

Connexin32 Is a Myelin-Related Protein in the PNS and CNS

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We have examined the expression of a gap junction protein, connexin32 (Cx32), in Schwann cells and oligodendrocytes. In peripheral nerve, Cx32 is found in the paranodal myelin loops and Schmidt-Lanterman incisures of myelinating Schwann cells, and the levels of Cx32 protein and mRNA change in parallel with those of other myelin-related genes during development, Wallerian degeneration, and axonal regeneration. In the central nervous system, Cx32 is found in oligodendrocytes and their processes, but not in compact myelin, and the levels of Cx32 protein and mRNA increase during development in parallel with those of the other myelin genes. Thus, Cx32 is expressed as part of the myelinating phenotype of both Schwann cells and oligodendrocytes, indicating that this gap junction protein plays an important role in the biology of myelin-forming cells.

[Key words: Cx32, gap junctions, Schwann cells, oligodendrocytes, myelin, incisures]

Myelin is the multi-lamellar structure that surrounds axons and increases axonal conduction velocity. It is formed by the spiral wrapping of the cell membrane of myelinating glia-oligodendrocytes in the CNS and Schwann cells in the PNS. The myelin sheaths produced by these two cell types are structurally similar, consisting mostly of compact myelin that is characterized by a unique but overlapping set of proteins (Lemke, 1992, 1993). Protolipid protein (PLP) and myelin basic protein (MBP) are the major structural proteins in the CNS, while protein zero (P₀), MBP, and peripheral myelin protein-22 kD (PMP-22) are the major proteins in the PNS. Each of these myelin proteins is essential for proper myelination, as mutations in PLP and MBP cause dysmyelination in the CNS, and mutations in P₀ and PMP-22 cause dysmyelination in the PNS (Hudson, 1990; Chance and Pleasure, 1993; Lemke, 1993; Snipes and Suter, 1995).

The compact myelin in the PNS contains periodic interruptions called Schmidt-Lanterman incisures or clefts (Peters et al., 1991). These incisures, as well as the paranodal regions of the

myelin sheath, contain a distinct group of proteins, including myelin-associated glycoprotein (MAG), connexin32 (Cx32), E-cadherin, and oligodendrocyte-myelin glycoprotein (Trapp et al., 1989; Bergoffen et al., 1993; Apostolski et al., 1994; Fannon et al., 1995). Incisures have also been reported in the CNS (Peters et al., 1991), but no molecules have yet been found to be preferentially localized to CNS incisures. The importance of the incisures to the normal function of myelinated axons was not appreciated until it was found that the X-linked form of Charcot-Marie-Tooth disease (CMTX) was caused by mutations in the Cx32 gene (Bergoffen et al., 1993). Cx32 is a gap junction protein and is expressed in many tissues, including oligodendrocytes and myelinating Schwann cells (Kumar and Gilula, 1986; Paul, 1986; Dermietzel et al., 1989, 1990; Bennett et al., 1991; Kumar and Gilula, 1992; Bergoffen et al., 1993). How Cx32 mutations cause peripheral neuropathy is unknown. One plausible explanation is that CMTX mutations alter the function of gap junctions at nodes and incisures, as Cx32-immunoreactivity colocalizes with gap junctions seen by freeze-fracture electron microscopy (Sandri et al., 1982; Bergoffen et al., 1993), and some mutations in Cx32 disrupt the formation of functional gap junctions (Bruzzone et al., 1994; Rabadan-Diehl et al., 1994). If CMTX mutations disrupted these gap junctions, this could interrupt the diffusion of ions and small molecules in a radial direction, directly across the myelin sheath, through the paranodes and incisures.

To learn more about the role of Cx32 in myelinating glia, we examined the localization and expression of Cx32 in Schwann cells and oligodendrocytes. In the PNS, Cx32 is found in the paranodal regions and incisures of myelinating Schwann cells. In the CNS, Cx32 is found in cell bodies and processes of oligodendrocytes, but not in compact myelin or Schmidt-Lanterman incisures. Axon-Schwann cell interactions *in vivo*, and cAMP analogs *in vitro*, increase Cx32 expression in Schwann cells. In the CNS, the level of Cx32 mRNA increases in different regions in parallel with those of the other myelin genes. Furthermore, in *jimpy* mice and *myelin-deficient* rats, which have PLP mutations that result in the failure of oligodendrocyte maturation (Hudson, 1990), the level of Cx32 mRNA, like those of other myelin-related genes, does not increase during the period of myelination. Thus, Cx32 is expressed as part of the program of myelin gene expression in both oligodendrocytes and Schwann cells, but is localized to different parts of each cell.

Materials and Methods

Surgery and collection of tissues. Using aseptic technique, the sciatic nerves of anesthetized (50 mg/kg pentobarbital i.p.), adult (10–13 week

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old) Sprague–Dawley rats were exposed at the sciatic notch. Permanent axotomy was accomplished by doubly ligating nerves, transecting between the ligatures with iridectomy scissors, and suturing the two nerve-stumps were at least 1 cm apart; this technique prevents axonal regeneration to the distal nerve-stump for at least 2 months. Nerve-crush was produced by tightly compressing the sciatic nerve at the sciatic notch with flattened forceps twice, each time for 10 sec; this technique causes all of the axons to degenerate, but allows axonal regeneration. At various times after nerve-injury, the animals were sacrificed by CO₂ inhalation, the distal nerve-stumps were removed, and the most proximal 2–3 mm were trimmed off. For transected nerves, the entire distal nerve-stump was taken from just below the lesion to the ankle (about 4 cm). For crushed nerves, the distal nerve-stump was divided into two equal segments, termed the proximal and distal segments, each about 2 cm long. The nerves were immediately frozen in liquid nitrogen and stored at –80°C. Unlesioned sciatic nerves and various brain regions were obtained from animals of different ages, from P1 (the day after birth) to P90. All animal protocols were approved by the Institutional Animal Care and Use Committee of The University of Pennsylvania.

Cell culture. Schwann cells were isolated from 3 d old rat pups (Brookes et al., 1979), and expanded on 10 cm plates coated with poly-L-lysine in DME supplemented with 10% FCS, a crude extract of glial growth factor (Brookes et al., 1980), and 2 μM forskolin (Porter et al., 1986). The cells were passaged three times, grown to confluence, then switched for 3 d to either DMEM + 10% FCS, or DMEM + 10% FCS supplemented with 4 μM forskolin. All of the cultures used in these experiments were >95% Schwann cells, as judged by staining for the low-affinity nerve growth factor receptor (NGFR; data not shown). Fibroblasts were obtained by culturing the perineurium in DMEM + 10% FCS on uncoated plastic plates, to which Schwann cells did not adhere. RNA and protein were isolated after the cells had been passaged three times.

Immunohistochemistry and immunocytochemistry. Nerve fibers were teased from fresh, unfixed nerves, or from nerves that had been fixed for 30 min in 4% paraformaldehyde or Zamboni's fixative. Teased fibers were dried on glass slides, then postfixed for 10 min with acetone. The best Cx32 labeling was obtained from tissues that were embedded in OCT (Miles, Elkhart, IN), without prior fixation or after fixation in 4% paraformaldehyde. Frozen sections of unfixed tissue were postfixed for 10 min in acetone. Teased fibers and sections were blocked for at least 1 hr in 10% fish skin gelatin containing 0.5% Triton X, and incubated 24–48 hr at 4°C with various combinations of primary antibodies. We used mouse monoclonal antibodies against rat Cx32 (M12.13, (Goodenough et al., 1988), P₀ (Archelos et al., 1993), MAG (Boehringer-Mannheim, Indianapolis, IN), myelin-oligodendrocyte glycoprotein (MOG; Linnington et al., 1984), growth-associated protein-43 kD (GAP-43; Schreyer and Skene, 1991), and rabbit polyclonal antibodies against rat Cx32 (Goodenough et al., 1988), MAG (Pedraza et al., 1990), PLP (Koeppen et al., 1988), P₀ (D'Urso et al., 1990), nestin (Friedman et al., 1990), GAP-43 (Curtis et al., 1992), and ED1 (Serotec; England). After incubating with the primary antibodies, the sections were washed, then incubated with the appropriate fluorescein-, rhodamine-, or biotin-conjugated donkey anti-rabbit and anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Fluorescein- or rhodamine-conjugated avidin was used to visualize biotin-conjugated antibodies.

The rabbit antiserum against Cx32 was an unfractionated serum generated against a synthetic peptide corresponding to amino acids 98–124 of Cx32 (Paul, 1986; Goodenough et al., 1988). This antibody recognizes Cx32 on Western blots and labels gap junctions by immunoelectron microscopy (Paul, 1986; Goodenough et al., 1988). Preincubation of the antiserum with the peptide against which it was raised abolishes Cx32 staining in several tissues (D. Paul, unpublished observations). The monoclonal antibody against rat Cx32 (M12.13) probably recognizes an epitope in the C-terminal cytoplasmic domain (Goodenough et al., 1988). In peripheral nerve and spinal cord both the polyclonal and the monoclonal antibodies gave identical patterns of staining. Furthermore, the preimmune rabbit serum, diluted to have the same protein concentration as the antiserum itself, did not label either peripheral nerve or spinal cord.

