EFFECTS OF BRAIN INJURY ON PRIMARY CILIA OF GLIAL CELLS AND PERICYTES

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Glial cells maintain homeostasis that is essential to neuronal function. Injury to the nervous system leads to the activation and proliferation of glial cells and pericytes, which helps to wall off the damaged region and restore homeostatic conditions. Sonic hedgehog is a mitogen which is implicated in injury-induced proliferation of glial cells and pericytes. The mitogenic effects of sonic hedgehog require primary cilia, but the few reports on glial or pericyte primary cilia do not agree about their abundance and did not address effects of injury on these cilia. Primary cilia are microtubule-based organelles that arise from the centrosome and are retracted before cells divide. Depending on cell type, proteins concentrated in cilia can transduce several mitotic, chemosensory, or mechanosensory stimuli. The present study investigated effects of stab wound injury on the incidence and length of glial and pericyte primary cilia in the area adjacent to the injury core.

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CHAPTER 1

EFFECTS OF BRAIN INJURY ON PRIMARY CILIA OF GLIAL CELLS AND PERICYTES

1.1 Introduction

Primary cilia are ubiquitous organelles whose role in the adult brain remains elusive. Primary cilia have a complex structure and concentrate components for various signaling pathways. Depending on the organ and developmental stage, primary cilia may transduce various sensory, mitogenic, or morphogenic signals. The cilia-mediated sonic hedgehog (Shh) pathway is of special importance during development and injury. Following brain injury and neurodegenerative diseases, glial cells respond to Shh, especially by proliferating and creating more glial cells which participate in several aspects of the recovery process. Thus it is likely that primary cilia are critical in the response to brain injury, yet these organelles have not been examined in that context.

In the central nervous system, glial cells - particularly astrocytes and polydendrocytes - maintain the environment required for proper neuronal signaling. Following injury, glial cells prevent the spread of insult by surrounding and containing the injured area. This is accomplished in part through proliferation of astrocytes, polydendrocytes and pericytes surrounding the injury. Pericytes play a crucial role in restoring blood vessel function following injury. Astrocytes, polydendrocytes and pericytes can respond to Shh. Considering that primary cilia mediate the mitogenic effect of Shh signaling; it is likely that proliferative astrocytes, polydendrocytes and pericytes that respond to Shh possess a primary cilium. At least some astrocytes and neural stem cells have a primary cilium. There are no reports of the presence of primary cilia in polydendrocytes or in pericytes in the central nervous system. This report examines

characteristics of astrocyte, polydendrocyte and pericyte primary cilia and the changes that follow injury-induced proliferation. This information is essential for understanding the role of primary cilia in these cell populations.

1.1.1 Classification

Primary cilia are microtubule based solitary organelles which project from the centrosome in most mammalian cell types. Primary cilia can range in size from 1-20 μm long (Chang et al., 2015). Cilia length can change due to a variety of reasons, including hypoxia (Verghese et al., 2008), mechanical loading (McGlashan et al., 2010), treatment with monovalent cation chlorides (Miyoshi et al., 2011) and autophagy (Tang et al., 2014). Based on microtubule doublet structure, cilia are classified as primary, motile, sensory or nodal. Primary cilia, found in most cell types in the body are non-motile and have microtubules with a 9+0 arrangement; the central microtubule pair and dynein arms are absent. Motile 9+2 cilia, are common on cells lining the brain ventricles, trachea, testis and fallopian tubes; they have dynein arms which facilitate sliding between the 9 microtubule doublets circling a central microtubule pair. Sensory 9+2 cilia are found in the vestibular and olfactory system; these cilia lack dynein arms and are non-motile. Nodal 9+0 cilia line the embryonic node; they have dynein arms and are motile, yet lack a central microtubule pair (Ibanez-Tallon et al., 2003, Michaud and Yoder, 2006). This classification is not absolute, since 9+0 primary cilia in the kidney were recently shown to be capable of actin directed movement (Battle et al., 2015). This thesis focuses on primary cilia, which are 9+0 and lack dynein arms.

1.1.2 Evolution

Primary cilia are organelles present in eukaryotes and absent in prokaryotes (Christensen et al., 2007). Among eukaryotes, primary cilia are present in vertebrates and some specialized sensory structures in invertebrates (Evans et al., 2006). Mitchell (2007) proposed that an early eukaryote ancestor possessed the microtubule machinery necessary for cilia formation prior to the separation of unikont (animals, fungi, choanozoa, amoebozoa) and bikont (plants, chromists, all other protozoa) clades. Development of 9+2 flagella gave this early ancestor unparalleled evolutionary advantage, considering all eukaryotes descended from this original ancestor. However it is unclear whether cell polarity, motility, chemosensation or some other factor was the driving force behind cilia formation (Jekely and Arendt, 2006).

1.1.3 Ciliogenesis

Primary cilia are templated/nucleated from the mother centriole, which is a modified centriole docked to the plasma membrane. The centrosome contains two centrioles, one of which becomes a modified mother-centriole capable of templating microtubules, while the other is an unmodified daughter-centriole that lacks this capacity (Bettencourt-Dias and Glover, 2007). The mother-centriole is the older of the centriole pair (Rieder and Borisy, 1982). A transition zone couples the cilium to the mother centriole (Jana et al., 2014). The 9 microtubule doublets in the axoneme elongate as a result of polymerization of α- and β-tubulin heterodimers, using energy derived from GTP hydrolysis (Haimo and Rosenbaum, 1981). Ciliary tubulin undergoes various post-translational modifications, acetylation being the most frequent, which modulate microtubule stability (Castro-Castro et al., 2012). Microtubule assembly and disassembly occur at the primary cilium distal tip and are tightly regulated (Johnson and Rosenbaum, 1992, Song

and Dentler, 2001). Primary cilia are resorbed in proliferating cells before mitosis; resorption is thought to be necessary to liberate the centrosome for nuclear translocation and subsequent mitotic spindle formation (Pan and Snell, 2007). Once the cell exits the cell cycle, the centrosome is free to migrate to the plasma membrane, where primary cilia are formed using the mother centriole as a template (Kim and Tsiokas, 2011). The resorption of primary cilia in relation to the cell cycle is not well understood in vertebrate cells *in vivo*.

1.1.4 Targeting of Ciliary Components

Primary cilia are associated with a complex system that targets specific proteins involved in ciliary function. The BBSome, a 450 kDa complex of Bardet-Biedl syndrome proteins (BBS 1/2/4/5/7/8/9), assembles a vesicular coat that traffics proteins within the vesicle, towards the cilium (Jin et al., 2010, Domire et al., 2011). Upon docking at the base of the primary cilium, cilia-bound cytoplasmic and membrane proteins conjugate with intraflagellar transport (IFT) complex components (Deane et al., 2001). Ciliary proteins can be transported anterogradely toward the tip via IFT complex B and kinesin motors, or retrogradely back to the base by means of IFT complex A and dynein motors (Kozminski et al., 1993, Kozminski et al., 1995, Pazour et al., 1999, Qin et al., 2005). IFT complex components move to the flagellar tip at 2 μm/s and back to the flagellar base at 3.5 μm/s, on average (Kozminski et al., 1993), suggesting that a protein can traverse the length of a cilium within a couple of seconds (Fig. 1).

Migration of proteins into the cilium is regulated through passive and active mechanisms analogous to those of the nuclear pore complex. This selective barrier resides in the transition zone, between the basal body and the ciliary axoneme (Hu et al., 2010, Williams et al., 2011, Kee et al., 2012). At the transition zone is a size-dependent diffusion barrier with pores of about

8-nm radius. These pores allow passive passage of cytoplasmic proteins into and out of the cilium. Rates of diffusion are dependent upon Stokes radii (Lin et al., 2013). Electron-dense transition fibers and Y-links project perpendicularly towards the plasma membrane, and are involved in establishing the selective barrier between cyto- and cilio-plasm (Garcia-Gonzalo and Reiter, 2012). Structures at the transition zone are analogous to the nuclear pore complex (Kee and Verhey, 2013) and consist of nucleoporins, importins and a Ran-guanosine triphosphate/diphosphate gradient (Nachury et al., 2007, Yoshimura et al., 2007, Dishinger et al., 2010, Knodler et al., 2010, Kee et al., 2012).

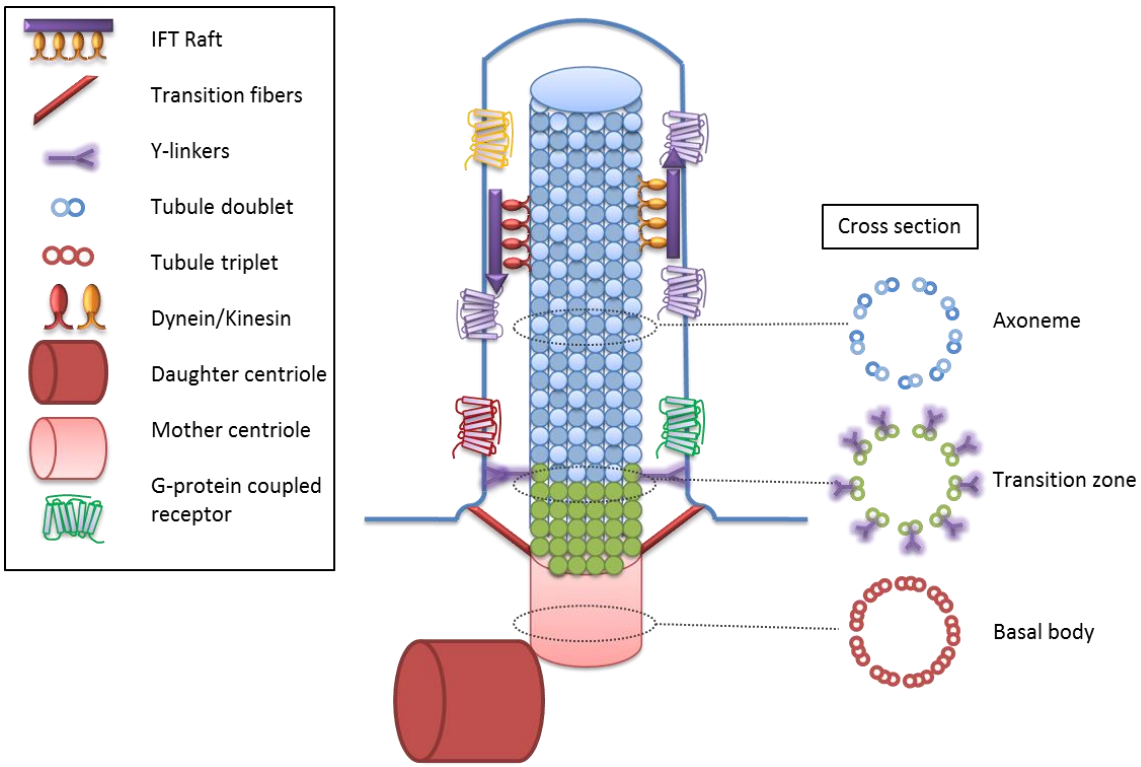


Figure 1: Model of primary cilia structure. Primary cilia have a 9+0 microtubule axoneme that serves as a scaffold for intraflagellar transport, which relays components necessary for proper cilia function. The cilium is nucleated from a modified centrosome, called the mother centriole. When the centrosome travels to the membrane and nucleates a cilium, it is called a basal body. Cilia components are transported through IFT rafts toward the cilium tip and back through kinesin and dynein motor proteins, respectively. Ciliary proteins must traverse the sieve mechanisms located at the transition fibers and Y-linker components of the transition zone.

Membrane and soluble proteins are compartmentalized in the cilium through several mechanisms. Membrane proteins are targeted to the base of the primary cilium through a ciliary localization sequence within the protein primary structure, which allows membrane proteins to traverse a septin barrier and transition fibers localized at the base of the cilium. The septin barrier at the ciliary base compartmentalizes the membrane constituents, limiting their diffusion in and out of the cilium (Hu et al., 2010). Transition fibers may serve as a selective barrier for soluble protein components (Williams et al., 2011, Chih et al., 2012, Breslow et al., 2013, Wei et al., 2015, Yang et al., 2015). Certain membrane and soluble proteins contain one of a handful of identified ciliary localization sequences, some of which are necessary and sufficient to traffic receptors into the primary cilium (Deretic et al., 1998, Geng et al., 2006, Berbari et al., 2008, Nagata et al., 2013). The primary cilium membrane contains high levels of lipid raft microdomain constituents, including high levels of sterol, glycolipids and sphingolipids (Tyler et al., 2009). These evolutionarily conserved features allow concentration of specific signaling components within the cilium, by at least 10^2-10^3 times compared to the cytoplasm (Nachury, 2014). Ciliary proteins differ between cell types (Bishop et al., 2007, Stanic et al., 2009), which suggests there are functional differences among cilia of different cell types.

1.1.5 Primary Cilia Function

Primary cilia are implicated in a variety of functions among different cell types. Primary cilia provide an environment separate from the cytoplasm for compartmentalization of signaling pathways, which can include chemosensory, mechanosensory, and mitogenic pathways. These functions are dependent on the signaling pathways hosted within the primary cilium structure.

1.1.5.1 Sensory Cilia

Retinal photoreceptor and olfactory neurons transduce sensory cues through G-protein coupled receptors (GPCRs) concentrated in the membrane of these specialized cilia. Upon photon or odorant binding to ciliary GPCRs, G-proteins are activated, leading to enzymatic activation that changes levels of cGMP and cAMP, respectively (Fesenko et al., 1985, Haynes and Yau, 1985, Nakamura and Gold, 1987) . The subsequent effects on ion flux through cyclic nucleotide gated ion channels causes a change in the cell membrane potential, thereby transducing the sensory signal into information that can be communicated by the neurons (Richardson, 1969, Glees and Spoerri, 1978).

1.1.5.2 Mechanosensory Cilia

Primary cilia in the kidney are mechanosensory, transducing fluid flow in the lumen of kidney tubules into an intracellular calcium signal, through the activation of mechanically gated receptors polycystin 1 and 2 (Hanaoka et al., 2000). The calcium ions entering through polycystin channels depolarize the cell, which is instrumental in regulation of fluid flow within kidney tubules (Schwartz et al., 1997). Primary cilia have also been shown to respond to interstitial fluid flow within bone (Delaine-Smith et al., 2014). Osteocyte primary cilia contribute to bone resorption in response to dynamic fluid flow, this mechanism is thought to be calcium-independent (Malone et al., 2007). Differences in cilia-mediated mechano-transduction suggest cilia allow transduction of mechanical signals through tissue-specific ciliary pathways.

1.1.6 Ciliary Morphogenic Pathways

Sonic hedgehog (Shh) is a mitogen and morphogen, and as such plays a vital role in organ and limb patterning during development. Components of the Shh pathway are found in the primary cilium, which is required for proper Shh signaling (Huangfu et al., 2003, Corbit et al., 2005, Haycraft et al., 2005, Huangfu and Anderson, 2005, Rohatgi et al., 2007, Goetz and Anderson, 2010). Primary cilia mutations lead to proliferative deficiencies, related to improper Shh pathway transduction, in various organs (Yu et al., 2002, Chizhikov et al., 2007, Han et al., 2008, Spassky et al., 2008).

The Wnt pathway is another embryonic signaling cascade that is affected by primary cilia (Lancaster et al., 2011). Cilia are involved in the transition between canonical and non-canonical Wnt pathways (Saito et al., 2015). In the canonical Wnt signaling pathway, Wnt ligand binds to a receptor of the frizzled family, leading to disheveled-directed stabilization of cytoplasmic β-catenin and its subsequent trafficking by Jouberin to the nucleus, where β-catenin causes changes in gene expression (Lancaster et al., 2009, May-Simera and Kelley, 2012). When the Wnt pathway is not activated, Jouberin and β-catenin are sequestered to the primary cilium, dampening the translocation of β-catenin to the nucleus (Lancaster et al., 2011).. When ciliary Kif3a, IFT88 or OFD1 are disrupted, primary cilia function is impaired, resulting in enhanced canonical Wnt pathway activity (Gerdes et al., 2007, Corbit et al., 2008), underscoring the role of primary cilia in proper Wnt signaling transduction. In contrast, the non-canonical Wnt pathway is β-catenin-independent and is involved in planar cell polarity (Wallingford and Mitchell, 2011, May-Simera and Kelley, 2012)

Platelet derived growth factor (PDGF) is involved in regulation of cell apoptosis, migration, proliferation and survival. The PDGF receptor has two isoforms α and β, which homo- or hetero-dimerize to transduce their signal. In fibroblasts in culture, the α receptor is present in cilia membranes, while the β receptor is not found on cilia. This localization was corroborated by demonstrating that cilia knockdown blocked PDGFα-induced cell cycle entry, while no change was observed in PDGFβ signaling (Schneider et al., 2005).

1.1.7 Cilia Function in the Nervous System

Primary cilia play essential roles during neurodevelopment and adult nervous system homeostasis. The central nervous system contains neurons, neural stem cells, macroglia (astrocytes, polydendrocyte and oligodendrocytes), vascular cells and microglia. Astrocytes and neural stem cells possess a primary cilium (Bishop et al., 2007, Han et al., 2008, Kasahara et al., 2014). The primary cilia of neural stem cells express ACIII (Amador-Arjona et al., 2011), while astrocyte primary cilia express ACIII and Arl13b (Bishop et al., 2007, Kasahara et al., 2014). There are no reports of primary cilia in polydendrocytes. Primary cilia in pericytes have been reported with the use of electron microscopy (Biscoe and Stehbens, 1966, Wandel et al., 1984).

Primary cilia are essential in the development of cerebral cortex, dentate gyrus and cerebellum (Chizhikov et al., 2007, Han et al., 2008, Wilson et al., 2012). In mice with genetic nestin-Cre deletion of Kif3a, a protein necessary for cilia formation, cortical progenitor cells pass through G_1 faster, leading to increased proliferation and an overgrown cerebral cortex. The decrease in cell cycle length is most likely mediated through Gli3 (Wilson et al., 2012). Mice lacking cilia proteins IFT88 or stumpy in neural stem cells have degenerate cilia, as well as a

hypomorphic cerebellum and dentate gyrus due to reduced neural progenitor proliferation. (Chizhikov et al., 2007, Han et al., 2008).

The following GPCRs are found in the membranes of neuronal cilia; somatostatin receptor 3, serotonin receptor 6, melanin concentrating hormone receptor 1, and dopamine receptor 1 (Handel et al., 1999, Brailov et al., 2000, Berbari et al., 2008, Domire et al., 2011). Behavioral deficits in knockout mice for the somatostatin receptor 3, localized almost exclusively in primary cilia, suggest that primary cilia play a role in object recognition although not spatial memory (Einstein et al., 2010). Mice null for ACIII, a downstream component of G-protein coupled receptor signaling also have object recognition deficits (Wang et al., 2011). ACIII is almost ubiquitous in neural cilia and likely participates in GPCR signaling. Primary cilia also regulate dendrite and synaptic integration of newborn hippocampal neurons in adult mice (Kumamoto et al., 2012). Unlike neurons, glial cells have not been reported to have GPCRs in their cilia. Identifying the signaling components native to glial cell primary cilia is essential for understanding cilia function.

1.1.8 Ciliopathies

Defects in primary cilia structure and function are involved in a number of genetically linked pathologies collectively called ciliopathies. Even though the roles of primary cilia have not been fully established, severe pathological phenotypes develop when primary cilia are defective, which is suggestive of the importance of primary cilia. The list of ciliopathies is long, with over 50 different genes responsible (Guemez-Gamboa et al., 2014) . Autosomal dominant polycystic kidney disease alone affects 1 in 1000 people (Tobin and Beales, 2009). In general, symptoms of ciliopathies include developmental abnormalities such as polydactyly, disturbances

of left-right asymmetry and central nervous system defects (Goetz and Anderson, 2010, Murdoch and Copp, 2010). Ciliopathies also include defects in cell cycle control leading to cyst formation (Gunay-Aygun, 2009) and insufficient generation of neural stem cells (Chizhikov et al., 2007). IFT88, essential for cilia formation, also regulates the G1-S and S-G2 transition (Robert et al., 2007). This is compatible with the idea that primary cilia play a role in cell cycle check point. Cognitive defects are characteristic of certain ciliopathies, suggesting that primary cilia are required for proper development and/or function of the adult brain. These observations reveal that cilia defects can translate to functional abnormalities at the systems level (Zaghloul and Katsanis, 2009, Armato et al., 2013).

1.1.9 Primary Cilia and Cell Proliferation

Primary cilia respond to proliferative cues in embryonic and adult cell populations. Cilia are considered postmitotic organelles found in G_0 cells (Tucker et al., 1979) and are resorbed before the cell reenters mitosis (Irigoin and Badano, 2011, Inoko et al., 2012). Cilia resorb prior to spindle formation in G_2/M (Rieder et al., 1979, Tucker et al., 1979) and arise from the mother centriole sometime in G_1/G_0 (Menco and Farbman, 1985a, b); (Fig.2). The ciliary protein IFT88 localizes to the centrosome during the cell cycle, and knocking down IFT88 promotes cell cycle progression (Robert et al., 2007). Aurora A regulates cell cycle reentry through phosphorylation of histone deacetylase 6 (HDAC6). Phosphorylation of HDAC6 destabilizes axoneme microtubules, leading to ciliary resorption (Pugacheva et al., 2007). A number of centrosomal mitotic kinases are involved in the kinase-dependent disassembly of primary cilia; aurora kinase A, NIMA related kinase and polo-like kinase1 are among the mitotic kinases whose levels

change in concert with cilia disassembly and the cell cycle (Pugacheva et al., 2007, Seeger-Nukpezah and Golemis, 2012).

