Acetylcholinesterase Gene Expression in Axotomized Rat Facial Motoneurons Is Differentially Regulated by Neurotrophins: Correlation with *trkB* and *trkC* mRNA Levels and Isoforms

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We examined the potential influences of muscle-derived neurotrophins on the acetylcholinesterase (AChE) gene expression of adult rat motoneurons. Seven days after facial nerve transection, both AChE mRNA and enzyme activity levels were markedly reduced in untreated and vehicle-treated facial motoneurons, suggesting positive regulation of motoneuron AChE expression by muscle-derived factors. Because skeletal muscle is a source of neurotrophin-3 (NT-3), NT-4/5, and BDNF, these neurotrophins were individually infused onto the proximal nerve stump for 7 d, beginning at the time of axotomy. The *trkB* ligands NT-4/5 and BDNF prevented the downregulation of AChE mRNA and enzymatic activity, as determined by *in situ* hybridization, biochemical assay, and histochemical visualization of enzyme activity. In contrast, NT-3 had limited effects, and NGF was without effect. Because motoneurons normally express both *trkB* and *trkC* receptors and the *trkC* ligand NT-3 is the most abundant muscle-derived neurotrophin, we investi-

gated possible reasons for the limited effects of NT-3. *In situ* hybridization and reverse transcription-PCR both revealed a downregulation of *trkC* mRNA in axotomized motoneurons, which contrasted the upregulation of *trkB* expression. Furthermore, isoforms of *trkC* were detected carrying insertions within their kinase domains, known to limit certain *trkC*-mediated signal transduction pathways. Because the changes in *trkB* and *trkC* mRNA levels were not significantly altered by neurotrophin infusions, it is unlikely they were induced by loss of muscle-derived neurotrophins. These results demonstrate that NT-4/5 and BDNF stimulate AChE gene expression in motoneurons and support the concept that muscle-derived *trkB* ligands modulate the cholinergic phenotype of their innervating motoneurons.

Key words: acetylcholinesterase; axotomy; BDNF; motoneurons; NT-4/5; NT-3; neurotransmitter enzymes; retrograde signals

Neurotrophic factors (NTFs) derived from axonal targets profoundly influence neuronal survival, differentiation, and synaptic function in the developing and mature nervous system (Schotzinger et al., 1994; Munson et al., 1997; for reviews, see Landis, 1990; Lowrie and Vrbova, 1992; Davies, 1994; Thoenen, 1995; Greensmith and Vrbova, 1996). After axonal injury, which interrupts the bidirectional flow of neuron–target interactions, the majority of mature cholinergic neurons survive but rapidly decrease their expression of the cholinergic neurotransmitter synthesizing enzyme choline acetyltransferase (ChAT) (Lams et al., 1988; Peterson et al., 1990; Kou et al., 1995). Consistent with a model of target regulation of neurotransmitter enzymes, infusion of pharmacological doses of putative target-derived NTFs restores ChAT immunoreactivity to axotomized cholinergic neurons (Hagg et al., 1989; Yan et al., 1994; Friedman et al., 1995).

A second enzyme associated with cholinergic neurotransmission is acetylcholinesterase (AChE). Although AChE is found to be coexpressed with ChAT in mature cholinergic neurons, it is also expressed in the absence of ChAT in certain noncholinergic, noncholinergic regions of the adult nervous system (Levey et al., 1983; Greenfield, 1984; Landwehrmeyer et al., 1993; Legay et al., 1993; Hammond et al., 1994; Bernard et al., 1995). Furthermore, expression of AChE mRNA, but not ChAT, is an early event during neuronal differentiation (Coleman and Taylor, 1996). These observations may be accounted for by a variety of nonacetylcholine-related functions for AChE (Krejci et al., 1991; Layer et al., 1993; Dupree and Bigbee, 1994, 1996; Webb et al., 1996; Srivatsan and Peretz, 1997; for review, see Appleyard, 1992; Massoulie et al., 1993; Layer and Willbold, 1995). Thus, coregulation of ChAT and AChE cannot be assumed per se, because these enzymes may be differentially regulated after injury or in response to different NTFs.

Previously, we have shown that adult rat facial motoneurons undergo a reduction in AChE enzymatic activity after axotomy (Tetzlaff and Kreutzberg, 1984). Because mature motoneurons express *trkB* and *trkC* high-affinity tyrosine kinase receptors (Henderson et al., 1993), muscle-derived members of the neurotrophin family of NTFs may play a role in AChE regulation in motoneurons. Adult muscle produces the *trkB* ligands neurotrophin-4/5 (NT-4/5) (Timmusk et al., 1993; Funakoshi et al., 1993; Koliatsos et al., 1993) and possibly low levels of BDNF (Funakoshi et al., 1993; Griesbeck et al., 1995). Several studies have

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Figure 1. Experimental paradigms to assess influence of axotomy and exogenously applied neurotrophins on AChE expression in adult rats. Group 1 underwent a single facial nerve axotomy (AX) to measure the relative change in FMN AChE expression compared with the uninjured contralateral (C) FMNs. Group 2 underwent axotomy of both facial nerves, with one side left untreated (AX) and the proximal stump of the opposite side receiving an osmotic pump containing vehicle solution (AX + vehicle pump); this allowed assessment of possible vehicle pump effects. Group 3 underwent axotomy of both facial

nerves, with one side receiving a vehicle pump (AX + vehicle pump) and the opposite side receiving a neurotrophin pump (AX + NT pump); this paradigm allowed assessment of neurotrophin effects. Group 4 received a single facial nerve axotomy, followed by implantation of a neurotrophin pump (AX + NT pump) to compare AChE expression after neurotrophin treatment with that in nonaxotomized contralateral (C) FMNs.

