Connexin32-Containing Gap Junctions in Schwann Cells at the Internodal Zone of Partial Myelin Compaction and in Schmidt–Lanterman Incisures

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In vertebrate peripheral nerves, the insulating myelin sheath is formed by Schwann cells, which generate flattened membrane processes that spiral around axons and form compact myelin by extrusion of cytoplasm and adhesion of apposed intracellular and extracellular membrane surfaces. Cytoplasm remains within the innermost and outermost tongues, in the paranodal loops bordering nodes of Ranvier and in Schmidt–Lanterman incisures. By immunocytochemistry, connexin32 (Cx32) protein has been demonstrated at paranodal loops and Schmidt–Lanterman incisures, and it is widely assumed that gap junctions are present in these locations, thereby providing a direct radial route for transport of ions and metabolites between cytoplasmic myelin layers. This study used freeze-fracture replica immunogold labeling to detect Cx32 in ultrastructurally defined gap junctions in Schmidt–Lanterman incisures, as well as in a novel location, between the outer two layers of internodal myelin, approximately every micrometer along the entire length of myelin, at the zone between compact myelin and noncompact myelin. Thus, these gap junctions link the partially compacted second layer of myelin to the noncompact outer tongue. Although these gap junctions are unusually small (average, 11 connexon channels), their relative abundance and regular distribution along the zone that is structurally intermediate between compact and noncompact myelin demonstrates the existence of multiple sites for unidirectional or bidirectional transport of water, ions, and small molecules between these two distinct cytoplasmic compartments, possibly to regulate or facilitate myelin compaction or to maintain the transition zone between noncompact and compact myelin.

Key words: Charcot-Marie-Tooth disease; connexon; freeze fracture; immunogold labeling; sciatic nerve; tight junction

Introduction

Myelin, which increases axon conduction velocity in the vertebrate CNS and peripheral nervous system (PNS), is formed by flattened glial cell processes that spiral around axons to form multiple layers of lipid-rich plasma membranes that, in effect, increase axonal membrane resistance and decrease axonal membrane capacitance. In the PNS, myelin is formed by Schwann cells, which ensheath axons along their entire lengths, except at nodes of Ranvier. Mature myelin consists of “compact” and “noncompact” (or “cytoplasmic”) myelin. Noncompact myelin is formed at paranodal loops (cytoplasmically expanded lateral margins of Schwann cells bordering nodes of Ranvier), at Schmidt–Lanterman incisures (continuous spirals of stair-stepped or overlapping cytoplasmic expansions within areas of otherwise compact myelin), and at the innermost and outermost cytoplasmic tongues. During initial myelin compaction, the aqueous components of cytoplasm are removed, and the intracellular and extracellular surfaces of the plasma membranes come into close contact, ultimately reducing or eliminating the intracellular and extracellular spaces. One major hypothesis for myelin compaction proposes that cytoplasm is mechanically extruded circumferentially from the advancing outer edge of compact myelin into the adjacent zone of noncompact myelin (Webster, 1971; Bunge et al., 1978) accompanied by tight adhesion of apposed membranes via myelin-specific proteins and cell adhesion molecules (Martini and Schachner, 1997). The existence of the circumferential pathway as the sole mechanism for removal of cytoplasmic constituents, particularly macromolecules, was presueme because, heretofore, there was no evidence for the existence of any type of radial pathway within internodal myelin, except at distant and infrequent Schmidt–Lanterman incisures.

Supplementary to the circumferential pathway proposed for extrusion of cytoplasmic contents during advance of compact myelin, we now provide evidence for an additional structural pathway that is potentially capable of direct radial transfer of ions, water, and small molecules [i.e., through gap junctions that link the cytoplasm of the partially compact (or “semicompact”) (Mugnaini et al., 1977) layer to the cytoplasm of the otherwise compact myelin).
cytoplasmic tongue]. As precedent, protein constituents of gap junctions ("connexins") are well documented in peripheral nerve Schwann cells (Bergoffen et al., 1993; Scherer et al., 1995; Yoshimura et al., 1996; Chandross et al., 1996; Mambetisava et al., 1999; Nagaoka et al., 1999; Zhao et al., 1999; Allevogt et al., 2002; Li et al., 2002). Immunocytochemical evidence is strong for connexin32 (Cx32) in paranodes, at Schmidt–Lanterman incisures, and along the "inner mesaxon" of the myelin sheath (Bergoffen et al., 1993; Scherer et al., 1995, 1998; Spray and Dermietzel, 1995). As a second precedent, myelin in Cx32-deficient mice and in humans suffering from the related human peripheral neuropathy, X-linked Charcot-Marie-Tooth (CMT-X) disease, which is primarily characterized by degeneration (Bergoffen et al., 1993; Anzini et al., 1997; Scherer et al., 1998). However, particularly in the mouse model, deficiencies in the ultrastructure of compact myelin have been observed also (Anzini et al., 1997).

Dye-transfer experiments performed on peripheral nerves suggest that gap junctions promote direct radial transport between cytoplasmic layers, primarily at Schmidt–Lanterman incisures (Balice-Gordon et al., 1998). Although gap junctions have been reported between myelin layers of paranodal loops in CNS myelin (Sandri et al., 1977), ultrastructurally defined gap junctions have not been demonstrated previously in PNS myelin of mammals. Nevertheless, a few ("less than ten") gap junctions were found between the two outermost myelin lamellae in chicken sciatic nerve, were reported to increase substantially in size and number in internodal regions during Wallerian degeneration, and were presumed to disappear ("no longer seen") during subsequent remyelination (Tetzlaff, 1982). Thus, precedent exists for gap junctions in internodal myelin in PNS, but no role could be assigned on the basis of the rarity of their observation.

Using antibodies to Cx32 and freeze-fracture replica immunogold labeling (FRIL), we now document Cx32 in ultrastructurally defined gap junctions at Schmidt–Lanterman incisures in mice, as well as more abundantly in a novel and unexpected location, between the outer two layers of internodal myelin, throughout the "zone of partially compact myelin" (also called semicompact myelin) (Mugnaini et al., 1977). In addition to radial transport through gap junctions as proposed at Schmidt–Lanterman incisures (Balice-Gordon et al., 1998), the newly discovered internodal gap junctions provide the structural basis for abundant, closely spaced, short-distance radial pathways, potentially allowing for metabolic communication between the outer two layers of myelin and for transport of water, ions, and small molecules between these two sequential cytoplasmic layers.

