from which mean and standard deviation was subsequently calculated. Mean and standard deviation were exported to GraphPad Prism to create dose−response curves. Curve fitting was done in GraphPad Prism with the variable slope—four-parameter model, apart from Figure 3C, in which the curves were fitted with the three-parameter model. Only curve fits >0.9 (i.e., correlation coefficient between data and fitted curve) were considered, if lower, or EC_{50} and Hill coefficients could not be calculated, "n.a." is stated. When either end of the 95% confidence intervals for EC_{50} or Hill coefficient could not be defined ("???" in Prism), "not defined" is stated. For Figure 3F, simpler linear regression in GraphPad Prism was used.[36] For Figure 1C, fold changes were calculated by dividing the induced-state value fluorescence intensity by the uninduced state fluorescence after calculating the mean of three median values as described previously. Sensor strains with fold-change values >1.4 were considered functional.

HPLC data were analyzed using Chromeleon CDS software. The snake plot was constructed using Protter[37] with the structure 5HT4R_HUMAN imported from UniProt.[9]

For the transcript heat map in Figure 1A, tissue isoform-RNA data was sourced from the Human Protein Atlas database (Uhlén et al. 2015). Data is available in proteinatlas.org, version 20.1. Several of the genes of interest produced splice variants. For this reason, the corresponding Ensembl transcript IDs were selected by amino acid similarity to the UniProt canonical sequence. In the case where several splice variants matched the canonical sequence, transcript levels were compared in GTEx, and the transcript with higher tissue expression was selected.[38] Nontissue samples were filtered out from the dataset. Heat maps were made with the R package "pheatmap" and normalized by row.[31,33−35] Transcript IDs for receptors and G alpha proteins can be found in Supporting Information Table S1.

All scripts for data analysis can be found at https://github.com/betlen/serosense.

cAMP Assay in COS7 Cells. The level of cAMP was monitored using bioluminescence resonance energy transfer (BRET). This method is based on a construct consisting of a cAMP-binding protein [exchange protein activated by cAMP (Epac)], which is flanked by a BRET pair, Renilla luciferase (Rluc) and yellow fluorescent protein (YFP). Together, this complex is called CAMYEL (cAMP sensor using YFP−Epac−Rluc) (Jiang et al. 2007). cAMP production is sensed as Epac change conformation in response to the increasing levels of cAMP, leading to a loss of BRET intensity. COS7 cells were plated in poly-D-lysine-coated white 96-well plates (20,000 cells/well). The following day, the cells were transfected in 100 μL of transfection medium/well for a total of 5 h and thereafter incubated in 100 μL of the growth medium O/N. On the subsequent day, the cells were washed twice with 100 μL/well Hanks' balanced salt solution (HBSS, GIBCO, Life Technologies) and preincubated for 30 min at 37 °C with 60 μL of HBSS. The luciferase substrate coelenterazine (Thermo Fisher) was added, and after a 5 min incubation, a baseline was measured. Ligands were added, and measurements were recorded every minute for 30 min using a CLARIOstar Plus plate reader. The BRET signal was calculated as the ratio of the emission intensity at 535 nm (citrine) to the emission intensity at 475 nm (luciferase). Determinations were made in triplicates. The primers, plasmids, and associated mammalian cell lines generated in this study are listed in Supporting Information Tables S7, S8, and S10.

■ RESULTS

Yeast Gα Library Screen Reveals pH-Dependent Signaling from Human Serotonin Receptors. In order to systematically investigate the potential to couple any of the 12 human serotonin receptors to the yeast mating pathway, we first mined the Human Protein Atlas database[39] for tissue- and organ-specific expression patterns of genes encoding the receptors in search of physiological biosensing parameters, which could be leveraged to confer signaling from these human receptors in a yeast cell.

Here, as several of the genes produced splice variants, we selected the corresponding Ensembl transcript IDs (Supporting Information Table S1) by amino acid similarity to the UniProt canonical sequence. From this analysis, it is evident that they all have different expression profiles (Figure 1A). The 5-HT6, 5-HT2C, 5-HT2A, and 5-HT5A receptors express most abundantly in the cerebral cortex, just as 5-HT7 expression is maximal in the parathyroid gland. In female reproductive tissues, 5-HT1B and 5-HT1F express at high levels in the placenta, 5-HT1A and 5-HT1E in the ovaries, and 5-HT2B in the endometrium and cervix most abundantly. In the gastrointestinal tract, 5-HT1D is most abundantly expressed in the small intestine and duodenum. Similarly, 5-HT4 shows high expression in the small intestine but at comparably lower levels in the rectum, colon, and duodenum. Previously, 5-HT4 has been identified to be highly expressed in the gastrointestinal tract and is a target for drugs for gastrointestinal disorders.[40,41] Taken together, the 5-HT class of receptors is expressed at different abundances and tissues.

Next, we performed a combinatorial library screen founded on 12 different Gα protein background strains expressing either a yeast-native Gpa1 Gα protein, one of 10 Gpa1/Gα chimeras, or a truncated Gpa1 (tGpa1) serving as a negative control.[11,16] Based on this platform, we transformed plasmids containing one of each of the 12 human serotonin GPCRs into the 12 different Gα background strains, creating a library of 144 serotonin GPCR/Gα strains. In this setup, successful coupling of human serotonin GPCRs with the yeast mating pathway will result in the activation of a synthetic transcription factor (LexA-PRD), which binds to a synthetic promoter
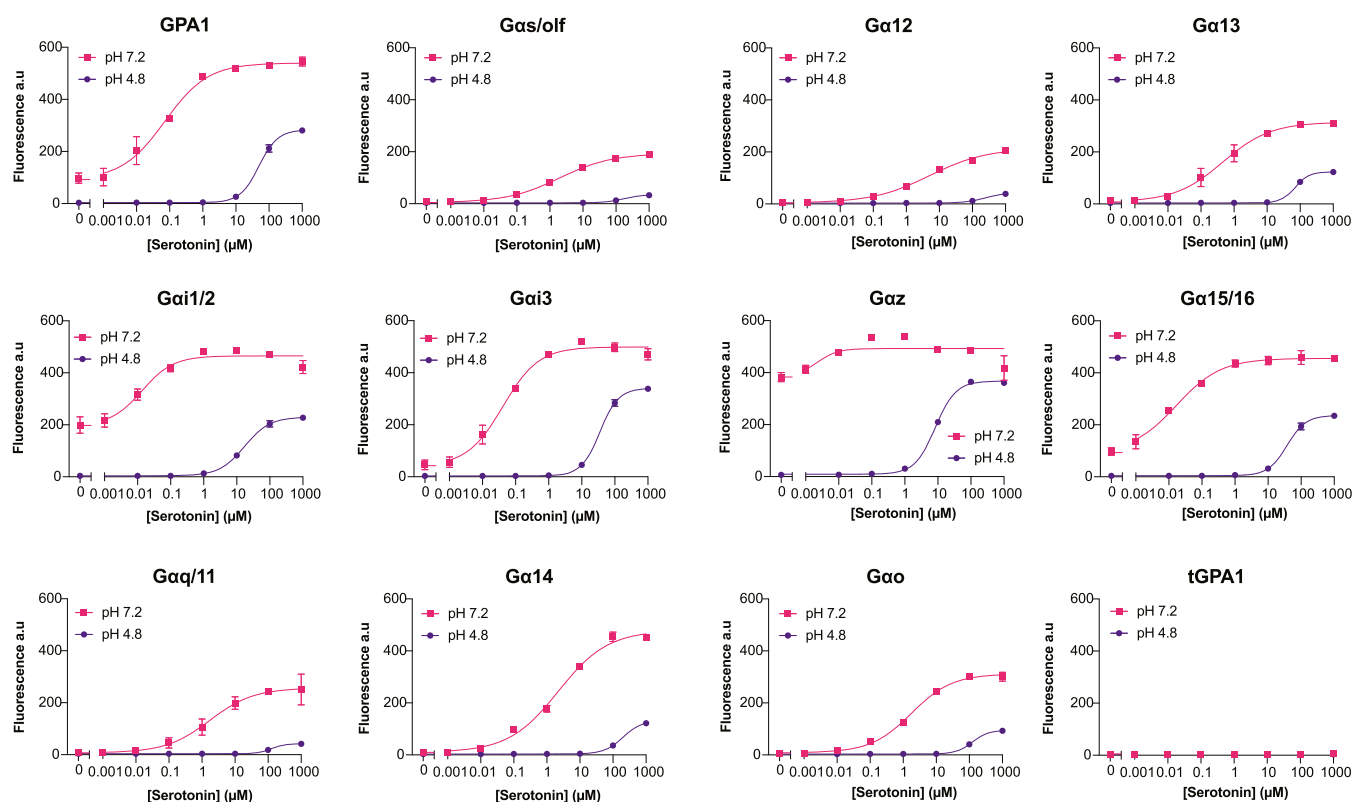
Figure 2. Dose−response curves of 5-HT4 coupling to chimeric Gα proteins. Yeast strains expressing 5-HT4 in combination with 12 different Gα backgrounds, namely, yeast Gpa1, tGpa1, or any of the 10 different Gpa1/Gα chimera.[11,16] Strains were cultivated in a control medium without serotonin, or 0.01−1000 μM serotonin, and sfGFP reporter outputs recorded following 4 h. All data points represent the median fluorescence intensity of three technical replicates (10,000 events each), of which the mean ± standard deviation was calculated. Data was fitted to a variable slope four-parameter curve fitting model, from which the EC$_{50}$ and Hill coefficient values were calculated, except for the tGPA1 background strain serving as a negative control. AU = arbitrary units. n.a. = not applicable.

