Myelin Loss and Axonal Ion Channel Adaptations Associated with Gray Matter Neuronal Hyperexcitability

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Myelination and voltage-gated ion channel clustering at the nodes of Ranvier are essential for the rapid saltatory conduction of action potentials. Whether myelination influences the structural organization of the axon initial segment (AIS) and action potential initiation is poorly understood. Using the cuprizone mouse model, we combined electrophysiological recordings with immunofluorescence of the voltage-gated Nav1.6 and Kv7.3 subunits and anchoring proteins to analyze the functional and structural properties of single demyelinated neocortical L5 axons. Whole-cell recordings demonstrated that neurons with demyelinated axons were intrinsically more excitable, characterized by increased spontaneous suprathreshold depolarizations as well as antidromically propagating action potentials ectopically generated in distal parts of the axon. Immunofluorescence examination of demyelinated axons showed that BIV-spectrin, Nav1.6, and the Kv7.3 channels in nodes of Ranvier either dissolved or extended into the paranodal domains. In contrast, while the AIS in demyelinated axons started more closely to the soma, ankyrin G, BIV-spectrin, and the ion channel expression were maintained. Structure–function analysis and computational modeling, constrained by the AIS location and realistic dendritic and axonal morphologies, confirmed that a more proximal onset of the AIS slightly reduced the efficacy of action potential generation, suggesting a compensatory role. These results suggest that oligodendroglial myelination is not only important for maximizing conduction velocity, but also for limiting hyperexcitability of pyramidal neurons.

Key words: axon; axon initial segment; demyelination; Kv7.3; Nav1.6; node of Ranvier

Introduction

The development and maintenance of voltage-gated ion channel clustering to nodes of Ranvier depend on multiple and complex interactions between neurons and glia cells, including clustering of NF186 by glia-derived extracellular matrix complexes, paranodal axoglial barriers, and stabilization of ion channel proteins by cytoskeletal scaffolds (Dzhashiashvili et al., 2007; Susuki et al., 2013). As a consequence, with the lack of oligodendroglial signals, such as in the neuroinflammatory disorder multiple sclerosis (MS) or in experimental animal models of demyelination, it is well established that nodal and juxtaparanodal voltage-gated ion channels diffuse laterally and can extend into the formerly myelinated internodal regions (Foster et al., 1980; Craner et al., 2004; Waxman et al., 2004). It is thought that these voltage-gated ion channel reorganizations compensate, in part, for the loss of saltatory conduction and slowing of action potential (AP) propagation in white matter axons (Bostock and Sears, 1978; Felts et al., 1997). Whether myelin loss affects the intrinsic excitability of cortical gray matter neuronal circuits, where large fractions of axons in the deeper layers are myelinated (Tomassy et al., 2014), remains poorly understood.

A major hallmark of gray matter axons is the axon initial segment (AIS), a ~40-μm-long excitable region responsible for the initiation of the fast sodium ion-mediated AP at the onset of axons (Rasband, 2010; Kole and Stuart, 2012). AISs share many of the same ion channel proteins and assembly mechanisms of the nodes of Ranvier, including ankyrin G, voltage-gated Nav and Kv channels, and cell adhesion molecules, such as neurofascin (Rasband, 2010). In contrast to nodes, the AIS develops independently of myelinating oligodendrocytes and its length is restricted by the position of submembranous intra-axonal boundaries (Mathis et al., 2001; Dzhashiashvili et al., 2007; Ogawa and Rasband, 2008; Galiano et al., 2012). To date, experimental demyelination studies have primarily focused on white matter axonal tracts and the role of myelin in the nodal organization, but the impact on the AIS is not understood.

To test the role of myelination in the structure and function of the AIS, we used the cuprizone model for demyelination (Kipp et al., 2009) and examined the myelinated axons of thick-tufted L5 pyramidal neurons in the somatosensory cortex. Demyelination relocated the position of the AIS toward the soma and reduced Nav1.6 channels but was also associated with increased Kv7.3 expression into the internodes, and AP initiation was only slightly

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impaired. Notably, denmyelination facilitated the initiation of ectopic APs antidromically propagating into the AIS and somatodendritic domain. These results suggest that oligodendroglial myelination is not only important for maximizing conduction velocity, but also for spatiotemporally confining AP generation to the AIS and limiting hyperexcitability of cortical pyramidal neurons.

Materials and Methods

Animals. Male C57BL/6 mice (Harlan) at 6 weeks of age (20 g) were fed ad libitum 0.2 or 0.3% (w/w) cuprizone (biscyclohexane oxaldihydrazone, Sigma-Aldrich Chemie) milled into mouse chow for over 9 weeks to induce acute and chronic demyelination, respectively. The body weight loss of cuprizone-treated mice was 20 ± 2.3% (n = 89) and 25 ± 2.0% (n = 39) with 0.2 and 0.3% cuprizone, respectively. Control mice received either powder or normal chow food. All experiments were done in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the Royal Netherlands Academy of Arts and Sciences animal welfare and ethical guidelines and protocols (DEC NIN 11.70).

Electrophysiological recording. At the end of the cuprizone feeding, mice were decapitated under deep isoflurane anesthesia (3%) and the brain was quickly removed from the skull and placed into ice-cold slicing artificial CSF (aCSF) of the following composition (in mM): 125 NaCl, 3 KCl, 25 glucose, 25 NaHCO3, 1.25 Na2H2PO4, 2 CaCl2, and 1 MgCl2. Whole-cell current-clamp recordings were first filtered with a high-pass filter (0.2 Hz), smoothed (10 points averaged with a moving average algorithm, Axograph X), and inspected for an ideal PSP template. The template was subsequently fitted with the function \( f(t) = [1 - \exp(-t/r_{rise})] \times \exp(-t/r_{decay}) \), where \( t_{rise} \) and \( t_{decay} \) are the rise and decay time constants. By setting a high amplitude detection threshold, the template was reiteratively applied to detect large events, which were then aligned and averaged to create the final template. The final template was then used to sweep and extract all events using a threshold-crossing protocol (\( \sim 5 \times \sigma_{noise} \)) to automatically detect events.

Immunohistochemistry. L5 pyramidal neurons were filled with Alexa 488 or 594 and 5 mg ml\(^{-1}\) biocytin during whole-cell configuration and fixed in −20°C methanol for 10 min (for Nav.6, βII-V spectrin, and Kv7.3 immunolabeling) or 4% paraformaldehyde (PFA) for 12 h at 4°C [for myelin basic protein (MBP)] and stored in 0.1 M PBS, pH 7.4. Sections were blocked in 5% normal goat serum (NGS) followed by 24 h incubation in primary antibodies diluted in 0.1 M PBS containing 5% NGS and 2% Triton X-100. The following primary antibodies were used: rabbit anti-Nav.1.6 and rabbit anti-βII-V spectrin (1:100; gift from M.N. Rasband, Baylor College of Medicine), guinea pig anti-Kv.7.3 (1:250; gift from E.C. Cooper, Baylor College of Medicine), and mouse anti-MBP (1:250; Covance). For triple immunolabeling of rabbit anti-ankyrin G (1:100; kind gift from M.N. Rasband, Baylor College of Medicine), mouse anti-a-PanNav (1:100; Sigma-Aldrich Chemie), and guinea pig anti-NeuN (1:1000; EMD Millipore), 300-μm-thick brain slices were fixed with 1% PFA for 30 min, then blocked with 10% NGS plus 0.2% Triton X-100 diluted in 0.1 M PBS for 2 h followed by 24 h incubation of primary antibodies, 10% NGS, and 0.2% Triton X-100 diluted in 0.1 M PBS.