demonstrated that exogenous NT-4/5 and BDNF can prevent the decrease in ChAT immunoreactivity after axotomy of adult motoneurons (Chiu et al., 1994; Yan et al., 1994; Friedman et al., 1995; Tuszynski et al., 1996). Based on these effects, neurotrophins are candidate muscle-derived factors regulating AChE expression *in vivo*. Neurotrophin application *in vitro* induces an increase in total AChE enzymatic activity in various populations of embryonic neurons (Greene and Rukenstein, 1981; Martinez et al., 1987; Raynaud et al., 1988; Alderson et al., 1990; Ojika et al., 1994), including embryonic motoneurons (Wong et al., 1993). Furthermore, a recent *in vivo* study reported that BDNF can maintain the survival of avulsed motoneurons, which normally undergo cell death, and that these rescued motoneurons express ChAT and AChE (Kishino et al., 1997).

Neurotrophins have been shown previously to stimulate survival (for review, see Sendtner et al., 1996) and electrophysiological (Munson et al., 1997) responses from motoneurons. In the present study, we assessed the possibility that AChE gene expression in motoneurons may be regulated by the neurotrophin family members found in muscle, i.e., NT-3, NT-4/5, and BDNF. We investigated whether the axotomy-induced reduction in motoneuron AChE enzymatic activity occurs at the mRNA or protein activity level, tested the abilities of the individual neurotrophins to prevent the axotomy-induced changes in AChE mRNA, biochemical activity, and histochemical localization, and examined the expression of trkB and trkC neurotrophin receptors in intact, axotomized, and neurotrophin-treated motoneurons.

MATERIALS AND METHODS

Experimental paradigms. Adult male Sprague Dawley rats (250–400 gm) were obtained from Charles River Laboratories (Quebec, Canada) and housed in a 12 hr light/dark cycle, with access to a standard diet and water *ad libitum*. The rats were divided into the following treatment groups, as shown schematically in Figure 1. In group 1, rats received a facial nerve resection involving removal of a 5 mm nerve segment to prevent reconnection to the distal stump, allowing us to determine the effects of axotomy on motoneuron gene expression. Gene expression in the axotomized facial motoneurons (FMNs) was compared with the contralateral uninjured FMNs. In group 2, one facial nerve was axotomized as for group 1. The contralateral facial nerve was likewise axotomized, and an osmotic pump containing vehicle was implanted onto the proximal nerve stump. This allowed us to measure any effect of vehicle compared with axotomy alone. In group 3, one facial nerve was axotomized, and an osmotic pump containing vehicle solution was implanted onto the proximal nerve stump. The contralateral facial nerve was also axotomized and received an osmotic pump containing one of the neurotrophin solutions. This permitted measurement of neurotrophin effects relative to contralateral vehicle treatment. In group 4, one facial nerve was axotomized and received a neurotrophin pump. The contralat-

eral facial nerve was left unoperated. This allowed comparison of gene expression in neurotrophin-treated axotomized FMNs to contralateral uninjured motoneurons. Thus, in groups 2 and 3 both facial nerves were axotomized, which the animals tolerated without weight loss. In addition, to avoid bias toward one particular side (left vs right), the left or the right side was randomly selected for neurotrophin treatment in different animals.

The experimental arrangements described for groups 1, 2, 3, and 4 involve comparisons between the left and right facial nuclei within individual animals. This intra-animal comparison was selected to preclude interanimal variabilities in gene expression, as well as to ensure that the populations of neurons compared undergo identical tissue fixation and hybridization conditions. Although it is possible that levels of AChE expression in motoneurons contralateral to axotomy may not be identical to those in motoneurons of completely uninjured rats, the magnitude of our findings are likely to err, if at all, on the slightly conservative side. This is because (1) our results are normalized against the contralateral nucleus so that any contralateral effects of axotomy–neurotrophin treatment resulting from release of diffusible factors would tend to reduce rather than increase the statistical significance of neurotrophin effects, and (2) both facial nerves were axotomized in groups 2 and 3 so that contralateral effects are internally controlled.

Animal surgery and pump implantation. All surgery was done according to the guidelines of the Canadian Council for Animal Care and approved by the local Animal Care Committee. The rats were anesthetized with a mixture of sodium pentobarbital (32 mg/kg) and chloral hydrate (150 mg/kg), and the facial nerve was exposed at its exit from the stylomastoid foramen. The buccal branch of the facial nerve was transected ~4–5 mm distal to the foramen, taking care not to injure the smaller auricular branch. In neurotrophin-treated rats, an osmotic minipump containing any one of the four neurotrophins (NGF, BDNF, NT-3, or NT-4/5) or vehicle was connected to a SILASTIC tubing (0.025 inches inner diameter × 0.047 inches outer diameter; Dow Corning), which was placed over the nerve stump and held in place with a 6.0 Prolene suture (Ethicon). SILASTIC silicone-like medical adhesive (Dow Corning) prevented leakage from the tubing. Penicillin–streptomycin solution (750 μ l at 5000 U/ml) was instilled into the wound area, and the skin was closed with wound clips.

