Modulation of Axon Diameter and Neurofilaments by Hypomyelinating Schwann Cells in Transgenic Mice

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Studies of peripheral nerves in two different lines of hypomyelinating transgenic mice support the hypothesis that myelinating Schwann cells exert a significant influence on key biological properties of axons. The mice contain transgenes combining the peripheral myelin protein zero gene (P_0) promoter and either the diphtheria toxin A chain gene product or the SV40 (simian virus 40) large T antigen. The consequences of peripheral nerve hypomyelination on axon diameter, neurofilament (NF) density, and NF phosphorylation were analyzed. The sciatic nerves of the P_0 diphtheria toxin A transgenic mice (DT) evidenced the most severe hypomyelination, and this was associated with a dramatic decrease in NF phosphorylation plus a marked increase in NF density. In contrast, the sciatic nerves in the P_0 SV40 large T antigen transgenic mice (SV40) were not as severely hypomyelinated and there was a milder decrease in NF phosphorylation plus a more modest increase in NF density. Further, the sciatic nerves in both lines evidenced a decrease in axonal caliber without any change in NF content. Taken together, these studies provide strong evidence indicating that myelinating Schwann cells exert a significant influence on axon caliber by modulating NF phosphorylation and NF packing density in the axons of peripheral nerves. Thus, key biological properties of axons are modulated by signals transmitted from myelinating Schwann cells to axons of peripheral nerves.

[Key words: neurofilaments, hypomyelination, transgenic mice]

In the PNS, Schwann cells and axons have complex interactions beginning in the developing nervous system and extending into adult animals. The Schwann cells develop into two different populations (i.e., one type myelinates large-diameter axons, while the other envelopes small-diameter axons but does not form myelin), and Schwann cells are thought to play an important role in determining the morphology and physiological functions of axons. Previous studies showed that PNS axons relayed in-

formation to the surrounding Schwann cells, leading to the induction of myelination by Schwann cells in contact with axons (Bray et al., 1981). Myelin sheath thickness and the internodal length were considered to be directly dependent on axonal caliber (Fraher, 1978). However, more recent experiments suggest that Schwann cells can cause local changes in the morphological properties of the axon. For example, the diameter of the axonal processes of cultured neurons is modified by the presence of Schwann cells (Windebank et al., 1985; Pannese et al., 1988), and the initiation of myelination by a Schwann cell will produce a local enlargement in axonal diameter relative to adjacent regions of the same axons that are in contact with nonmyelinating Schwann cells (Pannese et al., 1988). Further, Parhad et al. (1987) have reported changes in the organization of axonal NFs in segments where focal demyelination was produced.

Alterations in the properties of hypomyelinated axons in the mutant Trembler mouse also suggest that axons respond to signals from Schwann cells. For example, hypomyelination of the axons in these mice results in a reduction of axonal diameter in the hypomyelinated regions (Aguayo et al., 1977; Pollard and McLeod, 1980; Perkins et al., 1981), altered slow axonal transport, altered regeneration of the axons (de Waegh and Brady, 1990), decreased NF phosphorylation, and increased NF density (de Waegh et al., 1992). Hence, it now appears that Schwann cells modulate important properties of the neurons, including axon diameter.

The diameter of the axon is not predetermined and it changes during development and regeneration (Hoffman et al., 1985). Several studies correlating cytoskeletal composition and axonal diameter during neuronal development and regeneration suggest that the caliber of myelinated axons is primarily dependent on NF number (Friede and Samorajski, 1970; Lasek et al., 1983; Hoffman et al., 1985). Additionally, studies of the “quiver” mutant quail provide direct evidence of the importance of NFs in achieving and/or maintaining normal axon caliber (Yamasaki et al., 1992).

NFs belong to the family of intermediate filament (IF) proteins. NFs are made by the copolymerization of three proteins with predicted molecular weights of 62, 96, and 116 kDa in mice (Julien et al., 1986; Lewis and Cowan, 1986; Levy et al., 1987). The low- (NF-L), middle- (NF-M), and high- (NF-H) molecular-weight NF proteins share with each other and with other IF proteins a highly conserved central α helical rod domain of approximately 310 amino acid residues, which is responsible for the formation of coiled-coil structures in IFs. Antibody decoration experiments (Hirokawa et al., 1984) and partial proteolysis of NFs (Carden and Eagles, 1983; Julien and Mushynski,

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1983) suggested that the NF-L protein is part of the NF backbone, while NF-H and NF-M are involved in the formation of sidearm projections on the outside of the filament (Hisanaga and Hirokawa, 1988; Mulligan et al., 1991). A unique feature of NF-M and NF-H proteins is their long carboxy-terminal tail domains that contain multiple repeats of the sequence Lys-Ser-Pro (KSP), called KSP segments (Meyer et al., 1987; Lee et al., 1988a). These represent the major phosphorylation sites in NF proteins (Lee et al., 1988a,b). These charged amino acids at the periphery of the filament, and in sidearm projections may induce charge repulsions that increase the spacing between NFs (Carden et al., 1987; Lee et al., 1987, 1988a). The projections may also contribute to the control of axon caliber through changes in phosphorylation (de Waegh et al., 1992).