For immunolabeling of Nav.1.2 (1:100; Alomone Labs, catalog #ASC-002; 1:100; NeuroMab, catalog #73-024) antibodies, mice were perfused with 0.1 M PBS and incubated in the following secondary antibodies to visualize single axons for extracellular recordings, the epifluorescence signals were obtained with an excitation bandpass filter of 460–490 nm (Alexa 488; emission, 520 nm long-pass filter) or 510–550 nm (Alexa 594; emission, 590 nm long-pass filter), and collected concurrently with the bright-field image. Images were acquired and automatically overlaid with a CCD camera (CoolSNAP EZ, Photometrics) controlled by an Arduino board (http://www.arduino.cc/) and µManager software (http://www.micro-manager.org). Approximately 100–150 sweeps were off-line aligned at the somatic AP threshold and averaged. Average spike-triggered voltage transients were aligned to the peak dV/dt of the somatic AP and axosomatic delays calculated at 20% of the peak of each averaged axonal extracellular AP, relative to that of the peak in the somatic dV/dt. A 20% rise point was chosen because it is dominated by the onset of local sodium current in the AIS and nodes and not contaminated by other ionic currents, enabling reliable detection of the site of spike initiation (Palmer et al., 2010).

For analysis of spontaneous postsynaptic potentials (PSPs), individual recordings were first filtered with a high-pass filter (0.2 Hz), smoothed (10 points averaged with a moving average algorithm, Axograph X), and inspected for an ideal PSP template. The template was subsequently fitted with the function \( f(t) = [1 - \exp(-t/r_{rise})] \times \exp(-t/r_{decay}) \), where \( t_{rise} \) and \( t_{decay} \) are the rise and decay time constants. By setting a high amplitude detection threshold, the template was reiteratively applied to detect large events, which were then aligned and averaged to create the final template. The final template was then used to sweep and extract all events using a threshold-crossing protocol (\( \sim 5 \times \sigma_{noise} \)) to automatically detect events.
1.3) or 63× (numerical aperture, 1.4) oil-immersion objectives. To avoid bleed-through between emission wavelengths, automated sequential acquisition of multiple channels was used. Low-magnification (objective, 40×; digital zoom, 1×) images were collected; 10–25 images at 1 μm z steps and high-magnification (objective, 60×; digital zoom, 2–4×) images were collected with 0.5–1.0 μm z steps. Background noise was subtracted from each optical section before the analysis of individual AISs. The onset of the AIS was defined as the distance between the marker onset and to the edge of either the soma or a basal dendritic branch [somatic AIS, \( n = 74 \); (basal) dendritic AIS, \( n = 16 \)]. The length of the AIS fluorescence profile was measured by drawing a line from the AIS onset to the distal position. All image analysis was performed using Fiji (ImageJ) graphic software (v1.47p, National Institutes of Health).

**Compartmental modeling.** For conductance-based multicompartmental simulations, we used a biocytin-filled thick-tufted mouse L5 pyramidal neuron from the control group (cell number 20140421_c1, 24 weeks of age), scanned at 2048 × 2048 pixel resolution with confocal microscopy using 1 μm z steps (Leica SP8), three-dimensionally reconstructed with Neurolucida (v.10, MicroBrightField) and imported into the Neuron simulation environment (v. 7.3; Hines and Carnevale, 2001). Both the AIS and nodal domains were carefully incorporated into the model as distinct sections with the width, and length values based on the precise location of β-lactamase (see Fig. 3A–C). Cytoplasmic resistance (\( R_c \)) was set to 140 Ω cm throughout all compartments. The resting membrane potential (RMP) was adjusted with \( E_{\text{Na}} \) to −78 mV and temperature was set nominally at 33°C. Throughout the somatodendritic and nodal compartments, the membrane resistance (\( R_m \)) was set to 25 kΩ cm² and capacitance (\( C_m \)) 1.0 μF cm⁻². Myelination of internodal sections was represented by increasing internodal \( R_m \) to 100 kΩ cm² and decreasing \( C_m \) to 0.25 μF cm⁻², leading to a conduction velocity in the primary axons of 1.2 m s⁻¹ in accordance to the experimental value in control (−1.1 m s⁻¹; see Fig. 6E). “Demyelination” was simulated by setting \( R_m \) to 25 kΩ cm² and \( C_m \) to 1.0 μF cm⁻² in the internodal sections, leading to a continuous axonal conduction velocity of 0.32 m s⁻¹, consistent with experimental observations (−0.35 m s⁻¹; see Fig. 6E). Nav conductance was implemented by two separate eight-state allosteric models developed for the soma and the axon (Schmidt-Hieber and Bischoffer, 2010) and distributed linearly decreasing in density along the somatodendritic axis and locally increasing in peak densities in the AIS as described previously (Hallemann et al., 2012; Battefeld et al., 2014). Nav peak conductance values used were 500 pS μm⁻² in the soma, 45 pS μm⁻² in dendrites, 25 pS μm⁻² in the internodal membrane, 8000 pS μm⁻² in the nodes, and 9000 pS μm⁻² at the peak density in the AIS. Potassium (K⁺) and calcium-dependent K⁺ conductances were distributed as described recently (Battefeld et al., 2014; see also http://senselab.med.yale.edu/modeldb/ShowModel.asp?model=144526). Kv7 peak conductance was set at 150 pS μm⁻² in nodes of Ranvier and at the end of the AIS, 13 pS μm⁻² in axon collaterals and in the internodes, and 12 pS μm⁻² in dendrites. The K⁺ and Na⁺ equilibrium potentials were set to −85 and +55 mV, respectively. The hyperpolarization-activated cation channel model was exponentially increasing in the apical dendrites (Kole et al., 2006) but uniform in the axon (4 pS μm⁻²). The final input resistance of the model neuron was 25.4 MΩ (26.9 MΩ, experimentally). Since we were primarily interested in the rising phase of the AP, simulations were performed with a short time step of 5 μs. When overlaying and comparing with experimental recordings, we applied a 10 kHz Gaussian filter. In the experiments, APs were evoked with a 6 ms, 0.99 nA current injection (0.94 nA in the simulations).

**Statistics.** Statistical significance between multiple groups was tested with GraphPad Prism (v6.0d, GraphPad Software) and Matlab R2012b (v8.0.0.783, MathWorks). The following nonparametric statistical tests were used: Mann–Whitney, Wilcoxon, Kruskal–Wallis, and Friedman tests for pharmacological experiments and immunofluorescence analysis. All other data were analyzed using parametric statistical methods. Correlation analysis was performed using IBM SPSS (v22, IBM). The cutoff significance level (\( p \)) was 0.05.

**Results**

Cuprizone induces dose-dependent and time-dependent gray matter demyelination of L5 axons

To investigate the impact of demyelination at the level of single L5 axons, we recorded from visually identified L5 neurons in the primary somatosensory hindlimb region (S1HL) in slices from adult C57BL/6 mice, which were fed with 0.2% cuprizone for 5 weeks, a standard regime to obtain maximal oligodendrocyte death and loss of myelin protein expression (Kipp et al., 2009). Recorded cells were processed for biocytin and demyelination was assessed by immunofluorescence of MBP (essential for the formation of compact myelin; Boggs, 2006; Fig. 1A). Confocal z-projected images showed that although 0.2% cuprizone for 5 weeks reduced MBP expression prominently within the L5 and L6, patches of MBP could still be observed (Fig. 1A). Also when feeding mice for 9 weeks with 0.2% cuprizone, L6 was still immu-
nologically labeling with biocytin, indicating that 0.2% allows continuation of remyelination, consistent with previous work (Lindner et al., 2008). In contrast, 0.3% cuprizone feeding for 9 weeks induced a near-complete demyelination of the gray matter region (Fig. 1A).

