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Basic Biology and Mechanisms of Neural Ciliogenesis and the B9 Family

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Abstract Although the discovery of cilia is one of the earliest in cell biology, the past two decades have witnessed an explosion of new insight into these enigmatic organelles. While long believed to be vestigial, cilia have recently moved into the spotlight as key players in multiple cellular processes, including brain development and homeostasis. This review focuses on the rapidly expanding basic biology of neural cilia, with special emphasis on the newly emerging B9 family of proteins. In particular, recent findings have identified a critical role for the B9 complex in a network of protein interactions that take place at the ciliary transition zone (TZ). We describe the essential role of these protein complexes in signaling cascades that require primary (non-motile) cilia, including the sonic hedgehog pathway. Loss or dysfunction of ciliary trafficking and TZ function are linked to a number of neurologic diseases, which we propose to classify as neural ciliopathies. When taken together, the studies reviewed herein point to critical roles played by neural cilia, both in normal physiology and in disease.

Keywords Primary cilia · Neural ciliogenesis · Neural ciliopathy · B9-C2 family · Ciliary signaling · Stem cell · Progenitor

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A Brief History of Cilia: from van Leeuwenhoek to Today

The discovery of cilia is one of the earliest in cell biology, and ciliary motility is the first cellular function ever to be described. In a letter sent to the Royal Society of London, Antony van Leeuwenhoek wrote of his discovery of protozoa and their cilia in 1676 [1, 2]. He described "diverse incredibly thin little feet, or little legs, which were moved very nimbly… and wherewith, they brought off incredibly quick motions" [1–3]. Although nearly forgotten over time, later studies reported on the fibrillar structure of cilia in a variety of cells [4–6]. However, these observations were not validated until the advent of the electron microscope, which provided ultrastructural details of these enigmatic organelles and produced the first evidence of particular cell types containing cilia [6, 7]. Not surprisingly, this early visualization of ciliary structure led cell biologists to ponder the functional relevance of cilia in different cell types.

Over the past five decades, there has been a marked acceleration of insight into the biology of cilia in one such cell type—neurons. Seminal work in the field of neural cilia came from Hans Dahl. He and his colleagues utilized silver impregnation histochemistry to demonstrate that nearly all neurons in the cerebral cortex possessed a single cilium [8]. This key observation was subsequently validated in an

ultrastructural study [9], and we now know these structures as "neural cilia." While cilia are not unique to neurons, it is becoming appreciated that neural cilia govern a growing number of processes in the nervous system [10]. Moreover, it is increasingly clear that genetic disruptions of neural cilia cause complex human diseases, and pathways including sonic hedgehog (Shh) require neural primary (nonmotile) cilia for signaling [10]. Here, we highlight some of the critical roles that primary cilia play in neural development and homeostasis, with particular emphasis on the emerging B9 protein family. We also cover how ciliary dysfunction in the brain can lead to diseases that we collectively refer to as "neural ciliopathies."

Basic Ciliary Biology and Function

The vertebrate primary cilium is an antenna-like, microtubule-based organelle of about 1–10 μm in length. The cilium protrudes from the cell surface and is anchored by the basal body, a structure derived from the mother centriole. The axoneme, comprised of nine doublets of microtubules, emanates from the basal body and is surrounded by an external membrane that is continuous with the plasma membrane of the cell. Proteins permitted to enter the cilium migrate along the axoneme by intraflagellar transport (IFT), first described in pioneering studies from the laboratory of Joel Rosenbaum [11, 12]. IFT proteins form two complexes which carry cargo either retrogradely (complex A) or anterogradely (complex B) along the cilium. Complex A is driven by dynein motors which transport products back to the basal body of the cilium, the general area where IFT proteins are recycled. On the other hand, complex B particles utilize kinesin-2 (also known as the Kif3 motor complex) to transport cargo from the base to the tip of the cilium. IFTs are essential for the formation of cilia, and mutations in IFT genes lead to defects in the assembly of ciliated sensory neurons in *Caenorhabditis elegans* [13–17].

