

## Long-Term Effects of Axotomy on $\beta$ -Tubulin and NF Gene Expression in Rat DRG Neurons

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### SUMMARY

To compare the long-term recovery of gene expression in dorsal root ganglion (DRG) neurons under conditions of regeneration vs. non-regeneration, Northern blotting and *in situ* hybridization were used to assess steady-state neurofilament (NF) and beta tubulin mRNA levels 12 weeks following axonal injury. Adult male rats sustained either a crush lesion of the mid-sciatic nerve (regeneration occurs), or a cut lesion of the sciatic nerve combined with ligation of the proximal nerve stump and removal of a large segment of the distal nerve (regeneration does not occur). In the latter case, neuroma formation physically prevented axonal regeneration. Results of Northern blotting of total RNA obtained from the DRG indicated that NF-L and NF-M mRNA levels had largely returned to control levels at 12 weeks following crush axotomy but were still substantially depressed following cut/ligation injury of the sciatic nerve at that time. *In situ* hybridization studies indicated that both crush and cut/ligation axotomy resulted in significantly lower NF-L mRNA levels in large-sized ( $>1000 \mu\text{m}^2$ ) DRG neurons at 12 weeks post-axotomy. Discrepancies in the conclusions from Northern blotting and *in situ* hybridization experiments were also noted in the case of tubulin mRNA changes at long intervals after axotomy. *In situ* hybridization data derived from the large-sized DRG neurons using a coding region  $\beta$ -tubulin cDNA (which recognizes both  $\beta_{\text{II}}$  and  $\beta_{\text{III}}$  mRNAs) showed complete recovery of  $\beta$ -tubulin

mRNA levels in surviving large-sized DRG neurons after crush axotomy, but significantly elevated tubulin mRNA levels in surviving large DRG cells at 12 weeks after cut/ligation axotomy. In contrast, Northern blotting results indicated that  $\beta_{\text{II}}$ -tubulin mRNA levels in the crush axotomy condition remained elevated relative to control while they were substantially lower than control in cut/ligation axotomy samples. Results from analysis of  $\beta_{\text{III}}$ -tubulin mRNA changes were not conclusive. The lack of complete correspondence in the results from the two different methods of analysis of mRNA changes (blotting vs. *in situ*) is likely to be due to selective loss of large-sized DRG neurons in the long-standing cut/ligation injury condition. This would influence results from blotting data, where RNA is derived from the DRG as a whole, more so than *in situ* hybridization experiments which specifically focus on the surviving large-sized neurons. Overall, data from these experiments indicate that altered patterns of gene expression remain in the DRG for long intervals after axonal injury, whether or not axonal regeneration has been successful. However, recovery of "normal" patterns of cytoskeletal gene expression in the DRG is considerably more complete after crush injury than after cut/ligation injury.

### KEY WORDS

neurofilaments, microtubules, tubulin mRNA, neurofilament mRNA, cytoskeleton, sciatic nerve, regeneration

### INTRODUCTION

Different types of axotomy have clearly different long-term functional consequences. If the rat sciatic

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nerve is crushed, sprouting fibers from the proximal parent axon enter the distal nerve stump and elongate towards their normal target areas, ultimately reestablishing functional innervation. In contrast, if the nerve is transected (cut) and the proximal nerve is ligated, neuroma formation ensues and long-term functional deficits result /25/. In that case, the proximal axons develop swollen endings filled with membranous organelles and neurofilaments (NFs) /4,19/. In the case of peripheral sensory neurons in the DRG, consequences of long-term regenerative failure that have been well documented include cell death and atrophy of proximal axons. Estimates for cell death in the DRG after many weeks or months of regenerative failure range from 10-30% /1,10,23,26/. Axonal atrophy of DRG axons is reportedly more pronounced after cut lesions compared with crush lesions /1,10/. Axonal diameter is directly related to neurofilament (NF) number, and NF gene expression is a known regulator of axonal caliber /12,13,17/. Thus, different long-term changes in NF gene expression in DRG neurons after cut vs. crush injury may be involved in the different extent of axonal atrophy that occurs in these two lesion conditions.

A recent study compared the differential response of DRG neurons to axotomies that result in regeneration (crush lesion of sciatic nerve) with those that cause regenerative failure (cut lesion of sciatic with ligation of proximal nerve and resection of the distal nerve stump) at the "peak response" time, 2 weeks post-injury /15/. In that study, it was found that cut/ligation injuries had a more pronounced effect on  $\beta_{II}$  and  $\beta_{III}$  tubulin mRNA level upregulations than did crush axotomy /15/. Previous studies had also shown a more substantial reduction in the NF/tubulin protein ratio present in slow axonal transport in proximal DRG axons after transection of the sciatic nerve compared to crush lesion of the nerve /21/. However, the long-term effects of different types of axonal injury on cytoskeletal gene expression in the DRG had not been previously examined. The goal of the present study was to determine whether the extent of recovery of normal steady-state levels of  $\beta$ -tubulin and NF mRNAs in DRG neurons differs at long intervals after axotomy that is followed by regeneration vs. regenerative failure.

