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Sulfatide is required for organization of the paranode in the myelinated axon in the peripheral nervous system

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Sulfatide is required for organization of the paranode in the myelinated axon in the peripheral nervous system

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

by

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# TABLE OF CONTENTS

Acknowledgements ......................................................................................................................... ii

List of abbreviations ........................................................................................................................ viii

List of figures ................................................................................................................................... xii

List of tables ...................................................................................................................................... xiv

Abstract ........................................................................................................................................... xv

Introduction ...................................................................................................................................... 1

Communication within the nervous system ................................................................................... 1

  The neuron ...................................................................................................................................... 1

  The action potential ....................................................................................................................... 2

  Improving communication .............................................................................................................. 3

Myelin .................................................................................................................................................. 4

  CNS myelin ..................................................................................................................................... 4

  PNS myelin ..................................................................................................................................... 5

    Molecular aspects of PNS myelin ................................................................................................. 5

    Structural aspects of PNS myelin ............................................................................................... 6

  Schwann cells ................................................................................................................................. 9

    Classification of Schwann cells ................................................................................................. 12

Domains of the myelinated axon ............................................................................................... 13

  Node of Ranvier ........................................................................................................................... 16

  Paranode ....................................................................................................................................... 18
Juxtaparanode..................................................................................................................19
Internode..........................................................................................................................20
Myelin lipids and their role in organizing domains of the myelinated axon..................22
  *In vitro* studies: GalC and myelin formation.........................................................23
  *In vivo* studies: the CGT-knockout mouse ..............................................................24
  *In vivo* studies: the CST-knockout mouse ...............................................................25
Clinical implications........................................................................................................27
  CNS myelin diseases ....................................................................................................28
    Multiple sclerosis .....................................................................................................28
    Alzheimer’s disease ..................................................................................................29
    Metachromatic leukodystrophy ...............................................................................29
  PNS myelin diseases ....................................................................................................30
    Guillain Barré syndrome – acute motor axonal neuropathy ....................................30
Project aim .....................................................................................................................32

Materials and Methods................................................................................................33
  Animals ........................................................................................................................33
  Immunohistochemistry ..............................................................................................33
    Number of animals ..................................................................................................33
    Perfusion and tissue processing ..........................................................................33
    Immunohistochemistry protocol .................................................................34
    Visualization ..........................................................................................................35
    Quantification of protein clusters ......................................................................36
Quantification using Adobe Photoshop CS3 ............................................................37
Electron microscopy ...............................................................................................................38
Number of animals ..........................................................................................................38
Perfusion and tissue processing .......................................................................................39
Visualization ....................................................................................................................40
Quantification of 1 µm sections ......................................................................................40
Normalization of immunohistochemistry counts ............................................................41

Results ............................................................................................................................................42
Immunohistochemistry ...........................................................................................................42
15 days of age ..................................................................................................................42
1 month of age .................................................................................................................47
7 months of age ...............................................................................................................50
Preliminary results and ongoing studies .................................................................................53
Electron microscopy: 90 nm sections ..............................................................................53
15 days of age ...................................................................................................................53
4 months of age ...............................................................................................................58
Light microscopy: 1 µm sections ....................................................................................58
15 days of age ...................................................................................................................58
1 month of age ..................................................................................................................63
7-8 months of age ..............................................................................................................63
Immunohistochemistry: normalized Na, 1.6, gliomedin, Nfasc155, contactin data ........63
Normalized protein cluster averages at 15 days of age ..................................................68
Normalized protein cluster averages at 1 month of age ...........................................68
Normalized protein cluster averages at 7 months of age..........................................69
Immunohistochemistry: Na\textsubscript{v}1.6 & Nfasc186, Na\textsubscript{v}1.6 & ankyrin\textsubscript{G} ..................................70

Discussion ......................................................................................................................................75
Sulfatide is essential for paranodal organization........................................................................76
Sulfatide is differentially important for nodal protein organization depending on
    cell of origin .................................................................................................................................79
Initial clustering and long-term maintenance of nodal Na\textsubscript{v}1.6 channels utilize
different mechanisms..................................................................................................................80
Molecular and structural organization of the myelinated axon may not correspond
    in the absence of sulfatide ...........................................................................................................82
The density of myelinated axons is reduced in the absence of sulfatide ...............................83
    A reduced density of myelinated axons in the CST KO mice has an influence
        on immunohistochemistry counts.......................................................................................84
Sulfatide is more important for the organization of Na\textsubscript{v}1.6 clusters in the
    CNS than in the PNS ..................................................................................................................86
Preliminary data suggest a role for sulfatide in the initial clustering of nodal
    and paranodal proteins in the PNS ..........................................................................................87
Closing remarks ...............................................................................................................................88

References......................................................................................................................................89
LIST OF ABBREVIATIONS

Aβ ......................................................................................................................... amyloid-β peptide
AD ..................................................................................................................... Alzheimer’s disease
AIS .................................................................................................................... axon initial segment
AMAN ............................................................................................. acute motor axonal neuropathy
apoE ........................................................................................................................ apolipoprotein E
ASA........................................................................................................................... arylsulfatase A
ATPase ...................................................................................................... adenosine triphosphatase
Ca............................................................................................................................calcium
CAM ............................................................................................................. cell adhesion molecule
Caspr ..................................................................................................... contactin-associated protein
CGT.................................................................................................. ceramide galactosyltransferase
cST ................................................................................................... galactosylceramide (cerebroside) sulfotransferase
CNS............................................................................................................... central nervous system
Cx32........................................................................................................................ connexin32
EM..................................................................................................................... electron microscopy
EPSP ............................................................................................ excitatory post-synaptic potential
FOV............................................................................................................................... field of view
g................................................................................................................................................. gram
GalC .................................................................................................................... galactosylceramide
GalNAcT ........................................................................... β1, 4-N-acetylgalactosaminytransferase
GBS......................................................................................................................... Guillain-Barré syndrome
GPI ................................. glycophosphatidylinositol
IgSF .................................. immunoglobulin superfamily
IHC ..................................................... immunohistochemistry
IPL ........................................................ intraperiod line
IPSP ........................................................ inhibitory post-synaptic potential
K ................................................................. potassium
kDa .......................................................... kiloDalton
KO ............................................................... knockout
$K_v$ ..................................................... voltage-gated potassium
$K^+$ ......................................................... potassium ions
L-MAG ..................................................... large myelin-associated glycoprotein
M ................................................................. molar
MAG ....................................................... myelin-associated glycoprotein
MBP .......................................................... myelin basic protein
MCI .......................................................... mild cognitive impairment
MDL ........................................................ major dense line
mL ........................................................... milliliter
MLD ........................................................ metachromatic leukodystrophy
mm ........................................................ millimeter
MS ........................................................ multiple sclerosis
mV .......................................................... millivolts
Na ................................................................. sodium
$Na_v$ ...................................................... voltage-gated sodium
NAWM ................................................................................................................. normal appearing white matter
Na+ ............................................................................................................................... sodium ions
Nfasc .......................................................................................................................... neurofascin
Nfasc155 .................................................................................................................. neurofascin 155
Nfasc155H ............................................................................................................. neurofascin 155-high
Nfasc155L ............................................................................................................. neurofascin 155-low
Nfasc186 .................................................................................................................. neurofascin 186
nm .............................................................................................................................. nanometer
NrCAM ..................................................................................................................... neuron-glia related cell adhesion molecule
Necl ........................................................................................................................... nectin-like
NMJ .......................................................................................................................... neuromuscular junction
NMSC ..................................................................................................................... non-myelinating Schwann cell
OL .............................................................................................................................. oligodendrocyte
PBS ......................................................................................................................... phosphate buffered saline
PDZ ............................................................................................................................. post-synaptic density protein,

  Drosophila disc large tumor suppressor, zona occludens

PNS .......................................................................................................................... peripheral nervous system
PLP ............................................................................................................................ proteolipid protein
P0 ............................................................................................................................... protein zero
P2 ............................................................................................................................... protein two
SC ............................................................................................................................. Schwann cell
SLI ........................................................................................................................... Schmidt-Lanterman incisure
S-MAG ..................................................................................................................... small myelin-associated glycoprotein
SN ................................................................. sciatic nerve
TAG-1 ................................................................. transient axonal glycoprotein-1
TB ................................................................. transverse band
tSC ................................................................. terminal Schwann cell
µm ................................................................. micrometer
$V_{\text{extracellular}}$ ................................................................. extracellular voltage
$V_{\text{intracellular}}$ ................................................................. intracellular voltage
WT ................................................................. wild type
LIST OF FIGURES

Figure 1: Ultrastructure of PNS myelinated axons ........................................................................8
Figure 2: Schematic representation of Schwann cell myelination and the myelinated axon .......11
Figure 3: The structural and molecular domains of the PNS myelinated axon .............................15
Figure 4: Nodal proteins cluster normally while paranodal protein clustering is disrupted in CST KO mice at 15 days of age ..............................................................................................................44
Figure 5: The number of nodal protein clusters is comparable between the WT and CST KO mice, but the number of paranodal protein clusters is reduced at 15 days of age ............44
Figure 6: In contrast to Nfasc155, gliomedin, and contactin, Na\textsubscript{v}1.6 channels cluster normally in CST KO mice at 1 month of age .................................................................49
Figure 7: The number of gliomedin, Nfasc155, and contactin clusters is reduced in CST KO mice, while there remains no difference in Na\textsubscript{v}1.6 cluster numbers at 1 month of age ...........49
Figure 8: Abnormalities in Nfasc155, gliomedin, and contactin clusters persist in CST KO mice, while Na\textsubscript{v}1.6 clustering remains intact at 7 months of age ...........................................52
Figure 9: Na\textsubscript{v}1.6 cluster numbers are comparable between WT and CST KO mice, while gliomedin, Nfasc155, and contactin cluster numbers are reduced at 7 months of age .........52
Figure 10: The PNS myelinated axon in CST KO mice at 15 days of age exhibits loss of transverse bands and disorganization of Schwann cell microvilli.........................................................57
Figure 11: Normal ultrastructural characteristics of the PNS myelinated axon at 4 months of age include compact myelin, organized lateral loops, transverse bands, and properly oriented Schwann cell microvilli ..............................................................................60
Figure 12: Loss of transverse bands and disorganization of Schwann cell microvilli are
observed in the CST KO mouse at 4 months of age.............................................................62

Figure 13: The density of myelinated axons in WT and CST KO sciatic nerve at 15 days,
1 month, and 7 months of age was determined by light microscopy .................................65

Figure 14: At 7 months of age, all nodes in WT and CST KO mice exhibit colocalization
of Na\textsubscript{v}1.6 channels with Nfasc186..................................................................................72

Figure 15: At 7 months of age in the WT mice, but not the CST KO mice, Na\textsubscript{v}1.6-positive
nodes exhibit variable intensities of ankyrin\textsubscript{G} labeling ..................................................74
LIST OF TABLES

Table 1: Averages and standard deviations of nodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age.................................................................46

Table 2: Averages and standard deviations of paranodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age.........................................................46

Table 3: Nodal and paranodal cluster integrity in CST KO mice at 15 days, 1 month, and 7 months of age...........................................................................................................55

Table 4: Average density of myelinated axons in WT and CST KO mice at 15 days, 1 month, and 7 months of age.................................................................67
ABSTRACT

SULFATIDE IS REQUIRED FOR ORGANIZATION OF THE PARANODE IN THE MYELINATED AXON IN THE PERIPHERAL NERVOUS SYSTEM

By Eva Lynn Kwong, B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2011

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Myelin facilitates the timely, efficient conduction of action potentials along axons. Made by Schwann cells (SCs) in the PNS, myelin is unique in that it is composed of a high percentage of lipids, particularly galactolipids. Sulfatide, one such galactolipid, is made by cerebroside sulfotransferase (CST) and has been shown to play a role in organizing paranodal domains in myelinated axons. However less is known regarding the involvement of sulfatide in the establishment and maintenance of the node, of particular interest as it is responsible for the potentiation of action potentials along the axon. Using immunohistochemical and, to a lesser extent, electron microscopic techniques, we confirm that sulfatide is essential for organization of the paranode. Our data further shows that neuronal nodal clustering and maintenance is paranode-independent, thus not reliant on sulfatide, demonstrating that 1) distinct mechanisms
exist for nodal and paranodal organization 2) distinct mechanisms for nodal stability exist in the PNS versus the CNS. Interestingly, maintenance of the SC nodal protein, gliomedin, is sulfatide-dependent, indicating that sulfatide is differentially important for nodal organization depending on the cell of origin. Finally, we observe that despite compromised molecular organization of the nodal and paranodal domains in the absence of sulfatide, the gross structure is preserved, therefore a disconnect exists between molecular and structural organization.
INTRODUCTION

Communication within the Nervous System

The central nervous system (CNS) and the peripheral nervous system (PNS) are responsible for sensing external stimuli, relaying this information to the brain for processing, and generating appropriate responses to the environment. Communication within the nervous systems occurs between neurons, which are highly connected cells of the nervous system. For instance, if a body part comes in contact with an open flame, the heat and pain are detected by sensory neurons of the PNS. This sensory information is communicated to the CNS via neuron-neuron signaling, and after processing, the CNS sends the appropriate signals to the motor neurons to remove the body from the harmful stimulus. Thus, communication within the neural network plays a critical role in an organism’s ability to react and adapt to the external environment.

The neuron

A neuron is composed of a cell body, dendrites, and an axon, with each part playing a distinct functional role (Hille, 2001). Dendrites are branched projections that extend from the cell body and are responsible for receiving chemical signals from neurons and other cells (Ho and Rasband, 2010). The chemical signals received by the dendrites become integrated as electrical signals that can be either excitatory or inhibitory in nature, termed excitatory post-synaptic potentials (EPSPs) and inhibitory post-synaptic potentials (IPSPs), respectively (Rasband, 2010). These signals then temporally and spatially summate at the axon hillock; it is here that the decision is made whether or not the neuron will transmit a signal to the subsequent neuron. If the sum of the EPSPs and IPSPs is above a proper threshold, then an action potential is generated at
the axon initial segment (AIS), the region adjacent to the axon hillock and roughly the beginning of the axon (Hedstrom and Rasband, 2006; Ogawa and Rasband, 2008). The action potential is then propagated along the axon, a single projection arising from the soma. Once the action potential reaches the axon terminal, it modulates the release of neurotransmitters, the chemical signaling molecules, into the synaptic cleft. The neurotransmitters act as continuation of the signal, communicating with the post-synaptic neuron (Pitman, 1984).

**The action potential**

The axon is lined with a collection of transmembrane voltage-gated ion channels as well as adenosine triphosphatases (ATPases) such as sodium (Na)/potassium (K)-ATPase and calcium (Ca)-ATPase (Waxman and Ritchie, 1993). In particular, the voltage-gated sodium (Na\textsubscript{v}) channels are responsible for the propagation of action potentials (Hodgkin and Huxley, 1952; Hille, 2001; Quarles et al., 2006; Susuki and Rasband, 2008). In a resting neuron, the voltage difference between the inside of the neuron and the outside of the neuron ($V_{\text{intracellular}} - V_{\text{extracellular}}$), termed the membrane potential, is approximately -60 millivolts (mV). Generation of the action potential begins at the AIS when the infux of sodium ions (Na\textsuperscript{+}) through the Na\textsubscript{v} channels is enough to raise the neuronal membrane potential to threshold, which is around -55 mV. Once the threshold is reached, the neuron is depolarized, and an increasing number of Na\textsubscript{v} channels open, raising the membrane potential to approximately +40 mV. At this point, Na\textsubscript{v} channels begin to close and inactivate, and there is a net efflux of potassium ions (K\textsuperscript{+}) through the voltage-gated potassium (K\textsubscript{v}) channels as the neuron attempts to return the membrane potential to resting state. As the number of positive ions in the cell decreases, the membrane potential also decreases in the repolarization phase. There is a delay in the closing and
inactivation of $K_v$ channels however, resulting in a membrane potential of approximately -85 mV, which is more negative than the resting potential; this phase of the action potential is classified as hyperpolarization. Over time, the membrane potential returns to resting potential, and this portion of the axonal membrane is once again ready to propagate an action potential (Hodgkin and Huxley, 1952; Hille, 2001).

