Functional Gap Junctions in the Schwann Cell Myelin Sheath

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Abstract. The Schwann cell myelin sheath is a multilamellar structure with distinct structural domains in which different proteins are localized. Intracellular dye injection and video microscopy were used to show that functional gap junctions are present within the myelin sheath that allow small molecules to diffuse between the adaxonal and perinuclear Schwann cell cytoplasm. Gap junctions are localized to periodic interruptions in the compact myelin called Schmidt–Lanterman incisures and to paranodes; these regions contain at least one gap junction protein, connexin32 (Cx32). The radial diffusion of low molecular weight dyes across the myelin sheath was not interrupted in myelinating Schwann cells from cx32-null mice, indicating that other connexins participate in forming gap junctions in these cells. Owing to the unique geometry of myelinating Schwann cells, a gap junction-mediated radial pathway may be essential for rapid diffusion between the adaxonal and perinuclear cytoplasm, since this radial pathway is approximately one million times faster than the circumferential pathway.

Key words: myelin • gap junctions • glia • connexin • neuropathy • Schwann cell

The myelin sheath is a multilamellar structure that ensheathes axons in the peripheral nervous system (PNS) and central nervous system of vertebrates, increasing axonal conduction velocity by restricting the depolarizing ionic fluxes that generate action potentials to nodes of Ranvier (Salzer, 1997). In the PNS, Schwann cells make the myelin sheath, which is composed of two distinct domains, compact myelin and noncompact myelin, each containing distinct proteins. Compact myelin contains protein zero, peripheral myelin protein of 22 kD, and myelin basic protein. Noncompact myelin, found in paranodes and in periodic interruptions in the compact myelin called Schmidt–Lanterman incisures, contains myelin-associated glycoprotein (MAG), the gap junction protein connexin32 (Cx32, also known as β1 connexin), and E-cadherin (Scherer, 1996). E-cadherin is localized to adherens junc-

1. Abbreviations used in this paper: AGA, 18-α-glycerrhetinic acid; CMTX, X-linked Charcot-Marie-Tooth disease; Cx32, connexin32; MAG, myelin-associated glycoprotein; PNS, peripheral nervous system; SIT, silicon-intensified target.

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A portion of these results has appeared in abstract form (Bone, L.J., S.S. Scherer, and R.J. Balice-Gordon. 1996. The role of the gap junction protein connexin32 in myelinating Schwann cells. Soc. Neurosci. Abstracts. 22:1981).

The finding that Cx32 is localized to the incisures and paranodes, where putative gap junctions have been observed, led us to investigate whether incisures and paranodes have functional gap junctions. Such gap junctions could promote intracellular communication, since reflex-
ive gap junctions could provide a radial pathway directly across the myelin sheath. This radial pathway could facilitate the spatial buffering of extracellular potassium during action potential activity (Orkand et al., 1966) and the communication between the adaxonal cytoplasm and the nucleus. We used intracellular dye injection into living, myelinating Schwann cells ensheathing axons and video microscopy to demonstrate that functional, reflexive gap junctions mediate a radial pathway for diffusion of small molecules across the myelin sheath at incisures and paranodes. This radial pathway was also present in myelinating Schwann cells from cx32-null mice, indicating that other connexins participate in forming reflexive gap junctions. The gap junction-mediated radial pathway may be important for rapid diffusion between the adaxonal and perinuclear cytoplasm that would take approximately one million times longer via a circumferential route.

**Materials and Methods**

**Preparation of Teased Sciatic Nerve Fibers**

Young adult (7- or 8-wk-old) mice were anesthetized with CO2 and killed by cervical dislocation. Mouse sciatic nerves were removed and incubated in an ice-cold, oxygenated, buffered salt solution (137 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 29 mM NaHCO3, 1 mM Na2HPO4, 11 mM glucose, pH 7.4, at room temperature). To promote the adhesion of teased fibers and facilitate location of injected fibers after immunohistochemical processing, fibers were teased into small bundles on ~1 × 1-mm grids inscribed on tissue culture dishes with a scalpel blade. Nuclei were visualized after staining with 0.1 µg/ml bis-benzamide (Hoechst; Molecular Probes Inc., Eugene, OR) in Ringer’s for at least 5 min. Incisures and paranodes were visualized with polarized light, since they are isotropic and the doublet separated by 1 μm or less. The distance between the doublets was measured from iontophoretic injection data and was compared with the distance between the outermost peak.

