Kv1 channels identified in rodent myelinated axons, linked to Cx29 in innermost myelin: Support for electrically-active myelin in mammalian saltatory conduction

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Abstract:
Saltatory conduction in mammalian myelinated axons was thought to be well understood before recent discoveries revealed unexpected subcellular distributions and molecular identities of the K⁺-conductance pathways that provide for rapid axonal repolarization. Here, we visualize, identify, localize, quantify, and ultrastructurally-characterize axonal Kᵥ1.1/Kᵥ1.2 channels in sciatic nerves of rodents. Using light-microscopic immunocytochemistry and freeze-fracture-replica immunogold labeling electron microscopy, Kᵥ1.1/Kᵥ1.2 channels are localized to three anatomically- and compositionally-distinct domains in the internodal axolemmas of large myelinated axons, where they form densely-packed “rosettes” of 9-nm intramembrane particles. These axolemmal Kᵥ1.1/Kᵥ1.2 rosettes are precisely aligned with and ultrastructurally-coupled to Cx29 channels, also in matching rosettes, in the surrounding juxtaparanodal myelin collars and along the inner mesaxon. As >98% of transmembrane proteins large enough to represent ion channels in these specialized domains, ~500,000 Kᵥ1.1/Kᵥ1.2 channels define the paired juxtaparanodal regions as exclusive membrane domains for the voltage-gated K⁺ conductance that underlies rapid axonal repolarization in mammals. The 1:1 molecular linkage of Kᵥ1 channels to Cx29 channels in the apposed juxtaparanodal collars, plus their linkage to an additional 250,000-400,000 Cx29 channels along each inner mesaxon in every large-diameter myelinated axon examined, support previously-proposed K⁺ conductance directly from juxtaparanodal axoplasm into juxtaparanodal myeloplasm in mammalian axons. With neither Cx29 protein nor myelin rosettes detectable in frog myelinated axons, these data showing axon-to-myelin linkage by abundant Kᵥ1/Cx29 channels in rodent axons support renewed consideration of an electrically-active role for myelin in increasing both saltatory conduction velocity and maximum propagation frequency in mammalian myelinated axons.

Keywords: Cx29 channels; inner mesaxon; juxtamesaxon; juxtaparanode; Kᵥ1.1/Kᵥ1.2 channels.
For almost a century, neuroscientists have sought to identify commonalities and differences that might be relevant to how large vertebrates and invertebrates separately increased axonal conduction velocity in their fastest-conducting axons (Hodgkin and Huxley 1952). In their pioneering studies, Tasaki (1939) and Huxley and Stämpfli (1949) used dissected sciatic nerves of frogs to show that axonal action potentials in vertebrate myelinated axons result from inward electric current occurring solely at nodes of Ranvier (Fig. 1A; from Tasaki, 1939). Parallel studies of unmyelinated squid giant axons (Hodgkin and Huxley 1952) and myelinated frog axons (Hodgkin 1951) revealed that depolarizing “action currents” occurred in both organisms via Na⁺-selective “carrier” molecules that were diffusely distributed along unmyelinated axons but localized solely to nodes of Ranvier in the myelinated axons of vertebrates (Huxley and Stämpfli 1949; Hodgkin 1951). Likewise, rapid axonal repolarization was shown to occur via separate carrier molecules for K⁺ that were diffusely distributed along squid giant axons but whose distribution in frog myelinated axons could not be determined by methods then available (Hodgkin 1951). Despite the caution of the original investigators, later investigators proposed that: 1) voltage-gated K⁺ channels were co-localized with Na⁺ channels at nodes of Ranvier [Fig. 1B, from Purves (2012)]; 2) myelin passively increases conduction velocity by eliminating the internodal submyelinic extracellular space, thereby reducing axolemmal capacitance and, consequently, the amount of Na⁺ influx needed to overcome that capacitance during axonal depolarization [concepts summarized in Peles, et al. (2000)]; and 3) the presumed absence of submyelinic extracellular space into which ions could flow was thought to preclude the occurrence of any ion channels in the internodal axolemma, thereby presumably minimizing internodal current loss and increasing the axonal “length constant” (Figs. 1A,B; right side).

Three decades later, voltage-gated Na⁺ channels were localized solely to nodes of Ranvier in mammalian myelinated axons (Ritchie and Rogart 1977; Waxman and Ritchie...
However, neither voltage-gated $K^+$ currents nor $K^+$ channels could be detected at nodes of Ranvier in undamaged mammalian axons. Instead, voltage-gated $K^+$ currents were demonstrated in rodent optic nerve prior to myelination but became undetectable immediately after the first myelin layers were laid down (Connors et al. 1982), suggesting that $K^+$ efflux normally occurred within the internodes of adult mammals but was “masked” by even a few layers of myelin. In support, detachment of paranodal myelin loops by diverse mechanical and biochemical approaches (Brismar 1979;Sherratt et al. 1980;Bostock et al. 1981) allowed the first detection of internodal voltage-gated $K^+$ currents (Chiu and Ritchie 1980). Moreover, upon acute demyelination, the previously-detected internodal $K^+$ “leak” conductance (Chiu et al. 1979) appeared to be converted instantaneously into conventional voltage-gated $K^+$ current (Chiu and Ritchie 1980), but no mechanism was proposed to account for that rapid conversion. Instead, submyelinic $K^+$ currents were proposed to be masked by electrical barriers ascribed to the spiral septate junctions that link the paranodal loops of myelin to the axolemma (Livingston et al. 1973). However, that explanation was questioned because microperoxidase (mw 1,900) and colloidal lanthanum hydroxide applied outside intact mammalian axons rapidly diffused into and delineated the submyelinic extracellular space (Hirano and Dembitzer 1969, 1982;Feder 1971;MacKenzie et al. 1984), implying that smaller $K^+$ ions should even more readily bypass those leaky barriers for easy detection.

To identify possible anatomical/molecular pathways for the newly-discovered internodal $K^+$ efflux (Chiu and Ritchie 1980), early freeze-fracture studies from rat sciatic nerve revealed distinctive hexagonal “rosettes” of 11-nm intramembrane particles (IMPs) in the internodal axolemma, restricted to and defining the “juxtaparanodal” region (Stolinski et al. 1981). Similar IMPs were also localized to two narrow bands that followed the inner mesaxon from node to node (Stolinski et al. 1981, 1985). (Anatomical regions of myelinated axons described in this report are illustrated in Fig. 1C.) Based on their structural similarity to known ion channels and their localization to the juxtaparanodal and inner-mesaxonal axolemma, Stolinski et al. (1981)
proposed that the axonal rosette particles corresponded to the newly-detected internodal voltage-gated K⁺ channels (Chiu and Ritchie 1980). Remarkably, where the fracture plane stepped from axolemma to myelin within a rosette, the axolemmal rosette particles were directly apposed to and precisely aligned with matching 11-nm particles, also in rosettes, in innermost myelin, leading Stolinski et al. (1981) to propose direct ionic coupling of axoplasm with myeloplasm via these structurally-coupled channels.

Subsequent immunofluorescence labeling revealed that Kv1.1 and Kv1.2, the primary voltage-gated K⁺ channels of fast-conducting axons in the mammalian CNS and PNS, were not at nodes, as is still widely taught (Fig. 1C), but instead, are restricted to the juxtaparanodal region and to a narrow ribbon that follows the inner mesaxon (Wang et al. 1993; Vabnick et al. 1999; Chiu et al. 1999; Rasband and Shrager 2000; Bhat et al. 2001; Rasband et al. 2001; Altevogt et al. 2002; Rios et al. 2003), precisely where Stolinski et al. (1981; 1985) had mapped the particle rosettes. Curiously, Kv1.2 immunofluorescence was tightly co-localized with immuno-fluorescence for connexin29 (Cx29) (Altevogt et al. 2002), a 29 kDa connexin protein (equivalent to human GJC3) that is unique because it does not form gap junctions with itself or any other connexin (Altevogt and Paul 2004; Ahn et al. 2008) and, thus, has no known function. Independently, freeze-fracture replica immunogold labeling (FRIL) revealed that the rosettes in rodent myelin are composed of Cx29 (Li et al. 2002). However, the axolemmal rosette particles remained unidentified.

Here, we used immunofluorescence microscopy and FRIL to positively identify Kv1.1 within the axonal rosette particles in rodent sciatic nerve, and to demonstrate precise 1:1 molecular co-alignment of Kv1.1-containing channels with Cx29 channels in apposing myelin. Our data, in combination with published electrophysiological data, support the proposal of an electrically-active role for myelin in axonal saltatory conduction in mammals and suggest that axo-glial Kv1/Cx29 channels may underlie the faster axonal repolarizations and/or faster conduction velocity of mammalian myelinated axons.
METHODS

Animals. Animals used in this study included a total of 12 adult Sprague-Dawley rats from which tissues were taken for analysis by immunofluorescence. Three male wild-type (WT) and three male Cx29 knockout (ko) C57BL/6 mice (Eiberger et al., 2006) were generously provided by Klaus Willecke (Berlin, Germany) for tests of specificity of Cx29 detection by anti-Cx29 antibodies. For FRIL, sciatic nerve from two WT C57BL/6 mice (one male, one female) and from one male Cx29 ko mouse (courtesy of Dwight Bergles, Johns Hopkins University, Baltimore, MD) were fixed with 4% formaldehyde. In addition, segments of unfixed sciatic nerve were from a Cx32 knockout C57BL/6 mouse from a colony established at the Colorado State University animal facility (two breeding pairs provided by Carola Meier from animals provided by Klaus Willecke, then at the University of Bonn, Bonn, Germany) (Nelles et al. 1996), as reported (Meier et al. 2004). To assess possible species-specific or fixation-dependent differences in rosette morphology in FRIL samples in mice vs. the rosette morphology previously reported in glutaraldehyde-fixed sciatic nerve from rat (Stolinski et al. 1981), an additional WT mouse was fixed by perfusion with 2.5% glutaraldehyde for conventional freeze-fracture analysis. Animals were utilized according to protocols approved by the Central Animal Care Committee of the University of Manitoba, and the institutional Animal Care and Use Committees at Colorado State University and John Hopkins Medical Institutions, with minimization of pain and stress and minimization of the numbers of animals used.

Antibodies. The antibodies used for immunofluorescence studies (with dilution or concentration used, catalogue number, and source) included: polyclonal anti-Cx29 (2 μg/ml, 48-7700; ThermoFisher Corporation, Grand Island, NY, formerly Invitrogen/Zymed Laboratories); monoclonal anti-Kv1.2 and anti-Kv1.1 (2 μg/ml, catalog numbers 75-008 and 75-105, respectively; Antibodies Inc., Davis, CA); rabbit polyclonal pan anti-sodium channel antibody NaV (AB5210; Millipore, Temecula, CA); and monoclonal anti-caspr antibody,
which was generously provided by Dr. E. Peles (Weizmann Institute of Science, Rehovot, Israel).

**Fixation and sample preparation for light microscopic immunohistochemistry.** Rats were deeply anesthetized with Equithesin (3 ml/kg) and perfused transcardially with 40 ml of "prefixative" solution consisting of cold (4°C) 25 mM sodium phosphate buffer, pH 7.4, 0.9% NaCl (PBS), 0.1% sodium nitrite, and heparin (1 unit/ml). This was followed by perfusion with 1 ml per gram body weight of cold 0.16 M sodium phosphate buffer, pH 7.4, containing 4% formaldehyde, followed by perfusion with 40 ml of 25 mM phosphate buffer, pH 7.4, containing 10% sucrose. Sciatic nerves were removed and stored at 4°C for 48 h in cryoprotectant (10% sucrose in 25 mM phosphate buffer). Cryostat sections (10 μm thick) were collected on gelatinized glass slides, and processed for immunofluorescence with primary and secondary antibodies diluted in 50 mM Tris-HCl, pH 7.4, containing 1.5% NaCl, 0.3% Triton X-100 (TBST) and 4% normal donkey serum.

