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Restoration of Physiological Expression of 5-HT_6 Receptor into the Primary Cilia of Null Mutant Neurons Lengthens Both Primary Cilia and Dendrites [S]

Atom J. Lesiak, Matthew Brodsky, Nathalie Cohenca, Alexandra G. Croicu, and John F. Neumaier

Department of Psychiatry and Behavioral Science, Department of Pharmacology, University of Washington, Seattle, Washington

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ABSTRACT

5-HT_6 (serotonin) receptors are promising targets for a variety of neuropsychiatric disorders and have been linked to several cellular signaling cascades. Endogenous 5-HT_6 receptors are restricted to the primary neuronal cilium, a small sensory organelle stemming from the cell body that receives numerous extrasynaptic signals. Inhibition of 5-HT_6 receptors decreases cilia length in primary neuronal cultures, but the signaling mechanisms involved are still unclear. Intense overexpression of exogenous 5-HT_6 receptors increases the probability for receptors to localize outside the primary cilium and have been associated with changes in cilia morphology and dendritic outgrowth. In the present study, we explore the role of 5-HT_6R rescue on neuronal morphology in primary neuronal cultures from 5-HT_6R-KO mice, at the same time maintaining a more physiologic level of expression, wherein the receptor localizes to cilia in 80%–90% of neurons (similar to endogenous 5-HT_6R localization). We found that rescue of 5-HT_6R expression is sufficient to increase cilia length and dendritic outgrowth, but primarily in neurons in which the receptor is located exclusively in the primary cilia. Additionally, we found that expression of 5-HT_6R mutants deficient in agonist-stimulated cAMP or without the predicted Fyn kinase binding domain maintained constitutive activity for stimulating cAMP and still increased the length of cilia, and that the proposed Fyn kinase domain was required for stimulating dendritic outgrowth. These findings highlight the complexity of 5-HT_6R function and localization, particularly with the use of exogenous overexpression, and provide greater understanding and potential mechanisms for 5-HT_6R drug therapies.

Introduction

The 5-HT_6 serotonin receptor (5-HT_6R) is a promising target to treat a variety of neurologic and cognitive disorders, including cognitive impairment, Parkinson and Alzheimer diseases, obesity, schizophrenia, motor disorders, sleep, and depression (Mitchell and Neumaier, 2005; Hirst et al., 2006; Wesołowska and Nikiforuk, 2007; Ferguson et al., 2008; Morairty et al., 2008; King et al., 2009; Arnt et al., 2010; Carr et al., 2011; Meffre et al., 2012; de Bruin and Kruse, 2015; Aldrin-Kirk et al., 2016; Brodsky et al., 2016). Despite mounting evidence for the therapeutic value of this receptor, little is known about the specific mechanisms underlying 5-HT_6R signaling. 5-HT_6R is expressed nearly exclusively in the central nervous system, in a limited number of brain regions, including cortex, hippocampus, and most abundantly in the striatum (East et al., 2002; Hirst et al., 2003; Brodsky et al., 2017); however, localization of 5-HT_6R in vivo is notoriously difficult because commercially available antibodies with sufficient specificity have been inconsistently available. Additionally, the subcellular localization of the receptor can be easily overlooked because 5-HT_6R is the only serotonin receptor that localizes to the primary neuronal cilium (Brailov et al., 2000; Berbari et al., 2008; Domire and Mykytyn, 2009; Brodsky et al., 2017; Hu et al., 2017).

Primary cilia were described over a century ago and are ubiquitous to almost all nondividing mammalian cells, including neurons, yet these singular nonmotile appendages were frequently misinterpreted as vestigial organelles until recently (Fuchs and Schwark, 2004; Louvi and Grove, 2011). Now the primary cilium is recognized as an important regulator of cellular function by acting as a "cellular antenna," sensing extracellular signals in the extrasynaptic environment (Singla and Reiter, 2006; Green and Mykytyn, 2014). Trafficking of specific proteins into primary cilia is strictly regulated by the basal body and involves active transport along the central microtubule doublet that provides the cilia structure (Pazour and Bloodgood, 2008; Louvi and Grove, 2011; Stepanek and Pigino, 2016). Disruption of primary cilia function is linked with a variety of disorders termed ciliopathies, such as Bardet-Biedl syndrome, polycystic kidney disorder, polydactyly, hydrocephalus, obesity, disrupted neurogenesis, and cognitive disorders (Lee and Gleeson, 2011;

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ABBREVIATIONS: AC3, adenylyl cyclase III; ANOVA, analysis of variance; DIV, day in vitro; EV, empty vector; HA, hemagglutinin; HEK, human embryonic kidney; 5-HT, 5-hydroxytryptamine (serotonin); IP, immunoprecipitation; WT, wild type.

Louvi and Grove, 2011; Valente et al., 2014; Gazea et al., 2016; Schmidt et al., 2017; Trulioff et al., 2017).

Shortly after the cloning and identification of 5-HT_6Rs, expression of 5-HT_6Rs was reported to be faintly scattered throughout dendrites, particularly in striatum, but soon it was recognized that 5-HT_6Rs are predominantly found in the primary neuronal cilia (Ruat et al., 1993; Kohen et al., 1996; Hamon et al., 1999; Berbari et al., 2008; Brodsky et al., 2017). As a G protein-coupled receptor, 5-HT_6 is positively coupled with G proteins that stimulate production of cAMP, presumably through adenylyl cyclase III (AC3), the only adenylyl cyclase known to localize only to primary neuronal cilia (Sebben et al., 1994; Kohen et al., 2001; Kang et al., 2005; Bishop et al., 2007; Domire and Mykytyn, 2009). More recently, proteomic analysis of 5-HT_6R protein association has identified a variety of noncanonical signaling pathways, including CDK5, Fyn kinase, Jab1, and mTOR (Yun et al., 2010; Riccioni et al., 2011; Meffre et al., 2012; Duhr et al., 2014). 5-HT_6R displays a high level of ligand-independent constitutive activity, and this was proposed to regulate cortical neuronal migration and morphology (Grimaldi et al., 1998; Romero et al., 2007; Jacobshagen et al, 2014; Dayer et al., 2015). However, the mechanism by which 5-HT_6R signaling in cilia impacts morphology is still unclear, although several recent reports have attempted to elucidate this connection. Following in utero electroporation and heterologous overexpression, 5-HT_6R overexpression induced malformations and elongation of primary neuronal cilia and inhibited dendritic outgrowth (Guadiana et al., 2013). Interestingly, this study also found that overexpression not only caused AC3 to be excluded from the ciliary compartment but induced cilia branching, which is not typically observed (Guadiana et al., 2013). The same laboratory also found that expression of a range of mouse 5-HT_6R mutants in NIH3T3 cells, including nonfunctional mutants, all increased cilia length compared with controls (Guadiana et al., 2013). On the other hand, another study found a positive association between exogenous overexpression of human 5-HT_6R and an increase in dendritic outgrowth, whereas small-interfering RNA knockdown inhibited dendritic outgrowth (Duhr et al., 2014); the authors concluded that the Cdk5 interaction with 5-HT_6R was responsible, since inhibition of Gs-coupled cAMP signaling had no effect. Recently, in Alzheimer mouse models, 5-HT_6 was shown to have a potential role in regulating cilia and axon initial segment morphology (Hu et al., 2017).

Of note, most of these studies interrogated 5-HT_6R function using exogenous overexpression in wild-type (WT) animals rather than modulating endogenous receptor activity, and many did not focus on 5-HT_6R localization to primary neuronal cilia. Recently, we measured the effect of specific drugs on endogenous 5-HT_6R and found that selective antagonists shortened primary cilia, whereas none of the drug treatments increased dendritic outgrowth (Brodsky et al., 2017). During this study, we coincidentally found that increasing amounts of exogenous 5-HT_6R transfection led to drastically increased ectopic expression outside the primary cilia. Additionally, mutations that deleted a potential cilia-targeting sequence on the third intracellular loop decreased 5-HT_6R trafficking to cilia (Berbari et al., 2008; Brodsky et al., 2017). However, these mutations were unable to prevent cilia targeting entirely; highlighting the robust proclivity for 5-HT_6R to traffic into primary cilia.

In the present study, we investigated the effect of 5-HT_6R localization and signaling pathways on primary cilia and dendritic morphology, systematically accounting for receptor localization within each neuron. Using primary striatal neurons cultured from 5-HT_6R-null (5-HT_6R-KO) mice (Tecott et al., 2000), we found that rescue of 5-HT_6R expression was sufficient to increase cilia length and stimulate dendritic outgrowth, particularly when the receptor was restricted to primary cilia. Additionally, using a 5-HT-insensitive 5-HT_6R-mutant (D106A) and a mutant lacking the predicted Fyn kinase binding domain (426–431del), we support the idea that 5-HT_6R-dependent kinase cascades are essential for 5-HT_6R-dependent dendritic outgrowth. We hypothesize that many of the conflicting findings regarding the interplay of 5-HT_6R with neuronal morphology might be related to heterologous overexpression and extraciliary mis-localization. These findings highlight the careful consideration of expression level and subcellular distribution needed when studying 5-HT_6Rs and solidifies the predicted role of 5-HT_6Rs in neuronal morphology.

