Transgenic Expression of Human \textit{Connexin32} in Myelinating Schwann Cells Prevents Demyelination in \textit{Connexin32}-Null Mice

Steven S. Scherer, Yi-Tian Xu, Albee Messing, Klaus Willecke, Kenneth H. Fischbeck, Kenneth H. Fischbeck, and Linda Jo Bone Jeng

\textbf{Introduction}

Myelin sheaths are a multilamellar spiral of specialized membrane formed by Schwann cells in the PNS and oligodendrocytes in the CNS (Scherer et al., 2004; Trapp and Kidd, 2004). By limiting axonal depolarization to nodes of Ranvier, the periodic interruptions between myelin internodes, myelin increases axonal conduction velocity. PNS and CNS myelin sheaths are structurally similar but chemically distinct. Both consist mostly of lipids, including cholesterol and glycolipids, but each contains a unique but overlapping set of proteins. In the PNS, myelin protein zero (P0), peripheral myelin protein 22 kDa (PMP22), and myelin basic protein (MBP) are the main proteins in compact myelin; the main ones in the CNS are proteolipid protein and MBP. In addition to compact myelin, PNS myelin sheaths also have domains of noncompact myelin that contain myelin-associated glycoprotein (MAG) as well as the molecular components of adherens junctions, tight junctions, and gap junctions, including the gap junction protein \textit{connexin32} (Cx32). Although these junctions are classically described between apposed cells in various epithelia, they link apposed surfaces of the same cell in the PNS myelin sheath.

Inherited demyelinating neuropathies are a clinically and genetically heterogeneous group of diseases including the dominantly inherited, demyelinating forms of Charcot-Marie-Tooth disease (CMT1), as well as milder and more severe neuropathies (Dyck et al., 1993b; Lupski and Garcia, 2001; Wrabetz et al., 2004). Dominant mutations in \textit{PMP22}, \textit{MPZ/P0}, and \textit{GJB1/Cx32} cause CMT1A, CMT1B, and CMT1X, respectively. How these mutations cause demyelination is unknown, but it is likely that myelinating Schwann cells are affected first. For example, P0 mRNA and protein appear to be exclusively expressed by myelinating Schwann cells in adult animals, so that the demyelination is probably Schwann cell autonomous (Kirschner et al., 2004). Myelinating Schwann cells also express Cx32 (Scherer et al., 1995), but Cx32 is also expressed by some neurons and many other cell types (Yamamoto et al., 1991; Micevych et al., 1996; Bennett et al., 1999; Solomon et al., 2001). Because it is possible...
that the expression of Cx32 by other cell types contributes to the development of the demyelinating neuropathy, we expressed the human GJB1/Cx32 gene in transgenic mice using the rat Mpz promoter, which is expressed exclusively by myelinating Schwann cells (Lemke et al., 1988; Messing et al., 1992). Male transgenic mice were crossed with female Cx32-null mice; the resulting male offspring were all Cx32 null, and equal proportions were transgene positive (TG⁺) and negative (TG⁻). Myelinating Schwann cells expressed this transgene, and the human Cx32 protein was properly localized (to paranodes and incisures) and prevented the development of demyelination in Cx32-null mice. These findings provide strong evidence that the effects of GJB1/ Cx32 mutations are initially manifested in the myelinating Schwann cells themselves.

Materials and Methods

Generation of Cx32 mutant mice. The generation and initial characterization of Cx32-null (cx32⁻/⁻) female and cx32⁻/+ mice has been described (Nelles et al., 1996; Anzini et al., 1997; Scherer et al., 1998). Animals were generated from our colony at the University of Pennsylvania (Philadelphia, PA) by PCR analysis of genomic DNA isolated from tail clips (Anzini et al., 1997) and confirmed by Southern blot analysis (Abel et al., 1999).

The creation of mice expressing a GJB1 mutation (Arg175 frameshift) with a 1.1 kb rat Mpz promoter fused to the human GJB1 gene has been described (Abel et al., 1999). A genomic clone of GJB1 was isolated from a human X chromosome genomic library using probes for exon 1a and exon 2 (see Fig. 1A). Based on the characterization of the P2 promoter, which is active in peripheral nerve (Neuhauß et al., 1995, 1996; Söhl et al., 1996), a 5.3 kb Ndrl/Sacl fragment containing exon 1b, exon 2, and a 355 bp intron was isolated. A 1.1 kb fragment containing the rat Mpz promoter (Lemke et al., 1988) was purified and cloned upstream of the Ndrl/Sacl GJB1 fragment in the appropriate orientation; this removed the TATAA box of the P2 promoter. This construct was designated P0Cx32WT and is shown in Figure 1B.

