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Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: Induction of a specific β-tubulin isotype

(cDNA/mRNA/cytoskeleton/microtubule)

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ABSTRACT **We examined the differential expression of genes encoding three β-tubulin isotypes (classes I, II, and IV) and the 68-kDa neurofilament protein (NF68) in rat sensory neurons during development, maturation, and axonal regeneration. Expression of the specific β-tubulin gene encoding the class II isotype was induced to high levels during development and axonal regeneration, whereas the expression of genes encoding the two other isotypes (classes I and IV) remained comparable to mature levels. Conversely, expression of the NF68 gene was relatively low during development and regeneration. Thus, the developmental program for cytoskeletal gene expression is recapitulated during axonal regeneration. The high level of class II β-tubulin expression found in developing and regenerating neurons occurs during the longitudinal growth of axons. In contrast, induction of NF68 gene expression is associated with the radial growth of axons in maturing neurons.**

It has been proposed that developmental mechanisms for axonal growth are recapitulated during regeneration (1). After an axon has been interrupted by cutting or crushing a nerve (axotomy), the portion of the axon disconnected from the cell body (distal stump) degenerates and is replaced by outgrowth (elongation) of regenerating sprouts arising from the proximal stump. The major cytoskeletal proteins of the axon, tubulin and neurofilament (NF), participate directly in axonal growth (2, 3). Previous studies, in which axonal regeneration in rat lumbar sensory neurons was induced by crushing the sciatic nerve, demonstrated differential changes in the expression of these proteins; mRNA levels increased for tubulin and decreased for the 68-kDa NF protein (NF68) (4). These findings did not address the question of the extent to which these changes represent a recapitulation of the developmental pattern of gene expression.

There are five classes of β-tubulin that can be distinguished on the basis of their unique carboxyl-terminal amino acid sequences; each of these isotypes is encoded by a separate gene (5, 6). cDNA sequences for three of these isotypes (classes I, II, and IV) have been isolated from rat (7, 8). Using hybridization with cDNA probes that specifically recognize the mRNAs for each of these three classes, we investigated the expression of these β-tubulin isotypes and NF68 in regenerating, developing, and maturing sensory neurons of rat. The goal of these studies was twofold: to investigate whether the expression of a specific tubulin isotype is induced during axonal regeneration and to determine the extent to which the developmental pattern of gene expression is recapitulated during axonal regeneration.

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MATERIALS AND METHODS

***In Situ* Hybridization.** Sciatic nerves of anesthetized (chloral hydrate, 400 mg/kg, i.p.) 7-week-old male Sprague–Dawley rats were crushed twice for 30 sec at the L4–L5 junction with no. 7 Dumont forceps. Unfixed dorsal root ganglia (DRG), which contain sensory-neuron cell bodies, were removed from animals killed by overdose of anesthetic and immediately frozen in cryoprotective embedding medium. Cryostat sections (8 μm) of ganglia obtained 4 days after axotomy and from age-matched control animals were transferred to gelatin-coated slides before fixation with 4% paraformaldehyde. Tissue sections were hybridized with ^{35}S-labeled cDNA probes by a published method (9). Specific cDNAs encoding the 3′ untranslated regions of class I, class II, and class IV β-tubulin genes of rat (5) were subclones of RBT.3, RBT.1, and RBT.2, respectively (7); the cDNA encoding NF68 has been described (10). The density of autoradiographic silver grains (number per unit area) over either neurons or regions of ganglia containing connective tissue and myelinated nerve fibers was analyzed with a computer-based system (Loats Associates, Westminster, MD) (4).

RNA Blots. At intervals between 1 and 56 days after bilateral sciatic nerve crush, rats were killed by overdose of anesthetic and L4 and L5 DRG were removed. Ganglia were removed in a similar manner from 5-day-old and 10-week-old rats that had not undergone axotomy. Eight to 12 ganglia (from two or three rats, respectively) were pooled for RNA preparation (except for 5-day-old animals, from which 25–30 ganglia were pooled). A major advantage of this system is that the nerve cell bodies can be completely harvested by removal of the DRG and can be prepared free of contamination by other populations of neurons. RNA isolated from these ganglia (11) was fractionated by electrophoresis in agarose/formaldehyde gels and transferred to nylon membranes for hybridization with ^{32}P-labeled cDNA as described (4, 12). Labeled hybrids were detected autoradiographically and quantitated with a computer-based video densitometric system (Loats Associates). After hybridization with each cDNA probe, labeled hybrids were removed and blots were rehybridized successively with each probe (i.e., individual RNA blots were hybridized with each cDNA probe).

