Journal of Neuroscience Research 47:134–143 (1997)

Brain-Derived Neurotrophic Factor Spares Choline Acetyltransferase mRNA Following Axotomy of Motor Neurons In Vivo

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Choline acetyltransferase (ChAT) is a functional and specific marker gene for neurons such as primary motor neurons that synthesize and release acetylcholine as a neurotransmitter. In adult mammals, transection of the peripheral nerve results in a loss of immunoreactivity for ChAT in the injured motor neurons without affecting their cell number. Using a quantitative RNase protection assay, we have investigated dynamic changes in ChAT mRNA levels following axotomy of motor neurons in the brainstem of adult rats. One week after transection of the left hypoglossal nerve, levels of ChAT mRNA in the ipsilateral side of the hypoglossal motor nucleus decreased dramatically to around 10% when compared to the uninjured contralateral side. When cut axons were chronically exposed to brain-derived neurotrophic factor (BDNF) for 1 week, ChAT mRNA levels were maintained at 63% of control levels. Thus, BDNF can abrogate the injury-induced loss of ChAT mRNA in mature motor neurons in vivo. In contrast, neither neurotrophin 4/5 nor nerve growth factor could prevent the decrease in message. This effect of BDNF on ChAT mRNA levels following peripheral injury to motor neurons demonstrates the existence of regulatory pathways responsive to neurotrophic factors that can "rescue" or "protect" cholinergic gene expression. J. Neurosci. Res. 47:134–143, 1997.

Key words: neurotrophins; adult rats; neurotrophin 4/5; nerve growth factor; peripheral injury

INTRODUCTION

Neurons such as motor neurons that extend axons out of the protected environment of the central nervous system are particularly exposed to trauma resulting from accidental injury or disease. Their ability to survive and recover from injury is influenced by both the intrinsic properties of the neuron and the properties of the cellular environment surrounding the damaged cell. In recent years, a great deal of interest has focused on the role played by extrinsic signals such as soluble growth factors and the extracellular matrix in regeneration. Motor neurons straddle the peripheral and central nervous systems, their axons acting as conduits bypassing the blood-brain barrier. Molecules that are retrieved from target fields or along the peripheral nerve can be transported in the retrograde direction to their cell bodies within the spinal cord and brainstem (Miyata et al., 1986; Yan et al., 1988; DiStefano et al., 1992; Curtis et al., 1993, 1994). This system thus provides a unique opportunity to examine the effects of growth factors and trophic agents on neurons within the central nervous system.

The application of polymerase chain reaction (PCR) methodology has accelerated the discovery of families of neurotrophic factors—small proteins that can support the survival and differentiation of developing neurons (reviewed in Korsching, 1993; Lindsay et al., 1994)—and has raised hopes that they might also protect and promote the recovery of damaged neurons in adult animals. These molecules have been extensively investigated using cultured neurons derived from embryos (Hofer and Barde, 1988; Thanos et al., 1989; Martinou et al., 1992; Ohsawa et al., 1993; Pinco et al., 1993; Skaper et al., 1993; Wong et al., 1993; Kato and Lindsay, 1994). In vivo studies show that a variety of trophic agents can promote the survival of injured motor neurons to varying degrees in neonatal animals (Sendtner et al., 1990, 1992a; Hughes et al., 1993; Koliatsos et al., 1993; Neff et al., 1993; Yan et al., 1993, 1994; Cheema et al., 1994; Clatterbuck et al., 1994; Vejsada et al., 1995) and attenuate neuronal loss in adult rodent models of motor neuron disease (Sendtner et al., 1992c; Mitsumoto et al., 1994; Ikeda et al., 1995; Li et al., 1995; Sagot et al., 1995). Recently, our laboratory and others have found that exogenous application of neurotrophic molecules can exert a protective influence on motor neurons in mature animals and prevent the loss of

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Received 13 June 1996; Revised 15 August 1996; Accepted 16 August 1996.

choline acetyltransferase (ChAT) protein that is normally triggered by axotomy (Chiu et al., 1994, 1995a; Yan et al., 1994; Friedman et al., 1995). To investigate the molecular mechanisms underlying trophic regulation of injured motor neurons, we designed a simple method to measure ChAT mRNA levels in the hypoglossal motor nucleus. Using this procedure, we find that injury to the peripheral nerve causes a rapid and dramatic drop in the expression of ChAT mRNA in axotomized motor neurons. This loss persists for weeks but can be prevented by supplying brain-derived neurotrophic factor (BDNF) to the damaged axons, indicating that neurotrophic factors regulate gene expression at a pretranslational level in vivo. Our findings thus suggest that neurotrophic molecules can act to rescue or maintain the functional capabilities of cholinergic neurons such as motor neurons.