(LexO(6x)-pLEU2m),[16] to induce the expression of sfGFP in the presence of serotonin (Figure 1B). Given that gut-expressed receptors, for example, 5-HT1B, 5-HT1E, and 5-HT4, segregate from the other serotonin receptors (Figure 1A), perhaps serotonin receptors have evolved to work at different pHs, and thus we screened the GPCR/Gα library at both pH 4.8 and 7.2, spanning a physiologically relevant pH range for both human serum and yeast cultivation medium (Figure 1C). At both pHs, we cultivated the library in the absence of serotonin and in the presence of 100 μM serotonin and scored relative GFP reporter readouts following 4 h of induction.

From this screen, we found strains expressing 5-HT4 to be activated by serotonin, at both pH 4.8 and 7.2, and in all Gα backgrounds excluding the truncated Gα control (tGpa1) (Figure 1C). Fold inductions for the 5-HT4 receptor at pH 4.8 ranged between 1.8-fold for Gαs/olf coupling and 64-fold for Gαz coupling, followed by 48-fold Gαi3 and 46-fold for yeast-native Gpa1. Interestingly, looking at the four highest-induced Gα backgrounds for 5-HT4 at pH 4.8; Gαz, Gαi3, Gαi1/2, and Gpa1, they showed reduced fold inductions when using media at pH 7.2 (Figure 1C). The increase in background (OFF state) fluorescence at pH 7.2, rather than a drop in the maximal induced reporter output, was the main reason explaining the overall diminished fold-change for these four receptors at pH 7.2 (Supporting Information Figure S1). Interestingly, for all these four 5-HT4/Gα designs, the third position from the C-terminal of the Gα protein encoded a glycine residue (Supporting Information Figure S1). In

contrast, and in agreement with a recent study on proton-gated coincidence detection of GPCRs,[20] low-induced 5-HT4/Gα background strains tested at pH 4.8 showed high inductions at pH 7.2 with Gα14 at 51-fold, Gα13 at 33-fold, and Gαq/11 at 26-fold (Supporting Information Table S2 and Figure 1C).

Generally, the absolute induced signal was higher for all strains at pH 7.2 compared to pH 4.8 (Supporting Information Table S2). Interestingly, at pH 7.2, many poorly activated receptors at pH 4.8 showed an approximately 10-fold increase in the absolute fluorescence levels in the induced state, while the background was only modestly elevated. This was exemplified for 5-HT4 in the Gα14 background, where total induced reporter gene expression from 25 to 231 MFI units was observed, while the background fluorescence only increased from 3.03 to 4.56 MFI units, ultimately shifting the fold-change of 5-HT4 in the Gα14 background from 8.4 to 50.7 when comparing pH 4.8 and 7.2 (Supporting Information Table S2 and Figure 1C). Similar shifts could be observed for 5-HT4/Gα backgrounds (Gαs/olf, Gα12, Gα13, Gαq/11, Gα14, and Gαo) poorly induced at 4.8, which showed that fold changes increased by up to 10-fold at pH 7.2 (Supporting Information Figures S2 and S3 and Table S2 and Figure 1C).

Furthermore, at pH 7.2, 5-HT1B in the Gαi3 background and 5-HT1A in the Gαz background showed modest fold changes of 1.5-fold, while 5-HT1E in the Gαz background reached 1.7 fold-change (Figure 1C, Supporting Information Table S2). In comparison with previous serotonin receptor studies in yeast, 5-HT1A has been shown to couple to Gαo,
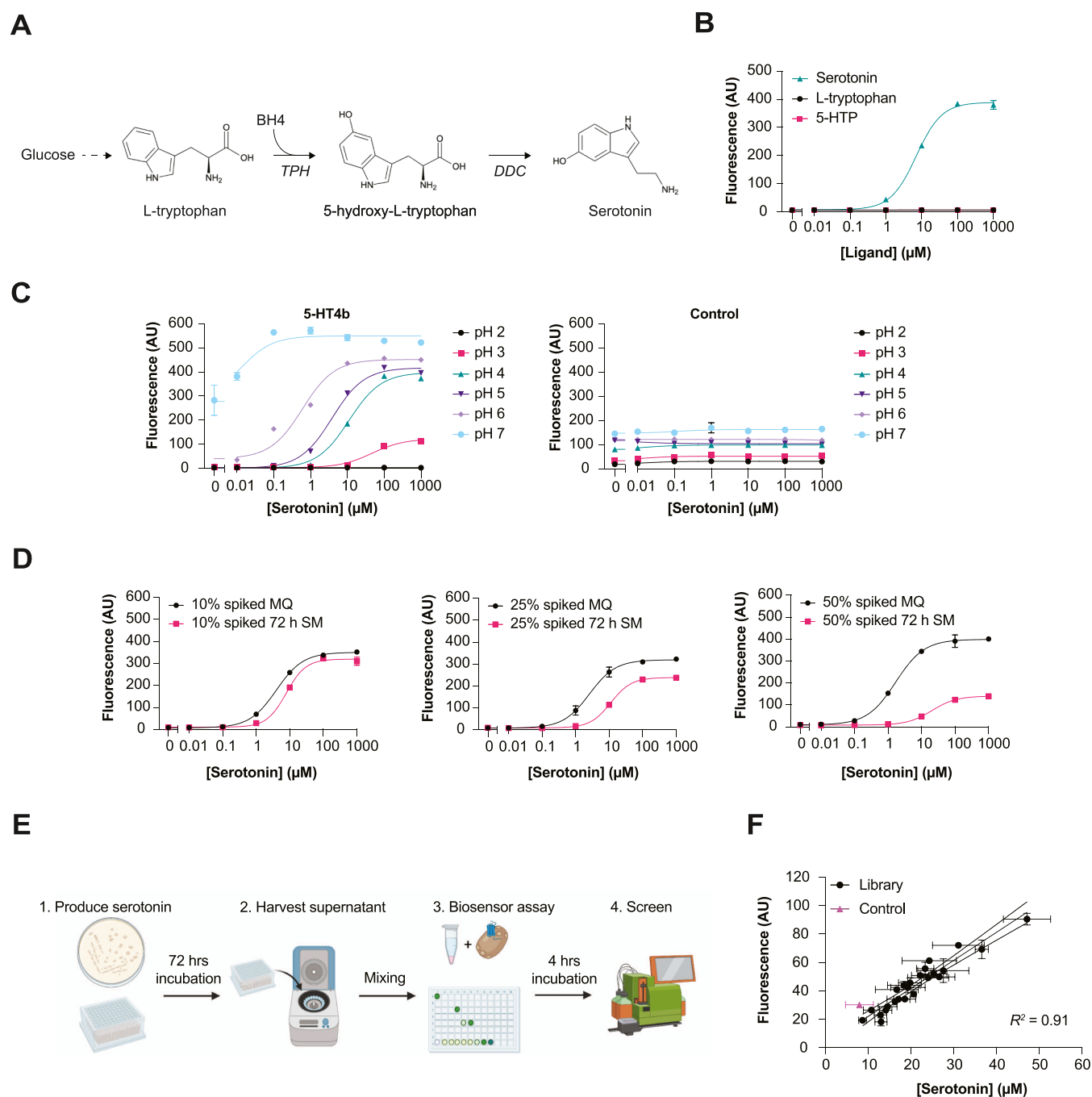
Figure 3. Workflow for semithroughput characterization of serotonin accumulation in engineered yeast cells. (A) Serotonin is produced from L-tryptophan and the BH4 cofactor *via* 5-hydroxy-L-tryptophan using TPH and 5-hydroxy-L-tryptophan decarboxylase (DDC) enzymes. (B) Dose–response curves of the 5-HT4/Gαz sensor strain upon induction with serotonin, L-tryptophan, or 5-HTP in media at pH 4.8. (C) Effect of media with different pHs on the Gαz + 5-HT4 sensor strain and a constitutively sfGFP expressing yeast base strain incubated with serotonin (D) dose–response curves of adding spiked spent media (72 h SM) or serotonin-spiked water (MQ) at different volumetric percentages added to the sensor strain. (E) Workflow for sensing yeast-produced serotonin. Serotonin-producing cells are incubated for 72 h; the supernatant is spun down, added to, and incubated with the sensor strain expressing the 5-HT4/Gαz sensor and sfGFP expression ultimately screened using a flow cytometer as a proxy for serotonin production. Created with BioRender.com. (F) Correlation of HPLC-based quantification of serotonin and sfGFP expression *via* Gαz + 5-HT4 of serotonin producing yeast strains carrying multiple copies of TPH (x *n*). Data were fitted with a simple linear regression model. For (B,D), each data point consists of technical triplicates of 10,000 events for (C) 6500 events were recorded and (F) 5000 events were analyzed for each triplicate. For all data panels, the median fluorescence of each triplicate was calculated and mean ± standard deviation shown.