RESULTS

Neuronal Expression of Class II β-Tubulin. Since tubulin is not a neuron-specific protein, we first asked whether the genes encoding class I, class II, and class IV β-tubulins are expressed in sensory neurons (as opposed to other cell types

Abbreviations: NF, neurofilament(s); NF68, 68-kDa NF protein; DRG, dorsal root ganglion or ganglia.
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in rat DRG, including perineural cells, connective tissue cells, and Schwann cells). Tissue sections of DRG were hybridized *in situ* with labeled cDNA probes complementary to the 3′ untranslated regions of these tubulin genes. This analysis demonstrated that each of these tubulin genes were expressed in neurons. The class I and class II isotypes were expressed predominantly in neurons, whereas the class IV isotype was expressed in all cell types in DRG. Neuronal expression of the class II isotype is illustrated in Fig. 1. Autoradiographic silver grains were preferentially associated with neurons; relatively few grains were located over lighter-staining areas containing connective tissue and myelinated nerve fibers. The density of silver grains was 21-fold greater over neurons than over connective tissue and myelinated nerve fibers in control ganglia [20.57 ± 2.0 vs. 0.95 ± 0.21 grains per 100 μm^2 (mean ± SEM)].

Recapitulation of the Developmental Pattern of Gene Expression After Axotomy. To determine whether the expression of specific β-tubulin genes is developmentally regulated in sensory neurons, RNA blots were used to examine levels of mRNA encoding these proteins in total RNA isolated from DRG of neonatal (5-day-old) and maturing (10-week-old) rats. Whereas relatively minor changes in the expression of class I and class IV β-tubulins were found during maturation

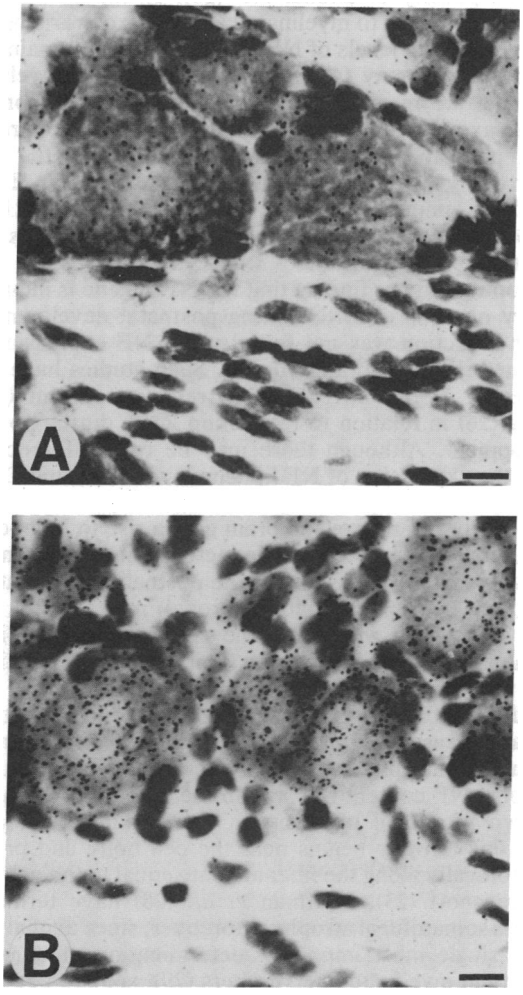

FIG. 1. Localization of class II β-tubulin mRNAs within sensory neurons. *In situ* hybridization of ^{35}S-labeled cDNA that specifically hybridizes with class II β-tubulin mRNAs was performed on tissue sections containing control sensory neurons from 10-week-old animals that had not undergone axotomy (*A*) and regenerating neurons 4 days after axotomy (*B*). Autoradiograms were exposed for 5 days. (Bars = 10 μm.)