MATERIALS AND METHODS

Surgery and Tissue Preparation

The effect of axotomy on ChAT expression was examined in the hypoglossal motor nucleus, a population of primary motor neurons within the brainstem that innervate muscles of the tongue. The left hypoglossal nerve was transected at a site under the digastric muscle in adult Sprague-Dawley female rats (120–180 g) and the cut end ligated to prevent regeneration. This treatment axotomizes all of the neurons on the ipsilateral side of the hypoglossal nucleus but leaves intact motor neurons on the contralateral side to serve as a built-in control (Chiu et al., 1994). Animals were killed at different times following surgery to determine the time course of axotomy-induced changes in ChAT mRNA levels and in immunoreactivity for the enzyme.

To examine the effect of neurotrophic factors, a solution containing 60 μg of the factor under investigation was applied to the site of nerve transection immediately after transection. Briefly, a 30 μl aliquot of test solution was applied to a piece of moistened, sterile gel foam, which was then wrapped around the proximal cut end of the axotomized peripheral nerve. Trophic factors with neutral isoelectric points were dissolved in 10 mM acetate buffer, pH 4.0, with 1% bovine serum albumin (BSA). Control animals received an equal aliquot of the appropriate vehicle solution or phosphate-buffered saline with BSA (PBS-BSA). To obtain tissue for RNase protection assays, the brainstem was removed quickly after killing the animal, rapidly frozen in Tissue Tek (O.C.T. Compound; Miles Inc., Elkhart, IN) (OCT), and sectioned in a cryostat to the level of the hypoglossal nucleus. Needle punches were taken from the left (lesioned) or right (control) half of the hypoglossal nucleus or from a nearby region of the brain stem where there were no cholinergic neurons. In all experiments, the uninjured neurons on the contralateral (right) side served as a built-in control for the axotomized side. To ensure that the two sides of the motor nucleus normally bear comparable levels of message, samples were taken from unoperated animals for comparison of the two halves.

To prepare tissue for immunohistochemistry, animals under deep anesthesia were perfused with 4% paraformaldehyde prior to removal of the brain stem. The tissue was further fixed in paraformaldehyde before cryoprotection in 30% sucrose, as previously described (Chiu et al., 1994). Consecutive 40 μm sections were cut on a cryostat and stored at 4°C in Sorenson's phosphate buffer until used. Immunohistochemistry was carried out using a monoclonal antibody specific for mammalian ChAT (antibody 1E6) (Crawford et al., 1982), as previously described (Houser et al., 1983; Chiu et al., 1993). Fixed sections were pretreated to inactivate endogenous peroxidase activity, then incubated overnight with the 1E6 antibody at 4°C. After extensive rinsing, sites of primary antibody binding were localized with biotinylated secondary antibodies and horseradish peroxidase-coupled avidin, and the peroxidase reaction product was visualized with diaminobenzidine.

Production of a Partial ChAT cDNA Clone

Forward and reverse synthetic oligonucleotide primers were derived from the spinal cord rat ChAT cDNA sequence reported by Ishii et al. (1990). Total RNA was isolated from rat spinal cord using the RNA isolation protocol from RNAzol™ B (Tel-Test, Friendswood, TX), then reverse-transcribed at 52°C for 30 min in a 10 μl reaction volume containing ChAT-specific primer, avian myeloblastosis virus reverse transcriptase (RT) (10 U, Anglian Biotec, Hoechst Ltd, UK), 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 10 U human placental ribonuclease inhibitor (Amersham, Arlington Heights, IL), and 1 mM deoxyribonucleoside triphosphates (dNTPs) (Pharmacia, Gaithersburg, MD). The reaction was inhibited by the addition of 5 mM EDTA, and RNA was hydrolyzed in the presence of 50 mM NaOH at 65°C for 30 min. Following neutralization with acetic acid, the volume was increased to 100 μl with 6 M NaI. The cDNA was purified with GENO-BIND™ (Clonetech, Palo Alto, CA) and precipitated by ice-cold 95% ethanol with glycogen and sodium acetate. The ChAT cDNA was dissolved in sterile diethylpyrocarbonate (DEPC)-treated H_2O.

PCR amplification was performed using a Stratagene (La Jolla, CA) DNA thermal cycler. Ten μl of RT products were diluted to 50 μl with 67 mM Tris-HCl, pH 8.8 (25°C), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 170 mg/ml BSA, 150 μM of each dNTP, 1 μM of each primer, and 0.5 U recombinant Taq DNA polymerase (Perkin-Elmer, Oak Brook, IL) in a

polypropylene microcentrifuge tube (0.5 ml; Sigma, St. Louis, MO). PCR was conducted for 25 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, with the final extension lengthened to 7 min. A 279 base pair PCR product was gel-purified and cloned into the XbaII-EcoRI restriction site of pBlue-script® SK (pBS-SK$^+$) (Promega, Madison, WI). The DNA sequence of this fragment was confirmed by the Sanger dideoxynucleotide chain termination method, using the Sequencase 2.0 kit (USB, Cleveland, OH).