**Immunolabeling.** For single labeling, sections were incubated for 24 h at 4°C with primary antibody, then washed for 1 h in TBST, and incubated for 1.5 h at room temperature with appropriate secondary antibody. For double immunofluorescence labeling, sections were incubated simultaneously with two different primary antibodies generated in different species, then washed in TBST for 1 h at room temperature and incubated simultaneously with appropriate secondary antibodies. Sections were washed for 20 min in TBST, followed by two 20 min washes in 50 mM Tris-HCl buffer, pH 7.4, and cover-slipped with antifade medium Fluoromount-G (SouthernBiotech, Birmingham, AL). To establish absence of inappropriate cross reactions of primary with secondary antibodies, control procedures included omission of one of the primary antibodies with inclusion of each of the secondary antibodies. Secondary antibodies included Cy3-conjugated goat or donkey anti-mouse IgG diluted 1:600 (Jackson ImmunoResearch Laboratories, West Grove, PA), Alexa Flour 488-conjugated goat or donkey anti-rabbit and anti-mouse IgG diluted 1:600
Light microscopic analysis. Immunofluorescence was examined on a Zeiss Axioskop2 fluorescence microscope, using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, Canada) for capturing images, and a Zeiss 710 laser scanning confocal microscope using ZEN 2010 image capture and analysis software. Data from wide-field and confocal microscopes were collected either as single scan images or z-stack images, with multiple scans capturing a thickness of 2 to 6 μm of tissue at z scanning intervals of 0.4 to 0.6 μm. Final images were assembled according to appropriate size and adjusted for optimal signal-to-noise presentation using CorelDraw Graphics (Corel Corp., Ottawa, Canada) and Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

Freeze fracture and FRIL: As freeze-fracture and FRIL techniques have traditionally not been published in this Journal, but are among the only current approaches that allow direct visualization, identification, quantification, ultrastructural characterization, and subcellular localization of membrane proteins such as those examined here, we provide relatively more detailed information regarding pertinent aspects of replica formation, immunogold labeling, and image interpretation. In initial FRIL experiments, we used unfixed tissues remaining from previous FRIL studies of Cx32 and Cx29 in sciatic nerve of Cx32 ko mice (Li et al. 2002;Meier et al. 2004) wherein we made preliminary determinations of: a) the axonal types and ultrastructural locations of Kv1.1 and Cx29 in myelinated axons; b) whether Kv1.1 forms either axolemmal P-face or E-face particles, or a mixture of particles on both E- and P-faces; c) whether antibody labeling efficiency (LE) was significantly reduced by formaldehyde fixation as compared with labeling in unfixed, rapidly-frozen samples, and d) whether formaldehyde fixation for FRIL accurately preserves the rosettes that previously had been demonstrated only in glutaraldehyde-fixed rat sciatic nerve (Stolinski et al. 1981, 1985). We then directly compared those unfixed Cx32 ko samples with samples from unfixed, formaldehyde-fixed, and
glutaraldehyde-fixed WT mice vs. formaldehyde-fixed and glutaraldehyde-fixed Cx29 ko mice.

**Perfusion fixation and cryoprotection.** Mice and rats were deeply anesthetized with ketamine and xylazine (80 mg/kg and 8 mg/kg, supplemented to effect). To determine whether formaldehyde fixation reduces FRIL labeling efficiency (LE) from that in unfixed samples, segments of either left or right sciatic nerve were removed prior to whole-body perfusion with 1%, 2%, or 4% formaldehyde (Ted Pella, Inc., Redding, CA) in 0.15 M Sorensen’s phosphate buffer (SPB), with the left and right sciatic nerves yielding both formaldehyde-fixed and unfixed samples from the same animal. Additional matched samples for high-resolution conventional freeze-fracture analysis were obtained by the same procedures, but using 2.5% glutaraldehyde instead of formaldehyde as the fixative. Because glutaraldehyde fixation results in formation of covalent bonds between cross-linked protein molecules (Johnson 1987), preventing most proteins from being washed from the replicas by SDS detergent, the resulting undigested tissue remnants completely block penetration of the electron beam that is used for image formation in transmission electron microscopy (Fujimoto 1995; Fujimoto 1997). Thus, glutaraldehyde-fixed tissues cannot be used for FRIL. Instead, glutaraldehyde-fixed samples were used to determine whether the IMP rosette in axons or myelin were altered or disrupted by formaldehyde fixation or by the procedures used to cryoprotect and freeze unfixed tissue. In all cases, a 10-mm incision was made in the skin overlying the “sciatic notch” in the upper thigh, the sciatic nerve was exposed, and a 10-mm segment of nerve was dissected free and either infiltrated with 30% sucrose (unfixed samples) or placed into buffered formaldehyde or glutaraldehyde for 1 h at 4°C, and then cryoprotected with 30% glycerol, as detailed below.

**Immersion fixation of frog sciatic nerves.** Two adult leopard frogs (*Rana pipiens*, 3.5” body length) and three adult American green tree frogs (*Hyla cinerea*; 1.2-2.3 gm) were anesthetized with MS222 (tricaine) in frog Ringer’s solution. The sciatic nerves were exposed and immersed in either 2.5% glutaraldehyde, 4% formaldehyde, or sucrose in Ringer’s solution,
then dissected free and prepared for freeze-fracture and FRIL by the same procedures used for mammalian tissues.

Determination of labeling efficiencies in formaldehyde-fixed vs. unfixed tissues. To compare FRIL LE’s and labeling specificity for K\textsubscript{v}1 and Cx29 in formaldehyde-fixed vs. unfixed tissues, the sciatic nerves of one Cx32 ko mouse and one WT mouse (see below) were removed without fixation, immersed for 30 minutes in SPB containing 30% sucrose as a cryoprotectant, and slam-frozen. Other samples, including samples from both formaldehyde-fixed and unfixed WT mouse, were infiltrated at 4°C with increasing concentrations of sucrose or glycerol (to 30% in SPB) as a “cryoprotectant”, then rapidly frozen (Chandler and Heuser 1979; Heuser et al. 1981) by contact with a liquid-nitrogen-cooled ultra-pure copper block (Ultra-Freeze MF7000; RMC Products, Tucson, AZ). Cx32 ko samples had been stored for 10-15 years in liquid nitrogen.

High-resolution freeze-fracture replication to resolve molecular substructure. Samples were freeze-fractured in a JEOL JFD-2 freeze-etch machine and replicated via unidirectional thermionic deposition of a 1-2 nm-thick “pre-carbon” coat, followed immediately by 1-1.5 nm of platinum/carbon, with both substances applied using separate electron beam guns. The “pre-carbon” coat slightly increases diameter of IMPS and decreases the size of membrane pits. However, in addition to decreasing the granularity of the platinum-replica film, thereby greatly improving imaging resolution (Kamasawa et al. 2006), it also has the advantage that it increases LE (Fujimoto 1995; Masugi-Tokita et al. 2007; Schlormann et al. 2007). After platinum shadowing, the replication process is completed by immediately coating the samples with ca. 20 nm of “post-carbon” applied with constant tilting and rotation.

SDS washing and blocking of sites for non-specific adsorption of antibodies. Replicated samples were prepared for FRIL [name coined by Gruijters et al. (1987)] according to our detailed procedures (Rash and Yasumura 1999; Kamasawa et al. 2005; Kamasawa et al. 2006), as modified from Fujimoto (1995; 1997). After freeze-fracture replication, samples were
digested in 1.7% SDS detergent in 30 mM sucrose and 15 mM Tris HCL pH 8.9 at 60°C for 12 h, rinsed briefly in distilled water, enzymatically digested for 6-8 h in 4% collagenase D (Roche Applied Science, Indianapolis, IN) in 0.15 M SPB at 44°C, followed by washing for an additional 10-12 h in 3% SDS solution (no sucrose added) pH 8.9 at 80°C, with intermittent agitation. To reduce non-specific adsorption of both primary and secondary antibody labels, washed replicas were rinsed for 3-12 h in “blocking buffer” (Dinchuk et al. 1987), consisting of 10% heat-inactivated goat serum plus 1.5% fish-gelatin digest (Sigma-Aldrich, St. Louis, MO) in 0.15 M SPB, and then immunogold labeled (Rash and Yasumura 1999; Kamasawa et al. 2006). [See Rash and Yasumura (1999) for methods to minimize background labeling “noise” and to discriminate “signal” from noise in FRIL.]

Analysis of primary antibodies directed against cytoplasmic vs. extracellular epitopes. For FRIL, primary antibodies included polyclonal rabbit antibodies against cytoplasmic epitopes of Cx29 (Catalog #48-7700; Invitrogen, Camarillo, CA) and monoclonal mouse anti-Kv1.1 separately directed against extracellular epitopes (# 75-105) and cytoplasmic epitopes (# 75-007). Because Kv1.1 and Kv1.2 must be co-expressed for stability and functionality of channels (Dodson et al. 2002; Al-Sabi et al. 2010), because both Kv1.1 and Kv1.2 were confirmed by fluorescence microscopic immunocytochemistry to be equally co-localized at juxtaparanodes and along inner mesaxons (Figs. 2-4), and because the antibodies currently available against Kv1.2 proved inadequate for FRIL, we used FRIL to determine the ultrastructural distribution of Kv1.1 as a proxy for both Kv1.1 and Kv1.2 in diverse axonal subdomains. In any case, ambiguities introduced by the “radius of uncertainty” of immunogold labeling (Kamasawa et al. 2006) do not yet permit determination of whether Kv1.1 and Kv1.2 are within the same intramembrane particle, or indeed within the same rosette. That determination is outside the scope of the current report.

Secondary antibodies and “dual double-labeling”. Secondary antibodies used in FRIL included goat anti-mouse IgG conjugated to 5-nm and 20-nm gold beads (dual-
labeling for a single protein), and goat anti-rabbit IgG conjugated to 10-nm and 30-nm gold beads (BBI Solutions, Madison, WI) (dual-labeling for a second protein), the combination reflecting “dual-double-labeling”. The 20- and 30-nm gold beads in each labeling cocktail allowed larger labels to be detected at low magnification (3,000x-5,000x), whereas the 5- and 10-nm gold beads, because of their much higher LE, were used to optimize LE and obtain high signal-to-noise ratios (Rash and Yasumura 1999). An additional advantage in using two sizes of gold beads for each labeled protein is that two independent labels against each primary antibody provides for internal verification of labeling specificity of both primary and secondary antibodies, as well as allows us to determine whether antibody clumping occurred for either the primary or secondary antibodies (Rash and Yasumura 1999).

Replicas were incubated simultaneously with both monoclonal (e.g., mouse anti-Kv1) and polyclonal antibodies (e.g., rabbit anti-Cx29), washed in SPB for 1 h at room temperature, and incubated simultaneously with appropriate gold-conjugated species-specific secondary antibodies. After rinsing and air-drying, samples were coated again with 20 nm of carbon on the labeled side to immobilize gold labels and to anneal thermal-expansion cracks formed when the replicated samples were warmed from −170°C to +22°C for subsequent immunolabeling. This secondary carbon layer provides additional structural support for the replica during removal of the Lexan film by ca. 10 hr immersion of the grids in 60-80°C dichloroethane.

Determination of “sidedness” of labeling for Kv1.1, Kv1.2, and Cx29. In SDS-washed FRIL replicas, antibodies cannot penetrate the atomic lattices of the thermionically-deposited platinum/carbon replica and therefore cannot label the epitopes on the replicated side of exposed proteins, but instead, are able to label only the unshadowed epitopes of those same proteins (Dinchuk et al. 1987; Rash et al. 1989; Fujimoto 1995; Fujimoto 1997; Rash and Yasumura 1999). Thus, to identify IMPs that might correspond to Kv1.1-containing channels in
sciatic nerve, it was first necessary to select appropriate antibodies for FRIL, which would depend on whether the corresponding IMPs remained adherent to the newly-created extracellular (extracellular) membrane leaflet (or E-face), to the complementary “protoplasmic” membrane leaflet (P-face), or fractured randomly to both E- and P-faces. [Membrane P- and E-fracture faces are designated according to the internationally-recognized terminology of Branton et al. (1975).] This topological feature provides a simple mnemonic: E-face particles label only with antibodies against extracellular epitopes, whereas P-face particles label only with antibodies against cytoplasmic epitopes.

“Complementarity” of membrane faces allows determination of total content of intramembrane proteins. A basic premise of freeze-fracture and FRIL, established by experiments spanning 30 years, is that all transmembrane proteins, including all ion channels, are cleaved intact, without breaking covalent bonds (Fisher and Yanagimoto 1986), leaving an IMP projecting from one fracture face and a corresponding pit in the fracture face that was cleaved away (Steere and Moseley 1969; Challcroft and Bullivant 1970; Ting-Beall et al. 1986; Li et al. 2008). [Energy released per unit area is insufficient to break covalent bonds; 50-250 kCal/mol vs. 0.1-1 kCal/mol to break Van der Walls and London dispersion forces during separation of transmembrane proteins from surrounding lipids and ice (Bailey et al. 1990; Lodish et al. 2000).] Moreover, by subtracting the platinum-shadow thickness, the diameter of each IMP has been shown to be proportional to the square root of the number of transmembrane alpha-helices multiplied by 1.3-1.4 nm per α-helix (Eskandari et al. 1998; Rash et al. 2004a), ranging from very small IMPs [ca. 2-3 nm for replicated proteins having a single transmembrane alpha-helix (Dinchuk et al. 1987), to 9-10 nm IMPs for 24-pass and 28-pass proteins, such as the hexameric arrangement of connexins within a connexon (Rash et al. 2004a), pentameric glutamate receptors (Rash et al. 2004b), and the tetrameric voltage gated K+ channels shown here. In replicas made with 1.0-1.5 nm of platinum, examining both E- and P-fracture faces from the same subcellular location allows us to reliably discern, count, and
measure ion channels as IMPs in one fracture face, and to verify those counts by quantifying the corresponding pits in the complementary fracture face. Thus, based on identification of Cx29 as the primary or sole large-diameter protein in innermost myelin P-faces (Li et al. 2002), we were able for the first time to predict the morphology and then to find and quantify the complementary rosettes of pits in the E-face of innermost myelin. Equally important, based on the current identification of K\textsubscript{v}1.1/K\textsubscript{v}1.2 as the primary or sole large-diameter protein in axolemmal E-faces, we were able to identify the complementary P-face of the juxtaparanodal axolemma, and from those images, to visualize and quantify the unexpectedly complex molecular architecture of the juxtaparanodal axolemmal P-face, and to distinguish large-diameter ion channels from their small-diameter trans-membrane, extracellularly-projecting “tether” molecules.

