Substitution of 5-HT$_{1A}$ Receptor Signaling by a Light-activated G Protein-coupled Receptor*

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Understanding serotonergic (5-HT) signaling is critical for understanding human physiology, behavior, and neuropsychiatric disease. 5-HT mediates its actions via ionotropic and metabotropic 5-HT receptors. The 5-HT$_{1A}$ receptor is a metabotropic G protein-coupled receptor linked to the G$_{i/o}$ signaling pathway and has been specifically implicated in the pathogenesis of depression and anxiety. To understand and precisely control 5-HT$_{1A}$ signaling, we created a light-activated G protein-coupled receptor that targets into 5-HT$_{1A}$ receptor domains and substitutes for endogenous 5-HT$_{1A}$ receptors. To induce 5-HT$_{1A}$-like targeting, vertebrate rhodopsin was tagged with the C-terminal domain (CT) of 5-HT$_{1A}$ (Rh-CT$_{5-HT1A}$). Rh-CT$_{5-HT1A}$ activates G protein-coupled inward rectifying K$^+$ channels in response to light and causes membrane hyperpolarization in hippocampal neurons, similar to the agonist-induced responses of the 5-HT$_{1A}$ receptor. The intracellular distribution of Rh-CT$_{5-HT1A}$ resembles that of the 5-HT$_{1A}$ receptor; Rh-CT$_{5-HT1A}$ localizes to somatodendritic sites and is efficiently trafficked to distal dendritic processes. Additionally, neuronal expression of Rh-CT$_{5-HT1A}$ but not Rh, decreases 5-HT$_{1A}$ agonist sensitivity, suggesting that Rh-CT$_{5-HT1A}$ and 5-HT$_{1A}$ receptors compete to interact with the same trafficking machinery. Finally, Rh-CT$_{5-HT1A}$ is able to rescue 5-HT$_{1A}$ signaling of 5-HT$_{1A}$ KO mice in cultured animals to inhibit neuronal and neural network excitability (9). Furthermore, we have shown that Rh can be exogenously expressed in non-visual cell types to activate downstream targets of G$_{i/o}$ signaling (9). Therefore, we have shown that Rh can be exogenously expressed in neurons both in primary culture and in intact animals to inhibit neuronal and neural network excitability (9). Based on these findings, we aimed to tailor the properties of Rh to manipulate other GPCR signaling pathways, namely, the 5-HT$_{1A}$-G$_{i/o}$ pathway. Here we describe the development of a chimeric light-sensitive GPCR that mimics the intracellular targeting and functional G$_{i/o}$-linked signaling of wild type 5-HT$_{1A}$. This receptor, which we call Rh-CT$_{5-HT1A}$, is able to functionally substitute for endogenous 5-HT$_{1A}$ receptors by exploiting the intracellular trafficking mechanisms used by the endogenous receptors. We show that Rh-CT$_{5-HT1A}$ distributes intra-neuronally to cell membrane sites normally occupied by 5-HT$_{1A}$.
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which then allows Rh-CT$_{5-HT_{1A}}$ to induce activation of the same downstream G$_{i/o}$ signaling targets with light stimulus.

**EXPERIMENTAL PROCEDURES**

**Generation of Plasmid Constructs for Transfection and Pseudovirion Production**—Rat Rh (RO4) and human 5-HT$_{1A}$ cDNA (GenBank™ accession numbers Z46957 and AF498978) clones were tagged C-terminally with mCherry immediately after the last coding codon using a two-step fusion PCR. Distal primers for Rh-mCherry were 5'-ATGGCTCGAGATGAAGGGCGAGAACGAGGCG-3' and 5'-GCTGTTCTTGTACAGCTCGTCCATGC-3'; distal primers for 5-HT$_{1A}$-mCherry were 5'-ATGGCTCGAGATGAAGGGCGAGAACGAGGCG-3' and 5'-GCTGTTCTTGTACAGCTCGTCCATGC-3'. Primers for the fusion site were 5'-AGGTGGCTCCAGCCATGGCTGGAGCCACCTGGCT-3' and 5'-CTGCACCATCTGGCGGCAGAACTTACACTTAAT-3'.

**Pseudovirion Production**—Rat Rh (RO4) and human 5-HT$_{1A}$ cDNA was cloned into the XhoI and NotI sites of pEGFP-N1 (Clontech) to generate HEK cell expression clones. Human 5-HT$_{1A}$ cDNA was appended immediately after the last coding nucleotide of Rh-mCherry by fusion PCR using the 5' distal primer for Rh-mCherry, the 3' distal primer for 5-HT$_{1A}$-mCherry, and the fusion primers 5'-GACATGGACGAGCTGTACAAGAACAAGGACTTTCAAAACGCG-3' and 5'-CTGACGACTTTCAAAACGCG-3'.

**Lentiviral Injections into the Dorsal Raphe Nuclei**—Lentivirus expressing Rh-CT$_{5-HT_{1A}}$ was injected into the dorsal raphe nucleus (DRN) of wild type (C57Bl/6J), ePet::YFP or 5-HT$_{1A}$ KO mice. 3-Week-old mice were anesthetized with 1–2% isoflurane in air delivered from a precision vaporizer (WPI) and mounted onto a stereotactic frame (Narishige). A sagittal incision along the midline was made to expose the cranium, and a burr hole was drilled 4.1 mm from the bregma. The tip of a micropipette attached to a 30-ml syringe was lowered into the dorsal raphe nucleus, and 3–4 μl of the virus was injected. Mice were housed for 7–10 days before performing immunohistochemistry or electrophysiological experiments.

