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Research report

Expression of β -calcitonin gene-related peptide in axotomized rubrospinal neurons and the effect of brain derived neurotrophic factor

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Abstract

The mRNA levels for α - and β -calcitonin gene-related peptide (CGRP) in rat rubrospinal neurons were studied by in situ hybridization 3, 7, 14, 28 and 56 days following cervical spinal hemisection. CGRP-like immunoreactivity (LI) in the rubrospinal neurons and the rubrospinal tract in cervical spinal cords were examined using immunohistochemistry. There was almost no signal for α - and β -CGRP mRNAs and undetectable level of CGRP-LI in the rubrospinal neurons ipsilateral to cervical spinal hemisection (control side). Fourteen days after spinal hemisection, the rubrospinal neurons contralateral to cervical hemisection (axotomized side) showed CGRP-LI in their cell bodies, and CGRP containing fibers were observed in the lateral funiculi just proximal, but not distal, to the injury sites. In situ hybridization showed upregulation of β -CGRP mRNA in a subpopulation of the rubrospinal neurons on the axotomized side. The proportion of β -CGRP mRNA-expressing neurons reached its maximum (approximately 19%) 4 days following axotomy and slowly decreased to about 5% 56 days after axotomy. The percentage of α -CGRP mRNA-expressing neurons was much lower than that of β -CGRP mRNA (maximum about 2.6% 4 days after axotomy) and not significantly different from the control side throughout the time period studied. These data indicate that axotomy induces de novo synthesis of the CGRP β -subtype in rubrospinal neurons and that the β -CGRP is transported to the injury site through the rubrospinal tract. In addition, we studied the effect of the intracerebral injections of brain derived neurotrophic factor (BDNF). BDNF treatment fully reversed the severe cell atrophy that followed axotomy and increased the number of neurons labeled for β -CGRP mRNA, but did not increase the percentage of rubrospinal neurons expressing β -CGRP mRNA. Thus, topical application of BDNF does not have direct modulatory effect on CGRP induction in axotomized neurons in the red nucleus. © 1997 Elsevier Science B.V.

Keywords: Rat; CGRP; Red nucleus; Axotomy; BDNF; In situ hybridization

1. Introduction

Calcitonin gene-related peptide (CGRP) was initially found to be generated by alternative RNA processing of the rat calcitonin gene [2]. This peptide was shown to coexist with acetylcholine in spinal and cranial motoneurons [9,27] and to have a synergistic action on the acetylcholine receptor at the neuromuscular junction [15,18]. Subsequently, a novel mRNA encoding a protein precursor of a peptide (β -CGRP) was identified in rat brain, which differs from the original CGRP (α -CGRP) by only a single amino acid [1]. The RNA encoding β -CGRP is the only mature transcript of the β -CGRP gene. α - and β -CGRP mRNAs have been demonstrated to have quantitatively

different distributions in cranial motor nuclei and spinal motoneurons [1,10,20].

A number of recent studies have indicated that CGRP increases following axotomy in spinal [3,4,16,19,22], facial [7,12,30], trochlear [35], and hypoglossal motoneurons [11]. Since α -CGRP mRNA is up-regulated, while β -CGRP mRNA is down-regulated or not affected in these axotomized motoneurons [19,21,22,28], the increase in CGRP seems to be attributed to the increase in synthesis of the α -CGRP subtype. All these motoneurons are 'extrinsic neurons' which have their cell bodies in the central nervous system (CNS) and project their axons to peripheral tissues. On the contrary, rubrospinal neurons have their cell bodies and axons confined within the CNS, so they are called 'intrinsic neurons'. Their axons reach spinal motoneurons mono- or poly-synaptically to regulate contralateral flexor tonicity. In general, mammalian extrinsic neu-

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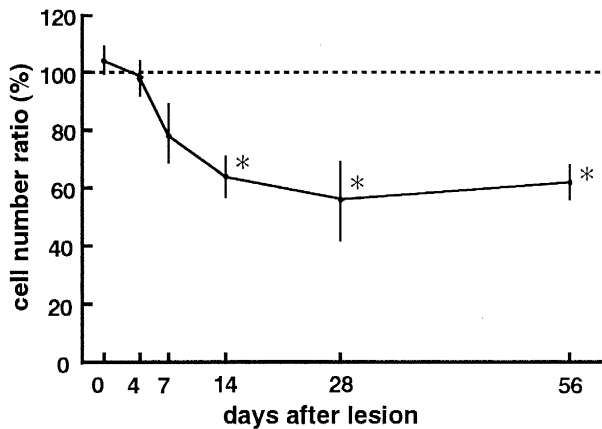


Fig. 1. Time course of the decrease in cell number ratio (axotomized side/control side) in the red nucleus following left cervical funiculotomy. Values are means \pm S.E. for 3 animals at each time-point. Asterisks indicate significant difference between lesioned and naive animals ($P < 0.05$).

rons are able to regenerate a new axon when lesioned, while intrinsic neurons typically do not regenerate. Nevertheless, it has been demonstrated that rubrospinal neurons, as well as motoneurons, have some prerequisites for axonal regeneration temporally, including increased mRNA levels for growth-associated protein (GAP)-43 and cytoskeletal proteins such as actin and α - and β -tubulin, as well as facial motoneurons [31]. The response of intrinsic neurons in terms of their neuropeptide phenotype, such as CGRP, following axotomy is not known. In order to compare the response to axotomy between intrinsic and extrinsic neurons with regard to the changes in expression of neuropeptides, we studied CGRP expression in rubrospinal neurons following axotomy. It has been reported that rubrospinal neurons express trkB, a high affinity BDNF receptor, and that intra-cranial injection of BDNF further increases GAP-43 mRNA expression in rubrospinal neurons following axotomy [33]. Therefore, we studied the effect of intra-cranial injection of BDNF on CGRP expres-

sion. Preliminary results have been presented in abstract form [8].

