Tight junctions potentiate the insulative properties of small CNS myelinated axons

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Claudin family proteins form the physical barriers of tight junctions (TJs) and regulate paracellular diffusion across polarized epithelia. In addition to these heterotypic TJs, claudin 11 forms autotypic TJs comprising the radial component of central nervous system myelin. The exact function of these TJs has been unclear, although their location at the membrane perimeter is well sited to regulate diffusion between the interstitium and intramyelinic space. In this study, we demonstrate that claudin 11 affords rapid nerve conduction principally for small diameter myelinated axons. Claudin 11–null mice have preserved myelin and axonal architecture, but as much as a 60% decrease in conduction. They also have increased action potential thresholds and activated internodal potassium channels. These data indicate that TJs modulate the biophysical properties of myelin. Computational modeling reveals that claudin 11 reduces current flow through myelin and moderates its capacitive charging. Together, our data shed new light on myelin structural components and our understanding of the biology and pathophysiology of this membrane.

Introduction

The ensheathment of neurons with myelin membrane is one of the key advances in vertebrate evolution (for review see Hartline and Colman, 2007) that has enabled rapid saltatory conduction, large reductions in axon diameter, and, ultimately, miniaturization of the central nervous system (CNS). Molecular and cell biology approaches over the last decade have unraveled several of the organizational and structural complexities of myelin sheaths that previously had been only glimpsed from morphological studies (for review see Schnapp and Mugnaini, 1978).

For example, the assembly of paranodal axoglial junctions (appearing as transverse bands in freeze-fracture replicas) requires association of the adhesion protein neurofascin-155 expressed by oligodendrocytes with the axonal caspr-contactin heteromer (Charles et al., 2002). Ablation of these junctions causes profound slowing of nerve conduction but minimally perturbs myelin compaction (Bhat et al., 2001; Boyle et al., 2001; Sherman et al., 2005). Together with evolutionary considerations (for review see Hartline and Colman, 2007), these data have led to the widespread notion that axoglial junctions form electrically tight barriers at paranodes to insulate the internodal axon segment, although the ultrastructure of transverse bands reveals an organization of intramembranous particles that is consistent with a permeable junction (Rosenbluth, 1999).

In contrast to axoglial junctions, the properties and functions of claudin 11 tight junctions (TJs) in CNS myelin are poorly understood. These junctions form the radial component observed in cross sections of CNS sheaths and may occlude the extracellular space at all noncompact myelin membrane surfaces (for review see Schnapp and Mugnaini, 1978; Peters, 1962). Early studies have suggested that TJs contribute to myelin stability or demarcate an immune privileged compartment to sequester myelin proteins (Mugnaini and Schnapp, 1974; Tabira et al., 1978). However, recent data from the lateral wall of the cochlear duct suggest a more traditional role for claudin 11 TJs...
in myelin physiology involving the generation and maintenance of chemical or electrical gradients (Gow et al., 2004; Kitajiri et al., 2004).

A detailed dissection of the physiological properties of myelin has been problematic, and this membrane is still viewed in a rudimentary manner as an amorphous stack of lipid bilayers (for review see Hartline and Colman, 2007; Waxman and Bennett, 1972). Computational models of myelinated fibers also reflect this simplistic view but reasonably account for its biophysical properties as an insulator with high resistance and low capacitance for large diameter fibers common to the peripheral nervous system (PNS). However, such models fall short in accounting for the properties of the small myelinated axons that are widely distributed in the CNS of humans and other mammals (Aboitiz et al., 1992; Rabi et al., 2007). Thus, current models may lack important structural features of CNS myelin sheaths.

In this study, we reveal novel functions and properties of two prominent structural components of murine myelinated fibers, TJs and axoglial junctions. First, an electrophysiological analysis in Claudin 11–null mice reveals severely slowed conduction velocities (CVs) and large juxtaparanodal potassium ion (K+) currents in small diameter fibers. These abnormalities do not arise from disruptions to myelin structure or the partitioning of ion channels in axons but from changes in the biophysical properties of myelin. Second, we demonstrate that peptide toxins gain access to juxtaparanodal voltage-gated potassium channels (Kv) in wild-type and mutant mice, revealing the permeability of axoglial junctions to large molecules. To elaborate on our findings, we developed a novel computational model based on small CNS myelinated fibers (unpublished data). This model incorporates TJs into compact and noncompact myelin and accords with the electrophysiological analysis of Claudin 11–null mice to a greater extent than current models based on double-cable designs (Blight, 1985). Our data are significant in two respects. First, they demonstrate that a major function of claudin 11 TJs in the CNS is to form a series resistance with myelin membrane and impede its capacitive charge. This function has greater impact for small diameter myelinated axons than for large fibers. Second, they indicate that axoglial junctions may not form a permeability barrier at paranodes. Together, these data shed new light on the general principles of saltatory conduction with implications for neural coding in disease states.