To quantitatively examine the differences in myelin loss across the groups, we used confocal image analysis of MBP immunofluorescence. Analysis of the myelin content in Layer 6 revealed that 0.3% cuprizone treatment for 9 weeks compared with control and 0.2% cuprizone for 5 weeks induced significantly more myelin loss (5.1 vs 50.4 and 13.9%, respectively; Fig. 1B,C). Furthermore, myelination of single axons was quantitatively measured by calculating the percentage of axon length covered with MBP. Control Layer 5 axons had an average onset of myelin at 45.7 ± 2.1 μm from the soma, at the end of the AIS, and myelination continued across the entire length examined (100%, n = 10; Fig. 1D). In contrast, 0.2%/5 weeks cuprizone treatment caused a significant ~70% loss of myelin and shifted the myelin onset to more distal locations in the axon (276.6 ± 40.6 μm distance from the soma, n = 9 axons, one-way ANOVA followed by Bonferroni’s post hoc test, p = 0.0002). Additionally, nearly ~35% (n = 5 of 14) of axons in 0.2%/5 weeks group were completely demyelinated. In comparison, within the 0.3%/9 weeks group, axons showed a ~97% loss of myelin and ~81% (n = 13 of 16) of the axons labeled with MBP were completely demyelinated (Fig. 1D). In three MBP-positive axons (n = 3), the myelin onset occurred at significantly more distal locations from the soma (0.3%/9 weeks, 448.3 ± 101 μm, n = 3 axons; compared with control, Bonferroni’s post hoc test, p < 0.0001; compared with 0.2%/5 weeks, p = 0.047). Despite the major loss of myelination, the axon morphology, as assessed by diameter, was not affected (axon diameter at 300 μm from soma: control, 1.03 ± 0.08 μm, n = 7; 0.2%/5 weeks, 0.76 ± 0.04 μm, n = 4; 0.3%/9 weeks, 0.92 ± 0.07 μm, n = 6; one-way ANOVA, p = 0.09).

Together, the observed spatial and temporal dynamics of the 0.2%/5 weeks and 0.3%/9 weeks cuprizone treatment indicates that the two paradigms cause an “acute” or “chronic” loss of myelination in Layer 5 axons, respectively.

Morphological changes of Layer 5 neurons in demyelination
To examine whether demyelinated L5 pyramidal neurons of the somatosensory cortex exhibit dendritic morphological differences, neurons were labeled with biocytin for post hoc analysis. Morphological analysis showed that targeted neurons were always of the thick-tufted L5 pyramidal neuron type (n = 38 neurons; Fig. 2A). All analyzed neurons extended apical dendrites that terminated in dendritic tufts near the pia, with laminar position of the bifurcation points of primary apical dendrites typically observed near the borders of Layer 2/3 and 4. Following chronic demyelination, L5 neurons had apical dendrites that were on average ~100 μm shorter compared with control and acute demyelination (Fig. 2B). The width of the apical dendritic tufts in neurons of cuprizone-treated animals was not different from that in control (Fig. 2B). Similarly, the diameter at the base of the apical dendrites of L5 neurons was not changed in cuprizone-treated animals (Fig. 2B).

We subsequently examined subdomains of the dendritic tree using Sholl plots. When comparing cuprizone-treated groups to control, it was found that chronic demyelination induced an increase in number of dendritic branches within a very small range spanning between 60 and 80 μm from the center of the cell body (Fig. 2C).

Demyelination-induced ion channel redistribution in the AIS
To examine whether loss of myelin affects the voltage-gated ion channel composition of the AIS, we performed immunofluorescence experiments on the slices in which L5 neurons were filled with Alexa Fluor during the whole-cell recordings (n = 97). Confocal images revealed an intense immunosignal for βIV-spectrin, an anchoring protein together with ankyrin G stabilizing Nav1.6 channels in the AIS (Rasband, 2010), and showed that it was associated with the plasma membrane (Fig. 3A). Three-dimensional analysis of z stacks showed that the average AIS onset of βIV-spectrin, relative to the edge of the soma, was 3.39 ± 0.36 μm in control cells (Fig. 3A,B). Acute and chronic demyelination led, however, to a significantly more proximal onset at 1.65 ± 0.32 μm and at 0.92 ± 0.19 μm, respectively (Fig. 3A,B). The average length of βIV-spectrin expression, however, did not change (Fig. 3C). Next, we labeled brain slices with a Nav1.6 antibody (Fig. 3D).
Figure 3. Demyelination-induced ion channel reorganization in the AIS. A, Single fluorescence channels and merged image of z-projected confocal scans of the soma and AIS morphology (Alexa Fluor 594 fill, red) and immunofluorescence labeling of βIV-spectrin (yellow). White arrowheads indicate the onset and extent of βIV-spectrin immunofluorescence identifying the AIS. B, C, Bar plots showing more proximal onset, but maintained length of expression of the AIS βIV-spectrin immunofluorescence. Kruskal–Wallis followed by Dunn post hoc test. Onset: *p = 0.042, ***p = 0.0003. Control, n = 8; acute, n = 13; chronic, n = 16. D–F, Nav1.6 immunofluorescence labeling (cyan) is significantly more proximal in its onset and changed in length in demyelinated axons. Onset: control versus acute, **p = 0.0025; control versus chronic, **p = 0.0043. Length: control versus acute, **p = 0.0023; acute versus chronic, *p = 0.039. Control, n = 14; acute, n = 23; chronic, n = 17. G–I, Confocal images of triple immunofluorescence of ankyrin G, α-PanNav, and NeuN. For both ankyrin G and α-PanNav expression, length was maintained in acute demyelination. Mann–Whitney test. Ankyrin G, p = 0.1647; α-PanNav, p = 0.099. Control, n = 44 AISs; acute, n = 63 AISs.

In control L5 neurons, the length of Nav1.6 immunosignal in the AIS was on average 34.3 ± 0.4 μm with a proximal onset of 4.2 ± 0.6 μm (Fig. 3E,F). In acutely demyelinated axons, the immunosignal length was significantly reduced and started at a more proximal site of onset (length, 31.3 ± 0.5 μm; onset, 2.11 ± 0.2 μm; Fig. 3E,F). Chronically demyelinated L5 axons also showed a more proximal onset of Nav1.6 at 2.0 ± 0.2 μm but were not different in length of expression (Fig. 3E,F).

In addition to βIV-spectrin and Nav1.6, we triple-immunolabeled control and acute brain slices with ankyrin G, α-PanNav, and NeuN (Fig. 3G). However, due to the required low-fixation regime, it was not possible to combine these two immunolabelings with neuron filling. Consistent with the βIV-spectrin results, the average length of ankyrin G in acute demyelination was similar to that in control (37.2 ± 0.3 μm, acute, 37.9 ± 0.4 μm, Fig. 3H). Interestingly, unlike the reduction of Nav1.6 expression in acute demyelination, we did not observe a reduction in α-PanNav expression in the acute group (Fig. 3I).

Further, when using triple fluorescence for Alexa 594, βIV-spectrin, and Kv7.3, we found that in both acutely and chronically demyelinated axons the Kv7.3 immunosignal spread widely into the formerly myelinated internodes (length: acute, 35.9 ± 4.2 μm; chronic, 35.8 ± 4.1 μm; Fig. 4B,C). Furthermore, more, when using triple fluorescence for Alexa 594, βIV-spectrin, and Kv7.3, we found that in both acutely and chronically demyelinated axons the Kv7.3 immunosignal spread widely into the first internode without detectable levels of βIV-spectrin (Fig. 4D).