Gαi2, Gαi3, Gα14, and Gpa1, while 5-HT1D couples to Gαi2 and Gαi3 when expressed from high-copy plasmids and using a β-galactosidase or ZnGreen reporter assays.[11,15] The observation that 5-HT1A couples to Gpa1 was not supported by Ehrenworth *et al.*,[14] and neither were we able to detect any

changes in the reporter output upon serotonin supplementation to strains expressing 5-HT1A, except in the Gαz background (Figure 1C). Furthermore, while we could not demonstrate the activation of the 5-HT1D receptor at pH 4.8 or 7.2 when the receptors were expressed from single-copy

plasmids, our library screen corroborated the recent study by Kapolka et al.,[20] showing the promiscuity of 5-HT4 in coupling to all chimeric Gα proteins tested.

Taken together, our results show the functionality of 5-HT1A, 5-HT1B, 5-HT1E, and 5-HT4 in different chimeric GPA1/Gα backgrounds, with up to 64-fold induction in the signaling output, largely determined by low-background reporter outputs. Also, our study illustrates increased background fluorescence at pH 7.2 for yeast expressing 5-HT4 together with some Gα proteins, of which most encode a glycine at position 3 of the Gα C-terminal (Supporting Information Figure S1).

Chimeric Gα Background Impacts EC_{50} and Sensitivity of 5-HT4 Biosensing. In humans, serotonin receptors are believed to couple and activate intracellular responses primarily through the Gαs protein.[42] Still, to further investigate the potential impact different chimeric Gα proteins could have on serotonin sensing parameters in yeast, we next studied the dose−response curves of the 5-HT4 receptor expressed together with the 12 different Gα background strains at pHs 4.8 and 7.2 (Figure 1B,C). As genomic integration of serotonin GPCRs shows a more homogeneous sensor signal compared to the plasmid-based expression of serotonin GPCRs (Supporting Information Figure S4), 5-HT4 was integrated into the 12 different Gα background strains and reporter gene outputs obtained with serotonin stimulation between 0.01 and 1000 μM of serotonin (Figure 2). Next, serotonin concentrations yielding the half-maximal reporter output (EC_{50}) and cooperativity of serotonin biosensing (Hill coefficient) were obtained. Here, at pH 4.8, 5-HT4 expressed together with the yeast native Gα protein Gpa1 showed an EC_{50} and a Hill coefficient of 49.6 and 1.53 μM, respectively (Figure 2, Supporting Information Table S13), while 5-HT4 expressed together with Gαz, Gαi1/2, and Gαi3 all yielded the lowest EC_{50} values of 8.33, 17.17, and 35.33 μM, respectively, and Gα12 and Gα14 showed the highest EC_{50} values of >200 μM (Figure 2 and Supporting Information Table S13). With respect to cooperativity, at pH 4.8, all designs had Hill coefficients >1, with the highest seen for 5-HT4 expressed with Gαq/11 (2.23), Gα13 (2.07), and Gαo (1.80). At pH 7.2, all 5-HT4/Gα designs showed drastically lowered EC_{50} values, with 5-HT4 expressed together with Gpa1, Gαi1/2, Gαi3, Gαz, and Gα15/16 showing the highest sensitivities to serotonin (Figure 2, Supporting Information Table S13), with EC_{50} values for the most sensitive 5-HT4/Gα designs decreased by more than 4 orders of magnitude compared to values obtained at pH 4.8. Concomitantly to the increased serotonin sensitivity, all 5-HT4/Gα designs showed lowered cooperativity at pH 7.2 compared to pH 4.8 (Supporting Information Table S13). Interestingly, for all the high-sensitivity designs at pH 7.2 (i.e., 5-HT4 expressed with either Gpa1, Gαi1/2, Gαi3, Gαz, or Gα15/16), reporter gene expression was activated even in the absence of serotonin (high off state) and thus inferred a lowered dynamic range (Figures 1C and 2). Furthermore, and in agreement with the increased off state (Supporting Information Figures S1 and S2), for all these designs, except for Gα15/16, a glycine residue at the third C-terminal position was observed together with another aliphatic residue at position 4 of the chimeric Gα proteins. However, while being interesting, glycine at position 3 together with another aliphatic residue at position 4 is not causal to the leaky activation observed at pH 7.2, as Gαo also has this design, yet it does not show high off state at pH 7.2

(Figure 2). Residue 3 of the Gα C-terminus, particularly as a glycine, has been identified as being an important residue for structural changes in the C-terminus and receptor interactions of G proteins.[43,44] Finally, at neither of the two tested pHs did the negative control, 5-HT4:tGpa1, induce reporter gene expression.

Taken together, at low pH, serotonin biosensing via chimeric Gα proteins spanned approximately 25- and 2-fold difference in EC_{50} and sensitivity, respectively, while at higher pH, the most sensitive designs were observed to have lowered EC_{50} over more than 4 orders of magnitude changes in serotonin concentrations.

Whole-Cell Biosensing Workflow for Serotonin. Based on the large operational range for GPCR-based biosensing of serotonin spanning almost 3 orders of magnitude, the low EC_{50}, and high dynamic range (Figures 1C and 2), the chimeric Gpa1/Gαz expressed together with 5-HT4 was next chosen as a platform design to explore the possibility of whole-cell biosensing of serotonin produced from yeast. Previously, it was shown that metabolically produced serotonin and melatonin can be sensed using their respective GPCRs[14,16] and also that 5-HT4 could be used as a biosensor to discriminate between the reporter outputs from a wild-type yeast and a serotonin-producing yeast, albeit with a modest sensor response of ~2-fold.[14] Here, we set out to (i) identify key parameters in developing a high-resolution and simple serotonin biosensing workflow using the biosensor based on chimeric G1/Gαz expressed together with 5-HT4 and (ii) construct a library of variant serotonin-producing yeast strains to validate biosensor performance.

Serotonin is produced from L-tryptophan via a 5-hydroxy-tryptophan (5-HTP) intermediate (Figure 3A).[30] To investigate possible activation of the 5-HT4 sensor by precursor products, the biosensing strain was first subjected to L-tryptophan and 5-HTP, as well as serotonin, as a positive control over a range of 0.01−1000 μM. Activation of the receptor, as inferred by the fluorescence output, was only observed for serotonin, confirming the specificity of 5-HT4 for serotonin over its precursors (Figure 3B).