Minipump preparation and neurotrophin solutions. Total volume required to fill each minipump (1 μ l/hr flow rate; model 2001; Alzet) and 6–8 cm of SILASTIC tubing was ~250 μ l. Vehicle solution consisted of 0.8% saline in 20 mM sodium phosphate buffer, pH 7.2, containing 0.5% rat serum albumin as a protein carrier and 100 U/ml each penicillin and streptomycin antibiotics. The neurotrophins (kindly provided by Regeneron Pharmaceuticals Inc.) were supplied as concentrates in acetate buffer and were then diluted in the vehicle solution to a final concentration of 5, 50, or 500 ng/ μ l. The BDNF concentration range chosen was based on a previous study by Friedman et al. (1995). All pumps were preincubated in 20 mM PBS, pH 7.4, for 12 hr at 37°C before surgery to initiate an even flow rate.

Animals were killed by lethal injection of chloral hydrate (1 gm/kg) 7 d after surgery and perfused transcardially with PBS, followed by 4% freshly hydrolyzed phosphate buffered paraformaldehyde. The brainstems were post-fixed in 4% paraformaldehyde for 18 hr, cryoprotected with 16 and 22% sucrose dissolved in 1× PBS, and quick-frozen in dry

ice-cooled isopentane. Postmortem analysis confirmed that the free end of the SILASTIC tubings had been maintained over the proximal nerve stump.

In situ hybridization. Each facial nucleus was cut in 12 μ m sections on a cryostat. Sections were collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), with each slide containing a section from group 1 together with a section from groups 2, 3, or 4. The oligonucleotide probe for AChE was complementary to nucleotides 1360–1409 (5'-CAAGTCAATGTGGAGGCACGGTGTTCAGATGTAGGCA-TAGACCCGAGC-3'; GenBank accession number S50879). The *trkC* probe corresponded to nucleotides 2109–2272 (excluding 2134–2250) (5'-GGAGCATGGTGTGTCCCTCCACCCCTGTAGTAATCAGTAGTACG-3'; GenBank accession number L14447), thereby bridging the potential insertion site in the tyrosine kinase domain (Valenzuela et al., 1993). Probes were 3' end-labeled by incubating 80 ng of the oligonucleotide with terminal transferase (24 U), 35 S-dATP (125 μ Ci), diethyl pyrocarbonate-treated water (6 μ l), and 5 $\times$ reaction buffer (5 μ l). The *in situ* hybridization (ISH) procedure and autoradiography was as described by Verge et al. (1992).

Quantification of ISH and data analysis. As described previously (Kobayashi et al., 1997), a computerized image analysis system (Northern Exposure; Empix Imaging Inc., Mississauga, Ontario, Canada) was used to quantify the ISH signals. Sections were stained in 0.01% ethidium bromide for 1 hr and rinsed under running demineralized H₂O for 1 hr. Sections from different treatment groups were randomly arranged on the microscope slides, which were then number coded to eliminate any bias during quantification (single blind analysis). Cells were visualized and traced under fluorescence illumination, and the area fraction occupied by grains (i.e., grain density) was then automatically measured from the corresponding dark-field image. Background autoradiographic signal from the white matter running through the facial nucleus was subtracted to obtain a corrected grain density. Axotomy and neurotrophin treatments induce significant increases in motoneuron size, which could dilute the mRNA density and obscure changes in levels of mRNA transcripts within a given neuron. Therefore, the mean ISH signal per neuron was obtained by multiplying the corrected grain density by the approximate cell volume, which was calculated from the cross-sectional area. The quantification was limited to those cells cut through the nucleus/nucleolus, resulting in data collection from ~25–30 cells per tissue section. For each section, the average ISH signal per neuron per section was expressed as percentage of the signal from the contralateral vehicle-treated motoneurons. At least three sections, >100 μ m apart, were quantified per facial nucleus, and the mean percentage was calculated for each animal.

Measurement of AChE enzyme activity. Facial nuclei were punched from fresh frozen brainstems using a blunted cannula. Each nucleus was homogenized on ice (2 $\times$ 15 sec) in 1 ml of a high salt–detergent buffer containing protease inhibitors (1.0 mg/ml bacitracin, 25 U/ml aprotinin). Homogenates were then centrifuged at 20,000 $\times$ g for 20 min at 4°C, and the supernatants were used immediately for reaction with acetylthiocholine as substrate for AChE (Ellman et al., 1961). Spectrophotometric measurement of AChE activity was performed in the presence of 10^{−5} M tetraisopropylpyrophosphoramide, a nonspecific cholinesterase inhibitor (Gisiger and Stephens, 1988; Jasmin and Gisiger, 1990). Nonspecific hydrolysis was determined by incubating the samples in the presence of 5-bis(4-allyldimethylammonium phenyl)pentanone dibromide (BW284c51), a specific acetylcholinesterase inhibitor. AChE activity per sample was normalized according to the amount of protein measured using a BCA protein assay reagent kit (Pierce, Rockford, IL). In each case, results were normalized against the acetylcholinesterase activity measured within the contralateral intact facial nucleus.

AChE histochemical staining. Coronal sections (12 μ m) through the facial nucleus of perfused brainstems were mounted onto Superfrost Plus slides (Fisher Scientific) and stored at −80°C. Sections were stained using the histochemical technique of Karnovsky and Roots (1964). Specificity of the staining procedure was confirmed by including BW284c51 in the reaction mixture, which eliminated all staining (data not shown).