A 6.4 kb transgene cassette for each clone was released from vector sequences by digestion with Apal and NsiI. The fragment was isolated, purified, and microinjected into the male pronucleus of fertilized eggs obtained from FVB/N mice (Taketo et al., 1991) (Taconic Farms, Germantown, NY), according to standard protocols (Brinster et al., 1985). Eggs that survived microinjection were then placed into pseudopregnant foster mice for gestation. Transgenic progeny were identified by PCR and line 96) that transmitted the transgene to their progeny. Transgenic progeny were identified by PCR with transgene-specific primers

**Immunohistochemistry.** Because fixation in paraformaldehyde reduces Cx32 immunoreactivity (Scherer et al., 1995), we teased nerve fibers from unfixed nerves; these were dried on glass slides (SuperFrost Plus; Fisher Scientific), postfixed for 10 min with acetone, blocked at room temperature for at least 1 h in 5% fish skin gelatin containing 0.5% Triton X-100 in PBS, and incubated for 24 – 48 h at 4°C with various combinations of primary antibodies. We used a mouse monoclonal antibody against rat Cx32 (7C6.67; diluted 1:1), which recognizes an epitope in the C-terminal tail (Li et al., 1997) or a rabbit antisemum against rat Cx32 [usually from Chemicon (Temecula, CA), diluted 1:200, but also from Zymed, diluted 1:200; both against a peptide from the cytoplasmic loop]. The anti-Cx32 antibodies were usually combined with either a rat monoclonal antibody (DECM; diluted 1:50; Sigma) or a rabbit antisemum (Fannon et al., 1995) (diluted 1:500) against E-cadherin or MG (Pedraza et al., 1990) (diluted 1:500). A rat antibody against nonphosphorylated neurofilament-heavy (NF-H; Ta51, diluted 1:10) (Lee et al., 1982, 1987) was used to visualize axons. A mouse monoclonal antibody (Y11) against myelin-oligodendrocyte glycoprotein (MOG) was used to visualize oligodendrocyte myelin sheaths. After incubation with the primary antibodies, the sections were washed and incubated with the appropriate fluorescein-, rhodamine-, and cyanine-5-conjugated donkey anti-rabbit, anti-mouse, and/or anti-rat secondary antibodies (Jackson Immunoresearch Laboratories). The preparations were imaged with epifluorescence tetramethylrhodamine isothiocyanate and FITC optics on a Leica DMR light microscope equipped with a cooled Hamamatsu (Bridgewater, NJ) camera or with a Leica TCS laser scanning confocal microscope, followed by image manipulation with Adobe Systems (San Jose, CA) Photoshop. Where appropriate, the images were made with comparable exposure times to allow better comparison between mice of different genotypes.

Light and electron microscopy. For the transgenic line 90, we examined litters of Cx32−/− mice at postnatal day 158 (P158) (two TG⁺), P250 (two TG⁺ and two TG⁻), and P365 (two TG⁺ and three TG⁻). Mice were deeply anesthetized with chloral hydrate and were then perfused with 0.9% NaCl followed by 3% glutaraldehyde in 0.1 M phosphate buffer. The spinal cord and attached roots, as well as sciatic and femoral nerves, were removed and placed in fresh fixative overnight at 4°C. For epoxy embedding, tissues were postfixed in 2% OsO4 in 0.1 M phosphate buffer, dehydrated in an ascending series of ethanol, and embedded in epoxy. Semithin sections were stained with toluidine blue; thin sections were stained with lead citrate and uranyl acetate. For line 96, we examined digestion was used to estimate the relative levels of the transgene/human and endogenous/mouse mRNAs.

