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5-HT₆ receptor blockade regulates primary cilia morphology in striatal neurons

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Abstract

The 5-HT₆ receptor has been implicated in a variety of cognitive processes including habitual behaviors, learning, and memory. It is found almost exclusively in the brain, is expressed abundantly in striatum, and localizes to neuronal primary cilia. Primary cilia are antenna-like, sensory organelles found on most neurons that receive both chemical and mechanical signals from other cells and the surrounding environment; however, the effect of 5-HT₆ receptor function on cellular morphology has not been examined. We confirmed that 5-HT₆ receptors were localized to primary cilia in wild-type (WT) but not 5-HT₆ knockout (5-HT₆KO) in both native mouse brain tissue and primary cultured striatal neurons then used primary neurons cultured from WT or 5-HT₆KO mice to study the function of these receptors. Selective 5-HT₆ antagonists reduced cilia length in neurons cultured from wild-type mice in a concentration and time-dependent manner without altering dendrites, but had no effect on cilia length in 5-HT₆KO cultured neurons. Varying the expression levels of heterologously expressed 5-HT₆ receptors affected the fidelity of ciliary localization in both WT and 5-HT₆KO neurons; overexpression lead to increasing amounts of 5-HT₆ localization outside of the cilia but did not alter cilia morphology. Introducing discrete mutations into the third cytoplasmic loop of the 5-HT₆ receptor greatly reduced, but did not entirely eliminate, trafficking of the 5-HT₆ receptor to primary cilia. These data suggest that blocking 5-HT₆ receptor activity reduces the length of primary cilia and that mechanisms that regulate trafficking of 5-HT₆ receptors to cilia are more complex than previously thought.

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Keywords

5-HT₆ receptors; Primary Cilia; Striatum; Serotonin; Serotonin-6; Primary cultured neurons

1. Introduction

Almost all mammalian cells, neurons included, have a primary cilium at some point during their life cycle (Bishop *et al*, 2007; Singla and Reiter, 2006; Wheatley *et al*, 1996). Primary cilia are microtubule-supported organelles that stem from the basal body and extend beyond the cell body into the extrasynaptic space (Arellano *et al*, 2012; Avasthi and Marshall, 2012; Louvi and Grove, 2011). Originally identified by the Swiss anatomist K.W. Zimmerman in 1898, primary cilia were neglected in biological research for many years and even misclassified as a vestigial organelle (Praetorius and Spring, 2003; Whitfield, 2004). Often referred to as “non-motile” cilia because they are not involved in motion generation, primary cilia are rich with receptors and receive both chemical and mechanical signals from the surrounding environment (Davenport and Yoder, 2005; Pazour and Witman, 2003; Praetorius and Spring, 2003). Signaling within primary cilia is transduced by a discrete set of membrane-bound receptors specifically localized to the primary cilium; while some receptors are found in both cilia and dendrites, others are exclusive to cilia and represent an interesting category of extra-synaptic receptor-mediated signals from the extracellular environment. Many studies have investigated the developmental impact of neuronal primary cilia, but their role in normal cognitive function is not well understood and there is some evidence that ciliary signaling throughout the lifespan modulates neuronal function and cognition (Berbari *et al*, 2008; Davenport and Yoder, 2005).

There is a rising interest in the function and significance of primary cilia, especially with regard to their role in a variety of disorders now known as “ciliopathies” (Ainsworth, 2007; Novarino *et al*, 2011). Some of the best characterized ciliopathies arise from mutations in or deletions of genes responsible for formation and function of primary cilia (Kim *et al*, 2010; Lee and Gleeson, 2011). Ciliopathies frequently involve malformations of primary cilia such as shortened, elongated or total ablation of the primary cilia (Avasthi *et al*, 2012; Marley and von Zastrow, 2012; Ou *et al*, 2009). Although, little is known about the function of primary cilia on neurons, common symptoms of ciliopathies include brain deformation and cognitive impairments (Green *et al*, 2012; Louvi and Grove, 2011). Even less is known about more subtle alterations in the signaling events that occur within cilia and how they affect health and disease, especially after early development.

The 5-HT₆ receptor is the only serotonin (5-HT) receptor that localizes to primary cilia in neurons (Berbari *et al*, 2007; Brailov *et al*, 2000). 5-HT₆ receptors are present during development and regulate neuronal migration (Dayer *et al*, 2015; Duhr *et al*, 2014; Grimaldi *et al*, 1998). These G_{α_s}-coupled metabotropic receptors couple to adenylyl cyclase and stimulate accumulation of cyclic adenosine monophosphate (cAMP) which, in most neurons, mediates a downstream excitatory effect (Duhr *et al*, 2014; Jacobshagen *et al*, 2014; Kohen *et al*, 2001). However, additional signal-transduction mechanisms distinct from the canonical G-protein signaling cascades have been recently described (Dayer *et al*, 2015;

Duhr *et al*, 2014; Yun and Rhim, 2011; Yun *et al*, 2007). 5-HT₆ receptors are expressed almost exclusively in the brain, especially densely in striatum, and have substantial similarities of sequence identity and pharmacological properties between rats and humans (Gerard *et al*, 1997; Hirst *et al*, 2003; Mitchell *et al*, 2007; Monsma Jr. *et al*, 1993; Ruat *et al*, 1993). 5-HT₆ receptors are considered to be important targets for modulating learning, memory, and reward-motivated behavior (Codony *et al*, 2011; Mitchell and Neumaier, 2005). Increased expression of 5-HT₆ receptors in neurons of the ventral striatum of rats blocks the reward-learning processes associated with cocaine (Ferguson *et al*, 2008). The specific cell type that expresses these receptors is critical as both compulsive behaviors and the sensitivity to cocaine reinforcement are altered by increasing 5-HT₆ expression in indirect pathway medium spiny neurons (Brodsky *et al*, 2016). Together these studies implicate 5-HT₆ receptors as a possible therapeutic target for drug addiction and possibly other cognitive disorders (Eskenazi *et al*, 2015a; Ferguson *et al*, 2011; Fukuo *et al*, 2010; Wilkinson *et al*, 2014).