Here, we studied the effect of hypomyelination on NF phosphorylation, NF density, and axonal caliber in two recently developed lines of transgenic mice. The promoter used to target the transgene expression to myelinating Schwann cells is the myelin protein zero gene (P_0). It is expressed only in myelinating Schwann cells (Brockes et al., 1980; Mirsky et al., 1980; Trapp et al., 1987; Martini et al., 1988) and it is positively regulated by contact with axons (Politis et al., 1982; Poduslo and Windebank, 1985; LeBlanc et al., 1987; Trapp et al., 1988), primarily at the level of gene transcription (Lemke and Chao, 1988; Trapp et al., 1988).

The P_0 promoter drove expression of the diphtheria toxin A chain gene (DT-A) to ablate myelinating Schwann cells selectively (Messing et al., 1992), as DT-A is a potent inhibitor of protein synthesis (Pappenheimer, 1977). The DT-A cassette was inserted into a P_0 -hGH gene that had previously been shown to be selectively expressed in myelinating Schwann cells, such that DT-A would be the first open reading frame (Fig. 1). This line of mice produces a hypomyelinating defect (Messing et al., 1992).

The second line of transgenic mice has an SV40 large T antigen tsA-1609 mutant gene after the P_0 promoter, such that the large T antigen gene would be the first open reading frame (see Fig. 1; Messing et al., 1994). This SV40 gene appears to trap developing Schwann cells in the proliferative phase, and the peripheral nerves contain Schwann cells that are unable to form myelin, resulting in a hypomyelinating phenotype (Messing et al., 1985; Small et al., 1986; Jensen et al., 1993).

Our data demonstrate that the hypomyelination of PNS axons in the two P_0 transgenic lines of mice results in decreased NF phosphorylation, increased NF density, and decreased axonal diameter like the Trembler mouse, which is due to a defect in the peripheral myelin protein-22 (Suter et al., 1992).

Materials and Methods

Transgenic animals. Transgenic mice carrying P_0 diphtheria toxin or P_0 SV40 transgenes have been described previously (Messing et al., 1992, 1994). Breeding lines of animals are maintained in a hybrid C57BL/6J $\times$ SJL background. The genetic designation for the lines of mice used in this study are Tg(Mpz, SV40E)Bri135 for the P_0 SV40 transgene and Tg(Mpz, DT-A)Bri167 for the P_0 diphtheria toxin transgene. The mouse colony is maintained in an AAALAC accredited animal facility at the University of Wisconsin-Madison.

Electrophoresis. The whole sciatic nerve was suspended in SUB buffer (0.5% SDS, 8 M urea, and 2% β -mercaptoethanol) as described by de Waegh and Brady (1990). Electrophoresis was performed on 0.75-mm-thick 6% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with approximately 10 μ g of protein from control and transgenic mouse sciatic nerves, and, as close as possible, equal amounts of NF-L were loaded in each lane on the gel. Then

Figure 1. Transgenes. Schematic diagrams of the P_0 -DT-A-hGH and P_0 -SV40-tsA-1609 cassettes used to generate transgenic mice. A 1.1 kb section of the rat P_0 promoter was subcloned upstream of these modules to direct expression exclusively to myelinating Schwann cells. DT-A sequences are inserted into the first exon of the hGH gene such that the DT-A open reading frame is translated (Messing et al., 1992). The SV40 tsA-1609 mutant gene was attached downstream of the P_0 promoter (Messing et al., 1994).

the relative levels of NF-M and NF-H phosphoisoforms were normalized to that of NF-L as determined by quantitative immunoblots. Four transgenic mice from each line (eight nerves) along with four age-matched controls from each line were used for biochemical analysis.