Reverse transcription-PCR for full-length *trkC* isoforms. Total RNA was isolated and reverse transcribed into cDNA as described by Kobayashi et al. (1996), with the following modifications. Full length *trkC* mRNAs were selectively amplified by PCR using previously described primers (Offenhauser et al., 1995) that bracket the potential insertion site within the *trkC* kinase domain. The amplified cDNA sequences corresponded to both noninserted and inserted full-length *trkC* mRNA species. Hence, the PCR products had sizes of 299, 341, 374, and 416 bp, corresponding

Figure 2. Reduced autoradiographic ISH signal for AChE mRNA in the axotomized facial nucleus. Seven days after resection of the buccal branch of the facial nerves of rats from group 1, dark-field micrographs reveal that ISH signals are reduced within the intermediate and lateral subgroups (*i, l*) of the axotomized facial nucleus (*A*) in comparison to the contralateral uninjured facial nucleus (*B*). Note that the ISH signal is not reduced in the medial subgroup (*m*), which projects via the nonaxotomized auricular branch of the facial nerve. Higher-magnification bright-field micrographs localize the specific ISH signals to the cresyl violet-stained axotomized (*C*) and contralateral (*D*) FMNs. Scale bar (in *D*), 30 μ m.

to the noninserted isoform, and 14, 25, and 39 amino acid inserted *trkC* isoforms, respectively. PCR amplification of *trkC* species was initially performed between 18 and 32 cycles (45 sec at 94°C, 1 min at 55°C, 1 min 30 sec at 72°C) and found to be within the linear range of amplification at 30 cycles. Serial dilutions from 12 to 0.75 ng of total RNA were amplified in 50 μ l PCR reactions. Amplification of cyclophilin mRNA was used to ensure equivalent amounts of input cDNA (Mearow et al., 1993). PCR products were visualized on 5% acrylamide gels.

Statistics. For each animal, data from the experimental facial nucleus was normalized against (i.e., expressed as a percentage of) the contralateral side. Within each treatment group, the normalized data from each animal was averaged and expressed as the mean $\pm$ SEM. To test for significant differences between treatment groups, we used a one-way ANOVA, followed by a multiple comparison of the group means with the Student–Newman–Keuls test.

RESULTS

Effect of axotomy on AChE mRNA expression

Using rats from group 1 (see Materials and Methods, Experimental paradigms) (Fig. 1), *in situ* hybridization for AChE mRNA, followed by autoradiography, was used to determine whether the decreased AChE activity found in axotomized motoneurons (Tetzlaff and Kreutzberg, 1984) was caused by downregulation of mRNA levels. Dark-field micrographs, which show the autoradiographic silver grains, clearly demonstrated reduced hybridization signal over the axotomized facial nucleus (Fig. 2*A*) when compared with the uninjured contralateral facial nucleus (Fig. 2*B*). In bright-field micrographs (Fig. 2*C, D*), the autoradiographic signal is localized to the facial motoneurons. Quantification of the ISH signal per cell determined that AChE mRNA expression by axotomized FMNs was reduced to 37 $\pm$ 4% SEM (n = 13 animals) compared with the noninjured contralateral side.

Figure 3. Dose–response analysis of axotomized FMNs to BDNF. Representative scatterplots showing AChE ISH signal per neuron in the facial nuclei of rats from group 3 receiving neurotrophin pumps for 7 d applications of either 5 (*A*), 50 (*B*), or 500 ng/μl/hr BDNF (*C*). Open circles represent the contralateral cells, which were axotomized and treated with vehicle. Filled squares represent the axotomized BDNF-treated FMNs. Sections were processed on the same slide for equal ISH conditions. Summary of dose–response data (*D*) demonstrates similar effects of 50 and 500 ng/μl BDNF treatments on AChE mRNA levels compared with contralateral axotomized FMNs treated with vehicle (normalized to 100%). *D*, Open circles represent the mean ISH signal per neuron per animal; filled circles represent the mean of each treatment group.

Dose–response analysis of axotomized FMNs to BDNF

To identify the concentrations at which axotomized FMNs would respond to neurotrophins in the osmotic pump infusion paradigm, we performed a dose–response analysis using 10-fold increments in BDNF concentration. Rats were treated as shown for group 3 (Fig. 1). Treatment with 5 ng/μl BDNF did not alter AChE mRNA levels in relation to contralateral vehicle infusion, as measured with *in situ* hybridization (Fig. 3*A*). However, infusion of either 50 (Fig. 3*B*) or 500 ng/μl BDNF (Fig. 3*C*) increased AChE mRNA hybridization signal ($171 \pm 17\%$ SEM; $n = 4$ animals; $196 \pm 15\%$ SEM; $n = 6$ animals, respectively). We also measured significantly larger soma sizes with BDNF treatment; importantly, however, the increases in AChE mRNA levels with 50 and 500 ng/μl BDNF treatments was several-fold greater than the 10–15% increase in soma volume (data not shown), indicating that the specific signal/cell volume ratio was increased. Further-

more, the stimulation of AChE mRNA levels does not represent a pleiotrophic effect on all motoneuron mRNAs, because we found that sections from the same animals did not exhibit a BDNF effect on the expression of *c-jun* mRNA (data not shown). Dose–response data are summarized in Figure 3*D*, illustrating the similar effects of 50 and 500 ng/μl BDNF treatments on AChE hybridization signal.

