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A role for primary cilia in glutamatergic synaptic integration of adult-born neurons

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Abstract

The sequential synaptic integration of adult-born neurons has been widely examined in rodents, but the mechanisms regulating the integration remain largely unknown. The primary cilium, a microtubule-based signaling center, plays essential roles in vertebrate development, including the development of the central nervous system. Here we examined the assembly and function of the primary cilium in the synaptic integration of adult-born hippocampal neurons. Strikingly, primary cilia are absent in young adult-born neurons but assemble precisely at the stage when newborn neurons approach their final destination, further extend dendrites and form synapses with entorhinal cortical projections. Conditional cilia deletion from adult-born neurons induced severe defects in dendritic refinement and synapse formation. Primary cilia deletion leads to enhanced Wnt/ β -catenin signaling which may account for these developmental defects. Taken together, our study identifies the assembly of primary cilia as a critical regulatory event in the dendritic refinement and synaptic integration of adult-born neurons.

Introduction

Adult hippocampal neural progenitors continuously give rise to dentate granule cells (DGCs) throughout life^{1–2}. The genesis of these DGCs is precisely controlled by many transcription factors^{3–4}, epigenetic modulators³ and physiological stimuli^{4–6}. After birth, newborn DGCs stereotypically integrate into existing neural circuits: they migrate into the granule cell layer², extend dendrites, and form functional synapses^{7–10}. This stepwise integration of newborn DGCs is regulated by mechanisms similar to those that regulate their

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Author contributions

N.K. engineered retroviral constructs. N.K. and J.W. did immunohistochemistry and confocal imaging analysis. Y.G. did all physiology analysis and took some images. S.J. helped analyzing some images and editing the manuscript. K.T. helped characterizing retroviral vectors. S.G. supervised the project. S.G. and J.L. wrote the manuscript. All authors read and discussed the manuscript.

initial genesis³⁻⁴. Despite the considerable progress in this area, many important questions remain, particularly regarding the mechanisms by which environmental or intrinsic signaling regulates sequential circuit integration.

One intriguing mechanism by which newborn DGCs could sequentially integrate into existing circuitry is via signaling cascades from the primary cilium, a highly specialized sensory organelle. Most vertebrate cells, including neural progenitors and neurons of the embryonic and adult brain, contain a primary cilium extending from a basal body. Specific architectural elements of primary cilia – including their polarized structure, high receptor and channel density, and close proximity to Golgi and vesicle transport systems¹¹⁻¹³ – suggest that they participate in fundamental biological processes during development and adult homeostasis. The importance of primary cilia is emphasized by the increasing number of human genetic diseases associated with cilia dysfunction¹⁴. In animals, mutations of genes involved in the assembly or maintenance of the primary cilia cause early embryonic developmental defects in left-right asymmetry and in the formation of several organs^{11,15}. Similar to the defects found in the developing nervous system^{11,13,16}, two recent studies found that disrupting primary cilia assembly in adult neural progenitors impairs the formation, self-renewal and differentiation of these cells¹⁷⁻¹⁸. Primary cilia mediate critical signaling pathways such as sonic hedgehog, platelet-derived growth factor receptor- α , integrin and Wnt^{11,14,17-19}. Disruption of these pathways may be the basis for the developmental abnormalities seen in both human diseases and animal models.

Here we asked whether primary cilia are present in adult-born DGCs and involved in regulating the sequential synaptic integration of adult-born neurons into functional circuits. We engineered inducible retroviral vectors to label and manipulate primary cilia in adult-born neurons during distinct phases of their development⁷⁻⁸. This approach allowed us to assess cell-autonomous functions of cilia without the confounding developmental defects or lethality common to germ-line knockout animals. We found that primary cilia begin to assemble when newborn DGCs complete their migration, refine dendritic arbors, and form glutamatergic synapses with entorhinal cortical (Ent) projections. Newborn DGCs lacking primary cilia display dendritic refinement defects and significantly diminished synaptic input from Ent projections. Moreover, we provide evidence that primary cilia deletion enhances Wnt/ β -catenin signaling and that this enhancement is required and sufficient to impact the dendritic refinement. Thus, we conclude that timely assembled primary cilia serve as a key structure to sense and transduce environmental signals critical for dendritic refinement of newborn DGCs and their proper synapse formation with Ent projections.

Results

The timing of primary cilia assembly in newborn DGCs

To examine the development of primary cilia in newborn DGCs and their potential functional roles, we used an oncoretrovirus-mediated approach to genetically label and manipulate these neurons *in vivo*⁷⁻⁸. We designed a retroviral vector (pCentrin-2, Fig. 1a) to co-express *dTomato* and *Centrin-2* tagged with enhanced green fluorescent protein (*EGFP*) using a 2A peptide sequence²⁰. High-titer retroviral stocks were injected into the hilar region of the hippocampus in adult mice. At 5, 14, 21 and 28 days post retroviral injection (dpi), we

stained hippocampal sections with an antibody to adenylyl cyclase III (ACIII), a specific marker of primary cilia²¹, and used confocal microscopy to reconstruct 3-dimensional images of newborn DGCs. Infected DGCs contained diffuse cytoplasmic *dTomato* and *EGFP*+ centrioles (Fig. 1b). We then determined whether *dTomato*+ DGCs were ciliated or not based on co-localization of ACIII with their *EGFP*+ centrosomes (Mother centriole, Supplementary Video 1). We found that primary cilia were absent from *dTomato*+ DGCs at 5dpi but present at 14 dpi. At 14dpi, $11 \pm 4.7\%$ contained primary cilia, with an average cilium length of $1.7 \pm 0.4 \mu\text{m}$ ($n=5$ of 48 newborn DGCs). At 21 and 28dpi, $99 \pm 1.9\%$ and $94 \pm 3.4\%$ of newborn DGCs contained primary cilia, averaging $2.8 \pm 0.5 \mu\text{m}$ and $3.0 \pm 0.4 \mu\text{m}$ in length, respectively (Fig. 1b, c).

To confirm that our ACIII staining was accurately and reliably birth-dating the formation of primary cilia, we constructed a retroviral vector to co-express intraflagellar transport protein 88 (*Ift88*), a component of the transport machinery required for primary cilia assembly²². As with *Centrin-2* experiments, *Ift88* was fused with *EGFP* and paired with *dTomato* using a 2A peptide sequence. We analyzed the percentage of *dTomato*+ DGCs with *EGFP*+ primary cilia at 5, 14 and 21 dpi, and again found that most cilia are generated between 14 and 21 days post-birth, consistent with ACIII staining results (Fig. 1c and Supplementary Fig. 1a, b). To further exclude any potential confounding effects of ectopic *Centrin-2* expression during cilia assembly, we then constructed an inducible retrovirus for *Centrin-2* and induced expression at 12dpi time point. Two days later, $13 \pm 3.1\%$ of newborn DGCs harbored ACIII + primary cilia, similar to the percentage of labeled cells having cilia after 14 days of continual *EGFP-Centrin-2* expression (Supplementary Fig. 1c), suggesting that ectopic *Centrin-2* expression is unlikely to alter cilia assembly. Together, these data show that most primary cilia assemble between 2 and 3 weeks after the birth of newborn DGCs.