**Materials and Methods**

**Sources of tissue.** Immunocytochemical and FRIL analyses were performed on C57/B6 mice (Charles River Laboratories, Wilmington, MA; Sulzfeld, Germany). Cx32–/– females and Cx32 X/– males (Nelles et al., 1996) were generated from a colony at the Colorado State University animal facility and established from one breeding pair (kindly provided by Klaus Willecke, University of Bonn, Bonn, Germany).

**Immunohistochemistry for light microscopy.** For immunocytochemical analyses, 6-month-old mice were deeply anesthetized and decapitated. Sciatic nerves were dissected, the epineurium were removed, and axon bundles were teased onto Superfrost plus glass slides (BDH Laboratory Supplies, London, UK). Samples were fixed in 100% ethanol (–20°C) for 15 min and washed in PBS. Non-specific binding sites were blocked by preincubation in 10% normal goat serum and 0.1% Triton X–100 in PBS (blocking solution) for 2 hr at 18–20°C. Monoclonal Cx32 antibody (Zymed; Zymed, Berlin, Germany) was diluted 1:200 in blocking solu-

ition. Incubation in primary antibody was performed for 16 hr at 18–20°C. Immunohistochemical analysis on Cx32-deficient tissues, as well as omission of the primary antibody from normal tissues, served as controls for nonspecific binding of primary and secondary antibodies, respectively. Incubation with secondary antibodies (goat anti-mouse, AL- EXA 488-conjugated immunoglobulins; Molecular Probes, Leiden, Netherlands) was performed for 2 hr at 18–20°C. After staining, nerves were mounted in anti-fade mounting medium (Molecular Probes) and photographed using confocal laser scanning microscopy (Zeiss LSM 510 Meta; Zeiss, Gottingen, Germany).

**Freeze-fracture immunolabeling.** All experiments were conducted according to the National Institutes of Health **Principles of Laboratory Animal Care.** For most ultrastructural studies, adult mice were anesthetized (90 mg/kg Ketamine, 8 mg/kg xylazine) and fixed for 10 min via transcardiac perfusion with 2% formaldehyde and 0.15 M Swann’s phosphate buffer (SPB) or were prepared without chemical fixation. (Formaldehyde-fixed tissue was used for samples shown in Fig. 2; all other photographs were from unfixed tissues.) Unfixed and formaldehyde-fixed tissues have different cleaving patterns for some classes of intramembrane particles (IMPs) as compared with the same structures in glutaraldehyde-fixed tissues (e.g., the IMPs in tight junctions) (Staehelin, 1974). However, because of the strong resistance to SDS washing, glutaraldehyde-fixed tissues cannot be used for FRIL (Rash and Yasumura, 1999). Schematic nerves were dissected, suspended in 10% gelatin solution, chilled to 4°C, and cut into 100 to 150 µm thick sections using a refrigerated Lancer (St. Louis, MO) 1000 Vibratome. Slices of fixed nerves were infiltrated with 30% glycerol in SPB, whereas slices of unfixed tissue were infiltrated with 2.5 M sucrose. Cryoprotected slices were frozen by pendrastically dipped rapidly cooled to −185°C cryogen (nitrogen) over a mirror (Phillips and Boyne, 1984). Samples were freeze-fractured in a JEOL (Peabody, MA) RFD9010 C freeze-fracture device, coated with 0.5–1 nm of carbon (Winkler et al., 2002), shadowed with 1 nm of platinum–carbon, and stabilized with 20 nm of carbon. Each replicated but unthawed sample was bonded to a gold "index" electron microscope grid in a thin film of 3% Lexan plastic (GE Plastics, Pittsfield, MA) by evaporating the dichloroethane solvent at −35°C (Rash et al., 1995). After thawing, cellular material was removed by vigorous washing with SDS detergent (2.5% SDS in D2O/Tris HCl, pH 8.9) for 24–29 hr, leaving a thin film of molecules adsorbed to the replica and available for immunogold labeling (Fujimoto, 1995; as modified in Rash and Yasumura, 1999). Before immunolabeling, nonspecific binding sites of the SDS–washed replicas were blocked by preincubation in labeling blocking buffer (LBB) containing 1% bovine serum albumin and 0.5% gelatin and 0.05% sodium azide in 150 mM SPB (Dinschau et al., 1987; Rash et al., 1990). Antibodies at a stock concentration of 1 mg/ml were diluted 1:100 in LBB, either using single antibodies or mixed antibodies of two species. Rabbit polyclonal antibodies to Cx43 were from Chemicon (Temecula, CA); rabbit polyclonal Cx26 and Cx29 antibodies, as well as the mouse monoclonal antibodies to Cx32 and Cx43, were from Zymed (San Francisco, CA).

Incubation with the primary antibody was performed for 1–1.5 hr at 24°C. Samples were rinsed in LBB (four times, 10 min each) and incubated for 12–16 hr at 24°C with species-specific secondary antibodies (goat anti-mouse; goat anti-rabbit) coupled to 6, 12, 18, or 20 nm colloidal gold (Chemicon; Jackson ImmunoResearch, West Grove, PA). Labeled replicas were rinsed in LBB (five times, 10 min each), twice in SPB and three times in distilled water, and were then air-dried. After application of a second "backing" backing gold coat on the labeled tissue side of the replica, the Lexan support film was removed by immersing the grid in dichloroethane (six changes in 1–1.5 hr). All samples were viewed in a JEOL 2000 EX-II transmission electron microscope and photographed at 10,000×, 30,000×, and 100,000×. Photographic stereopairs were exposed with an 8° included angle between images and viewed stereoscopically to distinguish specific labeling (occurring on tissue side only) from nonspecific labeling. We showed previously that for samples with low background, >90% of nonspecific immunogold beads were on the non-tissue, formerly Lexan-coated side of the replica (Rash and Yasumura, 1999). The numerical data presented below were assembled from six replicas from four wild-type mice (one male, three female) single- and double-labeled for Cx32 (one replica), Cx32 + Cx29 (four replicas), and Cx32 + Cx43 (one replica). Figures 2, 4, 5A–D, and 6 were derived from
Connexin32 in Schwann Cell Gap Junctions