**Immunoblot analysis.** Protein was isolated from snap-frozen sciatic nerves and livers of adult mice, crushed in a mortar and pestle on dry ice, suspended in Tris-buffered SDS lysis buffer (50 μM Tris, pH 7.0; 1% SDS, 0.017 mg/ml phenylmethylsulfonyl fluoride) with or without 6 μl urea, and sonicated (Sonic Dismembrator; Fisher Scientific, Pittsburgh, PA). Samples were spun at 4°C to pellet insolubles, and the supernatant was measured by a protein assay (Bio-Rad, Hercules, CA). One hundred micrograms of protein per lane were loaded onto 12% SDS-PAGE gels and run at 10 – 20 mA overnight at room temperature with size markers (Rainbow markers; Amersham Life Sciences, Buckinghamshire, UK). Semidy transfer (Fisher Biotech, Pittsburgh, PA) to a nylon membrane (Immobilon-P transfer membrane; Millipore, Bedford, MA) was completed following the recommendations of Millipore and analyzed by Western blot analysis (Scherer et al., 1995). A hybridoma supernatant of the mouse monoclonal antibody 7C6.67 (diluted 1:2) (Li et al., 1997) and a rabbit antisemum (diluted 1:1000; Zymed, San Francisco, CA) against Cx32 were diluted in 5% milk in Tris-buffered saline. The primary antibodies were detected with peroxidase-coupled goat anti-mouse IgG1 heavy chain (1:2500 dilution; Southern Biotechnology Associates, Birmingham, AL) or goat F(ab’)2 anti-rabbit IgG (1:2500 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Membranes were developed by established enhanced chemiluminescence methods (Amer- sham Biosciences, Arlington Heights, IL) and exposed to autoradiography film (X-OMAT AR; Kodak Scientific Imaging, Rochester, NY). Blots were re-probed with a rabbit antisemum against P0 (diluted 1:50,000) (D’Urso et al., 1990).

**Western blot analysis** (Scherer et al., 1995). Transgenic progeny were identified by PCR with transgene-specific primers

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semithin sections of animals at P138 (two TG  and two TG*). Color slides of representative images were scanned (Super Cool Scan;Nikon, Tokyo, Japan) and manipulated in Adobe Photoshop. Thin sections were examined with a Zeiss electron microscope.

Teased, osmicated fibers were prepared from one litter of P387 cx32 cx32  mice (two TG  and four TG* from line 96). After fixation and osmication as above, the nerves were placed in 33, 66, and 100% glycerol, each for at least 24 h at 45°C (Dyck et al., 1993a). Bundles of fibers were separated using fine forceps and a dissecting microscope, mounted in glycerin, and examined with differential interference optics with a Leica DMR microscope. To determine the proportion of normal and remyelinated axons, we counted myelinated axons that could be viewed for at least 2 mm. Myelinated axons that had internodes of uniform length, with myelin sheaths that appeared normally thick for the axonal caliber, were considered normal. Myelinated axons had internodes that were variable length, with myelin sheaths that were inappropriately thin for the axonal caliber, as well as supernumerary Schwann cells, were considered remyelinated. At least 100 myelinated fibers were examined in both the sensory and the motor branches of the femoral nerve.

Statistical analysis. For a quantitative analysis of demyelinated and remyelinated axons, digital images (OpenLab software; Improvision, Lexington, MA) were made of single transverse semithin sections femoral motor and sensory (saphenous) nerves. All demyelinated, remyelinated axons, and normally myelinated axons were counted by the same observer (S.S.S.) without knowledge of the animal’s genotype. Axons larger than 1 μm in diameter without a myelin sheath were considered demyelinated. Axons with myelin sheaths that were <10% of the axonal diameter as well as myelinated axons that were surrounded by “onion bulbs” (circumferentially arranged Schwann cell processes and extracellular matrix) were considered remyelinated. The rest of the myelinated axons were considered to be normally myelinated. Repeated counts of one affected nerve were essentially identical (within 1%). The proportion of demyelinated and remyelinated axons in TG  versus TG  animals was compared by the Wilcoxon rank–sum test (see Table 1). We also modeled the odds of observing an abnormally myelinated axon. In this context, odds were defined as the probability of an abnormally myelinated axon divided by the probability of a normally myelinated one. Odds ratios (ORs) were calculated using the Mantel-Haenszel statistic and the logit model; the Mantel-Haenszel statistic calculates ORs adjusted for or stratified by any confounding variables, and the logit model was used for this binomial outcome data because it also appropriately models probabilities (in the form of ORs). ORs were estimated using logit regression with adjustment for clustering. This adjustment changed the SEs and the values that depended on them including the p value and confidence intervals.