Newborn DGCs migrate radially into the granular cell layer during their initial development^{4,8,10}. In migrating cultured fibroblasts, primary cilia are found in the leading edge where they point in the direction of migration²³. To determine whether primary cilia play a similar role in the migration of newborn DGCs, we analyzed their cellular position and orientation (Supplementary Fig. 2a, b, c). While the majority of primary cilia protrude from the leading edge (between the nucleus and root of apical dendrite²⁴), they orient randomly. Moreover, when we analyzed the migration distance of newborn DGCs at different stages, we found that the majority of newborn DGCs had completed most of their migration into the DGC layer by 14dpi, prior to the elaboration of a primary cilium (Fig. 1a, d)²⁵. This analysis demonstrates that primary cilia assembly is initiated as newborn DGCs near their final destination. Thus, primary cilia, or primary cilia assembly, may not be required for migration or at least the initial phase of migration.

Synapse formation during primary cilia assembly

From 14dpi, newborn DGCs further extend their dendrites and form functional synapses with existing neural circuits^{7–10}. The coincident timing of cilia assembly and synapse formation suggested that cilia could play a role in synaptic integration.

A mature DGC receives glutamatergic synaptic inputs in a laminar pattern: the inner molecular layer is innervated by contra- and ipsi-lateral hilar mossy cell projections, while

the middle and outer layers receive medial and lateral Ent projections, respectively²⁶. These glutamatergic inputs become detectable at ~14 dpi in newborn DGCs^{8,10}. To examine the development of laminar glutamatergic innervations onto newborn DGCs and determine the specific roles of the primary cilium in this process, we dissected laminar glutamatergic inputs using optogenetic stimulation (Fig. 2a)²⁷. We infused the contralateral hippocampal hilus, medial entorhinal or lateral entorhinal cortex with an adeno-associated virus expressing *Channelrhodopsin 2* tagged with *EGFP* (AAV-*ChR2*; Fig. 2a; Methods). We showed sample axonal projections to the dentate gyrus from these areas (Fig. 2b). Fourteen days after AAV injections, laminar axonal projections were clearly visible and 473 nm blue-light stimulation could reliably induce evoked excitatory postsynaptic currents (eEPSCs) in randomly selected DGCs (Fig. 2c). Newborn DGCs were labeled with retrovirally expressed *dTomato* and analyzed at 7, 14, 21, and 28 dpi; for each of these time points, animals were injected with AAV-*ChR2* 14 days prior. Whole cell recordings were collected from *dTomato* + DGCs in acute brain slices (Methods)^{8,25}. Simultaneously, an additional recording was made from a *dTomato* negative DGC in the outer edge of the granule cell layer, where DGCs are considered fully mature^{28–29}. eEPSCs were elicited by short pulses of 473 nm light illumination in the presence of 5 μ M bicuculline to block GABAergic activity. The recordings from the mature DGCs confirmed that functional laminar excitation could be elicited by short pulses of blue light. Only when the mature DGC showed reliable eEPSCs was the newborn neuron included for subsequent analysis. We found that $43 \pm 10\%$ of the recorded *dTomato*+ DGCs at 14dpi received functional contralateral mossy cell projections (Fig. 2d). This proportion remained fairly constant until 28 dpi. In contrast, while $31 \pm 11\%$ of newborn DGCs showed responses to light stimulation of inputs from the medial entorhinal cortex at 14 dpi, this proportion increased significantly to $86 \pm 14\%$ at 21 dpi. A similar increase in the extent of innervations from the lateral entorhinal cortex also occurred between 14 and 21dpi ($33 \pm 10\%$ at 14dpi and $80 \pm 11\%$ at 21 dpi, Fig. 2d). These results show that robust glutamatergic synaptic development between newborn DGCs and Ent projections occurs 14–21 days after the neurons are born, a time coincident with cilia assembly (Fig. 2e).

Primary ciliogenesis is critical for synapse formation

We next asked whether primary cilia assembly in developing newborn DGCs plays a functional role in synapse formation with the Ent projections by deleting primary cilia of newborn DGCs during the period of synapse formation. Since ectopic expression of dominant-negative Kinesin II motor protein (*Kif3a*) or knockout of *Kif3a* prevents primary cilia assembly from various cell types, including adult neural progenitors^{17,19,30}, we constructed an inducible retroviral vector to express dominant-negative *Kif3a* (*dnKif3a*) in newborn DGCs. We co-injected an *EGFP*-tagged *Centrin-2* retrovirus with either the inducible *dnKif3a* retrovirus or an inducible retrovirus expressing *dTomato* as a control group (CTL) into the hilus of the adult dentate gyrus. We induced *dnKif3a* expression at 7–11 dpi with doxycycline (dox), during which time retrovirus-labeled DGCs have already formed stereotypical dendritic arbors and axonal projections^{4,8,10}. In this way, we avoided any possible confounding effects due to altered proliferation or differentiation caused by *dnKif3a*-induced primary cilia deletion^{17–18,31–32}. As expected, we found that dox-induced

ectopic expression of *dnKif3a* efficiently eliminated primary cilia in newborn DGCs at 14, 21 and 28 dpi (Fig. 3a).

We then measured functional synapse formation onto newborn DGCs by assessing the profile of evoked synaptic transmission. We performed whole cell recording in control and *dnKif3a*+ newborn DGCs at 21dpi while electrically stimulating the outer two-third of the molecular layer of the dentate gyrus to activate Ent projections (Fig. 3b)^{26,28}. Following electrophysiological recordings, biocytin-labeling confirmed that recorded cells were indeed retrovirus-labeled newborn DGCs (Fig. 3b). We analyzed eEPSCs in the presence of 5 μ M bicuculline as previously described^{8,10}. Successful synaptic transmission was recorded from 100% of control newborn DGCs but from only $75\pm5.6\%$ of *dnKif3a*+ DGCs. The mean peak amplitude of eEPSCs in responsive *dnKif3a*+ DGCs was smaller than controls (Fig. 3c, d, e). The frequency of spontaneous glutamatergic synaptic currents (sEPSCs) of control newborn DGCs was 0.82 ± 0.22 Hz as compared to 0.15 ± 0.05 Hz in *dnKif3a*+ DGCs. There was no significant difference in sEPSCs mean amplitude (Fig. 3f).

In addition to its ciliary role, *Kif3a* forms a motor complex involved in plus-end directed trafficking in cultured cells^{32–33}. Knock-down of *Kif3a* in epithelial cells causes endosome/lysosome clusters³³. It is unclear whether *Kif3a* knock-down might similarly affect minus-end directed endosome/lysosome trafficking in neuronal dendrites. To assess this, we briefly stained *dnKif3a*+ DGCs at 21 dpi with LAMP1 antibody, a marker for the late endosome/lysosome³³. We did not observe endosome/lysosome clusters in either the soma or proximal apical dendrite of *dnKif3a*+ DGCs (Supplementary Fig. 3). This suggests that *Kif3a* is likely not involved in minus-end directed endosome trafficking, consistent with a previous report³³. To further rule out possible non-ciliary effects of *dnKif3a* expression on synapse formation, we expressed *dnKif3a* in mature DGCs *in vivo* using AAV-*dnKif3a* and recorded dendritic synaptic activity by stimulating Ent projections. There was no difference in the dendritic synaptic responses from control and mature *dnKif3a*+ DGCs (Supplementary Fig. 4). Overall, these results suggest that the expression of *dnKif3a* causes defects in dendritic synapse formation in newborn DGCs and these defects arise due to the failure of cilia assembly. Thus, primary cilia assembly is required for the proper establishment of functional synapses between Ent projections and newborn DGCs.

