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Restoration of physiological expression of 5-HT$_6$ into the primary cilia of null mutant neurons lengthens both primary cilia and dendrites.

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5-HT$_6$R Rescue Lengthens Primary Cilia and Dendritic Length

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List of nonstandard abbreviations

CNS – Central Nervous System

GPCR – G-protein Coupled Receptor

DIV – Day in Vitro

NBA – Neurobasal A

GM – Growth Medium

WT - Wildtype

PFA/PHEMS – Paraformaldehyde/PIPES, HEPES, EGTA, $MgCl_2$ buffer

EV – Empty Vector

HA – Hemagglutinin

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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 extra-synaptic 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 likelihood for receptors to localize outside of 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, while maintaining a more physiological 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, maintain constitutive activity for stimulating cAMP and still increased the length of cilia, while the proposed Fyn kinase domain was required for stimulating dendritic outgrowth. These findings highlight the complexity of 5-HT_6R function and localization, particularly when using exogenous overexpression, and provide greater understanding and potential mechanisms for 5-HT_6R drug therapies.

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Introduction

The 5-HT_6 serotonin receptor (5-HT_6R) is a promising target to treat a variety of neurological and cognitive disorders, including cognitive impairment, Parkinson's, Alzheimer's, obesity, schizophrenia, motor disorders, sleep, and depression (Mitchell and Neumaier, 2005; Hirst *et al.*, 2006; Wesolowska and Nikiforuk, 2007; Ferguson *et al.*, 2008; King *et al.*, 2008; Morairty *et al.*, 2008; Arnt *et al.*, 2010; Carr *et al.*, 2010; Meffre *et al.*, 2012; M W J de Bruin and G 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 CNS, 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 non-dividing mammalian cells, including neurons, yet these singular non-motile 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 extra-synaptic environment (Singla, 2006; Green and

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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 (Louvi and Grove, 2011; Lee and Gleeson, 2011; Valente *et al.*, 2013; Gazea *et al.*, 2016; Trulioff *et al.*, 2017; Schmidt *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 non-canonical 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, Niquille, and Chaumont-Dubel, 2014; Dayer *et al.*, 2015). However, the mechanism by which 5-HT_6R signaling

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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, in this study they 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 lab also found that expression of a range of mouse 5-HT_6R mutants in NIH3T3 cells, including nonfunctional mutants, all increased cilia length compared to 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 siRNA knockdown inhibited dendritic outgrowth (Duhr *et al.*, 2014); they concluded that the Cdk5 interaction with 5-HT_6R was responsible since inhibition of Gs-coupled cAMP signaling had no effect. Recently, in Alzheimer's 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 wildtype 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, while 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 of the primary cilia. Additionally, mutations that

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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 extra-ciliary 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.

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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" (NIH publication No. 86-23, 1996). 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 co-cultured with a small number of cortical neurons (10% cortical and 90% striatal). Minced cortical and striatal tissues were dissociated independently using papain (Sigma-Aldrich, St. Louis, MO) and trituration through a fire-polished glass pipette. Cells were plated at a density of 7 x 10^4 cells per cm^2 in culture dishes pre-coated with poly-L-lysine (Sigma; molecular weight 300,000). Cultures were maintained in Growth Media (GM) consisting of Neurobasal A (NBA) medium (Life Technologies, Carlsbad, CA) supplemented with B27 and Glutamax (1X, Life Technologies) throughout treatment days. From the fourth day in vitro (DIV) until homogenization or fixation, culture media was supplemented with 1µM Ara-C (Sigma). This culturing method was adapted from previously described methods (Lesiak *et al.*, 2015; Brodsky *et al.*, 2017), and result in cultures consisting of approximately 70% neurons and 30% glia. Of note, glia cells have been shown not to express significant levels of 5-HT6R mRNA and were thus not imaged or included in our

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analysis (Gokce *et al.*, 2016). Cultures were maintained at 37ºC 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 wild-type 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 E2611S) and oligonucleotide primers (Sigma). 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 1µg DNA/well using a standard 24-well plate, with “transfection %” 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_6R plasmids were transfected at 15% of total transfected plasmid except in the dose-response experiments where they were transfected at a range from 0-80%. 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 Lab) 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 min), permeabilized with 1XPBS with 0.5% Triton-

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X100 (10 min), blocked with 10% BSA in 1XPBS, and stained overnight with corresponding primary antibodies diluted in 1%BSA in 1XPBS, as previously described (Brodsky *et al.*, 2017). Primary antibodies used were anti-HA rabbit (1:1000, Cell Signaling) and anti-Arl13b (1:1000, 73-287; NeuroMab, Davis, CA). Flourescent secondary antibodies, Alexa 488 anti-rabbit and Alexa 568 anti-mouse used at a dilution of 1:4000 (Invitrogen). All antibodies were diluted in 1%BSA in 1XPBS. Coverslips were mounted using ProLong Gold Antifade media containing DAPI (Invitrogen, Carlsbad, CA). Microscopic images used for morphology were acquired on a Leica inverted widefield fluorescence microscope using Metamorph software at the University of Washington Keck Microscopy Facility. 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) in order 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 Biosciences) were used as described in (Brodsky *et al.*, 2017), and cultures were treated for 48 hrs, 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

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primary neuronal cultures with receptor plasmids at 15% of the total DNA. Cultured cells were treated with 5-HT_6 agonist WAY-208466 (1µM) for 10 minutes before lysis.

Western Blot and Fyn-IP. HEK293 cells were plated and transfected with Lipofectamine using the same transfection and dose dependent expression as described above for primary cultures and treated with vehicle or 5-HT_6 agonist (WAY-208466, 1µM final concentration) diluted in culture media for time specified in each figure legend for the corresponding experiments. For western blot, samples were lysed using RIPA buffer supplemented containing 1:100 dilution of inhibitors for protease (Sigma P8340) and phosphatases (Calbiochem 524624), run on NuPAGE 4-12% Bis-Tris Gels (Life Sciences, NP0323) then transferred to PVDF membrane. Membranes were blocked for 1 hour with Aquablock (ab166952, Thermo Fisher) then primary antibodies were diluted in Aquablock (1:1000), and blots were incubated at 4°O/N. DyLight secondary antibodies were diluted in Aquablock 1:4000 (anti-Rabbit 800 5151S, anti-Mouse 680 5470S, Cell Signaling) for 1-2 hours. After washing, blots were scanned on an Olympus Odyssey Scanner, and blots were analyzed with Image Studio. Fyn-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 6-well plates were lysed in 500µl of 1X TBS with 1:100 dilution of Igepal, NaF, NaOv, protease inhibitor and phosphatase inhibitor (Sigma); a 50µl fraction of samples was saved as input. Samples were pre-incubated with 3µl of anti-Fyn antibody (Millipore MABT208) and rotated for 4 hours at 4°C. Anti-A/G magnetic beads (Pierce 88803, 100µl) 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

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other western blot samples. Primary antibodies used for western blot antigen detection were as follows: anti-HA (C294 Cell Signaling), anti-α-Tubulin (DM1A Millipore), anti-Fyn (EPR5500 Millipore), anti-phospho-Src (Tyr416) (clone 9A6 Millipore). 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).