The discovery of the *C. elegans* complex B subunit, IFT88, as the homologue of the murine *Tg737* gene product was a breakthrough that linked a structural abnormality of primary cilia to polycystic kidney disease (PKD) [18]. Mice harboring an insertional mutation in *Tg737* are IFT88 hypomorphs (known as the orpk mouse model of PKD) that also have defective left-right patterning and manifest retinal degeneration [18]. These disease phenotypes are associated with stunted growth of primary cilia in the kidney, at the embryonic node, and in the retina [18]. The initial experimental links between the primary cilium and PKD led to a massive effort to identify the genetics and cell biology of ciliopathies and also cilia-dependent aspects of neural development and function [10].

Another key function of IFTs is to facilitate trafficking of proteins involved in Shh signaling [19, 20]. In fact, myriad studies have investigated the association between primary cilia dysfunction and hedgehog signaling both in human ciliopathies and in mouse models of these diseases. For example, results from a mutation screen revealed that multiple components of the mouse IFT machinery are essential for specification of Shh-dependent ventral cell fate in the neural tube [21–23]. The genes disrupted in these mutants encode several IFT complex B proteins, including IFT88, IFT172, and cytoplasmic dynein 2 heavy chain 1, which is a subunit of the IFT retrograde motor. Additionally, disruption of the kinesin-2 motor (e.g., as in kinesin-like protein *Kif3a*-null embryos) results in defects in Shh-dependent neural patterning [21–23].

That IFTs and Shh signaling interact has raised an important question: How do IFT defects mechanistically lead to aberrant Shh pathway activity? The answer may come from recent studies, which indicate that neural primary cilia act as "cellular antennae" for Shh signaling. Activation of the Shh pathway is triggered by Shh binding to the patched-1 receptor (Ptch1), which, in the absence of Shh, functions to repress the activity of the seven-span transmembrane protein smoothened (Smo). However, when Shh is present, it binds to Ptch1, displacing this receptor from the cilium. This permits entry of Smo and, thereby, releases Shh pathway inhibition. The current hypothesis from genetic analyses is that IFT proteins act downstream of Ptch1 and Smo and upstream of the glioma-associated oncogene family homolog (Gli) transcriptional machinery [22]. Given that the activator or repressor forms of Gli proteins are dependent on IFT, it is becoming accepted that disruption of Shh-dependent neural patterning is owed to dysregulation of Gli protein processing in the primary cilium and associated compartments [24]. These studies demonstrate the impact of mutations in members of ciliary protein complexes and underscore the essential role of neural primary cilia in Shh signaling.

Despite an increasing awareness of the strong link between Shh signaling and the primary cilium, the precise cell biology of this relationship continues to be explored. For example, suppressor of fused (Sufu) is a molecule that has been considered to be antagonistic towards Gli activation, with the current thinking being that Smo inhibits the function of Sufu, allowing for the proper activation of Gli proteins.[25–31]. Support for these hypotheses comes from studies in *Ift88* mutant cells that lack cilia, where Sufu constitutively inhibits Gli activator function [32]. $Ift88^{-/-}/Sufu^{-/-}$ and $Smo^{-/-}/Sufu^{-/-}$ double-deficient mice exhibit Shh pathway activation in the form of spinal cord ventralization and increased *Ptch1* and/or *Gli* expression [32]. Thus, while the inhibitory function of Sufu is cilia-independent, Smo-mediated repression of Sufu requires cilia. Interestingly, despite the apparent cilia

independency of Sufu inhibitory function, the molecule localizes to the primary cilium in a Gli-dependent manner—accumulating at ciliary tips [33–35]. However, controversy surrounds whether this association is maintained in the presence of Shh, with one group reporting continued association [36] and others showing dissociation [34, 37]. As we will discuss below, this is just one issue related to the complex cell biology involved in trafficking and signaling among the golgi network, basal body-associated compartment, primary cilium, and endosome.