Results

Light microscopic immunohistochemistry

Immunocytochemical analysis of connexin expression in Schwann cells was performed on teased nerve preparations of wild-type and Cx32 null-mutant adult mice. In accordance with previous reports (Bergoffen et al., 1993; Scherer et al., 1995; Spray and Dermietzel, 1995), Cx32 protein was detected immunocytochemically in Schwann cells of wild-type mice using a monoclonal Cx32 antibody (Fig. 1), and this antibody was subsequently used for FRIL analysis. Immunohistochemistry resulted in prominent staining for Cx32 at the site of Schmidt–Lanterman incisures and at paranodal loops (Fig. 1A). Occasional punctate immunoreactivity was detected at the outermost Schwann cell surface or the outer mesaxon (Fig. 1A, white arrows; also see FRIL images below).

In tests for nonspecific binding of the primary antibody, immunocytochemical labeling for Cx32 on nerves of Cx32-deficient mice revealed no detectable immunofluorescence above background (Fig. 1B). Because omission of the primary antibody revealed no detectable staining within the nerve fibers of wild-type mice (Fig. 1C), nonspecific binding of the secondary antibody at Schmidt–Lanterman incisures or paranodal loops was excluded. However, outer margins of fibers revealed minimal background staining of the secondary antibody (Fig. 1C).

Detection of Cx32 in gap junctions at Schmidt–Lanterman incisures

Freeze-fracturing of biological membranes produces either of two views, the protoplasmic leaflet (P-face) or the extraplasmic leaflet (E-face). [The international standard for freeze-fracture nomenclature (Branton et al., 1975) is used in this report.] Freeze-fracture replicas of peripheral nerves reveal membranes from the outermost layer of the Schwann cell to the plasma membrane of the axon, as well as cross-fractured cytoplasm of Schwann cells and axons. Schmidt–Lanterman incisures were rarely fractured to expose their stair-stepped membranes (Fig. 2), and freeze-fractured paranodal loop membranes were not found, presumably because the fracture plane is usually diverted from areas of sharp membrane curvature to nearby membranes with a lower radius of curvature. However, in FRIL replicas labeled for Cx32, immunogold beads drew attention to minute but clearly resolvable gap junctions within Schmidt–Lanterman membranes (Fig. 2C–D). Gap junctions were identified on the basis of their distinctive, hexagonally arranged clusters of 9 nm IMPs on P-faces (illustrated below) or as clusters of 9 nm “pits” in membrane E-faces (Fig. 2C–D). The nearby E-face IMPs are not labeled, because they do not correspond to connexin IMPs, which are seen only in replicated P-faces, as shown in Figures 4, C–F, and 6A. Criteria for identifying gap junctions and their constituent connexons in freeze-fracture replicas are reviewed by Rash et al. (1997). [The ultrastructural feature designated as a connexon (Goodenough, 1975) is structurally equivalent to a “hemichannel” (regardless of functional state) and is used to refer to the P-face IMPs, whereas the E-face pits are referred to as “connexon imprints” or “connexon pits.” In each case, however, each E-face connexon pit overlies its companion connexon in the unfractioned membrane beneath.] Because E-face connexon pits are more difficult to recognize when printed with white shadows, selected images of gap junctions are also printed using the alternative black shadow convention (Fig. 2C′–D′, red overlays), which more closely resemble objects in nature when illuminated with white light (Steere et al., 1980). However, a minor disadvantage of images printed with black shadows is that gold beads appear white.

Although the existence of gap junctions at Schmidt–Lanterman incisures was proposed originally on the basis of light microscopic immunocytochemical data and on dye coupling experiments (Bergoffen et al., 1993; Scherer et al., 1995, Balice-Gordon et al., 1998), these are the first ultrastructural images of gap junctions at Schmidt–Lanterman incisures and the first ultrastructural documentation that Cx32 is present in these gap junctions. However, in these same replicas, immunogold labels revealed gap junctions in a newly discovered expression site, between the outer two wrappings of internodal myelin at the linear zone corre-
sponding to the area of overlap between the outer cytoplasmic tongue and the next inward layer of partially compacted (or semi-compact) myelin. In this study, we characterize their number, size, connexin composition, and subcellular distribution.

Interpretation of freeze-fracture replicas of internodal myelin

Interpretation of freeze-fracture replicas is somewhat more complicated for myelin membranes than for membranes of other cell types because: (1) successive layers are closely spaced (without intervening cytoplasm or extracellular space to act as landmarks for identifying fracture faces), (2) the abundance and preferential distributions of IMPs to P-faces (rather than to E-faces) is often replaced by areas in which both P- and E-faces are devoid of IMPs and pits, and (3) some areas of myelin membrane contain patches consisting of mixed IMPs and pits on both P- and E-faces (“reciprocal patches”) (Rash et al., 2001). Although glutaraldehyde fixation more consis-

