EFFECTS OF PERIPHERAL NERVE INJURY ON THE CELLS OF THE DORSAL ROOT GANGLION: A ROLE FOR PRIMARY CILIA

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Primary cilia are ubiquitous sensory organelles found on most cell types including cells of the dorsal root ganglia (DRG). The DRG are groups of peripheral neurons that relay sensory information from the periphery to the CNS. Other cell types in the DRG include a type of glial cell, the satellite glial cells (SGCs). The SGCs surround the DRG neurons and, with the neurons, form functional sensory units. Currently are no reports describing the numbers of DRG cells that have cilia. We found that 26% of the SGCs had primary cilia. The incidence of cilia on neurons varied with neuron size, a property that roughly correlates with physiological characteristics. We found that 29% of the small, 16% of the medium and 5% of the large neurons had primary cilia.

Primary cilia have been shown to have a role in cell proliferation in a variety of cell types. In some of the cells the cilia mediate the proliferative effects of Sonic hedgehog (Shh). In the CNS, Shh signaling through primary cilia affects proliferation during development as well as following injury, but no studies have looked at this function in the PNS. The SGCs and neurons of the DRG undergo complex changes following peripheral nerve injury such as axotomy. One marked change seen after axotomy is SGC proliferation and at later stages, neuronal death. We found that following axotomy there is a significant increase in the percentage of SGCs with primary cilia. We also found a significant increase in the percentage of medium-sized neurons with primary cilia. In other experiments we tested the idea that Shh plays a role in SGC proliferation. When Shh signaling was blocked following axotomy we found decreased proliferation of SGCs. This is the first report of a change in the percentage of cells with cilia following injury in the PNS, and the first report of a role for Shh in SGC proliferation following axotomy.

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LIST OF ABBREVIATIONS

ACIII	Adenylyl cyclase 3
Arl13b	ADP-riboslylation factor-like protein 13B
BrdU	5-bromo-2'-dioxyuridine
CNS	Central nervous system
DRG	Dorsal root ganglia
GFAP	Glial fibliary protein
Gli (1-3)	Glioma (1-3) transcription factor
IFT	Intraflagellar transport
KIF3A	Kinesin-like protein
GLAST	Glutamate aspartate transrporter
HDAC 6	Histone deacetylase 6
Nde-1	Nuclear distributing gene E-homolog 1
NG2	Chondroitin sulphate proteoglycan
Odf-1	Outer dense fiber protein-1
PC-1	Polycystin-1
PC-2	Polycystin-2
Ptc	Patched
PKD	Polycystic kidney disease
PNS	Peripheral nervous system
SGCs	Satellite glial cells
Shh	Sonic hedgehog
SK3	Anti-potassium channel SK3 antibody
Smo	Smoothened
Sst3	Somatostatic type-3
THM-1	TRP-containing hedgehog modulator 1

INTRODUCTION

Primary Cilia: an Overview

Primary cilia are solitary ubiquitous hair-like sensory organelles that arise from the plasma membrane on most vertebrate cell types. Primary cilia have a 9+0 arrangement of microtubule doublets composed of acetylated α-tubulin. They lack the central pair seen in motile cilia, which generally have a 9+2 microtubule arrangement (Satir and Christensen, 2007). Primary cilia are maintained by protein complexes called intraflagellar transport (IFT) particles that transport components up and down the cilium (Rosenbaum and Whitman, 2002).

A Brief History of the Primary Cilium

The first detailed description of primary cilia, was that of Zimmerman in the late 1800s (Zimmerman, 1894). Zimmerman's work (Bloodgood, 2009) contained some striking observations about primary cilia, as well as hypotheses regarding their functions. It would take quite some time, however, to begin to test his hypotheses. Zimmerman noted that the primary cilium (then referred to as the central flagellum - *Centralgeissel*), was always associated with a centriole, in particular the centriole that was furthest from the nucleus. I now know that this is the older, or mother centriole (Ishikawa et al., 2005). This observation was confirmed in 2005 with the demonstration that cells deficient in a protein (Odf-1) required for maintenance of the mother centriole, were unable to form primary cilia. It was concluded that the centriole that anchors the primary cilium must therefore be the mother centriole (Ishikawa et al., 2005). Zimmerman's hypothesis that some primary cilia may have a sensory role was confirmed beginning in 2001, when primary cilia in the kidney tubule were shown to act as mechanical flow sensors (described in detail below) (Praetorius and Spring, 2001).

Although primary cilia gained the attention of researchers from the late 1800s to the mid-early 1900s, there followed a gap where little work was reported from about 1910 to the mid 1950s. A few reports were made during this time, however, with some striking observations and predictions. One study was reported by Cowdry in 1921 in which he describes the presence of primary cilia (which he called flagella) on thyroid cells, but stated that they most likely had no adaptive value (Cowdry, 1921). With very few exceptions, this idea of no adaptive value was generally accepted by scientists well into the late 1970s. Interestingly, Alverdes (1927) reported the presence of primary cilia on epithelial cells of the human rete testis. He also noted the association of the primary cilium with the centriole farthest from the nucleus. Alverdes postulated a sensory role for primary cilia, and went even further to propose that they most likely acted as a cellular receptor which received information from the physiochemical composition of the contents of the lumen of the rete testes (Alverdes, 1927). The hypothesis that primary cilia play a sensory role, in particular by receiving information from the physiochemical composition of the surrounding environment, was impressive for its time because little data existed which addressed the ability for cilia to act as a signaling organelle. Support for this idea had to wait until 2005,when it was discovered that primary cilia have a critical role in the Sonic hedgehog (Shh) signaling pathway (described in more detail below) (Corbit et al., 2005; Huangfu and Anderson, 2005; Wong and Reiter, 2008).

It was not until around the mid 1950s that primary cilia made a substantial comeback in the literature. This was a result of the development of transmission electron microscopy. Around this time, several studies reported the presence of primary cilia on a variety of cell types in many different species. In 1957 Duncan was the first to describe solitary cilia on neuronal cells, of the spinal cord (Duncan, 1957). Then, in 1958, Sotelo and Trujillo-Cenoz, described in

great detail the presence of primary cilia on neural epithelium in the developing chick (Sotelo and Trujillo-Cenoz, 1958). They noted the presence of only one cilium per cell, and that the cilium had a 9+0 microtubule organization. The first report of cilia on adult neurons was by Palay in 1960, who noted the presence of primary cilia on preoptic nucleus neurons of the adult goldfish (Palay, 1960). Around the same time, a report by Taxi (1961) described primary cilia on sympathetic neurons of the frog (Taxi, 1961; Bloodgood, 2009). Many additional reports of primary cilia on a variety of cell types and in different species were made around this time (from the 1950s- to early 1980s). For instance, Dahl in 1965, identified the presence of cilia on the glial cells of mammals and also noted that primary cilia were commonly found on neurons (Dahl, 1965). Another important study from this era was conducted by Sorokin (1968), who provided a detailed description of ciliogenisis of primary cilia and motile cilia. Sorokin's contribution included coining the term 'primary cilia', which is now the accepted term (Sorokin 1968). Nevertheless, throughout this renaissance of cilia studies, the general consensus continued to be that the primary cilium must be a rudimentary organelle that is most likely an evolutionary remnant without a function (Bloodgood, 2009).

While the development of the electron microscope enabled scientists to study the structure of primary cilia, it was the development of additional techniques, such as immunohistochemistry, along with advances in light microscopy, which allowed scientists to study the distribution of cilia in greater detail. The search for the functions of primary cilia is ongoing. The recent explosion in of research on cilia function was ignited primarily by the discovery of a link between primary cilia and human disease. A deficiency in primary cilia was linked to some forms of polycystic kidney disease (PKD), and provided the first case of a role of primary cilia in human disease. Subsequently, more human diseases were linked to primary

cilia, and eventually the term “ciliopathies” was coined to encompass these diseases. But it was the initial discovery of the link between cilia and PKD that held promise for showing that primary cilia can have critical functions.

With the development of sophisticated genetic and molecular methods, scientists have been able to begin to address the question of what the functions of primary cilia are. Furthermore, understanding the dynamics of this organelle allowed scientists to devise experiments that could induce changes in the organelle, such as assembly and disassembly, which allowed the possibility of furthering our understanding of primary.

Primary Cilia Morphology and Maintenance

Primary cilia are maintained by IFT particles that travel along the microtubules within the primary cilium (Pazour et al., 2000). There are two complexes of IFT proteins: IFTA and IFTB. The IFTB complexes are powered by kinesin and move their cargo toward the tip of the cilium in an anterograde direction, whereas the IFTA complexes are powered by dynein, and move toward the cell in a retrograde direction (Rosenbaum and Whitman, 2002) (see Figure 1).

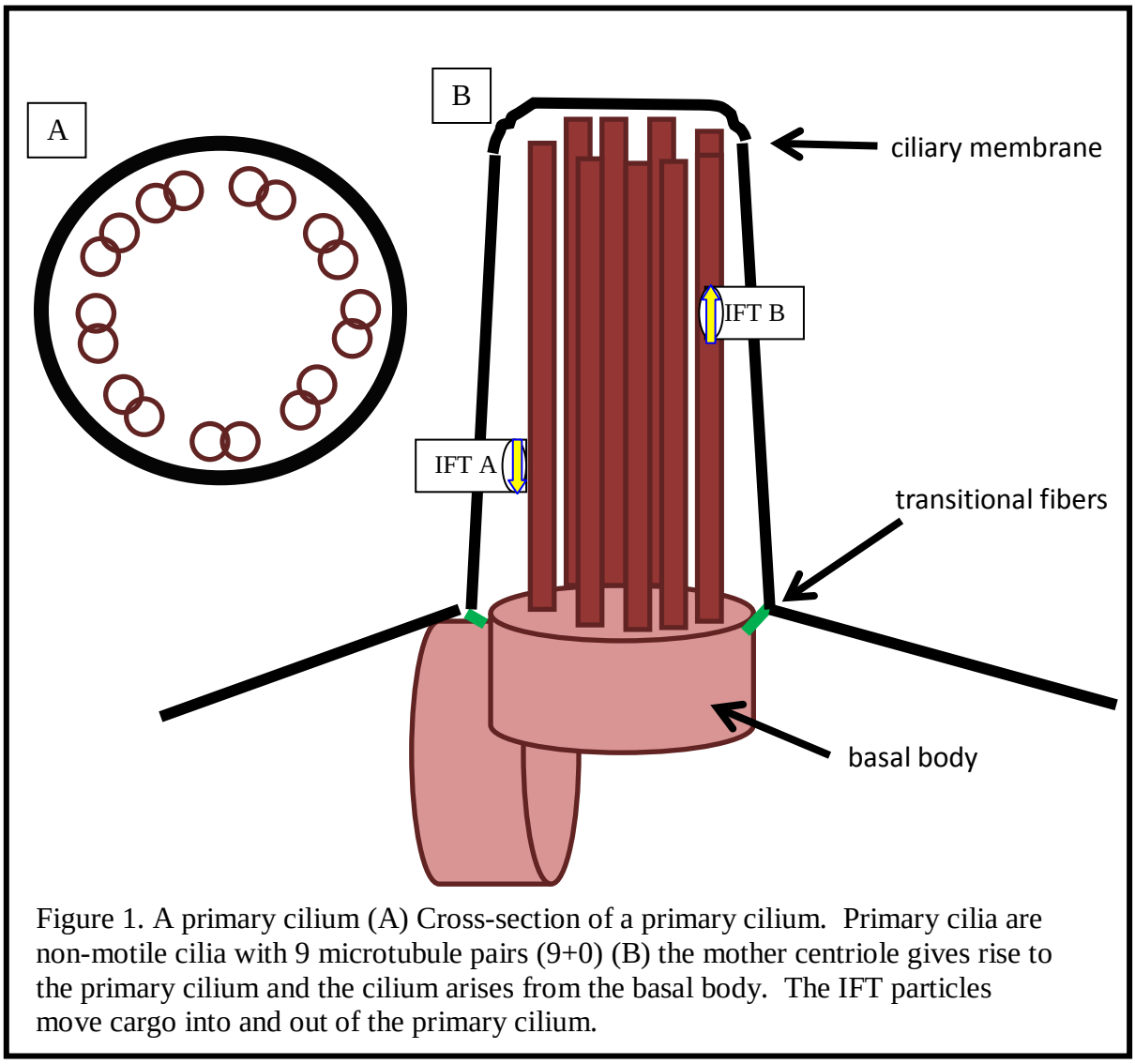

Figure 1. A primary cilium (A) Cross-section of a primary cilium. Primary cilia are non-motile cilia with 9 microtubule pairs (9+0) (B) the mother centriole gives rise to the primary cilium and the cilium arises from the basal body. The IFT particles move cargo into and out of the primary cilium.