Western blotting. Protein homogenates were obtained by pulverizing frozen tissues with a steel mortar and pestle on dry ice, homogenized in 50 mM Tris (pH 7.0) containing 1% SDS and 100 μM PMSE, then sonicated. Protein extracts were similarly prepared from cultured Schwann cells after scraping the cells into the same solution. A myelin

preparation was isolated from cerebrum, brainstem, and spinal cord of adult rats (Norton and Poduslo, 1973), lyophilized, and resuspended in the same solution. Insoluble material was removed by centrifugation in a microfuge for 15 min at 15,000 rpm, and the concentration of protein in the supernatant was measured with a Bio-Rad DC Assay kit according to the manufacturer's instructions. Equal amounts (25 μg) of protein were separated on 12% acrylamide, 0.1% SDS gels, transferred to Immobilon PVDf membrane (NEN Research Systems, Boston, MA), blocked (5% powdered milk in TRIS-buffered saline containing 0.5% Tween20) overnight at 4°C, then incubated with a rabbit antiserum against rat Cx32 (Goodenough et al., 1988), diluted 1:5000, for 24 hr at 4°C. The membranes were washed in blocking solution, then incubated at room temperature in peroxidase-coupled donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch Laboratory, West Grove, PA), diluted 1:10,000. After 1 hr, the membranes were washed, developed with chemiluminescence reagent (ECL kit, Amersham, Arlington Heights, IL), and exposed to film (Kodak X-OMAT AR imaging film). The membranes were reprobbed with a rabbit antiserum against a peptide encoded by exon 1 of human MBP (diluted 1:5000; DeFerra et al., 1985), a rabbit antiserum against P₀ (diluted 1:5000; D'Urso et al., 1990), or a rabbit antiserum against PLP (diluted 1:2000; Koeppen et al., 1988). To show the specificity of the primary antibodies, duplicate membranes were similarly prepared, but the primary antibody was omitted.

Northern blotting. RNA was isolated from rat sciatic nerves and Schwann cells by CsCl₂ gradient centrifugation (Chirgwin et al., 1979). Equal amounts (10 μg) of total RNA were electrophoresed in 1% agarose, 2.2 M formaldehyde gels, transferred to nylon membranes (Duralon, Stratagene) in 6× SSC, and u.v. cross-linked (0.12 joules). Blots were prehybridized, hybridized, and washed using standard techniques; the final stringency of the wash was 0.2× SSC at 65°C for 30 min (Sambrook et al., 1989). The following cDNAs were used as probes—a 1.1 kb fragment of rat Cx32 (Paul, 1986), a full-length cDNA of rat P₀ (Lemke and Axel, 1985), a 0.7 kb *Bam*HI fragment of rat NGFR (Radeke et al., 1987), and a full-length cDNA of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH; (Fort et al., 1985). Plasmid inserts were isolated after restriction endonuclease digestion by agarose gel electrophoresis, and purified by electroelution. ³²P-labeled cDNA probes with specific activities of 2–5 × 10⁹ cpm/μg were prepared by primer extension with random hexamers using the Prim-a-gene kit (Promega) according to the manufacturer's instructions.

Results

Cx32 is expressed by myelinating Schwann cells

In our initial report, we found that Cx32 appeared to be localized to the paranodes and Schmidt-Lanterman incisures of PNS myelin (Bergoffen et al., 1993). We have confirmed and extended this observation by double-labeling cryosections and teased fibers for Cx32 and MAG, pairing a mouse monoclonal antibody against Cx32 with a rabbit polyclonal antibody against MAG, and a rabbit polyclonal antibody against Cx32 with a mouse monoclonal antibody against MAG. Both combinations of antibodies gave similar results. In agreement with previous reports (Sternberger et al., 1979; Trapp and Quarles, 1982, 1984; Trapp et al., 1984), Figure 1*B* demonstrates that MAG-immunoreactivity was found around the entire adaxonal surface of myelinating Schwann cells (arrowheads), which apposes the axon, and in the incisures (arrows) and paranodes (not shown). Figure 1*A* demonstrates that Cx32-immunoreactivity colocalized with MAG at the incisures. At the adaxonal Schwann cell membrane, however, there was only a thin line of Cx32 staining, which probably corresponded to the inner mesaxon (Peters et al., 1991). We also performed double-labeling for Cx32 and P₀ (Fig. 2). P₀ was found throughout the compact myelin (Fig. 2*B*; Trapp et al., 1981), whereas Cx32 staining (Fig. 2*A*) was chiefly found at incisures (arrows) and paranodes (arrowheads). Thus, the localization of Cx32 in mature myelinated axons matches the localization of gap junctions in the PNS myelin sheath by freeze-fracture—at the paranodes, incisures, and inner mesaxon (Sandri

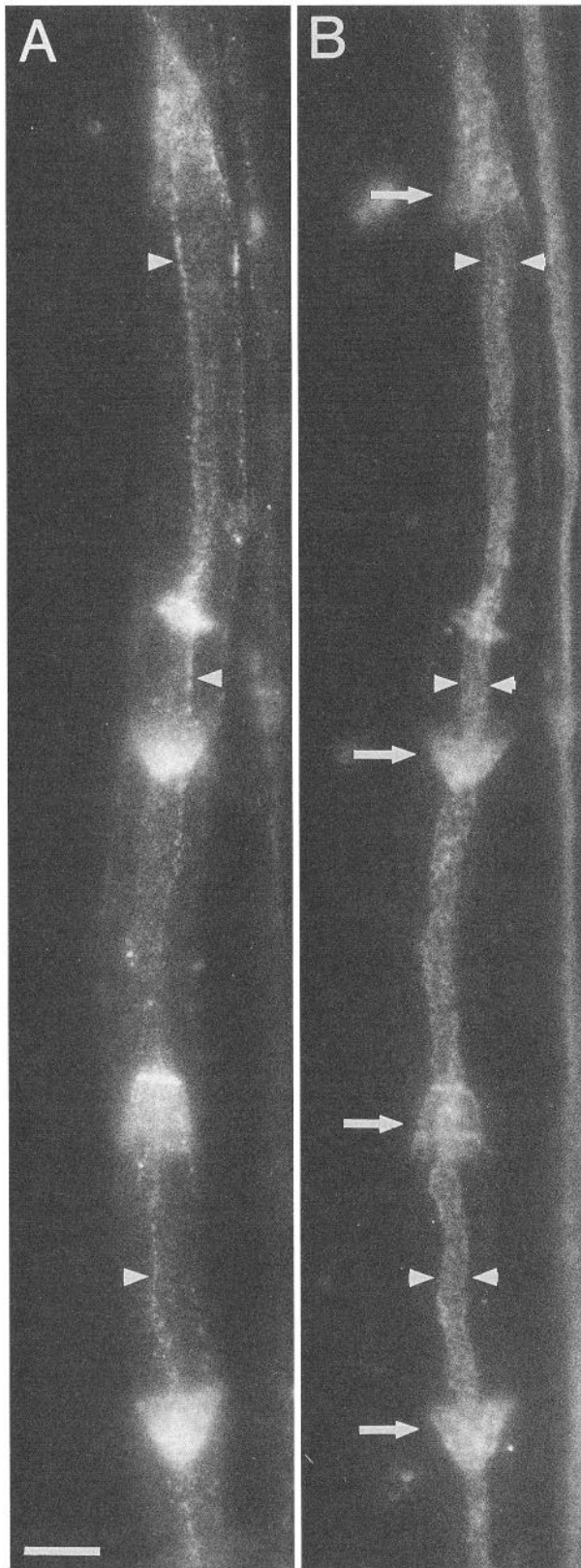


Figure 1. Immunohistochemical analysis of Cx32 and MAG in adult sciatic nerve. These are photomicrographs of a teased fiber that was labeled with a combination of a mouse monoclonal antibody against rat Cx32 (A) and a rabbit antiserum against MAG (B) and visualized with fluorescein- and rhodamine-coupled secondary antibodies, respectively. Cx32- and MAG-immunoreactivity colocalize at incisures (*arrows*), which are conical structures that traverse the unstained compact

et al., 1982). In addition, we also noted perinuclear Cx32 staining (data not shown), as has been noted for other components of the myelin sheath (Trapp et al., 1981).

To determine whether Cx32 is expressed in nonmyelinating Schwann cells, we examined teased fibers in the cervical sympathetic trunk, which is composed of unmyelinated axons, their associated (nonmyelinating) Schwann cells, as well as a few, thinly myelinated axons (Aguayo et al., 1976). As shown in Figure 3, A and B, there was an identical pattern of adaxonal Cx32- and MAG-immunoreactivity, respectively, in myelinating Schwann cells (*arrows*). Thus, Cx32-immunoreactivity was not confined to the inner mesaxon of these small myelinated fibers, but appeared to surround their entire adaxonal circumference. These small myelinated axons had internodal lengths of only 50–70 μm , and typically lacked incisures, like the fibers shown in Figure 3. When incisures were present, they were Cx32- and MAG-positive (data not shown). Nonmyelinating Schwann cells also had Cx32-immunoreactivity, although this staining was punctate and discontinuous, and overall significantly less than that seen in myelinating Schwann cells (Fig. 3A). We confirmed that these were nonmyelinating Schwann cells by double-labeling for both Cx32 and GAP-43, as GAP-43 labels unmyelinated axons and their associated nonmyelinating Schwann cells (data not shown; see Curtis et al., 1992).

To examine the onset of Cx32 expression during development, we examined teased fibers and sections of postnatal day 6 (P6) sciatic nerves. At this age, the sciatic nerve contains axons at many stages of ensheathment, including axons that have just begun to be myelinated (Webster and Favilla, 1984). There were many myelin sheaths in P6 nerves, and some had incisures that were MAG-positive, but there was little, if any, detectable Cx32 staining of incisures. The Cx32 staining that was present appeared to be associated with the inner mesaxon (data not shown). Thus, the onset of Cx32 expression in the incisures and paranodes may lag that of MAG.