Responsiveness of AChE mRNA expression to members of the neurotrophin family

Because NT-3 and NGF treatments elicited no detectable effects at 50 ng/μl, we used the highest neurotrophin dose (500 ng/μl/hr) to avoid false negative findings. High doses of neurotrophins did not elicit morphological or gene expression changes indicative of high dose toxicity (data not shown).

In animals operated as shown for group 3, low levels of AChE

Figure 4. Effects of neurotrophins on FMN ISH signal for AChE mRNA. Cresyl violet-stained sections from rats operated according to group 3. Shown are axotomized FMNs treated with vehicle (*A*) or 500 ng/ μ l of NGF (*B*), BDNF (*C*), NT-3 (*D*), or NT-4/5 (*E*). Note that BDNF- and NT-4/5-treated FMNs maintained AChE mRNA expression, whereas NT-3 and NGF were without obvious effect. Quantification of average ISH signal per neuron, normalized against contralateral vehicle-treated FMNs, confirms statistically significant effects ($p < 0.05$) of BDNF ($n = 6$) and NT-4/5 ($n = 4$) but not NT-3 ($n = 4$) or NGF ($n = 4$) (*F*). NT-3 had a marginal effect on AChE mRNA levels; however, this effect did not reach statistical significance. Scale bar (in *A*), 20 μ m.

ISH signal were observed over vehicle-treated axotomized FMNs contralateral to neurotrophin-treated FMNs, confirming consistent hybridization conditions and the absence of contralateral effects of neurotrophins (Fig. 4*A*). FMNs treated with NGF (Fig. 4*B*) or NT-3 (Fig. 4*D*) were not significantly different from their contralateral vehicle-treated controls. However, FMNs treated with BDNF or NT-4/5 exhibited high levels of cytoplasmic ISH signal (Fig. 4*C,E*, respectively). Interestingly, although treatment with NT-3 was ineffective on the FMN population as a whole, a

few FMNs appeared to express higher levels of AChE mRNA (data not shown).

Quantification of the ISH signals using computerized image analysis measurements (Fig. 4*F*) determined that treatment of axotomized FMNs with BDNF or NT-4/5 maintained the average ISH signal per neuron at 196 ± 15 ($n = 6$ animals) and $202 \pm 34\%$ SEM ($n = 4$ animals) respectively, or approximately twofold higher than vehicle-treated contralateral FMNs. ISH signal in response to NGF treatment ($113 \pm 26\%$ SEM; $n = 4$ animals) was

not significantly different from vehicle control treatment, whereas NT-3 treatment had an average ISH signal per neuron that was $141 \pm 18\%$ SEM ($n = 4$ animals) of contralateral. However, only BDNF and NT-4/5 treatments yielded statistically higher ISH signals than contralateral vehicle treatment ($p < 0.05$).

A few animals from group 4 were also used for *in situ* hybridization. In comparison to the contralateral nonaxotomized FMNs, AChE mRNA levels in FMNs from group 4 rats were reduced to $38 \pm 11\%$ (SEM) with NGF ($n = 2$) and $27 \pm 3\%$ (SEM) with NT-3 ($n = 2$) (data not shown). This was not significantly different from axotomy alone ($37 \pm 4\%$; $n = 13$) (Fig. 1). Axotomized motoneurons treated with BDNF, however, actually exhibited higher mRNA levels than the intact contralateral motoneurons ($147 \pm 10\%$; $n = 2$). Taking into account the $\sim 15\%$ hypertrophy, the specific signal/cell volume ratio was still $\sim 30\%$ higher in BDNF-treated FMNs than in intact motoneurons, indicating that BDNF actually restores AChE mRNA levels to supranormal levels.

Histochemical localization and biochemical measurement of AChE activity

To assess whether the BDNF- and NT-4/5-induced maintenance of AChE mRNA expression correlated with higher levels of AChE enzyme activity, sections through the facial nucleus from groups 1, 2, and 3 were processed for AChE histochemistry. Histochemical localization of AChE enzyme activity identified strong reactivity in FMN cell bodies contralateral to an axotomy (Fig. 5A), which was severely depleted in untreated (Fig. 5B) and vehicle-treated (Fig. 5C) axotomized FMNs. Some heterogeneity was noted in the response to NT-3 (Fig. 5D), because many NT-3-treated FMNs displayed moderate AChE activity. More significantly, after treatment with BDNF or NT-4/5, virtually all axotomized FMNs exhibited AChE staining within their cytoplasm (Fig. 5E,F, respectively).

To quantitatively measure the amount of AChE activity after treatment, facial nuclei were microdissected from the brainstems of axotomized group 1 and neurotrophin-treated group 4 rats, and the AChE biochemical activity was measured (Fig. 5G). In normal unoperated animals, there was no significant difference in total AChE activity between left and right facial nuclei ($102 \pm 9\%$ SEM; $n = 5$ animals), confirming a reproducible microdissection technique of the facial nucleus that was not biased to one side. At 7 d after axotomy, AChE activity was reduced to $66 \pm 9\%$ SEM ($n = 5$ animals) of the contralateral uninjured facial nucleus, in agreement with previous results (Tetzlaff and Kreutzberg, 1984). Treatment with 500 ng/ μ l of NT-3 had no significant effect ($62 \pm 7\%$ SEM; $n = 5$ animals) on the expression of total AChE activity, but treatment with 500 ng/ μ l BDNF ($92 \pm 5\%$ SEM; $n = 4$ animals) and NT-4/5 ($95 \pm 7\%$ SEM; $n = 2$ animals) prevented the axotomy-induced decrease in AChE activity. AChE-positive nerve terminals in the immediate vicinity of the FMN cell bodies were evident in all cases.