Results

Slowed conduction in small optic nerve axons from Claudin 11–null mice

The measurement of compound action potentials (APs [CAPs]) in isolated optic nerves is an effective method for examining the function of CNS white matter tracts (Harroch et al., 2000). In adult rodents, extracellular recordings reveal three CAP components (Fig. 1 A, arrows) that are assumed to derive from large, medium, and small myelinated fibers conducting APs at different velocities (Freeman, 1978). These data are quantitatively similar to published values for adult wild-type mice (Rasband et al., 1999; Devaux et al., 2003).

The CAPs from age-matched Claudin 11–null mice also exhibit three components; however, several abnormalities are apparent (Fig. 1 B). First, latencies of the second and third CAP components are markedly increased with respect to controls, indicating slower nerve conduction in medium and small fibers. A statistical analysis (Fig. 1 C) indicates that CVs for these components are statistically different (P < 0.01) and approach 50% of normal, whereas the first component is minimally
slowed (P > 0.05). Second, recruitment of the second and third components is right shifted between 7 and 20 V, demonstrating increased thresholds for AP generation in small axons (Fig. 1, D–F). Third, amplitudes of the second and third CAP components are diminished in the mutants (P < 0.001), whereas the first component amplitude is unchanged (P > 0.05). These changes are not associated with nerve conduction block or shifts in the refractory period (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1), which are hallmarks of demyelination. Thus, likely causes include slowed CV, temporal dispersion, or hyperpolarizing afterpotentials (Fig. 1 B, arrowhead).

**K⁺ channels are activated in Claudin 11-null mice**

Hyperpolarizing afterpotentials in the CAPs from mutant mice are strongly dependent on extracellular K⁺ (Fig. 2), demonstrating that they are mediated by a K⁺ conductance. Artificial cerebrospinal fluid (ACSF) used in the bath medium normally contains 3 mM KCl, and representative examples of the CAPs from wild-type (Fig. 2 A) and Claudin 11–null (Fig. 2 B) mice are shown. Decreasing extracellular KCl to 1 mM exacerbates hyperpolarization in the mutant optic nerves. Conversely, increasing the K⁺ to 6 or 12 mM reduces or eliminates the afterpotentials. Changing extracellular K⁺ has little effect on the wild-type CAPs (except at 12 mM K⁺), and, together, these data suggest that K⁺ channel activity is increased in the mutant mice in association with the absence of myelin TJ.

To identify the K⁺ conductance, we examined the effect on the CAPs of two channel-blocking agents. The Kᵥ channel blocker dendrotoxin-1 (DTX) is a specific inhibitor of the juxtaparanodal channels Kᵥ1.1 and Kᵥ1.2 and does not perturb CAP latencies or shape in wild-type mice (Fig. 3 A, top). In contrast, DTX significantly affects Claudin 11–null optic nerves by increasing the amplitude of the third component and eliminating the afterpotentials (Fig. 3 A, bottom). These data demonstrate that Kᵥ1.1 and Kᵥ1.2 channels generate the hyperpolarizing afterpotentials in the absence of TJs.

Importantly, our data do not imply that TJs impede access of peptide neurotoxins to juxtaparanodal K⁺ channels in wild-type fibers. Indeed, these channels reside in the axonal membrane and are sequestered underneath the myelin behind axoglial junctions, which are thought to serve as permeability barriers to the periaxonal space. However, the fluorescent conjugate 6-FAM-AEEAc–Stichodactyla helianthus toxin (SHK), which blocks Kᵥ1.1 and Kᵥ1.2 channels, readily diffuses through these junctions and binds to its targets in explants of adult wild-type mouse optic nerve (Fig. 3 B), Claudin 11–null optic nerve (not depicted), and rat spinal cord (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1). Thus, in contrast to published studies (for review see Hartline and Colman, 2007) suggesting that axoglial junctions serve as permeability barriers for the periaxonal space, our data show they are permeable to peptide toxins.

The broad spectrum membrane-permeant K⁺ channel blocker 4-aminopyridine (4-AP) strongly increases amplitudes of the second and third components of the CAPs from Claudin 11–null mice (Fig. 3 A, bottom), which is consistent with the DTX data. Similar to earlier experiments (Devaux et al., 2003), 4-AP also broadens the CAPs in wild-type mice, indicating that Kᵥ channels, possibly in the internode, are normally activated by APs in small fibers. The DTX and 4-AP inhibitors exert negligible effects on the latencies of CAP components (Fig. 3 C), indicating that Kᵥ channel activity in the mutants does not directly cause conduction slowing. Furthermore, Western blots of Kᵥ1.1 and Kᵥ1.2 in the optic nerve show that Kᵥ channels are expressed at normal levels in Claudin 11–null mice (Fig. 3 D).