2. Materials and methods

2.1. Animal procedures

A total of 35 male Sprague-Dawley rats (body weight 230–270 g) were used. All experimental procedures were done on animals deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, given i.p.). Additional doses of the anesthetics were given as needed. The spinal cord was exposed between C1-C2 and the left lateral funiculus was transected at the C2 level with a #11 scalpel blade. In some animals, a 1 μ l depot of 5% Fluoro-gold (Fluorochrome, Colorado) was applied to the lesion site by using a small piece of dye-soaked Gelfoam. The extent of the spinal cord lesion was examined following sacrifice, and only the animals with almost all left lateral funiculi transected were used following experiments. The axons of rubrospinal neurons are mostly (> 99%) crossed in the ventral tegmental decussation [6,13]. Thus, in this experimental model, the right red nucleus contains axotomized rubrospinal neurons and the left rubral neurons remain uninjured. Seven days after transection, 10 of the rats were implanted with 30 gauge cannulae into the vicinity of the right red nuclei and these were connected to osmotic minipumps (Alzet; 1 μ l/h for 7 days) filled with 0.1 M phosphate-buffered saline (PBS) or BDNF (500 ng/ml in PBS). All animal experiments were conformed to the regulations of the Hyogo College of Medicine Committee on Animal Research and were carried out in accordance with the guidelines of the NIH on animal care.

2.2. Immunohistochemistry

Fourteen days after transection, 5 rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phos-

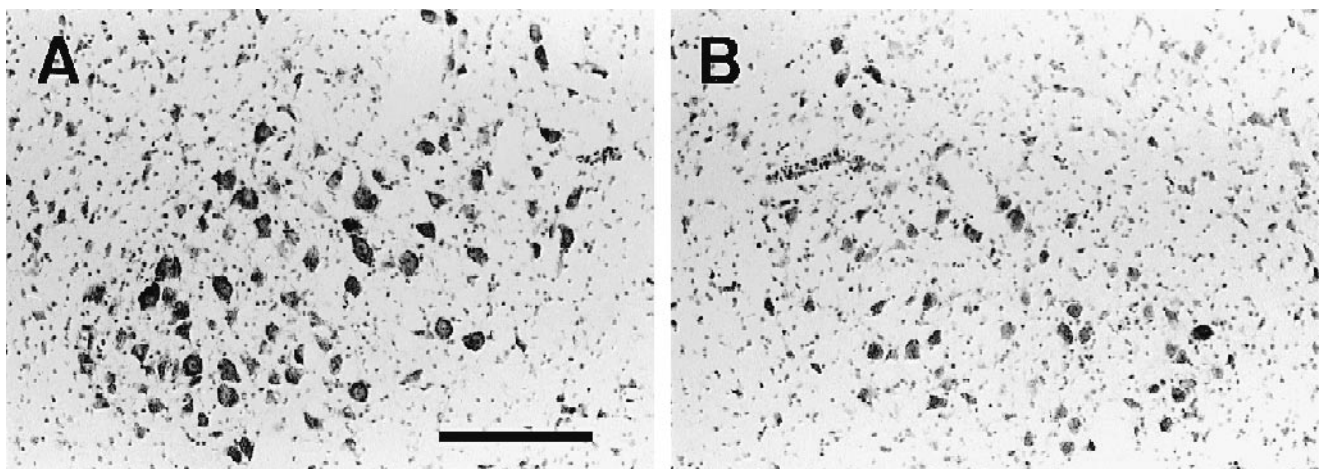


Fig. 2. Photomicrographs of rubrospinal neurons 14 days after unilateral cervical (C2) funiculotomy. The red nucleus on the axotomized side (B) contains fewer and smaller neurons than on the control side (A), owing to the severe cell atrophy evoked by axotomy. Scale bar = 200 μ m.

phate buffer (pH 7.4). The brain and a piece of cervical spinal cord containing the lesion site were dissected out and postfixed in the same fixative at 4°C overnight, followed by immersion in 20% sucrose in 0.1 M phosphate buffer at 4°C for cryoprotection. After a few days the tissues were frozen in powdered dry ice and cut transversely with a cryostat at 30 μm . The brain sections including the red nucleus were selected. The spinal cord sections were divided into 3 groups: rostral, caudal to the lesion site, and the lesion site. Sections were incubated in rabbit anti-CGRP polyclonal antiserum (1:6000; Amersham) for 48 h at 4°C and processed with an avidin-biotin kit (Vector, Burlingame, CA, USA). Tissue sections were incubated in biotinylated anti-rabbit immunoglobulin G (1:200; Vector) for 2 h at 4°C, followed by incubation in avidin-biotin complex for 60 min at room temperature, and finally reacted with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide. Sections were then washed, mounted on slides, dried, and coverslipped.