Materials and Methods

Animals. Animal procedures were approved by the University of Washington's Institutional Animal Care and Use Committee and carried out with NIH guidelines *Principles of Laboratory Animal Care* (*Guide for the Care and Use of Laboratory Animals, 8th Edition*, https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf). 5-HT_6KO mice on a C57BL/6 background were a generous gift provided by Dr. Lawrence Tecott (Bonasera et al., 2006). Breeding and genotyping of mice were carried out as previously described (Brodsky et al., 2017).

Cell Culture. Primary dissociated striatal cultures were generated from postnatal day 0–1 5-HT_6KO mice from both sexes. Crude membranes were removed prior to dissection and separation of striatum and cortical hemispheres. We and others have found that striatal neurons survive in primary culture longer when cocultured with a small number of cortical neurons (10% cortical and 90% striatal). Minced cortical and striatal tissues were dissociated independently using papain (MilliporeSigma, Burlington, MA) and trituration through a fire-polished glass pipette. Cells were plated at a density of 7×10^4 cells per cm^2 in culture dishes precoated with poly-L-lysine (molecular weight 300,000; MilliporeSigma). Cultures were maintained in growth media consisting of neurobasal A medium (Thermo Fisher Scientific, Waltham, MA) supplemented with B27 and GlutaMAX (1×; Thermo Fisher Scientific) throughout treatment days. From the 4th day in vitro (DIV) until homogenization or fixation, culture media was supplemented with 1 μM Ara-C (MilliporeSigma). This culturing method was adapted from previously described methods (Lesiak et al., 2015; Brodsky et al., 2017), and results in cultures consisting of approximately 70% neurons and 30% glia. Of note, glial cells have been shown not to express significant levels of 5-HT6R mRNA and were thus not imaged or included in our analysis (Gokce et al., 2016). Cultures were maintained at 37°C under 5% CO_2 from DIV0 until homogenization or fixation.

Plasmids/Transfection. The hemagglutinin (HA)-tagged rat 5-HT_6R (Brodsky et al., 2017)) was used as the WT receptor from which the following mutants were generated: HA-5-HT_6^{D106A} (single base-pair substitution) and HA-5-$HT_6^{406\text{-}411del}$ (removal of base pairs corresponding to PPPPTR, amino acids 406–411) using Gibson Assembly Master Mix (NEB cat. no. E2611S; New England BioLabs, Inc., Ipswich, MA) and oligonucleotide primers (MilliporeSigma). Rat WT and mutant 5-HT_6R plasmid maps can be found at Neumaier Lab website (http://depts.washington.edu/mnsl/). Primary neuronal cultures were transfected on DIV7 using Lipofectamine 2000 as previously described (Lesiak et al., 2015; Brodsky et al., 2017), wherein transfection efficiency is about 1%–10%. Total plasmid for

transfections consisted of 1 μg DNA/well of a standard 24-well plate, with the transfection percentage representing the proportion that each plasmid constituted relative to the 1 μg total transfected per well of a 24-well plate. When included, 5-HT$_6$R plasmids were transfected at 15% of total transfected plasmid except in the dose-response experiments where they were transfected at a range from 0% to 80%. Map2B-RFP plasmid (Wayman et al., 2008) was transfected as 30% of the total transfected plasmid to mark transfected neurons, since Map2B associates with the microtubules in the somatodendritic compartment of neurons and is excluded from the axon (Wayman et al., 2006). The remaining plasmid consisted of empty vector (EV; pCAGGS from Wayman Laboratory, Washington State University) and 5-HT$_6$ plasmid to reach the final 100% of total transfected plasmid.

Immunohistochemistry and Image Analysis. Cultured neurons were fixed on DIV10 with 32°C 4% PFA/PHEMS buffer (20 minutes; paraformaldehyde/PIPES, HEPES, EGTA, $MgCl_2$), permeabilized with 1× phosphate-buffered saline (PBS) with 0.5% Triton-X100 (10 minutes), blocked with 10% bovine serum albumin (BSA) in 1× PBS, and stained overnight with corresponding primary antibodies diluted in 1% BSA in 1× PBS, as previously described (Brodsky et al., 2017). Primary antibodies used were anti-HA rabbit (1:1000; Cell Signaling Technology, Danvers, MA) and anti-Arl13b (1:1000, 73–287; NeuroMab, Davis, CA). Fluorescent secondary antibodies, Alexa 488 anti-rabbit and Alexa 568 anti-mouse used at a dilution of 1:4000 (Invitrogen/Thermo Fisher Scientific). All antibodies were diluted in 1% BSA in 1× PBS. Coverslips were mounted using ProLong Gold Antifade media containing DAPI (Invitrogen/Thermo Fisher Scientific). Microscopic images used for morphology were acquired on a Leica inverted widefield fluorescence microscope using MetaMorph software at the University of Washington W. M. Keck Microscopy Center. Experimenters were blind to conditions during imaging and analysis. Transfected neurons were identified and selected on each coverslip using the red channel (Map2B-RFP expression) to avoid bias for receptor localization (anti-HA Green) or cilia presence (anti-Arl13b FarRed). Z-stack images were *z*-projected and analyzed using FIJI; dendrite length and branching and cilia length was analyzed using the NeuronJ plugin. Super-resolution images were acquired using a Zeiss LSM 880 confocal microscope with the Airyscan super-resolution detector and FAST module.

Drugs and Drug Treatments. The 5-HT$_6$-selective agonist WAY-208466 and antagonist SB-399885 (Tocris/Bio-Techne, Minneapolis, MN) were used as described in Brodsky et al. (2017), and cultures were treated for 48 hours, from DIV8–10 prior to fixation.

cAMP Accumulation Assay. cAMP accumulation assays were conducted on transfected IMCD-3 kidney cells (ATCC, Manassas, VA) as previously described (Brodsky et al., 2017) following the same transfection conditions described above for primary neuronal cultures with receptor plasmids at 15% of the total DNA. Cultured cells were treated with 5-HT$_6$ agonist WAY-208466 (1 μM) for 10 minutes before lysis.

Western Blot and Fyn-Immunoprecipitation. Human embryonic kidney (HEK)293 cells were plated and transfected with Lipofectamine, using the same transfection and dose-dependent expression described above for primary cultures, and treated with vehicle or 5-HT$_6$ agonist (WAY-208466, 1 μM final concentration) diluted in culture media for the time specified in each figure legend for the corresponding experiments. For Western blot, samples were lysed using RIPA buffer supplemented with 1:100 dilution of inhibitors for protease (cat. no. P8340; MilliporeSigma) and phosphatases (cat. no. 524624; Calbiochem/MilliporeSigma, Burlington, MA), run on NuPAGE 4%–12% Bis-Tris Gels (cat. no. NP0323; Thermo Fisher Scientific) then transferred to polyvinylidene fluoride membrane. Membranes were blocked for 1 hour with Aqua Block (cat. no. ab166952; Abcam, Cambridge, MA) primary antibodies were then diluted in Aqua Block (1:1000), and blots were incubated at 4°O/N. DyLight secondary antibodies were diluted in Aqua Block 1:4000 (anti-rabbit 800 5151S, anti-mouse 680 5470S; Cell Signaling Technology) for 1–2 hours. After washing, blots were scanned on an Olympus Odyssey Scanner, and blots were analyzed with Image Studio. Fyn-immunoprecipitation (IP) was initially conducted following the Fyn-IP protocol of (Riccioni et al., 2011) without detecting Fyn. For Fyn-IP, transfected HEK cells grown on six-well plates were lysed in 500 μl of 1× Tris-buffered saline with 1:100 dilution of IGEPAL, NaF, NaOv, protease inhibitor, and phosphatase inhibitor (MilliporeSigma); 50-μl fractions of samples were saved as input. Samples were preincubated with 3 μl of anti-Fyn antibody (cat. no. MABT208; MilliporeSigma) and rotated for 4 hours at 4°C. Anti-A/G magnetic beads (cat. no. 88803, 100 μl; Pierce/Thermo Fisher Scientific) were added and samples were rotated at 4°C O/N. Samples were washed with fresh lysis buffer, IP samples were eluted into RIPA buffer, and input fraction was added to RIPA buffer before being run as other Western blot samples. Primary antibodies used for Western blot antigen detection were as follows: anti-HA (C294; Cell Signaling Technology), anti-α-tubulin (cat. no. DM1A; MilliporeSigma), anti-Fyn (cat. no. EPR5500; MilliporeSigma), anti-phospho-Src (Tyr416) (clone 9A6; MilliporeSigma). For both Western blot and IP experiments, total protein concentrations were normalized across conditions prior to loading into the gel using Qubit and the Qubit Protein Assay Kit (Thermo Fisher Scientific).

