Neurotrophic factors enhance the survival of muscle fibers in EDL, but not SOL, after neonatal nerve injury

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Mousavi, Kambiz, Wilson Miranda, and David J. Parry. Neurotrophic factors enhance the survival of muscle fibers in extensor digitorum longus but not soleus after neonatal nerve injury. Am J Physiol Cell Physiol 283: C950–C959, 2002. First published May 22, 2002; 10.1152/ajpcell.00081.2002.—Neonatal sciatic nerve crush results in a sustained reduction of the mass of both extensor digitorum longus (EDL) and soleus (SOL) muscles in the rat. Type IIB fibers are selectively lost from EDL. We have investigated the effects of ciliary neurotrophic factor (CNTF) combined with neurotrophin (NT)-3 or NT-4 on muscle mass, as well as the number, cross-sectional area, and distribution of muscle fiber types and the number of motor neurons innervating EDL and SOL 3 mo after transient axotomy 5 days after birth. Both NT treatments prevented the axotomy-induced loss of muscle mass in both EDL and SOL and of total number of muscle fibers in EDL but not in SOL. Although IIB fiber loss was not prevented, both NT treatments resulted in altered fiber type distribution. Both NT combinations also reduced the loss of EDL motor neurons. These data suggest that a differential distribution of NT receptors on either motor neurons or muscle fibers may lead to different levels of susceptibility to neonatal axotomy.

motor neurons; myosin heavy chain; fiber size

NEONATAL NERVE INJURY in the rat results in a long-term loss of motor neurons, an 80% decrease in the number of motor neurons innervating the jaw muscles (5, 31, 38, 63), and a 50–60% decrease in the number of motor neurons innervating the hindlimb muscles (45, 60, 77). In contrast, transient injury in an adult rat results in the loss of <10% of the motor neuron population (9). The relative extent of reduction in the number of lumbar motor neurons after neonatal axotomy corresponds well with the extent of muscle atrophy and reduction of force generation (45, 47). In the hindlimb, muscle weight and maximum tetanic tension of tibialis anterior (TA) and extensor digitorum longus (EDL) are both reduced by ~50% 60 days after axotomy at postnatal day 5 (47). Soleus (SOL), on the other hand, showed only a 20% decrease in muscle weight and a 30% decrease in maximum tetanic tension after 2 mo of recovery (47). The extent of gastrocnemius muscle mass loss is similar to that of SOL (35), suggesting that posterior crural muscles (or the motor neurons innervating them) are less susceptible to neonatal axotomy than the anterior muscles of the hindlimb. Histochecmical studies of EDL and TA have shown the disappearance of fast glycolytic fibers (presumably type IIB) after transient neonatal axotomy (44).

Developing motor neurons are susceptible to target muscle ablation (24) and can be rescued with administration of muscle-derived neurotrophins (NTs) (40). Consequently, one possible explanation for susceptibility of a specific population of motor neurons and muscle fibers after neonatal axotomy is the transient block in retrograde transport of muscle-derived NTs to motor neurons. Of these factors, NT-3 (7), NT-4 (16), and brain-derived neurotrophic factor (BDNF) (23, 29) are expressed in skeletal muscle. Previous observations have shown prolonged survival of cultured motor neurons in response to the above-mentioned NTs (3, 29, 31). These NTs have also been reported to enhance the survival of motor neurons after neonatal nerve injury in rat (38, 40, 71, 77). Ciliary neurotrophic factor (CNTF) appears to have both myotrophic and neurotrophic activity and is expressed in Schwann cells (1, 14, 28, 49, 54, 62, 64, 65). However, histological analyses on mice null for NT-3, NT-4, and CNTF have revealed only a modest loss of facial motor neurons (6, 42, 43, 61).