Axotomy disrupts axon–Schwann cell interactions, causing the degeneration of the axons and their myelin sheaths, and a dramatic reduction in the expression of myelin-related proteins (Mirsky and Jessen, 1990; Scherer and Asbury, 1993). We examined Cx32 expression in nerves that were transected to cause permanent axotomy, as well as nerves were focally crushed, to cause Wallerian degeneration but promote axonal regeneration. In permanently transected nerves, Cx32 disappeared from myelinating Schwann cells in parallel with the loss of MAG (data not shown). The Schwann cells themselves, however, persisted, and could be labeled with an antibody against nestin (Fig. 4). At 58 d posttransection, Cx32-, MAG-, P_0 -, and MBP-immunoreactivity were found macrophages (Stoll et al., 1989), which were ED1-positive and nestin-negative (Fig. 4). In crushed nerves, Cx32-immunoreactivity was found in incisures and paranodal regions of remyelinated fibers by 24 d postlesion (data not shown). These data demonstrate that the expression of Cx32 protein is linked to the formation of the myelin sheath in Schwann cells.

To confirm that peripheral nerve contains Cx32, and to substantiate the changes in Cx32 protein expression after nerve-

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myelin sheath. MAG also surrounds the axon at the adaxonal Schwann cell surface (between *arrowheads* in B), whereas only a thin line of Cx32 staining is found at the adaxonal surface, probably at the inner mesaxon (*arrows* in A). Scale bar, 10 μm .

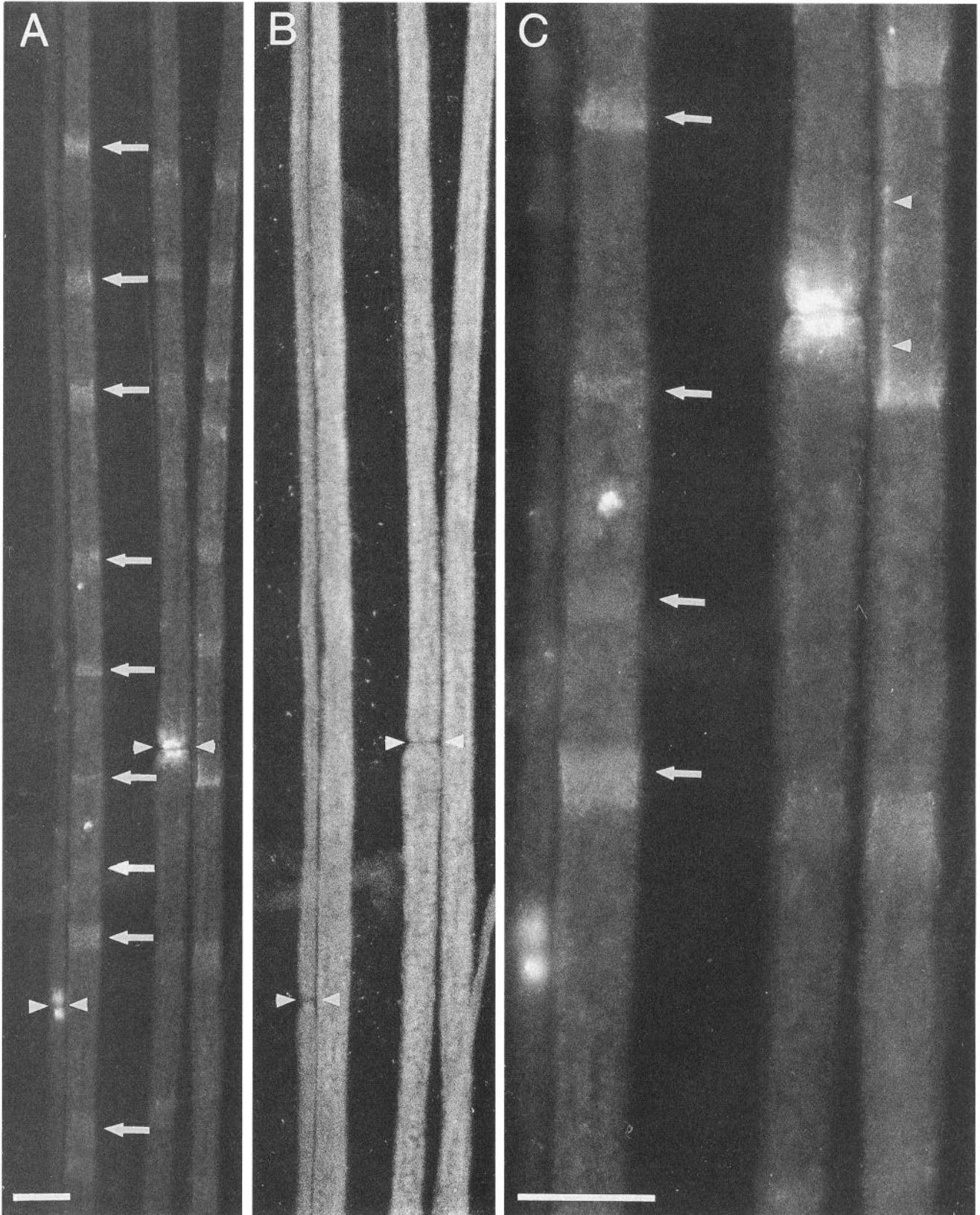


Figure 2. Immunohistochemical localization of Cx32 and P₀ in adult sciatic nerve. These are photomicrographs of teased fibers, double-labeled with a monoclonal antibody against rat Cx32 (*A,C*) and a rabbit polyclonal antibody against rat P₀ (*B*) and visualized with fluorescein- and rhodamine-coupled secondary antibodies, respectively. Note that Cx32 is predominantly found at the incisures (some of which are indicated by *arrows* in *A*) and paranodes (*arrowheads* in *A* and *B*), whereas the P₀ is found throughout the compact myelin sheath. *C* is an enlargement of the node shown in *A*, and shows Cx32 staining of the inner mesaxon (*arrowheads*). Scale bars, 10 μm.

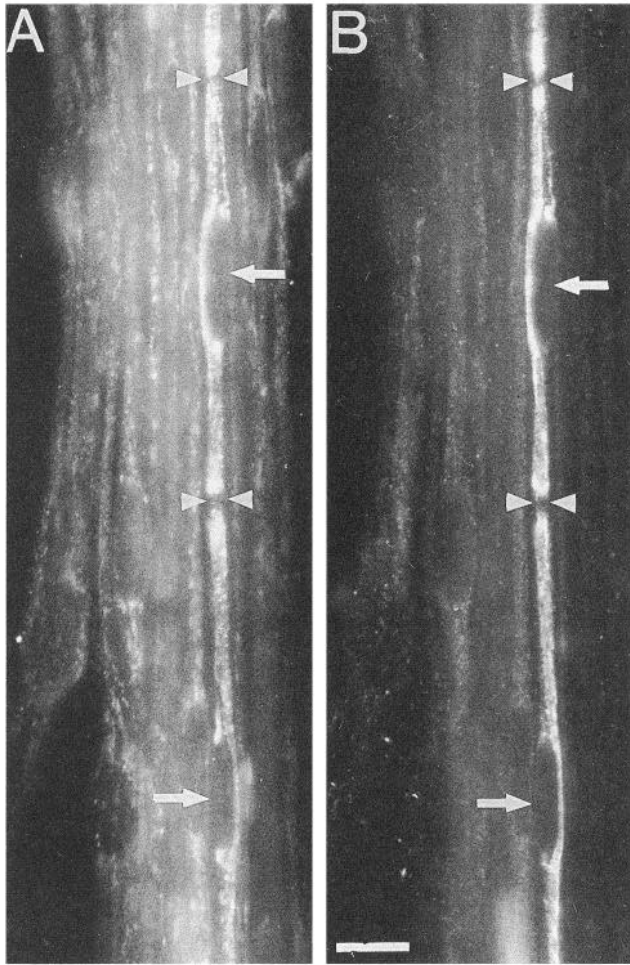


Figure 3. Immunohistochemical localization of Cx32 and MAG in adult cervical sympathetic trunk. These are photomicrographs of a bundle of teased fibers, double-labeled with a monoclonal antibody against rat Cx32 (*A*) and a rabbit polyclonal antibody against rat MAG (*B*) and visualized with fluorescein- and rhodamine-coupled secondary antibodies, respectively. There is a single myelinated fiber in this field, with two internodes (the *arrowheads* indicate the positions of the two Schwann cell nuclei), separated by nodes (*arrowheads*). The pattern of Cx32 and MAG staining of the two myelinating Schwann cells is essentially identical; neither one appears to have an incisure and the immunoreactivity is not restricted to the paranodes. Note that many nonmyelinating Schwann cells have some Cx32 staining, but little MAG staining. Scale bar, 10 μm .

injury, we performed Western blot analysis using a rabbit antibody against rat Cx32. As shown in Figure 5*A*, in unlesioned nerves (the lanes labeled "0"), this antibody recognized a protein of approximately 32 kDa molecular mass (arrowhead), as well as a dimer of Cx32 (double arrowhead), which results from the incomplete solubilization of Cx32 in SDS (Paul, 1986). The amount of Cx32 fell between 1 and 12 d posttransection, and did not increase thereafter. Prolonged exposure of the blot, however, demonstrated detectable Cx32 even at 60 d (data not shown). In crushed nerves, the amount of Cx32 fell between 1 and 12 d, as in transected nerves, but returned to near normal levels by 60 d post-crush. Reprobing the blots for P₀ demonstrated that the amount of this myelin protein also fell progressively in permanently transected nerves, and returned to normal in crushed nerves at 60 d (Fig. 5*B*). These data demonstrate that Cx32 is found in peripheral nerve, and that the amount of Cx32