Results

Generation of cx32-null TG  mice

Figure 1 shows the design of our transgenic construct. Because transgenes made with genomic DNA are expressed at higher levels than those made with cDNA (Brinster et al., 1988; Palmiter et al., 1991), we used the human GJB1/Cx32 genomic fragment containing exons 1b and 2 and the intervening intron. Other derivatives of this construct drive the expression of transgenes in myelinating Schwann cells (Abel et al., 1999; Pot et al., 2002; Leone et al., 2003). We generated three lines that expressed wild-type human Cx32 and selected the line (line 90) with the lowest expression of Cx32 protein (data not shown) for the experiments described below. To generate both TG  and TG  male mice that were also null at the endogenous cx32/Gjb1 locus (cx32^-/-), TG  male mice were crossed with cx32^-/- female mice (Nelles et al., 1996). The genotypes of the mice (TG  or TG* as well as cx32^-/- or cx32^-/-) were identified by PCR of genomic DNA (Abel et al., 1999). As expected, about one-half of the progeny were TG  mice, demonstrating that the TG was inherited in an autosomal pattern and that there was no excessive mortality in the either the TG  or TG* male mice (data not shown).

Expression of transgenic and endogenous Cx32 mRNA

To determine whether transgenic mRNA was expressed, we performed RT-PCR on peripheral nerves from single animals with transgene-specific primers (Fig. 1B, <P0> and <2>). RT-PCR amplified a 517 bp product, predicted to be the RNA-specific product, without any amplification of genomic DNA (predicted to be a 872 bp product) from each cDNA sample (data not shown). The amplification of a 553 bp product from the same cDNA samples with primers (<1> and <4>) that hybridize with a sequence that is identical in human and mouse Cx32/32. The undigested (U) PCR product is 553 bp; HhaI (H) cuts mouse cx32 but not human Cx32, MscI (M) cuts human Cx32 but not mouse cx32; the double digestion (D) proves that no full-length product remains. The bottom panel shows similarly digested RT-PCR products from adult transgenic sciatic nerve. Densitometric quantification of the mouse- and human-specific bands in the MscI-cleaved lane indicates that the ratio of transgenic/human Cx32 mRNA are —1 and 3 for lines 90 and 96, respectively. MK, DNA size markers.

Figure 1. A rat Mpz promoter/GJB1 transgene cassette is expressed in peripheral nerve. A, The structure of the human GJB1/Cx32 gene. In myelinating Schwann cells, Cx32 transcripts are initiated from the P2 promoter; in the liver, transcripts are initiated from the P1 promoter (Neuhau et al., 1995, 1996; Sohl et al., 1996). B, The structure of the transgene. The 1.1 kb rat Mpz/P0 promoter is joined upstream of exon 1b of the human GJB1/Cx32 gene. The positions of the primer pairs used to amplify cDNA, <1> and <4> and <P0> and <2>, are indicated. C, Analysis of endogenous versus transgene expression by semiquantitative RT-PCR. The top panel shows human/mouse Cx32/cx32 cDNA amplified with primers (<1> and <4>) that hybridize with a sequence that is identical in human and mouse Cx32/32. The undigested (U) PCR product is 553 bp; HhaI (H) cuts mouse cx32 but not human Cx32, MscI (M) cuts human Cx32 but not mouse cx32; the double digestion (D) proves that no full-length product remains. The bottom panel shows similarly digested RT-PCR products from adult transgenic sciatic nerve. Densitometric quantification of the mouse- and human-specific bands in the MscI-cleaved lane indicates that the ratio of transgenic/human Cx32 mRNA are —1 and 3 for lines 90 and 96, respectively. MK, DNA size markers.
threefold more transgenic/human Cx32 cDNA compared with endogenous/mouse cx32 cDNA for line 96, while line 90 had approximately equal amounts. These experiments were performed on two animals from each line (5 weeks and 6 months of age) with the same results.

**Immunoblot analysis**

To determine whether transgenic/human Cx32 protein was expressed, we performed immunoblot analysis using an antiserum against the intracellular loop of Cx32 (Fig. 2). The sciatic nerves of TG* Gjb1/cx32−/− male mice (lane 1) contained bands of Cx32 monomers and dimers, respectively (Scherer et al., 1995). The amount of Cx32 (monomer and dimer) was higher in TG* nerve than in wild-type nerve (lane 5). In addition, this antiserum recognized a lower molecular mass band in transgenic nerves, likely partially degraded Cx32 (perhaps caused by overexpression) or the result from a signal peptidase that cleaves the N terminus of Cx32 (Falk et al., 1994). In contrast, the liver from TG* (lane 2) or TG− (lane 4) mice, or the sciatic nerve from TG− mice (lane 5), did not contain Cx32. The bands that are found in cx32-null mice are “background bands” and do not represent Cx32. To show that these results are not confounded by unequal loading of the samples, the blot was reprobed for P0, and the gel was stained with Coomassie after transfer, as shown in Figure 2, B and C, respectively. We obtained similar results from another set of animals and confirmed these results with a monoclonal antibody (against intracellular loop of Cx32; data not shown). Identical blots probed with secondary antibody only showed no background bands (data not shown).

