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Sensory Neurons Selectively Upregulate Synthesis and Transport of the β_{III}-Tubulin Protein during Axonal Regeneration

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The effects of peripheral nerve injury on the content, synthesis, and axonal transport of the class III β-tubulin protein in adult rat dorsal root ganglion (DRG) neurons were examined. Recent reports of selective increases in the steady-state levels of the β_{III}-tubulin mRNA during axonal regeneration (Moskowitz et al., 1993) led to the hypothesis that upregulated levels of expression of the β_{III}-tubulin isotype that alter the composition of neuronal microtubules is important for effective axonal regrowth. If this is the case, the increases in mRNA levels must be translated into increased β_{III}-tubulin protein levels and subsequently modify the axonal cytoskeleton via axonal transport mechanisms. The present study assessed whether or not this occurs by examining β_{III}-tubulin protein content in adult rat lumbar DRG neurons at different times (1–14 d) after a distal sciatic nerve crush (~55 mm from the DRG) by Western blotting and immunocytochemistry with a β_{III}-tubulin specific monoclonal antibody. These studies showed substantial increases in β_{III}-tubulin content in DRG neurons, as well as in proximal regions of peripheral sensory axons (0–6 mm from the DRG), from 1–2 weeks after a distal nerve injury. Pulse labeling of DRG neurons with ^{35}S-methionine and ^{35}S-cysteine and immunoprecipitation of labeled β_{III}-tubulin indicated that the synthesis of β_{III}-tubulin was increased in the DRG after axotomy. Studies of axonal transport, wherein L5 DRG proteins were labeled with ^{35}S-methionine and ^{35}S-cysteine by microinjection, revealed that slow component b (SCb) of axonal transport conveyed more labeled tubulin moving at apparently faster rates through the intact regions of sciatic nerve axons in response to crush injury of the distal sciatic nerve. Immunoprecipitation experiments using proximal peripheral nerve segments showed that SCb in distally injured DRG neurons was enriched in the β_{III}-tubulin isotype. These findings demonstrate that the augmented synthesis of β_{III}-tubulin after axotomy alters the composition of the axonally transported cytoskeleton that moves with SCb. The increased amounts and rate of delivery of β_{III}-tubulin in axons of regenerating DRG neurons suggest that the altered pattern of tubulin gene expression that is initiated by axotomy impacts on the composition and organization of the axonal cytoskeleton in a manner that can facilitate axonal regrowth.

[Key words: microtubules, axon regeneration, cytoskeleton, nerve injury, axonal transport, tubulin, sensory neurons]

Metabolic alterations involving the axonal cytoskeleton are a large component of the neuronal response to injury. Increases in overall tubulin synthesis in peripheral neurons have been documented after axotomy (Perry and Wilson, 1981; Hall, 1982; Quesada et al., 1986; Oblinger and Lasek, 1988), and it is thought that upregulated levels of tubulin production and increased delivery of microtubules to regrowing axons are essential for effective regeneration after injury. There are multiple tubulin genes present in the vertebrate genome (Sullivan and Cleveland, 1986; Sullivan, 1988; Luduena, 1993) and it is not yet clear whether increases in all tubulins or only specific isotypes are essential for axonal regeneration. Evidence obtained using cDNA clones for *in situ* hybridization have suggested that some tubulin isotypes may be functionally more important than others during axonal regeneration. For example, preferential increases in the expression of the class II and III β-tubulin mRNAs occur in DRG neurons after peripheral axotomy, while little or no change in the levels of the class I and IV β- tubulin mRNAs occurs (Hoffman and Cleveland, 1988; Oblinger et al., 1989; Wong and Oblinger, 1990; Moskowitz et al., 1993). These observations support the idea that certain tubulin gene products are better able to meet the demands of regenerating neurons than are other isoforms. If this is the case, then the alterations in expression of specific tubulin mRNAs observed during regeneration must be translated in altered synthesis of specific tubulin isotypes and ultimately produce modifications in the composition of axons through axonal transport mechanisms.

Previous studies of peripheral mammalian axons have shown that distal axotomy results in decreases in the amount of labeled tubulin protein transported in slow component a (SCa) of axonal transport in motor and sensory axons (Hoffman and Lasek, 1980; Oblinger and Lasek, 1985, 1986; McQuarrie et al., 1986). In contrast, increases in the amount and rate of labeled tubulin transported in the faster of the two slow transport components, SCb, have been documented in axotomized motor neurons (Hoffman and Lasek, 1980; McQuarrie et al., 1986), but have not been previously examined in sensory neurons.

Since tubulin transport in SCa is not augmented in regenerating DRG neurons (Oblinger and Lasek, 1988), the increased synthesis of total tubulin that occurs in these neurons following axotomy must selectively affect the output of those microtubules that are conveyed in SCb. Recent studies of tubulin transport in rat motor neurons using isotype-specific antibodies have shown

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that β_{II} and β_{III}-tubulin transport are augmented in both amount and rate during regeneration (Hoffman et al., 1992). The leading front of the SCb wave, which is magnified in regenerating motor neurons, was shown to be enriched in β_{III}-tubulin (Hoffman et al., 1992). In regenerating sensory neurons, the question of whether differential induction and transport of β_{III}-tubulin occurs after injury and contributes to an alteration of the properties of the cytoskeleton has not been previously examined. Studies of this issue are of interest from a number of perspectives because of the unique properties of the β_{III}-tubulin isotype. β_{III}-tubulin is neuron specific in its expression pattern in avian species, and is nearly neuron specific in the rat where very low level expression of β_{III}-tubulin is also seen in the testis (Sullivan and Cleveland, 1986; Sullivan, 1988; Lee et al., 1990b). The β_{III} isotype is also subject to posttranslational modifications such as polyglutamylation and phosphorylation (Gard and Kirschner, 1985; Luduena et al., 1988; Alexander et al., 1991) that might influence the functional properties of microtubules enriched in β_{III}-tubulin. In the present study, the following questions concerning β_{III}-tubulin expression in regenerating rat DRG neurons were addressed: Does the content of β_{III}-tubulin in the DRG change after peripheral axotomy and, if so, is this due to alterations in the synthesis rate of the β_{III}-tubulin isotype? Do changes in β_{III}-tubulin synthesis modify the composition of DRG axons through axonal transport mechanisms after injury? Evidence was obtained showing that β_{III}-tubulin levels are elevated in axotomized DRG as a result of increased synthesis. In addition, data showing that SCb vector of slow transport conveys a modified cytoskeleton, enriched in β_{III}-tubulin, into the axons of regenerating DRG neurons were also obtained.

Materials and Methods

Animals. Adult male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 250–350 gm were used for these studies. Handling of the animals and surgical procedures were carried out in accordance with the *NIH Guide for the Care and Use of Laboratory Animals.* For all surgical procedures, rats were anesthetized with a mixture of sodium pentobarbital (27 mg/kg) and chloral hydrate (128 mg/kg) injected intraperitoneally, and given 0.2 cc atropine sulfate (Eli Lily and Company, Indianapolis, IN) by intramuscular injection to the gastrocnemius muscle. Using sterile procedures, unilateral sciatic nerve crushes were made at the midthigh level (~55 mm from the fifth lumbar DRG) by applying three successive 30 sec crushes to the nerve with #5 Dumont forceps as previously described (Wong and Oblinger, 1990). At scheduled tissue harvest times, animals were decapitated under deep ether anesthesia.