Figure 2. Cx32 immunogold labeling in freeze-fracture replicas of gap junctions in Schmidt–Lanterman incisures. A, Low-magnification view of an extensive stair-step arrangement of cytoplasmic expansions (blue shading) that are characteristic of Schmidt–Lanterman incisures. C, D, The inscribed areas are shown at higher magnification. B, Stereoscopic view of Schmidt–Lanterman incisures. C, D, High-magnification images of Cx32-labeled E-face gap junctions within the Schmidt–Lanterman incisure. Cx32 is labeled with 6 nm gold beads. C', D', High-magnification views of the same gap junctions, presented with black shadows (i.e., in reversed photographic contrast). At high magnification, white shadows (as seen in C and D) are unnatural and are difficult to interpret by most viewers (Steere et al., 1980). E-face pits of the gap junction are highlighted (red area). Immunogold beads are white in black shadow images. Gold beads smaller than 10 nm are difficult to detect or discriminate from shadowed IMPs without stereoscopic imaging. Scale bars (in electron micrographs), 0.1 μm; unless otherwise indicated.
myelin (Fig. 3), the next membrane encountered from the external plasma membrane of the outermost layer further designated as to fracture face (P-face or E-face). Starting designated 1ex and 1in (outer and inner membranes of the outer wrapping of myelin), 2ex and 2in (outer and inner layers of second wrapping), and so on. The fracture plane through a myelinated fiber cross-fractured the cytoplasm (gray) of the outer tongue and then sequentially exposed several membrane faces. Left, The fracture plane first exposed the E-face of the inner plasma membrane of the outer tongue (1inE) and then the P-face IMPS of a gap junction (GJ) and IMPS and pits of a tight junction (TJ) linking the inner plasma membrane of the outer tongue to the P-face of the outer plasma membrane of the second wrapping of myelin (2exP). In the center, the fracture plane exposed the E-face of the inner plasma membrane of the second wrapping of myelin (2inE) and then returned to the P-face of the outer plasma membrane of the first or outermost layer of myelin, which at that point is exposed beyond the tip of the Schwann cell outer tongue. Caveolae (Cv) are cross-fractured and surface-fractured (right margin). B, After SDS washing and immunogold labeling for Cx32, immunogold beads are found almost exclusively at gap junctions, which are identified as hexagonally packed clusters of 9 nm P-face IMPS (Fig. 4) or E-face pits (Fig. 5C).

To facilitate descriptions of myelin layers in freeze-fracture replicas, a numeric system is used (Fig. 3) in which each wrapping of the Schwann cell is numbered from outermost to innermost; each of the two membranes of each cytoplasmic layer is identified as “internal” or “external,” and each replicated membrane is then further designated as to fracture face (P-face or E-face). Starting from the external plasma membrane of the outermost layer of myelin [1internal (1ex)] (Fig. 3), the next membrane encountered is the internal membrane of the same outermost uncompacted layer [1internal (1in)]. The next step inward is the outer membrane of the first partially compacted or first completely compacted layer of myelin [2external (2ex)] followed by its inner membrane [2internal (2in)]. The external and internal membranes of each successive myelin layer are numbered in the same manner. To incorporate information regarding the fracture face of each membrane, the complete designations would be, for example, 1exP, 1inE, or 2exE, and this convention has been used to label each micrograph.

Freeze-fracture images of the outer layers of myelin
The outermost layer of myelin-forming Schwann cells is recognized by its apposition to the collagen-filled extracellular space, as well as by the presence of abundant cortical vesicle pits (Fig. 4A, black arrowheads), which are small cup-like membrane deformations that are characteristic of surface plasma membranes (Mugnaini et al., 1977; Smart et al., 1999). The inner plasma membrane of the same region of each outer tongue (layer 1in) also contains invaginations consistent with the incorporation of exocytotic vesicles (Fig. 4A, white arrowheads). Exocytotic pits represent the sites where cortical vesicles consisting of plasma membrane lipids and proteins fuse with and become incorporated into plasma membranes (Chen and Scheller, 2001). Endocytosis involving clathrin- and caveolin-coated vesicles may also occur, but no attempt was made to differentiate between endocytotic and exocytotic vesicles.

The Schwann cell outer tongue, which is uniformly filled with cytoplasm, is linked to the underlying second layer of myelin by tight junctions (Fig. 4A, white arrows) that run approximately parallel to the long axis of the axon cylinder. Away from the tip of the outer tongue and beyond the band of tight junctions, the remainder of the outer turn is characterized by areas of partially compacted cytoplasm, by reduced or absent cortical vesicle pits, and by the presence of areas resembling partially flattened exocytotic vesicles (Fig. 5A). These flattened invaginations are almost devoid of IMPS. Much of the plasma membrane in the outer turn, particularly near the transition zone to partially compact myelin, contains intermixed particle-free areas in shallow depressions, surrounded by raised areas enriched in small IMPS. Deeper layers of myelin are deficient in cortical vesicle pits and caveolae, and these layers are progressively or completely compacted

Myelin is one continuous spiral, from the outer mesaxon to the inner mesaxon. Beyond the point where the tip of the outer tongue overlaps the outermost layer, the (formerly) outermost plasma membrane becomes the outer membrane of the second layer of myelin. Thus, the seamless transition of the outermost layer of noncompact myelin (Fig. 4, 1exP, outlined in green) into its continuation as the second layer of myelin (Fig. 4, 2exP, green shading) is necessarily indistinct, but nevertheless, is detectable as the area where the density of cortical vesicles is abruptly reduced. In this image, the outer tongue of myelin was removed during fracture, and only a small remnant of the cross-fractured outer tongue is visible (Fig. 4A, blue shading, top center). However, the edge of its three-dimensional imprint is detectable in stereo-
scopic images (data not shown) as a continuous shallow depression of the outer myelin layer, corresponding to the area shaded in green.