Emergence of the B9 Complex in Ciliary Biology

The primary cilium is partially segregated from the rest of the cell membrane via the transition zone, which creates a diffusion barrier that effectively segregates the cilium from the rest of the cell [38–40]. This region appears to limit the diffusion of membrane proteins in and out of the cilium, but permits some classes of cytoplasmic proteins to freely move between the cilium and the rest of the cell [40]. Several recent reports have described a complex containing both cytoplasmic and transmembrane proteins that localize to the ciliary TZ [41–44]. This complex contains all three of the so-called B9-C2-containing proteins encoded by the human genome, and other proteins that collectively form a complex at the base of the cilium [45]. B9-C2 proteins were initially thought to have functional redundancy with nephrocystins in *C. elegans* to regulate the formation and maintenance of ciliated sensory neurons [46]. However, more recent studies have established the B9 complex as nonredundant for maintaining the cilia membrane as a compartmentalized signaling organelle in *C. elegans* [43] and in human cells [44]. Accordingly, disruption of the B9-C2 complex has been shown to decrease the amount of plasma membrane proteins including somatostatin receptor 3 (Sstr3) and serotonin receptor 6 (Htr6) within cilia [44]. Moreover, it has been demonstrated that all three members of the B9-C2 complex physically interact and play critical roles in ciliogenesis and ciliary protein localization [47]. In this scenario, B9-C2 proteins play at least two essential roles: (1) establishing connections between the ciliary membrane and axoneme at the TZ, and (2) forming the "ciliary gate" that regulates ciliary membrane composition [43].

We characterized a mouse B9-C2 family gene that we termed "Stumpy" (also known as B9 domain-containing protein 2, *B9d2*) and demonstrated that this gene is required for mammalian ciliogenesis [41]. Evidence for this came from conditional deficient mice lacking Stumpy exon 4 in the brain. Specifically, we crossed Stumpy floxed mice with the nestin-cre deleter mouse line, which recombines Stumpy in radial glia and their daughter lineages, including neurons, astrocytes, and oligodendrocytes [41, 48]. Strikingly, these mutants had near-complete loss of primary and motile cilia in the brain, and remaining cilia had a dysmorphic "stump-like" ultrastructural appearance [41]. Stumpy mutants manifested developmental abnormalities including perinatal hydrocephalus, depletion of forebrain neural progenitors, and hypoplastic cerebella characterized by lamination defects of cerebellar granule neuron progenitors (CGNPs), Purkinje cells, and Bergmann glia [41, 48].

Given the essential role of primary cilia in the proliferation of CGNPs [49, 50], we hypothesized that loss of cilia might underlie defective neural progenitor proliferation in the hippocampus and cerebellum of conditional Stumpy mutants. We localized two key Shh pathway mediators, Smo and the downstream transcriptional activator Gli1, to primary cilia on neural progenitors [48], and found that loss of cilia in Stumpy knockouts was associated with dysregulation of multiple Shh pathway molecules at the transcriptional level, including *Shh*, *Smo*, and *Gli1-3*. These data support the notion that a functional, intact cilium is required to enable proper Shh pathway function [48]. When taken together, these results suggest that loss of cilia in Stumpy deficient mice leads to dysregulation of the Shh pathway [48].

As mentioned above, in addition to Stumpy/B9d2, nearly every ciliated organism possesses two additional B9 proteins: Mks1 and B9d1 [51, 52]. Moreover, loss of *Mks1* or *B9d1* gene function alters ciliary protein localization and compromises ciliogenesis and Shh signal transduction [47, 53]. Interestingly, recent work investigating the role of Tectonic1 (Tctn1), a regulator of mouse Shh signaling, demonstrated that Tctn1 forms a complex with multiple TZ proteins, including Mks1 and B9d1 [42]. This complex is thought to control ciliogenesis and ciliary protein composition. The roles of B9 family members at the neural primary cilium are depicted in Fig. 1.

Primary Cilia Regulate Neural Development

The mammalian brain begins as a single layer of neuroepithelial cells that proliferate and form neural precursors, which differentiate and migrate to form complex, multilaminate structures [54]. The processes governing brain development are tightly regulated by diffusible factors (growth factors and morphogens) and juxtacrine cues (cell–cell and cell–matrix interactions) from the microenvironment [55]. Recently, two complimentary studies have delineated the role of primary cilia in neurogenesis [48, 56].

