Differential Regulation of the Expression of Neurotrophin Receptors in Rat Extraocular Motoneurons After Lesion

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ABSTRACT

Neurotrophins acting through high-affinity tyrosine kinase receptors (trkA, trkB, and trkC) play a crucial role in regulating survival and maintenance of specific neuronal functions after injury. Adult motoneurons supplying extraocular muscles survive after disconnection from the target, but suffer dramatic changes in morphological and physiological properties, due in part to the loss of their trophic support from the muscle. To investigate the dependence of the adult rat extraocular motoneurons on neurotrophins, we examined trkA, trkB, and trkC mRNA expression after axotomy by in situ hybridization. trkA mRNA expression was detectable at low levels in unlesioned motoneurons, and its expression was downregulated 1 and 3 days after injury. Expression of trkB and trkC mRNAs was stronger, and after axotomy a simultaneous, but inverse regulation of both receptors was observed. Thus, whereas a considerable increase in trkB expression was seen about 2 weeks after axotomy, the expression of trkC mRNA had decreased at the same post-lesion period. Injured extraocular motoneurons also experienced an initial induction in expression of calcitonin gene-related peptideand a transient downregulation of cholinergic characteristics, indicating a switch in the phenotype from a transmitter-specific to a regenerative state. These results suggest that specific neurotrophins may contribute differentially to the survival and regenerative responses of extraocular motoneurons after lesion.

INDEXING TERMS: axotomy; trk receptor; ChAT; CGRP; oculomotor; abducens

Neurotrophins are a family of growth factors that play essential roles, not only in neuron survival and the establishment of intercellular communication during development, but also in the maintenance and plasticity of the adult nervous system and in the neuronal response to lesion (for review, see Sofroniew et al., 2001; Chao, 2003; Allen and Dawbarn, 2006). Consequently, neuronal dependence on trophic support continues throughout the entire lifespan. In mammals, the neurotrophin family includes nerve growth factor (NGF; Levi-Montalcini, 1982), brain-derived neurotrophic factor (BDNF; Leibrock et al., 1989), neurotrophin-3 (NT-3; Jones and Reichardt, 1990), and neurotrophin-4/5 (NT-4/5; Ip et al., 1992). Their survival-promoting effects have been shown to be mediated by high-affinity receptors of the trk family of tyrosine kinases (Sendtner et al., 1996), which include trkA, trkB, and trkC receptors (Barbacid, 1994). Typically, NGF binds to and activates trkA, BDNF and NT-4/5 interact with trkB, and NT-3 interacts with trkC (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Besides these specific receptors, all neurotrophins bind the low-affinity receptor p75.

Trophic supply in the adult central nervous system may be interrupted by several types of insult, including axotomy, excitotoxicity, ischemia, or neurodegenerative diseases (Giehl et al., 1998; Venero et al., 2000, Boyd and Gordon, 2003). Indeed, both the neurotrophin requirement and the expression of different neurotrophin receptors are usually modified in lesioned neurons (Koliatsos

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et al., 1991; Connor et al., 1996; Canals et al., 1999; Duprey-D´ıaz et al., 2002). Motoneurons represent one of the most widely used models for the study of trophic dependence. Motoneurons are known to receive NGF, BDNF, and NT-3 from the muscle (DiStefano et al., 1992). In the adult, motoneurons survive after target disconnection, but with altered morphological and physiological properties, probably due to the lack of trophic factors supplied from the muscle. However, alternative sources of trophic support may arise through autocrine or paracrine pathways (Kobayashi et al., 1996).

Injury can also cause neuronal dedifferentiation and switching of the neuronal phenotype from a transmitter-specific to a regenerative state (Navarro et al., 2007), with the induction or up- and downregulation of numerous molecules that are related to neuronal survival and axonal regeneration (Costigan et al., 2002; Kubo et al., 2002; Schmitt et al., 2003; Yang et al., 2004). In motoneurons, a reduction in choline acetyltransferase (ChAT) expression, an enzyme involved in the synthesis of the neurotransmitter acetylcholine, often occurs after nerve damage (Lams et al., 1988; Matsuura et al., 1997; Wang et al., 1997). In contrast, other molecules like calcitonin gene-related peptide (CGRP), a protein related to rearrangement of synaptic connections, increase their expression after lesion (Calderó et al., 1992; Borke et al., 1993; Piehl et al., 1993; Fukuoka et al., 1999).

The oculomotor system constitutes a good model for the study of plastic changes induced by target loss; first, because it is a well-characterized system both anatomically and physiologically (Büttner-Ennever, 2006), and second because data are available that were obtained after different types of insult to both extraocular motoneurons and preoculomotor neurons. The motoneurons that innervate the eye muscles are located in three brainstem nuclei: 1) the oculomotor nucleus, innervating four of the extraocular muscles (the ipsilateral medial rectus, inferior rectus, and inferior oblique muscles, and the contralateral superior rectus muscle); 2) the trochlear nucleus innervating the contralateral superior oblique muscle; and 3) the abducens muscle innervating the ipsilateral lateral rectus muscle (Glicksman, 1980; Büttner-Ennever, 2006). The abducens nucleus also contains a group of premotor cells, the abducens internuclear neurons. In cats, these cells exhibit marked changes in firing properties after target removal, but return to normal after 1 month. This is likely due to the reestablishment of synaptic connections with novel targets within their terminal area (de la Cruz et al., 1994a,b, 1996, 2000; Pastor et al., 2000), although this also occurs with implanted neurons after embryonic graft (Benítez-Temiño et al., 2002; for review, see Benítez-Temiño et al., 2005).

The functional recovery of these neurons has been reported to be causally linked to the neurotrophic supply arising from the new targets. In line with this, the exogenous administration of different neurotrophins (BDNF, NT-3, or NGF), for which oculomotor nuclei possess receptors (Benítez-Temiño et al., 2004), to the proximal stump of cat axotomized abducens motoneurons has been described to differentially restore the electrophysiological and morphological changes induced by lesion (Davis-López de Carrizosa et al., 2009, 2010). In the present work, we have used the extraocular motor nuclei of adult rats as the anatomical substrate to investigate the changes induced by axotomy in the expression of several proteins that play a relevant role in the neural response to lesion. Our lesioning model consisted of enucleation, a procedure that involves both the injury to the extraocular motor nerves and the removal of target tissues within the orbit. In particular, we have characterized axotomy-induced changes in expression of the neurotrophin receptors trkA, trkB, and trkC, and of proteins related to either neurotransmission (ChAT) or regeneration (CGRP). This information will allow a better understanding of the process of structural and functional reorganization taking place in motoneurons after lesion, especially with respect to changes in trophic sensitivities and in the synthesis of distinct proteins whose functional role may reflect different operating modes of the motoneuron.

MATERIALS AND METHODS

Animals and surgical procedures

Wistar rats were used in accordance with the guidelines of the European Union (86/609/EU) and Spanish law (R.D. 120/2005 BOE 252/34367-91, 2005) for the use and care of laboratory animals. Protocols used in this study were approved by the ethics committee of the Universidad de Sevilla.

Adult rats (n ¼ 75) of either sex were monocularly enucleated under general anesthesia (sodium pentobarbital, 35 mg/kg, i.p.) as a method to axotomize extraocular motoneurons (Morcuende et al., 2005). The left eyeball was removed, the intraorbital tissues were eliminated, and the eyelids were sutured to close the cavity. Therefore, besides axotomy, the enucleation procedure also removed the target structures that could trophically supply the motoneurons. The analgesic pirazolone (0.1 g/kg, i.m.) was administered for 2 days, and the animals were monitored daily until healing for potential signs of distress. To analyze the time course of axotomy-induced changes in the expression of neurotrophin receptor mRNAs and in the expression of ChAT and CGRP proteins in extraocular motoneurons, animals were separated into five groups of different survival times after lesion (1, 3, 15, 30, and 60 days). Each experimental group comprised three to five animals.

trk mRNAs expression by in situ hybridization

In situ hybridization with oligonucleotide probes was performed as described by Wisden and Morris (2002). Oligonucleotide probes complementary to parts of mRNA encoding trkA, trkB, and trkC were synthesized (Genosys, Sigma, Saint Louis, MO) and radiolabeled with a-^{35}S-dATP (Amersham, Piscataway, NJ) at the 3′ end by using terminal deoxyribonucleotidyl transferase (Promega, Madison, WI). The specific activities of labeled probes used ranged from 200,000 to 300,000 cpm/1l. The following antisense DNA oligonucleotides were used: for rat trkA mRNA 5′-AGG GTT GAA CTC AAA AGG GTT GTC CAT AAA GGC AGC CAT GAT G-3′ (accession number M85214, bases 1,184–1,231; Meakin et al., 1992); rat trkB mRNA 5′-CTG CGA CTG CGT CAG CTC GGT GGG CGG GTT ACC CTC TGC CAT-3′ (accession number M55291, bases 2,576–2,617; Middlemas et al., 1991); rat trkC mRNA 5′-GCT CCT TAA GGA AGT GGC CGT TGA TGG TCT GGT TGG CTG TGC CCA G-3′ (accession number L03813, bases 1,201–1,246; Merlio et al., 1992). The trkB probe was complementary to mRNA encoding the tyrosine kinase domain of full-length trkB. The use of all these probes in the adult rat brain has been described elsewhere (Merlio et al., 1992; Piehl et al., 1994), and they generated the expected pattern.

Rat brains were dissected out and frozen on dry ice-isopentane. Serial coronal sections (12 1m) throughout the brainstem were cut on a Leica CM 1850 cryostat and mounted on poly-L-lysine-coated slides, fixed for 5 minutes in 4% paraformaldehyde, dehydrated through a graded ethanol series, and stored in 95% ethanol. Parallel sections were processed for in situ hybridization with the different a^{35}S-dATP 3′-end-labeled probes. Sections that were to undergo in situ hybridization were air-dried for 1–2 hours. Hybridizations were performed in a buffer containing 50% formamide, 4X sodium chloride-sodium citrated buffer (SSC), 10% dextran sulfate, 5X Denhardt's solution, 0.2 mg/ml salmon sperm DNA, 0.1 mg/ml polyadenilic acid, 0.025 M sodium phosphate (pH 7.0), and 1 mM sodium pyrophosphate. Sections were hybridized at 42°C in a humidified chamber for 16–18 hours with 100 1l of hybridization buffer, 0.02 M dithiothreitol (DTT), and 1 1l of the radioactive probe (5 ng/1l) per slide. Following hybridization, sections underwent three washes of decreasing stringency (two washes of 1X SSC at 55°C for 30 minutes and one wash of 0.1X SSC at room temperature for 1 minute), dehydrated in ethanol (70% and 95%, 1 minute each), and air-dried.

Sections were exposed to a radiosensitive Kodak Biomax MR X-ray film for 1 week in darkness, and afterward dipped in Amersham LM1 photoemulsion, exposed for 6 weeks at 4°C, developed in Kodak D-19 developer, fixed with Kodak Rapid Fixer, and counterstained with cresyl violet. Hybridization signals were regarded as positive when accumulation of silver grains clearly above background levels (statistical support discussed below) were found located over the soma of individual cells. Controls for the specificity of the in situ labeling were demonstrated by a concentration-dependent depletion of the signal, provided by a^{35}S-dATP-labeled probe, when the same unlabeled oligonucleotide was added in excess (100 fold) to the hybridization cocktail.

Data analysis of in situ hybridization signals

Tissues were visualized and analyzed by brightfield optic microscopy (Zeiss Axiophot, Oberkochen, Germany). The density of silver grains over the cytoplasm of each identified neuron was determined by quantifying the number of silver grains and measuring the area of each soma analyzed. Counts in the abducens, trochlear, and oculomotor nuclei were performed by using a 40× objective. For each section, background signal level was determined by averaging grain counts over three areas of neuropil lacking positively labeled cell bodies, and background labeling density was subtracted from the cell labeling. For each trk receptor and survival time, motoneurons were sampled from the abducens nucleus (n ¼ 38–52), the trochlear nucleus (n ¼ 20–38), and the rostral two-thirds of the oculomotor nucleus (n ¼ 85–106) because the caudal third preferentially contains the contralaterally projecting superior rectus motoneurons. Values for the lesioned side were expressed as percentages relative to the unlesioned (control) side. Statistical analysis for significant differences between the control and lesioned sides were performed by Student's t-test and between survival times by one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons. In all cases, the level of significance was $P < 0.05$.