**TG* myelinating Schwann cells but not oligodendrocytes express Cx32**

To determine whether the transgene was also expressed in oligodendrocytes, we examined the expression of Cx32 in sections of the spinal cord. To delineate the PNS/CNS boundary, we used an antibody against MOG, which labels both CNS and PNS axons. As shown in Figure 3C, a rabbit antiserum against the loop of Cx32 labeled myelinating Schwann cells in the ventral roots (vr) of TG* cx32−/− mice but not label the spinal cord (s.c.) itself. In TG− cx32−/− mice, there was no Cx32 staining in either the roots or in the CNS myelin sheaths (Fig. 3B), confirming the specificity of the Cx32 antibody, whereas wild-type mice had Cx32 in both locations (Fig. 3A). We observed the same results with another rabbit antiserum and a monoclonal antibody that were directed against the intracellular loop and the C terminus, respectively, of Cx32 (data not shown).

**The localization of human Cx32 in PNS myelin sheaths**

Human Cx32 is highly homologous to mouse Cx32, differing at only four amino acids in the extracellular loops (Scherer and Paul, 2004). Thus, it seemed plausible that human Cx32 would traffic like its murine counterpart, to the noncompact regions of the PNS myelin sheath. To determine the localization of human or mouse Cx32, we immunostained unfixed teased fibers from adult sciatic nerves. In wild-type mice, Cx32 was mainly localized to incisures and paranodes (Fig. 4A), with lesser amounts in the inner and outer mesaxons (Bergoffen et al., 1993; Scherer et al., 1995; Chandross et al., 1996; Balice-Gordon et al., 1998; Meier et al., 2004). No Cx32 immunoreactivity was seen in TG− cx32−/− nerves, whereas the localization of other components, such as E-cadherin (Fig. 4B) and MAG (data not shown) was unaffected. In TG* cx32−/− mice, in contrast, there was pronounced Cx32 immunostaining in paranodes and incisures, including aggregates that were much larger than those seen in wild-type nerves (Fig. 4C). Similar aggregates of Cx32 were colocalsized with strands of E-cadherin staining marking the outer mesaxons (Fan- non et al., 1995). These data show that although human Cx32 appears to be overexpressed in this transgenic line, it is properly localized.
A Cx32 transgene prevents demyelination in cx32\(^{-/-}\) mice

The above results demonstrate that the 1.1 kB rat \(Mpz\) promoter efficiently drives the expression of the human \(GJB1\) gene in myelinating Schwann cells, resulting in moderately higher levels of transgenic/human Cx32 protein than endogenous/murine Cx32 in wild-type mice. To determine whether selectively expressing normal human Cx32 in myelinating Schwann cells prevents demyelination in cx32\(^{-/-}\) mice, we analyzed semithin sections of the sciatic and femoral nerves, as well as the cauda equina (the ventral and dorsal roots) from TG\(^+\) and TG\(^-\) cx32\(^{-/-}\) littersmates, at P158, P250, and P365. These ages were selected because demyelination begins around P90 in cx32\(^{-/-}\) mice and becomes prominent by P158 (Anzini et al., 1997; Scherer et al., 1998). Because myelinated motor fibers are much more affected than are myelinated sensory fibers in cx32\(^{-/-}\) mice, we focused on the femoral motor nerve because it contains a higher proportion of motor axons than does the sciatic nerve (60 vs 20% of myelinated axons) (Boyd and Davey, 1968; Swett et al., 1986).

