Clustering of Na⁺ Channels and Node of Ranvier Formation in Remyelinating Axons

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Polyclonal antibodies were raised against a well conserved region of the vertebrate Na⁺ channel and were affinity purified for use in immunocytochemistry. Focal demyelination of rat sciatic axons was initiated by an intraneural injection of lysolecithin and Na⁺ channel clustering was followed at several stages of myelin removal and repair. At 1 week post-injection axons contained long, fully demyelinated regions. Na⁺ channel clusters appeared only at heminodes forming the borders of these zones, and at widely spaced isolated sites that may represent former nodes of Ranvier. Over the next few days proliferating Schwann cells adhered to axons and began to extend processes. Clusters of Na⁺ channels appeared at the edges of these structures. As the Schwann cells elongated, the clusters seemed to move with them, since they remained at edges and the distance between aggregates increased. Clusters associated with different Schwann cells ultimately approached each other and appeared to fuse. Na⁺ channels then coalesced further at these sites, forming new nodes of Ranvier in regions that previously were internodal. If Schwann cell proliferation were blocked by mitomycin, no new clusters of Na⁺ channels appeared within internodes. Under these conditions, heminodal clusters remained visible at 1 week postinjection, but by 2 weeks they were no longer detectable, suggesting that proliferating Schwann cells are required for their maintenance. Clusters at normal nodes of Ranvier remained. It is concluded that Na⁺ channel aggregation and mobility in demyelinated nerve fibers is controlled by adhering Schwann cells, resulting in the formation of stable new nodes of Ranvier during remyelination.

[Key words: sodium channel, remyelination, axon, Schwann cell, node]

The myelination of axons by Schwann cells and oligodendrocytes represents one of the most complex intercellular interactions in the nervous system. One essential aspect of this process involves the establishment of a marked heterogeneity in axonal ion channel distributions among nodal, paranodal, and inter-

nodal regions (Black et al., 1990). The density of Na⁺ channels, in particular, is about 25 times higher at nodes of Ranvier than at internodal sites (Shrager, 1989). There has been vigorous debate over the mechanism of Na⁺ channel clustering during myelination, particularly with respect to the role of Schwann cells, and studies have included both developing nerve and pathological conditions (Ellisman, 1979; Rosenbluth, 1979; Rosenbluth and Blakemore, 1984; Le Beau et al., 1987; England et al., 1990, 1991; Joe and Angelides, 1992, 1993). There remain many interesting questions, particularly regarding remodeling that occurs following myelin disruption. When axons are demyelinated, repair processes, especially vigorous in the peripheral nervous system, restore myelin but with altered structure. A series of short internodes results when new nodes of Ranvier are formed in regions that previously were internodal. It has been demonstrated electrophysiologically that these new nodes have densities of Na⁺ channels that are significantly higher than the original internodal density, and that they are functional during action potential conduction (Shrager, 1988, 1989; Shrager and Rubinstein, 1990). What determines the sites of the new nodes, and what is the origin of their Na⁺ channels? Do the original nodes survive as well? This article utilizes immunocytochemical labeling to attempt to answer these questions. The results suggest a unique role of Schwann cells in this process.

Materials and Methods

Axon preparation. Adult female Lewis rats were anesthetized with chloral hydrate/pentobarbital (0.3 ml/100 gm) and the sciatic nerve in one leg surgically exposed. Two or three branches were each injected with 1 μ l of 1% lysolecithin in sterile Locke's solution using a glass micropipette broken to a tip diameter of 18–20 μ m. The wound was closed and the animal allowed to recover. The injection was designed to demyelinate 50–75% of the axons within each branch, which allowed sufficient residual function. Remaining axons appeared to have normal morphology. In some experiments the lysolecithin solution included 400 μ g/ml mitomycin (Sigma Chemical Co.) to block Schwann cell proliferation. Seven to sixty days later, the rat was killed by CO₂ and the sciatic nerve was dissected, desheathed, and dissociated into single fibers with collagenase/dispase (Sigma Chemical Co., 3.5 mg/ml, 60 min at 35°C). Axons were gently teased with fine needles and spread over coverslips that had been coated in a few spots with Cell-Tak (Collaborative Biomedical Products). Fibers were placed to maximize the length between glue spots since adherent debris produced significant background fluorescence on the adhesive. The preparation was then fixed in 4% paraformaldehyde for 30 min, rinsed in 0.05 M phosphate buffer (PB), pH 7.4, and dried (5 min at 35°C).

In some experiments, in order to retract myelin from the node and expose the paranodal regions, axons were incubated in protease (Sigma bacterial, type XXIV), 1 mg/ml, 20 min at 35°C following the collagenase dissociation (Jonas et al., 1989).