ChAT and CGRP immunostaining

To prepare the tissue for immunocytochemistry, rats were perfused transcardially under deep anesthesia (sodium pentobarbital, 50 mg/kg, i.p.) with 100 ml of physiological saline followed by 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The brainstem was removed and cryoprotected by immersion in a solution of 30% sucrose in sodium phosphate-buffered saline (PBS) until it sank. The brainstem was then cut coronally at 40-1m-thick sections on a cryostat. Sections were divided in two adjacent series to be processed for ChAT or CGRP immunostaining, respectively.

Motoneurons in the abducens, trochlear, and oculomotor nuclei were identified by using an antibody against ChAT (polyclonal goat anti-ChAT, 1:100, AB144P, Chemicon, Temecula, CA; Table 1). Endogenous peroxidase activity was inactivated by a 10-minute incubation in a

TABLE 1.
Primary Antibodies Used in This Study

Antigen	Immunogen	Host/type	Manufacturer	Dilution
ChAT	Human placental ChAT enzyme	Goat polyclonal	Chemicon (Temecula, CA), cat. no. AB144P	1:100
CGRP	Synthetic peptide (H-Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH_2).	Rabbit polyclonal	Bachem/Peninsula (San Carlos, CA), cat. no. T-4032.	1:6,000

Abbreviations: ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide.

solution containing 10% methanol and 2% H_2O_2 in PBS. After washing with PBS, sections were incubated in block- ing solution (3% normal rabbit serum in PBS with 0.1% Tri-ton X-100, 40 minutes). Tissue was then incubated over- night in the primary antibody solution prepared in blocking solution with 0.05% sodium azide. After washing, tissue was exposed for 2 hours to the secondary antibody solution (biotinylated rabbit anti-goat, 1:250, Vector, Bur- lingame, CA). After rinsing, tissue was then incubated for 90 minutes in an avidin-biotin-horseradish peroxidase (HRP) complex (ABC, Vector). Motoneurons were finally made visible by using 3,3⁰-diaminobenzidine tetrahydrochloride (DAB) at 0.05% with 0.08% H_2O_2 diluted in PBS. Sections were mounted on glass slides, dehydrated, cleared and coverslipped.

For CGRP immunohistochemistry, the same protocol as above was used, but some additional steps were included. Nonspecific binding sites were blocked with 0.2 M glycine and lysine, 0.2% bovine serum albumin (BSA), 0.1% gelatin, and 10% normal goat serum in PBS with 0.1% Triton X-100. After 4 hours, tissue was incubated in a solution containing an antibody raised against CGRP (polyclonal rabbit anti-CGRP, 1:6000; T-4032, Peninsula/ Bachem, San Carlos, CA [previously available from Peninsula Laboratories, Belmont, CA, as ICH 6006]; Table 1) and then in the secondary antibody solution (biotinylated goat anti-rabbit, 1:250, Vector). After analysis for CGRP staining, coverslips were removed, and tissue was rehydrated in alcohols of decreasing concentrations, and then counterstained with toluidine blue in order to perform total cell counts in each nuclei.

Antibody characterization

The goat polyclonal anti-ChAT antibody was raised against human placental ChAT, which is identical to the brain enzyme (Bruce et al., 1985), and was affinity-purified (manufacturer's technical information). The antibody stained a single band of 68–70 kDa corresponding to the predicted molecular weight of ChAT on Western blot from rat tissue (Corcoran et al., 2004; Brunelli et al., 2005). Specificity has been previously demonstrated by using the blocking peptide (Márquez-Ruiz et al., 2007). All staining was abolished when the primary antibody was preincubated with 15 1g/ml of the blocking peptide (choline acetyltransferase, rat recombinant protein, Chemicon, cat. no. AG220) for 3 hours prior to being used on the tissue sections from rat and cat brain. In the present study, we have used this antibody as a marker of brainstem motoneurons in the III, IV, and VI nuclei, as it has been described to label cranial nerve motoneurons selectively (Geerling et al., 2010). With the same antibody, Wang and Morales (2009) observed a 100% correspondence between ChAT immunolabeling and ChAT mRNA labeling in the pedunculo-pontine nucleus.

The rabbit polyclonal antibody against CGRP was raised against a synthetic peptide (H-Ser-Cys-Asn-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asp-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Glu-Ala-Phe-NH_2). This antibody reacts 100% with rat a-CGRP and 79% with rat b-CGRP, but does not cross-react with rat amylin or calcitonin, as confirmed by radioimmunoassay (Peninsula Laboratories data sheet). It detects a single band of 4 kDa on Western blots of mouse trigeminal ganglion corresponding to the CGRP 32-amino acid peptide, and preadsorption with a-CGRP eliminated this band (Kosaras et al., 2009). Its specificity has been also tested by preabsorption with CGRP peptide, completely abolishing the immunostaining in rat nerve (Ma and Quirion, 2006) and rat brainstem (Wang et al., 2006).

Cell counting and analysis of immunostaining data

Cells immunolabeled for ChAT in the three extraocular motor nuclei (abducens, trochlear, and oculomotor nuclei) were visualized, and all motoneurons were counted by using a light microscope (Olympus BX61, Tokyo, Japan). Abercrombie's correction was then applied (Abercrombie, 1946). Photomicrographs were taken by brightfield optic microscopy (Olympus BX61). Pictures were adjusted for brightness and contrast with Adobe Photoshop 7.0 (San Jose, CA). For the abducens and trochlear nuclei, the ipsilateral and contralateral nucleus were the respective lesioned sides. For the oculomotor nucleus, however, it was necessary to apply another correction due to the presence of a contralaterally projecting group of motoneurons (Morcuende et al., 2005).

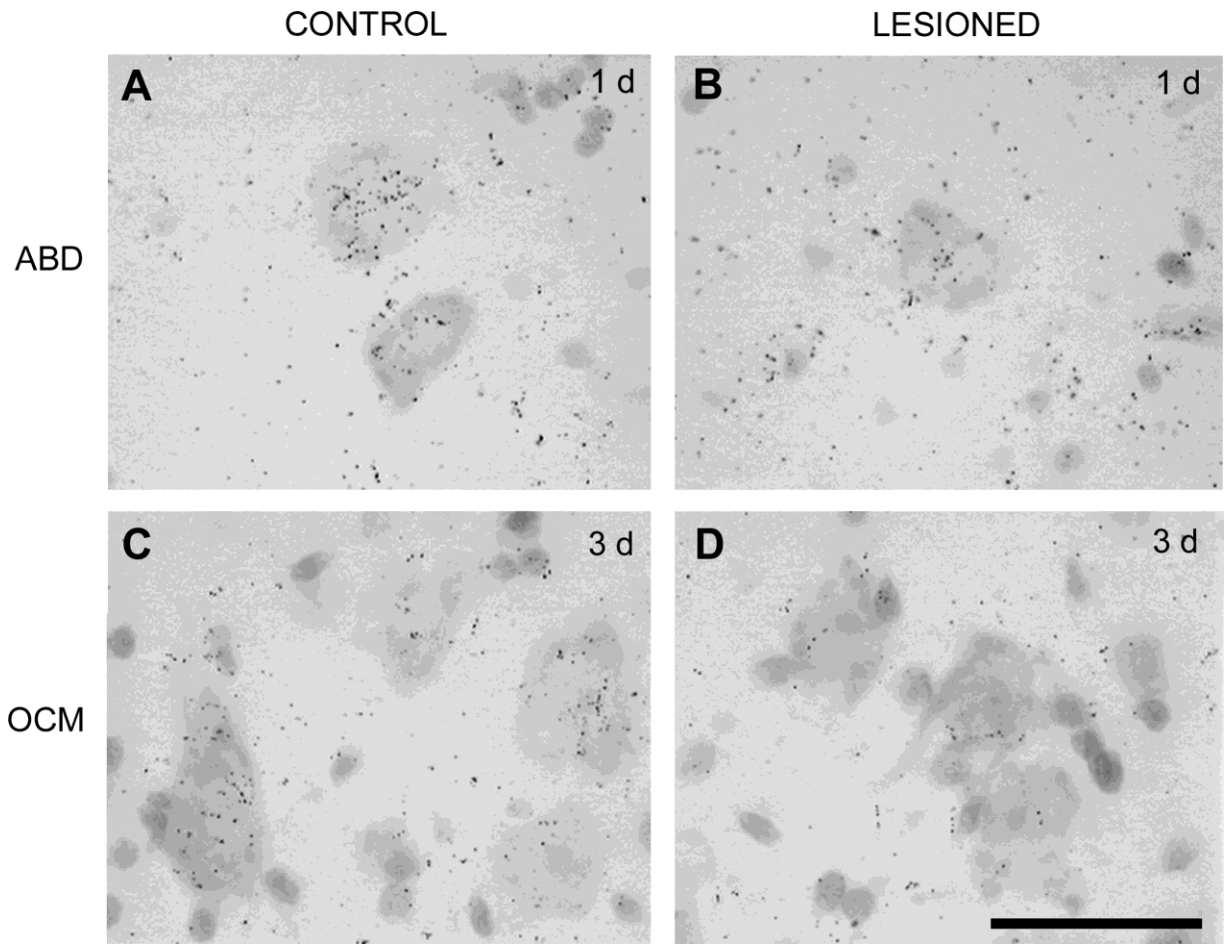

Figure 1. Modulation of trkA mRNA expression after axotomy. Images show the decrease in trkA mRNA in abducens and oculomotor neurons by in situ hybridization a short time after unilateral enucleation. Note the lower silver grain staining in lesioned neurons of both nuclei compared with control neurons. A: Image of control abducens neurons showing the silver grain labeling within the motoneuron cytoplasm. Cells were counterstained with cresyl violet for identification. B: Image of abducens neurons on the lesioned side showing a reduced level of trkA mRNA signal 1 day after axotomy. C,D: Neurons in the control oculomotor nucleus (C) also exhibited levels of trkA mRNA labeling higher than on the injured side (D) 3 days after lesion. Abbreviations: ABD, abducens nucleus; OCM, oculomotor nucleus. Scale bar ¼ 50 1m in D (applies to A–D).

Because the oculomotor nucleus contains four subnuclei of motoneurons, three ipsilateral and one contralateral for a given eye (Glicksman, 1980; Büttner-Ennever, 2006) with approximately equal number of motoneurons (Glicksman, 1980; Miyazaki, 1985), it was necessary to solve the following two equations to determine the number of lesioned and control cells: 3x þ y ¼ n_1 and 3y þ x ¼ n_2, where n_1 and n_2 are the ipsilateral and contralateral number of oculomotor nucleus motoneurons, respectively. Thus, we determined x and y, where x is the number of ChAT-immunoreactive cells per lesioned subnucleus (three groups ipsilateral and one contralateral) and y is the number of ChAT-immunoreactive cells per control subnucleus. To evaluate the effects of axotomy, the number of lesioned ChAT-labeled cells was expressed as the percentage relative to the number of control labeled cells. Comparisons between groups were carried out by using one-way ANOVA followed by post hoc multiple comparisons. When the comparison was performed between two groups, Student's paired t-test was used. In all cases, the level of significance was $P < 0.05$. Values were expressed as mean 6 SEM, and as percentages relative to the unlesioned side.

For CGRP immunostaining, all labeled motoneurons were counted, and Abercrombie's (1946) correction was then applied. In the oculomotor nucleus, the contralateral motoneurons expressing CGRP were considered to belong to the superior rectus group. No staining against CGRP was found in control motoneurons, so we could not express these data as percentages relative to the unlesioned side. Therefore, once CGRP counting was performed, tissue was counterstained by using toluidine blue, and all abducens, trochlear, and oculomotor Nissl-stained neurons were counted and corrected by using

Abercrombie's method. Data were then expressed as the percentage of the total Nissl-stained cells that expressed CGRP. Statistical analysis and comparisons at different time points for each nucleus were carried out by using one-way ANOVA followed by post hoc multiple comparisons. In all cases, the level of significance was $P < 0.05$.

RESULTS

Expression of mRNAs for neurotrophin receptors of the trk family in the extraocular motor nuclei

In situ hybridization against the mRNA of the three trk receptors was performed to assess their presence in the motoneurons that control the eye muscles. Positive labeling for trk mRNAs was found in the cell bodies of the neurons located in abducens, trochlear, and oculomotor nuclei of the unlesioned (control) side. The signal was mainly found over large cell bodies, most likely motoneurons (Figs. 1, 3, 5). The in situ hybridization labeling revealed clearly that the mRNA for trkB, the receptor for BDNF, was the most highly expressed trk neurotrophin receptor in control extraocular motoneurons. trkC mRNA and trkA mRNA were expressed in a lower degree, the latter being the trk receptor with the lowest signal expression (Figs. 1A,C, 3A,C,E, 5A,C,E). After enucleation and removal of the orbital contents, the trk receptor mRNA expression was analyzed at different time points after injury (1, 3, 15, 30, and 60 days) in the extraocular motoneurons.