Demyelination-induced ion channel redistribution reduces AIS excitability

The location of the AIS and its ion channel properties are major determinants of the AP voltage threshold and firing properties of neurons (Kole et al., 2008; Grubb and Burrone, 2010; Battefeld et al., 2010; Battefeld et al., 2014). To examine the functional impact on neuronal excitability we explored the resting membrane parameters and AP properties of the thick-tufted L5 neurons in the three groups. The RMP in control L5 pyramidal neurons was on average −67.9 ± 0.26 mV (n = 100), but neurons from both the acute and chronic demyelinated group showed a ∼1–2 mV more depolarized RMP (acute, −66.93 ± 0.31 mV, n = 93, p = 0.025;
chronic, $-66.22 \pm 0.46 \text{ mV, } n = 54$; one-way ANOVA followed by Bonferroni’s *post hoc* test, $p = 0.0008$). In the chronic demyelinated group, the input resistance was on average $49.5 \pm 1.1 \text{ M}\Omega (n = 54$; Bonferroni’s *post hoc* test, $p = 0.0396$), significantly higher compared with control (control, $45.3 \pm 0.9 \text{ M}\Omega, n = 10$; acute, $45.6 \pm 1.1 \text{ M}\Omega, n = 90$). This increase in input resistance was also accompanied by a lower rheobase of AP trains in chronic demyelinated neurons (control, $99.1 \pm 0.47 \text{ mV, } n = 77$, Bonferroni’s *post hoc* test, $p = 0.049$; chronic, $97.7 \pm 0.61 \text{ mV, } n = 54$, Bonferroni’s *post hoc* test, $p = 0.0001$) without change in half-width (control, $0.60 \pm 0.007 \text{ ms, } n = 98$; acute, $0.60 \pm 0.007 \text{ ms, } n = 77$; chronic, $0.58 \pm 0.012 \text{ ms, } n = 53$; one-way ANOVA, $p = 0.323$). The AP voltage threshold of demyelinated neurons showed a trend to depolarize following chronic cuprizone treatment (control, $-47.3 \pm 0.25 \text{ mV, } n = 98$; acute, $-47.2 \pm 0.34, n = 77$; chronic, $-46.3 \pm 0.40, n = 53$; one-way ANOVA, $p = 0.0682$). Closer examination of the AP rising phase showed that the initial component in the $dV_{m}/dt$, which reflects the current flow from the AIS during onset of the somatic AP voltage waveform, was selectively reduced in chronically demyelinated neurons (control, $383.8 \pm 6.4 \text{ V s}^{-1}, n = 65$; chronic, $356.6 \pm 7.2 \text{ V s}^{-1}, n = 33$; Bonferroni’s *post hoc* test, $p = 0.02$).

To examine whether these properties were a result of cuprizone or the associated myelin loss, we bath applied $100 \mu\text{m}$ cuprizone to control neurons in whole-cell current-clamp configuration *in vitro* and established the main properties. The RMP slightly hyperpolarized after 20 min of $100 \mu\text{m}$ cuprizone bath application (control, $-67.9 \pm 1.3 \text{ mV; cuprizone, } -69.0 \pm 1.1 \text{ mV, } n = 5$; paired Wilcoxon test, $p = 0.04$). However, bath application of cuprizone had no significant effects on AP properties, including AP amplitude (control, $104.4 \pm 1.5 \text{ mV; cuprizone, } 98.6 \pm 1.4 \text{ mV, } n = 5$; paired Wilcoxon test, $p = 0.17$).

Next, to test the hypothesis that the experimentally observed changes in AIS position or AIS length (Fig. 5) were associated with the group changes in AP generation, we took advantage of our correlated approach of recording APs from neurons that were also fluorescently labeled for βIV-spectrin or Nav1.6. We plotted the onset location of the AIS, varying between 0.5 and $8 \mu\text{m}$, against various parameters of excitability recorded from the same cell. The results showed that AIS onset significantly, and positively, correlated only with the AP amplitude in both control and demyelinated neurons (Fig. 5A, B). A linear fit of the pooled data from control and demyelinated neurons revealed a slope of $1.8 \text{ mV } \mu\text{m}^{-1}$ (Fig. 5B, red trace). As the AIS moved on average $-2 \mu\text{m}$, these results quantitatively predict a $3.6 \text{ mV}$ amplitude reduction, in accord with the $-3 \text{ mV}$ AP amplitude reduction in demyelinated L5 neurons.

To test whether AIS length or location suffices to reduce the somatically recorded APs, we generated a computational model of a control L5 neuron, reconstructed at high resolution from confocal microscopy z stacks (see Materials and Methods; Fig. 5C). We took particular care to reconstruct the AIS at the precise onset distance ($3.9 \mu\text{m}$) and length ($39 \mu\text{m}$) based on the overlaid βIV-spectrin distribution. Next, to simulate the experimentally recorded AP, the peak Nav conductance densities in the AIS and soma were adjusted to reproduce the AP rising phase shape recorded from the specific cell (AIS: $9000 \text{ pS } \mu\text{m}^{-2}$; soma: $500 \text{ pS } \mu\text{m}^{-2}$; Fig. 5D). Subsequently, both AIS onset location and length were varied between 0.1 and $12 \mu\text{m}$ and between 29 and $39 \mu\text{m}$, respectively. The result of changing these model parameters revealed that both onset and length reduction depolarized the somatic AP voltage threshold and thereby reduced the AP amplitude. Changes in AIS length alone had a larger impact compared with changes in onset location (Fig. 5E, F). The impact of onset location was 0.17 $\text{mV } \mu\text{m}^{-1}$ on AP amplitude and 0.4 $\text{mV } \mu\text{m}^{-2}$ on AIS length. While the computationally predicted geometrical relationship is less steep compared with the experimentally observed location dependence (1.8 $\text{mV } \mu\text{m}^{-1}$; Fig. 5B), the results are in accord with the hypothesis that demyelination-induced geometrical changes reduce the capacity of the AIS Nav channels to generate inward current for the rising phase of the AP. Furthermore, the simulations predicted that the AP in the AIS at the distal end increased in half-width duration by $-35\%$ (control, $290 \mu\text{s}$; demyelination, $390 \mu\text{s}$) when the AIS has an onset directly from the cell body (Fig. 5F).
Demyelination does not affect the AP initiation site in the AIS but reduces axonal conduction velocity

Based on the computationally predicted reduction in local AIS excitability, we next tested experimentally how local AP initiation in the AIS was affected by performing simultaneous patch-clamp recordings from the soma and axon. The axonal AP was assessed by recording the extracellular voltage (e-AP) at various distances from the soma along the axon during repetitive current injections (Fig. 6A; Palmer et al., 2010; Kole, 2011). The AP initiation site was identified by plotting the latency between the extracellular axonal AP onset and intracellular somatic AP, versus the distance of the axonal recording site (see Materials and Methods; Fig. 6B,C). In control neurons, the axonal initiation site of the AP was ~27 μm from the soma and not different from acutely demyelinated axons (~25 μm; Fig. 6D). The e-AP latencies from locations distal from the AIS (>50 μm) were fitted with a linear function yielding an estimate of axonal conduction velocity of 0.35 m s⁻¹ in axons from cuprizone-fed mice, a ~3-fold velocity reduction compared with the control velocity (1.1 m s⁻¹), consistent with the loss of myelin (Fig. 6E).