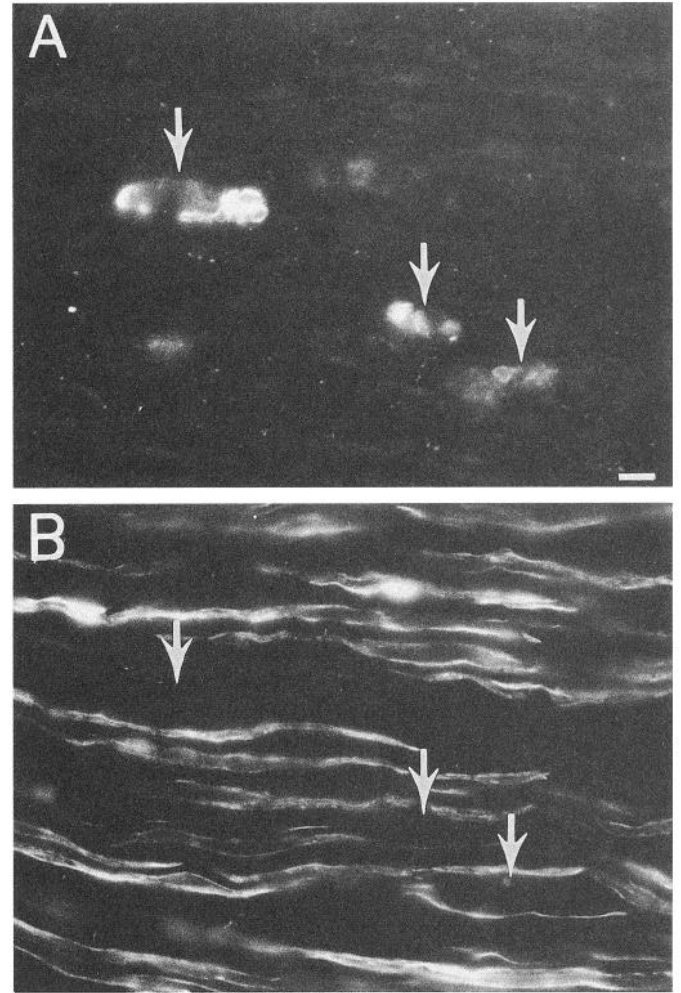


Figure 4. Immunohistochemical localization of Cx32 in permanently axotomized adult sciatic nerve. These are photomicrographs of the same field of a longitudinal section of nerve 58 d post transection, double-labeled for Cx32 (rhodamine, *A*) and (nestin; fluorescein, *B*). The arrows indicate three macrophages that have Cx32-immunoreactivity (*A*) but are nestin-negative (*B*). The nestin-positive structures are Schwann cell processes. Scale bar, 10 μm .

protein, like many myelin proteins, depends on the maintenance of axonal interactions (Mirsky and Jessen, 1990; Scherer and Asbury, 1993).

Cx32 mRNA is expressed in concert with other myelin-related genes and is regulated by axon-Schwann cell interactions and forskolin

Since the mRNAs of the major myelin genes accumulate in parallel during the development of the PNS (Stahl et al., 1990; Snipes et al., 1992), we examined the expression of Cx32 mRNA by Northern blot analysis. In the sciatic nerve, the level of P₀ mRNA increased dramatically after birth (Fig. 6). Reprobing the blot for Cx32 mRNA demonstrated that Cx32 mRNA changed during development in an identical pattern (the band indicated by the arrowhead in Fig. 6*A*), which is consistent with the idea that myelinating Schwann cells express Cx32 mRNA.

Since the maintenance of the myelinating phenotype, including the expression of high levels of myelin-related mRNAs, depends on the integrity of axon-Schwann cell interactions (Mirsky and Jessen, 1990; Scherer and Asbury, 1993), we examined

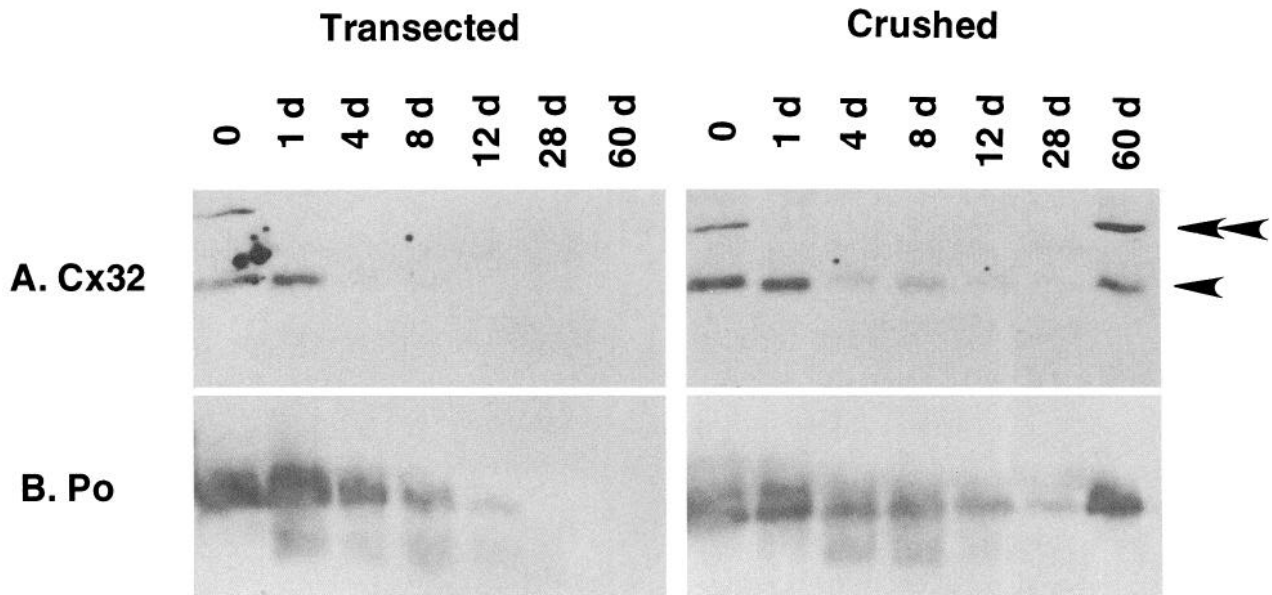


Figure 5. Western blot analysis of lesioned adult rat sciatic nerves. Each lane contains an equal amount (25 μ g) of protein homogenate from the distal nerve-stumps of sciatic nerves 1, 4, 8, 12, 28, or 60 d posttransection or crush; the "0" time point is from unlesioned nerves. The blots were hybridized together with a rabbit antiserum against rat Cx32, and then rehybridized with a rabbit antiserum against rat P₀. The blots were exposed to film for 30 min (Cx32) or 1 sec (P₀). The arrow marks the position of the Cx32 monomer, and the double arrowhead marks the position of the Cx32 dimer (Paul, 1986).

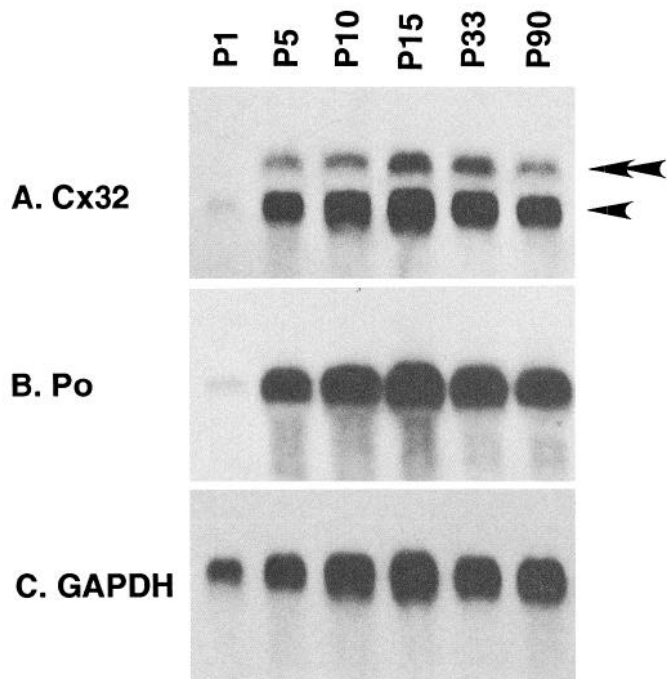


Figure 6. Northern blot analysis of developing sciatic nerve. Each lane contains an equal amount (10 μ g) of total RNA isolated from the distal stumps of sciatic nerves of various ages. The blots were successively hybridized with a radiolabeled cDNA probe for P₀ (B), Cx32 (A), and GAPDH (C), and exposed to film for 3 hr (P₀), 14 d (Cx32), and 1 d (GAPDH), respectively. The arrowhead marks the Cx32 mRNA; the double arrowhead indicates the signal from the previous hybridization for P₀ mRNA.

the expression of Cx32 mRNA after permanent axotomy and nerve-crush. The distal nerve-stumps of crushed nerves were divided into a proximal and a distal segment, to facilitate the analysis of how changes in Schwann cell gene expression depend on regenerating axons, as axons regenerate in a proximal to distal manner. In permanently transected nerves, the level of Cx32 mRNA fell sharply between 1 and 8 d postlesion and did not return even by 58 d (Fig. 7). In crushed nerves, the level of Cx32 mRNA also fell between 1 and 8 d postlesion, but then increased. This increase was first seen in the proximal segment of the distal nerve-stump at 12 d, and in the distal segment at 24 d. Reprobing the blots demonstrated that the level of P₀ mRNA in transected and crushed nerves followed essentially the same pattern as that of Cx32, except that the level of P₀ mRNA fell more promptly than that of Cx32 (Fig. 7). Reprobing the blots for the low-affinity nerve growth factor receptor (NGFR) mRNA demonstrated a reciprocal pattern to that of Cx32 and P₀, consistent with the evidence that myelinating Schwann cells do not express NGFR, whereas "denervated" Schwann cells express NGFR and not myelin-related genes (Mirsky and Jessen, 1990; Scherer and Asbury, 1993).