**Improving communication**

Two major mechanisms promote efficient neuronal communication: increased neuronal axon diameter and myelination. As axon diameter increases, the resistance the $Na^+$ face while traveling within the axon decreases (Gasser and Erlanger, 1927; Quarles et al., 2006; Hartline and Colman, 2007). This decrease in internal resistance results in an increase in conduction velocity that is proportional to the square root of the diameter (Hodgkin, 1954). In contrast, conduction velocity in myelinated axons is proportional to the diameter, thus myelin increases conduction velocity of the action potential while conserving space (Hartline and Colman, 2007).

In an unmyelinated axon, depolarization of adjacent segments of the axonal membrane and subsequent opening of $Na_v$ channels leads to propagation of the action potential. In a myelinated axon, myelin acts as a barrier to ion movement, also termed current, across the axonal membrane (Nave, 2010). By increasing transverse resistance to current, myelin restricts the depolarizing effect of the action potential to the next area devoid of myelin, classified as the node of Ranvier, which can be one millimeter (mm) or greater away (Quarles et al., 2006); this method of conduction is classified as “saltatory” from the Latin word *saltare*, meaning “to jump”, and greatly enhances the speed of the signal (Salzer, 1997). Also, myelin decreases the amount of charge the membrane can store, termed membrane capacitance, by increasing the distance
between the cytoplasmic and extracellular faces of the membrane (Hartline and Colman, 2007). A decreased membrane capacitance translates into less Na$^+$ stored at the membrane and a smaller change in membrane potential necessary to conduct an action potential. Furthermore, because the influx of Na$^+$ occurs selectively at the node and not across the entire membrane as in unmyelinated axons, less Na$^+$ movement during depolarization is required in myelinated axons, thus improving the energy efficiency of action potential propagation.

Myelin

A defining characteristic of myelin in comparison with other biological membranes is its composition. Myelin is composed of approximately 70% lipid and 30% protein, whereas other biological membranes are comprised of approximately 45% lipid and 55% protein (Baumann and Pham-Dinh, 2001; Quarles et al., 2006). Furthermore, myelin is not well hydrated, with white matter being comprised of approximately 40% water as compared to gray matter, which is approximately 80% water (Baumann and Pham-Dinh, 2001). The high lipid content and low water content together contribute to myelin being a nonconductive material, which suits its role as an insulating membrane of the nervous system. While myelin serves the same purpose in the CNS and PNS, the two systems begin to take on different identities as we investigate the finer details of myelin biology.

CNS myelin

The lipids in myelin can be categorized into three major classes: cholesterol, phospholipids, and glycosphingolipids, which exist in the CNS as molar ratios ranging from 4:3:2 to 4:4:2, respectively (Morell et al., 1994; Baumann and Pham-Dinh, 2001). The major proteins found in
CNS myelin are proteolipid protein (PLP) with its isoform DM-20 and myelin basic protein (MBP), which comprise approximately 50% and 30% of the CNS myelin protein content, respectively (Baumann and Pham-Dinh, 2001). PLP/DM-20 as well as MBP has been shown to play important roles in the proper compaction and structural organization of the myelin layers (Dupouey et al., 1979; Duncan et al., 1989; Boison and Stoffel, 1994). In addition to the characteristic myelin makeup of the CNS, there are specialized cells in the CNS responsible for the formation of the myelin sheath. These cells are known as oligodendrocytes (OLs). Depending on the region within the CNS, a single OL can extend up to 50 myelinating processes to produce multiple myelin segments (Bunge, 1968; Baumann and Pham-Dinh, 2001). There exist numerous exciting possibilities for investigation within the field of CNS myelin biology; however, this project involves study of PNS myelin biology. Hereafter, information provided and discussed refers to the PNS unless otherwise specified.

**PNS myelin**

*Molecular aspects of PNS myelin*

Of the PNS myelin lipid content, cholesterol, phospholipids, and glycosphingolipids exist in a molar ratio of approximately 2:4:2, respectively (Spritz et al., 1973; Garbay et al., 2000). In addition to the high lipid content of myelin, another defining characteristic is the striking percentage of galactocerebrosides, a subcategory of glycosphingolipids, which can constitute up to approximately 25% of all lipids within myelin (Spritz et al., 1973; Norton and Cammer, 1984; Garbay et al., 2000; Quarles et al., 2006). Galactocerebrosides have been shown both *in vitro* and *in vivo* to play a role in myelin formation, myelin stability, myelinating cell development, and organization of the myelinated axon, both structurally and molecularly (see below: Myelin...
lipids and their role in organizing domains of the myelinated axon). Protein zero (P0), MBP, and protein two (P2) are the major PNS myelin proteins, comprising approximately 50-70%, 5-15%, and 5-12% of the myelin protein content, respectively (Greenfield et al., 1973; Wiggins et al., 1975; Benjamins and Morell, 1978; Smith et al., 1979; Milek et al., 1981; Kadlubowski et al., 1984).

Structural aspects of PNS myelin

A cross section of myelin reveals periodicity, where there are alternating thick and thin lines, termed the major dense line (MDL) and the intraperiod line (IPL, also known as minor dense line), respectively (Figure 1) (Hildebrand, 1972). This patterning is due to the regular compaction of myelin, with the MDL formed by the close apposition of the cytoplasmic faces of the membrane while the IPL is formed by the apposition of extracellular faces of membrane (Figure 1) (Kirschner and Hollingshead, 1980; Garbay et al., 2000). Several proteins are responsible for the proper formation of these structures. MBP and the intracellular domain of P0 are responsible for the formation of the MDL (Kirschner and Ganser, 1980; Rosenbluth, 1980; Martini et al., 1995a; Quarles, 2005). P2, like MBP, is located on the cytoplasmic face of the membrane and while its specific function has not been confirmed, P2 is thought to play a role similar to MBP in forming the MDL (Quarles et al., 2006; Majava et al., 2010; Suresh et al., 2010). The extracellular domain of P0 is thought to contribute to proper compaction of the extracellular faces of the membrane to form the IPL (Giese et al., 1992; Martini et al., 1995b; Quarles, 2002).
Figure 1. Ultrastructure of PNS myelinated axons. PNS myelinated axons are depicted in a cross-section electron microscope (EM) image in panel A. Axons (A) are wrapped in multiple layers of myelin (M). Appearing on the outermost layer of myelin is cytoplasm of the Schwann cell (SC) (S), the myelinating cell of the PNS. Also present is the fibroblast (F). Panel B is a schematic representation of the major dense line (MDL) and intraperiod line (IPL). The MDL is formed by the apposition of the cytoplasmic faces of the membrane while the IPL is formed by the apposition of the extracellular faces of the membrane. Panel C shows a high magnification cross-sectional EM image of a myelinated axon in the PNS. The MDLs (arrows) and IPLs (arrowheads) make up the periodicity seen in myelin. Additionally, double arrows depict cytoplasmic SC collar and the double arrowheads indicate the axolemma, illustrating the close contact that occurs between the two cells. Scale bar: 1 µm in panel A, 0.1 µm in panel C.
Adapted from Martini et al. (1995a)

Adapted from Garbay and Cassagne (2000)

Adapted from Martini et al. (1995a)
Schmidt-Lanterman incisures (SLIs, also named Schmidt-Lanterman clefts; Peters et al., 1991) are structures that commonly occur in PNS myelin (Baumann and Pham-Dinh, 2001; Martini, 2005; Hoshi et al., 2007). A SLI is a cytoplasmic structure that spirals radially between the innermost to outermost layers of compact myelin (Sandri et al., 1977; Mugnaini et al., 1977). Because SLIs traverse the layers of myelin and provide a direct access route between the outer layers of myelin and the axon, they have been speculated to act as “highways” for transport (Bergoffen et al., 1993; Scherer et al., 1995; Balice-Gordon et al., 1998; Hoshi et al., 2007). Consistent with this idea, Balice-Gordon et al. (1998) showed that low molecular weight dyes diffuse rapidly from the outermost to innermost cytoplasm in a radial fashion, matching the path of the SLIs. This diffusion does not occur, however, when a pharmacological blocker of gap junctions is applied, suggesting that these reflexive gap junctions, meaning gap junctions that link layers of the same cell, in the SLI have functional roles. Connexin32 (Cx32, also known as β1 connexin) is one of the gap junction proteins observed in the SLI gap junctions (Bergoffen et al., 1993; Scherer et al., 1995), though the existence of other connexins is probable (Balice-Gordon et al., 1998).

**Schwann cells**

Schwann cells (SCs) are the cells responsible for formation of the myelin sheath within the PNS (Salzer, 1997). Prior to myelination, immature, myelinating SCs make contact with groups of axons, and the axons are bundled together and surrounded by a SC process (Friede and Samorajski, 1968). Then, individual SCs segregate single axons from the bundle, and each SC ensheaths the one axonal segment it will myelinate, thus resulting in a one-to-one ratio between the SC and an axonal segment. The process of radial sorting is possible only after the SC has
Figure 2. Schematic representation of Schwann cell myelination and the myelinated axon. During myelination, the Schwann cell (SC) extends a myelinating process and contacts the neuronal axon. As depicted in panel A, the distal most extent of the myelinating segment (“inner lip”) then crawls around the axon to form the myelin sheath. As myelination progresses, the SC process expands so that if unwrapped, it would resemble a spade-like shape, as seen in panel B. Coincident with myelination, distinct domains of the myelinated axon form. These domains are known as the node, paranode, juxtaparanode, and internode. Other structures of the myelinated axon are Schmidt-Lanterman incisures (SLIs), which are cytoplasmic channels that traverse compact myelin. These structures provide a more direct access route between the outermost and innermost layers of myelin. Gap junctions are observed in SLIs and have been implicated in transport within these structures.
A.

Adapted from Garbay and Cassagne (2000)

B.

Adapted from Arroyo and Scherer (2000)
produced the extracellular structure known as the basal lamina, which has been shown to drive SC development and myelination (Carey et al., 1986; Eldridge et al., 1989). After radial sorting, the basal lamina surrounds the axonal segment to be myelinated (Raine, 1984; Bunge et al., 1986; Baumann and Pham-Dinh, 2001; Simons and Trotter, 2007), and the myelinating process of the SC crawls around the axon in a spiraling fashion (Figure 2) (Webster, 1971; Bunge et al., 1989; Garbay et al., 2000). As myelination continues, the process being projected by the SC expands around and along the axon. If unwrapped, the myelin sheath would appear spade-like (Figure 2) because the width of the sheath progressively increases with each subsequent wrap. This progressive widening of the sheath results in the lateral edges of the myelinating process covering the lateral edges of the previous wrap. As the layers of myelin are deposited, compaction of the myelin occurs as SC cytoplasm is extruded, and with the distinct appearance of multi-lamellar, compact myelin, the axon is classified as myelinated.

In addition to the myelin segment, SCs also generate and extend microvilli. These finger-like projections are positioned at the node of Ranvier and directly contact the nodal axolemma (Raine, 1984; Ichimura and Ellisman, 1991; Baumann and Pham-Dinh, 2001; Melendez-Vasquez et al., 2001; Rios et al., 2003), placing the SC microvilli in an optimal location to facilitate the neuron-SC interactions at the node (Schafer and Rasband, 2006). However, the role of SC microvilli in nodal clustering and maintenance remains unclear, as nodes persist even in the presence of disorganized microvilli (Hoshi et al., 2007; Feinberg et al., 2010).

*Classification of Schwann cells*
Even among the SC population, there exists diversity. In addition to the myelinating SCs, there is another category of SCs that are mature and non-myelinating. These non-myelinating SCs (NMSCs) are associated with several structures, including Remak bundles and neuromuscular junctions (NMJs) (Griffin and Thompson, 2008). Remak bundles, first described by Remak in 1838, are PNS-specific structures that consist of small, unmyelinated axons ensheathed by a SC process and can be highly variable in the number and types of axons they include (Carlsen and Behse, 1980; Murinson and Griffin, 2004; Murinson et al., 2005). The characteristics that determine whether an axon is ensheathed or myelinated remains undetermined, though size dependency (Friede, 1972; Matthews, 1968; Friede and Bischhausen, 1982; Voyvodic, 1989), the quantity of neuronal cell surface proteins (Spencer and Weinberg, 1978), as well as specific axonal signaling (Michailov et al., 2004; Jessen and Mirsky, 2005; Taveggia et al., 2005; Birchmeier and Nave, 2008) are currently being investigated as potential candidates. There also exist NMSCs at the NMJ, the synapse between a motor neuron and muscle fiber, named terminal SCs (tSCs) (Couteaux, 1960). These tSCs have been speculated to play a role in synapse formation and stability (Son et al., 1996; Trachtenberg and Thompson, 1997; Lubischer and Thompson, 1999) as well as in the modulation of synaptic transmission (Robitaille, 1998).

While a noteworthy and important area of research, a complete discussion of the NMSC population is beyond the scope of this project; for this study, the focus will be the myelinating SC, and in particular, its interactions with the neuronal axon.

**Domains of the myelinated axon**
Figure 3. The structural and molecular domains of the PNS myelinated axon. Coincident with myelination, distinct structural and molecular domains, termed the node of Ranvier, paranode, juxtaparanode, and internode, form. Panels A & B are a schematic representation and longitudinal electron micrograph, respectively, which depict the different structural domains. The node of Ranvier is a myelin-bare region that is overlain with projections called Schwann cell microvilli (star, in panel A only) in the PNS. Regions adjacent to the node are termed the paranode and are defined by lateral loops (arrows). Adjacent to the paranode is the juxtaparanode, which is adjacent to the internode. Compact myelin is found along both the juxtaparanode and internode. The domains are also molecularly distinct, as illustrated by the immunohistochemical image in panel C. Nodal voltage-gated sodium channels are immunolabeled in green, paranodal contactin-associated protein in blue, and juxtaparanodal voltage-gated potassium channels in red. The internode is not pictured, but distinct proteins also localize to this domain. Scale bar: 1 µm in panel B, 10 µm in panel C.
Adapted from Miyamoto et al. (2005)

Adapted from Salzer et al. (2008)

Adapted from Miyamoto et al. (2005)

Adapted from Schafer and Rasband (2006)
The myelin sheath is by no means homogeneous; as myelination takes place, structurally and molecularly distinct domains form and take on specific roles (Figure 3) (Arroyo and Scherer, 2000; Peles and Salzer, 2000; Poliak and Peles, 2003; Salzer et al., 2008). These domains are known as the node of Ranvier, the paranode, the juxtaparanode, and the internode.