**Injection of Sciatic Nerve Fibers**

The perinuclear region identified by bis-benzamide staining was impaled with intracellular electrodes which were pulled from borosilicate glass with filament with fine tapers (1–4 n.a. oil immersion objectives). Z series were taken planes near the middle of the z series that contained the widest width of the axon diameter in addition to the myelin sheath were analyzed for the pattern of dye diffusion. Some fibers were subsequently fixed and processed for neurobiotin staining and/or immunohistochemistry and then analyzed again by confocal microscopy.

**Video Microscopy**

In some experiments, video microscopy was used to track dye diffusion across incisures. Current injection (5–10 nA, 100-ms pulses, 2 Hz for 2–3 min) was used to iontophoretically inject dye into the perinuclear cytoplasm (model Axoprobe 1A; Axon Instruments, Inc., Foster City, CA). Injections were considered to be successful if a resting potential below 4 mV and rapid longitudinal dye diffusion were observed: ~60–70% of injected fibers met this criteria, and only these fibers were analyzed further.

**Image Analysis**

Images were archived onto magneto-optical disks and printed using a dye sublimation printer (model Phaser 440; Tektronix, Wilsonville, OR). The pattern of dye distribution after injection was analyzed in single light micrographs or confocal images by mapping the intensities of pixels in a line 10–20 pixels wide, perpendicular to the long axis of the fiber (MetaMorph software; Universal Imaging Corp.). At least three regions were sampled in each image. In fibers judged to have been successfully injected (see above for criteria), one of two patterns was observed in each fiber analyzed, regardless of image contrast enhancement or the absolute intensity of labeling. In some cases, the line intensity histogram included a pair of single peaks separated by 8–12 μm. In other cases, the line intensity histogram included one or two peaks that were doublets, with each peak within the doublet separated by 1 μm or less. The distance between the doublets was compared with the distance between the outermost peak.

The rate of dye diffusion across incisures was measured from iontophoretic injection of myelinating Schwann cells from wild-type and cx32-

**Neurobiotin Histochemistry and Immunohistochemistry of Injected Sciatic Nerve Fibers**

After some injections, fibers were stained immunohistochemically for MAG after fixation in 4% paraformaldehyde and permeabilization with acid alcohol for 10 min. Fibers were blocked in 5% fish skin gelatin and 0.1% Triton X-100 and then incubated with a polyclonal rabbit anti-MAG antiserum (Pedraza et al., 1990), followed by rhodamine-conjugated donkey anti-rabbit secondary antibody as previously described (Scherer et al., 1995). The resulting staining pattern was evaluated using epifluorescence or confocal microscopy. To ascertain the localization of Cx32, we similarly immunostained adult rat teased fibers (fixed in Zamboni’s fixative [Zamboni, L., and C. de Martino. 1967. J. Cell Biol. 35:148a] for 30 min) with a monoclonal antibody for Cx32 (7C6.C7; Li et al., 1997) and a rabbit anti-aminoglutethimide and permeabilization with acetic alcohol. Fibers were blocked in 5% fish skin gelatin and 0.1% Triton X-100, labeled with 50 µg/ml rhodamine-conjugated streptavidin (Sigma Chemical Co.). Injected Schwann cells were then relocated using the etched grid as a guide (see Fig. 1, C and D) and imaged using confocal microscopy.

In some experiments, the viability of fibers was also evaluated by a live/dead assay according to the manufacturer’s instructions (Molecular Probes, Inc.). When viewed with the appropriate fluorescence optics, this technique stains living cells green (because they internalize and process calcine AM ester) but not red (because they exclude ethidium bromide homodimer from the nucleus).
null mice, in a set of experiments in which images were acquired at 30 frames/s from the onset of injection. Due to the limited spatial and temporal resolution of light video microscopy, we could not measure the rate of diffusion within incisures per se. Instead, we analyzed video frames (2–6 frames, 33 ms apart) in which we could clearly see an incisure in the field and measure the location of the dye front before, during and after the inner collar of cytoplasm filled.