Immunoblotting studies. Experimental and contralateral control side L4 and L5 ganglia and a 6 mm piece of peripheral spinal nerve attached to each DRG (designated "proximal" nerve sample) were obtained at 1, 7, and 14 d postaxotomy. At each time point, axotomized and contralateral control DRG and proximal nerve samples from four rats were pooled. Experiments were repeated using two different complete sets of pooled DRG and proximal nerve samples. DRG and proximal nerve samples were homogenized separately in 100 mM sodium phosphate buffer pH 7.4 using an Omni-1000 automatic microhomogenizer (Omni International). A 50 μl aliquot was removed from the sample homogenate and a standard Bradford protein assay was performed to determine total protein concentration. The remaining sample was diluted 1:1 with BUST (2% β- mercaptoethanol (BME), 8 M urea, 1% sodium dodecyl sulfate (SDS), 0.1 M Tris pH 6.8). Equal amounts of total protein (5 μg) from the various samples were loaded onto 7.5% polyacrylamide minislab gels and electrophoresed at 100 V for 1.5 hr. Proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a mini transblot apparatus (Bio-Rad Laboratories, Richmond CA) at 150 V for 40 min in a cooled chamber containing electroblotting buffer (20 mM Tris, 150 mM glycine, 20% methanol). Blots were washed in 1 M Tris-saline (pH 7.4) for 10 min and placed in milk-blocking buffer (5% nonfat Carnation dry milk in Tris-saline) for 30 min at room temperature. A mouse monoclonal antibody specific for β_{III}-tubulin, TuJ1 (Geisert and Frankfurter, 1989), was diluted 1:5000 in milk-blocking buffer and reacted with blots overnight at 4°C. Blots were then washed in Tris-saline and incubated with iodinated secondary antibody (^{125}I-labeled goat anti-mouse IgG from ICN Radiochemicals, Irvine CA) at a concentration of 0.5 μCi/gel lane for 2 hr at room temperature. Blots were washed four times 10 min each in Tris-saline with 0.5% Triton X-100 at room temperature and then in Tris-saline for 1 hr at room temperature. Autoradiographs were made by exposing Kodak XAR5 film to the air-dried blots at ambient temperature for 2–3 d.

Immunocytochemistry. Axotomy and contralateral control side L4 and L5 DRG were harvested from four animals at 14 d after sciatic nerve crush. The DRG were fixed in 4% paraformaldehyde for 1.5 hr, rinsed in 0.1 M phosphate-buffered saline (PBS) pH 7.4 for 2 hr, dehydrated in graded ethanols, and embedded in paraffin. Ganglia were then sectioned at 10 μm, mounted on gelatin chrome-alum subbed slides, and stored at room temperature until used. Immunostaining of the sections using the monoclonal antibody, TuJ1, was done as follows. Sections were deparaffinized in xylene and rehydrated in graded ethanols to PBS. Four percent normal goat serum (NGS) in PBS was applied to sections for 1 hr. Primary antibody to β_{III}-tubulin was diluted 1:10,000 in PBS containing 1% NGS and applied to sections for overnight incubation in a humid, room temperature chamber. Sections were then washed in PBS and a secondary biotinylated antibody (from the mouse ABC Vectastain Elite kit, Vector Laboratories, Burlingame, CA) was applied to sections (0.5% Ab, 1.5% NGS in PBS) and incubated for 1 hr. Sections were washed and then incubated in the Vectastain ABC reagent (2% reagent A, 2% reagent B in PBS) for 1 hr at room temperature. Finally, the sections were washed in PBS and the reaction product was visualized with 50 mM Tris-HCl (pH 7.6), 10 mM imidazole, 0.04% diaminobenzadine (DAB), and 0.01% hydrogen peroxide until desired staining was obtained (5–10 min). Slides were then washed in H_2O, passed through graded ethanol solutions to 100% ethanol, cleared in xylene, and coverslipped with Permount. To facilitate comparisons, sections from each experimental (axotomized) and control DRG were processed together under identical conditions in a given immunostaining run; two replications of the experiment were done.

Radiolabeling of newly synthesized DRG proteins. For *in vitro* labeling, L4 and L5 DRG from the experimental (axotomized) and contralateral control side were removed at 1, 7, and 14 d after unilateral sciatic nerve crush and pooled (axotomy and control side ganglia separately). The experiments were repeated using two additional pairs of pooled DRG samples. After quick removal from the animals, the ganglia were desheathed and the dorsal and ventral roots were trimmed away. The DRG were placed in 500 μl of a methionine, cysteine-free, Minimal Essential Media (MEM) solution made using a Select-Amine Kit (GIBCO, Grand Island, NY) and allowed to equilibrate at 37°C for 20 min with 95% oxygen bubbling. Next, the solution was replaced with methionine and cysteine-free MEM that contained 250 μCi of ^{35}S-Trans-label (a mixture of ^{35}S-methionine and ^{35}S-cysteine from ICN Radiochemicals, Irvine, CA), and the DRG were incubated at 37°C for 45 min with 95% oxygen bubbling. After labeling, the ganglia were rinsed and then frozen on powdered dry ice and stored at −70°C until used for immunoprecipitation.

Metabolic labeling of axonally transported proteins. Two groups of rats were used for these experiments. One group of rats sustained a distal sciatic nerve crush 14 d prior to labeling, and the other consisted of normal, untreated rats. In fully anesthetized rats, the L5 DRG was exposed unilaterally by a partial laminectomy using sterile procedures. Next, 2 μl of sterile saline containing 250 μCi of ^{35}S-Trans-label were injected into the midpoint of the DRG using a glass micropipette over a period of 10 min using a pressure injection system. After the injection, the DRG surface was flushed with saline and the muscle and skin incisions were closed with sutures. Animals were euthanized 3 or 5 d following injection to focus on the SCb component of transport, and the L5 DRG with the attached peripheral nerve system was removed, laid on a 3 × 5 index card, frozen with powdered dry ice, sealed in a plastic bag, and stored at −70°C until processed. In each condition (axotomy or normal, 3 or 5 d postlabeling) 3–4 rats were prepared.

SDS-PAGE/fluorography was used to analyze axonally transported proteins. The frozen nerves containing ^{35}S-labeled proteins were cut into consecutive 2 mm segments using a Mickel gel slicer (Brinkmann Instruments) and each piece was solubilized by homogenization in glass-glass microhomogenizers in Tris buffer containing 1% SDS, 8 M urea,

and 2 M BME. A 100 μl aliquot of each sample was subjected to SDS-PAGE on gradient slab gels (6–15%, 4% stack) as described previously (Oblinger and Lasek, 1988). After electrophoresis, the gels were processed for fluorography as described (Oblinger and Lasek, 1988). Gels were dried and exposed to Kodak XAR film to visualize labeled proteins found at successive 2 mm distances from the L5 DRG.

Immunoprecipitation. Immunoprecipitation was used to analyze newly synthesized β_{III}-tubulin in the DRG, and also to examine axonally transported β_{III}-tubulin. For these two studies, L4 and L5 DRG pulse-labeled *in vitro* for 1 hr (see above) or sections of frozen nerve that contained labeled, axonally transported proteins obtained 5 d after labeling (see above) were used. In the case of the nerve samples, the L5 spinal nerve and its continuation in the sciatic nerve was cut into 10 mm long segments. The samples (DRG or nerve pieces) were homogenized in 300 μl immunoprecipitation (IP) buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, aprotinin (0.2U/ml), antipain (10 mg/ml), pepstatin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 1% bovine hemoglobin), using an Omni microhomogenizer, and incubated on ice for 1 hr. During this incubation, Protein-A Sepharose CL-4B beads (Pharmacia LKB Biotech, Piscataway, NJ) were swollen in 1 ml dilution buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 0.1% bovine hemoglobin) for 20 min at 4°C, washed in dilution buffer, resuspended to produce a 1:1 (vol/vol) slurry of Protein-A beads in dilution buffer, and kept on ice until used. The DRG or nerve homogenates were next centrifuged at 3000 × *g* for 10 min at 4°C, and the supernatants were transferred to a new tube and centrifuged at 10,000 × *g* for 10 min at 4°C. Supernates were precleared with Protein-A Sepharose beads to remove nonspecifically adsorbing proteins by addition of 10 μl of swollen beads/200 μl supernatant and incubating for 1.5 hr on a nutator at 4°C. Samples were then centrifuged at 200 × *g* for 1 min and an aliquot of supernatant was removed to determine the total radioactivity in each sample by liquid scintillation counting. The volume of each supernatant that contained 10^5 (or 10^6) cpm was determined, and that amount was transferred to a reaction tube that was precoated with IP buffer. TuJ1 antibody (3 μg) and 10 μl of preswollen Protein-A beads were added to each reaction tube and the samples were incubated for 1.5 hr on a nutator at 4°C. Samples were then washed 2× with dilution buffer, once each with TSA buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl) and with 0.05 M Tris-HCl, pH 6.8 and then resuspended in 25 μl 2× sample buffer. Samples were then heated to 100°C for 5 min and equal volumes of immunoprecipitated protein from the various samples were loaded onto 10% polyacrylamide minislab gels and electrophoresed at 100 V for 1.5 hr. The gels were fixed in 7% acetic acid and 35% methanol and dried onto Whatman #1 paper. Autoradiographs were made by exposing Kodak XAR5 film to the dried gels at −70°C for 2–3 d to detect newly synthesized β_{III}-tubulin protein, and for 1–2 weeks to detect axonally transported β_{III}-tubulin.