**Immunofluorescence, Image Acquisition, and Data Analysis**—tsA201 cells were transfected with the indicated DNAs using Lipofectamine 2000, and hippocampal neurons (8–10 DIV) were transfected with using Sindbis viral stocks. HEK cells and neurons were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS 18 h and 12 h post-transfection, respectively. Anti-dsRed (Clontech; 1:300) and anti-5-HT$_{1A}$ (Clontech; 1:500) primary antibodies overnight at 4 °C. After extensive washes, cells were blocked with 10% normal goat serum and 3% BSA and incubated with secondary antibodies (Molecular Probes) for 30 min at room temperature. Cells were mounted in Prolong Gold antifade medium (Molecular Probes). Images were acquired with a Zeiss LSM 510 confocal microscope using 20× and 40× water objectives and analyzed by using VOLUMETRY (Improvision, Lexington, MA) and Zeiss LSM 5 software (Release 3.2). Z-stack images were acquired to image the entire cell and displayed as a projected image or single slice through the center of cell where indicated. For quantification of relative fluorescence intensity, imaging parameters were adjusted so that pixel intensity within neurites did not saturate. The line profile function in the LSM 5 software was used to trace the longest dendrite of each neuron analyzed. Dendrites were identified by both MAP-2 (dendritic marker) and GFP (positive infection) fluorescence. Fluorescence intensity was normalized to maximal intensity of each dendrite. For quantification of fluorescence along dendrite, piecewise linear interpolation was performed of each plot to and resuspended in HBSS. Viral titer was greater than 1 × 10$^{8}$ units per ml and stocked at −80 °C.

Continental culture of hippocampal neurons from P0-P3 rats and mice were performed by a modified Banker sandwich method as described (11, 12). The generation of 5-HT$_{1A}$ KO mice (13, 14) and genotyping methods (15) have been previously described. WT mice (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Handling and care of mice followed federal guidelines, and experimental methods were approved by the Case Western Reserve University Institutional Animal Care and Use Committee. For neuronal infection, 0.5–5 μl of thawed Sindbis virus suspension was added to cultured hippocampal neurons (9–14 DIV) on coverslips in 24-well plates. GFP expression was detected after 10 h and reached maximal expression after 24 h.
normalize the line profile distance to 1000 values between 0.0 and 1.0. Interpolated data were then grouped and plotted at the mean ± S.E. at each normalized point.

For immunohistology, adult mice were deeply anesthetized before transcardial perfusion with 4% paraformaldehyde in 0.1 M PBS for 20 min. The brain was then removed and fixed in paraformaldehyde for another 2 h at room temperature followed by cryoprotection in 30% sucrose (w/v) overnight at 4 °C. Tissue sections (16–20 μm) were prepared on a freezing microtome or cryostat and mounted on Superfrost Plus Microscope Slides (Fisher). Fluorescent immunohistochemistry was performed as described (16). Tissue sections were immunolabeled with rabbit anti-GFP (1:1,000, Invitrogen) and then FITC-conjugated secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). Fluorescent images were collected using a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus Optical BX51 microscope (Center Valley, PA).

**Electrophysiology and Data Analysis**—For GIRK channel recordings in HEK293 cells, human GIRK channel subunits (KCNJ3/5) and light-sensitive GPCRs or 5-HT1A receptor were coexpressed in tsA201 cells. GIRK subunit DNA was purchased from Genecopoeia (Rockville, MD). Cells were cultured and recorded in dark room conditions (red light only) after transfection. GIRK-mediated K⁺ currents were measured and analyzed as described previously (17). Absolute GIRK current was determined by brief application of a low K⁺ solution to abrogate GIRK current: 138 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-NaOH, pH 7.3 (KOH). The difference in current elicited with high K⁺ (external solution) and low K⁺ solutions was determined to be the absolute GIRK current. The external solution was as follows: 20 mM NaCl, 120 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES-NaOH, pH 7.3 (KOH). Patch pipettes (2–5 MΩ) were filled with internal solution: 100 mM potassium aspartate, 40 mM KCl, 5 mM MgATP, 10 mM HEPES-KOH, 5 mM NaCl, 2 mM EGTA, 2 mM MgCl₂, 0.01 mM GTP, pH 7.3 (KOH). Cells were incubated in external solution containing 1 μM 9-cis-retinal (Sigma) for 20 min before light stimulation. Cells were visualized using a transilluminated red light (590 nm filter) during experimental manipulations. GTPγS was added to the internal solution at a final concentration of 0.6 mM where indicated. Solutions containing agonist or low potassium were applied directly onto the recorded cells using a fast-flow perfusion system (ALA Scientific, Farmingdale, NY).

Cultured hippocampal neurons were recorded on days 10–14 in vitro and 14–20 h after Sindbis virus infection. Extracellular recording solution contained 125 mM NaCl, 2 mM KCl, 10 mM Hepes, 30 mM glucose, 3 mM CaCl₂, and 1 mM MgCl₂, pH 7.3 (NaOH); internal solution contained 97 mM potassium gluconate, 10 mM Hepes, 1 mM potassium-EGTA, 4 mM Mg-ATP, and 0.4 mM Na-GTP, pH 7.3 (KOH). Synaptic activity was silenced by adding 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (Tocris) and 10 μM SR 95531 hydrobromide (Gabazine, Tocris). Cells were perfused with 1 μM 9-cis-retinal (Sigma) for 2 min before light stimulation. 1 μM 8OH-DPAT (Calbiochem) and 50 μM baclofen (Sigma) were used in experiments where indicated.