2.3. *In situ* hybridization histochemistry

Animals were deeply anesthetized and killed by decapitation at 4 days ($n = 5$), 7 days ($n = 3$), 14 days ($n = 3$), 28 days ($n = 3$), and 56 days ($n = 3$) after hemisection of their cervical spinal cords. Brains were dissected out, rapidly frozen in powdered dry ice, and cut transversely on a cryostat at 16 μm . Serial sections including the red nucleus derived from each animal were thaw-mounted onto 8 slides coated with silane (3-aminopropyltriethoxysilane), and stored at -80°C until use. The animals which received cervical hemisection and infusion of BDNF ($n = 5$) or PBS ($n = 5$) into the vicinity of the right red nuclei, were anesthetized and killed by decapitation 14 days after lesion (7 days after injection). Brains were dissected out, and processed as described above.

2.4. Preparation of probes

We used α - and β -CGRP-specific probes consisting of 35-base oligonucleotides. The α -CGRP probe was complementary to bases 664–698 and the β -CGRP probe to bases 656–690 of the respective cDNAs [1]. These sequences were encoded in the 3'-noncoding exons unique to each mRNA. A DNA homology search ('DNASIS', Hitachi, Ltd., Japan) showed only 54.3% homology between the α -CGRP probe and the β -CGRP mRNA, and vice versa. The specificity of the probes used in this study was confirmed in previous reports [19,20]. These probes were labeled with ^{35}S -deoxyadenosine triphosphate (NEN, USA) and terminal deoxynucleotidyl transferase (Amersham, UK), giving a specific activity of $1.0\text{--}1.5 \times 10^9$ cpm/mg.

2.5. Hybridization procedure

Sections were hybridized after thawing, without any pretreatment, overnight at 42°C in humidified boxes with

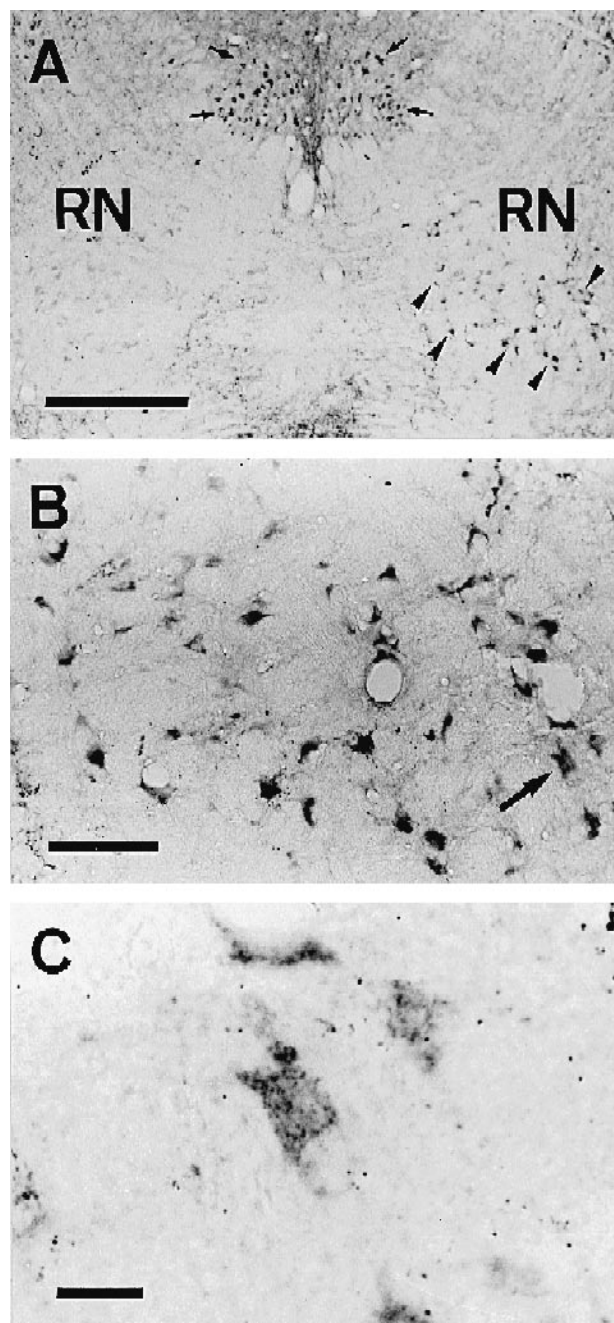


Fig. 3. Photomicrographs showing CGRP-immunoreactivity in the red nuclei 14 days after unilateral cervical (C2) funiculotomy. (A) Low-power photomicrograph of a transverse section at midbrain level. CGRP-immunoreactive cells can be seen in the red nucleus (RN) on the axotomized side (on the right-side, small arrowhead) and, bilaterally in the nuclei of the oculomotor nerve (arrows). CGRP is constitutively expressed in oculomotor neurons but not in red nucleus neurons (see the RN on the left-side). Scale bar = 400 μm . (B) Photomicrograph showing CGRP-immunoreactive cells in the red nucleus on the axotomized side 14 days after unilateral cervical (C2) funiculotomy. Scale bar = 100 μm . (C) High-power view of the CGRP-immunoreactive neuron marked with the arrow in (B). Note the granular appearance of the immunoreactivity in the cytoplasm. Scale bar = 20 μm .