It is unlikely that the 4-AP response stems from inhibiting nodal channels such as Kᵥ3.1 because of their low abundance in small CNS myelinated fibers (Devaux et al., 2003). Rather, the large effects of 4-AP (compared with DTX) on the Kᵥ1.1 and Kᵥ1.2 channels may stem from the small size and membrane-permeant properties of this inhibitor, which give it access to paranodal and internodal channels. Diffusion of DTX is more limited, and it would be unlikely to have significant access to internodal channels. However, we cannot preclude the possibility of other novel Kᵥ channels in the nodes that are blocked by 4-AP.
The ultrastructure of optic nerve myelinated axons is preserved in adult Claudin 11-null mice (Fig. 5, A–D). Transverse sections of ferrocyanide-treated wild-type optic nerves show the locations of radial TJs in most myelinated fibers (Fig. 5A, white arrowheads and arrows). These structures are absent in Claudin 11-null mice (Fig. 5B); nonetheless, compact myelin morphology, axon shape, and axoplasmic composition are indistinguishable from controls. In longitudinal optic nerve sections (Fig. 5, C and D), paranodes of wild-type and mutant mice are morphologically similar, particularly with regard to axoglial junctions (Fig. 5, C and D; black arrowheads). We also find little evidence of overall changes in g ratios from the mutant nerves (Fig. 5E). A frequency histogram of g ratios (Fig. 5F and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1) reveals a small population of fibers with thicker than normal myelin in Claudin 11-null mice (lower g ratios); however, such changes are unlikely to decrease CV and cannot account for the electrophysiological changes we observe.

The frequency distribution of axon diameters from mutant optic nerves is also very similar to controls (Fig. 5G), indicating that CNS axons are not significantly perturbed by the absence of TJs.

Small myelinated fibers are affected by the absence of Claudin 11 TJs

Our optic nerve CAP data reveal an important role for TJs predominantly in small caliber fibers. This is corroborated by recordings from ventral column explants of spinal cord, which
A novel computational model of optic nerve fibers incorporating TJs

To explore this hypothesis, we developed a computational TJ model (TJM) of CNS myelinated axons (unpublished data). This model is reminiscent of earlier double-cable model (DCM) designs that have been used to describe large fibers in the PNS and CNS (Halter and Clark, 1991; McIntyre et al., 2002), but it incorporates physical dimensions derived from optic nerve axons, experimentally determined conductances, and a TJ resistance in series with the axolemmal and myelin cables (see Materials and methods). The TJM is predicated on considering the myelin sheath as a modified polarized epithelium and satisfies five criteria that have been established empirically from electrophysiological and morphological data (Figs. 1–5): TJs enable conduction in axons of ∼0.5-μm diameter, TJs increase CV, TJs decrease AP threshold, TJs decrease juxtaparanodal K+ channel activity, and TJ function is substantially more important for small axons than for larger axons. The suitability of the TJM in fulfilling these criteria is demonstrated in Fig. 6 in a head to head comparison with simulations from a DCM based on previous models (Halter and Clark, 1991; McIntyre et al., 2002) but with physical dimensions and passive properties identical to the TJM (Fig. 6).

Slower CVs in small fibers in the absence of TJs

To derive physiological data from the optic nerve CAPs for comparison with the computational models, we estimated the mean axonal diameter corresponding to each of the CAP components (Fig. 1, A and B) using the axon diameter frequency distributions in Fig. 5 G. Thus, for both wild-type and Claudin 11–null mice (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1), the first CAP component corresponds to an axon diameter of ∼2.1 μm, the second component to 1.2 μm, and the third to 0.7 μm. The mean CVs for each of these components (Fig. 1 C) is expressed as a ratio of Claudin 11 null/wild type (CV+/CV−) and plotted as a function of axon diameter. These data highlight the strong reduction in CVs in the absence of TJs, particularly for small axons (Fig. 6 A, closed circles). Thus, CV in 0.7-μm axons is slowed by >50%, whereas the reduction is ∼20% for 2.1-μm axons.

Conduction slowing in TJM simulations for axons of 0.6–2.1-μm diameter (Fig. 6 A, open circles) closely fits the physiological data. DCM simulations also propagate APs over this range of axon diameters, and to determine whether the TJM data could arise from a simple decrease in myelin resistance, we reduced ρmy in the DCM by 30 (DCMα) or 300% (DCMb). In both cases, the DCM data poorly fit the physiological data. In the latter case, axons <0.9 μm in diameter also do not propagate APs. However, increasing myelin resistance by 300% (DCMc; unpublished data) does not improve the fit, and the data are very similar to DCMα.

Increased AP thresholds in small fibers in the absence of TJs

Recruitment of myelinated fibers also can be modeled using the TJM and DCM. In simulations, we define recruitment as the...
Increased juxtaparanodal $K^+$ currents in small fibers in the absence of TJs

The electrophysiology data in Figs. 2 and 3 indicate that $K_v$ channels, most likely juxtaparanodal, are strongly activated in the absence of TJs. To determine whether the TJM can model these data, we measured the juxtaparanodal $K^+$ currents generated from propagating APs and plotted them as a function of axon diameter (Fig. 6 C). In the absence of TJs, the peak $K^+$ currents increase sharply as axon diameter decreases. TJs strongly suppress this activity in axons of $\sim 0.7-0.9 \mu m$ diameter, but their effectiveness is relatively modest in smaller fibers. Furthermore, TJs have little influence over $K^+$ currents in axons $> 0.9 \mu m$, presumably because myelin thickness alone is sufficiently
Together, the data in Fig. 6 demonstrate that the TJM exhibits behavior that is similar in several respects to the electrophysiology data from Claudin 11–null optic nerves and superior to the DCM in most respects. The TJM satisfies five criteria: conduction in small fibers, the potentiating effect of TJs on CV, the reduction of AP thresholds by TJs, the suppression of juxtaparanodal K⁺ currents by TJs, and the axon diameter-dependent behavior with stronger effects in small fibers.