5-HT₆ receptors, along with most other cilia localizing GPCRs, have a five amino acid long ciliary targeting sequence (CTS) within their third intracellular (i3) loop that has been thought to be critical for trafficking into primary cilia (Berbari *et al*, 2008; Nachury *et al*, 2010; Nagata *et al*, 2013). Heterologously expressed 5-HT₆ receptors have been reported to alter cilia length and dendritic morphology (Duhr *et al*, 2014; Guadiana *et al*, 2013) but endogenous 5-HT₆ receptors in native neurons (such as medium spiny neurons) have not been examined in this manner.

In the present study, we asked whether the morphological properties of neuronal primary cilia are modulated by 5-HT₆ receptor localization and function. In particular we evaluated whether manipulating 5-HT₆ receptor activity and expression in striatal neurons alters neuronal primary cilia morphology and signaling. In both native brain tissue and primary cultured striatal neurons, 5-HT₆ receptors were localized to cilia in wild-type (WT) but not 5-HT₆ knockout mice (5-HT₆KO). Treatment of cultured neurons with a selective 5-HT₆ receptor antagonist reduced cilia length in striatal neurons in a concentration- and time-dependent manner, but agonism and antagonism of the endogenous receptor did not affect dendritic arborization. The level of heterologous expression of 5-HT₆ receptors modified the fidelity with which 5-HT₆ receptors localized to primary cilia in cultured neurons, but had no effect on primary cilia length. We targeted mutant 5-HT₆ receptors containing mutations within the CTS decreased, but failed to eliminate, targeting to the primary cilia or changes in primary cilia length. Suggesting, that the CTS is not exclusively responsible for 5-HT₆ receptor trafficking to primary cilia. Taken together, our findings suggest a role for endogenous 5-HT₆ receptor activity in the dynamic regulation of striatal primary cilia and indicate that regulation of cilia trafficking of 5-HT₆ receptors is more complex than previously thought.

2. Results

2.1 5-HT₆ receptors localize to WT mouse neuronal primary cilia both *in vivo* and *in vitro*

Previous studies have described endogenous 5-HT₆ receptors on “cilia-like” processes in rats with electron microscopy and peroxidase staining (Brailov *et al*, 2000; Hamon *et al*, 1999).

Here we confirmed that endogenous 5-HT₆ receptors co-localize with adenylyl cyclase 3 (AC3), an established marker for primary cilia, (Bishop *et al*, 2007; Wang *et al*, 2009), in sections of WT mouse striatum (Figure 1A) and CA1 of WT mouse hippocampus (Supplemental Figure 1A). Further, 5-HT₆ receptor immunostaining was absent in 5-HT₆KO sections despite continued presence AC3-positive cilia in striatum (Figure 1B) and CA1 of hippocampus (Supplemental Figure 1B). 5-HT₆ receptors also localized to primary cilia in WT primary cultured striatal neurons following dissociation and 10 days growth *in vitro* (Figure 1C); as expected, primary cultured striatal neurons from 5-HT₆ KO mice had cilia but lacked 5-HT₆ immunostaining altogether (Figure 1D).

2.2 5-HT₆ receptor antagonists shorten primary cilia of striatal neurons

To examine the effect of endogenous 5-HT₆ receptor activity on neuronal primary cilia morphology, we used primary cultured striatal neurons to facilitate precise control of drug concentration and ready imaging and measurement of individual primary cilia. Treatment of WT cultured striatal neurons with 1 μ M of selective 5-HT₆ receptor agonist (WAY-208466) for 24 hours had no effect on primary cilia length, but treatment with the 5-HT₆-selective receptor antagonist (SB-399885) for 24 hours significantly reduced the length of primary cilia (Figure 2A, $F(3,58) = 16.84$; $p < 0.0001$). Treatment with both 5-HT₆ receptor agonist and antagonist together had no effect. These drug treatments did not alter the percentage of primary neurons with primary cilia (ciliation) on the striatal neurons (Figure 2B, $F(3,33) = 0.113$; $p = 0.95$). Representative images of primary cilia on WT neurons treated with either vehicle or SB 399885 are shown in Figure 2C and 2D, respectively. Treatment with SB-399885 reduced the average primary cilia length in a concentration-dependent manner with an IC₅₀ value of 28 nM (Figure 2E), which is similar to the previously reported K_i for this drug (Hirst *et al*, 2006). The effect of 1 μ M SB-399885 treatments on WT cultured striatal neurons at different time intervals (0, 4, 12, and 24 hours) established that the antagonist-induced primary cilia shortening is also time dependent (Figure 2F). We observed a similar reduction in cilia length using another 5-HT₆-selective antagonist, SB-258585 (1 μ M, data not shown).