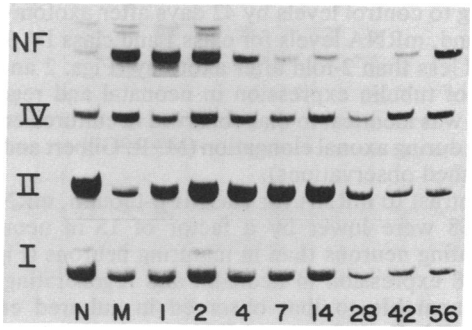

FIG. 2. RNA blot analysis of cytoskeletal protein mRNA levels in developing neurons and during axonal regeneration. Total RNA was harvested from DRG of neonatal rats (5 days old, lane N), maturing rats (10 weeks old, lane M), and at various times after the sciatic nerves of 7-week-old rats were crushed (in days, shown below each lane). mRNAs encoding NF68 and specific β-tubulin isotypes were identified by hybridization to appropriate cloned cDNAs. After hybridization with each cDNA probe, labeled probe was removed and the blot was rehybridized with the next probe. NF, autoradiogram of NF68 mRNAs; IV, II, and I, autoradiograms of mRNAs for β-tubulin isotypes IV, II, and I, respectively. Ten micrograms of RNA was analyzed in each lane. Autoradiograms were exposed for 1–3 days.

(expression decreased by a factor of 1.8 for class I and increased by a factor of 2.8 for class IV), the level of mRNA encoding class II β-tubulin in DRG from neonatal animals was ≈7 times higher than in maturing animals (Fig. 2). Thus, of the isotypes whose expression we have monitored, only class II β-tubulin showed a marked change in expression during the development of sensory neurons.

The expression of specific β-tubulin genes was also examined in RNA harvested from sensory neurons at various times after axonal regeneration was induced by crushing the sciatic nerve. A representative RNA blot is shown in Fig. 2; quantitation for this particular blot is illustrated in Fig. 3. Comparable results were obtained from RNA blots in four independent experiments. These analyses showed that class II β-tubulin mRNA levels were increased 2-fold 1 day after axotomy, and 6-fold 2 days after axotomy. Class II β-tubulin mRNA levels remained elevated between 4 and 14 days,

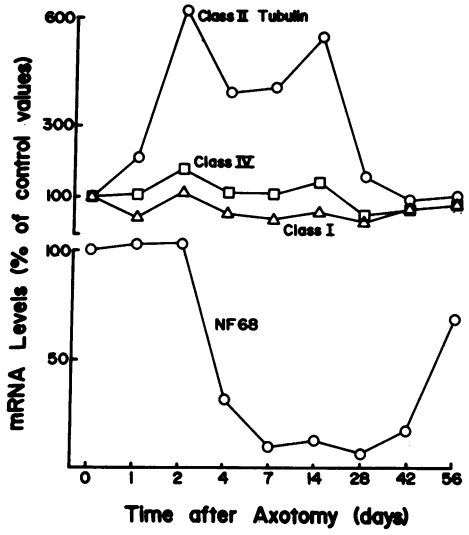

FIG. 3. Quantitation of mRNAs for NF68 and individual β-tubulin isotypes during axonal regeneration. Autoradiographic signals for the blots shown in Fig. 2 were quantitated with a computer-based video densitometry system (Loats Associates). Levels were normalized in relation to those in 10-week-old control animals (0 days) and plotted as a function of time after axotomy.

returning to control levels by 42 days after axotomy. On the other hand, mRNA levels for class I and class IV β-tubulins changed less than 2-fold after axotomy (Figs. 2 and 3). The pattern of tubulin expression in neonatal and regenerating neurons was identical to that observed in cultured embryonic neurons during axonal elongation (M. R. Gilbert and P.N.H., unpublished observations).