Synthesis of cRNA Probes

A ChAT cRNA probe was synthesized from the PBS-SK$^+$ recombinant plasmid. Antisense cRNA was transcribed with T3 RNA polymerase after linearization with EcoRI. The cRNA of a constitutively expressed enzyme, phosphoglycerate kinase (PGK), was used as an internal control in the RNase protection assay. The PGK probe was synthesized from a pGEM3Zf(+) recombinant plasmid generously provided by Dr. N. Mori (University of Southern California, Los Angeles, CA). The PGK plasmid was linearized with TaqI, and antisense cRNA was synthesized using SP6 RNA polymerase. Labeled probes were prepared with [^{32}P]-UTP (New England Nuclear, Boston, MA) and the MAXIscript in vitro transcription kit (Ambion, Austin, TX) to a specific activity of 1.0×10^9 cpm/μg. Antisense cRNA probes were purified by electrophoresis in a denaturing gel (8 M urea, 6% polyacrylamide); eluted in 0.5 M ammonium acetate, 0.2% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 37°C; and ethanol-precipitated.

Ribonuclease Protection Assay (RPA)

Total RNA was extracted from brainstem punches, using the RNAzol method (Bioprobe, Friendswood, TX) and resuspended in 0.1 mM EDTA. Quantitative estimates of ChAT and PGK mRNA were obtained from RPAs. The [^{32}P]-labeled CHAT antisense probe (2×10^5 cpm/tube), the ^{32}P-labeled PGK antisense probe (2×10^4 cpm/tube), and a nonlabeled PGK antisense probe (30 ng/tube) were added to each brain stem sample of total RNA, which was dissolved in 20 μl of hybridization buffer (80% formamide in 40 mM piperazine-*N*,*N*′-bis [2-ethanesulfonic acid], pH 6.4, 0.4 M NaCl, 1 mM EDTA). Following overnight incubation at 50°C, the hybridized mixture was digested with ribonuclease T, (400 U/200 μl; GIBCO BRL, Gaithersburg, MD) for 30 min at 37°C to digest single-stranded RNA. Ribonuclease digestion was terminated by the addition of 6 μl of 20% SDS and 2 μl of 20 mg/ml proteinase K (GIBCO BRL). Samples were extracted with 240 μl phenol/chloroform/isoamyl alcohol, then precipitated by cold ethanol. Each pellet was dissolved in gel-loading buffer and subjected to electrophoresis in a denaturing 8% polyacrylamide gel containing 8 M urea. The gel was electrophoresed at 250 V for 1.5 hr, air-dried, and exposed on to a phosphoimager screen (Molecular Dynamics, Sunnyvale, CA). The levels of ChAT and PGK mRNA were determined by densitometry with the Molecular Dynamics Phosphoimager, and pixel volumes were calculated using Image Quant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

RT was used to convert mRNA from rat spinal cord into single-stranded ChAT complementary DNA (ChAT cDNA), which was then amplified with standard PCR techniques. The PCR-amplified DNA fragment was cloned to serve as a template for the synthesis of labeled antisense RNA probes. The resulting probe was 320 bases long and protected a band of 279 bases, from +1158 to +1417, in the ChAT cDNA (Ishii et al., 1990) (Fig. 1). This ChAT-specific mRNA probe was subsequently used for solution hybridization experiments. To control for variability in RNA extraction efficiency and sample size, a probe for rat PGK was included in each experiment. This enzyme is present in all cell types; its expression does not appear to be modified in the axotomized hypoglossal motor nucleus. The PGK probe was 230 nucleotides long and protected a band of 210 bases (Fig. 1).

Levels of ChAT and PGK mRNA were detected concurrently in each sample of tissue as ChAT- and PGK-protected bands were clearly separated by electrophoresis (Fig. 2A). These protected fragments were also slightly smaller than their respective probes and could therefore be distinguished from them. In unoperated animals, no significant difference in the levels of either mRNA was observed between the two sides of the hypoglossal nucleus (Fig. 2A). Control samples, taken from areas of the brainstem without cholinergic neurons, showed equally high levels of PGK mRNA but no detectable ChAT message. In order to compare levels of ChAT mRNA between samples, the ChAT signal was standardized to its internal control, the PGK band. In unperturbed or sham-operated animals, the standardized levels of ChAT message (ChAT/PGK) on left (L) and right (R) sides of the motor nucleus were extremely similar (Fig. 2B), with a calculated L/R ratio of 0.99 ± 0.037 (average ± SEM, $n = 16$).