Data Analysis. For measurements illustrated in Fig. 2, seven to eight transfected neurons on two coverslips (15–16 total) were imaged and analyzed at each dose of 5-HT$_6$ transfection. Statistics in Fig. 2 used a *t* test of the slope of the regression line against a null-linear model. For measurements illustrated in Figs. 3–6 transfected neurons from each coverslip were imaged, and measurements of cilia and dendrites were averaged to generate each data point (*n*); three to six coverslips were analyzed from eight independent cultures for each experimental condition. For Fig. 6, only cultures including drug-treated conditions were included in analysis, and three to six neurons from one to three coverslips across six independent cultures were analyzed. For Fig. 6, individual neurons were treated as single data points (*n*) for analysis. Average cilia length remained constant across experiments, but average dendritic length varied significantly from culture to culture; therefore, average dendritic length of empty vector (EV)-controls were normalized across cultures. Cilia length data were analyzed using the Kruskal-Wallis test with Dunn multiple comparison post-hoc, dendritic outgrowth (total dendritic length and branches) data were analyzed using one-way analysis of variance (ANOVA) with Bonferroni post-hoc tests, and ciliation-dependent and receptor localization-dependent dendritic length analysis was analyzed using two-way ANOVA. All statistics were run using GraphPad Prism or Excel software, and all statistical values for experiments can be found in (Supplemental Table 1).

Results

HA-Tagged 5-HT$_6$R Localization to Primary Neuronal Cilia. For all experiments, primary striatal neuron cultures from 5-HT$_6$R-KO mice were transfected using lipofection with plasmids expressing Map2B-RFP and empty vector (pCAGGS, EV) or HA-tagged-5-HT$_6$Rs (rat WT or mutants) on the 7th day in vitro (DIV7), fixed on DIV10, and immunostained for HA-tag and a marker for primary cilia, Arl13b. We have previously confirmed overlap of cilia staining between Arl13b and adenylyl cyclase III in primary cultured striatal neurons (Brodsky et al., 2017). Super-resolution images of transfected neurons illustrate the presence of primary neuronal cilia on 5-HT$_6$-KO neurons (Fig. 1, A and C) and heterologously expressed HA-tagged receptor localizing exclusively to the Arl13b-marked primary neuronal cilia (Fig. 1, B and D). We did not observe 5-HT$_6$R expression (endogenous or transfected) in cells displaying glial morphology.

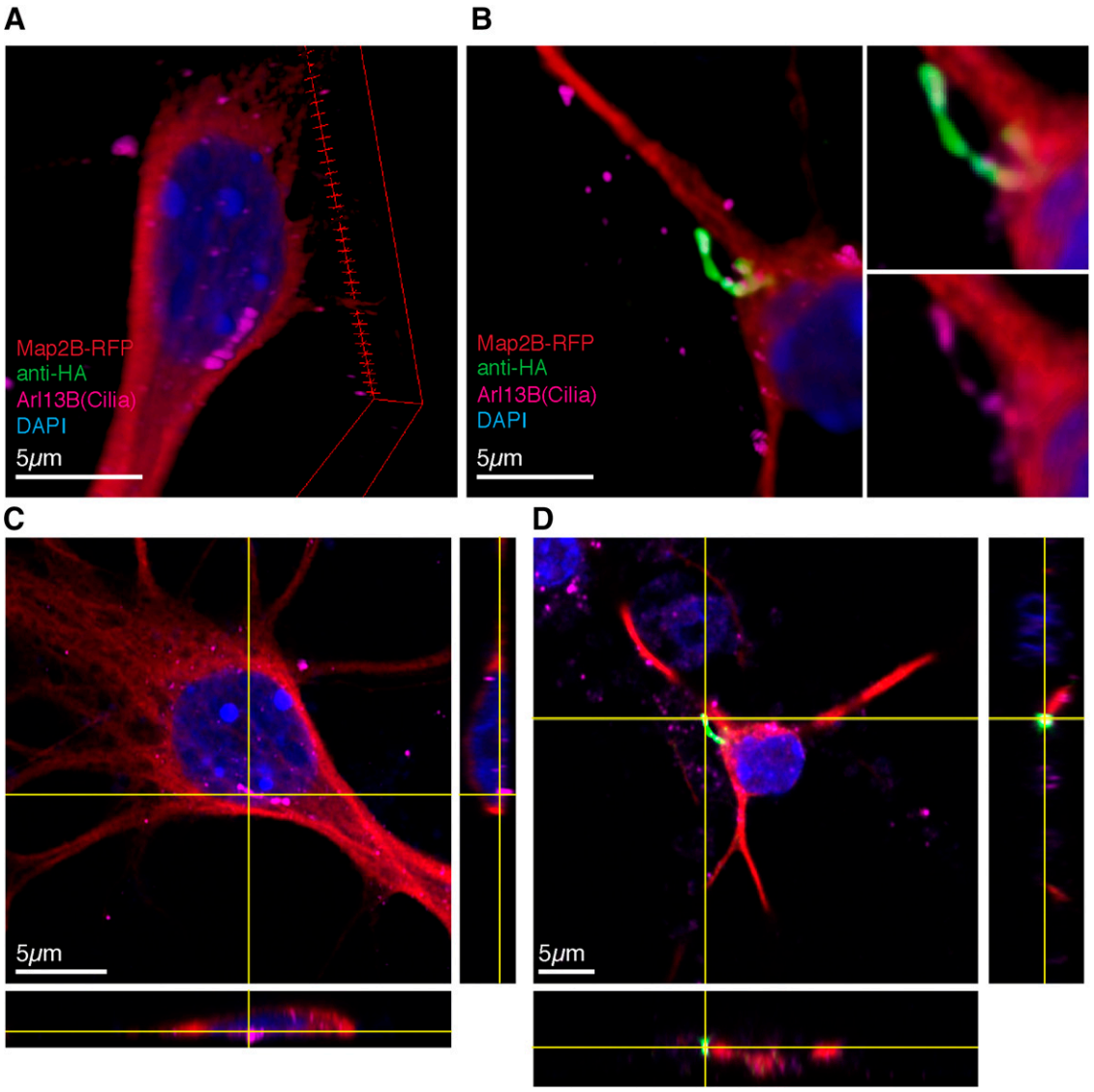

Fig. 1. Rescued 5-HT_6R localizes to the primary cilium in 5-HT_6R-KO neurons. Super-resolution images taken on Zeiss LSM 880 with Airyscan of primary striatal/cortical primary neuronal cultures. Primary cultures from 5-HT_6R-KO mice were transfected with 30% Map2B-RFP (Red) ± (A) empty vector or (B) 15% WT-HA-5-HT_6 receptor plasmid, fixed and imaged. (C and D) XYZ projected images demonstrating colocalization of 5-HT_6R (green) with Arl13B (cilia marker magenta) on the primary neuronal cilium.

Increased Expression of Exogenously Expressed 5-HT_6R Increases Extraciliary Localization and Aberrant Cilia Lengthening. Lipofection using increasing amounts of plasmid in primary cultures has been shown to lead to increased expression of exogenous mRNA and protein (Susa et al., 2008; Brodsky et al., 2017). Accordingly, as the proportion of HA-5-HT_6R plasmid transfected into HEK293 cells increased (balanced by corresponding empty vector), significantly greater levels of HA-tagged-5-HT_6R protein was expressed (Fig. 2A). Likewise, increasing the proportional amount of HA-5-HT_6R plasmid transfected into HEK293 cells led to significant increases in levels of 5-HT_6R mRNA, whereas expression of a stable amount of a transfected gene (GFP-CRE) and endogenous housekeeping genes did not change from condition to condition (Fig. 2B).

In primary neuronal cultures of 5-HT_6R-KO neurons, exogenously expressed HA-5-HT_6Rs localize exclusively to primary neuronal cilia in about 70% of transfected neurons; however, in some neurons, particularly those without primary cilia, the receptor is distributed throughout the entire neuron (Fig. 2, C–E). In a very small minority of neurons, despite the presence of Arl13b positive cilia, the receptor was expressed throughout the cell body (Fig. 2E).