Results

Axotomy-induced changes in β_{III}-tubulin content in DRG neurons

The effect of axotomy on β_{III}-tubulin levels in adult DRG was first examined by immunoblotting. Equal amounts of total protein isolated from axotomized DRG and from uninjured contralateral control DRG at 1, 7, and 14 d postaxotomy were electrophoresed, blotted to nitrocellulose, probed with a β_{III}-tubulin specific monoclonal antibody, and visualized by autoradiography. Densitometric evaluation of autoradiographs of these Western blots revealed consistent increases in the amount of immunoreactive β_{III}-tubulin in the DRG samples obtained at 7 and 14 d postaxotomy and decreases in the amount of immunoreactive β_{III}-tubulin in 1 d postaxotomy DRG samples (Fig. 1). β_{III}-tubulin levels in the axotomy DRG samples were reduced an average of 34% at 1 d after injury and then increased an average of 92% at 7 d and 21% at 14 d after injury compared to contralateral controls (Fig. 1).

Immunocytochemistry of histological sections of axotomy and contralateral control side DRG neurons at 14 d postaxotomy was done to confirm the changes observed in Western blotting experiments. The localization and level of immunoreactive β_{III}-tubulin was qualitatively compared in histological sections of axotomized and matched contralateral control DRG from four different animals. Overall, the results of immunocytochemistry experiments were concordant with those from the immunoblotting experiments. The following observations were consistently noted in all pairs of axotomy and control ganglia. First, the number of intensely immunoreactive DRG neurons appeared to be greater in sections of axotomized DRG (Fig. 2*B*) compared to contralateral control ganglia (Fig. 2*A*). Second, the relative intensity of β_{III}-tubulin immunoreactivity in the large-sized (>1000 μm²) DRG neurons was substantially greater in axotomized preparations as compared with contralateral controls.

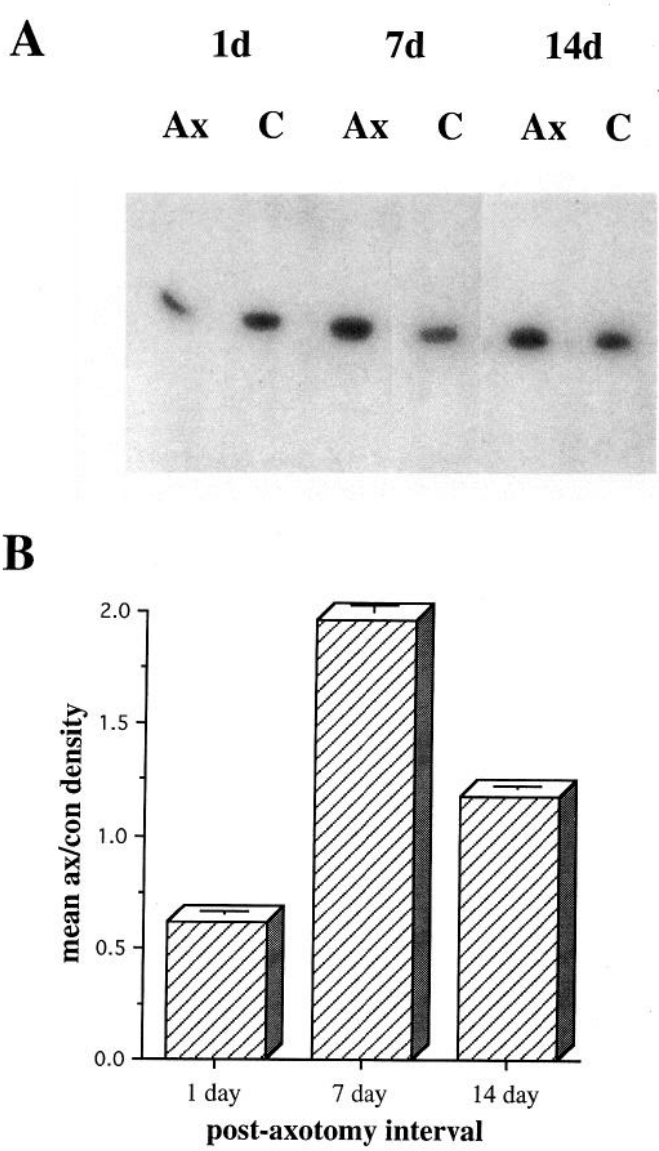

Figure 1. β_{III}-Tubulin protein content in the DRG is increased from 1 to 2 weeks after nerve injury. *A*, Representative autoradiograph of a Western blot of equal amounts (5 μg) of total protein isolated from axotomized (*Ax*) or contralateral control (*C*) side DRG at the indicated days postaxotomy. Blots were reacted using the TuJ1 antibody and visualized using ^{125}I-labeled secondary antibody and autoradiography. *B*, Densitometric analysis of β_{III}-tubulin content changes in DRG after axotomy. Mean ratios (and SEM) of β_{III}-tubulin band densities from the axotomy versus contralateral control side DRG at the indicated postaxotomy time points are plotted. $n = 2$ samples at each time point (where each sample represents four pooled L4 and L5 DRG).

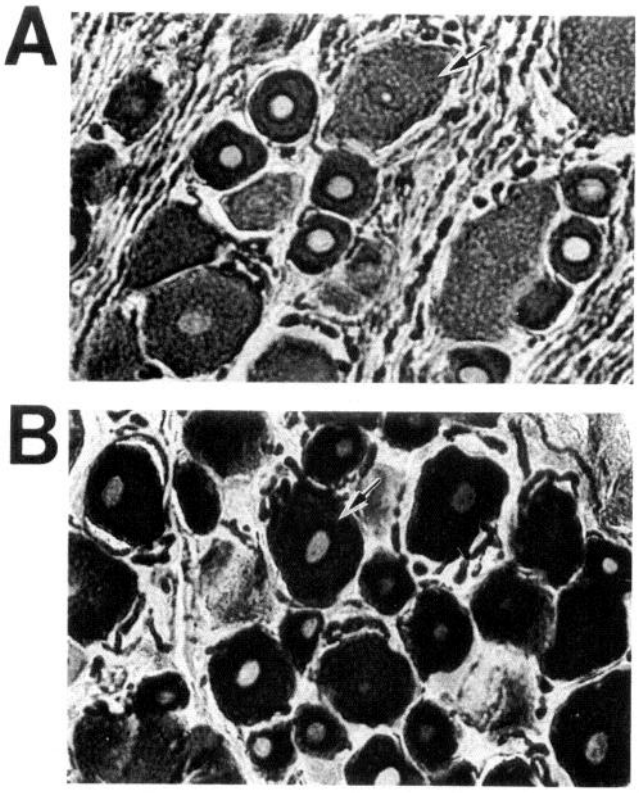

Figure 2. Immunocytochemistry of DRG neurons with a β_{III}-tubulin specific monoclonal antibody. At 14 d postaxotomy, histological sections of L4 and L5 DRG were obtained from *A*, contralateral control side or *B*, axotomy side and reacted with the TuJ1 antibody. Antigen-antibody complexes were visualized using the Vectastain ABC kit (peroxidase). In each panel, the *arrow* indicates a large DRG neuronal cell body.