Quantitative immunoblots. Following electrophoresis, proteins were transferred to nitrocellulose and probed with monoclonal antibodies (MAbs) that recognize phosphorylation-dependent (P+, P++, P+++) or dephosphorylation-dependent (dP) epitopes, as described by Lee et al. (1987, 1988a,b). Phosphorylation-independent MAbs that reacted with NF-H, NF-M, or NF-L epitopes regardless of phosphorylation state were used to quantitate total NF proteins on immunoblots of control and transgenic mice samples. All of the antibodies used here are summarized in Table 3 and have been characterized earlier (Carden et al., 1987; Lee et al., 1987, 1988a,b). To quantitate NF protein levels, the blots were washed five times for 3 min in GB buffer (50 mM triethanolamine, 0.5% Triton X-100, 0.1 M NaCl, 2 mM K-EDTA, 0.1% SDS, pH 7.5) and rinsed once in PTX buffer (10 mM NaPO₄, pH 7.5, 0.2% Triton X-100, 0.15 M NaCl, 1 mM EGTA). They were then incubated for 60 min in ¹²⁵I-labeled goat anti-mouse IgG (2 $\times$ 10⁶ dpm/ml) for the mouse MAbs, or protein A (2 $\times$ 10⁶ dpm/ml) for the rabbit polyclonal antibodies in 4% BSA-PTX, and finally washed five times for 3 min in GB buffer (Earnshaw et al., 1987).

Blots were then exposed to a phosphor screen for 5–24 hr and the amount of radioactivity for each protein band was quantified on a Phosphor Imager (Molecular Dynamics). The amount of radioactivity was expressed as a ratio of NF-H or NF-M isoforms to NF-L in the same lane. The degree of phosphorylation was then compared between transgenic and control samples on the same blot.

Electron microscopy. Sciatic nerves were examined by electron microscopy as described by de Waegh et al. (1992). Animals were killed with CO₂, and their sciatic nerves were exposed and immediately immersed *in situ* in freshly prepared fixative containing 2% glutaraldehyde and 2% formaldehyde in cacodylate buffer. Following 15 min of initial fixation, nerves were removed and reimmersed in fresh fixative. The nerves were stored in cacodylate buffer until they were analyzed. After three washes in fresh cacodylate buffer, each segment was dehydrated in a graded series of ethanol and embedded in Epon-Araldite resin. Ultrathin sections were stained for 1 min with uranyl acetate and 1 min in lead citrate, and examined in a Hitachi electron microscope. For the quantitative studies, the sciatic nerve was cut in cross section in the midhigh area and 8 $\times$ 10 inch photographs at a magnification of 60,000 were made of large-diameter axons cut in true cross section. In the electron microscopic analysis of the sciatic nerves, the use of the term “unmyelinated” refers to the large-diameter axons in the transgenic mice that do not have a myelin sheath. The small-diameter unmyelinated axons, grouped together with several axons ensheathed by one Schwann cell, were not used for the quantitative electron microscopic studies,

Table 1. Percentage of sciatic axonal area contributed by myelinated and unmyelinated large-diameter axons

	% relative area in sciatic nerve
DT transgenic (<i>n</i> = 4)	
Myelinated	34 ± 3%
Unmyelinated	66 ± 3%
DT litter mate control (<i>n</i> = 4)	100%
SV40 transgenic (<i>n</i> = 4)	
Myelinated	78 ± 5%
Unmyelinated	22 ± 5%
SV40 litter mate control (<i>n</i> = 4)	100%

although they were included in the biochemical analysis of the whole sciatic nerve. Axons with all of the NFs in true cross section represent approximately 5% of the axons in each cross section of the nerve. This representative sampling of axons was used for analysis that included the range of axonal calibers in each category (unmyelinated transgenic, myelinated transgenic, and myelinated control). We did not compare exactly equal size axons in each category because of the smaller size of all of the axons in the transgenic mice. In any event, it was not necessary to compare axons of the same caliber in each category because the NF density did not vary in relation to the caliber of the axons (an ANOVA test of this data revealed no significant relationship between axon caliber and NF density). The NFs were counted using a template of hexagons, each of which enclosed an area of 2.6 cm² (9 mm per side). All NFs were counted in every hexagon included within the axonal boundaries, as described earlier (de Waegh et al., 1992). We did not include any hexagons in the quantitative studies in which vesicular organelles occupied more than approximately 10% of the area of the hexagon or in which the NFs were not all in true cross section. Therefore, the densities reported are average densities per hexagonal area of axoplasm that was free of vesicular elements (Price et al., 1988). At this magnification, each hexagon represent a 5.8×10^{-2} m² region of axoplasm. Nerves from four mice from each line of transgenic mice and their age-matched controls were analyzed. Over 60 large-diameter axons in true cross section were selected in sections of transgenic and control sciatic nerve segments from the midhigh region. Over 300 hexagons were counted for each transgenic and each control mouse, with over 2500 NFs each. The DT mice were 26–30 d old, and the SV40 mice were all 20 d old.