We next investigated the local properties of the e-AP waveform within the AIS (binned into 0, 5, 15, 35 μm). Figure 6F shows the e-AP waveforms, reflecting primarily the sum of the local Na⁺ and K⁺ current densities, overlaid for distinct recording sites. Comparisons of e-AP waveforms from acute and chronic demyelinated neurons did not display any significant differences, and thus were pooled for further analysis. While the peak amplitudes of e-APs in demyelinated axons did not differ from the control e-AP amplitudes at any of the locations (0–35 μm), the e-AP half-width was selectively increased at 5 and 15 μm distance from the soma by ~30 and ~40%, respectively (Fig. 6F,G), consistent with the more proximal onset of Nav channel expression and impaired initiation of the local AIS AP (Fig. 5F).

Increased burst firing and spontaneous up-state-like events in demyelinated L5 neurons

If the AIS excitability is reduced, this may affect the input–output function of the demyelinated neurons. To test this we further analyzed the properties of subthreshold and suprathreshold excitability in both groups by current injections. The current-injection-evoked excitability of L5 neurons can be broadly divided into intrinsic bursting (IB) and regular spiking neurons with nonadapting spike intervals of ~100–200 ms (Hattox and Nelson, 2007; Kole, 2011). In control L5 neurons, steady current injections of just suprathreshold amplitudes either caused repetitive high-frequency bursts of 2–3 APs followed by regular spikes or a train of regular low-frequency APs only (Fig. 7A). IB of acutely demyelinated neurons was observed more frequently when compared with control and chronically demyelinated L5 neurons (Fig. 7B,C). Interestingly, not only current-evoked excitability but also spontaneous activity was enhanced in demyelinated neurons. When monitoring the baseline activity of L5 neurons in current-clamp mode (I = 0) in epochs of 20–30 min, it was observed that demyelinated L5 neurons showed spontaneous activity, characterized by transient suprathreshold depolarizations triggering multiple APs or subthreshold depolarizing envelopes, reminiscent of up-states reported for in vivo and in vitro recordings from L5 neurons (Fig. 7D; Sanchez-Vives and McCor-
Figure 6. Maintained AIS initiation site, increased half-width, and reduced conduction velocity of axonal APs in demyelinated L5 axons. A, Z-projection confocal image of a L5 pyramidal neuron illustrating the experimental design of whole-cell (W-C) configuration combined with e-AP recording. Locations of the recording sites for this example are indicated by blue arrowheads. e-AP traces (blue) from one typical recording aligned to the peak of the somatic dV/dt (black). Black dots indicate the time when 20% of the local e-AP maximum is reached. Asterisk (*) indicates the onset of AP. B, Pooled AP latency plotted versus axonal distance of the recording location in control (left, n = 30 neurons) and demyelinated axons (right, n = 26 acute and 2 chronic neurons). Red data point indicates the average initiation site. C, Top, Time derivative of APs from control (black) and acute demyelinated (red) L5 neurons aligned at peak amplitude. Bottom, e-APs recorded in demyelinated axons. Student’s t-test. Control, 26.6 ± 1.3 μm, n = 10; acute, 25.3 ± 1.3 μm, n = 12. E, Bar plot showing the decreased conduction velocity (CV) in demyelinated neurons. Control, 1.1 ± 0.2 m/s; demyelination (acute and chronic), 0.35 ± 0.1 m/s. F, Average e-APs recorded at 5 and 15 μm from the soma in control (black) and pooled demyelinated neurons (red, acute, and chronic). Note the broader e-AP half-widths of demyelinated neurons. 0.5 μm: control, n = 11; n = 14 acute and 9 chronic. 5 μm: control, n = 10; n = 8 acute and 9 chronic. 15 μm: control, n = 10; n = 10 acute and 9 chronic. 35 μm: control, n = 10; n = 10 acute and 9 chronic. G, Subpopulations of neurons with subthreshold up-state depolarizations increased in acute and chronic neurons. H, Overlaid and aligned up-state AP voltage traces from control (black) and demyelinated neurons (red) on an expanded time scale. Note the onset delay of the e-AP in the demyelinated axon due to reduction of conduction velocity as a consequence of myelin loss. Closed blue circles indicate the 20% onset of the local e-AP maxima. Asterisk (*) indicates the onset of AP. I, Bar plot showing a significantly increased subpopulation of acute and chronic demyelinated L5 neurons generating up-state APs.

Figure 7. Demyelination increases burst firing and up-state-like depolarizations in L5 neurons. A, Suprathreshold current injection (bottom) induced burst firing in control (left) and acute demyelinated (right) neuron. B, Overlaid voltage traces in A on an expanded time scale. Note the increase in number of APs in an acute demyelinated neuron (red) compared with control (black). C, Bar plot showing a significant increase in IB neurons in acute demyelination. χ²-test. D, Voltage traces of baseline activity at RMP (V_m) from chronic demyelinated L5 neurons (middle, red), showing the occurrence of spontaneous up-state APs and depolarizations (blue area), compared with control neuron (top, black). Bottom, Overlaid voltage traces of up-state APs and depolarizations (middle, blue area). E, Bar plot showing a significantly increased subpopulation of acute and chronic demyelinated L5 neurons generating up-state APs. χ²-test. Control, n = 5 of 61; acute, n = 25 of 79; chronic, n = 27 of 99. F, Subpopulations of neurons with subthreshold up-state depolarizations increased in acute and chronic neurons. χ²-test. Control, n = 11 of 61; acute, n = 38 of 79; chronic, n = 26 of 99. G, H, Overlaid and aligned up-state AP voltage traces from control (black) and demyelinated neurons (red) on an expanded time scale. Note the high firing frequency of the demyelinated (245.1 Hz) neurons compared with control (23.4 Hz). One-way ANOVA followed by Bonferroni’s post hoc test. Control, n = 4 neurons; acute, n = 19 neurons; chronic, n = 24 neurons. Co, control; Ac, acute; Ch, chronic. Data are presented as mean ± SEM.

Beltramo et al., 2013). Up-state-like events are a characteristic feature of L5 neurons and were observed in a larger population of neurons from cuprizone-treated animals (up-state AP: control, 8%; acute, 32%; chronic, 27%; up-state depolarization: control, 18%; acute, 48%; chronic, 26%; Fig. 7 E,F). These events also occurred more frequently within a single recording (up-state AP: control, 2.0 ± 0.33 mHz, n = 5; acute, 2.9 ± 0.41 mHz, n = 25; chronic, 4.8 ± 0.75 mHz, n = 27; one-way
ANOVAs, \( p = 0.039 \); up-state depolarization: control, \( 2.0 \pm 0.2 \) mHz, \( n = 11 \); acute, \( 2.1 \pm 0.1 \) mHz, \( n = 38 \); chronic, \( 2.7 \pm 0.3 \) mHz, \( n = 26 \); one-way ANOVA, \( p = 0.043 \) and the interspike frequency within the up-state AP was also significantly higher in demyelination \((\sim 100 \text{ Hz}; \text{Fig. 7G, H})\). Loss of myelination in the cuprizone model thus produces an increased excitability despite a decrease in AIS excitability.