Role of primary cilia in dendritic refinement

Newborn DGCs further elaborate their dendrites (dendritic refinement) between 14 and 21 dpi^{8,25}, during which they assemble primary cilia and form glutamatergic synapses with Ent projections. Accordingly, we next asked whether the assembly of primary cilia in newborn DGCs was required for dendritic refinement by measuring dendritic length and branching in *dnKif3a*+ and control newborn DGCs. Using confocal microscopy methods^{8,25}, we reconstructed the dendritic arborization of *dnKif3a*+ DGCs at 28 dpi (Fig. 4a and Methods). We then measured the total dendritic length of control and *dnKif3a*+ newborn DGCs. At 28 dpi, *dnKif3a*+ DGCs had significantly shorter total dendritic arbor length ($671\pm42\mu$ m) compared to control newborn DGCs ($807\pm51\mu$ m; Fig. 4b). In contrast, analysis of dendritic branching revealed that *dnKif3a*+ DGCs have 8.6 ± 1.4 branches in average, similar to control newborn DGCs (7.4 ± 1.5 , Fig. 4b). We further analyzed dendritic length and

branching of *dnKif3a*⁺ and control newborn DGCs at 14 and 21 dpi (Fig. 4c, d). At 14 dpi there was no difference in either total dendritic length or branch number between control and *dnKif3a*⁺ cells, but by 21dpi, defects similar to those observed at 28 dpi. The lack of an effect of *dnKif3a* expression at time prior to the formation of cilia but a robust effect after the period of cilia formation suggests that the failure to elaborate dendrites is due to the lack of a primary cilium and not due to a non-ciliary function of *Kif3a*.

To further rule out possible nonciliary function of *dnKif3a*, we expressed *dnKif3a* from 5 dpi and analyzed the dendrites of newborn DGCs at 7 dpi, during which time there were no primary cilia present (Fig. 1c). There were no significant defects in dendritic length or branching (Supplementary Fig. 5). These results indicate that defects in dendritic refinement in *dnKif3a*⁺ DGCs occurred indeed after the initiation of cilia assembly. To further confirm that the dendritic defects in *dnKif3a*⁺ DGCs are due to a failure of cilia assembly, we disrupted primary cilia assembly by an alternate approach, shRNA-mediated knockdown of *Ift88* (Supplementary Fig. 6a, b). We analyzed the dendrites of *shIft88#4*⁺ DGCs at 28dpi. Similar to the findings in *dnKif3a*⁺ DGCs (Fig. 4b, c), we found that *shIft88#4*⁺ DGCs had significantly shorter dendrites but similar dendritic branch number compared to control shRNA⁺ DGCs (Supplementary Fig. 6c, d). *Ift88* has been implicated in spindle formation and centrosome function^{34–35}. To exclude the possibility that these non-ciliary functions might alter dendritic refinement, we examined centrosome localization in *shRNA88#4*⁺ DGCs at 7, 14 and 21 dpi. Knock-down cells had normal centrosome localization (Supplementary Fig. 7a, b), suggesting no major effects of knock-down on either spindle or centrosome formation. Together, these results show that either the expression of *dnKif3a* or knock-down of *Ift88* disrupt cilia assembly and lead to defects in dendritic refinement. Furthermore, it is likely that in the absence of cilia, the resulting shorter dendrites reduces the number of active glutamatergic synapses from Ent projections; however, future studies will be required to confirm this hypothesis.

Enhanced Wnt/ β -catenin signaling following cilia loss

One function of primary cilia is the regulation of Wnt/ β -catenin signaling. In adult hippocampal neural progenitors, the Wnt/ β -catenin signaling pathway is active and regulates neuronal proliferation and differentiation³⁶. Whether Wnt/ β -catenin signaling is active in newborn DGCs, and whether it plays a role in dendritic refinement during cilia assembly, remains unknown. Thus, we first assessed Wnt/ β -catenin signaling activity in newborn DGCs using β -catenin reporter (BAT-gal) mice^{36–37}. At 14 dpi, 54 \pm 5.6% of newborn DGCs showed detectable β -gal signals, increasing to 64 \pm 6.3% by 21 dpi (Fig. 5a, b). We next asked whether primary cilia deletion alters Wnt/ β -catenin signaling activity in newborn DGCs as previously suggested in other cell types^{11,38}. At 21 dpi, β -gal signal was detectable in 85 \pm 6.8% of *dnKif3a*⁺ compared with 64 \pm 6.3% of control newborn DGCs. The mean β -gal intensity in *dnKif3a*⁺ DGCs was nearly 30% stronger than that of control DGCs (Fig. 5c), suggesting that not only is signaling active in more cells but that signal strength is enhanced. At 14 dpi, when most DGCs lack cilia, there was no difference in either the number of β -gal-positive cells (56 \pm 6.2% of *dnKif3a* versus 54 \pm 5.6% of control) or the intensity of the β -gal signal (Fig. 5b, c). This result shows that the expression of *dnKif3a* in newborn DGCs modulates Wnt/ β -catenin signaling only after primary cilia assembly.

Enhanced β -catenin signaling activity was likewise observed in *shIfi88#4*+ DGCs displaying disrupted cilia assembly and shorter cilia (Fig. 5b and Supplementary Fig. 6). We also found a significant increase of β -catenin signaling in *shIfi88#4*+ DGCs at 21 dpi but not 14 dpi. Together these results indicate that disrupted cilia assembly leads to enhanced β -catenin signaling in newborn DGCs.

We next asked whether the increased β -catenin signaling resulting from cilia loss is necessary for the impaired dendritic refinement. To test this, we expressed *dnKif3a* in β -catenin-deleted newborn DGCs and assessed their dendritic refinement. To eliminate Wnt/ β -catenin signaling, we co-injected inducible EGFP-tagged Cre recombinase with control or *dnKif3a* retroviruses into the hippocampus of conditional β -catenin^{-/-} (β -catenin^{fl/fl}) mice³⁹. Gene expression was induced beginning at 7 dpi and dendritic length and branching were measured at 21 dpi. The induction of *dnKif3a* in newborn DGCs with otherwise normal Wnt/ β -catenin signaling caused a significant decrease in mean total dendritic length (from 702±45µm in control newborn to 559±61µm in *dnKif3a*+DGCs) with no change in mean dendritic branching number (Fig. 5d, e, comparable to Fig. 4). In contrast, the specific induction of *dnKif3a* in newborn DGCs also deficient for Wnt/ β -catenin signaling resulted in a significant increase in mean total dendritic length (652±72µm compared to 559±61µm in cilia-deficient control animals). The mean number of dendritic branches in β -catenin^{fl/fl} and *Cre+dnKif3a*+ DGCs was not significantly different from that of β -catenin^{fl/fl} and *dnKif3a*+ DGCs (Fig. 5e). This data shows that knockout of β -catenin in newborn DGCs reversed the defective dendritic refinement caused by *dnKif3a* expression, suggesting that the increase in β -catenin signaling may contribute to defective dendritic refinement.