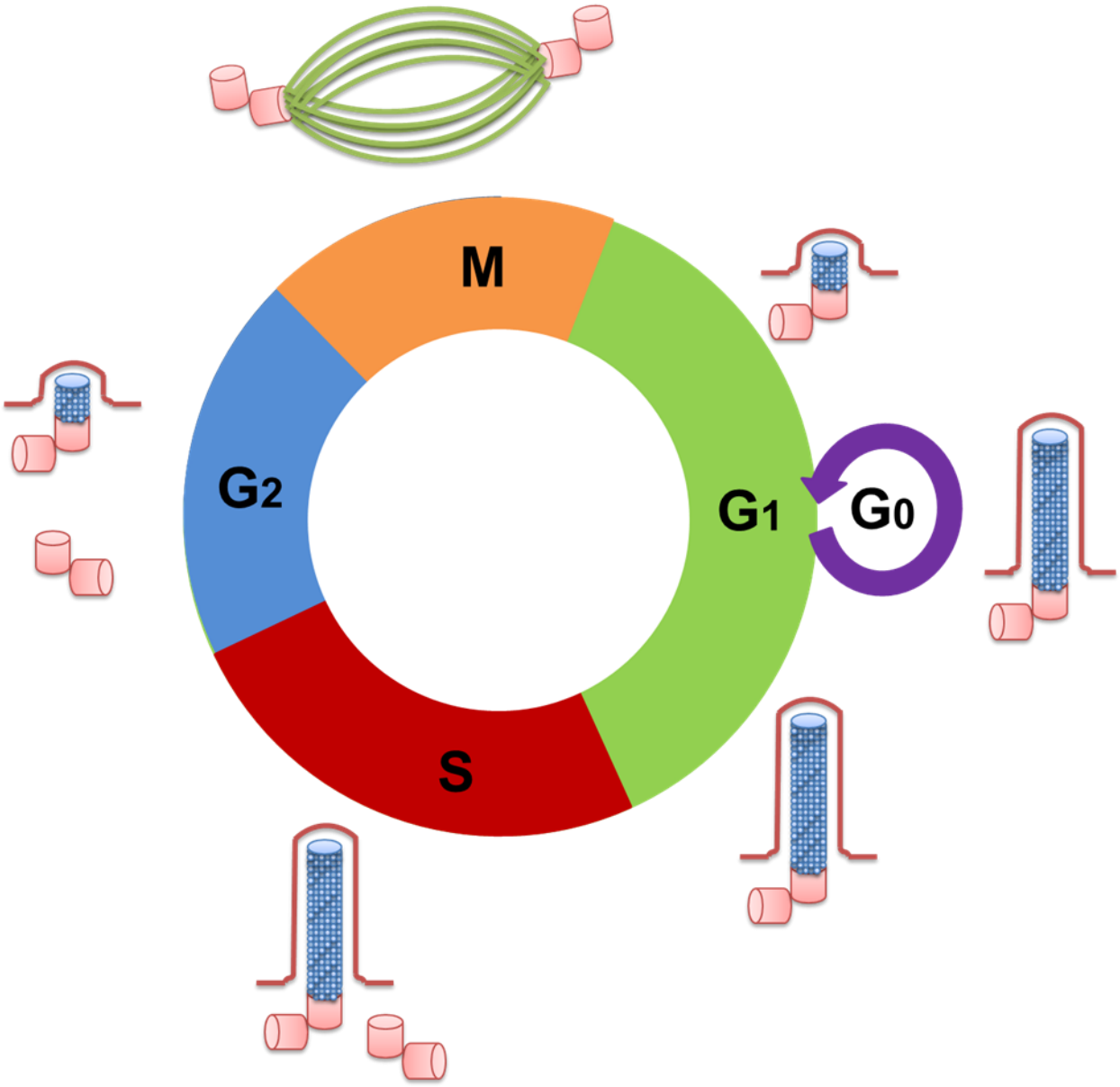


Figure 2: Primary cilia and centriole dynamics during the cell cycle: Centrosomes are composed of a centriole pair, surrounded by pericentriolar material. When a cell transitions from G_0 into the G_1 phase of the cell cycle, they still maintain a primary cilium. Centrioles duplicate during the S phase of the cell cycle. During G_2, prior to mitosis, the cilium is resorbed freeing the centrioles. During M phase the centrosome nucleates the microtubule mitotic spindle. Primary cilia are nucleated during G_1 or G_0, contingent on cell cycle exit.

In the central nervous system primary cilia mediate proliferative cues within neurogenic niches. With conditional ablation of primary cilia under the control of hGFAP promoter, the numbers of transit amplifying progenitor cells are markedly decreased in the subgranular zone of the dentate gyrus, although not in the subventricular zone (Han et al., 2008). Cilia-mediated Shh signaling has key mitogenic effects in the CNS (Chizhikov et al., 2007, Breunig et al., 2008, Han et al., 2008, Spassky et al., 2008). While the importance of primary cilia in normal development is becoming clear (Breunig et al., 2008, Han et al., 2008), less is known about the role of primary cilia in the proliferation that follows injury.

1.1.10 Primary Cilia and the Sonic Hedgehog Pathway

In most vertebrates the Shh signaling pathway is dependent on the presence of cilia as the site of signal transduction (Eggenschwiler and Anderson, 2007). Shh undergoes autoproteolytic cleavage in the lumen of the endoplasmic reticulum (Lee et al., 1994) before being released by the dispatched protein (Burke et al., 1999, Gallet et al., 2003). Once Shh reaches its target cell it interacts with patched (Ptc) in the cilia membrane (Marigo et al., 1996), causing Ptc to leave the cilium (Christensen et al., 2007, Rohatgi et al., 2007) and allowing smoothened (Smo) to enter the cilium (Corbit et al., 2005).

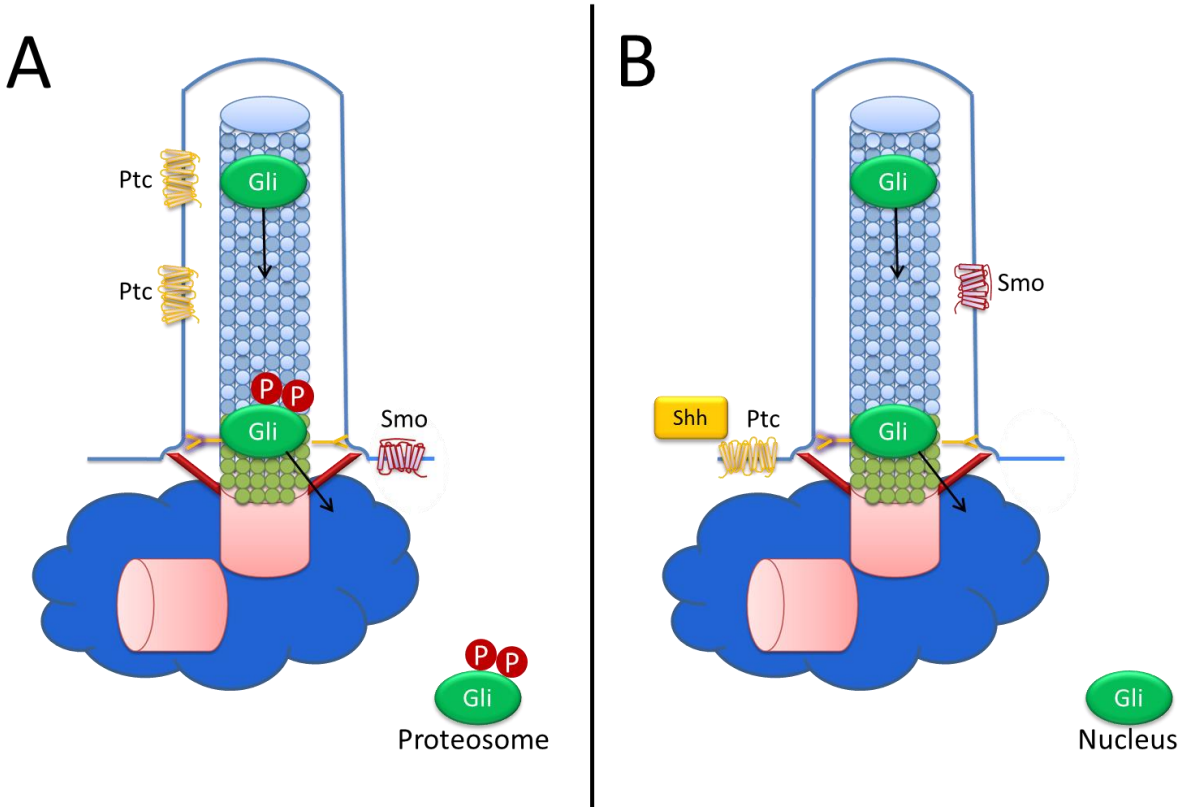


Figure 3: Primary cilia and the sonic hedgehog pathway: A. In the absence of Shh, the Shh ligand Ptc localizes to the ciliary membrane, inhibiting Smo translocation to the cilium. Ptc contributes to the phosphorylation of Gli transcription factors, which are subsequently degraded in the proteasome. B. In the presence of Shh, Ptc migrates out of the cilium and Smo enters the cilium. Gli transcription factors enter the nucleus and modulate gene expression. Ptc= patched; Gli= glioma factors; Shh= sonic hedgehog; Smo= smoothened. P= phosphorylation.

In the absence of Shh signal, Ptc biochemically inhibits Smo localization to primary cilia. When Ptc excludes Smo from the primary cilium the Suppressor of Fused (SuFu) protein interacts with glioma factors (Gli1, Gli2 and Gli3), forming a complex that retains glioma factors in the cytoplasm to be phosphorylated by casein kinase 1, glycogen synthase kinase 3 and cAMP-dependent protein kinase A (Wang and Li, 2006). Phosphorylation of Gli2 and Gli3 in the cytoplasm leads to their proteolytic cleavage by the proteasome into their repressor forms, inhibiting transcription of Shh targets (Sasaki et al., 1999, Wang et al., 2000, Pan et al., 2006). In the presence of Shh, Ptc leaves and Smo enters the cilium (Corbit et al., 2005). Ciliary Smo

allows the downstream translocation of the SuFu:Gli complex into the cilium, preventing the processing of Gli2 and Gli3 into their repressor forms (Tukachinsky et al., 2010, Wen et al., 2010, Hsu et al., 2011). Gli1 acts as an activator of gene expression; Gli1 does not possess a repressor domain and cannot be proteolytically cleaved to a repressor form (Fig. 3). Gli1 is an early gene expression target of Shh activity and can be activated as early as 3 hours following Shh activation (Dai et al., 1999). Glioma transcription factors regulate gene expression by associating with the consensus binding site (5'-GACCACCCA-3') in the promoter of target genes (Muller and Basler, 2000).

1.1.11 Glial and Neural Stem Cells of the Central Nervous System

Glial cells of the central nervous system have a wide variety of roles related to maintaining brain homeostasis. There is no consensus on the formal definition of glial cells, since they have diverse embryonic origins, and have various physiological and functional properties (Parpura and Verkhratsky, 2012). Glial cells in the adult brain can be divided into macroglia (astrocytes, oligodendrocytes, and polydendrocytes) and microglia. Macroglia are derived from the neuroectoderm (Kriegstein and Alvarez-Buylla, 2009) and play pivotal roles in the regulation of neuronal communication and the response to injury.

The subventricular zone and the dentate subgranular layer are the main neurogenic stem cell niches in the adult brain. Stem cell niches are comprised of a basement membrane, extracellular matrix and neurogenic cells (Lim and Alvarez-Buylla, 1999). The subventricular zone and the subgranular layer give rise to neurons destined for the olfactory bulb and the dentate gyrus respectively (Kriegstein and Alvarez-Buylla, 2009). In contrast, astrocytes and

polydendrocytes in the adult brain appear to rely on self-renewal, especially in response to injury (Bardehle et al., 2013, Hughes et al., 2013).

1.1.12 Glial Response to Injury

Following injury, glial cells proliferate and contain extravasation into the brain parenchyma. The concerted proliferation that follows insult involves mostly glial cells as opposed to neurons (Renner et al., 2003, Hampton et al., 2004). The time course of proliferation differs according to cell type. Within hours following stab injury, activated microglia (which do not have cilia) migrate and proliferate near the injury site, reaching peak proliferation 4 days after injury. As early as one day following injury, polydendrocytes begin to proliferate, reaching peak proliferation within the first two days after injury (Levine, 1994, Hampton et al., 2004). Astrocytes also proliferate as early as 1 day after injury, reaching peak proliferation 3 days after injury (Hampton et al., 2004, Komitova et al., 2011). Identifying characteristics of dividing glia could be beneficial in utilizing a source of stem cells to counteract brain injury and neurodegenerative diseases, where cell loss contributes to the pathology. Glial populations with protein composition similar to neural stem cells populations are prime candidates for cell populations that can potentially proliferate.

1.1.13 Anatomy of Nervous System Injury

In broad terms, the region of injury can be classified into three tissue compartments: injury core, glial scar border and penumbra (Sofroniew, 2009, Barreto et al., 2011, Huang et al., 2014a). The injury core is characterized by deposition of extracellular matrix molecules and it is composed mainly of perivascular fibroblasts and pericytes (Goritz et al., 2011, Soderblom et al.,

2013). In stroke models this area is also known as the ischemic core. The injury core is also characterized by the absence of GFAP immunoreactivity and is encapsulated by a GFAP glial scar border (Wanner et al., 2013). The glial scar border is composed of the end-feet of astrocyte processes, which form a glial limitans which contains the injury core and restores the blood-brain barrier (Yoshioka et al., 2010, Kawano et al., 2012). The penumbra region is mainly identified by astrocytes that visibly upregulate GFAP. This region is on average between 300-400 µm away from the injury core, depending on injury type (Alonso, 2005, Barreto et al., 2011). In the penumbra, the blood supply is constrained and energy metabolism is intermittently compromised (Ginsberg and Pulsinelli, 1994). Reactive astrocytes and other cells in the penumbra contain damage from spreading beyond the injury core (Pekny et al., 1999, Fisher and Bastan, 2012, Sofroniew, 2015) helping maintain the ionic balance, clearance of neurotransmitters, inflammatory response and free radical scavenging (Rolls et al., 2009). The penumbra region is also known as the region of reactive gliosis; in this region proliferation, cell morphology, gene expression and reactive gliosis is heterogeneous and dependent on distance from the injury core (Sofroniew and Vinters, 2010, Barreto et al., 2011, Kim et al., 2012).

1.1.14 Primary Cilia in Astrocytes, Polydendrocytes and Pericytes

Most ectoderm derived cell types, including neurons and their precursors, have primary cilia; however the prevalence of cilia in astrocytes, polydendrocytes and pericytes remains largely unexplored. Primary cilia have been observed in astrocytes (Kasahara et al., 2014) and pericytes (Biscoe and Stehbens, 1966, Wandel et al., 1984), but there are no reports of primary cilia in polydendrocytes. Considering that polydendrocytes may respond to Shh following injury

(Amankulor et al., 2009, Honsa et al., 2016) and that primary cilia mediate the effects of Shh, it may be that primary cilia are indeed present in polydendrocytes.

1.1.15 Primary Cilia and the Injury Response

At least some of the proliferation of astrocytes and possibly polydendrocytes following injury appears to occur in response to increase in Shh (Amankulor et al., 2009, Garcia et al., 2010), which as previously mentioned requires the primary cilium (Corbit et al., 2005, Eggenschwiler and Anderson, 2007, Rohatgi et al., 2007). This suggests that primary cilia may be present in proliferative glial populations. There are few reports of primary cilia in glial cells in healthy or injured nervous tissue (Bishop et al., 2007, Yoshimura and Takeda, 2012, Kasahara et al., 2014).

In the normal brain, neurons are the source of Shh (Garcia et al., 2010). However following injury, astrocytes release Shh, which is thought to induce proliferation of oligodendrocyte progenitor cells (Amankulor et al., 2009). These oligodendrocyte progenitors are most likely polydendrocytes, the most proliferative cell type in the brain parenchyma outside the neurogenic niches (Hampton et al., 2004). The effects on polydendrocytes might be also related to increased expression of Olig2 induced by Shh (Nery et al., 2001). Olig2 is a transcription factor necessary for the specification of oligodendrocytes (Nery et al., 2001). If polydendrocytes proliferate in response to Shh, it is likely that these cells have primary cilia.

The prevalence and role of primary cilia in glial cells and pericytes of the adult nervous system remains largely unexplored. Considering that primary cilia are involved in the transducing Shh signaling and that astrocytes, polydendrocytes and pericytes respond to Shh

after injury (Amankulor et al., 2009, Garcia et al., 2010, Fabian et al., 2012, Honsa et al., 2016), we hypothesize that this cell populations possess primary cilia. The role of primary cilia, like any other organelle, is given by their structural and chemical components. Immunohistochemistry will be used to explore the primary cilia and centrosome composition of astrocytes, polydendrocytes and pericytes. We will examine whether characteristics of primary cilia change following injury, by comparing the incidence and length of primary cilia in control versus injured somatosensory cortex.

CHAPTER 2

MATERIALS AND METHODS

2.1 Surgical Procedure and Ethical Statement

Experiments were done on 8-week-old male C57BL/6 mice in accordance with the NIH Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996). A unilateral cryofreeze stab-wound was made while the mouse was surgically anesthetized with isofluorane. The stab wound was made on the right hemisphere according to stereotaxic coordinates: 1.7 mm posterior to bregma, 1.0 mm from the midline and 2.2 mm deep. A small incision in the skin was made and a burr hole drilled through the skull to allow insertion of an 18-gauge needle. The needle was cooled for 30-seconds in liquid nitrogen and then inserted 2.2 mm vertically into the cortex, 1.0 mm from the midline suture and 1.7 mm posterior to bregma. The cooling and insertion procedure was repeated a total of three times at 30-second intervals. Animals were monitored regularly during recovery. After a 3-day survival, mice were deeply anesthetized with 20% urethane and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and post-fixed in 4% paraformaldehyde overnight at 4°C, then transferred to a cryoprotectant solution consisting of 30% sucrose in buffer for 24 hours at room temperature. Excised brains were frozen in -80 °C isopentane and stored at -80°C until use. Frozen sections were cut with a sliding microtome at 30 µm, collected in 0.1M Tris buffered saline (TBS; pH 7.6) and stored in glycerol-based cryoprotectant until staining. Unoperated mice were used as controls. A total of 7 mice were used in this project: 4 injured and 3 controls. Effects of “injury” in this report refer to the 3-day survival time mentioned above, unless otherwise specified.

2.2 Immunohistochemistry

All staining was done on tissue sections mounted on microscope slides. Sections were mounted on microscopes slides and allowed to air dry 4 hours, under the direct air flow of a fan. Antigen retrieval unmasking was done by immersing the slides in 0.1 M citric acid (pH 6.0) for 5 min at 90-100 ^{o}C, followed by 30- minutes at 85-95 ^{o}C. After sections cooled to room temperature, they were rinsed in 0.1 M TBS for 15-minutes. Non-specific binding of the secondary antibody was blocked by a 30-minute incubation in blocking solution #1 (TBS with 3% normal serum and 0.3% Triton X-100). Tissue sections were then incubated in blocking solution #1 containing primary antibodies, overnight at room temperature. Sections were then rinsed several times in TBS over a 30-minute period, and incubated for 15-minutes in blocking solution #2 (TBS with 3% normal serum from the host species of the secondary antibodies).

Antigen	Concentr.	Target	Host species	Supplier
Adenylate cyclase 3	1:1000	Primary cilia	Rabbit	Santa Cruz
Somatostatin receptor 3	1:1000	Primary cilia	Rabbit	Gramsch Labs
Arl13b	1:50	Primary cilia	Mouse	Neuromab
MCH receptor 1	1:500	Primary cilia	Goat	Santa Cruz
γ-Tubulin	1:500	Centrosomes	Goat	Santa Cruz
Pericentrin	1:1000	Centrosomes	Rabbit	Covance
GFAP	1:500	Astrocytes	Goat	Chemicon
NG2	1:1000	Polydendrocytes	Guinea Pig	Dr. W. Stallcup
Olig2	1:1000	Oligodendrocytes	Rabbit	Abcam
Ki67	1:1000	Proliferating cells	Rabbit	Santa Cruz
Cleaved caspase-3	1:300	Apoptotic cells	Rabbit	Cell Signaling

Table 1: Primary antibodies used in this study with their concentrations, targets, host species and supplier.

Labeling with secondary antibodies was done using different procedures depending upon the combination of primary antibodies that were used. The standard labeling procedure was incubation of the tissue in fluorophore-conjugated secondary antibodies in blocking solution #2 (1:500) for 90-minutes. When multiple primary antibodies raised in the same species were used, labeling was done using F(ab) fragments of the secondary antibodies. This procedure enhances specificity because the smaller F(ab) fragments can more efficiently cover all antigenic sites, thereby preventing subsequent secondary antibodies from binding to the previously F(ab) labeled primary. In this procedure, slides were incubated in fluorophore-conjugated F(ab) fragments in incubation solution #2 (1:500) for 90-minutes. Sections were then rinsed in 0.1 M TBS for 30-minutes, followed by a 30-minute incubation in blocking solution #1 and an overnight incubation with the second primary antibody in blocking solution #1. A 30-minute TBS wash was followed by incubation in blocking solution #2 for 15-minutes. The sections were then incubated for 90-minutes with a fluorophore-conjugated secondary antibody (1:500) in blocking solution #2. Unbound secondary antibodies were washed away for 30-minutes with TBS followed by a 10-minute incubation in DAPI (1:1000 in TBS). A final 10-minute wash was performed before coverslipping with Vectashield mounting medium. Several immunohistochemical amplification procedures were used when needed to enhance the signal for primary antibodies.

2.3 Microscopy and Data Analysis

Confocal fluorescence images were taken using a CsU-10 Yokogawa confocal scanner mounted on a Zeiss 200M inverted optical microscope at 400X magnification. A montage of 5 contiguous image stacks was taken next to the injury, through the depth of the cortex. Each image stack had dimensions 174x174x30 μm, and images were taken every 1 μm in the Z-axis.

Montages were taken just lateral to the stab-wound, starting at the beginning of the reactive astrocyte zone and extending from the most superficial neuronal layer (layer 1) through the depth of the cortex. An equivalent region was taken for control mice. Primary cilia length was measured from 3D reconstructed rotatable confocal stacks, using Bitplane Imaris software. Incidence and morphology data were acquired using FIJI (Fiji is just ImageJ) for Windows (http://fiji.sc/).

Cell populations were defined by the presence or absence of cell-type markers: Astrocytes were GFAP+/NG2-, polydendrocytes were NG2+/Olig2+, and pericytes were NG2+/Olig2-/GFAP- (Ligon et al., 2006, Birbrair et al., 2014). Pericyte criteria also included association with blood vessels. Nucleus sphericity in polydendrocytes was the ratio of length:width (FIJI software).