One week following unilateral axotomy and ligation of the peripheral nerve, ChAT mRNA levels on the lesioned left side decreased to around 10% of the value on the control side (Fig. 3). When gelfoam saturated with

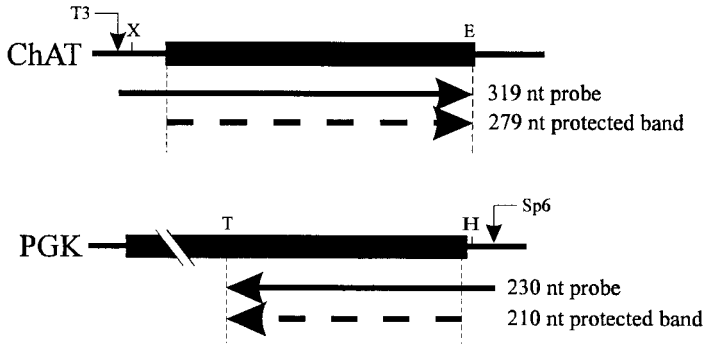

Fig. 1. Schematic representation of probes used for RNase protection. Clone inserts used as templates for making ChAT and PGK probes are shown. Black boxes indicate inserts, while solid lines linked to the black boxes designate vector sequences. Small arrows point to the position of the T3 and SP6 promoters. Solid arrows under the black boxes represent antisense cRNA probes; dashed arrows indicate protected fragments in RNase protection assays. X, XbaII; E, EcoRI; T, TaqI; H, HindIII.

PBS-BSA was applied to the cut peripheral nerve, the ratio of left to right sides of the nucleus (L/R ChAT mRNA) rose slightly to 0.2 ± 0.026 ($n = 14$). In all cases, no significant changes were observed in the level of PGK mRNA following injury (Fig. 2A). This dramatic loss of ChAT mRNA following lesion was partially prevented by the exogenous application of 60 μg of BDNF to the proximal nerve stump (Figs. 2, 3). With BDNF, the L/R ratio of ChAT mRNA was maintained at 0.63 ± 0.074 ($n = 4$). The n values refer to both the number of animals used and the number of assays conducted since each animal yielded a single sample for analysis.

We investigated the ability of other neurotrophins to attenuate the loss of ChAT mRNA following axotomy. Neurons supplied with nerve growth factor (NGF) were not significantly different from those receiving the vehicle alone (Fig. 3). Application of 60 μg of neurotrophin 4/5 (NT4/5) to the injured nerve produced an L/R ratio of 0.32 ± 0.009 ($n = 4$). However, this slight effect was not statistically significant based on Bonferroni analysis.

When animals were killed at different times following surgery, we found that message for ChAT fell rapidly during the days following transection of the peripheral nerve (Fig. 4). A decrease was evident as early as 1 day after axotomy, with ChAT mRNA levels reaching a minimum at around 1 week. These low levels persisted for 2 more weeks. However, the expression of ChAT mRNA returned with time, even when reinnervation was prevented. At 4 weeks the levels were about 60% of unoperated values, and they recovered to about 80–90% of normal by 6 weeks following surgery. When sections of the brainstem were examined for ChAT immunoreactivity during this period, the return of protein to injured neurons mirrored the rise in mRNA levels (Fig. 5).

DISCUSSION

Since ChAT catalyzes the synthesis of the neurotransmitter acetylcholine, the presence of ChAT immunoreactivity and the level of enzyme activity have been widely used to identify and localize cholinergic neurons and to assess their functional state in a variety of experimental paradigms (Houser et al., 1983; Tetzlaff and Kreutzberg, 1984; Davidoff and Schulze, 1988; Lams et al., 1988; Chiu et al., 1995b; Kou et al., 1995). Using immunoreactivity for ChAT as a criterion, a number of laboratories have reported that injury to the peripheral nerve can cause a loss of transmitter phenotype in motor neurons without incurring the loss of cells (Lams et al., 1988; Armstrong et al., 1991; Chiu et al., 1993). It is, however, unclear whether this deficit is the result of a loss of gene expression, a reduction in protein synthesis, or an increase in degradation of the enzyme. Furthermore, with the exception of direct enzyme assays, there is little quantitative information regarding ChAT expression following these types of experimental insult. Quantitative information is important not only for understanding the mechanisms that regulate gene expression but also for evaluating treatments that may attenuate injury-induced changes in motor neurons. With these goals in mind, we designed a reliable and sensitive method, using ribonuclease protection protocols, to quantify levels of ChAT message in primary motor neurons.