Primary cilia have been shown to respond to chemical as well as mechanical signals. In some cell types, particularly in the nervous system, disruption of the primary cilium results in deficient sonic hedgehog (Shh) signaling (Goetz and Anderson, 2010). Neuronal primary cilia contain other types of receptors as well, suggesting a role in signal transduction via these receptors. For example, primary cilia on (at least some) neurons have been shown to contain the following receptors: somatostatin receptor type 3 (sst3) (Handel et al., 1999; Stepanyan et al., 2003), patched (Ptc) (Corbit et al., 2005), melanin concentrating hormone receptor type 1 (Berbari et al., 2008), dopamine receptor type 1 (Domire et al., 2011), and serotonin receptor

type 6 (Brailov et al., 2000; Berbari et al., 2008). These receptors are much more heavily concentrated on the cilium than in the rest of the cell. While the roles of these ciliary receptors have not been fully elucidated, recent research has provided some insights. For instance, reduced cilium length on neuronal precursor cells of the adult mouse hippocampus impedes proliferation, presumably because these shorter cilia cannot fully transduce the Shh signal (Bruenig et al., 2008).

Primary Cilia and Cell Proliferation

It has been hypothesized for many years that the primary cilium may in some way, be linked to the cell cycle. The primary cilium contains microtubules and arises from the basal body, which is composed of the centrosome. The question which is then posed is; if the cilium is anchored by the centrosome, how can the cell undergo microtubule spindle formation as part of cell division? Although this question does not imply a direct role for the cilium in cell proliferation, a number of studies now suggest that primary cilia do play such a role. Insights into the function of the primary cilium in proliferation have been gained through the study of defects associated with cells and tissue with disrupted cilia. A very striking observation which provided evidence for a crucial role for primary cilia in development was the discovery that cilia disruption in the genome is embryonic lethal (Davenport and Yoder, 2005). This led to experiments which aimed to isolate the disruption of the cilia to only certain cell types and during certain time points in development, to parse out the role of the primary cilium in different cell types at different stages of development. For example, shortening of the cilia, specifically by mutating a genome involved in cilia formation (Tg737 ^{orpk}) which shortens the cilia of the epithelial cells in the kidney tubule, causes an increase in cell proliferation and the development of cysts (Pazour et al., 2000; Rosebaum and Whitman, 2002). Primary cilia apparently influence

proliferation in the kidney through their activities as mechanical flow sensors. The epithelial cells of the kidney tubules have primary cilia that extend into the lumen of the tubule and bend with movement of fluid through the renal ducts. When the cilium bends, mechanically sensitive Ca2+ channels on the cilium open and Ca2+ flows into the cell (Nauli et al., 2003). The Ca2+ enters through the polycystin-2 (PC-2) channel (Praetorius et al., 2001). In the absence of Ca2+ flow into the cell there is cleavage of polycystin 1 (PC-1), which activates STAT6 and P100 - proteins involved in transcriptional regulation and cellular proliferation (Low et al., 2006). Knockout of KIF3A (a subunit of kinesin) results in shortened cilia and an increase in duct cell proliferation (Lin et al., 2003). In vitro studies have shown that a lack of fluid movement leads to a decrease in Ca2+influx; conversely, moving the primary cilium by mechanically applied force increases Ca2+influx (Praetorius and Spring, 2001). It was hypothesized that cells with shortened cilia cannot fully respond to fluid movement, resulting in decreased Ca2+influx (Low et al., 2006) and then leads to an increased activation of transcription factors, which leads to increased cell proliferation. Therefore evidence points towards primary cilia having a role in proliferation of the cells in the kidney tubule.

Some proteins that are involved in the cell cycle also appear to be involved in the maintenance of the primary cilium. For example, HEF1 and Aurora A have been linked to the disassembly of the primary cilium. These proteins have previously been identified to be associated with molecules involved in the check-points associated with entry into mitosis, thus linking proteins involved in the cell cycle as well as in primary cilia disassembly. For instance, Aurora A has previously been shown to regulate by inducing mitotic entry by activating Cdk1-cyclin which organizes the mitotic spindle. The interactions between the HEF1 and Aurora A kinase cause phosphorylation and activation of HDAC6, which then promotes ciliary

disassembly. These proteins had been previously reported to be markedly up regulated in various types of cancers (Gritsko et al., 2003). Also, IFT27, a protein in the IFTA complex, has been shown to act as a Rab-like small G-protein (Qin et al., 2007). Rab-like small G proteins have been shown to be involved in the control of the cell cycle. Reducing IFT27 protein levels by RNA interference, reduces the activity of both the IFT A and B complexes and leads to defects in cytokinesis and a lengthening of the cell cycle, suggesting a role in regulating ciliation (Qin et al., 2007).

Primary cilia appear to play a somewhat different role in cell proliferation in the nervous system. It has recently been shown that primary cilia influence the proliferation of adult hippocampal progenitors in the dentate gyrus. This was shown by using a conditional knockout of the *Ift20* gene under the control of the specific mouse glial fibrillary acidic protein (GFAP), a protein known to be enriched in hippocampal progenitors. These mice had a decrease in learning and memory along with a decrease in proliferation of neural progenitors in the dentate subgranular zone (Amador-Arjona et al., 2011).

Other evidence linking primary cilia and proliferation has recently been reported. Two recently identified proteins, Nde1 and Tctx-1, appear to have a direct role in primary cilia disassembly and entry into the cell cycle (Li et al., 2011; Kim et al., 2011; Jackson, 2011). The centrosomal protein Nde1 is localized to the mother centriole and is highly expressed during mitosis but expressed at low levels when the cell is quiescent. Cells in vitro that lack Nde1 have longer cilia and display delayed re-entry into the cell cycle. Knock-down of Nde1 in zebrafish embryos results in increased cilium length and a suppression of cell division (Kim et al., 2011).

Tctex-1, when phosphorylated at Thr 94, is recruited to the ciliary transition zone just prior to S-phase entry. It also appears to play a role in ciliary disassembly, as shown in vitro.

Adding a phopho-mimic Tctex-1 mutation leads to disassembly of the primary cilium and accelerates entry into S-phase. Phosphorylated Tctex-1 also shortens G1 and has a role in fate determination of cortical neural progenitors in the mouse (Li et al., 2011). More experiments are necessary to identify the roles primary cilia play in neurons and glia, including how cilia influence cell proliferation. It is also important to further study the consequences of signal transduction through primary cilia. So far, no mechanoreceptive properties have been demonstrated for primary cilia of neurons and glia, as has been shown for cilia in kidney, liver, pancreas, vasculature, bone, and cartilage) (Anderson et al., 2008). Therefore, there must be another mechanism by which the primary cilium influences proliferation of the cells in the nervous system.

Sonic Hedgehog

Sonic hedgehog (Shh) can act as both a morphogen and mitogen that induces differentiation and proliferation, depending on the tissue and time in which signaling occurs (Ahn and Joyner, 2005 and Goetz and Anderson, 2010). Shh acts as a morphogen by diffusing to form a concentration gradient which influences proliferation and differentiation during development. Shh's role as a morphogen in the vertebrate has been most intensively studied in the embryonic development of the chick and mouse spinal cord, where Shh is particularly essential or ventralization in the spinal cord (Fuccillo et al., 2006). Shh is crucial for development and has also been shown to be present throughout life (Goetz and Anderson, 2010). Although the importance of Shh in the adult is still being elucidated, its importance in development is well established.

Shh is glycoprotein that is proteolytically cleaved into two secreted forms, the C- and N-terminal domains, the latter being referred to as SHH-N. A cholesterol is also added to the C-

terminus end of the N-terminal domain, aiding in the action of SHH-N as a diffusible signaling protein. The function of the C-terminal domain of the cleaved protein is still not clear (Bumcot et al., 1995). Shh (which is SHH-N, but from here on is referred to as Shh) acts by binding patched (Ptc), a 12 transmembrane protein. The binding of Shh to Ptc leads to inactivation of a 7 transmembrane G-protein coupled receptor, smoothened (Smo) (Murone et al., 1998), which then alleviates Smo's inhibition of Gli protein processing. The activated Gli proteins act as transcription factors which regulate the expression of proteins involved in proliferation and differentiation (Lum and Beachy, 2004; Robbins et al., 2012).

An example of Shh acting as a mitogenic molecule is the promotion of precursor proliferation in later stages of the dorsal brain, specifically in the neocortex, tectum and cerebellum (Dahmane and Ruiz i Altaba, 1999; Weschler-Reya and Scott, 1999; Wallace, 1999; Ruiz i Altaba et al., 2002). It has also been demonstrated that cilia-mediated Shh signaling has a role in proliferation of adult neuronal stem cells in the hippocampus (Ahn and Joyner, 2005; Bruenig et al., 2008) and developing postnatal cerebellum (Chizhikov et al., 2007; Han et al., 2008).

Shh has been shown to influence the ontogeny of the spinal cord and the dorsal root ganglion (DRG), even through the later stages of development. Shh has been shown to specifically be involved in the developmental specification of motor neurons (Ericson et al., 1996; Madden, 2006). Furthermore, the formation of the zebrafish DRG depends on the presence of Shh. Reduced Shh signaling in zebrafish, by genetic mutation or pharmacological inhibition of Shh, results in DRGs that are misshapen and contain reduced numbers of neurons and glial cells (Ungos et al., 2003). Shh regulates the expression of neurogenin 1, which is required for the differentiation of DRG precursors to become neurons or glia (Ungos et al.,

2003). These lines of evidence implicate Shh in the development of the DRG Shh also has a role in the patterning of the developing chick DRG in the later stages of development, by both influencing proliferation and differentiation of the neuronal cell types. Within the developing DRG there is a ventral-lateral gradient of the Shh morphogen that influences cellular proliferation and differentiation (Guan et al., 2008). Therefore, Shh influences the cellular organization of the DRG, both in the early and later stages of development.

Shh is up-regulated in various neural cells of the adult following injury. Following injury to peripheral nerves, the motor neurons of the ventral spinal cord begin to express Shh, as shown by immunohistochemistry. This up-regulation of Shh is thought to aid in motor neuron survival following injury, and can be blocked with the Shh antagonist, cyclopamine, or rescued with over-expression of Shh (Akazawa et al., 2004). Following traumatic brain injury in adult rodents, Shh is expressed by reactive astrocytes promotes the proliferation of neural stem cells (Amankulor et al., 2009). These studies implicate Shh in proliferation in the adult following injury.

Sonic Hedgehog and the Primary Cilium

A number of experiments have shown that primary cilia are required for Shh signaling (Huangfu et al., 2003; Huangfu and Anderson, 2005; Christensen and Ott, 2007; Rohatgi et al., 2007). Adult hippocampal progenitor cells with shortened cilia show decreased rates of proliferation, possibly due to their inability to respond to Shh (Bruening et al., 2008). Shh signaling has been shown to require IFT proteins (Huangfu et al., 2003; Huangfu and Anderson, 2005) and other ciliary proteins such as Arl13b (Caspary et al., 2007), as well as Rfx proteins which direct IFT127 transcription (Ashique et al., 2009), and Kif3a, which is part of the anterograde IFT machinery (Wong et al., 2009). Mutations in the retrograde IFT complexes (in

particular in THM1 mutants) cause enhanced Shh signaling via the over-activation of the Gli activators, Gli1 and Gli2 (Tran et al., 2008). It has also been shown that in some cancers, such as in basal cell carcinoma, the cilium mediates tumorigenesis through Shh signaling (Wong et al., 2009). Thus it appears that primary cilia play a pivotal role in Shh signaling.

The role of cilia in Shh signal transduction is beginning to be understood. Shh binds to Patched (Ptc), a 12 transmembrane protein, which is located on the primary cilium (Corbit et al., 2005; Bruenig et al., 2008). Then, by a still unknown process, Ptc releases inhibition of Smoothened (Smo), a 7-transmembrane domain protein that is typically located in a vesicle near the base of the cilium. Smo then appears to move into the cilium where it activates the transcription factors, Gli-1 and -2 (Tran et al., 2008). The activated Gli proteins then enter the nucleus where they activate transcription of proteins for proliferation and differentiation. While it now appears to be well established that Shh signal transduction requires the primary cilium, the cells and tissues where this occurs is not fully established, especially in the adult. Identifying the cells where Shh exerts its effects may further clarify its function in adult tissue. Studying ciliated cells that can be induced to proliferate may serve as good models to further test if Shh induces cellular proliferation in different cell types.

