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Desert hedgehog is a mediator of demyelination in compression neuropathies

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ABSTRACT

The secreted protein desert hedgehog (*dhh*) controls the formation of the nerve perineurium during development and is a key component of Schwann cells that ensures peripheral nerve survival. We postulated that *dhh* may play a critical role in maintaining myelination and investigated its role in demyelination-induced compression neuropathies by using a post-natal model of a chronic nerve injury in wildtype and $dhh^{-/-}$ mice. We evaluated demyelination using electrophysiological, morphological, and molecular approaches. *dhh* transcripts and protein are down-regulated early after injury in wild-type mice, suggesting an intimate relationship between the hedgehog pathway and demyelination. In $dhh^{-/-}$ mice, nerve injury induced more prominent and severe demyelination relative to their wild-type counterparts, suggesting a protective role of *dhh*. Alterations in nerve fiber characteristics included significant decreases in nerve conduction velocity, increased myelin debris, and substantial decreases in internodal length. Furthermore, in vitro studies showed that *dhh* blockade via either adenovirus-mediated (shRNA) or pharmacological inhibition both resulted in severe demyelination, which could be rescued by exogenous Dhh. Exogenous Dhh was protective against this demyelination and maintained myelination at baseline levels in a custom in vitro bioreactor to applied biophysical forces to myelinated DRG/Schwann cell co-cultures. Together, these results demonstrate a pivotal role for *dhh* in maintaining myelination. Furthermore, *dhh* signaling reveals a potential target for therapeutic intervention to prevent and treat demyelination of peripheral nerves in compression neuropathies.

1. Introduction

Compression neuropathies are highly prevalent, debilitating conditions with variable functional recovery following treatment. Evidence suggests that Schwann cells are the primary mediators of the disease process in compression neuropathies and undergo concurrent proliferation and apoptosis early (Gupta and Steward, 2003). These proliferating Schwann cells down-regulate myelin proteins, such as myelin-associated glycoprotein, leading to both demyelination and remyelination of the axon as well as axonal sprouting (Gupta et al., 2006). Interestingly, these early changes occur in the absence of both morphological and electrophysiological evidence of axonal damage (Pham and Gupta, 2009). Contrastingly, acute neural injuries, such as transection or crush injuries, are characterized by axonal injury followed by the ensuing Wallerian degeneration. Alternatively, compression neuropathies are characterized by a local demyelination in the absence of Wallerian degeneration (Gupta et al., 2012).

Central and peripheral nerve demyelination remains a major challenge for multiple neural conditions ranging from multiple sclerosis to compression neuropathies. After demyelination in the peripheral nervous system (PNS), Schwann cells remyelinate axons with a thinner layer of myelin, resulting in decreased conduction velocities and impulse propagation (Iwashita and Blakemore, 2000; Ludwin and Maitland, 1984; Schröder, 1972; Sherman and Brophy, 2005). Multiple cellular pathways have been implicated in demyelination and remyelination, including neuregulin (NRG), ERK/MAPK, Notch, and Sonic hedgehog signaling (Napoli et al., 2012; Tang et al., 2010; Taveggia et al., 2005; Woodhoo et al., 2009). Furthermore, in the context of the peripheral nervous system, all of these signaling cascades have been shown to originate from the Schwann cell (Hashimoto et al., 2008; Napoli et al., 2012; Woodhoo et al., 2009). After injury, high concentrations of NRG isoforms at the site of injury inhibit myelination and induce myelin degradation and Schwann cell proliferation and migration (Guertin et al., 2005; Zanazzi et al., 2001). Furthermore, following demyelinating injuries, there is a rapid and robust activation of

Abbreviations: CNC, chronic nerve compression; *dhh*, desert hedgehog; DRG, dorsal root ganglion; Ihh, Indian hedgehog; NCV, nerve conduction velocity; NRG, neuregulin; PNS, peripheral nervous system; *ptc*, patched; Shh, Sonic hedgehog.

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ERK signaling that is critical for controlling myelin thickness (Fyffe-Maricich et al., 2013; Ishii et al., 2012; Napoli et al., 2012). Interestingly, high levels of ERK activity have also been observed in Schwann cell models of various hereditary and infectious peripheral neuropathies (Stoll et al., 2002; Suter and Scherer, 2003). While there has been much work directed at mechanisms involved with hereditary, infectious, and lysolecithin-induced demyelination, acquired compression demyelinating neuropathies have not been as widely studied.

Desert hedgehog (*dhh*) is a signaling molecule produced by Schwann cells that ensures axonal survival and is required to regulate myelination in the PNS (Sharghi-Namini et al., 2006). *dhh* induces formation of nerve perineurium when bound to its receptor patched, *ptc*, on peripheral nerves (Parmantier et al., 1999). While mice lacking *dhh* exhibit normal function and gross phenotype, they exhibit abnormal perineurium development and mini-fascicle formation. Moreover, recent data has shown that *Sox10* activates *dhh* expression in Schwann cells via an enhancer and thereby increases *dhh* levels to promote formation of the perineurial sheath (Küspert et al., 2012). Decreased *dhh* has also been linked to diabetic neuropathies, as treatment via Sonic hedgehog (Shh) fusion proteins has shown recovery of conduction velocities (Calcutt et al., 2003). Interestingly, the three hedgehog signaling proteins, Sonic, Indian, and desert have all been well studied and shown to act through the same receptor and signaling cascade (Hahn et al., 1999; Parmantier et al., 1999; Stone et al., 1996; Varjosalo and Taipale, 2008; Xie et al., 1998). When Schwann cells are stimulated by the addition of *dhh* or smoothened agonists, formation of myelin segments occurs; however, in their absence, myelin segments were lacking (Yoshimura and Takeda, 2012).