Whole-cell patch clamp recordings of cultured neurons and tsA201 (18) were performed with an EPC9 amplifier (HEKA). Currents were digitized at 10 kHz and filtered with the internal 10-kHz three-pole Bessel filter (filter 1) in series with a 2.9-kHz 4-pole Bessel filter (filter 2) of the EPC9 amplifier. Series resistances were partially compensated between 70 and 90%. Leak and capacitive currents were subtracted by using hyperpolarizing pulses from −60 to −70 mV with the p/4 method.

**Brain Slice Recordings**—Coronal slices including dorsal raphe (250 μm thick) were cut from brains of the mice 8–12 days after lentivirus injection. Mice were anesthetized with isoflurane and decapitated. The removed brainstem was cooled and sliced in ice-cold solution containing 87 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.23 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose bubbled with 95% O₂ and 5% CO₂. Flourescent images were collected for at least 1 h at room temperature in a recording artificial cerebrospinal fluid containing 124 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.23 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose bubbled with 95% O₂ and 5% CO₂. Fluorescent mCherry-positive cells were visually identified under an upright microscope (DMLFLA, Leica) equipped with a monochromator system (Polychrome IV, TILL Photonics) flashing 585-nm excitation light. Whole-cell recordings were made at room temperature in the dark except for using infrared light to target the cell. Slices were preincubated at least 20 min and continuously perfused with the external solution including 25 μM 9-cis-retinal, 0.025% (±)-α-tocopherol (Sigma), 0.2% essentially fatty acid free albumin from bovine serum (Sigma), and 0.1% dimethyl sulfoxide. Patch pipettes (2–4 MΩ) were filled with an internal solution with the composition 140 mM potassium methyl sulfate, 4 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP, and 10 mM Tris-phosphocreatine, pH 7.3 (KOH). Membrane currents and voltages were recorded with an EPC10/2 amplifier (HEKA). The signals were filtered at 3 kHz and digitized at 50 kHz. The PatchMaster software (HEKA) was used for the controls of voltage and data acquisition, and off-line analysis was made with Igor Pro 6.0 software (Wavemetrics).

Statistical significance throughout the experiments was tested with ANOVA using Igor 6.0 software (Wavemetrics). S.E. are the mean ± S.E.

**RESULTS**

**Cloning of Rh-CT5-HT1A and Optimization of the Light Activation Paradigm**—Vertebrate Rh and 5-HT1A are G_ia/o-linked GPCRs belonging to the Class A (rhodopsin-like) family of seven transmembrane domain receptors. Because they both activate G_{a/o}-linked downstream signaling pathways, we hypothesized that we could functionally replace 5-HT1A receptor with a light-sensitive receptor by inducing the subcellular targeting of Rh to copy that of wild type 5-HT1A receptor. We did this by tagging Rh with the C-terminal domain of 5-HT1A receptor (CT), which has been shown to be critical for regulating correct intracellular trafficking of 5-HT1A via its interaction with the Yif1B (19). This receptor, which we call Rh-CT5-HT1A, consists of Rh-tagged C-terminally with mCherry and then with the CT
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A

GIRK

B

Rh + GIRK

C

Induced GIRK current

D

Maximal GIRK current

FIGURE 1. Vertebrate rhodopsin does not activate GIRK channels in the absence of light stimulus. HEK293 (tsA201) cells were co-transfected with either GIRK1/4 subunits alone (A) or GIRK1/4 and Rh (C), and GIRK currents were measured at a holding potential of −60 mV. 0.6 mM GTPγS present in the intracellular recording solution caused constitutive G protein activation and subsequent GIRK current enhancement. A low K$^+$ (2 mM) solution was applied for 10 s (white bars) at 5 s and 5 min after establishment of the whole-cell mode. B, absolute inward currents through GIRK channels were calculated as the difference between current in normal (high K$^+$) extracellular recording solution and low K$^+$ (2 mM). The current induced by GTPγS was calculated as the difference between absolute GIRK current at 5 min and 5 s. D, maximal GIRK current was determined by calculating induced GIRK current at 5 min after gaining access to the intracellular compartment. Quantification of GIRK current induced by GTPγS shows no significant difference between HEK293 cells transfected with Rh and GIRK1/4 or GIRK1/4 subunits alone. (mean ± S.E.; p > 0.05, ANOVA).

of 5-HT$_{1A}$ (see Fig. 2A). To determine whether this modified receptor retained G$_{i/o}$-linked GPCR activity, it was co-expressed with human GIRK1 and -4 subunits in HEK293 cells.

Accurate functional comparison of chimeric receptor with Rh and other GPCRs required modification of fluorescent tag, retinal loading, and recording conditions to improve assay consistency. GFP has been previously used as a CT tag to track functional expression in transgenic animals (20–22). However, when we expressed a similar construct, Rh-EGFP, in HEK293 cells, the amplitude of maximal GIRK channel activation we observed was at best only ~50% that of untagged or mCherry-tagged versions of Rh (data not shown). Furthermore, the consistency of responses to light stimulus of GFP positive cells was observed was at best only ~50% that of untagged or mCherry-tagged versions of Rh (data not shown). Furthermore, the consistency of responses to light stimulus of GFP positive cells was relatively low, 44.44% (12/27). Rh is maximally excited at 485 nm, which coincides almost exactly with the excitation wavelength of EGFP (488 nm). Thus, use of EGFP as a marker for positive transfection could cause inadvertent receptor activation as the rhodopsin apoprotein itself (even in the absence of retinal) exhibits weak activity (23). mCherry is an improved fluorescent tag because it has an excitation/emission profile of 587 nm/610 nm, which lies outside of the absorption spectrum of Rh (24).