**Node of Ranvier**

Prior to myelination, $\text{Na}_v$ channels are distributed uniformly along the axon. It is after direct contact with the SC that $\text{Na}_v$ channels begin to aggregate (Dugandzija-Novaković et al., 1995), eventually clustering to a density of approximately $1500/\mu\text{m}^2$, as compared to less than $25/\mu\text{m}^2$ elsewhere along the axonal membrane (Waxman and Ritchie, 1993). The approximately 1 µm long region where the clusters of $\text{Na}_v$ channels localize is classified as the node of Ranvier and exists between adjacent myelin segments (Arroyo and Scherer, 2000; Salzer et al., 2008). $\text{Na}_v$ channels are multimeric complexes that consist of various $\alpha$-subunits and one or more $\beta$-subunits (Catterall, 2000). Similar to other ion channels of the nervous system, $\text{Na}_v$ channels are encoded by multiple genes, and $\text{Na}_v$ channels with $\alpha$-subunits 1.2 and 1.6 are the predominant channel types that localize to the node in PNS axons (Caldwell et al., 2000; Krzemien et al., 2000; Schaller and Caldwell, 2000; Schafer et al., 2006). The temporal expression of the specific isoforms differs (Boiko et al., 2001); while both $\text{Na}_v1.2$ and $\text{Na}_v1.6$ are detected in unmyelinated axons, $\text{Na}_v1.2$ is detected at the node only during the first postnatal week while $\text{Na}_v1.6$ persists at the node throughout life (Schafer et al., 2006). The clusters of $\text{Na}_v$ channels at the node are responsible for propagating action potentials along the axon (Hille, 2001).
In addition to Nav channels, there is a plethora of proteins that are found specifically at the node and several of these proteins have been shown to contribute to Nav channel clustering and stabilization. The 186-kiloDalton (kDa) isoform of neurofascin (Nfasc186) is a transmembrane neuronal protein that belongs to the L1 subfamily of cell adhesion molecules (CAMs), which is part of the larger immunoglobulin superfamily (IgSF) (Volkmer et al., 1992; Davis et al., 1996). In addition to it being one of the first proteins to cluster at the presumptive node (Lambert et al., 1997), Nfasc186 has been shown to bind in cis with the β1 subunit of Nav channels, suggesting a direct role in the initial recruitment of Nav channels (Ratcliffe et al., 2001). More recently, this idea has been further supported using a Nfasc186-knockdown animal model, where nodal disorganization and loss of Nav channels was observed (Thaxton et al., 2011). AnkyrinG, a neuronal cytoskeletal protein, is another molecule of interest. AnkyrinG exists as two isoforms (480 kDa, 270 kDa) at the node (Kordeli et al., 1995), and it has been speculated to act as a scaffold to which other nodal proteins, including Nav channels, cytoskeletal protein βIV spectrin, and neuron-glia related CAM (NrCAM), bind (Grumet, 1997; Bennett and Lambert, 1999; Malhotra et al., 2000; Berghs et al., 2000; McEwen and Isom, 2004). AnkyrinG has further been shown to promote stable Nfasc186 expression specifically at the node and is required for proper maintenance of the nodal complex (Dzhashiashvili et al., 2007).

The SC also produces proteins that localize specifically to the node, the best characterized being gliomedin (Eshed et al., 2005). During early myelination, gliomedin is located along the edges of myelin segments and developing nodes, colocalizing with other nodal proteins such as Nav channels, NrCAM, and its neuronal binding partner Nfasc186 (Eshed et al., 2005; Schafer et al., 2006; Feinberg et al., 2010). In culture, gliomedin is sufficient to induce neuronal formation of
nodal-like clusters consisting of Na\textsubscript{v} channels, ankyrin\textsubscript{G}, and βIV spectrin, even in the absence of SC (Eshed \textit{et al.}, 2005), illustrating the possible involvement of gliomedin in the initial establishment of the node. After myelination has taken place, gliomedin is located in the SC microvilli, which directly contact the nodal axolemma (Ichimura and Ellisman, 1991). Interestingly, \textit{in vitro} studies have shown that NrCAM expressed by SCs possess a dual role of trapping gliomedin on the SC microvilli and enhancing binding of gliomedin to Nfasc186 (Feinberg \textit{et al.}, 2010). Gliomedin exists as part of a multi-protein complex that is in an ideal location for not only initial formation of the node but its continued maintenance as well.

**Paranode**

Immediately adjacent to the node is the paranode. The paranode is the region of the closest apposition between the neuronal axon and myelinating cell (Hirano and Dembitzer, 1969; Rosenbluth, 1987; Salzer \textit{et al.}, 2008) and has been speculated to be involved in bidirectional signaling between the neuron and SC (Traka \textit{et al.}, 2002). The paranode is defined by the presence of lateral loops, which are layers of noncompacted myelin formed by the overlapping lateral edges of the myelin sheath. These loops turn in towards the axon, and the outermost loop of myelin terminates most proximal to the SC microvilli. Proteins of both the axon and SC localize specifically to this domain. Contactin (also known as contactin-1) is a protein that is linked to the neuronal membrane by the glycolipid glycophasphatidylinositol (GPI) (Ranscht, 1988). Contactin binds in \textit{cis} to contactin-associated protein (Caspr), also named paranodin, a neuronal transmembrane protein belonging to the neurexin superfamily (Einheber \textit{et al.}, 1997, Menegoz \textit{et al.}, 1997; Peles \textit{et al.}, 1997), and this axonal complex is targeted to the neuronal paranode (Rios \textit{et al.}, 2000). The myelin binding partner to this axonal complex, specifically to
contactin, is the 155-kDa isoform of neurofascin (Nfasc155). Nfasc155 is another member of the L1 subfamily and is a transmembrane protein of myelinating cell origin that also localizes to the paranode (Volkmer et al., 1992; Tait et al., 2000; Gollan et al., 2003). Boyle et al. (2001) observed Nfasc155 at the paranode even in the absence of contactin, but not vice versa (Charles et al., 2002; Sherman et al., 2005), thus suggesting that Nfasc155 is required for proper stabilization of the contactin/Caspr complex at the paranode (Pillai et al., 2009). However, while Nfasc155 may be responsible for clustering of the paranodal complex, stabilization of Nfasc155 at the paranode is dependent on its interactions with the axonal contactin-Caspr complex (Boyle et al., 2001). Nfasc155 has become a particular protein of interest because of its clinical relevance to the demyelinating disease multiple sclerosis (MS) in the CNS (see below: Clinical implications).

Nfasc155-contactin/Caspr complexes are thought to make up transverse bands (TBs), hallmark structures of the paranode, as disruption of Nfasc155, contactin, or Caspr leads to disruption of the TBs (Bhat et al., 2001; Boyle et al., 2001; Charles et al., 2002; Rios et al., 2003). TBs appear to traverse the periaxonal space between the lateral loops and axonal membrane and have been speculated to play a role in stabilizing the junction between the myelin sheath and axon (Tao-Cheng and Rosenbluth, 1983; Peters et al., 1991; Rosenbluth, 1995; Marcus et al., 2002; Shepherd et al., 2010).

**Juxtaparanode**

The 10-15 µm-long domain adjacent to the paranode is called the juxtaparanode (Arroyo and Scherer, 2000). The juxtaparanode is characterized by dense clusters of Shaker-type K_+ channels,
specifically combinations of \( K_v \) channel subunits (Chiu and Ritchie, 1980; Wang et al., 1993; Rasband et al., 1998, 2001). The proteins Caspr2 and transient axonal glycoprotein (TAG-1, also named contactin-2) are also found in the juxtaparanode (Poljak et al., 1999; Traka et al., 2002). Caspr2 is of neuronal origin and was named based on its close homology to paranodal Caspr. TAG-1 is of both neuronal and myelinating cell origin, and it also exists in two forms: a GPI-linked form and a secreted form. Poljak et al. (2003) and Traka et al. (2003) concluded that Caspr2 and axonal TAG-1 interact in \textit{cis}, and this complex interacts with SC TAG-1 in \textit{trans}. This protein triplex has been hypothesized to associate with \( K_v \) channels in the juxtaparanode, as reduced juxtaparanodal \( K_v \) channels are observed in murine models lacking Caspr2 or TAG-1 (Poljak et al., 2003; Traka et al., 2003). However, the “link” by which \( K_v \) channels and Caspr2/TAG-1 interact has not been determined. One hypothesis proposed by Poljak et al. (1999) suggested that a protein containing at least one PDZ (Post synaptic density protein, \textit{Drosophila} disc large tumor suppressor, \textit{zonula occludens})-domain is responsible as mutation of the C-terminal PDZ domain in either \( K_v \) channels or Caspr2 disrupts binding. It is important to note that similar to the paranode and node, the juxtaparanode consists of close interactions between molecules of both neuronal and SC origin. These intimate connections contribute to the proper distribution and thus functioning of \( K_v \) channels, the roles of which remain unclear but are speculated to be involved in regulating action potential amplitude and resting membrane potential following an action potential (Wang et al., 1993; Hille, 2001).

**Internode**

The internode constitutes the largest domain of the myelinated axon, reaching up to 1 mm in length in adults (Abe et al., 2004). Structurally, the myelin is compacted along the internode, and
there is apposition of the axonal and SC membranes. The adhesion of these cells is established by cell surface proteins, as these contacts are disrupted with application of proteases (Yu and Bunge, 1975), and in particular, myelin-associated glycoprotein (MAG) as well as nectin-like (Necl) CAMs are being investigated as potential candidates. MAG is a transmembrane protein that, like the Nfasc proteins, belongs to the IgSF (Salzer et al., 1987; Lai et al., 1987; Trapp, 1990; Volkmer et al., 1992) and it comprises approximately 0.1% of the myelin protein content (Figlewicz et al., 1981). It exists in two isoforms: small-MAG (S-MAG) and large-MAG (L-MAG), with S-MAG comprising approximately 95% of the total MAG content (Pedraza et al., 1991; Miescher et al., 1997). MAG is located in the periaxonal SC membrane (Martini and Schachner, 1986; Quarles, 2002, 2005) and has been shown in vitro to specifically bind the gangliosides GD1a and GT1b, which are highly glycosylated and siacylated lipids found along the surface of the axonal membrane (Yang et al., 1996; Collins et al., 1997a,b); this interaction is a possible method by which MAG may facilitate SC-axon adhesion. In support of this hypothesis, mice deficient in the enzyme β1, 4-N-acetylgalactosaminyltransferase (GalNAcT, GM2/GD2 synthase) (Takamiya et al., 1996; Liu et al., 1999), which is required for the synthesis of complex gangliosides, including GD1a and GT1b, exhibit increased incidence of both dysmyelinated and degenerating axons (Sheikh et al., 1999; Chiavegatto et al., 2000; Pan et al., 2005). In contrast, studies involving MAG knockout (KO) mice report variable observations with regards to myelin ultrastructure. Both Li et al. (1994) and Montag et al. (1994) report only modest abnormalities in the MAG KO while Pan et al. (2005) report an increase in dysmyelinated and degenerating axons in the MAG KO, pathology similar to the GalNAcT KO (Liu et al., 1999; Sheikh et al., 1999; Chiavegatto et al., 2000). Further investigation is necessary
in order to elucidate the role of MAG and the MAG-ganglioside interaction in proper adhesion between the SC and axon.

Other proteins that localize to the internode are the Necl CAMs (also known as SynCAMs; Biederer et al., 2002) (Maurel et al., 2007; Spiegel et al., 2007). These internodal Necls are differentially expressed depending on cell type: neurons express Necl-1 and -2, while SCs express Necl-4 and lesser amounts of Necl-2, and specific binding occurs between axonal Necl-1 and SC Necl-4 (Maurel et al., 2007; Spiegel et al., 2007). Further supporting Necl proteins as possible mediators for axo-glial adhesion is the inhibition of myelination seen with Necl-4 knockdown or with disruption of Necl-1/Necl-4 interactions (Maurel et al., 2007; Spiegel et al., 2007). Interestingly, both MAG and SC Necl-2, and -4 are expressed in SLIs (Martini and Schachner, 1986; Maurel et al., 2007; Spiegel et al., 2007), where they may play comparable adhesive roles as in the internode.

**Myelin lipids and their role in organizing domains of the myelinated axon**

While there are no lipids exclusive to myelin, the most typical are the galactolipids galactocerebroside (GalC, also named galactosylceramide) and its sulfated derivative, sulfatide, which together can comprise a quarter of the myelin lipid content (Spritz et al., 1973; Norton and Cammer, 1984; Garbay et al., 2000; Quarles et al., 2006). GalC is generated by the enzyme uridine diphosphate (UDP) galactose:ceramide galactosyltransferase (CGT), which transfers a galactose from UDP-galactose to ceramide (Morell and Radin, 1969; Schulte and Stoffel, 1993; Stahl et al., 1994; Coetzee et al., 1996; Bosio et al., 1996), a combination of long-chain fatty acids and varying lengths of sphingosines. Sulfatide is generated by the enzyme 3’-
phosphoadenylylsulfate:galactosylceramide 3’-sulfotransferase (CST, also known as cerebroside sulfotransferase), which transfers a sulfate group from 3’phosphoadenosine-5’phosphosulfate to GalC (Ishizuka, 1997). Several studies (Ranscht et al., 1987; Owens and Bunge, 1990; Wood et al., 1990; Bosio et al., 1996; Coetzee et al., 1996, 1998; Dupree et al., 1998b,c; Dupree et al., 1999; Dupree and Popko, 1999; Hoshi et al., 2007) involving GalC and sulfatide sparked interest in these galactolipids and their possible role in the structural and molecular organization of the domains of the myelinated axon.

**In vitro studies: GalC and myelin formation**

The role of GalC in myelin formation was investigated by establishing co-cultures of SCs and neurons and exposing them to anti-GalC immunoglobulin during myelination (Ranscht et al., 1987; Owens and Bunge, 1990; Wood et al., 1990). The GalC antibody is hypothesized to remove GalC from the SC surfaces, an idea which is supported by the decreased levels of GalC observed in the anti-GalC-treated cultures. After exposure to the GalC antibody, the formation of myelin structures was greatly (99.3%) or completely inhibited, and myelin proteins P0 and MBP were detected in less than 5% of SCs. In contrast, the expression of MAG is not reduced in anti-GalC-treated SCs, suggesting that MAG is organized by a GalC-independent mechanism. Both Ranscht et al. (1987) and Owens and Bunge (1990) demonstrate that GalC plays a role in the formation of myelin, as anti-GalC-treated SCs enter the promyelin stage of development, where the basal lamina has formed and SCs have assumed the 1:1 ratio between the SC and axonal segment, but the process of myelination does not proceed past that point.
While these studies were useful to elucidate the importance of GalC in myelin development, there exist several caveats (Dupree et al., 1998a). First, while effective, in vitro studies take place in an “artificial” living environment and do not consider the numerous cell-cell interactions and events that normally occur within a living organism; thus, in vitro observations may be conditional. Also, the anti-GalC antibody used cross-reacts to the sulfated derivative of GalC, sulfatide, and so, changes in myelin formation due to alterations in sulfatide cannot be ruled out (Ranscht et al., 1987; Owens and Bunge, 1990; Dupree et al., 1998a). Lastly, the mechanism by which GalC mediates myelin formation is unclear and difficult to answer using the methods of this study, as the effect of the GalC antibody on GalC itself can only be hypothesized after observing the effects of experimental treatment (Dupree et al., 1998a). Utilization of in vivo transgenic models can begin to address these issues.