2.3 Effect of SB-399885 of primary cilia on striatal neurons is 5-HT₆ receptor specific

We confirmed that SB-399885 reduced cilia length via 5-HT₆ receptors using primary striatal neurons cultured from 5-HT₆KO mice; there was no effect of 5-HT₆-selective agonist (1 μ M WAY-208466), antagonist (1 μ M SB-399885) or both drugs together; none of these treatments altered primary cilia length (Figure 2G, $F(3,26) = 0.46$; $p = 0.71$) or ciliation (Figure 2H, $F(3,16) = 1.53$; $p = 0.25$).

2.4 5-HT₆ agonists and antagonists do not affect dendritic arborization

To assess the effect of endogenous 5-HT₆ receptor activity on general neuronal morphology, primary cultured striatal neurons from WT mice were transfected with microtubule-associated protein-2B fused to red fluorescent protein (Map2B-RFP) which labels the somato-dendritic domain (Figure 3A). Treatment with 5-HT₆-selective agonist (1 μ M WAY-208466), antagonist (1 μ M SB-399885) or both drugs together had no effect on either the average number of dendritic branches (Figure 3B, $F(3,68) = 0.09$; $p = 0.96$) nor the

average total dendritic length (Figure 3C, 0. $F(3,67) = 36$; $p=0.78$) of the primary cultured striatal neurons.

2.5 High levels of heterologous expression increased nonspecific localization of 5-HT₆ receptors

Previous studies of subcellular localization of 5-HT₆ receptors to primary cilia used heterologous expression of 5-HT₆ receptors (Berbari *et al*, 2008; Guadiana *et al*, 2013; Mahjoub and Stearns, 2012). HA-tagged 5-HT₆ receptor plasmid was transfected into primary striatal neurons along with a second plasmid encoding hSYN-RFP and an empty vector comprising a total of 1 μ g plasmid DNA per well (except the 100% 5-HT₆ condition which received an extra 250ng hSYN-RFP per well). Immunostaining of the transfected neurons revealed that the heterologous 5-HT₆ receptor protein did not always localize to neuronal primary cilia (Figure 4A), and sometimes localized throughout the cytoplasm (Figure 4B). This finding led us to vary the amount of 5-HT₆ plasmid to evaluate the extent to which increased levels of overexpression affected subcellular localization of the HA-5-HT₆ receptor (Susa *et al*, 2008). Each well of WT or 5-HT₆KO neurons was transfected with a total of 1 μ g of total plasmid DNA containing varying proportions of the 5-HT₆ receptor plasmid. At low levels of expression, most of the immunostaining for 5-HT₆ receptor was in the primary neuronal cilia, but as the amount of 5-HT₆ receptor plasmid increased, the percentage of transfected neurons with HA-tagged receptor localized outside of primary cilia increased significantly in both WT neurons (Figure 4C, $\chi^2(7, N=111) = 14.13$, $p=0.048$) and 5-HT₆KO neurons (Figure 4F, $\chi^2(7, N=169) = 22.26$, $p=0.0023$) neurons. Proportions of neurons where receptor was found only in primary cilia, only outside of primary cilia, or both were evaluated for both transfected WT (Figure 4D) and 5-HT₆KO (Figure 4G) neurons. Primary cilia length did not change with different amounts of transfected plasmid in either WT (Figure 4E, 1-way ANOVA, $p = 0.76$) or 5-HT₆KO (Figure 4H, 1-way ANOVA, $p = 0.12$) cultured striatal neurons.

2.6 Mutations to the CTS reduced cilia targeting

Previous studies of the 5-HT₆ and other GPCRs have suggested that a five amino acid long consensus sequence in the i3 loop acts as a cilia targeting sequence (CTS) (Berbari *et al*, 2008; Domire *et al*, 2011; Nagata *et al*, 2013). We took advantage of the CTS on the rat 5-HT₆ receptor and cloned two mutant forms of receptor: one in which the five amino acid CTS was removed (5-HT₆ No CTS, Figure 5A) and one in which the entire i3 loop was replaced with that of the 5-HT₇ receptor (5-HT₆ i3-7) (Figure 5B). The 5-HT₇ receptor was chosen because it is also a G α_s -coupled receptor, but does not localize to primary cilia (Berbari *et al*, 2008). We transfected WT 5-HT₆, 5-HT₆ No CTS, and 5-HT₆ i3-7 (25% of total 1 μ g DNA transfected per well) into 5-HT₆KO cultured neurons to test localization in the absence of endogenous 5-HT₆ receptors (to preclude possible WT-mutant heteromerization and thus prevent potential co-trafficking to primary cilia). These two mutants had significantly reduced trafficking to primary cilia but a modest proportion of the mutant receptors were still localized to primary cilia (Figure 5C, Fisher's Exact test; 5-HT₆ No CTS, $p=0.001$; 5-HT₆ i3-7, $p=0.007$). The mutant 5-HT₆ receptors did not affect the average length of primary cilia (Figure 5D, 1-way ANOVA; $F(2,109)=0.007$, $p = 0.6836$). Since it was possible that the functional properties of these mutants affected localization or

cilia morphology, we tested whether these mutants were still able to activate adenylyl cyclase using an enzyme-linked immunosorbent assay to measure cAMP accumulation in IMCD3 cells, which do not express 5-HT₆ receptors endogenously (data not shown). Both the WT 5-HT₆ and 5-HT₆ No CTS receptor showed constitutive activity relative to the empty vector control (Figure 5E, $F(3,16)=46.20$, $p<0.001$). Both the WT 5-HT₆ and 5-HT₆ receptor demonstrated agonist associated increased cAMP compared to their empty vector control (Figure 5E, $F(3,16)=46.20$, $p<0.001$); however, the addition of agonist did not further increase cAMP compared to vehicle for either of these receptors. However, the 5-HT₆ i3-7 receptor failed to activate cAMP accumulation. Thus, the 5-HT₆ i3-7 mutant was non-functional, at least in terms of the ability to increase adenylyl cyclase activity.