Antisera. Site-directed polyclonal antibodies were raised to an 18-mer peptide (EOIII) corresponding to a region highly conserved in all

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known vertebrate sodium channels. This segment (TEEQKKYYN-AMKKLGSKK) is found in a putative intracellular loop connecting homology domains III and IV and appears to play a role in sodium channel inactivation (Vassilev et al., 1988). EOIII was synthesized with the addition of a carboxy terminal cysteine by conventional solid phase methods in the institutional facility at the University of Colorado Medical School. Following purification by reverse-phase HPLC, EOIII was conjugated to maleimide-activated keyhole limpet hemocyanin (KLH) using the Imject kit obtained from Pierce. Antisera to KLH-EOIII conjugates were raised by immunizing rabbits at 4 week intervals. One of these antisera, designated 2944, exhibited very high titers to purified eel electroplax sodium channels in a sensitive radioimmunoassay (England et al., 1991). The radioimmunoassay signal was also diminished by pure EOIII peptide in the same manner as equivalent mole amounts of pure sodium channel. Conditions for preadsorbed controls could thus be established for immunocytochemistry (see below), and anti-EOIII concentrations in whole sera were estimated to be 1–3 mg/ml for various bleedings. Finally, 2944 antibodies were purified from antisera via affinity chromatography on an EOIII-coupled column (ImmunoPure Ag/Ab Immobilization Kit #2, Pierce); the resultant antibody fractions had anti-EOIII titers roughly one-half that of the original antisera. These purified antibodies were used in all subsequent immunolabeling experiments described below.

Immunocytochemistry. Axons were preincubated in 0.1 M PB, pH 7.4, containing 0.3% Triton X-100 and 10% goat serum (PBTGS) for 1.5 hr at room temperature (RT). For Na⁺ channel labeling, following preincubation axons were exposed to the primary antibodies at a dilution of 1/50 in PBTGS for 15 hr at RT. As controls, antibodies were preabsorbed with the peptide antigen (1 μ l of peptide/2 μ l of antibodies) overnight at 4°C. In some experiments an anti-S-100 polyclonal rabbit antibody (Dako) was used as the primary antiserum to label Schwann cells. Following overnight incubation, the slides were rinsed several times with PBTGS, and incubated with biotinylated goat anti-rabbit IgG (1/200, 1 hr, RT) followed by avidin-FITC (1/100, 1 hr, RT). The above were all in PBTGS, and the samples were rinsed (PBTGS, 3 $\times$, 15 min each) between incubations. Samples were rinsed with 0.05 M PB, dried, and mounted with DPX (BDH Supplies, Poole, England). Observations were made on a Nikon Labophot fluorescence microscope and on a Meridian ACAS laser scanning confocal instrument, each fitted with a 100 $\times$ oil-immersion objective.

Electron microscopy. Axons were fixed for 2 hr in 3% glutaraldehyde-H₂O₂ in cacodylate buffer (pH 7.4), followed by 2 hr in glutaraldehyde buffer alone (Peracchia and Mittler, 1972). Samples were postfixed in 2% OsO₄, buffered to pH 7.4 with cacodylate. They were stained in block with 0.5% uranyl acetate, dehydrated in graded alcohols, and embedded in Epon. Thin sections were cut on an LKB Ultratome and stained with uranyl and lead salts. They were examined on Zeiss 10 and 902 electron microscopes with calibrated magnifications.

Results

Structure of demyelinating and remyelinating axons

The initial damage to myelin following lysolecithin injection occurs rapidly, and is visible within the first day postinjection (Hall and Gregson, 1971; Shrager, 1988, 1989). Macrophages appear rapidly within the lesioned area and remove all myelin debris, a process that is typically complete after 1 week. Proliferating Schwann cells then appear in increasing numbers, adhere to the axons, and begin the process of remyelination. In *Xenopus*, initial myelin formation took place slowly during the second week postinjection, with the initial lamellae seen at 12 d and gaps indicative of presumptive new nodes of Ranvier visible after about 3 weeks (Shrager, 1988; Shrager and Rubinstein, 1990). In the rat, however, this transition occurred much more rapidly, with thinly remyelinated regions seen 9–10 d postsurgery in a limited number of axons. Figure 1 illustrates light micrographs of living, unstained cells. Figure 1*a* shows the edge of the injected zone, containing several heminodes (arrow). To the right of each heminode, axons are demyelinated and at this early stage only myelin debris and possibly occasional Schwann cells are visible. Most of the scattered round cells are macro-

phages. Figure 1*b* includes a similar zone, but at 9 d postinjection. The dark round cells are macrophages, and the lighter cells attached to the demyelinated axons are Schwann cells (arrow). Two axons at 14 d postinjection are illustrated in Figure 1*c*. The lower fiber has progressed to the point at which presumptive new nodes of Ranvier are visible as wide gaps in the new myelin (arrows), while the other is at an earlier stage, with several adherent Schwann cells. In the fluorescence images that follow, remyelinating axons were identified either by short internodes for a given diameter, or by following the fiber back to a heminode.

Ultrastructure is seen in Figure 2. A fully demyelinated axon surrounded by a disrupted basal lamina at 7 d postinjection is shown in Figure 2*a*. In Figure 2*b* an axon at 10 d is partially surrounded by a Schwann cell and its processes. A cell in a more advanced state of remyelination, 12 d postinjection, is seen in Figure 2*c,d*. Examining many light and electron micrographs, it was clear that at any particular time point there is considerable heterogeneity among individual axons.