**In vivo studies: the CGT-knockout mouse**

Mice lacking both GalC and sulfatide were generated by disrupting the cgt gene (Bosio et al., 1996; Coetzee et al., 1996). CGT KO mice begin to exhibit tremors at postnatal day 12-14 followed by progressive hindlimb paralysis, and most die between postnatal day 30-60, though some survive up to 90 days (Bosio et al., 1996; Coetzee et al., 1996, 1998). Unexpectedly, analysis of the PNS ultrastructure revealed little disruption in the structural domains of the myelinated axon of the CGT KO mice. Despite the lack of almost a quarter of the lipids present in myelin, there is no degeneration of the myelin sheath, proper myelin thickness is retained, and the lateral loops are properly inverted towards the axon (Dupree et al., 1998b,c; Dupree and Popko, 1999). In contrast to the other structural features that are maintained in the CGT KO mice, transverse bands at the paranode are absent (Dupree and Popko, 1999).
Unlike the mostly preserved ultrastructure observed in the CGT KO mice, the molecular organization is disrupted. The *Shaker*-type $K_v$ channels, which usually concentrate at the juxtaparanode, invade the paranode and are sometimes observed diffusely distributed along the internode, and accumulation of Caspr at the paranode is reduced in the CGT KO mice (Dupree *et al.*, 1999). In contrast, nodal formation is normal in the PNS of the CGT KO; $Na_v$ channels and ankyrin$_G$ cluster normally at the node (Dupree *et al.*, 1999). Electrophysiological studies reveal an approximately 50% reduction in compound action potential amplitude and 30-40% reduction in conduction velocity in the CGT KO mice (Dupree *et al.*, 1998b), which suggests that the gross structure of the myelin sheath by itself is not sufficient for normal functioning; the well-defined protein domains of the myelinated axon are necessary.

**In vivo studies: the CST-knockout mouse**

Studies involving the CGT KO mouse illustrate the importance of both GalC and sulfatide in the establishment of the domains of the myelinated axon, particularly the protein domains; however, it is not possible to discern the individual roles of these myelin galactolipids with this particular transgenic model. In 2002, Honke *et al.* generated a CST KO animal by disrupting the *cst* gene, leaving GalC formation intact while the conversion of GalC to sulfatide is disrupted. CST KO animals are sulfatide-null, but generate normal levels of GalC. The utilization of this model is one of the first steps towards elucidating the specific functions of sulfatide.

CST KO mice exhibit generalized tremors after two weeks of age followed by progressive hind limb paralysis; however, the progression of phenotypic pathology is much less severe as
compared to the CGT KO mice (Honke et al., 2002). Furthermore, the life expectancy of CST KO animals is approximately a year, making the model a useful tool to study the function of sulfatide. Analysis of the PNS ultrastructure again revealed little disruption in the structural domains of the myelinated axon, and in contrast to the CGT KO mouse, Hoshi et al. (2007) reported the presence of transverse bands at some paranodal loops. Previously not described in the CGT KO mouse, the SC microvilli, rather than being tightly aligned at the node, appear disorganized in the CST KO mouse (Hoshi et al., 2007). Furthermore, an increased incidence of SLIs in the compact myelin is detected, which correlates to observations made in several other mutants with abnormal SC-axon interactions such as the Caspr KO mouse (Bhat et al., 2001; Rios et al., 2003) and the shiverer mouse, an animal model with disrupted MBP formation, which exhibits a greater than a two-fold increase in the number of SLIs (Rosenbluth, 1980; Gould et al., 1995). This abnormality has been hypothesized to compensate for the disrupted interactions between the SC and neuron present in the shiverer mouse (Gould et al., 1995). However, greater incidence of SLIs is not always observed in mutant conditions where the SC-axon interaction is disrupted, and even when there is an increase of SLIs in the mutant, the mechanisms by which SLIs are regulated can be distinct (Sharghi-Namini et al., 2006), thus illustrating the complex nature of these structures.

Analysis of the molecular organization of the myelinated axon in the CST KO mice showed aberrant localization of Shaker-type $K_v$ channels as well as abnormal clustering of paranodal proteins Caspr and Nfasc155. $K_v$ channels were again observed at the paranode and not at the juxtaparanode. The number of Caspr and Nfasc155 clusters is decreased, and where Caspr and Nfasc155 do cluster, the paranodes are shorter (Hoshi et al., 2007). These are consistent findings
among other paranodal mutants such as the CGT KO mouse (Dupree et al., 1999), the Caspr KO mouse (Bhat et al., 2001; Rios et al., 2003), and the contactin KO mouse (Boyle et al., 2001). The invasion of $K_v$ channels is thought to occur because of the lack of an intact structural and molecular paranode, which normally acts as a “diffusion barrier”, preventing the lateral dispersion of proteins of the node as well as juxtaparanode (Rios et al., 2003); consistent with this idea, elongated nodes are seen in the CST KO mouse (Hoshi et al., 2007). Despite nodal lengthening, normal numbers of $Na_v$ channels form (Hoshi et al., 2007). Parallel with the increased incidence of SLIs observed in the CST KO mice, Cx32 expression is also increased (Hoshi et al., 2007). Electrophysiological studies show no differences in maximal conduction velocity in the CST KO mouse up to 18 weeks of age (Honke et al., 2002).

Studies involving the CST KO mice demonstrate that sulfatide is necessary for the proper establishment of the protein domains of the myelinated axon. In particular, the role of sulfatide in organizing the paranode has been previously shown, however less is known regarding the involvement of sulfatide in nodal organization. It is interesting to note that similar paranodal disruption is seen in the CGT/CST KO animals and paranodal mutant animals lacking contactin (Boyle et al., 2001), Caspr (Bhat et al., 2001; Rios et al., 2003), and Nfasc155 (Sherman et al., 2005; Pillai et al., 2009), the reason for which is still speculative. This correlation further supports the hypothesis that the establishment and/or maintenance of contactin, Caspr, and Nfasc155 proteins at the paranode is sulfatide-mediated.

**Clinical implications**
Because myelin has become so integral to the proper functioning of the neural network, pathology involving myelin and its organization can yield debilitating symptoms. Several myelin diseases exist in both the CNS and PNS that arise in part because of the misregulation of sulfatide and/or disorganization of the domains of the myelinated axon.

**CNS myelin diseases**

**Multiple sclerosis**

MS is the most common demyelinating disease of the CNS and is believed to be of autoimmune origin (Compston and Coles, 2002). MS is characterized by OL death, loss of myelin, and axonal degeneration. Symptoms include chronic pain, impaired motor/visual coordination, and cognitive dysfunction (Compston and Coles, 2002; Howell et al., 2006). While the cause of MS is unclear, Marbois et al. (2000) revealed an alteration in the distribution of sulfatide species in MS plaques and normal appearing white matter (NAWM), regions adjacent to MS lesions that do not exhibit demyelination or axonal degeneration, as compared to normal controls. Also, paranodal breakdown precedes demyelination as clustering of Nfasc155 is disrupted in NAWM (Howell et al., 2006). Furthermore, analyses reveal that in MS lesions, there is disruption in the structural and molecular paranode as paranodal loops are no longer inverted towards the axon (Suzuki et al., 1969), clustering of Caspr and Nfasc155 is disrupted (Wolswijk and Balestar, 2003; Howell et al., 2006), and juxtaparanodal Kv1.2 channels invade the paranode (Howell et al., 2006). It is also of note that two forms of Nfasc155 have been identified: Nfasc155-high (Nfasc155H) and Nfasc155-low (Nfasc155L), and the same pattern of a disruption in the Nfasc155H: Nfasc155L ratio is seen in MS lesions and the CST KO CNS (Pomicter et al., 2010).
**Alzheimer’s disease**

Alzheimer’s disease (AD) is a neurodegenerative disease of the CNS and is characterized by progressive loss of cognitive functions such as memory and language (Svennerholm and Gottfries, 1994; Han et al., 2002; Han, 2010). Hallmark histological pathologies of AD include neurofibrillary tangles and plaques composed of amyloid-β peptides (Aβ) and other plaque-associated proteins such as apolipoprotein E (apoE) (Svennerholm and Gottfries, 1994; Goedert and Spillantini, 2006; Han et al., 2002; Han, 2010). The role apoE has in the clearance of Aβ has been well established (Kang et al., 2000; Bales et al., 2002; Hartman et al., 2005; Bateman et al., 2006), and it has also been shown that sulfatide may be involved in the clearance of Aβ in the brain as well, as a dose-dependent decrease in Aβ following sulfatide treatment is seen in vitro (Zeng and Han, 2008). Zeng and Han (2008) further revealed that in vitro, effects on Aβ clearance by apoE3 in the absence of sulfatide are modest compared to the effects induced by apoE3 in the presence of sulfatide, suggesting that apoE and sulfatide contribute synergistically to Aβ clearance. Interestingly, studies comparing CNS lipid content in patients with a mild form of AD (mild cognitive impairment, MCI) and cognitively-normal controls revealed a marked decrease in sulfatide content, a decrease of up to 90% in cerebral gray matter and up to 50% in white matter, which suggests that sulfatide deficiency precedes and may contribute to the development of clinical symptoms (Han et al., 2002; Han, 2010).

**Metachromatic leukodystrophy**

Leukodystrophies are classified as a heterogeneous group of disorders in which the CNS white matter is affected (Lyon et al., 2006; Costello et al., 2009). Particularly of interest is the disease metachromatic leukodystrophy (MLD). Due to a lack of the lysosomal enzyme arylsulfatase A
ASA), which normally degrades sulfatide and other sulfolipids, symptoms of this progressive demyelinating disorder include severe neurological deficits and loss of motor control, culminating in paralysis (Molander-Melin et al., 2004; Lyon et al., 2006; Eckhardt et al., 2007; Costello et al., 2009). Due to a lack of ASA, there is a buildup of sulfatide and other sulfolipids in human MLD patients (von Figura et al., 2001), which is mimicked in the ASA KO murine model (Hess et al., 1996) as accumulation of sulfatide is detected within OLs and subpopulations of neurons (Molander-Melin et al., 2004; Eckhardt et al., 2007). This accumulation of sulfatide is thought to be responsible for the pathological symptoms observed in MLD patients. It is of note that several symptoms of MLD including severe myelin abnormalities, a reduction in nerve conduction velocity, and progressive paralysis are not observed in the ASA KO mice (Hess et al., 1996; Wittke et al., 2004). However, in the ASA KO mice that have transgenically enhanced myelinating cell CST activity, which resulted in increased production and storage of sulfatide, MLD symptoms not previously seen in the ASA KO model are exhibited (Ramakrishnan et al., 2007), thus suggesting the role of increased sulfatide storage in the development of MLD symptoms.

PNS myelin diseases

Guillain Barré syndrome – acute motor axonal neuropathy

Guillain Barré syndrome (GBS) is a disease of the PNS that is speculated to be of autoimmune origin. There are several subtypes GBS and GBS-related disorders, some which are demyelinating and others not (Hughes and Cornblath, 2005; Vucic et al., 2009). Acute motor axonal neuropathy (AMAN) is a subtype of GBS that is non-demyelinating, and it is characterized by a rapid and severe course of motor symptoms including acute limb weakness
and absent reflexes (Hughes and Cornblath, 2005; Vucic et al., 2009). AMAN is thought to result from the targeting of gangliosides GM1 and GD1a by the immune system, as GM1 and GD1a antibodies have been consistently detected in AMAN patients and macrophages are observed at the paranode and node where these gangliosides localize (Griffin et al., 1996; Hughes and Cornblath, 2005; Caporale et al., 2006; Susuki et al., 2007a,b; Vucic et al., 2009; McGonigal et al., 2010). Ultrastructural analyses of AMAN tissue reveal several nodal and paranodal abnormalities: nodal elongation, SC microvilli displacement from elongated nodes, disruption of paranodal myelin, and infiltration of macrophages into the paranodal periaxonal space advancing to the internode (Griffin et al., 1996). These disruptions appear to be concentrated in the nodal and paranodal regions as myelin along the internode is normal and little or no demyelination is observed (Griffin et al., 1996; Hafer-Macko et al., 1996; Hughes and Cornblath, 2005; Vucic et al., 2009). Additionally, immunoglobulin and complement deposition is observed on the nodal axolemma (Hafer-Macko et al., 1996).

These pathologies are exhibited by rabbits sensitized with GM1, thereby suggesting this animal model as a useful tool to study human AMAN (Yuki et al., 2001, 2004; Susuki et al., 2003; Caporale et al., 2006). In addition to the ultrastructural disruption of the node and paranode observed in the rabbit AMAN model, there is also molecular disruption; Susuki et al. (2007b) showed disruption or disappearance of Na\textsubscript{v} channels at elongated nodes and clustering of several node-stabilizing proteins, such as βIV spectrin and SC microvilli protein moesin at the node and Caspr at the paranode, is abnormal. It is also of note that nodal protein disorganization is observed in the presence of complement deposition and the absence of macrophages, suggesting that complement may contribute to the initial disruption of the node (Susuki et al., 2007b). In
line with this idea, complement-inhibitor treatment, which decreased complement deposition, provides protection from molecular disruption of the node in the rabbit AMAN model (Phongsisay et al., 2008).

**Project Aim**

Previous studies have determined that myelin galactolipids play an essential role in myelin formation, myelin stability, and the establishment and maintenance of domains of the myelinated axon. To date, the majority of these studies have focused on the CNS. Recently, Hoshi et al. (2007) investigated the role of sulfatide in the organization of the domains of the myelinated axon in the PNS. Their findings indicate that paranode formation is compromised in the absence of sulfatide, but Na<sub>v</sub> channel clustering is neither delayed nor unstable. However, this study was confined to mice between 1 and 5 months of age, and even though no significant reductions in Na<sub>v</sub> channel clustering were observed, the number of clusters was reduced by approximately 20% at each age studied. Furthermore, nodal lengths were elongated at each age, indicating abnormality. Based on these findings, we proposed that analysis of the CST KO mice at younger and older ages would reveal significant deficits in Na<sub>v</sub> clustering. Additionally, we investigated nodal clustering of the SC-specific protein, gliomedin, as it is possible that normal Na<sub>v</sub> clustering and maintenance is mediated not by the SC-axon interactions at the paranode but by the SC-axon interactions at the node. Also, we investigated paranodal proteins in the SC and neuron which mediate the interactions between these cell types to further our understanding of the involvement of the paranode in nodal formation and maintenance. Finally, a qualitative ultrastructural assessment was conducted to compare molecular and structural organization of the node and paranode.
MATERIALS AND METHODS

Animals
Cerebroside sulfotransferase (CST)-deficient mice were generated by gene targeting (Honke et al., 2002), and mice heterozygous for the cst gene were kindly provided by Dr. Koichi Honke (Kochi Medical School; Kochi, Japan). The mice were bred, and the resulting offspring were one of the following genotypes: wild type (WT, +/+), heterozygote (+/-), and CST KO (-/-). The polymerase chain reaction method was utilized as previously described (Marcus et al., 2006; Shroff et al., 2009; Pomicter et al., 2010), and WT and CST KO littermate mice were identified. Mice were bred and maintained in an AAALAC certified facility, and all protocols are approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Immunohistochemistry

Number of animals
The number of WT and littermate CST KO mice used for Na\textsubscript{v}1.6 and neurofascin (Nfasc) immunohistochemistry (IHC) is as follows: 15 days (n=3), 1 month (n=6), and 7 months (WT n=5; KO n=8). The number of WT and littermate CST KO mice used for contactin and gliomedin IHC is as follows: 15 days (n=3), 1 month (n=5), and 7 months (WT n=4; KO n=6). The number of WT and littermate CST KO mice used for Na\textsubscript{v}1.6 and Nfasc186 IHC as well as for Na\textsubscript{v}1.6 and ankyrin\textsubscript{G} IHC is as follows: 7 months (n=1).