Most granule neurons in the hippocampal dentate gyrus (DG) are generated in the early postnatal period [54]. Normally, granule neuron precursors translocate away from the primary germinal layer in the ventricular zone into the inner layer of the developing DG [57]. Upon reaching their

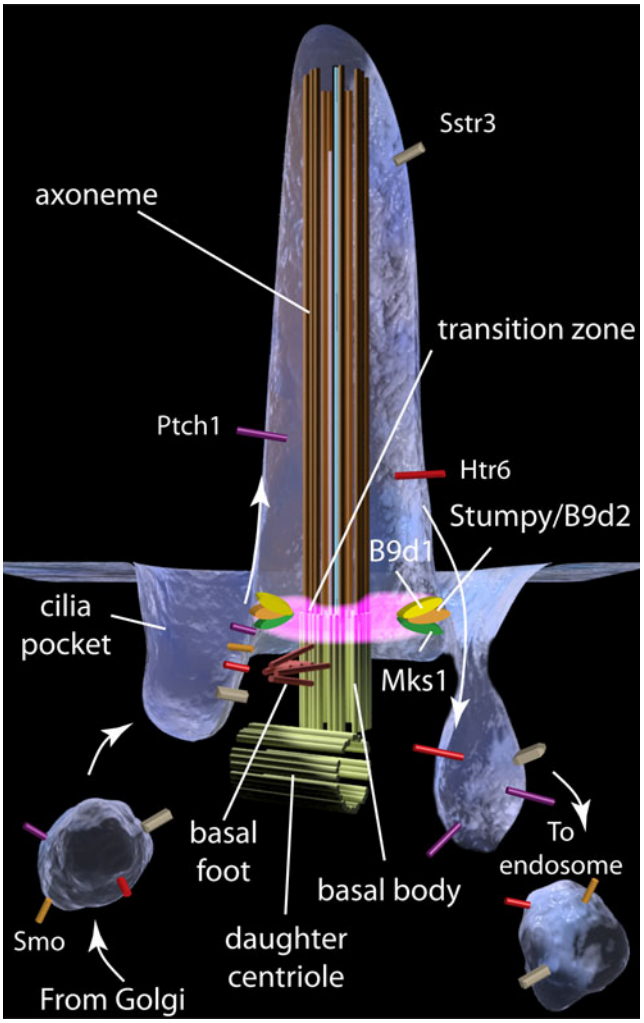

Fig. 1 B9-C2 proteins at the transition zone of the primary cilium. The axoneme of the primary cilium extends from the basal body of the centriole complex. It is from this junction of the basal body and axoneme that the transition zone extends into the plasma membrane. Three B9-C2 proteins: Stumpy (B9d2), B9d1, and Mks1, localize to this region and appear to participate in the formation and function of this region. The transition zone allows entry of membrane receptors into the axonemal membrane. These membrane receptors include the Shh receptor patched 1 (Ptch1), somatostatin receptor 3 (Sstr3), and serotonin receptor 6 (Htr6), which traffic from the golgi (or perhaps the recycling endosome) and dock at the ciliary pocket. The membrane protein smoothened (Smo) is prevented from entering the ciliary axoneme in the absence of sonic hedgehog

destination, these cells transform into postnatal neural stem cells that continue to produce new neurons throughout life [54], a process which may facilitate circuit plasticity, learning, and memory [58]. Others and we have shown that conditional mutant mice lacking neural cilia have decreased numbers of proliferating progenitor cells [48, 56], likely due to altered cell cycle exit kinetics [48]. Further, radial astrocytes, which function as primary progenitors in the postnatal brain, are likely exhausted in these animals [48, 56]. Moreover, an exciting new finding suggests that the primary cilium plays a role in glutamatergic synapse formation in newborn neurons in the DG. Specifically, deletion of neuronal primary cilia caused increased Wnt and β-catenin signaling that led to alterations in dendritic morphology and synaptogenesis [59]. These studies underscore the pleiotropic roles of primary cilia in neural development by demonstrating that these structures are required for the transition of embryonic to adult neural stem cells and integrating these neurons in the DG.