Na⁺ channel aggregation in normal and demyelinated axons

The Na⁺ channel antibody labeled axons in a distinctive manner. Sharply delineated zones of fluorescence were seen at nodes of Ranvier, and at particular internodal loci in demyelinated fibers. At these sites, the axon was not labeled uniformly across its diameter, but had brighter fluorescence at the outer edges. This is seen in Figure 3*a*₁, which is a photograph of a node of Ranvier in a remyelinating axon 18 d postinjection. This pattern is most likely due to the increased membrane surface in the field of view at the edges of the axon cylinder. Photography of labeled Na⁺ channels was difficult due to their relatively low density even at nodes of Ranvier, as compared with that of other surface antigens such as galactocerebroside (GalC), which constitutes about 12% of myelin lipids (Norton and Cammer, 1984). While aggregates were easily seen by eye with epifluorescence, the several seconds required for photographs generally resulted in significant bleaching. The laser scanning confocal microscope available to us reduced this problem significantly, but spatial resolution was limited and the double-line structure of these sites could be recorded in only a small number of cases. However, this instrument was fitted with an auxiliary Hg lamp, allowing visual identification of substructure. Thus, in the confocal images that follow, clusters of Na⁺ channels appear typically as single foci within the axon, whereas by eye they had the structure shown in Figure 3*a*. Figure 3, *a*₂ and *a*₃, represents a photograph and a confocal scan, respectively, of the same node of Ranvier. While the radial substructure was lost, the scan accurately localized the Na⁺ channel cluster along the axon length. The visual identification of the double-line structure was important, since it insured that artifacts due to debris that happened to adhere to or overlap axons were not mistaken for channel aggregates in scans. All sites described as Na⁺ channel clusters in confocal images below were first identified visually with this unique structure. Wherever possible we also show photographs of similar sites. It should also be noted that limitations in spatial resolution in our images precluded a determination of the precise extent of small Schwann cell processes along the axon.

Figure 3*b* illustrates some controls. Figure 3*b*₁ shows the result of labeling a normal node of Ranvier. The antibody appears to be restricted primarily to the nodal axolemma, with only a very low level detected on myelin and paranodal structures. We test-

Figure 1. Light micrographs of unstained, living cells. *a*, Phase microscopy of axons 7 d postinjection of lyssolecithin. Several heminodes (arrow) forming the border between myelin (white) and demyelinated axons (dark) may be seen. The large, bright, round cells are mainly macrophages. *b*, Bright-field microscopy of a similar transition zone (myelinated region at the bottom and left), 9 d postinjection. The dark round cells are macrophages, and the lighter cells adhering to demyelinated axons are Schwann cells (arrow). *c*, Two axons 14 d postinjection seen with Nomarski optics. Remyelination in the lower fiber has progressed to the point at which wide gaps may be seen (arrows). The upper axon is at an earlier stage and several Schwann cell bodies are visible. The large grainy cells are debris-laden macrophages. Scale, 50 μ m.

ed whether this pattern might be due simply to a lack of accessibility of the antibody to the paranodal axon membrane. In Figure 3*b*₂, paranodal myelin was retracted following incubation with protease, but despite this exposure the label remained focal. In Figure 3*b*₃, a retracted node was exposed to antibodies that had been preincubated with the peptide antigen. The strong nodal labeling is completely absent, while the low-level background myelin stain remains. This control has been repeated at each stage of remyelination that is examined below. In all cases, the focal double-line labeling that reflects clusters of Na⁺ channels is entirely eliminated by preincubation with the antigen. The low-intensity fluorescence seen over diffuse regions of myelin and Schwann cells typically remained. It is thus interpreted as nonspecific binding, and is not discussed further.

At about 1 week postinjection of lyssolecithin there are long regions of demyelinated internodes devoid of any adherent cells. At this stage clusters of Na⁺ channels were extremely rare. They were seen at heminodes marking the transition from normal to demyelinated at the edge of the injected zone, and occasionally

in the middle of a long demyelinated stretch. One such latter site is illustrated in Figure 3*c*₁. In some cases axons could be followed for up to 1200 μ m with no more than one such cluster visible. Thus, these sites may represent the original nodes of Ranvier present prior to demyelination, and at this stage no other aggregates of Na⁺ channels are seen. Beginning at about 8 d, and increasing rapidly in number over the next few days, proliferating Schwann cells were seen to adhere to demyelinated internodes, and foci of Na⁺ channels were found at their borders. Three examples are shown in Figure 3*c*. In Figure 3*c*₂, at one Schwann cell edge there is a bright, highly focal cluster of channels. At the other there is a labeled region that had the double-line form, but was weak and diffuse and could only tentatively be identified with Na⁺ channels. This polarity, with a higher density of Na⁺ channels at one end, was seen in many, but not all, cases of early Schwann cell adhesion. In Figure 3*c*_{3,4} bright foci are associated with both Schwann cell edges. At this stage the distance between these clusters was typically 10–50 μ m. In controls with antibody preincubated with the peptide antigen,