Importance of internodal K⁺ channels in minimizing depolarizing afterpotentials

The electrophysiology data in Fig. 3 demonstrate the importance of K⁺ channels in shaping the late phase of APs by minimizing depolarizing afterpotentials in small diameter fibers (Fig. 3 A). The TJM simulations reproduce several aspects of the electrophysiology in Fig. 3. They demonstrate that K⁺ channels underlie the myelin are normally active in small diameter axons irrespective of the presence or absence of TJs, TJs diminish K⁺ channel activity in intermediate axons, and K⁺ conductances have minimal influence on CV.
The mild phenotype of Claudin 11–null mice, which have a normal life span, is distinct from that of mice lacking axoglial junctions and is associated with a fine tremor and hind limb weakness. Currently, it is unclear to what extent these abnormalities are directly attributable to changes in the biophysical properties of myelin rather than to the absence of claudin 11 in other tissues (Gow et al., 1999); however, conceivably, they arise directly from slowed conduction in small myelinated axons.
of cerebellar Purkinje neurons and upper motor neurons of the corticospinal tract.

In contrast, the absence of axoglial junctions reduces or blocks conduction in all myelinated axons, resulting in severe CNS and PNS dysfunction and early death. Structural defects include the mislocalization of juxtaparanodal K\(^+\) channels to paranodes and nodes, the emergence of large K\(^+\) currents, and significant increases in the width of the periaxonal space as paranodal myelin loops become everted with respect to the axon (Coetzee et al., 1996; Bhat et al., 2001; Boyle et al., 2001; Sherman et al., 2005). Myelin TJMs remain intact in at least some of these mutants (Zonta et al., 2008), indicating that the observed conduction abnormalities stem from the axoglial junction defects.

From an evolutionary perspective, axoglial junctions (more generally known as septatelike junctions) and TJs have long been co-opted in the nervous system to serve as diffusion and possibly electrically tight barriers. For example, an antecedent of the caspr–neurexin IV–containing mammalian axoglial junction, the neurexin IV–containing septate junction of the Drosophila melanogaster blood–nerve barrier, is known to be impermeable to monovalent cations if not electrically tight (Baumgartner et al., 1996). This barrier is also dependent on the presence of the claudin family member sinuous for its structural organization and barrier properties (Wu et al., 2004); thus, it is unclear which pleated septate junction component confers barrier function.

Together, our data lead us to conclude that claudin 11 TJs form an electrically tight barrier in myelin, as in the cochlea (Gow et al., 2004; Kitajiri et al., 2004), and they complement the myelin membrane in preventing internodal depolarization. The model in Fig. 8 illustrates our view that axoglial junctions are not the only junctions involved in insulating the axon (for review see Hartline and Colman, 2007). Our finding that peptides are able to rapidly diffuse through axoglial junctions demonstrates that they do not form permeability barriers, but we do not know whether they are electrically tight. This issue not withstanding, our TJM simulations do indicate that myelin TJMs function differently and independently of axoglial junctions (Fig. S4, B and C).

**Implications for myelin TJs in remyelination**

In this study, we have focused on the importance of claudin 11 for small myelinated axons in the adult nervous system. However, TJ function may be also relevant for large axons under circumstances in which myelin is thin, such as during myelin deposition in development or for remyelinated axons denuded during neurodegenerative disease. Although the former instance is transient, the latter is permanent and can have major consequences for CNS function.

For example, the pathophysiology of multiple sclerosis (MS) involves focal demyelination in white and gray matter regions of the CNS. The majority of these lesions are subsequently repaired by remyelination of denuded axonal segments, but repeated demyelination/rymyelination cycles over many years may eventually result in the replacement of most myelin sheaths (Albert et al., 2007). Importantly, these secondary myelin sheaths rarely achieve the normal length and thickness of those generated during development, and remyelinated...
cortical axons in MS patients may only reach 60–70% of normal thickness. In this light, an axon of 2.1-μm diameter en- sheathed with 15 membrane wraps (the first component in Fig. 1) may achieve a myelin thickness of approximately nine wraps after remyelination (equivalent to the myelin thickness of the second component in Fig. 1). Thus, the range of axon diameters for which TJs are important for saltatory conduction is greater for demyelinating pathologies.