Along its entire length, the outer tongue of myelin is linked to the underlying membrane by tight junctions (Mugnaini et al., 1977; Sandri et al., 1977; Peters et al., 1991), binding membranes 1\textsubscript{m} to 2\textsubscript{ex} (Fig. 3A). In areas where the outer tongue of myelin was completely removed by fracturing, tight junctions were seen in myelin layer 2\textsubscript{exP} (Fig. 4A, white arrows). These tight junction strands, although located on a P-face, consist of rows of IMPs (Fig. 4B, black arrow) intermixed with linear grooves of pits (Fig. 4B, white arrow), a mixed morphology that is characteristic of tight junctions in un-
Figure 5. FRIL images of outer myelin layers after immunogold labeling for Cx32. A, Low-magnification image of myelin membrane 1exP, which is characterized by abundant caveolae. B, Layer 2exP, the outer membrane of the underlying Schwann cell wrapping, is characterized by the presence of rivulets containing residual cytoplasm, as documented at different tilt angles, adjacent. Two gap junctions are visible (inscribed areas B and C). B, A gap junction located where the fracture plane stepped from layer 2exP to 1inE consists of both P-face IMPs and E-face pits. The extracellular space (*) is narrowed to \( \frac{1}{11021} \) nm within the area of the gap junction. C, Gap junction in membrane 1inE. The E-face pits are immunogold labeled for Cx32. D, At a high tilt angle, rivulets at the margin of cross-fractured myelin (M) are seen to contain cytoplasm (blue shading). E, Removal of the top rivulet membrane (1exP) reveals a view of the E-face of the underlying membrane 1inE. Gap junctions frequently were found on rivulet membranes (inscribed area F). F, Cx32 immunogold-labeled gap junction (yellow arrowhead) in particle-rich myelin membrane 2inE. ECM, Extracellular matrix; 1exP, P-face of the outer membrane of the outermost Schwann cell wrapping; 1inE, E-face of the inner membrane of the outermost (first) Schwann cell wrapping; 2exP, P-face of the outer membrane of the second Schwann cell wrapping.
fixed and formaldehyde-fixed tissues used for FRIL (see Materials and Methods). Internodal tight junctions run approximately parallel to the long axis of the axons and parallel to the border of continuously compact myelin, except at Schmidt–Lanterman incisures, where they follow the Schmidt–Lanterman incisure internally (Mugnaini et al., 1977). In young adult mice, the first layer of continuously compact myelin usually occurs in the second or third turn. With tight junctions along one side and the border of compact myelin along the other side, these two diffusion barriers create an isolated osmotic compartment in the extracellular space between the outer two wrappings of myelin.

**Cx32 labeling of gap junctions in internodal myelin**

In Figure 4A, a total of four immunogold beads are visible on layer 2\textsubscript{ex}P. At higher magnification, the gold beads were seen associated with three of four distinctive clusters of closely spaced, hexagonally arranged 9 nm IMPs (Fig. 4C–E). All other areas were essentially devoid of clusters of large-diameter IMPs and of gold labels. On the basis of their close association with Cx32-immunogold beads, as well as on low nonspecific background seen elsewhere, these distinctive hexagonal arrays of 9 nm P-face IMPs were identified as gap junctions. Thus, as in other tissues, gap junction IMPs are clearly distinguishable from most other IMPs by their uniform larger diameter and regular hexagonal close packing (Fig. 4A, C–F). However, the gap junctions in this photograph are surprisingly small, ranging from 4 to 52 particles. Also within this patch of membrane, in approximate alignment with the labeled gap junctions is one unlabeled hexagonal cluster of seven IMPs (Fig. 4A, F), also identified on morphological grounds as a gap junction. On the basis of the observed labeling efficiency (see below), ~10% of gap junctions containing 10 IMPs would be expected to be unlabeled.

The four gap junctions seen in Figure 4, located on the same small area of layer 2\textsubscript{ex}P, have relatively regular spacings of ~1 \( \mu \text{m} \) (occasionally up to 4 \( \mu \text{m} \)) between junctions. This pattern of small, closely spaced gap junctions was repeated throughout the length of internodal myelin (see numerical data below). As many as seven gap junctions were seen in a single membrane patch along the boundary between noncompact and partially compact myelin, and in most cases, these were also separated by ~1 \( \mu \text{m} \). Therefore, we calculated that there were 500–1000 gap junctions per millimeter of internodal myelin. The locations and structural interrelationships of tight junctions, gap junctions, caveolae, and cytoplasmic rivulets are illustrated in Figure 3.

The color scheme introduced in Figure 4 is applied in Figure 5 also. The outermost myelin membrane (layer 1\textsubscript{in}P) is outlined in green, and the green shading designates its continuation where the outermost myelin tongue had advanced over it, thereby converting the extension of layer 1\textsubscript{in}P into layer 2\textsubscript{ex}P. This image also illustrates a different view of the membranes of the outer tongue (i.e., the E-face of its inner plasma membrane) (Fig. 5, layer 1\textsubscript{in}E, outlined in red). As noted above, cortical vesicle pits are abundant in the outermost Schwann cell plasma membrane (layer 1\textsubscript{ex}P, outlined in green), as well as in the inner membrane of the outer tongue (layer 1\textsubscript{in}E, outlined in pink). Immunogold beads indicating Cx32 labeling are present at several locations (Fig. 5B, C). These gold beads mark two gap junctions, one of which includes the step from E- to P-face within the margin of the gap junction (Fig. 5B). In such images, the narrowing of the extracellular space within the border of the gap junction (Fig. 5B, asterisk) provided a fourth distinctive feature for identifying gap junctions [criteria listed by Rash et al. (1997, 1998)]. Thus, for gap junctions between layers 1\textsubscript{in} and 2\textsubscript{ex} four freeze-fracture views are possible (1\textsubscript{in}P, 1\textsubscript{in}E, 2\textsubscript{ex}P, and 2\textsubscript{ex}E), with two of those views seen simultaneously where the fracture plane steps from one membrane face to the other within the gap junction (for example, 1\textsubscript{in}E to 2\textsubscript{ex}P) (Fig. 5B).

**Structural components defining the zone of partially compact myelin**

In areas of partially compact myelin, which occur within the outer few wrappings of internodal myelin, distinctive membrane P-face ridges and E-face furrows were observed (Fig. 5A, D). Although these furrows and ridges were described previously (Mugnaini et al., 1977; Sandri et al., 1977), neither group characterized them further. However, when traced to the edge where myelin is cross-fractured, cytoplasm was always found between the P-face ridges and the E-face furrows (Fig. 5D, blue areas). Because freeze-fractured membranes of deeper layers of compact myelin consist primarily of large areas that are essentially devoid of IMPs, and the IMPs in the remaining patches of membrane are primarily <6 \( \text{nm} \) in diameter, and because these areas enclose little or no cytoplasm, the cytoplasmic rivulets gain particular importance in that they represent areas of residual cytoplasm within areas of partially compact myelin. Cx32-labeled gap junctions were frequently found on such membrane ridges overlying cytoplasmic rivulets. In areas where the rivulet P-face membrane was removed over an area of underlying cytoplasm, the imprint of cytoplasm was also evident in the E-face of the underlying furrowed membrane (Fig. 5E), thereby allowing the meandering course and continuity of the cytoplasmic rivulets to be traced over large areas, often extending deep into the second turn of myelin. Close examination of such cytoplasmically expanded membranes revealed Cx32 gold beads beneath both E-face (Fig. 5F, arrow) and P-face images of gap junctions in these areas of partial compaction.