Our studies have established that reproducible ChAT mRNA analysis can be performed on small tissue samples of approximately 0.5 μl, selectively harvested from the rat brain. Since the ChAT gene transcript is present at relatively low levels, this method obviates the large amount of poly(A)$^+$ RNA that is required for detection by conventional Northern blot procedures. The levels of message within a specific motor nucleus, taken from a

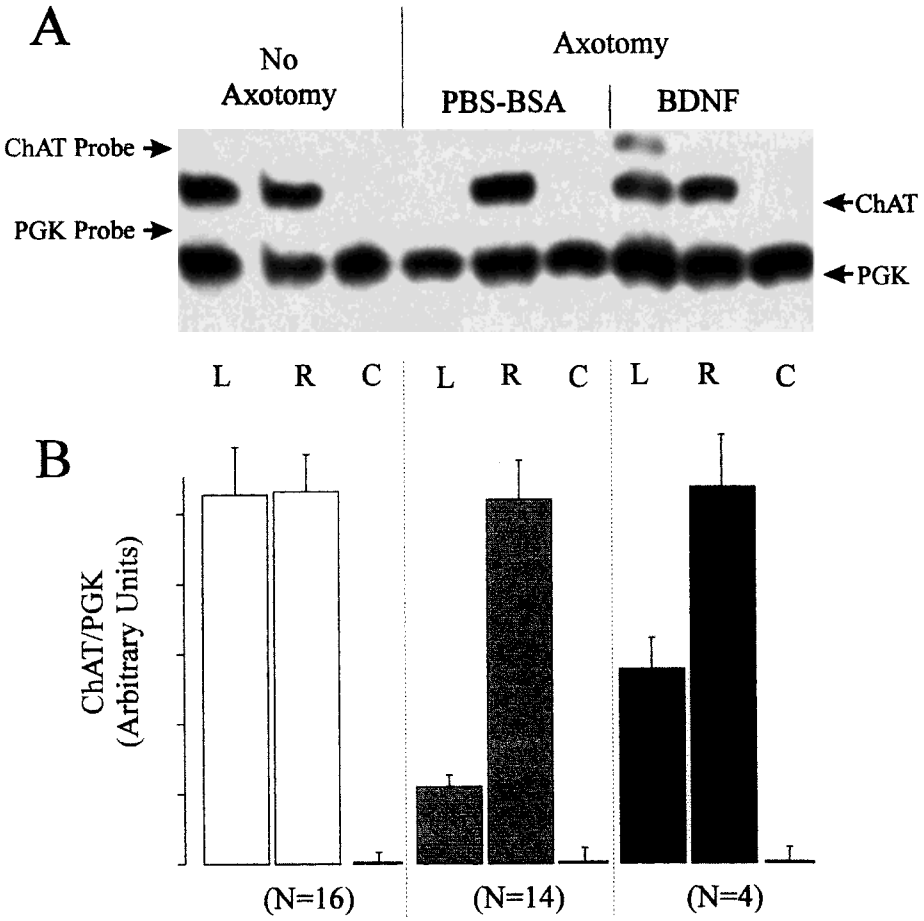

Fig. 2. Quantitation of ChAT and PGK mRNA. **A:** Representative phosphoimager scan showing protected bands for ChAT and PGK (indicated on right) as well as the position of cRNA probes (indicated on left). The first two lanes show that ChAT mRNA levels are the same in left (L) and right (R) halves of the hypoglossal nucleus. Note also that ChAT mRNA is not detectable in the control (C) sample taken from other regions of the brain stem (lane 3). One week after the left hypoglossal nerve was cut, the level of ChAT mRNA on the ipsilateral side (lane 4) is reduced to around 20% of levels present on the contralateral hypoglossal nucleus (lane 5). Application of BDNF prevents much of this loss of ChAT mRNA (lane 7). The PGK-protected band stays relatively constant in all samples. **B:** Densitometric quantitation of phosphoimager scans of samples from unoperated animals (open histograms) and animals killed 1 week after the left hypoglossal nerve was transected and the proximal nerve stump provided with PBS-BSA (shaded histograms) or with 60 μg of BDNF (dark histograms). Data have been normalized to account for different specific activities of the ChAT and PGK probes used in individual experiments. The ratio of ChAT to PGK levels is indicated (±SEM). N refers to the number of animals used and the number of assays conducted.

single animal, can now be evaluated. We find that ChAT gene expression is dramatically curtailed when motor neurons are axotomized; a reduction of ChAT mRNA is thus likely to be responsible for the loss of ChAT immunoreactivity observed after axotomy. This decrease in ChAT expression shows some specificity since PGK expression in the motor nucleus did not change significantly following injury. Moreover, the loss of ChAT mRNA can be partially prevented by BDNF but not NGF, suggesting the involvement of the trkB neurotrophin receptor. These results are consistent with previous reports that BDNF prevents the loss of ChAT immunoreactivity in axotomized cranial and spinal motor neurons (Chiu et al., 1994, 1995a; Yan et al., 1994; Friedman et al., 1995). Our current findings indicate that exogeneous application of trophic factors can "protect" transmitter phenotype by regulating gene expression in vivo.