Increasing amounts of HA-5-HT_6R transfected into primary neuronal cultures decreased the number of neurons with cilia-restricted HA-5-HT_6R localization but did not alter the proportion of neurons with detectable primary cilia (Fig. 2F). Interestingly, we found that, with increasing amounts of transfected HA-5-HT_6R, the length of cilia-associated HA-immunostaining significantly increased in neurons in which HA-5-HT_6R was restricted to the primary cilium, whereas the length of the Arl13b within the cilia remained constant (Fig. 2G, example neuron inset). In these cases, the cilia, as measured by HA immunostaining, was unusually long although the cilia-specific marker Arl13b was not detectable along the entire length of the presumed cilia. This may be a

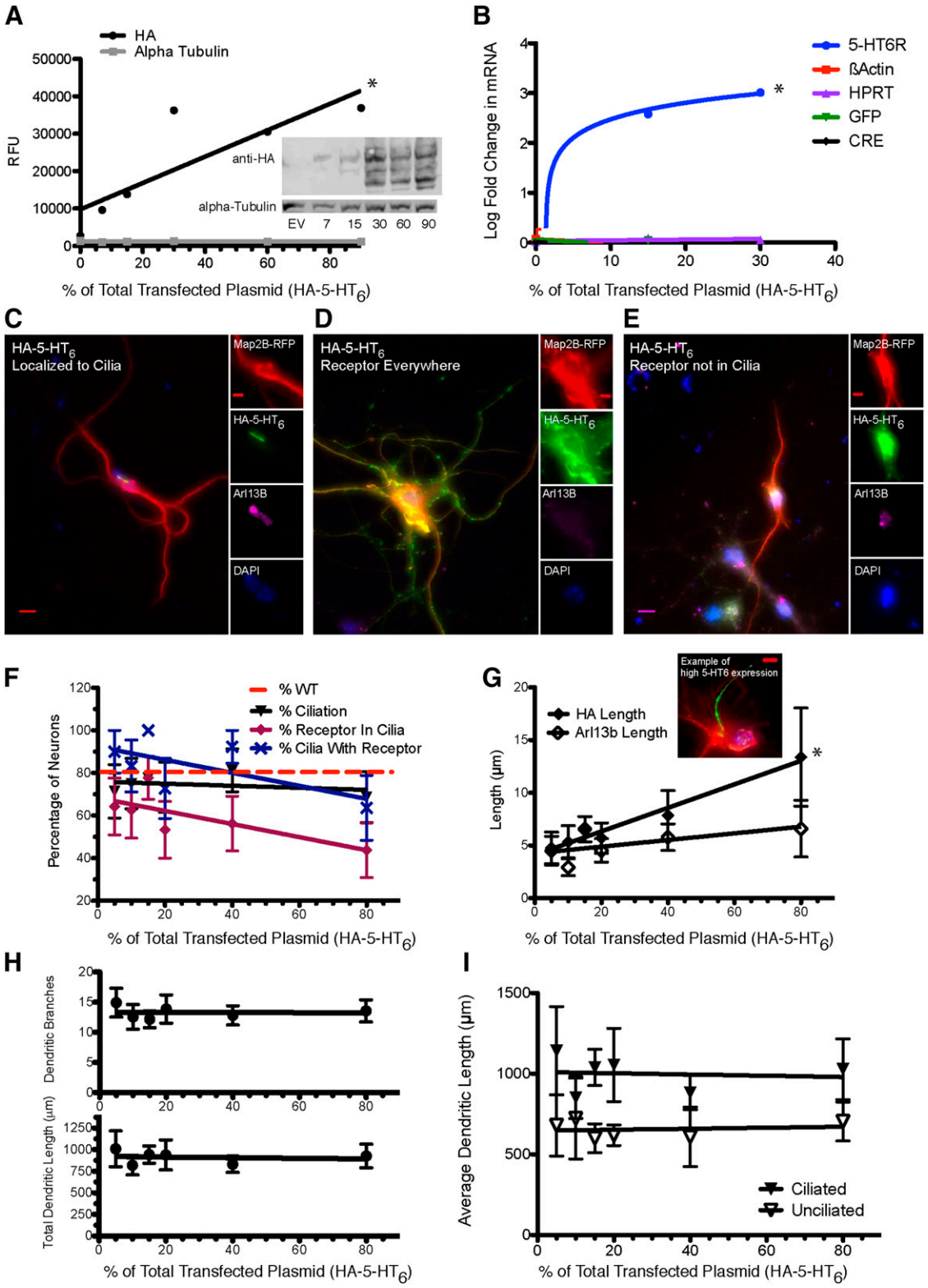

Fig. 2. Dose-dependent receptor expression alters 5-HT$_6$ receptor localization to primary cilia. (A) Graph and representative Western blot of HEK293 cells transfected with increasing amount of total transfected plasmid, demonstrating plasmid dose-dependent increase of exogenous protein expression. $n = 1$ sample per concentration of 5-HT$_6$. (B) Graph of qPCR data of mRNA expression following HEK293 cells transfected with increasing percentage of total transfected plasmid and 15% transfection of GFP-CRE, demonstrating increased expression of exogenous mRNA with increased plasmid transfection (GFP-CRE plasmid used to represent stable expression pattern of other exogenously expressed mRNA and HPRT and β-Actin mRNA used as housekeeping genes); $n = 3$ samples per concentration of 5-HT$_6$. (C–I) Primary neuronal cultures from 5-HT$_6$R-KO mice were transfected on DIV7 with 30% Map2B-RFP (Red) ± varying doses of HA-WT-5-HT$_6$R plasmid; on DIV10 they were fixed, mounted, then imaged. (C–E) Representative images of HA-WT-5-HT$_6$R localization in 5-HT$_6$R-KO primary neurons depicting (C) localization of receptor to primary cilium, (D) ectopic localization of receptor in neurons without primary cilium, and (E) ectopic localization of receptor in neurons with a primary cilium. (F) Graph depicting plasmid dose-dependent changes in percentage of transfected neurons with cilia, percentage of transfected neurons with receptor exclusively localized to cilia, and percentage of

pathologic change related to excessive 5-HT_6R trafficking and is similar to previously described effects of 5-HT_6R overexpression on cilia (Guadiana et al., 2013; Hu et al., 2017). However, we did not observe any receptor-dose effect on dendritic branching or total dendritic length in ciliated or unciliated neurons (Fig. 2, H and I). In a previous study we found that the percentage of ciliated striatal neurons cultured from wild-type animals containing endogenous 5-HT_6R was about 80% (Brodsky et al., 2017). Therefore, in subsequent experiments we decided to use a modest amount of receptor plasmid (15% of total transfected plasmid) to express exogenous receptors, because this level of transfection most closely replicated normal ciliation and endogenous receptor targeting to primary cilia in WT neurons (Fig. 2F).

HA-5-HT_6R Mutant Receptor Generation. In addition to investigating wild-type HA-5-HT_6R, we generated two mutants to investigate how 5-HT_6R signaling properties affect primary neuron morphology. The 5-HT_6R has extensive constitutive activity (Boess et al., 1998; Jacobshagen et al., 2014; Deraredj Nadim et al., 2016); therefore, we generated and tested a previously described mutant receptor that is insensitive to 5-HT and other 5-HT_6R agonists yet continues to display constitutive activity after heterologous expression in cell lines (5-HT_6^{D106A}). The second mutant, 5-$HT_6^{406–411del}$, is predicted to interrupt 5-HT_6 signaling associated with interactions with fyn kinase (Yun et al., 2007). Compared with IMCD3 cells transfected with EV plasmid, transfection with each of these three receptors increased cAMP levels in the absence of added agonist, consistent with constitutive activity of these receptors, although only the 5-HT_6^{D106A} mutant significantly increased cAMP compared with EV controls (Fig. 3A). Treatment of cells expressing WT-5-HT_6 or 5-$HT_6^{(406-411del)}$ with a 5-HT_6R agonist (WAY-208466, 1 μM) increased cAMP levels (Fig. 3B) and stimulated cAMP accumulation but was blocked by the further addition of the 5-HT_6R inverse agonist SB-399885 (1 μM). However, agonist treatment had no effect on cAMP levels in IMCD3 cells expressing the 5-HT_6^{D106A} receptor. Unfortunately, repeated attempts to demonstrate 5-HT_6R-mediated phosphorylation of fyn kinase or 5-HT_6R coimmunoprecipitation with CDK5 using previously published methods (Yun et al., 2007; Duhr et al., 2014) and several other strategies were unsuccessful (Fig. 3, C and D). We found that transfection with WT or mutant 5-HT_6R had no effect on Fyn expression or the phosphorylation of Fyn at Y-420, with or without agonist treatment (Fig. 3, C and D, and data not shown). Therefore, we could not confirm that the deletion of aa 406–411 in the 5-$HT_6^{(406–411del)}$ mutant affected fyn kinase phosphorylation or the direct interaction of 5-HT_6R with Cdk5. Additionally, we detected no difference in fyn phosphorylation, with or without agonist treatment, at low or high doses of receptor expression (Supplemental Fig. 1).