cAMP analogs mimic some of the effects of axon-Schwann cell interactions, such as increasing the expression of galactocerebroside, sulfatide, and P₀, and inhibiting the expression of NGFR and GAP-43 (Sobue and Pleasure, 1984; Lemke and Chao, 1988; Morgan et al., 1991, 1994; Scherer et al., 1994). To determine whether cAMP would increase the level of Cx32 mRNA, we performed Northern blot analysis of cultured rat Schwann cells treated for 3 d with 4 μ M forskolin, an activator of adenylate cyclase (Seamon et al., 1981). As shown in Figure 8, forskolin increased the levels of Cx32 and P₀ mRNA, but the level was not as high as in unlesioned sciatic nerve. Cultured perineurial fibroblasts, which are the other major cell type in peripheral nerve and coupled by gap junctions (Reale et al., 1975; Schiavinato et al., 1991), did not express Cx32 mRNA

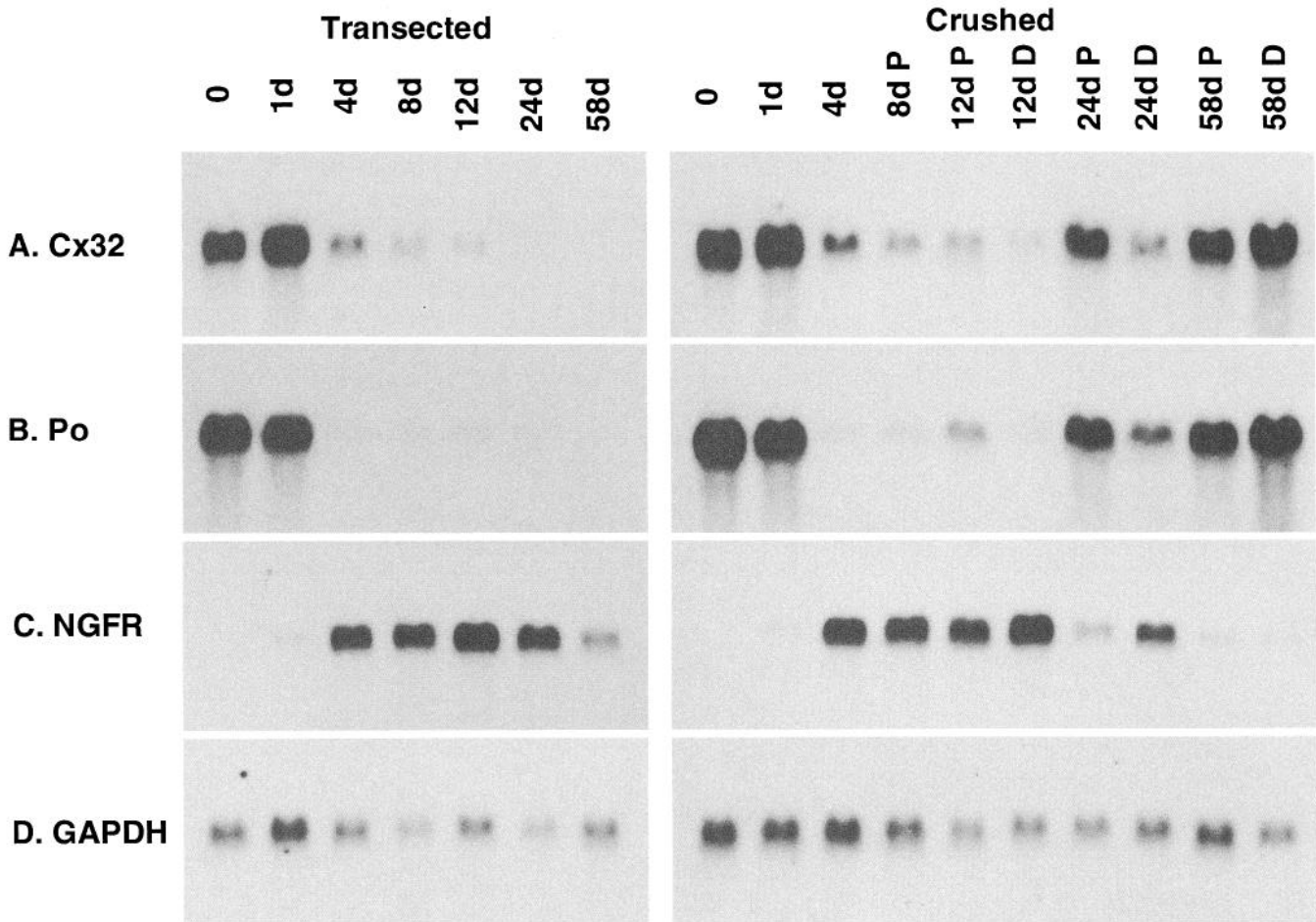


Figure 7. Northern blot analysis of normal and lesioned adult rat sciatic nerves. Each lane contains an equal amount (10 μ g) of total RNA isolated from the distal stumps of sciatic nerves that had been transected or crushed. The number of days after each of these lesions is indicated; the "0" time point is from unlesioned nerves. In crushed nerves, the distal nerve-stumps were divided into proximal (P) and distal (D) segments of equal lengths. The blots were successively hybridized with a radiolabeled cDNA probe for Cx32 (A), P₀ (B), NGFR (C), and GAPDH (D), and exposed to film for 14 d (Cx32), 2 hr (P₀), 1 d (NGFR), and 3 d (GAPDH), respectively.

even when treated with forskolin, but did express Cx43 mRNA (Fig. 8). To determine whether forskolin increased the expression of Cx32 protein, we prepared Western blots of Schwann cells that had been treated for 3 d with 0, 4, or 20 μ M forskolin. We did not detect Cx32 in either untreated or treated Schwann cells, whereas reprobing the blot for P₀ demonstrated a robust increase in P₀ protein in forskolin-treated cells (data not shown). Thus, forskolin increases the expression of Cx32 mRNA in a similar manner to other myelin-related genes.

Connexin32 is expressed by oligodendrocytes

Although Cx32 has been reported to be expressed by oligodendrocytes and neurons (Dermietzel et al., 1989; Micevych and Abelson, 1991; Yamamoto et al., 1991; Robinson et al., 1993), the clinical data indicate that CMTX patients do not usually have CNS abnormalities (Phillips et al., 1985; Rozear et al., 1987; Hahn et al., 1990; Ionasescu et al., 1992). Hence, even though Schwann cells and oligodendrocytes both express Cx32 and are the sole myelin-forming cells, oligodendrocytes may not be affected by mutations of Cx32.

We examined the expression of Cx32 in the developing rat spinal cord, which contains a number of tracts that myelinate at different times in postnatal development (Schwab and Schnell,

1989; Baron et al., 1993). At P1, Cx32 colocalized with MAG and PLP in the cell bodies of developing oligodendrocytes (data not shown). In adult spinal cord, the most prominent Cx32-immunoreactivity was found in the cell bodies and processes of oligodendrocytes, but the myelin sheaths themselves were not stained (Fig. 9A,C). To confirm and extend these findings, we double-labeled sections for Cx32 and MAG and for Cx32 and myelin-oligodendrocyte glycoprotein (MOG), as MAG and MOG are localized to the adaxonal and abaxonal surfaces of the myelin sheath, respectively (Sternberger et al., 1979; Brunner et al., 1989). Cx32 colocalized with MAG in oligodendrocyte cell bodies, but Cx32 labeling did not colocalize with that of MAG at the adaxonal surface of oligodendrocytes (data not shown). Cx32 colocalized with MOG in some oligodendrocyte processes, but the adaxonal surface of the myelin sheath was mostly Cx32-negative (Fig. 9). Thus, while Cx32 is a myelin-related protein in the PNS and CNS, it is distributed in different aspects of the myelinating cell.

The localization of Cx32 in the spinal cord suggested that it predominately expressed by oligodendrocytes. Since the onset and tempo of myelination in the CNS differ in the various myelinated fiber tracts of the CNS (Jacobson, 1963; Cohen and Guarnieri, 1976), we examined the accumulation of Cx32 pro-