## MATERIALS AND METHODS

### Animals and surgical procedures

Adult male Sprague-Dawley rats (250-350 grams) were anesthetized by intraperitoneal injection of a mixture of sodium pentobarbital (27 mg/kg) and chloral hydrate (128 mg/kg) for surgical procedures. Experimental animals received one of two types of sciatic nerve injury: (1) nerve crush (regeneration occurs), or (2) nerve transection followed by ligation of the proximal stump and removal of a segment of the distal nerve stump (no regeneration occurs). In both cases, the right sciatic nerve was injured at mid-thigh level (50-55 mm from the 5th lumbar DRG) using aseptic surgical techniques. For the crush axotomy condition, three successive 30-second crushes were applied to the sciatic nerve using #5 Dumont forceps. For the cut/ligation surgery, the sciatic nerve was transected with a small pair of scissors and a 1-1.5 cm piece of the distal sciatic nerve was excised. The proximal stump of sciatic nerve was then tightly ligated with #6 silk suture. At 84 days (12 weeks) post-axotomy the animals were decapitated while under ether anesthesia and L4 and L5 DRG from both sides of the animal were collected; the uninjured contralateral DRG served as controls for the axotomy-side ganglia. For the sham-operated condition, the sciatic nerve was exposed but not injured; only the contralateral L4 and L5 DRG from these animals were used. At sacrifice, the axotomy-side sciatic nerves of all experimental animals were visually examined. The presence of a neuroma and the absence of continuity between the proximal and distal stump portions of the sciatic nerve was confirmed in all of the animals in the cut/ligation axotomy condition. Conversely, physical continuity of the sciatic nerve and the absence of any neuroma formation was confirmed in all of the animals in the crush axotomy condition.

### cDNA probes

The probes used for *in situ* hybridization were a cDNA for  $\beta$ -tubulin (clone RBT1, /3/) and a cDNA for NF-L /18/. Additional clones used for Northern blot analysis included cDNAs for NF-M /16/ as well as cDNAs specific for the 3' untranslated region

(UT) of class  $\beta_{II}$ -tubulin (RBT1, 3'UT /3/) and rat class  $\beta_{III}$ -tubulin (KS-5, 3'UT). For hybridization specific to  $\beta_{III}$ -tubulin mRNA, we generated an Apa I fragment of the entire KS-5 clone (from Dr. Tony Frankfurter) that recognized the mRNA sequence for the terminal lysine and ~750 bp of 3'UT sequence (see /14/). For all hybridizations, the cDNA inserts were first isolated from the various plasmids using appropriate restriction enzymes and purified from agarose gels using Gene Clean (Bio 101). For Northern blot hybridizations, cDNA inserts were labeled with  $^{32}\text{P}$ -dCTP using the Prime-It kit (Stratagene, CA); unincorporated nucleotides were removed using NucTrap Push Columns (Stratagene, CA). For *in situ* hybridization, cDNA inserts were labeled with  $^{35}\text{S}$ -dCTP by nick-translation and unincorporated nucleotides were removed by centrifugation in Centricon 10 concentrator units (Amicon Corp.).

#### ***In situ* hybridization**

The L4 and L5 DRG were fixed in 4% paraformaldehyde in phosphate buffer overnight, and then embedded in paraffin. Sections of 10  $\mu\text{m}$  thickness were cut and mounted on gelatin chrome-alum subbed slides. After deparaffinization, the sections were hybridized with  $^{35}\text{S}$ -labeled cDNA probes for  $\beta$ -tubulin and NF-L at 37°C overnight exactly as described previously /14/. The sections were then washed in degraded concentrations of SSC containing 0.1% SDS and 0.1% 2-mercaptoethanol, air-dried, dipped in Kodak NTB2 emulsion and incubated in the dark at 4°C for 10 days to generate autoradiograms. After development, sections were stained with cresyl violet and coverslipped. Quantification of the *in situ* hybridization material was done as described previously /28/. Briefly, the density of silver grains overlying large-sized (> 1000  $\mu\text{m}^2$ ) DRG cells was determined in each of the experimental and control conditions using the Southern Micro Instruments Leading Edge image analysis system. Neurons were randomly selected for grain counting and only neurons with a nucleolus were analyzed. Eight different DRG from each of the experimental conditions (crush axotomy or cut/ligation axotomy) and the same number of matched contralateral control DRG in each condition were analyzed. Four

DRG from the sham-operated condition were used in hybridizations with the NF-L probe. From each DRG, 4-5 sections were prepared for autoradiography for each of the cDNA probes, and from each of these sections 20-30 large neurons were randomly selected for grain counting. Mean grain densities for the neurons in each experimental and control group were calculated and group differences were compared using Student's t-test.