Pharmacological Blockade of Gap Junctions
Halothane, octanol, and 18α-glycerrhetinic acid (AGA) were purchased from Sigma Chemical Co. Nerve fibers were incubated in 1–10% halothane, 1–100 μM octanol, or 1–100 μM AGA. AGA was dissolved in DMSO (100-mM stock solution) and diluted to 75 μM in oxygenated Ringer’s immediately before use. Nerve fibers were incubated in AGA for 40–105 min and then were injected iontophoretically with 5,6-carboxyfluorescein and were monitored optically for 1–4 h. All values are reported as mean ± SEM.

Generation of Cx32 Mutant Mice
The generation and initial characterization of cx32-null (cx32−/−; female and cx32Y/−) mice has been described (Nelles et al., 1996). Animals were generated from our colony at the University of Pennsylvania from two breeding pairs of cx32-null mice obtained from K. Willecke (University of Bonn, Bonn, Germany) (Nelles et al., 1996). Genotypes were established by PCR analysis of genomic DNA isolated from tail clips (Anzini et al., 1997), and confirmed by Southern blot analysis.

Results

Intracellular Dye Injection and Imaging of Living Myelinating Schwann Cells
To determine whether a radial pathway exists across the myelin sheath, mouse sciatic nerves were removed, incubated in physiological salt solution, and then teased into small bundles. Teased fibers were stained with bis-benzamide to identify the Schwann cell perinuclear region (Fig. 1 A, inset), which contains the largest amount of Schwann cell cytoplasm (Gould and Mattingly, 1990). Large myelinated fibers were selected for injection, and the perinuclear region was impaled with a microelectrode (Fig. 1 A) and injected with fluorescent dyes either by iontophoresis (Fig. 1, B and C) or by pressure. The pathway of dye diffusion was monitored with epifluorescence microscopy and the location of injected fibers was mapped using a grid etched into the bottom of the injection dish (Fig. 1 D). In iontophoresis experiments, Schwann cell resting membrane potential was monitored during and after iontophoretic injection as an index of cell viability. Schwann cell resting membrane potentials (−12.2 ± 1.9 mV; n = 33 fibers from 13 mice) were similar to those previously reported (Wilson and Chiu, 1993) and remained constant or improved during the course of the injection. Thus the dissection, injection, and imaging procedures did not seem to affect the viability of myelinating Schwann cells.

Low Molecular Mass Dyes Traverse the Myelin Sheath
Teased fibers were viewed with polarized light, revealing the locations of incisures, which are funnel-shaped, isotropic structures that interrupt the anisotropic compact myelin (Fig. 2 A). In some fibers, we directly demonstrated that these isotropic structures are incisures by fixing the fibers and immunostaining them for MAG, which is localized to incisures and the adaxonal surface of myelinating Schwann cells (Fig. 2 B). Finally, we double labeled teased fibers with antibodies against Cx32 and E-cadherin, which were colocalized at incisures and paranodes (Fig. 3). Thus, incisures can visualize in living teased fibers, and contain Cx32, MAG, and E-cadherin.

After injection of low molecular mass dyes, such as 5,6-carboxyfluorescein (376 Da), into the perinuclear region, we consistently observed a pattern of labeling similar to a pair of train tracks. Each track consisted of a double line of fluorescence, the lines separated by an unstrained space that corresponded precisely to the location of compact myelin (compare Fig. 2, A with C). A similar pattern was observed when dye was injected by iontophoresis (n = 30/33 fibers from 13 mice) or by pressure (n = 35/40 fibers from 24 mice). The train track pattern was observed in images that included the widest diameter of the axon, usually midway through the depth of the myelinated fiber. Cytoplasmic labeling was seen in planes above and below the planes containing the train track pattern (Fig. 2 D). Although dye diffusion was usually monitored near the site of injection, in most fibers, dye also rapidly diffused to each node, filling the paranodal region. The dynamics of dye diffusion were not routinely monitored in this location due to its distance from the site of injection, which precluded the paranodes from being visualized in the same field as the site of injection. Dye was not observed to pass from Schwann cells to axons nor to cross nodes of Ranvier (Fig. 1 C and Fig. 2 B), as previously reported (Konishi, 1990; David et al., 1993; Dezawa et al., 1998).