Third, the relative level of β_{III}-tubulin immunoreactivity in intraganglionic axons appeared to be greater in sections from the axotomy side as compared to the contralateral control side. These observations suggested that an increased level of β_{III}-tubulin was present in DRG neurons and their initial axons after distal sciatic nerve injury.

Synthesis of β_{III}-tubulin is increased in axotomized DRG 1–2 weeks after distal sciatic nerve injury

One possible explanation for the observed increases in the level of β_{III}-tubulin immunoreactivity and content in the DRG after axotomy is a selective change in the level of β_{III}-tubulin synthesis by DRG neurons. To examine whether or not axotomy affected the synthesis of β_{III}-tubulin, the amount of radioactivity incorporated into β_{III}-tubulin protein in the DRG during an *in vitro* pulse-labeling reaction was measured at 1, 7, and 14 d after peripheral crush axotomy. Axotomy and contralateral control side L4 and L5 DRG were excised, labeled *in vitro* with ^{35}S-Trans-label for 45 min, and then β_{III}-tubulin was immunoprecipitated from each sample and examined by PAGE/autoradiography. Densitometric evaluation of the autoradiographs revealed no change in the level of pulse-labeled β_{III}-tubulin in the DRG at 1 d postaxotomy (Fig. 3). However, at 7 and 14 d after injury, densitometric analysis of the autoradiographs showed average increases of 163% and 140% in the amount of radioactivity incorporated into β_{III}-tubulin in the axotomized DRG relative to contralateral controls (Fig. 3). These

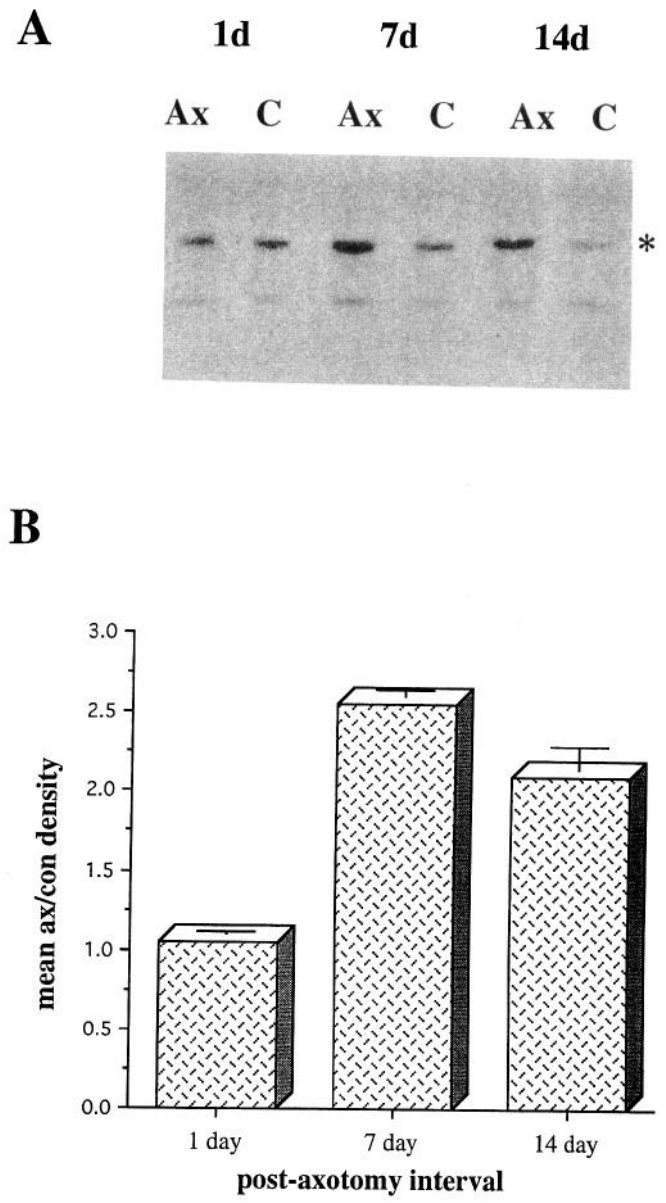

Figure 3. β_{III}-Tubulin protein synthesis is increased in the DRG from 1 to 2 weeks after nerve injury. *A*, Representative autoradiograph of a gel containing newly synthesized β_{III}-tubulin protein from the DRG on the axotomy side (*Ax*) or contralateral control side (*C*) at the indicated days postaxotomy. Proteins were labeled by incubating L4 and L5 DRG *in vitro* with ^{35}S-Trans-label (a mixture of ^{35}S-methionine and ^{35}S-cysteine) for 45 min. Equal counts of labeled DRG protein (10^6 c.p.m.) from each condition were then immunoprecipitated using the β_{III}-tubulin specific monoclonal antibody and equal volumes of the immunoprecipitates were loaded onto SDS/PAGE and visualized by autoradiography. *B*, Densitometric quantification of changes in β_{III}-tubulin protein synthesis. The mean axotomy/contralateral control ratios (with SEM) of immunoprecipitated β_{III}-tubulin (*asterisk*) in the DRG at the indicated postaxotomy times are plotted. n = 3 axotomized and three control ganglia at each time point.

experiments suggested that the rate of β_{III}-tubulin synthesis was increased in DRG neurons after axotomy.

β_{III}-Tubulin content is increased in initial regions of DRG axons 1–2 weeks after distal sciatic nerve injury

It was next of interest to determine if the β_{III}-tubulin synthesis changes that occurred in the neuronal cell bodies would be re-