Transmission electron microscopy and stereoscopic analysis. Freeze-fracture and FRIL replicas were examined in JEOL JEM2000 EX-II and JEM1400 transmission electron microscopes, both operated at 100 kV. To establish “sidedness” of labels [as an aid to distinguishing “signal” from “noise” (Rash and Yasumura 1999)], to analyze the three-dimensional topography, and to optimize image contrast, replicas were tilted up to ±60° and photographed as stereoscopic pairs having an included angle of 8° (Steere and Rash 1979). Electron microscope negatives from the JEOL 2000 were digitized using an ArtixScan 2500f digital scanner (Microtek, Carson, CA), whereas digital images were obtained from the JEOL 1400 using a 11 MB Orius SC1000 camera (Gatan, Inc., Pleasanton, CA). All images were processed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA), with “Levels” used for contrast expansion and “Brightness/Contrast” used to optimize image contrast. Extremes of photographic contrast of replica fragments and displaced sample debris (when present) were reduced using local area “dodging”, as indicated in appropriate images.
RESULTS

*Light microscopic immunocytochemistry.* As shown in overview (Fig. 2), the general features of immunofluorescence labeling for Cx29, Kv1.1, and Kv1.2 in rat and mouse sciatic nerve were similar to previous descriptions (Wang et al. 1993; Vabnick et al. 1999; Bhat et al. 2001; Altevogt et al. 2002; Rios et al. 2003), but with several significant additional details revealed. Cx29 labeling was most concentrated at juxtaparanodal regions (Fig. 2, arrows), with moderate labeling at internodal funnel-shaped collars that extended from the surface of myelin at their expanded end to a convergence on the axon at their narrow end (Fig. 2A1, arrowheads), suggestive of labeling of Schmidt-Lanterman incisures, extending the findings of Altevogt et al (2002). Additional internodal Cx29 was often localized to long filaments or strands of labeling associated with and running along the length of the axon (Fig. 2A1, double-headed arrows). Immunofluorescence labeling patterns of the two Kv1 channels (Kv1.1 and Kv1.2) were indistinguishable one from the other. As shown for Kv1.1 (Fig. 2A2), labeling was most dense at juxtaparanodes, followed by moderate labeling localized as narrow, intermittently occurring circumferential bands (“C-bands”) along internodal regions (arrowheads), and by labeling of variable intensity localized as thin strands running along internodal axons (double-headed arrows). Cx29 was co-localized with the Kv channels at many but not all of these regions (Fig. 2A3) (details described below).

Labeling patterns for Cx29 and the Kv1.1 and Kv1.2 channels observed in sciatic nerve of rat were also evident in the sciatic nerve of mouse, and all labeling patterns of Cx29 observed in sciatic nerve of WT mice (Fig. 2B) were absent in Cx29 ko mice (Fig. 2C), indicating specificity of labeling with the anti-Cx29 antibodies used and complete absence of Cx29 protein in the ko mice.

More detailed views of Cx29 and Kv channel labeling at regions adjacent to nodes of Ranvier are shown in Figure 3. In addition to the dense localization of Cx29 and Kv1 channels at the juxtaparanodal region, labeling for these proteins along internodal strands corresponding
to their localization to the inner mesaxon is seen extending into this region (Fig. 3A). Labeling for Cx29, together with faint labeling of both Kv1.1 and Kv1.2, is also seen in the paranodal region (Fig. 3B, double arrowhead). Occasionally, Kv1 labeling is resolved as one to three narrow bands straddling nodes of Ranvier (Fig. 3B2). The degree of Cx29 co-localization with the paranodal Kv1 bands was variable, from nearly total co-localization (Fig. 3C), only partial co-localization (Fig. 3D), or apparently separate, with Kv1 channels positioned closer to the node and Cx29 closer to juxtaparanode (Fig. 3E). Labeling of the transverse Cx29 bands was identified as intra-paranodal based on its co-localization with the paranodal marker caspr (Gordon et al. 2014) (Fig. 3F). The paranodal Kv1 labeling did not extend into the nodes of Ranvier, and no discernible labeling for either Kv1.2 (Fig. 3G) or Kv1.1 (Fig. 3H) could be detected at the nodal cleft, the exact location of which was identified by its dense labeling for NaV (Figs. 3G1 and 3H1). (Note: Image halation at high fluorescence amplification can produce some minor overlap of fluorescence signals.)

Localization of Cx29 and the Kv1 channels along internodal regions is shown in greater detail in Figure 4. Tortuous, often spiral strands of continuous labeling for these proteins, co-localized as thin filaments or ribbons at the inner mesaxon (Altevogt et al. 2002), could sometimes be followed for relatively long distances, including regions where the filaments seemed to maintain their continuity upon crossing the C-bands at the adaxonal extent of Schmidt-Lanterman incisures (Fig. 4A and Fig. 4B2, right arrowhead). The funnel-shaped incisures, delineated by their labeling for Cx29, were oriented with adjacent incisures having their narrow ends either pointing away from each other or pointing in the same direction (both configurations illustrated in Fig. 4B). Internodal labeling of Kv1 channels, localized as narrow “C-bands” encircling the axons (Fig. 4B1), were situated at the extreme narrow ends of the Cx29-positive Schmidt-Lanterman incisures (Fig. 4B2, arrows and arrowheads). The bands of Kv1 channels at these locations actually consisted of a doublet of bands in the case of both Kv1.1 (Fig. 4C1) and Kv1.2 (not shown), and it appeared that only one of the pair of bands
displayed overlap with labeling of Cx29 at the narrow end of incisures that converged on it (Figs. 4C2, 4D).

**FRIL analysis of Kv1.1 and Cx29.** In initial FRIL experiments, we first analyzed freeze-fractured samples of sciatic nerve that remained from an earlier study (Li et al. 2002; Meier et al. 2004) involving FRIL analysis of Cx32 and Cx29 in samples from sciatic nerve of unfixed Cx32 knockout (Cx32 ko) mice (Figs. 5B, 6A,B), and directly compared those images from sciatic nerve of formaldehyde-fixed WT mice (Figs. 6C, 7A,B) with images from unfixed sciatic nerve (not shown). All data presented are from large and medium-diameter myelinated axons (primarily alpha, beta and gamma motor axons and large primary 1A afferent sensory fibers), all of which were previously established to express both Kv1.1 and Kv1.2 but not Kv1.4 within their hetero-tetrameric channels, whereas the small-diameter A-delta myelinated “fast pain” fibers and unmyelinated C fibers express Kv1.4 as their primary K⁺ channels, usually co-expressed with Kv1.1 (Rasband et al. 2001).

There are 50-100 potential fracture planes within each large-diameter myelinated axon, but there is only one potential fracture plane -- along the lower surface of each axon (Fig. 5A) -- that exposes the E-face of the juxtaparanodal axolemma (JPAX), revealing its distinctive rosettes of E-face particles (Figs. 6, 8A), and there is only one fracture plane -- along the upper axonal membrane -- that reveals the previously-unrecognized JPAX P-face (JPAXᵣ), with its unexpected ultrastructural complexity (detailed below). Likewise, there is only one fracture plane that exposes the P-face of the adjacent juxtaparanodal innermost myelin (JPIM), revealing its distinctive rosettes of P-face particles (Figs. 6A, 7B), and only one fracture plane that exposes the complementary JPIM E-face, with its distinctive rosettes of pits (Figs. 7C).

From the few areas containing internodal axonal E-face and/or adaxonal myelin P-face that were encountered in each replica, only 1-2% of those were fractured within the 10-15 µm-long juxtaparanodal region, with the other 98% fracturing within the roughly 800 µm-long segment of internodal myelin or within deeper layers of compact myelin. Based on the relatively
rare fractures within juxtaparanodal membranes, several anatomically-instructive images containing almost all regions relevant to this study are from a fortuitous single small area from an unfixed sample from a Cx32 ko mouse sciatic nerve (Figs. 5B, 6A,B). We utilize those images primarily for anatomical reference purposes, because as documented below, the axolemmal regions and molecules identified in this Cx32 ko mouse were indistinguishable from those from similar areas in WT mice (Figs. 6C, 7). Moreover, this replica from Cx32-ko mouse had additional advantages, including a large area of juxtaparanodal apposition containing contiguous areas of both axolemmal E-face and innermost myelin P-face (Figs. 5B, 6A,B); an optimum shadow thickness, resulting in both high-resolution replication of IMPs and pits and high-visibility of the smallest gold beads (Fig. 6B; yellow arrowheads); and optimum LE for both Kᵥ1.1 and Cx29 (i.e., ca. 1:6, or ~1 gold bead per rosette) (Figs. 6A,B). Additional replicas from WT mice were prepared so as to have higher LE for quantitative analysis. (Figs. 7A,B). Although high numbers of gold beads potentially could obscure important replica details, those replicas provided positive demonstration that virtually all 9-nm IMPs in JPIM P-faces were immunogold labeled (Fig. 7B), as detailed below.

Nodes of Ranvier, identified by imprints of paranodal loops that mark the proximal and distal boundaries of the nodal plasma membrane, were devoid of gold beads labeling for either extracellular or cytoplasmic epitopes of Kᵥ1.1 on either P- or E-faces, thereby documenting complete absence of Kᵥ1.1 from rodent nodes of Ranvier, further contradicting proposals for voltage-gated K⁺ conductance within nodes (Fig. 1B). In contrast, juxtaparanodal regions of the axolemma were labeled at high density for Kᵥ1.1 (detailed next), whereas the paranodes had only a few axolemmal rosettes between imprints of paranodal loops, many of which were labeled for Kᵥ1.1 (not shown), consistent with faint immunofluorescence labeling for this protein within some paranodes (Fig. 3B2).

A low-magnification stereoscopic overview image from the juxtaparanodal region (Fig. 5B) is enlarged to show a portion of the axon E-face (Fig. 6A, AxE) and a relatively large
portion of the innermost myelin P-face (Fig. 6A, MyP), which is seen at still higher magnification in Figure 6B. The JPAX domain has distinctive, densely-packed hexagonal “rosettes” and irregular clusters of 9-nm E-face particles, many of which were dual-labeled by immunogold antibodies against extracellular epitopes of Kv1.1 (10-nm and 30-nm gold beads; ca. one gold bead per 12 IMPs). In this dual-double-labeled sample, myelin P-faces exhibited densely-packed IMP rosettes that were dual-labeled by antibodies against cytoplasmic epitopes of Cx29 (5-nm and 20-nm gold beads), consistent with our previous identification of the myelinic P-face rosette particles as Cx29 (Li et al. 2002). The red two-ended arrow in Figures 6A distinguishes 20-nm from 30-nm gold beads, and the yellow two-ended arrow in Figure 6A,B distinguishes 5-nm gold from 10-nm gold beads. The larger gold beads in each labeling combination acted as visual “flags” to facilitate finding labeled areas at 3,000X-5,000X “survey” magnifications, whereas the smaller gold beads, with their 10-fold higher LE but low detectability below 30,000X, were used for quantitative analysis. See METHODS, for additional rationale for “dual-double-labeling”.

In the apposed JPIM P-face, densely-packed rosettes and irregular clusters of 9-nm P-face particles are visible (Fig. 6A-C; aqua overlays), many of which were labeled for Cx29 (mostly 5-nm and a few 20-nm gold beads). In the JPIM collar of a heavily-labeled replica from a WT mouse (Figs. 7A,B), the LE for Cx29 was ca. 1:3 (i.e., ca. 2 gold beads per rosette). Approximately the same high LE also occurred beneath both dispersed 9-nm IMPs and those still clustered in rosettes and strings, demonstrating that during formaldehyde fixation, the almost pure population of Cx29 rosettes seen in glutaraldehyde-fixed samples (Fig. 7C) is variably dispersed, often into strings, clusters, and individual 9-nm IMPs, but invariably containing at least a few recognizable rosettes (Fig. 6B,C; 7A,B).