Phototransduction by Rh is initiated by the isomerization of the photosensitive pigment, 11-cis-retinal, by light. In the visual system, spent substrate (all-trans-retinal) is recycled by a series of transport and enzymatic reactions (25). HEK cells possess the intrinsic capability to regenerate 11-cis-retinal from all-trans-retinal or other analogs such as 9-cis or 13-cis-retinal but require an exogenous source of retinal (26). Another source of variability was the retinal loading conditions of Rh and variants, which was conditioned by the variability of serum used as a culture media supplement. Fetal bovine sera (FBS) contain retinal compounds as evidenced by the ability to activate transfected Rh in HEK293 cells cultured in media made with some but not all lots of FBS. This raised the possibility that ambient light could inadvertently activate the light-sensitive GPCRs. This in turn could lead to a decrease in receptor activity and/or desensitization, potentially confounding the experiments. Considering these complications, cells were kept in the dark after transfection and during experimental procedures. Furthermore, a 20-min preincubation of 1 μM 9 cis-retinal before recordings was used to yield the most consistent results, regardless of culture media composition. 90.48% (19/21) of mCherry-positive cells responded to light stimulus under these optimized conditions.

In vertebrate rod and cone cells, bleached vertebrate rhodopsin is able to transduce signal, and the pigment may remain in steady state of activation even after light stimulation is eliminated (27–29). Thus, we tested the possibility that light-activated receptors were active in heterologous expression systems even in the absence of light stimulus. This phenomenon could limit the extent of GIRK current modulation observed and could lead to constitutive, basal increases in G$_{i/o}$ activation. More importantly, because the ultimate goal is to exogenously express Rh-CT$_{5-HT_{1A}}$ in other cell types, this would limit the utility of the light-sensitive receptor as merely expressing it would affect function without light application. HEK293 cells transfected with GIRK1/4 subunits alone (Fig. 1A) or co-transfected with Rh (Fig. 1C) were analyzed with GTPγS (a non-hydrolyzable GTP analog) in the intracellular recording solution. GTPγS caused constitutive G protein activation and gradually led to maximal GIRK current induction. The absolute
Expression Pattern and Function of Rh-CT5-HT1A Resembles Fluorescently Tagged 5-HT1A in HEK Cells—mCherry-tagged versions of Rh, 5-HT1A, and Rh-CT5-HT1A were cloned into mammalian expression vectors (Fig. 2A). The determinants of G protein specificity and subcellular targeting are presumably contained within the intracellular domains of GPCRs. Based on this assertion, two different chimeric receptors were generated. The first consisted of Rh tagged C-terminally with mCherry and then the CT domain of 5-HT1A (Rh-CT5-HT1A). The CT domain of 5-HT1A has been shown to be necessary for its dendritic targeting via interaction with a putative ER/Golgi trafficking protein, Yif1B (19). The second was a mutant Rh receptor in which the intracellular domains were exchanged for those of 5-HT1A receptor. The rationale for this GPCR was that extracellular and transmembrane domains of Rh were retained, thus preserving responsiveness to light, but the intracellular domains of 5-HT1A would induce subcellular targeting and G protein coupling like 5-HT1A. The exact Rh residues retained and domain borders were analogous to the Rhβ2-adrenergic and Rh4α1-adrenergic receptors generated by the Khorana and co-workers (30) and Deisseroth and co-workers (31). However, this receptor revealed uncharacteristic activation kinetics and was constitutively active once light was applied (data not shown). We, therefore, did not perform a more precise analysis of this chimeric receptor but concentrated on the characterization of Rh-CT5-HT1A.

GIRK current was assessed by a short application of low K+ (2 mM), eliminating the inward K+ current. The GIRK current was then calculated as the difference between current immediately before (high K+) and during the low K+ treatment. The GIRK currents induced by GTPγS were not significantly different for HEK cells transfected with Rh and GIRK (410.919 ± 65.217 pA (n = 8)) versus GIRK subunits alone (375.958 ± 47.104 pA (n = 9)) (Fig. 1B), indicating that there was no appreciable activation of GIRK by Rh without light stimulus. Furthermore, the maximal GIRK current revealed by GTPγS was comparable in cells transfected with GIRK (771.722 ± 181.719 pA (n = 9)) or Rh with GIRK subunits (792.455 ± 79.213 pA (n = 8)) (Fig. 1D), suggesting that Rh co-expression did not interfere with GIRK expression level or targeting.