#### **Northern blotting**

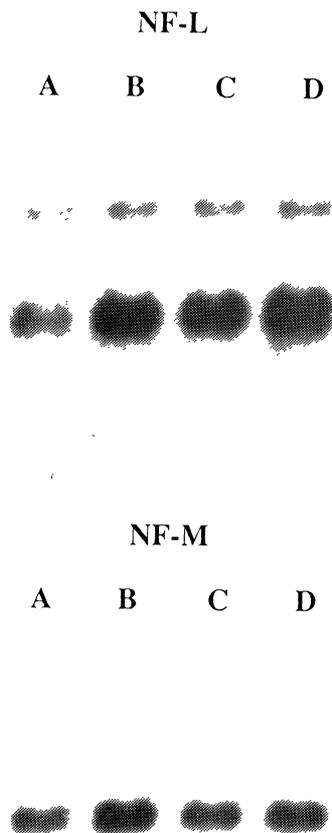
Eight animals were used to prepare RNA for Northern blotting studies. The experimental and contralateral control side L4 and L5 DRG were removed from the animals 12 weeks after either sciatic nerve crush or sciatic nerve cut/ligation surgery and rapidly frozen. For each RNA preparation, four ganglia were pooled (e.g. axotomy-side L4 and L5 DRG from two separate rats were pooled and used to prepare one RNA sample). Two independent sets of RNA samples for each of the experimental and control conditions were made. Total RNA was isolated as described /5/ and equal amounts (10  $\mu\text{g}$ ) of RNA from each of the different conditions were run on agarose gels and blotted to Nytran membranes as described previously /14/. The blots were hybridized with  $^{32}\text{P}$ -labeled cDNA probes, washed at high stringency, and exposed to Kodak X-OMAT film in cassettes with a single Cronex intensifying screen at -80°C for 1-5 days. Blots were stripped and then rehybridized with each of the four cDNA probes; each of the blots was hybridized using a different sequential order of the cDNAs. The film autoradiograms were scanned on a laser densitometer (LKB Instruments). In all cases, the autoradiograms selected for densitometry from various exposures of the blots were within the linear "gray" range that was established in initial experiments.

## **RESULTS**

### **NF mRNA changes at 12 weeks post-axotomy**

Northern blotting of total RNA was used to evaluate the extent of recovery of NF-L and NF-M mRNA levels in the DRG 12 weeks following

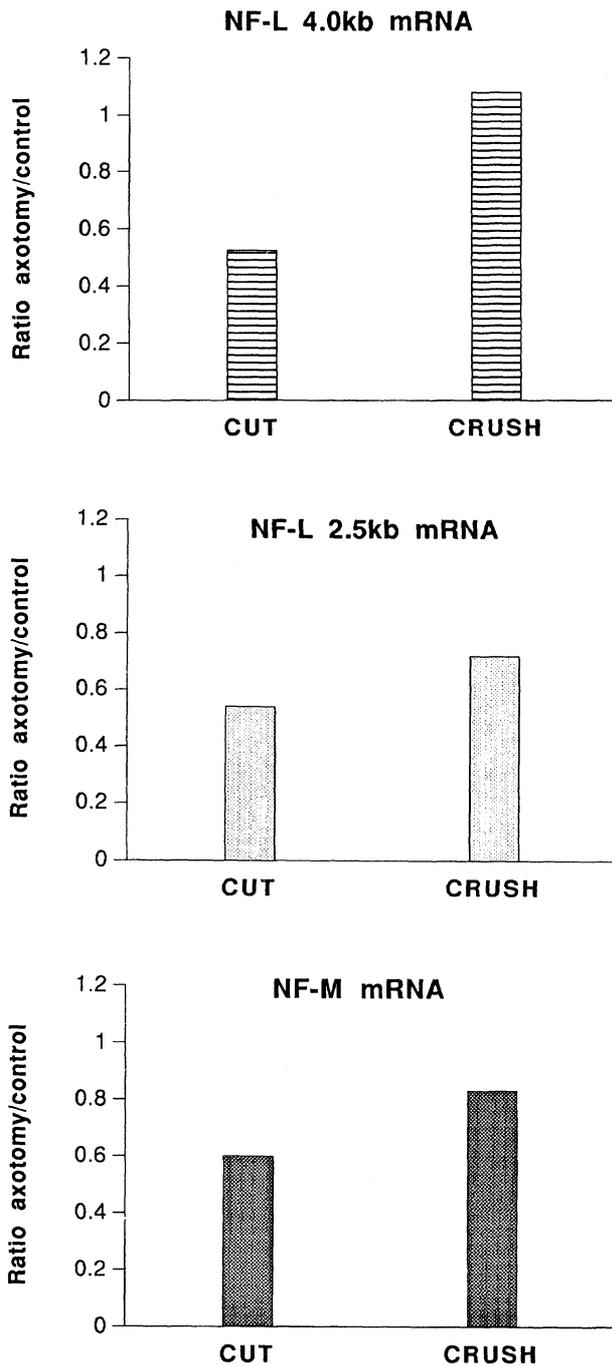
axotomies that were either followed by axonal regeneration (crush injury condition) or that resulted in neuroma formation and long-term failure of axonal regeneration (cut/ligation condition). Figure 1 shows autoradiograms of Northern blots probed with labeled NF-L and NF-M cDNAs. Qualitative evaluation of these blots suggested that NF-L and NF-M mRNA levels in the DRG



**Fig. 1:** Autoradiograms of a Northern blot showing changes in the levels of the two different NF-L mRNAs (2.5 kb and 4 kb) and the NF-M mRNA in the DRG at 12 weeks after either cut/ligation or crush axotomy. Equal amounts of total RNA (10  $\mu$ g) from (A) cut/ligation axotomized DRG; (B) contralateral control DRG; (C) crush-axotomized DRG, and (D) contralateral control DRG were loaded onto gel lanes. Blot was sequentially probed with the indicated cDNAs.