Kv1.1 rosettes label only with antibodies against extracellular epitopes. In multiple replicas from WT and Cx32 ko mice, axonal E-face particle rosettes were abundantly labeled by antibodies against extracellular epitopes of Kv1.1 (Figs. 5B, 6A,B). To test whether
antibodies directed against cytoplasmic epitopes of Kv1.1 also labeled the juxtaparanodal axolemmal rosettes or labeled any other IMPs in nodes of Ranvier or in any other regions of the axolemma, we dual-double-labeled other samples from WT mice using antibodies against cytoplasmic epitopes of Kv1.1, in combination with our standard antibodies against cytoplasmic epitopes of Cx29. In the JPAX E-face, Kv1.1-containing rosettes were abundant (Figs. 6C, 8A), but none were labeled by antibodies against their cytoplasmic epitopes, even though nearby myelin P-face rosettes were strongly labeled with antibodies against cytoplasmic epitopes of Cx29 (Figs. 6C). The latter area also served as an internal “control” to reveal that potentially excessive SDS washing had not removed other nearby proteins, which then remained for FRIL labeling. Also noteworthy, antibodies against cytoplasmic epitopes of Kv1.1 did not label axonal P-face IMPs at nodes, paranodes, inner mesaxon, or internodal axolemma. Thus, combined with strong labeling of axolemmal E-face rosettes (Figs. 6, 9A,B), the absence of P-face labeling confirms that most, if not all, freeze-fractured Kv1.1 channels remained as E-face particles within the internodal axolemmas in both the JPAX and juxtamesaxonal axolemma (JMAX), as further detailed below. Thus, these immunogold-labeling data reveal that the “particle partitioning coefficient” (Satir and Satir 1979) for Kv1.1-containing channels is 100% to the axolemmal E-face particles and 0% to any of the few particles found on the axolemmal P-faces. (JPAX P-face pits are described below). Because Kv1.1 and Kv1.2 must be co-expressed for stability and functionality of K+ channels (Dodson et al. 2002;Al-Sabi et al. 2010), and because both Kv1.1 and Kv1.2 were confirmed by immunocytochemistry to be co-localized at juxtaparanodes and inner mesaxons (Figs. 2-4), we used FRIL of Kv1.1 as a proxy for both proteins.

**Complementarity of membrane faces allows quantification of all ion channels in JPAX and JPIM.** To investigate the basis for the inability of previous investigators to find the complementary “pit” images of the JPAX and JPIM rosettes (Miller and Pinto da Silva 1977;Stolinski et al. 1981;Stolinski et al. 1985), and moreover, to determine if any other large
transmembrane proteins were present in those previously-unrecognized membrane faces, we examined conventional freeze-fracture replicas of glutaraldehyde-fixed (i.e., not labeled) sciatic nerve from WT mice. In stereoscopic images of the JPIM E-faces, we discovered densely-packed rosettes of myelin E-face pits (Fig. 7C), for the first time revealing the complementary impressions of the Cx29 P-face rosettes. These new images were extremely informative, revealing molecular details that could otherwise not be determined. Especially noteworthy was that very few IMPs of any size class were intermixed with the E-face rosettes of pits in the juxtaparanodal collars (Fig. 7C). Overall, there were 0-30 unidentified 6-10-nm-diameter IMPs per µm² in innermost JPIM collar E-faces vs. 3000 Cx29 particles per µm² in the complementary P-face. This means that the JPIM is a molecularly-homogeneous membrane domain, with Cx29 representing >98% if all particles large enough to be ion channels in the JPIM collars.

Based on these newly-recognized myelin E-face views, we surmise that replicas of JPIM E-face pits were not recognized in 1980-era replicas because the rosettes of membrane pits were partially filled in, either by a thin layer of water vapor contamination (Rash et al. 1979) or by the thicker platinum coats commonly used at that time. Either of those shadowing defects would have resulted in smooth, essentially particle-free myelin E-faces that would have resembled (and likely been mistaken for) one of the many deeper layers of particle-free compact myelin.

Although the JPAX E-face particle rosettes were abundant and easily recognized (Figs. 6, 8A), JPAX P-faces were especially difficult to recognize because we initially searched for membrane faces that were anticipated to be as smooth and as replete with rosettes of pits as were the JPIM E-faces (Fig. 7C). However, JPAX P-faces (Fig. 8B,C) had almost no large-diameter IMPs, but instead exhibited a fine “stubble” of 3-nm-diameter x 10-15-nm-long string-like IMPs, interspersed with densely-packed rosettes of pits (Figs. 8C, inset; blue arrowheads). Thus, the JPAX P-face pits were difficult to recognize, even in stereoscopic
images, because the pits were almost always camouflaged by an abundance of these 3 nm-
diameter, string-like particles (Fig. 8C, inset, green arrowheads) that projected upward from the
diameter (presumably slightly enlarged by the 1-1.5 nm Pt coat), each string-like molecule is
likely formed by a single transmembrane alpha-helix that extends from the axoplasm, through
the protoplasmic leaflet, around each axolemmal Kv1.1 particle, across the extracellular space,
apparently to link to Cx29 IMPs in apposed myelin (detailed below). Moreover, at the step from
myelin E-face to JPAX P-face, the string-like IMPs often spanned the extracellular space,
suggesting that these slender IMPs act as extracellular “tether” molecules that link and align
the Cx29- and Kv1.1-containing channels. Thus, we presume, but have not yet confirmed by
FRIL, that these string-like IMPs correspond to the Caspr2/Tag-1 complex that is thought to
localize Kv1.1/Kv1.2 subunits to the JPAX membrane (Poliak et al. 2003; Rasband 2004). Also
noteworthy, the absence of any other large-diameter IMPs in either JPAX P- or E-faces means
that there can be no significant numbers of ion channels other than Kv1.1/ Kv1.2 in the JPAX
domain. In contrast, small clusters of large diameter IMPs characterize the internodal
axolemma (Fig. 8D).

*Molecular co-alignment of Kv1.1 and Cx29 channels.* As originally shown by Stolinski et
al. (1981), the fracture plane occasionally steps from axolemmal E-face to myelin P-face within
a rosette (Figs. 9A-B; composite purple/aqua overlays). There, myelin P-face particles are
located precisely (±2 nm) where an axonal E-face rosette particle would have been predicted to
overlie the visualized myelin P-face particle had the axonal E-face particle not been fractured
away (Fig. 9B; yellow arrowhead in obliquely transecting box). This intercellular alignment of
rosette IMPs, seen in multiple examples, means either: *a*) that the apposed molecules are
structurally linked via short intercellular molecular tethers that maintain molecular alignment
(Figs. 8C, inset, green arrowheads) or *b*) that the mouths of the two types of apposed channels
are in direct molecular contact across the slightly narrowed extracellular space, as also occurs
when connexons align across a narrowed extracellular space during formation of gap junctions [Johnson et al. (2012), their Fig. 3C, red and blue arrowheads, and their Fig. 4A].

Cryptic labeling. In multiple areas where the fracture plane stepped from axolemmal E-face to myelin P-face within individual rosettes (Fig. 9B, purple overlays), the rosettes often were simultaneously double-labeled for Kv1.1 (10-nm gold beads; blue arrowheads) and Cx29 (5-nm gold beads, yellow arrowheads). Of the 19 gold labels shown here, three 5-nm gold beads for Cx29 (green arrowheads) beneath Kv1.1/Kv1.2 channels apparently represent “cryptic” labeling of the unvisualized Cx29 channels that remained adsorbed beneath the overlying and replicated Kv1.1 channels (interpretive drawing, Figs. 9C,D). [Note: Cryptic labeling of unvisualized connexin proteins beneath gap junction E-face pits is well documented (Fujimoto 1995; Fujimoto 1997), as confirmed in matched double-replicas (Li et al. 2008).]

If each paired rosette particle forms a composite channel, as proposed by Stolinski et al. (1981), these asymmetric Kv1/Cx29 channels would constitute an intercellular ion-conductance pathway that would likely have unique gating, ion selectivity, and ion conductance properties. Moreover, the proposed conductance properties of Kv1/Cx29 channels in mammals would likely be asymmetrically regulated by the cell expressing each protein -- Kv1.1/Kv1.2 by the axon and Cx29 by myelin. Although one might envision difficulties to be created by coupling a four subunit channel (Wang et al. 1999) to a six-subunit connexon channel (Cx29) (Unwin and Zampighi 1980), it should be noted that the four Kv1 channel monomers are “six-pass proteins”, whereas connexons are composed of hexamers of “four-pass proteins”, making both Kv1.1 and Cx29 equally-large “24-pass channels”. With both of these 24-pass channels having 12 extracellular loops, this also creates the potential for unique intercellular molecular coupling, potentially mediated by one or more of the cell-adhesion molecules localized to the JPAX/JPIM and JMAX/JMIM in mammalian myelinated axons (Peles and Salzer 2000; Poliak et al. 2003; Rasband 2004; Ogawa et al. 2008). In any case, the intercellular junctions formed by Kv1/Cx29 channels are “xenotypic” (i.e., unrelated proteins linking to form intercellular
junctions); however, demonstration of electrical coupling via Kv1/Cx29 channels and elucidation of their biophysical properties must await analysis of apposing cells separately expressing these proteins.

\[ \text{Kv1.1 and Cx29 resolved as tubular IMPs; their complementary pits have central pegs.} \]

Based on improvements in affordable high-vacuum and high-resolution shadowing technology (Rash and Yasumura 1992; Rash and Yasumura 1999), two-fold to four-fold finer details are resolved in these replicas, approaching 0.7 nm resolution (Rash et al. 1997; Rash et al. 2004a).

In the absence of a detectable layer of water-vapor contamination, which otherwise obscures membrane pits and enlarges IMPs (Rash et al. 1979), these thinner and finer-grained platinum shadows revealed new details of molecular morphology: a) the 11-12 nm rosette particles previously described (Miller and Pinto da Silva 1977; Stolinski et al. 1981; Stolinski et al. 1985) are now resolved as 9-nm IMPs; b) the rosettes of myelin E-face pits (Figs. 7C, 8C), which previously had never been observed, are now revealed to be complementary to the rosettes of Cx29-containing P-face particles (Figs. 6, 7B; 9A,B), and c) these higher-resolution complementary views reveal both the ice “peg” (Fig. 7C, insets, blue arrowheads; and explanatory drawing, Fig. 9C-E) extracted from within the pore of each aqueous ion channel, as well as the complementary “dimple” in the P-face IMP that represents the mouth of the pore of each ion channel (Fig. 6C; insets, yellow arrowheads). “[For details regarding visualization of open vs. closed connexons in deep etched samples and of the “etchability” of the water-filled central “pegs” in the complementary E-face pits of both gap junctions and AQP4 arrays, see Hirokawa and Heuser (1982) and Rash et al (2004a), respectively.] The insets are presented with both black shadows (Figs. 6C, 7C, 8C-D) and “white shadows” (Fig. 7B), because at high magnification, “life-like” black shadows are more easily interpreted than are images having unnatural “white shadows” (Steere et al. 1980).

\[ \text{Kv1.1 and Cx29 form rosettes along the inner mesaxon.} \] Along the entire internodal region of myelinated axons, the inner mesaxon represents the anatomical location where the
membrane of the innermost cytoplasmic tongue of myelin abuts the membrane of the cytoplasmic shoulder formed within the first complete layer of myelin (Figs. 10A,B). At this apposition, myelin is not compact, but instead forms two small, variable-bore cytoplasmic conduits, the juxtamesaxonal innermost myelin (JMIM) canals, which link the proximal juxtaparanodal innermost myelin (JPIM) collar to the distal JPIM collar (Mugnaini et al. 1977; Schnapp and Mugnaini 1978; Stolinski et al. 1985). (The physiological basis for the often-noted variation in volume of the JMIM inner tongue, which on occasion can extend around the entire circumference of the axon and become continuous with and terminate at the cytoplasmic shoulder of the first complete wrapping [see Figs. 6.4 and 6.5 in Peters et al., (1991)] is not yet established.) In FRIL images, the juxtamesaxonal innermost myelin (JMIM) conduit is recognized by the presence of one to three tight junction strands that link the inner tongue of myelin to the next overlying continuous layer of myelin (Fig. 10A,B), as described by others (Livingston et al. 1973; Mugnaini et al. 1977; Schnapp and Mugnaini 1978; Stolinski et al. 1985). In glutaraldehyde-fixed sciatic nerve axons, almost all of the 9-nm IMPs were present in rosettes in both inner mesaxonal axolemmal and inner mesaxonal myelin (Fig. 10A), confirming original descriptions of Stolinski et al. (1985). In contrast, formaldehyde fixation results in fewer rosettes, most of which were replaced by irregular clusters of 9-nm IMPs that, nevertheless, were immunogold labeled (Fig. 10B), thereby documenting that the rosettes disassemble into clustered 9-nm particles during fixation with formaldehyde. Except for localization of Cx29 at Schmidt-Lantermann incisures (see below), Cx29 was not detected elsewhere within compact myelin.