To determine whether enhanced β -catenin activity is sufficient to perturb dendritic refinement, we expressed constitutively active *S33Y β -catenin*⁴⁰ in newborn DGCs using an inducible retrovirus. To avoid any potentially confounding effects on the initial development of the newborn DGCs⁴¹, we induced *S33Y β -catenin* at 14dpi and analyzed dendrites at 21 dpi. *S33Y β -catenin*+ DGCs had significantly shorter dendrites (Fig. 5d, e), similar in length to *dnKif3a*+ DGCs (Fig. 4). There was no difference in dendritic branch number. This result demonstrates that an increase in β -catenin signaling can suppress dendritic refinement of newborn DGCs between 14 and 21 dpi.

In summary, these data show that the assembly of primary cilia, which occurs in newborn DGCs between 14 and 21 dpi, is required for proper dendritic refinement. Perturbing cilia assembly impairs dendritic refinement at least partially by enhancing Wnt/ β -catenin signaling.

Discussion

Here we have shown that primary cilia assemble in newborn DGCs during the time when these neurons are being actively innervated by the Ent projections, raising the possibility that cilia assembly could be essential to synaptic integration. Due to the embryonic lethality of cilia loss and technical limitations for labeling and controlling primary cilia, cilia function during neuronal development and the underlying mechanisms remain unknown. Recently, several transgenic mice with conditional deletion of primary cilia have revealed the essential

roles of primary cilia in neuronal proliferation and differentiation^{17–18,31}. Because of the limited availability of neuron-specific *Cre* transgenic lines and potential developmental compensation, however, these inducible lines have not been used to examine the role of primary cilia in neuronal development. Using an ‘*in vivo* single cell genetic approach’^{7–8}, we analyzed cilia formation in newborn DGCs and the developmental impact of cilia deletion. We found that primary cilia are absent from most young newborn neurons but form between 14 and 21 dpi, during which these neurons form synapses with Ent projections (Supplementary Fig. 9). Deletion of primary cilia during this period disrupted not only glutamatergic synapse formation but also dendritic refinement. Furthermore, we showed that these defects in dendritic refinement are likely due to elevated Wnt/ β -catenin signaling activity. These results demonstrate the requirement of primary cilia assembly in newborn DGCs for their successful synaptic integration.

Essential roles of primary cilia in neuronal development

In rodent embryos, disruption of primary cilia by altered expression of cilia-associated genes such as *Ift88*, *Kif3a*, *Smoothed* or *Stumpy* causes aberrant formation of numerous brain structures, presumably due to impaired expansion of different neural progenitor pools^{17–18,31,42}. These findings demonstrate that primary cilia regulate the early stages of brain morphogenesis, likely by regulating progenitor proliferation. In their knockout mice, there are a reduced pool of adult hippocampal neural stem cells^{17–18}, suggesting a role for primary cilia in maintaining neural progenitor cells.

Here we deleted primary cilia by expressing *dnKif3a* or shRNA against *Ift88* in newborn DGCs. Both *dnKif3a* expression and *Ift88* knock-down impaired dendritic refinement, while there were no apparent defects in non-ciliary functions such as neuronal polarization and endosomal trafficking. With these genetic manipulations, the development of DGCs was normal until 14 dpi, the period when cilia assembly commences. We consider it likely that the impaired dendritic refinement and synapse formation are the direct consequences of a failure to properly assemble and maintain a primary cilium.

The assembly of primary cilia in newborn DGCs occurs precisely between 14 and 21 dpi during which time newborn DGCs begin to form dendritic synapses with Ent projections. Primary cilia deletion severely disrupted this synaptic integration. Cells with disrupted cilia also displayed defective dendritic refinement, showing shorter dendrites. Although we found that the amplitude of sEPSCs was not affected by cilia loss, the frequency was reduced significantly. This suggests that individual synapses of cilia-deleted newborn neurons can form normally but are fewer in number. The decreased frequency is presumably due to a reduction in synapse number as would be expected from shorter dendrites, although the possibility of other dendritic defects remains to be examined. In the sholl analysis of dendrites in 14, 21 and 28 dpi (Fig. 4d), we did not find a significant change of dendritic branching. Branch formation and elongation may therefore be controlled differentially at least at this developmental stage. We also briefly analyzed dendritic length of control and *dnKif3a*+ newborn DGCs at 28 dpi in the inner molecular layer and granular cell layer, showing no significant change (data not shown). Since glutamatergic synapses in the inner molecular layer develop prior to cilia formation and the extent of glutamatergic synaptic

innervations remains relatively stable till 28 dpi (Fig. 2d, e), primary cilia may not be critical for synapses formed by contralateral projections to the dentate gyrus. Rather, cilia formation appears to be essential for newborn DGCs to form synapses with Ent projections.

Signaling pathways through primary cilia

It has become clear that various signaling pathways in vertebrate embryonic development require the presence of primary cilia¹³. In our studies, primary cilia deletion significantly enhanced Wnt/ β -catenin signaling, consistent with findings in other systems^{11,19,38}. Our results raise two important questions: 1) Does disrupting cilia assembly lead to enhanced Wnt/ β -catenin signaling (and how?); and 2) Is increased Wnt/ β -catenin signaling responsible for the observed reduced dendritic length? The data (Fig. 5) shows a significant increase of Wnt/ β -catenin signaling in *dnKif3a* or *Ift88* shRNA expressing newborn DGCs at 21 dpi. Since there is no significant increase before cilia formation, *Kif3a* or *Ift88* likely modulates this pathway through its ciliary function rather than both ciliary and non-ciliary functions, as previously reported¹⁹. Furthermore, the lack of *Smoothened* expression in the granule cell and molecular layers in the dentate gyrus makes it unlikely that hedgehog signaling is involved in dendritic refinement during this period^{43–44}. With regard to the second question, the data (Fig. 5) show that genetic removal of β -catenin in newborn DGCs rescues the dendritic defects caused by *dnKif3a* expression and that the expression of a constitutively active form of β -catenin is sufficient to cause shorter dendritic length in newborn DGCs. In summary, we surmise that the assembly of primary cilia between 14 and 21 dpi in newborn DGCs is essential for dendritic refinement at least in part through regulating Wnt/ β -catenin signaling. Indeed dendritic development is regulated in other types of neurons by several different Wnt/ β -catenin signaling pathways^{45–47}. Thus in addition to its function in other brain circuits, Wnt/ β -catenin signaling may play complex roles in the formation and maintenance of entorhinal cortex-dentate gyrus circuitry.

Primary cilia may sense local environmental signals to regulate neuronal development. For example, sonic hedgehog signaling requires the presence of primary cilia and is essential for the establishment, proliferation and differentiation of adult neural progenitor cells¹⁶. Our data suggests that the Wnt/ β -catenin signaling pathway is altered by the assembly of primary cilia and in turn impacts the stepwise synaptic integration of newborn DGCs (Supplementary Fig. 9). Thus, different signaling pathways are mediated by primary cilia at distinct developmental stages. Furthermore, we found that there is a sustained period of neuronal migration during which newborn neurons lack primary cilia and show no response to experimental manipulations of cilia-associated proteins. Such stage-specific differences in the pathways requiring functional primary cilia may explain why other studies found that primary cilia play no predominant roles in transducing canonical Wnt signaling in developing organisms^{48–49}. Thus, further study of the precise time course of cilia development and associated signaling pathways is required.