**Glutamate receptor-dependent up-state-like depolarizations in demyelinated L5 neurons**

What underlies the increased excitability? We next aimed to examine whether up-state depolarizations after demyelination are dependent on network activity. Simultaneous recording from two horizontally adjacent demyelinated L5 neurons \((\sim 50 \mu \text{m apart}, n = 3 \text{ pairs})\) showed that up-state events could occur temporally correlated, consistent with synchronized network activity (Fig. 8A). To pharmacologically block synaptic activity, we bath applied glutamate receptor blockers and measured the impact on spontaneous AP generation. We first recorded a 10–20 min baseline activity in slices from cuprizone-treated mice and subsequently bath applied CNQX, a competitive AMPA/kainate receptor antagonist, which reduced the number of events (baseline, \( \sim 7.1 \) mHz; CNQX, \( \sim 0.42 \) mHz; Fig. 8B, C). Subsequent addition of D-AP5, a selective NMDA receptor antagonist, led to a complete block (CNQX and D-AP5, 0 mHz; Fig. 8B, C). Bath application of CNQX and D-AP5 also significantly reduced the number of up-state depolarizations in demyelinated neurons \([\text{baseline}, 4.0 \pm 1.0 \text{ mHz}; \text{CNQX}, 1.7 \pm 0.53 \text{ mHz}; \text{CNQX and D-AP5}, 0.33 \pm 0.33 \text{ mHz}, n = 5 \text{ (acute)}; \text{paired Friedman followed by Dunn post hoc test, } p = 0.0089\])

Based on these results, we hypothesized that synaptic glutamate receptor activity may be increased following demyelination. There is evidence that cuprizone, which is a copper chelator, may have also a direct impact on neurons by decreasing the free level of copper and thereby inducing persistent opening of the NMDA receptor (Stys et al., 2012). On the other hand, cuprizone-induced demyelination for 6 weeks reduces AMPA receptor expression in the hippocampus, which may reduce the synaptic drive (Dutta et al., 2013). To test whether synaptic properties of L5 neurons were altered, we examined the interevent interval frequency and amplitudes of spontaneous postsynaptic potentials. The results showed, in contrast, that the postsynaptic potential frequency in demyelinated L5 neurons was not affected (control, \( 1.62 \pm 0.33 \) Hz, \( n = 7 \); demyelination, \( 1.54 \pm 0.15 \) Hz, \( n = 6 \) acute and 6 chronic; Student’s \( t \) test, \( p = 0.801 \)). The postsynaptic potential amplitude in demyelinated neurons \((0.99 \pm 0.06 \text{ mV}, n = 12; \text{Student’s } t \text{ test, } p = 0.177)\) was also similar to that in control \((0.85 \pm 0.08 \text{ mV}, n = 7)\).

To examine the role of direct cuprizone exposure on NMDA synaptic currents, we extracellularly stimulated intracortical fibers and recorded NMDA currents in the presence of 20 \( \mu \text{M} \) CNQX and 2 \( \mu \text{M gabazine} \) (SR 95531). In both control conditions and in the presence of 100 \( \mu \text{M} \) cuprizone bath application, we did not observe any changes in amplitude (control, \( 379 \pm 36 \text{ pA} \); with cuprizone, \( 364 \pm 73 \text{ pA}, n = 7 \); paired Wilcoxon test, \( p = 0.813 \); Fig. 8D, E) or decay time constant \((\tau); \text{control, } 34.5 \pm 3.2 \text{ ms}; \text{with cuprizone, } 37.9 \pm 3.0 \text{ ms}, n = 7; \text{paired Wilcoxon test, } p = 0.297; \text{Fig. 8D}) of the NMDA currents. These results show that both long-term demyelination by cuprizone treatment and acute application of cuprizone do not change synaptic transmission of L5 pyramidal neurons.

Studies in the visual cortex showed that spontaneous pacemaker-like activity in the neocortex may be generated by the persistent Na\(^+\) current (Le Bon-Jego and Yuste, 2007), and persistent Na\(^+\) current has been implicated in axonal pathology in demyelination (Kapoor et al., 1997; Crainer et al., 2004; Waxman, 2008). To test whether up-state APs were dependent on a subthreshold persistent Na\(^+\) current in the demyelinated axon, we
applied 20 nM of tetrodotoxin (TTX), TTX significantly reduced the rate of up-states but did not fully block them, suggesting that intrinsic conductances may contribute in part to the demyelination-induced increased up-state AP event rate (baseline, 2.8 mHz; plus 20 nM TTX, 1.1 mHz; Fig. 8F,G). Bath application of 20 nM TTX neither blocked nor modulated the event frequency of up-state depolarizations (baseline, 2.3 ± 0.21 mHz; plus 20 nM TTX, 2.25 ± 0.64 mHz; acute, n = 2; chronic, n = 5; paired Wilcoxon test, p = 0.8125). Thus, persistent Na⁺ current may contribute to the amplification of the up-state depolarizations in demyelinated axons.

**Demyelination causes ectopic AP generation**

In the course of the long-duration recordings (Figs. 7, 8), it was furthermore observed that nearly ~15% of the demyelinated L5 neurons exhibited ectopic APs (acute, 16%; chronic, 14%; Fig. 9A–C). Consistent with the presumed ectopic origin, these APs had a nearly instantaneous voltage deflection from the RMP, reflecting axial current flow initiated distally in the axon and invading the AIS and somatodendritic region antidromically (Fig. 9B). Furthermore, when the soma was ~25 mV hyperpolarized, the AIS-somatodendritic component of the ectopic AP could be activated and isolated from the spontaneous distal axonal AP, appearing as a small ~1 mV spikelet at the soma (Fig. 9A). Ectopic APs were never observed in control myelinated L5 neurons (0%; 0 of 61 neurons; Fig. 9C). The average ectopic AP event rate during a recording was ~7–10 mHz in both acute (7.1 ± 1.7 mHz, n = 13) and chronic demyelination (10.1 ± 3.6 mHz, n = 14; Mann–Whitney test, p > 0.999).

To further examine whether demyelinated L5 axons also have higher propensity of pharmacologically induced ectopic AP generation, we applied 75 μM 4-aminopyridine (4-AP, a blocker of Kv1 and Kv3 voltage-gated K⁺ channels) during current-clamp baseline recordings (Fig. 9D). Bath application of 4-AP induced ectopic APs in control neurons at rate of ~8 mHz. However, in acutely demyelinated L5 axons, 4-AP induced nearly a ~7-fold increase in the rate of ectopic AP (Fig. 9E). In contrast, chronic demyelinated L5 axons displayed comparable sensitivity to 4-AP-induced ectopic AP generation with control. Ectopic AP generation has previously been described in demyelinated axons from the sciatic nerve (Baker and Bostock, 1992; Felts et al., 1995) and has been thought to rely on activation of persistent Na⁺ current at the site of demyelination (Kapoor et al., 1997). To test this, we bath applied 20 nM TTX, which depolarized the somatic AP voltage threshold by 17.2 ± 1.2 mV (n = 2 acute and 6 chronic) and reduced the AP amplitude by 45.7 ± 6.4 mV (n = 8). At this concentration, TTX blocked the initiation of ectopic APs (baseline, 8.13; with TTX, 0.0 mHz; Fig. 9F,G), suggesting that activation of axonal Na⁺ current is critically involved in the generation of demyelination associated ectopic APs.

Together, these results indicate that demyelination leads to decreased AP generation evoked from somatodendritic current inputs in the AIS while facilitating generation of Na⁺-dependent ectopic APs in axonal regions distally from the AIS.