recovered to nearly control levels in the crush axotomy condition, but showed somewhat less recovery in the cut/ligation axotomy condition DRG (Figure 1). The two blots made with independent RNA samples from different animals were highly similar. Densitometric evaluation of film autoradiograms from the two blots also indicated that the steady-state levels of NF-L mRNA in the crush axotomy condition were returning to the levels present in contralateral control DRG but that the NF-L mRNA levels in the cut/ligated DRG were lower compared to controls (Figure 2). Of interest was the finding that the two different sized NF-L mRNA species (2.5 and 4 kb) exhibited somewhat different degrees of recovery. For example, the levels of the 4 kb NF-L mRNA species in the axotomy-side DRG were 108% of contralateral control-side levels at 12 weeks after crush axotomy, but only 53% of control side DRG levels in the cut/ligation axotomy condition (Figure 2). In contrast, the 2.5 kb NF-L mRNA species was present at 70% of control levels in the crush axotomized DRG but at only 56% of control levels in the long-term cut/ligated DRG (Figure 2). The steady-state NF-M mRNA levels 12 weeks after axotomy were 84% of contralateral control levels in the crush-axotomized DRG and 60% of control in the cut/ligation axotomy condition (Figure 2).

*In situ* hybridization was used to examine long-term changes in NF-L mRNA levels specifically in the large-sized DRG neurons (> 1000  $\mu$ m<sup>2</sup> diameter) after axotomy. Figure 3 illustrates representative examples of large neurons in both of the axotomy conditions compared with uninjured contralateral control neurons. It should be noted that the *in situ* hybridization method does not distinguish between the two different sized NF-L mRNA species, and thus the grains in the autoradiograms reflect steady-state levels of both NF-L mRNA species. Results of quantitative evaluation of the *in situ* hybridization material by grain counting are shown in Figure 4. This analysis revealed that NF-L mRNA levels had returned to 82% of control levels in the large-sized DRG neurons 12 weeks after crush axotomy. In the large DRG cells in the non-regenerating condition (cut/ligation) the steady-state NF-L mRNA levels were 69% of those in contralateral control DRG neurons. Statistical comparisons of grain densities



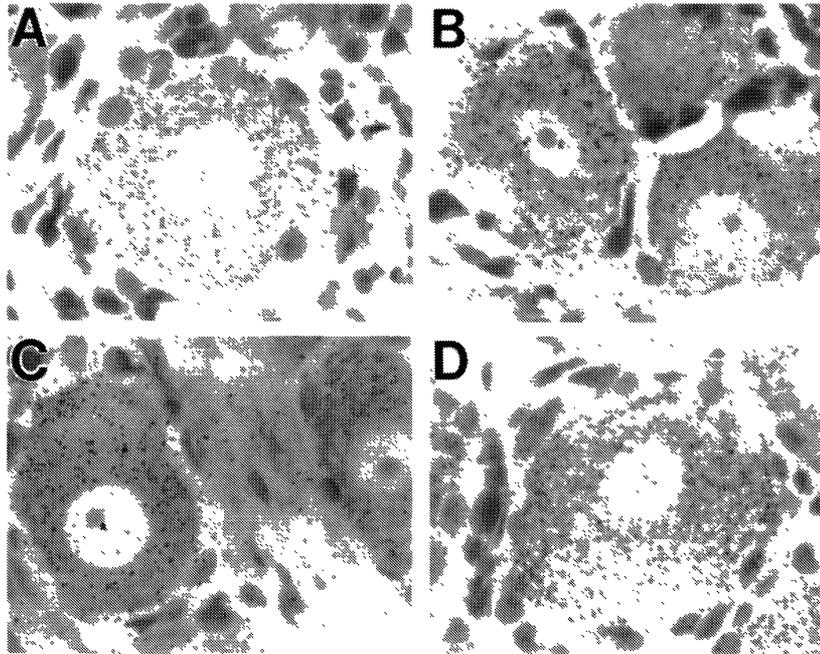
**Fig. 2:** Densitometric analysis of NF-L and NF-M mRNA levels in axotomized DRG neurons at 12 weeks post-axotomy. Autoradiograms of Northern blots were scanned and mean density values ( $n = 2$ ) in axotomized DRG samples are expressed as a ratio of contralateral control side values for each of the two axotomy conditions.

over the DRG neurons revealed that both axotomy groups differed significantly from their contralateral control groups ( $p < 0.01$ ), but did not differ significantly from each other (Figure 4). Also, there were no significant differences between the contralateral control neurons in the two treatment conditions (Figure 4) and no differences between either of the contralateral control groups and sham-operated control DRG neurons (data not shown).

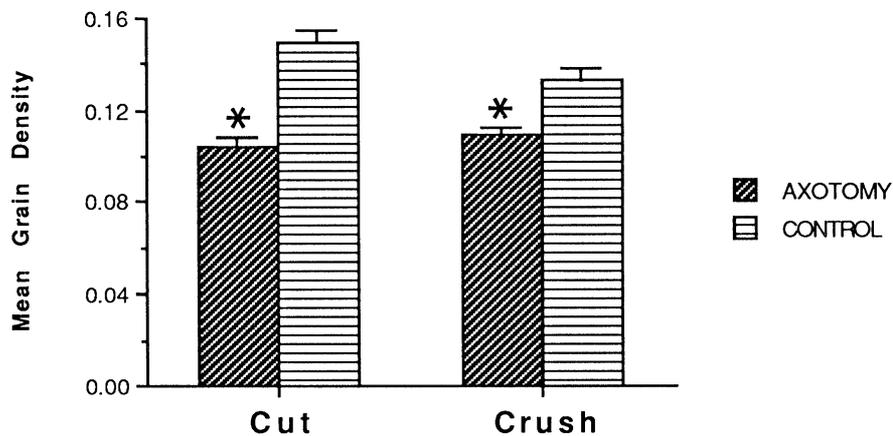
#### Tubulin mRNA changes at 12 weeks post-axotomy

