Response of Facial and Rubrospinal Neurons to Axotomy: Changes in mRNA Expression for Cytoskeletal Proteins and GAP-43

Wolfram Tetzlaff,¹ Sarah W. Alexander,¹ Freda D. Miller,² and Mark A. Bisby³

¹Departments of Anatomy and Pathology, University of Calgary, Calgary, Alberta, Canada T2N 4N1, ²Departments of Anatomy and Cell Biology, University of Alberta, Edmonton, Alberta, Canada T6G 2R7, and ³Department of Physiology, Queen's University, Kingston, Ontario, Canada K7L 3N6

Neurons confined within the mammalian CNS usually do not regenerate after axonal injury, while axonal regeneration is the rule in the PNS. It has been hypothesized that this may be related to differences in the microenvironment of the PNS versus CNS and to differences in the neuronal response to injury. In order to test the latter hypothesis, we compared changes in gene expression after axotomy in two populations of neurons: rat facial motoneurons and rat rubrospinal neurons. *In situ* hybridization with cDNA probes for the medium and light neurofilament protein revealed a reduced mRNA content in both facial and rubrospinal neurons at all times investigated (i.e., 1, 2, and 3 weeks after axotomy). On the other hand, mRNAs for actin and tubulin were increased in both neuronal populations during the first week after axotomy. While this increase was sustained in facial motoneurons for several weeks, total tubulin mRNA and actin mRNA were decreased in rubrospinal neurons at 2 and 3 weeks after axotomy, coincident with their atrophy. The developmentally regulated α 1 tubulin mRNA, which was previously shown to be reexpressed in facial motoneurons after axotomy, was elevated severalfold in axotomized rubrospinal neurons, and increased levels persisted in some rubrospinal neurons as late as 7 weeks after axotomy. Similarly, the developmentally regulated GAP-43 mRNA increased in both axotomized facial and rubrospinal neurons, and increased levels were sustained in some axotomized rubrospinal neurons for at least 7 weeks. The response of rubrospinal neurons to axotomy in the cervical spinal cord is, in the first week, qualitatively similar to the response of facial motoneurons. However, by 2 weeks after axotomy there is a generalized reduction in mRNA levels for all three cytoskeletal proteins that is associated with neuronal atrophy. During this period, mRNA levels for the two specific markers of the

growth state, α 1 tubulin and GAP-43, remain elevated. Thus, axotomy of rubrospinal neurons appears to set in motion two independent events. First, an axotomy signal initiates a cell-body reaction similar to that of PNS neurons, including increased mRNA levels for α 1 tubulin and GAP-43. Later, a generalized cellular atrophy and decrease in mRNA levels occur without reversing the specific responses of α 1 and GAP-43 to axotomy. We conclude that the failure of rubrospinal neurons to regenerate is not due to a failure to initiate gene-expression changes characteristic of regenerating peripheral neurons. However, the inability to sustain increased tubulin and actin expression may be related to the abortion of initial sprouting responses of the injured axons.

Mammalian neurons projecting into the PNS (extrinsic neurons) are usually able to regenerate a new axon when lesioned, while those confined within the CNS (intrinsic neurons) typically do not regenerate (Ramon y Cajal, 1959). The reasons for this are poorly understood, and neuronal factors as well as environmental factors are probably involved (Barron et al., 1976, 1977, 1985, 1986, 1989; Richardson et al., 1980; Skene and Willard, 1981; Berry et al., 1986; Politis and Spencer, 1986; Vidal-Sanz et al., 1987; Villegas-Peres et al., 1988; for review, see Aguayo, 1985, 1987; Barron, 1983a,b, 1989; Caroni and Schwab, 1988a,b; Bray and Aguayo, 1989). Extrinsic neurons, which are capable of regeneration, respond to axonal lesions with a variety of biochemical and morphological changes, commonly referred to as cell-body response or axonal reaction (for review, see Lieberman 1971, 1974; Grafstein and McQuarrie, 1978; Kreutzberg, 1982; Barron, 1983a,b, 1989; Grafstein 1983, 1986; Tetzlaff et al., 1986). In regenerating extrinsic neurons and regenerating neurons of lower vertebrates, this cell-body response includes dramatic changes in cytoskeletal protein synthesis (Hall et al., 1978; Burrell et al., 1979; Hall, 1982; Quesada et al., 1986; Tesser et al., 1986; Greenberg and Lasek, 1988; Oblinger and Lasek, 1988; Tetzlaff et al., 1988) and cytoskeletal mRNA expression (Neumann et al., 1983; Hoffman et al., 1987; Wong and Oblinger, 1987; Goldstein et al., 1988; Hoffman and Cleveland, 1988; Hoffman, 1989; Miller et al., 1989; Oblinger et al., 1989; Muma et al., 1990; Verge et al., 1990b), as well as the expression of growth-associated proteins such as GAP-43 (Skene and Willard, 1981; Redshaw and Bisby, 1984a; Bisby, 1988; Hoffman, 1989; Tetzlaff et al., 1989; Van der Zee et al., 1989; Verge et al., 1990a; for review, see Benowitz and Routtenberg, 1987; Skene, 1989). In contrast, most studies on intrinsic neurons of the mammalian CNS did not find an increased expression of GAP-43 after axotomy. Axotomy of corticospinal

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Correspondence should be addressed to Dr. Wolfram Tetzlaff, Department of Anatomy, University of Calgary, 3330 Hospital Drive Northwest, Calgary, Alberta, Canada T2N 4N1.