Data Analysis. For measurements illustrated in Figure 2, 7-8 transfected neurons on 2 coverslips (15-16 total) were imaged and analyzed at each dose of 5-HT_6 transfection. Statistics in Figure 2 used a T-test of the slope of the regression line against a null-linear model. For measurements illustrated in Figures 4-5, 3-6 transfected neurons from each coverslip were imaged and measurements of cilia and dendrites were averaged to generate each data point (n); 3-6 coverslips were analyzed from 8 independent cultures for each experimental condition. For Figure 6, only cultures including drug-treated conditions were included in analysis, and 3-6 neurons from 1-3 coverslips across 6 independent cultures were analyzed. For Figure 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 was analyzed using the Kruskal-Wallis test with Dunn's multiple comparison post-hoc, dendritic outgrowth (total dendritic length and branches) data was analyzed using 1-way ANOVA with Bonferroni post-hoc tests, and ciliation-dependent and receptor localization-dependent dendritic length analysis was analyzed using 2-Way ANOVA. All statistics were run using GraphPad Prism or Excel

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software, and all statistical values for experiments can be found in (Supplemental Table 1).

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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 wild-type (WT) or mutants) on the seventh 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 (AC3) 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 (Figure 1A,C) and heterologously expressed HA-tagged receptor localizing exclusively to the Arl13b marked primary neuronal cilia (Figure 1B,D). We did not observe 5-HT$_6$R expression (endogenous or transfected) in cells displaying glial morphology.

Increased expression of exogenously expressed 5-HT$_6$R increases extra-ciliary 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$_6$R plasmid transfected into HEK293 cells increased (balanced by corresponding empty vector), significantly greater levels of HA-tagged-5-HT$_6$R protein was expressed (Figure 2A). Likewise increasing the proportional amount of HA-5-HT$_6$R plasmid transfected into HEK293 cells led to significant increases in levels of 5-HT$_6$R mRNA, while expression of

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a stable amount of a transfected gene (GFP-CRE) and endogenous housekeeping genes did not change from condition to condition (Figure 2B).

In primary neuronal cultures of 5-HT$_6$R-KO neurons, exogenously expressed HA-5-HT$_6$Rs 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 (Figure 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 (Figure 2E).

Increasing amounts of HA-5-HT$_6$R transfected into primary neuronal cultures decreased the number of neurons with cilia-restricted HA-5-HT$_6$R localization but did not alter the proportion of neurons with detectable primary cilia (Figure 2F). Interestingly, we found that, with increasing amounts of transfected HA-5-HT$_6$R, the length of cilia-associated HA-immunostaining significantly increased in neurons in which HA-5-HT$_6$R was restricted to the primary cilium, while the length of the Arl13b within the cilia remained constant (Figure 2G, example neuron inset). In these cases, the cilia, as measured by HA immunostaining, was unusually long while the cilia-specific marker Arl13b was not detectable along the entire length of the presumed cilia. This may be a pathological change related to excessive 5-HT$_6$R trafficking and is similar to previously described effects of 5-HT$_6$R 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 (Figure 2H-I). In a previous study we found that the percentage of ciliated striatal neurons cultured from wild-type animals containing endogenous 5-HT$_6$R was about 80% (Brodsky *et al.*, 2017). Therefore, in

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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 (Figure 2F).

HA-5-HT$_6$R mutant receptor generation

In addition to investigating wild type HA-5-HT$_6$R, we generated two mutants to investigate how 5-HT$_6$R signaling properties affect primary neuron morphology. The 5-HT$_6$R has extensive constitutive activity (Boess *et al.*, 2002; Jacobshagen, Niquille, and Chaumont-Dubel, 2014; Nadim *et al.*, 2016); therefore, we generated and tested a previously described mutant receptor that is insensitive to 5-HT and other 5-HT$_6$R agonists yet continues to display constitutive activity after heterologous expression in cell lines (5-HT$_6^{D106A}$). The second mutant, 5-HT$_6^{406\text{-}411del}$, is predicted to interrupt 5-HT$_6$ signaling associated with interactions with fyn kinase (Yun *et al.*, 2007). Compared to 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 when compared to EV controls (Figure 3A). Treatment of cells expressing WT-5-HT$_6$ or 5-HT$_6^{(406\text{-}411del)}$ with a 5-HT$_6$R agonist (WAY-208466, 1μM) increased cAMP levels (Figure 3B) and stimulated cAMP accumulation was blocked by the further addition of the 5-HT$_6$R inverse agonist SB-399885 (1μM). 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$_6$R-mediated phosphorylation of fyn kinase or 5-HT$_6$R co-immunoprecipitation with CDK5 using

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previously published methods (Yun *et al.*, 2007; Duhr *et al.*, 2014) and several other strategies were unsuccessful (Figure 3C,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 (Figure C-D, and data not shown). Therefore, we could not confirm that the deletion of aa406-411 in the 5-$HT_6^{(406\text{-}411del)}$ 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 Figure 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% wild-type or either of the two mutant receptors (Figure 4A). In all measured neurons, regardless of whether wild-type or mutant 5-HT_6 receptors were expressed, approximately 60-70% of neurons displayed exogenous receptor localized exclusively to the cilia (Figure 4B). In ciliated neurons under these conditions, wild-type or mutant 5-HT_6 receptors were localized exclusively to the cilia about 90% of the time (Figure 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 to empty vector controls (Figure 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 (Figure 4E). These findings highlight that neurons expressing exogenous 5-HT_6R have longer cilia compared to

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neurons lacking 5-HT$_6$R, and that this effect was not ligand-activation dependent nor requiring the presence of the predicted Fyn Kinase binding domain. Cilia in neurons transfected with the WT or 5-HT$_6^{406\text{-}411del}$ mutants were neither longer nor shorter than those transfected with the 5-HT insensitive 5-HT$_6^{D106A}$ mutant (Figure 4D-E).

5-HT$_6$R rescue increases dendritic length

Map2B is exclusively localized to dendrites (Wayman *et al.*, 2012), so we co-transfected Map2B-RFP with wild-type and mutant 5-HT$_6$Rs and then measured dendritic morphology in the same transfected neurons used to measure cilia length and receptor localization as described above. Rescue of wild-type 5-HT$_6$R expression in these neurons cultured from 5-HT$_6$ KO mice significantly increased average total dendritic length compared to empty vector controls, without changing dendritic branching (Figure 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 (Figure 5 A-C). On the other hand, the average dendritic length of the 5-HT$_6^{406\text{-}411del}$ mutant was not different from negative controls (Figure 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$_6$R, the presence of cilia was associated with an increase in total dendritic length (Figure 5D). 5-HT$_6$R rescue significantly increased dendritic outgrowth compared to empty vector controls in ciliated neurons. Expression of the 5-HT$_6^{406\text{-}411del}$ mutant had no effect on dendritic length

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regardless of ciliation, but the 5-HT$_6^{D106A}$ receptor expression, like WT 5-HT$_6$, significantly increased dendritic outgrowth compared to empty vector controls in ciliated neurons. Ciliation did not significantly change total dendritic length in neurons expressing WT or 5-HT$_6^{406\text{-}411del}$ (Figure 5D), suggesting that 5-HT$_6$R activity altered dendritic growth whether or not the receptor was exclusively localized in cilia.