3. Discussion

The 5-HT₆ receptor has become an important target for drug development (Fone, 2008) and at least one 5-HT₆ antagonist is in later stages of development for the treatment of Alzheimer's disease (Wilkinson *et al*, 2014). However, the implications that 5-HT₆ receptors localize to primary neuronal cilia has received scant attention. Primary cilia are enriched with signaling proteins such as receptors and second messenger systems that allow them to function as neuronal sensory organelles that sample the extrasynaptic space (Koemeter-Cox *et al*, 2014; Louvi and Grove, 2011; Whitfield, 2004). This could be an important mechanism for integrating extracellular signals over a different time scale than that associated with synaptic neurotransmission. 5-HT₆ receptors on neuronal primary cilia are well positioned for detecting the extrasynaptic effects of 5-HT (Whitfield, 2004), rendering them unique targets for modulating neuronal plasticity without interfering with momentary synaptic events. Here, we demonstrate that 5-HT₆ receptors preferentially localize to primary cilia and regulate primary cilia morphology. We also find that the level of 5-HT₆ receptor expression dictates receptor localization, and that the mechanism leading to the unique neuronal primary cilia localization is more complicated than previously described.

We focused on striatal neurons since nearly all striatal medium spiny neurons express 5-HT₆ receptors in rats (Helboe *et al*, 2015) although, this has not been directly measured in mouse brain, and due to the importance of 5-HT₆ receptors in drug reward and procedural learning mechanisms (Brodsky *et al*, 2016; Eskenazi *et al*, 2015b). We observed no effect of the addition of a 5-HT₆ agonist on primary cilia length, but 5-HT₆ antagonists reduce striatal primary cilia length in a time- and concentration-dependent manner. There are at least two possible explanations for this. First, the 5-HT₆ receptor has been reported to have constitutive activity (Brouard *et al*, 2015; Duhr *et al*, 2014; Jacobshagen *et al*, 2014; Sebben *et al*, 1994) and there may be a “ceiling effect” in which additional receptor activation produces no further elongation of primary cilia. The lack of effect of 5-HT₆ overexpression or added agonist on cilia length is consistent with this hypothesis. Second, the primary neurons were cultured in the presence of B27 supplement and NeurobasalA, which can contain a small amount of serotonin. Because 5-HT₆ receptors have moderately high affinity for 5-HT (Monsma Jr. *et al*, 1993), they might be activated by trace levels of serotonin. While we observed constitutive activity in our functional assay of cAMP accumulation following heterologous overexpression of the receptor, constitutive activity at 5-HT₆ receptors has been described even in native tissues recently (Deraredj Nadim *et al*, 2016).

Regulation of cilia length is thought to modulate the impact of ciliary signaling (Marley and von Zastrow, 2012), with longer cilia allowing greater signaling capacity by the receptor systems within. The effect of 5-HT₆ receptors on primary cilia morphology is particularly interesting because altered primary cilia length is regulated during key stages in development and is often associated with genetic ciliopathies (Armato *et al*, 2013; Chakravarthy *et al*, 2012; Pan and Snell, 2007). Another phenotype often seen in ciliopathies is a reduction in the number of neurons possessing primary cilia (Kulaga *et al*, 2004; Lee and Gleeson, 2011; Novarino *et al*, 2011; Ross *et al*, 2005). 5-HT₆ receptor antagonism did not change the proportion of neurons bearing primary cilia nor did it affect dendritic outgrowth. This lack of effect of 5-HT₆ on dendritic morphology differs from results in a previous report (Duhr *et al*, 2014), perhaps because of different culturing conditions and that expression of endogenous 5-HT₆ receptors is significantly less than the dramatic exogenous overexpression in prior studies.

Using cultured 5-HT₆KO neurons, 5-HT₆ drugs did not alter cilia length, indicating that the effects of SB-399885 were dependent upon endogenous 5-HT₆ receptors on the WT neurons. Although we were able to confirm that the antagonist effects were absent in striatal neurons cultured from 5-HT₆KO mice, these germ-line null mutants had normal cilia length, perhaps due to developmental compensation that has been described for 5-HT₆ effects on neuronal migration (Dayer *et al*, 2015). These data implicate 5-HT₆ receptors in the dynamic regulation of neuronal primary cilia length, and provide another target, along with lithium, for studying inducible changes to primary cilia morphology (Miyoshi *et al*, 2009; Ou *et al*, 2009).