Next, as we previously observed a strong pH-dependent effect on overall fluorescence and background fluorescence output from yeast strains expressing 5-HT4 together with Gαz (5-HT4/Gαz) (Figure 1C, Supporting Information Figures S1 and S2, Table S2), we sought to investigate the effect of pH on serotonin dose−response curves over a wider pH range. Consequently, the sensor strain was subjected to media with the pH ranging from pH 2 to pH 7. A yeast strain carrying only sfGFP under a TDH3 promoter served as a control and was subjected to the same pH conditions. Overall, the lowest EC_{50} value was observed at pH 7 (0.01 μM), with the EC_{50} values showing an inverse proportional relationship with pH (Figure 3C). The broadest operational ranges were observed for the biosensing strain cultivated at pH 5 and pH 6, spanning from 0.1 to 100 and 0.01 to 10 μM, respectively. At pH 7, the sensor strain reported changes in serotonin concentrations from 0.01 to 0.1 μM, while at pH 2, no changes in the reporter output were observed over the applied range of serotonin concentrations (Figure 3C). Of importance, background fluorescence in the absence of serotonin was generally low for the conditions tested (Supporting Information Table S3), although, at pH 6, and especially at pH 7, background fluorescence increased, as also observed with strains having plasmid-based expression of GPCRs (Figure 3C, Supporting
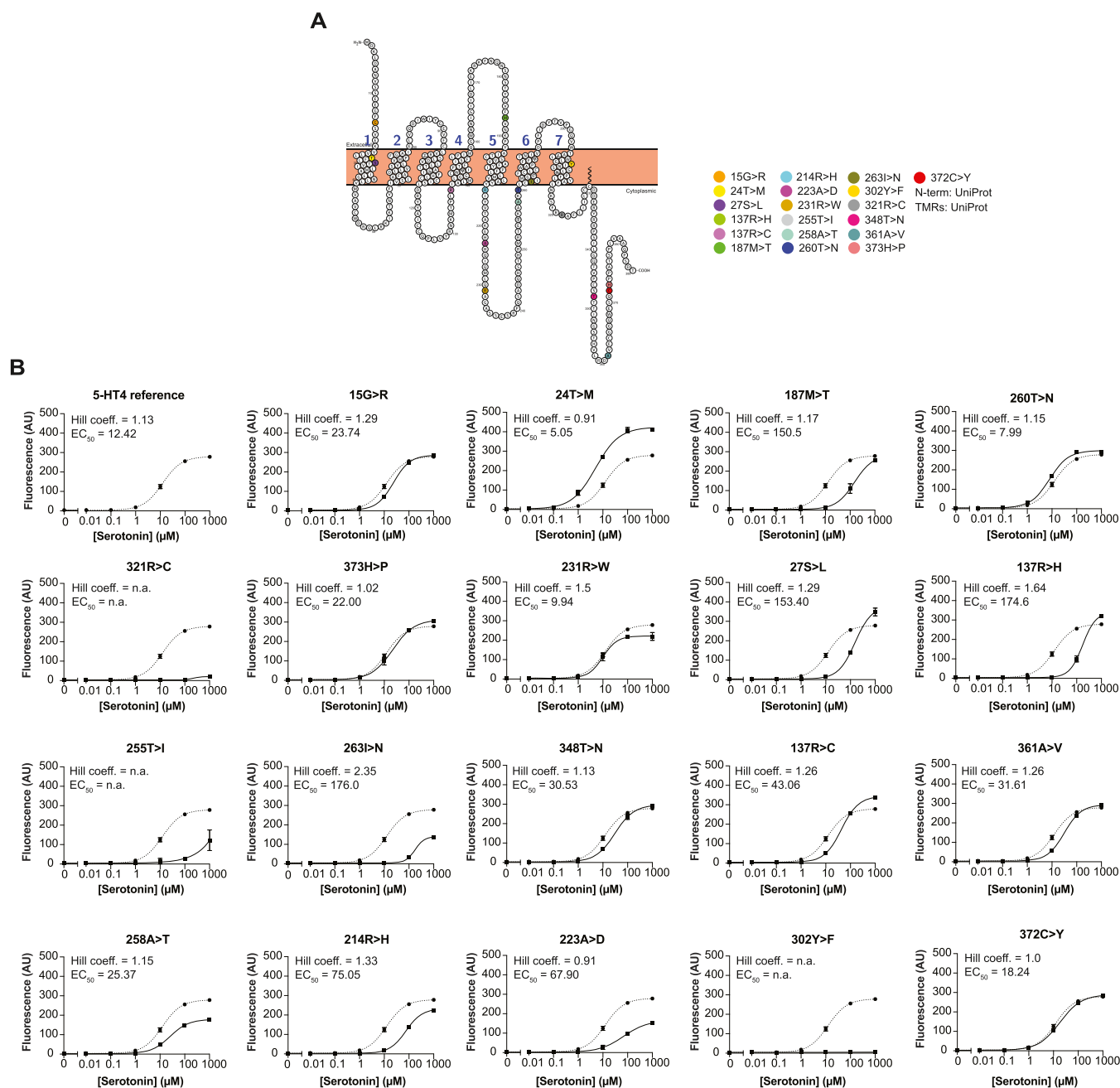
Figure 4. Characterization of 5-HT4 serotonin GPCR variants from human genomes. (A) Snake plot of the 5-HT4 isoform b mutational landscape showing the location of analyzed variants on the protein. All screened variants had a single mutated amino acid, except for residue 137, for which two mutants were screened. (B) Dose responses for the 5-HT4b isoform reference receptor and 19 variants in the Gαs background. Yeast strains expressing single-amino acid variant GPCRs were induced with 0.01−1000 μM serotonin in addition to a noninduced "0" control. sfGFP was measured following 4 h of incubation with serotonin. All data points represent the median fluorescence intensity of three technical replicates (10,000 events each), from which the mean ± the standard deviation was calculated. AU = arbitrary units, n.a. = not applicable. Data was fitted to a variable slope four-parameter curve fitting model, from which EC_{50} and Hill coefficients values were calculated. Dose−response curves show the tested variant (red line) and the reference receptor (black line).

Information Figure S4). Finally, the sfGFP control strain showed no serotonin-dependent increase in fluorescence with increasing pH (Figure 3C).

In addition to assessing pH effects, and acknowledging that serotonin produced from yeast cells is secreted into the cultivation medium,[30] we tested if adding serotonin in yeast spent media would influence the signaling behavior of the 5-HT4/Gαz sensor strain. For this purpose, the spent medium from a yeast base strain (BY4741) cultivated for 72 h (72 h

SM) was spiked with different concentrations of serotonin. Adding serotonin-spiked SM at 10, 25, or 50% of the volume in the plate dilution step allowed us to evaluate spent medium effects versus a control with serotonin spiked into water (MQ) (Figure 3D). At 10%, only a slight decrease in fluorescence was observed between the cultivations with MQ control and 72 h SM, as well as a modest increase in the EC_{50} from 4.00 to 7.88 μM. At 25% SM, an EC_{50} increase from 2.59 to 11.21 μM was observed, while most notably, the EC_{50} increased from 1.70 to

20.41 μM when adding the ligand at 50% SM (Figure 3D, Supporting Information Table S4). Thus, taking into consideration the expected concentration of the ligand produced, the ratio at which the SM supernatant is added to the medium with the sensor strain should enable a simple biosensing workflow with adjustable EC_{50} values to the application of interest. However, it deserves to be mentioned that the general acidification spent medium from cultivated yeast is an important parameter to consider when establishing and optimizing biosensing workflows, especially when using GPCRs with proton-gated coincidence detection such as 5-HT4.[51] Indeed, we observed a general lowering of the pH with increasing ratio of the spent medium added to the medium with the sensor (Supporting Information Figure S5).