In contrast to mRNA for class II β-tubulin, mRNA levels for NF68 were lower by a factor of 15 in neonatal and regenerating neurons than in maturing neurons (Figs. 2 and 3). NF68 expression in neonatal and regenerating neurons was comparable to that observed in cultured embryonic neurons (M. R. Gilbert and P.N.H., unpublished observations). Reduction in NF68 mRNA levels was first seen 4 days after axotomy; maximal reduction occurred 28 days after axotomy (Figs. 2 and 3). A significant increase towards control levels of NF68 mRNA was seen 56 days after axotomy (Figs. 2 and 3). Previous work showed that NF68 expression in these neurons returns to control levels by 70 days after nerve crush (4). Thus, during axonal regeneration both NF68 and class II β-tubulin reverted to levels of gene expression characteristic of neonatal (as compared to maturing) neurons. Furthermore, alterations in the expression of NF68 and class II β-tubulin genes followed different time courses, suggesting that they are independently regulated.

In situ hybridization studies demonstrated that the neuronal expression of class II β-tubulin increased after axotomy (Fig. 1); at 4 days after axotomy the density of autoradiographic silver grains was 2-fold greater over regenerating neurons than over control neurons (44.29 ± 3.97 vs. 20.57 ± 2.0 grains per 100 μm^2). Previous work showed a factor-of-3 reduction in the density of silver grains over neurons hybridized *in situ* with NF68 cDNA 2 weeks after axotomy (4). The results from *in situ* hybridization and RNA blots therefore show parallel changes, but the magnitude of the change is somewhat different for the two techniques. In both cases, RNA blots demonstrated changes in mRNA levels that were ≈3-fold greater than those found by *in situ* hybridization; that is, RNA blots demonstrated a 6-fold increase in class II β-tubulin mRNA levels at 4 days after axotomy and a factor-of-10 reduction in NF68 mRNA at 2 weeks (Fig. 3).

DISCUSSION

Tubulin Expression and Axonal Growth. The expression of a specific tubulin gene encoding a distinct β-tubulin isotype (i.e., class II) is selectively induced during neuronal development and axonal regeneration. This is consistent with the previous observation that the class II isotype is expressed at higher levels in the brains of neonatal rats than in the brains of adult rats (8). It is not surprising that tubulin is expressed at high levels (compared to mature neurons) during development and regeneration since microtubules, which are composed primarily of tubulin, are the principal cytoskeletal elements in both developing axons and regenerating sprouts (13). In fact, tubulin is known to participate directly in the mechanism of axonal elongation (14).

Since axons are devoid of ribosomes, axonal proteins are synthesized in the neuron cell body (soma) before undergoing somatofugal translocation within the axon. Cytoskeletal proteins are conveyed in the slow component of axonal transport (15). Increased expression of the class II isotype after axotomy correlates with increases in regenerating nerve fibers in both the total amount of pulse-labeled tubulin undergoing axonal transport and the relative amount of this tubulin conveyed in the faster phase of slow axonal transport (SCb) (3, 16). This suggests that increased amounts of class II β-tubulin may be transported in SCb in regenerating nerve fibers, thereby increasing the delivery of this isotype to regenerating sprouts.

The functional significance of the increased neuronal expression of the class II β-tubulin isotype after axotomy remains to be determined. An extensive series of genetic and biochemical experiments have shown that, in some instances, tubulin isotypes appear to be functionally indistinguishable (refs. 17 and 18; reviewed by Cleveland in ref. 19) and that, in other cases, a single isotype is multifunctional (20). Thus, it is possible that the specific induction of class II β-tubulin during axonal regeneration merely reflects the enhanced activation of the class II gene. On the other hand, the present evidence is consistent with the view that the class II isotype plays a special role in the outgrowth of regenerating sprouts. This possibility is further strengthened by the finding that in neurites extended *in vitro* by PC12 pheochromocytoma cells, the class II β-tubulin isotype is polymerized more efficiently than the class III, IV, and V isotypes (H. C. Joshi and D.W.C., unpublished observations).