The Dorsal Root Ganglia

The dorsal root ganglia (DRG) are groups of cell bodies of neurons in the peripheral nervous system (PNS) that carry sensory information from the periphery to the central nervous system (CNS). Surrounding the neuron cell bodies are glial cells called satellite glial cells (SGCs) (see Figure 2). These SGCs form a sheath, which together with a sensory neuron, form a functional sensory unit. The SGCs play important roles in the DRG, including modulating the activity of sensory neurons. The SGCs may be a unique class of glia, although they share

characteristics with other types of glia, which will be discussed further later. Three populations of neurons have been defined in the DRG based on conduction velocity and soma size. The C-cells have small non-myelinated axons and have smaller cell bodies (less than 30 µm in diameter). The Aδ-cells have medium diameter myelinated axons and medium sized cell bodies (30-40 µm in diameter), and the Aβ-cells have larger diameter myelinated axons and larger cell bodies (greater than 40 µm in diameter) (Scoggs and Fox, 1992). These neuron classes also generally correspond to the sensory properties they convey. The small neurons carry mostly nociceptive (painful stimuli) and temperature information, the medium neurons carry touch and temperature information, and the larger neurons (Aβ-fibers) carry touch and proprioceptive information (Scoggs and Fox, 1992).

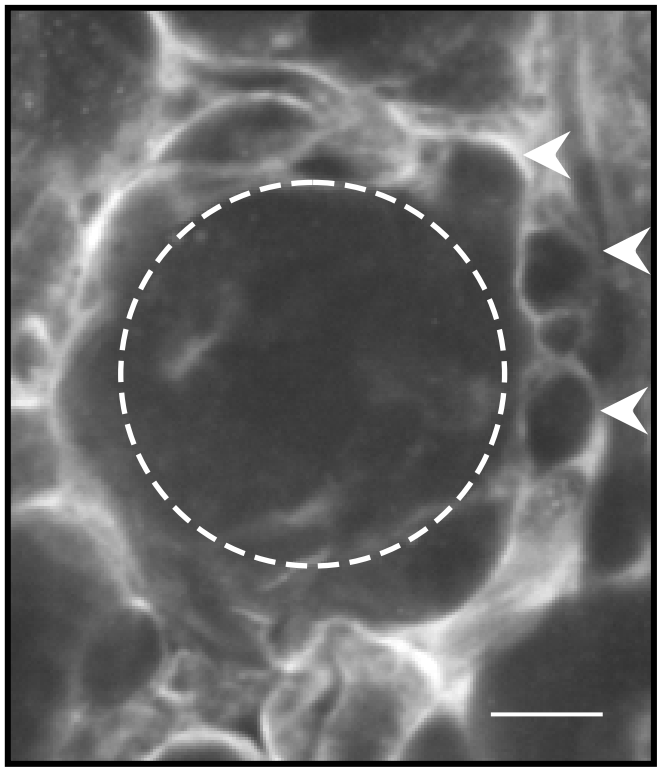

Figure. 2. A neuron in the dorsal root ganglion (dotted line) surrounded by satellite glial cells (arrow heads). SGCs membranes are labeled with an antibody to GFAP, which then outlines the cell body of the neuron they surround. Scale bar= 5 µm

The SGCs have been described as similar to both astrocytes and Schwann cells, even though the latter two glial types are quite different from each other in structure and function. The SGCs are similar to astrocytes in how they respond to injury, as well as in some key proteins expressed by both astrocytes and the SGCs. Injury to a peripheral nerve causes a physiological response of the SGCs surrounding the injured neuron that is analogous to the physiological response of astrocytes in the CNS following different models of injury (Ohara et al., 2009). In both SGCs and astrocytes, this response includes proliferation (Guenard et al., 1996). SGCs have been compared to Schwann cells in that these glial cell types both act by tightly associating with neurons as well as some proteins both expressed on SGCs and Schwann cells such as Krox-20 (Haggerdorn et al., 2000).

SGCs are perhaps best considered a glial cell type of their own class. Functions for this cell type are not fully known. Interestingly, SGCs express many different proteins that are also found on various glial cells in both the CNS and PNS. Identifying the proteins SGCs express is a first step in developing hypothesis as to their function. Among the proteins expressed by SGCs are GFAP, which is also characteristically expressed by astrocytes, neural stem cells, and ependymal cells (Takeda et al., 2007); NG2, expressed by oligendrocyte precursors (Rezajooi et al., 2004); glutamate synthetase, expressed by astrocytes (Ohara et al., 2009); and L-glutamate transporter protein (GLAST), expressed in astrocytes (Ohara et al., 2009). Some of the proteins expressed by SGCs are associated with neural progenitor cells; included are Sox 10 (Aquino et al., 2006), and nestin (Hockfield and McKay, 1985). SGCs in culture migrate and form neurospheres (a characteristic of stem cells and progenitors) and can be coaxed to become neurons when the proper growth factors are applied in vitro (Li et al., 2007). The fact that SGCs express proteins similar to those found in other cell types in the CNS and PNS, have

characteristics of glial progenitors, and when cultured can differentiate into different neuronal cell types, creates many possibilities as to what functions these cells may have in the DRG.

SGCs undergo complex and varied responses to pain or direct axonal injury. Following axonal damage, SGCs proliferate. This response occurs following axotomy (Lu and Richardson, 1991; Wen et al., 1994), inflammation near the cell bodies (Lu and Richardson 1991; Hanani, 2005), and even following a scratch of the skin (Elson et al., 2004). A greater understanding of SGCs and the signals involved in their proliferation following injury can provide insight into their functions within the DRG and possibly into mechanisms of pain.

Sensory neurons in the DRG also respond to injury of a peripheral nerve. For example, following axotomy the number of small neurons is significantly reduced compared to medium or large neurons (Vestergaard et al., 1997). These cells are thought to die by apoptosis (Groves et al., 1997). This decrease in neuronal numbers reaches significant levels by 12 days following axotomy, with cell death detected as early as four days (Vestergaard et al., 1997). Thus axotomy presents an interesting functional model to study what may seem like responses but are perhaps causally related events in the DRG: the death of sensory neurons and the proliferation of SGCs.

The Role of Primary Cilia in the DRG

Primary cilia are present on both sensory neurons (Tan et al., 2007) and SGCs (Pannese, 1969; and 1981). These cilia may play a role in communication between the neurons and their surrounding glia. For example, primary cilia on SGCs might receive neuronal signals that induce proliferation.

Shh plays a role in DRG development (Ungos et al., 2003 and Guan et al., 2008) and has been shown to influence cell proliferation via the primary cilium (Goetz and Anderson, 2010). Following injury to a peripheral nerve the SGCs surrounding the injured neurons proliferate.

Moreover, Shh mRNA increases in injured neurons of the DRG following chronic constriction of a peripheral nerve (So et al., 2006). It is reasonable to hypothesize that the increase in Shh promotes proliferation of SGCs in the DRG following injury, and primary cilia in the SGCs might mediate the mitogenic effects of the Shh. In the present study, I test this hypothesis, by examining the incidence of primary cilia in the normal and axotomized DRG, and evaluate effects of a Shh antagonist to test if Shh signaling is involved in the proliferative response. I found that the Shh antagonist cyclopamine led to a decrease in proliferation of SGCs in the injured DRG following axotomy, suggesting that Shh promotes proliferation of SGCs in the injured DRG. Axotomy led to a significant increase in the percentage of SGCs with primary cilia, supporting the novel possibility that ciliogenesis may enhance the proliferative response of SGCs. To our knowledge, this is the first report of primary cilia having a role in proliferation in the PNS, and the first evidence that Shh promotes proliferation of the SGCs in the injured adult DRG.

The Main Goals for This Thesis are as Follows:

1. Determine the incidence of primary cilia on the SGCs and neurons of the DRG.
2. Determine if there is a change in incidence of primary cilia on the SGCs and neurons of the DRG following peripheral nerve injury.
3. Determine if Shh could be a signaling molecule involved in the proliferation of the SGCs that occurs following peripheral nerve injury.

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PRIMARY CILIA AND THE EFFECTS OF AXOTOMY IN THE DORSAL ROOT GANGLIA

Introduction

Primary cilia are solitary sensory organelles that arise from the plasma membrane on most cell types, and are anchored by the mother centriole (Satir and Christensen, 2007). In the central nervous system (CNS), primary cilia are found on most neurons, neuronal precursors (Fuchs and Schwark, 2004; Ahn and Joyner, 2005; Breunig et al., 2008), and glial cells, such as astrocytes (Yoshimura et al., 2011). A few reports have described the *in vivo* presence of primary cilia on cells of the peripheral nervous system (PNS) including neurons (Tan et al., 2007), satellite glia cells (SGCs) (Pannese, 1981), and Schwann cells (Grillo and Palay, 1963). These studies in the PNS, which were based on electron microscopy, were unable to reveal the abundance of cilia in the various cell types.

The mitogenic signaling molecule, sonic hedgehog (Shh), requires primary cilia (Huangfu and Anderson, 2005). Disrupting primary cilia of neuronal precursors in the developing or adult brain results in decreased rates of proliferation (Bruenig et al., 2008; Han et al., 2008; Spassky et al., 2008; Amador-Arjona et al., 2011). In addition, injury to the adult brain results in proliferation of progenitors, a response that requires both primary cilia and Shh (Bruenig et al., 2008 and Berbari et al., 2009). Therefore, in the CNS, primary cilium-mediated Shh signaling appears to play an important role in regulating proliferation. It is not known if primary cilia play similar roles in the PNS.

The dorsal root ganglia (DRG) are groups of cell bodies of sensory neurons in the PNS. There are three general populations of neurons found within the DRG that can be characterized chemically, physiologically, and by size. In the adult rat, each sensory neuron is surrounded by a

group of about 7 SGCs, although these numbers vary in proportion to neuron size (Hanani, 2005; Durham and Garrett, 2010). Together, a neuron with its surrounding SGCs, appear to form a functional unit surrounded by a basement membrane. A variety of factors have been found to underlie communication between neurons and SGCs (Hanani, 2005 and Pannese, 2010). Although all of the functions of the SGCs have yet to be fully elucidated, it has been proposed that they may have a role in modulating the extracellular environment of the sensory neurons and may contribute to mechanisms of pain (Hanani, 2005; 2010). SGC half life has been estimated to be about 600 days in the mouse, and is therefore a slowly replicating population of cells (Elson et al., 2003).

Following injury to a peripheral nerve, the SGCs in the affected DRGs proliferate (Lu and Richardson, 1991). It has also been shown that peripheral nerve crush results in increased Shh mRNA in the injured DRG neurons (So et al., 2006). Since the SGCs of the DRG have primary cilia, and primary cilia have been shown to have a role in cell proliferation in the CNS, specifically through the Shh pathway, I investigated the incidence of cilia on the SGCs and the neurons in the uninjured DRG as well as in the injured DRG.

I observed primary cilia on both SGCs and neurons in the DRG. I observed a significant increase in percentage of SGCs and medium-sized neurons with primary cilia following nerve injury. This is the first report detailing the abundance of primary cilia in the neurons and SGCs of the DRG.

Materials and Methods

Animals and Surgical Procedures

All experimental procedures were done in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee. Subjects were adult male

Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing approximately 200-240 g. Animals were maintained on a 12:12 light:dark cycle and given food and water *ad libitum*. Axotomies were done while the animals were surgically anesthetized with isoflurane (Baxter, Deerfield, IL). An incision was made through the skin parallel to the femur to expose the sciatic nerve and a retrograde tracer (0.05% Fluoro-ruby or Fluoro-gold in saline) was injected into the proximal nerve stump to label axotomized DRG cells. After a 7-day survival the animals were given an overdose of 20% urethane and were perfused through the heart with saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.5. The spinal cords with attached DRGs, were removed and immersed in the fixative solution for 24 hours and then placed in 30% sucrose. After equilibration in 30% sucrose, the tissue was frozen and stored at -80°C.

Tissue Preparation and Immunohistochemistry

Spinal cords with attached DRGs were cut longitudinally in 25-µm sections on a cryostat-microtome. Sections were mounted on gelatin-subbed slides and stored at -20°C until stained. An antibody raised against adenylyl cyclase type 3 (ACIII) (rabbit polyclonal, lot # E1809, Santa Cruz Biotechnology, 1:500) was used to identify primary cilia. An antibody raised against K_{Ca}-2.3 (SK3) which labels potassium channels found on SGCs (rabbit polyclonal, lot #AN-04, Alomone labs, 1:1000) and was used to label the cell membranes of SGCs. Sections were pre-incubated for 30 minutes in a blocking solution consisting of 3.0% normal serum and 0.03% Triton X-100 in 0.1 M Tris-buffered saline (TBS), pH 7.6. They were then incubated in this solution with the addition of the primary antibodies. Tissue sections were incubated in the primary antibodies sequentially. Since both primaries were raised in the same species, after incubation with the first primary antibody the sections were incubated in labeled fluorescent

labeled Fab fragments of a secondary antibody, in order to label and block, antigenic sties on the first primary antibody. Next, the sections were incubated in the second primary antibody, followed by incubation with a labeled secondary antibody. Incubations with primary antibodies were done in a humidified chamber at room temperature for 24 h. Following a series of TBS rinses, the sections were incubated for 2.5 h in TBS with 3.0% normal serum and fluorescent secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA). Sections were then rinsed and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). Slides were coverslipped with Vectashield (Vector Laboratories, Burlingame, CA) and stored at 4^{o}C.