Modulation of trkA mRNA expression in extraocular motoneurons after lesion

In the extraocular motoneurons, in situ hybridization signal for trkA mRNA decreased significantly, reaching approximately 65% of control values on days 1 and 3 after lesion in the abducens nuclei (day 1: 65.60 6 6.87 %, $P < 0.05$; day 3: 72.23 6 5.62%, $P < 0.01$; day 30: 58.13 6 11.39%, $P < 0.05$) and oculomotor nuclei (day 1: 63.79 6 8.03%, $P < 0.01$; day 3: 68.10 6 9.40%, $P < 0.05$; Figs. 1, 2). A similar decay was also observed in trochlear motoneurons in this period, but this decrement did not reach statistical significance compared with the control side (e.g., day 3: 57.61 6 14.63%, $P > 0.05$; Fig. 2B). The decrease observed in trkA mRNA after lesion was transient because trkA expression in the oculomotor and trochlear nuclei recovered to control values between 1 and 2 months after the lesion (Fig. 2). Therefore, injury of extraocular nerves produced a short-term decrease in the mRNA expression of NGF receptor in the axotomized neurons, despite the scarce but evident basal expression of trkA mRNA in control extraocular motoneurons.

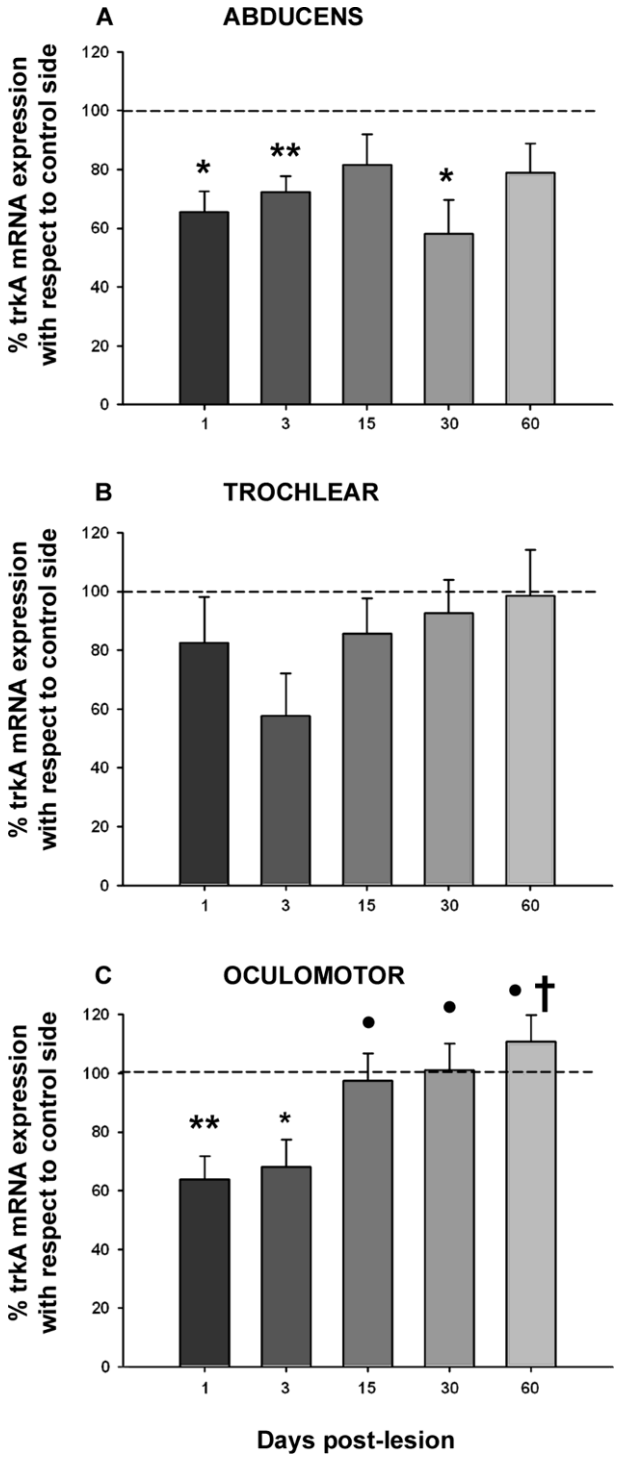

Figure 2. A–C: Time course of changes in the percentage of trkA mRNA expression in lesioned neurons with respect to control in the abducens (A), trochlear (B), and oculomotor (C) nuclei. The control value (100%) is represented by the dashed horizontal line. Data are mean 6 SEM. Asterisks represent statistically significant differences compared with the control side (*, $P < 0.05$; **, $P < 0.01$; Student's t-test). l and † represent statistically significant differences compared with day 1 and day 3, respectively ($P < 0.05$, ANOVA test).

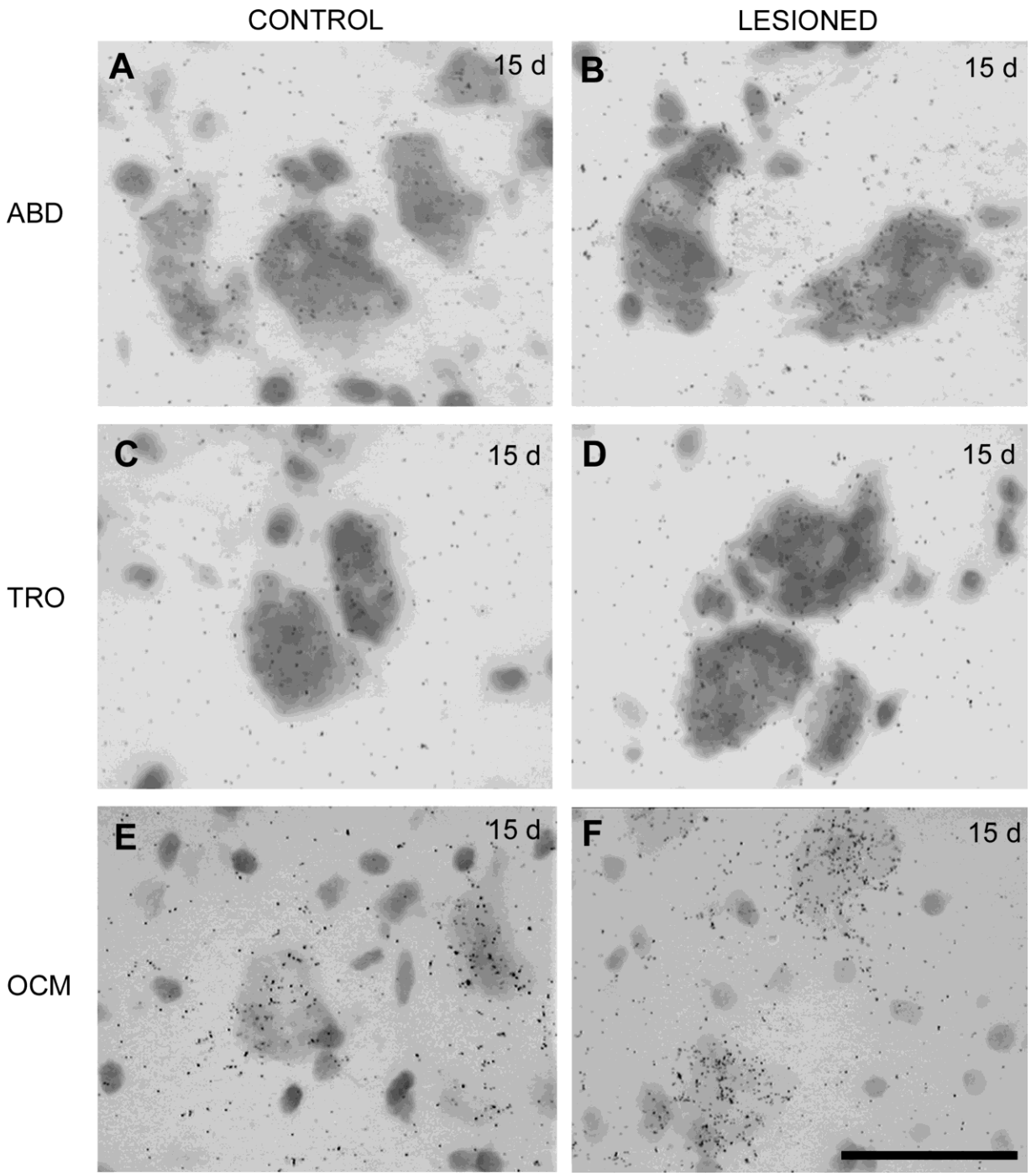

Figure 3. Modulation of trkB mRNA expression after axotomy. Images show the increase in trkB mRNA expression in abducens, trochlear, and oculomotor neurons by in situ hybridization 15 days after unilateral enucleation. Note the high levels of signal for trkB mRNA expres- sion in the control neurons and even higher levels in axotomized neurons, particularly in the oculomotor nucleus. A,C,E: Neurons in the control abducens (A), trochlear (C), and oculomotor (E) nuclei. B,D,F: Images corresponding to their respective lesioned side. Abbrevia- tions: ABD, abducens nucleus; OCM, oculomotor nucleus; TRO, trochlear nucleus. Scale bar ¼ 50 1m in F (applies to A–F).

Modulation of trkB mRNA expression in extraocular motoneurons after lesion

As illustrated in Figure 3, labeling of trkB was conspicuous in the motoneurons of the abducens, trochlear, and oculomotor nuclei of the control side. Hybridization with the trkB mRNA probe demonstrated a strong, but transient, increase in labeling over the extraocular neurons after lesion compared with control neurons. In the abducens nucleus, a significant elevation in trkB mRNA signal was detected 3 days after lesion, and was about 150% of

control values on day 15. However, it returned to control levels by 1 month post lesion (day 3: 124.61 6 9.39%, $P < 0.05$; day 15: 157.53 6 10.36%, $P < 0.01$; Figs. 3A,B, 4A). Similarly, in trochlear neurons the change in trkB mRNA expression was significantly different from the control side at 3 and 15 days after axotomy (day 3: 149.90 6 28.01%, $P < 0.05$; day 15: 144.33 6 10.51%, $P < 0.01$; Figs. 3C,D, 4B), but was at control level by 2 months. However, in oculomotor neurons, the increase in labeling was delayed and smaller compared with the other two nuclei. Thus, we did not find any change in trkB expression at short time intervals after lesion (1–3 days), but a significant increase was measured between 15 days and 1 month (day 15: 118.86 6 7.15%, $P < 0.05$; day 30: 128.24 6 9.36%, $P < 0.05$), returning to control levels by 2 months (Figs. 3E,F, 4C). These results showed a significant upregulation in the expression of the BDNF receptor, trkB, in the motoneurons innervating the eye muscles of the adult rat after axotomy, which was consistently found in the three extraocular motor nuclei by 2 weeks after lesion. Control levels of expression were recovered in all these structures by 2 months.

Modulation of trkC mRNA expression in extraocular motoneurons after lesion

A noticeable downregulation in trkC mRNA expression was observed in the three motor nuclei by 15 days after axotomy (Figs. 5, 6). In the abducens nucleus, we could observe only a 54.27 6 3.01% level of expression in the experimental side compared with the expression observed in control abducens neurons ($P < 0.001$; Fig. 6A). At the same time point, the oculomotor neurons showed an equally significant diminution. This loss was sustained for longer, as was evident, but not significant, on day 3, and was still present on day 30 (day 15: 69.45 6 3.55%, $P < 0.001$; day 30: 87.97 6 3.23%, $P < 0.05$; Fig. 6C). The expression of trkC mRNA in lesioned trochlear motoneurons was also depressed on days 3–30, but not at 15 days post lesion (day 3: 63.34 6 4.47%, $P < 0.001$; day 15: 68.21 6 9.89%, $P > 0.05$; day 30: 72.43 6 8.48%, $P < 0.05$; Fig. 6B). In all cases, the signal regained normal values by 2 months (Fig. 6). Thus, the in situ hybridization revealed that trkC mRNA levels in the extraocular motor nuclei of the adult rat decreased between 3 and 30 days after axotomy in a nucleus-dependent fashion.