**Demyelination-induced ion channel distribution in nodes of Ranvier**

The results above indicate that Nav channel-dependent distal axonal excitability is increased. While the majority of demyelinated L5 neurons generating ectopic APs had axons terminating in deep L6 or corpus callosum, four axons were cut between ~150 and 300 μm distance from the soma (237.5 ± 28.9 μm), indicating that the source of ectopic APs in demyelinated L5 axons may be in the proximity of the first two nodes of Ranvier. To examine whether axonal hyperexcitability is caused by a reorganization of nodal ion channels, we investigated the expression of Nav1.6 at nodes of Ranvier in L5 axons, which were filled with Alexa 594 (Fig. 10A). Control nodes, identified by the position of branch points in the primary axon, were always positive for Nav1.6 (100%, n = 17 of 17 branch points, 6 axons; range: 74–
392 \mu m distance from the soma). In contrast, in demyelinated L5 axons nearly 63% of the nodes lacked Nav1.6 expression (Nav1.6-positive branch points: \( n = 13 \) of 35, 18 axons; \( \chi^2 \) test, \( p < 0.0001 \); Fig. 10B). Interestingly, 39% (\( n = 7 \)) of the axons contained both Nav1.6-positive and Nav1.6-negative branch points, indicating that the impact of demyelination leads to a large heterogeneity in nodal expression of ion channels. When Nav1.6 was present in distal axons, it was expressed over a significantly longer region compared with the more focal expression of control myelinated axons (Fig. 10C).

We also examined the expression of \( \beta IV \)-spectrin and Kv7.3 (range: 87–327 \mu m distance from the soma; Fig. 10D–F). While \( \beta IV \)-spectrin was detected in all control branch points, \(~48\%\) of demyelinated branch points showed no \( \beta IV \)-spectrin clustering (\( \beta IV \)-spectrin-positive branch points: control, \( n = 11 \) of 11, 4 axons; demyelination, \( n = 14 \) of 27, 9 axons; \( \chi^2 \) test, \( p = 0.0045 \)). In accord with \( \beta IV \)-spectrin and Nav1.6 labeling, in demyelinated axons Kv7.3 was not present in 46% of the branch points (Kv7.3-positive branch points: control, \( n = 14 \) of 14, 5 axons; demyelination, \( n = 14 \) of 26, 8 axons; \( \chi^2 \) test, \( p = 0.0024 \)). In addition, \( \beta IV \)-spectrin and Kv7.3 also displayed increased length of expression into the paranodal domains (Fig. 10D–F). Similar to Nav1.6 nodal heterogeneity, examination of \( \beta IV \)-spectrin and Kv7.3 branch point expression revealed that a large percentage of axons contained both negative and positive branch points (\( \beta IV \)-spectrin, \( n = 6 \) of 9 axons; Kv7.3, \( n = 5 \) of 8 axons). Furthermore, in some demyelinated axons we observed that both \( \beta IV \)-spectrin and Kv7.3 were also diffusely expressed along internodal regions but at the same time lost from branch points (\( n = 2 \) axons; Fig. 10G). In addition to the observation in fluorescently identified L5 axons, other nonfilled axons in L6 showed similar continuous \( \beta IV \)-spectrin and Kv7.3 expression along internodal regions. However, brain sections of cuprizone-treated mice immunolabeled for ankyrin G (\( n = 9 \)), Nav1.6 (\( n = 20 \)), Nav1.2 (\( n = 8 \)), and \( \alpha \)-PanNav (\( n = 9 \)) did not reveal continuous internodal expression (data not shown).

Together, these data show that Nav1.6, \( \beta IV \)-spectrin, and Kv7.3 can both be lost and gained within nodal and paranodal regions but, in contrast to Nav1.6, the Kv7.3 channels redistribute extensively into the demyelinated internodes.

Neurons with demyelinated axons are more susceptible to anomalous AP generation

The observed divergence of nodal and internodal voltage-gated channel expression (Fig. 10) may lead to a highly heterogeneous excitability of the axon with longer diffuse expression of Nav1.6 channels underlying ectopic AP generation (Fig. 9). Demyelination exposes primary axons to the extracellular environment and possibly to local activity-driven K\(^+\) and glutamate accumulation (Rasinsky, 1978; Kapoor et al., 1993). To investigate this possibility, we applied high concentrations of K\(^+\) at fluorescently identified branch points and internodes (10 ms pulse, [K\(^+\)] = 140 mM; Fig. 11A). The high-K\(^+\)-evoked AP displayed ectopic characteristics with more hyperpolarized voltage threshold re-
The present study shows that cuprizone-induced myelin loss affects voltage-gated ion channel expression in the nodes of Ranvier and the AIS. To the best of our knowledge, these results provide the first evidence for a functional impact of myelin loss on the AIS and the occurrence of spontaneous intrinsic and network excitability of pyramidal neurons in the demyelinated neocortex.

Loss of myelin causes highly heterogeneous changes in nodes of Ranvier
By taking advantage of fluorescently identifying single gray matter L5 axons, allowing a longitudinal distribution profile analysis, and identifying nodal domains morphologically by their branch points, we found that Nav1.6 and Kv7.3 channels are highly heterogeneously affected. Even at neighboring branch points within one and the same demyelinated axon β1IV-spectrin, Nav1.6 and/or Kv7.3 could extend into the paranodes or disappear. The heterogeneity of the molecular properties of demyelinated nodes was consistent with the locally evoked nodal APs often failing at branch points. Surprisingly, in none of the L5 internodes did we find evidence for a Nav channel upregulation; that is, neither for α-PanNav, nor Nav1.6, nor Nav1.2. Dispersion of the nodal Nav channel expression into paranodes and internodes is a hallmark of demyelinated axons, both in experimental models (Dupree et al., 2004; Crawford et al., 2009) and in the spinal cord and optic nerve of MS patients (Crane et al., 2004). While the immunosignals of Nav1.6 and α-PanNav were always detected in the AIS, these primary antibodies do not show signals in the somatodendritic region, which is known to contain low densities of Nav channels. Therefore, the Nav antibodies used may not be able to detect expression of Nav channels at very low densities (<20 channels/μm²) in demyelinated internodal regions. Even a modest Nav channel density increase in the internodes (∼4% of the nodal density) can be sufficient to restore conduction of AP in demyelinated axonal regions (Shragar and Rubinstein, 1990). Additionally, it is also possible that upregulation of Nav channels in demyelinated internodes may be axon specific or occur at more chronic stages of demyelination.

In contrast to Nav channels, in the internodes of demyelinated L5 axons there was substantial evidence for diffuse Kv7.3 expression. A role of Kv7 channels in demyelinated axons has been computationally predicted and experimentally reported in axotomy-induced demyelination in peripheral axons (Coggan et al., 2010; Roza et al., 2011). Furthermore, on basis of in vivo axonal recordings in MS patients, the I\(_{\text{Ks}}\) (or I\(_{\text{KCl}}\)), mediated by Kv7.2/7.3 channels, is predicted to be upregulated (Ng et al., 2008). The functional role of Kv7 in demyelinated axons remains to be further examined. Increased internodal Kv7, which is active at resting potential, may for example via a membrane potential hyperpolarization increase the availability of the transient Nav channels (Battefeld et al., 2014), ensuing continuation of axonal AP conduction.

Compensatory ion channel expression in the AIS
Two of our main findings were that despite the extensive myelin loss and dissolution of nodes of Ranvier, the AIS in the same axon always remained strongly immunopositive for Nav1.6 and Kv7.3, and AP generation from currents injected in the soma was only slightly impaired. The AIS structure changed, however, its location and reduced in length following acute, but not chronic, myelin loss. Recent studies showed that altered network activity, injury, or disease change the geometrical position of the AIS, leading to a homeostatic regulation of excitability (Kuba et al., 2006; Grubb and Burrone, 2010; Buffington and Rasband, 2011; Hinman et al., 2013). These results are consistent with a compen-
Implications for therapeutic ion channel treatment in MS

Cuprizone-induced lesions resemble some, but certainly not all, of the histopathological presentations of gray matter lesions in MS patients (Kipp et al., 2009; Clarner et al., 2012). The present evidence for axonal and neuronal hyperexcitability in the gray matter of cuprizone-treated mice is, however, noteworthy to relate to clinical observations. Demyelinating lesions and axonal injury in the gray matter are typically observed in 50% of MS patients (Geurts et al., 2005; Geurts and Barkhof, 2008). MS patients often suffer from cognitive symptoms, including memory impairments, attention deficits, and slow sensory processing, which correlate with the extent of lesion load in the gray matter (Chiarevalloti and DeLuca, 2008; Fisniku et al., 2008). The finding of spontaneous APs generated outside of the AIS region may significantly debilitate the capacity to spatiotemporally encode sensory stimuli precisely. Identifying pharmacological means to prevent spontaneous ectopic generation of APs in gray matter lesions may thus benefit axonal function and/or injury and may alleviate some of the cognitive impairments.