Next, having confirmed the possibility to discriminate serotonin concentrations in the spent medium, we applied the workflow to screen a panel of yeast cells engineered to produce different levels of serotonin by randomly integrating variable numbers of expression cassettes for the tryptophan hydroxylase (TPH) enzyme into genomic Ty2 retrotransposon sites (Figure 3E,F).[30] In brief, following random integration of open reading frames for TPH expression in transposable elements of the yeast genome,[30,45] 24 randomly sampled colonies were grown for 72 h before harvesting and adding supernatants to the sensor strain, followed by incubation for 4 h and measurement of sfGFP fluorescence (Figure 3E). In parallel, the supernatants were analyzed using HPLC to validate sfGFP reporter output as a proxy for absolute serotonin concentrations in the spent medium.

Taking into consideration acidification of yeast media over prolonged cultivations of strain BY4741,[46] and the observed negative impact of acidified spent media on maximum reporter output (Figure 3D), the reporter output from this screen was expected to be diminished compared to the serotonin-spike in titrations (Figure 2). Therefore, for the biosensing workflow, we decided to use supernatants from each of the 24 randomly sampled colonies of yeast strains engineered for serotonin production added at only 10% volume. While adding the spent medium at 10% infers a 10-fold dilution, the biosensor was able to resolve fluorescence outputs in these lower serotonin ranges (Figure 3F). From plotting biosensor fluorescence outputs against serotonin quantification as inferred from HPLC, a linear model fitted HPLC-measured serotonin concentrations and biosensor fluorescence from the 24 sampled strains ($R^2 = 0.91$) (Figure 3F).

In summary, the engineered 5-HT4/Gαz biosensing strain specifically senses serotonin and can reliably detect serotonin in a facile and easily adjustable (*e.g.*, pH and volume of the spent medium) workflow compatible with high-throughput screening of libraries of yeast cells engineered to produce serotonin or other biological samples.

Characterization of Human 5-HT4 Variants in Yeast. GPCR single-nucleotide polymorphisms are known to impact EC_{50} and agonist sensitivities in humans,[47] and human variants of 5HT1a, rhodopsin, and MOR1 expressed in yeast have previously shown to reproduce Gα-dependent sensitivities to serotonin, light, and morphine, respectively, as reported from mammalian cells.[15,21,48]

Based on the biosensing platform developed in this study, we therefore next sought to examine the canonical isoform b of human 5-HT4, in comparison to human receptor variants or "protein haplotypes" sourced from the 1000 Genomes Project using the Haplosaurus tool browser *via* Ensembl.[24,25] From

this data mining, 20 5-HT4 receptor variants were identified, of which 19 were cloned into yeast (Figure 4A). Nine of the tested variants were in the intracellular loops (including two on residue 137), four were in the transmembrane domains, four in the C-terminus, one in the extracellular loop, and one in the N-terminus (Figure 4A).[49]

Filtered data from Ensembl's Haplosaurus tool showed the distribution of variants across five different populations: African, American, East Asian, European, and South Asian (Supporting Information Table S14). From this distribution, there were 218 nonreference genomes present in the dataset, showing different frequencies of variants in different populations (Supporting Information Table S14). Of these variants, some were computationally predicted to have deleterious or possibly damaging effects on receptor functions ("D", Supporting Information Table S14). Because of this, we decided to introduce all the receptor variants into the Gαs background as 5HT4 natively couples to this Gα protein in humans. For the 5-HT4 dose−response study (Figure 4B), receptor variants were assayed for serotonin responsiveness from 0.01 to 1000 μM serotonin in addition to noninduced control without supplemented serotonin. The EC_{50} values and Hill coefficients ranged from 1.31 μM to 25.00, and 0.61 to 1.48, respectively (Figure 4B). The 5-HT4 isoform b reference strain had an EC_{50} of 2.42 μM and a Hill coefficient of 0.82, while the variants 258A > T (2.34 μM), 361A > V (1.83 μM), and 373H > P (1.31 μM) resulted in decreased EC_{50} values. Additionally, the 231R > W, 361A > V, and 373H > P variants had an increased operational range as compared to the reference (Figure 4B, Supporting Information Table S5). The variants 15G > R, 27S > L, 137R > C, 214R > H, 231R > W, 260T > N, 348T > N, and 372C > Y resulted in minor increases in the EC_{50} values (3.32−8.48 μM), while variants 187M > T and 137R > H resulted in notably higher EC_{50} values (>15 μM) as compared to the reference receptor. Interestingly, the 137R > H variant had a considerably higher EC_{50} value than the 137R > C variant (25.00 *vs* 7.61 μM) despite their shared residue location. For the variants 223A > D, 255T > I, 263I > N, 302Y > F, and 321R > C, no EC_{50} or Hill coefficient could be calculated due to their low curve fit and almost complete loss of function (Figure 4B, Supporting Information Table S5).

In order to investigate if the observed EC_{50} and Hill coefficients of the selected 5-HT4 variants toward serotonin in yeast cells would be valid proxies for 5-HT4 signaling in mammalian cells, we cloned the 5-HT4 reference GPCR and two variants, 5-HT4_231R > W and 5-HT4_302Y > F, into the COS7 cells and measured cAMP productivity as a proxy for their responsiveness to serotonin (Jiang *et al.* 2007). Here, we found that while the 231R > W receptor variant also displayed increased sensitivity and cooperativity in the COS7 cells compared to the reference 5-HT4 receptor, the variant 302Y > F, shown to be nonfunctional in yeast, showed the best signaling characteristics of all three receptors tested in COS7 cells, including the lowest EC_{50}, highest Hill coefficient, and highest maximum cAMP production (Supporting Information Figure S6). Furthermore, for the reference 5-HT4 receptor, the EC_{50} was approximately 25-fold lower in COS7 cells compared to yeast cells (2.42 *vs* 0.097 μM) (Supporting Information Table S5 and Figure S6).

Taken together, this study enabled reporting of serotonin dose−response parameters for 19 5-HT4 variants found in human populations spanning five demographic regions.

Importantly, in yeast, variants stimulated with serotonin displayed large differences in the EC_{50} values (1.31−25.00 μM), cooperativity, as inferred from Hill coefficients, and maximum reporter output, compared to the reference receptor. Furthermore, while serotonin signaling observed in yeast cells of the 5-HT4_231R > W variant could be recapitulated in mammalian cells, disparate findings were observed when comparing serotonin signaling through the 5-HT4_302Y > F variant, as well as serotonin sensitivity of the 5-HT4 reference GPCR.

CONCLUSIONS

Here, we showed a combinatorial serotonin GPCR/Gα library screened at different pHs and found 5-HT4, 5-HT1A, 5-HT1B, and 5-HT1E GPCRs functional in yeast. Apart from the observation that 5-HT4 coupled to all Gα protein backgrounds, only modest fold inductions (i.e., 1.5−1.7) were observed for 5-HT1B in the Gαi3 background and 5-HT1A and 5-HT1E in the Gαz background. In agreement with Ehrenworth et al.,[14] we found that, when coupled to yeast-native Gpa1, the 5-HT1A, 5-HT1D, 5-HT2B, 5-HT5A, and 5-HT6 receptors were nonfunctional at both pHs tested in this study. Surprisingly, while Brown et al. have previously shown the activation of 5-HT1D in chimeric and wild-type Gpa1 Gα proteins,[11] we were not able to demonstrate the activation of the 5-HT1D receptor and also not 5-HT1 in the Gpa1 and Gαi3 background as previously reported.[11,15] Moreover, pH-dependent coupling of 5-HT4 has recently been reported.[51] Interestingly, while Kapolka et al. observed 5-HT4 to signal through all Gα proteins at pH 7, and only Gαz and Gαi1/2 at pH 5, we observed coupling to all tested Gα proteins at both pH 4.8 and 7, albeit with Gαz, Gαi1/2, and Gαi3 to a higher dynamic range at pH 4.8 compared to pH 7 (Supporting Information Table S3). While there is a good agreement between our results and the data presented by Kapolka et al. at low pH (pH 4.8−5.0), we consider some of the divergent findings at high pH to potentially be attributable to different genetic backgrounds of sensor strains (Supporting Information Table S11) and call for adoption of assay standards regarding the cultivation medium, timescales, and background strains when characterizing GPCRs in heterologous hosts such as yeast.