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neurons and retinal ganglion cells at long distances from their cell bodies failed to produce increased amounts of axonally transported GAP-43 (Skene and Willard, 1981; Redshaw and Bisby, 1984b; Kalil and Skene, 1986; Reh et al., 1987). This correlates with the failure of these neurons to regenerate into peripheral nerve transplants, which provide a permissive growth environment (Richardson et al., 1982, 1984). Recently, Lozano et al. (1987) reported increased GAP-43 immunoreactivity in retinal ganglion cells when axotomized close to the eye, and a significant number of retinal ganglion cells regenerated their axons into peripheral transplants when these were offered close to the retina (Vidal-Sanz et al., 1987). These observations support the hypothesis that the expression of growth-associated proteins, such as GAP-43, represents a prerequisite for axonal regeneration and that the failure to express these genes by CNS neurons after injury relates causally to their failure to regenerate (Skene and Willard, 1981; for reviews, see Barron, 1983a,b, 1989; Skene, 1989). In order to test this hypothesis further, we have now compared changes in cytoskeletal and GAP-43 mRNAs in axotomized neurons of the facial nucleus (extrinsic) and the red nucleus (intrinsic) of the rat using *in situ* hybridization techniques. The neuron cell bodies of both systems are located within the CNS and are therefore exposed to a common CNS microenvironment.

The facial nerve projects into the PNS and its axons regenerate successfully when crushed at the stylomastoid foramen (Tetzlaff and Bisby, 1989). A further reason for choosing the facial nucleus was that it can easily be isolated by microdissection and analyzed with biochemical techniques (Tetzlaff and Kreutzberg 1984, 1985). In agreement with studies of peripheral ganglia (Hoffman et al., 1987; Greenberg and Lasek, 1988; Oblinger and Lasek, 1988), we found an increased ^{35}S -methionine incorporation into actin and tubulin and a decreased incorporation into neurofilament proteins after axotomy (Tetzlaff et al., 1988). Here, we will show that these previously determined changes in methionine incorporation correlate closely with changes in mRNA levels within the axotomized motoneurons using quantitative *in situ* hybridization and Northern blot analysis.

These results provide essential baseline data for comparison with the responses of the intrinsic neurons of the red nucleus to axotomy. The rubrospinal system of the rat was chosen because it is composed of a circumscribed, easily identifiable group of neurons within the midbrain amenable to image analysis. The axons are almost completely (>99%) crossed (Brown, 1974; Huisman et al., 1981), and thus only those rubral neurons contralateral to a spinal hemisection are axotomized. The caudal magnocellular pole is anatomically well defined (Reid et al., 1975a,b), and the comparison with a nonaxotomized contralateral side within the same brainstem section is possible. The rubrospinal axons do not regenerate successfully across a spinal cord lesion in the adult (Barron et al., 1989), but they do display some regenerative potential, sending out numerous sprouts soon after injury (Barron et al., 1989), and some can regenerate into the permissive environment of a peripheral nerve transplant (Richardson et al., 1984). Nevertheless, previous investigations of injured rubral neurons have so far indicated that their response to injury is "regressive" in nature: no increase in RNA synthesis is seen (Barron, 1983a,b, 1989; Barron et al., 1976, 1989); the cells shrink, but little neuronal death occurs (Prendergast and Stelzner, 1976; Barron et al., 1989; Xu and Martin, 1990). With this background, we expected to see major differences in the expression of cytoskeletal proteins and GAP-43

mRNAs in rubrospinal axons after axotomy, when compared with facial motoneurons. Our results show that the initial response to axotomy is qualitatively similar in the two types of neurons, but that during the second week after axotomy, rubrospinal neurons reduce expression of tubulin and actin mRNAs to subnormal values. Although these intrinsic neurons initially respond appropriately to axon injury by increased expression of regeneration-associated genes, this is not sufficient to permit their regeneration in the absence of a supportive environment.

Materials and Methods

The cDNA clones were a generous gift of Dr. J.-P. Julien [medium-molecular-weight neurofilament protein (NFM); see Julien et al., 1986], Dr. N. Cowan (NF68; see Lewis and Cowan, 1985; $\text{M}\alpha 1$ α -tubulin and $\text{M}\beta 2$ β -tubulin; see Lewis et al., 1985), Dr. G. Schulz (actin; see Minty et al., 1981), and Dr. Pate Skene (GAP-43; Basi et al., 1987). Fluorogold was obtained from Fluorochrome Inc., Englewood, Colorado, and fast blue was from Sigma, St. Louis, Missouri.

Animal surgery. Male Sprague-Dawley rats (230–300 gm) were used throughout this study. They were kept in a 14-hr:10-hr light/dark cycle and fed a standard diet and water ad libitum.

Facial nerve lesions. The rats were anesthetized with sodium pentobarbital (32 mg/kg) plus chloral hydrate (150 mg/kg), and the left facial nerve was exposed at the stylomastoid foramen. For *crush lesions*, the nerve was crushed twice with a pair of Dumont #5 forceps for 30 sec, 2–3 mm distal from the stylomastoid foramen (about 12–14 mm distal to the motoneurons in the brainstem). In selected animals, facial nerves distal to the lesion were processed for immunocytochemical staining for neurofilaments 2 d after lesioning to verify that the nerves were completely crushed (data not shown). For *nerve resections*, the nerve was transected 2–3 mm distal from the stylomastoid foramen, and a 1-cm segment distal to the transection was excised in order to impede regeneration. The animals were killed between 12 hr and 28 d after facial nerve lesion by intracardiac perfusion with 0.1 M phosphate-buffered (pH 7.4) 4% paraformaldehyde under deep chloral hydrate anesthesia. In some cases, the brainstem was removed fresh and rapidly frozen.