Neural Ciliopathies

In recent years, an ever-increasing number of human-inherited neurologic diseases have been shown to arise from or to be associated with defects in the structure or function of primary cilia [60–62]. The causative genes for these syndromes all seem to be involved in ciliary protein trafficking, TZ function, and/or ciliogenesis. One of the perplexing features of ciliopathies is the widely variable clinical phenotypes that are characteristic of these disorders. One possible explanation for this is that ciliary protein complexes have numerous functions, affecting protein trafficking, TZ function, and ciliogenesis to varying extents [47]. Recent examples of brain disorders that are now thought to be etiologically linked to loss or dysfunction of neural cilia are detailed to follow.

It is becoming increasingly appreciated that mutations in B9 family genes are linked to neural ciliopathies. For example, it is clear that mutations in human *Mks1* cause Meckel syndrome (MKS), a severe ciliopathy characterized by occipital encephalocele, liver ductal plate malformations, polydactyly, and kidney cysts [52]. MKS is a rare pleiotropic neonatal lethal autosomal recessive disorder, considered to be the most common syndromic neural tube defect [52]. Recently, genetic screening for mutations in *B9d1* and *B9d2* in human MKS fetuses identified a homozygous mutation in *Stumpy/B9d2* [47]. This mutation compromises Stumpy/B9d2 function, resulting in disruption of interaction with Mks1 protein [47]. Collectively, these reports indicate that mutations in B9 family member genes cause a syndrome that is clinically indistinguishable from MKS [47].

Further evidence suggests that genes associated with developmental disorders may be connected to ciliary function. For example, Bardet–Biedl syndrome (BBS) has a wide range of clinical manifestations, including mental retardation, retinal degeneration, polydactyly, kidney cysts, and obesity. BBS can be caused by mutations in any of 14 known genes of the *BBS* family that are linked with ciliary dysfunction, and so this disease is now regarded as a ciliopathy [63]. Additional neural ciliopathies include oral-facial-digital type 1 syndromes caused by mutations in the ciliary *Ofd1* gene [64] and microcephalic primordial

dwarfism, etiologically linked to mutations in *Pericentrin*, a gene which encodes a centrosomal protein [65, 66].

The Primary Cilium as a "Gatekeeper" for Cell Division

In addition to being uniquely present during development in neural progenitors, primary cilia regrow in postmitotic neurons [67, 68], in astrocytes throughout the brain parenchyma [48, 67], and in choroid plexus cells [41, 67]. That primary cilia are found on a significant proportion of eukaryotic cells, suggests, at least from an evolutionary perspective, that they have broader functional relevance. One such role seems to be in cell division. In this regard, it is interesting that the cilium is anchored to the cell membrane by the older (mother) centriole [69]. In order to move from G1- to S-phase, the centriole must duplicate, and so this event represents an initial mitotic checkpoint [70]. Another checkpoint is proposed to exist between G2- and M-phase, where the daughter centrioles must be liberated from the cilium, allowing centriole maturation to form the mitotic spindle in M-phase [70]. After the completion of mitosis, the centriole is captured, where it forms the basal body of the extended cilium during interphase (G1) (Fig. 2). Thus, the primary cilium is resorbed before mitosis, establishing a relationship between cell division and ciliogenesis.

Furthermore, it has recently been shown that segregation of the mother centriole may be a key to cell fate determinations made during asymmetric divisions of neural stem cells [71]. Specifically, neural stem cells retain the mother centriole, while differentiating daughter cells inherit the "new" mother centriole, which is derived from the daughter centriole in the originally dividing cell [71]. This "younger" mother centriole, for a time, lacks all of the properties inherent to a more mature mother centriole such as the ability to extend a cilium. Given that primary cilia play key roles in cell proliferation and differentiation, their prominent role in developmental disorders and cancer is becoming increasingly appreciated.