2.4 Statistics

IBM SPSS Statistic 20 for Windows was used. All datasets were analyzed for normal distribution using Pearson's analysis. All datasets that met the criteria for Gaussian distribution were then analyzed for statistical differences using independent samples t-tests and were considered significant at alpha <0.05. Mann-Whitney U-tests were reserved for data that were not normally distributed. Multiple comparison analyses were done with one-way ANOVAs followed by Student Newman Keuls post-hoc analysis. Groups were considered significant at alpha< 0.05. Graphs show mean ± standard error of the mean

CHAPTER 3

EFFECTS OF BRAIN INJURY ON PRIMARY CILIA OF ASTROCYTES

3.1 Introduction

3.1.1 Astrocytes and the Central Nervous System

Astrocytes are distributed throughout the brain parenchyma and modulate neuronal communication at the synapse through energy balance, gliotransmitter release, protection against reactive oxygen species, and synapse regulation (Halassa et al., 2007, Oberheim et al., 2012, Xu et al., 2014). Glial fibrillary acidic protein (GFAP) is the most widely used astrocyte marker, although it is not a pan-astrocyte marker. GFAP is expressed highly in fibrous astrocytes of the white matter and expressed in low levels in protoplasmic astrocytes (Cahoy et al., 2008, Zhang and Barres, 2010). The use of a GFP reporter for GFAP has revealed regional heterogeneity in levels of GFAP, in the spinal cord 97% of astrocytes labeled with a Cre-loxP reporter of mGFAP also label with the antibody against GFAP (Herrmann et al., 2008). In the injured brain, GFAP labels most astrocytes responding to injury, while in the healthy brain GFAP is not as detectable (Sofroniew and Vinters, 2010). The most reliable astrocyte marker is aldehyde dehydrogenase 1 family member 1 (Aldh1L1). The use of multiple protein markers identifies astrocytes as a heterogeneous population. Some of these markers include S100β (a calcium-binding protein), glutamine synthetase, and glutamate/aspartate transporter (Cahoy et al., 2008, Khakh and Sofroniew, 2015). Based on location astrocytes fall into two broad categories; protoplasmic and fibrous, which are associated with gray and white matter, respectively (Cajal, 1909).

3.1.2 Astrocyte Function

Astrocytes complement neuronal energy supply, synaptic transmission, free radical clearance and ion buffering. Unlike neurons, astrocytes can store energy in the form of glycogen; neuronal activity unfolds a cascade of events that activates glycolysis in astrocytes. Astrocytes produce and release lactate and to a lesser extent pyruvate, which is made available to neurons for oxidation and production of ATP (Bouzier-Sore et al., 2002). This energy is mainly consumed in presynaptic and postsynaptic products (Harris et al., 2012).

Astrocytes release gliotransmitters to which neurons can respond, suggesting that astrocytes can actively influence synaptic transmission. Astrocytes can affect long term potentiation and synaptic depression through ATP and glutamate release. This most likely occurs through the synchronization of synaptic responses, leading to Hebbian modulation of synapses (Guthrie et al., 1999, Anlauf and Derouiche, 2005, Perea and Araque, 2006). Populations of astrocytes respond synchronously to neuronal activity through the propagation of calcium waves, which travel through gap junctions (Garcia-Segura and McCarthy, 2004, Bains and Oliet, 2007). Astrocytes also participate in the glutamate/GABA-glutamine cycle, in which astrocytes take up glutamate or GABA and release glutamine to be taken up by neurons as a precursor for glutamate or GABA, critical for neurotransmission (Bak et al., 2006). Taken together, these data suggest that astrocytes are active players in the process of synaptic communication.

Astrocytes provide protection against reactive oxygen species. Due to high oxidative metabolic activity and myelin fatty acids, the CNS is particularly prone to damage from reactive oxygen species. Cultured neurons survive reactive oxygen and nitrogen radical insult more readily when astrocyte-conditioned medium is also present (Tanaka et al., 1999). Astrocytes

express pro- and anti-inflammatory factors which can interact with superoxide dismutase and catalases concentrated in neurons (Tanaka et al., 1999, Cahoy et al., 2008, Wu et al., 2014).

Astrocytes also take up ions at the synapse in a process deemed spatial buffering. Sodium, potassium, chloride and hydrogen are among the ions buffered by astrocytes (Simard and Nedergaard, 2004). For example, when potassium levels reach critical levels, receptors in astrocytes take up excess potassium and dilute the intracellular concentrations by transferring through gap junctions to adjacent astrocytes (Holthoff and Witte, 2000).

3.1.3 Astrocyte Precursor Cells

During prenatal development astrocytes arise from radial glia and polydendrocytes. Mature and immature astrocytes express markers GFAP, vimentin, S100B, Aldh1L1 and SOX2 (Sofroniew, 2009, Robel et al., 2011). The astrocyte-like nature of neural stem cells is highlighted by the presence of these markers in neural stem cells (Doetsch et al., 1999, Seri et al., 2004, Suh et al., 2007, Kriegstein and Alvarez-Buylla, 2009, Kuegler et al., 2010, Foo and Dougherty, 2013). Recent reports suggest that fully differentiated astrocytes can proliferate following injury (Buffo et al., 2008, Gadea et al., 2008, Bardehle et al., 2013, Martin-Lopez et al., 2013).

3.1.4 Astrocytes and the Injury Response

Astrocytes have a dynamic role following injury, which involves proliferation, reactive gliosis and containment of the necrotic injury core. After traumatic brain injury, astrocytes reach peak proliferation at 3 days; most astrocyte proliferation occurs adjacent to blood vessels (Komitova et al., 2011, Robel et al., 2011). The proliferation of astrocytes is inversely correlated

with distance from the injury core (Barreto et al., 2011). Astrocytes become reactive and surround the injury site, creating a barrier between healthy and necrotic tissue (Wanner et al., 2013). Reactive astrocytes upregulate the intermediate filaments GFAP, vimentin, nestin (Correa-Cerro and Mandell, 2007, Kamphuis et al., 2012, de Pablo et al., 2013) and undergo hypertrophy; these changes are dependent on injury type and intensity, among other factors (Wilhelmsson et al., 2006)**.** One highlight of this zone of reactive gliosis is the appearance of new astrocytes, whose exact source remains controversial, with some studies demonstrating *in vivo* proliferation of fully differentiated astrocytes (Miyake et al., 1988, Guizzetti et al., 2011, Bardehle et al., 2013) , while others deny that astrocytes proliferate (Qu and Jakobs, 2013).

3.1.5 Astrocyte Primary Cilia

Primary cilia are present in astrocytes. Bishop et al. (2007) deemed "rare" the occurrence of ACIII+ astrocyte cilia *in vivo.* A recent report found that 88% of astrocytes contain an Arl13b+ primary cilium and 44% contain an ACIII+ cilium. Kasahara also found that astrocyte cilia are on average 3.3μm in length (Kasahara et al., 2014). A few examples of motile cilia with (9+2) microtubule pattern have also been observed in astrocytes (Chung and Keefer, 1976). The present report examines cilia and centrosomes of astrocytes using several antibodies. We will also measure the incidence and length of astrocyte primary cilia in healthy and injured mouse cortex. The prevalence of paired mirror astrocytes in control and injured mice will be explored as well as the incidence of primary cilia in paired mirror astrocytes in the injured mice.

3.2 Results

3.2.1 Primary Cilia Were Ubiquitous in Astrocytes

In this study astrocytes were classified as GFAP+/NG2- cells, where NG2 was used to exclude NG2 immunoreactive polydendrocytes from the astrocyte category. In the mouse somatosensory cortex examined here, GFAP immunoreactivity appeared to be exclusively in astrocytes, and essentially no colocalization of GFAP and NG2 was observed in the penumbra region. A previous study identified rare colocalization between GFAP and NG2 in injured cortex, mainly in the glial scar border (Alonso, 2005), although we did not find any in this study. Cells in the intact cortex apparently do not co-express NG2 and GFAP (Leoni et al., 2009). GFAP immunoreactivity was particularly strong in astrocytes adjacent to blood vessels in control and injured astrocytes. GFAP+ astrocytes appeared to be uniformly distributed throughout the neocortex. GFAP+ astrocytes showed a highly polarized distribution of GFAP (Fig. 4A, C). Astrocytes maintain non-overlapping territory separated from neighboring astrocytes (Bardehle et al., 2013), with the exception of paired mirror astrocytes, whose cell bodies lie adjacent to each other with their processes polarized opposite each other (Fig. 4).

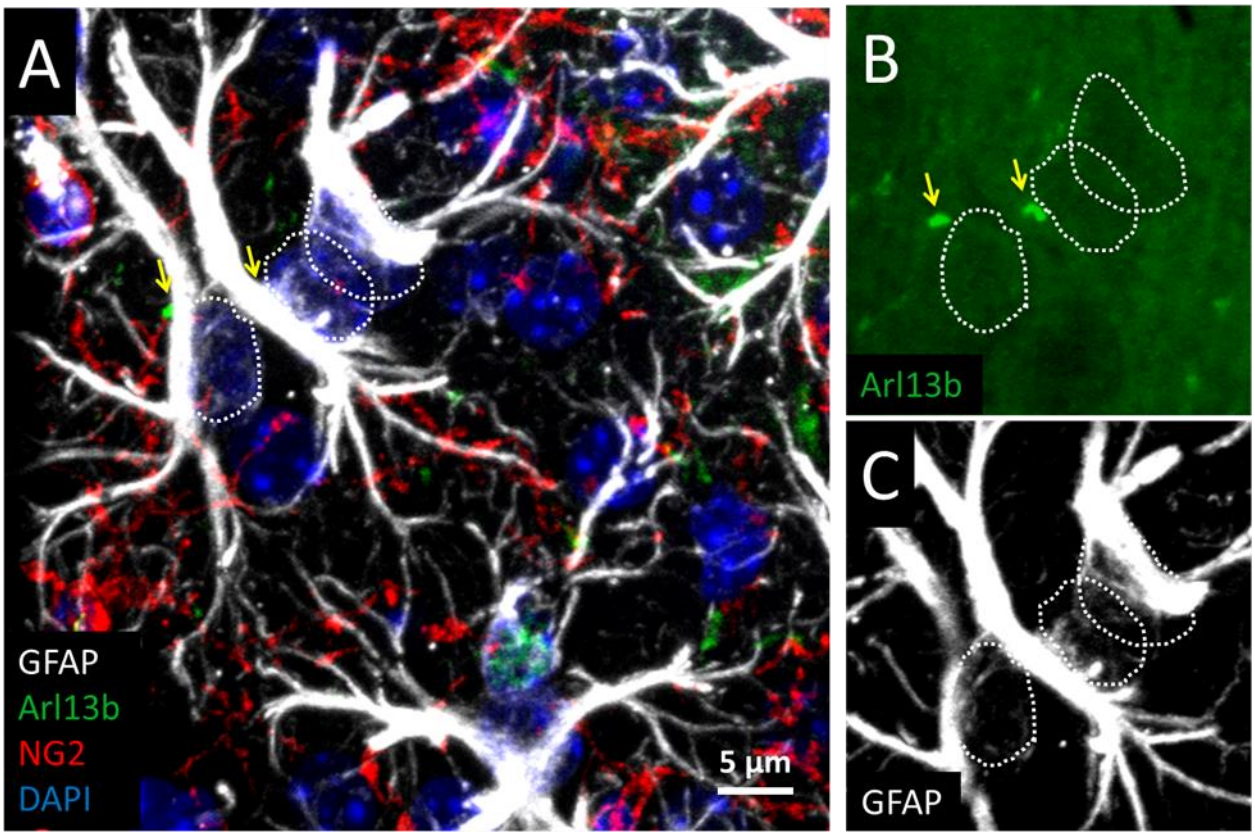


Figure 4: Primary cilia of astrocytes. A-C. Nuclei of GFAP+/NG2- astrocytes are outlined and channels separated in B and C. B. Separation of channels allows a clear view of the Arl13b+ primary cilia (yellow arrows), which are next to the astrocyte nuclei (outlined in white). C. The two nuclei furthest to the right are in slightly different Z-planes, but their processes appeared to be mirror images of each other when viewed in 3D. One of these mirror astrocytes was ciliated (B, yellow arrow on the right), while its counterpart lacked a primary cilium.

Using 3D reconstructions of confocal stacks, we analyzed the presence of primary cilia as labeled by the markers Arl13b and ACIII, in astrocytes of the somatosensory cortex from 8-week-old C57BL/6 mice. Arl13b+ primary cilia were found in 96 $\pm$ 1.9% of GFAP+/NG2- astrocytes, while ACIII+ primary cilia were found in 92 $\pm$ 2.0% of GFAP+/NG2- astrocytes (Fig. 5). The incidence of Arl13b+ versus ACIII+ primary cilia in astrocytes was not significantly different ($p < 0.18$; independent samples t-test; n=3 mice, 163 total cilia). In astrocyte cilia, ACIII immunoreactivity was faint in comparison to Arl13b (compare Fig. 6A with Fig. 6B).

Confocal microscopy often revealed astrocyte ACIII+ cilia that were difficult to identify when viewed through an epifluorescence microscope.

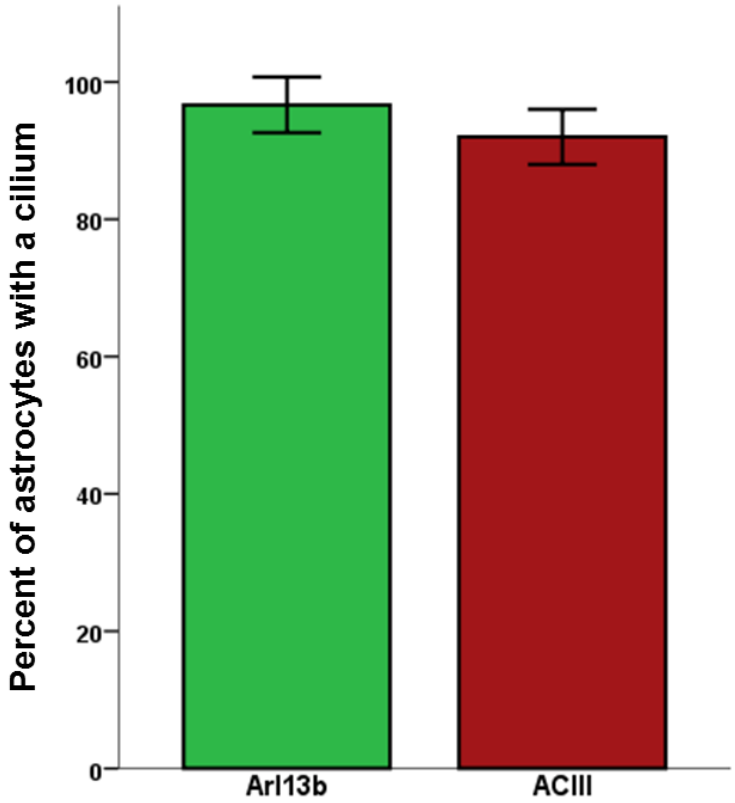


Figure 5: Incidence of GFAP+/NG2- astrocytes with Arl13b+ or ACIII+ primary cilia. The difference between these means was not significant ($p < 0.18$). Mean $\pm$ SEM, based on n= 3 mice.

3.2.2 Proteins in Astrocyte Primary Cilia and Centrosomes

Validated neuronal ciliary markers were used to identify proteins in astrocyte primary cilia (Fig. 6). Primary cilia in neurons of the central nervous system are reported to contain MCHR1 (Nagata et al., 2013), SstR3 (Handel et al., 1999), ACIII (Bishop et al., 2007) and Arl13b (Kasahara et al., 2014). Primary cilia in astrocytes contain ACIII (Bishop et al., 2007) and Arl13b (Kasahara et al., 2014). In the present study, primary cilia of astrocytes showed

ubiquitous label of ACIII and Arl13b (Fig. 6A, B), and unlike neurons, astrocyte primary cilia showed no immunoreactivity for the GPCRs SstR3 or MCHR1 (Fig 6C, D).

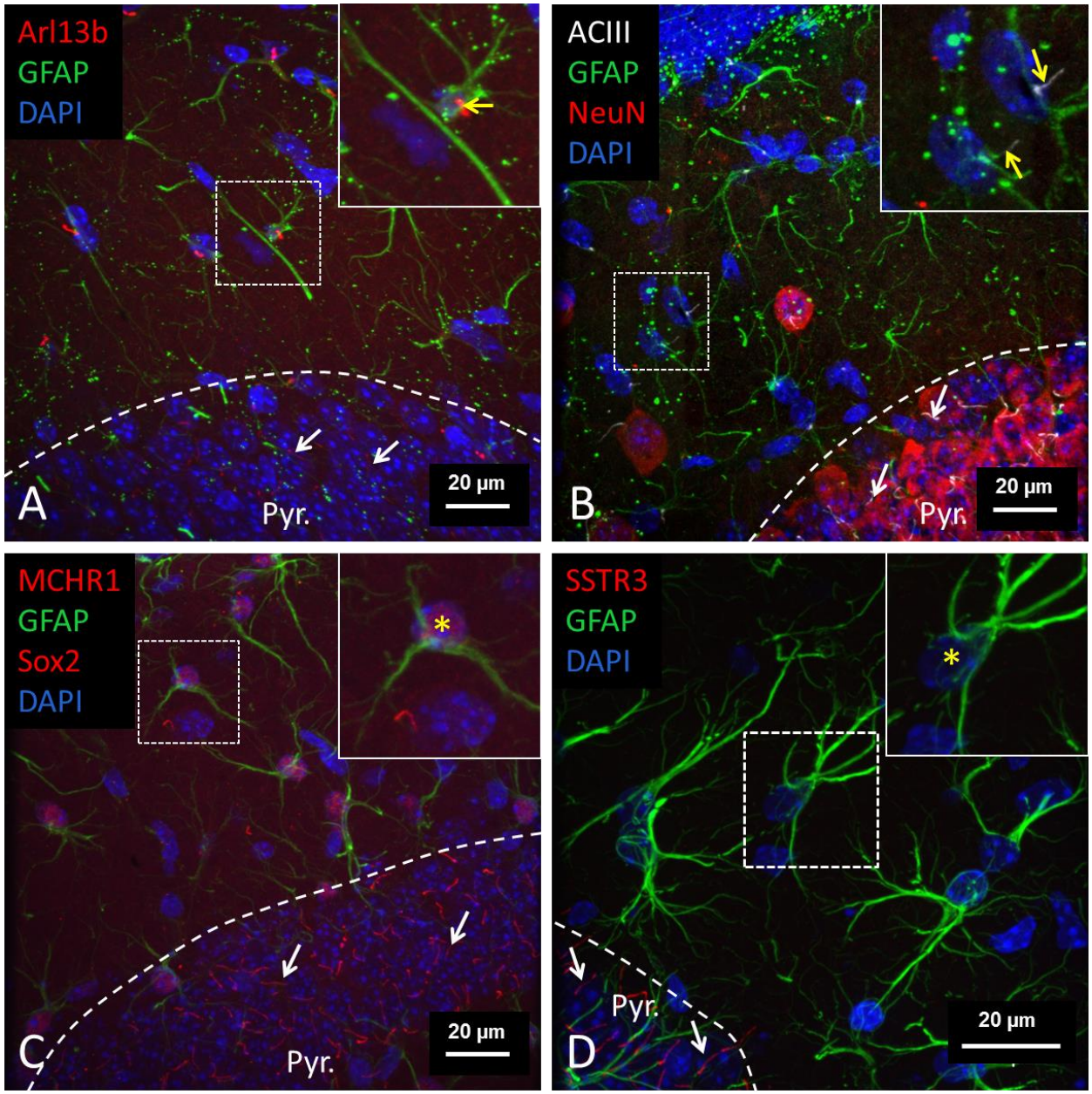


Figure 6: Primary cilia protein profile of GFAP+ astrocytes. A-B. Arl13b+ and ACIII+ primary cilia were present in astrocytes. Examples are enlarged in the insets are designated by yellow arrows. C-D. MCHR1 and SstR3 were absent from cilia in astrocytes, which are highlighted in the insets by yellow asterisks. A-D. By contrast, neurons with these ciliary GPCRs (white arrows) were common in the pyramidal layer (Pyr) of the underlying hippocampus.

The centrosomal markers γ-tubulin and pericentrin were also analyzed in astrocytes. Pericentrin and γ-tubulin are concentrated in the centrosomes of astrocytes *in vitro* (Diaz-Corrales et al., 2005, Peng et al., 2013, Shashikala et al., 2013). Pericentrin has been used *in vivo* to localize centrosomes in astrocytes in a mouse model of Alexander's disease (Sosunov et al., 2013). Pericentrin and γ-tubulin immunoreactivity were both found in the centrosomal region (at the base of primary cilia) of GFAP+ astrocytes. γ-Tubulin immunoreactivity was noticeably more intense than that of pericentrin, in astrocytes under the present staining conditions (Fig. 7B, D). Centrosomes were found in close proximity to the nuclei, and were located according to cell polarity, in the sense that they were closest to the region of the astrocyte having the most GFAP immunoreactivity (Fig. 7).

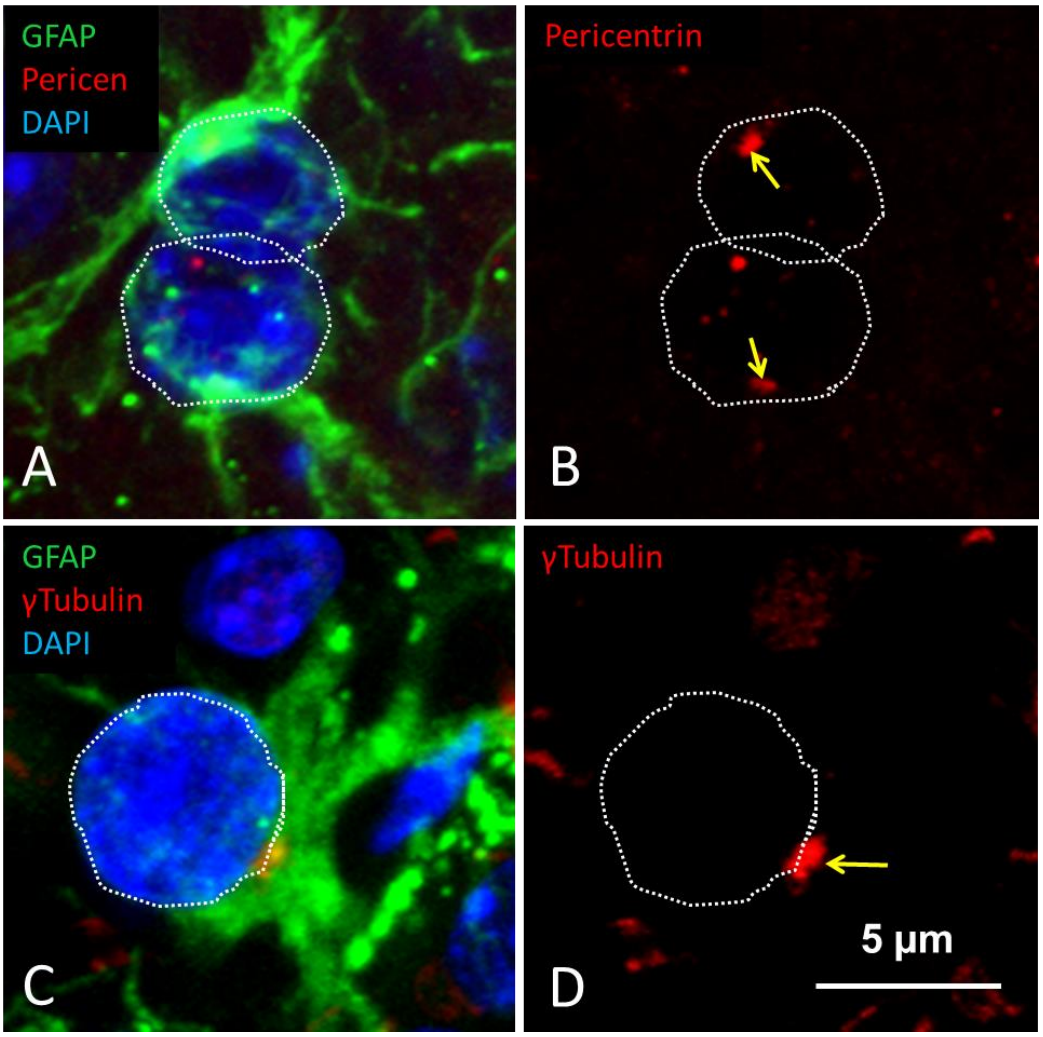


Figure 7: Pericentrin and γ-tubulin in the centrosome of astrocytes. A-D. Centrosomes in all GFAP+ (green) astrocytes were immunoreactive for pericentrin (A,B) and γ-tubulin (C,D), shown by yellow arrows. In A and B, the pericentrin in paired mirror astrocytes, indicate centrosomes occupy positions opposite each other.