5-HT_6R Rescue Elongates Primary Neuronal Cilia. As previously reported (Brodsky et al., 2017), approximately 80% of primary striatal neurons cultured from 5-HT_6R -KO had primary cilia, similar to neurons from WT mice, and this was not altered by transfection with 15% WT or either of the two mutant receptors (Fig. 4A). In all measured neurons, regardless of whether WT or mutant 5-HT_6 receptors were expressed, approximately 60%–70% of neurons displayed exogenous receptor localized exclusively to the cilia (Fig. 4B). In ciliated neurons under these conditions, WT or mutant 5-HT_6 receptors were localized exclusively to the cilia about 90% of the time (Fig. 4C), highlighting the proclivity of 5-HT_6 receptors to localize to the cilia when a cilium is present. Expression of WT and both mutant 5-HT_6Rs significantly increased the length of cilia (as defined by Arl13b staining) compared with empty vector controls (Fig. 4D). The length of cilia when defined by HA staining of primary cilia in neurons where HA-5-HT_6R was exclusively localized in cilia was the same as when measured by Arl13b staining (Fig. 4E). These findings highlight that neurons expressing exogenous 5-HT_6R have longer cilia compared with neurons lacking 5-HT_6R, and that this effect was not ligand-activation dependent nor required the presence of the predicted Fyn kinase binding domain. Cilia in neurons transfected with the WT or 5-$HT_6^{406–411del}$ mutants were neither longer nor shorter than those transfected with the 5-HT insensitive 5-HT_6^{D106A} mutant (Fig. 4, D and E).

5-HT_6R Rescue Increases Dendritic Length. Map2B is exclusively localized to dendrites (Wayman et al., 2012), so we cotransfected Map2B-RFP with WT and mutant 5-HT_6Rs and then measured dendritic morphology in the same transfected neurons used to measure cilia length and receptor localization as described above. Rescue of WT 5-HT_6R expression in these neurons cultured from 5-HT_6 KO mice significantly increased average total dendritic length compared with empty vector controls, without changing dendritic branching (Fig. 5, A–C). This increase in average total dendritic length did not appear to depend on receptor sensitivity to 5-HT, as the 5-HT insensitive 5-HT_6^{D106A} receptor mutant still increased average dendritic length (Fig. 5, A–C). On the other hand, the average dendritic length of the 5-$HT_6^{406–411del}$ mutant was not different from negative controls (Fig. 5, A–C), suggesting that constitutive activation of cAMP production is insufficient to impact dendritic outgrowth, but perhaps another signaling event that is disrupted by the deletion of residues 406–411 is involved in regulating dendritic outgrowth.

Even in transfected neurons lacking 5-HT_6R, the presence of cilia was associated with an increase in total dendritic length (Fig. 5D). 5-HT_6R rescue significantly increased dendritic outgrowth compared with empty vector controls in ciliated neurons. Expression of the 5-$HT_6^{406–411del}$ mutant had no effect on dendritic length regardless of ciliation, but the 5-HT_6^{D106A} receptor expression, like WT 5-HT_6, significantly increased dendritic outgrowth compared with empty vector controls in ciliated neurons. Ciliation did not significantly change total dendritic length in neurons expressing WT or 5-$HT_6^{406–411del}$

ciliated transfected neurons in which the receptor localized exclusively to cilia. Red dashed line represents percentage of neurons with cilia in WT cultures (Brodsky et al., 2017). (G) Graph depicting average cilia length in transfected neurons as determined by HA and Arl13B staining (inset representative image of extreme cilium lengthening and Arl13B exclusion at high doses of HA-5-HT_6R expression). (H) Graph depicting average number of dendritic branches and average total dendritic length of transfected neurons at each receptor dose. (I) Graph depicting average total dendritic length of ciliated and unciliated transfected neurons at each receptor dose. For (F–I), n = 15–16 neurons/condition. All statistical measures used t test on the slope of the regression line against a null-linear model. *$P < 0.05$.

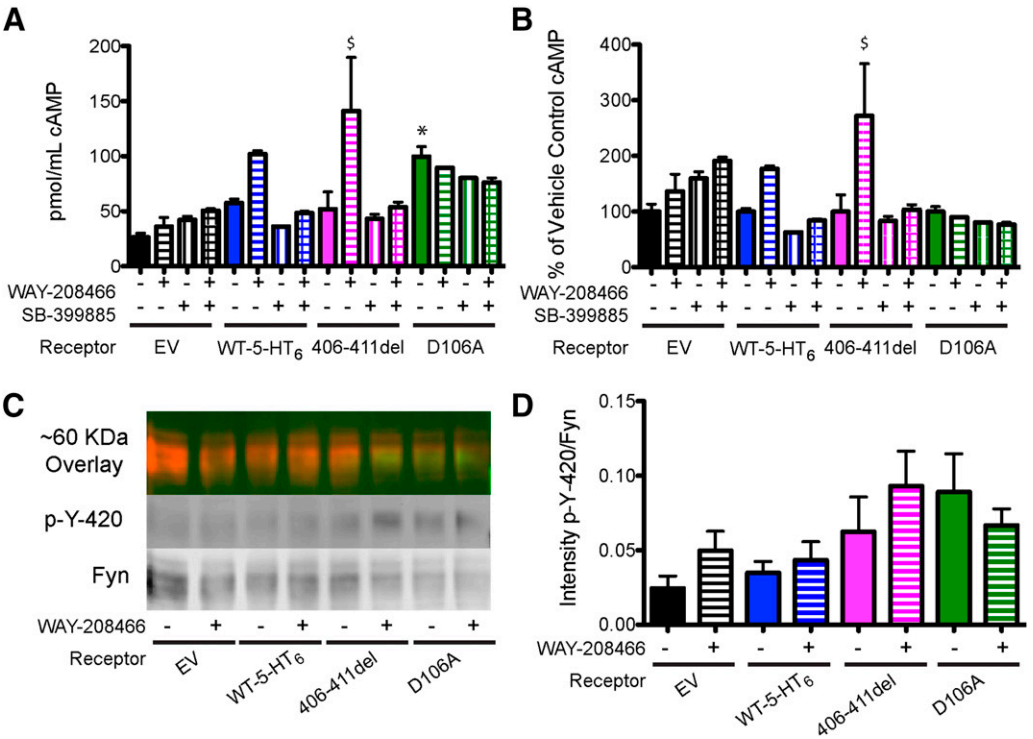

Fig. 3. Signaling properties of 5-HT$_6$ receptor mutants. IMCD3 cells were transfected with empty vector ± 5-HT$_6$R plasmid (WT-5-HT$_6$R, 5-HT$_6$R^{D106A}, 5-HT$_6$R$^{del406–411}$) and treated with vehicle, 1 μM WAY-208466 (agonist) or 1 μM SB-399885 (antagonist), or 1 μM concentration of both drugs before cell lysis and tissue harvest for cAMP assay. (A) Absolute cAMP levels and (B) cAMP relative to corresponding vehicle controls are shown after 10 minutes of drug treatment. (C and D) HEK 293 were transfected with empty vector ± 5-HT$_6$R plasmid or mutant receptors and treated with vehicle or 1 μM WAY-208466 for 15 minutes. Immunoprecipitation from cell lysates was conducted using anti-Fyn Ab, bound material was eluted, subjected to SDS-PAGE, and then immunoblotted for Fyn and phosphorylated Fyn (p-Y420). (C) Representative Western blot of IP-isolated Fyn and Fyn phosphorylated at Y-420. (D) Graph depicting p-Y-420 intensity/Fyn intensity. One-way ANOVA, $n = 3$ biologic replicates for each experiment. Bonferroni post-hoc. $^*P < 0.05$. $^\$P < 0.01$ compared with receptor vehicle control.

(Fig. 5D), suggesting that 5-HT$_6$R activity altered dendritic growth whether the receptor was exclusively localized in cilia.

In transfected neurons with discernable cilia, we found that cilia-restricted WT 5-HT$_6$R and 5-HT$_6^{D106A}$ expression increased total dendritic length compared with ciliated control (Fig. 5E). The 5-HT$_6^{406–411del}$ mutant receptor continued to have no effect on dendritic outgrowth regardless of localization. The 5-HT$_6^{D106A}$ mutant, like the WT receptor, only increased dendritic outgrowth in neurons where the receptor localized exclusively to cilia (Fig. 5E). Of note, owing to the receptors targeting outside the primary cilia in only ~10% of ciliated neurons, the number of ciliated neurons with cell-wide receptor expression included in the analysis was very low.