In transfected neurons with discernable cilia, we found that cilia-restricted wild-type 5-HT$_6$R and 5-HT$_6^{D106A}$ expression increased total dendritic length compared to ciliated control (Figure 5E). The 5-HT$_6^{406\text{-}411del}$ 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 (Figure 5E). Of note, due to the receptors targeting outside of 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, while selective agonists had little effect on cilia length (Brodsky *et al.*, 2017). Of note, in order to maintain primary neuronal culture integrity, cultures were grown in the presence of serum, which contains 5-HT that might potentially activate 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). Similarly, neither the selective agonist (1μM WAY-208466) nor antagonist (1μM

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SB-399885) significantly changed dendritic outgrowth or primary cilia length compared to vehicle controls (Figure 6), with the exception that neurons expressing WT-5-HT_6Rs treated with SB-399885 had a small but significant decrease in total dendritic length (Figure 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 as compared to the corresponding ciliated neurons, replicating our results from Figure 4 (Figure 6 C and O). While there was no overall interaction between the localization of transfected receptors (inside vs. outside the cilia) with drug treatments in any cases, extra-ciliary localization of the 5-$HT_6^{406\text{-}411del}$ and 5-HT_6^{D106A} mutants significantly decreased dendritic outgrowth (Figure 6 L and P), suggesting that the trafficking of 5-HT_6R may be an important determinant of its effects on total dendritic length.

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Discussion

Our findings support several conclusions that impact the interpretation of 5-HT_6R studies. First, receptor location matters, and careful attention needs to be paid to whether 5-HT_6R are being appropriately trafficked to primary cilia, as has been well established for wild-type 5-HT_6R (Hamon *et al.*, 1999; Brailov *et al.*, 2000; Berbari *et al.*, 2008)3. 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 receptor inverse agonists for drug development (Duhr *et al.*, 2014). Third, the extent of heterologous expression contributes to extra-ciliary localization of 5-HT_6Rs and malformation of primary cilia. This effect is potentially due 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 extra-ciliary 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 extra-ciliary 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

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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 in order 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 with ligand-mediated signaling or signaling dependent on residues 406-411. Recently, in a similar study, strong overexpression of WT 5-HT_6R stimulated cilia lengthening and branching, but 5-HT_6R mutants that were deficient in cAMP production did not induce cilia lengthening (or produce abberant 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, while in other reports mutations affecting 5-HT_6R function had little to 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 of cilia when drawing conclusions about the contribution of different signaling pathways to 5-HT_6R actions in neurons. At more physiologically-relevant levels of receptor rescue, cilia were never branched, and in ciliated neurons (about 75% using

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Arl13b staining as a criteria) 5-HT_6Rs overwhelmingly co-localized with Arl13b as expected (~95% of ciliated neurons).

High rates of constitutive activity of exogenously expressed 5-HT_6Rs have been reported, albeit not when localized exclusively to primarily cilia (Duhr *et al.*, 2014). This is partly due to the limitations of using 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_6R in KO 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_6R with SB-399885 decreased cilia length in some but not all expected cases, whereas we previously observed that this antagonist shortened cilia length in wild-type mouse neurons expressing endogenous 5-HT_6R (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_6R on cAMP or possibly other signaling pathways. Exogenous expression, even at modest levels, results in significantly more mRNA and protein production compared to 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_6R and 5-$HT_6R^{406\text{-}411del}$ to some extent. Another interesting dimension is the impact of rescuing 5-

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HT_6R at different developmental stages considering that this receptor has tremendous impacts on cell migration and maturation(Jacobshagen, Niquille, Chaumont-Dubel, *et al.*, 2014). Cultured wild-type neurons will express 5-HT6R throughout in vitro development, while in the present study 5-HT6R was only present in 5-HT6-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 manners, especially since trafficking of GPCRs in and out of the primary cilium involves a complex interaction between intraflagellar transport complexes, “BBsome” proteins that are involved in complex network of interacting proteins that continues to be elucidated (Schou *et al.*, 2015; Ye *et al.*, 2017).

Previous studies reported that exogenous overexpression of $5\text{-}HT_6Rs$ in NG108-15 cells and neuronal explants stimulated neurite outgrowth, while *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 $5\text{-}HT_6R$ had the greatest impact on dendritic outgrowth when they were in the cilia. These findings highlight the importance of monitoring $5\text{-}HT_6$ receptor localization, as we found that rescue of $5\text{-}HT_6R$ 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 (Figure 5 D-E).

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Interestingly, this effect was not observed for the 5-$HT_6^{406\text{-}411del}$ 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_6R 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 ß-Arrestin association are potentially disrupted. CDK5 was previously identified as important for 5-HT_6R-dependent dendritic outgrowth and constitutive activity of 5-HT_6R (Duhr *et al.*, 2014). ß-Arrestins, particularly ß-Arrestin 2, has been shown to play an important role for 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-terminal did not also affect ß-Arrestin association with 5-HT_6R (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_6R 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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Author Contributions

Participated in Research Design: Lesiak, Brodsky, and Neumaier

Conducted Experiments: Lesiak

Contributed New Reagents or Analytic Tools: Lesiak, and Brodsky

Performed Data Analysis: Lesiak, Cohenca, and Croicu

Wrote or contributed to the writing of the manuscript: Lesiak and Neumaier

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Footnotes

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Figure 1: Rescued 5-HT$_6$R localizes to the primary cilium in 5-HT$_6$R-KO neurons. Super-resolution images taken on Zeiss LSM 880 with Airyscan of primary striatal/cortical primary neuronal cultures. Primary cultures from 5-HT$_6$R-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-D) XYZ projected images demonstrating co-localization of 5-HT$_6$R (Green) with Arl13B (cilia marker magenta) on the primary neuronal cilium.

Figure 2: Dose-dependent receptor expression alters 5-HT$_6$ receptor localization to primary cilia. A) Graph and representative Western Blot of HEK 293 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 HEK 293 cells transfected with increasing % 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 ß-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 and on DIV 10, 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

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receptor in neurons with a primary cilium. F) Graph depicting plasmid dose-dependent changes in % of transfected neurons with cilia, % of transfected neurons with receptor exclusively localized to cilia, and % of ciliated transfected neurons in which the receptor localized exclusively to cilia. Red dashed line represents % 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.

Figure 3: Signaling properties of 5-HT_6 Receptor Mutants. IMCD3 cells were transfected with empty vector ± 5-HT_6R plasmid (WT-5-HT_6R, 5-HT_6R^{D106A}, 5-$HT_6R^{del406-411}$) and treated with vehicle, 1µM WAY-208466 (agonist), or 1µM SB-399885 (antagonist), or 1µM 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 min of drug treatment. C-D) HEK 293 were transfected with empty vector ± 5-HT_6R plasmid or mutant receptors and treated with vehicle or 1µM 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

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Fyn and Fyn phosphorylated at Y-420. D) Graph depicting p-Y-420 intensity/Fyn intensity. 1-Way ANOVA, n=3 biological replicates for each experiment. Bonferroni post-hoc. *p<0.05, **p<0.01, ***p<0.001 compared to EV control. $ p<0.01 compared to receptor vehicle control.