Figure 1. Dye injection into living, teased myelinated fibers from mouse sciatic nerve. Shown are images of a single fiber that was iontophoretically injected with 5,6-carboxyfluorescein. (A) Polarized light in combination with fluorescent bis-benzamide staining (inset) shows an electrode near a Schwann cell nucleus. (B) Diffusion of 5,6-carboxyfluorescein during iontophoretic injection. (C) Diffusion of dye from node to node was observed within 0.5–5 min (this image was captured at ~1 min into injection). (D) After imaging of dye diffusion, injected fibers were mapped at lower magnification relative to grid lines (same field as C) to facilitate identification for confocal microscopic analysis. Bars, 10 μm.
The pattern of 5,6-carboxyfluorescein filling indicates that this dye filled the cytoplasmic space on both sides of the myelin sheath. To analyze this further, we mapped the intensities of pixels in line perpendicular to the long axis of the fiber; this analysis is illustrated in each figure. In fibers injected with 5,6-carboxyfluorescein (Fig. 2 E), the line intensity histogram showed two peaks, each of which contained a second, inner peak (Fig. 2 F). The distance between the outermost peaks was 10.2 ± 0.7 μm, which corresponds to the diameter of large, myelinated fibers. The doublets were separated by 1.7 ± 0.4 μm; n = 19 fibers analyzed), which corresponds well to the thickness of the myelin sheath of the same fibers viewed by polarized optics (1.8 ± 0.5 μm; not significantly different, Student’s t test). This analysis supports the idea that the myelin sheath separates the inner and outer collars of cytoplasm filled by dye. These data agree well with the thickness of the myelin sheath in adult peripheral nerve; the myelin sheath has been reported to account for nearly 40% of the total fiber diameter (Friede and Bischhausen, 1980; Berthold and Rydmark, 1995). In the present experiments, the thickness of the compartment bracketed by double train tracks was 37 ± 4% of the total fiber diameter (not significantly different, Student’s t test).

To determine directly whether the double train track pattern of dye diffusion resulted from dye passing from the outer/abaxonal collar of Schwann cell cytoplasm to the inner/adaxonal collar of cytoplasm, the path of dye diffusion was monitored continuously during injection. Selected planes from one such sequence are shown in Fig. 4. From the onset of injection, dye spread rapidly along the myelin sheath, initially remaining within the outer/abaxonal collar near the nucleus (Fig. 4; 0 s). In most fibers, the dye appeared to collect at nearby incisures (Fig. 4; 15 s, arrowhead), partially filling the inner/adaxonal collar of cytoplasm immediately and more completely within a few seconds (Fig. 4; 30 s, arrow). The dye continued to fill these compartments, with each line becoming progressively brighter in intensity (Fig. 4; 80 and 147 s), and also spread longitudinally, usually reaching the nodes of Ranvier within a few minutes. Injections that were monitored with continuous acquisition onto videotape confirmed the above sequence of events, and, in addition, showed that...
A doublet is apparent in the only clearly visualized on the left side of the fiber. The train pattern is seen further away from the injection site, but in this case is in terms of absolute intensity (across the same region of the fiber over time could be compared camera so that changes in the line histogram intensity mapped dye diffusion were obtained using manual gain settings of the SIT camera, allowing quantitative comparisons of changes in intensity mapped across the same region of the fiber over time (Fig. 4; right-hand panels). This analysis confirmed that the intensity of the outer and inner cytoplasmic incisures increased over time, and that the inner incisural collar filled after the outer one. Thus, the temporal sequence of the train track pattern indicates that 5,6-carboxyfluorescein diffuses from the outer/abaxonal to the inner/adaxonal cytoplasm across incisures.