Perfusion and tissue processing
Mice were deeply anesthetized with an intraperitoneal injection of 2.5% 2, 2, 2 tribromoethanol (Avertin, Sigma-Aldrich) in 0.9% NaCl (VWR International; West Chester, PA) at 0.016 milliliter (mL) per gram (g) of body mass. Mice were individually stabilized on a dissecting block ventral side up, and an incision was made from the abdomen to the sternum. The right atrium of the heart was cut, and a needle was inserted into the left ventricle. A 0.9% NaCl solution was continuously flushed until the perfusate ran clear. The mouse was then transcardially perfused with 0.1 molar (M) Millonig’s buffer containing 4% paraformaldehyde (Electron Microscopy Services; Hatfield, PA) (pH 7.3) for ten minutes. The sciatic nerves (SN) were immediately harvested, cryopreserved in 30% sucrose in phosphate buffered saline (PBS), frozen in Optimal Cutting Temperature compound (Sakurak; Torrance, CA), and sectioned at ten µm thickness. Sections were stored at -80°C until used.

**Immunohistochemistry protocol**

Sections were immunolabeled with antibodies directed against pan-Nfasc and Na\(_{v1.6}\) channels. Different sections were also immunolabeled with other antibody pairs including contactin and gliomedin, Na\(_{v1.6}\) and Nfasc186, and Na\(_{v1.6}\) and ankyrin\(_{G}\). First, sections were air-dried, then immersed in -20°C acetone (Fisher Scientific; Pittsburg, PA) for ten minutes, washed in PBS, then blocked in a solution of PBS, 10% cold water fish gelatin (Electron Microscopy Services; Hatfield, PA), and 0.1% Triton X-100 (MP Biomedicals; Solon, OH) for 15 minutes at room temperature. The NFCT pan-Nfasc primary antibody (Rat, kindly provided by Dr. Manzoor Bhat, University of North Carolina at Chapel Hill) was used at a concentration of 1:500. The Na\(_{v1.6}\) primary antibody (Rabbit, acquired from Alomone Labs) was used at a concentration of 1:200. The contactin primary antibody (Goat, acquired from R&D systems) was used at a
concentration of 1:200. The gliomedin primary antibody (Rabbit, kindly provided by Dr. Elior Peles, Weizmann Institute of Science, Israel) was used at a concentration of 1:500. The Nfasc186 primary antibody (Guinea pig, kindly provided by Dr. Manzoor Bhat, University of North Carolina at Chapel Hill) was used at a concentration of 1:1000. The ankyrin G primary antibody (Rat, kindly provided by Dr. Manzoor Bhat, University of North Carolina at Chapel Hill) was used at a concentration of 1:200. Sections were incubated overnight at 4°C in the appropriate primary antibodies diluted in blocking solution. The following day, sections were washed in PBS and blocked a second time. Sections were incubated with appropriate fluorescent labeled secondary antibodies (Invitrogen; Carlsbad, CA) at 1:200 for 90 minutes. For instance, slides treated with Na\textsubscript{v}1.6 primary antibody were incubated with an Alexafluor goat anti-rabbit 594. Then, sections were washed in PBS, mounted with VectaShield (Vector Laboratories; Burlingame, CA) to minimize photo-bleaching, and stored at -20°C until imaged.

**Visualization**

Sections were visualized first with a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, NY) to ensure IHC was effective. Then, all images for quantification were taken with a Leica TCS-SP2 AOBs laser confocal microscope (Leica Microsystems Inc.; Exton, PA). Images were collected with maximum projection of Z stack images compiled from eight optical sections using a pin hole of one Airy unit with a resolution of 1024 x 1024 pixels. All images were collected using a PL APO 63x oil immersion objective (numerical aperture of 1.3) with a digital zoom of two and a line average of four. Each microscopic field dimension was 119 μm x 119 μm x 3.1 μm, and each FOV collected had negligible area without tissue present. For 488 nm
excitation, emission wavelengths between 505-530 nm were collected. For 594 nm excitation, emission wavelengths between 610-650 nm were collected.

For image collection, the end of the SN section was found, and images were collected starting one field of view (FOV) away. Images were taken from either three of four different areas along the SN, and one or two non-overlapping images were taken from each area. If two pieces of tissue were present on a given section, the end of one piece of tissue was found, and images were collected starting one FOV away and from two different areas along that nerve, with a total of three or four images being taken from the one piece of tissue. The same protocol was repeated for the other piece of SN on the section. For sections immunolabeled with pan-Nfasc and Na\textsubscript{v}1.6, a total of 212 images was collected and the total number of FOV per age group is as follows: 15 days (WT n=21; KO n=21), 1 month (WT n=40; KO n=40), and 7 months (WT n=35; KO n=55). For sections immunolabeled with contactin and gliomedin, a total of 182 images was collected and the total number of FOV per age group is as follows: 15 days (WT n=21; KO n=21), 1 month (WT n=35; KO n=35), and 7 months (WT n=28; KO n=42). For sections immunolabeled with Na\textsubscript{v}1.6 and Nfasc186, a total of 14 images was collected and the total number of FOV per age group is 7 months (WT n=7; KO n=7). For sections immunolabeled with Na\textsubscript{v}1.6 and ankyrin\textsubscript{G}, a total of 14 images was collected and the total number of FOV per age group is 7 months (WT n=7; KO n=7). Microscopy was performed at the Virginia Commonwealth University Department of Anatomy and Neurobiology Microscopy Facility.

Quantification of protein clusters
The pan-Nfasc antibody recognizes both nodal Nfasc186 and paranodal Nfasc155 (Schafer et al., 2006), thus strict criteria is needed to classify specific isoforms. Quantification was conducted as previously described with several modifications (Shepherd et al., 2010). A positive paranodal Nfasc155 cluster was identified as two adjacent clusters separated by a small unlabeled region or a single cluster between 5-11 µm in length. A long single cluster between 5-11 µm corresponds to the labeling of a paranodal paired cluster and an intervening node of Ranvier. A single cluster of less than 5 µm can be nodal labeling of Nfasc186 only or an unpaired paranode, thus not quantified as a positive Nfasc155 cluster. A positive Na\textsubscript{v}1.6 channel cluster was identified as a single cluster between 0.5-1.5 µm in length. A positive contactin cluster was identified as two adjacent clusters separated by a small unlabeled region. A positive gliomedin cluster was identified as a single cluster between 0.5-1.5 µm in length. A positive Nfasc186 cluster was identified as a single cluster between 0.5-1.5 µm in length. A positive ankyrin\textsubscript{G} cluster was identified as a single cluster between 0.5-1.5 µm in length. Clusters that were not entirely within the FOV were excluded.

**Quantification using Adobe Photoshop CS3**

Quantification was conducted using Adobe Photoshop CS3 computer software (Adobe Systems Inc.; San Jose, CA) provided by the Virginia Commonwealth University Department of Anatomy and Neurobiology Microscopy Facility. For Nfasc/Na\textsubscript{v}1.6 IHC sections, both Nfasc155 and Nfasc186 clusters were counted independent of Na\textsubscript{v}1.6 channel clusters. Then, Na\textsubscript{v}1.6 clusters were counted independent of Nfasc clusters. Lastly, the overlay image was used to determine if there were any Nfasc155 clusters present in the absence of Na\textsubscript{v}1.6 channel clusters. For example, the number of Nfasc155 and Nfasc186 clusters was quantified in the 488 nm channel. Then, the
number of Na$_v$1.6 channel clusters was quantified in the 594 nm channel. An overlay of the 488 and 594 nm channels was then used to identify the existence of any Nfasc155 cluster without a corresponding Na$_v$1.6 channel cluster. For contactin/gliomedin IHC sections, contactin and gliomedin clusters were counted independent of one another. For example, the number of contactin clusters was quantified in the 488 nm channel. Then, the number of gliomedin clusters was quantified in the 594 nm channel. For Na$_v$1.6/Nfasc186 IHC sections, overlay images in conjunction with the individual 488 and 594 nm channels were used to identify the number of clusters that fit the following categories: Na$_v$1.6-positive and Nfasc186-positive clusters, Na$_v$1.6-positive and Nfasc186-negative clusters, and Na$_v$1.6-negative and Nfasc186-positive clusters; the same protocol was followed for the Na$_v$1.6/ankyring$_G$ IHC sections.

The number of protein clusters was averaged per mouse for each protein. Then, an average of these averages was determined per genotype at each age. For instance, three 15 day old WT sections were immunolabeled for pan-Nfasc, and seven images were taken for each mouse. The counts from each set of seven images were averaged then an average of the three averages was determined for an overall average for the 15 day WT counts. WT and CST KO averages at each age were statistically compared using a two-tailed Student’s $t$-test with significance was defined as $p < 0.05$.

**Electron microscopy**

**Number of animals**

The number of WT and littermate CST KO mice used for longitudinal electron microscopy (EM) is as follows: 15 days (KO n=1), 4 months (n=1). The number of WT and littermate CST KO
mice used for transverse EM is as follows: 15 days (WT n=3; KO n=2), 1 month (WT n=1; KO n=2), 7-8 months (WT n=1; KO n=3).

**Perfusion and tissue processing**

Mice were anesthetized and perfused using the protocol as previously described with minor modifications. Mice were transcardially perfused with a 0.1 Millonig’s buffer containing 4% paraformaldehyde and 5% glutaraldehyde (Electron Microscopy Sciences; Hatfield, PA) (pH 7.3) for 20 minutes. Mice were then submerged in the fixative solution for two weeks at 4°C to ensure optimal fixation.

The SN was then harvested and rinsed in 0.1 M cacodylate buffer (Electron Microscopy Sciences; Hatfield, PA) overnight to remove aldehydes. Tissue was post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences; Hatfield, PA) in 0.1M cacodylate buffer for one hour with continuous agitation. The tissue was then rinsed three times in 0.1 M cacodylate buffer followed by dehydration in a series of dilutions in ethanol (30% - 100%). Samples were incubated in propylene oxide (Polysciences Inc.; Warrington, PA) twice for 20 minutes each time, then left overnight in a 1:1 mixture of propylene oxide and PolyBed (Polysciences Inc.; Warrington, PA). Tissue samples were then placed in 100% PolyBed for at least six hours and then embedded in freshly prepared PolyBed. Blocks were polymerized at 55°C for 48 hours and sectioned at 1 μm for transverse light microscopic analysis and 90 nanometers (nm) for longitudinal EM analysis using a Leica EM UC6 microtome (Leica Microsystems Inc.; Exton, PA). The 1 μm sections were allowed to dry on a warm hot plate, stained with a toluidine blue solution, rinsed with water, dried, and coverslipped with Permount (Fisher Scientific; Pittsburg,
PA). The 90 nm sections were collected on EM grids and allowed to dry. The grids were placed in a solution of 5% uranyl acetate in 50% methanol for seven minutes, rinsed three times in distilled water, then placed in Reynold’s Lead Citrate solution (Fisher Scientific; Pittsburg, PA), a mixture of lead nitrate, sodium citrate, and distilled water. The sections were removed after five minutes then rinsed three times in distilled water and allowed to dry.

**Visualization**

The 90 nm sections were visualized using a JEOL JEM1230 transmission EM (JEOL Ltd.; Tokyo, Japan) equipped with a Gatan Ultrascan CCD camera (Gatan Inc.; Pleasanton, CA). 1 µm sections were visualized using a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, NY) equipped with a RT Slider Spot camera (Diagnostic Instruments Inc.; Sterling Heights, MI), and all images of the 1 µm sections were collected using a 100x oil immersion objective. Each microscopic field dimension had a width of 118.4 µm and a height of 88.8 µm. Several transverse tissue sections existed on each slide, and images were collected from at least two pieces of tissue. Image collection started at the edge of a piece of tissue, and six non-overlapping images were taken from each slide. For 1 µm sections, a total of 78 images was collected and the total number of FOV per age group is as follows: 15 days (WT n=24; KO n=12), 1 month (WT n=6; KO n=12), 7 months (KO n=6), and 8 months (WT n=6; KO n=12). Microscopy was performed at the Virginia Commonwealth University Department of Anatomy and Neurobiology Microscopy Facility.

**Quantification of 1 µm sections**
Quantification of 1 µm sections was conducted as previously described (Forrest et al., 2009). Briefly, the number of myelinated axons was counted per mouse at each age and genotype. Based on previous work, only axons with a minimum of four myelin wraps would be resolved using this technique.

**Normalization of immunohistochemistry counts**

Using the 1 µm transverse data, the number of myelinated axons per field was averaged per mouse. Then, an average of these averages was determined per genotype at each age. For instance, two 15 day old CST KO EM sections were counted, and six images were taken for each mouse. The counts from each set of six images were averaged then an average of the two averages was determined for an overall average for the 15 day CST KO 1 µm counts. This average is the number of myelinated axons per FOV, or density of myelinated axons, specific for the 15 day CST KO. Each density was then used to normalize the Na1.6, gliomedin, Nfasc155, and contactin protein cluster counts determined by IHC. The average IHC count for each mouse at a particular age and genotype was divided by the associated density and a normalized average for each genotype at each age was determined. For instance, each of the three 15 day old WT pan-Nfasc averages was divided by the density determined for the 15 day old WT. Statistical analyses of normalized IHC data were not conducted as sample sizes for 1 µm data were insufficient.
RESULTS

**Immunohistochemistry**

15 days of age

Hoshi et al. (2007) determined that the number of Na\(_v\) clusters was not altered at 4 weeks of age in the CST KO mouse; however the nodal length was abnormally elongated, which is reminiscent of an immature node. Thus, by looking at an earlier time point, the CST KO mice could potentially exhibit fewer Na\(_v\)\(_{1.6}\) clusters than WT animals as development may be delayed. To determine whether PNS nodal clustering of Na\(_v\)\(_{1.6}\) channels is altered in CST KO mice at 15 days of age, immunohistochemical techniques were employed to compare the number of Na\(_v\)\(_{1.6}\) clusters between WT and littermate CST KO mice. There is no difference in the number of Na\(_v\)\(_{1.6}\) clusters between the WT and CST KO mice (8.2 ± 1.8, 6.5 ± 1.3, \(p = 0.258\); Figure 4, 5; Table 1). To investigate whether the developmental clustering of nodal proteins correlates between neurons and SCs, immunolabeling of gliomedin, the SC nodal protein, was conducted. Similar results are observed: there is no difference in the number of gliomedin clusters between the WT and CST KO mice at 15 days of age (8.8 ± 1.4, 9.5 ± 0.5, \(p = 0.506\); Figure 4, 5; Table 1).

The paranodal proteins Nfasc155 and contactin are also of interest as mice that lack contactin, Caspr, or Nfasc155 have disrupted paranodal junctions, which are thought to play a role in the proper assembly of protein domains of the myelinated axon. To investigate both the role the paranode plays in formation of the node during development as well as the role of sulfatide in the
Figure 4. Nodal proteins cluster normally while paranodal protein clustering is disrupted in CST KO mice at 15 days of age. Immunohistochemistry of nodal voltage-gated sodium (Na\textsubscript{\textit{v}}1.6) channels (\textbf{panels A, E}) and gliomedin (\textbf{panels C, G}) is shown in red, paranodal Nfasc155 (\textbf{panels B, F}) and contactin (\textbf{panels D, H}) is shown in green. Na\textsubscript{\textit{v}}1.6 and gliomedin clusters in the CST KO mice are comparable to the WT mice, while Nfasc155 and contactin clusters are reduced in the CST KO mice as compared to the WT mice. Scale bar = 10 µm

Figure 5. The number of nodal protein clusters is comparable between the WT and CST KO mice, but the number of paranodal protein clusters is reduced at 15 days of age. Quantitative analysis of nodal proteins is shown in red and paranodal proteins in green. There is no difference in the number of Na\textsubscript{\textit{v}}1.6 or gliomedin clusters between the WT and CST KO mice. In contrast, the CST KO mice exhibit a decrease in the number of Nfasc155 and contactin clusters. * indicates p < 0.05
Nodal and paranodal protein clustering at 15 days of age
Table 1. Averages and standard deviations of nodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age.