Figure 4: 5-HT$_6$R rescue elongates primary neuronal cilia. Primary striatal/cortical co-cultures from 5-HT$_6$RKO pups were transfected on DIV7 with Map2B-RFP ± 70% empty vector or 55% EV + 15% WT-5-HT$_6$, 5-HT$_6^{406\text{-}411del}$, 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's multiple comparison post-hoc. Data measured from 3-6 neurons per coverslip and pooled into a single data point. 3-6 coverslips were analyzed across 8 experiments (n coverslips, n=EV-31, WT-32, 5-HT$_6^{406\text{-}411del}$-31, 5-HT$_6^{D106A}$-33). *p<0.05, **p<0.01, ***p<0.001.

Figure 5: 5-HT$_6$R rescue increases dendritic length. Primary striatal/cortical co-cultures 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$_6^{406\text{-}411del}$, 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

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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 3-6 neurons per coverslip (same cells and cultures as in Figure 4) and pooled into a single data point. 3-6 coverslips were analyzed across 8 experiments. B-C) (n coverslips, n=EV-31, WT-32,5-$HT_6^{406\text{-}411del}$-31, 5-HT_6^{D106A}-33), 1-way ANOVA, Bonferroni post-hoc. D) n coverslips=ciliated/unciliated, EV n=30/20, WT n=29/24, 5-$HT_6^{406\text{-}411del}$-29/22, 5-HT_6^{D106A}-30/22), 2-way ANOVA, Bonferroni post-hoc. E) n coverslips=cilia with receptor/ cilia without receptor, EV n=0/31, WT n=27/26, 5-$HT_6^{406\text{-}411del}$-29/27, 5-HT_6^{D106A}-30/23). Post-hoc analysis, *$p<0.05$, $p<0.01$ compared to EV (ciliated or cilia without receptor control) and $ $p<0.05$ compared to EV ciliated or EV receptor without cilia control.

Figure 6: Pharmacological regulation of 5-HT_6R in 5-HT_6-KO neurons. Primary striatal/cortical co-cultures from 5-HT_6R KO pups were transfected on DIV7 with Map2B-RFP ± 70% empty vector or 55% EV + 15% WT-5-HT_6, 5-$HT_6^{406\text{-}411del}$, or 5-HT_6^{D106A}. On DIV9 cultures were treated with vehicle, either 1µM WAY-208466 (agonist) or 1µM SB-399885 (antagonist), or 1µM of both drugs until DIV 10 when cultures were fixed, immunostained for Arl13B and HA-tag then imaged and analyzed. For analysis, 3-6 neurons individual neurons were measured from 1-3 coverslips across 6 independent experiments, statistical analysis on cilia was completed using Kruskal-Wallis with Dunn's multiple comparison posthoc, on dendrites 1-way ANOVA with Bonferroni posthoc, and on dendritic measures separating cilia and receptor localization 2-way ANOVA with Bonferronni posthoc. For average cilia length and dendritic length, n=

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neurons, A,B) Empty Vector, n=103,52,45,45. E,F) WT-5-HT6R, n=120, 48, 47,39. I,J) 5-$HT_6^{406\text{-}411del}$, n=115, 42, 39, 31. M,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_6R, Veh: n=87/33, WAY: n=36/12, SB=37/10, Both=30/9. K) 5-$HT_6^{406\text{-}411del}$, 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_6R, Veh: n=80/40, WAY: n=34/14, SB=32/15, Both=30/9. L) 5-$HT_6^{406\text{-}411del}$, Veh: n=78/37, WAY: n=31/11, SB: n=29/10, Both: n=19/12. O) 5-HT_6^{D106A}, Veh: n=95/28, WAY: n=39/9, SB: n=26/14, Both: n=35/6. *p<0.05, **p<0.01, ***p<0.001.