Functional expression and characterization of Rh-CT5-HT1A in HEK293 cells. A, shown are schematic representations of GPCRs C-terminal-tagged with mCherry used for exogenous expression. The chimera Rh-CT5-HT1A contains the C-terminal domain of human 5-HT1A receptor after the fluorescent tag. GPCRs (left, red) and GIRK1/4 (center, green) channels target efficiently to the cell membrane. Right, overlay of left and right panels shows colocalization of transfected GPCRs and GIRK channels indicated by a yellow color (scale bar = 10 μm). Time course of GPCR-induced GIRK current increase demonstrates that the increase in GIRK current by Rh-mCherry (C) and Rh-CT5-HT1A (D) in response to a 10-s light pulse of 485 nm (green bars) is comparable with the GIRK current increase mediated by 5-HT1A (E) or 5-HT1A-mCherry (F) when activated by 10 s application of 8OH-DPAT (1 μM) (black bars). GIRK currents were measured at a holding potential of −60 mV. G, average light and agonist-induced GIRK current increase is shown. Light-sensitive GPCRs were activated with a 10-s light pulse (485 nm), and 1 μM 8OH-DPAT was applied to cells transfected with 5-HT1A receptors. H, shown is a comparison of the time constants of the GPCR-induced GIRK current changes before and after GPCR activation. Throughout the experiments the number in parentheses indicates the number of experiments and statistical significance as indicated (mean ± S.E.; * indicates activation time constant was significantly different from Rh, p < 0.05; ** indicates activation time constant was significantly different from Rh, p < 0.001, ANOVA).
When co-transfected into HEK293 cells, exogenously expressed Rh-mCherry, Rh-CT\textsubscript{5-HT\textsubscript{1A}}, and 5-HT\textsubscript{1A}-mCherry targeted efficiently to the cell membrane and colocalized with GIRQ channel subunits (Fig. 2B). Functionally, Rh-mCherry and Rh-CT\textsubscript{5-HT\textsubscript{1A}} were able to activate GIRK current when exposed to light at 485 nm (Figs. 2, C and D). The extent of GIRK activation was not significantly different from untagged Rh and was similar to responses induced in tagged and untagged 5-HT\textsubscript{1A} receptors (Figs. 2, E and F) by the selective 5-HT\textsubscript{1A} agonist, 8OH-DPAT (Fig. 2G). It is important to note that the GIRK current induction for all GPCRs tested is similar to that induced by GTP\textgamma{}S (Fig. 1B), suggesting that both agonist and light application caused near maximal induction of GIRK for 5-HT\textsubscript{1A} and light-sensitive receptors, respectively. The time constants for onset of GIRK channel activation and deactivation were also similar between Rh, Rh-CT\textsubscript{5-HT\textsubscript{1A}}, and 5-HT\textsubscript{1A} receptors (\(\tau\textsubscript{on} \approx 2–10\) s, \(\tau\textsubscript{off} \approx 30–50\) s, Fig. 2H), although activation of 5-HT\textsubscript{1A} and 5-HT\textsubscript{1A}-mCherry were significantly faster (\(\tau\textsubscript{on} = 1–2\) s) than the light-activated receptors 5-HT\textsubscript{1A}-mCherry localized somatodendritically in neurons (Fig. 3, M–P), which is consistent with the \textit{in vivo} distribution of 5-HT\textsubscript{1A} observed in serotonergic neurons (2, 3, 32). Likewise, Rh-CT\textsubscript{5-HT\textsubscript{1A}} showed an analogous intracellular trafficking pattern (Fig. 3, I–L). No dsRed+/GFP+/MAP-2 negative processes were seen in neurons infected with 5-HT\textsubscript{1A} and Rh-CT\textsubscript{5-HT\textsubscript{1A}} viruses. Rh-CT\textsubscript{5-HT\textsubscript{1A}} and 5-HT\textsubscript{1A} fluorescence was absent from the axonal processes of positively infected neurons (Tau-1+/GFP+) at described expression conditions. Axonal Rh-CT\textsubscript{5-HT\textsubscript{1A}} was only observed when the receptor was expressed at very high levels by using 2–10-fold higher titer virus and allowing Sindbis infection to occur for greater than 24 h (data not shown). At these conditions, toxicity effects and cell death were observed most likely due to inhibition of host protein synthesis by excessive virally driven expression (33). Axonal targeting and toxicity were similarly observed with high expression of wild type 5-HT\textsubscript{1A}. Taken together, the data show that the CT of 5-HT\textsubscript{1A} is sufficient to promote somatodendritic

\(\tau\textsubscript{on} \approx 9–10\) s). The kinetics of inactivation for all GPCRs tested were not significantly different, suggesting that the addition of mCherry and CT tags to Rh does not interfere with function.

\textit{Subcellular Targeting of Rh-CT\textsubscript{5-HT\textsubscript{1A}} Resembles That of 5-HT\textsubscript{1A}—Exogenous expression of Rh targets to both somatodendritic and presynaptic sites when expressed in rat hippocampal neurons (9). We confirmed this by immunolabeling neurons infected with Sindbis virus driving the expression of Rh-mCherry and also EGFP under the control of a second subgenomic promoter. GFP was expressed throughout the entire cell and proved to be a valuable tool for identifying and matching axons, dendrites, and soma of infected neurons. Rh-mCherry (Fig. 3A) colocalized with the dendritic marker, MAP-2 (Fig. 3C), but was also expressed in processes that were presumably axons because they were GFP-positive (Fig. 3B) but lacked MAP-2 labeling (Fig. 3, C and D). To confirm this, neurons were stained with anti-Tau-1 antibody (axonal marker), and we observed Rh-mCherry fluorescence that coincided with processes labeled by Tau-1 (Fig. 3, E–H). This indicated that Rh targeted both to axons and dendrites, confirming our previous findings (9). In contrast, virally expressed