Northern blotting of total RNA obtained from the DRG 12 weeks after sciatic crush or cut/ligation axotomies was used to examine long-term changes in steady-state levels of the  $\beta_{II}$  and  $\beta_{III}$ -tubulin mRNAs (Figure 5). Autoradiograms revealed an apparent decrease in  $\beta_{II}$  mRNA levels in the cut/ligation DRG relative to control and a slight increase in the  $\beta_{II}$ -tubulin mRNA levels in the crush-axotomized DRG relative to control. Densitometry of the autoradiograms revealed that the cut/ligation axotomy DRG samples contained an average of only 52% of the  $\beta_{II}$ -tubulin mRNA levels present in contralateral control DRG (Figure 6). The crush axotomy condition DRG samples contained 34% more  $\beta_{II}$ -tubulin mRNA than did the contralateral control DRG (Figure 6). The two separate blots used in this analysis showed virtually identical patterns of change in  $\beta_{II}$ -tubulin mRNA levels.

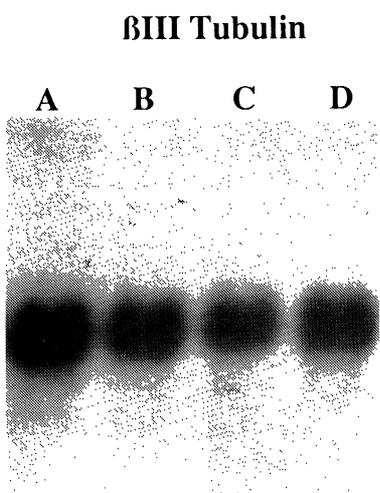
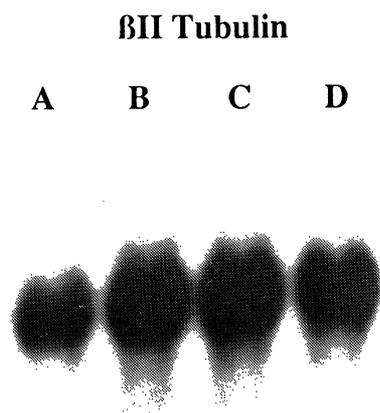
The  $\beta_{III}$ -tubulin mRNA changes that were observed using Northern blotting were more variable than the  $\beta_{II}$ -tubulin or NF mRNA patterns. One of the blots showed an apparent increase in  $\beta_{III}$ -tubulin mRNA levels in the cut/ligation axotomy sample but not in the crush axotomy DRG relative to controls (Figure 5). The second blot showed an apparent decrease in  $\beta_{III}$ -mRNA levels in the cut/ligation axotomy sample relative to controls (data not shown). Thus, the averaged densitometric values indicated no change in  $\beta_{III}$ -mRNA levels in the cut/ligation axotomy condition relative to control (average ratio of 1.07). The crush axotomy DRG  $\beta_{III}$ -mRNA levels were also similar to control levels at 12 weeks post-axotomy (average ratio of 1.08, Figure 6). The lack of consistency in the  $\beta_{III}$ -tubulin mRNA expression pattern in cut/ligation axotomy DRG samples between the two independent Northern blots (each made using 4



**Fig. 3:** Autoradiograms of DRG neurons after *in situ* hybridization with a  $^{35}\text{S}$ -labeled NF-L cDNA probe. (A) uninjured contralateral DRG neurons from the cut/ligation axotomy condition; (B) Axotomized neurons from the cut/ligation condition at 12 weeks after the lesion; (C) uninjured contralateral DRG neurons from the crush axotomy condition; (D) crush axotomized DRG neurons 12 weeks after lesion.

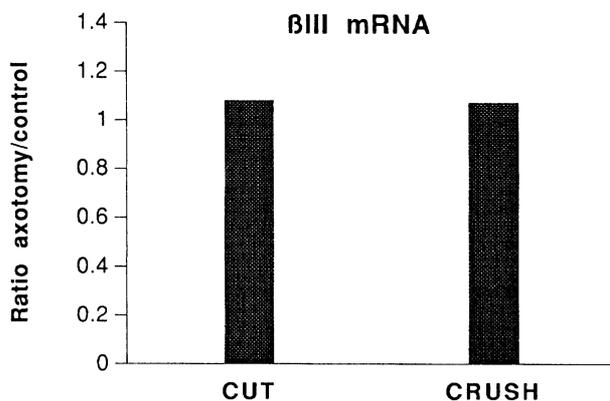
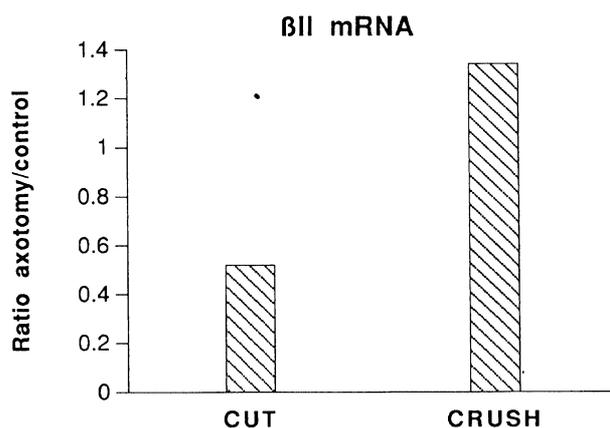


**Fig. 4:** NF-L mRNA levels in axotomized and control side DRG neurons at 12 weeks post-axotomy as assessed by *in situ* hybridization. Average grain densities and standard errors of the large-sized ( $> 1000 \mu\text{m}^2$ ) DRG neurons in the axotomy side and contralateral control side are plotted; asterisks indicate significant differences ( $p < 0.01$ ) between axotomy and contralateral control groups.