5×10^5 cpm of labeled probe per 100 μ l of a mixture containing $4 \times$ SSC, 50% formamide, 0.12 M phosphate buffer, $1 \times$ Denhardt's solution, 0.2% sodium dodecyl sulfate, 250 μ g/ml yeast tRNA, 10% dextran sulfate, and 100 mM dithiothreitol. After hybridization, the sections were rinsed 4 times for 15 min each at 55°C in $1 \times$ SSC,

dipped in distilled water, transferred through 60, 80, and 95% ethanol, and then air-dried. For autoradiography, the sections were coated with NTB-3 emulsion (Kodak; diluted 1:1 with distilled water at 45°C) and exposed for 2–3 weeks in light-tight boxes at 4°C . After development in D19 (Kodak) and fixation in 24% sodium thiosulfate,

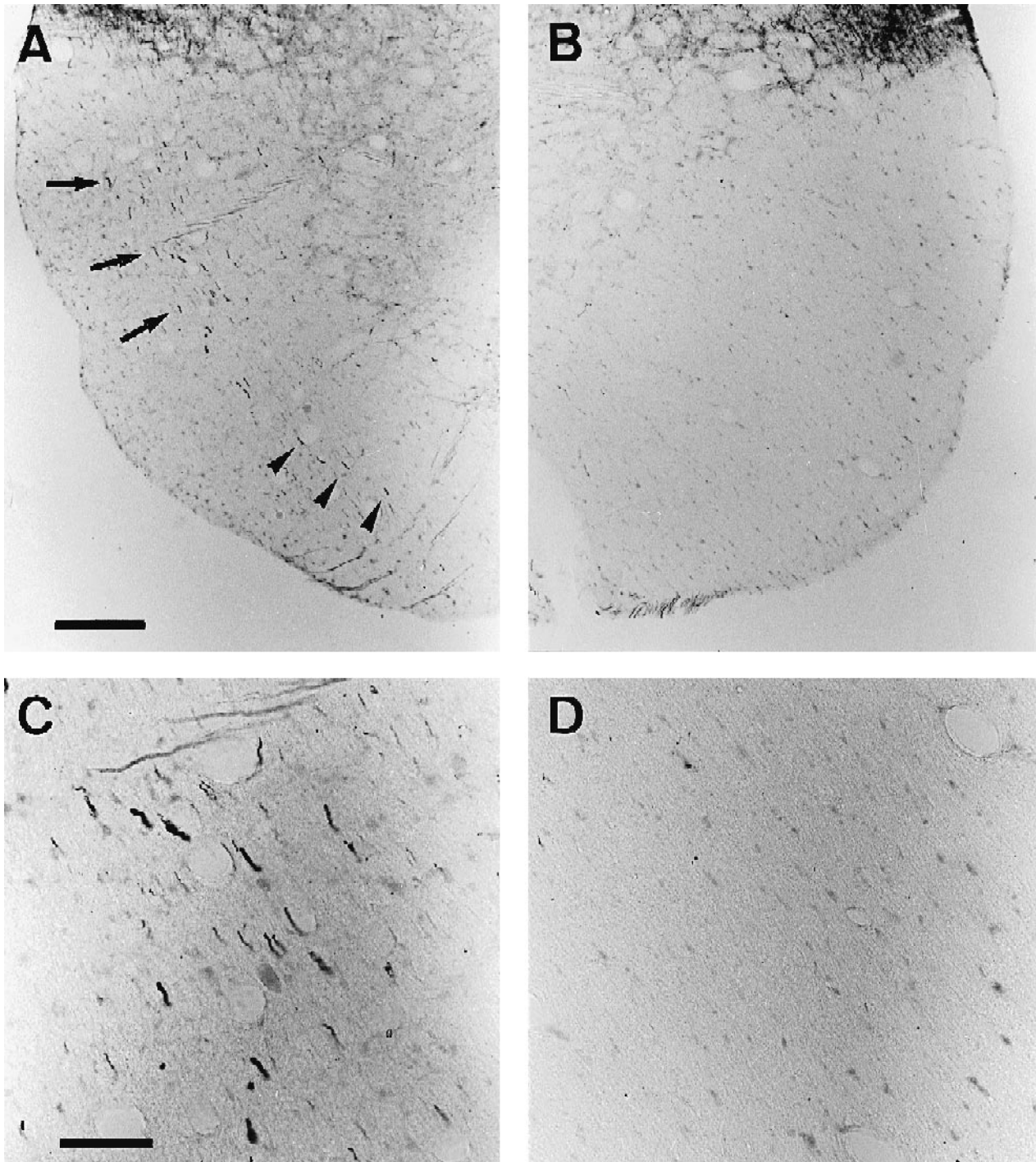


Fig. 4. Transverse sections of cervical spinal cord at the C2 level rostral to the lesion site 14 days after unilateral cervical (C2) funiculotomy. On the side ipsilateral to the lesion site (A,C), CGRP-immunoreactive fibers are seen in the dorsomedial aspect of the lateral funiculus (arrows), which contains the rubrospinal tract. CGRP-immunoreactive fibers are also seen in the ventral aspect of the lateral funiculus and in the ventral funiculus (arrowheads). No such fibers are seen on the side contralateral to the lesion site (B,D). Scale bars = 200 μ m for (A,B), and 100 μ m for (C,D).

the sections were rinsed in distilled water, counter-stained with neutral red, dehydrated in a graded ethanol series, cleared in xylene, and coverslipped.