The effect of individual NTs on the survival of specific populations of motor neurons (those innervating fast vs. slow muscle fibers) after neonatal nerve injury has not been investigated. However, Sterne et al. (68) have shown that treatment with NT-3 decreased the loss of type IIB fibers in denervated gastrocnemius of adult rats. This could result from enhanced survival of the motor neurons innervating type IIB fibers or, alternatively, modification of the properties of the remaining motor neurons. NT-3 and NT-4 exhibit distinct temporal and spatial patterns of expression in the developing hindlimb (16, 23, 29). Prenatal expression of NT-3 is observed throughout the developing muscle, whereas NT-4 expression is restricted to the epidermis at this stage (23). In the adult, NT-3 is expressed in

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were anesthetized with isoflurane. Day of birth. Animals were housed in the University of Ottawa animal 

MATERIALS AND METHODS

Neonatal Sciatic Nerve Crush

Thirteen-day-gestation Sprague-Dawley rats were obtained from Charles River Laboratories (Quebec, Canada). Animals were housed in the University of Ottawa animal care facility with 12-12-h dark-light cycle and unlimited food and water. All surgical procedures were approved by University of Ottawa Animal Care and Use Committee. Day of birth of pups was taken as postnatal day 0 (P0). Five-day-old pups were anesthetized with isoflurane gas, and the right sciatic nerve was exposed at midthigh level by carefully parting surface muscles (biceps femoris and gluteus maximus). With fine forceps, the sciatic nerve was crushed for 5 s. The wound was sutured with thin prolene suture, and the pups were returned to their mother. The animals were housed in the Animal Care facility for a period between 3 and 5 mo, after which they were subjected to motor unit counting and muscle analysis. Here we decided to investigate whether there is preferential survival of muscle fiber types and motor neurons with administration of different NTs after neonatal nerve injury.

NT Application

NTs were provided by Regeneron (Tarrytown, NY). A piece of gelfoam soaked in sterile phosphate-buffered saline (PBS) containing the various NTs was placed on the site of the crush. The following doses were used: in CNTF + NT-3-treated animals, 5 μg of CNTF plus 6.25 μg of NT-3; in CNTF + NT-4-treated animals, 5 μg of CNTF plus 1.5 μg of NT-4. NTs were then injected daily under the skin of the back (28) in a volume of 0.005 ml of sterile PBS per gram of body weight for a period of 10 days. Seven pups were administered a daily injection of 0.3 μg of CNTF and 1.5 μg of NT-3 per gram of body weight. In the CNTF + NT-4 group, three pups were administered 0.3 μg of CNTF and 1.5 μg of NT-4 per gram of body weight. These doses were selected based on those reported in the literature. For example, Helgren et al. (28) used doses of CNTF in the range of 0.3–1 μg/g body wt. Funakoshi et al. (16) implanted genetically modified 3T3 cells, which secreted NT-4 at a rate of 150 ng/day, into the gastrocnemius muscle; given that the mass of this muscle is ~0.6% of total body mass, a dose of 30 μg of NT-4 into rat pups with a mean body weight of 20 g, as in our experiments, would yield values of NT-4 at the level of the muscle comparable to those obtained by Funakoshi et al. (16). Furthermore, doses higher than these have been shown, in preliminary experiments, to induce cachexia. Indeed, because of the death of a second group of three pups injected with this dose of CNTF + NT-4, the amounts of CNTF and NT-4 given an additional two pups in this group were reduced to 0.08 and 0.38 μg/body wt, respectively.

Electrophysiological Counting of Motor Units in EDL and SOL

After the survival period, the animals were weighed and then anesthetized with halothane gas. A catheter was placed in the animal’s tail vein for continuous anesthesia with injection of pentobarbital sodium (Somnotol; MTC Pharmaceuticals) at a dose of 0.06 mg/kg body wt−1·h−1. The animals’ legs and backs were shaved, and the muscles were exposed by a surgical incision from the ankle to the knee. TA muscle was slightly displaced such that the branches of the deep peroneal nerve innervating this muscle were exposed and crushed, after which the superficial peroneal nerve was exposed and transected to denervate the remaining peroneal muscles. Subsequent electrical stimulation of the common peroneal nerve resulted in EDL contraction only. The tibial and sural branches of sciatic nerve were also cut to denervate all posterior hindlimb muscles. In the case of SOL, all muscles of the hindlimb were denervated except SOL. Electrodes were placed near the sciatic nerve at mid-thigh level for whole muscle stimulation. The skin overlaying the muscle was loosely sutured after denervation. A laminectomy was performed, exposing the spinal cord from L1 to L6. Gauze moistened with PBS was placed on the exposed part of spinal cord to avoid dehydration. The distal tendon of EDL or SOL was attached to a stainless steel wire loop with silk thread. The ankle was secured, the distal tendon was cut, and the loop was attached to a transducer (FT03; Grass Medical Instruments, Quincy, MA) for force measurement. Muscle length was adjusted to yield a maximal tetanic tension at a stimulation frequency of 100 Hz for 400 ms. The skin on the back was raised to make a pool containing mineral oil for electrical insulation. The dura mater was removed; L4, L5, and L6 ventral roots were cut at the point of exit from the cord and placed on electrodes for stimulation of EDL or SOL. Roots containing axons innervating EDL or SOL were split with fine forceps to yield filaments, which, upon stimulation, generated ~30% of the whole muscle force. Each of these filaments was stimulated with incrementally increasing voltage, and the number of motor units was determined by the number of increments in recorded force.