ChAT expression was downregulated transiently in extraocular motoneurons after lesion

Motoneuronal ChAT expression can be altered by several types of insult, including axotomy. We analyzed this point in the adult rat extraocular motoneurons by means of immunostaining following enucleation. No change was

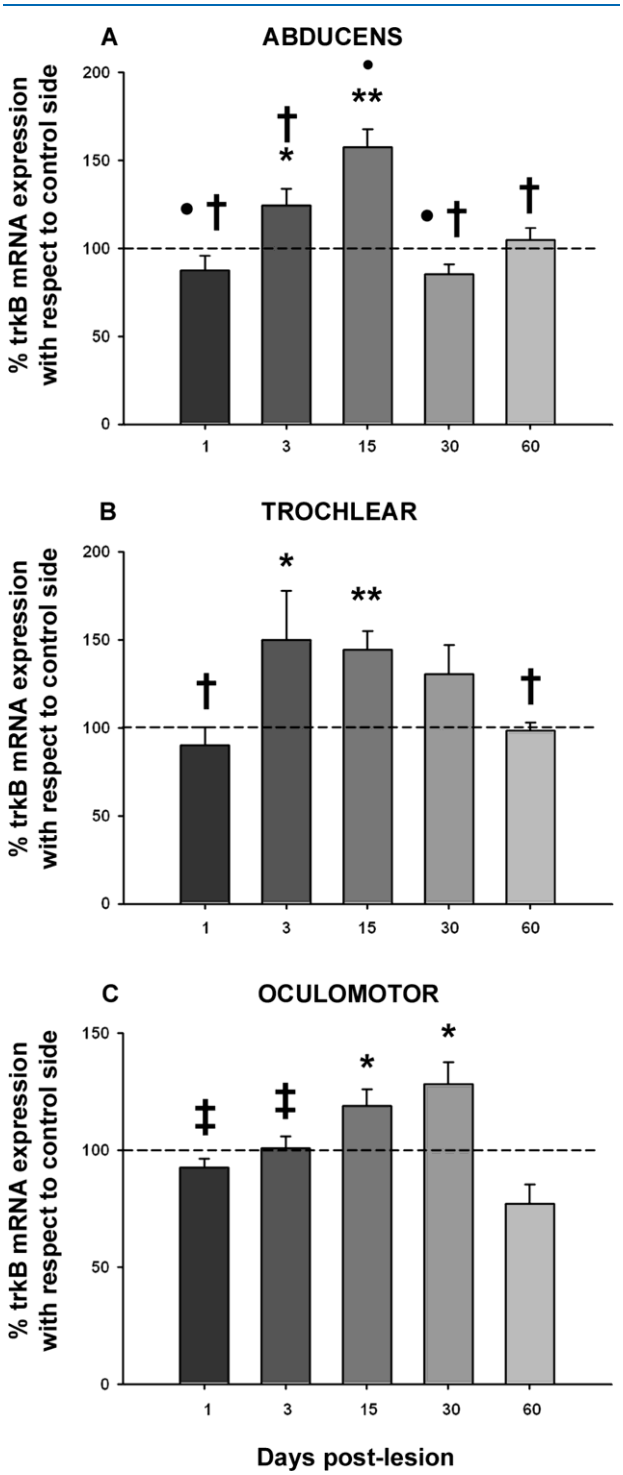

Figure 4. A–C: Time course of changes in the percentage of trkB mRNA expression in lesioned neurons with respect to controls (100%, dashed horizontal line) in abducens (A), trochlear (B), and oculomotor (C) nuclei. Data are mean 6 SEM. Asterisks represent statistically significant differences compared with the control side (*, $P < 0.05$; **, $P < 0.01$; Student's t-test). ●, †, and ‡ represent statistically significant differences compared with day 3, day 15, and day 30, respectively ($P < 0.05$, ANOVA test).

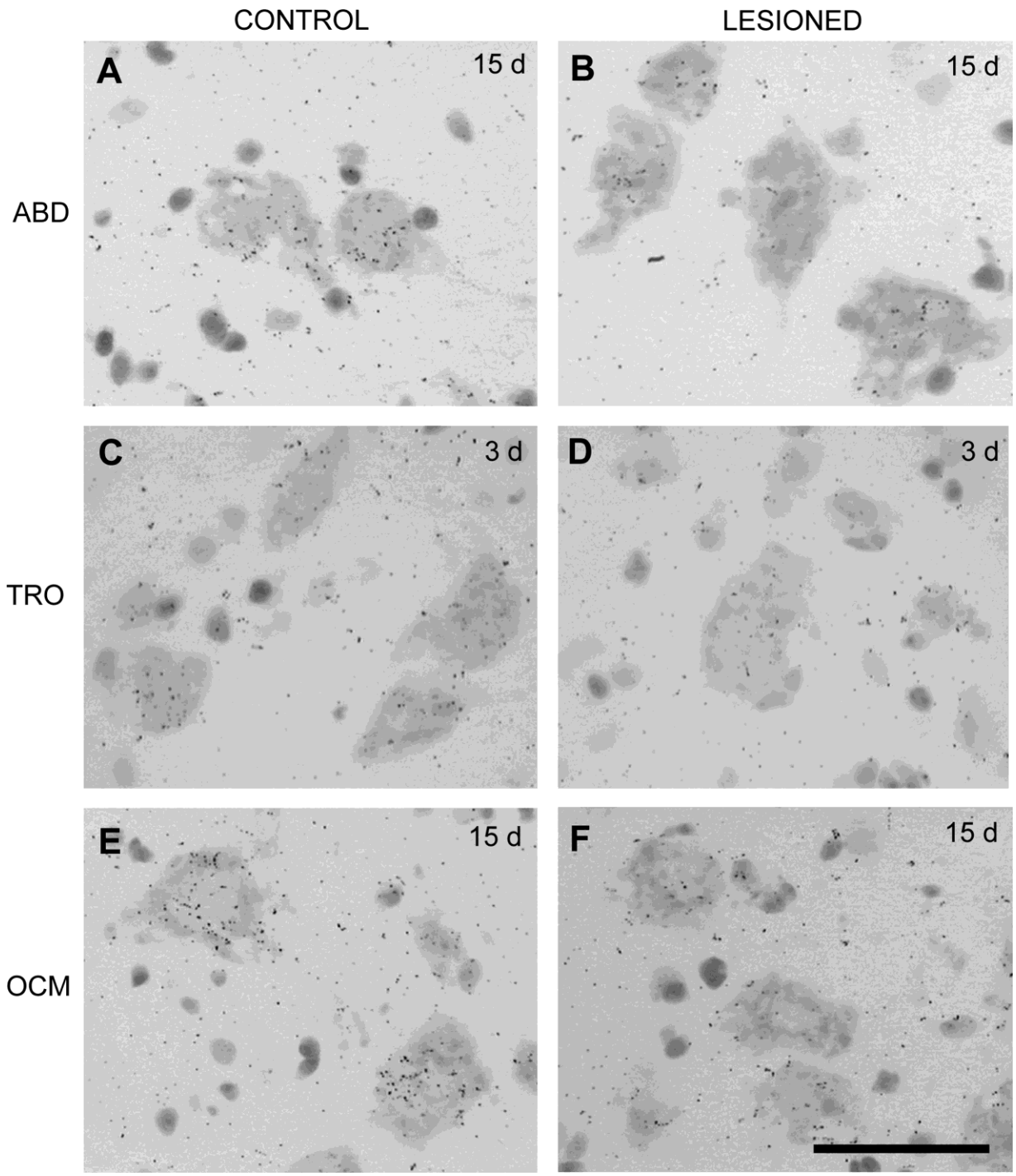

Figure 5. Modulation of trkC mRNA expression after axotomy. A–F: Images show the decrease in trkC mRNA expression in abducens, trochlear, and oculomotor neurons by in situ hybridization either 3 (C,D) or 15 days (A,B,E,F) after unilateral enucleation. Downregulation of trkC expression can be appreciated in the lesioned abducens (B), trochlear (D), and oculomotor (F) nuclei compared with their respec- tive control side (A,C,E). Abbreviations: ABD, abducens nucleus; OCM, oculomotor nucleus; TRO, trochlear nucleus. Scale bar ¼ 50 1m inF (applies to A–F).

observed at short time intervals after lesion (1 and 3 days), as a similar number of ChAT-positive neurons was found on both sides of each motor nucleus (Figs. 7A–C, 8). However, by 15 days post lesion, the number of ChAT-immunoreactive neurons was significantly reduced to levels around 65% (abducens: 72.44 6 6.51%; trochlear: 67.63 6 9.56%; oculomotor: 66.96 6 6.94%) of the control value ($P < 0.05$, paired t-test, one-way ANOVA test; Figs. 7D–F, 8). This decrease was transient, with the number of motoneurons immunoreactive for ChAT recovering by 1 month

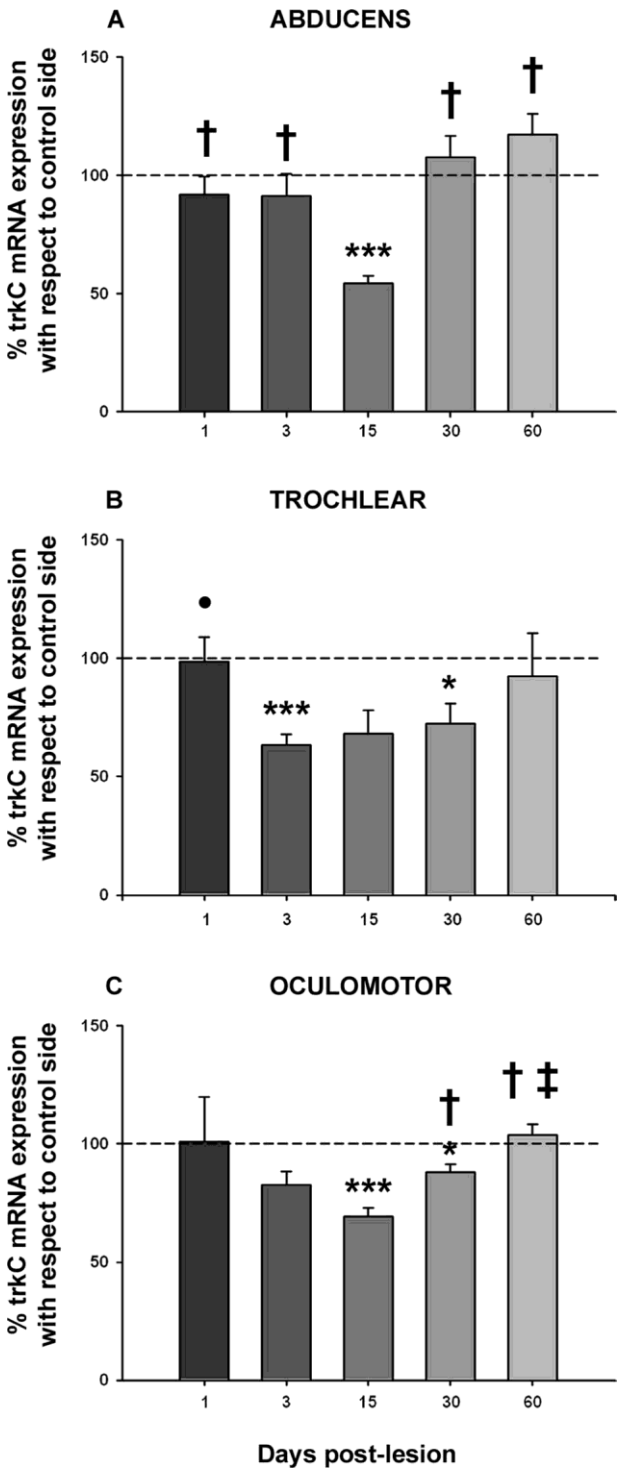

Figure 6. A–C: Time course of changes in the percentage of trkC mRNA expression in lesioned neurons with respect to control (100%, dashed horizontal line) in abducens (A), trochlear (B), and oculomotor (C) nuclei. Data are mean 6 SEM. Asterisks represent statistically significant differences compared with the control side (*, $P < 0.05$, ***, $P < 0.001$; Student's t-test). 1, †, and ‡ represent statistically significant differences compared with day 3, day 15, and day 30, respectively ($P < 0.05$, ANOVA test).

after enucleation. No significant neuronal death occurred as a consequence of target muscle loss or axonal injury, because by 2 months post lesion, the number of motoneurons in the lesioned nuclei labeled with the antibody against ChAT was similar to that on the control side (Figs. 7G–I, 8).