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Figure 1

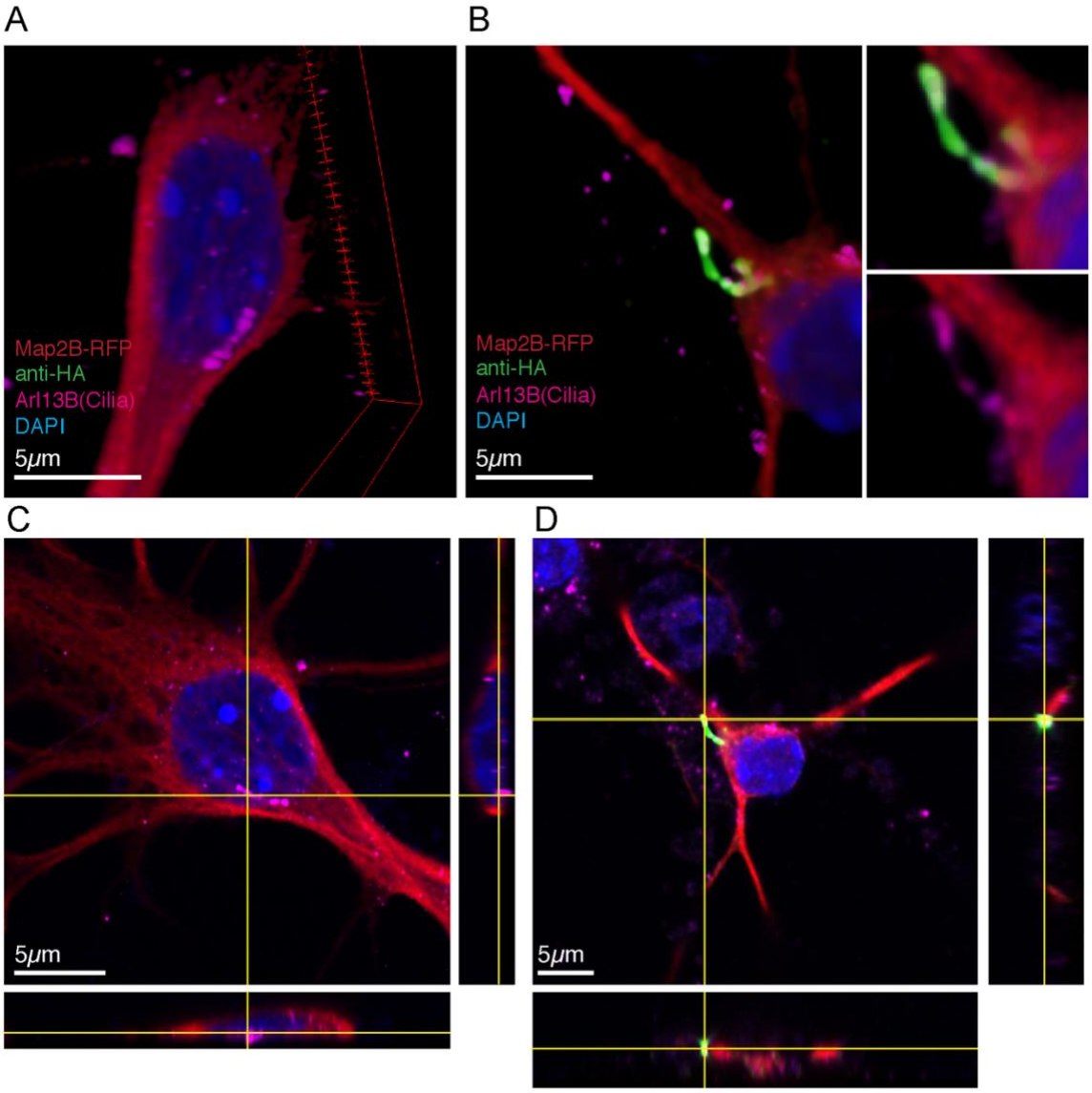

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Figure 2

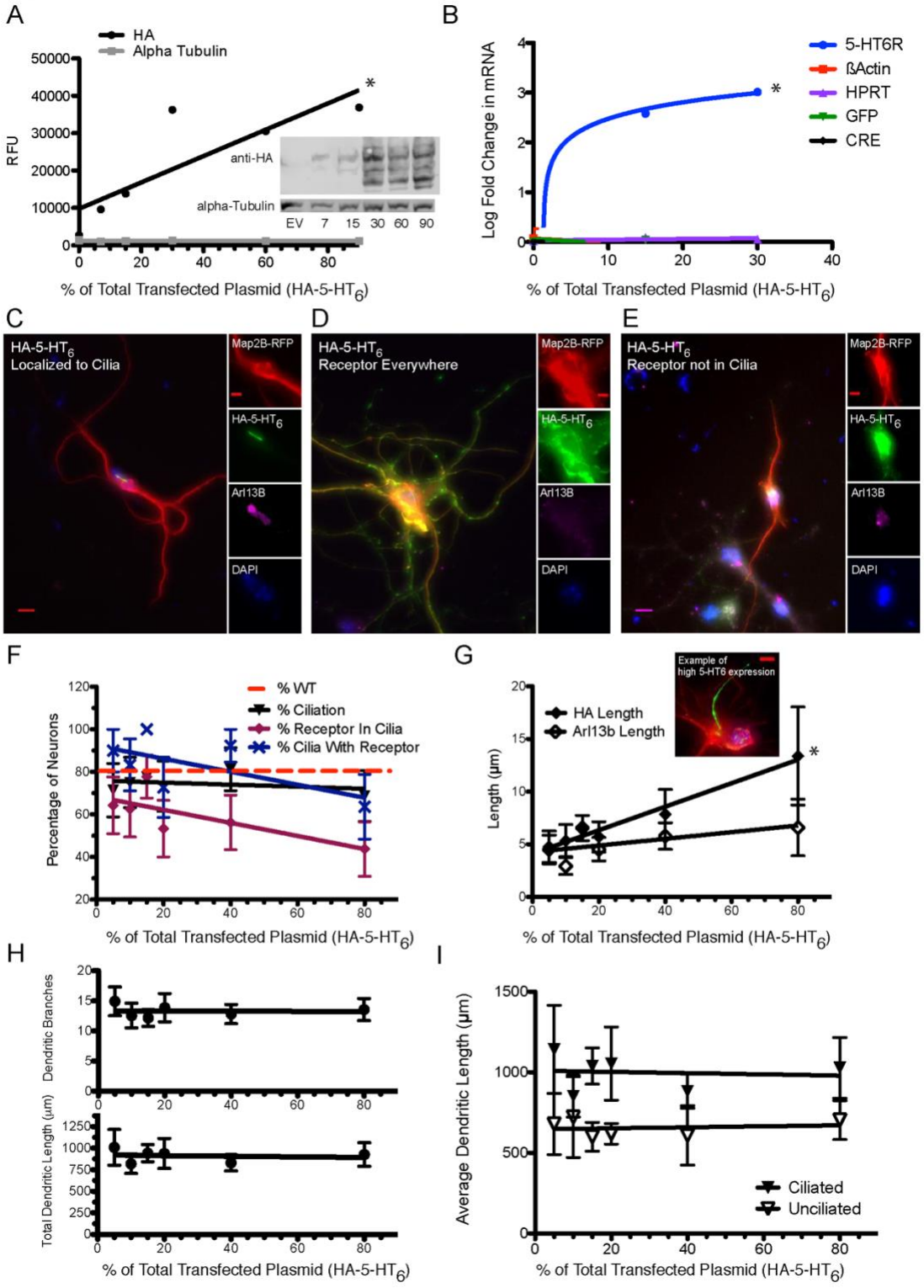

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Figure 3