Rubrospinal tract lesions. The rats were deeply anesthetized with sodium pentobarbital (32 mg/kg) plus chloral hydrate (150–180 mg/kg) and under strictly aseptic conditions the C3 vertebrae were laminectomized to expose the spinal cord. The left lateral funiculus of the spinal cord was transected with a #11 scalpel blade. In some animals, a 1- μl depot of Fluorogold (5%) or fast blue (3–5%; Kuypers and Huisman, 1984) was applied to the lesion site by using a small piece of dye-soaked Gelfoam. This application allowed the positive identification of rubrospinal neurons through retrograde transport from the lesion site and is compatible with the *in situ* hybridization procedure. The gap in the dura was covered with Gelfoam, and subsequently the muscular layers and the skin were sutured. During the postoperative period, bladder function was usually not impaired, but was replaced by manual expression of urine if necessary. In rare cases of wound infections, the rat was immediately killed. The extent of the spinal cord lesion was examined post-mortem in some animals. In most animals, it included the lateral, anterior, and parts of the dorsal funiculi. Thus, the lesion was in most cases an incomplete hemisection and included in all cases the lateral funiculus.

In situ hybridization. The protocol of Miller et al. (1987) was used, with some modifications. The rats were killed by paraformaldehyde perfusion, and the cryoprotected brains were rapidly frozen in dry ice-cooled isopentane, then cut on a cryostat at 10 μm . In the rubrospinal series, only those sections of the caudal magnocellular pole of the red nucleus (most caudal 400 μm) were selected and analyzed. Prehybridization solution contained 50% formamide, 25 mM PIPES (Boehringer Mannheim) buffer (pH 6.8), 25 mM EDTA, 0.75 M NaCl, 5 $\times$ Denhardt's solution (Maniatis et al., 1982), 100 mM dithiothreitol, 250 $\mu\text{g}/\text{ml}$ tRNA from yeast, and 250 $\mu\text{g}/\text{ml}$ salmon sperm DNA (denatured). For hybridization, the same solution was used containing 10–20 ng/ml ^{35}S -radiolabeled cDNA probe prepared by random hexanucleotide priming (Feinberg and Vogelstein, 1983).

Controls were incubated with ^{35}S -labeled plasmid DNA and revealed low background labeling with no specific pattern. Moreover, pretreatment of the slides with RNase A eliminated the signals over the neurons and left behind background labeling.

Radioautography. After *in situ* hybridization, the slides were exposed to Kodak XAR5 film for 1–3 d. These film radioautographs gave an idea of the strength of the radioactive signal and allowed an estimation of an appropriate exposure time for emulsion radioautography. The latter was done according to routine protocols by dipping the slides in Kodak NTB2 emulsion diluted by 1:1 with distilled water at 42°C. After 1–4 weeks exposure, the slides were developed in D19 for 3 min at 20°C and fixed in Kodak Rapidfix. The facial nucleus slides were subsequently stained with cresyl violet, dehydrated, and embedded in Permount or DePeX (BDH). Because the fluorescent retrograde tracers used in the rubrospinal study were easily washed out during hybridization, staining, and dehydration, these sections were analyzed first in the fluorescence microscope (Leitz Orthoplan, Ploemopak Filter A) in order to identify and map the labeled rubrospinal neurons. After *in situ* hybridization, prior to staining, photography using epifluorescence for the label combined with dark-field illumination for the silver grains was carried out.

Quantification of *in situ* hybridizations. Silver grains were quantitated by measuring the relative fraction of the cytoplasmic area of individual neurons that was occupied by emulsion grains using a computerized image analysis system that we have used in previous studies (Verge et al., 1990a,b). Because the selection of density levels of cells used for the measurement of the area fraction occupied by grains could be subject to bias on the part of the observer, all identification tags on the slides were obliterated after coding; thus, the observer was unaware of experimental procedure, survival time, and probe used. The mean values obtained by two independent observers from the same section differed by less than 20%. The cytoplasmic and nuclear areas were measured for each neuron, permitting the calculation of the cytoplasmic volume, assuming that the cell and nucleus were approximately spherical. Because neurons change size after axotomy, the same amount of signal per cell can appear diluted (smaller fraction of the measured area occupied by grains) if the cell swells, or can appear concentrated if the cell shrinks. In order to get an estimate of the total grain signal per cell, the fractional area occupied by grains was multiplied by the cytoplasmic volume. This calculated signal per cell (counts per cell) was plotted against the calculated cytoplasmic volume of the cell. Finally, the mean counts per cell of measured neurons from the experimental side (injured) were expressed as a percentage of the equivalent mean value from the control side. Thus, each point in the diagrams showing the percentage of control is based on 20–30 cells from each side of an individual animal. In the case of the red nucleus, the analysis was restricted to the caudal 400 μ m of the red nucleus, which contains mainly magnocellular neurons that project to the spinal cord. In cases where retrograde tracers had been applied, only labeled neurons were analyzed. These sections confirmed that the majority (>90%) of magnocellular neurons were axotomized by cervical lesions at C3. Because the caudal pole of the red nucleus contains fewer neurons than the facial nucleus, only 10–30 cells from each animal could be quantitated after hybridization with one of the seven different cDNA probes used. Student's *t* test was applied to the mean counts per cell values (not the normalized data) to detect significant changes between experimental and control neurons. To detect significant differences between percentage changes for different probes (e.g., α -tubulin vs. β -tubulin), the nonparametric Wilcoxon–Mann–Whitney test was used. The criterion for significance was $p < 0.05$.

The comparison with the contralateral side implies that the effects of injury are entirely unilateral, which remains to be shown for each mRNA analyzed here. Variations between animals and between slides did occur, and in order to compare two animals, sections of each have to be on the same slide and processed identically. Thus, a control series of four unoperated and four unilaterally lesioned (facial nerve) rats was generated separately in order to search for possible contralateral effects of axotomy. Sections from one unlesioned and one lesioned animal were mounted on the same slide and subjected to identical hybridization conditions. There was no indication of a contralateral increase 7 d after facial nerve axotomy (data not shown). Because this result contrasted

with the film-radioautographic study on α -tubulin expression in the rat facial nucleus by Pearson et al. (1988), we performed film radioautography, light microscopical analysis, and RNA dot blot analysis on facial nuclei of operated versus unoperated rats and could not find any evidence for a contralateral effect at 7 d after lesion (data not shown). Therefore, we consider the expression of our data in the form of “percentage of contralateral” is justifiable. If a small, nondetectable contralateral effect existed, the axotomy-induced changes presented in this study would be even greater.