CGRP expression was increased in extraocular motoneurons shortly after lesion

Immunohistochemistry for CGRP revealed the absence of labeling in control extraocular motoneurons of the adult rat (Fig. 9; see the control side without the arrow). In contrast, at 1 and 3 days post lesion, labeled CGRP-positive cells appeared within the extraocular motor nuclei (Fig. 9; lesioned side indicated by the arrow). Due to the absence of control labeling, computation of the number of CGRP-positive neurons was expressed relative to the total number of Nissl-stained cells and referred to in percentages (Fig. 10). Thus, by 1 and 3 days post lesion, the percentage of CGRP-immunoreactive neurons in the abducens nucleus of the affected side was 80.02 6 3.93% and 75.81 6 8.05%, respectively, of the total population. Similarly, in the trochlear nucleus, these values were 79.38 6 2.32% at day 1 and 86.34 6 8.90% at day 3, and in the oculomotor nucleus they were 81.62 6 7.52% and 83.76 6 5.64% by 1 and 3 days post lesion, respectively (Fig. 10). At longer time intervals, the number of CGRP-labeled cells in the lesioned nuclei was significatly reduced compared with days 1 and 3 ($P < 0.05$, ANOVA test, Fig. 10). However, CGRP immunostaining never disappeared completely as, unlike the control side, the nuclei on the lesioned side showed some CGRP-positive cells even 60 days after axotomy. It is noteworthy that at 30 days post lesion there were no differences in the percentage of CGRP-immunoreactive neurons compared with early days after lesion in both the trochlear and oculomotor nuclei.

DISCUSSION

The data presented here show a differential regulation of the expression of trk receptors in the extraocular motor nuclei of the adult rat following a lesion that severs their axons and destroys their targets. Extraocular motoneurons experienced a decrease in trkA expression shortly after axotomy, in a period of time coincident with an increase in expression of CGRP, a protein related to regeneration. Two weeks after lesion, trkB receptor expression was considerably increased, whereas both trkC and expression of the cholinergic phenotype were significantly reduced (Fig. 11A). Together, these results point to changes in neurotrophin dependence of lesioned motoneurons that might be related to temporal switches from a neurotransmitter-specific to a regenerative state (Fig. 11).

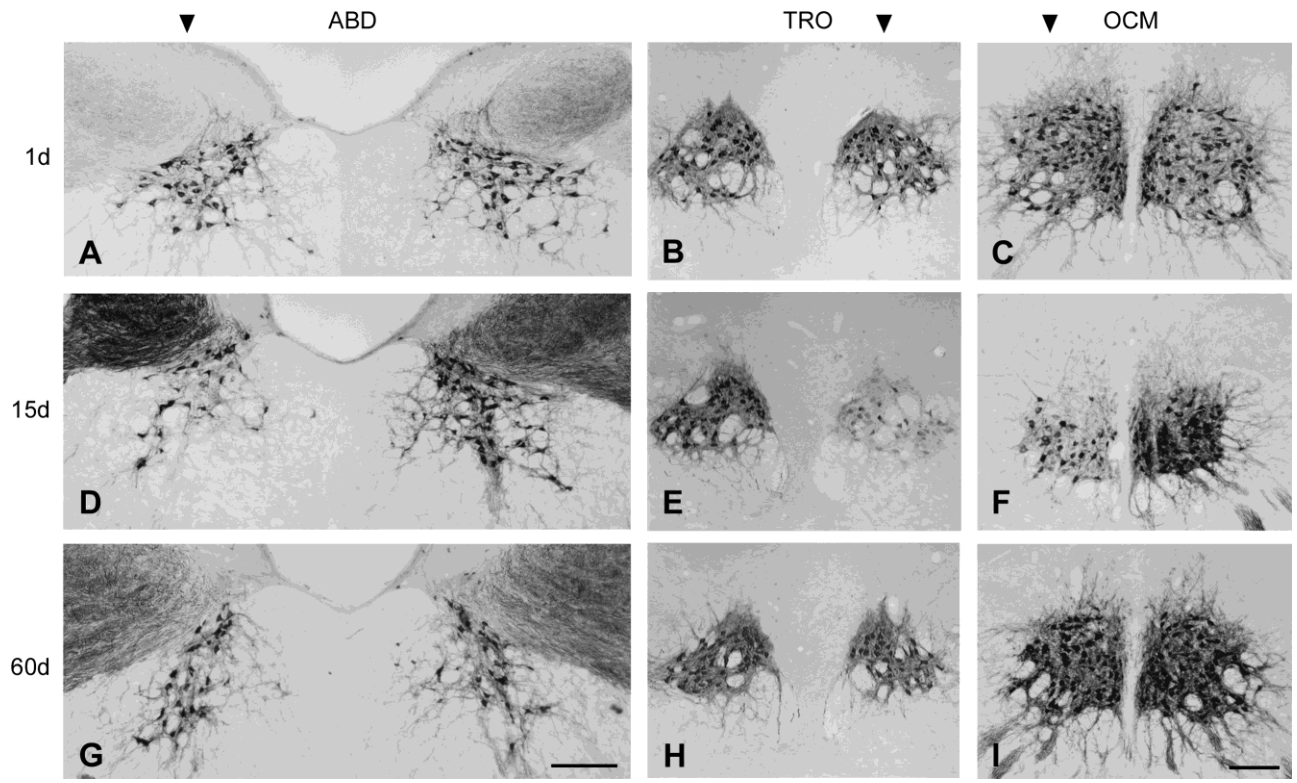

Figure 7. ChAT immunostaining of extraocular motoneurons after left orbit enucleation. A–C: One day after lesion the axotomized motoneurons kept their cholinergic phenotype. D–F: A noticeable decrease in ChAT immunostaining was observed in the abducens (D), trochlear (E), and oculomotor (F) nuclei of the lesioned side after 15 days. G–I: However, 60 days after lesion, the level of ChAT immunoreaction was similar on both sides of the abducens (G), trochlear (H), and oculomotor (I) nuclei. Arrows point to the affected side. Abbreviations: ABD, abducens nucleus; OCM, oculomotor nucleus; TRO, trochlear nucleus. Scale bar ¼ 100 1m in G (applies to A,D,G); 200 1m in I (applies to B,C,E,F,H,I).

Changes in expression of trk receptors after axotomy

It is well known that motoneurons deprived of retrograde neurotrophic support from the muscle experience profound changes in their physiological and morphological properties, including synaptic stripping (Sumner, 1975; de la Cruz et al., 1996). It has been suggested that axotomized motoneurons might compensate for the lack of target-derived support by enhancing trophic factor delivery via autocrine/paracrine pathways (Giehl et al., 1998). Nevertheless, the lack of the essential muscle-derived trophic support triggers a biochemical readjustment in the expression of neurotrophin receptors, as well as other molecules required for survival. These provide for the maintenance of synaptic contacts and maintain the deafferented cells, albeit with reduced firing rates (Fig. 11B-2; de la Cruz et al., 2000; Pastor et al., 2000). In the present study, we have analyzed how extraocular motoneurons modify the expression of trk receptors after permanent disconnection from their target muscles.

Motoneurons innervating the extraocular muscles expressed the high-affinity receptors for neurotrophins trkA, trkB, and trkC, with trkB being the most abundantly expressed trk receptor. In contrast to other spinal or cranial motoneurons, trkA mRNA expression in adult rat ocular motoneurons was readily detectable above background level. Moreover, we have recently shown that axotomized abducens motoneurons in the cat recover their modified firing rate pattern and sensitivity to eye movements after application of NGF, acting via both the p75 and the trkA receptors, and exhibit an increase in both types of receptors by 15 days after axotomy (Davis-López de Carrizosa et al., 2010). We noticed a small decrease in trkA receptor 1–3 days after axotomy in the rat. Although in our physiological study in the cat we did not study the expression of trkA at short time intervals (i.e., 3 days) after axotomy, there could be species-specific differences or a different time course of changes. The normal expression of trkA receptor in the rat after 15 days is more in line with our previous findings in the cat. Taken together, these findings suggest that adult extraocular motoneurons are endowed with the machinery to respond to NGF under both normal and axotomized conditions, in contrast to spinal motoneurons, in which the

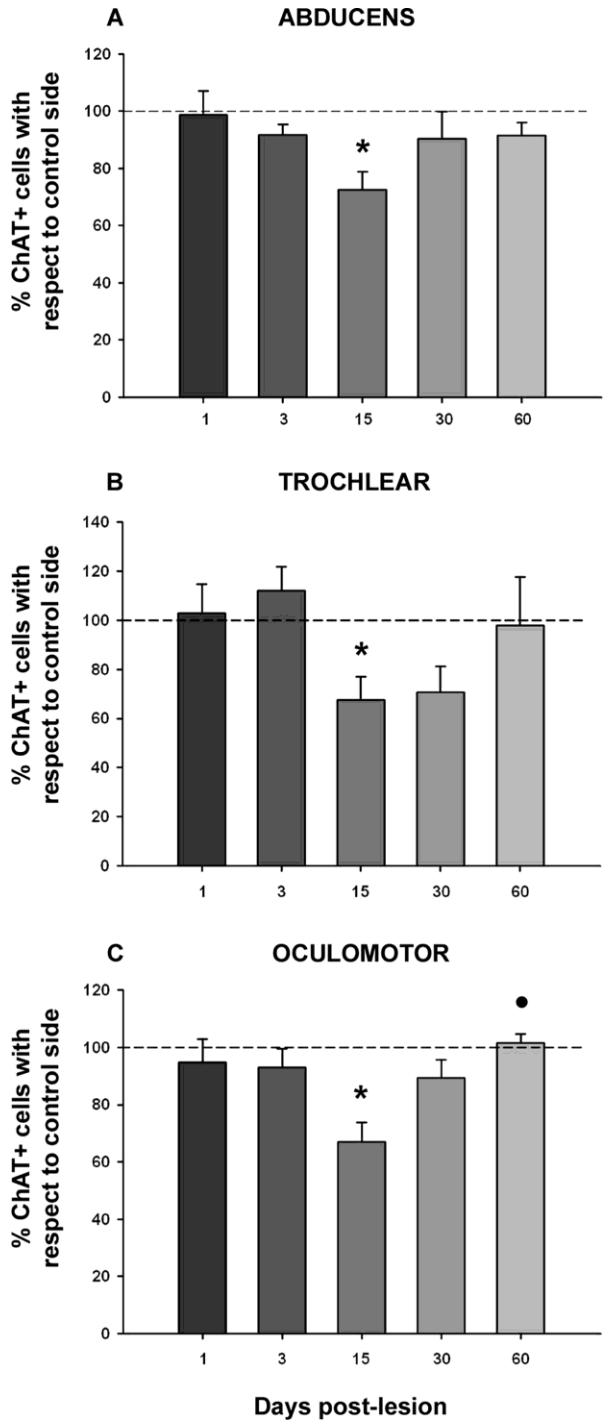

Figure 8. Transient decay in ChAT content in extraocular motoneurons following axotomy. A–C: The histograms show the time course of changes in the percentage of ChAT-immunoreactive motoneurons in the abducens (A), trochlear (B), and oculomotor (C) nucleus of the lesioned side with respect to the control side (considered as 100%, dashed horizontal line). Note that the number of ChAT-immunoreactive neurons was significantly decreased 15 days after axotomy in the three nuclei and recovered to control values 1 month after lesion. Data are mean 6 SEM. Asterisks represent statistically significant differences compared with the control side (*, $P < 0.05$, Student's t-test). ● represents statistically significant differences compared with day 15 ($P < 0.05$, ANOVA test).

basal expression of trkA receptor in the adult is negligible (Curtis et al., 1998).

We have found that extraocular motoneurons transiently increased their expression of trkB mRNA encoding the full-length tyrosine kinase receptor after axotomy. This finding strongly supports a general role for BDNF in the motoneuron response to a lesion, as it occurs in other cranial and spinal motoneurons as well (Oppenheim et al., 1992; Koliatsos et al., 1993; Phiel et al., 1994; Kobayashi et al., 1996). In spinal motoneurons, this increase results in an accumulation of trkB receptor in the axonal growth cone, which could make motoneuronsmore sensitive to local or target-derived neurotrophins (Phiel et al., 1994). These results, together with previousstudies that have demonstrated increased BDNF synthe- sis by Schwann cells in injured nerves (Meyer et al., 1992; Funakoshi et al., 1993; Hughes et al., 1993; Yanet al., 1994; Boyd and Gordon, 2003), strongly supportthe idea that BDNF may be important for survival and/or regeneration of motoneurons after axon injury. In agreement with our results, other brainstem motoneurons, like those in the facial and hypoglossal nuclei, also show a robust increase in trkB expression after injury, which may enhance their responsiveness to BDNF (Kobayashi et al., 1996; Tuszynsky et al., 1996). Expression of BDNF in the proximal stump of axotomized motoneurons is low compared with the distal stump (Meyer et al., 1992). In our model of enucleation the distal stump was also removed (i.e., only the proximal stump remained), indicating that the strong upregulation of trkB expression detected in extraocular motoneurons could be related to an increase in the availability of BDNF from other sources, e.g., via autocrine/paracrine mechanisms or synaptic inputs or from central glial cells (Giehl et al., 2001; Fig. 11B3, arrows).