While 5-HT1A, 5-HT1B, 5-HT1E, and 5-HT4 were demonstrated to be functional in this study, reasons for nonfunctionality of the other eight serotonin GPCRs in yeast could be manifold. The key points to be considered for future mitigations are endoplasmic reticulum processing issues, improper membrane localization, lack of coupling of receptors to Gα proteins, and suboptimal sterol environment compared to native human host cells. While adding signaling sequences seems to yield mixed results,[52,53] changing the lipid composition from yeast native ergosterol to sterol synthesis has yielded promising results on opioid receptors in yeast.[21] Likewise, coincidence detection,[51] and even biased signaling,[54] affect GPCR signaling and should be considered for further investigation of 5-HT receptor signaling in yeast.

Furthermore, we demonstrated the impact of 19 different polymorphisms of 5-HT4. Previously, a few 5-HT4 variants have been studied in mammalian heterologous systems. For instance, when the 5-HT4 isoform g was expressed in COS7 cells, the 302Y > F variant decreased the affinity of the selective 5-HT4 receptor antagonist GR113808 to the receptor by 13-fold but did not affect serotonin-induced activity, although it

decreased receptor expression.[55,56] Interestingly, in our *S. cerevisiae* platform, the receptor variant 302Y > F resulted in a complete loss of function in the presence of serotonin (Figure 4), in accordance with the hypothesis that residue 302Y is an important residue for agonist and antagonist binding to the 5-HT4 receptor.[55,57]

Lastly, yeast GPCR assays have previously been successfully used as drug discovery prescreens for 5-HT4 agonists in human colon cells.[50] Also, comparison of binding affinities of ligands to a human adrenergic receptor in yeast and COS7 reported similar values for four different agonists,[10] while Kapolka et al. recently confirmed yeast-based findings for two out of three GPCRs in human embryonic kidney cells.[20] In our study, the comparisons of 5-HT receptor signaling in the yeast cells versus COS7 cells clearly show that more research is needed for "humanizing" yeast as a platform for studying human variants of the 5-HT receptor class. While the yeast platform currently cannot be 1:1 paralleled to human GPCR signaling, differences in activity and EC_{50} values in these receptor variants could indicate important shifts in receptor activity and expression.

For future directions, we envision the study of polymorphisms of receptor variants for better sequence- and structure-guided engineering of GPCRs to flourish. As demonstrated herein, changes of single residues can vastly influence the signaling behavior of GPCR receptors, and we consider yeast a relevant chassis for large-scale mutational studies coupled to machine learning approaches, ultimately enabling better understanding of GPCR specificity and pharmacokinetic properties. Additionally, further establishing yeast as a platform to study mammalian receptor polymorphisms could provide a high-throughput platform for flagging variant-related drug activity impacts and thus serve a purpose in hit-to-lead drug discovery regimes.[50] In terms of human health, such findings could then further extend yeast GPCR biosensing to real-life applications, as recently shown by probiotic yeast for the destruction of extracellular ATP in mice guts.[16,58] We anticipate that such opportunities will be explored further and form the basis for many more GPCR-based biosensors to be developed and applied soon.

ASSOCIATED CONTENT

ⓈI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.1c02061.

> Increased off-state signaling at pH 7.2 for yeast strains expressing 5-HT4 together with Gpa1/Gα chimera; 12 yeast strains with different Gα protein backgrounds, transformed with 5-HT4 plasmids; distribution of expression of genes encoding Gα proteins in human tissues; fluorescence of yeast biosensors strains with different Gα backgrounds, either having 5-HT4 integrated (purple and green) genomically or expressed from a plasmid (blue or yellow); measurements of pH in the medium used for serotonin sensing; dose−response curves of 5-HT4 receptors expressed in COS7 cells in response to serotonin; Ensembl transcript IDs; data corresponding to the heat map in Figure 1C; EC_{50}s and R^2 for pH data; R^2, EC_{50}, and Hill coefficient from the spent medium test; overview of EC_{50}s and Hill coefficients for all 19 tested human 5HT4b variants; gBlocks for serotonin GPCRs, based on UniProt entries,

including the DNA sequence; overview of primers used, including description, the amplification template they were used with, new plasmid to be created, description, purpose, antibiotic and yeast marker, and reference if used previously; overview of plasmids used, including the plasmid name, description, purpose, antibiotic and yeast marker, and reference if used previously; overview of the yeast strains used, including the strain name, description, plasmid used for integration, in which figure it was used, and reference if used previously; overview of mammalian strains; comparison between the papers that used the 5-HT4 receptor in yeast-sensing strains; the solvent gradient for serotonin analysis using HPLC; EC_{50} and Hill coefficients from serotonin dose−response curves of 5-HT4 coupling to all 12 different chimeric Gα proteins at two different pHs when expressed in yeast; and demographic distribution of human 5-HT4 non-synonymous polymorphisms (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Michael K. Jensen − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; orcid.org/0000-0001-7574-4707; Email: mije@biosustain.dtu.dk

Authors

Bettina Lengger − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Emma E. Hoch-Schneider − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Christina N. Jensen − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Tadas Jakočiūnas − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark; orcid.org/0000-0003-1264-173X

Anja A. Petersen − Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark

Thomas M. Frimurer − Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, DK-2200 Copenhagen, Denmark

Emil D. Jensen − Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Complete contact information is available at:
https://pubs.acs.org/10.1021/acssensors.1c02061

Author Contributions

B.L., E.D.J., and M.K.J. conceived the study. B.L., E.E.H.-S., A.A.P., and T.M.F. performed all the experiments and performed all the data analysis. B.L., E.E.H.-S., E.D.J., T.J., and C.N.J. designed and constructed all plasmids and strains. B.L., E.E.H.-S., and M.K.J. wrote the manuscript. All authors approved the manuscript.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Berger, M.; Gray, J. A.; Roth, B. L. The Expanded Biology of Serotonin. Annu. Rev. Med. 2009, 60, 355−366.

(2) Nichols, D. E.; Nichols, C. D. Serotonin Receptors. Chem. Rev. 2008, 108, 1614−1641.

(3) Zhang, D.; Zhao, Q.; Wu, B. Structural Studies of G Protein-Coupled Receptors. Mol. Cells 2015, 38, 836.

(4) Syrovatkina, V.; Alegre, K. O.; Dey, R.; Huang, X.-Y. Regulation, Signaling, and Physiological Functions of G-Proteins. J. Mol. Biol. 2016, 428, 3850−3868.

(5) Leberer, E.; Dignard, D.; Harcus, D.; Thomas, D. Y.; Whiteway, M. The Protein Kinase Homologue Ste20p Is Required to Link the Yeast Pheromone Response G-Protein Beta Gamma Subunits to Downstream Signalling Components. EMBO J. 1992, 11, 4815−4824.

(6) Marinissen, M. J.; Gutkind, J. S. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol. Sci. 2001, 22, 368−376.

(7) Pándy-Szekeres, G.; Munk, C.; Tsonkov, T. M.; Mordalski, S.; Harpsøe, K.; Hauser, A. S.; Bojarski, A. J.; Gloriam, D. E. GPCRdb in 2018: Adding GPCR Structure Models and Ligands. Nucleic Acids Res. 2018, 46, D440−D446.

(8) Lengger, B.; Jensen, M. K. Engineering G Protein-Coupled Receptor Signalling in Yeast for Biotechnological and Medical Purposes. FEMS Yeast Res. 2020, 20, foz087.

(9) UniProt Consortium. UniProt: The Universal Protein Knowledgebase in 2021. Nucleic Acids Res. 2021, 49, D480−D489.

(10) King, K.; Dohlman, H. G.; Thorner, J.; Caron, M. G.; Lefkowitz, R. J. Control of Yeast Mating Signal Transduction by a Mammalian Beta 2-Adrenergic Receptor and Gs Alpha Subunit. Science 1990, 250, 121−123.

(11) Brown, A. J.; Dyos, S. L.; Whiteway, M. S.; White, J. H. M.; Watson, M.-A. E. A.; Marzioch, M.; Clare, J. J.; Cousens, D. J.; Paddon, C.; Plumpton, C.; Romanos, M. A.; Dowell, S. J. Functional Coupling of Mammalian Receptors to the Yeast Mating Pathway Using Novel Yeast/mammalian G Protein Alpha-Subunit Chimeras. Yeast 2000, 16, 11−22.