Implications for claudin 11 loss of function in other neurological diseases
Morphometric studies show that small diameter myelinated axons are widely distributed in the CNS of mammals, including humans (Aboitiz et al., 1992; Rabi et al., 2007). As summarized in Fig. S5 (available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1), 45–70% of myelinated axons in human corpus callosum are <1 μm in diameter, and we envisage that the absence of claudin 11 TJs would significantly lower CVs of most fibers in this white matter tract. Corpus callosum is the major conduit for interhemispheric communication, and small myelinated axons dominating the fiber population in anterior regions (e.g., the genu) mainly connect the prefrontal cortices, whereas those in posterior regions (anterior two-thirds of the splenium) connect the associative cortices (Hofer and Frahm, 2006).

The approximate length of collostral fibers in humans is 100–130 mm, and the transit time for APs through small diameter axons is 50–100 ms. A 40–60% reduction in CV in the absence of claudin 11 TJs would increase AP latencies by at least 20 ms, thereby perturbing information processing and integration in higher order cortical circuits with disastrous consequences for Hebbian long-term potentiation (Caporale and Dan, 2008). Thus, a CLAUDIN 11 hypomorphic phenotype conceivably could include altered executive brain function, associative behavior, learning, and memory.

In this vein, several microarray studies show reduced expression of CLAUDIN 11 and several other myelin-specific genes in cingulate and temporal cortices and the hippocampus in postmortem tissue from schizophrenia patients (Katsel et al., 2005; McCullumsmith et al., 2007). It is tempting to speculate that the disturbances in perception that feature in the clinical phenotype of schizophrenia may be in part associated with the distribution of small diameter myelinated fibers in the CNS. Interestingly, schizophrenia endophenotypes also are manifest in several demyelinating disorders, including metachromatic leukodystrophy, X-linked adrenoleukodystrophy, and MS, for which multifocal or diffuse myelin pathophysiology is known (Stewart and Davis, 2004). The convergent pathology in these diseases is thinner than normal myelin, which would reduce membrane resistance, increase capacitive charging of the myelin, and reduce CVs, perhaps analogously to the absence of claudin 11 TJs in Claudin 11−null mice. Thus, our analyses suggest a plausible link between congenital or acquired TJ dysfunction and several neurological disorders.

TJs in PNS myelin
The satisfying account of claudin 11 function in central myelinated fibers in this study has important implications for peripheral myelin. Schwann cells in the PNS localize claudin 19 TJs between paranodal loops, inner and outer mesaxons, and Schmidt-Lantermann incisures (Miyamoto et al., 2005). These sites are analogous to the locations of TJs in the CNS where the extracellular space around the entire perimeter of the myelin sheath is occluded. However, axons of <1-μm diameter that are most dependent on TJs in the CNS are rarely myelinated in the PNS (Waxman and Bennett, 1972); thus, it seems unlikely that PNS and CNS TJs function analogously. Indeed, several mis- sense mutations identified in the human CLAUDIN 19 gene are associated with renal failure and polycystic kidney disease but not with peripheral neuropathy (Konrad et al., 2006; Lee et al., 2006). Furthermore, nerve conduction measurements in sciatic nerves from Claudin 19−null mice (Miyamoto et al., 2005) fail to convincingly demonstrate slower than normal CVs.

Materials and methods

Animals
Claudin 11−null mice were generated as previously described (Gow et al., 1999) and maintained on an interbred 129 Sv/Ev-C57BL/6Tac background at Wayne State University facilities. These mice and wild-type littersmates were used for experiment in 4–6 mo of age. Animals were handled and killed according to Wayne State University and University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

Electrophysiology

Ventral columns from spinal cord were also recorded as described previously (Shi and Blight, 1996). In brief, animals were anesthetized with ketamine xylazine and perfused transcardially with oxygenated ice-cold ACSF containing 126 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgSO₄, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM dextrose, pH 7.4–7.5, at 4°C equilibrated with 95% O₂/5% CO₂ (flow rate = 1–2 ml/min). The vertebral column was rapidly excised and transferred into oxygenated ice-cold ACSF, and the ventral white matter tracts were isolated with scissors (3-cm segment). After 60 min, ventral white matter tracts were placed in a triple-compartment recording chamber. The central compartment was perfused with ACSF (at 1–2 ml/min) equilibrated with 95% O₂/5% CO₂ at 35°C. The outer compartments were filled with ACSF containing 120 mM isotonic KCl and were isolated from the central compartment by a sucrose gap (isotonic sucrose solution perfused at 1 ml/min) and sealed with Vaseline. Electrodes were placed in the central and outer compartments to trigger and record CAPs. Optic nerves were quickly dis- sected from killed mice and transferred into ice-cold ACSF equilibrated with 95% O₂/5% CO₂ (flow rate = 1–2 ml/min). Optic nerves were al- lowed to equilibrate for 60 min in the chamber before electrophysiological measurement at 35°C, and suction electrodes were used to record CAPs (Devaux et al., 2002).