Previous studies have demonstrated that BDNF and NT-3 act as opposing, sometimes complementary, factors. For instance, they have contrary effects in regulating cortical dentritic growth (McAllister et al., 1997) and survival of corticospinal axotomized neurons (Giehl et al., 2001). It has also been shown that full-length trkB is upregulated in adult rat spinal and facial motoneurons after peripheral axotomy (Piehl et al., 1994; Kobayashi et al., 1996), whereas, in the same lesion models, trkC mRNA is downregulated (Fernandes et al., 1998; Johnson et al., 1999; Hammarberg et al., 2000). Our results are in agreement with those studies, because trkB was upregulated and the expression of NT-3 receptor trkC was downregulated in injured ocular motoneurons (Fig. 11). Reciprocal changes in the trkB and trkC receptors have also been shown in axotomized facial motoneurons (Kobayashi et al., 1996). In fact, the time course of these modifications resembled that described for axotomized facial

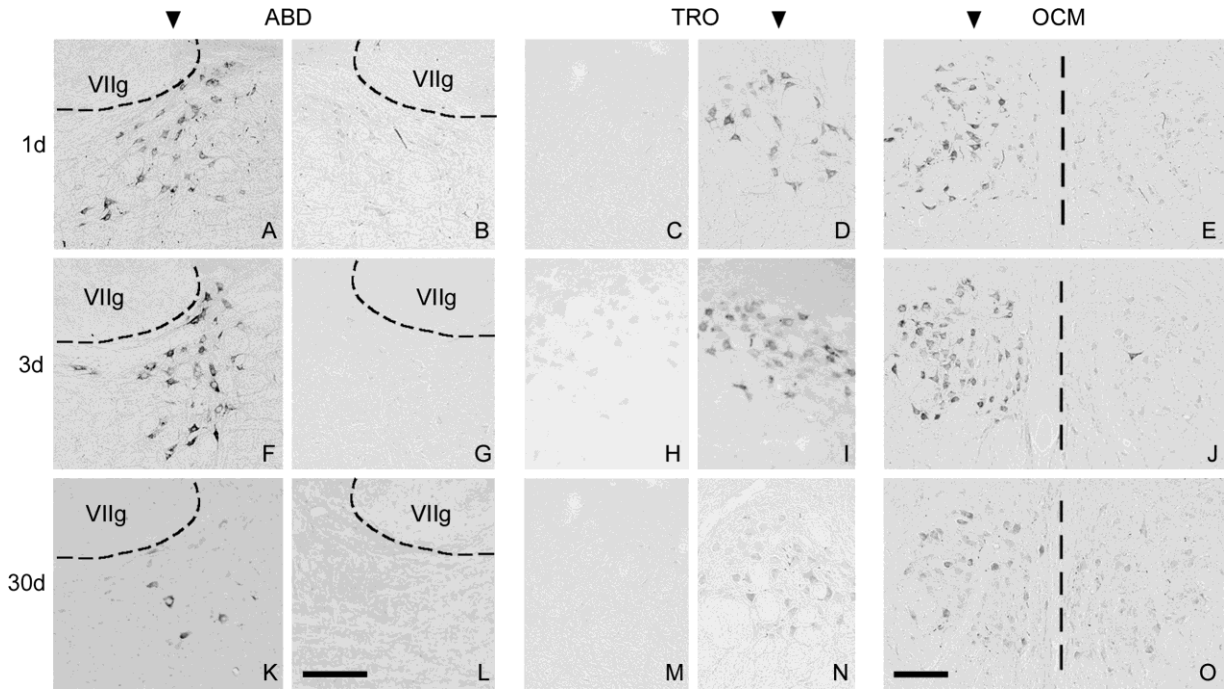

Figure 9. CGRP immunostaining in the extraocular motor nuclei after left orbit enucleation. A–O: A marked increase in CGRP immunoreactivity was observed on the lesioned side of the abducens, trochlear, and oculomotor nuclei 1 day (A,D, left panel in E, respectively) and 3 days after axotomy (F,I, left panel in J, respectively) compared with their respective unlesioned sides (1 day: B,C, right panel in E; 3 days: G,H, right panel in J). Note that the increase in CGRP immunolabeling was less noticeable 30 days after lesion (K–O) when compared with shorter survival times (A–J). Dashed lines in abducens panels delimit the genu of the facial nerve. The dashed line in E, J, and O separates the left, affected side, from the right, control side, of the oculomotor nucleus. Note the lack of signal in the control sides of the three nuclei. Arrows point to the affected side. Abbreviations: ABD, abducens nucleus; OCM, oculomotor nucleus; TRO, trochlear nucleus; VIIg, genu of the facial nerve. Scale bar ¼ 200 1m in L (applies to A,B,F,G,K,L [abducens nuclei]; 200 1m in O (applies to C–E,H–J,M–O [troch-lear and oculomotor nuclei]).

motoneurons, peaking at 1–2 weeks after lesion, and lasting for no more than 1 month (Kobayashi et al., 1996). Several studies have reported that NT-3 expression does not increase after a lesion in denervated muscle (Piehl et al., 1994; Omura et al., 2005) and is actually decreased in the injured nerve (Funakoshi et al., 1993). Moreover, the infusion of NT-3 is not as efficient as that of BDNF in preventing the death or downregulation of cholinergic phenotype in injured motoneurons (Sendtner et al., 1996; Tuszynsky et al., 1996). In view of these findings, it seems that NT-3 might not be the mediator of an important role in the survival of extraocular motoneurons after lesion.

In contrast, our recent findings showed that BDNF and NT-3 have complementary roles in regulating the firing patterns of axotomized abducens motoneurons in the cat. Thus, we have shown that the exogenous administration of BDNF supports tonic firing, whereas NT-3 supports phasic firing, with both neurotrophins enhancing afferent connectivity (Davis-López de Carrizosa et al., 2009). It might also be possible that the reduction in NT-3 availability after muscle disconnection induced the downregulation in trkC receptors observed in these motoneurons. Therefore, we believe that both neurotrophins are important regulators for axotomized motoneurons. The opposing regulation of the receptors found in the present work might be an intrinsic feature of these cells that is dependent on the levels of neurotrophin availability (Suneja et al., 2005). Thus, changes in the availability of a given neurotrophin might in turn alter the expression levels of the receptor in presynaptic inputs.

Trophic dependence of the cholinergic phenotype in motoneurons

In adult mammals, transection of the peripheral nerve results in a drop in ChAT immunoreactivity in the injured motoneurons, without affecting their cell number (Lams et al., 1988; Fernandes et al., 1998; Morcuende et al., 2005). The downregulation of the cholinergic phenotype seems to be a common phenomenon, as it is described in other spinal and cranial motoneurons (Lams et al., 1988; Tuszynsky et al., 1996; Wang et al., 1997; Fernandes et al., 1998). Among neurotrophins, BDNF has been shown to attenuate the decrease of ChAT expression in

axotomized motoneurons (Yan et al., 1994; Friedman et al., 1995; Tuszynsky et al., 1996). Tuszynsky and colleagues (1996) showed that in axotomized hypoglossal motoneurons BDNF prevents downregulation of the cholinergic phenotype, whereas NGF or NT-3 have no effect, in accordance with an induction of trkB, but not of trkA or trkC. Similar results have been obtained by Wang et al. (1997), who observed that ChAT levels are maintained after administration of BDNF, but not when NGF or NT-4/5 are applied to the transected hypoglossal nerve. Those results are in agreement with ours and with those of Fernandes et al. (1998), who observed in facial motoneurons support for ChAT expression by BDNF linked to an increase in the expression of trkB and a decrease in trkC receptors. The increase in trkB expression described for the axotomized ocular motoneurons in our study might then be related to a determinant role for BDNF in restoration of the cholinergic phenotype.

Because removal of retrograde trophic support could be one cause of the downregulation of ChAT, then other sources of trophic support (anterograde, autocrine, and paracrine as well as those derived retrogradely from post-traumatic neuromas [Kryger et al., 2001; Kotulska et al., 2006] or from Schwann cells in the proximal stump [Meyer et al., 1992]) could explain the recovery of ChAT re-expression. In line with our findings, ChAT re-expression occurs in models of peripheral nerve axotomy, even when the nerve is ligated and thus blocked from reinnervation (Wang et al., 1997; Matsuura et al., 1997; Okura et al., 1999). Moreover, if reinnervation into the muscle is allowed, the timing of ChAT expression after recovery is not coincident with the reinnervation of the muscle (Borke et al., 1993). Thus, even when reinnervation is impeded, or even aborted, regeneration recapitulates the program of axonal growth in search of a target (Pastor et al., 2000).

In summary, the present data suggest that the recovery of ChAT expression (and that of trk receptors) after lesion is not dependent on target reinnervation and therefore it likely represents either a truncated regenerative cell program or a response to the increased availability of trophic factors from alternative sources. This is not the case for other neuronal properties like firing pattern and synaptic

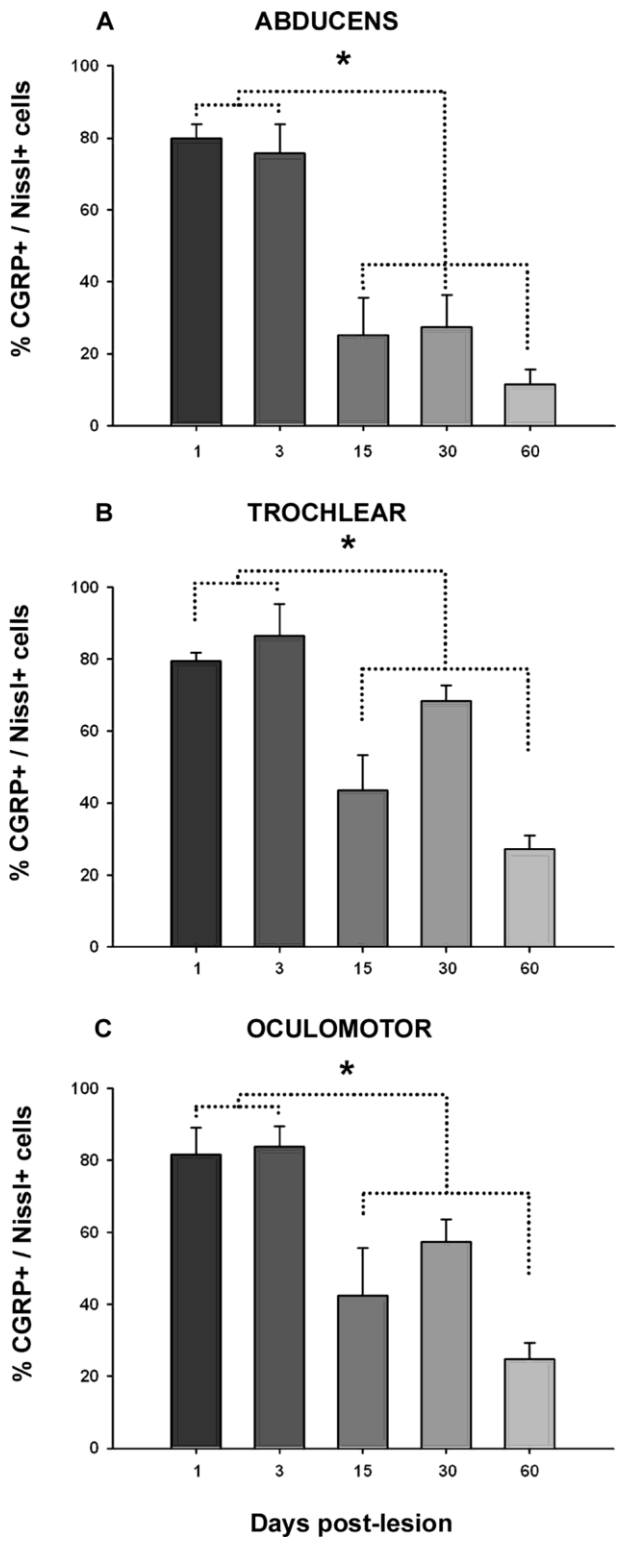

Figure 10

Figure 10. Increase in the number of CGRP-immunoreactive neurons at short time intervals after axotomy in the extraocular motor nuclei. Control motoneurons showed absence of CGRP immunolabeling. A–C: Therefore, histograms compare the percentage of axotomized neurons positive for CGRP in abducens (A), trochlear (B), and oculomotor (C) nuclei relative to the total number of Nissl-stained cells and at different survival times after lesion. One and 3 days after axotomy, a significant increase in the percentage of CGRP-immunoreactive neurons was observed in the three lesioned nuclei in relation to longer survival times. Data are mean 6 SEM. Asterisks represent statistically significant differences ($P < 0.05$, ANOVA test).