Comparison of the overall IMP densities in rivulet membranes versus nearby compact myelin revealed that rivulet membranes contained many more IMPs than compact myelin (Fig. 5D), and almost all of those IMPs were <6 \( \text{nm} \) in diameter. Moreover, in rivulet E-faces, there were no large pits other than the few hexagonal clusters identified as imprints of gap junctions, and >90% of these clusters of pits were immunogold labeled (Fig. 5F, arrowhead). The gap junction in Figure 5F, located on membrane 1\textsubscript{in}E, was connected to the external membrane of an almost completely compacted rivulet in the second turn of myelin (membrane 2\textsubscript{ex}E). Beneath the gap junction, the remnants of successive stacked rivulets were identified on the basis of their high density of small IMPs, whereas adjacent stacks of compact smooth myelin were devoid of IMPs. Thus, gap junctions linking rivulets in the outer two turns of myelin provide direct radial pathways between the layer of partially compacted myelin and the overlying layer of noncompact myelin (~20 \( \text{nm} \)), whereas the cytoplasm of the rivulets provides a circuitous circumferential pathway (~10 \( \mu \text{m} \)) to the noncompacted, caveolae-rich portions of the outer tongue. On the basis of these structural differences from other areas of myelin, the continuous area between the band of tight junctions in the outer tongue of myelin and the edge of continuously compacted myelin in the second or third turn is defined as the zone of partially compact myelin (also called the area of semicom pact myelin) (Mugnaini et al., 1977). The zone of partially compact myelin contains mixed areas of compact and noncompact myelin, often traversed by cytoplasmic rivulets. Many of these cytoplasmic rivulets were linked to the overlying noncompact myelin by micro gap junctions, and most of these were labeled for Cx32.
Number, locations, and sizes of gap junctions in internodal myelin

In six replicas single-labeled for Cx32, an estimated 500 myelin-ated segments and >2000 patches of membrane from all layers of myelin were examined for immunogold labeling. Ultimately, 152 gap junctions consisting of 1688 connexons were found, and these were labeled by 316 immunogold beads. Some individual IMPs and pits were difficult to discern because of overlapping shadows or local defects in the shadowing film (Figs. 2C, 5B, respectively). Overall, we calculated a labeling efficiency (LE) of one gold bead per 5.3 connexons (LE = 1:5.3). The LE for E-face pits was 1:5, and the LE for P-face connexons was 1:6. Note that labeling of E-face pits is actually against connexins of the hemichannel of the unreplicated but intact hemiplaque of the underlying cell (Fujimoto, 1995; Rash et al., 2001). Incomplete detergent washing leaves sufficient connexins adsorbed to the platinum–carbon replica to permit essentially equal labeling of both E- and P-face images of gap junctions but, in either case, to epitopes in the cytoplasm of the underlying cell or cell process (Fujimoto, 1995; Rash and Yasumura, 1999).

Significantly, these six replicas had a signal-to-noise ratio (SNR) of 15,000:1, which is ~30- to 50-fold higher than previously reported for CNS gap junctions (Rash and Yasumura, 1999; Rash et al., 2001; Pereda et al., 2003). (SNR is defined as the density of gold beads per square micrometer of gap junction divided by the density of gold beads per square micrometer on external plasma membranes and nuclear membranes of the same replica.) In these replicas having a high LE and very low nonspecific background, the resulting extraordinarily high SNR allowed detection of even the smallest gap junctions (Figs. 3C,D, 6B–E).

Six of these labeled gap junctions contained only two IMPs–pits (Fig. 6B,D). Such small gap junctions would not have been detected or identified by any other approach.

Gold beads were not detected near any of the few single isolated 9 nm IMPs, nor were loose aggregates of unlabeled 9 nm IMPs detected anywhere in myelin. Thus, despite extensive searches, we found no evidence for single isolated connexons or distributions consistent with aggregation or dispersal of connexons.

Overall, replicated internodal gap junctions contained an average of 11 connexons, ranging from 2 to 76 connexons (Fig. 6A–D). This largest internodal gap junction, which consisted of 36 P-face particles plus 40 E-face pits, was labeled by five gold beads (Fig. 6A). Also evident in stereoscopic images is the distinctive narrowing of the extracellular space at the area of gap junction contact (Figs. 5B, 6A). Moreover, fractures that stepped from P- to E-face within a gap junction (Fig. 6A) revealed that the apposed P-face IMPs and E-face pits were aligned across the extracellular space, supporting the notion that the connexons were assembled into intact intercellular channels. Because these gap junctions link two cytoplasmic regions within the same cell, they are considered to be reflexive or autologous (Larsen, 1983).

A total of 152 gap junctions was identified in internodal myelin, 140 of which were immunogold labeled, and 12 were unlabeled but were nevertheless identified as gap junctions on the basis of morphological criteria (Fig. 4F). Of these 152 gap junctions, 59 could not be localized to a specific membrane layer or region (i.e., to Schmidt–Lanterman incisures vs internodal myelin) (Fig. 6F shows a gap junction on an isolated pedestal of...
myelin). Of the remaining 93 gap junctions that could be mapped to a particular myelin layer, 95% (88 gap junctions) were localized to the apposition of layers 1\textsubscript{p} and 2\textsubscript{c} (i.e., to the contact between the outermost and second outermost wrappings of myelin). The remaining five gap junctions were deeper within membrane stacks of compacting or compact internodal myelin (Fig. 6E). Because gap junctions were detected on the basis of their proximity to high-visibility immunogold labels, and because the search strategy included searches of all myelin layers (including much larger numbers and areas of internal myelin layers), the infrequent detection of gap junctions in deeper myelin layers suggests that Cx32-containing gap junctions and their constituent connexons are rarely stranded or orphaned in the deeper layers of myelin.

Are other connexins expressed at internodal gap junctions?