We do not as yet understand how axotomy triggers a loss of ChAT mRNA and how neurotrophic factors act to attenuate this loss. Neurotrophic factors have been shown to promote the survival of embryonic motor neurons and to enhance ChAT enzyme activity in vitro, demonstrating

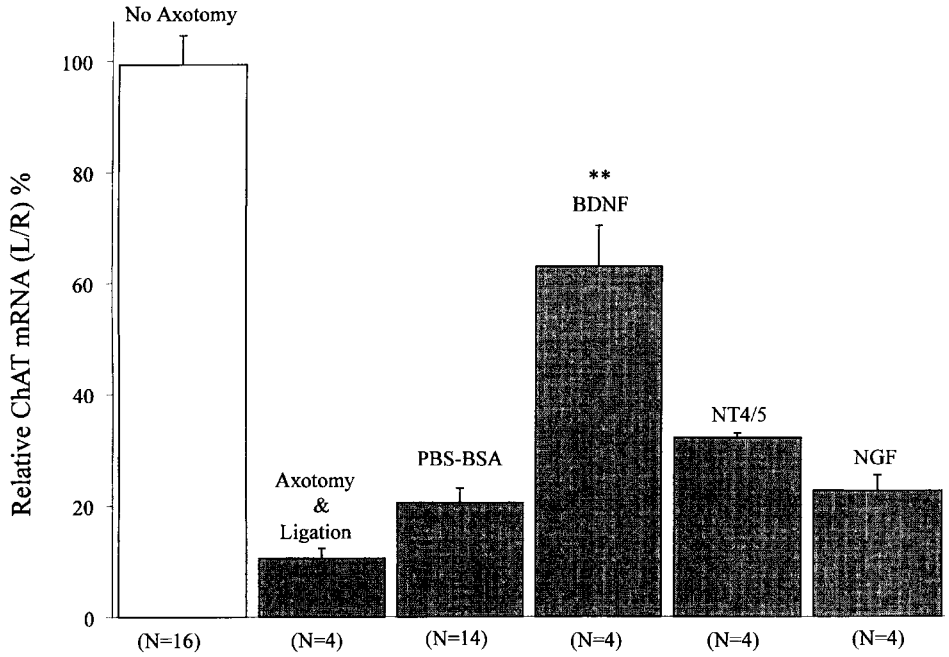

Fig. 3. Motor neurons respond selectively to the application of different neurotrophins. Unoperated animals show similar levels of ChAT mRNA in the left and right hypoglossal nucleus. One week after unilateral transection and ligation of the hypoglossal nerve, the levels of ChAT mRNA in the axotomized neurons decrease dramatically to around 10% of the level in the contralateral hypoglossal neurons. When the cut nerve end is exposed to PBS-BSA, ChAT mRNA levels are 20% of normal. Nerve exposure to BDNF for 1 week results in significantly higher ChAT mRNA levels (63% of normal; $^{**}P < 0.001$). In contrast, when the nerve is exposed to NGF, no difference is seen compared to the level observed for PBS-BSA. Treatment with NT4/5 results in neurons expressing 32% of the normal levels of ChAT mRNA; however, this is not statistically different from control ($P < 0.06$). Data are shown as the mean $\pm$ S.E.M. ($n > 4$).

that a direct route or mode of action is possible (Wong et al., 1993; Kato and Lindsay, 1994). Studies conducted in vivo show that both developing and mature motor neurons are responsive to the exogenous application of BDNF, NT3, and NT4/5 (Sendtner et al., 1992a; Yan et al., 1992, 1994; Henderson et al., 1993; Koliatsos et al., 1993, 1994; Chiu et al., 1994, 1995a; Qin-Wei et al., 1994; Friedman et al., 1995; Vejsada et al., 1995; reviewed in Lindsay et al., 1994 and Houenou et al., 1994) and can accumulate a variety of neurotrophic molecules by receptor-mediated uptake and retrograde transport (Yan et al., 1988; Wayne and Heaton, 1988; DiStefano et al., 1992; Curtis et al., 1993, 1994). Furthermore, the two currently identified receptors for BDNF, p75 and trkB, are upregulated in motor neurons following axotomy (Ernfors et al., 1989; Armstrong et al., 1991; Saika et al., 1991; Chiu et al., 1993; Piehl et al., 1994; Yan et al., 1994). All neurotrophins bind with similar affinity to p75, the nondiscriminating neurotrophic receptor; specific and high-affinity binding is mediated by different members of the trk family of tyrosine kinase receptors (Ip et al., 1993; reviewed in Bothwell, 1995). The selective upregulation of p75 and trkB may enable injured motor neurons to retrieve circulating trophic molecules, which in turn act to promote the recovery of ChAT gene expression.