The cross-sectional area of all individual large-diameter axons (i.e., those not grouped together and ensheathed by a single Schwann cell) was determined from 8×10 inch photographs at a magnification of $2000\times$ from the same section of sciatic nerve. The length and width of each axon (not including the axon sheath) was measured and then the area was determined, using these measurements to approximate an oval (Tables 1, 2). This cross-sectional area is used to compare the percentage of unmyelinated versus myelinated large-diameter axons in the sciatic nerves in Table 1. Cross-sectional area was used instead of the number of unmyelinated versus myelinated axons because the number does not reflect the true amount of NF protein involved with the smaller size of the unmyelinated axons.

Statistical analysis. Statistical analysis of the quantitative immunoblots, NF density, and cross-sectional area was done with the *t* test on Microsoft EXCELL software. The variability in all of the data presented represents the standard error in the data from the *t* test analysis. An ANOVA test on the cross-sectional area in Table 2 demonstrated that each category on the table had a statistically significant difference in mean of $p < 0.005$ or better. An ANOVA test of the NF density demonstrated no statistical relationship between NF density and axonal caliber.

Table 2. Average cross-sectional area of large-diameter axons in sciatic nerves

	μm ²
DT transgenic (<i>n</i> = 4)	
Myelinated	3.62 ± 0.08
Unmyelinated	2.42 ± 0.04
Transgenic (myelinated and unmyelinated)	2.83 ± 0.08
DT litter mate control (<i>n</i> = 4)	6.53 ± 0.02
SV40 transgenic (<i>n</i> = 4)	
Myelinated	3.04 ± 0.02
Unmyelinated	1.84 ± 0.02
Transgenic (myelinated and unmyelinated)	2.77 ± 0.02
SV40 litter mate control (<i>n</i> = 4)	4.59 ± 0.05

Results

Electron microscopy and morphometry

The percentage of myelinated axons versus unmyelinated axons, based on the cross-sectional area, in each line of transgenic mice demonstrated that the DT mice had much more extensive hypomyelination than the SV40 mice (Fig. 2, Table 1). Of the large-diameter axons, 66% of the cross-sectional area was unmyelinated in the DT mice versus only 22% of the area in the SV40 mice ($p < 0.005$; Table 1). This difference in the amount of hypomyelination correlates with the quantitative immunoblot data that demonstrates the reduction in NF phosphorylation (see below).

The density of NFs was greater in the unmyelinated large-diameter axons in each line of transgenic mice when compared to myelinated axons in age-matched controls (Figs. 2, 3). The NF densities were determined in cross sections through internodal axons of sciatic nerve of age-matched control mice and each of the transgenic lines. Nodal and paranodal axonal regions as well as regions with Schmidt–Lanterman clefts were excluded from the analyses because NF densities are altered in normal axons in these areas. Unmyelinated axons from both lines of transgenic mice showed an increase in NF density compared to controls. Myelinated axons from both lines of transgenic mice also showed an increase in NF density compared to controls, but not as large as the unmyelinated axon (Fig. 4). This may be due to some disruption of normal myelination in all of the large-diameter axons. NFs were randomly distributed throughout cross sections of transgenic and control mice and the frequency distribution of densities was consistent with those seen by Price et al. (1988). In the DT mice, the myelinated axons had 145.4 ± 6.7 NFs/μm² and the unmyelinated axons had 235.9 ± 7.0 NFs/μm², while the controls had 90 ± 2.4 NFs/μm² ($p < 0.005$; Fig. 4A,C,E). In the SV40 mice, the myelinated axons had 114.45 ± 3.3 NFs/μm² and the unmyelinated axons had 156.25 ± 7.2 NFs/μm², while the control axons had 93.9 ± 2.5 NFs/μm² ($p < 0.005$; Fig. 4B,D,F).

The average axonal cross-sectional area of axons in the sciatic nerves of transgenics and controls was determined, and this

Figure 2. Electron micrographs of normal (A, B), SV40 transgenic (C, D), and DT transgenic (E, F) sciatic nerves. A, C, and E are transverse sections of sciatic nerve at $2000\times$ magnification. B, D, and F are longitudinal sections of sciatic nerve at $60,000\times$ magnification. These axons are representative of the changes seen in either the myelinated control axons or the unmyelinated axons from the transgenic mice. They are not the same caliber because the unmyelinated axons from the transgenic mice are smaller. As stated in the Discussion, the density of NFs in each category did not vary significantly with axonal caliber. Arrowheads mark large-diameter axons that are unmyelinated.