Heterologous expression of 5-HT₆ receptors via *in utero* electroporation of embryonic neocortical neurons was previously reported to dramatically elongate primary cilia and change dendritic morphology (Guadiana *et al*, 2013). We expressed heterologous 5-HT₆ receptors in both WT and 5-HT₆KO striatal primary neurons after seven days of culture using lipofection and found the subcellular distribution of heterologously expressed 5-HT₆ receptors was dependent on the amount of receptor plasmid introduced to the neurons, while primary cilia length was unaffected. The previous report studied neocortical neurons that were transfected by electroporation in utero and then cultured; they found that even mutant, nonfunctional receptors produced elongation of up to several hundred percent, along with other morphological and phenotypic anomalies such as branching cilia and a loss of AC3 expression. We did not observe these effects and others have shown that loss of AC3 markedly elongates olfactory cilia (Challis *et al*, 2016). Therefore, it is possible that altering 5-HT₆ receptor expression alone has more modest effects on cilia morphology than disrupting Gα_s signaling in cilia altogether.

We found that transfection with increasing amounts of exogenous 5-HT₆ receptor led to localization outside of primary cilia in both WT and 5-HT₆KO cultured striatal neurons. Other investigators have used heterologous overexpression of ciliary receptors to study the effects on primary cilia, but to our knowledge the observation of gene dose-dependent mislocalization of protein has not been previously addressed. Here, we find that moderate to high levels of expression impact the fidelity of ciliary localization in primary neurons, perhaps due to overloading of the ciliary protein trafficking machinery. This is consistent

with the notion that the extent of localization to primary cilia is more dependent on the trafficking machinery than on the total amount of protein expressed (McGlashan *et al*, 2010). Additionally, cilia length was unaffected by reintroducing 5-HT₆ receptors into 5-HT₆KO striatal neurons or increasing 5-HT₆ expression in WT neurons. It is possible that cilia localization depends on the maturity of the neurons, and so studying them in neonatal neurons grown in culture for 10-14 days provides new perspective on 5-HT₆ trafficking and signaling, as we did not observe the lengthening that others saw even with receptors deemed as nonfunctional. It is also possible that the effect of overexpression of 5-HT₆ receptors in primary cilia may have cell type-specific effects, as we did not observe disrupted cilia morphology in striatal neurons as has been observed in neocortical neurons.

A five amino acid consensus sequence on the i3 loop, denoted the CTS, has been found in many GPCRs that traffic into primary cilia (Berbari *et al*, 2008; Nachury *et al*, 2010; Nagata *et al*, 2013). The 5-HT₆ receptor is the only serotonin receptor that contains this motif and no other serotonin receptors have been reported to traffic to cilia. Based on these findings, we designed two mutant 5-HT₆ receptors that disrupted the CTS in different ways and were predicted to block cilia trafficking in order to study the effect of 5-HT₆ receptors signaling inside or outside of neuronal primary cilia. By deleting the CTS or by swapping the i3 loop from a non-cilia localizing receptor into the 5-HT₆ receptor, we found that these changes significantly reduced, but did not entirely abolish, trafficking of the receptor to cilia. Additionally, cilia length was unaffected by reintroducing these mutant 5-HT₆ receptors into 5-HT₆KO striatal neurons. One possible explanation for how mutant receptors might gain access to cilia is through dimerization with endogenous 5-HT₆ receptors; however, trafficking of the mutant 5-HT₆ receptors in 5-HT₆KO neurons was not substantially different than in WT neurons, so dimerization (at least with endogenous 5-HT₆ receptors) is unlikely to explain trafficking to cilia. It remains possible that 5-HT₆ receptors heterodimerize with some other cilia-localizing GPCR but that seems less likely since receptors heterodimerize rather freely but very few receptors gain access to cilia.

3.1 Conclusions

Together, our results implicate a role of 5-HT₆ receptor signaling in primary cilia morphology. Our findings suggest that 5-HT₆ receptor research should consider relative levels of receptor expression when evaluating their impact on primary cilia function. Future studies can also examine how 5-HT₆ receptor signaling alters neuronal function and interacts with signaling via other cilia-localizing receptors; together such studies may provide novel avenues for treating a variety of neuropsychiatric diseases (Marley and von Zastrow, 2012).

4. Experimental Procedures

4.1 Animals

All animal procedures were approved by the University of Washington's Institutional Animal Care and Use Committee and were carried out in accordance with National Institutes of Health guidelines of the "Principles of Laboratory Animal Care" (NIH publication no. 86-23, 1996). Care was taken to minimize animal discomfort. 5-HT₆KO mice on a C57BL/6 background (generously provided by Dr. Lawrence Tecott) (Bonasera *et al*, 2006) or WT

C57BL/6 mice (Jackson Labs, Sacramento, CA) were housed for breeding with two females and one male per cage with access to food and water available *ad libitum*. 5-HT₆KO mice were genotyped by polymerase chain reaction performed on tail DNA utilizing 5'GCCATGCTGAACGCGCTG as the forward primer upstream of the Htr6 null mutation and either 5'GCACCCAGGATGAGCGC or 5'TGCCCCAAAGGCCTACCCGCTTCC as the reverse primer for WT or 5-HT₆KO, respectively.