The finding that NGF was unable to prevent the loss of ChAT mRNA is not surprising given that several other lines of evidence suggest that motor neurons are not responsive to NGF. First, motor neurons do not express trkA, the high-affinity NGF receptor. Second, NGF is unable to rescue immature motor neurons in culture or following axotomy in neonatal rats (Miyata et al., 1986; Wong et al., 1993; Houenou et al., 1994; Kato and Lindsay, 1994; Vejsada et al., 1995). In fact, treatment with NGF may even enhance the loss of damaged motor neurons (Miyata et al., 1986). This could be a consequence of NGF binding to p75, receptor sites that might otherwise be available to acquire other members of the neurotrophin family, such as BDNF. Retrograde transport of NGF by motor neurons may merely reflect this ability to bind to p75 (Yan et al., 1988). More surprising is the observation that BDNF generated a robust response, while an equivalent dose of NT4/5 did not since both ligands bind to the same receptors (Klein et al., 1991; Soppet et al., 1991; Bothwell, 1995). No difference in ChAT immunoreactivity was noted between these two factors in studies of neurotrophin dose dependence (Chiu

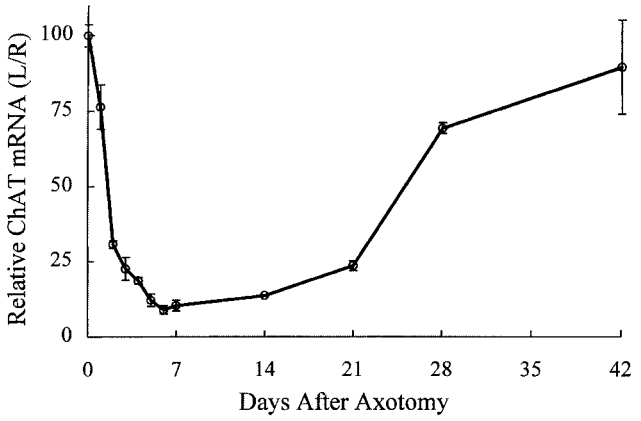

Fig. 4. Time course of axotomy-induced changes of ChAT mRNA levels in hypoglossal motor neurons. The level of ChAT mRNA drops rapidly during the first 2 days after axotomy and reaches a minimum at about 1 week. Levels of mRNA increase slowly during the next 4 weeks, returning to about 80–90% of control levels at 6 weeks after surgery. Data are expressed as means ± S.E.M. ($n > 3$).

et al., 1995a; Friedman et al., 1995). However, the relationship between mRNA and protein levels may not be a linear one, as discussed below. Davies and colleagues (1993) also noted that NT4/5 was far less potent than BDNF in supporting the survival of chick sensory neurons but suggested that these results may be due to species differences in the structure of the ligands. An alternate possibility is the existence of an as yet undiscovered receptor(s) that distinguishes between these two ligand molecules, thus enabling certain populations, such as chick sensory neurons and rodent motor neurons, to be more responsive to levels of BDNF.

We find that lesion-induced changes in ChAT mRNA precede changes in immunoreactivity for ChAT protein. In immunocytochemical studies, a decrease in ChAT-positive neurons was not observed until 3 days after surgery (Armstrong et al., 1991; Borke et al., 1993; Chiu, unpublished results). In contrast, we could detect a significant reduction in ChAT mRNA level within 1 day after axotomy, and by 3 days mRNA levels were reduced by 75% on the injured side. Quantitation of mRNA levels