Data Collection and Analysis

The incidence of primary cilia was quantified on a sample of approximately 15-50 neurons per DRG section, on at least 3 full sections per DRG, and in two DRGs per animal. The numbers of neurons sampled per animal within experimental condition ranged from 100 to 300. The neurons were chosen using an epifluorescence microscope (Nikon Optiphot 2) at low magnification (100X). To be selected for analysis, the neuron's nucleus had to be contained within the section. Once a neuron was selected for analysis and the presence or absence of a cilium noted, the SGCs surrounding that neuron were analyzed. An SGC was evaluated for association with a particular neuron by identifying the membrane of the SGC (labeled with SK3) and determining whether the membrane was in juxtaposition to that neuron. Neurons were grouped into one of three categories based on soma size. The major axes were measured at the largest diameter profile containing the nucleus. Categories were according to Scoggs and Fox (1992) as follows: large (> 40 µm at the largest axis), medium (30-40 µm) and small (< 30 µm).

Based on SK3 immunostaining as well as cellular and nuclear morphologies, neurons and SGCs could be readily distinguished.

Neuronal primary cilia always had at least one end of the cilium within the neuronal cell membrane. The identities of neuronal primary cilia were confirmed on a subset of neuronal cilia using 3D reconstruction of confocal image stacks. Primary cilia of SGCs were always located next to the nuclei of the SGCs and never crossed the plasma membranes of the neurons.

Because many of the sensory neurons were larger than the section thickness, corrections were applied to obtain a better estimate of the proportion of ciliated neurons. To estimate the average amount of membrane lost by sectioning for each category of neuron, I first calculated the average nuclear and soma diameters. Next, assuming that the nuclei were located at the center of the neuron somata (this is generally the case for primary sensory neurons) and that they were entirely contained within the section (a criterion for our analysis), I was able to calculate the average height of the "caps" cut off by sectioning. Finally, I calculated the surface area of the caps and subtracted it from the average surface area of the neurons in each category. Similar corrections were not applied to SGC data because of their small size (approximately 6 μm diameter).

Comparisons between treatments groups were based on paired t-tests using SPSS (α=0.05). N was based on the number of animals per group. The contralateral DRGs were controls and the axotomized DRGs were the experimental DRGs.

Results

Neurons with primary cilia were distributed uniformly across different areas of the DRG. In general, neuronal cilia were longer (on average 2.1 μm) than those cilia on the SGCs (on

average 1.2 µm). Figure 3A micrograph illustrates a neuronal primary cilium in the DRG. The cilia on neurons were found to originate near the nucleus of the neuron. The primary cilia on SGCs were not always seen to reside between the neuron and the SGC. Figure 3B micrograph illustrates cilia on SGCs of the rat DRG. There was no bias in location of the origin of the cilium on the SGCs are they were located either on the neuronal or extracellular interface (Figure 3B). I occasionally observed primary cilia on Schwann cells in the DRG, but did not study them further. The primary cilia on the Schwann cells were found within in the nerve root and were not found near the cell bodies of the neuron, and thus were easily distinguishable from those of SGCs.

Primary Cilia on the Neurons and SGCs of Normal DRGs in the Rat

Parametric data from neurons were collected from between 100 to 300 neurons per animal. The average soma diameter of the neurons in each size category were 49 ± 7.1 µm (large), 34 µm ± 2.7 (medium) and 24 ± 4.1 µm (small). The average nuclear diameters in each category were 13.9 ± 2.6 µm (large), 11.3 ± 2.1 µm (medium) and 8.8 ± 1.6 µm (small). Based on these measures, I derived correction factors of 0.51 (large), 0.70 (medium) and 0.84 (small) to adjust for loss of the portions of somata extending outside of the section. The observed proportions of ciliated neurons in each category were divided by these factors to get a better estimate of the true proportion of ciliated neurons. I found ACIII+ primary cilia on 29% (small), 16% (medium) and 5% (large) neurons.

In the contralateral DRGs, 33% of the neurons were large, 35% percent were medium and 31% were small. Neurons and their surrounding SGCs are known to create functional units surrounded by a basement membrane. To test for a relationship between neuronal ciliation and

SGC ciliation, I determined the percentage of ciliated SGCs around ciliated and non-ciliated neurons. Overall, 26% of the SGCs in the normal DRG had primary cilia. The percentage of SGCs with primary cilia around neurons without primary cilia (35%) was significantly higher than the comparable measure for SGCs around neurons with primary cilia (26%) (ttest; $p<0.05$).

Primary Cilia on the Neurons and SGCs of Injured DRGs in the Rat

Within the affected DRGs, axotomized versus non-injured neurons were examined for the presence of primary cilia. I used the presence of an eccentric nucleus as an indication that the neuron had been injured (Cragg, 1970). Neurons with eccentric nuclei were less likely to have a primary cilium than neurons with centrally positioned nuclei (8% vs. 29%; paired ttest $p = 0.01$;n = 4 rats) (Figure 6).

In the injured DRG, 40% of the neurons were large, 32% percent were medium and 28% were small. There was a significant increase in the percentage of medium-sized neurons with primary cilia (16% of the medium neurons were ciliated in the contralateral DRG to 22% of the medium neurons were ciliated in the axotomized DRG; paired ttest; $p=0.048$; n= 6 rats) (Figure 5). Although ciliation in the other classes of neurons also increased, these changes were not statistically significant (small: 29% to 37% (paired ttest; p=0.269); large: 5% to 9%; (paired ttest; $p=0.264$; n=6) (Figure 5). The number of neurons counted in the injured DRGs ranged from 125-849 per animal.

The percentage of SGCs with primary cilia increased following axotomy. Figure 4 illustrates a greater percentage of SGCs with primary cilia following sciatic nerve axotomy compared to the contralateral DRG (paired ttest $p= 0.001$; 26.3% to 48.7%; n= 6 rats) (Figure 7). Unlike the case for the contralateral DRG, following axotomy there was no significant difference

in percentage of ciliated SGCs associated with ciliated or non-ciliated neurons (48% vs. 50%, ttest; $p>0.5$; n=6). I also studied the percentage of ciliated SGCs around neurons by neuron size and by neuronal ciliation in the contralateral and axotomized DRG (Table 1).

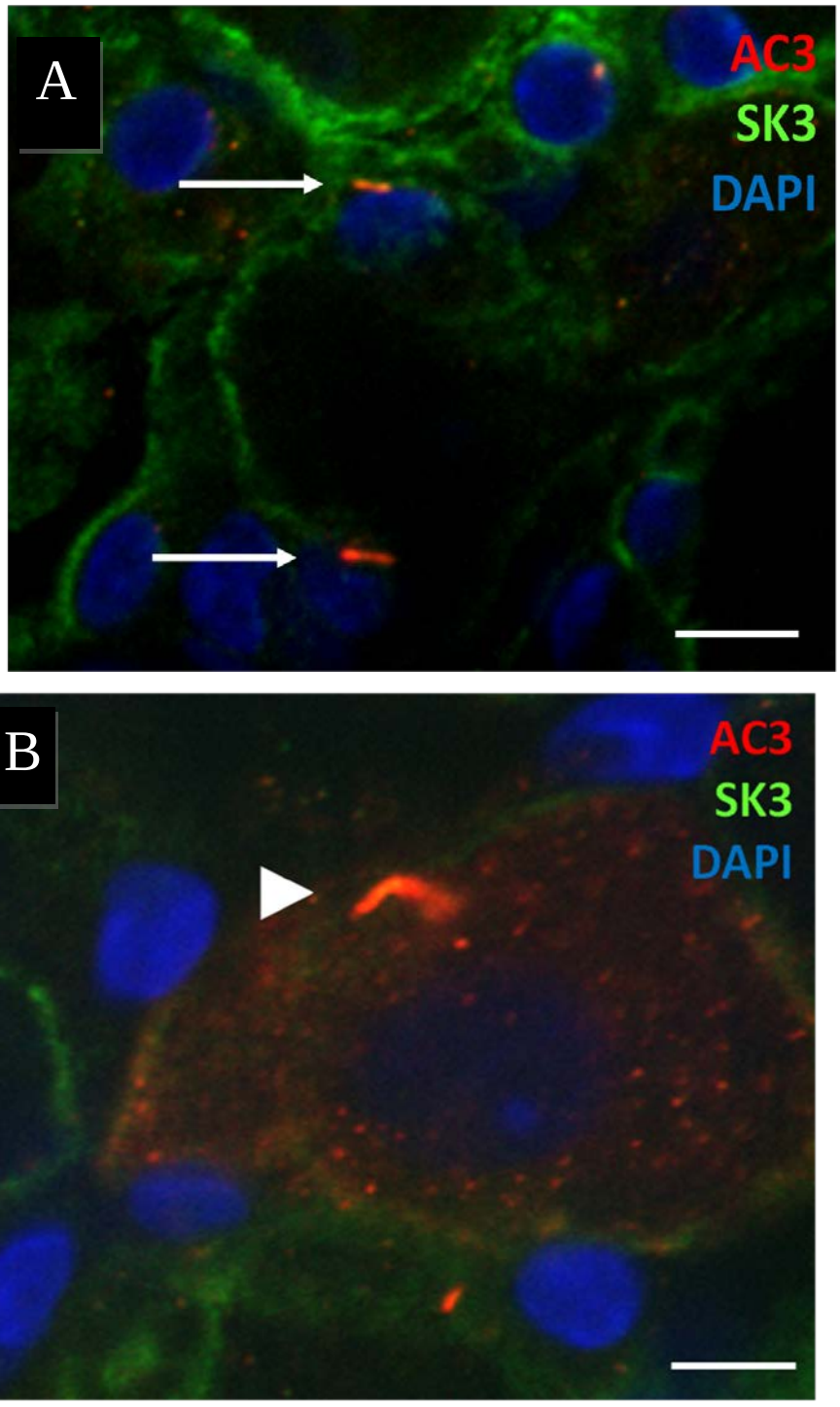

Figure. 3. **Primary cilia on cells of the DRG.** SK3 (green) labeled the membranes of SGCs, and as a result, outlined the sensory neurons. Immunostaining of ACIII (red) was used to label primary cilia. A.)Primary cilium (triangle) on a sensory neuron. These cilia typically originated near the nuclei of the neurons. Scale bar = 5µm B. Primary cilia on SGCs. These cilia tended to be shorter than those on neurons. Note the position of the cilia near the nuclei. Some primary cilia (lower arrow) on SGCs were located near the neuron whereas others were not (upper arrow). Scale bar = 5 µm

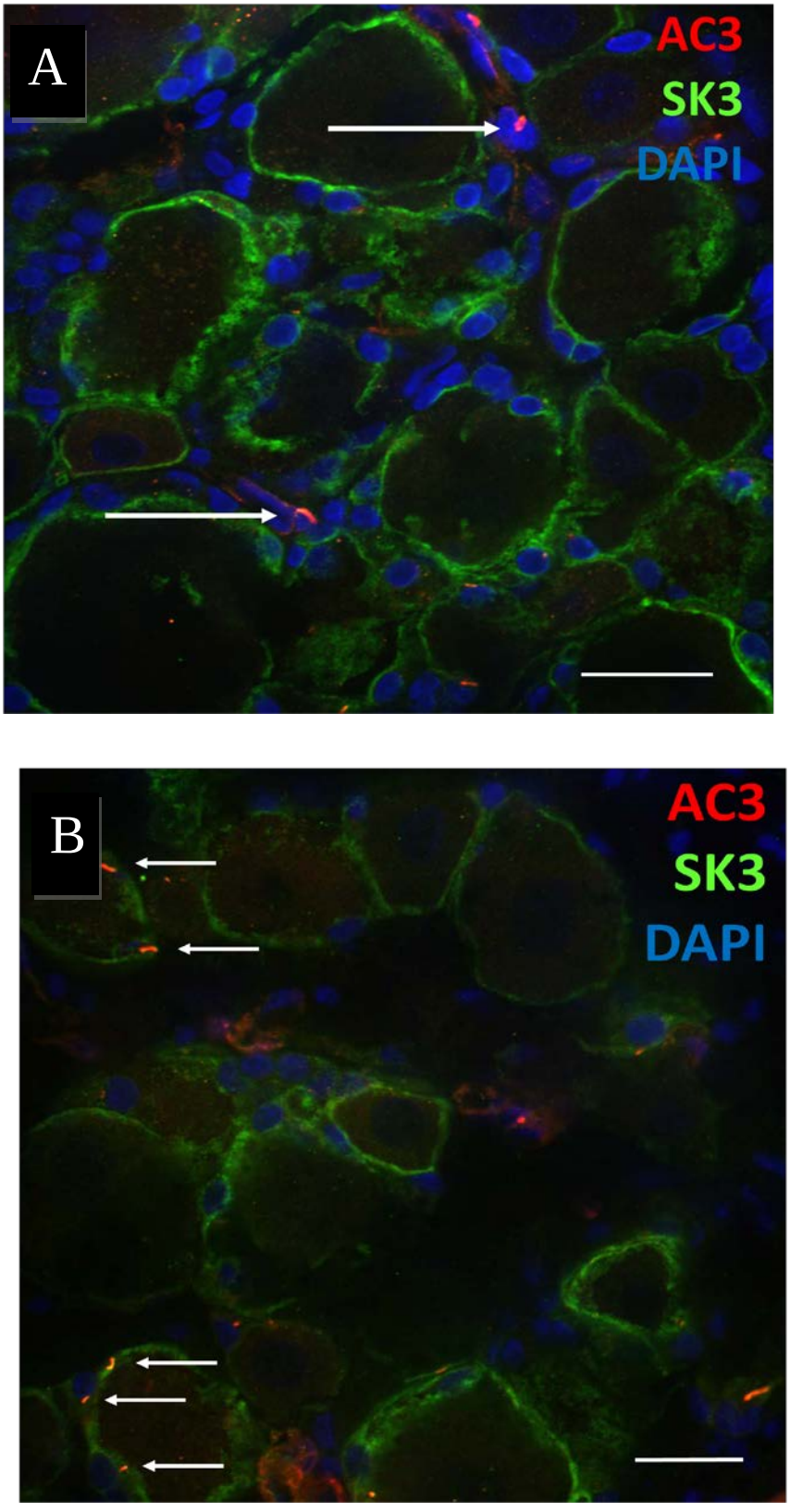

Figure. 4. **Primary cilia on SGCs of the DRG.** Immunostaining as in Fig. 3. (A) Contralateral DRG. (B) Axotomized DRG. Scale bars = 30µm