The motor unit experiment, TA, EDL, gastrocnemius, and SOL were excised, weighed, and frozen in isopentane chilled in liquid nitrogen. The muscle blocks were kept at −80°C and subsequently cryosectioned at 10-μm thickness and placed on positively charged slides (FisherBrand Suprathrost Plus).

Myosin Heavy Chain Immunohistochemistry

Sectioned muscles were blocked with 5% skim milk solution in PBS for 30–60 min. Primary antibodies for myosin heavy chain (MHC; see below) were placed on the slides in 1:5 dilution for duration of 4 h to overnight. Slides were washed three times for 15 min in PBS followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:100 dilution for 1–2 h). After washes in PBS, slides were placed in 1% diaminobenzidine (DAB) and 0.01% (vol/vol...
vol) 30% hydrogen peroxide (H₂O₂) in PBS in the dark for 5–10 min for color development and were then mounted with Permount and coverslips. MHC monoclonal primary antibodies were A4–840 for type I (Developmental Studies Hybridoma Bank at the University of Iowa), SC-71 for type IIA, and BP-F3 for type IIB (two latter types from German Collection of Microorganisms and Cell Cultures). These antibodies have previously been described (32, 58).

Fiber Counting in EDL and SOL

Fiber measurements were performed by using a Zeiss microscope (Axioptophot) and a computer equipped with Northern Eclipse (Empix Imaging). Clearly identifiable degenerated fibers (<5 μm in diameter) in operated EDL were individually counted. All other fibers were counted in five different sample areas within the cross sections of muscles at ×200 magnification. By measuring whole muscle surface area and the mean surface area of the sample field, the estimated total fiber number was calculated as

\[
\text{Mean fiber number per field} = \frac{\text{Mean area of field} \times \text{muscle cross-sectional area} + \text{degenerating fibers}}{\text{Muscle surface area}}
\]

A similar protocol was applied elsewhere (59). The mean number of fibers per field averaged 114–187 in the control EDL, 106–187 in the EDL of the operated leg, 88–108 in the control SOL, and 76–86 in the SOL of the operated leg. In control EDL, in which fiber type distribution is essentially random, the number of type IIB fibers was estimated in the same way as the total number of fibers. Type I and IIA fibers, which were relatively sparse, were individually counted in the entire muscle section. Type IIX fibers were estimated by subtracting the number of type I, IIA, and IIB fibers from the total number of fibers. Type I fibers in control SOL were estimated similarly to the total number of fibers; type IIA fibers were individually counted. To verify that there was no selection bias due to type grouping of fibers in the operated muscles, calculated and individually counted type IIA fiber numbers were compared in some EDL muscles, and these values were not significantly different.

The cross-sectional area of 100 fibers per type per section was measured. The average cross-sectional area for each fiber type was compared between the operated and contralateral muscles and also between the untreated and NT-treated groups.

Motor Neuron Labeling

Retrograde labeling of motor neurons innervating TA with HRP was done as previously described (70). Briefly, after 2–3 wk of unilateral P5 sciatic nerve crush, TA muscles in both legs were exposed and injected with 20 μl of 20% HRP (Sigma; type VI) solution in sterile PBS. To avoid labeling motor neuron pools of the other muscles, care was taken to clean any leakage of HRP solution. After 24–36 h, the animal was perfused with 50 ml of sterile PBS, followed by 50 ml of 4% paraformaldehyde in sterile PBS. The spinal cord was then excised and incubated in 1:1 solution of OCT (Tissue Tek) and 30% sucrose for 1–2 h, cryosectioned at 40-μm thickness, and placed on gelatin-coated slides. The protocol of Mesulam (50) was followed for visualizing the motor neurons. To avoid multiple counting of labeled neurons, only those with visible nucleoli were counted.

Statistical Analyses

All values were normalized against contralateral control side (except for motor unit values) and compared by using one-way ANOVA with least square difference (LSD).