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![FIGURE 3. The C-terminal domain of 5-HT\textsubscript{1A} is sufficient to induce targeting of vertebrate rhodopsin somatodendritically in neurons. Confocal immunofluorescence images were taken of cultured rat hippocampal neurons (8 DIV) infected with Sindbis virus driving expression of Rh-mCherry (A–H), Rh-CT\textsubscript{5-HT\textsubscript{1A}} (I–L), and 5-HT\textsubscript{1A}-mCherry (M–P). All Sindbis virus vectors also induced expression of EGFP (B, F, J, and N) under the control of a second subgenomic promoter. Neurons were stained with anti-dsRed antibody (A, E, I, and M) to delineate the distribution of mCherry-tagged receptors and co-labeled with the dendritic marker, MAP-2 (C), or axonal marker, Tau-1 (G, K, and O). Rh-mCherry targets to both axons and dendrites in cultured neurons. Neurons infected with Rh-mCherry virus showed processes with dsRed (A), GFP (B), and MAP-2 (C) staining. Rh-mCherry and GFP were also found in processes that lack MAP-2 expression, suggesting that both proteins are targeted to axons (C). These processes are labeled by \textit{white arrows}. D, overlay of dsRed, GFP, and MAP-2 staining shows colocalization pattern. Rh-mCherry targeting to axons was revealed by the presence of processes colabeled with anti-dsRed (E) and anti-Tau-1 (G) antibodies. H, overlay of dsRed, GFP, and Tau-1 labeling shows colocalization. The \textit{white arrow} points to the Rh-mCherry-, GFP-, and Tau-1-positive axon. Rh-CT\textsubscript{5-HT\textsubscript{1A}} (I) expressed in hippocampal neurons is present in dendrites but is not targeted to GFP-positive (J) and Tau-1-positive (K) processes. \textit{White arrows} mark these axons. L, overlay of dsRed in neurons infected with Rh-CT\textsubscript{5-HT\textsubscript{1A}} virus shows colocalization of Tau-1-positive and GFP-positive but not Rh-CT\textsubscript{5-HT\textsubscript{1A}} processes. 5-HT\textsubscript{1A}-mCherry (M) also is not targeted to GFP-positive (N) and Tau-1-positive (O) processes. \textit{White arrows} mark one of these processes. P, overlay of dsRed, GFP, and Tau-1 images shows colocalization of Tau-1- and GFP-positive but 5-HT\textsubscript{1A}-mCherry negative processes. 10, 12, and 8 neurons expressing Rh-mCherry, Rh-CT\textsubscript{5-HT\textsubscript{1A}}, and 5-HT\textsubscript{1A}-mCherry, respectively, were analyzed. The scale bar represents 20 μm. Images are representative z-stack images projected to two dimensions.}
trafficking away from axons in an analogous manner to wild type 5-HT$_{1A}$.

Another similarity between 5-HT$_{1A}$-mCherry and Rh-CT$_{5-HT_{1A}}$ was their efficient targeting to the distal ends of dendrites. In comparison to Rh-mCherry (Fig. 4A), 5-HT$_{1A}$-mCherry and Rh-CT$_{5-HT_{1A}}$ fluorescence is observed much further away from the soma (Fig. 4, B and C). To quantify dendritic fluorescence distributions, neurons infected with Sindbis virus were stained with anti-dsRed (red) and anti-MAP-2 (not shown) antibodies to enhance and delineate mCherry tagged receptors and dendrites, respectively. Images taken at 20× magnification reveal that dsRed fluorescence is observed much more distally from the soma for neurons expressing Rh-CT$_{5-HT_{1A}}$ (B) and 5-HT$_{1A}$-mCherry (C) when compared with neurons expressing Rh-mCherry (A).

E, normalized fluorescence of the longest dendrite of a given neuron was quantified as a function of distance from the soma. Rh-CT$_{5-HT_{1A}}$ (red) and 5-HT$_{1A}$-mCherry (green) (mean ± S.E.; n = 16). F, Rh-CT$_{5-HT_{1A}}$ and 5-HT$_{1A}$-mCherry target further along the extent of dendrites. Normalized fluorescence of the longest dendrite was plotted against normalized dendritic length. The length of each dendrite analyzed was normalized from 0 to 1.0 by piecewise linear interpolation. Interpolated data were pooled, and the mean ± S.E. was plotted against normalized distance (n = 16). D, lengths of dendrites analyzed were not significantly different (n = 16; p > 0.05, ANOVA).

**FIGURE 4.** The C-terminal domain of 5-HT$_{1A}$ receptor promotes distal targeting within dendrites of hippocampal neurons. Confocal images were taken of cultured rat hippocampal neurons (8 DIV) infected with Sindbis virus driving expression of Rh-mCherry (A), Rh-CT$_{5-HT_{1A}}$ (B), and 5-HT$_{1A}$-mCherry (C). These neurons were immunolabeled with anti-dsRed (red) and anti-MAP-2 antibodies to enhance and delineate mCherry tagged receptors and dendrites, respectively. Images taken at 20× magnification reveal that dsRed fluorescence is observed much more distally from the soma for neurons expressing Rh-CT$_{5-HT_{1A}}$ (B) and 5-HT$_{1A}$-mCherry (C) when compared with neurons expressing Rh-mCherry (A).
signaling and induce hyperpolarization in a neuronal context. mCherry-tagged Rh (Fig. 5A) as well as the Rh-CT$_{5-HT1A}$ (Fig. 5B) caused a 8–9-mV membrane hyperpolarization (postsynaptic effect) in response to a 1-s light pulse. Hyperpolarization was sustained for the duration of longer (10 s) light stimulus protocols, and cells showed rapid reversal of membrane voltage change after the light was turned off. Uninfected hippocampal neurons were also assessed for their ability to respond to baclofen, a GABAB agonist that served as a positive control for Gi/o activation, and the selective 5-HT$_1A$ agonist, 8OH-DPAT. For quantification of biophysical properties, a 1-s light or agonist application was used that was long enough to induce maximal activation of GPCR and induce hyperpolarization but short enough so that GPCR desensitization was not observed. The resulting change in membrane voltage for Rh-mCherry and Rh-CT$_{5-HT1A}$ stimulated by light was not significantly different from neuronal responses to activation of endogenous GABAB or 5-HT$_1A$ receptors (Fig. 5C). The time constants for hyperpolarization and recovery by GPCR activation in neurons were much faster than in HEK293 cells (Fig. 5, D versus H). This is most likely due to the effect of proteins endogenous to neurons, such as RGS proteins, which potentiate the GTPase activity of G proteins.