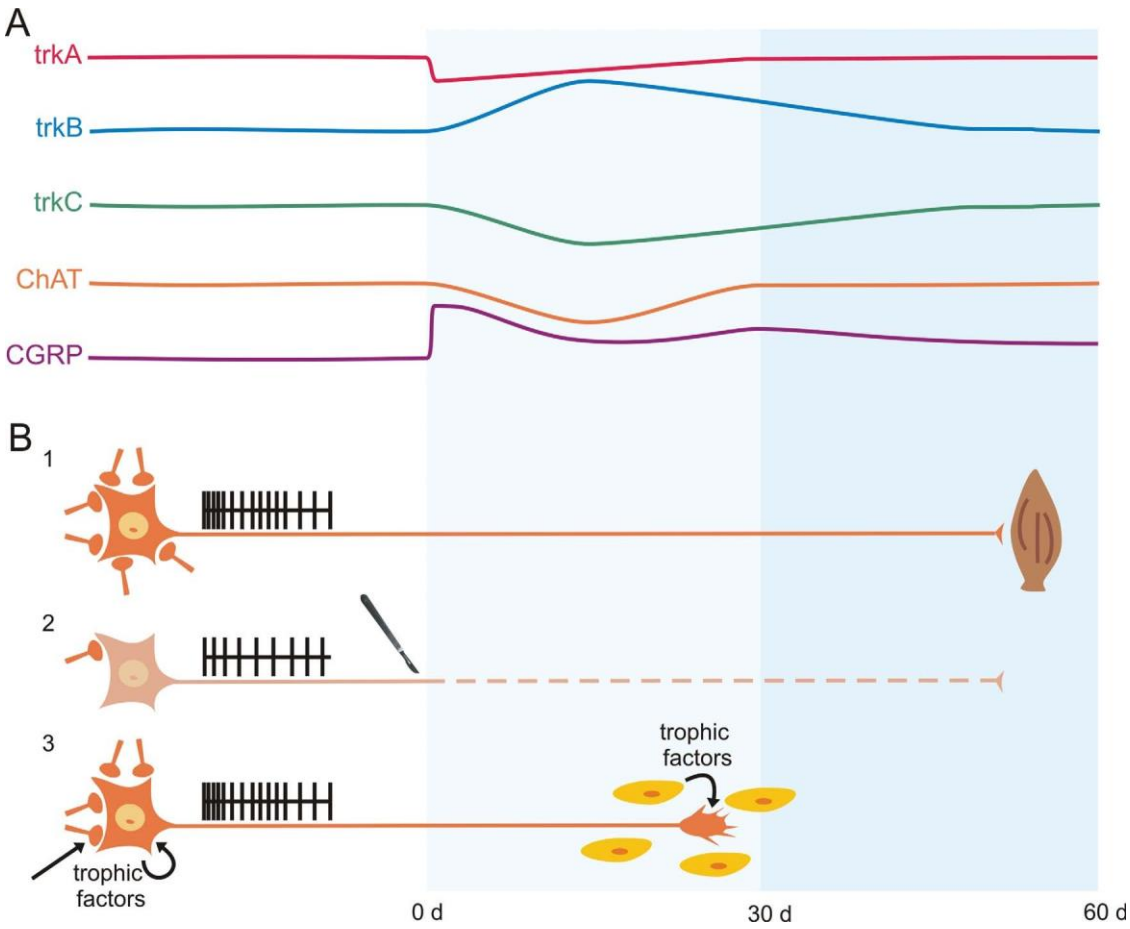

Figure 11. Schematic diagram indicating alterations in extraocular motoneurons after axotomy. A: Time course of changes in the expres- sion of trk receptors, choline acetyltransferase (ChAT), and calcitonin gene-related peptide (CGRP) in extraocular motoneurons after axot- omy. B-1: The connected neuron shows a normal phenotype in afferent synaptic activity and firing rate. B-2: The axotomized neuron has reduced levels of firing rate and afferent synaptic activity. B-3: The neuron switches from a transmitter to a regenerative phenotype whereby the autocrine/paracrine and anterograde trophic support gains relative importance with respect to the retrograde trophic sup-port. Appropriate levels of retrograde trophic factors delivered from different sources would encourage restoration of normal values in the altered molecules.

inputs that show a high degree of dependence on target reinnervation for their recovery from the axotomized state (Delgado-Garc´ıa et al., 1988; de la Cruz et al., 1996; Pastor et al., 2000; Benítez-Temiño et al., 2005).

Induction of CGRP expression by axotomy

The upregulation of CGRP, a protein involved in the formation and maintenance of neuromuscular junctions and axonal regeneration, seems to be a common response of cranial motoneurons following axotomy (Fukuoka et al., 1999). Its expression is regulated in an activity-dependent manner (González-Forero et al., 2002). We have observed and quantified a clear induction of its expression in the extraocular motor nuclei at short time intervals (1 and 3 days) after lesion. This early induction has been suggested to exert a trophic influence on the lesioned neurons in severe environmental insult (Dumoulin et al., 1992). Thirty days after lesion, we observed a new peak in CGRP expression (Fig. 10). Chang et al. (2004) obtained similar results, and argued a relation between the expression of this molecule and innervation of a new target. In line with this, CGRP has been involved in the formation of synaptic processes (Sala et al., 1995). Increased CGRP staining has been shown to persist for a long time, and to be still evident when nearly complete reinnervation has been achieved, even when levels of neurotrophin receptors have returned to control levels (Piehl et al., 1993). Therefore, the fact that CGRP protein was expressed in extraocular motoneurons after lesion might suggest that a process of axonal regrowth and reinnervation has been at least initiated (Fig. 11A). Nevertheless, in our lesion model (i.e., enucleation), the chances for reinnervating a new target are scarce, and this could explain the slow, albeit incomplete, reduction in CGRP we observed over 2 months.

In conclusion, the present study shows that differential regulation of trk receptor expression occurs in extraocular motoneurons after injury, with the most significant change being the robust increase in trkB expression. We can hypothesize that a period of axonal regrowth might take place shortly after axotomy, suggested by the induction in CGRP protein. Injured motoneurons may be less dependent on NGF and NT-3, as indicated by the decrease in trkA and trkC expression. In contrast, the increase in trkB expression might suggest a relevant role for BDNF in the survival and recovery of the cholinergic phenotype in extraocular motoneurons after lesion. Together, these findings suggest a concerted action of trophic and programmed signals through the course of neuronal restoration after lesion that under less severe conditions would lead the neuron to resume normal firing and express normal levels of neurotransmitter in conjunction with renewed connections to targets.

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LITERATURE CITED

Abercrombie M. 1946. Estimation of the nuclear population from microtome sections. Anat Rec 94:239–247.

Allen SJ, Dawbarn D. 2006. Clinical relevance of the neurotrophins and their receptors. Clin Sci (Lond) 110:175–191.

Barbacid M. 1994. The Trk family of neurotrophin receptors. J Neurobiol 25:1386–1414.

Benítez-Temiño B, de la Cruz RR, Pastor AM. 2002. Firing properties of axotomized central nervous system neurons recover after graft reinnervation. J Comp Neurol 444: 324–344.

Benítez-Temiño B, Morcuende S, Mentis GZ, de la Cruz RR, Pastor AM. 2004. Expression of Trk receptors in the oculomotor system of the adult cat. J Comp Neurol 473: 538–552.

Benítez-Temiño B, de la Cruz RR, Tena J, Pastor AM. 2005 Cerebellar grafting in the oculomotor system as a model to study target influence on adult neurons. Brain Res Brain Res Rev 49:317–329.

Borke RC, Curtis M, Ginsberg C. 1993. Choline acetyltransferase and calcitonin gene-related peptide immunoreactivity in motoneurons after different types of nerve injury. J Neurocytol 22:141–153.

Boyd JG, Gordon T. 2003. Neurotrophic factors and their receptors in axonal regeneration and function recovery after peripheral nerve injury. Mol Neurobiol 27:277–324.

Bruce G, Wainer BH, Hersh LB. 1985. Immunoaffinity purification of human choline acetyltransferase: comparison of the brain and placental enzymes. J Neurochem 45:611–620.

Brunelli G, Spano P, Barlati S, Guarneri B, Barbon A, Bresciani R, Pizzi M. 2005. Glutamatergic reinnervation through peripheral nerve graft dictates assembly of glutamatergic synapses at rat skeletal muscle. Proc Natl Acad Sci U S A 102:8752–8757.

Büttner-Ennever JA. 2006. Neuroanatomy of the oculomotor system. Progress in brain research, vol. 151. Amsterdam: Elsevier.

Calderó J, Casanovas A, Sorribas A, Esquerda JE. 1992. Calcitonin gene related peptide in rat spinal cord motoneurons: subcellular distribution and changes induced by axotomy. Neuroscience 48:449–461.

Canals JM, Checa N, Marco S, Michels A, Perez-Navarro E, Alberch J. 1999. The neurotrophin receptors trkA, trkB and trkC are differentially regulated after excitotoxic lesion in the rat striatum. Brain Res Mol Brain Res 69:242–248.

Chang HM, Wei IH, Tseng ChY, Lue JH, Wen CY, Shieh JY. 2004. Differential expression of calcitonin gene related peptide (CGRP) and choline acetyltransferase (ChAT) in the axotomized motoneurons of normoxic and hypoxic rats. J Chem Neuroanat 28:239–251.

Chao MV. 2003. Neurotrophins and their receptors: a convergence point for many signaling pathways. Nat Rev 4: 299–309.

Connor B, Young D, Lawlor P, Gai W, Waldvogel H, Faull RL, Dragunow M. 1996. Trk receptor alteration in Alzheimer's disease. Brain Res Mol Brain Res 42:1–17.

Corcoran JPT, So PL, Maden M. 2004. Disruption of the retinoid signalling pathway causes a deposition of amyloid in the adult rat brain. Eur J Neurosci 20:896–902.

Costigan M, Befort K, Karchewski L, Griffin RS, D'Urso D, Allchorne A, Sitarski J, Mannion JW, Pratt RE, Woolf CJ. 2002. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. BMC Neurosci 3:16.

Curtis R, Tonra JR, Stark JL, Adryan KM, Park JS, Cliffer KD, Lindsay M, DiStefano PS. 1998. Neuronal injury increases retrograde axonal transport of neurotrophins to spinal sensory neurons and motor neurons via multiple receptor mechanisms. Mol Cell Neurosci 12:105–118.

Davis-López de Carrizosa MA, Morado-Díaz CJ, Tena JJ, Benítez-Temiño B, Pecero ML, Morcuende S, de la Cruz RR, Pastor AM. 2009. Complementary actions of BDNF and neurotrophin-3 on the firing patterns and synaptic composition of motoneurons. J Neurosci 29:575–587.

Davis-López de Carrizosa MA, Morado-Díaz CJ, Morcuende S, de la Cruz RR, Pastor AM. 2010. Nerve growth factor regulates the firing patterns and synaptic composition of motoneurons. J Neurosci 30:8308–8319.

de la Cruz RR, Pastor AM, Delgado-Garc´ıa JM. 1994a. Effects of target depletion on adult mammalian central neurons: morphological correlates. Neuroscience 58:59–79.

de la Cruz RR, Pastor AM, Delgado-Garc´ıa JM. 1994b. Effects of target depletion on adult mammalian central neurons: functional correlates. Neuroscience 58:81–97.

de la Cruz RR, Pastor AM, Delgado-Garc´ıa JM. 1996. Influence of the postsynaptic target on the functional properties of neurons in the adult mammalian central nervous system. Rev Neurosci 7:115–149.

de la Cruz RR, Delgado-Garc´ıa JM, Pastor AM. 2000. Discharge characteristics of axotomized abducens internuclear neurons in the adult cat. J Comp Neurol 427: 391–404.