RESULTS

Effect of Transient Axotomy at P5

Muscle mass and force generation. Transient denervation of the hindlimb at P5 results in long-lasting muscle atrophy. The anterior crural muscles of the hindlimb (EDL and TA) are affected to a greater extent than the posterior crural muscles (gastrocnemius and SOL). At 3 mo after sciatic nerve crush at P5, the mass of TA and EDL were each reduced by ~50%, whereas gastrocnemius and SOL decreased in weight by 30 and 20%, respectively (Fig. 1). At this time, maximum tetanic force decreased by 64% in EDL and 23% in SOL. When the maximum tetanic force was normalized per gram of muscle mass (specific force), EDL specific force was reduced by 30% relative to normal control muscle. In contrast, SOL specific force was significantly increased (Table 1).

To determine whether the reduced muscle mass and absolute force generation reflect a decrease in the number of motor neurons that reinnervated the hindlimb muscles, the number of motor units in EDL and SOL muscles of rats 3 mo after sciatic nerve crush at P5 was assessed by a conventional ventral root-splitting technique. In control EDL muscles, the average number of motor units was found to be about 36, whereas only 18 motor units were present in the transiently axotomized EDL. A similar loss of motor neurons innervating the TA muscle was observed by using a retrograde labeling procedure (156 ± 16 for control rats and 83 ± 17 following P5 sciatic nerve crush; mean ± SE). In the case of SOL muscle, no loss of motor units was detected following the transient axotomy (Table 1).

Fig. 1. Effects of postnatal day 5 (P5) sciatic axotomy on hindlimb muscle mass. The right sciatic nerve of P5 rat pups was crushed for 5 s. After a 10–12-wk recovery period, the muscles of the hindlimb were excised and weighed. The weight of the muscles from the operated leg was normalized against the contralateral side (n = 7). Values are means ± SE. EDL, extensor digitorum longus; TA, tibialis anterior; GAS, gastrocnemius; SOL, soleus.
Muscle fiber number, size, and type distribution. TA and EDL muscles, which had been transiently denervated at P5, exhibited a marked reduction in the total numbers of muscle fibers as detected with MHC immunohistochemistry. Fiber counts were made in EDL muscles of both the operated hindlimb and the contralateral unoperated hindlimb. The total fiber number was significantly lower in the transiently axotomized EDL (2,570 ± 159) than in the contralateral EDL (3,116 ± 206), with a mean loss of 18%. The transiently denervated EDL muscles contained a mean of 153 degenerating fibers (6 ± 1% of the total fiber number). Because these atrophic fibers did not express embryonic MHC (data not shown), we assumed that they were not regenerating fibers. Furthermore, relatively few degenerating fibers (mean = 24 ± 8) were detected in transiently denervated EDL muscles in 5 of 7 rats that were examined 8 mo after sciatic nerve crush at P5. In SOL, P5 transient denervation resulted in a 25% decrease in the total number of fibers from 2,520 ± 126 in contralateral control side to 1,884 ± 61 in the operated side. Degenerating fibers were rarely seen in the transiently denervated SOL.

In the axotomized group, unoperated EDL muscle contained a mean of 1,494 ± 88 IIB fibers, whereas this fiber type was almost completely absent from the EDL on the operated side (Fig. 2). Type I fibers did not change significantly in number (204 ± 76 in operated and 137 ± 26 in contralateral control side). There was a significant increase in the number of type IIA fibers from a control value of 714 ± 68 to 1,406 ± 217 in the transiently axotomized EDL, and because there was no difference in the number of type IIX fibers, this suggested that approximately one half of the fibers destined to express type IIB MHC were lost and, in the remaining fibers, MHC expression was changed. Transient axotomy at P5 resulted in hypertrophy of type I and type IIA fibers in EDL (from 1,069 ± 96 to 2,448 ± 694 μm² and 1,101 ± 124 to 2,139 ± 221 μm², respectively), presumably as a compensatory response to the loss of type IIB fibers.