3.2.3 Effects of Cortical Injury on Cilia Length and Incidence in Astrocytes

Following cortical stab wound injury in mouse cortex, primary cilia incidence and length significantly decreased in astrocytes. The changes in astrocyte primary cilia length and incidence were quantified using Arl13b. Injured and unoperated controls were all 8-week old male C57BL/6 mice. The length of primary cilia was obtained in 3D reconstructed confocal stacks

from GFAP+ astrocytes in the penumbra region, adjacent to the injury core. The penumbra extends 300-400 μm from the injury core border (Barreto et al., 2011), and we evaluated cilia within the column of confocal stacks that extended 174 μm from the border of the injury core. Measurements were made from 5 consecutive sampling sites in the penumbra (parallel to the needle track), where reactive gliosis and cell proliferation is strongest (Alonso, 2005, Barreto et al., 2011). The incidence of primary cilia in astrocytes 3 days after injury decreased by 17% compared with astrocytes of control mice; from 96 $\pm$ 1.9% in control to 80 $\pm$ 5.0% after injury (Fig. 8A; $p < 0.02$; independent samples t-test; n=7 mice, 262 total astrocytes). Cilia length decreased by 20% in the injury group compared with the control, uninjured mice; from 5.5 $\pm$ 0.3 μm in control mice to 4.4 $\pm$ 0.1 μm after injury (Fig. 8B; $p < 0.05$; independent samples t-test; n=7 mice, 242 total cilia).

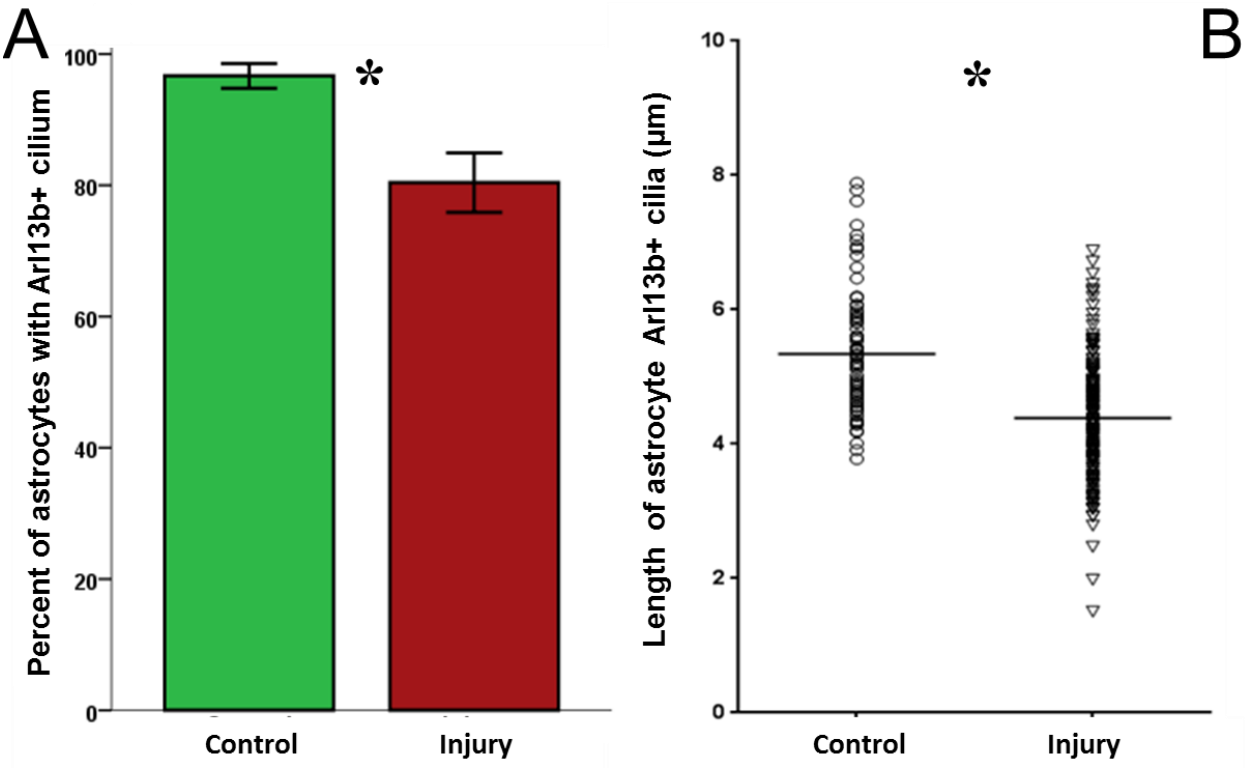


Figure 8: Decreased astrocyte Arl13b+ cilia incidence and length in the injured cortex. A. Astrocyte primary cilia decreased in incidence by 17% (*p< 0.02) 3 days after injury. B. Cilium length declined by 20% (*p< 0.05). Each data point represents one cilium. Mean ± SEM, based on n= 7 mice; 3 control and 4 injured mice.

A tendency to show an injury-associated decrease in cilium incidence was found using ACIII as the cilia marker. Although ACIII also labeled the centrosome in astrocytes, centrosomes and cilia could be readily distinguished based on morphology and the presence or absence of the centrosomal marker γ-tubulin. In the penumbra, parallel to the injury core, the incidence of ACIII+ astrocyte primary cilia decreased non-significantly by 11%; from 92 ± 2.0 % in controls to 81 ± 3.6 % in injured cortex (Fig. 9; p< 0.067; independent samples t-test; n=6 mice, 155 total cilia).

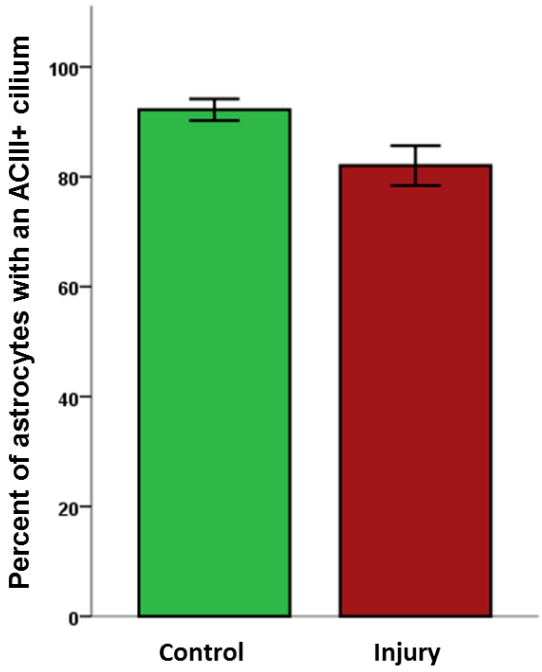


Figure 9: ACIII+ cilia incidence in astrocytes following injury. The incidence of ACIII+ primary cilia tended to decline 3 days after injury, but the difference was non-significant ($p<0.067$). Mean $\pm$ SEM, based on n= 6 mice; 3 control and 3 injured mice.

3.2.4 Primary Cilia in Paired Mirror Astrocytes

In general, astrocytes maintain clear non-overlapping borders with neighboring astrocytes (Salisbury, 2004), with the exception of a small population of paired mirror astrocytes, in which two astrocytes lie in close proximity to each other with their processes opposite each other (Bushong et al., 2002, Xu et al., 2014). Previous studies suggest that astrocytes can arise from cell division of differentiated astrocytes following injury (Buffo et al., 2008, Gadea et al., 2008, Bardehle et al., 2013); these recently divided astrocytes are likely the paired mirror astrocytes observed in the present study. In control animals, paired mirror astrocytes were a rarity. In injured animals, paired mirror astrocytes were more common, and were found with and without primary cilia; in some paired mirror astrocytes both astrocytes were ciliated (Fig. 10B), others

had no cilia in either mirror astrocyte (Fig. 10A), while others had cilia in only one of the paired mirror astrocytes (Fig. 4).

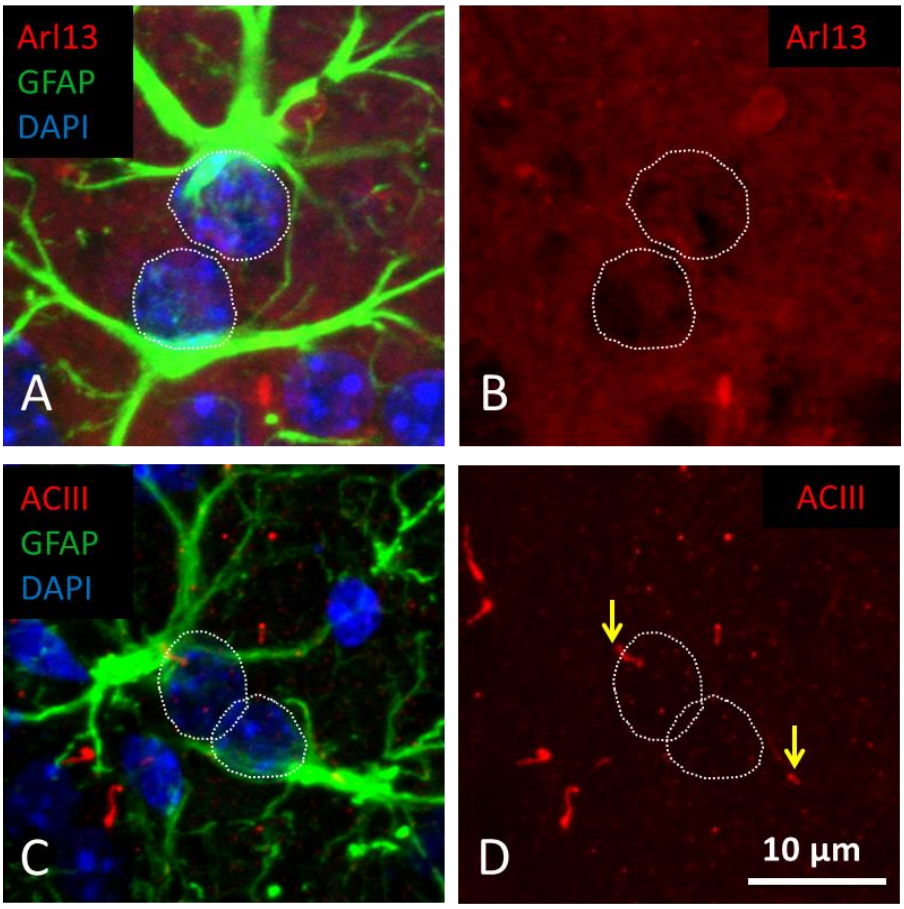


Figure 10: Primary cilia of paired mirror image astrocytes in the injured brain cortex. GFAP+ astrocyte (green) pairs with mirror image morphology were found with a variety of cilia configurations. A, B. These images from injured cortex show two non-ciliated GFAP+ astrocytes. C, D. Both of these paired mirror image astrocytes had an ACIII+ cilium. One cilium is adjacent to the nucleus, while the other cilium is further from the nucleus. Each image consists of 5 consecutive merged confocal slices, each 1 µm apart.

The number of GFAP+ paired mirror astrocytes increased by 7-fold after injury. In 5 confocal stacks of dimensions 174 µm x174 µm x 30 µm each, control mouse cortex had 0.7 $\pm$ 0.7 (SEM) paired mirror astrocytes compared to 6.0 $\pm$ 0.0 in the penumbra region of injured

cortex (Fig. 11; p< 0.018; Mann-Whitney U non parametric analysis; n=7; 26 total mirror image astrocytes).

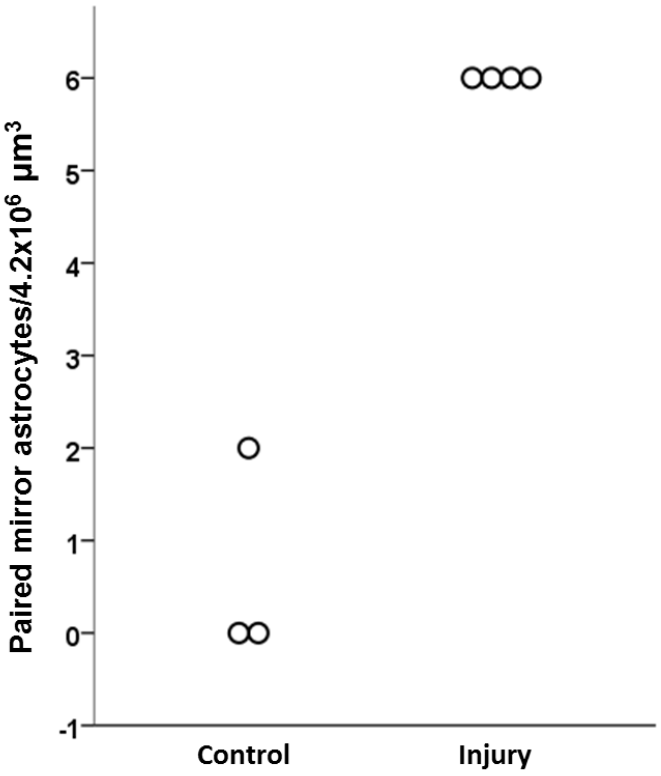


Figure 11: Paired mirror astrocytes in control and injured mice. There are more paired mirror astrocytes after injury compared to control (p< 0.018). Circles indicate mean number of paired mirror astrocytes per mice, based on n= 7 mice; 3 control and 4 injured mice.

We evaluated the incidence of ciliation in single astrocytes vs. paired mirror astrocytes in the injured brain. The incidence of paired mirror astrocytes had a non-statistically significant decrease of 26% compare to isolated single astrocytes. In the injured cortex, 82 $\pm$ 4% of the non-paired, single astrocytes, were ciliated, while 61 $\pm$ 25 % of the paired mirror astrocytes were ciliated (Fig. 12; p < 0.16; independent sample t-test; n=3 mice, 178 total astrocytes).

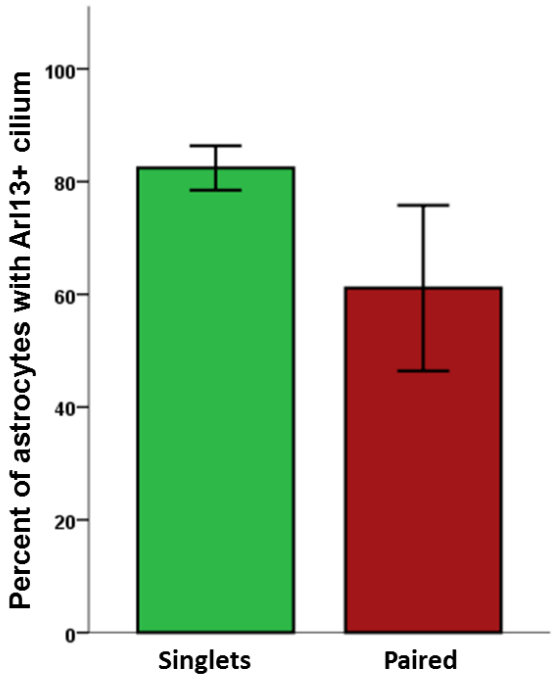


Figure 12: Incidence of cilia in single and paired mirror astrocytes. In the injured mice there was a non-statistically significant decrease of 26% in Arl13b+ cilia in paired mirror astrocytes (paired), compared to single astrocytes (singlets), ($p < 0.16$). Mean $\pm$ SEM, based on n= 3 mice.

To determine if any of the paired mirror image astrocytes were engaged in the cell cycle, we immunostained for the proliferation marker Ki67, but found no Ki67+ cells among paired mirror astrocytes in 5 confocal stacks (174 µm x174 µm x 30 µm/stack) per mouse. The neurogenic subventricular zone was examined to verify that Ki67+ cells could be identified.

The contribution of cell death to changes in cilia incidence was evaluated using the apoptotic marker cleaved-caspase 3. No apoptotic cells were found in the examined cortical area, which consisted of 5 confocal stacks (174 µm x174 µm x 30 µm/stack) per mouse.

3.2.5 History of Gli1 Expression in Astrocytes

We stained brain sections from C57BL/6 Gli1$^{CreER/+}$; R26$^{tdTom/tdTom}$ mice provided by Vivian Allahyari from Dr. Denise Garcia's lab (Drexel University, Philadelphia, PA). In these mice, exposure to injected tamoxifen activated Cre recombinase expression under the control of the Gli1 promoter (Ahn and Joyner, 2005), which led to removal of the floxed stop signal that prevented the red signal, Rosa26-tdTomato, from being expressed constitutively. In these mice tamoxifen injections were given daily for 3 consecutive days. 14 days after the beginning of tamoxifen injections, a unilateral cortical injury was made by scalpel blade. 1 week post-lesion the mice were sacrificed and perfused, and the brains were later sectioned for immunohistochemistry. Thus, cells that were expressing Gli1 during tamoxifen induction and all progeny cells resulting from subsequent proliferation were permanently filled with the red fluorescent tdTomato reporter. During development of the nervous system all Gli1 expression is dependent on Shh (Bai et al., 2002). Gli1 provides a read-out of Shh signaling in many cell types, including astrocytes (Bai et al., 2002, Bai et al., 2004, Garcia et al., 2010, Fabian et al., 2012).

Some of the GFAP+ astrocytes had Gli1-tdTomato red fluorescence, in both the injured and contralateral somatosensory cortex. Gli1-tdTomato staining was intense in the nuclei of these astrocytes, and cytoplasmic levels were somewhat less intense (Fig. 13 A). Gli1-tdTomato+ astrocytes from the injured area (Fig. 13C) showed morphological signs of hypertrophy and reactive gliosis when compared to astrocytes on the contralateral side (Fig. 13A). The Gli1-tdTomato+ astrocytes in the injured area appeared to be less arborized, and their nuclei were enlarged, as compared to the tdTomato+ astrocytes on the contralateral side. There

were no Gli1-tdTomato+ somata in the injury core, which is the ischemic area with a compromised blood brain barrier. The lack of Gli1-tdTomato+ somata in the ischemic core is consistent with the lack of GFAP+ astrocytes in the injury core.

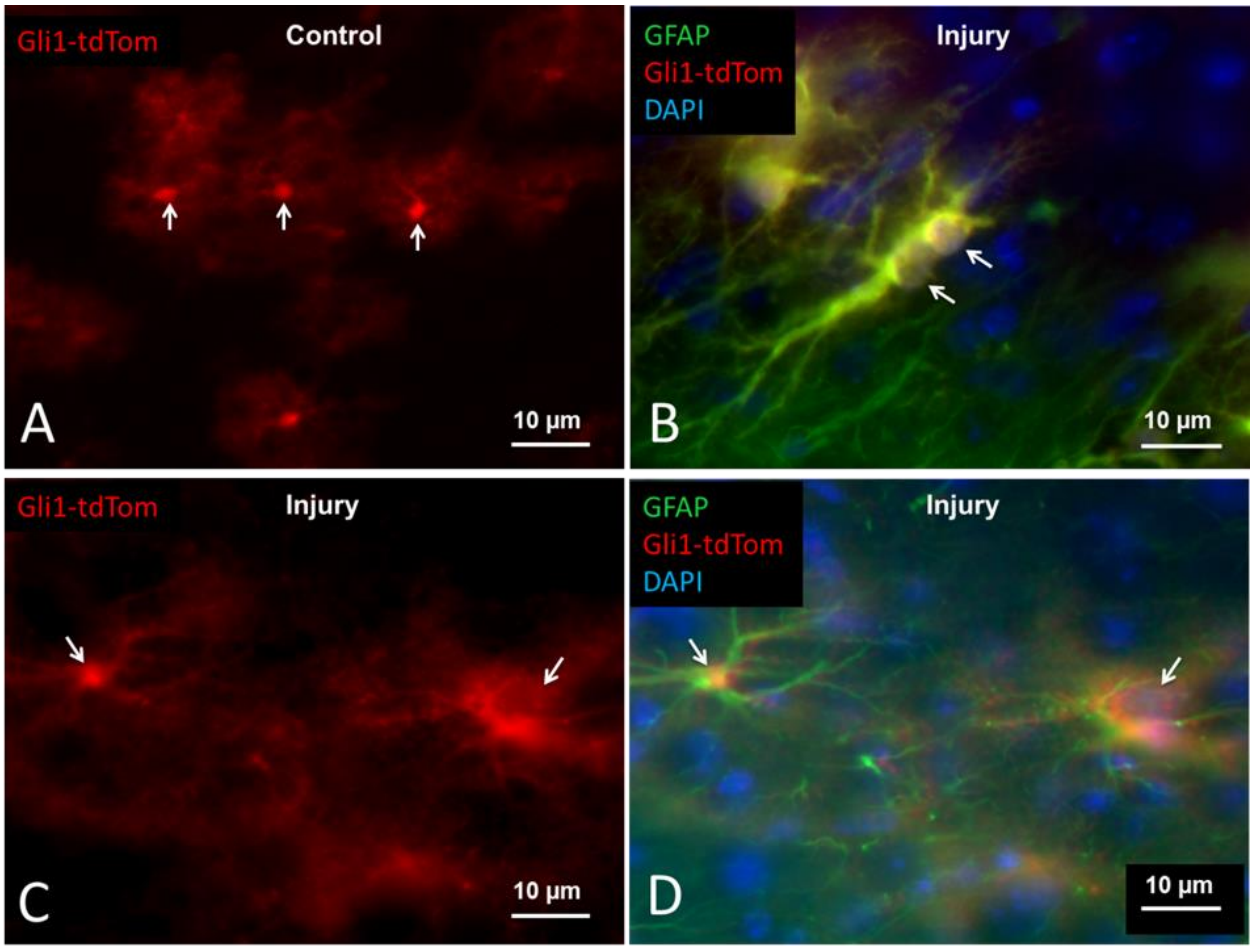


Figure 13. Gli1-tdTomato in cortical astrocytes. A. In the contralateral, uninjured cortex, Gli1-tdTomato appeared strong in the nuclei, surrounded by a cloud of cytoplasmic Gli1-tdTomato expression. B. A GFAP+ paired mirror astrocyte (white arrows) with Gli1-tdTomato in the nuclei and cytoplasm appears yellow, reflecting the colocalization of red tdTomato with green GFAP. C-D. A section from injured cortex, showing just the Gli1-tdTomato channel (C) and the composite image with Gli1-tdTomato, GFAP and DAPI (D). GFAP+ astrocytes (white arrows) and Gli1-tdTomato (C). The colocalization of tdTomato with GFAP and DAPI is shown in D.