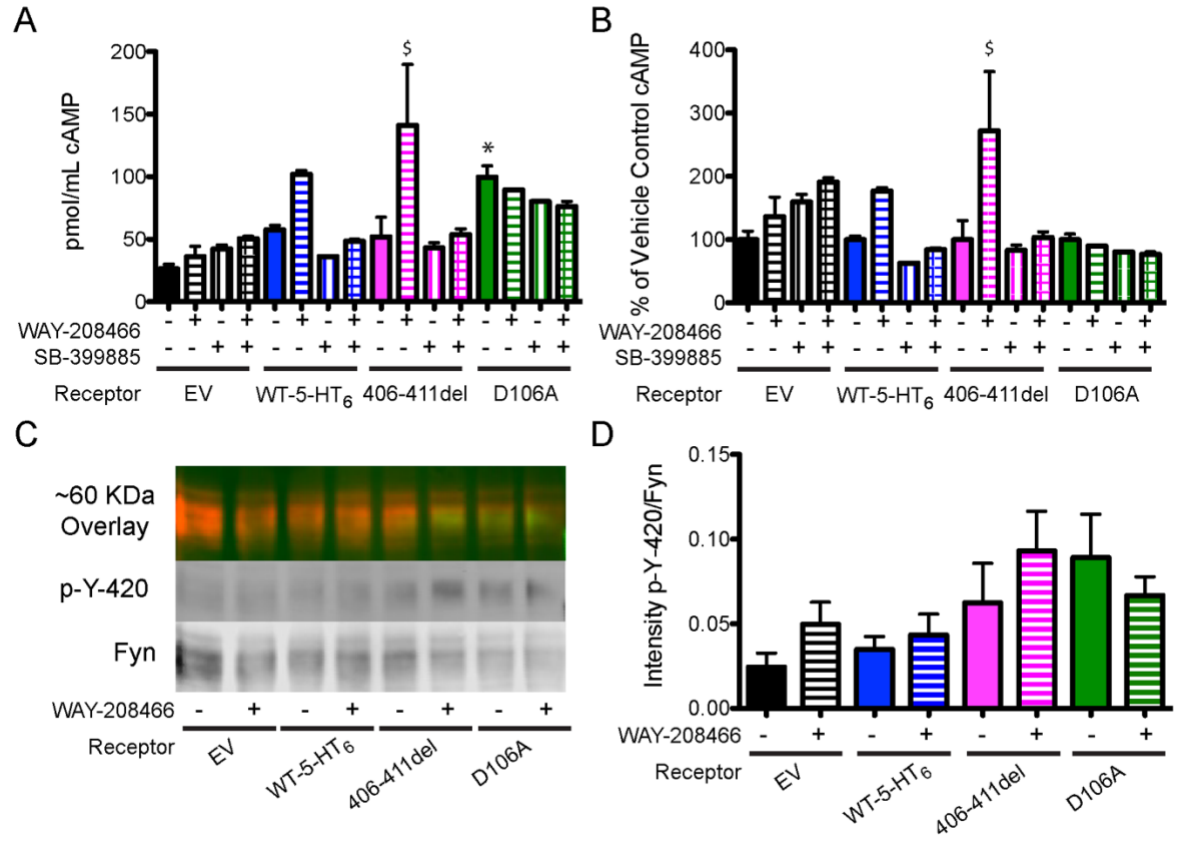

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Figure 4

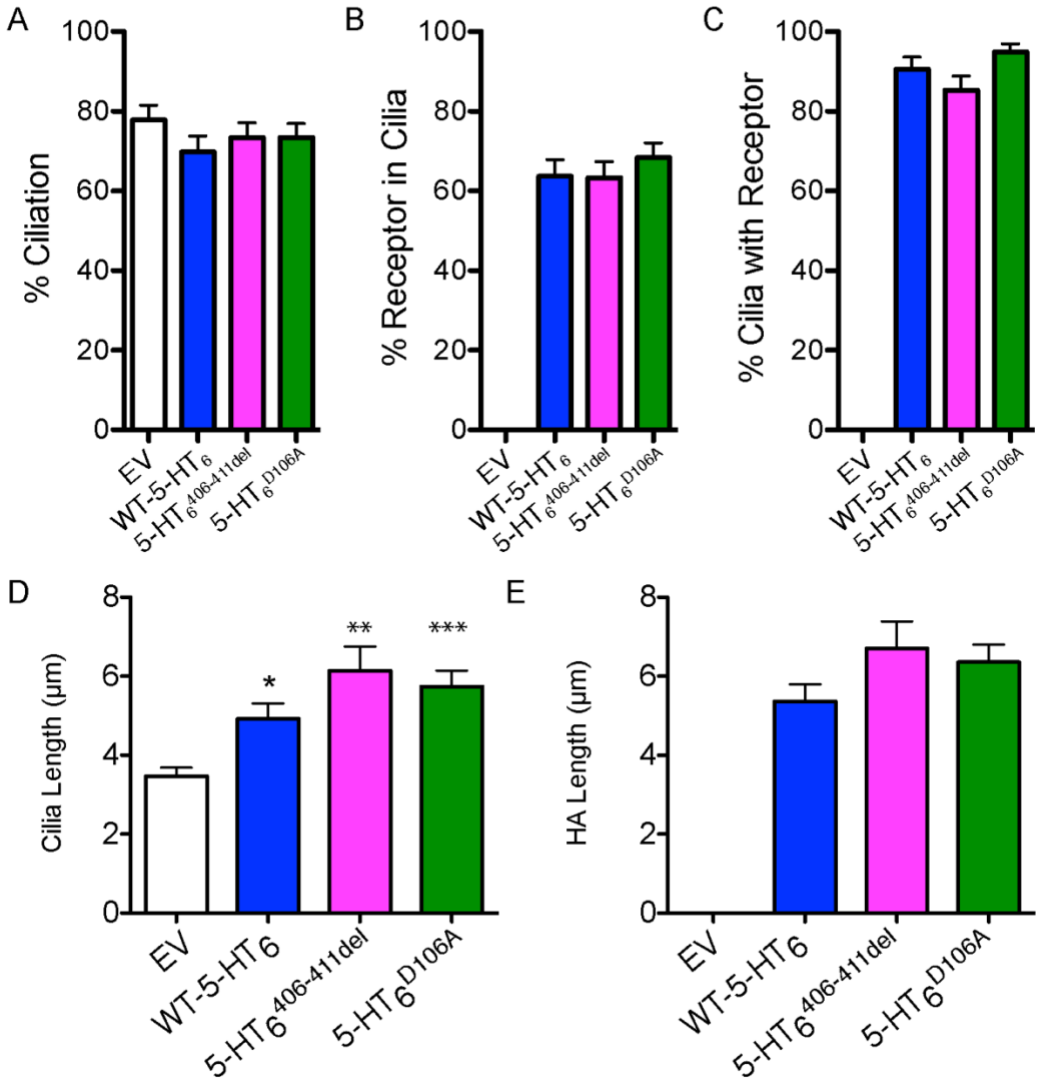

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Figure 5

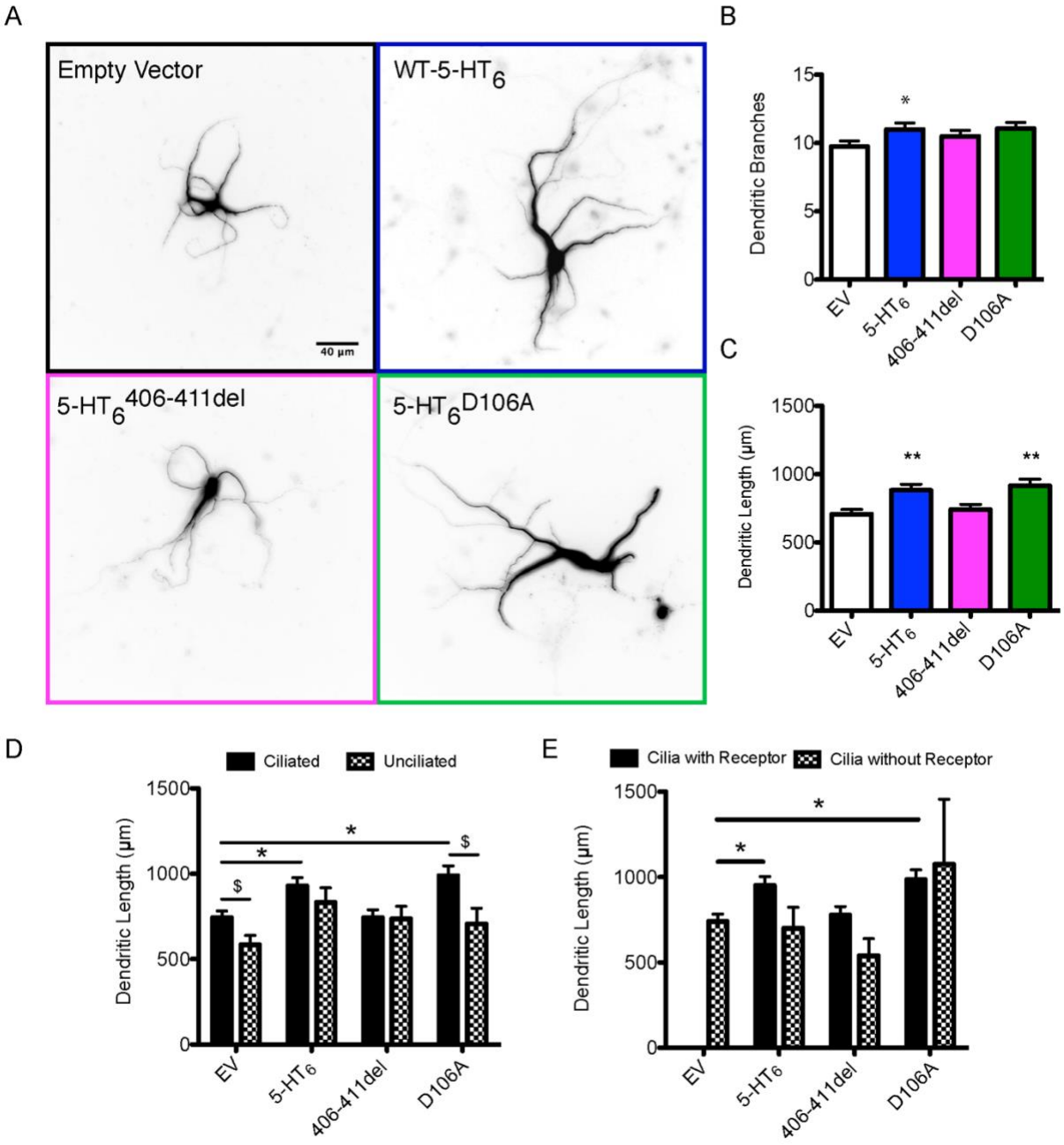