Defects in primary cilia are associated with several human diseases, including retinal blindness, brain dysgenesis and neurocognitive impairments¹⁴. In ciliopathies such as Joubert syndrome and related disorders, there are numerous structural brain lesions. Patients with Meckel-Gruber or Bardet-Biedl syndrome also display structural defects in the central

nervous system and defective neocortical development is associated with cilia disorders^{12,14,50}. Thus, cilia function is likely required for many aspects of brain development, although the detailed signaling pathways and mechanisms remain to be determined. Here we identified a new role for the primary cilium in the sequential synaptic integration of adult-born neurons *in vivo*. This study also sheds light on the mechanisms of neural development in the adult organism, and possibly on the etiology of cilia associated-brain disorders.

Methods

Labeling and manipulation of newborn DGCs

Engineered self-inactivating murine oncoretroviruses were used to deliver genes of interest and to express shRNAs against targeted gene transcripts specifically in proliferating cells and their progeny⁷⁻⁸. The retroviral packaging vectors and cell line were kindly provided by Fred Gage's laboratory (The Salk Institute). The retroviral vectors were originally obtained from Dr. Hongjun Song's laboratory (The Johns Hopkins University). *dTomato*, *Centrin-2*, *dnKif3a*, *Cre* and short hairpin sequences were inserted into different retroviral vectors as described in the text. Four shRNAs against different regions of *Ift88* and a control shRNA not against any known genes were produced (Supplementary Fig. 6). To validate the specificity and efficiency of shRNAs, retroviral shRNA vectors and expression constructs for EGFP-tagged full-length mouse *Ift88* were co-transfected into 293 cells. Cell lysates were then prepared for Western blot analysis using anti-GFP antibodies.

High titers of engineered retroviruses were stereotactically injected into ~5 weeks old adult female C57BL/6 mice (Charles River), β -gal reporter mice³⁷ and β -catenin^{fl/fl} mice (The Jackson laboratory) housed under standard conditions⁸. Doxycycline, where used, was administered daily through drinking water with 5% sucrose. All animal procedures were in accordance with institutional guidelines.

Genetical delivery of Channelrhodopsin-2 and dnKif3a

Adeno-associated viral vector expressing Channelrhodopsin-2 (AAV-*ChR2*) was kindly provided by Karl Deisseroth's laboratory. High-titer AAV-*ChR2* or *dnKif3a* was produced and purified by the University of North Carolina Vector Core Facility. The AAV injection procedure was the same as used for retroviral injections. For the AAV-*ChR2*, the contralateral hilus received two injections (0.1 μ l each, 1×10^{12} viral particles/ml) along the anterior-posterior axis to infect nearly the entire hilus of the hippocampus. Lateral or medial entorhinal cortex received only one injection per hemisphere. As described in the text, 14 days after AAV-*ChR2* injection, mice were used for electrophysiological tests. The AAV-*dnKif3a* was injected into the dentate gyrus to infect DGCs with the same procedure as that for AAV-*ChR2* injection.

Immunostaining, confocal imaging and image analysis

Coronal brain sections (40 μ m thick) were prepared from different retrovirus-injected mice and processed for immunostaining, as previously described⁸, using ACIII primary antibody (goat, 1:250, Santa Cruz). Images were acquired on an Olympus FV1000 confocal system

using a multi-track configuration. For analysis of primary cilia and cell morphology, Z-series stacks of 640×640 pixel images were taken with a 60X/1.4 NA oil objective using a Z-resolution of 0.5 μm . For dendritic analysis, Z-series stacks of 640×640 pixel images were taken using a 40X/1.0 NA oil objective using a Z-resolution of 1 μm . The resulting three-dimensional images were analyzed using the Imaris V6.4.

For analysis of neuronal positioning, single section confocal images of *dTomato*+ neurons counterstained with 4',6-diaminodino-2-phenylindole (DAPI, 1:5000) in the mounting media were used to localize the cell, as previously reported²⁵. We defined the inner or outer granule cell layer by drawing a tangential line across three DAPI-stained cells approximately 50 μm apart in the inner or outermost cell layer. We then measured the position of these neurons as defined above (Fig. 1a). For analysis of primary cilia development and length, we used reconstructed 3D primary cilia images of newborn DGCs. All the co-localization of primary cilia and centrosome were confirmed by rotating these images. Using the above measurement definition (supplementary Fig. 2a), we measured cilia position and angle with single Z-plane 2D images containing two centrioles. A minimum of 25 neurons from randomly chosen sections of at least 3 animals were analyzed for each experimental condition. For analysis of dendritic refinement, 3D reconstructions of entire dendritic arbors were made from Z-series stacks of confocal images using Imaris. Those *dTomato*+ dentate granule cells with largely intact dendritic trees were analyzed for dendritic length and branch number (Fig. 4b, c), the same as previously reported methods^{8,25}. Data shown are from at least 17 individual *dTomato*+ neurons from 3 or more animals for each condition.

The β -gal antigen was stained with an antibody from MP Biomedicals, LLC (Rabbit, 1:500). LAMP-1 was stained with an antibody from Saint Cruz (Rabbit, 1:250). To minimize variation between samples, CTL and experimental brain sections were immunostained side by side and imaged on the same day using identical imaging parameters. Ten scans across the cell were collected, and the image with the largest nuclear area was analyzed. When any procedure failed, the selected cell was discarded. The intensity of cellular β -gal or LAMP-1 was measured with Image J software (NIH).

Significance in all statistical tests was determined using either ANOVA or the Kolmogorov-Smirnov test.

Electrophysiology

Mice were housed under standard conditions and processed at 7, 14, 21 and 28 dpi for electrophysiological recordings at 32–34°C, as previously described⁸. To examine contralateral mossy cell and lateral or medial entorhinal cortical glutamatergic inputs, cells were held at –65 mV and exposed to short pulses of blue light from a 473 nm laser launched into a Zeiss upright microscope through the epifluorescence light path. The 50mW laser was controlled by a standard TTL board; ending power on brain slices was ~5 mW. Electrical stimulation experiments of the entorhinal perforant path used standard bipolar electrodes to determine evoked glutamatergic synaptic transmission in cells held at –65 mV (Fig. 3b). The stimulus intensity was maintained for all tests. Any failure to evoke a response in recorded neurons was further confirmed by increasing the stimulus intensity⁸(Supplementary Fig. 8).