In formaldehyde-fixed WT mice viewed toward the JMIM P-face (Fig. 10B), abundant 5-nm gold beads for Cx29 are present, paralleling the inner mesaxon (Fig. 10B; yellow arrowhead), reflecting the FRIL correlate of the immunofluorescence ribbons along the inner mesaxon (Figs. 2, 3). Notably, immunogold labeling is absent where the second layer of myelin is overlapped and covered by the inner tongue of myelin (Figs. 10B; to the left of the tight
This confirms that Cx29 rosettes and Cx29 labeling occurs only in the areas of direct apposition of the axolemma with the adaxonal layer of myelin on either side of the inner mesaxon, where direct structural coupling of axonal $K_v1$ to myelin Cx29 can occur. Overall, we estimate that there were 30-50 rosettes per linear micrometer of inner mesaxon or 240,000-400,000 $K_v1$/Cx29 channels per 800-µm-long JMIM, which in addition to the JPIM, provide a second, stereotypically-distributed configuration of axo-glial junctions in mammals.

**Cx29 ko mice: $K_v1$ rosettes occur without apposed Cx29 rosettes.** To begin our investigations of factors regulating structural assembly of axolemmal and myelin rosettes, we examined FRIL replicas of sciatic nerve from Cx29 ko mice. Notably, internodal axolemmas of myelinated axons of Cx29 ko mice exhibited E-face $K_v1$ rosettes (Fig. 10C) that had particle sizes and particle spacings identical to those in WT mice (Figs. 6C, 8A, 10A). However, apposed JMIM P-faces of Cx29-deficient mice were devoid of the normally-abundant rosettes of 9-nm IMPs (Fig. 10C); and there appeared to be no compensatory migration into or insertion of other classes of ion channels into the JPIM collar of Cx29 ko mice, which remained remarkably particle free. Thus, the assembly of $K_v1.1$-containing rosettes in Cx29 ko mice reveals that the formation of axolemmal rosettes is an inherent property of $K_v1.1/K_v1.2$ channels, and does not depend on linkage with Cx29.

**Cx29 but not $K_v1.1$ in Schmidt-Lanterman incisures.** Cross-fractures of myelin frequently exposed the stair-stepped Schmidt-Lanterman incisures. In en face views of myelin that included one or more steps within a Schmidt-Lanterman incisure (Fig. 11), labeling for Cx29 (5-nm and 20-nm gold beads) was robust on P-faces (Fig. 11C), which were enriched in 9-nm IMPs, and moderate on E-faces (Fig. 11B), where 9-nm IMPs were at low density. In contrast, labeling for $K_v1.1$ (10-nm and 30-nm gold beads) was entirely absent on both E- and P-faces of the Schmidt-Lanterman incisure, accounting for the funnel-like distribution of Cx29 and absence of immunofluorescence labeling for $K_v1$ in immunofluorescence images of Schmidt-Lanterman incisures (Figs. 2, 4). Although Cx29-immunogold-labeled 9-nm IMPs are
abundant in Schmidt-Lanterman incisures, these particles are not arranged in hexagonal rosettes, even in glutaraldehyde-fixed tissues, as noted by Stolinski et al. (1985), further supporting the conjecture that Cx29 does not form rosettes without apposed K_\text{V}_{1.1}/K_\text{V}_{1.2} coupling partners. Notably, however, clusters of hexagonally-arranged 9-nm P-face IMPs and corresponding E-face pits, both labeled for Cx32 and therefore positively identified “reflexive” gap junctions (Larsen 1983), were occasionally encountered in Schmidt-Lanterman incisures in both formaldehyde-fixed and unfixed sciatic nerve (Meier et al. 2004). These regular hexagonal arrays of Cx32-containing gap junction particles in both glutaraldehyde-fixed and formaldehyde-fixed sciatic nerve are thus easily distinguished from the irregularly-distributed Cx29 particles described above.

**Cx29 but not K_\text{V}_{1.1} in the outer surface of myelin.** In WT and Cx32 ko mice, P-faces of outermost myelin of large-diameter axons were immunogold labeled at moderate density for Cx29 (Fig. 12). Overall, gold beads for Cx29 were at a density of ca. 10-50/\mu m^2 of Schwann cell outer P-face in large-diameter myelinated fibers vs. ca. 0.1/\mu m^2 as background “noise” in other areas, such as nodes of Ranvier, E- and P-faces of fibroblasts, and on replicated extracellular ice (Ex). Thus, the signal-to-noise ratio for Cx29 in outer plasma membrane P-faces of large-diameter myelinating Schwann cell was 100:1 to 1000:1 (vs. >5000:1 in the JPIM and JMIM). Thus, diffuse immunofluorescence labeling for Cx29 along the surface of fibers was considered to be authentic based on low level of labeling for Cx29 in WT mice vs. large depletion of labeling in Cx29 ko mice (Fig. 2B vs. 2C). In contrast, there was no detectable FRIL labeling for K_\text{V}_{1.1} on either P- or E-faces of the outermost layer of myelin. In support, the area of overlap of the outermost tongue of myelin, adjacent to the tight junctions of the outer mesaxon (Meier et al. 2004), was not labeled for Cx29 (not shown), with the abrupt termination of labeling at this normal biological landmark further supporting specificity of labeling of Cx29 in the outer surface of myelin. However, none of the 9-nm IMPs in the outer myelin P-face were arranged in rosettes in either glutaraldehyde-fixed or formaldehyde-fixed samples. Thus, the
absence of rosettes in outermost myelin is further consistent with proposed inability of Cx29 to form rosettes in the absence of apposed Kv1 coupling partners.

The overall density of labeling for Cx29 was moderately high over the entire length of outermost myelin (ca. 10/μm² x 800 μm long by 10 μm in diameter x pi = 240,000 labels per internode outer surface). Thus, we estimate that the number of immunogold labels on the outer surface of myelin is ca. 35% of the total number of immunogold labels in innermost myelin. This moderately high density of Cx29 channels along myelinated fibers could imply that in PNS myelin, a portion of the ionic and osmotic load arising from high-frequency axonal activity may be dispersed temporally and spatially into the surrounding endoneurial space by Cx29 hemichannels in the Schwann cell outer plasma membrane. [This potential for local release of K⁺ in the PNS is to be contrasted with proposed long-distance K⁺ siphoning via the parglial syncytium in the CNS (Rash 2010).]

Axolemmal E-face rosettes are abundant in lower vertebrates, but P-face rosettes are not detected in their myelin. Teleost myelinated axons [from Electrophorus electricus, see Fig. 4A in Rash (2010), and from Salmo truta (Nancy Shinowara, personal communication)] have abundant axolemmal Kv1-like rosettes in their juxtaparanodes and along their internodal axolemmas. However, they apparently lack myelin Cx29 rosettes, either at JPIM or along the JMIM. Likewise, the antibodies against Cx29 used for both immunocytochemistry and FRIL do not detect Cx29 in frog myelinated axons, due to either: a) the absence of the Cx29 gene in frogs (see Discussion) or b) to complete lack of cross reactivity of the antibodies used with the frog ortholog of mammalian Cx29. If the former is true, Cx29 would represent a late evolutionary addition to myelinated axons, where its direct structural coupling to Kv1.1 channels may contribute to the faster axonal conduction velocity and/or faster repolarization/higher frequency of axonal action potential generation that characterizes the myelinated axons of mammals (see Discussion). With frog myelinated axons apparently lacking Cx29 yet propagating action potentials at 13-20 m/sec (Raymond 1979) [vs. up to 120 m/Sec in
mammals (Buchthal and Rosenfalck 1966; Waxman 1980)], it is not surprising that deletion of Cx29 in mice (Eiberger et al. 2006) apparently had no detectable effect on myelin formation and no statistically-significant effect on the few electrophysiological properties of axons that were monitored. Rather, measurements pertinent to $K^+$ conductance into myelin, as proposed by Stolinski et al. (1981), were not monitored in that study. Based on additional data provided above, we suggest that future studies of Cx29 ko mice include: **a)** analysis of the maximum sustainable rate of impulse conduction (related to altered rate of axonal repolarization by interrupting flow of $K^+$ into myelin) and **b)** resistance to axonal fatigue during repetitive stimulation (as related to proposed rapid sequestration of $K^+$ in myelin).
DISCUSSION

Anatomical and molecular underpinnings of faster saltatory conduction velocity in mammals. Voltage-gated Na\(^+\) and K\(^+\) channels, Cx29 hemichannels, and their anchoring, scaffolding, and intercellular adhesion molecules (Srinivasan et al. 1988; Kordeli et al. 1995; Peles and Salzer 2000; Komada and Soriano 2002; Poliak et al. 2003; Rasband 2004; Ogawa et al. 2008) are localized to and define 11 distinct subcellular domains of myelinated axons (Fig. 13A,B): 1) voltage-gated Na\(^+\) channels are localized solely to nodes of Ranvier (Rosenbluth 1976; Ritchie and Rogart 1977; Caldwell et al. 2000); 2-3) K\(_{V1.1}/K_{V1.2}\) voltage-gated K\(^+\) channels, plus cell adhesion molecules such as Caspr2 and Tag-1, are localized to the proximal and distal juxtaparanodal axolemma (JPAX) plus 4-5) to two thin bands along the juxtamesaxonal axolemma (JMAX) (Gordon et al. 2014) and to the intermodal “C-bands” (Poliak et al. 2003), whereas 6-7) Cx29 channels are localized to the proximal and distal juxtaparanodal innermost myelin (JPIM) collars and to 8-9) the twin-bore juxtamesaxonal innermost myelin (JMIM) conduits formed by the “inner tongue” of myelin and by the cytoplasmic shoulder of myelin to which the inner tongue is attached. Finally, immunofluorescence labeling for Caspr, contactin, and NF155 [reviewed in Peles and Salzer (2000)] are used to define 10-11) the paranodal axolemma and paranodal loops of myelin.

Although axolemmal E-face rosettes similar to those identified in mammals as K\(_{V1}\) are abundant at juxtaparanodes in fish axons [Fig. 4A in Rash (2010)], similar rosettes have not been detected along fish JMAX (Nancy Shinowara, personal communication), nor have we detected them along the JMAX of frog axons. Likewise, immunofluorescence labeling for K\(_{V1.2}\) was robust at frog juxtaparanodes (Rasband 2004) but was not visible along the JMAX of those same axons. Also noteworthy, neither Cx29 immunolabeling nor Cx29 rosettes were detected by us along the JMIM in frog myelin. With no direct genomic evidence so far for the presence of a Cx29 gene ortholog in fish, amphibia, birds, or reptiles, and synteny analyses suggesting absence of the Cx29 ortholog in fish [Ingo Braasch, personal communication], and with Cx29
protein not reported in the axons of those infra-mammalian species, mammals appear to have evolved an additional axo-glial coupling mechanism that is not present in more basal vertebrates. If so, the molecular mechanisms underlying saltatory conduction in mammals appear to be modified from those in frogs, potentially accounting for the faster conduction velocity, higher sustainable rates of firing, and/or greater metabolic efficiency of saltatory conduction in mammalian myelinated axons.

The immunocytochemical and ultrastructural data provided in this and recent reports, combined with electrophysiological observations made over the past two decades proposing electrical coupling of axoplasm to myeloplasm in mammalian myelinated axons (summarized below), require revision of the traditional “nodal-conductance/passive-myelin” model that had been derived from studies of frog axons, and reconsideration of an alternative “dual-circuit/active-myelin” model that incorporates a direct axo-glial pathway for K+ ions (Stolinski et al. 1981;Stolinski et al. 1985). Based on the current demonstration of the abundance and highly-reproducible distribution of molecularly-linked Kv1/Cx29 channels in the internodal axonal and glial membranes of mammalian myelinated axons, we propose the rudiments of a modified model for saltatory conduction in mammals in which Kv1/Cx29 axo-glial junctions contribute to one or more of the following: 1) increased speed of axonal repolarization/shorter absolute refractory period; 2) local (intrasegmental) recycling of K+; and 3) precocious activation of multiple successive nodes of Ranvier via K+ flux from distal JPIM into distal JPAX/nodal axoplasm, resulting in increased conduction velocity.