At P158 and P250, the femoral nerves (Figs. 5A, C, 6A), ventral roots (Fig. 6C), and sciatic nerves (data not shown) of TG\(^+\) cx32\(^{-/-}\) mice looked normal. There were rare demyelinated axons (axons without a myelin sheath; \(>1\) \(\mu\)m in diameter) or remyelinated axons (axons \(>1\) \(\mu\)m in diameter with inappropriately thin myelin sheaths for their axonal caliber, often associated with onion bulbs, crescents of Schwann cells processes). In contrast, in their age-matched, TG\(^-\) littersmates, there were numerous demyelinated axons, remyelinated axons, and onion bulbs, as well as occasional myelin sheaths that were disrupted, split, or separated from their axon and macrophages containing myelin debris and/or lipid droplets within the endoneurium or apposed to the inner surface of the perineurium (Fig. 6B, D), as described previously (Anzini et al., 1997; Scherer et al., 1998). These pathological findings were much more prominent in motor axons, in the motor but not the sensory branch of the femoral nerve, in fascicles of large myelinated axons (presumably motor axons) in the sciatic nerve, and in the ventral/motor (Fig. 6D) but not the dorsal/sensory (data not shown) lumbar roots, except that onion bulbs were not prominent in the roots. At P365, however, there appeared to be more demyelinated and remyelinated axons than in younger TG\(^+\) cx32\(^{-/-}\) mice, although still not as many as in P365 TG\(^-\) cx32\(^{-/-}\) mice (Fig. 5E, F). This increase in pathologically affected myelinated axons was more pronounced in the femoral motor branch (Fig. 5E, F) than in the sciatic nerve, ventral roots, or dorsal roots (Fig. 6 and data not shown).

To confirm and extend the above findings, we teased osmiated fibers from a litter of 1-yr-old cx32\(^{-/-}\) mice (four TG\(^+\) and
two TG−). A 1 cm segment of the femoral motor branch was teased into small bundles and examined by light microscopy without knowledge of the animal’s genotype. Nerves from two animals had a modest proportion of remyelinated axons (at least 20% of myelinated fibers; >100 fibers examined per animal), whereas remyelinated axons were relatively scarce in the other four animals (between 1 and 5%; >100 fibers examined per animal). These results proved to be in complete accord with the genotypes of the mice; TG− mice had few remyelinated axons. In all animals, the largest axons were the most affected, probably because they contain the highest proportion of motor axons. Most large myelinated axons from TG+ mice had uniformly spaced, long internodes with myelin sheaths that appeared to be appropriately thick for the axonal caliber (Fig. 7B). In contrast, many large myelinated fibers from TG− mice had internodes of variable lengths, some of which had abnormally thin myelin sheaths, often with associated supernumerary nuclei and Schwann cell processes (Fig. 7A). Some of the myelin sheaths of these remyelinated axons were split or separated from their axon, as noted previously (Scherer et al., 1998). Furthermore, most myelin internodes belonging to the same fiber appeared to be either normal or affected, indicating that single fibers are affected over most of their length.

We suspected that the small myelinated axons, and even many of the unaffected large myelinated sensory fibers in the femoral motor branch, were unaffected because they are mainly sensory axons. To pursue this issue, we also examined teased fibers from the sensory branch of the femoral nerve, the saphenous nerve. In both TG− and TG+ mice, nearly all (>99%) of myelinated fibers in the saphenous nerve were normally myelinated (data not shown). These results, taken together, provide additional evidence that cx32−/− mice have a demyelinating neuropathy that primarily affects motor axons (Anzini et al., 1997; Scherer et al., 1998). As the disease progresses, motor axons are demyelinated and remyelinated throughout their length, and this can be mostly prevented or at least delayed by the expression of normal human Cx32 in myelinating Schwann cells.

**Statistical analysis**

The above data demonstrate that expression of human Cx32 can prevent/delay the onset of demyelination in motor axons of cx32−/− mice. To substantiate this point, we performed a quantitative analysis of the femoral nerve, because it has a tractable number of axons for counting and a partial separation of motor and sensory axons. We counted all of the demyelinated, remyelinated, and normally myelinated axons in a single transverse section of the femoral sensory and motor branches at P158, P250, and P365, combining the counts from the left and right sides for individual animals where possible. The image in Figure 6 depicts examples of what we considered to be demyelinated and remyelinated axons. As shown in Table 1, there were essentially no demyelinated or remyelinated axons in the sensory branch, as expected (Anzini et al., 1997; Scherer et al., 1998), whereas the motor branch in TG− mice had more abnormal fibers (demyelinated or remyelinated) than did those in TG+ mice (Fig. 8). By the Wilcoxon rank-sum test, the overall difference in the proportion of abnormally myelinated fibers between TG− and TG+ was 0.0012, but this was less compelling at any individual time point because of small sample sizes.