During experiments, white matter tracts were stimulated continu- ously with square wave pulses of 40-μs duration up to 80 V at a frequency of 0.25 Hz. Recorded signals were amplified, digitized at 500 kHz, and stored on a hard disk. Drugs were applied to the central compartment, and electrophysiological measurements were recorded after the effects of the drugs reached steady state, typically 30–60 min after application.

DTX was purchased from Alomone Laboratories, and 4-AP was purchased from Sigma-Aldrich. For recruitment analysis, CAP amplitudes were mea- sured and plotted as functions of stimulation intensity. Maximal amplitudes from Sigma-Aldrich. For recruitment analysis, CAP amplitudes were mea- sured and plotted as functions of stimulation intensity. Maximal amplitudes were measured and plotted as functions of the delay interval between the two stimuli.

EM
Mice were anesthetized with avertin and perfused with 0.9% NaCl followed by freshly prepared 2% PFA/0.5% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, for 1 h. Optic nerves were kept overnight

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in situ at 4°C and dissected, rinsed, and postfixed in 1% osmium in 0.15 M cacodylate buffer, pH 7.4. Alternately, 1.5% ferrocyanide was included with the osmium treatment to visualize the myelin TJs. Optic nerves were dehydrated with an ascending series of ethanol and embedded in epon. Thin sections were cut, stained with filtered lead citrate and uranyl acetate, and photographed at 8,000 g using a microscope (EM 1010; JOEL Ltd). Images in Fig. 5 have been contrast enhanced and sharpened using the unsharp mask tool (radius of 3.3 pixels) in Photoshop (CS2; Adobe).

Axon circumference and myelin thickness were measured from wild-type and Claudin 11–null littermate mice (three mice per genotype, 100 myelinated fibers per mouse) using the advanced measurements module in OpenLab (PerkinElmer), and g ratios were calculated (g ratio = axon diameter/fiber diameter).

**Immunofluorescence**

Mice were perfused with 4% PFA in 0.1 M sodium phosphate buffer, pH 7.2. Optic nerves and cervical spinal cords were dissected, cryoprotected, and embedded for cryostat sectioning. Cryostat sections are permeabilized with methanol for 10 min and blocked in 2% goat serum albumin and 0.1% gelatin.

The following overnight primary antibodies were used: guinea pig anti-caspar (1:500; Bhat et al., 2001), mouse anti-Na1.2 (1:250; Rasband et al., 1999), rabbit anti-Na1.6 (1:400; Rasband et al., 1999), rabbit anticonnectin (1:500; Rios et al., 2000), rabbit anti-K1.1 (1:60; Alomone Laboratories), rabbit anti-K1.2 (1:60; Alomone Laboratories), rabbit anti–neurofascin-186 (1:500; Southwood et al., 2004), and rabbit anti–neurofascin-155 (1:500; Southwood et al., 2004). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Invitrogen, and SouthernBiotech. Sections were visualized using a microscope (DMRA2; Leica). Photographs were captured through 100x 1.4 NA and 1.6x intermediate lenses using a digital camera (ER; Orca) driven by Openlab software imported into Photoshop (CS2) as 16-bit grayscale files, combined, and the contrast was adjusted. Images were converted to 24-bit RGB and cropped.

**6-FAM-SHK labeling**

Freshly dissected optic nerves (unfixed) were incubated for 1 h into oxygenated ACSF maintained at 35°C and containing 500 nM 6-FAM-SHK (Bachem; Beeton et al., 2003). Optic nerves were rinsed three times in ACSF and quickly frozen in optimal cutting temperature, and 5–10 μm cryostat sections were cut, thaw mounted on glass slides, and cover slipped. Unfixed nonpermeabilized spinal cord sections were also labeled with 0.05–500 nM 6-FAM-SHK for 1 h in TBS containing 5% nonfat milk. Sections were mounted using a microscope (DMRA2) with a 63x 1.3 NA lens, and images were captured using a camera (Hamamatsu Photonics) driven by Openlab (version 2.2). Images were contrast enhanced using Photoshop (CS2).

**Western blotting**

Optic nerves from wild-type and Claudin 11–null mice were dissected into small pieces and dissolved in 5% SDS, 0.5% β-mercaptoethanol, 0.125 M Tris, pH 6.8, 20% sucrose, and pronaseol and heated to 90°C for 1 min. The insoluble material was removed by centrifugation at 10,000 rpm for 10 min. Protein concentrations were determined using a Bio-Rad Laboratories kit. Samples (100 μg of protein) were separated in a 10% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk in PBS for 1 h, the membranes were incubated with rabbit antibodies against K1.1 or K1.2 (1:1,000) at 4°C overnight, rinsed several times, and incubated with horseradish peroxidase–conjugated donkey anti–rabbit secondary antibody (1:5,000; Jackson Immuno-Research Laboratories). Finally, the membranes were washed several times, and the signal was developed using ECL Plus (GE Healthcare) and their densities were derived from published studies (Frankenhaeuser and Huxley, 1964; Schwarz and Eikhof, 1987; Halter and Clark, 1991). The resting potential is set to 75 mV, and the temperature is 37°C. Integrations of differential algebraic equations are solved for variable time steps (absolute tolerance = 0.001) using the differential algebraic solver with preconditioned Krylov incorporated into the Neuron simulation environment.