The rate of dye diffusion was estimated in a subset of iontophoretic injection experiments in which images were acquired at 30 frames/s from the onset of injection (n = 6 fibers from 5 mice). This was accomplished by measuring the difference in the location of the dye front in consecutive images, immediately before and immediately after an incisure was filled and the train track pattern appeared. The mean rate of diffusion estimated in this way was 4.25 ± 0.49 μm/s, average ± SEM; range 2.27–6.06). We could not measure a rate of diffusion within incisures themselves owing to the limited temporal and spatial resolution of video microscopy.

We also evaluated whether other low molecular mass compounds known to cross gap junctions also filled the inner/adaxonal collar of Schwann cell cytoplasm. Fluorescence from other commonly used compounds, such as Lucifer yellow, propidium iodide, and ethidium bromide, was not as intense as that from 5,6-carboxyfluorescein, making it difficult to monitor dye movement by video microscopy. These dyes also have broad-spectrum emission that interfered with staining in other fluorescence channels. Moreover, since propidium iodide and ethidium bromide are impermeant to Cx32 channels in transfected cells (Elfgang et al., 1995), we selected Lucifer yellow and neurobiotin. Lucifer yellow injected into myelinating Schwann cells produced a double train track pattern of staining (n = 6 fibers from two mice; data not shown). Neurobiotin, a nonfluorescent, low-mass compound that crosses gap junctions, was coinjected iontophoretically with 5,6-carboxyfluorescein to monitor the injection (Fig. 5 A). The fibers were examined by confocal microscopy (Fig. 5 B). Evaluation of serial single confocal planes through the z axis confirmed the double train track pattern of labeling of 5,6-carboxyfluorescein, and demonstrated that this pattern was distinct from cytoplasmic labeling (Mugnaini et al., 1977; Gould and Mattingly, 1990). Fibers then were fixed, permeabilized, stained with rhodamine-conjugated strepavidin to visualize the neurobiotin, and then were reexamined by confocal microscopy (Fig. 5 D; same fiber as in A–C), although the structure of the myelin sheath was distorted by this processing. In nine out of

**Figure 4.** Rapid diffusion of 5,6-carboxyfluorescein across incisures to the inner/adaxonal collar of Schwann cell cytoplasm. Right panels, from the same fiber at the onset (0 s), and 15, 30, 80, and 147 s after the onset of iontophoretic injection. The locations of an incisure are indicated with an arrowhead and were confirmed by viewing the fiber with polarized light. At the onset of injection, dye immediately fills the outer collar of Schwann cell cytoplasm. By 15 s, an incisure is labeled and a faint train track is apparent on one side of the myelin sheath. The train track pattern and incisure are more apparent at 30 s. At longer times (80 and 147 s), more incisures become filled and the train track pattern is seen further away from the injection site, but in this case is only clearly visualized on the left side of the fiber. Left panels, quantitative analysis of pattern of dye distribution. The intensity of pixels in a line perpendicular to the long axis of the fiber was mapped at the position indicated by the black arrowheads in the corresponding photomicrograph. The light microscopic images of dye diffusion were obtained using manual gain settings of the SIT camera so that changes in the line histogram intensity mapped across the same region of the fiber over time could be compared in terms of absolute intensity (right panels; 0–255 intensity levels). A doublet is apparent in the left side of the histogram by 30 s after the onset of injection (black arrow; corresponding location in the 30-s image is indicated by white arrow). Within the doublet, the incisures themselves were labeled as the dye front moved down the myelin sheath. In some fibers, including the one illustrated in Fig. 4, light microscopic images of dye diffusion were obtained using manual gain settings of the SIT camera, allowing quantitative comparisons of changes in intensity mapped across the same region of the fiber over time (Fig. 4; right-hand panels). This analysis confirmed that the intensity of the outer and inner cytoplasmic incisures increased over time, and that the inner cytoplasmic collar filled after the outer one. Thus, the temporal sequence of the train track pattern indicates that 5,6-carboxyfluorescein diffuses from the outer/abaxonal to the inner/adaxonal cytoplasm across incisures.

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12 fibers, both 5,6-carboxyfluorescein and neurobiotin stained in a double train track pattern, indicating that both compounds filled the inner collar of Schwann cell cytoplasm. Thus, several low molecular mass compounds known to pass through gap junctions appeared to diffuse across the myelin sheath.