RNA blotting. Total cytoplasmic RNA was extracted from four pooled isolated facial nuclei according to the phenol/chloroform/isoamyl alcohol technique of Schibler et al. (1980). Equal amounts of RNA were fractionated by electrophoresis on 1.2% agarose gels in the presence of 1 M formaldehyde (Rave et al., 1979) and blotted onto nitrocellulose by capillary transfer (Thomas, 1980). The filters were covered for 2 hr at 43°C in prehybridization solution (see above). Hybridization was performed for 12–16 hr using the same solution with the addition of a 32 S-labeled cDNA probe (5×10^6 cpm/ml, 10–20 ng probe/ml; see above). Posthybridization washes were carried out with 4x, 2x, 1x, 0.5x, and 0.1x SSC (Maniatis et al., 1982) at 45°C. Dried filters were exposed to Kodak XAR5 x-ray film for 1–2 weeks and developed. The signals were quantified by densitometry using an LKB laser densitometer.

Results

Changes in neurofilament mRNA

Facial motoneurons. Changes in mRNA levels for the light-molecular-weight neurofilament (NFL) and NFM were analyzed between 12 hr and 21 d after facial nerve axotomy. An example of *in situ* hybridization to NFM is displayed in Figure 1, *A* and *B*. The axotomized motoneurons are increased in size and contain a lower density of silver grains (Fig. 1*A*).

Figure 2, *A* and *B*, shows the results of quantification of the *in situ* hybridizations. The relative decrease of the NFL and NFM hybridization signals was already apparent 12 hr after axotomy, when levels were at 74–80% of control; however, none of these values were significantly different from contralateral. By 24 hr, the average neuronal neurofilament mRNA content was between 45% and 92% of contralateral, and this difference was significant ($p < 0.05$) in some animals. NFM and NFL hybridization signals dropped further to reach levels between 10% and 40% on days 5 and 7. In cases of successful regeneration (i.e., after a crush lesion), the values recovered to between 43% and 82% by 14 d and 71% and 113% (78–145%, NFL) by 21 d. This is in contrast to those cases where resection prevented successful regeneration; for these, the signal levels stayed between 5% and 30% for both messages.

Northern blot analysis for NFM (Fig. 3) revealed a decrease to 20.5% of the control side 7 d after resection (Fig. 2*B*, solid star) and to 66% after crush (open star).

Rubrospinal neurons. The *in situ* hybridization signal for NFM and NFL mRNA was decreased at all time points. This is obvious in the dark-field micrograph shown in Figure 4, *A* and *B*, taken 10 d after rubrospinal tract lesion and in the bright-field micrographs in Figure 4, *C* and *D*. (The combination of fast blue and NFM *in situ* hybridization is displayed in Fig. 9*A,B*.) Most labeled cells display reduced grain densities. The quan-

Figure 1. Light microscopic radioautography of facial motoneurons after *in situ* hybridization with probes to NFM (*A, B*), actin (*C, D*), α -tubulin (*E, F*), and GAP-43 (*G, H*). The axotomized facial motoneurons are displayed on the left, and their contralateral counterparts are presented on the right. *A* and *G* were taken at 7 d after resection; *C* and *E*, at 5 d after resection. The perikarya are enlarged on the axotomized side. Note the decreased NFM hybridization signals over the axotomized motoneurons (*A*), while the actin (*C*), α -tubulin (*E*), and GAP-43 (*G*) hybridization signals are increased. Little signal is associated with glial cells. The arrow in *B* points to a presumed dendritic profile. Scale bars: 50 μ m for *A, B, E–H*; 20 μ m for *C, D*.

Figure 2. Quantification of *in situ* hybridizations of facial motoneurons with cDNA probes to NFL (A), NFM (B), actin (C), α -tubulin (D, E), and GAP-43 (F). D gives an example of the analysis of individual motoneurons. The cell volume is the cytoplasmic volume and is plotted against the number of counts per cell. In D, solid circles represent individual axotomized neurons, and open circles, contralateral intact neurons. Note the increase in cell volume and counts/cell for α -tubulin mRNA, 5 d postcrush. The boxes in D indicate mean $\pm$ SEM for volume and counts. Each symbol in A–C, E, and F is based on a similar scatterplot (>25 cells) from individual animals and represents the mean counts/cell for axotomized neurons expressed as percentage of mean counts in contralateral neurons from the same animal. Open symbols, facial nerve crush; solid symbols, resection; vertical bars, SEM. Values above the small broken lines were significantly different from contralateral (t test, $p < 0.05$). The stars represent percentage change of the Northern blot signal of pooled facial nuclei taken 7 d after nerve crush; Northern blots are shown in Figure 3.

tification of these neurofilament hybridizations revealed values around 25–65% during the first week (Fig. 4E,F). In all except one animal, significant reductions in neurofilament mRNA were found at 14 d and 21 d. By this time, values around 20–40% of contralateral were typically observed (Fig. 4F).