Spontaneous synaptic activity was examined in the presence of 1 μ M TTX during five-minute continuous sweeps recorded under voltage-clamp at -65 mV in the presence of 5 μ M bicuculline. All chemicals used were purchased from Sigma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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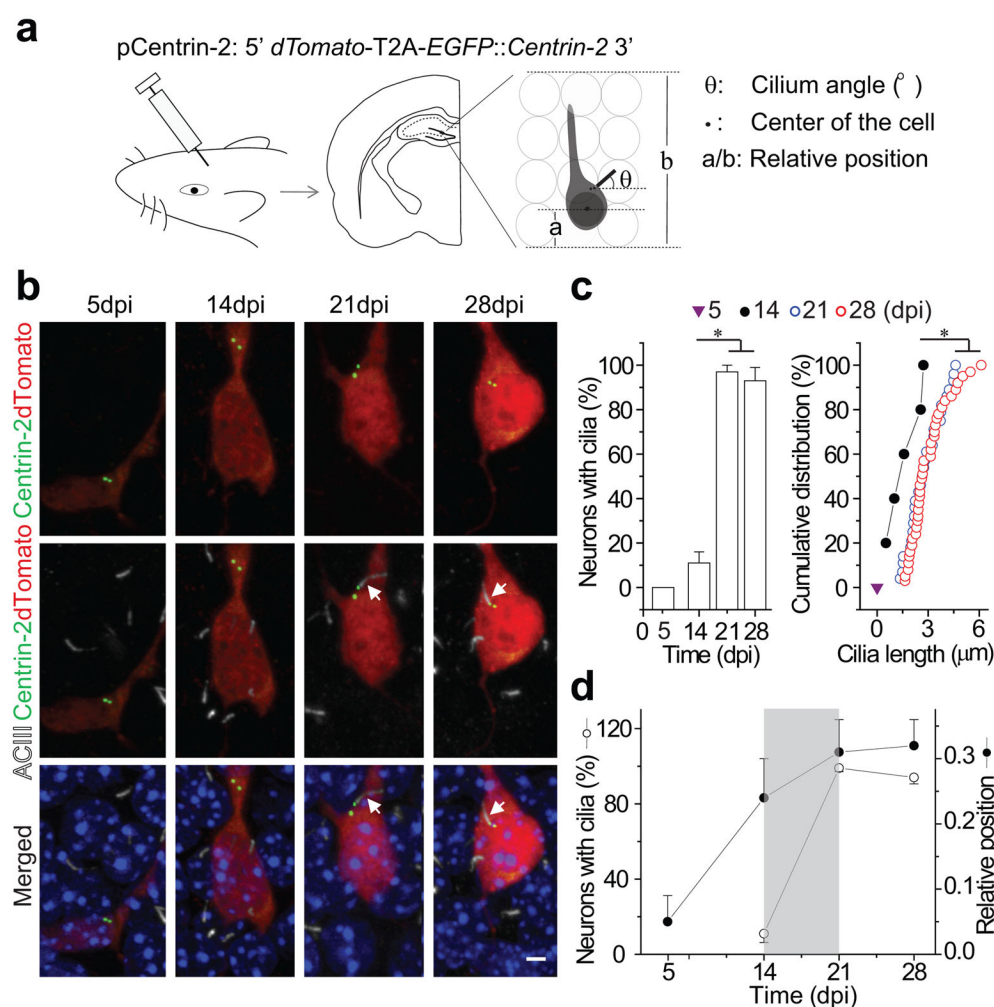


Figure 1. Primary cilia assemble in developing adult-born neurons

(a) A schematic diagram of the retroviral vector (pCentrin-2), retroviral injection, a sample brain section and the parameters measured from reconstructed images. (b) Confocal images of cilia formation in developing adult-born neurons showing *EGFP* (*Centrin-2*), *dTomato*, DAPI and immunostaining for ACIII. Arrows point to primary cilia associated with centrosomes. Scale bar: 3 μ m. (c) Quantification of percentage of labeled adult-born neurons with primary cilia (left) and the distribution of ciliary length (right) at 5, 14, 21 and 28 dpi. (d) Percentage of retrovirally labeled neurons with primary cilia and their relative position within the dentate gyrus granule layer at 5, 14, 21 and 28 dpi. Values represent mean \pm SEM (n=32–48 neurons; *: $p < 0.01$, ANOVA or Kolmogorov-Smirnov test). The analyses in c and d are from the same group of cells.

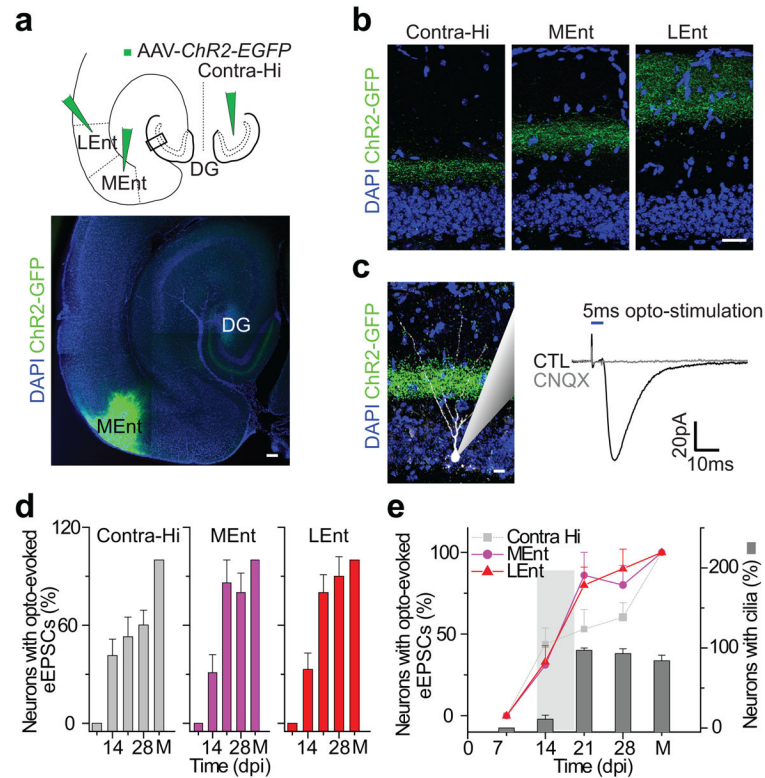


Figure 2. Entorhinal cortical innervations of adult-born neurons accompany primary cilia assembly

(a) A schematic diagram showing the AAV-*ChR2-EGFP* injection sites in the contralateral hilus (Contra-Hi), lateral (LEnt) and medial (MEnt) entorhinal cortex. The sample image (*EGFP*) shows the MEnt injection site and axonal projections in the molecular layer of the dentate gyrus. Scale bar: 150 μ m. (b) Laminar glutamatergic innervation pattern in the molecular layer of the dentate gyrus. The images, taken from the location marked by a rectangle in a, show axon terminals (*EGFP*) in the molecular layer originating from the contralateral hilus, medial and lateral entorhinal cortex, respectively. Scale bar: 50 μ m. (c) Opto-evoked glutamatergic synaptic transmission in a randomly picked DGC using a 5ms pulse of 473 nm light stimulation. Shown to the left are MEnt axonal terminals (*EGFP*) and a recorded DGC (white). A recording trace is shown to the right, which could be completely blocked by 50 μ M CNQX. Scale bar: 20 μ m. (d) Functional glutamatergic synapse formation in the developing adult-born neurons. Shown are the percentages of recorded mature and adult-born DGCs at 7, 14, 21 or 28 dpi with detectable evoked eEPSCs after opto-stimulation of a single layer. (e) Correlations of functional entorhinal cortical innervations with primary cilia assembly. Shown is a summary of the contralateral, lateral and medial entorhinal cortical innervations and cilia assembly of adult-born DGCs at 7, 14, 21 and 28 dpi. Values represent mean \pm SEM ($n=10-12$ neurons). M, mature DGCs.