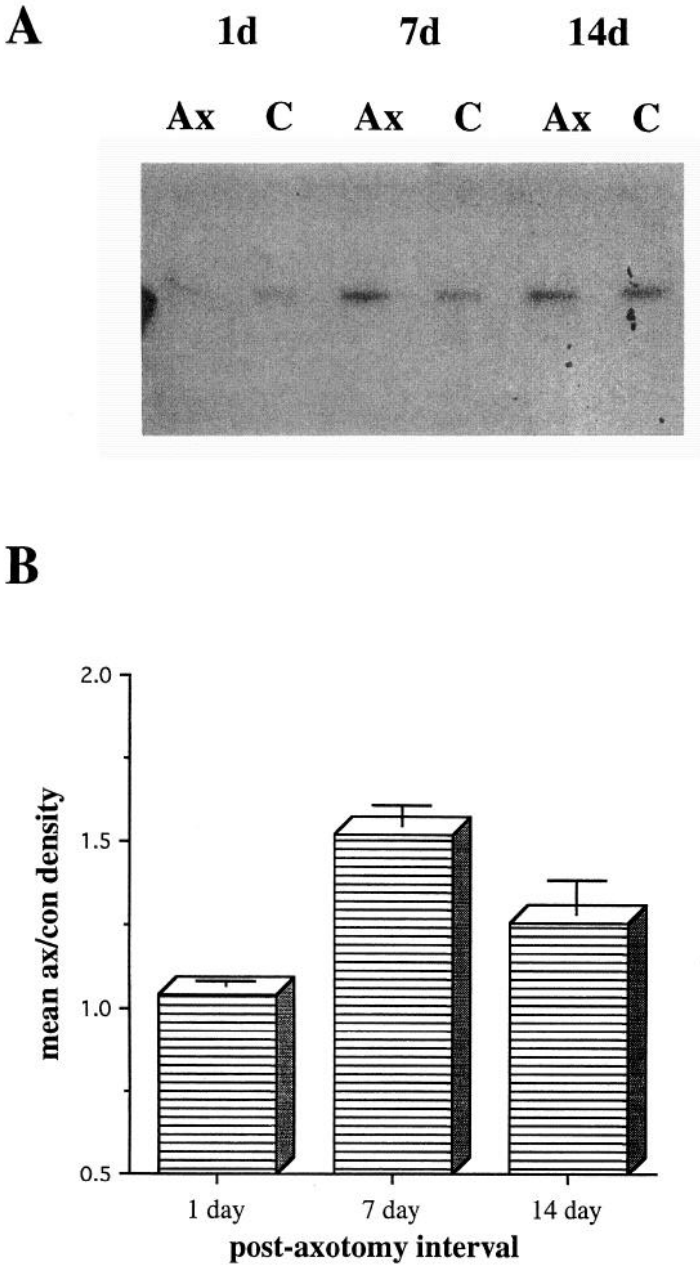

Figure 4. β_{III}-Tubulin protein content is increased in proximal DRG axons from 1 to 2 weeks postaxotomy. *A*, Representative autoradiograph of Western blot of equal amounts of total protein (5 μg) isolated from L4 or L5 spinal nerves (0–6 mm segments) on the axotomy side (*Ax*) or contralateral control side (*C*) at the indicated days postaxotomy. Blots were reacted with the TuJ1 antibody and visualized using iodinated secondary antibody and autoradiography. *B*, Densitometric analysis of changes in β_{III}-tubulin protein content in proximal regions of DRG axons. Mean ratios (and SEM) of β_{III}-tubulin band densities from the axotomy versus contralateral control side nerve segments at the indicated postaxotomy time points are plotted. $n = 2$ samples at each time point (where each sample represents four pooled spinal nerves).

flected in DRG axons. We first examined the relative content of β_{III}-tubulin in pooled proximal regions of L4 and L5 spinal nerves (6 mm segments) by Western blotting. Figure 4 shows the relative levels of β_{III}-tubulin protein in the initial nerve segments on the axotomy and control side at different postinjury times. Densitometric analysis revealed that β_{III}-tubulin levels in initial peripheral nerve segments of axotomized DRG preparations were increased an average of 10%, 51%, and 22% at 1, 7, and 14 d after injury, respectively. While the initial regions of the peripheral nerves examined in these experiments contain both sensory and motor axons, it is probable that the augmented signal observed in these experiments primarily reflects changes in the composition of the sensory axons from DRG neurons. These regions are close to the DRG cell bodies, unlike the motor axons, which are ~30 mm from their cell bodies at the sampling site used.

Overall tubulin transport in slow component B (SCb) is increased in amount and rate in proximal regions of DRG axons after distal nerve injury

To examine how axotomy alters the axonal transport of tubulin in DRG axons, the L5 DRG was microinjected with ^{35}S-Translabel in normal rats or in animals that had sustained a distal nerve crush (at 55 mm from the L5 DRG) 14 d earlier. Labeled protein transport profiles in the regions of the sciatic nerve proximal to the injury site (0–52 mm) were examined 3 and 5 d after labeling. These relatively early postinjection times were specifically selected to enrich for SCb proteins. Previous studies indicated that the slower, NF-enriched SCa wave, which moves at a mean velocity of 1–1.5 mm/d in sensory axons, would be just entering sciatic nerve axons while the faster moving SCb wave, which moves at a mean velocity of ~4 mm/d, would be prominent in the nerve at 3–5 d postlabeling (Wujek and Lasek, 1983; Oblinger et al., 1987). Representative fluorographs of labeled proteins present in intact sciatic nerve segments at 3 and 5 d postinjection are shown in Fig. 5. Visual analysis of the fluorographs revealed that tubulin (indicated by small arrows at "T") was the predominant SCb protein in both normal and axotomized DRG preparations. It was also apparent that the amount of labeled tubulin transported in SCb was increased and that more labeled tubulin was present at more distal positions in the sciatic nerve in the axotomized condition than in the normal control condition (compare Fig. 5*A* and *B*; *C* and *D*).

To enable comparisons between different preparations, the total tubulin radioactivity (d.p.m) in each sciatic nerve was determined. The tubulin region was excised from consecutive gel lanes, solubilized, counted, and summed. In each case, the 100% level represented the total d.p.m. present in the initial 52 mm of sciatic nerve extending from the L5 DRG. The percentage of total tubulin radioactivity in each nerve that was present in each 2 mm segment of nerve was next determined for each animal and then data from all preparations in a given condition were averaged and plotted. At both 3 and 5 d after labeling, the labeled peak of SCb tubulin was found to be located further from the L5 DRG in axons of distally axotomized neurons than it was in axons of normal DRG neurons at the comparable postlabeling interval (Fig. 6*A*,*B*).

The transport of tubulin in the axons was also evaluated by determining the distance from the L5 DRG that defined the 50% level of total labeled nerve tubulin. Cumulative plots of the labeled tubulin present in successive 2 mm sciatic nerve pieces were made. The 100% level was defined as the total d.p.m of tubulin in the entire 52 mm of nerve that was examined. Nerves from injured and normal preparations were evaluated individually and then percentage data from all animals in a given condition were averaged. Figure 7 shows that more of the total labeled tubulin was located further from the DRG in axotomized preparations than was the case in normal uninjured neurons at both 3 and 5 d after labeling. Fifty percent of the transported