Figure 2. Electron micrographs of rat sciatic axons at several stages. *a*, A fully demyelinated axon surrounded by the disrupted basal lamina, 7 d postinjection. *b*, An axon (right) partially enveloped by a Schwann cell, 10 d postinjection. *c*, A remyelinating axon and Schwann cell, 12 d postinjection. *d*, Detail of *c*. Scale bars: *a*, *b*, *c*, 0.5 μ m; *d*, 0.1 μ m.

the diffuse label over the Schwann cell body remained, while the clusters at the edges disappeared (Fig. 3*c*₅).

As remyelination progressed, Schwann cells continued to elongate over the demyelinated internode, and the distance between the Na⁺ channel clusters at their edges increased. In these actively remyelinating fibers these distances could vary considerably along single axons. In one fiber at 12 d postinjection, 10 clusters were found over an 850 μ m length. In some cases, denoted below by SC, a Schwann cell was clearly seen between them, and in others, denoted by AX, there was only demyelinated axon between them (the two clusters were associated with different Schwann cells). In other cases the morphology did not allow an unambiguous determination. Distances between them were measured consecutively to be 85 SC, 102, 75 SC, 127, 187, 57 AX, 84, 94, and 35 AX μ m. As this process continued, in many cases, two Schwann cells were seen to approach each other. Clusters of Na⁺ channels were located in close proximity, with no apparent intervening myelinating cell. Figure 3*d*₁ shows an example in which the distance between clusters is 30 μ m. With further Schwann cell elongation, regions were seen that

were suggestive of the fusion of two clusters, forming sites that appeared to be presumptive new nodes of Ranvier. The confocal scan in Figure 3*d*₂ seemed to represent such a merging of two Na⁺ channel clusters. The resulting long "nodes" persisted for a time, as in the case shown in Figure 3*d*₃ at 20 d postsurgery. At 60 d postinjection, the longest times examined in these studies, remyelination had progressed to what was likely to be a stable state. New nodes of Ranvier were distributed at fairly regular intervals, averaging about 270 μ m (268 ± 19 μ m, $n = 19$), and Na⁺ channels were no longer spread over a wide gap, but rather were tightly aggregated within nodes. Figure 4*a*₁ illustrates one such region. A magnified view of one of these nodes is shown in Figure 4*a*₂. Figure 4*a*₃ represents a preabsorbed control at 60 d.

Figure 4*b* illustrates photographs of axons at stages similar to those scanned above. All of these fibers were from the same preparation, 13 d postinjection, indicating the variation in rate of remyelination seen among different axons. In Figure 4*b*₁, a Schwann cell separates Na⁺ channel clusters 40 μ m apart. Figure 4*b*₂ shows Na⁺ channel clusters in close proximity, and in Figure

Figure 3. Na⁺ channel clustering in normal and demyelinated axons. *a*₁ and *a*₂ are photographs. All others are confocal images. *a*₁, New node of Ranvier in a remyelinating axon at 18 d. *a*₂ and *a*₃ are both of the same node, and illustrate the difference in appearance between photographs and confocal images. Scale bars: *a*₁, *a*₂, 12 μm. *a*₃, 10 μm. *b*, Nodal labeling. *b*₁, Normal node. *b*₂, Myelin enzymatically retracted from the paranodes. *b*₃, Control node, antibody preincubated with the peptide antigen. Scale, 10 μm. *c*, Fibers from full demyelination to early stages of remyelination.

4b, the pattern of antibody staining suggests the possible fusion of two clusters. A long diffuse cluster is present in Figure 4b₄.

We have ascribed the labeling that we observe at the edge of a remyelinating Schwann cell to the axolemma. However, rat Schwann cells have been shown to contain Na⁺ channels (Chiu et al., 1984; Shrager et al., 1985), although it is controversial whether they are present in myelinating cells (Chiu, 1987, 1993; Howe and Ritchie, 1990). In order to test whether the clusters we observe are axonal in origin, remyelinating axons were exposed to protease in order to retract the newly forming myelin. Figure 4c illustrates one such fiber, and it can be seen that the Na⁺ channel label remained on the axon following retraction of the Schwann cell/myelin.

The dynamic picture of new node formation suggested above has been drawn from observations on different axons at each time point, and thus does not represent a necessarily unique interpretation. As one further test of this hypothesis we have consolidated the data as histograms for three different cases. In Figure 5a we plot the number of isolated (presumed original) Na⁺ channel clusters seen in demyelinated axons *versus* the time postinjection of lysolecithin. In Figure 5b we present a similar plot for the case of clusters at opposite edges of a Schwann cell, and in Figure 5c we show the numbers of sites in which aggregates were in close proximity with no intervening Schwann cell, as though about to fuse. Isolated clusters are not seen beyond 7 d as large numbers of Schwann cells adhere. We were unable to judge if these original clusters disappeared or became associated with Schwann cells. Channel aggregates bordering short Schwann cells appeared as rapidly as the cells themselves, increased in number rapidly, and then fell off over days 10–17. In contrast, the “fusing” clusters appeared a bit later, and were still increasing in number by day 17. The statistical picture thus supports the suggested temporal progression of events. It remains important to note, however, that an unambiguous determination requires continuous observations on single fibers over several days, something not possible with the present system.