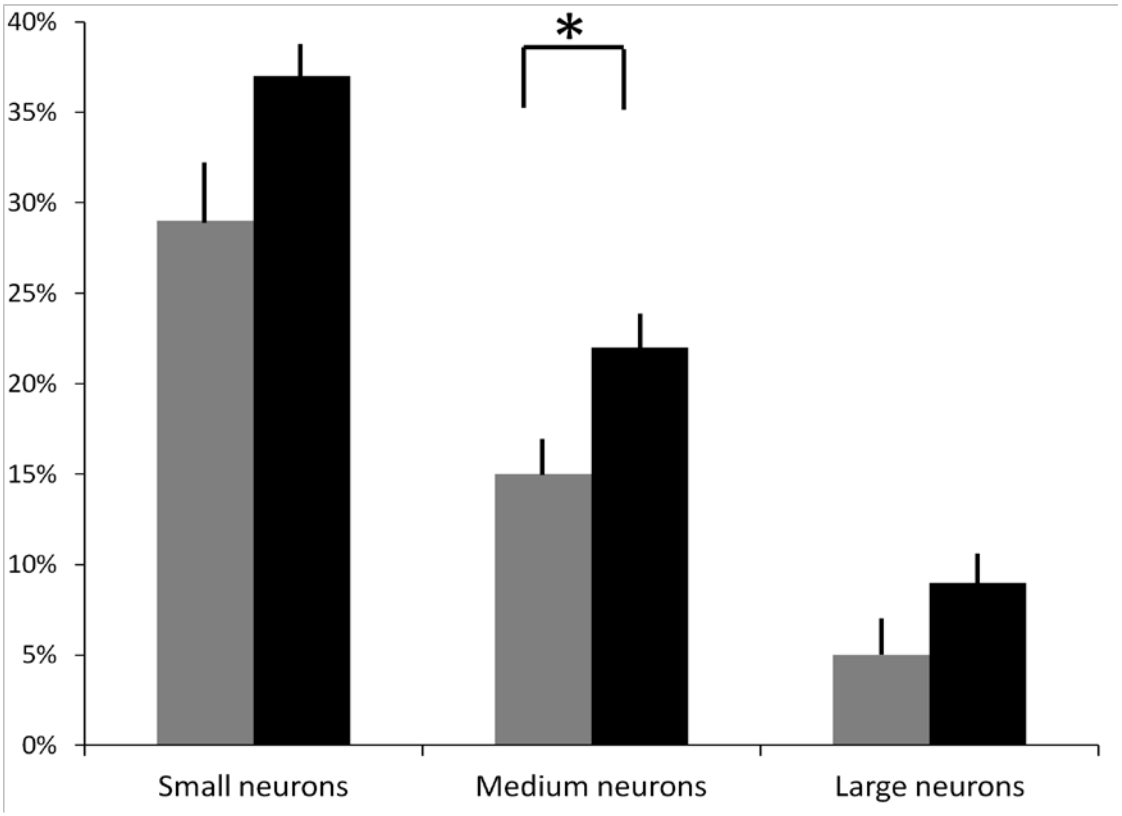

Figure. 5. Seven days after axotomy, the percentage neurons with cilia increased significantly in the medium category (30-40 µm; * p=0.048) but not in the small neurons (<30 µm) (p=0.269) or large neurons (>40 µm; p=0.264). Gray bars represent the contralateral DRGs and black bars represent the axotomized DRG. Standard error bars; n=6 rats.

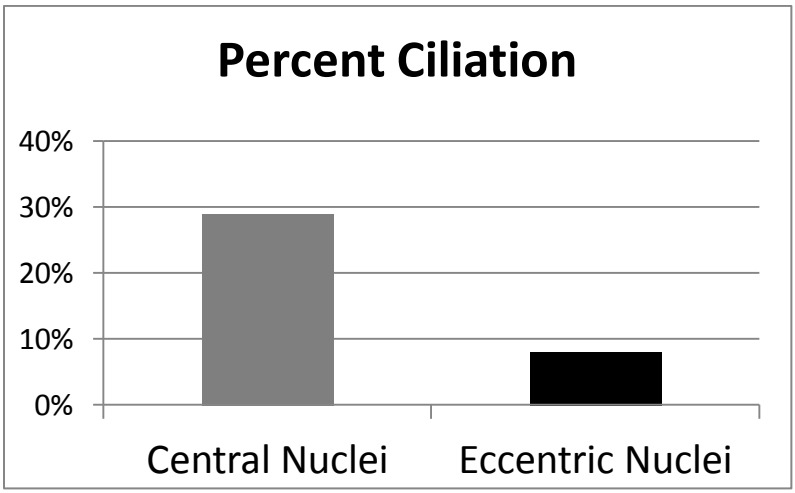

Figure. 6. Injured neurons (as indicated by eccentric nuclei) were significantly less likely to have primary cilia than non-injured neurons (central nuclei) Gray bars indicate non-damaged neurons and black bars injured neurons Stander error bars, n=6 rats.

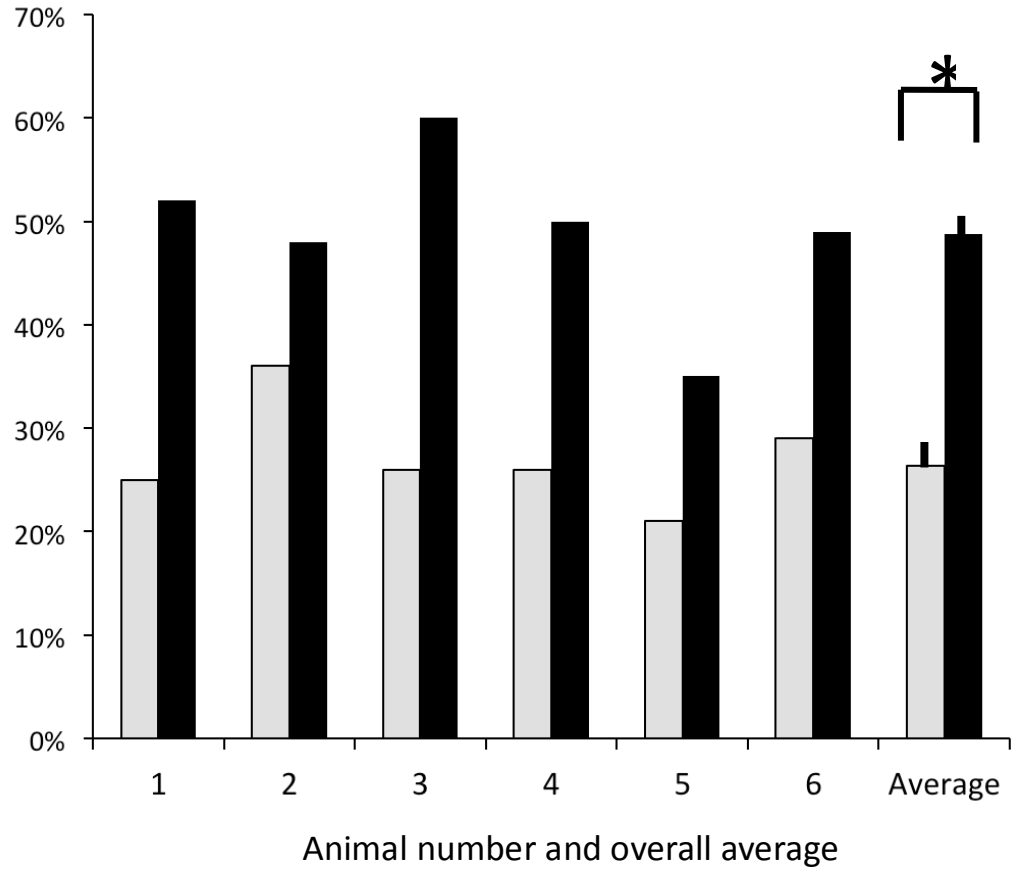

Figure. 7. Percentage of SGCs with primary cilia increased 7 days after axotomy in each individual rat, and the increase was significant for the average of all 6 rats (*p=0.003). Gray bars indicate contralateral and black bars indicate axotomized DRG. Standard error, n=6 rats.

Table 1. Percentage of ciliated SGCs around neurons. Paired ttest, α=0.05; n= 6 rats

Percentage of ciliated SGCs around neurons			
Contralateral (n=6)			
	non-ciliated neurons	ciliated neurons	
small	48%	29%	p=0.003
medium	24%	26%	p=0.612
large	16%	17%	p=0.814
Axotomized (n=6)			
	non-ciliated neurons	ciliated neurons	
small	68%	55%	p=0.059
medium	52%	50%	p=0.906
large	40%	25%	p=0.087

Discussion

Although the presence of primary cilia on the cells of the PNS has been recognized for many years (Grillo and Palay, 1963; Pannese 1981; Tan et al., 2007), currently there are no reports quantifying the percentage of those cells in the DRG that have primary cilia. Furthermore, proliferation of the SGCs in the DRG following peripheral nerve damage is well established, yet very little research has addressed what mechanisms may signal for that proliferation. Primary cilia have been shown to have a role in proliferation in the CNS (Bruenig et al., 2008; Han et al., 2008; Spassky et al., 2008; Amador-Arjona et al., 2011), as well as in other tissues such as kidney epithelial cells (Praeterious and Spring, 2001). In this study I investigated the potential change in percentage of cells with primary cilia following axotomy, to identify if this organelle could then be further studied as a potential mediator of proliferation in the PNS.

Primary Cilia in the Normal DRG

Fewer than half of the cells of the different cell types I studied (SGCs and neurons) of the normal DRG had primary cilia. As noted by others (Pannese, 2010), there was no preferential orientation of the primary cilia of the SGCs (See Figure 3B). Although the cilia were always found near the SGC nuclei, and were oriented in parallel with the major axis of the cell, there was no bias in location (toward the adjacent neuron or toward extracellular space). It seems likely that one function of primary cilia on SGCs is to communicate with the neurons they surround. The lack of a positional bias in the SGC primary cilia suggests that they may also receive signals from the extracellular milieu.

There appeared to be a relationship between whether a sensory neuron was ciliated and how many of its surrounding SGCs were ciliated. Approximately 35% of the SGCs surrounding non-ciliated neurons were ciliated, whereas only 26% of those surrounding ciliated neurons had

cilia. This analysis was extended to neurons grouped according to size and found that the difference in ciliation was due mainly to SGCs that surrounded small neurons (approximately 48% of the SGCs were ciliated around non-ciliated neurons, and 29% of the SGCs were ciliated around ciliated neurons). The reasons for these differences remain obscure. Although, it could be that the smaller neurons, which have an increased susceptibility to cell death following peripheral nerve injury, are also more likely to release signaling molecules which could bind on primary cilia of surrounding cells. The increase in likelihood for SGCs to have primary cilia around the smaller neurons could increase the chance that the primary cilia on those SGCs could respond to cues released from injured neurons, but I have yet to test this idea.