**CVs**

CVs were derived from the latencies of APs propagated between 16 simulated myelin sheaths divided by the aggregate length of those sheaths. Current thresholds used in Fig. 6 B were derived iteratively by injecting square current pulses of varied amplitudes (100 μs) into node of Ranvier #9. The minimum current required to elicit an AP was defined as the threshold current, I. Peak juxtaparanodal K+ currents elicited by a propagating AP (Fig. 6 C) were measured just distal to the paranode–juxtaparanode boundary at the proximal end of myelin sheath #5.

**Statistical analyses**

Data in this study are presented as means and standard deviations. Analysis of the CAP latencies was performed using an analysis of variance with Bonferroni posttests. Comparisons between other groups were performed using a Student’s t test assuming two-tailed distributions and unequal variances. Differences are considered statistically significant at the P < 0.05 level.

**Online supplemental material**

Fig. S1 demonstrates that the Claudin 11–null phenotype is not a result of conduction block in the CNS. Fig. S2 shows that fluorescently labeled 6-FAM-SHK passes through the axoglial junctions in rat spinal cord myelinated fibers. Fig. S3 shows that CAPs in Claudin 11–null ventral spinal cord are only modestly slowed compared with controls and only in small fibers. In addition, the recruitment and refractory periods are normal in the absence of TJs. Fig. S4 shows the analysis of axon diameter in the optic nerve of wild-type and Claudin 11–null mice for use in TJM and DCM simulations. In addition, TJM simulations reveal the dependence of CV in the absence of TJs as functions of axon diameter or resistance of axoglial junctions. Fig. S5 shows a morphometric analysis of the myelinated axon diameter distribution in human corpus callosum. Table S1 shows percentiles for the g ratio and axon diameter morphometric analyses in optic nerves from wild-type and Claudin 11–null mice. Table S2 shows quantitative characteristics of CAPs from spinal cord ventral column vectors of wild-type and Claudin 11–null mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200808034/DC1.

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References


Figure S1. **Absence of conduction block in Claudin 11-null mice.** (A) Mean CAP area (n = 6) from wild-type (+/+) and Claudin 11-null mice (−/−) recorded as a function of temperature (35–25°C) and expressed as a percentage of the CAP area at 35°C. No differences between wild-type and mutant optic nerves are observed (two-tailed unpaired t-test; P < 0.05). Lowering the bath temperature does not significantly improve CAP amplitudes or areas in mutant nerves, thereby ruling out any possibility that the diminished CAP amplitude is related to conduction block in the mutants. (B–D) The refractory period in Claudin 11-null optic nerves is normal, indicating that AP duration and repolarization are not affected by the absence of TJs. The supranormal period in the third component from Claudin 11-null mice corresponds temporally to the hyperpolarizing afterpotentials observed in Fig. 1 B. Error bars indicate SD.
Figure S2. **Fluorescent peptide toxins bind to juxtaparanodal K⁺ channels.** 10-µm cryostat sections of unfixed nonpermeabilized rat spinal cord are incubated in 50 pM–50 nM 6-FAM-SHK in PBS. At these concentrations, the toxin only labels juxtaparanodes and is optimal at 50 nM. Above 50 nM, some background staining is apparent. Nodes of Ranvier are not labeled. Spcd, spinal cord.
Nerve conduction in spinal ventral column is moderately affected in Claudin 11–null mice. (A and B) Recordings from spinal cord ventral columns in wild-type (A) and Claudin 11–null mice (B) treated with DTX and 4-AP as indicated. Although the CAP delays and amplitudes are similar for both genotypes, the duration of the CAPs is significantly longer in Claudin 11–null mice, which is indicative of conduction slowing in small axons. DTX has no effect on either wild-type or mutant animals. 4-AP broadens the CAPs from wild-type and null spinal cords similarly. No hyperpolarizing after-potentials are detected. Gray shading indicates CAPs from untreated spinal cords for comparison with drug treatments (n = 7; two-tailed nonpaired t test; P < 0.05). (C) Normal recruitment period in spinal cord ventral columns from Claudin 11–null mice. Recruitment of APs to the CAPs from wild-type (+/+) and Claudin 11–null mice (−/−) are not significantly different (n = 7; one-tailed nonpaired t test; P < 0.05). (D) Normal recruitment and refractory period in spinal cord ventral columns from Claudin 11–null mice. AP refractory period from wild-type (+/+) and Claudin 11–null mice (−/−) are not significantly different (n = 7; one-tailed nonpaired t test; P < 0.05). Error bars indicate SD.
Figure S4. **Estimation of mean axon diameters corresponding to each CAP component in optic nerve.** TJs and axoglial junctions (AJ) serve different and independent functions in CNS myelinated axons. (A) To derive physiological data from the electrophysiological analysis of optic nerves in Claudin 11-null mice for comparison with the TJM and the DCM, we first determined the areas under the curve for each of the three components in the CAPs (Fig. 1, A and B). These areas approximate the proportions of the CAPs contributed by axons of different diameters in the optic nerves. Next, we generated cumulative frequency histograms from the optic nerve frequency histograms in Fig. 5 G and used the peak areas from the CAPs to calculate mean axon diameters for each component. (B) The ratio of CVs in the absence and presence of TJs is shown as a function of axon diameter. The solid curve is derived from Fig. 6 A (open circles) and represents TJM simulations using an axoglial junction resistance equivalent to twice that of the periaxonal space (i.e., based on 50% occupancy of the periaxonal space by the protein complexes forming the axoglial junctions). The dashed line represents TJM simulations when axoglial junction resistance is 100-fold higher than the periaxonal space (axoglial junctions are effectively electrically tight). These simulations demonstrate that the loss of TJs results in diminished CV independently of the electrical barrier properties of axoglial junctions, particularly in small axons. (C) The relative changes in CV from different fibers as a function of the resistance of axoglial junctions. These data are normalized to the CV for an axoglial junction that is equal to the resistance of the periaxonal space (i.e., no axoglial junctions). For axons of all diameters, CV increases significantly as axoglial junction resistance increases. These data are consistent with experimental data from axoglial junction mutant mice in which CV is reduced or blocked in all axons. These data reveal a major difference with TJs, which only significantly affect small fibers.
Figure S5. Predominance of small myelinated axons in human corpus callosum. Schematic of human corpus callosum in the sagittal plane showing division into five regions (Aboitiz, F., A.B. Scheibel, R.S. Fisher, and E. Zaidel. 1992. *Brain Res.* 598:143–153). In contemporary literature (Hofer, S., and J. Frahm. 2006. *Neuroimage.* 32:989–994), these regions approximately correspond to the Genu, which comprises fibers connecting prefrontal cortices of the left and right hemispheres; the anterior body (Ant.), which connects premotor and supplemental motor cortices; the midbody (Mid), which connects primary motor cortices; the posterior body (Post.), which connects primary sensory cortices; and Splenium, which connects higher order processing areas of the parietal and temporal lobes (anterior two thirds) and visual cortex (posterior one third). The graph details morphometry from electron micrographs of human corpus callosum adapted from Fig. 4 in Aboitiz et al. (Aboitiz, F., A.B. Scheibel, R.S. Fisher, and E. Zaidel. 1992. *Brain Res.* 598:143–153). The frequency distribution of small, medium, and large diameter axons is shown for each of the five regions of corpus callosum.