revealed that the average size of the large-diameter axons in the transgenic mice was smaller than the average of the control axons (Table 2). The axons in the DT transgenic mice showed a greater difference than those in the SV40 mice. In the DT mice the average size of the axon was 43% of the age-matched control axons, contrasted to 60% in the SV40 mice (Table 2). There also was a difference between myelinated and unmyelinated axons within each line of mice. In the DT mice, the unmyelinated axons had the smallest average cross-sectional area ($2.42 \pm 0.04 \mu\text{m}^2$), the myelinated axons had larger areas ($3.62 \pm 0.08 \mu\text{m}^2$), and the age-matched controls had the largest axonal area ($6.53 \pm 0.02 \mu\text{m}^2$, $p < 0.005$; Table 2). The SV40 mice showed a similar relationship; the unmyelinated axons were the smallest ($1.84 \pm 0.02 \mu\text{m}^2$), the myelinated axons were larger ($3.04 \pm 0.02 \mu\text{m}^2$), and the age-matched controls were the largest ($4.59 \pm 0.05 \mu\text{m}^2$, $p < 0.005$; Table 2). Overall, the SV40 axons were smaller and this is probably due to the fact that these mice were younger (20 d old) than the DT mice (26–30 d old). It is interesting to note that the SV40 mice were phenotypically more affected (i.e., they showed more severe trembling) than the DT mice.

Biochemical analysis

Several anti-NF antibodies that distinguish between phosphorylated isoforms of NF proteins (Table 3) were used to determine biochemical differences between the transgenic and control sciatic nerves. MAbs that recognize the phosphorylation state of NF isoforms were used to determine the level of phosphorylation, while phosphorylation-independent antibodies were used to quantify total NF subunit proteins (Lee et al., 1987, 1988a,b; de Waegh et al., 1992).

For each antibody, six lanes of transgenic sciatic nerve were compared to six lanes of control sciatic nerve on the same gel. Approximately the same amount of NF-L for each of the samples was loaded and then the ratio of either NF-H or NF-M was compared to NF-L in the same lane (Fig. 5). A *t* test was run on each gel to determine if there was a significant difference between the control and transgenic nerves. Figure 6 shows representative data produced with each antibody for both transgenic and control sciatic nerves, and a summary of the data for each antibody is shown in Figure 7. The largest difference in NF protein levels was seen with the antibody to dephosphorylated NF-H (RMdO9), which showed an increase of $90 \pm 32\%$ in the DT mice and an increase of $27 \pm 9\%$ ($p < 0.05$) in the SV40 mice. The antibody to highly phosphorylated NF-H (RMO24) showed a decrease of $28 \pm 13\%$ ($p < 0.05$) in the DT mice, but no significant difference in the SV40 mice (Fig. 5). The antibodies to poorly phosphorylated NF-M (RMO108) and to phosphorylated NF-M (RMO55) showed a modest decrease in the DT mice, but it was not statistically significant. The SV40 mice showed similar statistically significant increases in RMdO9 binding, but no significant differences with most of the other antibodies, presumably because the level of hypomyelination was too low to cause a detectable change in the levels of the NF isoforms (Fig. 7).

Table 3. Antibodies to NFs raised against rat epitopes that have been previously characterized

Subunit	Phosphorylation state recognized	Antibody name
NF-H	P+++ (6)	RMO24
	P+ (2)	Ta51
	dP (0)	RMdO9
	Pind	Polyclonal
NF-M	P++	RMO55
	Pind (core)	RMO189
	poorly phosphorylated	RMO108
NF-L	Pind	Polyclonal

P+, P++, and P+++ recognize epitopes corresponding to increasing levels of phosphorylation at the multiple phosphorylation domain. For NF-H, P+, and P+++, antibodies bind to epitopes corresponding to two and six consecutive phosphorylated repeats, respectively, within the multiphosphorylation domain. dP recognizes NF-H when six consecutive repeats within the multiphosphorylation repeats are not phosphorylated. Pind recognizes epitopes distinct from the multiphosphorylation domain and binds to a specific NF subunit independently of the level of phosphorylation. All antibodies have been previously characterized and were shown to be specific for different levels of phosphorylation of a given NF subunit (Lee et al., 1987, 1988a,b).

Finally, the amount of NF-H, NF-M, and NF-L detected with phosphate-independent antibodies did not appear to vary between the control and transgenic sciatic nerves, which implies that the dorsal root ganglia neurons synthesize and transport similar amounts of NF proteins despite the hypomyelination of the transgenic sciatic nerves.

Discussion

Although mechanisms that regulate axon caliber have not been elucidated, several factors have been correlated with axon caliber including number of NFs, target size, and the thickness of the myelin sheath (Friede and Samorajski, 1970). NF density varies between axons in different white matter tracts (Szaro et al., 1990), and even along a single axon (Berthold, 1982; Price et al., 1988), implying that local regulation of axon cytoskeletal organization must occur. Since cytoskeletal elements undergo a variety of posttranslational modifications, these may regulate cytoskeletal dynamics and organization as well as axon caliber.