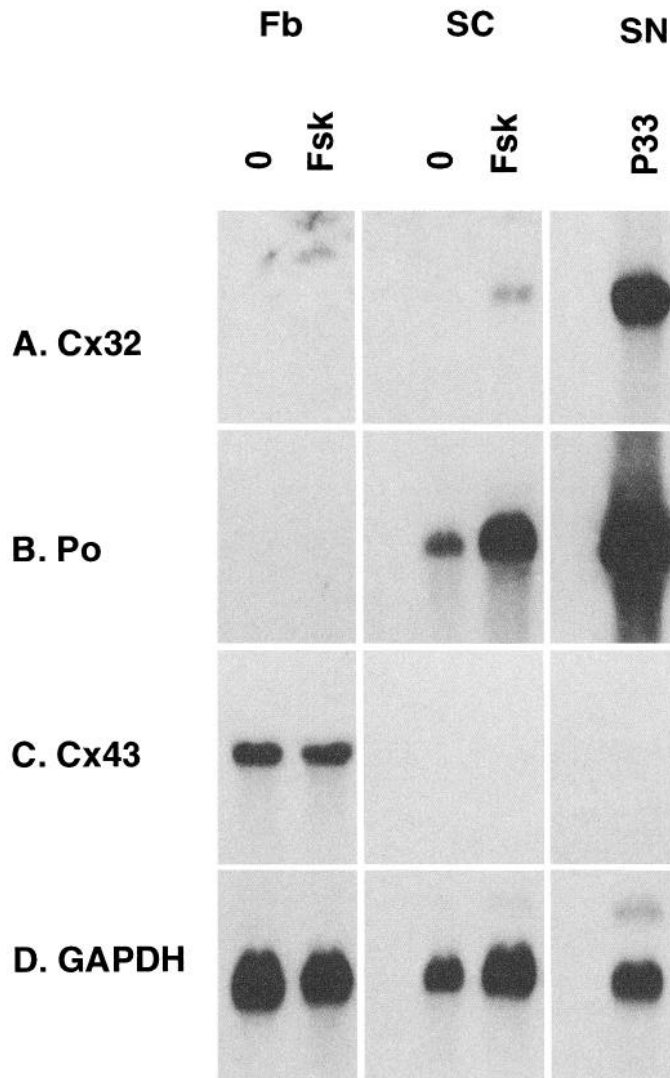


Figure 8. Northern blot analysis of cultured Schwann cells and fibroblasts. Each lane contains an equal amount (30 μ g) of total RNA isolated from Schwann cells or fibroblasts cultured for 3 d in the absence (lane 0) or presence of 4 μ M forskolin. For comparison, a sample of normal sciatic nerve RNA was run in an adjacent lane (marked SN). The blot was successively hybridized with a radiolabeled cDNA probe for Cx32 (A), P₀ (B), GAPDH (D), and Cx43 (C), and exposed to film for 12 d (Cx32), 2 hr (P₀), 4 d (Cx43), and 3 d (GAPDH).

tein in different CNS regions by Western blot analysis. Protein homogenates of spinal cord, brainstem, cerebellum, and cerebrum were prepared from rats of various ages, from P1 to P90. The blots were probed with a rabbit antiserum against Cx32, then the same blots were reprobed with a rabbit antiserum against PLP. Cx32 and PLP were more abundant in the brainstem and spinal cord (Fig. 10) than in the cerebrum and cerebellum (not shown), and the amount of Cx32 and PLP in each brain region increased from P1 to P90 (Fig. 10). We also made a myelin preparation of adult cerebrum, brainstem, and spinal cord, to enrich for membrane-related proteins, in order to compare the amount of Cx32 in the myelin fractions to that in homogenates of the corresponding brain regions. Western blot analysis demonstrated that in each brain region, the myelin fraction contained relatively more Cx32, and that PLP was enriched to a similar extent (Fig. 10). These results demonstrate that Cx32

accumulates in parallel with other myelin proteins during development.

Cx32 mRNA is expressed in concert with other myelin-related genes in oligodendrocytes

Like the myelin proteins, the myelin-related mRNAs accumulate in parallel during the development of the CNS, with modest differences between different brain regions (Kanfer et al., 1989; Scherer et al., 1994). Thus, we compared the expression of Cx32 mRNA in the cerebrum, cerebellum, and brainstem to that of PLP (Fig. 11). In each region, the levels of Cx32 and PLP mRNA were parallel, with a similar onset and peak. Prolonged overexposure of the blot demonstrated, however, a low level of Cx32 mRNA expression prior to the onset of PLP mRNA expression (data not shown), consistent with the idea that neurons express Cx32 prior to oligodendrocytes.

The above findings indicate that most of the Cx32 mRNA in the CNS originates from oligodendrocytes, which is consistent with the *in situ* hybridization localization of Cx32 mRNA in adult rat CNS (Micevych and Abelson, 1991). To further substantiate this idea, we analyzed Cx32 mRNA expression in *myelin-deficient* rats and *jimpy* mice, which have mutations in the PLP gene, and drastically reduced levels of all myelin-related mRNAs (Hudson, 1990). Northern blot analysis of affected male *myelin-deficient* rats and their age-matched normal male littermates revealed that affected males had much lower levels of Cx32 mRNA at every age (Fig. 12). The levels of Cx32 mRNA in the brains of *jimpy* mice were also much lower than those in the brains of age-matched normal mice as well as *shiverer* mice, which have a recessively inherited mutation in the MBP gene (Hudson, 1990). Reprobing the blots for PLP mRNA demonstrated that the levels of PLP mRNA were much lower at every age in both *myelin-deficient* rats and *jimpy* mice than in non-mutant animals and *shiverer* mice (Fig. 12). The changes in myelin-related mRNA expression in the mutant animals were not confined to Cx32 and PLP, as MBP and MAG are similarly affected (data not shown; see also Roth et al., 1985; Gardinier and Macklin, 1988; Kumar et al., 1988; Kumar et al., 1990; Macklin et al., 1991). Thus, the parallel reduction in Cx32 mRNA and other myelin-related mRNAs in these PLP mutants provides strong evidence that most Cx32 mRNA in the CNS is derived from oligodendrocytes.

Discussion

The finding that mutations in Cx32 cause CMTX (Bergoffen et al., 1993) led us to investigate the role of Cx32 in myelinating glia. In this article, we have shown that Cx32 is expressed in myelinating Schwann cells and oligodendrocytes, but is localized to different parts of each cell. In the PNS, Cx32 is found mainly in the incisures and paranodal regions of myelinating Schwann cells, whereas in the CNS, it is found in oligodendrocyte cell bodies and processes. In both Schwann cells and oligodendrocytes, Cx32 protein and mRNA are expressed in a coordinate manner with those of other myelin genes. In Schwann cells, the expression of Cx32 mRNA and protein is developmentally regulated and depends on the integrity of axon-Schwann cell interactions. In oligodendrocytes, Cx32 is expressed in concert with other myelin genes, both in normal development and in *jimpy* mice and *myelin-deficient* rats. Thus, Cx32 is expressed as part of the myelinating phenotype of both Schwann cells and oligodendrocytes.

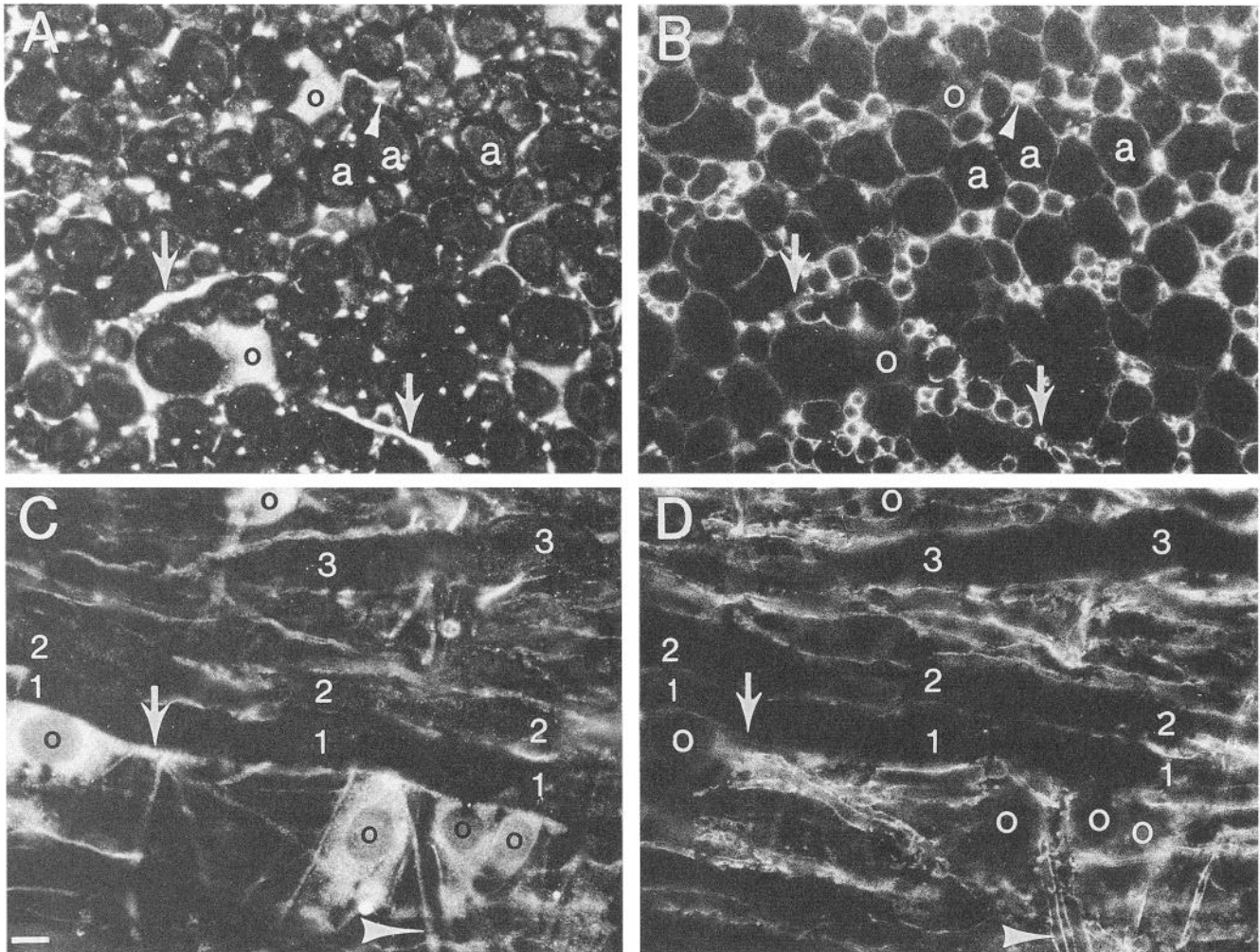


Figure 9. Immunohistochemical analysis of Cx32 in the ventral funiculus of adult rat spinal cord. These are photomicrographs of transverse (*A,B*) and longitudinal sections (*C,D*), double-labeled with a rabbit antiserum against Cx32 (*A,C*) and a mouse monoclonal antibody against MOG (*B,D*), and visualized with rhodamine- and fluorescein-coupled secondary antibodies, respectively. Cx32-immunoreactivity is found in oligodendrocytes (*o*) and their processes, some of which are indicated by arrows. MOG-immunoreactivity is found on the external/abaxonal surface of the myelin sheath, around a few oligodendrocyte processes (*arrowheads*), but not in the oligodendrocytes themselves (*o*). Some myelinated axons are indicated (*a* in *A* and *B* and *1,2,3* in *C* and *D*); the myelin sheath is unlabeled by Cx32 or MOG. Scale bar, 10 μ m.