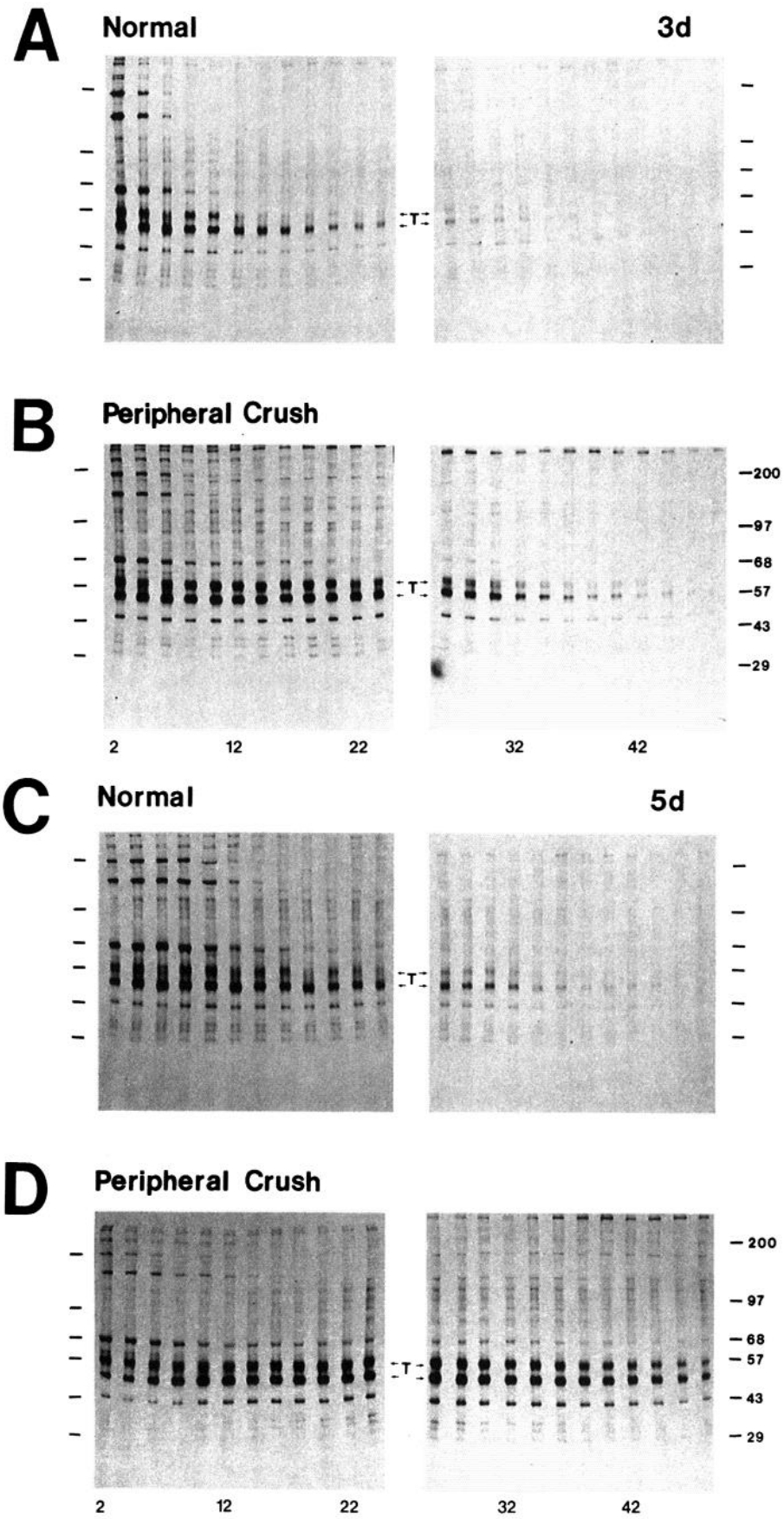

Figure 5. Representative fluorographs of gels showing labeled proteins present in consecutive 2 mm segments of peripheral L5 nerve in normal and regenerating DRG systems at 3 or 5 d after labeling the L5 DRG with ^{35}S-Trans-label. *A,* Normal DRG system at 3 d after labeling. *B,* Axotomized system (labeled 14 d after distal sciatic crush) at 3 d after labeling *C,* Normal DRG system 5 d after labeling. *D,* Axotomized system 5 d after labeling. The distance (mm) from the DRG that each nerve sample was obtained is indicated by *numbers below the fluorographs.* In all cases, transported proteins are from intact regions of DRG axons, since the crush injury was at ~55 mm from the L5 DRG. The position of the alpha (*top*) and beta (*bottom*) tubulin proteins are indicated by *arrows* and the "*T*" in the middle of each panel. Molecular weight standards are indicated by *dashes* to both sides of each gel panel; from the top, these are 200, 97, 68, 53, 48, and 29 kDa.

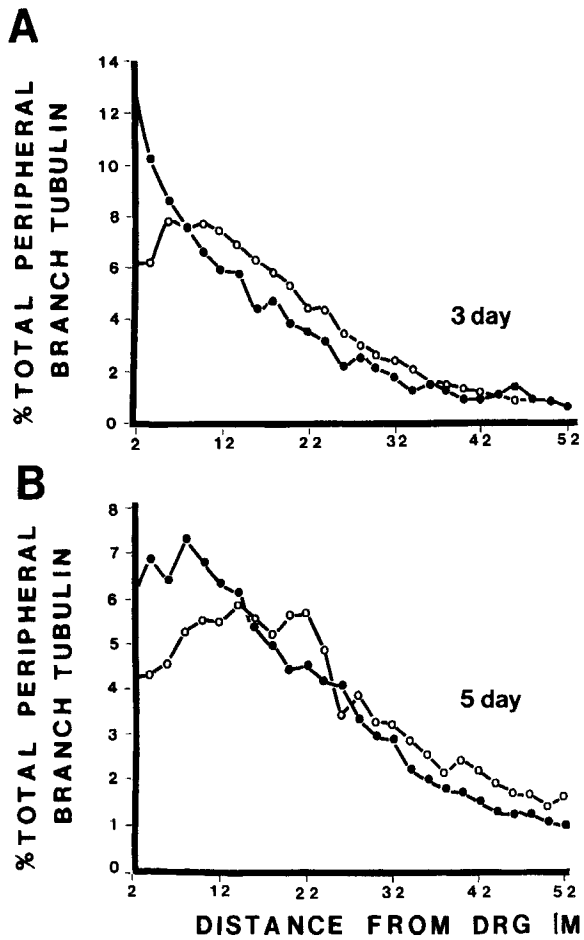

Figure 6. Quantitation of tubulin transport in normal L5 DRG axons and in proximal regions of peripheral L5 DRG axons after a previous distal crush axotomy. Samples were obtained at either 3 or 5 d after labeling the L5 DRG with ^{35}S-Trans-label. The total tubulin radioactivity (d.p.m.) in the sciatic nerve (from 0–52 mm) was determined by excising the tubulin region from the gels using the fluorographs as templates, dissolving the gel pieces, and then assessing radioactivity by liquid scintillation counting. The percentage of the total tubulin radioactivity for each nerve that was present in each 2 mm segment of nerve was calculated, and averaged data from all animals in a given condition are plotted. Normal DRG axons at 3 and 5 d after labeling are depicted by *solid dots,* axons of previously injured DRG neurons are depicted by *open circles.* At 3 d, $n = 3$ for both normal and axotomy; at 5 d after labeling, $n = 4$ for both normal and axotomy groups.

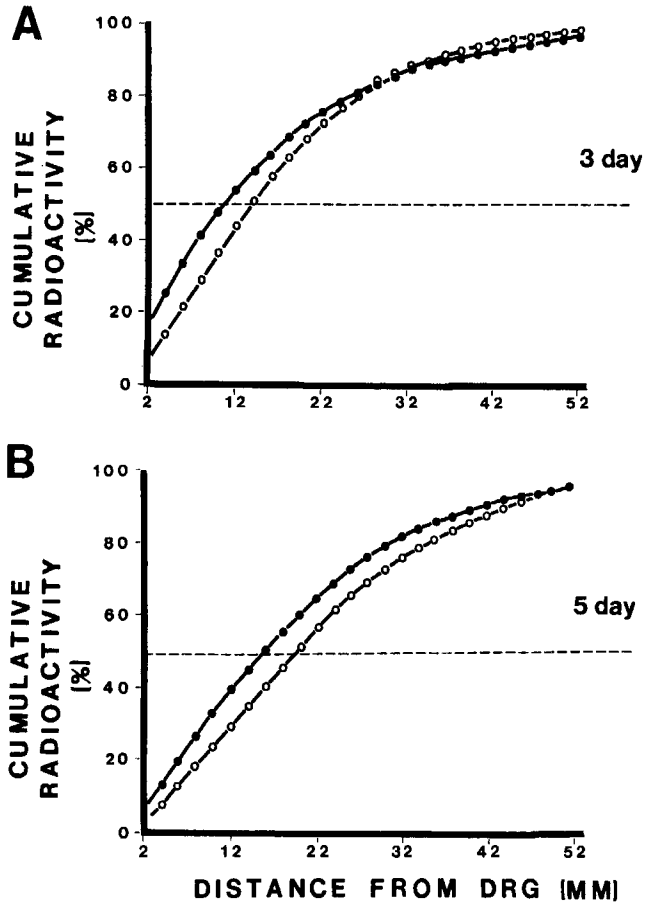

Figure 7. Cumulative plots depicting the transport of SCb tubulin in DRG axons. Tubulin bands from SDS/PAGE containing consecutive 2 mm segments of nerve were excised and the radioactivity in each segment was determined. The total tubulin d.p.m. in each nerve (from 0–52 mm) was defined as 100%, and the progressive cumulative percentages towards this total were calculated for each nerve segment. Arithmetic averages were obtained from animals in a given condition and the mean cumulative percentages were plotted. *A,* Data from 3 d after labeling; $n = 3$ for both axotomy and normal conditions. *B,* Data from 5 d after labeling, $n = 4$ for both axotomy and normal conditions. The *solid dots* represent data from normal DRG preparations, and the *open circles* represent data from axotomized preparations.

tubulin was beyond 14 mm from the L5 DRG at 3 d after labeling in the axotomized system, compared to 11 mm in normal control DRG axons (Fig. 7*A*). At 5 d postlabeling, 50% of the labeled, transported tubulin had moved beyond 23 mm from the L5 DRG in axotomized DRG axons (Fig. 7*B*). In contrast, 50% of the labeled nerve tubulin in control DRG preparations had moved past 16 mm from the L5 DRG (Fig. 7*B*). This method of analysis indicated that more of the total transported tubulin was shifted distally in axotomized systems, supporting the conclusion that overall tubulin transport was accelerated in intact, proximal regions of sensory axons after a distal nerve injury.