Previously, changes in hypomyelinated axons have been studied in the Trembler mouse, in which the hypomyelination is produced by a defect in the peripheral myelin protein-22 (Suter et al., 1992). These studies showed a reduction of axonal diameter (Aguayo et al., 1977; Pollard and McLeod, 1980; Perkins et al., 1981), altered slow axonal transport, altered regeneration of the axons (de Waegh and Brady, 1990), decreased NF phosphorylation, and increased NF density (de Waegh et al., 1992) in the hypomyelinated regions.

Because the hypomyelination produced in the two lines of transgenic mice studied here have resulted from two different mechanisms distinct from those in the Trembler mice, the results of this study give strong support to the hypothesis that the Schwann cell has a significant influence on the phosphorylation

Figure 3. Neurofilament density in the axons of the sciatic nerve in control, SV40 transgenic, and DT transgenic mice. *A* and *B*, Age-matched myelinated control axons. *C* and *D*, Unmyelinated SV40 transgenic axons; and *E* and *F*, Unmyelinated DT transgenic axons. *A*, *C*, and *E* each contain an entire axon in the sciatic nerve shown in transverse section to demonstrate the distribution of NFs through the entire axon ($30,000\times$ magnification). *B*, *D*, and *F* illustrate NF density of control and transgenic axons ($60,000\times$ magnification). This is the magnification at which the NF counts were done to determine density.

Figure 4. Neurofilament density distribution in sciatic nerve axons. *A*, *C*, and *E* compare NF densities in DT transgenic mice. *B*, *D*, and *F* compare NF densities in SV40 transgenic mice. *A*, Comparison of NF density in the axons of control versus unmyelinated DT transgenic. The average number of NFs/hexagon was 5.3 ± 0.14 in control and 14.3 ± 0.41 in unmyelinated axons in DT transgenic. This correlates to 90 ± 2.4 NFs/ μm^2 and 235.9 ± 7.0 NFs/ μm^2 , respectively. These numbers represent a greater than twofold increase in NF density. *C*, Comparison of NF density in the axons of myelinated versus unmyelinated DT transgenic. The average number of NFs/hexagon was 8.6 ± 0.40 in myelinated and 14.3 ± 0.41 in unmyelinated axons in DT transgenic. This correlates to 145.4 ± 6.7 NFs/ μm^2 and 235.9 ± 7.0 NFs/ μm^2 , respectively. This represents approximately a 1.6-fold increase in NF density even between myelinated and unmyelinated large-diameter axons in the same nerve. *E*, Comparison of NF density in the axons of control versus all of the axons (myelinated and unmyelinated) in the DT transgenic. The average

Figure 5. Immunoblot analysis of NF phosphorylation state in transgenic and control sciatic nerves. A representative immunoblot of a 6% SDS-PAGE gel of control and transgenic sciatic nerves labeled with RMdO9 (NF-H, dP), RMO 108 (NF-M, P+), and a rabbit polyclonal antibody to NF-L (Pind). Ten micrograms of total protein was loaded in each lane. Six lanes from one control nerve and six lanes from one DT transgenic nerve were loaded on one gel, to eliminate variation in loading and quantitation. NF-L was loaded as equally as possible for each sample. A ratio of either NF-H or NF-M to NF-L was used for comparison to eliminate any variability in loading each lane.

of axonal NFs. As proposed by de Waegh et al. (1992), there may be transmembrane signals that modulate the balance between kinase and phosphatase activities in axons, changing phosphorylation levels for NF and possibly for other neuronal proteins, and this transmembrane signal from the Schwann cell may depend on normal myelin formation. Thus, when myelination is disturbed (i.e., by diphtheria toxin-induced lysis of Schwann cells, SV40 large T antigen disruption of normal growth of the Schwann cells, or by a natural mutation in the peripheral myelin protein-22 in Trembler mice), the axon does not receive the signal to phosphorylate the NF sidearms. However, the precise nature of this signaling pathway has not been determined.

A correlation was seen between the degree of perturbation of normal myelination and the increased density of NFs. NF densities increase significantly (Figs. 3, 4) in the transgenic mouse axons versus the age-matched controls in both lines of transgenic mice. This increased density of NFs was greater in the DT mice compared to the SV40 mice. In DT mice, the unmyelinated transgenic axons showed a 2.6-fold increase in NF density compared to controls. In SV40 mice, the difference was only a 1.7-fold increase in NF density (Fig. 4). This larger difference in densities correlates with the more significant disruption in myelination that is seen in the DT mice. This difference is also seen when all of the axons, myelinated and unmyelinated, in the transgenics are compared to the control mouse axons. It