Morphological Correlates of NF Expression. NF are major intrinsic determinants of axonal caliber in myelinated nerve fibers, where NF number correlates directly with axonal cross-sectional area (13, 21–23). Prior to undergoing radial growth, developing axons are small in caliber (<1 μm in diameter), are highly enriched in microtubules, and contain relatively few NF (13, 24). Thus, the low level of NF expression in developing neurons is consistent with the observed paucity of axonal NF (24). In mammals, radial growth is restricted to myelinated axons. Axons arising from neurons with low levels of NF gene expression remain small throughout ontogeny (4) and include normal unmyelinated fibers that are morphologically indistinguishable from embryonic axons (13). The induction of NF gene expression during postnatal development correlates with the radial growth of myelinated nerve fibers (N. A. Muma, E. H. Koo, and P.N.H., unpublished observations), a process that begins at ≈10 days of age and continues until at least 6 months of age in rat (25).

In contrast to our finding that the NF68 gene is induced in sensory neurons of DRG during postnatal development, a similar induction was not found when NF expression was examined in whole brain (10, 26). Such studies have found that levels of NF mRNA either remain constant (10) or decline (26) in relation to total brain RNA during postnatal development. Although there may be real differences between the expression of NF in neurons of brain and DRG, it is also possible that the induction of NF (in the relatively small number of neurons in brain giving rise to large-caliber axons) could be masked by the increase in total brain RNA that presumably occurs during the postnatal proliferation of cells (both neuronal and non-neuronal) in brain (27). Resolution of this issue will require the use of *in situ* hybridization to evaluate NF gene expression (4) in specific populations of brain neurons during postnatal development.

Reduced NF gene expression after axotomy is associated with a decrease in axonal caliber in the proximal stump (4). This axonal atrophy correlates with proportional reductions in axonal NF content (23) and in the amount of NF protein undergoing axonal transport in the proximal stump (16). Since this atrophy begins near the cell body and proceeds somatofugally along the fiber at a rate equal to the velocity of NF transport (23), Hoffman *et al.* (28) have termed this process somatofugal atrophy. Moreover, since axonal caliber is the single most important determination of conduction velocity in myelinated nerve fibers (29), somatofugal axonal atrophy is associated with reduced conduction velocity in the proximal stumps of regenerating nerve fibers (30, 31).

Regulation of Cytoskeletal Gene Expression. Maintenance of the maturational pattern of cytoskeletal gene expression (i.e., relatively high levels of gene expression for NF and low levels for class II β-tubulin) may be regulated by neuron–target interactions. Somatofugal atrophy, the morphological

correlate of reduced NF gene expression (4) (i.e., associated with recapitulation of the developmental pattern), is initiated when a sensory neuron is disconnected from its peripheral targets (23, 32). If the nerve is cut and regeneration is prevented, the proximal stumps of axons remain atrophic throughout the life of the animal (33). On the other hand, if the nerve is crushed and effective regeneration is allowed, caliber recovers in the proximal stump (23). The recovery of normal caliber in the proximal axon, which correlates with restoration of the maturational pattern (i.e., the return of NF and class II β-tubulin expression to pre-axotomy levels), coincides with the reinnervation of target tissues (34). Thus, specific interactions between neurons and their targets may play important roles in the induction of NF gene expression and the down-regulation of class II β-tubulin expression. The transition from the developmental to the maturational pattern of gene expression at ≈10 days of age in rat sensory neurons (N. A. Muma, E. H. Koo, and P.N.H., unpublished observations) coincides in a general manner with the time when sensory axons establish their mature patterns of peripheral innervation (35).

Recapitulation of the Developmental Program for NF and Tubulin Expression During Axonal Regeneration. The longitudinal growth and radial growth of axons correlate with distinct patterns of NF and class II β-tubulin gene expression. The developmental pattern, in which expression is relatively high for class II β-tubulin and low for NF, correlates with the longitudinal growth of developing axons. Similarly, recapitulation of the developmental pattern during axonal regeneration correlates with the outgrowth of regenerating sprouts (and the cessation of radial growth). In contrast, the inverse pattern of gene expression (i.e., relatively high for NF and low for class II β-tubulin) in maturing neurons correlates with the radial growth of axons (28).

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