Table 2 summarizes proteins in cilia and centrosomes of neurons (images not shown), astrocytes and neural stem cells. Astrocytes and neural stem cells share similar primary cilia and

centrosome protein characteristics. This observation may be relevant to the proliferative capacity of astrocytes after injury.

Marker	Astrocytes	Stem Cells	Neurons
ACIII	+	+	++++
Arl13b	++++	++++	+
MCHR1	-	-	++++
SstR3	-	-	++++
γ-Tubulin	++++	++++	-
Pericentrin	++	+++	-

Table 2: Protein markers of primary cilia and centrosomes from astrocytes, neural stem cells, and neurons. Primary cilia and centrosomes of astrocytes and neural stem cells have similar proteins. Postmitotic neurons have different protein markers compared to astrocytes and neural stem cells. The GPCRs (MCHR1 and SstR3) are present in some but not all neuronal cilia, but were found in none of the polydendrocytes or stem cells. Observations on neural stem cells (adult SGZ) from Samip Bhattarai Doctoral Thesis (2015)

3.3 Discussion

Most astrocytes possessed a primary cilium. Astrocytes are identified by their star shaped morphology and the expression of GFAP (Cahoy et al., 2008). GFAP is expressed highly in fibrous astrocytes of the white matter and is expressed in low levels in protoplasmic astrocytes (Cahoy et al., 2008, Zhang and Barres, 2010). The low levels of GFAP in protoplasmic astrocytes were detectable using confocal microscopy. Arl13b+ primary cilia were identified in 98% of GFAP+ astrocytes in the healthy mouse cortex.

Astrocytes and neural stem cells share similar cilia/centrosome proteins, transcription factors and electrophysiological responses (Fukuda et al., 2003, Kriegstein and Alvarez-Buylla, 2009). Cilia of both astrocytes and neural stem cells were characterized by high levels of Arl13b and low levels of ACIII, and absence of SstR3 and MCHR1. The centrosome markers γ-tubulin and pericentrin showed noticeable differences in astrocytes and neural stem cells (higher γ-tubulin staining intensity, lower pericentrin). Astrocytes and neural stem cells express SOX2 and musashi1, which are transcription factors present in cells with proliferative capacity (Komitova and Eriksson, 2004, Wagner et al., 2013). In neural stem cells primary cilia are thought to respond to proliferative signals (Chizhikov et al., 2007, Han et al., 2008, Spassky and Aguilar, 2008), whilst the role of primary cilia in astrocytes remains unknown. The similarities in expression of primary cilia markers in astrocytes and neural stem cells, suggests similarities in functions of primary cilia in these populations. Astrocytes can proliferate in response to injury, and their cilia and centrosome characteristics may be of importance in that role. Considering that injuries can induce astrocyte proliferation in any region of the brain, it is likely that most astrocytes are capable of proliferating. Alternatively, there may be a small population of as yet unidentified astrocyte progenitors, dispersed evenly throughout the brain parenchyma contributing new astrocytes *in-situ* following injury (Holmin et al., 1997, Fawcett and Asher, 1999), since migration doesn't appear to contribute new astrocytes found near the injury site (Bardehle et al., 2013).

The primary cilia of astrocytes did not express the ciliary GPCRs SstR3 or MCHR1, whose presence might otherwise suggest a role for these organelles in detection of neurotransmitters or hormones. Primary cilia of astrocytes did, however, contain ACIII, which is

a common target of GPCR activation. In certain populations of hippocampal neurons an inhibitory G-protein (Gpr175), which modulates Shh signal through cAMP, is also found in primary cilia (Singh et al., 2015), further suggesting the idea that G-protein coupled receptor signaling may be a hallmark of some primary cilia. Although the presence of ACIII suggests that primary cilia may be involved in GPCR signal transduction of astrocytes, the ligands and corresponding cilia-localized receptors remain to be identified.

Following stab injury, Arl13b+ primary cilia of astrocytes adjacent to the injury core decreased in length and incidence. Primary cilia were most likely resorbed in astrocytes that entered the cell cycle, following injury. A similar tendency towards an injury-induced decrease in incidence was observed in ACIII+ primary cilia of astrocytes (not statistically significant). There could be a differential loss in ACIII and Arl13b in cilia of astrocytes following injury, with ACIII having a more fundamental role and hence being retained longer. Future studies using colocalization of both primary cilia markers in astrocytes will help elucidate the dynamics of ciliary ACIII and Arl13b following injury. Assuming that primary cilia resorption occurs in astrocytes entering the cell cycle, resorption could occur through active or passive mechanisms. An active mechanism might involve a negative feedback that serves to stop further activation of proliferative pathways localized in primary cilia. In this model, a proliferative cue might trigger resorption of the primary cilium. The phosphorylation of the tubulin deacetylase HDAC6 through the kinase Aurora A, leads to ciliary disassembly (Pugacheva et al., 2007); a similar mechanism might be involved in the cilia loss observed after injury. An alternative mechanism for cilia loss involves resorption of cilia as an inadvertent byproduct of the dramatic cytoskeletal transformations that proliferating astrocytes experience. Considering that centrosomes assemble

microtubules for the mitotic spindle in proliferating cells, resorption of the microtubule-based cilium may be related to centrosome-mediated cytoskeletal remodeling.

While this study was in progress, Kasahara et al. (2014) published a study addressing the incidence and length of primary cilia in astrocytes using the markers Arl13b and ACIII in the somatosensory cortex of 8-week-old C57BL/6 mice, similar to the control mice used in the present study. Kasahara found that in S100β+ astrocytes, the incidence of primary cilia is 88% for Arl13b and 48% for ACIII. These numbers were lower than our observed incidences of 98% and 92% for Arl13b and ACIII, respectively. Kasahara also found that the length of primary cilia in astrocytes is approximately 3.3 µm, which is 40% shorter than the 5.5 µm cilia length we identified. These discrepancies may be related to differences in immunostaining or microscopy techniques. Kasahara used epifluorescence microscopy, whereas the present study used confocal microscopy. Confocal microscopy provides a better signal-to-noise ratio through reduction of stray fluorescence, thereby improving the detection of weaker signals. This would preferentially increase the detection of ACIII+ cilia, since the intensity of ACIII is considerably weaker compared to Arl13b in astrocyte cilia. In addition, while we measured cilium incidence and length from 3D confocal stacks, Kasahara apparently collected data on cilium incidence while viewing tissue sections through an epifluorescence microscope, and measured length from 2D photographs of each cilium. Using confocal microscopy we identified 48% more ACIII+ primary cilia and 10% more Arl13b+ primary cilia in astrocytes compared to Kasahara's epifluorescence study. Another point of contrast between this study and Kasahara's is the astrocyte marker used; Kasahara used S100β compared to our use of GFAP. GFAP is a more specific marker for astrocytes than S100β (Steiner et al., 2007). In the cortex, 98.4% of GFAP+ cells express S100 β

in the 8-week-old mouse (Raponi et al., 2007). However, S100β stains not only astrocytes, but also oligodendrocytes and microglia (Dyck et al., 1993, Adami et al., 2001, Deloulme et al., 2004). The lower incidence of ciliation encountered in Kasahara's results might be attributed to the expression of S100β in microglia and oligodendrocyte lineage, two populations that are less ciliated than astrocytes.

Astrocytes maintain separate, non-overlapping domains with neighboring astrocytes. Exceptions to this pattern are a small population of paired mirror astrocytes (Bushong et al., 2002, Xu et al., 2014). The nuclei of paired mirror astrocytes are positioned near each other with cytoplasmic processes polarized away from each other. Paired mirror astrocytes were more prevalent in injured mice compared to control. The present study is the first characterization of paired mirror astrocytes following injury. Paired mirror astrocytes are probably recently divided cells (Ge et al., 2012), which share adjacent territories immediately following mitosis, as visualized *in vivo* (Bardehle et al., 2013). These results are consistent with the idea that primary cilia have been resorbed before cells enter mitosis. There is evidence of astrocyte proliferation following injury using the cell cycle marker BrdU (Hampton et al., 2004). Although previous results from our lab have found the proliferation marker Ki67 in some paired mirror astrocytes, the present study found Ki67 to be absent in the examined single or paired mirror astrocytes. The discrepancy with the results of the BrdU study could be attributed to a short lived expression of Ki67+ in proliferating astrocytes post injury, due to a short cell cycle duration (Ernst and Christie, 2006). During embryonic retinal development the length of the cell cycle is inversely proportional to rates of proliferation, most likely due to changes in length of the S and G_1 phases (Alexiades and Cepko, 1996), it is possible that astrocytes following injury revert to embryonic

dynamics of proliferation, including a shortened cell cycle. In the mice with induced expression of Gli1-tdTomato, the presence of label in paired mirror and single astrocytes suggests that astrocytes and/or their progenitors in both the injured and control cortex had recently responded to Shh signal, considering that Gli1 is a marker of Shh activity (Ferent et al., 2013). Future studies are necessary to understand the relationship of paired mirror image astrocytes, the cell cycle and primary cilia. The proliferation marker PCNA is upregulated in astrocytes following injury and is known to be expressed longer than Ki67 during the cell cycle (Bologna-Molina et al., 2013, Liu et al., 2014). The use of PCNA will help capture a wider window of the cell cycle in proliferating astrocytes.

Astrocytes have been shown to respond to Shh following injury (Sirko et al., 2013). It is likely that Shh signaling is mediated by primary cilia in astrocytes, as demonstrated in many other cell types, although the direct involvement of primary cilia in the astrocyte response has not been shown. Also found in the primary cilia of astrocytes is the GTPase Arl13b, which has been linked to Shh signaling. In the absence of Arl13b, Smo is constitutively enriched in cilia, suggesting that the presence of Arl13b in astrocytes might serve as a brake on the mitogenic Shh pathway (Larkins et al., 2011). The presence of Arl13b in astrocytes may have a role in cell cycle reentry by regulating the entrance of Smo into the cilium and the subsequent activation of Smo within the cilium by the ligand Shh. The idea that primary cilia resorption acts as a checkpoint for cell cycle reentry has been proposed (Jackson, 2011, Goto et al., 2013, Ke and Yang, 2014).

In the CNS, the sources and targets of Shh signaling may depend on region, developmental age and tissue injury. During embryonic development of the nervous system, ventral tissues secrete Shh, resulting in a dorso-ventral gradient of Shh that is necessary for

proper morphogenesis. In the adult mouse cortex, the sources of Shh are neurons, and astrocytes are the chief target (Garcia et al., 2010). Astrocyte function and gene expression is influenced by the release of Shh by neurons (Farmer et al., 2016). Following injury astrocytes upregulate Shh and proliferate in response to Shh (Amankulor et al., 2009, Bardehle et al., 2013, Sirko et al., 2013). In the injured mice, astrocytes start expressing Shh (Becher et al., 2008, He et al., 2013) and Gli1, a downstream component of the Shh pathway (Amankulor et al., 2009, Pitter et al., 2014). Increased Shh production by astrocytes *in vitro* leads to dedifferention of astrocytes, which involves downregulation of mature astrocyte markers (Yang et al., 2012). Expression of Shh by injured astrocytes recapitulates the expression of Shh by neural stem cells from the neurogenic regions of the brain (Lai et al., 2003, Machold et al., 2003, Jiao and Chen, 2008), possibly creating the niche microenvironment necessary for proliferation (Sirko et al., 2013).

Failure to provide trophic support of Shh from neurons to astrocytes may lead to astrocyte reactive gliosis. Blocking Shh signaling in astrocytes of postnatal mouse through Cre-mediated ablation of Smo under the control of the mGFAP promoter, leads to reactive gliosis in astrocytes. Reactive gliosis is a response usually reserved to injured brains. This effect is most likely mediated through the overproduction of the repressor form of Gli3, since double knockdowns of Smo;Gli3 do not trigger reactive gliosis (Petrova et al., 2013). Reactive gliosis in $Smo^{loxP/loxP}$/mGFAP-Cre suggests that a constant trophic support of neuronal Shh is necessary to maintain astrocyte homeostasis (Garcia et al., 2010). When the Shh trophic support is disrupted, such as during the initial stages of mechanical injury, astrocytes will undergo reactive gliosis and eventually serve as their own source of Shh, which in turn creates a microenvironment that will allow them to proliferate and contain the injury. The idea that Shh

acts as trophic support of astrocytes through a primary cilia-mediated pathway could be tested using several models of primary cilia knockdowns. Astrocytes in these mice should display a higher level of constitutive reactive gliosis.

Astrocytes are a heterogeneous cell population (Oberheim et al., 2012, Schitine et al., 2015). This heterogeneity was not systematically investigated, but no evidence of heterogeneity was found in the cilia and centrosomes of astrocytes with the antibodies used in this study. Future research may uncover heterogeneity among astrocytes in proliferation potential, responsiveness to Shh, and dependence on signaling by primary cilia

CHAPTER 4

EFFECTS OF BRAIN INJURY ON PRIMARY CILIA OF POLYDENDROCYTES

4.1 Introduction

4.1.1 Polydendrocytes of the Central Nervous System

Polydendrocytes are identified mainly by the expression of NG2 chondroitin sulfate proteoglycan (NG2) and PDGFRα (Nishiyama et al., 1996, Rivers et al., 2008). In the central nervous system 98% of NG2+ cells also express Olig2 (Ligon et al., 2006). Polydendrocytes are uniformly distributed throughout the white and gray matter of the CNS (Dawson et al., 2003, Nishiyama et al., 2009), suggesting a widespread function.

4.1.2 Polydendrocyte Function

Polydendrocytes are considered oligodendrocyte precursor cells (OPC), since they mainly give rise to oligodendrocytes in the adult. During development polydendrocytes are also capable of giving rise to astrocytes (Zhu et al., 2008). Polydendrocyte proliferation throughout development correlates with the rate of oligodendrocyte production (Hill and Nishiyama, 2014). During embryonic development Olig2 is required for polydendrocyte specification; when Olig2 is deleted, a complete fate switch from oligodendrocytes to astrocytes occurs (Zhu et al., 2012). Polydendrocytes are also known to receive neuronal inputs and depolarize; however they do not produce action potentials, hence they are considered a non-excitable population (Bergles et al., 2000). Polydendrocytes contain receptors for the neurotransmitters GABA and glutamate (Lin and Bergles, 2004). Polydendrocytes occupy contiguous, non-overlapping regions, and appear to

be contact inhibited (Hughes et al., 2013). Polydendrocytes are dispersed throughout the brain parenchyma; some authors suggest this distribution is due to a role of polydendrocytes in the integration of communication signals (Funk et al., 2015).

4.1.3 Polydendrocyte Proliferative Capacities

Polydendrocytes retain proliferative capacities through adulthood and are the major neural proliferative cell population outside the neurogenic niches (Dawson et al., 2003, Robel et al., 2011, Bardehle et al., 2013). Polydendrocytes comprise approximately 70% of cycling cells in the CNS (Dawson et al., 2003). During perinatal development polydendrocytes give rise to mainly oligodendrocytes; about 40% of their progeny become astrocytes in the gray matter of the ventral forebrain (Zhu et al., 2008, Huang et al., 2014b). The capacity for polydendrocytes to produce astrocytes rapidly decreases after birth (Zhu et al., 2011, Huang et al., 2014b, Nishiyama et al., 2014). Postnatal polydendrocytes give rise to oligodendrocytes as well as resident polydendrocytes. These polydendrocytes are oligopotent *in vitro* depending on the growth conditions (Kondo and Raff, 2000) and *in vivo* (Rivers et al., 2008, Zhu et al., 2008). Genetic manipulations can be used to coerce polydendrocytes into producing functional astrocytes and neurons through the deletion of Olig2 and the upregulation of NeuroD1, respectively (Zhu et al., 2012, Guo et al., 2014).

4.1.4 Polydendrocyte Response to Injury

Polydendrocytes have a dynamic response following injury. Polydendrocytes can undergo drastic morphological and proliferative changes in response to demyelinating (Di Bello et al., 1999, Watanabe et al., 2002), mechanical (Hampton et al., 2004), ischemic (Zhang et al.,

2013), and excitotoxic injury (Wennstrom et al., 2004). As early as one day following injury, polydendrocytes begin to proliferate, reaching peak proliferation within the first two days after injury (Levine, 1994, Hampton et al., 2004)

4.1.5 Polydendrocyte Primary Cilia

Primary cilia respond to the mitogen Shh in certain proliferative populations, even though polydendrocytes proliferate, there are no reports of primacy cilia in polydendrocytes. The present report examines the incidence and length of polydendrocyte primary cilia in healthy and injured mouse brains. We will also explore the protein composition of cilia and centrosomes of polydendrocytes using several protein markers.

4.2 Results

4.2.1 Primary Cilia Were Ubiquitous in Polydendrocytes

NG2 is currently the best polydendrocyte marker, although it is not exclusive to polydendrocytes, nor does it label all polydendrocytes (Nishiyama et al., 1996, Ligon et al., 2006). In this study, the presence of both nuclear Olig2 and cytoplasmic NG2 was used as the criterion to identify a polydendrocyte (Kitada and Rowitch, 2006, Ligon et al., 2006). Nuclear Olig2 helped to identify the soma of those polydendrocytes with widespread, diffuse NG2 staining in the cytoplasm, which can otherwise make their identification ambiguous (Fig. 14). NG2+ polydendrocytes are distributed uniformly throughout the brain cortex (Hughes et al., 2013).

Polydendrocytes maintain non-overlapping territories separated from neighboring polydendrocytes, with the exception of paired mirror polydendrocytes (Xu et al., 2014). Using reconstructed 3D confocal stacks, we analyzed the incidence of Arl13b+ primary cilia in polydendrocytes. Primary cilia were found in 87 ± 3.6% of NG2+/Olig2+ polydendrocytes in the control somatosensory cortex (Fig. 17).

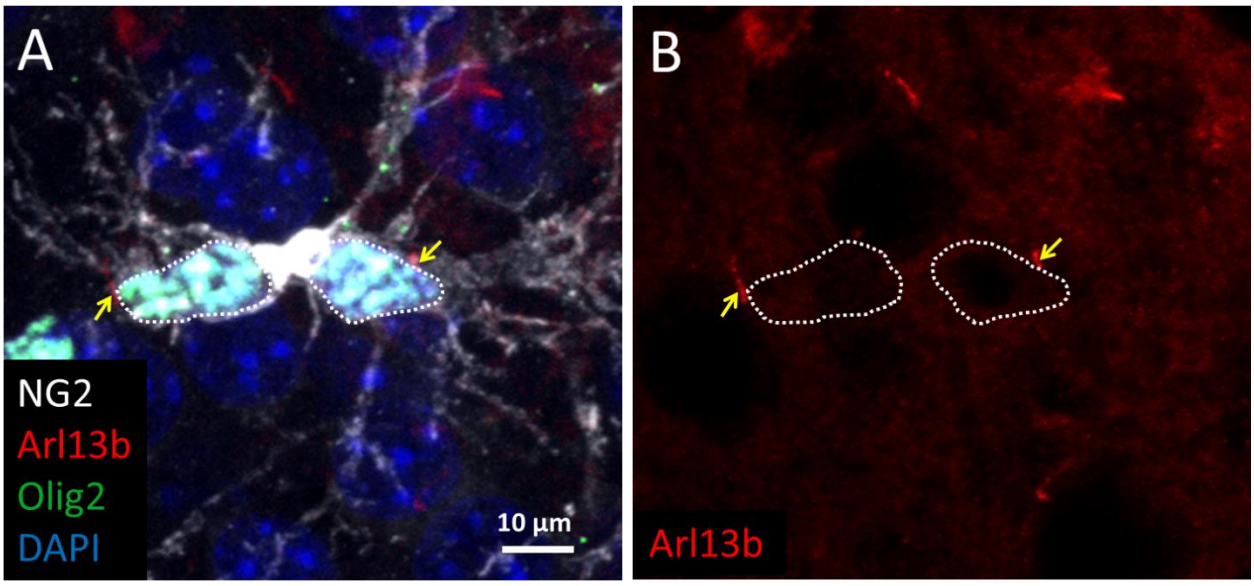


Figure 14: Primary cilia of polydendrocytes. A-B. The DAPI stained nuclei of NG2+/Olig2+ polydendrocytes are outlined. A. Paired mirror polydendrocytes with adjacent nuclei. Both paired mirror polydendrocytes are ciliated. B. Yellow arrows point to Arl13b+ (red) primary cilia in these polydendrocytes.

4.2.2 Proteins in Polydendrocyte Primary Cilia and Centrosomes

ACIII+ and Arl13b+ primary cilia were present in the great majority of polydendrocytes (Fig. 15A, B). SstR3 and MCHR1, which are GPCRs concentrated in neuronal cilia, were absent in polydendrocyte primary cilia (Fig. 15C, D). The staining intensity of Arl13b was slightly stronger than that of ACIII in polydendrocyte cilia. Ciliary Arl13b staining was more intense in astrocytes compared to polydendrocytes, while ACIII staining was more intense in polydendrocytes compared to astrocytes.