Effects of axotomy and neurotrophins on trkB and trkC receptor expression

Because it was unclear how axotomy and neurotrophin treatments might have altered the responsiveness of FMNs to neurotrophins, we examined the expression of trkB and trkC receptors. Axotomy was found to induce opposing changes in expression of full-length trkB and trkC receptors. Consistent with previous reports, we detected an approximately twofold to threefold increase in full-

length trkB mRNA within axotomized FMNs (Piehl et al., 1994; Kobayashi et al., 1996), which was not altered by infusion of BDNF or NT-4/5 in our paradigm (data not shown).

In contrast, we identified an axotomy-induced downregulation of trkC mRNA (Fig. 6A,B). Because multiple isoforms of the trkC receptors exist, we used an ISH probe that spans the potential insertion site within the kinase domain and is therefore specific for the full-length noninserted trkC isoform (trkCni). The expression of trkCni was reduced in the axotomized FMNs 7 d after axotomy, and the downregulation appeared uniform, as illustrated by scatterplot analysis of ISH signals (Fig. 6C). To determine whether this downregulation was directly caused by the loss of target-derived NT-3 ligand, we used the probe specific for trkCni for *in situ* hybridization to sections from the NT-3-treated rats. The decline in trkC hybridization signal was not prevented by NT-3 infusions at concentrations of 500 (Fig. 6D) and 50 ng/ μ l (data not shown).

The trkC receptor profile within the facial nucleus was further characterized using total RNA extracted from microdissected unoperated and axotomized facial nuclei. Reverse-transcription (RT), followed by PCR using primers bracketing the potential insertion site in the trkC kinase domain, demonstrated the presence of the trkCni mRNA, as well as of inserted full-length trkC isoforms (Fig. 6E). Multiple comparisons of serial dilutions containing 12, 6, and 3 ng of template indicated an approximate twofold to threefold reduction in mRNA for trkCni mRNA, as well as for the larger inserted trkC isoforms. Inserted trkC isoforms appeared to include the 14, 25, and 39 amino acid insert variants, the presumed 14 amino acid variant yielding the strongest band after PCR amplification. Bands for trkCni and the 14 amino acid inserted trkC variant appeared equally intense.

DISCUSSION

In the present study, we have shown that axotomy of FMNs results in a reduction in their mRNA expression, as well as their total enzyme activity, for the neurotransmitter hydrolyzing enzyme acetylcholinesterase. Although FMNs express both trkB and trkC neurotrophin receptors, only the trkB ligands BDNF and NT-4/5 prevented the decrease in AChE mRNA expression, biochemical enzyme activity, and somal histochemical staining, and stimulated hypertrophy of the FMN cell bodies when infused onto the proximal nerve stump. In contrast, NT-3 and NGF did not have significant effects. The effects of BDNF and NT-4/5 on FMN gene expression and soma size were consistent with the axotomy-induced upregulation of the full-length trkB receptor. In the case of NT-3, the trkC receptor profile was found to consist of comparable levels of the noninserted and inserted isoforms of the full-length receptor, particularly the 14 amino acid variant, and expression of both the noninserted and inserted isoforms was sharply reduced after axotomy, correlating with the lack of NT-3 effects. Injury-induced changes in the neurotrophin receptor mRNA expression was not detectably altered by infusion of the respective ligands.

Regulation of acetylcholinesterase by neurotrophins

Our results confirm and extend our earlier report that axotomy of adult rat facial motoneurons reduces AChE activity to $\sim 60\%$ of levels in the uninjured contralateral motoneurons (Tetzlaff and Kreutzberg, 1984). We detected a concomitant decline in AChE mRNA to levels of 30–40% of contralateral. The more pronounced reduction in mRNA levels than enzyme activity is likely

Figure 5. Histochemical and biochemical analyses of AChE enzyme activity in the facial nucleus. Rats were operated as shown for groups 1, 2, and 3. In group 1 rats, abundant histochemical reaction product clearly identified the cell bodies of the nonaxotomized FMNs contralateral to axotomy (*A*), whereas the axotomized FMNs were depleted of AChE activity (*B*). In group 2 rats, axotomized FMNs (*B*) that received vehicle pump implantation were also depleted of AChE activity (*C*). However, in group 3 rats, virtually all FMNs that received a BDNF (*E*) or (Figure legend continues)

explained by a slower turnover of protein than mRNA or by the presence of AChE activity in cholinergic afferent terminals (Li et al., 1995) that were visible on the FMN cell bodies by enzyme histochemistry. However, we cannot exclude the possibility of posttranslational modifications that increase enzyme specific activity.