A small number of myelinated fibers from rats (n = 12 fibers from seven rats) and frogs (n = 8 fibers from two Rana pipiens) were also injected with 5,6-carboxyfluorescein. Incisures were present in both species. In both rats and frogs, a double train track pattern identical to that observed in mice was observed (data not shown), indicating that the incisures in these species also contain functional gap junctions.

**High Molecular Mass Dyes Do Not Traverse the Myelin Sheath**

Since gap junctions have been reported to have a perme-
atation limit at ~1,000 Da (Bruzzone et al., 1996), we determined whether dyes of higher molecular mass would diffuse in a pattern similar to that observed with low molecular mass compounds. Pressure injection was used to inject large molecular mass dyes because iontophoretic injection was unreliable. Dye passage through the high resistance electrodes was difficult, and many injections were judged to have failed, either because dye did not diffuse beyond the perinuclear region or because the electrodes clogged during the injection ($n$ = six out of nine injections from six mice). When 3,000-Da rhodamine-conjugated dextran was successfully injected into the perinuclear region ($n$ = three fibers from three mice), dye spread longitudinally in a similar fashion to 5,6-carboxyfluorescein, except that fluorescence was not observed in the inner collar of Schwann cell cytoplasm. Even after allowing 1–4 h for additional diffusion (Fig. 6 A), dye remained confined to the outer collar of Schwann cell cytoplasm. Subsequent confocal analysis of the same fibers verified the absence of a double train track pattern of dye labeling (Fig. 6 B). Image analysis of intensity mapped along a line perpendicular to the long axis of injected fibers showed that no doublet in the intensity peaks was observed in any of the injected fibers (Fig. 6, A and B, bottom). Similar results were obtained in rats (data not shown) after injection of 3,000-Da fluorescein-conjugated dextran ($n$ = three fibers from two rats) or 10,000-Da rhodamine-conjugated dextran ($n$ = two fibers from one rat). Thus, low molecular mass, but not high molecular mass, compounds diffuse radially across the myelin sheath.

**Dye Diffusion Across the Myelin Sheath Is Prevented by Pharmacological Blockade of Gap Junctions**

To further evaluate whether functional gap junctions were present in incisures, teased nerve fibers were incubated in 1–10% halothane, 1–100 μM octanol, or 1–100 μM AGA, all of which have been shown to block gap junctions (Davidson and Baumgarten, 1988; Bruzzone et al., 1996; Goldberg et al., 1996; Goodenough et al., 1996). Schwann cell toxicity was assayed by measuring resting potential and by visually determining whether structural changes in the membrane occurred during the incubation. In some experiments, a fluorescence assay was used to evaluate cell viability, by the criteria of exclusion of ethidium bromide homodimer from the nucleus and internalization and processing of calcein AM ester which results in cytoplasmic labeling (live/dead assay, Molecular Probes, Inc.). These three criteria were used to evaluate Schwann cell viability in the course of these experiments.

Incubation in >1% halothane or >1 μM octanol rapidly induced loss of resting potential, widening of incisures, and disorganization of the Schwann cell membrane (data not shown). Teased fibers maintained in 1–100 μM AGA, however, retained their structural integrity, internalized and then processed calcein AM ester, and excluded ethidium bromide homodimer from the nucleus for more than 4 h. Thus, these concentrations of AGA did not appear to be toxic, and 75 μM AGA was used for subsequent experiments. Nerve fibers were incubated in 75 μM AGA for 40–105 min, then injected iontophoretically with 5,6-carboxyfluorescein and monitored optically for 1–4 h. In pilot experiments, we determined that 30–40 min of incubation in AGA was required to block radial dye transfer in myelinating Schwann cells. The length of time required is probably due to the geometry of the myelin sheath as well as the probable inaccessibility of putative gap junctions to bath applied AGA. In addition to retaining their structural integrity, myelinating Schwann cells incubated in AGA had resting potentials near the normal range ($-8.1 \pm 1.6$ mV, $n = 15$ fibers from seven mice). Myelinating Schwann cells incubated in AGA did not have a double train track pattern after dye injection ($n$ = six or seven fibers from five mice); the dye remained in the outer collar of cytoplasm (Fig. 7, A and B) and no doublet in the intensity histogram was observed (Fig. 7, A and B, bottom panels). Nerve fibers incubated in 0.15% DMSO (the carrier solution used to dissolve AGA) for a similar length of time also had resting potentials near the normal range ($-9.5 \pm 1.1$ mV, $n = 11$ fibers from six mice), but 11 out of 11 fibers showed the double train track pattern after dye diffusion, which was confirmed by the presence of a doublet in the intensity histogram on at least one side of the fiber (Fig. 7 C). Thus, AGA prevented dye from filling the inner collar of Schwann cell cytoplasm, suggesting that functional gap