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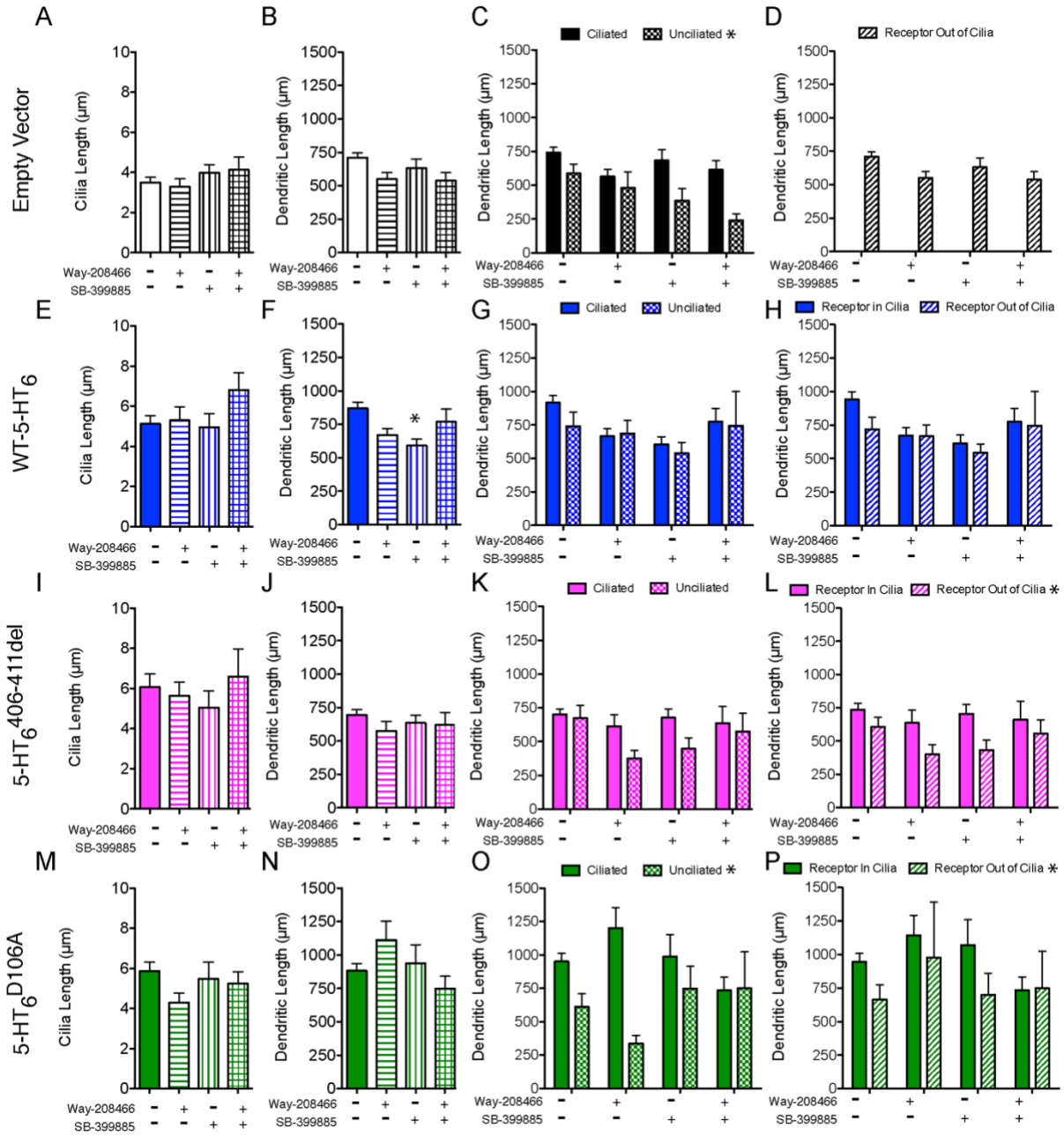
Figure 6

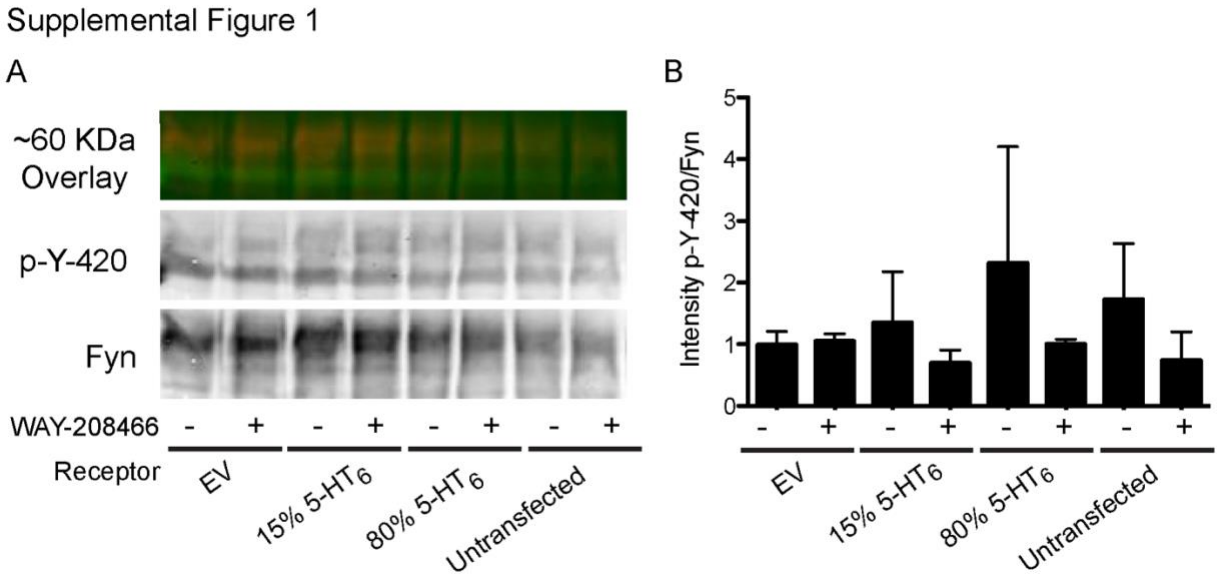


Restoration of physiological expression of 5-HT$_6$ into the primary cilia of null mutant neurons lengthens both primary cilia and dendrites.