Primary Cilia in the DRG Following Axotomy

Axotomy has a number of effects on the DRG. Peripheral nerve injury tends to preferentially affect smaller neurons (Vestergaard et al., 1997 and Abdualla and Smith, 2001). These axons of these neurons are small C-type fibers that play an important role in pain sensation. Following nerve injury, small DRG neurons display increased excitability (Abdulla and Smith, 2001) and are more likely to die, presumably by apoptosis (Vestergaard et al., 1997). Data from the present experiments are consistent with these observations. In the normal DRG 31% of the neurons were small, while after axotomy this percentage decreased to 28%. Within this same population of neurons, there was a tendency toward increased ciliation: 29% of the small neurons in the normal DRG were ciliated, whereas 37% were ciliated after axotomy. Similar increases in proportions of ciliated neurons were seen for all size classes of DRG neurons. These data suggest the possibility that neurons with primary cilia have a better chance to survive axotomy. This effect might be related to Shh. Shh signaling depends on the presence of primary cilia (Huangfu et al., 2003; Huangfu and Anderson, 2005; Rhoatig et al.,

2006; Christensen et al., 2007). Shh has also been suggested to increase neuronal survival in the CNS. Shh expression increases following axotomy of motor neurons, and over-expression of Shh in neonatal rats following axotomy increases neuronal survival (Akazawa et al., 2004). Shh also appears to protect against oxidative stress in injury induced ischemia in the adult brain (Ji et al., 2012). Therefore, Shh could increase neuronal survival in those small neurons by binding to receptors on their primary cilia and increasing survival. However, more research is needed to further address this idea.

An alternative possibility to explain the changes in neuronal ciliation following axotomy might be that there is a tendency for neurons to become ciliated as a result of a stress response to axotomy. Arguing against this is our observation that neurons with eccentric nuclei (thought to be an indicator of stress) were much less likely to be ciliated compared to those with centrally-located nuclei. These data suggest that cilia may instead be retracted as part of the stress response to axotomy. These are among the first data to suggest such a response to cell stress.

The percentage of SGCs with cilia increased after axotomy. It is possible that this increase in ciliation in a population of proliferating cells reflects an increase in the expression of Shh. It has been reported that, following peripheral nerve damage, Shh mRNA expression increases in DRG neurons (So et al., 2006). Increased ciliation of SGCs would presumably allow more of these cells to respond to Shh, and thereby undergo proliferation. Alternatively (or perhaps in addition), Shh might act as the stimulus for increased ciliation in SGCs. However, evidence for such a role for Shh does not currently exist.

Our experimental results reinforce the idea that primary cilia are dynamic organelles that respond to changes in the local milieu. A variety of biochemical signals have been linked to primary cilia, typically through expression of receptors. Shh signaling through primary cilia has

been extensively studied, and may well be involved in the results described here. However it is likely that additional, as yet to be identified, signaling pathways may also be involved. It will be useful to directly test the role of Shh in this experimental paradigm, as well as to identify other signaling pathways within primary cilia of the DRG.

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SONIC HEDGEHOG INFLUENCES SATELLITE GLIAL CELL PROLIFERATION FOLLOWING PERIPHERAL NERVE DAMAGE

Introduction

The glycoprotein sonic hedgehog (Shh), functions as a morphogen or a mitogen and can induce cellular proliferation or differentiation, depending on the tissue and time in which signaling occurs (Ahn and Joyner, 2005; Goetz and Anderson, 2010). Shh plays an important role in development of the spinal cord, specifically the specification and differentiation of motor neurons (Ericson et al., 1996; Madden 2006). In the adult rat, Shh expression is up-regulated in spinal motor neurons following injury to a peripheral nerve (Akazawa et al., 2004). Intravenous injections of Shh in adult mice with spinal cord injury induce proliferation of neural precursors and oligodendrocytes (Bambakidis et al., 2010).

Shh also plays a role in development of the peripheral nervous system (PNS). Shh is required for the formation of the neural crest (Patten and Placzek, 2000) and has been shown to play a critical role in development of the dorsal root ganglia (DRG) in zebrafish and chicks (Ungos et al., 2003; Guan et al., 2008). Reduced Shh signaling in the zebrafish results in DRGs that are misshapen and have reduced numbers of neurons and glial cells (Ungos *et al*, 2003). A potential mechanism for this effect may be Shh's influence on the expression of neurogenin 1, which is required for the differentiation of DRG precursors (Ungos et al., 2003). In the chick, Shh plays a role in DRG patterning at later stages of development by influencing both the proliferation and differentiation of the neuronal cell types (Guan et al., 2008).

Shh signaling depends on the presence of primary cilia (Huangfu et al., 2003; Huangfu and Anderson, 2005; Rohatgi et al., 2006; Christensen et al., 2007). Primary cilia are ubiquitous

hair-like sensory organelles that arise from the basal body and extend from the plasma membrane in most vertebrate cell types. There is one primary cilium per cell. Like other cilia, primary cilia contain a patterned arrangement of microtubules, but this pattern differs from that seen in motile cilia. Whereas motile cilia contain 9 microtubule doublets surrounding a central pair of single microtubules, primary cilia lack the central pair in an arrangement referred to as "9 + 0" (Satir and Christensen, 2007).

Shh exerts its effects by binding to its receptor, Patched (Ptc), which is localized to the primary cilium (Corbit et al., 2005; Bruenig et al, 2008). Shh binding to Ptc releases Ptc-mediated ongoing inhibition of smoothened (Smo). Smo then activates the glioblastoma transcription factors, Gli1-3 (Tran et al., 2008). The Gli proteins are localized in the primary cilium, and once activated, they translocate to the nucleus where they affect, either by repressing or stimulating the transcription of proteins for proliferation and differentiation (Haycraft et al., 2005).

In the central nervous system (CNS) it has been shown that injury, such as ischemia, can induce proliferation of neural progenitors of the hippocampus, and that Shh appears to -promote proliferation (Sims et al., 2009). Neurons of the hippocampus were shown to up-regulate the expression of Shh and are most likely the source of the Shh that induces proliferation (Sims et al., 2009). In addition, animals that were treated with cyclopamine (a Shh inhibitor) had more marked neurological deficits following a model of ischemic stroke. The same study also showed that cyclopamine administration led to a decrease in levels of Shh-activated proteins such as Gli1 (Ji et al., 2012). Furthermore, rodents treated with Shh demonstrated a significant increase in behavioral functional recovery following ischemic stroke. These animals also had an increase in

nestin positive cells in the sub-ventricular zone (SVZ) (Bambakidis et al., 2012). These data suggest a role for Shh in recovery processes in adult brain following injury.

In the DRGs of the PNS, injury also induces proliferation, but here the cells that proliferate are primarily the satellite glial cells (SGCs). The time course for proliferation of SGCs following injury varies by type of injury and model. For example, beginning at four days following nerve transection, there is evidence of proliferation. In cell culture explants, SGCs proliferate as early as 27 hours following explantation (Lu and Richardson, 1991; Wen et al., 1994; Hanani 2005; 2010). It is not yet clear if Shh plays a role in stimulating this proliferation. One report has shown that Shh mRNA increases in injured neurons of the DRG following nerve crush (So et al., 2006). As described above, Shh has been shown to signal via primary cilia in the CNS. Since SGCs of the DRG have primary cilia and proliferate following axotomy, I hypothesize that Shh is a signal involved in this proliferation.

In order to test this hypothesis, I examined the effects of cyclopamine, a Shh antagonist, on SGC proliferation following transection of the sciatic nerve (axotomy). I observed a decrease in the number of 5-bromo-2'-dioxyuridine (BrdU)-positive cells in the cyclopamine-treated animals. These results suggest that Shh may play enhance SGC proliferation following peripheral axotomy.

Materials and Methods

Animals, Surgical Procedures, and Drug Administration

All experimental procedures were done in accordance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee. Subjects were C57BL male

mice weighing approximately 24-30 g (Jackson Laboratories). Mice were kept on a 12:12 light:dark cycle and given food and water *ad libitum*.

Axotomies were done while the animals were surgically anesthetized with isoflurane (Baxter, Deerfield, IL). An incision was made in the skin parallel to the femur, and blunt dissection was used to expose the right sciatic nerve. After the nerve was cut, retrograde tracer was injected into the nerve stump (5 µl of a solution of 5% Fast Blue or 10% Fluoro-gold in saline). The mice were randomly assigned to one of two groups. The experimental group received daily i.p. injections of cyclopamine (1mg/1ml cyclopamine in 45% 2-hydropropyl-β-cyclodextrin (HBC) in 0.1 M phosphate buffered saline (PBS), pH 7.6) at a dose of 10mg/kg/day for 7 consecutive days. The control group received equivalent injections of the vehicle solution (45% HBC in 0.1 M PBS). During the 7-day survival period all animals were given *ad libitum* access to water containing 5-bromo-2'-dioxyuridine (BrdU; 0.8 mg/ml) (Elson et al., 2003). At the end of the survival period the animals were given an overdose of 20% urethane and perfused through the heart with saline followed by 4% paraformaldehyde in PBS. The spinal cords, with attached DRGs, were removed and post-fixed in 4% paraformaldehyde for 24 hours and then placed in a 30% sucrose solution. After equilibration in 30% sucrose the tissue was frozen and stored at -80°C. Stomach tissue was used as a control for BrdU uptake and staining, as well as for confirmation that cyclopamine blocked Shh.

Tissue Preparation and Immunohistochemistry for BrdU

Spinal cords with attached DRGs were cut longitudinally at 25-µm on a cryostat. Sections were mounted on gelatin subbed slides and stored at -20°C until stained. An antibody raised against BrdU (anti-rat monoclonal, Abcam, lot # 489683, 1:500) was used to identify

dividing cells according to the following procedures. Slides were incubated in 4% paraformaldehyde for 30 minutes; then rinsed in 0.1 M PBS, pH 7.5, for 15 minutes. The DNA was denatured using 0.1 M HCl, pH 2, for 30 minutes at 37°C. The acidic pH was then neutralized using 0.1 M borate buffer, pH 8, for 15 minutes. The slides were rinsed in 0.1 M Tris-buffered saline (TBS), pH 7.6, and pre-incubated for 30 minutes in a solution consisting of 3.0% normal serum and 0.03% Triton X-100 in TBS. They were then incubated in this solution containing primary antibodies for 24 h. After three TBS rinses, the sections were incubated for 2.5 h in 3.0% normal serum in TBS with fluorescent secondary antibodies (1:500, Jackson ImmunoResearch, West Grove, PA). Following another three TBS rinses, coverslips were mounted on the slides using Vectashield (Vector Laboratories, Burlingame, CA) and the slides were stored at 4^{o}C.

Data Collection and Analysis

Sections of DRG were selected for analysis based on the presence of Fast-blue (FB)-positive neurons. BrdU-positive cells surrounding neurons were counted and the total number of labeled cells per section was tallied. All sections identified to have FB-positive neurons were used. Approximately four sections per DRG and two DRGs per animal (cyclopamine group, n=5; vehicle group, n=4) were used for data collection. The average number of BrdU-positive cells per area of each DRG was quantified for each animal. The means of the cyclopamine treated and the control groups were compared by an independent t-test (SPSS).

Results

Although formal behavioral tests were not performed on the mice, it was noted that there were substantial differences in behavior between the animals injected with cyclopamine

compared to those animals that received vehicle injections. It is commonly noted that animals which have undergone some sort of peripheral nerve damage exhibit behaviors such as autotomy (limb chewing) (Wall et al., 1979). The physiological explanation behind this behavior is unclear, but it is often used as an indication that the animal has suffered peripheral nerve damage and the behavior may also be an indication of pain. It was noted in our study that the animals that were injected with cyclopamine substantially had substantially less autotomy and were less jumpy when handled for daily injections. There was not a significant difference in weight or general activity between animals treated with cyclopamine or vehicle injections (p=0.29).

In order to determine if Shh-induced proliferation was successfully blocked by cyclopamine, as well as to determine if BrdU was incorporated in dividing cells, the appearance of BrdU-positive cells was analyzed in the stomach. In control mice, BrdU-labeled cells were located in the gastric pits of the stomach, where Shh is known to influence proliferation (Osawa et al., 2006). In the cyclopamine-injected mice there were fewer BrdU-positive cells in the gastric pits of the stomach. This decrease in BrdU-positive cells in the stomach confirmed that the BrdU had been successfully taken up by dividing cells and that cyclopamine administration had reduced the Shh induced proliferation.