As a proof of principle for using *dhh* for therapeutic interventions with demyelinating neuropathies, we investigated the role of *dhh* signaling in demyelination by using an acquired form of demyelination with a compression-induced injury. We investigated the effects of *dhh* deletion on the myelination of peripheral nerves in both in vivo and in vitro models of CNC injury and identify de-activation of *dhh* expression being the pivotal event in demyelination. Furthermore, we show that *Dhh* has a vital role against mechanical stimuli in our in vitro model of compression neuropathies. Finally, loss of *dhh* with demyelination provides evidence for considering the protein as a regulator of myelination as well as a future therapeutic target for compressive neuropathies and possibly other demyelinating diseases.

2. Materials and methods

2.1. Animal model

All procedures involving living animals were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. A previously described model of chronic nerve compression was used in these experiments with 6 week old, male C57BL/6 mice (Harlan Laboratories, UK) and *dhh* null (B6; 129-Dhh^{tm1Amc}/J) mice (Jackson Laboratories, Bar Harbor, ME) with genotyping performed by Transnetyx, Inc (Transnetyx, TN) (Gupta et al., 2012). Mice were anesthetized by intraperitoneal injection of ketamine/xylazine. A dorsal gluteal-splitting approach was used to isolate and dissect each sciatic nerve free from its surrounding tissue. A 3-mm biologically inert silastic tube (Cole-Palmer, Vernon Hills, Illinois) was placed atraumatically around the ipsilateral nerve below the level of the sciatic notch. All tubing was placed in a Petri dish and soaked in 70% ethanol overnight prior to surgery and allowed to dry in a vacuum hood to minimize the inflammatory response. After the experimental nerve was returned to the host bed, the tube readily glided along the nerve.

2.2. Electrodiagnostic evaluation

Electrodiagnostic studies of nerve conduction velocity (NCV) were performed on all animals pre-operatively to establish their baseline values and serially at bi-weekly time points by an experienced blinded evaluator (TM). Animals were anesthetized with ketamine/xylazine anesthesia and nerve conduction studies were recorded in the ipsilateral experimental and contralateral limbs (Sierra LT; Cadwell Laboratories, Kennewick, WA). The sciatic nerve was electrically stimulated at the sciatic notch and popliteal region using monopolar EMG electrodes (Ambu Inc., Glen Burney, MD), with the reference for stimulating electrodes placed in the paraspinal muscles. A recording subdermal EEG electrode (Ambu Inc., Glen Burney, MD) was placed in the tibialis anterior muscle, with the reference electrode placed in the footpad. Latency, compound motor amplitude, conduction velocity, and electrical threshold needed to obtain a response were recorded.

2.3. Preparation for RT-PCR analysis

Total RNA was obtained from uninjured and CNC sciatic nerves using Trizol reagent (Invitrogen, Grand Island, NY) and reverse transcribed with SuperScript III First-strand synthesis system (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. RT-PCR was performed to assess the *dhh* mRNA expression in *dhh* heterozygous and homozygous mice following the protocols previously described (Parmantier et al., 1999).

The quantitative RT-PCR reactions were performed using the Taqman method. The amplification reactions were made in triplicate, using 96-well plates with 1 μl of primers and probe, 6 μl of cDNA, 3 μl of DEPC water and 10 μl of master mix (Invitrogen, Grand Island, NY). The thermal cycling profile consisted of an initial temperature of 95 °C for 20 s and then 40 successive cycles at 95 °C for 1 s and 60 °C for 20 s. The Taqman assay of mouse *dhh* gene expression was used and Taqman Endogenous Control of 18S rRNA (Invitrogen Cat # 4352930E) was used to normalize the *dhh* mRNA expression. Results are expressed as the fold change of *dhh* gene cDNA in normal sciatic nerves relative to CNC sciatic using the $2-\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

2.4. Preparation for histology and electron microscopy

dhh null mice nerves both ipsilateral and contralateral to CNC injury were harvested and rinsed in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, rinsed in ddH_2O for 2 × 10 min, dehydrated in increasing serial dilutions of ethanol (70%, 85%, 95%, 100% × 2) for 10 min each, placed in propylene oxide for 2 × 10 min, incubated in propylene oxide/Spurr's resin (1:1 mix) for 30 min, and in Spurr's resin overnight. Sections were flat-embedded and polymerized overnight at 60 °C. Molds were cut with an ultramicrotome to obtain 1 μm sections and stained with toluidine blue and whole nerve maps of cross-sections were captured at 100× magnification using an inverted microscope (1X71; Olympus) for histology. Ultrathin sections of 60 nm thickness were cut, mounted on copper grids and viewed using a JEOL 1400 electron microscope for EM.

2.5. Morphometric analysis

To assess changes in myelination, g-ratios were calculated as the ratio of axon diameter to the total fiber diameter for 500 axons per group per time point. Total axon counts and number of myelinated axons were evaluated in uninjured and injured *dhh* null samples. Distribution of axon diameter (d) was also evaluated in uninjured and compressed specimens and fibers were categorized as either small ($d \leq 3$ μm), medium (3 μm $\leq d \leq 6$ μm), or large ($d \geq 6$ μm). All measurements were taken using VisioPharm software (VisioPharm, Broomfield, CO).