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

Molecular Pharmacology

Supplemental Materials



Supplemental Figure 1: 5-HT$_6$R dose does not affect Fyn phosphorylation in HEK 293 cells. HEK 293 cells were transfected with empty vector, 5-HT$_6$R plasmid at different doses, or untransfected, then treated with vehicle or 1µM 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). A) Representative western blot of IP isolated Fyn and Fyn phosphorylated at Y-420. B) Graph depicting p-Y-420 intensity/Fyn intensity. 1-Way ANOVA, n=3 biological replicates for each experiment.

Supplemental Table 1: Statistical Data for Figures

Supplemental Table 1: Table of Statistical Analysis

Figure 2	Panel	Test	Protein	n	r^2	t	df	p-value
	A	T-test on Regression Against a Null Linear Model	5-HT6	5	0.864	2.976	3	0.058
			CRE	5	0.112	0.196	3	0.856
		Test	**mRNA**	**n**	**r^2**	**t**	**df**	**p-value**
	B	T-test on Regression Against a Null Linear Model	5-HT6	3	0.999	505.613	1	0.001
			CRE	3	-0.991	-7.569	1	0.083
			ß-Actin	3	-0.905	-2.134	1	0.278
			HPRT	3	0.659	0.878	1	0.541
			GFP	3	-0.802	-1.344	1	0.407
		Test	**Measure**	**n**	**r^2**	**t**	**df**	**p-value**
	F	T-test on Regression Against a Null Linear Model	%Ciliation	6	-0.302	-0.634	4	0.561
			%Receptor in Cilia	6	-0.749	-2.267	4	0.085
			%Cilia with Receptor	6	-0.638	-1.657	4	0.172
		Test	**Measure**	**n**	**r^2**	**t**	**df**	**p-value**
	G	T-test on Regression Against a Null Linear Model	HA Length	6	0.983	10.88	4	0.0004
			Arl13b Length	6	0.621	1.587	4	0.187
			Measure	**n**	**r^2**	**t**	**df**	**p-value**
	H	T-test on Regression Against a Null Linear Model	Dendritic Branches	6	-0.043	-0.087	4	0.934
			Dendritic Length	6	-0.136	-0.275	4	0.796
			Measure	**n**	**r^2**	**t**	**df**	**p-value**
	I	T-test on Regression Against a Null Linear Model	Ciliated Dendritic Length	6	-0.097	-0.196	4	0.854
			Unciliated Dendritic Length	6	0.163	0.332	4	0.756

Figure 3	Panel	Test		F	r^2	df(treatment)	df(residual)	p-value
	A	ANOVA, Bonferroni Post-Hoc		5.327	0.7141	15	32	<0.0001
	B	ANOVA, Bonferroni Post-Hoc		4.263	0.666	15	32	0.0003
			Protein					
	D	ANOVA, Bonferroni Post-Hoc	Fyn	0.908	0.284	7	16	0.524
			p-Y-420 (pFyn)	0.64	0.219	7	16	0.716
			p-Y-420/Fyn	2.075	0.475	7	16	0.107

Figure 4	Panel	Test	H	p-value				
	A	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	2.209	0.5302				
	B	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	1.043	0.5937				
	C	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	5.767	0.0559				
	D	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	19.35	0.0002				
	E	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	1.449	0.4845				

Figure 5	Panel	Test		F	r^2	df(treatment)	df(residual)	p-value
	B	ANOVA, Bonferroni Post-Hoc		2.645	0.06	3	123	0.0522
	C	ANOVA, Bonferroni Post-Hoc		5.246	0.113	3	123	0.0019
		Test		**F**	**dF**	dF (residual)	**% of total variance**	**p-value**
	D	2-Way ANOVA	Interaction	2.419	3	198	3.25	0.0674
			Ciliation	5.613	1		2.51	0.0188
			Receptor Expression	4.244	3		5.69	0.0062
	E	2-Way ANOVA	Interaction	2.288	3	216	2.84	0.0795
			Receptor Localization	6.966	1		2.88	0.0089
			Receptor Expression	3.896	3		4.84	0.0097

Figure 6	Panel	Test	Receptor	H	p-value			
Cilia Length	A	Kruskal-Wallis and Dunn's Multiple Comparison Post-hoc	EV	2.645	0.482			
	E		WT 5-HT_6	5.246	0.203			
	I		5-$HT_6^{406\text{-}411del}$	0.936	0.816			
	M		5-HT_6^{D106A}	3.141	0.37			
		Test		**F**	**r^2**	**df(treatment)**	**df(residual)**	**p-value**
Dendritic Length	B	ANOVA, Bonferroni Post-Hoc	EV	3.063	0.0384	3	231	0.028
	F		WT 5-HT_6	4.674	0.0531	3	250	0.0034
	J		5-$HT_6^{406\text{-}411del}$	0.902	0.012	3	223	0.4405
	N		5-HT_6^{D106A}	1.958	0.023	3	249	0.12
		Test		**F**	**dF**	**dF (residual)**	**% of total variance**	**p-value**
Dendritic Length	C	EV	Interaction	0.968	3	227	1.17	0.408
Ciliation Dependent		2-Way ANOVA	Ciliation	11.45	1		4.62	0.0008
			Receptor Expression	2.134	3		2.58	0.0968
	G	WT 5-HT_6	Interaction	0.299	3	246	0.35	0.8256
		2-Way ANOVA	Ciliation	0.67	1		0.26	0.4137
			Receptor Expression	2.069	3		2.45	0.1049
	K	5-$HT_6^{406\text{-}411del}$	Interaction	0.468	3	219	0.62	0.704
		2-Way ANOVA	Ciliation	2.972	1		1.31	0.0861
			Receptor Expression	0.996	3		1.32	0.3952
	O	5-HT_6^{D106A}	Interaction	1.664	3	244	1.95	0.175
		2-Way ANOVA	Ciliation	6.196	1		2.42	0.0135
			Receptor Expression	0.144	3		0.17	0.933
		Test		**F**	**dF**	**dF (residual)**	**% of total variance**	**p-value**
Dendritic Length	D	EV	Interaction	Not capable of statistical analysis because Receptor Never in Cilia				
Receptor Localization		2-Way ANOVA	Receptor Localization					
			Receptor Expression					
	H	WT 5-HT_6	Interaction	0.456	3	246	0.54	0.713
		2-Way ANOVA	Receptor Localization	1.256	1		0.49	0.263
			Receptor Expression	2.281	3		2.68	0.079
	L	5-$HT_6^{406\text{-}411del}$	Interaction	0.3368	3	219	0.44	0.7988
		2-Way ANOVA	Receptor Localization	6.95	1		3.03	0.009
			Receptor Expression	0.8478	3		1.11	0.4691
	P	5-HT_6^{D106A}	Interaction	0.4248	3	244	0.51	0.7354
		2-Way ANOVA	Receptor Localization	2.448	1		0.97	0.1189
			Receptor Expression	1.166	3		1.39	0.3232