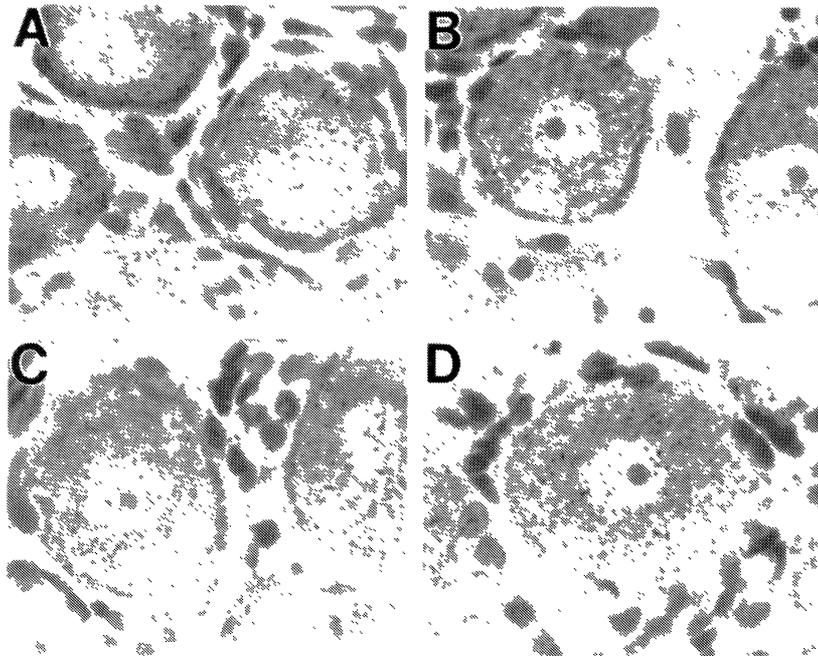
**Fig. 5:** Autoradiograms of Northern blots showing changes in  $\beta_{II}$  and  $\beta_{III}$ -tubulin mRNA levels in the DRG 12 weeks after either cut/ligation or crush axotomy. Equal amounts of total RNA (10  $\mu$ g) were loaded and probed with the indicated cDNAs.

pooled DRG from different animals for a given RNA sample) was curious since virtually identical patterns of change were observed using these same blots in hybridizations with NF-L, NF-M and  $\beta_{II}$ -tubulin probes. Reprobings of the blots with  $\beta_{III}$  probe showed virtually identical patterns as the initial hybridizations. Thus, the results from  $\beta_{III}$  experiments were not conclusive.



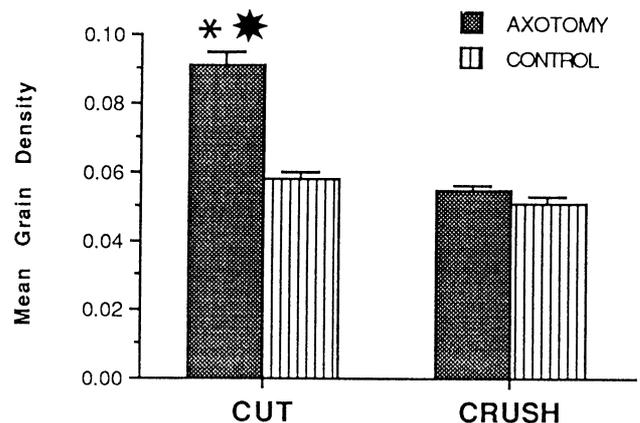
**Fig. 6:** Densitometric analysis of  $\beta_{II}$  and  $\beta_{III}$  mRNA levels in axotomized DRG neurons at 12 weeks post-injury. Autoradiograms of Northern blots were scanned and mean density values ( $n = 2$ ) in axotomized DRG samples are expressed as a ratio of contralateral control side values for each of the two axotomy conditions.

*In situ* hybridization was used to examine the long-term changes in  $\beta$ -tubulin expression specifically in the large-sized ( $> 1000 \mu\text{m}^2$ ) DRG neurons. The cDNA probe (RBT1) used for *in situ* hybridization is a coding region probe that potentially recognizes all  $\beta$ -tubulin mRNA species. However, on Northern blots washed to the high stringency conditions used in our *in situ*



**Fig. 7:** Autoradiograms of DRG neurons after *in situ* hybridization with a  $^{35}\text{S}$ -labeled  $\beta$ -tubulin cDNA probe. (A) uninjured contralateral DRG neurons from the cut/ligation axotomy condition; (B) axotomized DRG neurons from the cut/ligation axotomy condition 12 weeks after the lesion; (C) uninjured contralateral DRG neurons from the crush axotomy condition; (D) DRG neurons from the crush axotomy condition at 12 weeks after lesion.