Pharmacological Regulation of 5-HT$_6$R in 5-HT$_6$-KO Neurons. In prior studies examining endogenous expression of 5-HT$_6$Rs in primary cultures, we observed that a 5-HT$_6$R-selective antagonist decreased the length of neuronal primary cilia in a dose and time-dependent manner, whereas selective agonists had little effect on cilia length (Brodsky et al., 2017). Of note, to maintain primary neuronal culture integrity, cultures were grown in the presence of serum containing 5-HT, which potentially could have activated the WT and mutant 5-HT$_6$ receptors following transfection. In that study we did not observe agonist or antagonist effects on the average dendritic length in WT primary striatal neuron cultures (Brodsky et al., 2017). Likewise, neither the selective agonist (1 μM WAY-208466) nor antagonist (1 μM SB-399885) significantly changed dendritic outgrowth or primary cilia length compared with vehicle controls (Fig. 6), with the exception that neurons expressing WT-5-HT$_6$Rs treated with SB-399885 had a small but significant decrease in total dendritic length (Fig. 6F). These pharmacological treatments did not change dendritic length in either ciliated or unciliated neurons. However, unciliated neurons transfected with empty vector or 5-HT$_6^{D106A}$ had significantly shorter dendrites compared with the corresponding ciliated neurons, replicating our results from (Figs. 4C and 6O). Although there was no overall interaction between the localization of transfected receptors (inside vs. outside the cilia) with drug treatments in any cases, extraciliary localization of the 5-HT$_6^{406–411del}$ and 5-HT$_6^{D106A}$ mutants significantly decreased dendritic outgrowth (Fig. 6, L and P, respectively), suggesting that the trafficking of 5-HT$_6$R may be an important determinant of its effects on total dendritic length.

Discussion

Our findings support several conclusions that impact the interpretation of 5-HT$_6$R studies. First, receptor location matters, and careful attention needs to be paid to whether 5-HT$_6$R are being appropriately trafficked to primary cilia, as has been well established for WT 5-HT$_6$R (Hamon et al., 1999; Brailov et al., 2000; Berbari et al., 2008). Second, these receptors display substantial constitutive activity (at least when ectopically localized and exogenously expressed), and this complicates the interpretation of pharmacological manipulation, emphasizing the potential importance of 5-HT$_6$

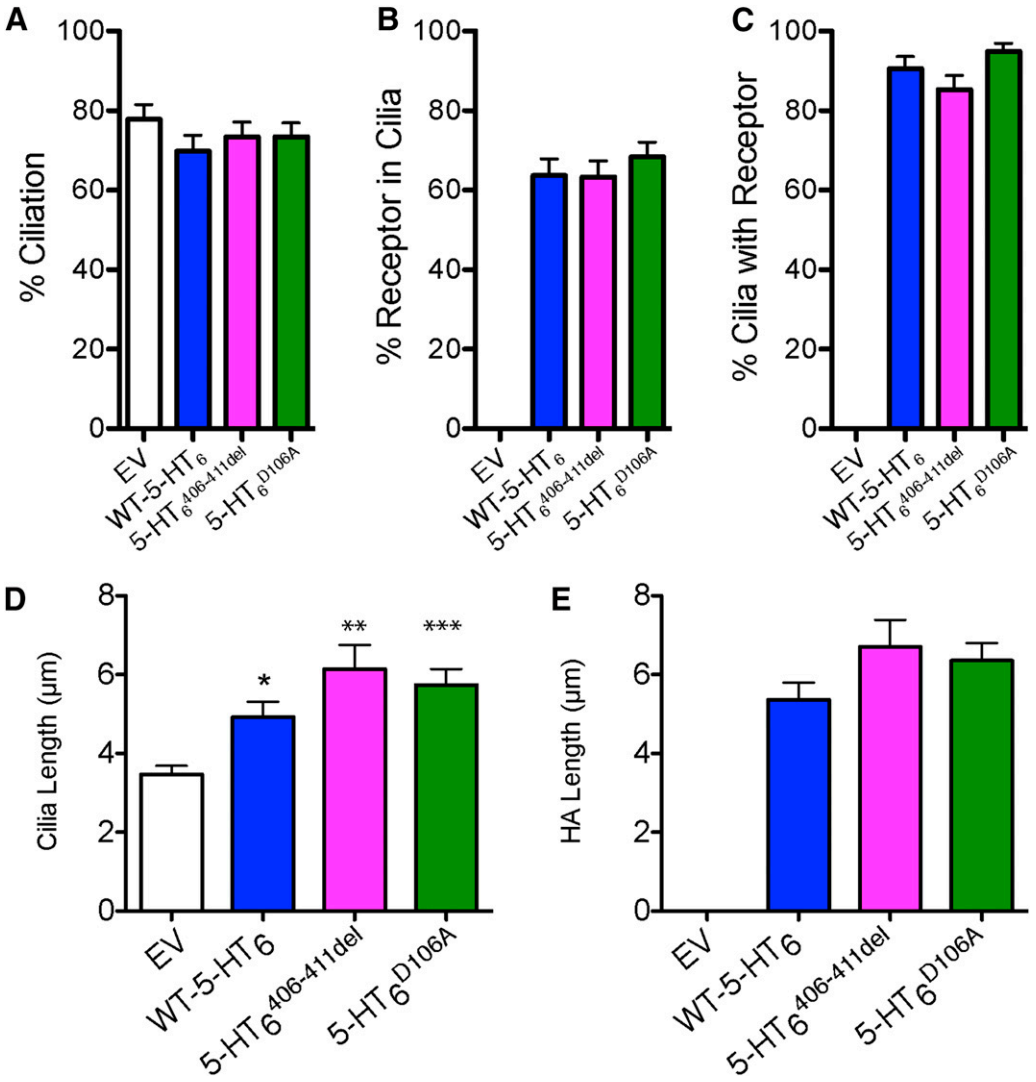

Fig. 4. 5-HT_6R rescue elongates primary neuronal cilia. Primary striatal/cortical cocultures from 5-HT_6RKO pups were transfected on DIV7 with Map2B-RFP ± 70% EV or 55% EV + 15% WT-5-HT_6, 5-$HT_6^{406–411del}$, or 5-HT_6^{D106A}. On DIV10, cultures were fixed, immunostained for Arl13B and HA, then imaged and analyzed. (A) Average percentage of neurons with cilia. (B) Average percentage of neurons in which the receptor is exclusively in the primary cilium. (C) Average percentage of ciliated neurons in which the receptor is exclusively in the primary cilium. (D) Average cilia length. (E) Average HA length. Statistical analysis was conducted using Kruskal-Wallis and Dunn multiple comparison post-hoc. Data measured from three to six neurons per coverslip and pooled into a single data point. Three to six coverslips were analyzed across eight experiments (n coverslips, n = EV-31, WT-32, 5-$HT_6^{406–411del}$-31, 5-HT_6^{D106A}-33). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

receptor inverse agonists for drug development (Duhr et al., 2014). Third, the extent of heterologous expression contributes to extraciliary localization of 5-HT_6Rs and malformation of primary cilia. This effect is potentially attributable to disrupted trafficking and this may in turn alter the availability of signaling partners that are generally localized to primary cilia, for example AC3 (Guadiana et al., 2013; Hu et al., 2017). Likewise, exogenous and heterologous overexpression of 5-HT_6R increases extraciliary targeting, and this may lead to interactions with signaling molecules that are not typical partners with 5-HT_6R within the primary cilium. Finally, the presence of cilia, and 5-HT_6R within these cilia, has important implications for the regulation of neuronal morphology.

One important finding from our study is that drastic overexpression of 5-HT_6R causes radical cilia elongation and leads to increased rates of extraciliary receptor trafficking and cilia malformation. Interestingly, in both the present report and Brodsky et al. (2017), Arl13b length did not change with high levels of HA-5HT_6R overexpression, but in many cases the overexpressed receptor accumulated in the cilia and dramatically extended the cilia compartment as measured by HA immunostaining. Previous studies have observed aberrant cilia formation after overexpression (Guadiana et al., 2013; Hu et al., 2017); however, these studies described extensive cilia branching but did not measure the length of a cilia marker (like Arl13b or AC3) and did not quantify the extent to which they overexpressed 5-HT_6-eGFP. As such, we used low levels of heterologous expression to rescue receptor expression in a more physiologically relevant manner, and we assessed ciliation and receptor localization to recapitulate endogenous receptor function. We did not demonstrate whether increased cAMP production or other signaling events mediated the increase in cilia length, as we tested mutants that interfered