Treatment of the axotomized FMNs with the *trkB* ligands BDNF or NT-4/5 maintained AChE mRNA expression and prevented the axotomy-induced reduction of AChE activity. The mechanism underlying the neurotrophin-induced maintenance of AChE mRNA levels has not been established. *In vitro* studies have demonstrated that the RNA synthesis inhibitors can abolish the NGF-induced stimulation of AChE expression in PC12 cells (Greene and Rukenstein, 1981); however, this may indicate either a direct effect of NGF on AChE transcription or an indirect effect via expression of stabilizing RNA-binding proteins. A recent study in PC12 cells demonstrated that NGF can activate the transcription factor Sp1 (Yan and Ziff, 1997), whose recognition site appears essential for transcriptional activation of the AChE promoter (Getman et al., 1995). On the other hand, stabilization of existing AChE mRNA transcripts was recently shown to underlie the increased AChE mRNA expression that occurs when P19 embryonic carcinoma cells undergo neuronal differentiation (Coleman and Taylor, 1996). Further studies are necessary to differentiate between these possibilities in axotomized motoneurons.

Comparison of neurotrophin effects on AChE and ChAT in motoneurons

Studies examining neurotrophin effects on ChAT expression in adult motoneurons have used a variety of application paradigms, including local treatment at the lesion site (Chiu et al., 1994; Yan et al., 1994; Friedman et al., 1995; Wang et al., 1997), subcutaneous injections (Yan et al., 1994; Friedman et al., 1995), intravenous injection (Yan et al., 1994), and intracerebroventricular infusion (Yan et al., 1994; Tuszynski et al., 1996). With some exceptions (Clatterbuck et al., 1994; Piehl et al., 1995; Wang et al., 1997), which may be attributable to variations in experimental protocols, the majority of these results have demonstrated that pharmacological doses of BDNF or NT-4/5 maintain ChAT expression in axotomized adult motoneurons. A recent quantitative RNase protection study demonstrated that BDNF maintained ChAT mRNA levels in axotomized hypoglossal motoneurons (Wang et al., 1997); thus, the neurotrophin effects on ChAT expression are likely to be mediated by increases in mRNA transcription or stability rather than rate of translation or specific activity of the enzyme. These results parallel the present findings that BDNF and NT-4/5 maintain AChE mRNA expression and enzyme activity. Collectively, the evidence indicates that signaling in response to either of the *trkB* ligands BDNF or NT-4/5 can regulate both of the enzymes associated with the cholinergic

phenotype at the level of their mRNA expression in facial motoneurons.

In comparison to BDNF and NT-4/5, NT-3 treatment only stimulated a small increase in AChE mRNA, which did not reach statistical significance. NT-3-treated axotomized FMNs were heterogeneously stained by AChE histochemistry and, unlike axotomy alone or axotomy plus vehicle treatment, included a small number of clearly AChE-positive motoneurons. The reason for the heterogeneous NT-3 effect is unclear. NT-3 has been reported to be the most abundant muscle-derived neurotrophin in adult muscle, with mRNA levels of approximately two to three times those of NT-4/5 and 20 times those of BDNF (Funakoshi et al., 1995; Griesbeck et al., 1995). Furthermore, potent effects of NT-3 on motoneurons during embryonic and neonatal stages of development have been described (Wong et al., 1993; for review, see Sendtner et al., 1996). However, there are few reported effects of NT-3 on mature motoneurons (Haase et al., 1997), and to our knowledge, regulation of motoneuron transmitter enzymes in adult rats by NT-3 has not been reported. The limited NT-3 effect that we detected was not attributable to the quality of NT-3, because the same batch was effective in parallel investigations on other neurons within the CNS (Giehl et al., 1995; Giehl and Tetzlaff, 1996). One possibility for the minor NT-3 effects is that only sensory afferents and γ -motoneurons might be exposed to muscle-derived NT-3, because the main source of NT-3 in mature muscle appears to be intrafusal muscle spindles (Coprav and Brouwer, 1994); since the facial motonucleus is known to contain only α -motoneurons, it is possible that they are not normally responsive to NT-3. However, mature α -motoneurons have been shown to express *trkC* receptors (Merlio et al., 1992; Henderson et al., 1993; Johnson et al., 1996) and are capable of retrogradely transporting iodinated NT-3 (Di Stefano et al., 1992). We therefore investigated whether the partial NT-3 effects on axotomized motoneurons may be attributable to the composition of, or injury-induced changes in, the *trkC* receptor profile.

Axotomy differentially regulates *trkB* and *trkC* receptor expression

Axotomy of FMNs has been shown to lead to an upregulation of mRNA for the full-length *trkB* isoform (Piehl et al., 1994; Kobayashi et al., 1996), which is consistent with the responsiveness of axotomized motoneurons to treatment with BDNF or NT-4/5. We found that this twofold to threefold mRNA upregulation was not detectably altered by the infusion of either BDNF or NT-4/5 onto the nerve stump (data not shown). In other studies, BDNF infusion into the midbrain of adult rats also did not alter *trkB* mRNA levels and resulted in a prolonged analgesia (Frank et al., 1997); thus, it is possible that findings of reduced *trkB* immunoreactivity after BDNF treatments (Dittrich et al., 1996; Frank et al., 1996, 1997) may be attributable to increased receptor turnover