2.6. Quantitative analysis

Three sections were picked for each probe from every eighth section within the caudal 400 μm of the red nucleus of each animal. These sections contain mainly magnocellular neurons in the red nucleus which project to the spinal cord. At least 9 sections derived from 3 different animals in each experimental group were analyzed. Measurements of the density of silver grains over selected tissue profiles were performed using a computerized image analysis system (IBAS; Zeiss). At a magnification of $\times 400$ with bright-field illumination, upper and lower thresholds of gray level density were set such that only silver grains were accurately discriminated from the background in the outlined cell or tissue profile and read pixel-by-pixel by the computer. Subsequently, the area of discriminated pixels was measured and divided by the area of the outlined profile, giving a grain density for each cell or tissue profile. To reduce the risk of biased sampling of the data owing to varying thickness of the emulsion, we used a signal/noise (S/N) ratio for each cell in each tissue. Cells with a grain density of fivefold the background level or higher (S/N ratio ≥ 5) were considered positively labeled.

The number of positively labeled rubrospinal neurons was divided by the number of neuronal profiles counted in the red nucleus, and the 'percentage of the cells positively labeled for α - or β -CGRP mRNA' was calculated. The total number of neuronal profiles on the axotomized side was divided by the corresponding number on the control side. The calculated ratio, referred to as 'cell number ratio' in the text below, provides an index of cell atrophy. Data are expressed throughout as means \pm S.E. To detect significant difference among multi-time point data (left vs. right or α -CGRP vs. β -CGRP), the values were tested using two-way factorial ANOVA, followed by Fisher's least significant difference test. Student's *t*-test was applied to detect significant changes between the BDNF- and PBS-treated groups. Two-tailed *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Cervical funiculotomy

When Fluoro-gold was applied to the rostral stump of the cervical funiculotomy site, almost all rubral neurons on the side contralateral to the lesion were labeled for Fluoro-gold. Thus, the majority ($> 90\%$) of magnocellular neurons in the red nucleus were axotomized by lesions at C2 in the present study. After left cervical funiculotomy,

the cell number ratios decreased over time; they were 97.2, 79.6, 66.2, 58.7, and 61.3% on days 4, 7, 14, 28, 56, respectively (Fig. 1). Previous investigations indicate that injured rubral neurons shrink, but little neuronal death occurs [5,24,31,36]. Therefore, these decreases in cell number are due to severe atrophy of the axotomized rubrospinal neurons (Fig. 2).

3.2. CGRP-LI in the red nucleus and lateral funiculi

CGRP-LI could not be demonstrated in any cell bodies in the red nucleus of naive animals. Fourteen days after cervical spinal hemisection, CGRP-LI-labeled cell bodies were observed in the right red nucleus (axotomized side) (Fig. 3A,B). The CGRP-LI exhibited a granule-like staining in the cytoplasm (Fig. 3C). Moderately to strongly stained cells were mainly observed in the ventrolateral part of the caudal half of the nucleus (Fig. 3A). These cells seem to correspond to the large cells in the caudal magnocellular part of the red nucleus.

In the cervical spinal cord, scattered fibers labeled for CGRP-LI, descending in the left lateral funiculus, were observed just rostral to the lesion site 14 days after spinal

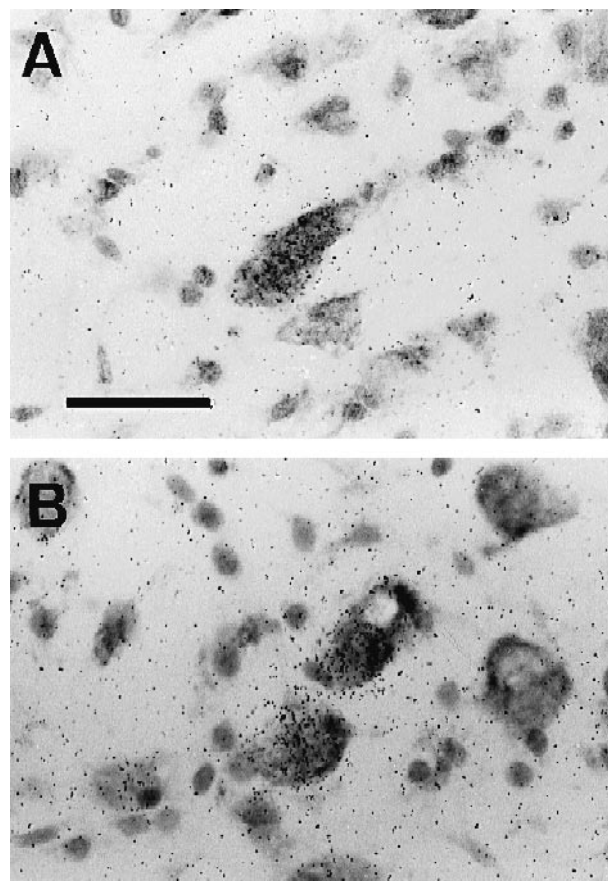


Fig. 5. Bright-field photomicrographs of rubrospinal neurons on the axotomized side showing in situ hybridization signals for α -CGRP (A) and β -CGRP (B) mRNAs 4 days after unilateral cervical (C2) funiculotomy. Scale bar = 50 μm .

hemisection. A few CGRP-LI-labeled fibers were also seen in the ventral funiculus (Fig. 4A,C). No such fibers were seen in the right lateral funiculus (Fig. 4B,D) or in the lateral funiculi caudal to the lesion site on either side.