While large numbers of axons utilized the pattern of Na⁺ channel clustering and new node formation outlined above, other fibers appeared to follow a modified route. In Figure 4d₁, at 13 d postinjection, a Schwann cell 15 μ m long separates immunoreactive zones with the appearance of nodes. In the axon scanned in Figure 4d₂, Na⁺ channel clusters were separated by only about 70 μ m, but were bordered on both sides by structures likely to be new myelin or Schwann cell processes. As shown in Figure 2, at this stage some fibers already have several lamellae. The structures illustrated in Figure 4, d₁ and d₂, are not likely to be stable since at 60 d, as noted above, internodal distances in remyelinated regions were always much longer.

Block of Schwann cell proliferation

In one group of animals the mitotic inhibitor mitomycin C was included in the lysolecithin injection (Hall and Gregson, 1974). Demyelination proceeded as before, but Schwann cell proliferation

and remyelination were virtually completely eliminated. As a means of identification, Schwann cells were labeled with an antibody against S-100 protein (Weiss et al., 1983; Clemence et al., 1989). In Figure 6, a₁ and a₂, axons were injected with lysolecithin alone (no mitomycin) 14 d earlier. The yellow-labeled cells are proliferating Schwann cells adhering to demyelinated axons. In Figure 6a₁, gaps between Schwann cell processes suggest possible presumptive new nodes. Figure 6a₂ is a control in which the primary antibody was absent. The diffuse green fluorescence thus corresponds to nonspecific background label. Mitomycin-treated axons, also at 14 d postinjection, are seen in Figure 6a₃, labeled with anti-S-100. No Schwann cells are visible, and axons appear thin since they have no new myelin.

In axons that were treated with mitomycin and examined at 7 d postinjection, a time prior to the expected appearance of Schwann cells, Na⁺ channel clustering was similar to that in untreated fibers at the same stage. Heminodes were labeled (Fig. 6b₁) and isolated clusters were seen in demyelinated regions, separated by long (>600 μ m) distances (Fig. 6b₂). On the other hand, at 14 d results were very different. Axons exposed to mitomycin and probed with the anti-Na⁺ channel antibody at the latter time point showed that there was virtually no channel clustering in demyelinated regions. Figure 6c₁ illustrates a section of a demyelinated internode and is devoid of label. These regions could be followed for long distances in the confocal microscope. In seven cases, single axons were observed over lengths in excess of 1 mm (largest, 1.8 mm) with no Na⁺ channel clusters seen. Further, Na⁺ channels were virtually absent from heminodes at 14 d. Of 10 such structures examined, seven had no visible label (Fig. 6c₂), one had a very slight diffuse signal, and two had just a small focal spot of label remaining (Fig. 6c₃). This result was not due to a loss of Na⁺ channels by mitomycin nor to an interference with antibody binding, since nondemyelinated nodes of Ranvier in the same preparation were labeled normally (Fig. 6c₄). In contrast, in axons not treated with mitomycin, high densities of Na⁺ channels remained at heminodes at 14 d postsurgery (Fig. 6c₅).

Discussion

Our results suggest that the distribution of axonal Na⁺ channels is markedly altered during remyelination under the influence of Schwann cells. Except for the case of 7 d axons in which Schwann cells are largely absent, clusters of Na⁺ channels appear typically at the edges of Schwann cells or their paranodal processes. Given the limitations in resolution inherent in light microscopy, what is the evidence that these clusters are axonal rather than of Schwann cell origin? We showed that following enzymatic retraction of the sheath the clusters appeared identical to those in nonretracted fibers, suggesting an axonal locus. Wilson and Chiu (1990) have demonstrated that at sites retracted by collagenase plus mechanical stress, some spotty GalC-positive material remained at the paranodal region. However, at these sites Na⁺ currents were recorded from axonal membranes, but not the GalC-positive membranes (identified via Lucifer yellow injection).

c₁, A Na⁺ channel cluster within a long demyelinated segment, 7 d postinjection. c₂, Clusters at the edges of a rounded Schwann cell adhering to a demyelinated axon, 10 d. The region with blue label on the right had the double-line structure (unlike that in c₁), but was spread over a wide region. c₃, Clusters at both ends of an elongating Schwann cell, 8 d. c₄, Highly focal clusters and Schwann cell, 14 d. c₅, Control: adherent Schwann cell, preabsorbed antibody, 10 d. Scale, c₁, 10 μ m; c₂, 6 μ m; c₃, c₄, 10 μ m; c₅, 6 μ m. d, Intermediate stages of node formation. d₁, Na⁺ channel clusters associated with two different Schwann cells, 30 μ m apart, 10 d postinjection. d₂, Apparent merging of two clusters, 14 d. d₃, A diffuse cluster between two Schwann cells, 10 d. Scale, d₁, 8 μ m; d₂, 5 μ m; d₃, 3 μ m.