Changes in actin mRNA

Facial motoneurons. Actin mRNA hybridization signals were increased in facial motoneurons after axotomy. By 7 d, the axotomized motoneurons were larger and more basophilic, but

Figure 3. Northern blot analysis of total RNA from four pooled facial nuclei transferred onto nitrocellulose and probed with the α -tubulin (α -Tub), actin (ACT), NFM, and GAP-43 cDNA probes. In case of actin and NFM, the same membrane was hybridized twice, and RNA from an equal volume of tissue from the reticular formation located between the facial nuclei was loaded in the *middle lane*. All probes give a single hybridization band at 1.8 kb, 1.7 kb, 3.4 kb, and 1.4 kb, respectively. The intensity of the actin, α -tubulin, and GAP-43 signal increased in the axotomized (crush) facial nucleus, while the NFM signal decreased. The percentage changes are displayed as stars in Figure 2. *ax*, axotomized (crush), 7 d; *c*, contralateral, intact.

did not display the classical appearance of chromatolysis (Fig. 1C,D). They contained significantly more silver grains per cell than the contralateral counterparts (Fig. 1C,D). Only a few grains were associated with glial cells (Fig. 1C,D). Quantification of the actin *in situ* hybridization data is displayed in Figure 2C. Actin *in situ* hybridization signals were between 95% and 117% of contralateral 12 hr after lesion. The values varied between 100% and 145% of contralateral on day 1 and increased to around 200% (range, 106–269%) on days 2, 3, 5, and 7. With the exception of two animals (7 d after crush), data from all others showed significantly higher values for axotomized neurons than for contralateral unoperated neurons. At 14 d, actin mRNA signal was between 193% and 413% of contralateral (all $p < 0.02$), with no obvious difference between animals that received facial nerve crush or resection. At 21 d, values between 90% and 275% were found. Regardless of the type of lesion, the mRNA levels appeared to be returning to normal.

Densitometric analysis of Northern blots of four pooled facial nuclei revealed an increase to 165% of contralateral 7 d after crush. This is represented as the star in Figure 2C. The Northern blot is shown in Figure 3 and demonstrates that only a single message size (≈ 1.7 kilobases) hybridized with the actin probe, which corresponds to the size of the α -actin mRNA (Minty et al., 1981).

Rubrospinal neurons. Seven days after lesion, rubrospinal neurons revealed a clear increase of the silver grains reflecting actin hybridization signal over the axotomized cells when viewed under dark-field illumination (Fig. 5A,B). In bright-field illumination, these neurons clearly showed central chromatolysis and higher grain density (Fig. 5C,D). Silver grain quantifications revealed a significant increase by day 8 to about twofold (Fig. 5E,F). However, this actin mRNA increase was not seen in all

cells (Fig. 5E), and in animals with retrograde labeling from the lesion site, some labeled cells did not display increased *in situ* hybridization signals (data not shown). At 14 d and 21 d, the majority of the rubral neurons displayed lower actin hybridization signals than those on the nonaxotomized contralateral side. At these later time points, the cytoplasmic volume of the neurons on the axotomized side was significantly reduced to 65% (14 d) and 63% (21 d; $p < 0.01$), and though the grain density was only slightly reduced or even similar to the contralateral side, the grain counts per cell were significantly ($p < 0.02$) reduced compared to contralateral (Fig. 5F), and also compared to normal rubrospinal neurons from nonoperated animals (processed together on the same slide).

Changes in total tubulin and T α 1 tubulin mRNA

Facial motoneurons. *In situ* hybridization of facial motoneurons with probes for α -tubulin and β -tubulin revealed increased neuronal mRNA levels for both tubulin families; α -tubulin is shown in Figure 1, E and F. Both tubulin probes used (M α 1 and M β 2) contain conserved sequences shared between different genetic tubulin isotypes (Lewis et al., 1985; Villasante et al., 1986) and therefore hybridize with all α -tubulin and β -tubulin messages, respectively. The predominant neuronal localization of the tubulin mRNAs is obvious; only a few glial cells reveal higher grain levels than the tissue background. Quantification of tubulin *in situ* hybridizations revealed for most animals levels higher than contralateral (Fig. 2D). However, one animal with a nerve resection was not different from contralateral by 24 hr. All other values at this and the later time points were significantly elevated. The relative increase for β -tubulin was significantly higher than for α -tubulin at days 7, 14, and 21 when compared within individual animals. There was no obvious

Figure 4. Light microscopic radioautography of rubral neurons after *in situ* hybridization to a radiolabeled NFM cDNA probe (*A–D*). The axotomized side is displayed in *A* and *C*. Dark-field illumination in *A* and *B* shows a marked decrease in hybridization signal in the caudal red nucleus (*RN*; arrowheads) at 10 d after axotomy. Bright-field illumination in *C* and *D* shows decreased density of silver grains over chromatolytic rubral neurons (*C*). *C* and *D* were taken from a section adjacent to *A* and *B*. *N3* is the oculomotor nucleus nerve. Scale bars: 100 μm for *A* and *B*; 50 μm for *C* and *D*. *E* shows a scatter plot of quantitative *in situ* hybridization of rubrospinal neurons to an NFL cDNA probe, 8 d after rubrospinal tractotomy at C3. Symbols are as in Figure 2*D*. *F* shows changes in mean counts/cell shown as axotomized neuron values as a percentage of uninjured contralateral neuron values. The vertical bars indicate SEM for >20 cells from individual animals. Values below the short broken lines were significantly different from uninjured (*t* test).