Table 2. Averages and standard deviations of paranodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age.
Average number of nodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age

<table>
<thead>
<tr>
<th></th>
<th>Na\textsubscript{v}1.6</th>
<th>gliomedin</th>
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<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>15 days</td>
<td>8.2 ± 1.8</td>
<td>6.5 ± 1.3</td>
</tr>
<tr>
<td>1 month</td>
<td>7.4 ± 1.8</td>
<td>6.0 ± 1.2</td>
</tr>
<tr>
<td>7 months</td>
<td>3.5 ± 0.9</td>
<td>2.6 ± 0.4</td>
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Average number of paranodal protein clusters in WT and CST KO mice at 15 days, 1 month, and 7 months of age

<table>
<thead>
<tr>
<th></th>
<th>Nfasc155</th>
<th>contactin</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>15 days</td>
<td>8.2 ± 1.6</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>1 month</td>
<td>5.8 ± 1.5</td>
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<tr>
<td>7 months</td>
<td>3.4 ± 1.3</td>
<td>1.5 ± 0.5</td>
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clustering of paranodal proteins, immunolabeling of Nfasc155 and contactin was conducted. In contrast to the normal clustering of Na\textsubscript{v}1.6 channels and gliomedin observed at 15 days of age in the CST KO mice, immunolabeling of paranodal proteins Nfasc155 and contactin shows a reduction in the clustering of these proteins in absence of sulfatide. The number of Nfasc155 clusters is decreased in the CST KO by 41\% compared to the WT (4.8 \pm 1.1, 8.2 \pm 1.6, p = 0.043; Figure 4, 5; Table 2), and the number of contactin clusters is decreased by 44\% in the CST KO compared to the WT (4.6 \pm 0.3, 8.1 \pm 1.3, p = 0.040; Figure 4, 5; Table 2).

**1 month of age**

By 1 month of age, myelination in the mouse has already reached its peak (Friede and Samorajski, 1968; Webster, 1971). Na\textsubscript{v}1.6 clusters set up properly in the CST KO mouse, as indicated by the results at 15 days of age. To investigate whether Na\textsubscript{v}1.6 clusters are susceptible to breakdown in the CST KO mouse, immunohistochemical techniques were utilized to compare the number of Na\textsubscript{v}1.6 clusters between WT and CST KO mice at 1 month of age. Similar to the results at 15 days of age, there is no difference in the number of Na\textsubscript{v}1.6 clusters between the WT and CST KO mice (7.4 \pm 1.8, 6.0 \pm 1.2, p = 0.163, Figure 6, 7; Table 1). Although nodal clustering of neuronal Na\textsubscript{v}1.6 channels was not disrupted in the absence of sulfatide, quantitation of the gliomedin clusters revealed a significant reduction in the CST KO mice as the mutants exhibited 32\% fewer clusters compared to their WT littermates at 1 month of age (4.6 \pm 0.5, 6.7 \pm 1.7, p = 0.048; Figure 6, 7; Table 1).

Clustering of paranodal proteins Nfasc155 and contactin is decreased at 15 days of age in the CST KO mouse. To determine if this abnormality is corrected with age, immunolabeling of the
Figure 6. In contrast to Nfasc155, gliomedin, and contactin, Na\textsubscript{v}1.6 channels cluster normally in CST KO mice at 1 month of age. Immunohistochemistry of nodal voltage-gated sodium (Na\textsubscript{v}1.6) channels (panels A, E) and gliomedin (panels C, G) is shown in red, paranodal Nfasc155 (panels B, F) and contactin (panels D, H) is shown in green. Similar to 15 days of age, the Na\textsubscript{v}1.6 clusters are comparable between WT and CST KO mice (panels A, E). Nfasc155 and contactin clusters again appear reduced in the CST KO mice at 1 month of age (panels B, D, F, H). In contrast to 15 day data, gliomedin clusters appear reduced in the CST KO mice at 1 month of age (panels C, G). It is of note that a pan-Nfasc antibody was used to immunolabel paranodal Nfasc155. In the CST KO mice, there is an increased incidence of nodal labeling, which likely represents nodal Nfasc186 rather than paranodal Nfasc155 (panel F). Scale bar = 10 µm

Figure 7. The number of gliomedin, Nfasc155, and contactin clusters is reduced in CST KO mice, while there remains no difference in Na\textsubscript{v}1.6 cluster numbers at 1 month of age. Quantitative analysis of nodal proteins is shown in red and paranodal proteins in green. The CST KO mice show a decrease in the number of Nfasc155 and contactin clusters at 1 month of age. In contrast to observations at 15 days, the CST KO mice show a decrease in gliomedin clusters. There remains no difference in the number of Na\textsubscript{v}1.6 clusters between the WT and CST KO mice. * indicates p < 0.05
Nodal and paranodal protein clustering at 1 month of age
paranodal proteins was conducted at 1 month of age. At this time point, disruption of paranodal clustering of Nfasc155 and contactin is again observed. The number of Nfasc155 clusters is decreased in the CST KO by 50% compared to the WT (2.9 ± 1.8, 5.8 ± 1.5, p = 0.011; Figure 6, 7; Table 2), and the number of contactin clusters is decreased by 51% in the CST KO compared to the WT (2.9 ± 1.2, 5.9 ± 2.2, p = 0.033; Figure 6, 7; Table 2).

7 months of age

Hoshi et al. (2007) studied PNS nodal clustering in the CST KO mouse up to 22 weeks of age and reported normal numbers of Na\(\text{v}\) clusters. However, a CST KO mouse has an expected life expectancy of one year, and susceptibility of Na\(\text{v}\) clusters to break down may be observed only at older ages. Thus, to determine whether PNS nodal maintenance of Na\(\text{v}\)1.6 channels is altered in CST KO mice, immunohistochemical techniques were employed to compare the number of Na\(\text{v}\)1.6 clusters between WT and CST KO mice at 7 months of age. Similar to the results at 15 days and 1 month of age, there is no difference in the number of Na\(\text{v}\)1.6 clusters between the WT and CST KO animals (3.5 ± 0.9, 2.6 ± 0.4, p = 0.078; Figure 8, 9; Table 1). It is noteworthy, however, the differences between the WT and CST KO mice in nodal Na\(\text{v}\)1.6 clustering progressively increased from 15 days to 7 months of age (p at 15 days = 0.258; p at 1 month = 0.163; p at 7 months = 0.078). In contrast, because gliomedin clustering was disrupted at 1 month of age, it would be interesting to determine if there continues to be disruption or if there is rescue of gliomedin clustering even in the absence of sulfatide. At 7 months of age, the clustering of gliomedin is disrupted with clusters decreased by 25% in the CST KO compared to the WT (3.3 ± 0.6, 4.4 ± 0.6, p = 0.041; Figure 8, 9; Table 1).
Figure 8. Abnormalities in Nfasc155, gliomedin, and contactin clusters persist in CST KO mice, while Na,1.6 clustering remains intact at 7 months of age. Immunohistochemistry of nodal voltage-gated sodium (Na,1.6) channels (panels A, E) and gliomedin (panels C, G) is shown in red, paranodal Nfasc155 (panels B, F) and contactin (panels D, H) is shown in green. Similar to analyses at 15 days and 1 month of age, Na,1.6 clusters are comparable between WT and CST KO mice (panels A, E). Again, Nfasc155, gliomedin, and contactin clusters are disrupted in the CST KO mice (panels B-D, F-H). Nodal labeling by the pan-Nfasc antibody is observed in the CST KO mice, which likely represents immunolabeling of Nfasc186 rather than Nfasc155 (panel F). Scale bar = 10 µm

Figure 9. Na,1.6 cluster numbers are comparable between WT and CST KO mice, while gliomedin, Nfasc155, and contactin cluster numbers are reduced at 7 months of age. Quantitative analysis of nodal proteins is shown in red and paranodal proteins in green. Similar to 15 day and 1 month analyses, the CST KO mice show a decrease in the number of Nfasc155, contactin, and gliomedin clusters. There is no difference in the number of Nav1.6 clusters between the WT and CST KO mice. * indicates p < 0.05
Nodal and paranodal protein clustering at 7 months of age

Percent of wildtype

<table>
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<tr>
<th>Protein</th>
<th>WT KO Na\textsubscript{v}1.6</th>
<th>WT KO gliomedin</th>
<th>WT KO Nfasc155</th>
<th>WT KO contactin</th>
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* indicates statistical significance.
At 1 month of age, Na\textsubscript{v1.6} channels are clustering in normal numbers in the CST KO mice compared to the WT mice. However, to investigate whether the paranode plays a role in long-term maintenance of the node, Nfasc155 and contactin clustering must be investigated in the CST KO animals at older ages. At 7 months of age, the clustering of the paranodal proteins Nfasc155 and contactin continues to be disrupted. The number of Nfasc155 clusters is decreased by 56% in the CST KO compared to the WT (1.5 ± 0.5, 3.4 ± 1.3, \( p = 0.029 \); Figure 8, 9; Table 2), and the number of contactin clusters is decreased in the CST KO by 58% compared to the WT (1.7 ± 0.3, 4.0 ± 0.4, \( p = 0.0002 \); Figure 8, 9; Table 2).

A summary of nodal and paranodal protein integrity in the CST KO mice at 15 days, 1 month, and 7 months of age is detailed in Table 3.

**Preliminary results and ongoing studies**

**Electron microscopy: 90 nm sections**

**15 days of age**

By 2 weeks of age, the CST KO mice begin to exhibit pathological symptoms such as hindlimb weakness and tremors. To determine the structural organization of the myelinated axon in the CST KO mouse, as abnormal structure may contribute to the observed symptoms, longitudinal EM at 15 days of age was qualitatively assessed. Preliminary results reveal that gross myelin structure is intact as compact myelin is observed along the juxtaparanode and internode and lateral loops, defining the paranode, are inverted towards the axon (Figure 10). It is of note that the node is myelin-bare, and the gross structure of the node appears normal. However, transverse
Table 3. Nodal and paranodal protein cluster integrity in CST KO mice at 15 days, 1 month, and 7 months of age.
Nodal and paranodal protein cluster integrity in CST KO mice at 15 days, 1 month, and 7 months of age

<table>
<thead>
<tr>
<th></th>
<th>15 days</th>
<th>1 month</th>
<th>7 months</th>
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<tbody>
<tr>
<td>node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na\textsubscript{v}1.6</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>gliomedin</td>
<td>✓</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>paranode</td>
<td></td>
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</tr>
<tr>
<td>contactin</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nfasc155</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

✓: no difference between WT and CST KO mice
X: reduction in cluster number in CST KO mice
Figure 10. The PNS myelinated axon in CST KO mice at 15 days of age exhibits loss of transverse bands and disorganization of SC microvilli. Assessment of ultrastructure was conducted by examining longitudinal EM. In panel A, normal, compacted myelin is observed along the internode and juxtaparanode. Lateral loops of the paranode are organized and properly efface the axon (panel B), however transverse bands are absent (panel C, arrows). Panel D shows that the node appears grossly normal, but the SC microvilli are disorganized as cross sections of the microvilli are observed (arrows). Scale bars = 1 µm in panel A; 0.5 µm in panels B, D; 0.1 µm in panel C.
bands normally at the paranode are absent and the SC microvilli at the node appear disorganized as cross sections of the SC microvilli are observed.

4 months of age

To determine the effect of age on nodal and paranodal ultrastructure, longitudinal EM of the WT and CST KO mice at 4 months of age was qualitatively evaluated. Preliminary observations include compact myelin along the juxtaparanode and internode, lateral loops that are inverted towards the axon, myelin-bare nodes, and transverse bands that appear to traverse the periaxonal space (Figure 11). Furthermore, SC microvilli appose the nodal axolemma in proper orientation as longitudinal sections of the SC microvilli are observed, characteristically resembling spokes of a wheel. Similarly in the CST KO mice, gross myelin structure appears intact, as compact myelin and lateral loops are properly oriented and observed in their respective domains, and nodes are myelin-bare (Figure 12). However, similar to observations seen in the 15 day old CST KO animals, transverse bands are once absent and SC microvilli are disorganized as cross sections of SC microvilli are abundantly observed at the node.

Light microscopy: 1 µm sections

To determine whether the WT and CST KO animals have a comparable density of myelinated axons, the number of myelinated axons per FOV (width: 118.4 µm, height: 88.8 µm) in WT and CST KO animals was counted at 15 days, 1 month, and 7-8 months of age.

15 days of age
Figure 11. Normal ultrastructural characteristics of the PNS myelinated axon at 4 months of age include compact myelin, organized lateral loops, transverse bands, and properly oriented SC microvilli. Assessment of ultrastructure was conducted by examining longitudinal EM. In panel A, normal, compacted myelin is observed. Panel B exhibits organized lateral loops of the paranode that properly efface the axon, and transverse bands, structures that appear to traverse the periaxonal space, are observed in panel C (arrows). Panel D shows that the node appears grossly normal as it is myelin-bare. Also in panel D, the SC microvilli are depicted as finger-like projections contacting the nodal axolemma and are properly organized as longitudinal sections of the SC microvilli are observed (arrows). Scale bars = 1 µm in panel A; 0.5 µm in panels B, D; 0.1 µm in panel C.
Figure 12. Loss of transverse bands and disorganization of SC microvilli are observed in the CST KO mouse at 4 months of age. Assessment of ultrastructure was conducted by examining longitudinal EM. In panel A, normal, compacted myelin is observed. Panel B exhibits organized lateral loops of the paranode that properly efface the axon. Similar to qualitative analysis of the CST KO at 15 days of age, transverse bands are not observed (panel C, arrows). Panel D shows that the node appears grossly normal as it is myelin-bare. However, in contrast to the WT, SC microvilli are not seen contacting the nodal axolemma, indicating abnormality, and cross sections of the SC microvilli are commonly seen (arrows). Scale bars = 1 µm in panel A; 0.5 µm in panels B, D, 0.1 µm in panel C.
Myelinated axons from cross sections of three 15 day old WT animals were counted, yielding averages of 431.3, 474.3, and 391.3 per microscopic FOV per mouse, with an overall average of 432.3 myelinated axons per FOV (Figure 13; Table 3). Myelinated axons from cross sections of two 15 day old CST KO animals were counted, yielding averages of 407.3 and 400.5, with an overall average of 404.9 myelinated axons per FOV.

1 month of age
Myelinated axons from cross sections of one 1 month old WT animal were counted, yielding an average of 392.8 myelinated axons per FOV (Figure 13; Table 3). Myelinated axons from cross sections of two 1 month old CST KO animals were counted, yielding averages of 286.3 and 268.5, with an overall average of 277.4 myelinated axons per FOV.

7-8 months of age
Myelinated axons from cross sections of one 8 month old WT animal were counted, yielding an average of 263.8 myelinated axons per FOV (Figure 13; Table 3). Myelinated axons from cross sections of one 7 month old and two 8 month old CST KO animals were counted, yielding averages of 213.5, 225.5 and 222.6, with an overall average of 220.5 myelinated axons per FOV.