In unoperated SOL, 86% of fibers were type I fibers (2,174 ± 127) and the rest type IIA (347 ± 40). After P5 axotomy, the number of fibers expressing type I MHC decreased by 42% to 1,258 ± 120. Type IIA fibers increased (from 347 ± 40 in contralateral control side to 439 ± 70 in the operated side), which was twofold in proportion to the total number of fibers (Fig. 5). Type IIX fibers were not present in contralateral control SOL, whereas in operated SOL these fibers constituted between 5–10% of total fibers (Fig. 5). Transient axotomy at P5 resulted in hypertrophy of type I fibers in SOL (from 3,648 ± 177 μm² to 4,742 ± 395 μm²), presumably as a compensatory response to the loss of fibers, whereas type IIA did not change significantly in size (2,617 ± 241 μm² in operated and 2,874 ± 234 μm² in contralateral control).

Table 1. Effects of P5 sciatic nerve crush on force generation and number of motor units of EDL and SOL and the effects of neurotrophins on EDL after P5 sciatic nerve crush

<table>
<thead>
<tr>
<th>Muscle</th>
<th>n</th>
<th>Specific Force, N/g muscle mass</th>
<th>Number of MU</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL Control</td>
<td>8</td>
<td>15.5 ± 0.8</td>
<td>36.2 ± 1.8</td>
</tr>
<tr>
<td>P5 axotomized</td>
<td>12</td>
<td>11.2 ± 0.9*</td>
<td>17.9 ± 1.6*</td>
</tr>
<tr>
<td>CNTF + NT-3</td>
<td>7</td>
<td>12.9 ± 1.0*</td>
<td>24.7 ± 4.1*</td>
</tr>
<tr>
<td>CNTF + NT-4</td>
<td>5</td>
<td>17.7 ± 3.3†</td>
<td>30.6 ± 3.9†</td>
</tr>
<tr>
<td>SOL Control</td>
<td>13</td>
<td>9.2 ± 0.6</td>
<td>31.6 ± 2.8</td>
</tr>
<tr>
<td>P5 axotomized</td>
<td>8</td>
<td>14.0 ± 0.8*</td>
<td>34.5 ± 2.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 denotes significance when compared to control values. †P < 0.05 denotes significance when compared to axotomized values.
Effects of NTs Following Transient Axotomy at P5

Muscle mass and force generation. When NTs were administered individually, no reduction was observed in the extent of loss of muscle mass relative to that occurring after axotomy alone. When expressed as a percentage of unoperated contralateral muscle, the mass of axotomized EDL and SOL, respectively, were 55 ± 4%, 82 ± 5% with CNTF treatment; 46 ± 1%, 87 ± 4% with NT-3; and 55 ± 3%, 73 ± 1% with NT-4. In no case were these values significantly different from axotomized muscles without NT treatment.

Because the combined application of NTs has been shown to have a synergistic effect in culture (75, 78), we decided to administer combinations of CNTF + NT-3 or CNTF + NT-4 to determine the effect of muscle-deprived NTs (NT-3 and NT-4) on muscle and motor neuron survival after neonatal axotomy. CNTF + NT-3 reduced the loss of EDL mass to 62 ± 9% of contralateral muscle mass, and CNTF + NT-4 had an even greater effect; the transiently axotomized EDL mass was 72 ± 7% of the contralateral control (Fig. 3). These values are both significantly different from the EDL muscles after P5 sciatic nerve section without administration of NTs (50 ± 11% contralateral muscle mass). In the case of SOL, the axotomy-induced loss of muscle mass (82 ± 3% of contralateral control) was completely prevented by treatment with CNTF + NT-3 (99 ± 4%) and CNTF + NT-4 (102 ± 7%). The combined administration of NTs at the doses given did not affect the mass of the contralateral control muscles, which remained ~0.05% of the body weight as in the normal rat.

Specific tetanic force (per gram of muscle mass) generated by EDL decreased by 30% 3 mo after P5 sciatic crush, whereas specific force declined only 20% in CNTF + NT-3 group, and no loss in specific force was observed in CNTF + NT-4-treated group (Table 1).

This partial rescue of contractile force was accompanied by a reduction in the loss in the number of motor units in EDL of both CNTF + NT-3 and CNTF + NT-4 groups. The EDL motor neuron pool comprised 25 ± 4 for CNTF + NT-3 and 31 ± 4 for CNTF + NT-4, although only in the case of CNTF + NT-4 was this value significantly different from untreated group (17.9 ± 1.6) and not significantly different from the control value (36.2 ± 1.8).