3.3. *In situ* hybridization for α -, β -CGRP mRNAs

No α - or β -CGRP mRNA-expressing cells were detected in the red nucleus of normal animals ($n = 3$). There

was almost no CGRP mRNA-labeled neurons in the left red nucleus (control side) of experimental animals at any time point. Four days after cervical spinal hemisection, some rubrospinal neurons on the axotomized side expressed α - or β -CGRP mRNA hybridization signal (Fig. 5A,B). Silver grain quantification revealed that about 18% (range, 10.9–27.7%, $n = 5$) of the counted rubrospinal neurons on the axotomized side were labeled (S/N ratio ≥ 5) for β -CGRP mRNA 4 days after axotomy (Fig. 6).

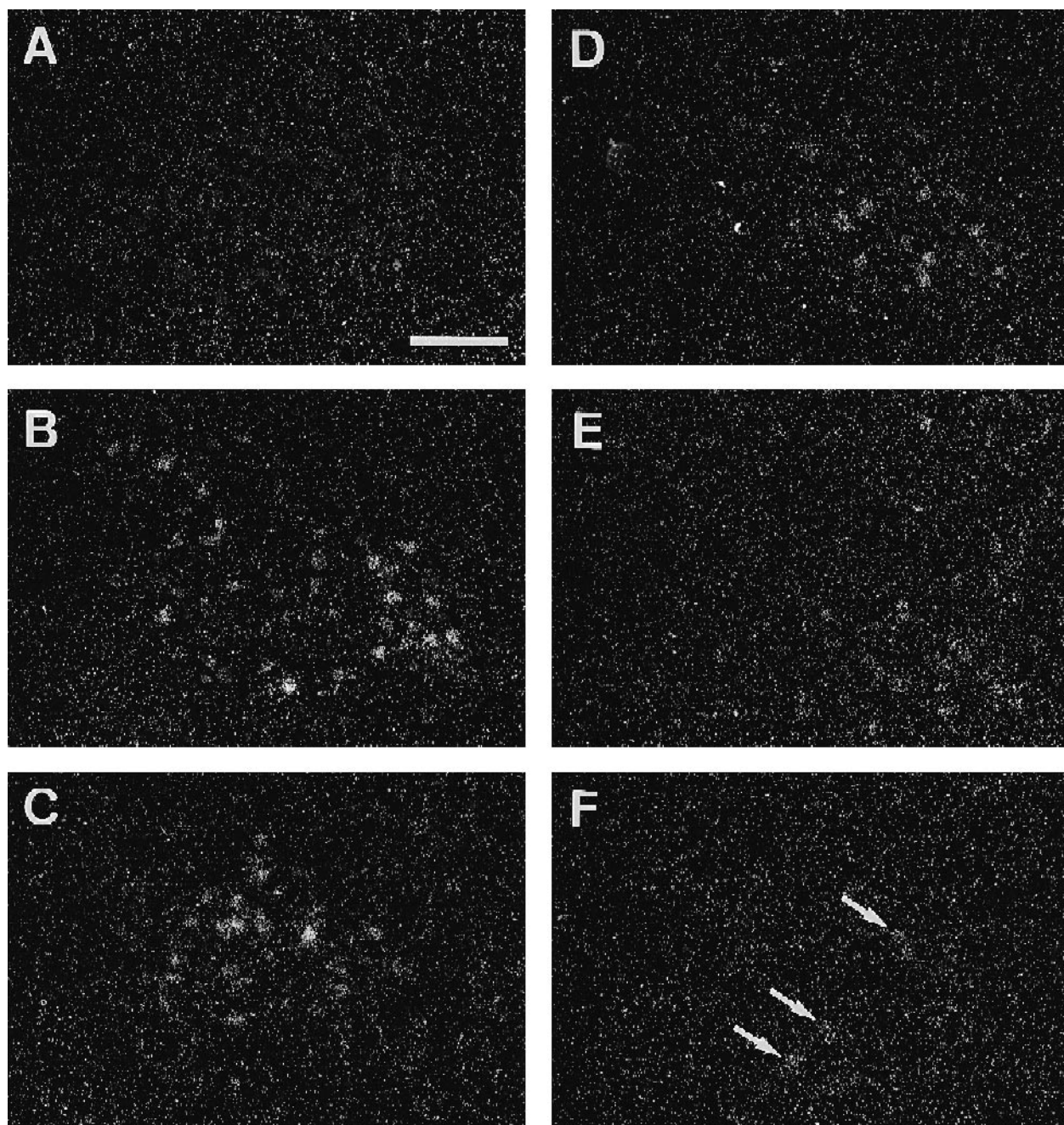


Fig. 6. Dark-field photomicrographs showing *in situ* hybridization signals for β -CGRP mRNA in the red nuclei on the control side (A) and axotomized side (B–F). Four days after unilateral cervical (C2) funiculotomy, β -CGRP mRNA-expressing neurons were seen on the axotomized side (B), but not on the control side (A). At longer survival times, labeled cells gradually decreased in number and signal intensity (C; 1 week, D; 2 weeks, E; 4 weeks, F; 8 weeks). Several weakly labeled neurons were still seen 8 weeks after injury (arrows). Scale bar = 200 μ m.