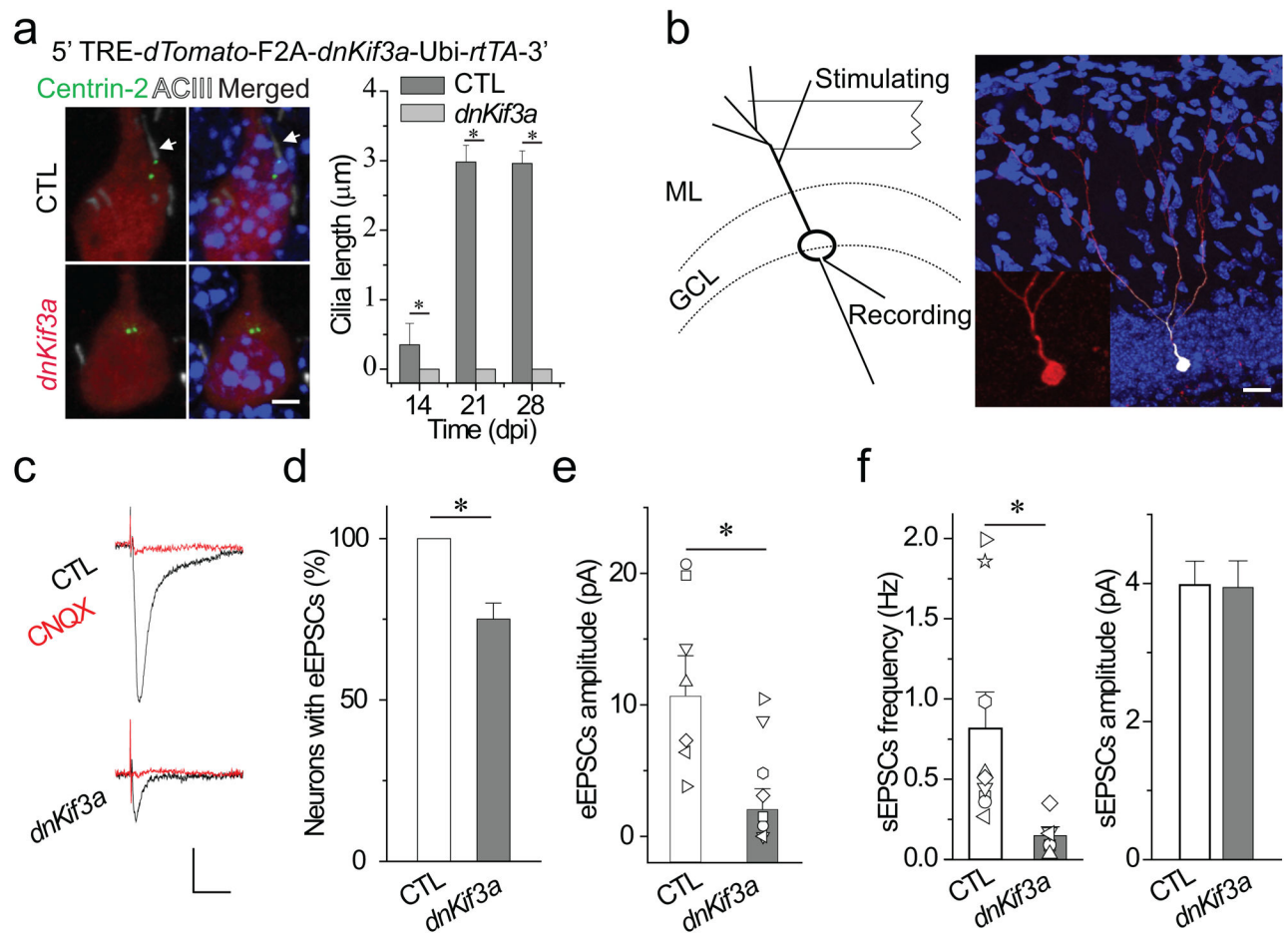


Figure 3. Primary cilia deletion caused by expressing *dnKif3a* severely disrupts functional glutamatergic synapse formation in adult-born neurons

(a) Primary cilia deletion in adult-born DGCs by *dnKif3a* ectopic expression. At top, a diagram of the inducible retroviral vector (*dnKif3a*), below, typical adult-born DGCs labeled with markers of cilia (White, ACIII staining), centrioles (Green, *Centrin-2-EGFP*) and adult-born DGCs (Red, *dTomato*). Arrows point to centrosome. Scale bar: 5 μm . At right, a comparison of cilia length in control and *dnKif3a*+ adult-born DGCs. (b) A schematic diagram of the electrophysiological testing and an image of a recorded newborn DGC filled with biocytin through the recording pipette. The stimulating electrode was placed in the outer molecular layer to excite principally the entorhinal cortical projections. The inset is an enlarged image of the recorded adult-born DGC with *dTomato* signal. GCL, granule cell layer. ML, molecular layer. Scale bar: 25 μm . (c–f) Glutamatergic synaptic transmission recorded from control and *dnKif3a*+ adult-born DGCs at 21 dpi. c) Sample traces of glutamatergic synaptic transmission in the presence of 5 μM bicuculline. Cells were held at $V_m = -65$ mV. Scale bars: 10 pA and 15 ms. d) The percent of recorded newborn DGCs with detectable synaptic transmission. e) eEPSCs amplitude. Shown are individual and averaged eEPSCs amplitudes from control and *dnKif3a*+ adult-born DGCs. f) Spontaneous glutamatergic synaptic transmission, frequency and amplitude. In a, d–f, all values represent mean \pm SEM ($n = 7$ –12 neurons; *: $p < 0.01$, ANOVA).