Figure 4. Remyelinating axons. *a*, Confocal scans of late stages of remyelination. *a*₁, An internode bordered by two new nodes of Ranvier, 60 d postinjection. *a*₂, Magnified view of a new node, 60 d. *a*₃, Preabsorbed control, new node, 60 d. Scale bars, *a*₁, 21 μm ; *a*₂, *a*₃, 4 μm . *b*, Photographs at intermediate stages of remyelination, 13 d postinjection. *b*₁, An adherent Schwann cell with two clusters of Na^+ channels at its edges. *b*₂, Clusters associated with two different Schwann cells in close proximity. *b*₃, Apparent fusion of two clusters. *b*₄, A long, diffuse cluster. Scale, 12 μm . *c*,

tion). Further, Safronov et al. (1993) showed that following a protease retraction almost identical to that used here, Lucifer yellow injection from patch pipettes was invariably into the axon, suggesting that Schwann cell processes do not remain over an appreciable portion of the paranodal axolemma. On the other hand, Ritchie et al. (1990) have demonstrated Na⁺ channel immunoreactivity in perinodal processes of Schwann cells. Thus, while the weight of available evidence points to an axonal locus, some questions remain which will probably require ultrastructural localization.

Much of the work that has been done to date on Na⁺ channel cluster formation concerns developing axons or fibers in hypomyelinated mutant animals. Early efforts examined some unique ultrastructural features of the nodal/paranodal region, but left some issues unresolved. Rosenbluth (1976) studied the dense cytoplasmic undercoating and clusters of intramembranous particles that are characteristic of normal nodes. In amyelinated axons of dystrophic mice he found that these nodal specializations occurred only in regions of close contact with Schwann cells (Rosenbluth, 1979). Results were similar in chronically demyelinated CNS fibers (Rosenbluth and Blake-more, 1984). Rosenbluth (1981, 1988) has suggested further that the paranodal axoglial junctions may displace membrane glycoproteins and act as a diffusion barrier to limit Na⁺ channels to the node. On the other hand, Ellisman (1979) reported node-type particle distributions in dystrophic mice that were independent of glial association, and Le Beau et al. (1987) described a similar situation in regenerating nerve. Further, examined *in vivo*, Wiley-Livingston and Ellisman (1980) demonstrated nodal particle specialization preceding myelination, while dorsal root ganglion (DRG) neurons in culture failed to develop these structures in the absence of glial cells (Bigbee and Foster, 1989).

Recently, more specific methods for localizing ionic channels have been employed. England et al. (1990, 1991) demyelinated goldfish peripheral nerve with doxorubicin, a DNA-intercalating agent that kills Schwann cells. Remyelination in this preparation is much slower than in the lysolecithin lesion, and early stages are seen at about 4 weeks (England et al., 1988). At about 3 weeks, long (~50 μ m) internodal zones labeled positively for Na⁺ channels with a polyclonal antibody to the purified TTX binding protein. These regions developed in the absence of Schwann cells. Judged by radioimmunoassay, the Na⁺ channel density in these axons rose about threefold at 3–4 weeks post-injection, suggesting new synthesis and insertion. In the mitomycin-treated axons in this study we did not see such regions, although the experimental period was limited to 14 d. Black et al. (1991) studied Na⁺ channels immunocytochemically in rat spinal cords chronically demyelinated by ethidium bromide and irradiation. Examined within the lesion 25–35 d later, no Na⁺ channel sites were seen. At the edges of the injected region, focal zones of Na⁺ channels were seen at sites of glial cell abutment, and broad, essentially continuous regions of label were seen in ensheathed axons. The lack of channels within the lesion at this stage corresponds with our results in mitomycin-treated fibers at 14 d, but continuous stretches of label were not observed in our preparation at any level of remyelination.

Joe and Angelides (1992, 1993) labeled cultured rat DRG

Figure 5. The distribution of Na⁺ channel cluster sites over time post-injection of lysolecithin. *a*, Single clusters in demyelinated axons. *b*, Sites with clusters at both edges of a single Schwann cell. *c*, Sites with aggregates associated with neighboring Schwann cells (and with no intervening Schwann cell) in close proximity (interpreted as "fusing clusters").

neurons with an antibody against Na⁺ channels and found that clustering occurred only after the addition of Schwann cells. Using a rhodamine-labeled toxin, they measured Na⁺ channel mobility from the kinetics of fluorescence recovery after photobleaching. On the time scale of the experiment (1–4 min) it was found that 80% of the Na⁺ channels were immobile ($D < 10^{-12}$ cm²/sec) independent of Schwann cell adhesion. The remaining 20% were mobile with $D \sim 10^{-10}$ cm²/sec. Finally, they have provided evidence that Na⁺ channel mobility may be restricted through association with ankyrin and spectrin (Srinivasan et al., 1988).