Delgado-Garc´ıa JM, del Pozo F, Spencer RF, Baker R. 1988. Behavior of neurons in the abducens nucleus of the alert cat. III. Axotomized motoneurons. Neuroscience 24:143–160.

DiStefano PS, Friedman B, Radziejewski C, Alexander C, Boland P, Schick CM, Lindsay RM, Wiegand SJ. 1992. The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8:983–993.

Dumoulin FL, Raivich G, Haas CA, Lazar P, Reddington M, Streit WJ, Kreutzberg GW. 1992. Calcitonin gene-related peptide and peripheral nerve regeneration. Ann N Y Acad Sci 657:351–360.

Duprey-D´ıaz MV, Soto I, Blagburn JM, Blanco RE. 2002. Changes in brain-derived neurotrophic factor and trkB receptor in adult *Rana pipiens* retina and optic tectum after optic nerve injury. J Comp Neurol 454:456–469.

Fernandes KJL, Kobayashi NR, Jasmin BJ, Tetzlaff W. 1998. Acetylcholinesterase gene expression in axotomized rat facial motoneurons is differentially regulated by neurotrophins: correlation with trkB and trkC mRNA levels and isoforms. J Neurosci 18:9936–9947.

Friedman B, Kleinfeld D, Ip NY, Verge VMK, Moulton R, Boland P, Zlotchenko E, Lindsay RM, Liu L. 1995. BDNF and NT-4/5 exert neurotrophic influences on injured adult spinal motor neurons. J Neurosci 15:1044–1056.

Fukuoka T, Tokunaga A, Kondo E, Miki K, Tachibana T, Noguchi K. 1999. Differential regulation of alpha- and beta-CGRP mRNAs within oculomotor, trochlear, abducens, and trigeminal motoneurons in response to axotomy. Brain Res Mol Brain Res 63:304–315.

Funakoshi H, Frisén J, Barbany G, Timmusk T, Zachrisson O, Verge VMK, Persson H. 1993. Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. J Cell Biol 123:455–465.

Geerling JC, Shin JW, Chimenti PC, Joel C, Loewv AD. 2010. Pararaventricular hypothalamic nucleus: axonal projections to the brainstem. J Comp Neurol 518:1460–1499.

Giehl KM, Schütte A, Mestres P, Yan Q. 1998. The survival-promoting effect of glial cell line-derived neurotrophic factor on axotomized corticospinal neurons *in vivo* is mediated by an endogenous brain-derived neurotrophic factor mechanism. J Neurosci 18:7351–7360.

Giehl KM, Röhrig S, Bonatz H, Gutjahr M, Leiner B, Bartke i, Yan Q, Reichardt LF, Backus C, Welcher AA, Dethleffsen K, Mestres M, Meyer M. 2001. Endogenous brain-derived neurotrophic factor and neurotrophin-3 antagonistically regulate survival of axotomized corticospinal neurons*in vivo*. J Neurosci 21:3492–3502.

Glicksman MA. 1980. Localization of motoneurons controlling the extraocular muscles of the rat. Brain Res 188:53–62.

González-Forero D, de La Cruz RR, Delgado-García JM, Álvarez FJ, Pastor AM. 2002. Correlation between CGRP immunoreactivity and firing activity in cat abducens motoneurons. J Comp Neurol 451:201–212.

Hammarberg H, Piehl F, Risling M, Cullheim S. 2000. Differential regulation of trophic factor receptor mRNAs in spinal motoneurons after sciatic nerve transection and ventralroot avulsion in the rat. J Comp Neurol 426:587–601.

Hughes RA, Sendtner M, Thoenen H. 1993. Members of several gene families influence survival of rat motoneurons *in vitro* and *in vivo*. J Neurosci Res 36:663–671.

Ip NY, Ibáñez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa R III, Squinto SP, Persson H, Yancopoulos GD. 1992. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci U S A 89:3060–3064.

Johnson H, Hökfelt T, Ulfhake B. 1999. Expression of p75(NTR), trkB and trkC in nonmanipulated and axotomized motoneurons of aged rats. Brain Res Mol Brain Res 69:21–34.

Jones KR, Reichardt LF. 1990. Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc Natl Acad Sci U S A 87:8060–8064.

Kaplan DR, Miller FD. 2000. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10:381–391.

Kobayashi NR, Bedard AM, Hinchke MT, Tetzlaff W. 1996. Increased expression of BDNF and TrkB mRNA in rat facial motoneurons after axotomy. Eur J Neurosci 8:1018–1029.

Koliatsos VE, Crawford TO, Price DL. 1991. Axotomy induces nerve growth factor receptor immunoreactivity in spinal motor neurons. Brain Res 549:297–304.

Koliatsos VE, Clatterbuck RE, Winslow JW, Cayouette MH, Price DL. 1993. Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons *in vivo*. Neuron 10:359–367.

Kosaras B, Jakubowski M, Kainz V, Burstein R. 2009. Sensory innervation of the calvarial bones of the mouse. J Comp Neurol 515:331–348.

Kotulska K, Larysz-Brysz M, Marcol W, Jozwiak S, Lewin-Kowalik J. 2006. The role of trkB receptor in the formation of post-traumatic neuroma. Folia Neuropathol 44:221–227

Kryger GS, Kriger Z, Zhang F, Shelton DL, Leneaweaver WC, Buncke HJ. 2001. Nerve growth factor inhibition prevents traumatic neuroma formation in the rat. J Hand Surg Am 26:635–644.

Kubo T, Yamashita T, Yamaguchi A, Hosokawa K, Tohyama M. 2002. Analysis of genes induced in peripheral nerve after axotomy using cDNA microarrays. J Neurochem 82: 1129–1136.

Lams BE, Isacson O, Sofroniew MV. 1988. Loss of transmitter-associated enzyme staining following axotomy does not indicate death of brainstem cholinergic neurons. Brain Res 475:401–406.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde Y-A. 1989. Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149–152.

Levi-Montalcini R. 1982. Developmental biology and the natural history of nerve growth factor. Annu Rev Neurosci 5: 341–362.

Ma W, Quirion R. 2006. Increased calcitonin gene-related peptide in neuroma and invading macrophages is involved in the up-regulation of interleukin-6 and thermal hyperalgesia in a rat model of mononeuropathy. J Neurochem 98: 180–192.

Márquez-Ruiz J, Morcuende S, Navarro-López JD, Escudero M. 2007. Anatomical and pharmacological relationship between acetylcholine and nitric oxide in the prepositus hypoglossi nucleus of the cat: functional implications for eye-movement control. J Comp Neurol 503:407–20.

Matsuura J, Ajiki K, Ichikawa T, Misawa H. 1997. Changes of expression levels of choline acetyltransferase and vesicular acetylcholine transporter mRNAs after transection of the hypoglossal nerve in adult rats. Neurosci Lett 236:95–98.

McAllister AK, Katz LC, Lo DC. 1997. Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. Neuron 18:767–778.

Meakin SO, Suter U, Drinkwater CC, Welcher AA, Shooter EM. 1992. The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc Natl Acad Sci U S A 89:2374–2378.

Merlio JP, Ernfors P, Jaber M, Persson H. 1992. Molecular cloning of rat trkC and distribution of cells expressing messenger RNAs for members of the trk family in the rat central nervous system. Neuroscience 51:513–532.

Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. 1992. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: differential mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol 119:45–54.

Middlemas DS, Lindberg RA, Hunter T. 1991. trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol Cell Biol 11:143–153.

Miyazaki S. 1985. Location of motoneurons in the oculomotor nucleus and the course of their axons in the oculomotor nerve. Brain Res 348:57–63.

Morcuende S, Benítez-Temiño B, Pecero ML, Pastor AM, de la Cruz RR. 2005. Abducens internuclear neurons depend on their target motoneurons for survival during early postnatal development. Exp Neurol 195:244–256.

Navarro X, Vivó M, Valero-Cabré A. 2007. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 82:163–201.

Okura Y, Arimoto H, Tanuma N, Matsumoto K, Nakamura T, Yamashima T, Miyazawa T, Matsumoto Y. 1999. Analysis of neurotrophic effects of hepatocyte growth factor in the adult hypoglossal nerve axotomy model. Eur J Neurosci 11: 4139–4144.

Omura T, Sano M, Omura K, Hasegawa T, Doi M, Sawada T, Nagano A. 2005. Different expressions of BDNF, NT3 and NT4 in muscle and nerve after various types of peripheral nerve injuries. J Peripher Nerv Syst 10:293–300.

Oppenheim RW, Yin QW, Prevette D, Yan Q. 1992. Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature 360:755–757.

Pastor AM, Delgado-García JM, Martínez-Guijarro FJ, López-Garc´ıa C, de la Cruz RR. 2000. Response of abducens internuclear neurons to axotomy in the adult cat. J Comp Neurol 427:370–390.

Patapoutian A, Reichardt lf. 2001. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11:272–280.

Piehl F, Arvidsson U, Johnson H, Cullheim S, Dagerlind A, Ulfhake B, Cao Y, Elde R, Pettersson RF, Terenius L, Hökfelt T. 1993. GAP-43, Afgf, CCK and alpha- and beta-CGRP in rat spinal motoneurons subjected to axotomy and/or dorsal root severance. Eur J Neurosci 5:1321–1333.

Piehl F, Frisen J, Risling M, Hökfelt T, Cullheim S. 1994. Increased trkB mRNA expression by axotomized motoneurons. Neuroreport 5:697–700.

Sala C, Andreose JS, Fumagalli G, Lomo T. 1995. Calcitonin gene-related peptide: possible role in formation and maintenance of neuromuscular junctions. J Neurosci 15: 520–528.

Schmitt AB, Breuer S, Liman J, Buss A, Schlangen C, Pech K, Hol EM, Brook GA, Noth J, Schwaiger FW. 2003. Identification of regeneration-associated genes after centraland peripheral nerve injury in the adult rat. BMC Neurosci 4:8.

Sendtner M, Hotmann G, Hughes RA. 1996. The response of motoneurons to neurotrophins. Neurochem Res 21:831–841.

Sofroniew MV, Howe CL, Mobley WC. 2001. Nerve growth factor signaling, neuroprotection, and neural repair. Annu Rev Neurosci 24:1217–1281.

Sumner BE. 1975. A quantitative analysis of boutons with different types of synapse in normal and injured hypoglossal nuclei. Exp Neurol 49:406–417.

Suneja SK, Yan L, Potasher SJ. 2005. Regulation of NT-3 and BDNF levels in guinea pig auditory brain stem nuclei after unilateral cochlear ablation. J Neurosci Res 80:381–390.

Tuszynsky MH, Mafong E, Meyer S. 1996. Central infusion of brain-derived neurotrophic factor and neurotrophin-4/5, but not nerve growth factor and neurotrophin-3, prevent loss of the cholinergic phenotype in injured adult motor neurons. Neuroscience 71:761–771.

Venero JL, Vizuete ML, Revuelta M, Vargas C, Cano J, Machado A. 2000. Upregulation of BDNF mRNA and trkB mRNA in the nigrostriatal system and in the lesion site following unilateral transection of the medial forebrain bundle. Exp Neurol 161:38–48.

Wang HL, Morales M. 2009. Pedunculopontine and laterodorsal tegmental nuclei contain distinct populations of cholinergic, glutamatergic and GABAergic neurons in the rat. Eur J Neurosci 29:340–358.

Wang H, Wei F, Dubner R, Ren K. 2006. Selective distribution and function of primary afferent nociceptive inputs from deep muscle tissue to the brainstem trigeminal transition zone. J Comp Neurol 498:390–402.

Wang W, Salvaterra PM, Loera S, Chiu AY. 1997. Brain-derived neurotrophic factor spares choline acetyltransferase mRNA following axotomy of motor neurons in vivo. J Neurosci Res 47:134–143.

Wisden W, Morris BJ. 2002. In situ hybridization with oligonucleotide probes. Int Rev Neurobiol 2002; 47:3–59.

Yan Q, Matheson C, Lopez OT, Miller JA. 1994. The biological responses of axotomized adult motoneurons to BDNF. J Neurosci 14:5281–5291.

Yang L, Zhang FX, Huang F, Lu YJ, Li GD, Bao L, Xiao HS, Zhang X. 2004. Peripheral nerve injury induces trans-synaptic modification of channels, receptors and signal pathways in rat dorsal spinal cord. Eur J Neurosci 19:871–883.