Rh-CT$_{5-HT1A}$ but not Rh-mCherry decreases endogenous 5-HT$_1A$-induced hyperpolarization without affecting GABA$_B$ responses. A, extent of membrane hyperpolarization induced by 5-HT$_1A$ activation is decreased in neurons expressing Rh-CT$_{5-HT1A}$ but not Rh-mCherry. Cultured rat hippocampal neurons (21–22 DIV) were infected with Sindbis virus, driving the expression of Rh-mCherry or Rh-CT$_{5-HT1A}$. Voltage changes induced by a 10-s application of the 5-HT$_1A$ agonist, 8OH-DPAT (1 µM), in the presence of Rh-CT$_{5-HT1A}$ (top) and Rh-mCherry (bottom) are shown. B, relative changes in membrane voltage for 5-HT$_1A$ (8OH-DPAT) and GABA$_B$ (baclofen) activation in the absence or presence of Rh-CT$_{5-HT1A}$ and Rh-mCherry compared with the agonist-induced responses in uninfected (WT) cells (mean ± S.E.; **, p < 0.01, ANOVA).
affected 80OH-DPAT responses (103.5% ± 13.6 of uninfected neuron (n = 11)), indicating that neither viral infection nor exogenous expression of GPCRs affects endogenous 5-HT1A responses. This effect is consistent with the experiments showing that co-expression of the CT reduces 5-HT1A in distal dendrites in cultured neurons (19). Application of baclofen resulted in comparable hyperpolarization for uninfected versus Sindbis virus-treated neurons (Rh-mCherry = 90.8% ± 17.3 of uninfected neuron (n = 13); Rh-CT5-HT1A = 92.1% ± 15.6 of uninfected neuron (n = 9)), ruling out the possibility that virus application interrupted targeting and expression for all endogenous GPCRs.

Rh-CT5-HT1A Compensates for the Loss of 5-HT1A Signaling in Cultured Hippocampal Neurons of 5-HT1A Null Mice—To demonstrate that Rh-CT5-HT1A could functionally substitute for 5-HT1A receptors, we sought to determine whether Rh-CT5-HT1A could functionally “rescue” 5-HT1A signaling in neurons from 5-HT1A KO mice. As expected, 5-HT1A immunostaining was absent from neurons from 5-HT1A null mice (13), but hippocampal neurons of wild type mouse showed robust staining throughout dendrites (Fig. 7A). Functionally, the application of 1 μM 8OH-DPAT (5-HT1A agonist) onto neurons of KO mice failed to elicit a hyperpolarization response, even with longer agonist applications (10 s) (Fig. 7E). WT mouse neurons hyperpolarized when exposed to 1 μM 8OH-DPAT with a comparable response to what was seen in hippocampal neurons cultured from wild type rats (Fig. 7, C and H, versus Fig. 5C). The response to baclofen remained intact in the KO neurons, suggesting that the mutation is specific to 5-HT1A and does not affect G_{i/o} signaling broadly (Fig. 7, D and H). 5-HT1A-mCherry and Rh-CT5-HT1A expressed by Sindbis virus vectors localized to the dendrites of KO neurons (Fig. 7A). The hyperpolarization defect in KO neurons was rescued by exogenous expression of both 5-HT1A-mCherry (with agonist application, Fig. 7D) and Rh-CT5-HT1A (with light, Fig. 7G). The loss of function phenotype was completely compensated for, as activation of exogenously expressed 5-HT1A (7.270 ± 1.175 (n = 8)) and Rh-CT5-HT1A (8.437 ± 1.271 (n = 7)) was indistinguishable from wild type mouse neuron response to 8OH-DPAT.

Rh-CT5-HT1A Functionally Substitutes for 5-HT1A Signaling in Dorsal Raphe Neurons in Brain Slices from 5-HT1A Null Mice—We next wanted to demonstrate if Rh-CT5-HT1A was capable of modulating serotonergic neurons of the dorsal raphe compared with endogenous 5-HT1A. Again, these neurons are important neural regulators for the generation of anxiety and depression. Lentivirus expressing Rh-CT5-HT1A was stereotactically injected into the DRN of ePet::YPF or 5-HT1A/−/− mice. As indicated in Fig. 8A, Rh-CT5-HT1A was expressed in 5-HT neurons (labeled with YFP in ePet::YPF mice) and revealed a punctate distribution most prominently in the soma. Notably, Rh-CT5-HT1A was absent from YFP+ long processes most likely representing axons of 5-HT neurons. We next determined if Rh-CT5-HT1A could rescue the phenotype in serotonergic neurons of 5-HT1A KO mice. Similar to what was observed in cultured hippocampal neurons, application of the 5-HT1A agonist, 8OH-DPAT (1 μM), onto brainstem slices failed to elicit a hyperpolarization response in the dorsal raphe nucleus of KO mice (data not shown). This defect can be rescued by expression of Rh-CT5-HT1A and subsequent activation by light (Fig. 8, B–F). Light stimulus in these neurons caused a decrease in spontaneous action potential firing rate (Fig. 8D). In 10 of 15 cells expressing Rh-CT5-HT1A, the interspike interval was increased in response to a 3-s light stimulus on average from 202 ± 21 ms (n = 10) to 313 ± 58 ms and returned to 207 ± 25 ms 13–16 s after cessation of light application (Fig. 8, E and F). The change in firing rate could be attributed to enhancement of K+ conductance (most likely mediated by GIRK channels) revealed by increased inward rectification with light (Fig. 8, B and C). Eliciting light responses in brainstem slices required sufficient 9-cis-retinal loading with fatty acid-free-BSA-supplemented extracellular solution, facilitating retinal delivery as previously suggested (35). These results indicate that Rh-CT5-HT1A can functionally replace 5-HT1A in the neurons of the dorsal raphe nucleus.