**Juxtaparanodal domains further defined.** “Juxtaparanodal” domains were first defined in freeze-fracture images showing abundant rosettes of IMPs linking the JPAX with the surrounding JPIM collar (Stolinski et al. 1981;Stolinski et al. 1985). Subsequently, immunocytochemistry revealed the localized distribution of Kv1.1 and Kv1.2 to the juxtaparanodal domains in mammals (Wang et al. 1993;Vabnick et al. 1999;Chiu et al. 1999;Bhat et al. 2001;Altevogt et al. 2002;Rios et al. 2003;Rasband 2004). However, it remained unknown whether significant
numbers of any other ion channels might also be present in those specialized domains. Here, we showed that in adult mice, as in rats (Stolinski et al. 1981; Stolinski et al. 1985), Kv1.1 channels that define the JPAX, and Cx29 channels that define the JPIM collars, are densely-packed to the virtual exclusion of all other classes of transmembrane proteins large enough to be ion channels. Based on the presence of almost a million Kv1/Cx29 channels per internode and their highly-reproducible localization to the JPAX/JPIM and JMAX/JMIM in mammalian myelinated axons, we suggest that these axo-glial junctions are sufficiently abundant and appropriately localized to provide for the more rapid axonal depolarizations and repolarizations of mammals. By way of comparison to mammalian neuronal gap junctions, whose high-speed electrical communications are critically important for a host of neuronal functions in a wide variety of brain regions, neuronal gap junctions contain 10-4,000 connexons (Nagy et al. 2004; Rash et al. 2005; Rash et al. 2007), whereas there are 750,000-900,000 Kv1/Cx29 channels per internode, or >100-fold to 10,000-fold more Kv1/Cx29 channels than connexin channels in a typical neuronal gap junction, suggesting the importance of the function(s) subserved.

*Electrophysiological evidence for K⁺ conductance from axoplasm to myeloplasm.* David et al. (1993) used his newly-developed “ultrasharp” electrodes to discover that precisely coincident with axonal action potentials, strong action-potential-like depolarizations occurred within the deepest layers of myelin, decreasing in amplitude in successively-external myelin layers. Those “prolonged negative potentials” (PNPs) in inner myelin persisted for hundreds of milliseconds after each action potential, resulting in virtually-continuous myelin depolarization during repetitive axonal firing. Importantly, the myelin PNPs were blocked by tetraethyl ammonium (TEA), a specific blocker of voltage-gated K⁺ channels, thereby demonstrating that: a) PNPs resulted from axon-to-myelin K⁺ flux, b) through pharmacologically-identified voltage-gated K⁺ channels, and c) these K⁺ channels remained conductive for hundreds of milliseconds after the axonal resting potential was restored, effectively voltage-clamping axoplasm to an
undefined compartment within deepest myelin. Based on the present results, we reinterpret those findings to suggest that these continuously-open, TEA/4AP-sensitive K\(^+\) channels correspond to Kv1/Cx29 channels within the internode. Moreover, the prolonged “leak” conductance, occurring even at resting potential [also (Chiu et al. 1979)], implies that these K\(^+\) channels are not “voltage gated” in the classical sense, and that most remain conductive at rest. This apparent lack of voltage-dependent gating of Kv1 channels may have profound implications for axonal physiology in mammals.

Evidence for bidirectional conductance between axoplasm and myeloplasm: The TEA-sensitive K\(^+\) channels of mammalian myelinated axons were shown to be bi-directionally conductive for multiple cations, including K\(^+\), Rb\(^+\), and Na\(^+\) (David et al. 1993) and apparently, even Ca\(^{++}\) (Zhang et al. 2006). In mouse optic nerve axons [which, although CNS, have similar juxtaparanodal and internodal distributions of Kv1.1, Kv1.2, and Cx29 (Bhat et al. 2001;Altevogt et al. 2002;Rios et al. 2003)], Zhang et al. (2006) noted that “during each action potential, ‘retrograde’ Ca\(^{++}\) conductance occurs from myeloplasm into axoplasm” (emphasis added). Moreover, this intercellular Ca\(^{++}\) conductance was not blocked by ryanodine or nifedipine, which are specific blockers of Ca\(^{++}\)-induced Ca\(^{++}\)-release channels and of L-type voltage-gated Ca\(^{++}\) channels, respectively. Instead, myelin-to-axon Ca\(^{++}\)-conductance was blocked by TEA and 4-aminopyridine (4-AP), both of which are blockers of voltage-gated K\(^+\) channels. Thus, bidirectional conductance of Ca\(^{++}\) appears to occur via the classical K\(^+\)-conductance pathway. Current FRIL data show that Kv1.1/Cx29 channels are distributed in the JPAX/JPIM domains and in the JMAX/JMIM along the entire internode, where internodal Ca\(^{++}\) influx was reported. Moreover, based on the general cation permeability of other connexons (Bennett and Goodenough 1978;Palacios-Prado and Bukauskas 2009;Kanaporis et al. 2010), we propose broad ion permissiveness of the Kv1.1/Cx29 channels, as determined, in part, by the conductance and gating properties of the connexon-side of these ultrastructurally-linked “xenotypic” channels.
Rudiments of a modified model for electrically-active myelin in mammalian saltatory conduction. Based on the explicit presumption that tight structural coupling of \( K_{V1.1} \) channels with Cx29 channels connotes functional coupling, we support the proposal (Stolinski et al. 1981, 1985) that during an axonal action potential, \( K_{V1.1}/\text{Cx29} \) rosettes provide pathways for conventional outward \( K^+ \) currents, with \( K^+ \) efflux occurring from juxtaparanodal axoplasm directly into the JPIM collar (Fig. 13D). Moreover, we propose that during depolarization toward threshold, the rapidly-increasing voltage difference between JPAX and JPIM augments \( K^+ \) efflux from the depolarizing axoplasm into the less-depolarized proximal JPIM collar. The proposed capacitance-augmented \( K^+ \) efflux would likely reduce the time between successive depolarizations \((i.e.,\) reduce the absolute refractory period\)), and correspondingly increase the maximum-attainable frequency of action potential generation.

Does increased conduction velocity in mammalian myelinated axons arise in part from axo-glial coupling? The speed of saltatory conduction in frogs was originally proposed to be limited primarily by distance between nodes [2.3 mm in frogs (Tasaki et al. 1943); also (Raymond 1979)] and the time needed to reach threshold at each succeeding node [100 µSec (Tasaki and Frank 1955; Marks and Loeb 1976)], with that simple numerical ratio proposed to account for the observed conduction velocity of 23 m/Sec in frogs \((i.e.,\) 2.3 mm/node ÷ 0.1 mSec/node = 23 m/Sec). However, that numerical relationship likely was a mere coincidence, because in humans, nodal separations average \(~0.8\) mm (Hiscoe 1947; Friede et al. 1981), and the time to reach threshold is 80 µSec (Paintal 1966; Marks and Loeb 1976). Thus, the same simple numerical ratio would predict a maximum conduction velocity of only 10 m/Sec in humans \((i.e.,\) 0.8 mm/node ÷ 0.08 mSec/node = 10 m/Sec). Yet conduction velocity of large myelinated fibers in human PNS is an order of magnitude faster than predicted by that numerical ratio \([i.e.,\) \(~120\) m/Sec vs. 10 m/Sec (Buchthal and Rosenfalck 1966; Waxman 1980)]. Also relevant, this 120 m/Sec conduction velocity corresponds to an average delay of 8 µSec between successive nodes, which would seem to require that 10 nodes [to as many as 30...
nodes (Debanne et al. 2011)] be activated simultaneously under a nominal 80-µSec nodal
depolarization to threshold. Such microsecond events are too fast to be measured by current
electrophysiological methods using either sharp electrodes or patch-clamp electrodes
(Sontheimer and Ransom 2002; Sakmann and Neher 2009). Thus, to analyze microsecond
changes in voltage within innermost myeloplasm during saltatory conduction, we envision using
high-speed optical detection methods (>100,000 frames/Sec or >1,000,000 line scans/Sec) to
measure changes in fluorescence intensity of voltage-indicator dyes injected into or genetically
expressed in the innermost cytoplasm of oligodendrocytes and Schwann cells.

**Evidence that K⁺ efflux in mammals does not occur into the submyelinic extracellular space.** Early suggestions that K⁺ efflux in mammals occurs into the submyelinic extracellular space (Chiu et al. 1979) were contradicted by experiments investigating the effects of repetitive stimulation and of K⁺ channel activation on saltatory conduction, neither of which produced the predicted swelling of the sub-myelinic extracellular space, but instead resulted in swelling of innermost and outermost myelin cytoplasm (see below) or in swelling of the nodal axoplasm (Love and Cruz-Höfling 1986). As predicted by the “electrically-active myelin” model, when K⁺ transport pathways within the “panglial syncytium” (Rash 2010) were disrupted by mutations or genetic knockouts of glial connexins (Cx32, Cx43, Cx26), GFAP or Kir4.1, swelling of innermost myelin cytoplasm was substantial, often resulting in myelin sclerosis (Senderek et al. 1999; Menichella et al. 2003; Odermatt et al. 2003; Lennon et al. 2004; Menichella et al. 2006; Lutz et al. 2009). Those observations are consistent with the proposal (Rash 2010) that K⁺ and obligatorily-associated osmotic water normally utilize Kv1.1/Cx29 channels to enter innermost myeloplasm, with disruption of K⁺ exit pathways resulting in the observed myelin swelling of CNS myelin.

**Proposed local recycling of K⁺.** In early models of saltatory conduction, K⁺ efflux was presumed to occur into the perinodal extracellular space, where K⁺ was thought to diffuse away freely, thereby requiring axoplasmic replacement, presumably by the action of Na⁺/K⁺ATPase
As another alternative, K$^+$ efflux into the submyelinic extracellular space (Chiu and Ritchie 1980; David et al. 1993), with either diffusion across the paranodal septate junctions, or direct return to the axoplasm via axolemmal Na$^+$/K$^+$ATPase, would require equally substantial expenditure of energy. In mammals, however, linked Kv1/Cx29 channels provide a plausible axo-glial coupling pathway for efficient intrasegmental recycling of K$^+$ – from axoplasm to myeloplasm to axoplasm – which would greatly reduce this energy expenditure.

**Alternative explanation for the detection of voltage-gated K$^+$ conductance after acute demyelination.** Structural breakage of linked Kv1.1/Cx29 by diverse demyelinating procedures (Brismar 1979; Sherratt et al. 1980; Bostock et al. 1981) would result in K$^+$ efflux through the newly-separated Kv1 channels, directly into the peri-internodal extracellular space and its immediate detectability by K$^+$-sensitive electrodes. The proposed separation of Kv1 channels from Cx29 channels, with resultant removal of allosteric influence by Cx29, would account for the reported instantaneous conversion of submyelinic K$^+$ “leak” current into voltage-gated K$^+$ current (Chiu and Ritchie 1980).

**Potential function(s) for two JPAX/JPIM domains in each internode.** In PNS axons, which normally conduct only unidirectionally, it is relevant to consider the invariant presence of two ionically-active juxtaparanodal domains, one on each side of each node of Ranvier. One explanation could be that this arrangement would provide twice as many Kv1/Cx29 channels in close proximity to each node for more rapid repolarization. However, that would not explain why, in mammals but not in frogs, the axoplasm is linked to the internodal myeloplasm along the entire internode via the Kv1/Cx29 channels of the JPAX/JMIM (Figs. 10A,B, 13A,B,E; also see Figs. 2A3 and 4A3). As one potential additional explanation, we propose that at the distal JPIM, return K$^+$ flux occurs directly into the distal juxtaparanodal axoplasm (Fig. 13D,E; right sides). In turn, this would provide for precocious, capacitance-driven initiation of depolarization (“K$^+$ kindling”) of the next node, even before the preceding node had reached threshold. In the
resulting wave of depolarization, occurring almost simultaneously across a dozen or more nodes, this proposed long-distance "K⁺ kindling" would be expected to increase conduction velocity several fold over that permitted by separate, temporally-delayed saltatory activations of successive individual nodes, as previously envisioned.