hybridization protocol, only a 1.8-2 kb band is seen (data not shown). Previous studies have shown that the  $\beta_{\text{II}}$  and  $\beta_{\text{III}}$ -tubulin mRNA species in the DRG are both within this size range while the  $\beta_{\text{I}}$  and  $\beta_{\text{IV}}$ -tubulin mRNAs are larger [14]. Thus, we assume that the *in situ* signal reflects mainly the contribution from both the  $\beta_{\text{II}}$  and  $\beta_{\text{III}}$ -tubulin mRNAs. Figure 7 shows representative autoradiograms of experimental and control neurons in the two different axotomy conditions. Grain counting analysis of this material showed that the average grain density of the large DRG neurons 12 weeks after cut/ligation axotomy was 156% of that in contralateral control neurons (Figure 8). Statistical evaluation indicated that this difference was significant ( $p < 0.01$ ). On the other hand, overall  $\beta$  tubulin mRNA levels were essentially the same in the large DRG neurons at 12 weeks after crush axotomy condition as they were in contralateral control neurons (mean grain density ratio in axotomy: control neurons of 1.08, Figure 8).



**Fig. 8:** Quantification of  $\beta$ -tubulin mRNA levels in axotomized and contralateral control DRG neurons at 12 weeks post-injury by *in situ* hybridization. Average grain densities and standard errors of the large-sized ( $> 1000 \mu\text{m}^2$ ) DRG neurons in the axotomy side and contralateral control side are plotted. Asterisk indicates a significant difference ( $p < 0.01$ ) between axotomy and control; star indicates a significant difference ( $p < 0.01$ ) between the two axotomy groups.

## DISCUSSION

This study examined the extent to which recovery of normal patterns of gene expression occurred in DRG cells after axotomizing injuries that resulted in either axonal regeneration or long-term regenerative failure due to neuroma formation. The comparisons made were of steady-state NF and tubulin mRNA levels in the DRG at 12 weeks following a crush lesion of the sciatic nerve (regeneration occurs) with those in DRG neurons at the same interval after a cut/ligation injury of the sciatic nerve with removal of a segment of the distal nerve stump (no regeneration occurs). Comparisons of cytoskeletal gene expression in the DRG after these two types of lesion conditions had recently been made at earlier times after axotomy when most cytoskeletal changes are maximal /15/. In that study, the responses of DRG cells to cut/ligation axotomy were found to be more pronounced than the responses to crush axotomy. Comparisons of long-term changes in gene expression in the DRG system after various axotomies had not been made previous to this study. At the 12 week post-injury time examined in this study, it is likely that regeneration of DRG axons after crush lesion was completed since the fastest growing fibers are known to elongate in the sciatic nerve at 4 mm/day /20,29/. However, many axons do grow somewhat more slowly than this /20/, but even that axon population can be assumed to have traversed the distal nerve and reached appropriate targets by 12 weeks post-crush. The present study indicates that the recovery of "normal" patterns of cytoskeletal gene expression in the DRG is considerably more complete after crush injury than after cut/ligation injury. However, even at long intervals after nerve crush, aspects of the cytoskeletal expression pattern in the DRG (NF mRNA levels in large-sized neurons) remained subnormal. This suggests either that these components never recover fully following axonal injury, or that the maturation of regenerated axons in the crush injury condition was still not complete 12 weeks after the injury.

Results of Northern blotting experiments in the present study showed that NF-L and NF-M mRNA levels in the DRG were approaching control levels at 12 weeks after crush axotomy, but were still substantially reduced at this time after cut/ligation

injury. The results of *in situ* hybridization experiments to specifically examine NF-L mRNA levels in the large-sized DRG neurons showed a significant reduction in both lesion conditions with no significant difference between the two lesion conditions. This modest discrepancy between the Northern blotting experiments which examined mRNA changes in the DRG as a whole and *in situ* hybridization experiments which focused only on the large cells and not the medium or small-sized DRG neurons might be due to a differential cell loss in the DRG after the two types of lesions. Theoretically, loss of many large-sized DRG neurons (which contain the majority of the NF mRNA in the DRG) in the cut/ligation, non-regenerating, condition would affect Northern blotting results more than *in situ* hybridization results. This is because *in situ* hybridization studies examined NF mRNA expression only in large cells that had survived the injury while the Northern blotting experiments utilized total RNA extracted from whole DRGs which, in the cut/ligation condition, may have proportionately less "signal" than normal contributed by large cells (due to cell loss).

At the outset of this study, a number of earlier observations fueled our hypothesis that the recovery of gene expression in DRG cells at long intervals after axotomy would differ depending on whether or not regeneration occurred. For example, crush lesions of peripheral nerves are followed by axonal regeneration without significant neuronal cell loss while cut injury results in significant neuronal loss /2,27/. In the DRG system, estimates of 10-30% neuronal cell death after sciatic transection (cut) have been reported /1,10,23,26/. In addition, axonal atrophy of DRG axons is reportedly more pronounced after cut lesions compared with crush lesions /1,10/. Interestingly, sensory neurons appear to be more vulnerable than motor neurons to long-standing cut axotomies. For example, axonal atrophy and reduction in NF numbers /7/, and decreases in conduction velocity /11/ are more prominent in sensory nerve fibers than in motor fibers of the sciatic nerve after transection when regeneration is prevented. Cytoskeletal gene expression, and NF gene products in particular, are known to regulate axon caliber /12,13,17/. The long-term changes in cytoskeletal gene expression

in DRG neurons after cut vs. crush injury are likely to be directly involved in generating the axon caliber changes that have been observed in DRG axons at long times after different types of axonal injury.