Fig. 4. Fiber-type distribution in EDL after transient axotomy at P5 with and without subsequent treatment with NTs. The operated side is expressed as a proportion of the contralateral control side. Type I (open), type IIA (light shaded), type IIB (dark shaded), and type IIX (solid). Type IIB fibers are almost absent in all groups. Values are means ± SE; n = 7 (axotomized and CNTF + NT-3) and 5 (CNTF + NT-4).

Fiber number, size, and type distribution. Because it has been demonstrated that various NTs can save motor neurons from axotomy-induced death, we decided to see whether these NTs would be able to prevent the loss of muscle fibers. In addition, the loss of type IIB fibers from TA and EDL raised the possibility that the transient axotomy at P5 resulted in the death of a specific population of motor neurons which normally innervated type IIB fibers. In the CNTF + NT-3- and CNTF + NT-4-treated groups, the total number of fibers in operated EDL was 3,258 ± 261 and 2,984 ± 250. These muscles had 20 and 11% more fibers, respectively, than the contralateral unoperated muscles. The transiently denervated EDL in the CNTF + NT-3 treated rats contained a mean of 296 degenerating fibers (9 ± 3% of the total number), whereas degenerating fibers were only found in 3 of 5 of the transiently denervated EDL muscles of CNTF + NT-4-treated rats (mean number = 13 ± 10).

In the CNTF + NT-3 group, the proportion of type I in EDL was the same as in the untreated group (two-fold higher than the contralateral control side), whereas in the CNTF + NT-4 group the proportion of type I fibers was not different from contralateral control EDL (Fig. 4).

In both NT-treated groups, there was an increase in proportion of type IIA compared with the untreated group (Fig. 4). Type IIB fibers were not present in EDL of either NT-treated groups (Fig. 2), and type IIX remained the same in proportion relative to the contralateral control EDLs (Fig. 4). Treatment with either of the NT combinations reduced the hypertrophic response of the type I fibers in EDL (1,524 ± 100 μm² for CNTF + NT-3 and 1,264 ± 353 μm² for CNTF + NT-4), although type IIA fibers remained...
enlarged \(2,168 \pm 151 \mu m^2\) for CNTF + NT-3 and \(2,366 \pm 114 \mu m^2\) for CNTF + NT-4.

In operated SOL of animals treated with CNTF + NT-3, the total number of fibers was \(1,852 \pm 111\) (77% of contralateral control side). In this group, the number of type I fibers was \(1,052 \pm 164\) (a 54% decrease compared with contralateral control side), whereas the number of type IIA fibers increased fourfold from \(141 \pm 40\) in the contralateral control side to \(561 \pm 73\) in the operated side (Fig. 5). Type IIX fibers were absent in contralateral control side of CNTF + NT-3-treated group, whereas in the operated SOL, there were \(240 \pm 156\) type IIX fibers. In CNTF + NT-4-treated animals, the total number of fibers was \(2,290 \pm 43\) (86% of the contralateral control side). The number of type I fibers decreased by 24% to \(1,826 \pm 40\), whereas the number of type IIA fibers increased slightly to \(388 \pm 32\). In this experimental group, only the operated SOL of one animal had 376 type IIX fibers, whereas the rest of the animals had no type IIX fibers in the operated SOL (Fig. 5). In the NT-treated animals, type I fibers in SOL remained hypertrophied \((4,923 \pm 318 \mu m^2\) for CNTF + NT-3 and \(4,414 \pm 422 \mu m^2\) for CNTF + NT-4), as in the untreated groups. Type IIA fibers did not change in size compared with contralateral control SOL and also the untreated group \((2,570 \pm 174 \mu m^2\) for CNTF + NT-3 and \(2,334 \pm 202 \mu m^2\) for CNTF + NT-4).

**DISCUSSION**

As previously reported (44), neonatal sciatic nerve crush in the rat results in extensive skeletal muscle atrophy, especially of the anterior crural muscles (EDL and TA) of the hindlimb. The posterior crural muscles (gastrocnemius and SOL) are affected to a significantly lesser extent. The greater loss of muscle mass in EDL and TA is associated with the virtually complete loss of type IIB fibers, which form the largest proportion of EDL fibers in control rats but which are absent from SOL. In the present experiments, the total number of fibers 3 mo after nerve crush was 18 and 25% lower in EDL and SOL, respectively, compared with the contralateral control side. The loss of fibers in EDL was prevented by the administration of CNTF with NT-3 or NT-4 after neonatal axotomy. In SOL, the restoration of muscle mass by NTs was due largely to hypertrophy of the surviving fibers (Fig. 6). These data suggest the possibility of selective effects of NTs on specific subpopulations of developing skeletal muscle fibers.