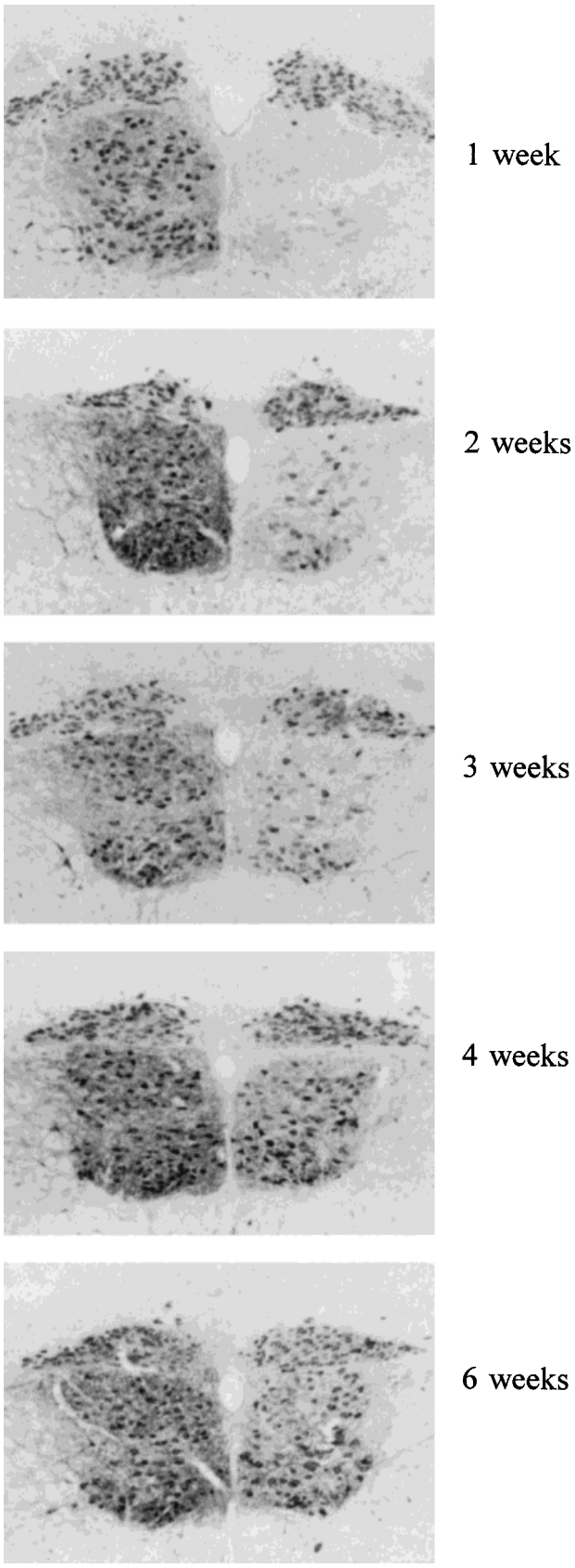

Fig. 5. Return of ChAT protein to motor neurons following chronic axotomy. If the hypoglossal nerve is unilaterally transected and ligated to prevent regeneration, ChAT immunoreactivity is initially lost from the injured neurons (1 week) but gradually returns, and by 6 weeks most of the neurons on the axotomized side (shown on right) bear high levels of ChAT protein. The preganglionic neurons of the dorsal nucleus of the vagus sit above the two halves of the hypoglossal nucleus; since they are also cholinergic, these neurons are immunopositive for ChAT at all stages of this experiment.

may therefore be a more sensitive and direct measure of the response of motor neurons to trauma. The decrease in ChAT immunoreactivity may result from ChAT protein loss without a sufficient mRNA pool to replace it.

Other laboratories have reported that the initial decrease in ChAT protein following transection of the hypoglossal nerve is transient (Wooten et al., 1978; Borke et al., 1993). We now find that both ChAT mRNA and protein eventually return to motor neurons, even if the transected axons are prevented from regenerating and reinnervating a target. Since we have shown that exogenous BDNF can regulate ChAT gene expression, one possibility is that BDNF or other trophic agents are produced and released by cells in the vicinity of the regenerating axons. Two potential sources are Schwann cells and denervated muscles. In fact, following damage to the peripheral nerve, Schwann cells gradually upregulate their expression of a variety of trophic molecules, including BDNF, NGF, glial-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (Eckenstein et al., 1991; Meyer et al., 1992; Sendtner et al., 1992b; Funakoshi et al., 1993; Trupp et al., 1995). The rise in BDNF mRNA is detectable at 3 days after injury, reaching maximal levels 3–4 weeks later (Meyer et al., 1992). Moreover, the peak expression of BDNF after lesion coincides with a period of maximal recovery for ChAT mRNA. By gradually raising endogenous levels of BDNF within the injured peripheral nerve, Schwann cells may contribute to the gradual return of ChAT in motor neurons. Increased levels of BDNF mRNA have also been observed in denervated muscles (Meyer et al., 1992; Funakoshi et al., 1993); the availability of neurotrophic factors may promote the return of functional motor axons to their denervated target muscles.

ACKNOWLEDGMENTS

We thank Dr. M. Glicksman (Cephalon, Inc., West Chester, PA) and Dr. A. Rosenthal (Genentech, South San Francisco, CA) for generously providing us with BDNF and NT4/5, respectively.

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