In order to determine if cyclopamine decreased the proliferation of SGCs in the DRG following axotomy, the numbers of BrdU-positive cells per section of DRG from all sections with FB-positive neurons were counted. BrdU-positive cells were counted if they were found in close proximity to a neuron within an injured DRG (confirmed by the presence of FB- positive neurons). No BrdU-positive neurons were observed. Occasionally there were BrdU-positive cells in the connective tissue sheath surrounding the DRG, as well as some BrdU-positive Schwann cells that were in close proximity to axons running through the DRG, but those cells

were not counted. Following cyclopamine injections there was a significant decrease in the number of BrdU-positive cells per total area of DRG from 2.12 to 0.31 per mm^2 (p=0.02) (Figure 8). Figure 9 shows a micrograph illustrating fewer BrdU-positive cells in the cyclopamine treated animal compared to the control).

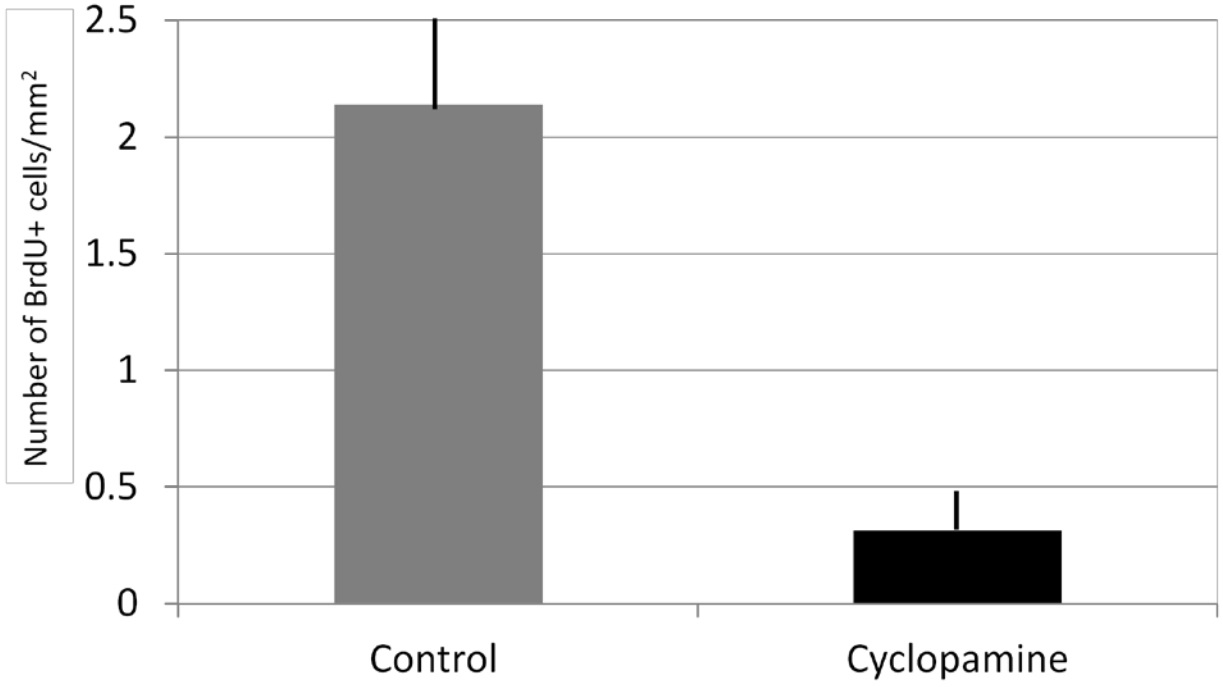

Figure. 8. **Cyclopamine treated mice had a significantly fewer BrdU-positive cells than animals injected with vehicle**. Data were collected from 5 experimental (cyclopamine) mice and 4 control (vehicle) mice, p=0.02; error bars= SEM

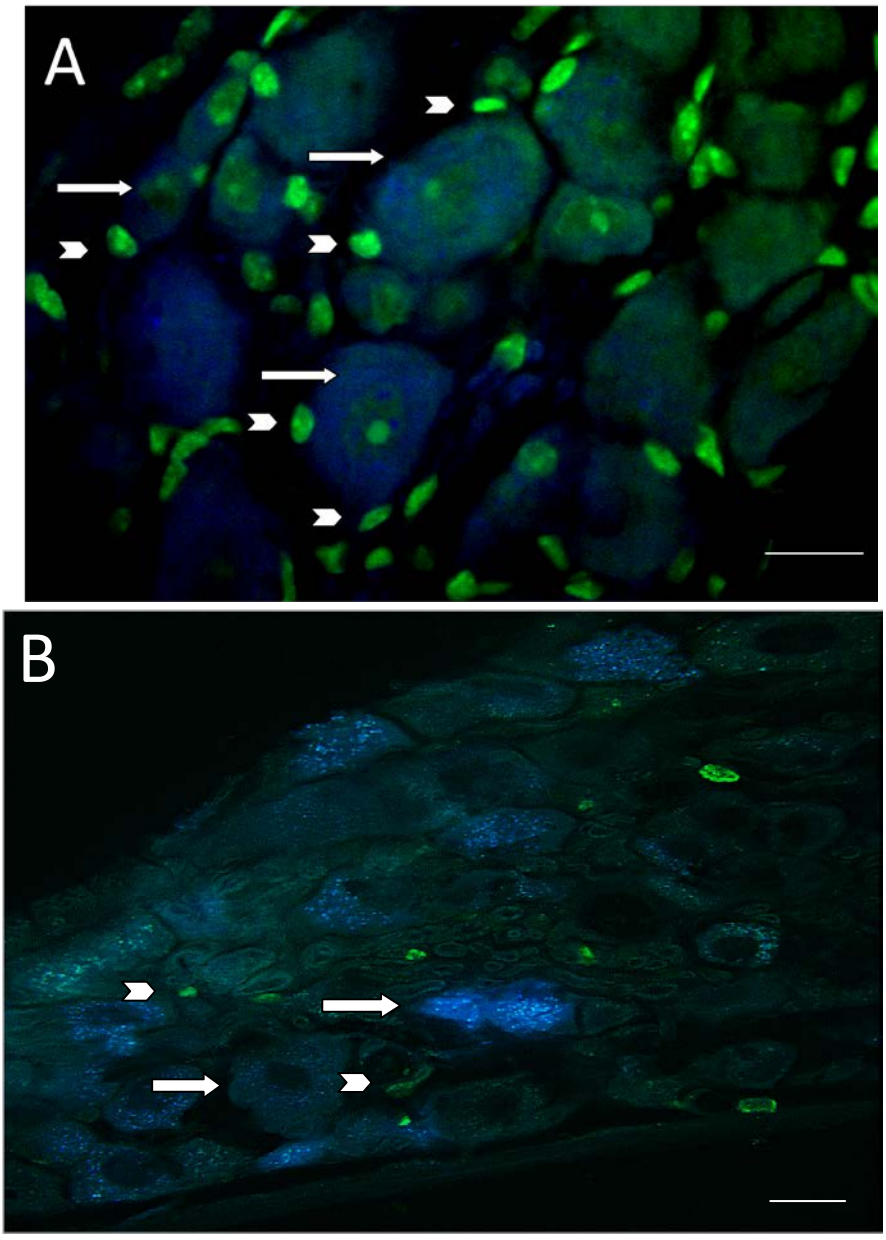

Figure. 9. **The Shh inhibitor cyclopamine decreases proliferation of SGCs following sciatic nerve section.** Immunostaining of BrdU (green) labeled the nuclei of proliferated SGCs and Fast-blue (blue) indicates injured neurons. A. DRG section with Fast-blue positive neurons from a mouse injected with vehicle (control) Scale bar = 50 µm. (B) DRG section with Fast-blue positive neurons from a cyclopamine-treated mouse, which had fewer BrdU-positive cells than control mice. Scale bar = 30 µm; arrows indicate FB positive neurons; arrow heads indicate BrdU-positive SGCs.

Discussion

Although it is well established that SGCs of the DRG proliferate following axotomy (Hanani 2005), mechanisms that signal this proliferation have not been elucidated. In the present study, blocking Shh signaling led to a significant decrease in proliferation of the SGCs, as assessed 7 days after sciatic nerve cut (axotomy). These results suggest that Shh is involved in injury-induced SGC proliferation.

To assess cellular proliferation, BrdU was administered in the drinking water, a technique that has been successfully used by others to study SGC proliferation following introduction of the herpes simplex virus (Elson et al., 2003). I did not use a specific marker of SGCs here, but I feel confident the BrdU-positive cells I counted were SGCs. I could readily identify SGCs based on the size, morphology, and location, and from our previous experience identifying SGCs that were immunoreactive for SK3, which specifically labels the SGCs in the DRG. Moreover, it has been reported that the SGCs are the only BrdU-positive cells seen near the soma of the neurons of the DRG following axotomy, an observation that was confirmed by colocalizing SGC markers with BrdU (Jasmin et al., 2010). Therefore, I am confident the BrdU-positive cells I counted were SGCs.

Shh influences proliferation and differentiation of the neurons and glia in the developing DRG (Guan et al., 2003; Ungos et al., 2004). Shh reduction (by genetic mutation or pharmacological inhibition of Shh) in zebrafish results in DRGs that are misshapen and have reduced numbers of neurons and glial cells (Ungos et al., 2003). In the chick, Shh patterns the DRG by influencing both proliferation and differentiation of neuronal cell types during the early postnatal stages of development. Shh creates a gradient within the DRG and has a role in both the proliferation and programmed cell death that occurs during the later stages of development.

Increasing or decreasing that gradient can then alter the proliferation or programmed cell death, depending on location within the gradient (Guan et al., 2008).

Studies have revealed roles for Shh following injury in the CNS and the PNS. In the CNS, Shh expression is up-regulated following injury, and has been shown to influence proliferation. Shh expression increases in the motor neurons following axotomy of the facial nerve in adult rats. This increase in Shh may increase neuronal survival following injury (Akazawa et al., 2004). Over-expressing Shh in neonatal rats led to increased neuronal survival following facial nerve axotomy (Akazawa et al., 2004). Furthermore, intravenous injections of Shh in adult mice with spinal cord injury induce proliferation of neural precursors and oligodendrocytes (Bambakidis et al., 2010). Shh also regulates proliferation of neural progenitors in the hippocampus following ischemia (Sims et al., 2009). The neural precursors proliferate following ischemia and this proliferation can be blocked with cyclopamine and increased by Shh over-expression, by genetic mutation (Sims et al., 2009). In addition, intrathecal administration of Shh protein results in an increase in recovery of behavioral function, as well as increase in proliferation of nestin positive cells in the SVZ, following a rodent model of ischemic stroke (Bambikidis et al., 2012).

In the adult PNS, Shh expression has been shown to increase following injury, but no evidence for Shh having a role in proliferation has been reported. In the DRG it has been demonstrated that Shh mRNA expression increases in the injured neurons following peripheral nerve injury (So et al., 2006). It is also well established that SGCs proliferate following injury (Hanani 2005). Since there is evidence that Shh influences injury-induced proliferation in the CNS following injury, and Shh expression increases in the neurons of the DRG following injury, Shh may influence injury-induced proliferation of the SGCs in the PNS as well.

Shh influences proliferation by signaling via the primary cilium (Huangfu et al., 2003; Huangfu and Anderson, 2005; Rohatgi et al., 2006; Christensen et al., 2007). In another study (Chapter 2 of this thesis) I investigated the percentage of cells with primary cilia in axotomized and non-axotomized DRGs. Both the SGCs and neurons of the DRG have primary cilia. Since substantial percentages of the SGCs of the DRG have primary cilia, these cells may be targets of Shh. In the current study I found a decrease in the number of BrdU-positive cells in animals treated with the Shh antagonist cyclopamine. Taken together, these data suggest that the injured neurons in the DRG might release Shh, which may then signal via the primary cilia on the surrounding SGCs to induce proliferation.

Since neurons of the DRG have primary cilia as well, this injury-induced release of Shh may also have an autocrine affect. Shh may be released from the injured neurons and then bind to the primary cilia on those same neurons, which may aid in neuronal survival. Others have demonstrated a role for Shh in neuronal survival in the neonatal spinal cord of rats following injury (Akazawa et al., 2004) as well as a role for Shh protecting against oxidative stress in injury induced ischemia in the adult brain (Ji et al., 2012). Shh might play a similar role in the adult DRG following injury, but I have not yet tested this idea.

In the present study, animals injected with cyclopamine displayed less autotomy (hind limb chewing). This behavior is commonly observed following peripheral nerve injury. It is unclear why mice exhibit autotomy, although it has been suggested that it may be due to pain (Wall et al., 1979). The decrease in autotomy in the animals injected with cyclopamine could indicate that they were in less pain. Reinforcing this idea, the mice that were injected with cyclopamine were easier to handle and were less jumpy when handled for daily injections. These observations suggest that reduced Shh signaling leads to behavioral changes consistent

with a reduction in pain. It is not clear whether this is due to a decrease in SGC proliferation or to changes elsewhere. There was not a significant difference in weight between groups; therefore this decrease in pain behavior is probably not due to illness produced by the cyclopamine injections.