Gap junctions in Schwann cells

Since Cx32 forms gap junctions in many tissues, its localization in the incisures and paranodal regions indicates that gap junctions should be found at these locations. Small collections of hexagonally packed particles of the appropriate size to be gap junctions have been found in the incisures and paranodes of the PNS myelin sheath by freeze-fracture electron microscopy (Sandri et al., 1982). As these collections of particles seen by freeze-fracture are characteristic of gap junctions, and Cx32 is found at these same sites, it is reasonable to propose that these gap junctions contain Cx32. Since they link apposed layers of the same cell, these could be called "reflexive" gap junctions, which have also been described in the kidney and other tissues (Majack and Larsen, 1980). Why typical gap junctions have not been noted in incisures and paranodes is unclear, as these structures have been extensively studied by transmission electron microscopy (Thomas and Ochoa, 1984; Peters et al., 1991).

More typical gap junctions, between different Schwann cells, have also been described. Electrophysiological recordings and tracer studies have revealed gap junctions between nonmyeli-

nating Schwann cells (but not between myelinating Schwann cells) in peripheral nerve (Konishi, 1990). In peripheral nerve undergoing Wallerian degeneration, gap junctions between adjacent Schwann cells have been observed by transmission electron microscopy (Tetzlaff, 1982), and their existence has also been inferred by recent electrophysiological observations (Brunet and Jirounek, 1994). Finally, gap junctions have been observed between rat Schwann cells cultured in the absence of forskolin (Chanson et al., 1993). The connexin(s) that forms gap junctions between nonmyelinating, denervated, and cultured rat Schwann cells has not been identified. Since Cx32 appears to be expressed by nonmyelinating Schwann, it may form the gap junctions in these cells. The connexin expressed by Schwann cells cultured in the absence of forskolin is unlikely to be Cx32, which we find to be expressed at very low levels under these conditions, and which has different electrophysiological properties than those described by Chanson et al. (1993).

How do CMTX mutations cause neuropathy?

Many different mutations in the Cx32 gene cause CMTX. To date, 33 different mutations, affecting nearly every portion of

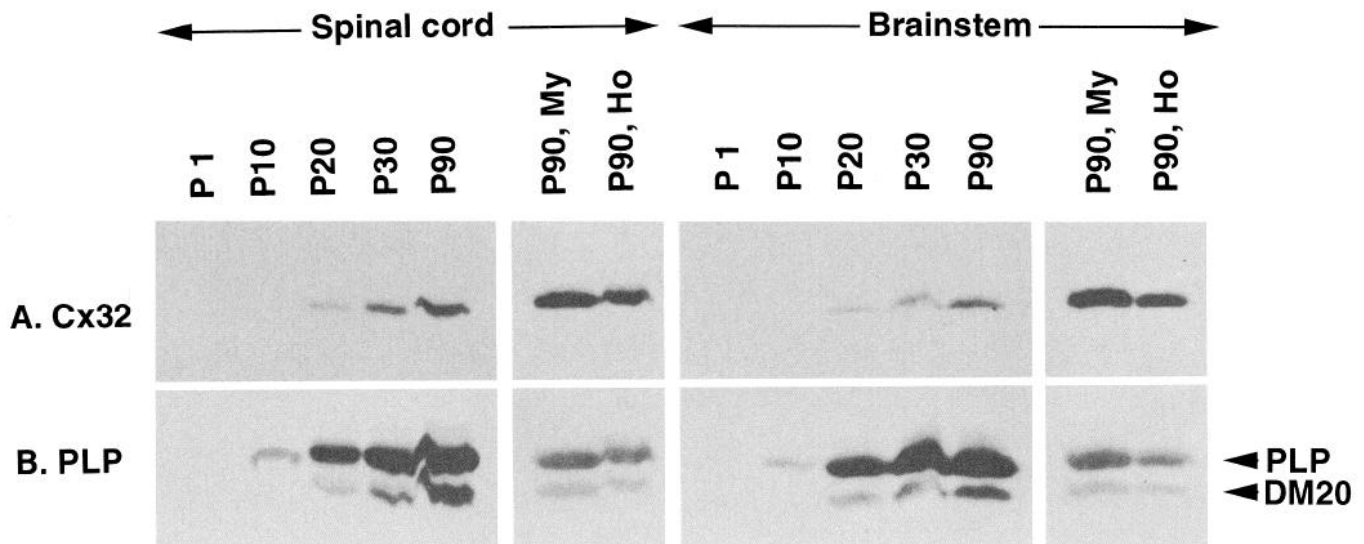


Figure 10. Western blot analysis of Cx32 in the CNS. Each lane contains 25 μ g of protein homogenate (Ho) from developing rat spinal cord or brainstem at the indicated ages, or from a myelin preparation (My) of adult rat spinal cord or brainstem. The blots were hybridized with a rabbit antiserum against rat Cx32, then rehybridized with a rabbit antiserum against rat PLP. The blots of developing spinal cord and brainstem were hybridized together and exposed to film for 5 min (Cx32) and 1 min (PLP). The blots of spinal cord and brainstem myelin were hybridized together and exposed to film for 3 min (Cx32) and 10 sec (PLP). The arrowheads indicate the position of PLP and its alternatively spliced isoform, DM20.

the Cx32 protein, have been found in 39 kindreds (Bone et al., 1995). To determine whether these mutations result in a loss of function, Bruzzone et al. (1994) expressed three different CMTX genes, as well as the wild-type Cx32 gene, in *Xenopus* oocytes. Whereas the wild-type Cx32 formed gap junctions, no oocytes expressing any of the three CMTX cDNAs formed functional gap junctions with oocytes expressing Cx26 or wild-type Cx32. Using a similar approach, Rabadan-Diehl and colleagues (1994) found that another CMTX mutation (Arg 200 \rightarrow stop codon) did not destroy channel activity, indicating that CMTX mutations may disrupt gap junctions in other ways besides a simple

loss of function. To determine whether Cx32 mutations could also have a dominant-negative effect, Bruzzone et al. (1994) coinjected the same three CMTX mutations described above with wild-type Cx26, which is often coexpressed with Cx32 (Nicholson et al., 1987; Meda et al., 1993; Zhang and Nicholson, 1994). Oocytes that were coinjected with wild-type Cx26 and wild-type Cx32 cDNAs formed gap junctions when apposed to oocytes that expressed wild-type Cx26 cDNA (Barrio et al., 1991). Oocytes that were coinjected with wild-type Cx26 and the CMTX mutations, however, showed decreased junctional conductance (Bruzzone et al., 1994). These data suggest that

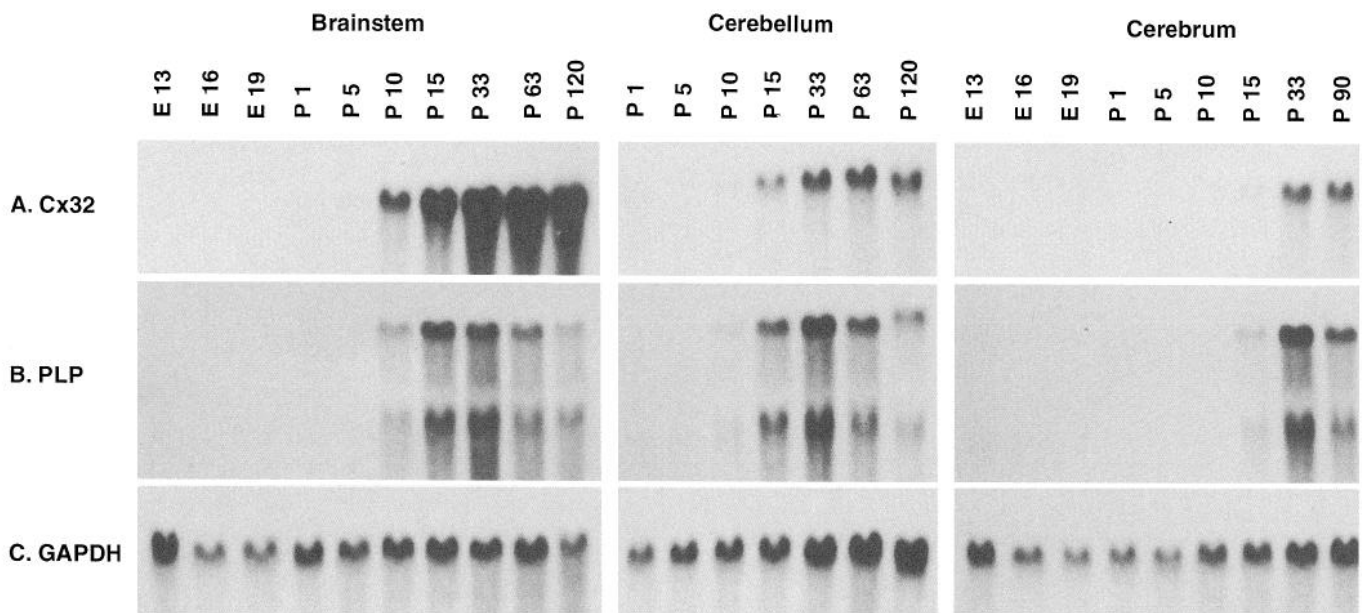


Figure 11. Northern blot analysis of Cx32 mRNA in developing brain. Each lane contains an equal amount (10 μ g) of total RNA isolated from the cerebrum, cerebellum, and brainstem. The blots were successively hybridized with a radiolabeled cDNA probe for Cx32 (A), PLP (B), and GAPDH (C), and exposed to film for 15 d (Cx32), 2 hr (PLP), and 16 hr (GAPDH), respectively.