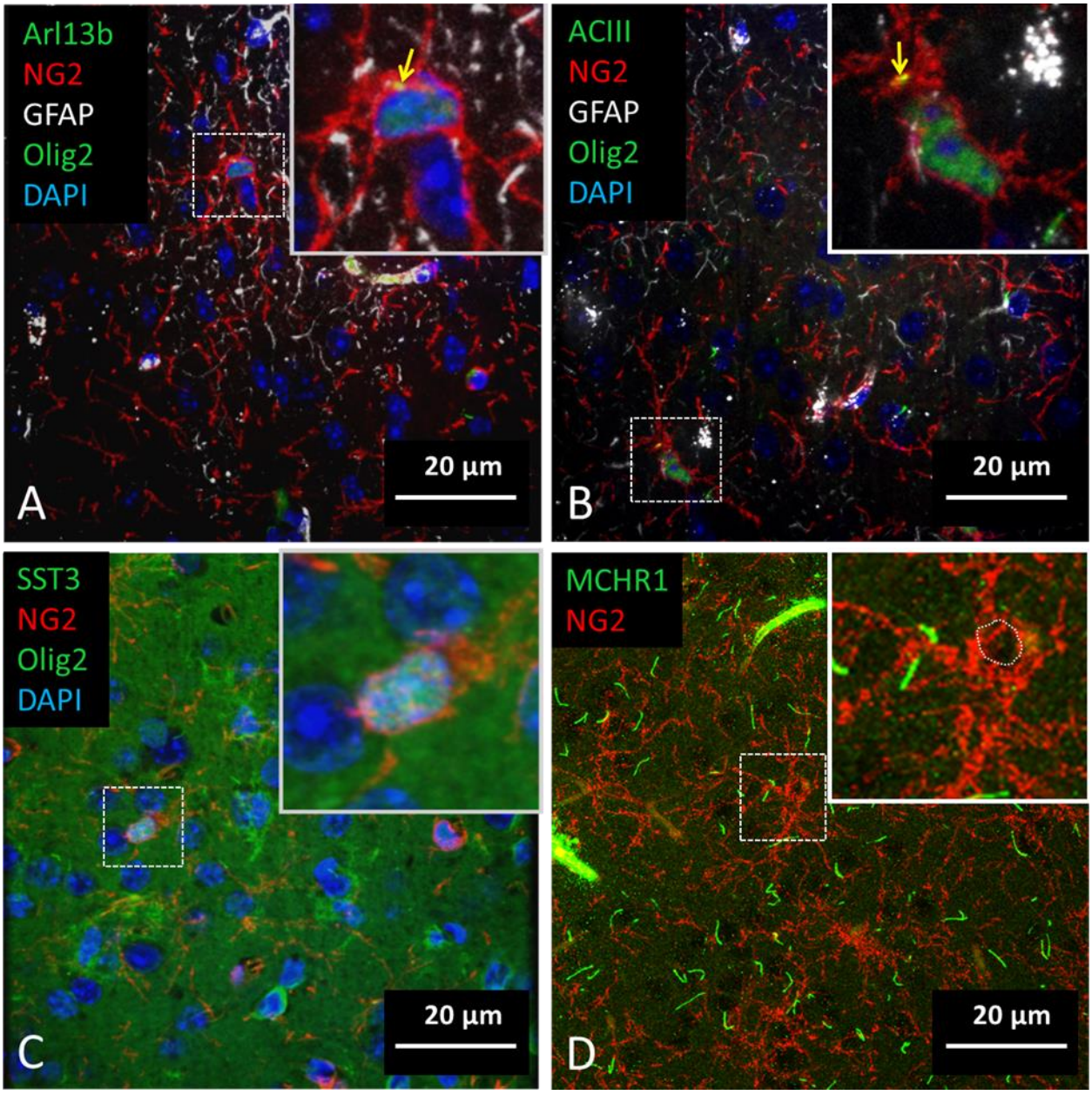


Figure 15: Primary cilia proteins of polydendrocytes. These confocal images show polydendrocytes, which are NG2+/Olig2+. The presence or absence of immunostained cilia in each cell was verified in 3D reconstructions of image stacks. A. NG2+ cells had Arl13b+ cilia (arrow). B. ACIII was also present in cilia of polydendrocytes (arrow). C, D. Polydendrocytes did not have SstR3+ (C) or MCHR1+ (D) cilia. The immunopositive cilia in C and D most likely belong to neurons.

We also analyzed the presence of two widely used centrosome markers, γ-tubulin and pericentrin, which have not yet been described in polydendrocytes. Both pericentrin and γ-

tubulin were found in the centrosome of polydendrocytes (Fig. 16). The intensity of γ-tubulin staining was consistently stronger than that of pericentrin, similar to the results in astrocytes and opposite to what is observed in neurons.

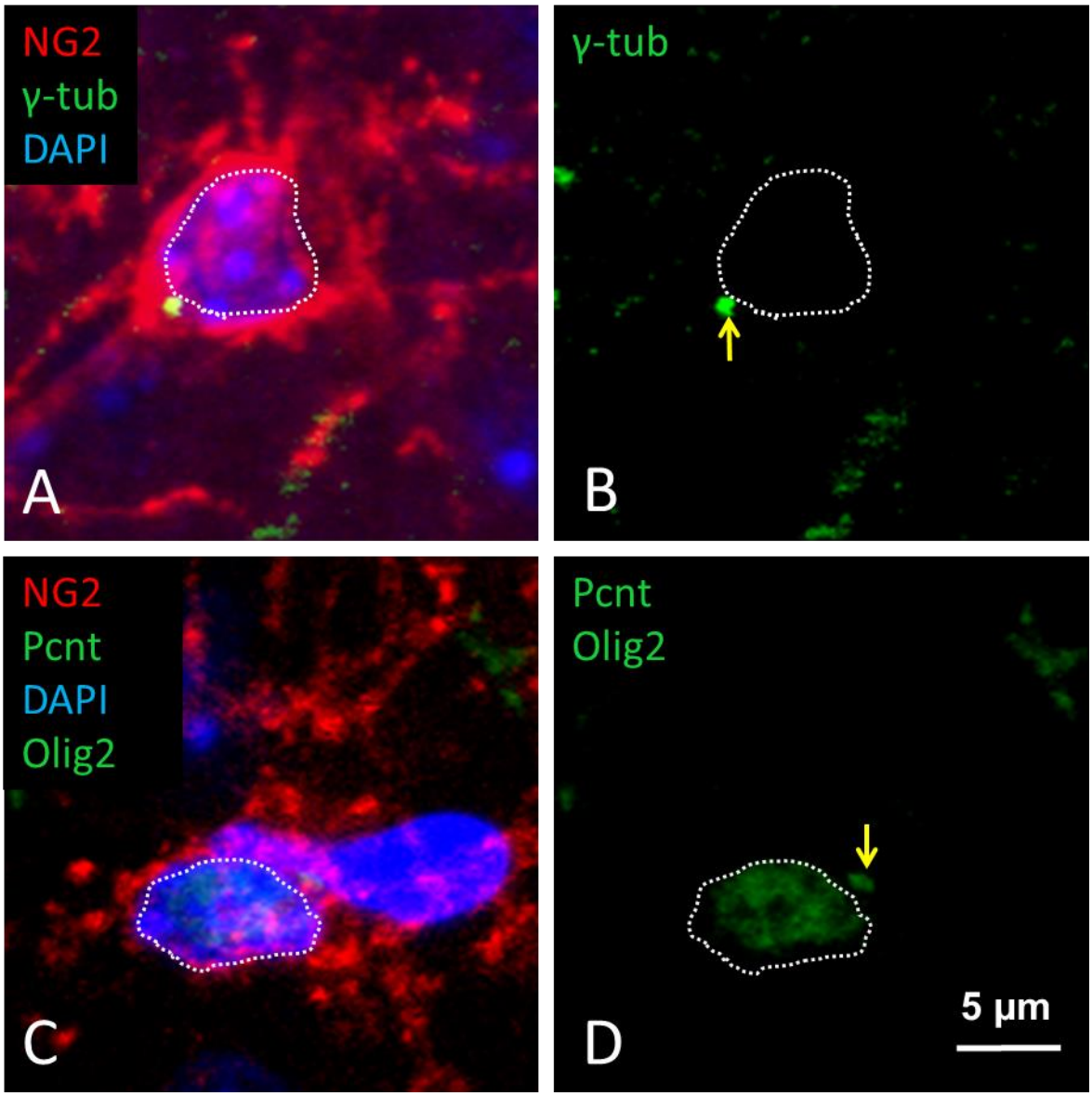


Figure 16: Pericentrin and γ-tubulin in the centrosome of polydendrocytes. A-B. Centrosomal staining (arrows) was present near the nucleus. γ-Tubulin (green) was expressed in all polydendrocytes examined (red). C-D. Pericentrin (green) was expressed in all polydendrocytes examined (red). Staining for γ-tubulin was noticeably brighter than for pericentrin. Staining for γ-tubulin was considerably less bright in polydendrocytes compared to astrocytes. Pcnt = Pericentrin; γ-tub=γ-tubulin

4.2.3 Effects of Cortical Injury on Cilia Length and Incidence in Polydendrocytes

Three days after cortical stab wound injury, there was a significant decline in incidence but not length, of primary cilia in polydendrocytes. Cilium incidence decreased by 41% after injury, from 87 ± 3.6% of the NG2+/Olig2+ polydendrocytes in control mice to 51 ± 7.1% in the injured cortex (Fig. 17A; $p < 0.011$; independent samples t-test; n=7, 109 total polydendrocytes). Cilia length increased non-significantly by 11%, from 2.9 ± 0.1 µm in control cortex to 3.2 ± 0.2 µm in injured cortex (Fig. 17B; $p < 0.117$; independent samples t-test n=7 mice; 67 total cilia).

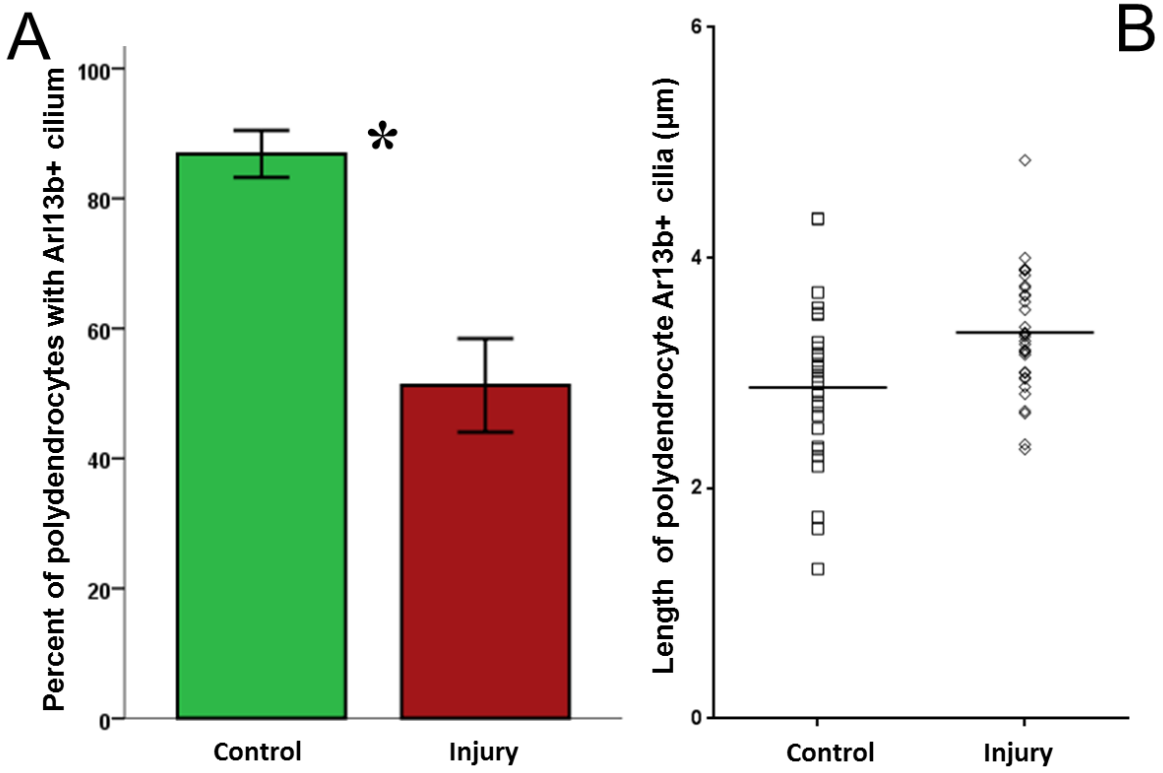


Figure 17: Changes in polydendrocyte primary cilia length and incidence following injury. A. Polydendrocyte primary cilia incidence decreased significantly by 41% (*$p < 0.011$) following injury. B. The 11% increase in cilia length was not statistically significant ($p < 0.117$). Each point represents one cilium. Mean ± SEM, based on n= 7 mice; 3 control and 4 injured mice.

4.2.4 Primary Cilia Incidence in Proliferating Polydendrocytes

Primary cilia incidence was significantly lower in proliferating polydendrocytes vs. non-proliferating polydendrocytes in the injured cortex. Using the proliferation marker Ki67, we analyzed the incidence of ciliation of polydendrocytes undergoing the cell cycle and compared it to non-cycling polydendrocytes. Ki67 labels cells from G_1 through the end of mitosis (Prayson, 2002). Most Ki67+ polydendrocytes were in the interphase stage of the cell cycle, based on the absence of condensed chromatin (Fig. 18B). In injured mice, the incidence of cilia in polydendrocytes that were Ki67+ decreased by 57% compared with those that were Ki67-, from 83 ± 16 % in Ki67- cells to 36 ± 11 % in Ki67+ cells (Fig. 18C; $p < 0.015$; independent samples t-test; n=3 mice; 57 total polydendrocytes).

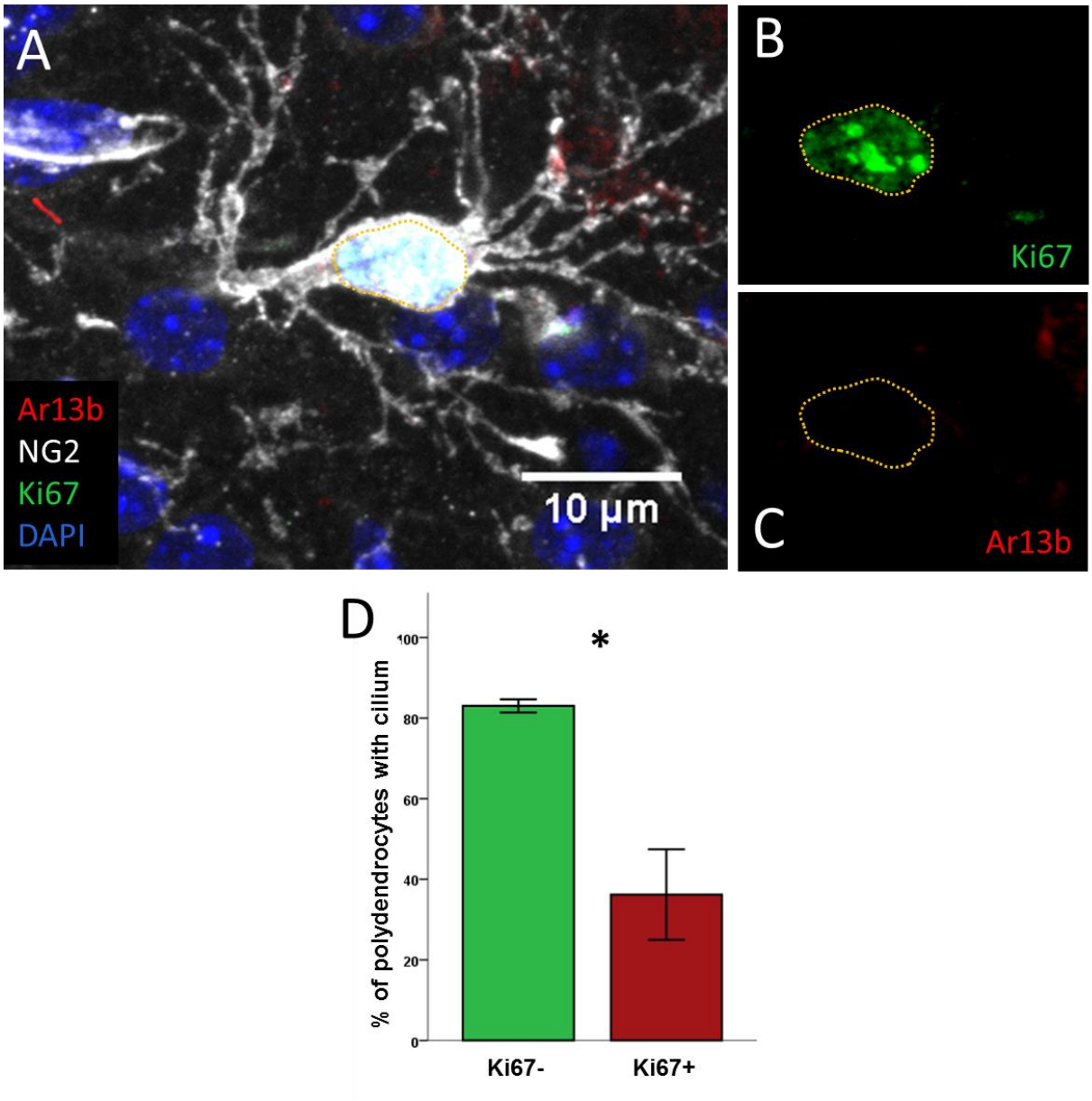
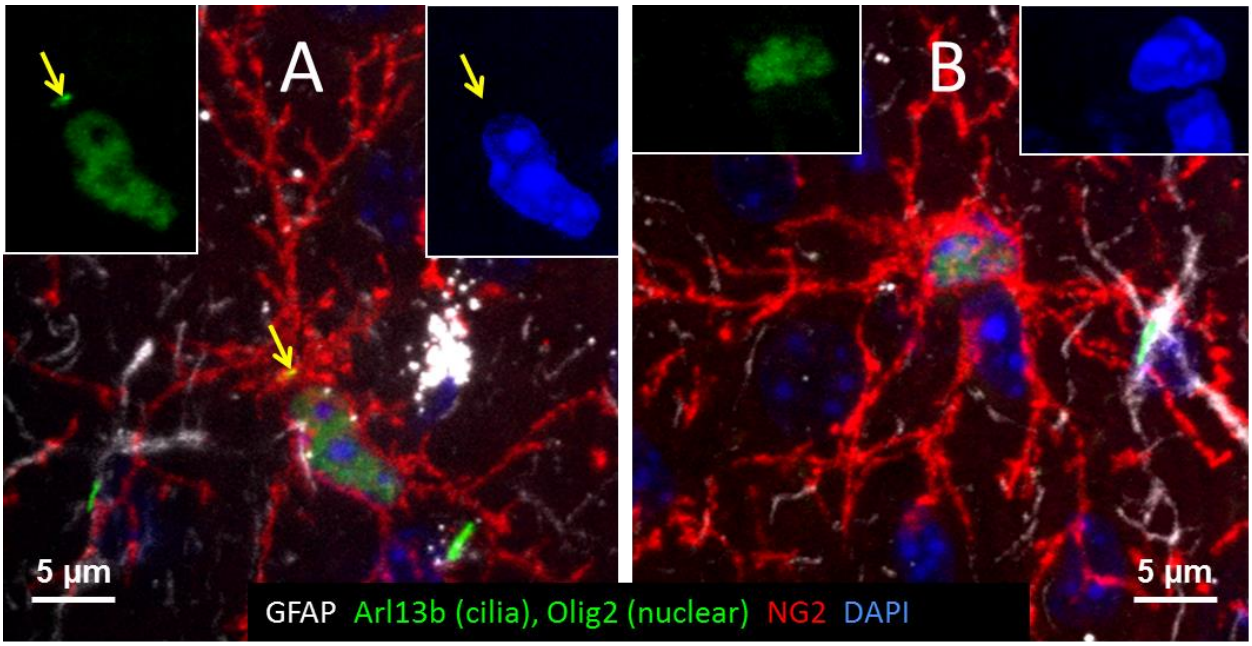


Figure 18: Primary cilia in proliferating and non-proliferating polydendrocytes after stab injury. A-C. Like most cycling polydendrocytes, this one lacked an Arl13b+ primary cilium. Nuclear Ki67+ (green) indicates that this NG2+ (white) polydendrocyte was in the cell cycle. D. Bar chart shows that the incidence of cilia was significantly lower in proliferating (Ki67+) compared to non-proliferating (Ki67-) polydendrocytes (*$p < 0.015$). Mean $\pm$ SEM, based on n= 3 mice.

4.2.5 Morphological Changes in Polydendrocytes Following Injury

Polydendrocytes possess diffuse stellate cytoplasmic neurites, and nuclei of various shapes. Polydendrocyte cytoplasm was stellate with no obvious polar asymmetry. The nuclei presented a wide range of shapes, with sphericity ratio (length/width) ranging from 1.2 - 3.6 (nearly spherical to quite elongated) in control mouse cortex (Fig. 19A, B). The variety in nuclear shapes might reflect a yet undetermined function, property or category of polydendrocytes. In order to further explore the significance of nuclear shape, we examined the sphericity ratio of ciliated and non-ciliated polydendrocytes in control and injured cortex. In control mice, nuclear sphericity was similar in ciliated and non-ciliated polydendrocytes (2.5$\pm$ 0.16 SEM and 2.3 $\pm$ 0.17 SEM, respectively). However, in the injured mice, sphericity was smaller (i.e., rounder nuclei) in non-ciliated compared to ciliated polydendrocytes (1.7 $\pm$ 0.12 SEM and 2.7 $\pm$ 0.10 SEM, respectively); (Fig. 19C; $p < 0.05$; ANOVA followed by Student-Newman-Keuls post-hoc analysis; n=7; 96 polydendrocytes).