β_{III}-tubulin levels in axonal transport are augmented in axons of regenerating DRG neurons

In order to examine the contribution of β_{III}-tubulin to the total tubulin transport profiles in axons of regenerating (14 d postaxotomy) and normal DRG neurons, nerves containing labeled, axonally transported proteins were sectioned into four consecutive 10 mm pieces and β_{III}-tubulin was isolated from each piece by immunoprecipitation. Again, to enrich for SCb in the nerves, a relatively early postinjection interval (5 d postlabeling) was used in the experiments. Densitometric analysis of the resulting fluorographs from the transport experiment gels was done to determine the mean level of labeled, immunoprecipitable β_{III}-tubulin in the four consecutive nerve segments. Figure 8 shows that in normal, as well as in axotomized DRG axons, the first two 10 mm segments of sciatic nerve contained nearly equivalent amounts of labeled β_{III}-tubulin. However, at 30 and 40 mm from the L5 DRG, the axons of axotomized DRG neurons contained substantially more labeled β_{III}-tubulin than did those of normal neurons. These more distal regions of the sciatic nerve are devoid of labeled NF proteins at 5 d postlabeling (see Fig. 5), indicating that they contain primarily SCb proteins. Thus, the elevated amounts of β_{III}-tubulin in those regions in the axoto-

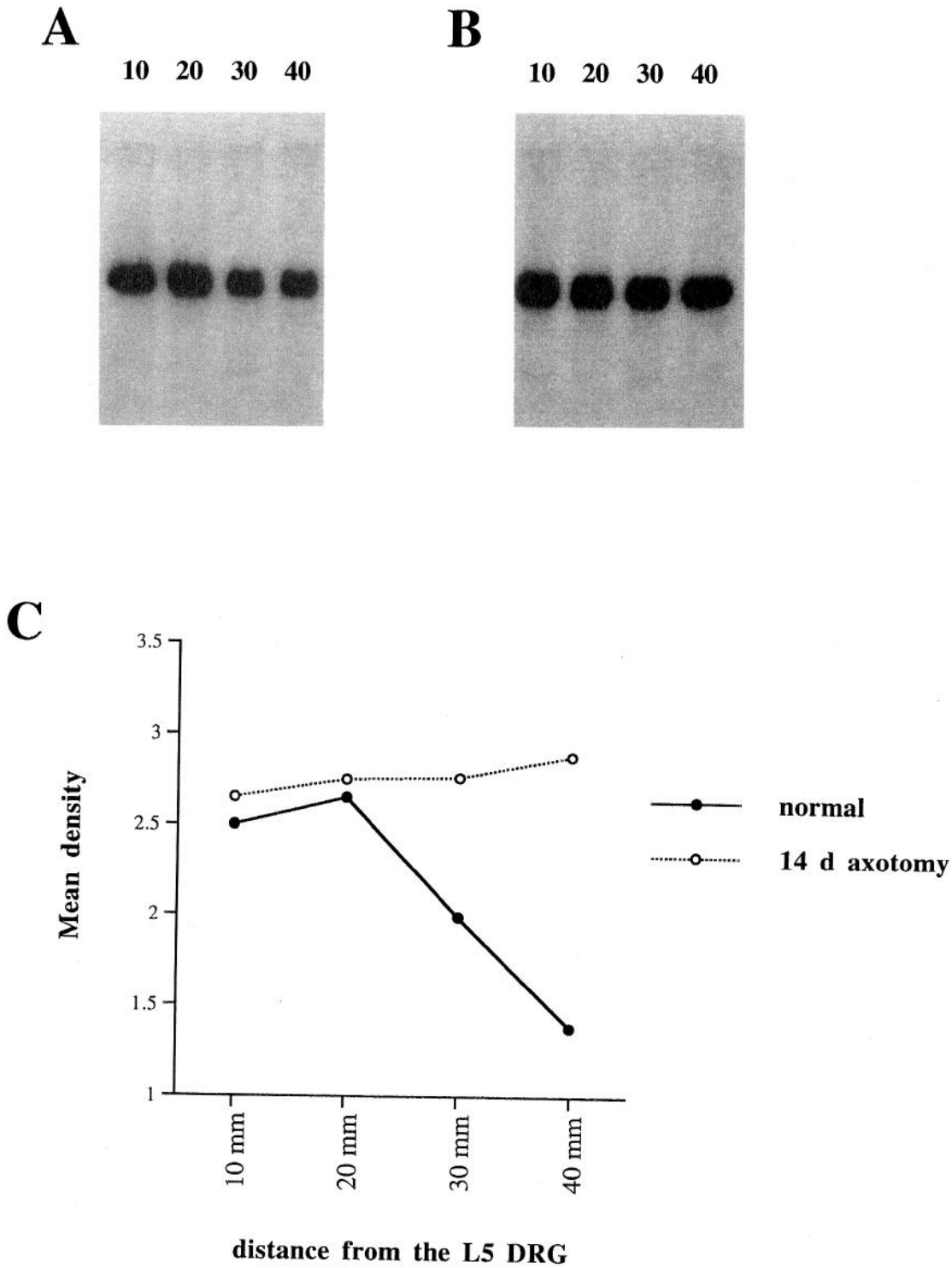

Figure 8. Axons of DRG neurons that are regenerating distally are enriched in axonally transported β_{III}-tubulin. Fluorographs of representative gels showing the labeled β_{III}-tubulin protein that was immunoprecipitated from consecutive 10 mm regions of L5 spinal nerve/sciatic nerve of normal (*A*) and axotomized (*B*) preparations at 5d after labeling the L5 DRG with ^{35}S-Trans-label *in vivo*. The axotomy group was labeled 2 weeks following a crush lesion of the distal sciatic nerve (~55 mm from the DRG). Distances from L5 DRG from which the samples were obtained are indicated above gel lanes. In all cases, equal counts (10^5 c.p.m.) from each sample were immunoprecipitated using TUJ1 antibody and loaded on SDS/PAGE gels. *C*, Densitometric analysis of the fluorographs showing the mean density values for immunoprecipitable, axonally transported β_{III}-tubulin in normal DRG axons (*closed dots*) and in previously injured DRG neurons (*open circles*). $n = 3$ axotomized and three normal preparations.

mized preparations indicates that β_{III}-tubulin transport in SCb is augmented in DRG neurons after a prior axotomy.

Discussion

The present study examined the regulatory dynamics of the microtubule network in regenerating sensory neurons. A vectorial process that alters the axonal microtubule network in regenerating neurons by shifting the tubulin isotype balance towards a more β_{III}-tubulin dominated profile was documented. These findings complement those in a previous study, wherein increases in steady-state levels of β_{III}-tubulin mRNA were found in axotomized sensory neurons at 1–2 weeks postinjury, following a transient reduction in β_{III}-tubulin mRNA levels at 1 d postinjury (Moskowitz et al., 1993). In order for the prolonged increases in β_{III}-tubulin mRNA levels that occur after axotomy to impact on the process of axonal regeneration, synthesis and axonal transport vectors must translate and transfer the change in mRNA status to the axonal compartment. In DRG neurons, no previous studies had examined this issue for specific tubulin isotypes. The present study documents the events that enable an orderly transformation of the microtubule network to occur in regenerating sensory neurons. First, the synthesis of β_{III}-tubulin in the DRG, as assessed by the incorporation of ^{35}S during a pulse-labeling period, is augmented within a week of axotomy. This translational increase fuels an increase in the content of β_{III}-tubulin in the DRG neuron and its axon during the initial weeks after distal nerve injury. The SCb vector of slow axonal transport is the vehicle by which the augmented β_{III}-tubulin translation products are conveyed to distal regions of peripheral sensory axons.