Table 1
BDNF and PBS effects on cell atrophy

Treatment	Cell number ratio (%)	<i>n</i> =	<i>P</i>
BDNF	100.9 ± 6.7	5	< 0.01
PBS	54.9 ± 4.3	5	

The number of rubrospinal neurons on the axotomized side in BDNF- and PBS-treated animals 14 days after cervical funiculotomy. The treatment was given from day 7 to day 14. Data are normalized to the control side. Note that the decrease in the cell number in PBS-treated animals was fully reversed in BDNF-treated animals.

The values remained around 15% (6.6–33.3%, *n* = 3) of the counted cells on days 7 and 14, and decreased to about 11% (3.2–18.2%, *n* = 3) and 5% (range, 3.8–7.4%, *n* = 3) on days 28 and 56, respectively (Figs. 6 and 7). By contrast, the increase in the α -CGRP mRNA hybridization signal was quite small. The percentage of α -CGRP mRNA-expressing neurons in the right (axotomized) red nucleus was 0–4.2% of the counted cells through the first 4 weeks, and returned to 0% by 8 weeks after the lesion (Fig. 7).

3.4. BDNF treatment

On day 14 after lesion, the rubral cell number of the axotomized side decreased to 54.9% (range, 42.6–65.4%, *n* = 5) of that of the uninjured control side in PBS-treated animals. This reflects the severe atrophy which occurs

Table 2
BDNF and PBS effects on CGRP mRNA expression

Treatment		Positively labeled cells (%)	<i>n</i> =	<i>P</i>
α -CGRP	BDNF	4.1 ± 2.2	4	0.10
	PBS	0.0 ± 0.0	4	
β -CGRP	BDNF	10.8 ± 1.0	5	0.65
	PBS	12.3 ± 3.3	4	

The percentage of rubrospinal neurons positively labeled for α -CGRP and β -CGRP mRNAs in BDNF- and PBS-treated animals 14 days after cervical funiculotomy. The treatment was given from day 7 to day 14. Data indicate the percentage of axotomized rubrospinal neurons expressing α - or β -CGRP mRNA. Note the small increase in α -CGRP mRNA-labeled neurons in BDNF-treated animals, though it was not significantly different from PBS-treated animals. For β -CGRP mRNA, there was no difference between BDNF- and PBS-treated rats.

between 7 and 14 post injury [5,31]. BDNF treatment fully prevented the decrease. The cell number of the axotomized side was preserved: 100.9% (range, 82.0–117.3%, *n* = 5) of that of the control side (Table 1).

In BDNF-treated rats the percentage of α -CGRP mRNA positive neurons on the axotomized side slightly increased to 4.1%, though it was not significantly different from PBS treatment (0.0% *P* = 0.10). BDNF injection increased the number of neurons expressing β -CGRP mRNA in the red nucleus; $22.2 \pm 3.2/3$ sections from each BDNF-treated rat (*n* = 5) vs. $13.5 \pm 2.6/3$ sections from each PBS-treated rat (*n* = 4). However, the percentage of rubral neurons labeled for β -CGRP mRNA on the axotomized side was not significantly different between BDNF- and