Immunohistochemistry: normalized Na,1.6, gliomedin, Nfasc155, contactin data
The density of myelinated axons determined by light microscopic assessment was factored into the corresponding data to ensure that any differences seen in the number of protein clusters between WT and CST KO mice were not due to differences in the number of myelinated axons
Figure 13. The density of myelinated axons in WT and CST KO sciatic nerve at 15 days, 1 month, and 7 months of age was determined by light microscopy. Panels A, C, and E represent cross sections of the WT sciatic nerve (SN) at 15 days, 1 month, and 7 months of age, respectively. Panels B, D, and F represent cross sections of the CST KO SN at 15 days, 1 month, and 7 months of age, respectively. Investigation is ongoing to determine if there is a difference in the density of myelinated axons between the WT and CST KO at these ages. Scale bar = 10 µm
Table 4. Average density of myelinated axons in WT and CST KO mice at 15 days, 1 month, and 7 months of age.
### Average density of myelinated axons in WT and CST KO mice at 15 days, 1 month, and 7 months of age

<table>
<thead>
<tr>
<th>Time</th>
<th>Average number of myelinated axons per field of view (FOV)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
</tbody>
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| **15 days** | 432.3 ± 41.5  
  n=3               | 404.9 ± 4.8     
  n=3               |
| **1 month** | 392.8        
  n=1               | 277.4 ± 12.5    
  n=2               |
| **7 months** | 263.8        
  n=1               | 220.5 ± 6.2     
  n=3               |
per FOV. Preliminary results suggest that a consistent decrease in the number of myelinated axons per FOV in the CST KO mice at each age may account for differences seen in nodal and paranodal protein clustering, however further assessment is ongoing and necessary to verify this observation.

**Normalized protein cluster averages at 15 days of age**

After normalization of data at 15 days of age, the average number of $\text{Na}_v 1.6$ clusters in the WT and CST KO mice is $0.0191 \pm 0.0042$ and $0.0162 \pm 0.0032$, respectively. The average number of gliomedin clusters in the WT compared to the CST KO mice after normalization at this age is $0.0204 \pm 0.0033$ and $0.0219 \pm 0.0012$, respectively. The normalized 15 day averages of $\text{Na}_v 1.6$ and gliomedin clusters appear to suggest no difference in clustering of these proteins between the WT and CST KO mice.

After normalization, the average number of Nfasc155 clusters in the WT and CST KO mice at this age is $0.0189 \pm 0.0036$ and $0.0119 \pm 0.0026$, respectively. The average number of contactin clusters in the WT versus the CST KO mice after normalization is $0.0187 \pm 0.0031$ and $0.0106 \pm 0.0006$, respectively. The difference observed in Nfasc155 clustering between WT and CST KO mice prior to normalization seems to disappear, while there still remains a difference in number of contactin clusters between the WT and CST KO mice. This conclusion cannot yet be made however, as the sample size for the 1 µm counts is insufficient.

**Normalized protein cluster averages at 1 month of age**
After normalization, the average number of Na\textsubscript{v}1.6 clusters in the WT and CST KO mice is 0.0187 ± 0.0047 and 0.0216 ± 0.0042, respectively. The average number of gliomedin clusters in the WT compared to the CST KO mice after normalization is 0.0170 ± 0.0044, 0.0165 ± 0.0018, respectively. The normalized 1 month data appear to suggest that Na\textsubscript{v}1.6 channels and gliomedin clustering is normal in the CST KO mice.

After taking into account the density of myelinated axons, the average number of Nfasc155 clusters in WT and CST KO mice is 0.0147 ± 0.0037 and 0.0104 ± 0.0063, respectively. The average number of contactin clusters in the WT and CST KO mice is 0.0151 ± 0.0056, 0.0104 ± 0.0042, respectively. It appears that there exists no difference in the clustering of paranodal proteins Nfasc155 and contactin between the WT and CST KO mice at 1 month of age; however this observation needs further verification.

**Normalized protein cluster averages at 7 months of age**

The average normalized number of Na\textsubscript{v}1.6 clusters in the WT and CST KO mice is 0.0133 ± 0.0035 and 0.0116 ± 0.0018, respectively, while the average number of gliomedin clusters in the WT and CST KO mice after normalization is 0.0165 ± 0.0022 and 0.0148 ± 0.0028, respectively. Preliminary assessment of the normalized 7 month data suggest no difference between the WT and CST KO mice in Na\textsubscript{v}1.6 or gliomedin nodal clustering.

After normalization, the average number of Nfasc155 clusters in the WT and CST KO mice is 0.0068 ± 0.0020 and 0.0130 ± 0.0051, respectively. The average number of contactin clusters in the WT versus the CST KO mice is 0.0077 ± 0.0015 and 0.0152 ± 0.0014, respectively. In
contrast to preliminary observations of the normalized data at 15 days and 1 month of age, the data suggest that clustering of paranodal proteins Nfasc155 and contactin in the 7 month old CST KO mice may be abnormal.

**Immunohistochemistry: Na\(_{\text{v}}\)1.6 & Nfasc186, Na\(_{\text{v}}\)1.6 & ankyrin\(_G\)**

Non-normalized IHC data demonstrate that paranodal clustering of Nfasc155 and contactin is disrupted in the CST KO PNS while nodal clustering of Na\(_{\text{v}}\)1.6 channels is preserved. Therefore, additional nodal proteins may regulate maintenance of Na\(_{\text{v}}\)1.6 clusters at the node. Two possible candidates are Nfasc186 and ankyrin\(_G\), which have both been previously shown to be involved in the maintenance of Na\(_{\text{v}}\) channel clusters (Dzhashiashvili et al., 2007; Thaxton et al., 2011). Immunohistochemical techniques investigating colocalization of Nfasc186 and ankyrin\(_G\) with Na\(_{\text{v}}\)1.6 clusters at 7 months of age were conducted. In both the 7 month WT and 7 month CST KO sections immunolabeled with Na\(_{\text{v}}\)1.6 and Nfasc186, all nodes strongly label for both Na\(_{\text{v}}\)1.6 and Nfasc186 (Figure 14). A different pattern is seen with IHC involving ankyrin\(_G\). In the 7 month WT section immunolabeled with Na\(_{\text{v}}\)1.6 and ankyrin\(_G\), there are Na\(_{\text{v}}\)1.6-positive nodes with varying intensities of ankyrin\(_G\) labeling (Figure 15). In contrast, the 7 month CST KO section immunolabeled with Na\(_{\text{v}}\)1.6 and ankyrin\(_G\) exhibited strong labeling of both Na\(_{\text{v}}\)1.6 channels and ankyrin\(_G\) at all nodes observed.
Figure 14. At 7 months of age, all nodes in WT and CST KO mice exhibit colocalization of $\text{Na}_{v1.6}$ channels with $\text{Nfasc186}$. $\text{Na}_{v1.6}$ channels are immunolabeled red, and $\text{Nfasc186}$ is immunolabeled green. All nodes in the WT and CST KO mice show colocalization of $\text{Na}_{v1.6}$ channels and $\text{Nfasc186}$. Scale bar: 5 µm
Figure 15. At 7 months of age in the WT mice, but not the CST KO mice, Na\textsubscript{v}1.6-positive nodes exhibit variable intensities of ankyrin\textsubscript{G} labeling. Na\textsubscript{v}1.6 channels are immunolabeled red, and ankyrin\textsubscript{G} is immunolabeled green. In the WT mice, there are Na\textsubscript{v}1.6-positive nodes that have variable intensities of ankyrin\textsubscript{G} labeling. In contrast, the CST KO mice exhibit nodes strongly labeled for both Na\textsubscript{v}1.6 channels and ankyrin\textsubscript{G}. Scale bar: 5 µm
DISCUSSION

The goal of this study is to elucidate the role of sulfatide in organizing the well-delineated structural and molecular domains of the myelinated axon within the PNS. Data gathered from immunohistochemical and, to a lesser extent, electron microscopic techniques suggest that the establishment and maintenance of these myelin and axonal domains are differentially dependent on sulfatide. In the absence of sulfatide, paranodal clustering of Nfasc155 and neuronal contactin is disrupted between 15 days and 7 months of age but nodal clustering of Na\textsubscript{v}1.6 channels is unaffected, suggesting the existence of distinct mechanisms responsible for organization of the node versus paranode. Our data further imply that sulfatide is differentially important for nodal organization depending on the cell type. In the absence of sulfatide, SC gliomedin and neuronal Na\textsubscript{v}1.6 clusters form normally, but neuronal Na\textsubscript{v}1.6 clusters remain intact with age while gliomedin clusters are prone to breakdown. Coincident with normal Na\textsubscript{v}1.6 clustering, other neuronal nodal proteins, Nfasc186 and ankyrin\textsubscript{G}, are normally localized in the 7 month old CST KO, further indicating that neuronal nodal stability is independent of sulfatide. Ultrastructural assessment of nodal and paranodal domains supports the conclusions drawn from IHC. While neuronal nodal structures are grossly normal, the myelin-associated nodal structures, known as SC microvilli, are disorganized and paranodal TBs are absent in CST KO animals. Thus, in addition to demonstrating that the specific myelin and axonal domains are differentially dependent on sulfatide, these findings also demonstrate that despite compromised nodal and paranodal molecular organization, the structure of these domains can be preserved. This disconnect between structural and molecular organization is an important and novel observation. Finally, since nodal organization is dependent on sulfatide in the CNS (Ishibashi et al., 2002),
our study shows that distinct mechanisms regulate nodal stability in the CNS as compared to the PNS.

**Sulfatide is essential for paranodal organization**

At 15 days, 1 month, and 7 months of age, the number of Nfasc155 and contactin clusters is decreased in the absence of sulfatide. This finding is consistent with the report of Hoshi et al. (2007), who reported that CST KO SN exhibited a reduction in paranodal clusters of Caspr, the *cis* binding partner of contactin. Sulfatide is thus crucial for the proper localization of these proteins at the paranode, though the method by which sulfatide accomplishes this remains unclear. One possible method by which sulfatide may facilitate paranodal protein clustering is through the regulation of intracellular trafficking. Our laboratory has observed an increased accumulation of vesicles containing Nfasc within the OL cell body in the CST KO CNS between 7 and 30 days of age (Shroff and Dupree, unpublished data). These findings suggest a misregulation in the trafficking of Nfasc, which may be attributable to the absence of sulfatide.

One possible mechanism by which sulfatide could mediate protein sorting within the SC is by mediating intracellular protein trafficking through the regulation of lipid raft formation. This hypothesis, at least in regard to sphingolipids in general, has been previously proposed (Simons and Ikonen, 1997; van IJzendoorn *et al*., 1997; Brown and London, 1998; van IJzendoorn and Hoekstra, 1999; Simons and Toomre, 2000; Helms and Zurzolo, 2004). Lipid rafts, also called membrane rafts, are specialized membrane domains that assemble in the plasma membrane or in the Golgi apparatus before being trafficked to the plasma membrane (Brown and London, 1998; Brown and London, 2000; Helms and Zurzolo, 2004). Lipid rafts consist of cholesterol,
sphingolipids, and proteins with lipid modifications (Brown and London, 2000; Simons and Toomre, 2000; Helms and Zurzolo, 2004). Both Nfasc155 and contactin contain post-translational lipid modifications (Davis et al., 1996; Labasque and Faivre-Sarrailh, 2010), thus making them candidates for raft recruitment. Through selective recruitment and exclusion, lipid rafts provide an optimal site for facilitating intermolecular interactions and adhesion complex assembly (Harris and Siu, 2002). Additionally, sphingolipids display a distinct distribution pattern in polarized cells; for instance, glucosylceramide is specifically targeted to the apical plasma membrane while sphingomyelin is targeted to the basolateral plasma membrane (van IJzendoorn et al., 1997). The selective distribution of these sphingolipids may be instrumental to protein sorting, as a specific composition and distribution of proteins exists in these plasma membrane domains (van IJzendoorn and Hoekstra, 1999). Indeed, inhibition of sphingolipid synthesis has been shown to result in mislocalization of GPI-linked proteins (Mays et al., 1995; Ledesma et al., 1998). Furthermore, Schafer et al. (2004) demonstrate that a population of Nfasc155 is present in lipid rafts, and CGT KO mice, animals that lack both galactocerebroside and sulfatide, exhibit reduced association of Nfasc155 with lipid rafts correspondingly with a reduction in paranodal clustering of Nfasc155. These findings strongly support the idea that sulfatide plays a role in targeting Nfasc155 to the paranode via a lipid raft-dependent mechanism (Schafer et al., 2004).

It is of note, however, that while the number of Nfasc155 and contactin clusters are reduced in the absence of sulfatide, there are some clusters of Nfasc155 and contactin detected at the paranode. Several interpretations of this observation can be made. It is possible a sulfatide-independent mechanism exists that can somewhat compensate for deficits in the clustering of
paranodal proteins. It is also possible that the initial paranodal clustering of Nfasc155 and contactin/Caspr does not require sulfatide, but sulfatide is essential for maintaining the paranodal clusters. Another possibility is that the lack of sulfatide may alter lipid raft integrity, which could potentially disrupt the lipid raft-dependent mechanism involved in paranodal organization. Yet another interpretation is that the lack of sulfatide preferentially decreases the expression of Nfasc155. Preliminary, unpublished observations are consistent with this possibility as western blot analysis reveals a greater than 50% reduction in levels of Nfasc155 in CST KO SN (Pomicter and Dupree, unpublished). Because Nfasc155 has been shown to be essential for the stabilization of the contactin/Caspr complex at the paranode (Charles et al., 2002; Sherman et al., 2005; Pillai et al., 2009), decreased Nfasc155 potentially translates into decreased contactin/Caspr observed at the paranode; therefore, the deficit in contactin/Caspr clustering may be due to decreased Nfasc155 expression rather than a direct effect of the absence of sulfatide. However, it is also possible that the reduced contactin/Caspr clusters detected at the paranode are directly due to a lack of sulfatide, but sulfatide produced by neurons rather than the SCs. Pernber et al. (2002), by utilizing a sulfatide antibody in IHC, reported immunolabeling in subpopulations of neurons in the CNS. Additionally, in human MLD, accumulation of sulfatide in neurons is observed, suggesting that neurons may produce sulfatide normally, but in the absence of ASA, it is not broken down properly (Molander-Melin et al., 2004; Eckhardt et al., 2007). However, these studies have their limitations. The Sulph1 antibody used in Pernber et al. (2002) also binds seminolipid, and while the antibody may have a higher affinity for sulfatide and seminolipid concentrations are far less than that of sulfatide in the brain, immunolabeling of seminolipid cannot be excluded. Also, while neuronal sulfatide accumulation is observed in human MLD, the pathological condition may not necessarily represent an exaggerated version of
the normal condition. However, if a neuronal population of sulfatide exists, several questions come to mind. Are the cell-specific sulfatide populations responsible for directing their respective paranodal protein(s) to the paranode? Are the different populations of sulfatide capable of indirectly regulating the localization of the other cell type’s paranodal protein(s)? Is the expression of one population of sulfatide somehow involved in the expression or stabilization of the other? These are all questions that can fuel potential lines of study.

**Sulfatide is differentially important for nodal protein organization depending on cell of origin**

Hoshi *et al.* (2007) reported normal numbers of Na\(_v\) channels in the CST KO mouse between 4 and 22 weeks of age. However, at all time points investigated, the node is elongated, reminiscent of immature nodes and indicating abnormality. Furthermore, Ishibashi *et al.* (2002) reported a breakdown in Na\(_v\) clusters by 6 weeks of age in the CST KO CNS. Based on these findings, we hypothesized that nodal Na\(_v\)1.6 clusters would be unstable in the CST KO SN and that by expanding the time course used by Hoshi *et al.* (2007), we would observe a similar instability of Na\(_v\)1.6 clusters. Additionally, since Hoshi *et al.* (2007) reported elongation of Na\(_v\) clusters, we hypothesized that nodal clusters were immature and that analysis of younger ages would reveal a delay in clustering. Unexpectedly, throughout all time points investigated, the number of neuronal Na\(_v\)1.6 channel clusters is comparable between the WT and CST KO mice; the clustering and maintenance of neuronal Na\(_v\)1.6 channels is not reliant on sulfatide.