Although it is conceivable that the reduced fiber number after neonatal sciatic nerve crush could reflect a failure of new fiber generation, there is no evidence of any such increase in fiber number in either EDL (20) or SOL (20, 53) after 5 days of age. In fact, the loss of muscle fibers in transiently denervated EDL (and also TA) is paralleled by the loss of a significant proportion of motor neurons normally innervating these muscles. The SOL motor neuron pool, by contrast, was not affected by the transient axotomy, and this is reflected in the smaller reduction of mass in this muscle. There have been several previous reports of loss of a significant proportion of motor neurons following neonatal axotomy (5, 31, 38, 45, 60, 63, 77). We suggest that the relatively specific susceptibility of type IIB fibers, which we have seen after neonatal nerve injury, may be correlated with the loss of a specific population of

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**Fig. 6.** Values for the muscle mass (open), the total number of fibers (shaded), and the average fiber cross-sectional area (solid) in EDL (A) and SOL (B) in different experimental groups. In EDL of the untreated axotomized group, both the number of fibers and average fiber cross-sectional area contribute to the loss of the mass observed. In CNTF + NT-3 \(n = 7\) and CNTF + NT-4 \(n = 5\) groups, only average fiber size contributes to the loss of the muscle mass. In SOL of the untreated axotomized group, only the loss of fibers contributes to the loss of the mass observed. In CNTF + NT-3 and CNTF + NT-4 groups, average fiber cross-sectional area increased by 20%, whereas the total number of fiber remained \(\sim 80\%\) of contralateral control side. *Significantly different \((P < 0.05)\) from axotomy alone.

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**Fig. 5.** Fiber-type distribution in SOL after transient axotomy at P5 with and without subsequent treatment with NTs. The operated side is expressed as proportion of total number of fibers. Type I (open), type IIA (shaded), and type IIX (solid). Values are means ± SE; \(n = 7\) (axotomized and CNTF + NT-3) and 5 (CNTF + NT-4).
motor neurons innervating these fibers in EDL and TA. Transient axotomy after 21 days of age does not result in the extensive death of motor neurons or the disappearance of type IIB fibers (9, 46, 55). We have also noted that sciatic nerve crush in 14-day-old rats results in only a 25% loss of TA and EDL muscle mass (data not shown) and that these transiently axotomized muscles contain a nearly normal complement of type IIB fibers that do not appear to be atrophic. Therefore, there is a period, before 14 days of age, during which these motor neurons are vulnerable to transient target removal.

Neonatal motor neurons die within 6 days after neonatal nerve crush, i.e., before reinnervation (46), and can be rescued by the administration of NTs (30, 40), demonstrating their target-derived trophic dependency. Combinations of CNTF with either NT-3 or BDNF have been reported to enhance survival of motor neurons in mice with genetic mutations that result in motor neuron death (26, 51). We have shown a similar NT-dependency in the population of rat EDL motor neurons that are susceptible to transient neonatal axotomy (Table 1). It is worth emphasizing, however, that the different effect of neonatal axotomy on EDL and SOL is not due to a differential sensitivity of fast vs. slow muscle, because the gastrocnemius muscle also was relatively unaffected by the transient axotomy. One possible explanation for this could be a slower rate of maturation of the anterior vs. posterior crural muscles or the motor neurons innervating them. However, there does not appear to be any evidence to this effect, and, indeed, it has been reported that motor neurons are born and develop to maturity in a rostral (EDL and TA) to caudal (gastrocnemius and SOL) fashion during embryonic development (19). Similarly, using dopamine (L-DOPA) to induce overstimulation and apoptosis in the first 12 days after birth, Sanusi et al. (57) have shown that motor neurons innervating SOL are more susceptible to excitotoxicity than those motor neurons innervating EDL, indicative of SOL motor neurons being less mature than those of EDL.