DISCUSSION

Various strategies have been developed to control GPCRs and G protein pathways to dissect the function in vitro and in vivo. Receptors activated solely by synthetic ligands (RASSL) (36) and designer receptors exclusively activated by designer drugs (DREADDs) (37) are GPCRs that are activated by organic compounds but not by the endogenous ligands such as serotonin or acetylcholine. The basic idea is to use these GPCRs to control the corresponding intracellular pathway by a drug, which specifically only activates the modified GPCR. RASSLs and DREADDs have been used successfully in vitro and in vivo and have the potential to study GPCR function in a tissue- and cell-type-specific manner (38). However, for the investigation of fast spatial-temporal control of GPCRs, for example in brain slice preparations or in vivo recordings, the application of chemical compounds to activate the GPCR is slow and almost irreversible. It is difficult to control the ligand concentration in particular in brain tissues, where the chemical compound has to diffuse through various cellular layers and can not be washed out. As a consequence of continuous ligand application, receptor desensitization may occur, and precisely controlled repetitive stimulation of the GPCR pathway is not possible. Therefore, light-activated GPCRs became of interest to us for controlling signaling events in particular in neurons (9, 39). In our first approach we used vertebrate Rh to control the pre- and postsynaptic G_{i/o} signaling in cultured neurons and the embryonic chicken spinal cord. We now became interested in developing this GPCR further. We engineered a chimeric light-activated receptor that targets and functions in 5-HT1A receptor signaling domains. With the addition of mCherry and the CT domain of 5-HT1A, vertebrate Rh retains its ability to activate G_{i/o}-coupled signaling, causes subsequent GIRK channel activation, and induces membrane hyperpolarization. When expressed in neurons, Rh-CT5-HT1A traffics to somatodendritic compartments and to distal dendritic segments, where endogenous and exogenously expressed 5-HT1A receptors are found and functionally substitutes for the missing 5-HT1A receptors in both cultured hippocampal neurons and neurons of the dorsal raphe in hindbrain slices from HT1A KO mice.
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with unusual activation and inactivation kinetics (data not shown). The reason for the altered receptor kinetics will be investigated biophysically in future studies and may shed additional light onto the coupling between GPCR, G protein, and the intracellular signaling cascade.

Another important aspect of our studies is that Rh-CT⁵-HT₁₆ could functionally and competitively substitute for 5-HT₁₆ signaling responses in hippocampal neurons. Tagging with critical targeting domains to drive differential intracellular localization has, therefore, the potential to induce competitive substitutions of the endogenously expressed proteins. The strategy of targeting domain tagging of exogenous receptors depends on their interaction with chaperone proteins normally binding and trafficking with endogenous receptors. Therefore, exogenously expressed proteins expressed at high enough levels could create an analogous situation to overexpression of the target domain alone. This could result in a dominant negative effect by direct competition with endogenous receptors. We reason that this would occur as there are a finite number of trafficking proteins, and a finite number of positions GPCRs can occupy at a given submembrane locale. Because our goal is to utilize Rh-CT⁵-HT₁₆ as a functional substitute of 5-HT₁₆, the pseudo-knockdown of endogenous 5-HT₁₆ signaling would be desirable. For correlative and causal studies linking 5-HT₁₆-like signaling of Rh-CT⁵-HT₁₆ to behavior, the compensatory effects from endogenous 5-HT₁₆ signaling would be minimized.

Taken together, our findings suggest that light activation of Rh-CT⁵-HT₁₆ serves as a suitable proxy for agonist-induced 5-HT₁₆ receptor activation in wild type and 5-HT₁₆ KO animals for understanding the function of 5-HT₁₆ signaling in mice. Thus, our results suggest that Rh-CT⁵-HT₁₆ can act in place of endogenous 5-HT₁₆ receptor.

In developing Rh-CT⁵-HT₁₆, we have gained some important understanding for using light-activated GPCRs to control specific GPCR pathways. We learned that the intracellular trafficking of Rh can be directed to specific subcellular domains by the addition of critical targeting domains of other GPCRs. The successful application of this type of strategy may depend on the presence of specific binding motifs on the donor receptor in addition to the targeting tag. Our efforts to further replace the intracellular domains of Rh using the corresponding 5-HT₁₆ intracellular domains of other GPCRs. The success of this approach depends on their interaction with chaperone proteins normally binding and trafficking with endogenous receptors. Therefore, exogenously expressed proteins expressed at high enough levels could create an analogous situation to overexpression of the target domain alone. This could result in a dominant negative effect by direct competition with endogenous receptors. We reason that this would occur as there are a finite number of trafficking proteins, and a finite number of positions GPCRs can occupy at a given submembrane locale. Because our goal is to utilize Rh-CT⁵-HT₁₆ as a functional substitute of 5-HT₁₆, the pseudo-knockdown of endogenous 5-HT₁₆ signaling would be desirable. For correlative and causal studies linking 5-HT₁₆-like signaling of Rh-CT⁵-HT₁₆ to behavior, the compensatory effects from endogenous 5-HT₁₆ signaling would be minimized.

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*in vitro* and *in vivo* preparations. Thus, Rh-CT$_{5-HT1A}$ adds a new engineered GPCR as a tool to control intracellular signaling events.

Acknowledgments—We thank Dr. Lawrence Tecott (University of California, San Francisco) for the 5-HT$_1A$ knock-out mice. We also express our gratitude to Dr. Gary Landreth for critically reading the manuscript and providing laboratory space to complete this manuscript.

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