We also modeled the odds of observing an abnormally myelinated axon, defining odds as the probability of an abnormally myelinated axon divided by the probability of a normally myelinated axon, defining odds as the probability of an abnormally myelinated axon divided by the probability of a normally myelinated one. An OR of 1.0 means the odds of the outcome occurring is not different than the odds of the outcome not occurring. By estimating the ORs with the logit regression model, we could adjust the logit model to account for the clustering of data within animals, in addition to controlling for confounders and stratify-
ing the analyses when there were significant interactions. Table 2 shows the ORs of abnormally myelinated axons, separately by age group, from the logit regression model with an interaction for age and adjustment for clustering of fibers within mice. Adjustment for clustering only affected the SEs and the values that depended on them, including the p value and confidence intervals. The odds of observing an abnormally myelinated axon in the P158 TG group was 90 times higher than the odds of finding one in the P158 TG group ($p < 0.00$); the OR at P250 was also large ($67; p < 0.00$). The OR at P365 was 8 ($p < 0.00$), still large, but less than at the younger ages. Additional comparisons showed that the OR for the P158 group was significantly different from that of the P365 group ($p = 0.003$) but not more than that of the P250 group ($p = 0.711$). These analyses indicate that there was not one overall OR that characterized a genotype (TG or TG); the relationship differed depending on age. The interaction between age and genotype was statistically significant (likelihood ratio test; $\chi^2 = 65.99; p > 0.00$; the effect of genotype varied significantly by age.

**Discussion**

**Is CMT1X primarily a demyelinating or axonal neuropathy?**

According to criteria that were developed to distinguish the demyelinating (CMT1) from the axonal (CMT2) forms of CMT (Harding and Thomas, 1980), CMT1X is more like CMT2 than CMT1. Nerve conduction velocities are typically in 30–40 m/s affected males and 30–50 m/s in affected females (Nicholson and Nash, 1993; Rouger et al., 1997; Birouk et al., 1998; Hahn et al., 1999; Nicholson and Nash, 1993; Rouger et al., 1997; Birouk et al., 1998; Senderek et al., 1999); this is faster than the 20 m/s typically seen in CMT1A patients (Birouk et al., 1997). In addition, electrophysiological studies pronounced loss of distal motor axons in CMT1X (Rozear et al., 1987; Hahn et al., 1990, 1999; Nicholson and Nash, 1993; Rouger et al., 1997; Birouk et al., 1998; Senderek et al., 1999). Finally, nerve biopsies show more axonal loss and less remyelination than is typically seen in CMT1A or CMT1B (Sander et al., 1998; Hahn et al., 2001). These data have lead to the frequent supposition that CMT1X is an axonal neuropathy (Hahn et al., 1990; Timmerman et al., 1996; Birouk et al., 1998).

Other data indicate that CMT1X is primarily a demyelinating neuropathy, albeit with important secondary axonal loss. Myelinating Schwann cells express Cx32, and its expression appears to be regulated at the level of transcription in concert with many myelin-related genes (Scherer et al., 1995; Chandross et al., 1996; Sohl et al., 1996). Cx32 is prominently localized in noncompact myelin and may help form a radial pathway that mediates the diffusion of small molecules and ions directly across the layers of the myelin sheath (Balice-Gordon et al., 1998). Finally, demyelination is the first pathological alteration in Cx32-null mice, preceding axonal loss (Anzini et al., 1997; Scherer et al., 1998). Why axonal loss appears to be more pronounced in CMT1X than in other kinds of CMT1 that have even slower conduction is unknown; this discrepancy underscores our lack of understanding of the mechanisms of axonal loss in demyelinating diseases.

To address this issue, we selectively expressed human Cx32 in myelinating Schwann cells of cx32−/− mice. The human and mouse Cx32 proteins differ at four amino acids (Scherer and Paul, 2004), but none of these are known to be affected by missense mutations that cause CMT1X (http://www.molgen.ua.ac.be/CMTMutations/DataSource/MutByGene.cfm), so that human and mouse proteins may be functionally equivalent. Using a rat Mpz promoter, we were able to show that mouse myelinating Schwann cells express human Cx32, that human Cx32 is properly localized to incisures and paranodes, and that human Cx32 “rescues” the demyelinating phenotype in cx32−/− Schwann cells. That the selective expression of human Cx32 in myelinating Schwann cells is sufficient to rescue the phenotype of cx32−/− mice indicates that demyelination initiates the complex events of neuropathy, which includes immune-mediated damage and axonal loss (Kobsar et al., 2002, 2003).
Cell autonomy and the pathogenesis of demyelinating neuropathies