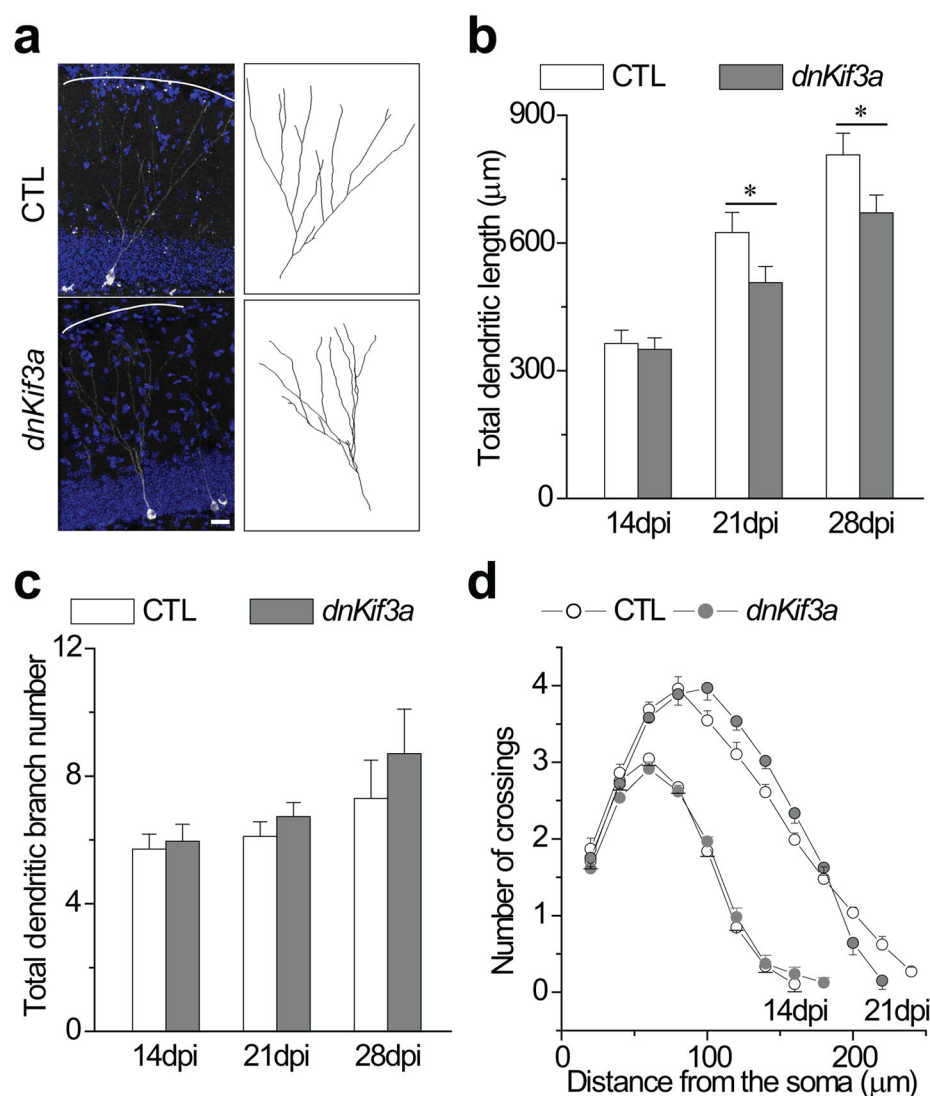


Figure 4. Primary cilia deletion in adult-born neurons results in defective dendritic refinement (a) Defective dendritic refinement of *dnKif3a*+ DGCs. Shown on the left are the images of control and *dnKif3a*+ DGCs at 28 dpi. Solid lines mark the edges of the outer molecular layer. Shown to the right are drawings from the images. Scale bar: 20 μm . (b) A summary of total dendritic length for control and *dnKif3a*+ adult-born DGCs at 14, 21, and 28 dpi. (c) Dendritic branch number for control and *dnKif3a*+ adult-born DGCs at 14, 21, and 28 dpi. (d) Sholl analysis of the dendritic tree of control and *dnKif3a* adult-born neurons at 14 and 21dpi. (For b, c and d, $n=25-38$ neurons; for b and c, *: $p<0.01$, ANOVA; for d, statistical significance was determined by Student's t-test). All values represent mean \pm SEM.

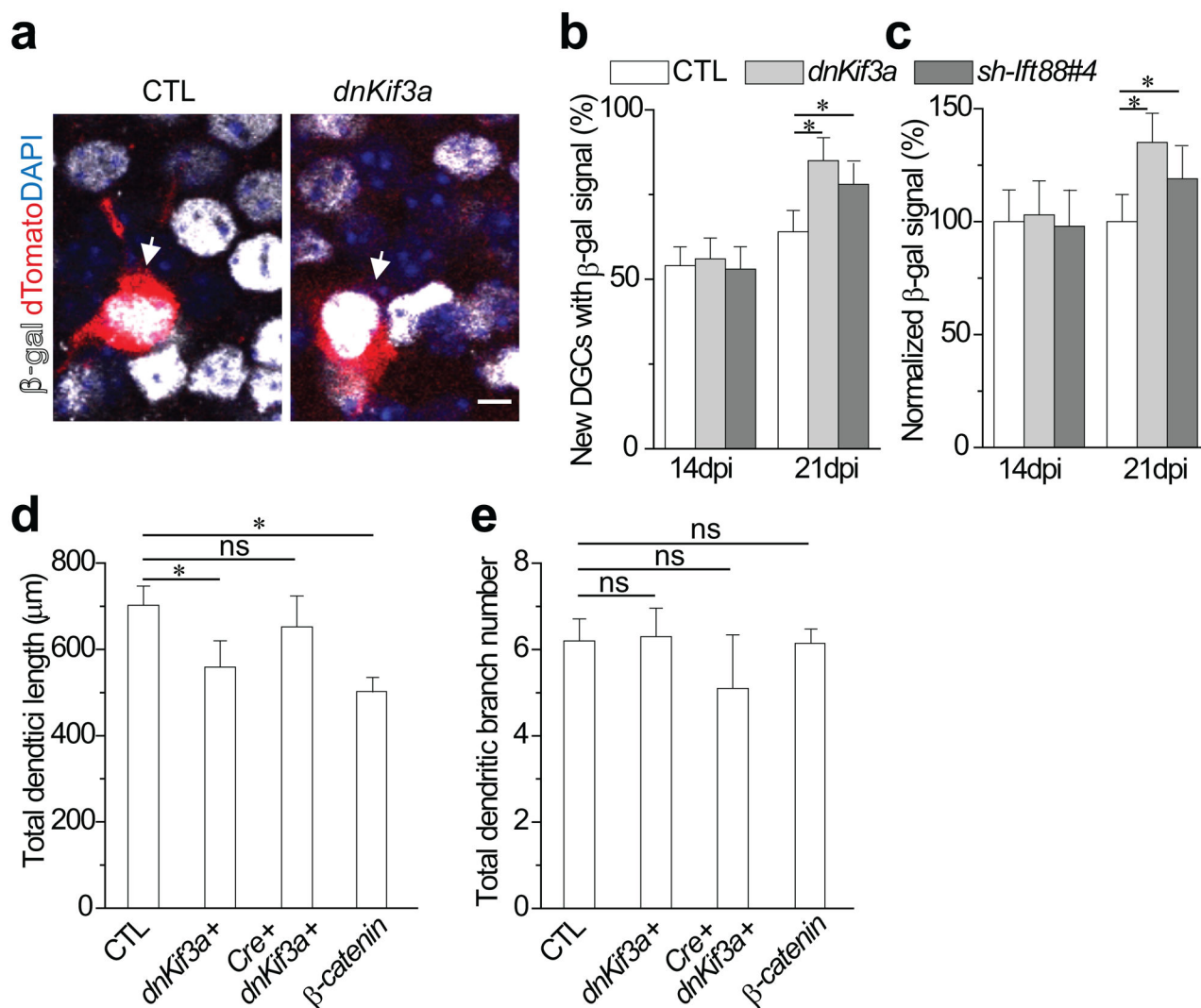


Figure 5. Altered Wnt/β-catenin signaling activity upon primary cilia deletion regulates the dendritic refinement of adult-born neurons

(a) Wnt/β-catenin signaling in developing adult-born DGCS. Shown are sample single plane images of β-gal signal in control and *dnKif3a*⁺ DGCS at 21 dpi using BAT-gal reporter mice. Arrows point to newborn DGCS. Scale bar: 10μm. (b, c) Enhanced β-gal signal upon primary cilia deletion at 21 dpi. Shown in (b) is a summary of the percent of the control or *dnKif3a*⁺ DGCS having β-gal signal. Shown in (c) is a summary of normalized intensity of the β-gal signal for control or *dnKif3a*⁺ adult-born DGCS at 21 dpi. All values represent mean±SEM (n=22–29; *, *p*<0.01, ANOVA). (d) Cre-mediated excision of β-catenin restores normal dendritic length in *dnKif3a*⁺ adult-born neurons at 21 dpi. β-catenin^{fl/fl} mice were injected with *dnKif3a* and inducible Cre retroviruses as described in the Methods and dendritic lengths measured at 21 dpi. S33Y β-catenin was induced at 14 dpi and dendritic lengths determined at 21 dpi. (e) Dendritic branch number for the same cohort of neurons shown in d. All values represent mean±SEM (n=35–40; *, *p*<0.01, ANOVA; ns represents no significance).