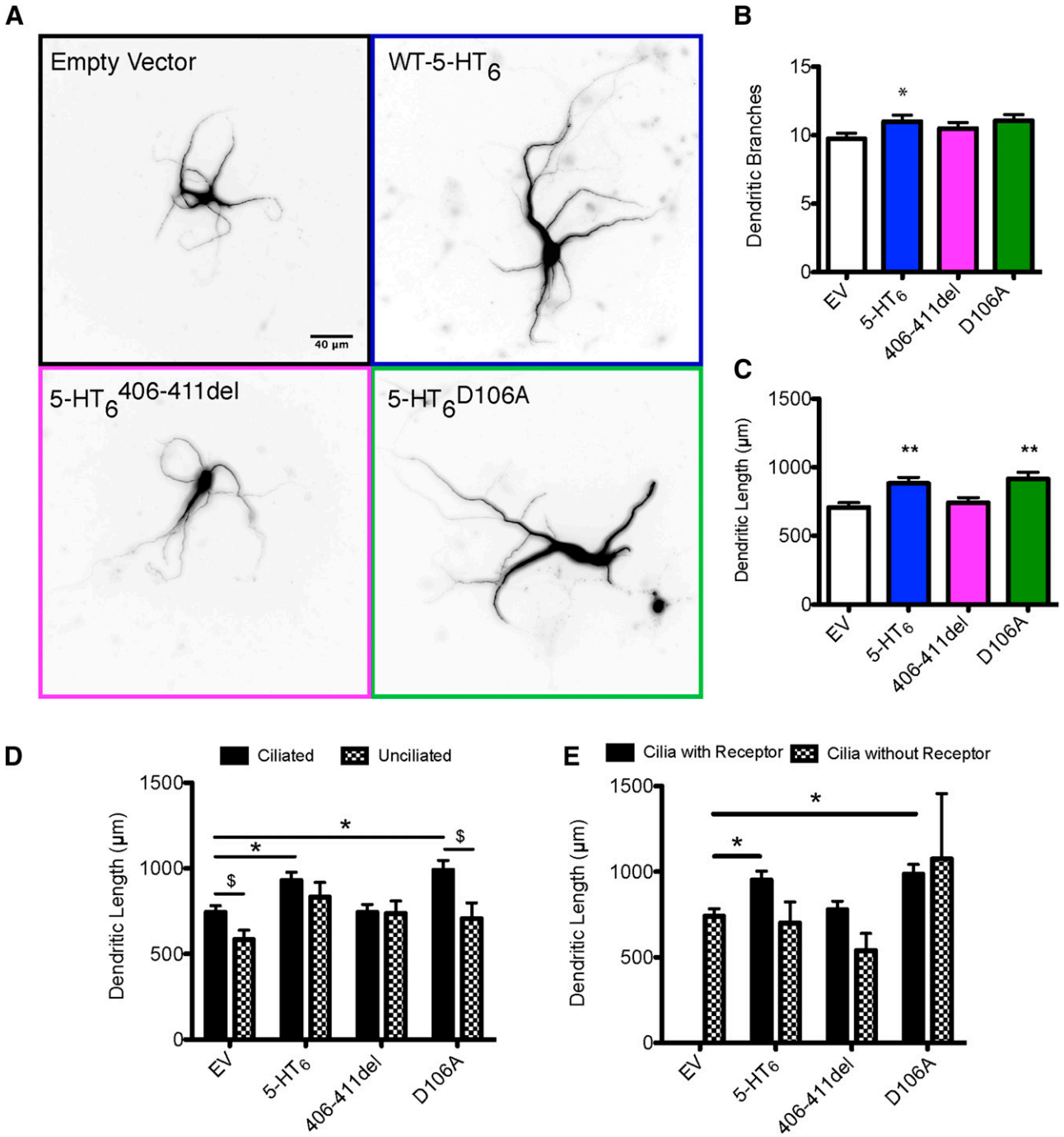

Fig. 5. 5-HT$_6$R rescue increases dendritic length. Primary striatal/cortical cocultures from 5-HT$_6$R KO pups were transfected on DIV7 with Map2B-RFP ± 70% empty vector or 55% EV + 15% WT-5-HT$_6$, 5-HT$_6^{406–411del}$, or 5-HT$_6^{D106A}$. On DIV10, cultures were fixed, immunostained for Arl13B and HA-tag, then imaged and analyzed. (A) Representative images of neurons transfected with different 5-HT$_6$ receptors and mutants. (B) Average number of dendritic branches. (C) Average dendritic length. (D) Average dendritic length of ciliated vs. unciliated neurons. (E) Average dendritic length of ciliated neurons with and without the receptor in cilia. Data measured from three to six neurons per coverslip (same cells and cultures as in Fig. 4) and pooled into a single data point. Three to six coverslips were analyzed across eight experiments. (B and C) (n coverslips, n = EV-31, WT-32,5-HT$_6^{406–411del}$-31, 5-HT$_6^{D106A}$-33), one-way ANOVA, Bonferroni post-hoc. (D) n coverslips = ciliated/unciliated, EV n = 30/20, WT n = 29/24, 5-HT$_6^{406–411del}$-29/22, 5-HT$_6^{D106A}$-30/22), two-way ANOVA, Bonferroni post-hoc. (E) n coverslips = cilia with receptor/cilia without receptor, EV n = 0/31, WT n = 27/26, 5-HT$_6^{406–411del}$-29/27, 5-HT$_6^{D106A}$-30/23). Post-hoc analysis, $^*P < 0.05$; $^{**}P < 0.01$ compared with EV (ciliated or cilia without receptor control), and $^{\$}P < 0.05$ compared with EV ciliated or EV receptor without cilia control.

with ligand-mediated signaling or signaling dependent on residues 406–411. Recently, in a similar study, strong overexpression of WT 5-HT$_6$R stimulated cilia lengthening and branching, but 5-HT$_6$R mutants that were deficient in cAMP production did not induce cilia lengthening (or produce aberrant cilia morphology) (Hu et al., 2017). These results are still puzzling because previous reports have observed that overexpression of both Gs- and Gi-coupled receptors (5-HT$_6$ and SSTR3, respectively) caused cilia elongation, whereas in other reports mutations affecting 5-HT$_6$R function had little to