An active role proposed for myelin in increasing velocity of saltatory conduction. We propose that in each internode, myelin is segmented into two biological capacitors (the proximal and distal JPIM collars), linked in series by the twin-bore JMIM conduits. These cylindrical capacitors are envisioned to store and release potassium ions, which flow into and between at least three distinct cytoplasmic myelin compartments (Figs. 13B,D,E): 1) During initial depolarization toward threshold, capacitance-augmented K⁺ efflux is proposed to occur from proximal JPAX into the proximal JPIM via the estimated 250,000 Kv1/Cx29 channels within each JPIM collar. In contrast to electronic circuits, where capacitance discharge via electrons is virtually instantaneous, limited only by the resistance and capacitance of the discharging circuit and the rate of flux of electrons in copper conductors, biological systems utilize Na⁺ and K⁺ ions as charge carriers and cytoplasm as the conductive medium. As a result, ionic capacitance discharge of the proximal JPIM through abundant but tiny conductors having finite resistance (i.e., Kv1/Cx29 channels) may be slightly slower than in conventional electronic circuits – perhaps requiring several microseconds to sequentially depolarize and repolarize each successive JPIM compartment. Likewise, even though they are electrically connected by the narrow twin-bore inner mesaxonal cytoplasms, the relatively long-distances between proximal and distal JPIM collars (nominally 800 μm) likely means that these two partially-isolated compartments will be depolarized and repolarized rapidly -- but not at precisely the same instant. This relatively brief delay arising from proposed capacitance-augmented repolarization in mammalian axons may have profound implications for both velocity and maximum frequency of saltatory conduction. As envisioned, the depolarization of the proximal JPIM collar during an action potential is proposed to lag the depolarization of the proximal JPAX by a few
microseconds. Likewise, the rapidly-rising depolarization of the distal JPIM collar is envisioned to slightly lead (by a few microseconds) the depolarization of the distal JPAX. Also according to this model, capacitance-augmented discharge of the distal JPIM collar would occur faster than the action potential depolarization could be conducted through the internodal axoplasm.

**Proposed additional role for the distal JPIM collars: “K⁺ kindling”**. We envision that an approaching action potential (still many nodes upstream) would first be manifest as increased peri-nodal axoplasmic K⁺ influx, from the upstream distal JPIM collar via continuously-open Kv1/Cx29 channels, creating a small inward “K⁺ kindling current” sufficient to begin depolarizing the upstream node by a few mV, even before arrival of the axoplasmically-conducted action potential. Upon reaching threshold (ca. −40 mV; requiring ~80 microseconds) and before formation of a full-amplitude action potential at the upstream node, axonal K⁺ efflux into the upstream proximal JPIM collar would be strongly augmented by its nominal −70 mV resting potential. Nearly simultaneously at the downstream distal juxtaparanode, as electrically linked by the JMIM cytoplasm (Figs. 13D-E), we envision that capacitance-augmented K⁺ efflux occurs from the now-rapidly-depolarizing distal JPIM collar, into the still-polarized downstream distal JPAX axoplasm, thereby precociously initiating depolarization (K⁺ “kindling”) of the next node. With each successive distal JPAX axoplasm proposed to be capacitance-coupled via abundant Kv1/Cx29 channels to its surrounding JPIM collar, rapid K⁺ kindling would also be initiated in a graded fashion at many successive downstream nodes, thereby almost simultaneously kindling multiple additional nodes in advance of the axonal action potential, thereby increasing conduction velocity of large-diameter myelinated axons in mammals.

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**Summary:**

490 In mammals, Kv1.1/ Kv1.2 channels are confined to the internodal axolemmal and not to nodes of Ranvier, as previously proposed. Moreover, K⁺ conductance apparently does not occur into the submyelinic extracellular space, as previously envisioned, but instead, is proposed to occur
into innermost myeloplasm, which is strongly electrically isolated from the nodal extracellular space by the myelin plasma membrane. By FRIL, we showed that the $K_v$1.1-containing channels are tightly structurally-coupled and therefore are likely functionally-coupled to Cx29 channels in innermost myelin, creating “xenotypic” intercellular junctions between the JPAX/JPIM and JMAX/JMIM domains. These xenotypic channels would provide direct, possibly bi-directional, intercellular molecular pathways for $K^+$, from axoplasm into myeloplasm and return. $K_v$1/Cx29 channels, pulled tightly together and potentially sealed by surrounding Caspr2/Tag-1 cell adhesion molecules, would account for failure to detect $K^+$ efflux at intact nodes but its ready detection following acute detachment of myelin and consequent physical separation of $K_v$1 channels from Cx29 channels, which would result in pathological release of $K^+$ into the submyelinic extracellular space. In addition, we propose that $K_v$1/Cx29 channels provide for downstream, capacitance-augmented “$K^+$ kindling” that precociously and almost simultaneously activates depolarizations at multiple successive nodes, thereby increasing both velocity and maximum frequency of saltatory conduction in mammalian myelinated axons. Finally, return flux of $K^+$ from myeloplasm to axoplasm would provide for efficient local recycling of all or a portion of the $K^+$ used for axonal repolarization.
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Figure Legends:

Fig. 1. Early model of saltatory conduction in frogs (A), recent version attributed to frogs and mammals (B), and diagram of the major axonal regions described in this report (C). A: Original model of saltatory conduction in frog sciatic nerve [modified from (Tasaki 1939)], illustrating inward current at nodes of Ranvier (red arrow), induced outward currents (blue arrows), also at nodes of Ranvier, and “nerve impulse” (green arrows) conducted in cytoplasm. B: Recent diagram showing co-localization of voltage-gated Na⁺ channels and voltage-gated K⁺ channels within nodes of Ranvier, with temporal but not spatial separation of Na⁺ and K⁺ currents. [Modified from Purves (2012)]. C: Diagram delineating functional domains of mammalian myelinated axons described in this report. Node of Ranvier, red; paranode, green (para = both sides of). Juxtaparanodal axolemma, medium blue (juxtaparanode = adjacent to both paranodes). Internodal axolemma, light blue. Inner mesaxon, dark blue line (mesaxon = mesentery-like support for axon). SLI, Schmidt-Lanterman incisure.
Fig. 2. Low magnification overview of immunofluorescence labeling of Cx29 and Kv1.1 in adult rat sciatic nerve and Cx29 in adult WT mouse and Cx29 ko mouse (C). A: Three images of the same field, showing similar patterns of labeling for Cx29 (A1) and Kv1.1 (A2) at juxtaparanodal regions (arrows), distinct patterns of labeling are seen at Schmidt-Lanterman incisures (arrowheads), and overlapping patterns are seen along longitudinally oriented strands at internodal regions corresponding to location of inner mesaxon (double arrows), as shown in overlay (A3). **B,C:** Immunofluorescence labeling of Cx29 seen in sciatic nerve of adult WT mouse (B) is absent in sciatic nerve of Cx29 ko mouse (C). Scale bars are as indicated on each group of panels.
Fig. 3. Immunofluorescence labeling of Cx29 and Kv1.1 at regions surrounding nodes of Ranvier in adult rat sciatic nerve. Proteins labeled and color code for labeling is indicated in the upper left. Images with the same lower left lettering show the same field. A: Labeling for both Cx29 (A1) and Kv1.1 (A2) is dense in the juxtaparanodal region (arrows), faint in the paranodal region (arrowheads), and can be seen extending up to and into the juxtaparanodal region along the inner mesaxon (double arrows), with overlap of labeling in most of these regions (A3). B: Similar co-localized labeling of Cx29 (B1,B3) in relation to Kv1.2 (B2,B3) is seen at juxtaparanodal (arrows), paranodal (arrowhead) and mesaxonal (double arrows) regions. At paranodes, labeling of Kv1.2 is resolved as several transverse bands (B2, double arrowhead). C-E: Overlays of labeling for Cx29 and Kv1.1 at the paranodal/juxtaparanodal region, showing variable degrees of overlap between the transverse bands of Kv1.1 labeling at paranodes and dense (C), moderate (D) or weak (E) labeling for Cx29 (arrows) extending into this region. F, Labeling of Cx29 in the paranodal area (F1, arrows) overlaps with labeling for the paranodal marker caspr (F2, arrows), as shown in overlay (F3, arrows). G,H: Labeling for Kv1.2 (G1) and Kv1.1 (H1) at paranodes (arrows) does not extend into nodes of Ranvier, identified by their labeling of sodium channels (Nav) (G2,H2, arrowheads) sandwiched between the paranodal transverse Kv bands (G3,H3). Scale bars are as indicated on each group of panels.
Fig. 4. Immunofluorescence double-labeling of Cx29 and Kv channels along myelinated axons in sciatic nerve of adult rat. Proteins labeled and color code for labeling is indicated in the upper left. Images with the same lower left lettering show the same field. A: Magnification of internodal regions, showing both Cx29 (A1) and Kv1.2 (A2) localized at myelinated fibers as a single near continuous linear strand of labeling (arrows) running along the internodal segment in an area overlying the axon, with Cx29/Kv1.2 co-localization along these strands shown in overlay (A3, arrows). B: Internodal regions of myelinated fibers, showing Kv1.2 localized as intermittent narrow transverse bands along axons (B1, arrowheads), and Cx29 at funnel-shaped Schmidt-Lanterman incisures (B2, arrows), with the Kv1.2 bands situated at the narrow ends of the Cx29-immunopositive incisures (B2, arrowheads). C,D: Magnifications of Schmidt-Lanterman incisures, showing labeling for Kv1.1 localized as transverse bands along axons (C1, arrowheads), with the bands consisting of a doublet of bars (arrowheads) overlapping with the narrow end of the incisures labeled for Cx29 (C2, arrows), as seen in overlay (arrowheads) and as shown at higher magnification (D). Scale bars are as indicated on each group of panels.
Fig. 5. Eight of the 10 principal fracture planes examined in this study (A) and stereoscopic low-magnification image from dual-double-labeled FRIL replica from mouse sciatic nerve revealing the paranodal and juxtaparanodal membranes (B). A: Two freeze-fractured myelinated axons (blue axoplasm), with the fracture plane (purple line) exposing a node of Ranvier, the E-face of the paranodal axolemma, E-face of the juxtaparanodal axolemma and P-face of juxtaparanodal innermost myelin (Box 6/9), pitted E-face of juxtaparanodal myelin (Box 7), P-face of the juxtaparanodal axolemma (Box 8), inner mesaxonal myelin E- and P-faces (blue overlay and Box 10), Schmidt-Lanterman incisures, and P-face of outer surface of myelin. Boxes correspond to numbered figures. For simplicity, only 10 of the usual 25-50 layers of myelin are indicated. Kv1.1-enriched JPAX (Box 8, dark aqua) and IMAX = blue ribbon (Box 10). Axon (light blue), myelin (beige), node (red), paranodes (green), juxtaparanodes (purple). B: Low magnification overview of the paranodal and juxtaparanodal membranes in a single axon, with boxed area shown at higher magnification in succeeding figures. At this magnification, three of the four sizes of gold beads used for dual-double labeling are discernible (10-nm and 30-nm gold for Kv1.1, and 20-nm gold beads for Cx29) inside the yellow box. Abundant 5-nm gold beads (also for Cx29) cannot be discerned, but are detected when Box 6 is further enlarged as Fig. 6. A pair of gold beads (barred circles at top edge of yellow box) on top of the replica are positively identified as “noise”. Purple overlay at top, axolemmal E-face, with impressions of paranodal loops of myelin; purple overlay at bottom, E-face of the JPAX region within the same complexly-fractured myelin sheath (M). Aqua overlays (middle and bottom) are portions of underlying P-face of innermost myelin, including tips of paranodal loops. Green overlays, cytoplasm of paranodal loops. Local area “dodging" used to reduce the photographic intensity of superimposed replica fragments and of areas of folded replica. Scale bar, 1 µm.
Fig. 6. Stereoscopic images of particle rosettes in JPAX E-face and JPIM P-face from Cx32 ko mouse (A,B) and from WT mouse (C). A,B: E-face rosettes (purple overlays) in the axolemma are labeled for $K_v1.1$, and P-face rosettes (aqua overlays) in innermost myelin are labeled for Cx29. Four sizes of gold beads are used -- two for $K_v1.1$ (10-nm and 30-nm gold) and two for Cx29 (5-nm and 20-nm gold). Double-ended red arrow compares 30-nm vs. 20 nm gold beads; double-ended yellow arrow compares 10-nm vs. 5-nm gold beads. Ax E, axolemmal E-face; My P, innermost myelin P-face. Green overlays, tongues of myeloplasm at inner mesaxon. Apposing strips of axolemmal E-face (orange overlay) and myelin P-face (yellow overlay) are deficient in rosettes, whereas rosettes are abundant on either side of this band. B: High-magnification stereoscopic image of axolemmal E-face (enlarged from Box in A). Most axonal rosettes are labeled by one to four 10-nm gold beads (blue arrowheads) for $K_v1.1$. In lower right quadrant, the fracture plane dropped from the axonal E-face to the myelin P-face, where the rosettes and clusters of 9-nm particles are labeled for Cx29 by 5-nm gold beads (yellow arrowheads). Purple overlays, rosettes of axolemmal 9-nm E-face particles. Rosettes of P-face particles (aqua overlays) in innermost myelin are labeled for Cx29. C: Fracture from axolemmal E-face (Ax E) to myelin P-face (My P) in a WT sample that was dual-double-labeled for Cx29 (ca. 18 10-nm and six 30-nm gold beads) and for cytoplasmic epitopes of $K_v1.1$ (5-nm and 20-nm gold; none present). As here, cytoplasmic epitopes of Kv1.1-containing particle rosettes were never labeled. Purple overlays, unlabeled axonal rosettes. Aqua overlays, clusters and rosettes of 9-nm IMPs labeled for Cx29. Left inset, myelin P-face particles labeled for Cx29 (from top yellow box), presented with black shadows to reveal the central “dimples” (yellow arrowhead). Right inset, axolemmal E-face rosette (from bottom yellow box), also presented with black shadows to reveal the central “dimple” in each $K_v1.1$-containing IMP (yellow arrowhead). Scale bar, 0.1 µm; 10 nm in insets.
Fig. 7. Stereoscopic images of labeled JPIM P-face \((A,B)\) and unlabeled JPIM E-face \((C)\). \(A\).