4.2 Neuronal cell culture

Primary dissociated striatal cultures containing a small number of cortical neurons were generated from either postnatal day 0–1 C57BL/6 mice or postnatal day 0–1 5-HT₆ KO mice (Bonasera *et al*, 2006). Dissociation and culture method were adapted from previously described protocols (Brewer, 1997; Lesiak *et al*, 2015; Pratt *et al*, 2011). Cells were plated at a density of 7×10^4 cells per cm² on culture dishes pre-coated with poly-L-lysine (Sigma; molecular weight 300,000). Cultures were maintained in growth media consisting of Neurobasal-A (NBA) medium (Life Technologies, Carlsbad, CA) supplemented with B27 and Glutamax (Life Technologies) throughout treatment days. From the fourth day *in vitro* (DIV) until homogenization or fixation, culture medium was supplemented with 1 μ M Ara-C (Sigma), a mitotic inhibitor. This results in cultures consisting of approximately 70% neurons and 30% glia. Cultures were maintained at 37°C in 5% CO₂ from DIV0 until homogenization or fixation.

4.3 Drugs and Drug Treatments

Selective 5-HT₆ receptor agonist WAY-208466 (*N*-[2-[3-(3-fluorophenylsulfonyl)-1*H*-pyrrolo[2,3-*b*]pyridin-1-yl]ethyl]-*N,N*-dimethylamine) and selective 5-HT₆ receptor antagonists SB-399885 (*N*-[3,5-dichloro-2-(methoxy)phenyl]-4-(methoxy)-3-(1-piperazinyl)benzenesulfonamide) HCl and SB-258585 (4-Iodo-*N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]benzenesulfonamide) were obtained from Tocris Biosciences. Unless noted otherwise, drugs were diluted in warm neuronal growth medium and administered directly to primary neuronal cultures on DIV9.

4.4 Transfection/Plasmids

Cultured neurons were transfected on DIV7 using Lipofectamine 2000 (Thermo Fisher Scientific). Lipofectamine 2000 was added to warm NBA media (0.02 μ L/ μ L) and incubated for 5 minutes before combining with pre-mixed plasmid DNA (50 μ L/well). Total plasmid DNA for transfections always consisted of 1 μ g/well (24-well plate), unless otherwise noted. The percentage of total plasmid transfected for each plasmid-combination used in the experiments was as follows: 25% 5-HT₆ receptor or mutant 5-HT₆ receptors (unless specified otherwise), 25% fluorescent protein reporter (hSyn-RFP or Clover), and for each condition empty plasmid vector (pCAGGS) was added to the plasmid mix to reach 100% of total transfected plasmid. For dendritic analysis pCAGGS-Map2B-RFP plasmid was used in place of fluorescent reporter to identify dendritic branching. Lipofectamine 2000/NBA/DNA mix was incubated at room temperature for 20 minutes, while native culture media was collected and kept at 37°C and replaced with growth medium. Lipofectamine 2000/NBA/DNA mix was added to plates (50 μ L/well), and incubated for 35–40 minutes before transfection media was aspirated and replaced with original culture media. The

plasmids containing the varied 5-HT₆ receptors (Figure 5) were all tagged with a hemagglutinin (HA) epitope tag on the N-terminus of the receptor sequence via PCR cloning (Ferguson *et al*, 2008; Mitchell *et al*, 2007), and express under the CMV promoter.

4.5 Immunohistochemistry

Cultured neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) / PHEMS (Sigma-Aldrich) buffer on DIV10 for 20 minutes at room temperature. Neurons were then permeabilized with 1×PBS/0.5% Triton-×100 for 10 minutes at room temperature, and blocked in 5% Bovine Serum Albumen (BSA) in PBS for 1 hour at 4°C. Cultured cells were incubated overnight at 4 °C with either of the following primary antibodies in PBS with 1% BSA: anti-adenylyl cyclase III (AC3) rabbit polyclonal antibody (sc-588; Santa Cruz Biotechnology, Santa Cruz, CA), used at 1:1000, anti- ADP-ribosylation factor-like protein 13B (Arl13b) mouse monoclonal antibody (73-287; NeuroMab, Davis, CA), used at 1:1000, anti-SR-6 (5-HT₆) (A-20) raised in goat (sc-26668; Santa Cruz Biotechnology, Santa Cruz, CA), used at 1:1000, or anti-HA (1:1000, rabbit, Cell Signaling). Next, coverslips were rinsed briefly four times in PBS and incubated in species-appropriate Alexa 488 (green) and/or Alexa 568 (red)-conjugated donkey secondary antibodies (1:400, Invitrogen) for 1-2 hours at room temperature. Coverslips were washed 3 times in PBS, mounted on slides and using ProLong Gold Antifade media containing DAPI (Invitrogen, Carlsbad, CA).

Floating sections (40 µm) from adult mice (12-16 weeks old) were washed in 0.5% Triton-X/PBS for 10 min, then blocked in 10% normal goat serum (NGS)-Triton-X/PBS for 1 h. Sections were then incubated in 5% NGS-Triton-X/PBS containing anti-adenylyl cyclase III (ACIII) rabbit polyclonal antibody (sc-588; Santa Cruz Biotechnology, Santa Cruz, CA), used at 1:400 and anti-SR-6 (5-HT₆) (A-20) raised in goat (sc-26668; Santa Cruz Biotechnology, Santa Cruz, CA), used at 1:400 with gentle agitation at 4°C overnight. Next, sections were rinsed 4 times in PBS and incubated with Alexa 488 or Alexa 568, conjugated goat anti-rabbit secondary antibody (1:250, Invitrogen, Carlsbad, CA) for 2 h. Sections were washed 3 times in PBS, mounted on slides and cover-slipped with ProLong Gold Antifade mounting medium.