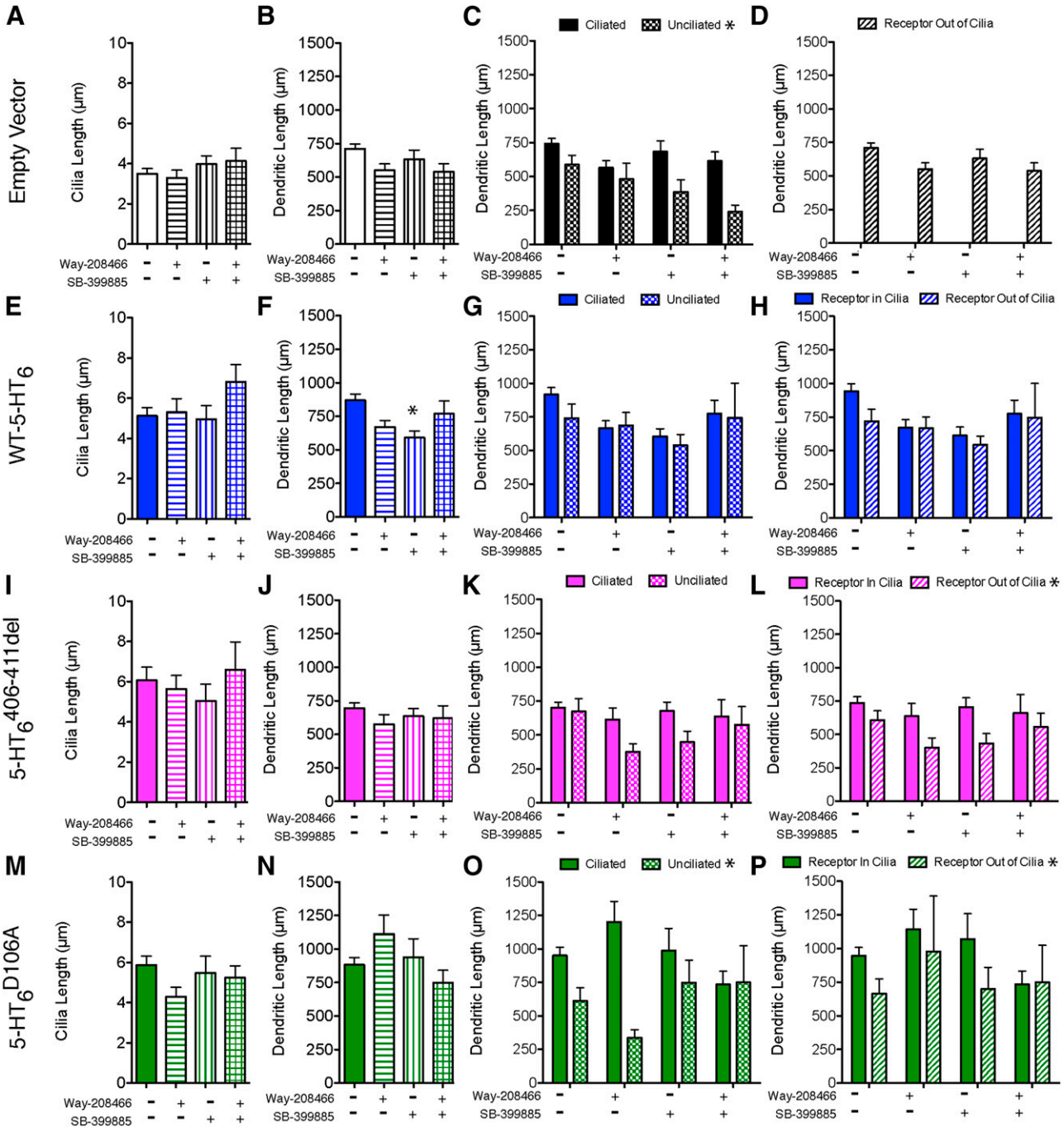

Fig. 6. Pharmacological regulation of 5-HT$_6$R in 5-HT$_6$-KO neurons. Primary striatal/cortical cocultures from 5-HT$_6$R KO pups were transfected on DIV7 with Map2B-RFP ± 70% empty vector or 55% EV + 15% WT-5-HT$_6$, 5-HT$_6^{406–411del}$, or 5-HT$_6^{D106A}$. On DIV9 cultures were treated with vehicle, either 1 μM WAY-208466 (agonist) or 1 μM SB-399885 (antagonist), or 1 μM concentration of both drugs until DIV10 when cultures were fixed, immunostained for Arl13B and HA-tag, then imaged and analyzed. For analysis, three to six individual neurons were measured from 1 to 3 coverslips across six independent experiments, statistical analysis on cilia was completed using Kruskal-Wallis with Dunn multiple comparison post-hoc, on dendrites one-way ANOVA with Bonferroni post hoc, and on dendritic measures separating cilia and receptor localization two-way ANOVA with Bonferroni post-hoc. For average cilia length and dendritic length, n = neurons, (A and B) empty vector, n = 103, 52, 45, 45. (E and F) WT-5-HT6R, n = 120, 48, 47, 39. (I and J) 5-HT$_6^{406–411del}$, n = 115, 42, 39, 31. (M and N) 5-HT$_6^{D106A}$, n = 123, 48, 40, 42. For effect of cilium presence on dendritic length, n = neurons ciliated/unciliated, (C) empty vector, veh: n = 83/20, WAY: n = 43/9, SB: n = 37/8, both: n = 28/7. (G) WT-5-HT$_6$R, veh: n = 87/33, WAY: n = 36/12, SB = 37/10, both = 30/9. (K) 5-HT$_6^{406–411del}$, veh: n = 91/24, WAY: n = 35/7, SB: n = 32/7, both: n = 22/9. (O) 5-HT$_6^{D106A}$, veh: n = 98/25, WAY: n = 43/5, SB: n = 32/8, both: n = 35/6. For effect of receptor localization on dendritic length, n = neurons cilia with receptor/without, (D) empty vector (H) WT-5-HT$_6$R, veh: n = 80/40, WAY: n = 34/14, SB = 32/15, both = 30/9. (L) 5-HT$_6^{406–411del}$, veh: n = 78/37, WAY: n = 31/11, SB: n = 29/10, both: n = 19/12. (P) 5-HT$_6^{D106A}$, veh: n = 95/28, WAY: n = 39/9, SB: n = 26/14, both: n = 35/6. *$P < 0.05$.

no impact on preventing this elongation (Guadiana et al., 2013; Hu et al., 2017). However, in these previous findings, strong overexpression of 5-HT$_6$ disrupted localization of other important ciliary proteins (e.g., AC3 and Arl13b), and caused cilia to branch, which is not observed naturally. We suggest that it is critical to report the rates of neuronal ciliation and whether heterologously expressed receptors localize inside or outside cilia when drawing conclusions about the contribution

of different signaling pathways to 5-HT$_6$R actions in neurons. At more physiologically relevant levels of receptor rescue, cilia were never branched, and in ciliated neurons (about 75% using Arl13b staining as a criteria) 5-HT$_6$Rs overwhelmingly colocalized with Arl13b as expected (~95% of ciliated neurons).

High rates of constitutive activity of exogenously expressed 5-HT$_6$Rs have been reported, albeit not when localized exclusively to primarily cilia (Duhr et al., 2014). This is partly owing to the limitations of biochemical measurements of cAMP in this and other reports that cannot readily determine the rate of ciliation or fidelity of trafficking. Nevertheless, constitutive activity could potentially explain why more physiologically relevant rescue of 5-HT$_6$R in knockout cultures increased cilia length in each of the mutations we tested, and why strong exogenous overexpression resulted in excessively long cilia. Expression of other receptors that occasionally traffic to primary cilia, like type 1 dopamine receptors, have also been associated with increased cilia length (Avasthi et al., 2012; Schou et al., 2015).

We found that inhibition of exogenously expressed 5-HT$_6$R with SB-399885 decreased cilia length in some but not all expected cases, whereas we previously observed that this antagonist shortened cilia length in WT mouse neurons expressing endogenous 5-HT$_6$R (Brodsky et al., 2017). One interpretation is that SB-399885 is not a full inverse agonist in this experimental system and is unable to entirely reverse the effects of the strong expression of exogenous 5-HT$_6$R on cAMP or possibly other signaling pathways. Exogenous expression, even at modest levels, results in significantly more mRNA and protein production compared with endogenous expression. Additionally, since the culture media was not dialyzed, residual 5-HT present in the culture medium could have contributed to cAMP production by WT 5-HT$_6$R and 5-HT$_6$R$^{406–411del}$ to some extent. Another interesting dimension is the impact of rescuing 5-HT$_6$R at different developmental stages considering that this receptor has tremendous impacts on cell migration and maturation (Jacobshagen et al., 2014). Cultured WT neurons will express 5-HT6R throughout in vitro development, whereas in the present study 5-HT6R was only present in 5-HT$_6$-KO neurons from DIV7–10. This difference could have led to differences in responsiveness to pharmacological manipulation at the time of drug treatment on DIV9. Finally, intense overexpression of cilia-targeted receptors may alter the biology of cilia in unpredictable ways, especially since trafficking of G protein-coupled receptors in and out of the primary cilium involves a complex interaction between intraflagellar transport complexes, "BBsome" proteins that are involved in a complex network of interacting proteins that continues to be elucidated (Schou et al., 2015; Ye et al., 2018).

Previous studies reported that exogenous overexpression of 5-HT$_6$Rs in NG108-15 cells and neuronal explants stimulated neurite outgrowth, whereas in utero electroporation of the receptor led to aberrant cilia formation and inhibited dendritic outgrowth (Guadiana et al., 2013; Duhr et al., 2014). The relevance of cilia length on neuronal physiology continues to be unclear, and we found no correlations between cilia length and dendritic morphology. On the other hand, we found that dendritic morphology was correlated with the presence of a cilium and the localization of the receptor to the cilium and that 5-HT$_6$R had the greatest impact on dendritic outgrowth when they were in the cilia. These findings highlight the importance of monitoring 5-HT$_6$ receptor localization, as we found that rescue of 5-HT$_6$R increased dendritic outgrowth significantly only in neurons with identifiable cilia. This effect was further amplified in neurons with the receptor exclusively localized to primary cilia (Fig. 5, D and E). Interestingly, this effect was not observed for the 5-HT$_6^{406–411del}$ mutant receptor that deleted the predicted Fyn kinase binding domain (Yun et al., 2007), and unaffected by inhibiting ligand-dependent receptor activation with 5-HT$_6^{D106A}$ expression. Although, we were unable to detect agonist stimulation of Fyn phosphorylation in cells expressing WT 5-HT$_6$R and confirm the predicted effect of deleting residues 406–411 on Fyn kinase, this deletion may interfere with other protein interactions and signaling cascades. For example, CDK5 and β-arrestin association are potentially disrupted. CDK5 was previously identified as important for 5-HT$_6$R-dependent dendritic outgrowth and constitutive activity of 5-HT$_6$R (Duhr et al., 2014). β-Arrestins, particularly β-arrestin 2, has been shown to play an important role in trafficking activated somatostatin receptor 3 out of the cilium in neurons, so we cannot rule out the possibility that the 406–411 deletion in the C-terminus did not also affect β-arrestin association with 5-HT$_6$R (Green et al., 2015).

Taken together, our findings highlight the complexity of 5-HT$_6$ receptor signaling on neuronal physiology and support the idea that this receptor modulates neuronal morphology. We suggest that future studies and experiments should take into consideration receptor localization and the nuances of exogenous overexpression as they seek to clarify the mechanisms underlying the role of 5-HT$_6$R and other proteins. It is increasingly clear that 5-HT$_6$ receptors are targets of promising therapeutics; however, interpretation of the mechanism by which they exert their effect on neuronal function and morphology remains elusive.

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Authorship Contributions

Participated in research design: Lesiak, Brodsky, Neumaier.
Conducted experiments: Lesiak.
Contributed new reagents or analytic tools: Lesiak, Brodsky.
Performed data analysis: Lesiak, Cohenca, Croicu.
Wrote or contributed to the writing of the manuscript: Lesiak, Neumaier.

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Address correspondence to: Dr. John F. Neumaier, Department of Psychiatry and Behavioral Sciences, University of Washington, Harborview Medical Center, Box 359911, 325 9th Avenue, Seattle, WA 98104. E-mail: neumaier@uw.edu