Using longitudinal current analysis, Bostock and Sears (1978) showed that in rat spinal roots demyelinated by diphtheria toxin inward currents could be recorded from broad internodal zones following action potential stimulation. In contrast, when these

Figure 6. Schwann cell proliferation blocked by mitomycin. *a*, Photographs of Schwann cell labeling with anti-S-100. *a*₁, *a*₂, Axons 14 d postinjection with lyssolecithin alone. Schwann cells and their processes are labeled yellow. *a*₃, Control, no primary antibody, same experiment as *a*₁, *a*₂. *a*₄, Anti-S-100 labeling of mitomycin-treated axons, 14 d. Scale, 12 μm . *b*, Na^+ channel labeling of mitomycin-treated axons at 7 d postinjection. Confocal scans. *b*₁, A heminode, 7 d. *b*₂, Fully demyelinated site, 7 d. Scale bars: *b*₁, 12 μm ; *b*₂, 10 μm . *c*, Na^+ channel clusters at 14 d following loss of

same axons were demyelinated by lysolecithin, Smith et al. (1982) found new discrete foci of inward current. These sites, named ϕ -nodes, were seen as early as 4 d postinjection, a time at which axons were associated with Schwann cells, but prior to the appearance of myelin. The maximum spatial resolution in these studies was about 80 μm , and spacings similar to those seen in the present work at the earliest stages of remyelination would not have been seen. Irradiated roots in which Schwann cells were rare also had ϕ -nodes, and Smith et al. concluded that Schwann cell contact was not required for the formation of these structures. However, since the electrical measurements were made in undissected roots, individual axons were not observed. It is possible that the timing of events in spinal roots differs from that in the sciatic nerve. In our observations, new Na^+ channel clusters were always associated with Schwann cells and appeared beginning at 8 d. Given the similarities in the preparations, it is likely that the aggregates of Na^+ channels seen immunocytochemically correspond to ϕ -nodes determined physiologically.

In these studies, we have shown that following demyelination there is no evidence for internodal foci of Na^+ channels prior to the appearance of Schwann cells. At 1 week postinjection of lysolecithin, aside from heminodes, the only clusters seen were very widely spaced, and were likely to be sites of original nodes. On the other hand, 1–2 d later, as Schwann cells first began to adhere and before they had elongated appreciably, Na^+ channel clusters were seen at their edges, often with one site highly focused and the other more diffuse, as though in the process of formation. Over the next few days, as Schwann cells extended laterally along the internode and began to elaborate myelin, the clusters appeared with increasing distance, suggesting that they followed closely the progression of the ensheathment. Ultimately at many sites, presumably driven by the lateral movement of their respective Schwann cells, two clusters appeared to merge. We suggest that this may represent a mechanism for the formation of at least some new nodes of Ranvier. Other schemes may also be at work. In some cases, clusters seemed to be bordered on both sides by Schwann cells/myelin at very early stages and with very short (10–40 μm) intervening distances. Since the final internodal lengths are about 10 times greater, either these sites must also be capable of moving laterally, or, alternatively, some may disappear. Far more Schwann cells adhere to a demyelinated internode at 8–9 d postinjection than will ultimately form myelinated internodes. Presumably, some of these are eliminated competitively during myelination, and they may have associated Na^+ channel clusters which then become reorganized.

In mitomycin-treated nerves, demyelination proceeded normally over the first week postinjection. Sites of high Na^+ channel density were also indistinguishable from those of fibers injected with lysolecithin alone. Clusters were present at both heminodes and at isolated internodal sites assumed to be fully demyelinated nodes. However, at 14 d, Schwann cell proliferation was almost completely absent, and virtually no Na^+ channel clusters could be seen at any sites along demyelinated fibers. Thus, not only were Schwann cells required for the formation of new aggregates of Na^+ channels, but they also played an essential role in the

maintenance of existing clusters. At heminodes, the presence of myelin on one border was insufficient to prevent the loss of these channels. On the other hand, it is not certain that this myelin was normal since while the primary action of mitomycin is the inhibition of DNA synthesis, some delayed reduction in RNA levels is also seen (Schwartz et al., 1963). Schwann cell bodies and their associated myelin could thus have been altered, although at the light microscope level no changes could be seen at nodes. It was not possible to judge with certainty the mechanism by which the nodal channels disappeared. There was some hint of possible lateral diffusion in some heminodes at 7 d, but this was not a consistent finding.