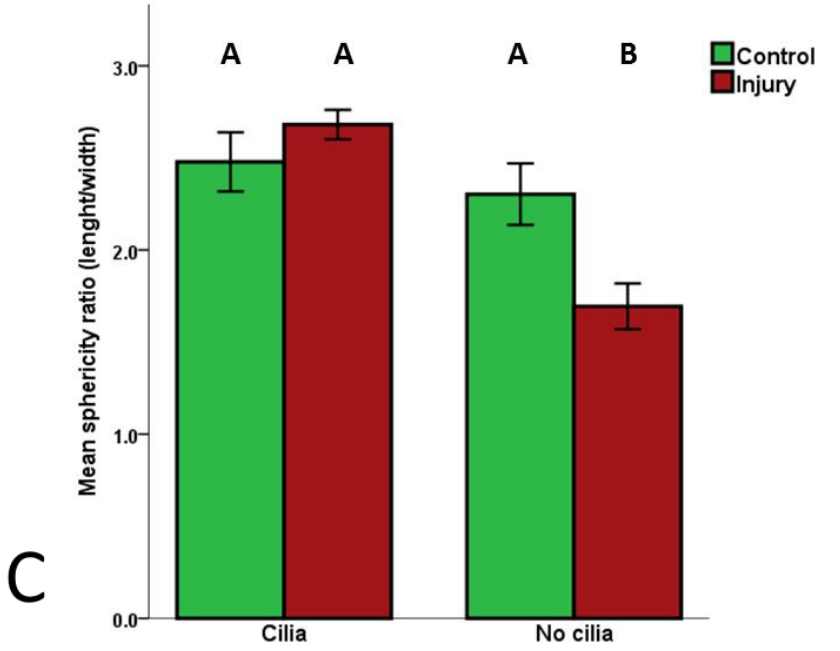


Figure 19: Sphericity ratios of ciliated and non-ciliated polydendrocytes following injury. A. Polydendrocytes (red) with elliptical nuclei were more common and usually possessed a primary cilium (green). B. Polydendrocyte nuclei can also have spherical profiles; this polydendrocyte lacks primary cilia as highlighted by the inset. C. In control mice, ciliated and non-ciliated cells showed no significant difference in sphericity ratio. Following injury non ciliated cells showed a significant decrease in sphericity ratio ($p < 0.05$ between A and B groups).

4.2.6 Gli1-tdTomato Was Not Found in Polydendrocytes

In the conditional Gli1 reporter mice from Dr. Garcia's lab, Gli1-tdTomato was not observed in NG2+ polydendrocytes. None of the polydendrocytes analyzed were labeled with Gli1-tdTomato in the somatosensory cortex (Fig. 20), on either the contralateral or injured side. However this deserves further investigation, since it was difficult to separate the strong Gli1-tdTomato signal from the NG2 signal, in the epifluorescence microscope, adding some uncertainty to the interpretation of colocalization. Nevertheless, based on identification of polydendrocytes by cell morphology, it appeared that none of the polydendrocytes were labeled with Gli1-tdTomato, even in the area of injury. As described earlier, most Gli1-tdTomato cells appeared to be astrocytes.

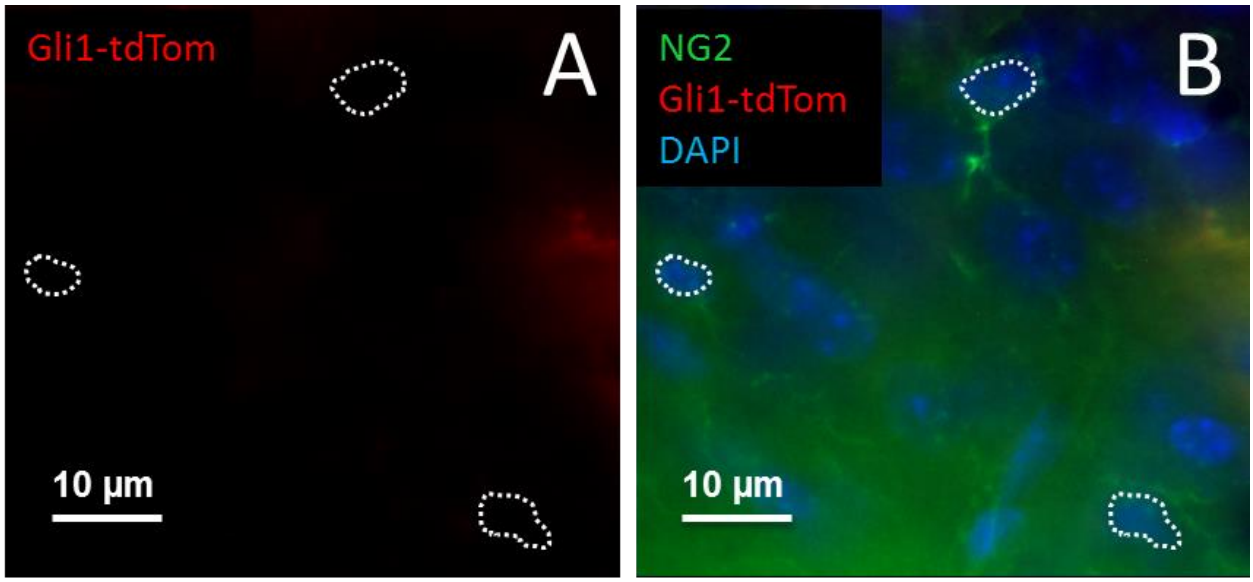


Figure 20: Gli1-tdTomato was not found in NG2+ polydendrocytes. Shown here are epifluorescence images from the somatosensory cortex contralateral to the injured side. A. No colocalization between NG2 and Gli1-tdTomato was observed in contralateral or injured cortex. Gli1-tdTomato (red) was expressed in cells that were most likely astrocytes. B. NG2+ polydendrocyte nuclei are outlined in white, without colocalization of Gli1-tdTomato (red channel, A).

Table 3 summarizes proteins in cilia and centrosomes of neurons (images not shown), polydendrocyte and neural stem cells. Polydendrocytes and neural stem cells share similar primary cilia protein characteristics. This observation may be relevant to the proliferative capacity of polydendrocytes.

Marker	Polydendrocytes	Stem Cells	Neurons
ACIII	++	+	++++
Arl13b	+++	++++	+
MCHR1	-	-	++++
SstR3	-	-	++++
γ-Tubulin	+++	++++	-
Pericentrin	++	+++	-

Table 3: Protein markers of primary cilia and centrosomes from polydendrocytes, neural stem cells and neurons. Primary cilia and centrosomes of polydendrocytes and neural stem cells have similar protein characteristics. The cilia and centrosomes of postmitotic neurons have different proteins compared to polydendrocytes and neural stem cells. The GPCRs (MCHR1 and SstR3) are present in some but not all neuronal cilia, but were found in none of the polydendrocytes or stem cells. Observations on neural stem cells (adult SGZ) from Samip Bhattarai Doctoral Thesis (2015)

4.3 Discussion

This is the first report of the presence of primary cilia in polydendrocytes. Polydendrocytes were identified by the immunoreactivity of cytoplasmic NG2 and nuclear Olig2 (Ligon et al., 2006). 87% of polydendrocytes (NG2+/Olig2+/GFAP-) contained an Arl13b+ primary cilium, which is a remarkably high incidence for a previously unreported organelle in polydendrocytes.

The primary cilia of polydendrocytes were similar to those of neural stem cells in that they had Arl13b and ACIII, but not SstR3 or MCHR1. The presence of ACIII suggests that these primary cilia are involved in signal transduction, since ACIII is involved in second messenger systems. The centrosome markers γ-tubulin and pericentrin were found in the centrosomes of polydendrocytes. Thus, the cilia and centrosome proteins of polydendrocytes were similar to that of neural stem cells and astrocytes, and different from those of neurons. The comparisons are consistent with the idea that primary cilia and centrosomes have characteristics that serve transduction of proliferative signals, since neural stem cell and polydendrocytes are proliferative cell populations and neurons are not.

The apparent resorption of polydendrocyte primary cilia is most likely due to cell cycle reentry. Polydendrocytes are the most proliferative macroglia in the brain and are known to divide robustly in response to injury (Nishiyama et al., 2014, Susarla et al., 2014). It is possible that resorption of cilia is involved in a negative feedback mechanism triggered by proliferative signals to limit proliferation, or cilia resorption may occur as part of the drastic reorganization in microtubule structure that occurs with cell division. Our results show that in polydendrocytes cilia are lost principally on proliferating cells, which express the proliferation marker Ki67. Unlike most neural stem cell populations, which are localized to particular niches, polydendrocytes are distributed throughout the brain. The widespread distribution of polydendrocytes across the brain might help contain injuries through proliferation, considering that injuries can occur at any region of the brain parenchyma.

Shh is involved in the proliferation of polydendrocytes during development and following injury (Ferent et al., 2013). Increasing Shh signaling leads to increased myelination *in*

vitro, through a mechanism which involves the cilium (Yoshimura and Takeda, 2012). Our results suggest that Gli1, a transcription factor downstream of Shh activation, is not expressed in polydendrocytes of healthy mice. Gli1tdTomato upregulation was also examined following injury, but the high signal of Gli1tdTomato and low NG2 signal made the results ambiguous. To the best of our knowledge we did not observe Gli1tdTomato following injury in polydendrocytes.

In order to further characterize polydendrocytes that lacked cilia, we evaluated nuclear sphericity. Unlike most glia and neurons, polydendrocytes have a variety of nuclear shapes. Our results indicate that polydendrocytes are most likely spherical in injured cortex, especially in the non-ciliated polydendrocytes. This analysis was performed in an effort to further delineate polydendrocyte subpopulations, but the results obtained might simply be a consequence of the cytoarchitectural dynamics that accompany cell division. In that polydendrocytes lose their elliptical shape when they enter the cell cycle, acquiring a round nuclear shape.

CHAPTER 5

EFFECTS OF BRAIN INJURY ON PRIMARY CILIA OF PERICYTES

5.1 Introduction

5.1.1 Pericytes of the Central Nervous System

Pericytes were first described in 1873 and were initially named Rouget cells. Due to their close apposition to endothelial cells surrounding blood vessels Rouget cells were renamed pericytes in 1923 (Dore-Duffy and Cleary, 2011). Brain pericytes are derived from both the mesoderm and neuroectoderm depending on their final location (Etchevers et al., 2001, Korn et al., 2002, Kurz, 2009). They are not classified as glial cells, and are associated with the vasculature Pericytes are a diverse population, with no known pan-specific marker. Pericytes are identified by their morphology, proximity to blood vessels, and expression of particular proteins (Birbrair et al., 2015). Pericytes are known to express NG2 and PDGFRβ (Krueger and Bechmann, 2010).

5.1.2 Pericytes and the Blood Brain Barrier

Pericytes help form the blood brain barrier (BBB), which limits entrance of blood-borne molecules to the brain parenchyma. Pericyte function is tightly linked to the regulation of the BBB through tight and adherens junctions (Hermann and ElAli, 2012). Pericytes have long processes that run parallel to blood vessels and ensheath endothelial cells, which communicate with pericytes through gap junctions (Bergers and Song, 2005, Krueger and Bechmann, 2010). Pericytes can also interact with endothelial cells through peg-socket cell to cell contacts (Rucker

et al., 2000). These cell to cell contacts contain N-cadherin and connexin-3 hemichannels that allow nutrient exchange between pericyte and endothelial cells (Gerhardt et al., 2000).

Pericytes surround endothelial cells in capillary arterioles and venules. Pericytes and endothelial cells form the basement membrane that surrounds blood vessels (Mandarino et al., 1993), which is part of the blood brain barrier (BBB). The BBB allows the passage of water, gases and lipid soluble molecules through diffusion. Other molecules necessary for brain function, including glucose, require selective transporters (Mandarino et al., 1993, Bergers and Song, 2005).

5.1.3 Pericyte Function

Pericytes play a role in regulation of capillary diameter, blood flow, and BBB formation and maintenance (Kutcher and Herman, 2009, Hamilton et al., 2010, Dore-Duffy et al., 2011). Pericyte number is positively correlated with blood pressure (Shepro and Morel, 1993). The regulation of capillary diameter occurs through calcium mediated pericyte contractility (Hamilton et al., 2010). The brain receives up to 20% of blood flow (Girouard and Iadecola, 2006). Pericytes help prevent the diffusion of potentially toxic molecules from the circulation into the brain. Pericytes have also been hypothesized to act as pluripotent stem cells.

5.1.4 Pericyte Precursors and Fates

A population of brain pericytes arises from the neuroectoderm during development. In the adult NG2+ pericytes can express nestin, these pericytes are angiogenic and multipotent in vitro and in vivo (Alliot et al., 1999, Crisan et al., 2009, Diaz-Flores et al., 2009, Birbrair et al., 2014, Nakagomi et al., 2015). Pericytes may be a source of mesenchymal stem cells in multiple

tissues, including brain, skeletal muscle, pancreas, adipose and placental tissues, making these cells a ubiquitous potential source of stem cells (Crisan et al., 2008, da Silva Meirelles L, 2016). Pericytes from human cerebral cortex can be reprogrammed into neuronal cells through induction of Sox 2 and Mash1 (Karow et al., 2012).

5.1.5 Pericyte Response to Injury

Pericytes are essential for glial scar formation and neovascularization in response to injury. The main cell type that forms part of the glial scar following injury is pericytes. Blocking the generation of pericyte progeny impedes scar formation (Popa-Wagner et al., 2006, Goritz et al., 2011, Dulauroy et al., 2012). Proliferation of pericytes increases in response to pathological stimuli (Ozerdem and Stallcup, 2003). Pericytes are necessary during vascularization, especially during development or in response to injury (Nakagomi et al., 2015). Neovascularization is triggered by hypoxia due to increased metabolic needs or due to injury related ischemia (Lee et al., 2011). Neovascularization occurs through the sprouting of endothelial tubes from existing blood vessels. Endothelial precursors called angioblasts give rise to new endothelial tubes forming a primitive vascular network (Winkler et al., 2011). Newly differentiated endothelial tubes release signals that recruit pericytes (Bautch and James, 2009). Pericytes are known to contribute to glial scaring and fibrosis following injury (Chen et al., 2011, Goritz et al., 2011). Following injury, pericytes can migrate into parenchymal tissue from the capillary wall (Dore-Duffy et al., 2000).

In certain injury models, such as brain ischemia, pericytes are initially lost in local blood vessels (Hall et al., 2014). Loss of pericytes leads to leaky capillaries, which leads to infiltration of neurotoxins, hypoxic induced loss of dendrites, and memory impairment (Bell et al., 2010).

Following injury, pericytes upregulate NG2, desmin and PDGFRβ. Pericyte proliferation can be observed as early as 1 day following insult, reaching peak proliferation 3-days after middle cerebral artery occlusion (Zehendner et al., 2015). During kidney fibrosis Shh induces proliferation of pericytes (Fabian et al., 2012), though the contribution of pericyte primary cilia following injury remains unexplored (Dore-Duffy et al., 2006).

5.1.6 Pericyte Primary Cilia

Pericytes are known to possess primary cilia (Biscoe and Stehbens, 1966, Wandel et al., 1984), and respond to Shh during development and following injury (Fabian et al., 2012, Yang, 2014, Rauhauser et al., 2015). Considering that primary cilia mediate Shh signaling and that Shh contributes to the injury response of pericytes, it is worth investigating the role of pericyte primary cilia following stab injury.

5.2 Results

5.2.1 Primary Cilia Were Present in Most Pericytes

Pericytes are known to express NG2, although NG2 t is not exclusive to pericytes, nor does it label all pericytes (Armulik et al., 2011, Birbrair et al., 2014). In this study we classified pericytes as NG2+/Olig2-/GFAP- cells adjacent to blood vessels with dense cytoplasm, unlike the diffuse cytoplasm of NG2+ polydendrocytes. The absence of Olig2 in pericytes also allowed us to distinguish pericytes from polydendrocytes, which areNG2+/Olig2+. Most pericytes contain a round nucleus visibly enveloped by a thin cytoplasm, dense with NG2. No specific blood vessel staining was used; however the end-feet of GFAP+ astrocytes and NG2+ polydendrocytes outlined the position of blood vessels (Fig. 21). Using 3D reconstructions of

confocal stacks, we analyzed primary cilia labeled with Arl13b. Primary cilia were present in 78 ± 13% of NG2+/Olig2-/GFAP- pericytes in the cortex of control mice.

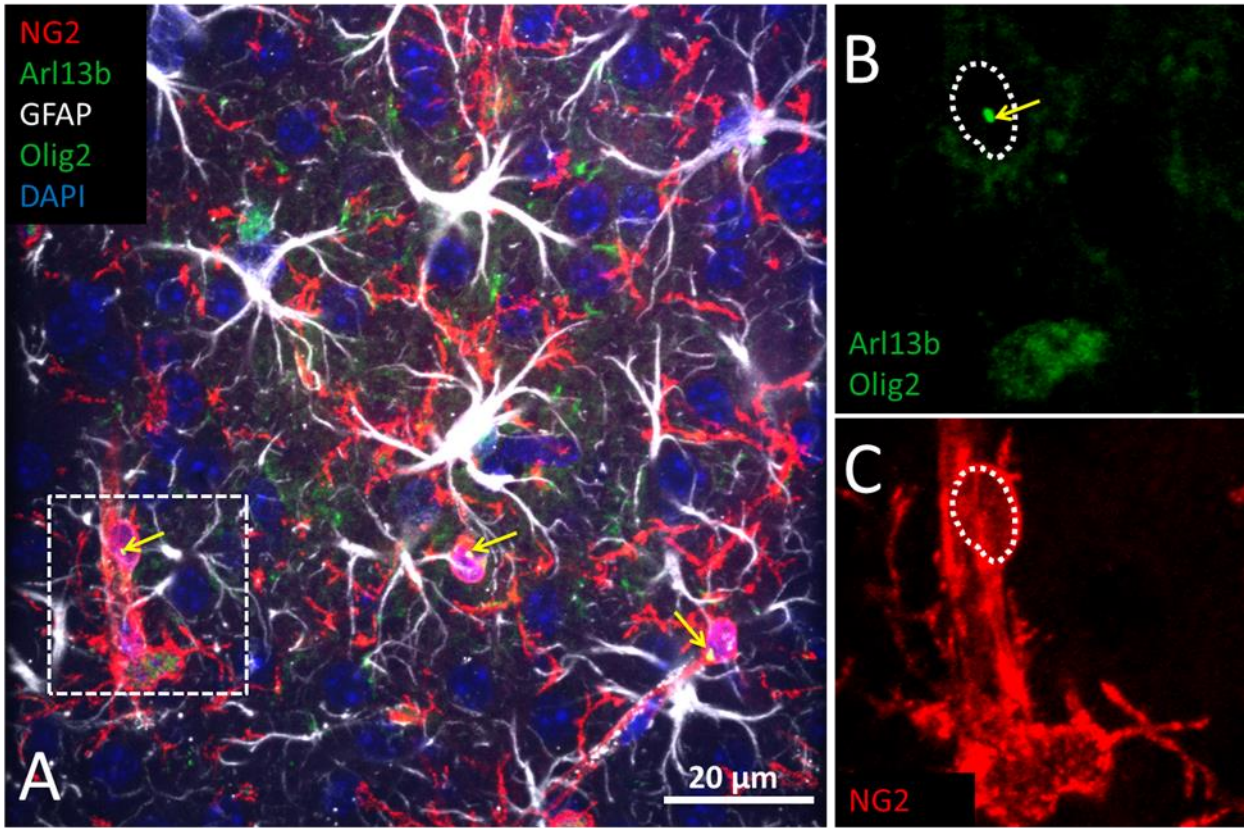


Figure 21: Primary cilia of pericytes. A-C. NG2+/Olig2-/GFAP- cells associated with blood vessels are pericytes (red), most of them were ciliated. A. The primary cilia of three pericytes are shown by yellow arrows. B. The dotted box is enlarged in B and C, which show separate channels. The nucleus of this ciliated pericyte is outlined, and the Arl13b+ primary cilium is shown by a yellow arrow. C. The nucleus of an NG2+ pericyte adjacent to a blood vessel is outlined.

5.2.2 Proteins in Pericyte Primary Cilia and Centrosomes

Authenticated cilia antibodies were used to study proteins in pericyte primary cilia (Fig. 22). Primary cilia in some neurons contain MCHR1 (Berbari et al., 2008) and/or SstR3 (Handel et al., 1999, Green et al., 2012), and the cilia of many neurons contain ACIII and Arl13b (Bishop et al., 2007, Kasahara et al., 2014). The primary cilia of pericytes were immunoreactive for

ACIII and Arl13b (Fig. 22A, B), and lacked immunoreactivity for the G-protein coupled receptors SstR3 and MCHR1 (Fig 22C, D).

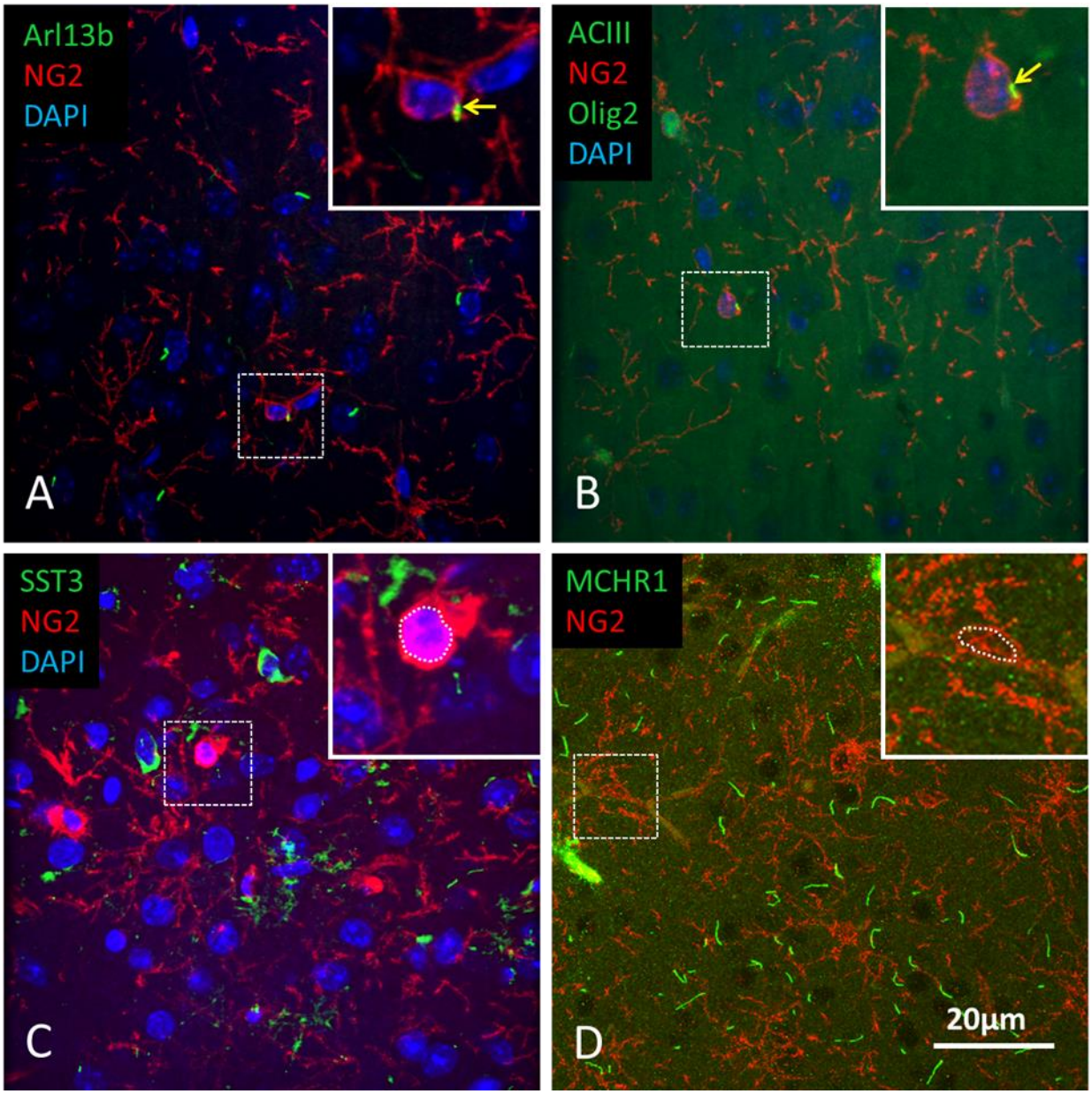


Figure 22: Primary cilia protein prolife of NG2 positive pericytes. A-B. Arl13b and ACIII primary cilia were present in pericytes. Cilia are shown in the insets with yellow arrows. C-D. MCHR1 and SstR3+ primary cilia were absent in pericytes (inset), but are present in nearby cells, which are most likely neurons.