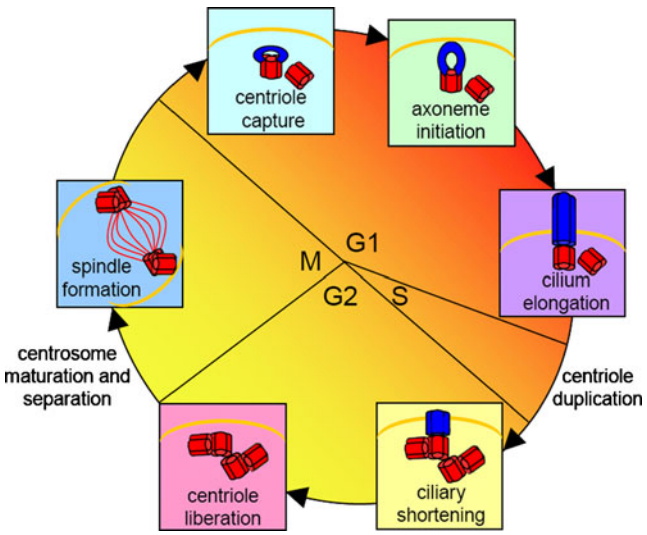

Fig. 2 The cilium–centriole–centrosome cycle. This cartoon illustrates the relationship between ciliogenesis and cell cycle checkpoints (adapted from [70])

Primary Cilia and Developmental Brain Cancer

In addition to developmental brain abnormalities, mounting evidence suggests that dysfunction of neural cilia may also be linked to childhood brain cancer. Medulloblastomas are primitive neuroectodermal tumors that originate in the cerebellum, a brain region where primary cilia are known to orchestrate proliferation of progenitor cells [50]. Interestingly, the presence or absence of cilia correlates with specific variants of medulloblastoma [72], the most common malignant brain tumor in children [73, 74]. Importantly, several reports have identified *Ptch1* mutations in patients with sporadic medulloblastoma, and it is now generally accepted that abnormal activation of Shh signaling leads to medulloblastoma formation [75]. However, it is becoming clear that there are at least four subgroups of medulloblastoma, and not all forms of this developmental brain cancer are etiologically linked to Shh pathway dysregulation. For example, medulloblastomas that exhibit activated Shh signaling contain neural primary cilia, but these structures are typically not detected in medulloblastomas belonging to the other distinct subgroups [72].

Primary cilia have also recently been demonstrated to play critical roles in Shh signaling-driven medulloblastoma in genetically engineered mice [72]. Genetic ablation of primary cilia blocks mouse medulloblastoma driven by constitutively active Smo protein (SmoM2), which promotes runaway Shh signaling. Conversely, a constitutively active form of Gli2 induces medulloblastoma only when primary cilia are genetically ablated [72]. Thus, depending on the initiating oncogenic event, it seems as though primary cilia can either facilitate or inhibit medulloblastoma formation [72]. These reports indicate that the primary cilium may play opposing roles in tumorigenesis and demonstrate that defects in primary cilia have oncogenic potential.

Concluding Remarks

In this review, we have focused on basic biology and mechanisms of neural ciliogenesis and the B9 family of cilia-related proteins. This field has enjoyed rapid expansion over the past 5 years, owed in no small part to the myriad of neurologic diseases that have just recently been ascribed to

loss/dysfunction of cilia. These "neural ciliopathies" have led us to delve more deeply into the fascinating biology of these tiny, underappreciated organelles. One of the key protein families that orchestrate the complex process of ciliogenesis is the B9 family of proteins, including B9d1, Stumpy/B9d2, and Mks1. The current awareness is that these proteins exist as a macrocomplex that is essential to form the ciliary TZ to endorse axonemal extension that is a defining event for ciliogenesis. As the field continues to move forward at a rapid pace, it will be interesting to understand more about the complex relationship amongst the B9 family, IFTs, and neural ciliogenesis. One thing is certain—that additional surprises remain regarding the important roles of neural cilia in health and in disease.

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