Since clinical trials with sodium channel blockers failed to prevent neurodegeneration in MS (Waxman, 2008; Kapoor et al., 2010), the diffuse axonal Kv7.3 subunit channel expression along demyelinated gray matter internodes may provide interesting new directions for pharmacological intervention in demyelinating diseases. Kv7 channels are abundantly expressed in axons, are activated in the subthreshold voltage range, and strongly regulate AP frequencies. Such channels thus represent a promising alternative approach to modify demyelination-induced axonal excitability. Ezogabine (retigabine outside of the United States), a nonselective opener of Kv7.2-containing channels, has been approved for clinical use as an adjunctive treatment against partial epilepsy in adults (Stafstrom et al., 2011) and has been demonstrated to block ectopic APs in peripheral nerves and the neocortex (Straub et al., 2001; Roza and Lopez-Garcia, 2008). Conversely, XE-991 is clinically used as a cognitive enhancer and a highly selective Kv7.2/7.3 blocker (Wang et al., 2000). Examination of axonal excitability in the cuprizone model may thus provide a powerful experimental paradigm for exploration and development of rational therapies for gray matter axonal injury and the debilitating neurological and cognitive symptoms associated with demyelinating CNS disorders.

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Demyelination-induced AIS ion channel expression changes thus may have a marginal impact on the overall neuronal excitability.

Ectopic AP generation in demyelinated gray matter axons

The present findings suggest that hyperexcitability is common in demyelinated gray matter axons. Ectopic APs have been previously reported during in vivo recordings within central and peripheral demyelinated axons (Rasminsky, 1978; Baker and Bostock, 1992; Felts et al., 1995; Kapoor et al., 1997) but never before in the cell bodies in the neocortex of demyelinating lesions. The physiological mechanisms underlying ectopic APs in demyelinated axons may include increased activation of persistent Nav channels, K⁺ ion accumulation in the periaxonal/extracellular space, or an ephaptic coupling between the bare internodes (Rasminsky, 1978; Kapoor et al., 1993). Interestingly, while ectopic APs were observed in both cuprizone-treated groups, application of 4-AP induced a significantly larger increase of ectopic AP generation in acute demyelinated neurons, which were partially myelinated (Fig. 9E). These findings are consistent with the idea that ectopic APs are triggered during increased periaxonal K⁺ accumulation in the vicinity of the MS lesion (Kapoor et al., 1993). In agreement with these mechanisms, a low concentration of TTX or local K⁺ application to demyelinated gray matter internodes was sufficient to eliminate and evoke ectopic APs, respectively.

While the occurrence of spontaneous ectopic APs in our neocortical slices was rare, on average ~0.1 min⁻¹, under in vivo conditions with increased activity, significantly higher local extracellular K⁺ concentrations may accumulate and trigger ectopic APs and aberrant increased network activity. Furthermore, given the integrated role of astrocytes and oligodendrocytes in removing K⁺ around nodal domains during axonal conduction (Rash, 2010), the loss of oligodendrocytes may lead to an impaired glial-mediated K⁺ buffering in the demyelinated cortex. In addition to ectopic APs, the L5 neurons from cuprizone-treated mice also showed increased spontaneous excitability reminiscent of paroxysmal depolarizations reported at the soma from cortical pyramidal neurons in the connexin-32 knock-out mouse, which shows myelin defects (Sutor et al., 2000). The L5 axon provides both a recurrent excitatory feedback signal into the supragranular layers within the same column and in neighboring columns via the horizontally layered collaterals synchronizing activity and generating sensory-evoked network activity (Sanchez-Vives and McCormick, 2000; Oberlaender et al., 2011; Beltramo et al., 2013). Spontaneous hyperexcitability, conduction failure, and/or ectopic APs in demyelinated axons may thus have a widespread impact on the intracortical computational functions of neocortical pyramidal neurons, the extent of which remains to be examined in vivo.

Implications for the therapeutic ion channel treatment in MS

Cuprizone-induced lesions resemble some, but certainly not all, of the histopathological presentations of gray matter lesions in MS patients. The extent of AIS relocation in demyelination (~2 µm) was, however, insufficient to compensate for the increased intrinsic and network activity; both ectopic and spontaneously driven APs occurred more frequently in the L5 neurons in both experimental groups. Demyelination-induced AIS ion channel expression changes thus may have a marginal impact on the overall neuronal excitability.

Ectopic AP generation in demyelinated gray matter axons

The present findings suggest that hyperexcitability is common in demyelinated gray matter axons. Ectopic APs have been previously reported during in vivo recordings within central and peripheral demyelinated axons (Rasminsky, 1978; Baker and Bostock, 1992; Felts et al., 1995; Kapoor et al., 1997) but never before in the cell bodies in the neocortex of demyelinating lesions. The physiological mechanisms underlying ectopic APs in demyelinated axons may include increased activation of persistent Nav channels, K⁺ ion accumulation in the periaxonal/extracellular space, or an ephaptic coupling between the bare internodes (Rasminsky, 1978; Kapoor et al., 1993). Interestingly, while ectopic APs were observed in both cuprizone-treated groups, application of 4-AP induced a significantly larger increase of ectopic AP generation in acute demyelinated neurons, which were partially myelinated (Fig. 9E). These findings are consistent with the idea that ectopic APs are triggered during increased periaxonal K⁺ accumulation in the vicinity of the MS lesion (Kapoor et al., 1993). In agreement with these mechanisms, a low concentration of TTX or local K⁺ application to demyelinated gray matter internodes was sufficient to eliminate and evoke ectopic APs, respectively.

While the occurrence of spontaneous ectopic APs in our neocortical slices was rare, on average ~0.1 min⁻¹, under in vivo conditions with increased activity, significantly higher local extracellular K⁺ concentrations may accumulate and trigger ectopic APs and aberrant increased network activity. Furthermore, given the integrated role of astrocytes and oligodendrocytes in removing K⁺ around nodal domains during axonal conduction (Rash, 2010), the loss of oligodendrocytes may lead to an impaired glial-mediated K⁺ buffering in the demyelinated cortex. In addition to ectopic APs, the L5 neurons from cuprizone-treated mice also showed increased spontaneous excitability reminiscent of paroxysmal depolarizations reported at the soma from cortical pyramidal neurons in the connexin-32 knock-out mouse, which shows myelin defects (Sutor et al., 2000). The L5 axon provides both a recurrent excitatory feedback signal into the supragranular layers within the same column and in neighboring columns via the horizontally layered collaterals synchronizing activity and generating sensory-evoked network activity (Sanchez-Vives and McCormick, 2000; Oberlaender et al., 2011; Beltramo et al., 2013). Spontaneous hyperexcitability, conduction failure, and/or ectopic APs in demyelinated axons may thus have a widespread impact on the intracortical computational functions of neocortical pyramidal neurons, the extent of which remains to be examined in vivo.
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