Figure 5. Light microscopical radioautography of rubral neurons after *in situ* hybridization to a radiolabeled actin cDNA probe. The axotomized side is displayed in *A* and *C*. Dark-field illumination in *A* and *B* shows a marked increase in hybridization signal in the caudal portion of the red nucleus at 8 d after axotomy (arrowheads in *A*). Bright-field illumination in *C* and *D* shows increased density of silver grains over chromatolytic rubral neurons 8 d after axotomy (*C*). Scale bars: 100 μm for *A* and *B*, 50 μm for *C* and *D*. *E* shows a scatter plot of quantitative *in situ* hybridization of rubrospinal neurons to an α -actin cDNA probe, 8 d after spinal hemisection at C3. Symbols are as in Figure 2*D*. *F* displays changes in mean counts/cell. Values above (8 d) and below (14 d and 21 d) the short stippled lines were significantly different from contralateral.

difference between the tubulin mRNA increase in crushed versus resected motoneurons at early time points, but by 21 d the mRNA levels were lower in crushed than in resected facial motoneurons. The regenerating axons of the crushed motoneurons reach their targets by 11–14 d after facial nerve crush, as shown by the return of whisker movement. The relative α -tubulin mRNA increase in Northern blots of four pooled facial nuclei are displayed as stars in Figure 2E. The signal increased to 164% of contralateral after resection and 158% after crush (both at 7 d), which was in good agreement with the *in situ* hybridization data.

Rubrospinal neurons. We found an increased *in situ* hybridization signal for α - and β -tubulin mRNA (total α -tubulin, total β -tubulin) in the axotomized rubrospinal neurons during the first week after the lesion (Fig. 6A–D; see also Fig. 9C,D). As seen in the scatter plot in Figure 6G, this involved the majority of the rubrospinal neurons within the caudal, magnocellular part of the nucleus from which the measurements were taken. A small increase in volume to $108 \pm 4.9\%$ was observed during this first week. At both 14 d and 21 d, the axotomized rubral neurons displayed lower *in situ* signals per cell than the contralateral not axotomized neurons (Fig. 6E,F). As with actin, this was mainly due to a reduced cellular volume, which in combination with an equivalent or slightly diminished grain density resulted in a significant reduction of hybridization signals per injured cell (Fig. 6H).

The fall of actin and total tubulin expression after 8–14 d could be the result of a general depression of RNA synthesis or indicate a failure to sustain the specific changes in gene expression associated with the cell body reaction to injury. Therefore, we analyzed the expression of T α 1 tubulin mRNA, which represents a genetic α -tubulin isotype (Lemischka et al., 1981) and is highly expressed in developing brain (Miller et al., 1987). Recently, we demonstrated that it is reexpressed in adult facial motoneurons after axotomy (Miller et al., 1989). Using a cDNA probe generated from the 3' nontranslated sequences unique to this isotype, we found an increase in T α 1 tubulin mRNA in axotomized rubrospinal neurons (Fig. 7E,F). This increase is sustained beyond the period when actin and total tubulin mRNA levels decrease (Fig. 8B,C). Even at 7 weeks, some, but not all, axotomized rubrospinal neurons display increased signals with the T α 1 tubulin probe (Fig. 9G,H).

Changes in GAP-43 mRNA

GAP-43 (also known as B50, F1, pp46, neuromodulin) has previously been shown to be expressed in the developing nervous system, and in regenerating extrinsic neurons, but most studies analyzing its expression in intrinsic CNS neurons have reported no increase after axotomy (for review, see Skene, 1989).

Facial motoneurons. Intact motoneurons showed little more than background levels of silver grains, while the axotomized cells displayed high levels of GAP-43 *in situ* hybridization signal (Fig. 1G,H). Quantification of the *in situ* signal demonstrated a

6–10-fold increase compared to the contralateral side by 24 hr after axotomy (Fig. 2F). This increase peaked at 8–60-fold at 7 d postlesion. mRNA levels remained elevated 21 d after injury, that is, after the regenerating axons had reached their targets. Northern blot analysis (Fig. 3) revealed an increase to about 11-fold by 7 d after nerve crush (Fig. 2F, star).

Rubrospinal neurons. In normal adult midbrain, we found significant GAP-43 hybridization signals in neurons of the substantia nigra, prerubral field, and some smaller neurons of the red nucleus. The normal magnocellular rubrospinal neurons displayed only low signals (Fig. 7B,D). After axotomy, however, the GAP-43 hybridization signal was significantly ($p < 0.01$) increased by 10–20-fold (Figs. 7A,C, 9E,F), and like T α 1 tubulin mRNA, this other marker for the cell-body reaction remained elevated beyond the period when actin and total tubulin mRNA levels decreased (Fig. 8). This increased signal was seen for over 7 weeks, though fewer cells appeared to have elevated GAP-43 mRNA as time progressed (data not shown).

Discussion

In the present study we have demonstrated that axotomized facial motoneurons exhibit decreased hybridization signals with neurofilament probes and increase their hybridization signal when probed with cDNAs specific for actin, α - and β -tubulin, and GAP-43 mRNAs. The quantitative Northern blot analysis of total cytoplasmic RNA from facial nuclei is in good agreement with these quantitative *in situ* hybridization data and with our previously published 35 S-methionine incorporation measurements (Tetzlaff et al., 1988). These findings confirm and extend previous findings on axotomized peripheral ganglia (Hoffman et al., 1987; Wong and Oblinger, 1987; Goldstein et al., 1988; Hoffman and Cleveland, 1988) and motoneurons (Miller et al., 1989; Muma et al., 1990). In addition, they emphasize that *in situ* hybridization consistently detects altered mRNA expression and is a suitable tool to analyze changes in gene expression even within the complexity of the CNS. We demonstrate here that rubrospinal neurons show initial changes in cytoskeletal protein and GAP-43 mRNA expression that are similar to those occurring in facial motoneurons. During the first week after lesion, neuronal content of actin and tubulin mRNAs increased, while the neurofilament messages decreased. However, during the second and following weeks after axotomy, the cellular levels of actin and total tubulin mRNAs decreased to subnormal values, and cell volumes decreased. Nevertheless, the sustained expression of T α 1 tubulin and GAP-43 mRNA during this period indicates that the rubrospinal neurons maintained aspects of the “normal” axotomy response despite the superimposed reduction in overall cytoskeletal mRNA levels and resulting cellular atrophy. These findings were unexpected and are the first evidence that intrinsic mammalian neurons that are entirely confined within the CNS can show initially similar changes in cytoskeletal gene expression to those found in extrinsic facial motoneurons, which regenerate avidly.