4.6 Image Analysis

Immunostained primary cultured neurons or brain sections were imaged as stacks using either a Leica inverted widefield fluorescence microscope and associated Metamorph software or a Leica TCS SL Confocal at the University of Washington Keck Microscopy Facility. Stacked images were analyzed as maximum z-projections and images were analyzed using the NeuronJ plugin of the ImageJ software for cilia length or dendritic branching (Schneider *et al*, 2012; Wayman *et al*, 2012). Unless noted otherwise, primary cilia measurements from neurons were averaged from neurons on each culture coverslip and those coverslip averages were used as each biological replicate.

4.7 Cyclic AMP analysis

Cyclic adenosine monophosphate (cAMP) accumulation was measured using IMCD-3 kidney cells (ATCC, Manassas, VA) as they possess primary cilia (Mai *et al*, 2005); they

were maintained in Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12) media supplemented with 10% FBS, 1.2 g/l of sodium bicarbonate, and 0.5 mM sodium pyruvate (Invitrogen). Cells were plated on 6-well plates at 100,000 cells per well and allowed to grow to overnight. On the second day IMCD3 cells were transfected using lipofectamine 2000 with plasmids containing 5-HT₆ receptors or our mutants and allowed to grow in the absence of serum for two days. The cells were then lysed with 0.1 M HCl, and then cellular cAMP levels were measured using an enzyme-linked immunosorbent assay-based detection kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's directions.

4.8 Data Analysis

Statistical analysis, non-linear regression modeling, and IC₅₀ calculations were performed using GraphPad Prism. Significance for multiple comparisons was assessed with one-way analyses of variance (ANOVA) followed by Tukey post hoc tests. For analysis involving only two samples we used a two-tailed t-test. Chi-square (χ^2) and Fisher's exact test were used where appropriate to evaluate contingency tables of categorical data done in GraphPad Prism. For all comparisons, we used an alpha value of 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-HT	5-hydroxytryptamine (serotonin)
5-HT₆	5-hydroxytryptamine ₆
AC3	Adenylyl Cyclase 3 (marker of primary cilia)
cAMP	Cyclic adenosine Monophosphate
hSyn	Human Synapsin-1 promoter
RFP	Red fluorescence protein
WT	Wild-type
5-HT₆KO	5-HT ₆ knockout
CMV	Cytomegalovirus promoter
pcDNA	Plasmid cytomegalovirus promoter deoxyribonucleic acid
CTS	Ciliary targeting sequence
i3 loop	Third intracellular loop
DIV	Days <i>in vitro</i>
NBA	Neurobasal-A medium
PBS	Phosphate buffered saline
BSA	Bovine serum albumin
Arl13b	ADP-ribosylation factor-like protein 13B (marker of primary cilia)
HA	Hemagglutinin tag
5-HT₆ No CTS	5-HT ₆ receptor mutated to remove CTS
5-HT₆ i3-7	5-HT ₆ receptors mutated to swap i3 loop with that of 5-HT ₇ receptor
IMCD3	Inner medullary collecting duct
FBS	Fetal bovine serum

- 5-HT₆ receptor antagonists reduce the length of primary cilia in striatal neurons.
- High levels of heterologous expression impact trafficking of 5-HT₆ receptors to cilia.
- Mutations in the 5-HT₆ receptor's third cytoplasmic loop alter cilia trafficking.

Figure 1. 5-HT₆ receptors localize to mouse striatal primary cilia both *in vivo* and *in vitro*
 A) Images of WT mouse striatum stained against cilia marker AC3 (green), 5-HT₆ receptors (red), and both (also shown at higher magnification). B) Images of 5-HT₆ receptors mouse striatum stained against cilia marker AC3, 5-HT₆ receptors, and both (also shown at higher magnification). C) Images of WT primary mouse striatal neurons stained against cilia marker AC3, 5-HT₆ receptors, and both (also shown at higher magnification). D) Images of 5-HT₆ receptor KO primary mouse striatal neurons stained against cilia marker AC3, 5-HT₆ receptors, and both (also shown at higher magnification).

Figure 2. 5-HT₆ receptor antagonism shortens primary cilia of striatal neurons

A) Effect of 5-HT₆ receptor-selective drugs on primary cilia lengths (Mean ± SEM) of WT primary striatal neurons in culture. Vehicle (n=18 coverslips), WAY (n=17 coverslips), SB (n=18 coverslips) WAY+SB (n=8 coverslips). B) Effect of 5-HT₆ receptor-selective drugs on percentage of WT striatal neurons with primary cilia (Mean ± SEM). Vehicle (n=10 coverslips), WAY (n=10 coverslips), SB (n=9 coverslips) WAY+SB (n=8 coverslips). C) Primary cilia length in response to different concentrations of antagonist SB-399885 (Mean ± SEM); Vehicle (n=25 cilia across 5 coverslips), 10 nM (n=36 cilia across 5 coverslips), 100 nM (n=29 cilia across 5 coverslips) and 1 μM (n=30 cilia across 5 coverslips). D) Time-dependent reduction of cilia length by SB-399885 (Mean ± SEM); 0 (n=6 coverslips), 4 (n=7 coverslips), 12 (n=5 coverslips), and 24 hours (n=7 coverslips). * = P < 0.05, ** = P < 0.001, *** = P < 0.0001; one-way ANOVA followed by post hoc Tukey's HSD test. Effect of SB-399885 on primary cilia length of striatal neurons is 5-HT₆ receptor specific. G) Effect of 5-HT₆ receptor selective drugs on primary cilia lengths (Mean ± SEM) of 5-HT₆ KO primary striatal neurons in culture. Vehicle (n=5 coverslips), WAY (n=6 coverslips), SB (n=8 coverslips) WAY+SB (n=10 coverslips). H) Effect of 5-HT₆ receptor selective drugs on percentage of 5-HT₆KO striatal neurons with primary cilia (Mean ± SEM). Vehicle (n=5 coverslips), WAY (n=5 coverslips), SB (n=5 coverslips) WAY+SB (n=5 coverslips). *P < 0.05, **P < 0.01; one-way ANOVA followed by post hoc Tukey's HSD test.