In loose patch-clamp measurements on both rat and frog demyelinated axons, we were able to demonstrate the presence of Na^+ channels in the internodal membrane and to estimate their density at about 4% of the nodal level (Shrager, 1987, 1988, 1989). Unusual instabilities in ionic currents in response to step depolarizations were recorded adjacent to heminodes, and were interpreted as resulting from the nearby sharp gradient in Na^+ channel density (Shrager, 1987, 1988, 1989). These currents, which were seen in seven of the 12 paranodal sites examined (and in none of the 68 internodal sites tested), persisted throughout the period of remyelination followed in those experiments for up to 1 month in the rat and 5 months in the frog. This was interpreted to suggest that nodal Na^+ channels did not simply break up and diffuse laterally into the internode. A similar conclusion was reached by Brismar (1981) in rat axons demyelinated with diphtheria toxin. The present study confirms this prediction, at least over a 2 month time scale. In fibers not exposed to mitomycin, clusters of Na^+ channels at heminodes persisted over this period. Presumably, proliferating Schwann cells play an important role in channel maintenance, since in mitomycin-treated axons heminodal channels were lost. This work suggested further that fully demyelinated nodes may also survive. Isolated clusters remained at least 7 d with no bordering myelin. During the second week the appearance of new clusters at the edges of adherent Schwann cells made it impossible to distinguish the original nodes and their fate is thus uncertain.

What is the origin of the Na^+ channels that aggregate in response to Schwann cell adhesion? We consider three possibilities: (1) *de novo* synthesis, axonal transport, and insertion; (2) transfer from Schwann cell to axolemma; and (3) uniform distribution in the internode at low density prior to Schwann cell proliferation. The time scale places some constraints on the first of these mechanisms. Once a Schwann cell binds to an axon, cluster formation seems to occur in less than 1 d. At 7 d we saw only widely spaced sites with no neighboring Schwann cells (presumed to be original nodes), and at 8 d there were already adherent Schwann cells with clusters at each edge. Fast axoplasmic transport, the route utilized by Na^+ channels (Lombet et al., 1985), is in excess of 400 mm/day (Ochs, 1982), much faster than needed for a signal to reach the soma and for synthesized channels to return. On the other hand, we have shown via transected nerves that, at least in the frog, synthesis seems not to be required for new node formation (Rubinstein and Shrager, 1990). It remains possible that an axoplasmic pool of channels contributes to the clusters. The suggestion that Na^+

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Schwann cell proliferation. *c*₁, Confocal image of a section of a demyelinated internode. *c*₂, Heminode, confocal scan. *c*₃, Photograph of a heminode with a small remnant of label remaining. *c*₄, Confocal scan of an undemyelinated node in the same preparation as *c*₂. *c*₅, Heminode in an axon treated with lysolecithin alone (no mitomycin), 14 d. Confocal image. Scale bars: *c*₁, 12 μm ; *c*₂, 10 μm ; *c*₃, 12 μm ; *c*₄ and *c*₅, 5 μm .

channels may be transferred from Schwann cell to axon (Shrager et al., 1985) remains highly speculative but is not inconsistent with our results. As noted above, Na⁺ channels have been demonstrated immunocytochemically in perinodal processes of Schwann cells (Ritchie et al., 1990), but electrophysiological evidence is lacking (Wilson and Chiu, 1990).

The simplest explanation is that the internodal channels provide a pool that can be caused to aggregate by Schwann cells, but is lateral diffusion quantitatively consistent with the results? The density of Na⁺ channels in the internodal axolemma has been measured to be about 4% of the nodal value (Shrager, 1989), and, from the results of Joe and Angelides (1993), 20% of these are mobile with a diffusion coefficient of 10⁻¹⁰ cm²/sec. This system can then be approximated as diffusion from two semi-infinite regions, separated by a surface held at constant concentration. The surface corresponds to the newly forming node, and its concentration is set to zero since all channels within it are considered to be bound. The total amount of diffusing material can then be calculated as $4C_0(Dt/\pi)^{1/2}$, where C_0 corresponds to the initial mobile internodal density (Crank, 1975). Three and one-half days would then be required for a new 1 μm node to be fully populated with Na⁺ channels. The early clusters forming in response to Schwann cell adhesion may not be fully mature. In *Xenopus*, early new nodes had about 1/3 the total number of Na⁺ channels of original nodes (Shrager, 1988). The diffusion model suggests that significant channel levels could be reached in relatively short times. For example, to attain 1/3 of the final density only 9.5 hr are needed. At 7 d we saw only widely spaced sites with no neighboring Schwann cells (presumed to be original nodes), and at 8 d there were already adherent Schwann cells with clusters at each edge. Further, each internode in a remyelinated region, with a length of about 270 μm, contains twice the number of mobile channels required to fully populate the new nodes. Thus, lateral diffusion and recruitment of internodal Na⁺ channels by Schwann cells is in principle a possible mechanism for new node formation.

Another question of fundamental interest concerns the mechanism through which ionic channels cluster. These studies do not directly address this issue, but they suggest possibilities for certain aspects of the process. Na⁺ channels became concentrated at the outer edges of adherent Schwann cells. Thus, either the processes of these cells selectively attract axonal Na⁺ channels, or the Schwann cell body exerts an influence to exclude these channels from the underlying axolemma. A number of cell surface recognition molecules have been implicated in modulating interactions between neurons and glia (Schachner, 1991), and these may be attractive or inhibitory.

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