(12) Versele, M.; Lemaire, K.; Thevelein, J. M. Sex and Sugar in Yeast: Two Distinct GPCR Systems. EMBO Rep. 2001, 2, 574−579.

(13) Mukherjee, K.; Bhattacharyya, S.; Peralta-Yahya, P. GPCR-Based Chemical Biosensors for Medium-Chain Fatty Acids. ACS Synth. Biol. 2015, 4, 1261−1269.

(14) Ehrenworth, A. M.; Claiborne, T.; Peralta-Yahya, P. Medium-Throughput Screen of Microbially Produced Serotonin via a G-Protein-Coupled Receptor-Based Sensor. Biochemistry 2017, 56, 5471−5475.

(15) Nakamura, Y.; Ishii, J.; Kondo, A. Applications of Yeast-Based Signaling Sensor for Characterization of Antagonist and Analysis of Site-Directed Mutants of the Human Serotonin 1A Receptor. Biotechnol. Bioeng. 2015, 112, 1906−1915.

(16) Shaw, W. M.; Yamauchi, H.; Mead, J.; Gowers, G.-O. F.; Bell, D. J.; Öling, D.; Larsson, N.; Wigglesworth, M.; Ladds, G.; Ellis, T. Engineering a Model Cell for Rational Tuning of GPCR Signaling. Cell 2019, 177, 782−796.

(17) Conklin, B. R.; Bourne, H. R. Structural Elements of Gα Subunits That Interact with G$\beta\gamma$, Receptors, and Effectors. *Cell* **1993**, *73*, 631−641.

(18) Dohlman, H. G.; Song, J.; Ma, D.; Courchesne, W. E.; Thorner, J. Sst2, a Negative Regulator of Pheromone Signaling in the Yeast Saccharomyces Cerevisiae: Expression, Localization, and Genetic Interaction and Physical Association with Gpa1 (the G-Protein Alpha Subunit). *Mol. Cell. Biol.* **1996**, *16*, 5194−5209.

(19) Price, L. A.; Kajkowski, E. M.; Hadcock, J. R.; Ozenberger, B. A.; Pausch, M. H. Functional Coupling of a Mammalian Somatostatin Receptor to the Yeast Pheromone Response Pathway. *Mol. Cell. Biol.* **1995**, *15*, 6188−6195.

(20) Kapolka, N. J.; Taghon, G. J.; Rowe, J. B.; Morgan, W. M.; Enten, J. F.; Lambert, N. A.; Isom, D. G. DCyFIR: A High-Throughput CRISPR Platform for Multiplexed G Protein-Coupled Receptor Profiling and Ligand Discovery. *Proc. Natl. Acad. Sci. U.S.A.* **2020**, *117*, 13117−13126.

(21) Bean, B. D. M.; Mulvihill, C. J.; Garge, R. K.; Boutz, D. R.; Rousseau, O.; Floyd, B. M.; Cheney, W.; Gardner, E. C.; Ellington, A. D.; Marcotte, E. M.; Gollihar, J. D.; Whiteway, M.; Martin, V. J. J. Functional Expression of Opioid Receptors and Other Human GPCRs in Yeast Engineered to Produce Human Sterols. **2021**, bioRxiv: 2021.05.12.443385.

(22) Luo, X.; Reiter, M. A.; d'Espaux, L.; Wong, J.; Denby, C. M.; Lechner, A.; Zhang, Y.; Grzybowski, A. T.; Harth, S.; Lin, W.; Lee, H.; Yu, C.; Shin, J.; Deng, K.; Benites, V. T.; Wang, G.; Baidoo, E. E. K.; Chen, Y.; Dev, I.; Petzold, C. J.; Keasling, J. D. Complete Biosynthesis of Cannabinoids and Their Unnatural Analogues in Yeast. *Nature* **2019**, *567*, 123−126.

(23) Galanie, S.; Thodey, K.; Trenchard, I. J.; Filsinger Interrante, M.; Smolke, C. D. Complete Biosynthesis of Opioids in Yeast. *Science* **2015**, *349*, 1095−1100.

(24) Auton, A.; Auton, A.; Brooks, L. D.; Durbin, R. M.; Garrison, E. P.; Kang, H. M.; Korbel, J. O.; Marchini, J. L.; McCarthy, S.; McVean, G. A.; Abecasis, G. R.; Genomes Project Consortium. A Global Reference for Human Genetic Variation. *Nature* **2015**, *526*, 68−74.

(25) Spooner, W.; McLaren, W.; Slidel, T.; Finch, D. K.; Butler, R.; Campbell, J.; Eghobamien, L.; Rider, D.; Kiefer, C. M.; Robinson, M. J.; Hardman, C.; Cunningham, F.; Vaughan, T.; Flicek, P.; Huntington, C. C. Haplosaurus Computes Protein Haplotypes for Use in Precision Drug Design. *Nat. Commun.* **2018**, *9*, 4128.

(26) Madeira, F.; Park, Y. M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A. R. N.; Potter, S. C.; Finn, R. D.; Lopez, R. The EMBL-EBI Search and Sequence Analysis Tools APIs in 2019. *Nucleic Acids Res.* **2019**, *47*, W636−W641.

(27) Jessop-Fabre, M. M.; Jakočiūnas, T.; Stovicek, V.; Dai, Z.; Jensen, M. K.; Keasling, J. D.; Borodina, I. EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into Saccharomyces cerevisiae via CRISPR-Cas9. *Biotechnol. J.* **2016**, *11*, 1110.

(28) Jakočiūnas, T.; Bonde, I.; Herrgård, M.; Harrison, S. J.; Kristensen, M.; Pedersen, L. E.; Jensen, M. K.; Keasling, J. D. Multiplex Metabolic Pathway Engineering Using CRISPR/Cas9 in Saccharomyces Cerevisiae. *Metab. Eng.* **2015**, *28*, 213−222.

(29) Gietz, R. D.; Schiestl, R. H. High-Efficiency Yeast Transformation Using the LiAc/SS Carrier DNA/PEG Method. *Nat. Protoc.* **2007**, *2*, 31−34.

(30) Germann, S. M.; Baallal Jacobsen, S. A.; Schneider, K.; Harrison, S. J.; Jensen, N. B.; Chen, X.; Stahlhut, S. G.; Borodina, I.; Luo, H.; Zhu, J.; Maury, J.; Forster, J. Glucose-Based Microbial Production of the Hormone Melatonin in Yeast Saccharomyces Cerevisiae. *Biotechnol. J.* **2016**, *11*, 717−724.

(31) Team, RC *R: A Language and Environment for Statistical Computing*, Version 4.0. 2 (Taking Off Again); R Foundation for Statistical Computing: Vienna, Austria, 2020.

(32) RStudio Team *RStudio: Integrated Development Environment for R*; RStudio, PBC.: Boston, MA, 2020.

(33) Wickham, H.; Averick, M.; Bryan, J.; Chang, W.; McGowan, L.; François, R.; Grolemund, G.; Hayes, A.; Henry, L.; Hester, J.; Kuhn, M.; Pedersen, T.; Miller, E.; Bache, S.; Müller, K.; Ooms, J.; Robinson, D.; Seidel, D.; Spinu, V.; Takahashi, K.; Vaughan, D.; Wilke, C.; Woo, K.; Yutani, H. Welcome to the Tidyverse. *J. Open Source Softw.* **2019**, *4*, 1686.

(34) Hahne, F.; LeMeur, N.; Brinkman, R. R.; Ellis, B.; Haaland, P.; Sarkar, D.; Spidlen, J.; Strain, E.; Gentleman, R. flowCore: A Bioconductor Package for High Throughput Flow Cytometry. *BMC Bioinf.* **2009**, *10*, 106.

(35) Kolde, R. *Pheatmap: Pretty Heatmaps. 2015, R Package Version*, 2017.

(36) *GraphPad Software*: San Diego, California USA.

(37) Omasits, U.; Ahrens, C. H.; Müller, S.; Wollscheid, B. Protter: Interactive Protein Feature Visualization and Integration with Experimental Proteomic Data. *Bioinformatics* **2014**, *30*, 884−886.