To investigate the possibility that the 12 unlabeled gap junctions detected in this study indicate that some gap junctions in the outer layers of internodal myelin contain connexins other than Cx32, FRIL analysis of sciatic nerves was performed using antibodies to other connexins reported to be expressed in glial cells, including Cx26, Cx29, and Cx43. Although immunogold labeling for Cx26 and Cx43 was found in gap junctions in nearby perineurial cells (data not shown), and immunogold labels to Cx29 were found in gap junctions in the innermost layers of Schwann cell myelin (Li et al., 2002; this study, data not shown), none of the other anti-connexin antibodies resulted in specific labeling of gap junctions within the outer layers of internodal myelin. Thus, these data suggest that internodal gap junctions between the outer two layers of myelin contain Cx32 but not Cx26, Cx29, or Cx43. However, the presence of as yet unidentified Schwann cell connexins is not excluded.

Cx32 knock-out mice and the possibility of compensatory upregulation of other connexins

To investigate further the hypothesis that internodal gap junctions are composed solely of Cx32, Cx32 knock-out mice were examined for the possibility of compensatory overexpression of other connexin isofoms in Schwann cells. Although Cx32-deficient mice were shown previously not to express Cx32 mRNA or protein (Nelles et al., 1996), nonspecific binding of Cx32 antibodies was investigated in FRIL replicas of liver and sciatic nerve in Cx32 knock-out mice. Cx32 antibodies produced no labeling of gap junctions in myelin or liver from these animals, confirming the absence of Cx32 expression in knock-out mice, as well as the lack of cross-reactivity of the Cx32 antibodies used in this investigation. However, in the livers of Cx32-deficient mice, fewer and much smaller gap junctions were seen than in wild-type animals, and these remaining gap junctions were labeled for Cx26 but not Cx32 (images not shown). Thus, compensatory upregulation of Cx26 was not detected in the livers or myelin of Cx32 knock-out mice.

Sciatic nerves from the same knock-out mice, labeled for Cx32, Cx26, Cx29, and Cx43, were also examined for labeled and unlabeled gap junctions. Neither labeled nor unlabeled gap junctions were detected in outer myelin membranes in Cx32 null-mutant animals.

Discussion

Using FRIL and immunogold antibodies to Cx32, we found abundant but extremely small gap junctions in myelin of both young adult and mature mouse peripheral nerves. In addition to three examples of Cx32 immunogold-labeled gap junctions in Schmidt–Lanterman incisures, we found 152 gap junctions in internodal membranes, primarily between the uncompacted outer tongue and the second layer of semicompact or partially compacted myelin.

Cx32 in gap junctions at Schmidt–Lanterman incisures

Although gap junctions have been documented in CNS myelin at paranodal loops (Sandri et al., 1977) and linking the outermost layer of myelin with adjacent astrocyte processes (Dermietzel et al., 1978; Massa and Mugnaini, 1982; Rash et al., 1997, 2001), ultrastructurally defined gap junctions have not been found previously at Schmidt–Lanterman incisures in the CNS or PNS. This study provides the first ultrastructural evidence for gap junctions at Schmidt–Lanterman incisures in CNS myelin and documents Cx32 in those junctions, extending previous reports from immunocytochemical and dye coupling experiments that have suggested the existence of gap junctions at that location (Spray and Dermietzel, 1995; Balice-Gordon et al., 1998; Scherer et al., 1998). Although immunostaining for Cx32 is also present at paranodal loops, pointing to the presence of gap junctions at these locations, neither we nor others have observed freeze-fracture evidence for gap junctions at paranodal loops in peripheral nerve myelin, primarily because of the limitations of fracturing areas with sharp membrane curvature, which occurs at Schmidt–Lanterman incisures and paranodal regions.

Cx32, gap junctions, and the zone of partially compact myelin

FRIL revealed the existence and widespread distribution of gap junctions in a newly discovered expression site, between the outer two layers of internodal myelin. Moreover, these gap junctions contained Cx32 but did not contain detectable levels of Cx26, Cx29, or Cx43. The internodal gap junctions were abundant and relatively closely spaced (~1 μm apart). Although much smaller than conventional gap junctions in other tissues, averaging 11 channels per plaque, these gap junctions nevertheless represent >22,000 connexons (and >132,000 connexin molecules) per millimeter of Schwann cell myelin (calculations are based on 12 connexins per functional gap junction channel and one gap junction per micrometer). Even though Cx32 has been documented immunocytochemically in Schwann cells at Schmidt–Lanterman incisures and paranodal loops, the existence of Cx32-containing micro gap junctions in internodal myelin between the outer two wrappings of Schwann cell myelin was not predicted on the basis of light microscopic observations. Although gap junctions in partially compact internodal myelin are abundant, they contain too few connexins to have been detected reliably by immunofluorescence microscopy (but see Fig. 1A). In contrast, the threshold of detectability of FRIL is approximately two connexons. Similarly, analysis of dye transfer cannot, and has not, allowed separate discrimination of dye in two adjacent layers of myelin, which are not separately distinguishable because of inherent limits of resolution of light microscopy, nor can dye diffusion be resolved as occurring through the gap junctions that link two successive outer layers of internodal myelin.

Internodal gap junctions were localized to a distinctive zone that begins adjacent to the band of tight junctions that forms the diffusion barrier between the outermost and the second partially compacted wrapping of myelin and ends at the margin of continuously compact myelin. These internodal gap junctions were located primarily in membranes overlying rivulets of residual cytoplasm within a clearly demarcated zone of partially compact myelin. On the basis of established roles of gap junctions in water transport, ions and small molecules (for review, see Kumar and...
Gilula, 1996), the abundance of gap junctions in areas where cytoplasm was markedly reduced but not eliminated suggests a role for gap junctions in direct radial transport for removal of aqueous components of cytoplasm, presumably from the areas of partial compaction in the second turn to the cytoplasmic, uncompacted, outer tongue of myelin.