Interestingly, these observations are not seen with SC gliomedin. At 15 days of age, the number of gliomedin clusters is not reduced in the CST KO mice. However, at 1 month and 7 months of
age, the CST KO mice exhibit a reduction in the number of gliomedin clusters, indicating that the absence of sulfatide has a deleterious effect on the maintenance of gliomedin. Assessment of the CST KO nodal ultrastructure at 15 days of age reveals disorganization of the microvilli such that they do not contact the nodal axolemma, a similar pattern of pathology to that observed in the gliomedin KO, Nfasc186-knockdown, and NrCAM KO mice (Feinberg et al., 2010; Thaxton et al., 2011). Because gliomedin is found specifically associated with SC microvilli, structural disruption may precipitate molecular disruption, resulting in improper maintenance of existing gliomedin clusters. From these observations, it can be concluded that sulfatide is differentially important for the organization of nodal proteins based on their cell of origin.

**Initial clustering and long-term maintenance of nodal Na\textsubscript{v}1.6 channels utilize different mechanisms**

Despite the absence of sulfatide, the number of Na\textsubscript{v}1.6 clusters is comparable between the WT and CST KO mice at all ages studied. It is of note that the number of paranodal protein clusters is reduced in the CST KO during these same time points, indicating that the molecular paranode is not essential for the clustering Na\textsubscript{v}1.6 channels. If the paranodal proteins Nfasc155 and contactin are not responsible for the establishment of nodal Na\textsubscript{v}1.6 clusters, what other proteins are possible candidates? Feinberg *et al.* (2010) first proposed the existence of a paranode-independent mechanism involved in clustering Na\textsubscript{v} channels that is mediated by gliomedin. Supporting this hypothesis, gliomedin has been shown to induce neuronal formation of nodal-like clusters that include Na\textsubscript{v} channels, ankyrin\textsubscript{G}, and βIV spectrin in the absence of SCs, thereby demonstrating its potential role in the clustering of nodal Na\textsubscript{v} channels (Eshed *et al.*, 2005). In
agreement with these ideas, at 15 days of age, we observe normal clustering of gliomedin in the CST KO mice.

Gliomedin clusters break down by 1 month of age in the CST KO, yet Naᵥ1.6 clusters remain intact, which makes gliomedin unlikely to be responsible for the maintenance of Naᵥ1.6 clusters. What is maintaining Naᵥ1.6 channels at the node then? Likely, the mechanism involves nodal proteins, as nodal abnormalities observed in the CST KO mice are minimal and may be a subsequent effect of paranodal disruption. The neuronal nodal protein Nfasc186 is one such candidate. Previously, both in vitro and in vivo studies utilizing Nfasc186-knockdown techniques have shown loss of nodal proteins, including Naᵥ channels and ankyrinG (Dzhashiashvili et al., 2007; Thaxton et al., 2011). Our preliminary study investigating Nfasc186 and Naᵥ1.6 channels in 7 month old animals show Nfasc186 always colocalizing with Naᵥ1.6 in both the WT and CST KO animals. These observations collectively suggest that the maintenance of Nfasc186 is unaffected in the absence of sulfatide and also that Nfasc186 is a possible facilitator of Naᵥ1.6 cluster stability.

AnkyrinG is another candidate that may be involved in Naᵥ1.6 channel maintenance at the node. Like Nfasc186, previous in vitro studies utilizing ankyrinG-knockdown techniques have shown loss of nodal components, including Naᵥ channels and Nfasc186 (Dzhashiashvili et al., 2007). Our preliminary data, resulting from the investigation of ankyrinG and Naᵥ1.6 channel clusters in 7 month old animals, show ankyrinG always colocalizing with Naᵥ1.6 in the CST KO mice, while Naᵥ1.6-positive nodes colocalize with variable concentrations of ankyrinG in the WT mice. AnkyrinG, similar to Nfasc186, appears to be unaffected by the lack of sulfatide and may play a
role in the stabilization of Na\textsubscript{v}1.6 channels at the node in the absence of sulfatide. Interestingly, our preliminary observations suggest that ankyrin\textsubscript{G} may play a lesser role in the maintenance of Na\textsubscript{v}1.6 clusters in certain populations of nodes under “normal” conditions. Perhaps only under extraordinary stress, such as the absence of sulfatide, is ankyrin\textsubscript{G} absolutely necessary for nodal Na\textsubscript{v}1.6 cluster stability. It is of note that preliminary study of Nfasc186 and ankyrin\textsubscript{G} verify these proteins as potential mediators of Na\textsubscript{v}1.6 cluster stability, however further investigation must be conducted to verify these conclusions. Additionally, Nfasc186 and ankyrin\textsubscript{G} may also be involved in the establishment of Na\textsubscript{v}1.6 clusters, along with gliomedin. Additional investigation at younger time points can begin to address this hypothesis.

**Molecular and structural organization of the myelinated axon may not correspond in the absence of sulfatide**

Preliminary ultrastructural assessment of the domains of the myelinated axon in the CST KO mice at 15 days and 4 months of age reveals generally normal structure. Compact myelin is detected along the juxtaparanode and internode, and the lateral loops at the paranode are properly organized and turn in towards the axon. In contrast to the preserved structural organization, the molecular paranode and juxtaparanode (Hoshi et al., 2007) is disrupted, thus demonstrating that the molecular and structural state of the myelinated axon may not always correspond. While mostly conserved, there exists some structural abnormalities in the CST KO mice such as the absence of TBs at the paranode at both 15 days and 4 months of age. Interestingly, the absence of TBs is consistent with the decrease in the number of Nfasc155 and contactin clusters detected at the paranode. TBs are mysterious, as their precise function in paranodal organization is unclear. As evidenced by our preliminary data, paranodal structure is maintained, even in the absence of
TBs. It is of note that while TBs may not be necessary for formation or maintenance of gross paranodal structure, their role in stabilizing axon-myelinating cell adhesion has been postulated to become increasingly important with age, as a reduction in TBs seen in the CNS of aged animals may facilitate myelin degeneration (Shepherd et al., 2010).

Disorganization of the SC microvilli is also observed in the CST KO mice at 15 days and 4 months of age. Longitudinal EM analysis of the nodal regions in the CST KO mice reveals SC microvilli cut transversely. At the node, SC microvilli are no longer oriented parallel to the axon, and instead, turn 90 degrees towards the axon such that a longitudinal section of the node depicts SC microvilli as finger-like projections contacting the nodal axolemma. In the CST KO mice, however, the SC microvilli are frequently cut in transverse section, indicating that the microvilli are not oriented towards the axon; instead, they are positioned parallel to the axon. These observations suggest that sulfatide facilitates proper SC microvilli orientation. Interestingly, disorganized SC microvilli are also seen in the gliomedin KO, Nfasc186-knockdown, and NrCAM KO animals (Feinberg et al., 2010; Thaxton et al., 2011), thereby suggesting that the proper clustering of gliomedin may also be essential, but not sufficient, to orient the SC microvilli. Despite disorganization of the SC microvilli seen at 15 days of age in the CST KO mice, clustering of gliomedin remains normal until 1 month of age, signifying that molecular and structural organization of the myelinated axon may not always correspond.

**The density of myelinated axons is reduced in the absence of sulfatide**

Preliminary results investigating the density of myelinated axons in the WT and CST KO mice suggest a decrease at 15 days, 1 month, and 7-8 months of age in the mutants. This observation
has several possible implications. Potentially, fewer axons are myelinated in the absence of sulfatide, and this could be due to SC abnormalities. In vitro studies show that SC myelin formation is greatly reduced after treatment with a GalC antibody, which also reacts with sulfatide (Ranscht et al., 1987; Owens and Bunge, 1990; Wood et al., 1990), which suggests that in the absence of sulfatide, the myelinating ability of the SC may be compromised. On the other hand, the reduction in myelin formation may lead to an upregulation of SCs. Previous in vitro work suggests that sulfatide negatively regulates OL differentiation (Bansal et al., 1999), which is supported by an increase in OL numbers observed in the CST KO CNS (Hirahara et al., 2004; Shroff et al., 2009) and CGT KO CNS (Bansal et al., 1999). The number of SCs could similarly be increased in the absence of sulfatide, resulting in SCs occupying space that would otherwise be filled by myelinated axons, giving the illusion of a reduced density of myelinated axons; however, this remains to be investigated. The decreased density of myelinated axons could also result from a reduction in the total number of axons in the CST KO mouse. Further study is ongoing and necessary to test these possibilities.

**A reduced density of myelinated axons in the CST KO mice has an influence on immunohistochemistry counts**

Because preliminary indications suggest that the density of myelinated axons is reduced in the CST KO SN, all IHC data were normalized based on myelinated axon density. After normalization of Na\textsubscript{v},1.6 data, the number of Na\textsubscript{v},1.6 clusters remains normal in the CST KO mice at all ages investigated, which supports our previous conclusion that Na\textsubscript{v},1.6 clustering is independent of sulfatide.
The number of gliomedin clusters in the CST KO mice, after normalization of data, is no longer reduced at 15 days, 1 month, or 7 months of age. The normalized gliomedin counts suggest that the apparent reduction in gliomedin clusters seen in the CST KO animals at 1 and 7 months of age may not be a reflection of impaired gliomedin maintenance resulting from the lack of sulfatide. Instead, the original observation that gliomedin clusters are reduced in the CST KO mice at older ages may be due to fewer myelinated axons. Thorough quantitative investigation is ongoing to determine whether the number of myelinated axons is significantly reduced. It is of note that the normalized gliomedin data continues to support the hypothesis that molecular and structural organization may not correspond in the absence of sulfatide, as the SC microvilli are disorganized as early as 15 days of age in the CST KO mice, but the gliomedin clustering may be normal between 15 days and 7 months of age.

After normalization of Nfasc155 data, the number of Nfasc155 clusters is reduced in the CST KO mice at 7 months of age only, suggesting that sulfatide is essential for the maintenance, but not establishment, of Nfasc155 clusters. Unexpectedly, normalization of contactin data resulted in a dissimilar pattern. Preliminary assessment reveals that the number of contactin clusters is decreased in the CST KO mice at 15 days and 7 months of age, but not at 1 month of age. This observation potentially suggests that the delay in contactin clustering lasts longer than that observed with Nfasc155, suggesting that Nfasc155 and contactin clustering do not always correspond. However, the reduction in the number of normalized Nfasc155 clusters seen in the CST KO mice at 15 days of age appears to near significance ($p = 0.058$). This suggests that Nfasc155 clusters may in fact be reduced in the CST KO mice at 15 days of age, though it is not detectable due to the limited sample size of the density counts. Furthermore, it is again unclear if
the deficits in contactin clustering are a secondary effect of disrupted Nfasc155 clustering due to the lack of SC sulfatide, or a direct effect of potentially the lack of neuronal sulfatide. Additional investigation is necessary to clarify these observations.

**Sulfatide is more important for the organization of Na\textsubscript{v}1.6 clusters in the CNS than in the PNS**

With regards to nodal organization in the myelinated axon, sulfatide is differentially important between the CNS and PNS. Ishibashi *et al.* (2002) reported that in the CNS, Na\textsubscript{v} channels cluster properly but break down prematurely by 6 weeks of age in the CST KO mice, thus suggesting that sulfatide contributes to the stabilization of Na\textsubscript{v} clusters at the node. Interestingly, we do not observe a reduction of Na\textsubscript{v} channel clusters in the CST KO PNS between 15 days and 7 months of age. This difference demonstrates that the proper localization of Na\textsubscript{v}1.6 channels is less reliant on sulfatide in the PNS versus the CNS. These observations also show that the compensatory Nfasc186/ankyrin\textsubscript{G}-mediated mechanism involved in neuronal nodal maintenance in the PNS is not reliant on sulfatide. It is important to mention that our data implicate sulfatide as a contributor to maintenance of gliomedin clusters in the PNS. However, because a CNS equivalent of gliomedin has not been discovered, a comparison of the role of sulfatide in properly organizing SC/OL nodal proteins cannot be made.

While the role of sulfatide in nodal formation and maintenance differs between the CNS and PNS, sulfatide appears to play a similar role in organization of the paranode between the two systems. In our study of the PNS, we observe a reduction of Nfasc155 and contactin clusters at the paranode in the CST KO mice, and Ishibashi *et al.* (2002) reported a reduction in Caspr
clustering in the CST KO CNS. It is of great interest though how the paranode differs in its contribution towards nodal organization between the CNS and PNS. In Nfasc KO mice (Sherman et al., 2005), transgenic Nfasc155 expression rescues paranodal organization and nodal assembly in the CNS (Zonta et al., 2008), but recapitulation of the paranodal complex by Nfasc155 expression is not sufficient to rescue the node in the PNS (Sherman et al., 2005). Additionally, establishment of the paranode occurs prior to localization of nodal proteins in the CNS (Rasband et al., 1999; Schafer et al., 2004). These observations together suggest that the paranode plays a greater role in the organization of Na\textsubscript{v} channel clusters, which coincides with greater involvement of sulfatide in Na\textsubscript{v} cluster organization as mediated by the paranode, in the CNS than in the PNS. It is of note that the paranode is not solely responsible for formation and maintenance of the node. In paranodal mutants (Bosio et al., 1996; Coetzee et al., 1996; Bhat et al., 2001; Boyle et al., 2001; Honke et al., 2002; Rios et al., 2003; Sherman et al., 2005; Pillai et al., 2009), Na\textsubscript{v} channels are able to cluster, which demonstrate the existence and importance of paranode-independent mechanisms.

**Preliminary data suggest a role for sulfatide in the initial clustering of nodal and paranodal proteins in the PNS**

Independent of the current study, investigation is currently underway to assess Na\textsubscript{v}1.6, gliomedin, Nfasc155, and contactin clustering in the WT and CST KO mice at 4 days of age; this time point corresponds to the initial clustering of these proteins and can potentially clarify the role of sulfatide during development. Additionally, the density of myelinated axons in the WT and CST KO mice will be determined at this age for similar normalization analyses to be conducted. Based on preliminary qualitative assessment, the number of Na\textsubscript{v}1.6 channels,
gliomedin, Nfasc155, and contactin clusters is reduced in the CST KO mice at 4 days of age (Herman and Dupree, unpublished). These preliminary results suggest that sulfatide plays a role in the proper localization of proteins from different cell origins and protein domains. However, it is possible that the lack of sulfatide is adversely affecting developmental processes involved in the clustering of these protein domains, rather than sulfatide playing a direct role in mediating protein distribution. For instance, a lack of sulfatide may disrupt SC myelination (Ranscht et al., 1987; Owens and Bunge, 1990; Wood et al., 1990), resulting in a delay of SC-neuron contact and/or fewer myelinated axons. Further investigation is necessary in order to elucidate a potentially distinct role of sulfatide in the initial organization of protein domains of the myelinated axon in the PNS.

**Closing remarks**

The present investigation has furthered our existing knowledge regarding the beautifully complex study of PNS myelin biology. We have clarified and also raised additional questions about the role of sulfatide in organization of the domains of the myelinated axon in the PNS. Additionally, we were able to conclude that despite seemingly similar molecular and structural characteristics of aforementioned domains in the PNS and CNS, these are functionally distinct and highly regulated systems. By better understanding the intricate workings of myelin biology can we continue to progress towards the hopeful goal of utilizing our research in clinical applications.
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