High labeling specificity, high LE (> 1:3), and high signal-to-noise ratio for Cx29 in innermost adaxonal myellemma of a large-diameter axon in mouse sciatic nerve. Box in \(A\) is further magnified as panel \(B\). Slight local area “dodging” used to reduce the photographic intensity of the clump of antibodies outside the upper left of the Box. \(B\): At higher magnification, the excessively high LE is seen to result in potential obscuring of some particles. \textit{Yellow boxes} are enlarged to reveal the greater LE for small gold beads. \(C\): E-face imprints of densely-packed Cx29 rosettes (aqua overlays) in JPIM of glutaraldehyde-fixed \((i.e., \text{not labeled})\) sciatic nerve from WT mouse. Most rosettes are complete rings, but a few incomplete rings contain only four or five pits. Most pits contain a central peg (blue arrowhead in inset; shown with black shadows). Pegs represent the frozen water-filled matrix extracted from the ion channel during cleaving (Hirokawa and Heuser 1982; Rash et al. 2004a), implying that most Cx29 channels are open when fixed. Paucity of E-face IMPs indicates virtual absence of all other types of ion channels in E-faces. Likewise, in the complementary P-faces \((B)\), there are few if any particles other than Cx29. Scale bars, 10 µm \((A)\), 0.1 µm \((B,C)\), and 10-nm \((B \text{ and } C \text{ insets})\).
Fig. 8. Stereoscopic images comparing JPAX E-face rosettes of particles (A), JPAX P-face rosettes of pits (B,C), and internodal axolemmal P-face particles (D). A: At high magnification, the JPAX E-face from formaldehyde-fixed WT mouse sciatic nerve contains densely-packed rosettes of K\textsubscript{v}1.1/K\textsubscript{v}1.2 particles (purple overlays), with almost no additional free particles that are larger than 6-nm in diameter. WT sciatic nerve axon dual-double-labeled with antibodies against cytoplasmic epitopes of K\textsubscript{v}1.1 and K\textsubscript{v}1.2 (10-nm and 30-nm gold beads; none present) and Cx29 (not present here). Axonal E-face rosettes are densely packed in juxtaparanodal regions, typically ranging from 200-270 rosettes/µm\textsuperscript{2} [see also Stolinski et al. (1981;1985)]. In this ca. 0.4 µm\textsuperscript{2} area, there are 270 rosettes per µm\textsuperscript{2}. Ax = axoplasm. B: Low-magnification stereoscopic view of axon and surrounding myelin. Boxed area is shown at higher magnification in C. Ax = axoplasm; Ax P = axolemmal P-face; My E = innermost myelin E-face; M = cross-fracture myelin. C: JPIM E-face collar is recognized by its characteristic high density of E-face rosettes of pits (a few delineated by yellow circles). The underlying JPAX P-face is not smooth, as we originally expected based on densely-packed rosettes of IMPs in the JPAX E-face (A), but instead, has a fine “stubble” of 2-3-nm diameter string-like IMPs that are interspersed with and therefore partially disguise the rosettes of pits (blue box; enlarged as inset). String-like IMPs (inset, green arrowheads) jut from the left side (leeward cleaving side) of each 7-9-nm pit (blue arrowhead). The vertically oriented string-like IMPs are delineated by the extremely electron-opaque platinum coat as viewed along its vertical axis, here seen as intense white dots in black shadow image. Each pit has a faint central peg (blue arrowhead). (See Fig. 12C for diagram of “tether” proteins.) D: Small clusters of large IMPs characterize the internodal axolemma, which has no rosettes of P-face pits, except along the juxtamesaxon (Fig. 10A). Blue box enlarged as inset, with clustered medium- to large-diameter IMPs. Ax, axoplasm; M, compact myelin, Scale bars, 1 µm in B, 0.1 µm in A, C, and D, and 10 nm in insets.
Fig. 9. High-magnification FRIL images (A,B) and correlative diagrams (C-E) showing molecular co-alignment of immunogold-labeled $K_v1$ and Cx29 channels along the JMAX/JMIM. A: Rosettes of axonal E-face particles (purple overlays) are labeled by ca. 15 10-nm gold beads and by one 30-nm gold bead (red arrow at left edge). At the step from axolemmal E-face to myelin P-face, both proteins remain adsorbed following SDS washing, as inferred from presence of labels for both $K_v1$ and Cx29. Ax, axoplasm (orange overlay), Ax E, axolemmal E-face (light purple overlay); P1, innermost myelin P-face. ME1 and M3-11+, succeeding layers of myelin (light aqua overlay). B: Stereoscopic (left pair of images) and reverse stereoscopic images (right pair of images) from boxed area in A, revealing “direct” labeling of Cx29 (yellow arrowheads) and $K_v1.1$ IMPs (blue arrowheads) vs. “cryptic” labeling for Cx29 (green arrowheads) beneath $K_v1.1$ rosettes. In reverse stereo, gold beads appear to be on top of the replica, making them easier to discern. Where the fracture plane stepped from axonal E-face to myelin P-face within a rosette (left and right sides of B), axonal E-face particles form one side of rosette (purple portions of circular overlays), and underlying myelin P-face particles complete the rosette pattern (aqua portions of same circular overlays). Fracture step in Box D is diagrammed as Fig. 10D. C-E: Diagrams showing complementarity of composite $K_v1.1$/Cx29 particles and their respective pits, with the icy “pegs” (E, arrows) corresponding to the central “dimple” (mouth of ion channel) in each IMP (D, arrows). C: Diagram of fracture plane (red line) in B. D,E: Complementary fracture faces. Unreplicated proteins remain attached to the replicated IMPs, allowing cryptic labeling (Fujimoto 1995; Fujimoto 1997; Rash and Yasumura 1999). Axonal E-face particles (blue channels) directly appose myelin P-face particles (yellow channels). “Direct” labeling occurs where the target protein is visualized by replication with Pt/C, whereas “cryptic” labeling occurs where the target protein is not visualized by replication but nonetheless, is inferred based on immunogold labeling of its strongly-bound and platinum-replicated coupling partner. Thus, some axonal E-face particles appear to be labeled for both $K_v1$ and Cx29. “Y”-shaped gray linkers, primary and secondary antibodies. Scale bars, 0.1 µm.
Fig. 10. Juxtamesaxonal membranes in glutaraldehyde-fixed (A) and formaldehyde-fixed WT mouse sciatic nerve (B), and in glutaraldehyde-fixed Cx29 ko mouse (C). A: Rosettes of myelin P-face particles in glutaraldehyde-fixed JMIM (MP₁, light aqua overlay), and absence of rosettes where the second wrapping of myelin is exposed (MP₂). Orange overlay and Ax, axoplasm; pink overlay, tight junction; purple overlay, axon E-face; M, compact myelin. B: Abundant 9-nm IMPs along the JMIM expansion (to the right side of the tight junction, arrow and pink overlay) but almost no 9-nm IMPs on the continuation of the same membrane, which becomes the second wrapping of myelin to the left of the tight junction. Cx29 is labeled with 10-nm gold beads (yellow arrowhead). Ax, axoplasm (orange overlay). E, E-face of the JMAX (purple overlay). P, myelin P-face. Dark aqua, myelin E-face. Lower green overlay, cytoplasm of myelin inner tongue. Upper green overlay, juxtamesaxonal myeloplasm. C: Stereoscopic image of axolemma of myelinated axon from formaldehyde-fixed Cx29 ko mouse. Rosettes of 9-nm Kᵥ1 E-face particles (purple overlays) are present, even though their normal coupling partners (Cx29 rosettes) are absent from the apposed myelin P-face (P; aqua overlay) (none present here or elsewhere). Also noteworthy, there are essentially no other large P-face particles (<50/μm²) in the innermost myelin juxtaparanodal membrane, consistent with the proposal that normally, there are virtually no channels other than Cx29 in the JPIM collars. Scale bars, 0.1 μ and 1 μm.
Fig. 11. Cross-fracture (A) and en face fractures (B,C) through Schmidt-Lanterman incisures (SLI; green overlays) from dual-double-labeled sciatic nerve of Cx32 ko mouse. A: Cross-fractured SLIs are only weakly immunogold-labeled for Cx29. B,C: Fractures that include en face views of one of the steps in the SLI E-face (B) and P-face (C) are strongly labeled for Cx29 (5-nm gold beads [arrowheads] and 20-nm gold), but they are not at all labeled for KV1 (10-nm and 30-nm gold beads, none present in either E-face or P-face views) (See Figs. 2, 4 for immunofluorescence imaging correlates). E-face labeling represents “cryptic” labeling of Cx29 in the subjacent membrane of another step in the SLIs. No rosettes are present in E- or P-faces of SLIs in either formaldehyde-fixed (here) or glutaraldehyde-fixed tissue [also see Stolinski et al. (1985)], suggesting that Cx29 is present as singlet IMPs that do not form rosettes without KV1.1-containing coupling partners. Gold beads in barred circles in B and C are identified as “noise” because they are on top of the replica, where no labeling is possible. Ax, axoplasm; E, E-face of myelin within the SLIs. P, P-face. Green overlays (* in B) mark cytoplasmic expansions that characterize the SLIs. Scale bar, 0.1 µm in C.
Fig. 12. Outer surface of myelin, showing moderate labeling for Cx29 (5-nm and 20-nm gold beads) but absence of immunogold labeling for Kv1.1 (10-nm and 30-nm gold beads; none present). Box enlarged as inset shows relative abundance of 5-nm (arrowheads) vs. 20-nm gold beads. Note absence of gold beads on extracellular ice (Ex) and on cross-fractured myelin and cross-fractured axoplasm (Ax). Arrows, collagen fibers. Scale bars, 1 µm
Fig. 13. Diagrams showing relationship of JPAX to JPIM and JMAX to JMIM (A), comparative sizes of JMIM conduits (B), proposed coupling of Kv1 to Cx29 rosettes (C), proposed intramyelinic pathways for K⁺ along the JMIM conduits that link proximal and distal JPIM collars (D), and of myelin unwrapped from its axon (E). A: Diagram showing distribution of NaV channels (red) at nodes of Ranvier, intercellular contactin/Caspr proteins (green) that link paranodal loops (beige) to the axolemma (blue), and axonal Kv1 channels (dark blue circles) concentrated in the JPAX region and in two thin bands along the JMAX. Cx29 channels (yellow rosettes) are restricted to the JPIM and JMIM. The trapezoidal blue box in A is diagrammed in B. B: Cytoplasmic expansions of myelin on both sides of the mesaxon (i.e., the paired JMIM) are continuous with the stair-stepped cytoplasmic expansions of the Schmidt-Lanterman incisures (SLI). (See Figs. 2-4 for immunofluorescence images.) The JMAX (purple arrow to purple segment of axolemma), opposite the inner mesaxon, is defined by its content of Kv1 rosettes (blue ovals), whereas the JMIM (aqua arrow to aqua line segments) is defined by its high density of Cx29 rosettes (yellow ovals) and by tight junctions linking the inner tongue with the second layer of myelin (Fig. 10). C: Models for tight vs. loose coupling of Cx29 to Kv1 in mammals (C1 vs. C2) and their corresponding freeze-fracture images (C3 vs. C4). Cx29 channel (yellow) aligns precisely with Kv1.1-containing channel (blue). It is not yet determined whether the channels are in molecular contact (C1), or whether there are tether proteins loosely linking the two channels (C2), where they may contribute to a molecular seal. D, E: Diagrams of wrapped (D) and unwrapped myelin (E) showing the cytoplasmic expansions (beige cylinders) surrounding the axolemma (blue), with its densely-packed NaV channels (red) at nodes of Ranvier. Axolemmal Kv1 channels (dark blue rosettes) are concentrated in the JPAX and in two thin bands within the JMAX. Cx29 channels (yellow rosettes) are concentrated in the JPIM and JMIM. Schmidt-Lanterman incisures (SLI) are stair-stepped expansions of myeloplasm that usually extend from the juxtamesaxonal expansions to outermost cytoplasmic myelin.