Dominant mutations in PMP22, MPZ, EGR2, LITAF/SIMPLE, and GJB1, as well as recessive mutations in GDAP1, MTMR2, MTMR13, KIAA1985, NDRG1, PRX, and EGR2 cause demyelinating neuropathies (http://www.molgen.ua.ac.be/CMTMutations/DataSource/MutByGene.cfm). Myelinating Schwann cells express these genes, many of which (PMP22, MPZ, EGR2, GJB1/Cx32, NDRG1, and PRX) appear to be similarly regulated at the level of transcription by axon–Schwann cell interactions (Wrabetz et al., 2004). Mutations in these genes have been considered to have cell-autonomous effects that result in demyelination, but some of these genes (PMP22, GJB1/Cx32, GDAP1, MTMR2, MTMR13) are also expressed by neurons (Suter and Scherer, 2003). A formal evaluation regarding whether neuronal expression of these genes contributes to the pathogenesis of these neuropathies, as we have shown here for Cx32, remains to be performed.

Effects of Cx32 overexpression

By immunoblotting and immunostaining, transgenic/human Cx32 was overexpressed in myelinating Schwann cells compared with the endogenous/mouse Cx32. The cause of this overexpression is unclear, because the steady-state levels of transgenic and endogenous mRNA appeared to be similar. The larger than normal aggregates of Cx32 could be the cause or the result of overexpressed protein. A cx32-null background ameliorates the effect of Cx32 overexpression, and breeding line 90 or 96 into a wild-type background causes a gain-of-function phenotype (splitting of the myelin sheath) (L. J. B. Jeng, unpublished observations). Wrabetz et al. (2000) have reported a similar experience with transgenic lines that overexpress P0, there is an “overexpression phenotype” that is partially ameliorated by breeding the transgene into a Mpz-null background.

Transgenic models of CMT1X

The ability to selectively express a transgene in myelinating Schwann cells provides a straightforward means of determining the effects of various mutants that cause CMT1X. Given the large number of GJB1 mutations, this strategy is much more efficient than “knocking in” the corresponding mutation. We have used the Mpz-Cx32 construct to express other mutations as transgenes: 175 frameshift (Abel et al., 1999), Arg142Trp (Jeng, unpublished observations), and P365A (Scherer et al., 2003). P365A causes a gain-of-function phenotype (splitting of the myelin sheath) (L. J. B. Jeng, unpublished observations). Wrabetz et al. (2000) have reported a similar experience with transgenic lines that overexpress P0, there is an “overexpression phenotype” that is partially ameliorated by breeding the transgene into a Mpz-null background.

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published observations), Cys280Gly, and Ser281stop (S. Scherer, unpublished observations); each of these has distinct effects from published observations). The unique effects of these mutations can be ascribed to the Cx32, the unique effects of these mutations can be ascribed to the Cx32, the unique effects of these mutations can be ascribed to the Cx32.

## Table 2. Odds ratios for abnormally myelinated fibers for TG⁻ or TG⁺ femoral motor nerves.

<table>
<thead>
<tr>
<th>Age (days post-natal)</th>
<th>Odds ratio</th>
<th>Robust SE</th>
<th>( z )</th>
<th>( p &gt; z )</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>P158</td>
<td>89.9</td>
<td>66.9</td>
<td>6.04</td>
<td>0.000</td>
<td>20.9–387</td>
</tr>
<tr>
<td>P250</td>
<td>67.1</td>
<td>17.4</td>
<td>16.2</td>
<td>0.000</td>
<td>40.3–112</td>
</tr>
<tr>
<td>P365</td>
<td>8.08</td>
<td>2.41</td>
<td>7.01</td>
<td>0.000</td>
<td>4.50–14.5</td>
</tr>
</tbody>
</table>

Figure 8. The proportion of abnormally myelinated axons in TG⁻ or TG⁺ femoral motor nerves. This plot shows the proportion of abnormally myelinated axons in the femoral motor nerves at P158, P250, and P365. The means are shown at each age, separately for TG⁻ and TG⁺ animals.

Table 2. Odds ratios for abnormally myelinated fibers for TG⁻ or TG⁺, stratified by age group and adjusted for clustering.

**References**


Scherer et al. • A Cx32 Transgene Prevents Demyelination