Figure 6. Composite of immunoblot analysis for each of the antibodies used to determine the phosphorylation state of NF-H and NF-M in transgenic and control sciatic nerves. Immunoblots of 6% SDS-PAGE gels of sciatic nerves from transgenic mice (T) and control (C). RMdO9 (NF-H, dP), TA51 (NF-H, P++), RMO24 (NF-H, P+++), RMO189 (NF-M, Pind), RMO108 (NF-M, P+), RMO55 (NF-M, P++), and rabbit anti-NF-L (Pind) were used to probe the blots. A, Immunoblots for DT transgenic mice. Although not obvious by visual inspection of the blots, the statistical analysis revealed that TA51 (NF-H P++) and RMO24 (NF-H P+++) reactivities are greater in control nerves than in transgenic nerves. This suggests a decrease in NF phosphorylation in the transgenic. B, Immunoblots for SV40 transgenic mice, using the same antibodies as A.

appears that the more the Schwann cell's normal myelination is perturbed the more this affects NF density.

For the NF density to increase 90% in the DT mice and 27% in the SV40 mice relative to normal axons, changes must occur in interactions between NFs and other structures in the axon. The NF sidearms formed by NF-M and NF-H carboxy termi-

number of NFs/hexagon was 5.3 ± 0.14 in control and 12.1 ± 0.32 in the in DT transgenic mice. This correlates to 90 ± 2.4 NFs/ μm^2 and 202.1 ± 5.5 NFs/ μm^2 , respectively. These numbers also show a greater than twofold increase in NF density when both myelinated and unmyelinated axons are compared to the age-matched control axons. This represents the entire nerve that is used for the biochemical analysis for NF phosphorylation. B, Comparison of NF density in the axons of control versus unmyelinated SV40 transgenic. The average number of NFs/hexagon was 5.5 ± 0.15 in control and 9.1 ± 0.42 in unmyelinated axons in SV40 transgenic. This correlates to 93.9 ± 2.5 NFs/ μm^2 and 156.2 ± 7.2 NFs/ μm^2 , respectively. These numbers represent a 1.7-fold increase in NF density. D, Comparison of NF density in the axons of myelinated versus unmyelinated SV40 transgenic. The average number of NFs/hexagon was 6.7 ± 0.19 in myelinated and 9.1 ± 0.42 in unmyelinated axons in SV40 transgenic. This correlates to 114.5 ± 3.3 NFs/ μm^2 and 156.2 ± 7.2 NFs/ μm^2 , respectively. This represents approximately a 1.4-fold increase in NF density. F, Comparison of NF density in the axons of control versus all of the axons (myelinated and unmyelinated) in the SV40 transgenic. The average number of NFs/hexagon was 5.5 ± 0.15 in control and 7.4 ± 0.19 in the in SV40 transgenic mice. This correlates to 93.9 ± 2.5 NFs/ μm^2 and 126.6 ± 3.3 NFs/ μm^2 , respectively. These numbers represent a 1.3-fold increase in NF density in the entire nerve. This smaller increase is reflected in the changes that are seen in the biochemical analysis for NF phosphorylation.

A Quantitative Immunoblot Analysis of the Difference in NF Antibody Reactivity in Diphtheria Toxin Transgenic vs Control Sciatic Nerves

Figure 7. Quantitative immunoblot analysis of the difference in NF antibody reactivity of four transgenic versus four control sciatic nerves. Relative transgenic reactivities are measured by calculating the ratio of radioactivity bound to nitrocellulose blots of sciatic nerves from transgenic over the radioactivity incorporated in control. An asterisk indicates that the data obtained from each blot of transgenic versus control was statistically significant. **A**, Comparison of antibody reactivity in the DT transgenic mice. RMdO9, which labels the dephosphorylated epitopes of NF-H, was increased $90 \pm 32\%$ in the transgenic versus control. The phosphorylated epitopes of NF-H were decreased. TA51 (P+) showed a decrease of $15 \pm 22\%$ and RMO 24 (P+++) showed a decrease of $28 \pm 13\%$. The antibody to poorly phosphorylated NF-M (RMO108) showed a tendency to increase and the phosphorylated NF-M (RMO55) showed a tendency to decrease but the data was not statistically significant. The rabbit polyclonal anti-NF-H and RMO189, which labels the core of NF-M, are both phosphorylation independent and showed no change between transgenic and control. **B**, Comparison of antibody reactivity in the SV40 transgenic mice. RMdO9, which labels the dephosphorylated epitopes of NF-H, was increased $27 \pm 9\%$ in the transgenic versus control. TA51 (P+) showed a tendency to decrease but was not statistically significant. The remainder of the antibodies tested showed no significant difference. With the low percentage of unmyelinated large-diameter axons, it was not expected to detect a significant difference with many of the antibodies that show differences in the phosphorylation state of NFs.