Figure 3. 5-HT₆ receptor agonists and antagonists do not affect dendritic arborization

Primary striatal cultures were transfected on DIV7 with Map2B-RFP plasmid to identify dendritic branching, and treated with vehicle, 5-HT₆ agonist (1μM Way-181187), 5-HT₆ antagonist (1μM SB399885), or 1μM of Both from DIV9-10, fixed, and imaged. A) Representative photomicrograph of Map2B-RFP transfected neuron. B) Average number of dendritic branches (Mean ± SEM); Vehicle (n=24 neurons), WAY (n=17 neurons), SB (n=14 neurons) WAY+SB (n=17 neurons). *P < 0.05, **P < 0.01; one-way ANOVA followed by post hoc Tukey's HSD test. C) Average total dendritic length (Mean ± SEM); Vehicle (n=24 neurons), WAY (n=17 neurons), SB (n=14 neurons) WAY+SB (n=17 neurons). *P < 0.05, **P < 0.01; one-way ANOVA followed by post hoc Tukey's HSD test.

Figure 4. High levels of heterologous expression increases nonspecific localization of 5-HT₆ receptors

A) Representative images of heterologous 5-HT₆ receptor expressed in neuronal primary cilia. Red = hSynRFP (marker for neuronal specific transfection), Green = HA (HA-tagged 5-HT₆ receptors), Blue = Arl13b (marker for primary cilia). B) Representative image of transfected 5-HT₆ receptor expressed outside of neuronal primary cilia and cytoplasmically. Red = hSynRFP (marker for neuronal specific transfection), Green = HA (HA-tagged 5-HT₆ receptors), Blue = Arl13b (marker for primary cilia). C) Receptor localization from gene-dose transfection of WT 5-HT₆ receptor in WT primary culture neurons (counts). 0% (n=15 cells), 3% (n=13 cells), 6% (n=11 cells), 12% (n=13 cells), 25% (n=12 cells), 50% (n=17 cells), 75% (n=16 cells), 100% (n=14 cells). D) Proportion of total WT neurons transfected with varied amount of 5-HT₆ receptor plasmid where HA is found either in primary cilia, outside of primary cilia, or both. E) Average primary cilium lengths resulting from gene-dose transfection of WT 5-HT₆ receptor in WT primary culture neurons 0% (n=29 cells), 3% (n=10 cells), 6% (n=10 cells), 12% (n=10 cells), 25% (n=17 cells), 50% (n=6 cells), 75% (n=28 cells), 100% (n=13 cells). F) Receptor localization gene-dose transfection of 5-HT₆ receptor in 5-HT₆KO primary culture neurons (count). 0% (n=15 cells), 3% (n=13 cells), 6% (n=17 cells), 12% (n=25 cells), 25% (n=24 cells), 50% (n=26 cells), 75% (n=21 cells), 100% (n=28 cells). G) Proportion of total 5-HT₆KO neurons transfected with varied amount

of 5-HT₆ receptor plasmid where HA is found either in primary cilia, outside of primary cilia, or both. H) Average primary cilia lengths resulting from gene-dose transfection of 5-HT₆ receptor in 5-HT₆KO primary culture neurons 0% (n=54 cells), 3% (n=19 cells), 6% (n=16 cells), 12% (n=20 cells), 25% (n=43 cells), 50% (n=17 cells), 75% (n=20 cells), 100% (n=29 cells). *P < 0.05, **P < 0.01; Chi-square Test.

Figure 5. Mutations to the CTS reduces cilia targeting

A) Representative diagram of mutation on 5-HT₆ receptor to create a receptor without the primary cilia targeting sequence (5-HT₆ No CTS). B) Representative diagram of mutation on 5-HT₆ receptor to create a receptor with 5-HT₇ receptor intracellular loop 3 (IC3) (5-HT₆ i3-7). C) Proportion of receptor localized in and out of primary cilia in 5-HT₆KO cultured neurons transfected with 25% receptor plasmid: WT 5-HT₆ (n=39 neurons), 5-HT₆ No CTS (n=15 neurons), or 5-HT₆ i3-7 (n=18 neurons). D) Average primary cilia lengths when the mutant 5-HT₆ receptors localize to cilia: WT 5-HT₆ (n=43 neurons), 5-HT₆ No CTS (n=28 neurons), or 5-HT₆ i3-7 (n=41 neurons). E) Relative levels of cAMP accumulation in IMCD3 cells transfected with empty vector (pCAGGS), WT 5-HT₆, 5-HT₆ No CTS, or 5-HT₆ i3-7; both in the presence and absence of WAY-208846 (n=3 independent biological replicates). * = P < 0.05, ** = P < 0.001, *** = P < 0.0001; 2-way ANOVA followed by post hoc Bonferroni test.