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Figure 6. Light microscopical radioautography of rubral neurons after *in situ* hybridization to a radiolabeled β -tubulin cDNA probe (A–F). The axotomized side is displayed on the left (A, C, E). Dark-field illumination in A and B shows a marked increase in hybridization signal in the caudal red nucleus at 8 d after axotomy (arrows). Bright-field illumination in C–F shows increased density of silver grains over axotomized chromatolytic rubral neurons (C) at 8 d after axotomy compared to contralateral (D). At 14 d after axotomy (E), the axotomized neurons are shrunken, and the signal per cell is reduced compared to contralateral uninjured neurons (F). Scale bars: 100 μ m for A and B, μ m for C–F. G, Scatterplot of quantitative *in situ* hybridization of rubrospinal neurons to an α -tubulin cDNA probe, 8 d after spinal hemisection at C3. Symbols are as in Figure 2D. In H, values above (8 d) or below (14 d and 21 d) the short broken lines are significantly different from contralateral.

Figure 7. Light microscopical radioautography of rubrospinal neurons after *in situ* hybridization to a radiolabeled cDNA probe specific for GAP-43 (A–D) and Tα1 tubulin (E, F). The axotomized side is displayed on the left (A, C, E). The GAP-43 *in situ* hybridization signals over axotomized rubral neurons were significantly increased at 8 d (A) and 21 d (C) after axotomy. Note a decrease in cell size after 21 d. The Tα1 tubulin signal is clearly increased over axotomized rubrospinal neurons at 8 d after lesion (E, F). Scale bars, 50 μm.

Figure 8. *A* and *B*, Scatter plot of quantitative *in situ* hybridization of rubral neurons to a cDNA probe containing the noncoding region of the $T\alpha 1$ tubulin mRNA 8 d (*A*) and 21 d (*B*) after spinal hemisection at C3. Symbols are as in Figure 2*D*. Note the increase in counts/cell but not in cell size at 8 d and the increase in counts but decrease in cell size at 21 d. *C*, Changes in mean counts/cell as in Figure 2*A–C*. *, significantly different from contralateral. *D* and *E*, Scatter plot of quantification of *in situ* hybridization of rubral neurons to a GAP-43 cDNA probe, 8 d and 21 d after spinal hemisection at C3. Note the increase in counts/cell but not in cell size at 8 d and the increase in counts but decrease in cell size at 21 d. *F*, Changes in mean counts/cell as in Figure 2*F*. Note the logarithmic scale. All points are significantly different from contralateral.

Until now, the response of injured rubrospinal neurons was thought to be largely “regressive” in character (Barron, 1983a,b, 1989). These neurons react with central chromatolysis or chromophobia after cervical lesion and show typical signs of cellular atrophy: cytoplasmic, nuclear, and nucleolar shrinkage (Barron, 1983a,b). However, they do not die (Prendergast and Stelzner, 1976; Egan et al., 1977; Barron et al., 1989). Axotomized rubral

neurons incorporate less tritiated leucine from 5 d on than their nonaxotomized counterparts (Barron et al., 1976). Similarly, there is no increased deoxyglucose incorporation in the axotomized red nucleus at any timepoint 1–30 d after a high cervical lesion (Rodichok et al., 1984), in contrast to the increase observed in most injured neurons projecting into the PNS (Kreutzberg and Emmert, 1980; Singer and Mehler, 1980; Smith et al., 1984).

Quantitative microspectrophotometry used for RNA measurement in rat and feline rubral neurons (Barron et al., 1977; Barron, 1989) showed progressive reductions in cytoplasmic and nucleolar RNA content after axotomy. Whether the onset of the cytoplasmic, nuclear, and nucleolar atrophy is already detectable by the first few days postlesion (Barron et al., 1989), or whether a transitory increase in cell size occurs (Egan et al., 1977), is controversial. The early findings by Cole and collaborators (Hanson et al., 1976) supported a transitory regenerative response of rubral neurons after axonal lesions. These authors described an increase of TPNH diaphorase reaction during the first week after axotomy followed by a decrease at later time points. Our present study clearly emphasizes and extends this view of an initial regenerative response. We demonstrated that the reaction of rat rubral neurons to axotomy is not initially regressive, but "regenerative," with the initial mRNA expression changes qualitatively identical to those of facial motoneurons. These regenerative changes may not be apparent when total RNA changes are measured (Barron et al., 1977, 1989) because the increase in actin and tubulin mRNAs may be masked by the decrease in neurofilament mRNAs. Thus, total mRNA levels and protein synthesis may not change initially or may even decrease, depending on the ratio of synthesis of actin plus tubulin versus neurofilament proteins. The reduced mRNA levels for all three cytoskeletal proteins at later time points are consistent with reduced total RNA levels in rubral neurons (Barron, 1989). Nevertheless, the sustained expression of α 1 tubulin and GAP-43 clearly indicate that the regeneration response is not aborted at later stages. Thus, this developmental mode of gene expression typically found in regenerating peripheral neurons (Hoffman and Cleveland, 1988; Miller et al., 1989) can also be expressed and sustained in intrinsic CNS neurons.