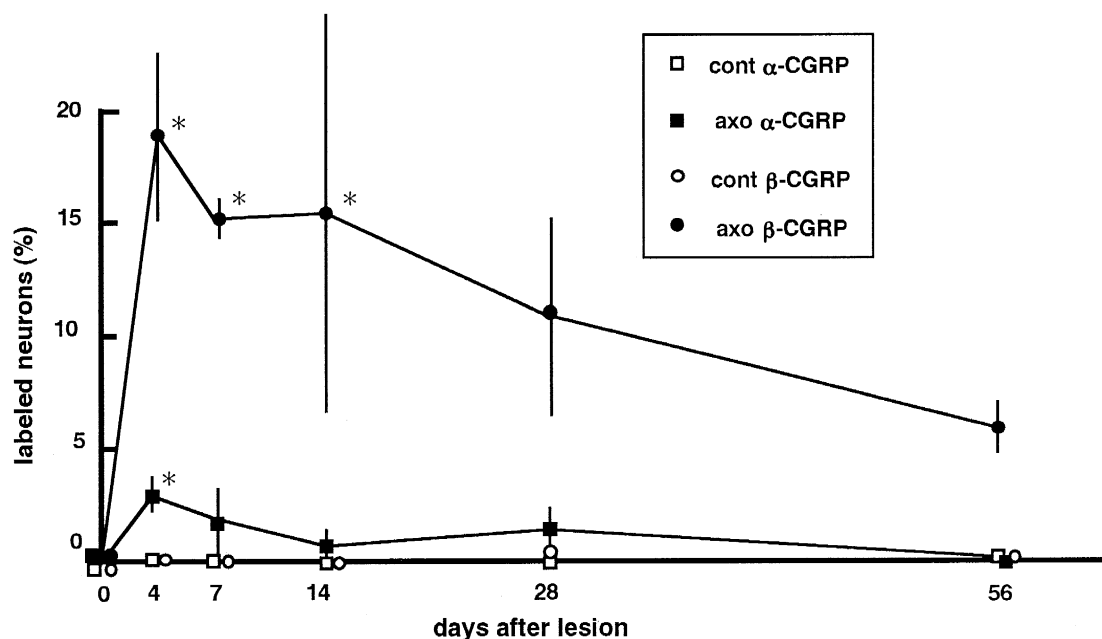


Fig. 7. Time course of quantitative in situ hybridization for α -CGRP (squares) and β -CGRP (circles) mRNAs in rubrospinal neurons on the control side (open symbols) and the axotomized side (closed symbols) after cervical axotomy. Each data point represents the mean of 3 animals (\pm S.E.) except day 4 (*n* = 5). Asterisks indicate significant difference between lesioned animals and naive animals. Note the significant increase in β -CGRP mRNA on the axotomized side during the first 2 weeks after cervical lesion. In contrast, the increase in α -CGRP mRNA is less pronounced. Almost no signals for α -CGRP and β -CGRP mRNA were seen on the control side.

PBS-treated rats (10.8% in BDNF-treated rats vs. 12.3% in PBS-treated rats, $P = 0.65$) (Table 2).

4. Discussion

In this study, we demonstrated that axotomy induced an upregulation of CGRP synthesis in rubrospinal neurons. The CGRP induced in axotomized rubrospinal neurons was mainly the β -subtype. CGRP-immunoreactive fibers were observed in the left lateral funiculus, including the rubrospinal tract, proximal to the lesion (Fig. 4). This means that the CGRP synthesized in the axotomized rubrospinal neurons was transported into the axons projecting to the spinal cord. Spinal and bulbar motoneurons express CGRP-LI constitutively. The α - and β -CGRP mRNAs in these motoneurons are differentially regulated following axotomy: α -CGRP mRNA is up-regulated while β -CGRP mRNA is down-regulated or not affected [19,21,22,28]. The up-regulation of α -CGRP following axotomy may reflect a role for this neuropeptide in axonal regeneration at the site of axon injury, supported by the presence of CGRP-LI in the motor axon proximal to the lesion, as reported previously [22,25]. Since β -CGRP mRNA is decreased in axotomized spinal and bulbar motoneurons, these two types of CGRP have been suggested to serve different functions [19,28].

Rubrospinal neurons do not express CGRP-LI nor other neuropeptides constitutively [14,29]. In this study, however, we observed that they became CGRP positive and they expressed predominantly β -CGRP mRNA following axotomy. Therefore, β -CGRP could have some role in injured axons in this system. Rubrospinal axons have some regenerative potential, sending out numerous sprouts soon after injury [5], and some can regenerate into the permissive environment of a peripheral nerve transplant [26], but they do not regenerate successfully across a spinal cord lesion in the adult [5]. If β -CGRP has a weaker supportive effect on axonal regeneration in comparison to α -CGRP, the failure of rubrospinal neurons to regenerate may be explained in part by the insufficiency of α -CGRP up-regulation in these neurons. However, CGRP consists of 37 amino acids and only 1 amino acid is different between the α - and β -CGRP subtypes. The functional difference between the subtypes is not clear, except a report that α -CGRP is slightly more potent than β -CGRP in relaxing colonic smooth muscle [17]. Since the cranial motor nuclei express α - and β -CGRP mRNAs, but the ratio of the expression of the two mRNAs varies considerably [1], β -CGRP could be a substitute for α -CGRP. Examinations of the change in CGRP expression in other intrinsic and extrinsic neurons following axotomy may provide some clues to this question. The CGRP synthesized in axotomized rubrospinal neurons was transported along the rubrospinal tract toward the lesion site. Since similar CGRP-LI-labeled fibers were also observed in the ventral

part of the lateral funiculus and in the ventral funiculus (Fig. 4), CGRP could be expressed in CNS neurons projecting through the other descending spinal tracts. We observed some reticulospinal neurons expressing CGRP mRNA in medulla oblongata (unpublished data).

Tetzlaff et al. have demonstrated that rubrospinal neurons express trkB and trkC but not trkA [34], and that BDNF treatment prevents the neuronal atrophy and stimulates the expression of the growth associated protein (GAP)-43 following cervical hemifuniculotomy [32,33]. In the present study, we examined the effect of BDNF treatment on CGRP expression, starting 7 days after lesion and lasting for 7 days. Local injection of BDNF fully prevented the decrease in the number of rubral neurons 2 weeks after lesion, which is consistent with previous studies [32,33]. However, the percentage of neurons positively labeled for α - and β -CGRP mRNAs on the axotomized side was not significantly different between BDNF- and PBS-treated rats. Recently, Piehl et al. reported that local application of BDNF did not modulate the lesion-induced changes in the pattern of expression of α - and β -CGRP in adult spinal motoneurons, but BDNF was able to somewhat increase α -CGRP and decrease β -CGRP expression in embryonic motoneuron cultures [23]. The absence of a specific effect of BDNF on the lesion-induced expression of the β -CGRP subtype in this study might reflect the changes in sensitivity of rubral neurons to neurotrophic factors during development. The small increase in α -CGRP mRNA-labeled cells in BDNF-treated rats in this study may reflect the BDNF effect in vitro.

In conclusion, our results provide the first evidence that rubrospinal neurons confined within the CNS (intrinsic neurons) are able to change their neuropeptide phenotype following axotomy. The changes in the expression of other neuropeptides following axotomy remain to be investigated.

Acknowledgements

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