Table S1. Morphometric analysis in optic nerves from wild-type and Claudin 11-null mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>g ratio</th>
<th>Axon diameter</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>++/−</td>
<td>+/−</td>
<td>−/−</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.75</td>
<td>0.42 µm</td>
<td>0.44 µm</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.81</td>
<td>0.73 µm</td>
<td>0.75 µm</td>
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<tr>
<td>Median</td>
<td>0.83</td>
<td>0.96 µm</td>
<td>0.94 µm</td>
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<tr>
<td>75th percentile</td>
<td>0.85</td>
<td>1.26 µm</td>
<td>1.23 µm</td>
</tr>
<tr>
<td>Maximum</td>
<td>0.91</td>
<td>2.81 µm</td>
<td>3.31 µm</td>
</tr>
<tr>
<td>n</td>
<td>328</td>
<td>328</td>
<td>325</td>
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</tbody>
</table>

Table S2. Characteristics of CAPs recorded from spinal cord ventral columns of wild-type and Claudin 11-null mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>+/−</th>
<th>−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude V_{max} (mV)</td>
<td>2.95 ± 1.7</td>
<td>3.06 ± 1.29</td>
</tr>
<tr>
<td>Delay V_{max} (ms)</td>
<td>0.48 ± 0.06</td>
<td>0.55 ± 0.15</td>
</tr>
<tr>
<td>Delay V_{1/2} (ms)</td>
<td>0.31 ± 0.05</td>
<td>0.35 ± 0.08</td>
</tr>
<tr>
<td>Duration V_{1/2} (ms)</td>
<td>0.6 ± 0.06</td>
<td>1.02 ± 0.35a</td>
</tr>
<tr>
<td>Conduction velocity V_{1/2} (ms⁻¹)</td>
<td>23.3 ± 3.7</td>
<td>21.3 ± 5.2</td>
</tr>
<tr>
<td>Conduction velocity V_{max} (ms⁻¹)</td>
<td>14.8 ± 1.8</td>
<td>13.5 ± 3.5</td>
</tr>
<tr>
<td>Conduction velocity V_{fall} (ms⁻¹)</td>
<td>7.8 ± 0.8</td>
<td>5.6 ± 1.9a</td>
</tr>
<tr>
<td>Area</td>
<td>2,556 ± 1,054</td>
<td>3,817 ± 1,152</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

*Significantly different from controls (two-tailed nonpaired t test; P < 0.05).