(38) Carithers, L. J.; Ardlie, K.; Barcus, M.; Branton, P. A.; Britton, A.; Buia, S. A.; Compton, C. C.; DeLuca, D. S.; Peter-Demchok, J.; Gelfand, E. T.; Guan, P.; Korzeniewski, G. E.; Lockhart, N. C.; Rabiner, C. A.; Rao, A. K.; Robinson, K. L.; Roche, N. V.; Sawyer, S. J.; Segrè, A. V.; Shive, C. E.; Smith, A. M.; Sobin, L. H.; Undale, A. H.; Valentino, K. M.; Vaught, J.; Young, T. R.; Moore, H. M.; GTEx Consortium. A Novel Approach to High-Quality Postmortem Tissue Procurement: The GTEx Project. *Biopreserv. Biobanking* **2015**, *13*, 311−319.

(39) Uhlén, M.; Fagerberg, L.; Hallström, B. M.; Lindskog, C.; Oksvold, P.; Mardinoglu, A.; Sivertsson, Å.; Kampf, C.; Sjöstedt, E.; Asplund, A.; Olsson, I.; Edlund, K.; Lundberg, E.; Navani, S.; Szigyarto, C. A.-K.; Odeberg, J.; Djureinovic, D.; Takanen, J. O.; Hober, S.; Alm, T.; Edqvist, P.-H.; Berling, H.; Tegel, H.; Mulder, J.; Rockberg, J.; Nilsson, P.; Schwenk, J. M.; Hamsten, M.; von Feilitzen, K.; Forsberg, M.; Persson, L.; Johansson, F.; Zwahlen, M.; von Heijne, G.; Nielsen, J.; Pontén, F. Proteomics. Tissue-Based Map of the Human Proteome. *Science* **2015**, *347*, 1260419.

(40) Manabe, N.; Wong, B. S.; Camilleri, M. New-Generation 5-HT4 Receptor Agonists: Potential for Treatment of Gastrointestinal Motility Disorders. *Expet Opin. Invest. Drugs* **2010**, *19*, 765−775.

(41) Wong, B. S.; Manabe, N.; Camilleri, M. Role of Prucalopride, a Serotonin (5-HT(4)) Receptor Agonist, for the Treatment of Chronic Constipation. *Clin. Exp. Gastroenterol.* **2010**, *3*, 49−56.

(42) Berumen, L. C.; Rodríguez, A.; Miledi, R.; García-Alcocer, G. Serotonin Receptors in Hippocampus. *Sci. World J.* **2012**, *2012*, 823493.

(43) Conklin, B. R.; Farfel, Z.; Lustig, K. D.; Julius, D.; Bourne, H. R. Substitution of Three Amino Acids Switches Receptor Specificity of Gq Alpha to that of Gi Alpha. *Nature* **1993**, *363*, 274−276.

(44) Conklin, B. R.; Herzmark, P.; Ishida, S.; Voyno-Yasenetskaya, T. A.; Sun, Y.; Farfel, Z.; Bourne, H. R. Carboxyl-Terminal Mutations of Gq Alpha and Gs Alpha That Alter the Fidelity of Receptor Activation. *Mol. Pharmacol.* **1996**, *50*, 885.

(45) Maury, J.; Germann, S. M.; Baallal Jacobsen, S. A.; Jensen, N. B.; Kildegaard, K. R.; Herrgård, M. J.; Schneider, K.; Koza, A.; Forster, J.; Nielsen, J.; Borodina, I. EasyCloneMulti: A Set of Vectors for Simultaneous and Multiple Genomic Integrations in Saccharomyces Cerevisiae. *PLoS One* **2016**, *11*, No. e0150394.

(46) Murakami, C. J.; Wall, V.; Basisty, N.; Kaeberlein, M. Composition and Acidification of the Culture Medium Influences Chronological Aging Similarly in Vineyard and Laboratory Yeast. *PLoS One* **2011**, *6*, No. e24530.

(47) Marti-Solano, M.; Crilly, S. E.; Malinverni, D.; Munk, C.; Harris, M.; Pearce, A.; Quon, T.; Mackenzie, A. E.; Wang, X.; Peng, J.; Tobin, A. B.; Ladds, G.; Milligan, G.; Gloriam, D. E.; Puthenveedu, M. A.; Babu, M. M. Combinatorial Expression of GPCR Isoforms Affects Signalling and Drug Responses. *Nature* **2020**, *587*, 650−656.

(48) Scott, B. M.; Chen, S. K.; Bhattacharyya, N.; Moalim, A. Y.; Plotnikov, S. V.; Heon, E.; Peisajovich, S. G.; Chang, B. S. W. Coupling of Human Rhodopsin to a Yeast Signaling Pathway Enables Characterization of Mutations Associated with Retinal Disease. *Genetics* **2019**, *211*, 597−615.

(49) Briejer, M. R.; Bosmans, J.-P.; Van Daele, P.; Jurzak, M.; Heylen, L.; Leysen, J. E.; Prins, N. H.; Schuurkes, J. A. J. The in Vitro

Pharmacological Profile of Prucalopride, a Novel Enterokinetic Compound. *Eur. J. Pharmacol.* **2001**, *423*, 71−83.

(50) Yasi, E. A.; Allen, A. A.; Sugianto, W.; Peralta-Yahya, P. Identification of Three Antimicrobials Activating Serotonin Receptor 4 in Colon Cells. *ACS Synth. Biol.* **2019**, *8*, 2710−2717.

(51) Kapolka, N. J.; Rowe, J. B.; Taghon, G. J.; Morgan, W. M.; O'Shea, C. R.; Isom, D. G. Proton-Gated Coincidence Detection Is a Common Feature of GPCR Signaling. *Proc. Natl. Acad. Sci. U.S.A.* **2021**, *118*, No. e2100171118.

(52) Fukutani, Y.; Nakamura, T.; Yorozu, M.; Ishii, J.; Kondo, A.; Yohda, M. The N-Terminal Replacement of an Olfactory Receptor for the Development of a Yeast-Based Biomimetic Odor Sensor. *Biotechnol. Bioeng.* **2012**, *109*, 205−212.

(53) Dirnberger, D.; Seuwen, K. Signaling of Human Frizzled Receptors to the Mating Pathway in Yeast. *PLoS One* **2007**, *2*, No. e954.

(54) Choudhary, P.; Loewen, M. C. Evidence of a Role for S. Cerevisiae α-Arrestin Art1 (Ldb19) in Mating Projection and Zygote Formations. *Cell Biol. Int.* **2016**, *40*, 83−90.

(55) Rivail, L.; Giner, M.; Gastineau, M.; Berthouze, M.; Soulier, J.-L.; Fischmeister, R.; Lezoualc'h, F.; Maigret, B.; Sicsic, S.; Berque-Bestel, I. New Insights into the Human 5-HT4 Receptor Binding Site: Exploration of a Hydrophobic Pocket. *Br. J. Pharmacol.* **2004**, *143*, 361−370.

(56) Vass, M.; Podlewska, S.; de Esch, I. J. P.; Bojarski, A. J.; Leurs, R.; Kooistra, A. J.; de Graaf, C. Aminergic GPCR-Ligand Interactions: A Chemical and Structural Map of Receptor Mutation Data. *J. Med. Chem.* **2019**, *62*, 3784−3839.

(57) Mialet, J.; Dahmoune, Y.; Lezoualc'h, F.; Berque-Bestel, I.; Eftekhari, P.; Hoebeke, J.; Sicsic, S.; Langlois, M.; Fischmeister, R. Exploration of the Ligand Binding Site of the Human 5-HT(4) Receptor by Site-Directed Mutagenesis and Molecular Modeling. *Br. J. Pharmacol.* **2000**, *130*, 527−538.

(58) Scott, B. M.; Gutiérrez-Vázquez, C.; Sanmarco, L. M.; da Silva Pereira, J. A.; Li, Z.; Plasencia, A.; Hewson, P.; Cox, L. M.; O'Brien, M.; Chen, S. K.; Moraes-Vieira, P. M.; Chang, B. S. W.; Peisajovich, S. G.; Quintana, F. J. Self-Tunable Engineered Yeast Probiotics for the Treatment of Inflammatory Bowel Disease. *Nat. Med.* **2021**, *27*, 1212.