Is the increased β_{III}-tubulin content in regenerating DRG neurons and their axons incorporated into microtubules? While direct evidence for this was not obtained in this study, the fact that SCb is markedly enriched in β_{III}-tubulin in regenerating DRG axons strongly supports this idea. Because of the absence of protein synthetic machinery in mature mammalian axons, proteins are delivered to the axonal compartment from their site of synthesis in the cell soma. Unlike the NF proteins, which are conveyed exclusively in SCa, tubulin proteins are transported in both the SCa and SCb components of slow axonal transport in DRG neurons (Wujek and Lasek, 1983; Oblinger et al., 1987). The consensus from much of the previous literature on slow axonal transport is that tubulin transport is in the form of assembled microtubules (Hoffman and Lasek, 1975; Brady et al., 1984; Brady and Black, 1986; Lasek, 1988; Reinsch et al., 1991). Thus, the augmented levels of β_{III}-tubulin in SCb found in the present study suggests that axons of regenerating sensory neurons contain an increased number of β_{III}-tubulin-enriched microtubules.

One of the issues that is of interest is the partitioning of altered synthesis products into the faster of the two slow axonal transport rate components, SCb. The marked increase in both total tubulin and β_{III}-tubulin transport in SCb in intact regions of peripheral sensory axons after distal nerve injury had not been previously documented. Our present findings on this issue in the sensory neuron differ from those in a previous study done in motor neurons (Hoffman et al., 1992). In motor neurons, axotomy results in an increased level of tubulin transport (including of β_{III}-tubulin) in SCb, but no apparent increase in the axonal content of specific tubulin isotypes. This discordance between the profiles of labeled, transported tubulins and total tubulin content in the nerve was hypothesized to be due to slow transport conveying only a small amount of the total tubulin through motor axons at any point in time with the bulk of the axonal tubulin remaining relatively stationary (Hoffman et al., 1992). However, alternative explanations could also be considered. For example, it should be noted that a previous study in motor neurons had shown that SCa tubulin levels decline in motor axons during regeneration (Hoffman and Lasek, 1980). It is considered likely that the reduced tubulin transport in SCa balances the increased transport of tubulin in SCb in motor neurons, resulting in no net changes in overall axonal tubulin content in those cells. Our present findings in DRG neurons show that slow transport changes are correlated with axonal content changes for β_{III}-tubulin during regeneration. Both the amounts of β_{III}-tubulin in SCb and the content of β_{III}-tubulin relative to total axonal protein was augmented in regenerating DRG neurons. This data argues that axonal tubulin content is, indeed, sensitive to changes in slow transport parameters. It should be noted that earlier studies in the DRG system had reported no change in total tubulin transport in the SCa transport component, but SCb had not been previously examined (Oblinger and Lasek, 1988). Because of this previous finding and reports of increased tubulin mRNA levels in the DRG (Hoffman and Cleveland, 1988; Wong and Oblinger, 1990; Moskowitz et al., 1993), a question that has been extant for some time is: where do the products of increased tubulin synthesis go in DRG neurons? With the present study, it is now clear that SCb receives and conveys the bulk of the augmented tubulin synthesis products in regenerating DRG neurons.

When axonal regeneration is initiated at a considerable distance from the cell body, as was the case in the present study, the microtubules needed for formation of regrowing axonal sprouts will be recruited from existing supplies in the parent axon. Are the changes in synthesis and slow transport of β_{III}-tubulin rapid enough to affect distal DRG axons? It is known that sprouting occurs at about 2 d postinjury in the DRG system (Oblinger and Lasek, 1984) and, thus, the changes in β_{III}-tubulin synthesis and transport need to be put into this time perspective. Evaluation of the autoradiograms of SCb transport in the DRG system in this study revealed that tubulin transport in SCb is quite rapid during regeneration. While the rate of translocation of the "peak" of a given SCb protein is typically noted to be in the range of ~4 mm/d in the normal DRG system (Wujek and Lasek, 1983; Oblinger et al., 1987), it is clear that a substantial portion of labeled tubulin moves at much faster rates, particularly in distally axotomized systems. For example, labeled tubulin was readily detectable at 40 mm from the DRG within 3 d of labeling an axotomized DRG preparation in the present study. This indicates that a portion of the SCb tubulin is moving at rates exceeding 10 mm/d. The time at which the synthesis of β_{III}-tubulin is first significantly increased after axotomy in the DRG has not yet been established, but it clearly occurs within the first week of axotomy. Thus, augmented supplies might be available in the cell body at the time of distal axonal sprouting (2 d postinjury) and these increased supplies could reach a distal axotomy site by SCb within several days. This time frame suggests that the augmented supply of β_{III}-tubulin–enriched microtubules to distal regions of the DRG axonal arbor may not be rapid enough to support the initial stages of axonal sprouting; however, they could arrive in time to help sustain the elongation and support the initial maturation of the regrowing sensory axons, even after distal axotomy.

What are the functional consequences of increased β_{III}-tubulin

levels in axotomized and regenerating DRG neurons? One of the potential consequences would be a shift in the lability of the microtubule network in regenerating axons. Previous studies of P19 embryonal carcinoma cells that were differentiated into a neuronal phenotype showed that β_{II} and β_{III}-tubulin isotypes sort into colchicine-stable and colchicine-labile microtubule populations, respectively (Falconer et al., 1992). A greater lability of microtubules enriched in β_{III}-tubulin to colchicine pressure does not necessarily signify a greater intrinsic instability of such microtubules. However, it is quite possible that an increase in the β_{III}-tubulin content of microtubules could push the axonal balance of microtubules towards a generally more labile phenotype. It is known that SCa and SCb microtubules differ in their solubility in the face of depolymerizing stimuli, with SCb microtubules being more labile (Brady and Black, 1986). These solubility characteristics may reflect a unique tubulin composition in SCa and SCb due to differential sorting of tubulin isotypes into different transport compartments. Quantitative studies examining the tubulin isotype distribution in SCa versus SCb have not yet been done in DRG neurons, but recent studies on this issue in motor neurons indicate that SCb is enriched in the β_{III}-tubulin isotype.

It is possible that alterations in the content of β_{III}-tubulin could either directly influence microtubule properties or do so as a result of selective MAP binding by different tubulin isotypes. Falconer et al. proposed the idea that unique MT-MAP interactions could result in a dynamic array of MTs that are high in β_{III}-tubulin content with fewer MAPs bound to them and a less dynamic array of β_{II}-rich microtubules with a high MAP content. While β_{II}-tubulin contains sequence that has a high affinity for MAP2 and tau binding (Littauer et al., 1986; Luduena et al., 1988; Banerjee et al., 1990), the corresponding β_{III}-tubulin sequence has much lower affinity for these MAPs (Falconer et al., 1992). Thus, a shift in the balance of various tubulins and MAPs during regeneration may either increase or decrease the plasticity of the microtubule network during different phases of regeneration. Recent observations of downregulation of tau mRNA and protein levels in DRG neurons during regeneration provide support to this argument (Oblinger et al., 1991). Clearly, additional information will be required to sort out the mechanisms by which augmented or decreased levels of various tubulins and MAPs produce alterations in the functional properties of microtubule networks.

Because β_{III}-tubulin is subject to a number of posttranslational modifications such as phosphorylation (Gard and Kirschner, 1985; Luduena et al., 1988; Lee et al., 1990a,b) and polyglutamylation (Lee et al., 1990a,b), changes in posttranslational modifications may further alter the propensity of β_{III}-tubulin enriched microtubules to bind MAPs or otherwise alter the properties of the microtubules containing such modified forms. Future studies to determine the extent at which β_{III}-tubulin is phosphorylated or otherwise modified in regenerating neurons, and their axons may further increase our understanding of the role of β_{III}-tubulin in axon regrowth. It is also important that studies that directly assess the plasticity of the microtubule network in regenerating versus nonregenerating sensory neurons be done. Such studies would provide more direct evidence of the functional consequences of tubulin isotype shifts during regeneration and the mechanisms by which changes in the composition and organization of the axonal microtubule network can facilitate axonal regrowth.

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