These findings may help to explain the regenerative capacity of rubrospinal neurons. A significant number of rubrospinal neurons regenerated into peripheral nerve transplants when these were implanted into the lesioned cervical spinal cord (Richardson et al., 1984). The present study supports the hypothesis that those CNS neurons able to initiate the regeneration-associated changes in gene expression are able to grow their axons into a permissive environment. For GAP-43, the correlation between its expression and capacity to regenerate has been reported in a variety of examples in the PNS, while in the CNS this correlation was largely supported by negative examples (for review, see Skene, 1989). Lack of increased axonal transport of GAP-43 has been observed in the optic nerve and corticospinal tract of rodents (Skene and Willard, 1981; Kalil and Skene, 1986; Reh et al., 1987; for review, see Skene, 1989), supporting the view that the failure of axonal regeneration within these CNS tracts is related to the failure to express regeneration-associated proteins like GAP-43. Indeed, peripheral nerve transplants to the intracranial optic nerve or to the spinal cord failed to support regeneration of retinal ganglion cells or corticospinal cells, re-

spectively (Richardson et al., 1982, 1984). However, two preliminary reports (Freeman et al., 1986; Lozano et al., 1987) indicated that GAP-43 can be expressed by axotomized retinal ganglion cells in rodents. The reason for discrepancies with earlier negative findings is most likely the effect of the distance from the lesion site to the cell bodies. The study of Lozano et al. (1987) on retinal ganglion cells was done with intraorbital lesions of the optic nerve, while previous investigators used intracranial lesions (Skene and Willard, 1981). Rat retinal ganglion cells can grow into nerves transplanted directly behind the eye (Vidal-Sanz et al., 1987). Our present report shows a clear increase in GAP-43 mRNA in rubrospinal neurons after a cervical lesion of the spinal cord, but preliminary findings indicate that this is not seen with thoracic lesions (Tetzlaff et al., 1990). A significant number of rubrospinal neurons regenerate into cervical but not into thoracic transplants (Richardson et al., 1984). Thus, even within the same CNS neuronal system, the response to axotomy might be largely dependent on the distance from the lesion to the cell body and/or the associated sparing of sustaining collaterals. Although none of these neuronal systems regenerate within the CNS, their capacity to grow into a peripheral nerve transplant appears to be correlated with their ability to express GAP-43. It is now necessary to understand the molecular mechanisms that trigger the regeneration-associated response in gene expression, in order to enhance the neuronal propensity for growth into an environment that has been rendered permissive.

The signal for axotomy that leads to the so-called "cell-body reaction" is not known, and a variety of possibilities have been considered for peripheral neurons (Cragg, 1970). We recognize that different components of this "cell-body reaction" are probably regulated from several separately controlled genes that respond to different molecular signals (Verge et al., 1990a,b). These signals may include positive signals such as factors from the injured peripheral stump or negative signals such as the interruption of a normally retrogradely transported factor, or the premature return of an anterogradely transported substance (for review, see Bisby, 1984). The present study indicates that for cytoskeletal proteins and for GAP-43 these axotomy signals are likely to be similar in the lesioned CNS and PNS. Thus, the signal(s) for axotomy is apparently not a factor specific to the PNS. This is an important finding because the microenvironment of the PNS is permissive to axonal outgrowth, and transplanting peripheral nerve segments into the CNS might itself have triggered a growth response. However, the present study demonstrated that at least those parameters of the neuronal cell body response tested so far were induced by the axotomy.

The second major finding of this study is the decreased expression of cytoskeletal mRNAs in rubrospinal neurons during the second and third weeks after axotomy. This is in distinct contrast to facial motoneurons. It is unclear whether this decrease is a cause or an effect of the failure of rubrospinal neurons to regenerate within the spinal cord. It is conceivable that the

Figure 9. Retrograde labeling of axotomized rubrospinal neurons combined with *in situ* hybridization for NFM (*A, B*), α -tubulin (*C, D*), GAP-43 (*E, F*), and α 1 tubulin (*G, H*). The axotomized side is on the left, and the uninjured contralateral side is on the right. Fluorescence illumination was used to photograph the fast blue (*A, C, G*) or Fluoro-gold (*E*) label, and simultaneously dark-field illumination was used to reveal the silver grains representing the *in situ* hybridization signal. One neuron in *B* is both labeled and displaying lower levels of NFM mRNA than the adjacent unlabeled neurons. Apparently, its axon was not crossed, and so it was axotomized and labeled, representing an exception. *A–F* are 7 d, and *G* and *H*, 7 weeks after axotomy. Note the increased α 1 tubulin label (silver grains) in some but not all fast blue-labeled neurons (*G*). Some cells in *G* show increased silver grain density, but were only faintly labeled with fast blue and lost their label during hybridization. Scale bar, 50 μ m.

lack of actin and tubulin supply from the cell body leads to an aborted axonal sprouting response during the second week. We do not know the reason for this "regressive" cell-body response and can only presume that either a lack of some trophic factor or the active depression by some inhibiting factor is involved. Growth inhibiting molecules have recently been identified (Caroni and Schwab, 1988a,b), and treatment with antibodies to these 35-kDa and 250-kDa proteins enhanced outgrowth of some axons in the lesioned spinal cord of postnatal rats (Schnell and Schwab, 1990). These inhibitory molecules, which have been shown to act locally at the growth cone, may also inhibit directly or indirectly the neuronal gene expression response to injury. Our study indicates that such inhibitory factors within the CNS do not interfere with the signal for axotomy and the initiation of a regenerative response, but they could interfere with the maintenance of this response.

Alternatively, the regressive cell-body response may be due to the fact that trophic support of rubrospinal neurons has been interrupted by disconnecting them from their target. The CNS environment might not provide enough trophic substitute compared to the PNS, where Schwann cells in the distal nerve stump and neuroma upregulate the production of NGF and other trophic molecules (Richardson and Ebendal, 1982; Heumann et al., 1987). Those rubrospinal neurons with regenerating axons into peripheral nerve grafts did not appear to be atrophic (Richardson et al., 1984). It will be interesting to determine whether regeneration of rubrospinal neurons into a peripheral nerve graft is associated with a sustained increase in tubulin and actin expression. The combination of *in situ* hybridization with retrograde labeling through a peripheral nerve graft will allow us to investigate this question.

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