![Figure 7. Pharmacologic blockade of gap junctions with AGA prevents the diffusion of 5,6-carboxyfluorescein across incisures.](image-url)
junctions within incisures mediate the diffusion of dye into the inner collar of cytoplasm.

Dye Diffusion across the Myelin Sheath in Cx32-null Mice

Since Cx32 is the only gap junction protein known to be localized to incisures, we evaluated the possibility that Cx32 is necessary for the diffusion of small molecular mass dyes across the myelin sheath by injecting myelinating Schwann cells from cx32-null mice. We teased myelinated fibers from 4-mo-old mice, when few sciatic fibers are demyelinated (Anzini et al., 1997; Nelles et al., 1996; Scherer et al., 1998), avoiding any fibers that appeared abnormal, and injected them with 5,6-carboxyfluorescein by iontophoresis. When viewed with polarized light, myelinated fibers in cx32-null mice had incisures, which was confirmed by MAG-immunostaining (Scherer et al., 1998). The mean resting potential ($-6 \pm 3$ mV) in myelinating Schwann cells from cx32-null mice was similar to that observed in wild type mice. Double train tracks of fluorescence (Fig. 8, left) and doublets in each peak of the line histogram analysis (Fig. 8, right) were observed in all of the successfully injected fibers ($n = six$ fibers from three mice). Moreover, the mean rate of diffusion was estimated to be $4.09 \pm 0.67$ mm/s ($n = five$ fibers from three mice; average $\pm$ SEM; range 2.02–7.58). This rate is not significantly different from that observed in myelinating Schwann cells from wild type mice (Student’s t test). Thus, the absence of Cx32 does not appear to affect the radial pathway of dye diffusion across the myelin sheath, indicating that there are functional gap junctions in the incisures of cx32-null mice.

Discussion

The results of our injections provide the first functional evidence that gap junctions mediate a radial pathway of diffusion across the myelin sheath. Low molecular mass compounds diffused rapidly across the myelin sheath, from the outer/abaxonal cytoplasm to the inner/adaxonal cytoplasm, whereas high molecular mass compounds remained confined to the outer/abaxonal cytoplasm. Furthermore, the inner/adaxonal cytoplasm was not labeled after injection of low molecular mass compounds in the presence of pharmacological blockers of gap junctions. The gap junctions mediating the diffusion of low molecular mass dyes are probably localized in Schmidt–Lanterman incisures, since these structures appear to fill with 5,6-carboxyfluorescein during injections, contain Cx32-immunoreactivity (Bergoffen et al., 1993; Chandross et al., 1996a; Scherer
These results are summarized in Fig. 9, which is a schematic view of dye diffusion in myelinating Schwann cells after perinuclear dye injection. One myelinating Schwann cell has been unrolled to reveal the regions of compact myelin, incisures, and paranodes. The continuous lines represent rows of tight junctions; two or more rows form a circumferential belt around the perimeter of the cell and are also found in incisures (Tetzlaff, 1978; Shinowara et al., 1980; Sandri et al., 1982; Stolinski and Breathnach, 1982). These tight junctions are probably leaky, since they are not well developed as in tight epithelia (Friend and Gilula, 1972; Claude and Goodenough, 1973). Gap junctions are depicted as ovals and are found between the rows of tight junctions. The left Schwann cell has been injected with a low molecular mass compound, which we demonstrated diffused across incisures to fill the inner collars of Schwann cell cytoplasm. This resulted in a double train track pattern whose width closely matched the width of the myelin sheath. The right Schwann cell has been injected either with a high molecular mass compound or with a low molecular mass compound in the presence of a pharmacological blocker of gap junctions. In both cases, the dye diffused within the outer collar of Schwann cell cytoplasm, but did not reach the inner collar of cytoplasm.