The long-term effects of axonal injuries which are followed by axonal regeneration vs. non-regeneration on tubulin gene expression in the DRG were also examined in this study. For the tubulin mRNA changes, apparent discrepancies in the results of Northern blotting and *in situ* hybridization experiments were found. *In situ* hybridization data derived from the large-sized DRG neurons using a coding region  $\beta$ -tubulin cDNA which recognizes both  $\beta_{II}$  and  $\beta_{III}$  mRNAs indicated that tubulin mRNA levels had returned to control levels in surviving large-sized DRG neurons after crush axotomy but were significantly higher than control in surviving large DRG cells 12 weeks after cut/ligation axotomy. In contrast, Northern blotting results indicated that  $\beta_{II}$ -tubulin mRNA levels in the DRG as a whole were substantially lower than control in cut/ligation axotomized samples while  $\beta_{III}$ -tubulin mRNA levels were quite variable. Northern blotting also indicated that  $\beta_{II}$  mRNA levels in the crush axotomy condition remained elevated relative to control. Several factors may account for the differences observed using the two methods to examine cytoskeletal mRNA levels. Again, the argument that long-standing cut/ligation injury leads to preferential cell death of the largest-sized DRG neurons compared with the smaller-sized cells could be raised. Substantial levels of tubulin mRNA are expressed in the large DRG neurons and preferential loss of these cells could alter the overall DRG expression pattern that is observed when total DRG-derived RNA is examined by Northern blotting. Such an event would be unlikely to affect the *in situ* analysis where only large cells are examined. Careful examination of published data from a study that examined long-term cell loss in the DRG when sciatic nerve transections were done in young rats reveals a more substantial loss of the largest-sized DRG cells compared with smaller neurons (see Figure 3 in /24/). However, in another study, no evidence for a selective loss of large neurons in the L5 DRG was found when adult neurons were axotomized by cut/ligation, although a significant loss of large cells was observed when

such lesions were made in neonatal rats /10/. Further study of the issue of cell survival in the DRG after different types of injuries is needed to validate the selective cell loss argument.

Another possibility for the discrepancies in the Northern blotting and *in situ* hybridization results in the present study is that the molecular response profile of the small-sized DRG cells differs from that of larger cells at long times after axotomy. In fact, different responses of small vs. large DRG cells to axotomy have already been reported at shorter intervals after axotomy (2 weeks) in the case of peripherin gene expression in the DRG /22/. Since we did not examine tubulin mRNA expression in the small cells by quantitative grain counting of the *in situ* hybridization material in the present study, this possibility for the tubulin mRNA level changes remains to be examined in the future.

The finding from *in situ* hybridization experiments that the surviving large-sized DRG cells after cut/ligation injury exhibit increased levels of  $\beta$ -tubulin mRNA 12 weeks after injury is of interest. It is possible that these large neurons are continuing to mount a regenerative effort even at these long intervals after growth has been blocked by neuroma formation. Lower than normal NF mRNA levels and higher tubulin mRNA levels in the surviving cut/ligated large DRG neurons may reflect the continued presence of signals that perpetuate a "growth state" in these cells for long intervals after injury. In contrast, the recovery of tubulin mRNA levels in the large cells at 12 weeks after crush injury (*in situ* hybridization data) indicates that such signals have subsided, presumably because regeneration was completed and the neurons had returned to a non-growing state. Is there evidence that DRG neurons are still trying to regrow axons even in long-standing neuromas after cut/ligation injury? In fact, studies have shown that massive sprouting efforts from preterminal axons entrapped in neuromas occurs 2-3 months into the post-operative period /9/. The significance of this late sprouting phenomenon to the clinical dilemma of pain associated with long-standing neuromas /25/ is still unclear. However, the occurrence of such late sprouting events is consistent with the findings of long-term changes in cytoskeletal gene expression in the DRG after cut/ligation injury of the sciatic nerve in the present study.

Future studies directed at elucidating the factors/signals responsible for the long-term changes in gene expression in the DRG following various types of injury are needed. Clearly, the role of neurotrophic factors (or lack thereof) will be of interest since such molecules are known to mediate a variety of cellular responses when complexed with their receptors. Examinations of the distribution of neurotrophic factor receptors in the DRG have shown that large vs. small DRG cells have a different complement of receptors /6,8/. This in turn may affect the expression of neurotransmitters and perhaps cytoskeletal genes in these neurons under normal conditions as well as after injury. For example, small neurons in the DRG which express substance P and have high affinity NGF receptors may respond differently to long-term removal of normal levels of NGF after axotomies than do large-sized DRG cells to withdrawal of their specific trophic factor(s). Future studies that attempt to modulate gene expression in different classes of DRG cells with therapeutic doses of specific neurotrophic factors will be of great interest. Such approaches may also serve to modify the cell survival profiles after traumatizing injuries and thus will be useful in elucidating the role of various classes of DRG cells to the long-term recovery patterns of gene expression after crush vs. cut/ligation injuries.

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