It has also been reported that cyclopamine can prolong the effects of morphine. Cyclopamine administered together with morphine, causes morphine's analgesic effects to last longer (Babcock et al., 2011). Also, animals with reduced Shh, levels through a mutation in the processing of Shh, fail to develop thermal allodynia and hyperalgesia (Babcock et al., 2011). The authors suggest that the effects of Shh mutations on pain thresholds could not be due to proliferative effects, but they based this suggestion on the behavior of DRG neurons. Injured neurons in the adult DRG are thought to be unable to proliferate in response to Shh because neurons in the adult DRG are thought to be terminally post-mitotic (Babcock et al., 2011). Given the results of the present study, it seems possible that injury-induced SGC proliferation may play a role in pain behavior. Further studies addressing Shh's role in modulating pain will be necessary to further understand these observed behaviors.

This is the first report suggesting that Shh plays a role in proliferation of PNS cells following injury. Further studies are needed to directly test the involvement of primary cilia in SGC injury-induced proliferation. If SGC proliferation plays a role in pain, identifying molecules involved with this signaling might lead to the development of therapies for pain and other peripheral neuropathies.

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DISCUSSION

Primary cilia are sensory organelles found on most vertebrate cell types. The presence of occasional cilia on cells of most cell types has been recognized since the 1800s, but only recently has the ubiquity of these organelles within cell types been recognized (Bloodgood, 2009). Moreover, it is now apparent that these organelles are essential to normal cell function. Disrupted function of primary cilia in the embryonic node can lead to situs inversus (Nonaka et al., 1998), as well as other variants, depending on the disruption of the cilia. In addition, primary cilia knockouts are embryonic lethal (Pazour and Rosenbaum, 2002). Disrupted cilia function has recently been recognized as the basis for a number of disease states, now referred to as 'ciliopothies'. These ciliopothies include polycystic kidney disease, nephronophthisis, retinitis pigmentosa, Bardet–Biedl syndrome, Joubert syndrome, and Meckel syndrome (Schwartz, 2011).

A variety of functions for primary cilia have been identified. One of the initial roles was described in the kidney. Here, primary cilia extend into the lumen of the tubules, where they act as mechanosensors. Primary cilia influence proliferation in the kidney by acting as mechanical flow sensors. The epithelial cells of the kidney tubules have primary cilia that extend into the lumen of the tubule and sense movement of fluid through the renal ducts. When the cilium bends due to fluid movement, mechanoreceptors on the cilium that are associated with calcium influx are activated and calcium flows into the cell (Nauli et al., 2003). The calcium enters through the polycystin-2 (PC-2) channel (Praetorius et al., 2001). In the absence of calcium flow into the cell there is cleavage of polycystin 1 (PC-1) which activates STAT6 and P100 - proteins involved in transcriptional regulation and cellular proliferation (Low et al., 2006). In vitro studies have shown that a lack of fluid movement leads to decreased Ca+ influx; conversely,

moving the primary cilium by mechanically applied force increases Ca+ influx (Praetorius and Spring, 2001). It was hypothesized that cells with shortened cilia cannot fully respond to fluid movement, resulting in decreased calcium influx (Low et al., 2006) and increased activation of transcription factors, which leads to increased cell proliferation.

These lead to changes in cell proliferation, seen as the development of cysts. It has been demonstrated that ciliary defects underlie polycystic kidney disease (Praetorius and Spring, 2001). Primary cilia on other cell types may also serve as mechanosensors. For example, primary cilia on osteocytes are thought to sense mechanical deformation of bone, and perhaps play a role in subsequent bone remodeling. Mice with defected primary cilia of the osteocytes showed a decrease in new bone formation following ulnar stress, compared to control mice (Temiyasathit et al., 2012).

Primary cilia have been identified in the central nervous system, on neurons (Fuchs and Schwark, 2004) astrocytes (Bishop et al., 2007) and adult neural progenitor cells (Fuchs and Schwark 2004). A mechanosensitve role for these cilia has not yet been discovered. Instead, they appear to be essential for transducing Shh's effect on cell proliferation (Huangfu et al., 2003; Huangfu and Anderson, 2005; Christensen and Ott, 2007; Rhoatig et al., 2007). Little is known about the characteristics of primary cilia in the PNS. A few reports, based on electron microscopy, have described cilia on neurons (Tan et al., 2007), satellite glia cells (SGCs) (Pannese, 1981), and Schwann cells (Grillo and Palay, 1963). From these reports, however, it was not clear how many of each of the cells types have primary cilia. The experiments described in this thesis determined that 14% of sensory neurons in the DRG had primary cilia. Because the neuronal population of the DRG is comprised of neurons which differ in their morphology and physiology, I evaluated the expression of cilia on neurons after they were grouped according to

size. I found that 29% of the large neurons were ciliated, 16% of the medium neurons were ciliated and 5% of the small neurons had primary cilia. It is not yet clear why the expression of primary cilia varies with neuronal size. Future experiments might begin to address this question by, for example, examining the nature of the sensory deficits that follow cilia disruption. One experiment of this type has been reported. One study reported that if the basal body of the primary cilia were disrupted then those animals showed thermo- and mechano-sensory deficits. Using techniques to identify the root of the deficit, they were able to identify that the sensory deficits were most likely due the mutated primary cilia of the neurons in the DRG, but the exact mechanisms were not identified (Tan et al., 2007).

I found that 26% of SGCs of the DRG have cilia. It has been proposed that a neuron and its surrounding SGCs form a functional unit. This represents a unique organization of closely interacting, yet different cell types in which to examine how cilia might contribute to the interactions. It might be expected, for example, that SGC primary cilia would lie between the somata of the SGCs and the neuron in order to facilitate communication between these cell types. However, in agreement with a previous report (Pannese, 2010) I found that while some cilia were in this position, others were positioned between the SGC somata and the surrounding basement membrane. It therefore appears that primary cilia on SGCs might provide a route for neuron-SGC communication as well as a route to sample the extracellular environment surrounding the neuron-SGC unit. It has been shown that SGCs react to changes in neuronal physiology (Hanani, 2005). The positioning of the primary cilia suggests that the SGCs might respond not only to the neuron within their functional unit, but to neurons outside the unit as well.

The expression of primary cilia on SGCs in functional units containing small neurons varied according to whether or not the neuron was ciliated. More of the SGCs had cilia if the neuron in their functional unit was not ciliated (48% vs. 29%). Because I still know very little about the functions of primary cilia in the DRG, it is difficult to speculate about the reasons for this difference.

An important function of the cells of the DRG is to respond to peripheral sensory stimulation. When this stimulation becomes noxious or physically damaging, the cells' responses become accentuated and can lead to pathological states such as chronic pain. One model that has been used to study such responses is axotomy by transaction of a peripheral nerve. Following axotomy, the neurons increase in excitability, (Abdualla and Smith, 2001) release ATP (Zhang et al., 2007), and increase expression of many genes (Xiao et al., 2002). Neurons have also been shown to die, presumably by apoptosis, following axotomy (Vestergaard et al., 1997). The SGCs also respond to axotomy. Following injury to the peripheral nerve there is an increase in gap junction formation between SGCs surrounding the injured neuron (Hanani et al., 2002), and increases in the number of potassium channels (Zhang et al., 2009). SGCs also increase in GFAP expression following injury (Hanani, 2005) as well as proliferate (Lu and Richardson, 1991). It is tempting to speculate that an increase in SGC number provides increased buffering around neurons that are rapidly firing action potentials following the injury, though this has not been demonstrated. Because some aspects of the response to axonal injury involve communication between neurons and SGCs, I looked for changes in primary cilia in the DRG following axotomy. Following axotomy, more DRG neurons had primary cilia. Increased ciliation was seen in all neuron sizes, although only the increase for medium-sized neurons (from 16% to 22%) was statistically significant. Primary cilia also increased in SGCs (from 26% to

almost 49%). These results are consistent with the idea that signaling mediated by primary cilia increases following axotomy. While it is not clear what signals may be involved, I will discuss below the possibility that it may involve Shh.

With significant peripheral nerve damage (such as axotomy), DRG neurons undergo a stress response and, in some cases, die. Stressed neurons have been identified by their expression of the transcription factor ATF3, or simply by the eccentric position of the nucleus within the soma. In the present experiments, neurons with eccentric nuclei were much less likely to have a primary cilium. This suggests that part of the stress response might include withdrawal of the primary cilium, possibly as a prelude to cell death. Unpublished work from our lab is consistent with this idea: neurons expressing caspase 3 rarely have primary cilia (Coronel, unpublished data).

In the CNS, proliferative responses appear to involve Shh signaling which depends on primary cilia. For example, experimentally-induced ischemia induces proliferation of neural progenitors in the hippocampus, a response that is mediated by Shh which apparently is released from hippocampal neurons (Sims et al., 2009). In a model of stroke-induced ischemia, blocking Shh signaling leads to increased severity of neurological symptoms (Ji et al., 2012). Following traumatic brain injury, reactive astrocytes begin to express Shh, and Shh signaling promotes the proliferation of neural stem cell precursors (Amankulor et al., 2009). Following axotomy of the facial nerve in adult rats, motor neurons begin to express Shh. Blockade of Shh signaling in these animals results in motor neuron death, while overexpression of Shh in neonates enhances motor neuron survival (Akazawa et al., 2004). Many or all of these effects of Shh may be mediated by primary cilia. It has been demonstrated that disruption of primary cilia in neuronal precursors in developing or adult brain results in decreased rates of proliferation, an effect

normally mediated by Shh (Han et al., 2008; Spassky et al., 2008; Bruenig et al., 2008; Amador-Arjona et al., 2011). Thus, there is good evidence that Shh plays a role in stimulating injury-induced cell proliferation in the CNS, that Shh signaling depends on intact primary cilia, and that Shh might have protective effects on damaged neurons.

Shh plays an important role in development of the peripheral nervous system, where it is required for the formation of the neural crest (Patten and Placzek, 2000). At later stages, Shh influences the development of the spinal cord and the DRG. In the spinal cord, Shh is specifically involved in motor neuron development (Ericson et al., 1996; Madden 2006). In zebrafish, reduced Shh signaling results in DRGs that are misshapen and have reduced numbers of neurons and glia. Shh has also been shown to influence the expression of neurogenin 1, which is required for the differentiation of DRG precursors (Ungos et al., 2003). Shh plays a role in the patterning of the developing chick DRG during later stages of development by influencing the proliferation and differentiation of neuronal cell types. These influences can be seen in a dorsomedial-to-ventrolateral gradient of Shh effects (Guan et al., 2008).

As described above, axotomy stimulates proliferation of SGCs in the DRG. Moreover, damage to peripheral axons leads to increased expression of Shh in DRG sensory neurons (So et al., 2006). In the experiments described in this thesis, I found that blocking Shh signaling resulted in decreased proliferation of SGCs. It seems likely that this effect of Shh is mediated by primary cilia on the SGCs. It is not clear if primary cilia might play a similar role in DRG neurons. There is some evidence that new neurons replace those that die following axotomy (McKay-Hart et al., 2004) but this takes approximately 3-6 months, well past the survival time examined in the present experiments. Moreover, the source of these new neurons has not been identified. Another possibility is that Shh might act on primary cilia of DRG neurons to enhance

their survival following axotomy. Evidence for an autocrine effect of Shh is seen in motor neurons following facial nerve damage (Akazawa et al., 2004).

The present research raises a number of questions for future research. For example, it would be useful to determine if any cells other than neurons begin to express Shh, and to determine the time course of Shh expression in the DRG following axotomy. It would also be interesting to find out if direct damage (axotomy) is required to elicit Shh expression, or if other injury models also increase Shh expression. It has been reported that skin abrasion is sufficient to induce SGC proliferation (Elson et al., 2004). Although such a model would presumably entail less damage to the DRG neurons, I would still predict that the neurons would begin to express Shh to elicit a proliferative response in the SGCs. I also would like to further study the idea that primary cilia play a role in protecting cells from damage. I have evidence that the injured cells are more likely not have a primary cilium than those that are not injured, but I do not know if those cells merely lost their cilia due to stress or if they were more susceptible to stress because they were lacking primary cilia when the injury occurred. Genetic tools such as conditional ablation of primary cilia in the mature DRG, will be useful for uncovering additional answers to these questions.

The Main Conclusions for this Thesis are as Follows:

1. Twenty six percent of the SGCs in the normal DRG are ciliated
2. Twenty nine percent of the small neurons, 16% of the medium neurons and 5% of the large neurons in the normal DRG are ciliated.
3. There is a significant increase in percentage of SGCs with primary cilia following axotomy.

4. There is an increase in percentage of neurons with primary cilia following axotomy, but this increase in only significant for the medium neurons.
5. The proliferation of the SGCs that follows as a result of axotomy can be decreased by blocking the Shh pathway, using cyclopamine, a Shh antagonist.

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