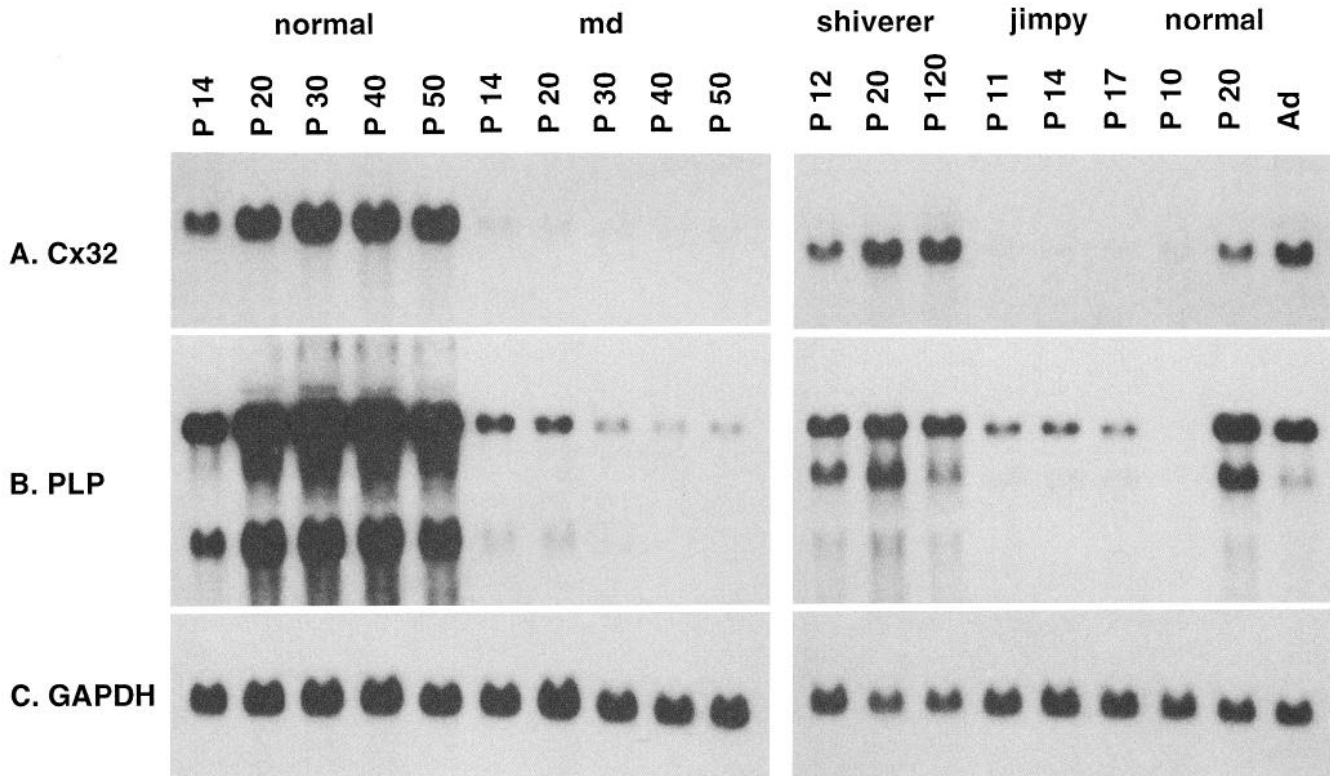


Figure 12. Northern blot analysis of Cx32 mRNA expression in the brains of myelin mutants. Each lane contains an equal amount (10 μ g) of total RNA isolated from the whole brains (cerebrum, cerebellum, and brainstem) of individual animals of the indicated ages—*myelin-deficient* rats and their normal littermates, *shiverer* mice, and *jimpy* mice. RNA from cerebra of normal mice is shown for comparison. The blots were successively hybridized together with a radiolabeled cDNA probe for Cx32 (A), PLP (B), and GAPDH (C), and exposed to film for 14 d (Cx32), 1 d (PLP), and 16 hr (GAPDH), respectively. Note that in rats, the major PLP transcripts in rats are 3.2 and 1.6 kb; in mice, 3.2 and 2.8 kb are main transcripts.

many CMTX mutations cause a loss of functional Cx32 protein, and that at least some CMTX mutations can also have a dominant-negative effect. Yet, in spite of the large number of different mutations, and the evidence that different mutations may have different functional effects, there is no compelling evidence that different mutations cause different clinical phenotypes.

The localization of Cx32 in the Schwann cell myelin sheath, and the loss of function of CMTX mutations in oocytes, strongly suggest that at least some CMTX mutations cause a loss of functional gap junctions in the incisures and paranodes. Why these gap junctions are critical to the normal health of myelinated axons, however, remains to be determined. One potentially important consideration is that the geometry of the myelin sheath itself. Myelin is a fundamental adaptation of jawed vertebrates, and is formed by the enormous expansion of Schwann cell and oligodendrocyte membrane (Peters et al., 1991). Gap junctions in the paranodes and incisures would allow ions and small molecules to diffuse radially, directly traversing the myelin sheath, instead of circumferentially through the Schwann cell cytoplasm, which would be a much longer pathway. In the largest myelin sheaths of mammals, this potential radial pathway would be more than 1000-fold shorter than the circumferential pathway, as the unrolled myelin sheath is more than 4 mm long, whereas the compact myelin sheath is less than 4 μ m thick (Friede and Bischhausen, 1980). If CMTX mutations interrupt the function of these gap junctions, then this radial pathway would be abolished. At this time, we can only speculate as to what are the crucial molecules that pass through these gap junctions, or even

whether the interruption of diffusion towards or away from the axon is more important.

A related issue is why mutations of Cx32 selectively affect myelinating Schwann cells. Patients who have CMTX have not been reported to have significant abnormalities in other tissues that express Cx32. The level of Cx32 expression does not seem to be critical, as several tissues, such as the brain and spleen, express levels of Cx32 mRNA that are comparable to peripheral nerve, and liver expresses much higher levels (Nishi et al., 1991; Bergoffen et al., 1993; Meda et al., 1993). Myelinating Schwann cells may be uniquely susceptible to Cx32 mutations because Cx32 is the only connexin they express. In support of this idea, we have not detected Cx43, Cx40, or Cx26 mRNA in rat Schwann cells treated with forskolin (Fig. 8 and data not shown). Cultured sciatic fibroblasts express Cx43 mRNA (Fig. 8), and adult sciatic nerve expresses a low level of Cx43 mRNA that is not modulated by axotomy (data not shown), indicating Cx43 mRNA in nerve is derived from fibroblasts and not myelinating Schwann cells. On the other hand, there is a growing body of evidence that most cell types express more than one connexin. The liver, for example, expresses Cx32 and Cx26 (Nicholson et al., 1987; Meda et al., 1993; Kuraoka et al., 1993), and epidermal cells express four different connexins (Goliger and Paul, 1994).

Cx32 in CNS myelin

Patients who have CMTX are not known to have CNS abnormalities, although oligodendrocytes, the only other myelin-form-

ing cells, have been reported to express Cx32 mRNA and protein (Dermietzel et al., 1989; Micevych and Abelson, 1991). In agreement with previous reports (Naus et al., 1990; Belliveau et al., 1991), we found regional and developmental differences in the expression of Cx32 mRNA. We extended these observations by demonstrating a close association between Cx32 mRNA expression and that of other myelin genes, both in normal development and in PLP mutants. Western blot analysis of Cx32 also demonstrated regional and developmental differences, as well as decreased expression in PLP mutants. Cx32 protein was more abundant in the pons and spinal cord than in the cerebrum and cerebellum, and the amount of protein accumulated in parallel with PLP. These results differ from those of Belliveau et al. (1991), who did not find a developmental increase in Cx32 protein in the cerebrum or hindbrain. The good agreement between the levels of Cx32 mRNA and protein both in development and in PLP mutants, nevertheless, indicates that most of the Cx32 mRNA and protein in the CNS is derived from oligodendrocytes.

The localization of Cx32 in oligodendrocytes has received relatively little attention. Several workers have examined the localization of Cx32 in the neurons of the spinal cord without mentioning oligodendrocyte labeling (Carr et al., 1991; Yamamoto et al., 1991). Dermietzel et al. (1989) reported Cx32-immunoreactivity in the perinuclear region of oligodendrocytes, but did not systemically evaluate the localization of Cx32 during development. We found that in older animals, Cx32 is predominantly found in cell bodies and processes, and was not found in compact myelin, in contrast to the recent report of Spray and Dermietzel (1995). These observations generally agree with the localization of gap junctions in by freeze-fracture electron microscopy (Massa and Mugnaini, 1982; Sandri et al., 1982; Robinson et al., 1993). Whether Cx32 is also found in the paranodal regions of CNS myelin sheaths, which contains gap junctions by freeze-fracture, remains to be determined.

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