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NT-4/5 pump (*F*) maintained strong cytoplasmic AChE staining. FMNs treated with an NT-3 pump (*D*) exhibited a heterogeneous response that included both moderately stained (*bold arrows*) and unstained (*thin arrowheads*) FMNs. In all groups, AChE-positive afferent terminals were visible as punctate reaction product around the perimeter of the FMN cell bodies. Animals from groups 1 and 4 were also used for biochemical measurement of total tissue AChE activity within the microdissected facial nuclei (*G*). Control unoperated rats had no significant difference in AChE activity between left and right sides (*Uninjured*; *n* = 5). Axotomized facial nuclei from group 1 animals (*AX*; *n* = 5), which did not receive a pump, and axotomized facial nuclei from group 4 animals, which did receive an NT-3 pump (*AX* + *NT-3*; *n* = 5), both had significantly reduced AChE activity compared with the contralateral nonaxotomized nuclei. In contrast, axotomized facial nuclei from group 4 animals treated with BDNF (*AX* + *BDNF*; *n* = 4) and NT-4/5 (*AX* + *NT-4/5*; *n* = 2) maintained their AChE expression, showing no significant difference from the nonaxotomized contralateral nuclei. Scale bar (in *A*), 80 μ m.

Figure 6. Analysis of the trkC receptor profile in axotomized and neurotrophin-treated FMNs. Autoradiographic ISH signal for noninserted full-length trkC receptor mRNA in nonaxotomized contralateral (*A*) and axotomized (*B*) FMNs. Representative scatterplots illustrate the axotomy-induced downregulation of noninserted full-length trkC mRNA (*C*), which is not significantly changed by 7 d infusion of NT-3 ligand onto the proximal nerve stump (*D*). Representative serial dilutions of RT-PCR products generated using primers that bracket the kinase insertion site (*E*). Noninserted (*large arrow*) and inserted (*small arrowheads*) isoforms of full-length trkC mRNAs are detected in the facial nucleus 7 d after axotomy. Note the axotomy-induced twofold to threefold reduction in levels of the noninserted, as well as the inserted, trkC mRNAs. The apparent band sizes are consistent with the full-length trkC isoform sizes of 299 bp (noninserted), as well as 341, 374, and 416 bp, representing the 14, 25, and 39 amino acid insertion variants, respectively. Scale bar (in *A*), 20 μm .

rather than downregulation of trkB expression (Frank et al., 1997).

In contrast to the upregulation of the full-length trkB receptor, we found mRNA for the noninserted full-length trkC receptor to

be downregulated after axotomy, offering one explanation for the limited NT-3 effects on AChE expression. Infusion of NT-3 did not prevent this trkC downregulation. To further characterize the trkC expression, we tested for the presence of inserted isoforms

of full-length trkC receptors, which carry insertions of 14, 25, or 39 amino acids within their kinase domain (Tsoulfas et al., 1993; Valenzuela et al., 1993). The presence of such inserts within the trkC kinase domain appears to limit its signaling capabilities (Tsoulfas et al., 1996; Gunn-Moore et al., 1997) by interfering with the sustained activation of the mitogen-activated protein kinase pathway (Gunn-Moore et al., 1997). *In vitro* studies have shown that PC12 cells respond to NT-3 with neurite outgrowth when transfected with the noninserted full-length trkC but not when transfected with inserted full-length trkC variants (Lamballe et al., 1993; Tsoulfas et al., 1993; Valenzuela et al., 1993). The use of RT-PCR primers that bracket the potential kinase insertion site allowed us to identify the presence of both noninserted and inserted trkC receptor isoforms within the microdissected facial nucleus, as well as demonstrate that both inserted and noninserted full-length trkC isoforms are downregulated after axotomy.

Because we used RNA extracted from the microdissected facial nucleus, we cannot exclude a non-neuronal contribution to the expression of inserted trkC isoforms. However, we believe them to be predominantly of neuronal origin, because (1) virtually all ISH signals were located over neurons when using the extracellular domain probe common to all trkC isotypes (data not shown), and (2) the inserted trkC isoforms were downregulated in a manner similar to the noninserted trkC isoform. Because NT-3 binding to trkC is followed by receptor dimerization and mutual phosphorylation, the presence of inserted isoforms may exert a dominant negative effect on noninserted trkC signaling pathways, contributing to the mitigated responsiveness of axotomized motoneurons to NT-3. Further studies will be required to determine whether a differential distribution of inserted and/or truncated trkC receptors accounts for the heterogeneous effects of NT-3.

Conclusions

The roles of endogenous muscle-derived neurotrophins for mature motoneurons have not been clearly established. NT-4/5 is expressed in an activity-dependent manner in skeletal muscle, and NT-4/5 injection into muscle elicits motoneuron sprouting (Funakoshi et al., 1995). Recently, inhibition of endogenous trkB ligands in muscle has been shown to reduce motoneuron conduction velocity, further suggesting a dynamic regulation of motoneuron properties by endogenous neurotrophins (Munson et al., 1997). The present findings showing the potent effects of NT-4/5 and BDNF on AChE mRNA and total enzyme activity, together with their reported effects on ChAT expression, support the concept of a broad influence of trkB ligands on the cholinergic phenotype of mature motoneurons. We also demonstrated that the most abundantly expressed neurotrophin in muscle, NT-3, appears to have only limited effects on neurotransmitter enzyme expression in adult motoneurons after axotomy and that this may be attributable to the presence of inserted trkC receptor isoforms that can limit the responsiveness to NT-3. Furthermore, axotomized motoneurons downregulate their trkC expression, which most likely renders them less responsive to NT-3 after injury.

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