Expression of centrosome markers pericentrin and γ-tubulin was also analyzed in pericytes. Pericentrin and γ-tubulin are present in the centrosomes of some cell types (Salisbury, 2004). Pericentrin and γ-tubulin were found in the centrosomes of NG2+ pericytes (Fig. 23). Centrosomes, like primary cilia, were found in close proximity to the nuclei of pericytes.

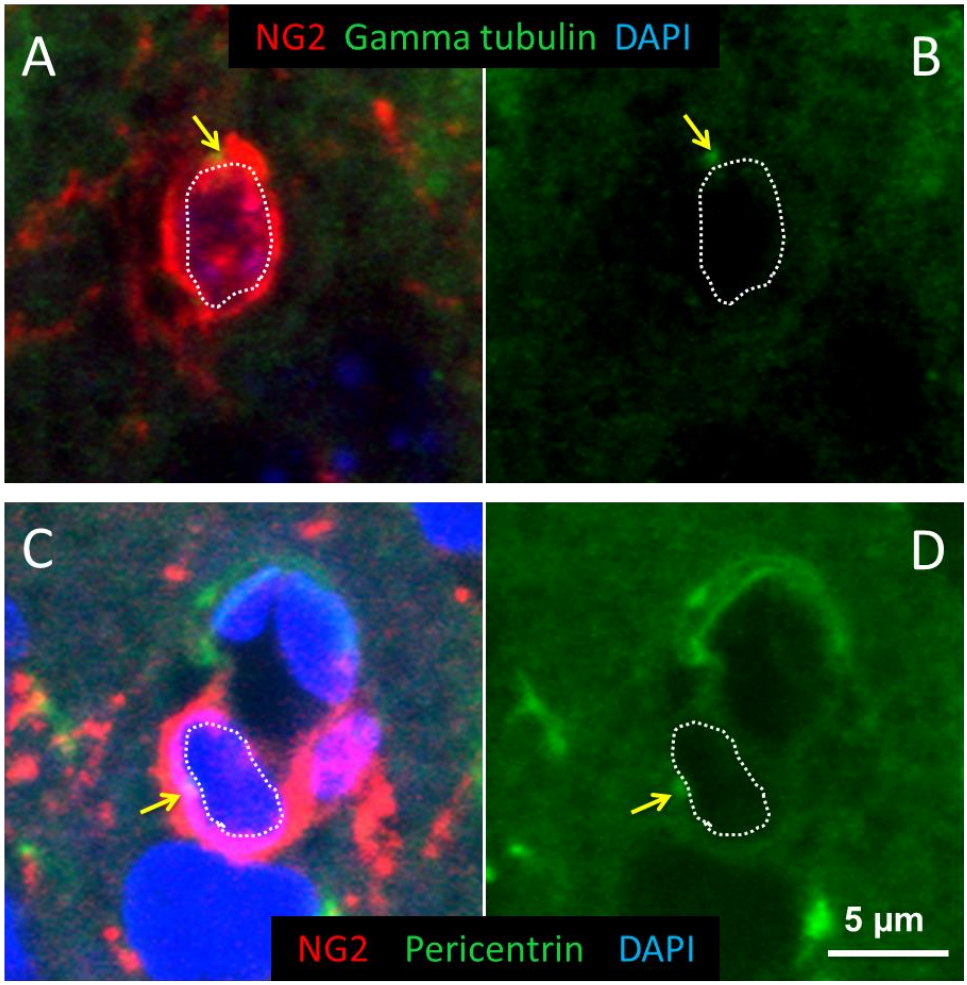


Figure 23: Pericentrin and γ-tubulin in the centrosome of pericytes. The centrosomal markers γ-tubulin and pericentrin were found in the centrosomes of NG2+/Olig2-/GFAP- cells associated with blood vessels. A-B. γ-Tubulin was in close proximity to the nuclei of pericytes. C-D. Pericentrin was also found adjacent to the pericyte nuclei.

5.2.3 Effects of Cortical Injury on Pericyte Primary Cilia Length and Incidence

Three days after stab injury in the mouse cortex, primary cilia incidence and length were not changed significantly. Pericyte primary cilia length and incidence were quantified using Arl13b. Primary cilia of injured mice were compared with those of age-matched unoperated controls. The length of primary cilia was obtained using 3D reconstructed confocal stacks. Cells from the injured cortex were evaluated in the penumbra region, within 174 μm parallel to the injury core. The incidence of pericyte cilia from the injured cortex had a non-significant increase of 12% when compared with pericytes from control uninjured mice. Of the NG2+ pericytes in control mice, 78 ± 13% were ciliated, while 3 days after injury this percentage was 87 ± 13% (Fig. 24A; $p < 0.224$; independent samples t-test; n=7 mice; 84 pericytes total). Pericyte primary cilia length from stab injured mice had a non-significant 3% decrease from 3.05 ± 0.31 μm in control to 2.96 ± 0.09 μm in injured cortex (Fig. 24B; $p < 0.798$; independent samples t-test; n=7 mice; 85 cilia total).

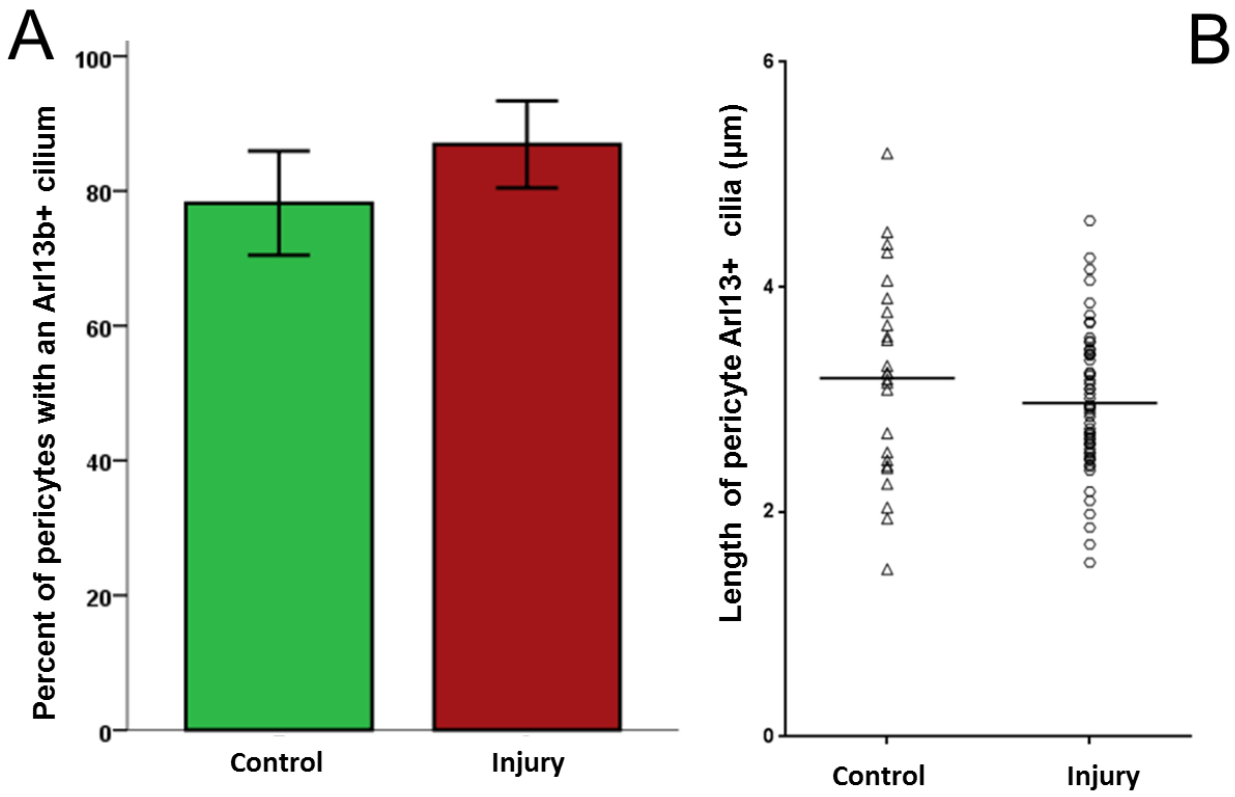


Figure 24: Incidence and length measurements of pericyte primary cilia in control and injured mice. A. Primary cilia incidence ($p < 0.224$) did not change significantly in pericytes following injury when compared to controls. B. Primary cilia length did not change significantly in pericytes following injury when compared to controls ($p < 0.798$). Each point represents one cilium. Mean + SEM, based on n= 7 mice; 3 control and 4 injured mice.

5.2.4 History of Gli1-tdTomato Expression in Pericytes

We stained brain sections provided by Dr. Garcia (Drexel University, Philadelphia, PA), from mice that conditionally express the red fluorescence reporter tdTomato under the control of the Gli1 promoter upon administration of tamoxifen. In adult C57BL/6 mice, a daily tamoxifen injection was given for 3 consecutive days. 2-weeks after the first tamoxifen injection a unilateral cortical scalpel-blade injury was made under anesthesia (Ahn and Joyner, 2005). 1-week post-lesion, the mice were sacrificed and perfused, and were later sectioned for immunohistochemistry. Thus, cells that were expressing Gli1 during tamoxifen induction and

any progeny cells resulting from their proliferation were permanently filled with the red fluorescent tdTomato reporter.

A small population of NG2+cells adjacent to blood vessels, which are presumably pericytes, had Gli1-tdTomato red fluorescence in the contralateral somatosensory cortex of injured mice. Gli1-tdTomato staining was intense in the nuclei of these pericytes (Fig 25A). Pericytes in the ipsilateral cortex also appeared to have Gli1-tdTomato label, though this interpretation was less clear due to the overwhelming Gli1-tdTomato signal from astrocytes.

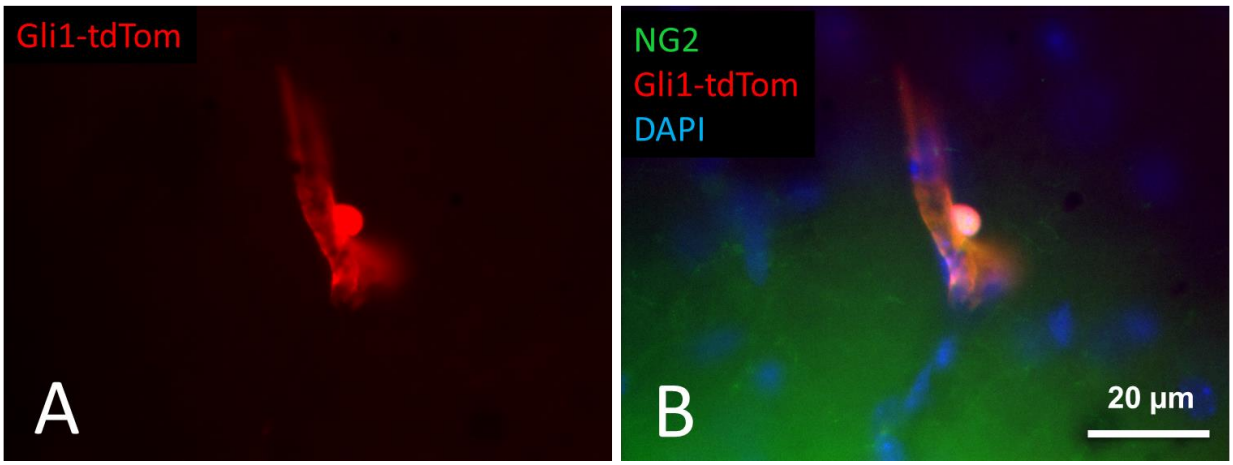


Figure 25: Gli1-tdTomato in pericytes. A. A small population of pericytes (green) in the vasculature show strong expression of Gli1-tdTomato (red). Gli1-tdTomato was present in the nuclei and cytoplasm of some NG2+ pericytes.

Table 4 summarizes proteins in cilia and centrosomes of neurons (images not shown), pericytes and neural stem cells. Pericytes and neural stem cells share similar primary cilia protein characteristics. This observation may be relevant to the proliferative capacity of pericytes.

Marker	Pericytes	Stem Cells	Neurons
ACIII	+++	+	++++
Arl13b	++++	++++	+
MCHR1	-	-	++++
SstR3	-	-	++++
γ-Tubulin	++	++++	-
Pericentrin	++	+++	-

Table 4: Protein markers of primary cilia and centrosomes from pericytes, neural stem cells and neurons. Primary cilia and centrosomes of pericytes and neural stem cells have similar protein characteristics. The cilia and centrosomes of postmitotic neurons have different proteins compared to pericytes and neural stem cells. The GPCRs (MCHR1 and SstR3) are present in some but not all neuronal cilia, but were found in none of the polydendrocytes or stem cells. Observations on neural stem cells (adult SGZ) from Samip Bhattarai Doctoral Thesis (2015)

5.3 Discussion

Pericytes are a diverse population that reside in the basement membrane of blood vessels and play an integral role in the healing process that follows brain injury. Pericyte populations are delineated by the expression of several markers. In our analysis we focused on NG2+ pericytes. Most pericytes in our sample - close to 80% - possessed a primary cilium.

Primary cilia appeared to be directed towards the brain parenchyma rather than the lumen of the blood vessel in most observed cases. This is unlike the results previous EM results which identified pericyte(s) directed towards the blood lumen (Biscoe and Stehbens, 1966). The lack of quantification makes contrasting these results challenging. It could be the case that the primary cilia of pericytes are directed towards the lumen of blood vessels as well as towards the brain

parenchyma. It is thought that in the healthy brain, neurons are the main sources of Shh (Garcia et al., 2010), which is congruent with the idea that pericyte primary cilia survey the brain parenchyma for Shh proliferative cues.

This is the first report of primary cilia in brain pericytes. The large incidence of primary cilia in brain pericytes suggest they have a function, which remains unexplored. To further elucidate the roles of brain pericytes we surveyed the presence of several cilia and centrosome markers.

Pericytes expressed ACIII and Arl13b, and lack the G-protein coupled receptors SstR3 and MCHR1. The absence of ACIII and Arl13b has been evaluated in the systemic operation of the brain (Wang et al., 2011, Higginbotham et al., 2012), but little attention has been paid to the indirect effects of these manipulations on the blood brain barrier. Leaks in the blood brain barrier are thought to contribute to neurodegenerative diseases such as Alzheimer's and Parkinson's. Understanding the composition of the pericyte primary cilium adds a new level of complexity to previously reported data.

The incidence and length of pericyte primary cilia did not change significantly following injury. These results are contrary to the expected primary cilia loss by proliferating pericytes after injury (Zehendner et al., 2015). The lack of significant change in cilia incidence after injury could be attributed to low rates of proliferation of pericytes in the injury penumbra. In a model of cortical controlled contusion injury and ischemic stroke, proliferating pericytes were mainly encountered in the injury core (Fernandez-Klett et al., 2013, Zehendner et al., 2015). If these pericytes migrate into the penumbra region following injury (Dore-Duffy et al., 2000), they will most likely acquire markers of maturity including a primary cilium.

We identified a small population of Gli1tdTomato + pericytes. Primary cilia are involved in a large number of signaling pathways; it is possible that the primary cilia of pericytes are not involved in the transduction of the Shh pathway, save for a small population of Gli1tdTomato+ pericytes. Future studies can shed light on the role of primary cilia in the proliferation potential of pericytes. Pericytes are classified as type 1 (NG2+/nestin-) and type 2 (NG2+/nestin+). Type-2 pericytes can proliferate while type-1 does not proliferate (Birbrair et al., 2014). It is likely that pericyte precursor cells or type-2 pericytes have a primary cilium that responds to Shh, resulting in pericytes expressing Gli1tdTomato. Understanding the role that a nearly ubiquitous organelle such as the primary cilium plays in the function of pericytes could prove a pivotal therapeutic target following brain insults.

CHAPTER 6

OVERALL CONCLUSION

Given the diverse protein composition of primary cilia, proteins targeted towards the primary cilium of individual cell types likely reflect the function of the primary cilium within that specific cell type. In astrocytes, polydendrocytes and pericytes we identified the presence and absence of components of similar signaling pathways. In the cilia, ACIII and Arl13b were present, while SstR3 and MCHR1 were absent. One of the advantages of concentrating signaling pathways in primary cilia is the 10^2-10^3 increase in concentration of proteins, compared to the cytoplasm (Nachury, 2014). For example ACIII, which is part of a second messenger signaling cascade, is concentrated 50-fold in olfactory neuron cilia compared to the total cell extract (Mayer et al., 2008). In olfactory neurons, the activation of G-protein coupled receptors found in the ciliary membrane is sufficient to depolarize the olfactory neuron and elicit an action potential. Another advantage of concentrating signals within the cilium is the use of targeting and sifting mechanisms, using protein primary structure and protein size (Stokes radii) respectively, which allow specific transport of proteins into and out of the cilium (Berbari et al., 2008, Lin et al., 2013). This compartmentalization provides an ideal environment for signal transduction. Pericentrin and γ-tubulin were present in the centrosomes of astrocytes, polydendrocytes and pericytes. Recruitment of pericentriolar materials, such as γ-tubulin is important for the formation of the microtubule ring complex in quiescent and cycling cells (Khodjakov and Rieder, 1999). It is possible that glial cells readily maintain a stock of γ-tubulin to respond promptly to insults through proliferation, similar to neural stem cells that reside in the neurogenic niches of the brain.

The present results suggest that both astrocytes and polydendrocytes may respond to injury by resorbing their primary cilia. Considering that both glial populations proliferate following injury, it is likely that cilia are resorbed due to the centrosome's active role in microtubule nucleation during the cell cycle. In polydendrocytes this was supported by the reduced incidence of cilia in cells with the proliferation marker Ki67. Unfortunately, we could not find as many proliferating astrocytes to confirm this observation for astrocytes. This is likely due to low rates of astrocyte proliferation and/or short cell cycle duration at survival day 3. Injections of Shh into the neocortex engenders a 30-fold increase in BrdU+ cells, 80% being GFAP+ astrocytes and 10% NG2+ polydendrocytes (Jiao and Chen, 2008). This proliferative cue is most likely transduced by the primary cilium.

There are several factors that can influence cilia length, including ciliary receptor activation and injury. Endothelial cells elongate their primary cilia in response to the activation of dopamine receptors found on their primary cilia (Abdul-Majeed and Nauli, 2011). Upregulation of the serotonin receptor 6 leads to a marked increase in cilia length (Guadiana et al., 2013), leading to the idea that increased activation of ciliary pathways can lead to an increase in cilia length in non-injured animals. The underlying mechanism of ciliary length control may be mediated through the cAMP second messenger system (Besschetnova et al., 2010). Previous studies have demonstrated that in other cell types injury can lead to an increase in primary cilia length. Primary cilia on renal epithelial cells sense fluid flow in the lumen of kidney tubules, and can increase in length after injury (Verghese et al., 2008, Wang et al., 2008). An Increase in primary cilia length is thought to increase the sensory capacity of cilia to counteract loss of function due to injury (Miyoshi et al., 2011). On the other hand over excitation of cilia in bone

cells may lead to cilia shortening (Delaine-Smith et al., 2014). In this study cilia of astrocytes and polydendrocytes shortened following injury. This is most likely as a consequence of cell cycle reentry, but could also involve stimulus-dependent changes in cilia length.

Astrocytes, polydendrocytes and pericytes have a dynamic response after brain injury that includes proliferation and changes in gene expression. The response of astrocytes and polydendrocytes to cortical stab wound injury is thought to be mediated in part by Shh (Amankulor et al., 2009), whose signaling components are located in the primary cilium. Survival of primary astrocytes in culture increases when they are exposed to Shh after serum starvation, through a primary cilium mediated mechanism (Yoshimura et al., 2011). Glial cells of the peripheral nervous system are also known to transduce the Shh signal through their primary cilium. Shh facilitates myelination in Schwann cells *in vitro* (Yoshimura and Takeda, 2012). Even though primary cilia are thought to be responsible for transduction of the Shh signal, no analyses of the involvement of glial primary cilia in brain injury *in vivo* have been published. The present study characterizes for the first time effects of brain injury on primary cilia in relation to cell type and proliferation. Primary cilia are potential therapeutic targets in afflictions of the nervous system ranging from cell death in neurodegenerative diseases, to uncontrolled glial proliferation in cancers.

The presence of primary cilia in proliferative cell populations is thought to block entrance to the cell cycle (Ke and Yang, 2014) but also detects proliferative signals (Han et al., 2008, Spassky et al., 2008, Li et al., 2011). The shortening of primary cilia observed in proliferative cells such as injured astrocytes and polydendrocytes may remove the block and allow cells to enter the cell cycle (Kim and Tsiokas, 2011, Li et al., 2011). Our results are also consistent with

the idea that primary cilia are resorbed in response to injury, but the specific pathways that mediate this response remain unclear.

Regulation of proliferation rates and subsequent glial scar formation play an important role in containing the injury site to promote neuronal survival following injury and return to homeostasis. Understanding the role of primary cilia in the sequence of events that unfolds following injury may lead to the development of treatments that will yield a better prognosis for a variety of nervous system insults.

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