**Functional Significance of Gap Junctions in the Myelin Sheath**

The importance of gap junctions in the myelin sheath was not known until mutations in Cx32 were shown to be associated with X-linked Charcot-Marie-Tooth disease (CMTX), the eponym for dominantly inherited neuropathies (Bergoffen et al., 1993). CMTX is the second most common form of inherited demyelinating neuropathy, and more than 130 mutations in Cx32 have been described (Bone et al., 1997). The importance of Cx32 in the Schwann cell myelin sheath may owe to its unique geometry: the unrolled spiral of myelin membrane may be as long as 4 mm, whereas it is less than 4 μm in the radial dimension (Friede and Bischhausen, 1980). A radial channel through the myelin sheath would thus be roughly 1,000 times shorter than the circumferential pathway. This difference translates to a million-fold shorter diffusion time, since diffusion in a plane is proportional to the square of the distance (Berg, 1983). It is possible that this pathway may be compromised in CMTX, in which the failure of ions and small molecules to diffuse across the myelin sheath damages myelinating Schwann cells and their axons, resulting in demyelination and axonal loss (Rozear et al., 1987; Sander et al., 1998; Hahn et al., 1990).

Using video microscopy, we estimated that the rate of radial diffusion of 5,6-carboxyfluorescein across incisures was ~4 μm/s. This raises a potential problem, because if the rate of diffusion within incisures (circumferentially, within the cytoplasm) was 4 μm/s, then circumferential diffusion of 5,6-carboxyfluorescein should have been observed by 4 h in the myelinating Schwann cells that were incubated in AGA. We could not measure the rate of diffusion within the incisures, however, and this may be substantially slower than 4 μm/s. Our observation that a 3,000-kD fluorescent dye also does not reach the adaxonal cytoplasm within 4 h provides additional support for this explanation.

We observed that the myelin sheaths of cx32-null mice have a radial pathway for diffusion that does not appear to be significantly slower than that observed in wild-type mice. The presence of functional gap junctions in cx32-null mice indicates that myelinating Schwann cells express at least one other gap junction protein, and perhaps several. One candidate is Cx46, although Cx46 has only been shown to be expressed by denervated Schwann cells (Chandross et al., 1996a,b); whether myelinating Schwann cells also express Cx46 remains to be demonstrated. Another candidate is Cx43 (Chandross et al., 1996a; Scherer et al., 1995; Yoshimura et al., 1996), although this connexin has not yet been localized to incisures or paranodes. The other putative connexin(s), however, is not sufficient to preserve the functional or structural integrity of myelinating Schwann cells, as cx32-null mice develop a demyelinating neuropathy (Anzini et al., 1997; Scherer et al., 1998). This could be an indication that the gap junctions formed by other connexins are not permeant to the same molecules as gap junctions formed by Cx32 (Elfgang et al., 1995). Alternatively, Cx32 can interact with other connexins, forming heteromeric hemichannels and heterotypic channels that may have different voltage sensitivities and conductances (Bruzzone et al., 1996), so that both connexins may be required to form the proper channels in myelinating Schwann cells.

Our work provides the first demonstration of the functional importance of incisures, which are an ancient and fundamental adaptation of the myelin sheath. The gap junction plaques observed within incisures and paranodes may play a functional role in intracellular communication. Because of the unique geometry of myelinating Schwann cells, reflexive gap junctions across the myelin sheath are likely to be important for communication between the adaxonal cytoplasm and nucleus. In the absence of Cx32, intracellular communication mediated by the remaining gap junction proteins may be compromised, leading to demyelination and axonal damage.

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**References**

Anzini, P., D.H.-H. Neuberg, M. Schachner, E. Nelles, K. Willecke, J. Zielasek, K. Toyka, U. Suter, and R. Martini. 1997. Structural abnormalities and deficient maintenance of peripheral nerve myelin in mice lacking the gap junc-