B Quantitative Immunoblot Analysis of the Difference in NF Antibody Reactivity in SV40 Transgenic vs Control Sciatic Nerves

nals are believed to play a major functional role in determining surface properties of NFs. The NF carboxy-terminal sidearms project at regular 22 nm intervals along the filament. They contain highly conserved phosphorylation sites of NFs with multiple repeats of KSP sequences (Breen et al., 1988; Lee et al., 1988a,b). NF phosphorylation at these sites is currently believed to control how closely the NFs can pack together in the axon (de Waegh et al., 1992). Recently, antibodies specific for phosphorylated epitopes on NF-H and NF-M demonstrated a re-

duction in the amount of phosphorylation at the node of Ranvier (Mata et al., 1992). When the KSP sites are phosphorylated, a large number of negative charges are added to the carboxyl-terminal regions of NF-M and NF-H, which is speculated to cause the sidearms to extend away from the main coiled-coil region of the filament. These negatively charged extensions will then prevent the NFs from moving close together, which decreases their density in the axon (Carden et al., 1987b).

A correlation was also seen between the degree of hypomye-

lination and the dephosphorylation of the NFs. The degree of phosphorylation of NFs in these mice was compared with MABs that are specific for different NF phosphorylation states (Table 3). For example, the RMD09 antibody binds the repeated KSPAEEA sequence on NF-H only when the serines in six consecutive tandem repeats are not phosphorylated (Clark and Lee, 1991), and this MAB showed a direct correlation to the observations obtained in the EM morphometry studies of the NFs. Specifically, RMD09 showed the largest increase in labeling in the DT transgenic mice over the control. The labeling was also significantly increased in the SV40 mice, but the increase was not as large. This is the only MAB that showed a significant change in the SV40 mice. This is probably due to the very low level of hypomyelination (only 22% of the cross-sectional area of the large-diameter axons are unmyelinated) observed in these mice. The RMD24 MAB, which binds the same sequence as RMD09 only when six consecutive tandem repeats of this motif are phosphorylated (Clark and Lee, 1991), was significantly decreased in the DT mice (Fig. 6). These results support the current hypothesis that NF phosphorylation controls spacing. Further, the change in the NF phosphorylation and NF density in the axons in turn leads to a morphologic change in the size of the transgenic axons. The average size of the large-diameter axons in the transgenic mice was smaller than the average of the age-matched control axons (Table 2). This demonstrates a correlation between the degree of hypomyelination, NF dephosphorylation, and decrease in axonal size. Also, for each transgenic line the unmyelinated axons are smaller than the myelinated axons in the sciatic nerve. This also correlates with the NF density, which is higher in the unmyelinated versus the myelinated axons in each transgenic line (Table 2).

This study indicated that NF synthesis is independent of myelination, since NF content appears to be unchanged. The total amount of NF-H, NF-M, and NF-L did not appear to be changed in the transgenic mice compared to the control mice (Figs. 6, 7). Thus, the mechanisms that determine the amount of NF protein in the axons do not seem to be regulated by Schwann cells. The control of axonal diameter is believed to be dependent on a variety of factors, including synthesis of proteins in the cell body, presence of growth factors, the neuron's developmental program, as well as factors that affect the density and distribution of microtubules and NFs in the axon (Friede and Samorajski, 1970). The developmental program of neurons and the synthesis of NF proteins did not seem to be affected in these two lines of transgenic mice.

Control of cellular mechanisms by phosphorylation or dephosphorylation of proteins is used extensively in eukaryotic cells. Alterations in NF phosphorylation may be important in normal nerves and pathologic events in nerves (Nixon, 1993). Indeed, NF phosphorylation has been shown to be important for regulating axonal transport and increasing the stability of NFs in neuronal processes (Griffin et al., 1988; Lewis and Nixon, 1988). Alterations in NF protein phosphorylation also have been documented in regenerating axons and in various neuropathies. For example, increases in NF density can be seen in diabetic neuropathy (Medori et al., 1988a,b), which suggests that the increased NF density may result from altered patterns of NF phosphorylation. Heavily phosphorylated NF epitopes are found in Alzheimer's tangles (Lee et al., 1988b) and Lewy bodies in Parkinson's disease (Hill et al., 1991; Trojanowski et al., 1993). Some neurotoxic substances induce abnormal accumulations and increased NF phosphorylation (Bizzi and

Gambetti, 1986; Carden et al., 1986, 1987a; Johnson and Jope, 1988). It is unclear if the accumulation of NFs is a cause or a consequence of changes in NF phosphorylation, but recent studies showed that the overexpression of NF-H or NF-L in transgenic mice triggers neurodegenerative processes (Cote et al., 1993; Xu et al., 1993).

Insights into the mechanisms whereby glial cells modulate axonal properties normally and in disease states are beginning to emerge and we anticipate that the transgenic mice described here will be important model systems for further studies of these mechanisms.

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