The following are additional defining features of the zone of partially compact myelin. (1) In the extracellular compartment, lateral edges of the zone of myelin compaction are delineated by a continuous band of tight junctions on one side, and on the other side, by the edge of the continuously occluded extracellular space of the first continuous layer of compact myelin. (2) In the intracellular compartment, some areas are devoid of cytoplasm, whereas other areas contain meandering rivulets of cytoplasm. (3) In plasma membranes, between the tight junctions and compact myelin, gap junctions are restricted almost exclusively to the cytoplasmic patches and rivulets.

Possible relevance of internodal gap junctions to myelin compaction

In the PNS (sciatic nerve), formation of compact myelin is maximal during early postnatal development and occurs at a moderate pace from the third to the sixth month (the period from which most of our images were obtained) (Webster and Favilla, 1984; for review, see Peters et al., 1991). The zone of partially compact myelin was observed in all of our replicas, which were derived from young as well as old animals (mice 3–19 months of age), indicating that this zone is maintained throughout peripheral nerve development and maturity.

Autoradiographic studies have revealed that both lipids and proteins are added throughout the length of the forming myelinated segment, presumably by incorporation of exocytotic vesicles into the plasma membrane (Webster, 1971). Flattened membrane invaginations devoid of large IMPs and, hence, resembling remnants of fused exocytotic vesicles were found in cytoplasmic myelin in the zone of partial compaction. These invaginations may represent areas of recent addition of membrane characteristic of mature myelin, as suggested by Mugnaini et al. (1977). Moreover, those myelin components appear to be added as particle-free membrane patches, thus resembling preformed compact myelin within the zone of semicompact myelin (Mugnaini et al., 1977). The maintenance of the zone of partial compaction throughout life might therefore point to its functional relevance. However, analyzing the mechanisms and sites of myelin compaction is outside the scope of this FRIL characterization of newly discovered gap junctions in internodal myelin. Nevertheless, we call attention to the various ultrastructural components present in the outer two turns of myelin. A summary diagram (Fig. 7) illustrates the layers of myelin and the location of gap junctions with respect to exocytotic vesicles, tight junctions, cytoplasmic ridges, and the transition zone between noncompact and compact myelin. The drawing also reveals that the gap junctions and tight junctions on layer 2in are precise mirror images of gap junctions and tight junctions on layer 1in.

Differential distribution of Cx32 and Cx29

On the basis of light microscopic evidence, the majority of detectable Cx32 and Cx29 immunoreactivity was reported to be present within Schmidt–Lanterman incisures and paranodal loops (Altevogt et al., 2002; Li et al., 2002). However, investigations of the subcellular distribution of these connexins at the electron microscopic level revealed Cx32 and Cx29 at additional sites. By FRIL, Cx32 was found to be concentrated in gap junctions of the nuclear (abaxonal) end of the myelin (this study), whereas Cx29 was
found to be concentrated in the adaxonal end (Li et al., 2002). The asymmetric subcellular distributions of Cx32 and Cx29 imply that connexins with different properties are required at different subcellular locations, and that there are functional differences in the apical and basal Schwann cell compartments. These functional differences may be related to Cx32-specific activity in the outer layers of myelin versus an unidentified function for Cx29 in the innermost layer of myelin (Li et al., 2002).

Gap junctions as conduits for water and ions

Although Cx32-containing gap junctions in intermodal myelin contain an average of only 11 connexons, the abundance of these junctions may provide sufficient numbers of well distributed channels for the efficient radial transport of water, ions, and small molecules between the partially compact layer and the noncompacted outer tongue. Moreover, the near absence of gap junctions (and very low density of dispersed 10 nm IMPs and absence of gold labels for Cx32) in more inward layers of compact myelin implies that Cx32-containing gap junctions are not present and, hence, not required at these other subcellular sites. Moreover, the rarity of gap junctions in deeper layers suggests the existence of an efficient but fallible mechanism for removing connexons from compacting layers before complete compaction. However, once stranded within deeper myelin layers, absence of cytoplasm may prevent endocytosis and recycling of the few remaining orphaned gap junctions. Finally, the near absence of gap junctions in deeper layers indicates that there is little or no lateral movement of connexons into compact myelin.

Relevance of internodal gap junctions to human neurological disease

Mice with a null mutation in the Cx32 gene have myelinated axons, albeit with distinctive abnormalities, leading to the hypothesis that Cx32 is not required for myelin formation or compaction per se (Nelles et al., 1996; Anzini et al., 1997; Scherer et al., 1998). However, “severe structural abnormalities,” including “abnormally organized noncompacted aspects of myelinating Schwann cells,” have been reported in myelin sheaths of these mice (Anzini et al., 1997). The current demonstration of Cx32 in a newly discovered internodal expression site for gap junctions, as well as in gap junctions at Schmidt–Lanterman incisures, suggests a functional role for Cx32 at these locations. In myelin sheaths of Cx32-deficient mice labeled for Cx32, Cx26, Cx29, and Cx43, neither labeled nor unlabeled gap junctions were detected in outer myelin membranes, indicating either the lack of compensatory expression of any other connexins between the outer two layers of internodal myelin or our inability to find them by FRIL because of the presence of connexins other than those tested. In that regard, the expression of other connexins at Schmidt–Lanterman incisures has been predicted on the basis of dye transfer studies (Balice-Gordon et al., 1998). Schwann cell connexins other than Cx32 might include Cx29 and Cx43, and their expression at Schmidt–Lanterman incisures has not been ruled out by this study.

Deletion of Cx32 resulted in the absence of detectable gap junctions in internodal myelin (this study). Absence of otherwise abundant internodal gap junctions would be predicted to result in altered water and ion homeostasis in the zone of partially compact myelin, which may provide a partial explanation for the deleterious alterations in the CMT-X neuropathy. Because substantial myelin compaction occurs in the absence of gap junctions in Cx32-deficient mice (Scherer et al., 1998), Cx32-containing gap junctions appear not to be required for compaction per se.

Nevertheless, the abundance and regular distribution of internodal gap junctions in normal animals (this study) is consistent with an essential role for these junctions in the unidirectional or bidirectional interlamellar transport of water and ions between the two outer layers of myelin, possibly for the process of myelin compaction or maintaining the border of compact myelin at the transition zone between compacted and noncompact myelin.

References