Our data indicate that NT-3 and NT-4, together with CNTF, may induce some new fiber generation, because the total number of fibers of normal size in these muscles was 6–10% greater than in unoperated control muscles. However, the fiber-type distribution of these muscles was altered. Rescued fibers did not express type IIB MHC, at least up to 5 mo after the transient axotomy. The number of type IIA fibers increased, largely offsetting the loss of IIB fibers and suggesting that fiber-type conversion had occurred. Although this may simply reflect a response to the overload placed on the IIA fibers, it is known that changes in the electrophysiological properties of motor neurons, resulting in an altered firing pattern, may in turn modify MyHC expression (52, 69). Gonzales and Collins (17) have reported that application of BDNF (a TrkB ligand) to intact gastrocnemius muscles of the rat produced a shift of the properties of the motor neurons, which are normally “fast”, toward those typical of SOL (“slow”) motor neurons. Our findings are consistent with a role for NT-4 (and possibly BDNF) not only in aiding the survival of motor neurons after transient axotomy but also in modifying the electrophysiological characteristics of EDL motor neurons such that the fibers they innervate switch their MyHC expression toward the slow end of the continuum of MHC expression (i.e., IIB ↔ IIX ↔ IIA ↔ I). It has been reported that NT-4 expression in muscle is activity dependent (16, 73), indicative of higher expression in more active muscle fibers. Indeed, Funakoshi et al. (16) observed higher expression of NT-4 in slow fibers (type I) of SOL, which would tend to support this hypothesis (see Ref. 56).

We suggest that these target-derived NTs are already expressed at P5 and not only ensure survival of those motor neurons innervating these fibers but also specify them to a slow type (cf. Ref. 17). We further hypothesize that at P5, the secondary myofibers (i.e., those that will eventually express type IIB MHC) are relatively immature and do not express NTs, with the result that the motor neurons innervating these fibers are in some way more susceptible to the axotomy and, furthermore, have not been specified to either a fast or slow type. As a consequence, this population of motor neurons dies, and a proportion of the fibers that would have been innervated by them also degenerate, although others within this cohort are reinnervated by branches of the surviving slow motor neurons. Application of NT-4 at the time of axotomy results in the entire motor neuronal population receiving the NT, which in turn enhances their chances of survival and also specifies them to the slow type. As a result of this, virtually all of the muscle fibers can be reinnervated but will only express type I or IIA MHC.

During development in rodents, expression of both BDNF and NT-3 in muscle is highest during the late fetal and early postnatal stages and then progressively falls to very low levels by 3 wk of age (16, 23). By contrast, expression of NT-4 is low at birth and progressively rises to reach the adult level at about 5 wk (16, 23). Thus the effect of NT-4 which we have shown may normally be carried out in vivo by BDNF, also acting via TrkB receptors. In our experiments, the contralateral unoperated SOL in the NT-treated rats also appeared to undergo a slight (though not statistically significant) change in fiber type distribution. The unoperated SOL of the NT-4-treated group, compared with that of the untreated rats, exhibited a reduction in the ratio of type IIA to I fibers, indicative of a switch towards type I MHC expression in these muscles.

The present study suggests that the EDL muscle fibers and/or motor neurons that die after transient nerve crush and are rescued by NTs may be expressing TrkB and/or TrkC together with CNTFR-α during the neonatal period. Because TrkB and TrkC, along with their putative ligands (NT-4 and NT-3, respectively), are expressed in skeletal muscle and in motor neurons (8, 12, 18, 29, 33, 56), NT-3 and NT-4 may have autocrine as well as paracrine effects. For example, TrkB is localized to both the postsynaptic (18, 74) and the presynaptic region of the neuromuscular junction (11, 77), and postsynaptic TrkB activation stabilizes ster-
nomastoid muscle ACh receptors in mouse (18). In addition, ACh receptors are disrupted in NT-4-null mice (4). Similarly, NT-3, perhaps through TrkC, increases ACh aggregates on myotubes in nerve-muscle cocultures (15), and it also increases the ACh release from presynaptic terminals (72). Given that ACh receptor blockade in neonates leads to motor neuron death (21, 22), these observations raise the importance of NTs in maintaining the integrity of neuromuscular properties that are essential for the survival of motor neurons and muscle fibers. Furthermore, TrkB null mice display extensive motor neuron loss in the lumbar region of the spinal cord (37), although it is not clear whether this loss was restricted to any specific population of motor neurons. Heterogeneity within the motor neuronal population is suggested by the following observations: 1) in mice lacking the receptors for either CNTF or leukemia inhibitory factor, only a proportion of motor neurons are lost (10, 41); 2) c-Met, the receptor for hepatocyte growth factor/scatter factor, which also has neurotrophic activity, is only expressed by a fraction of cultured rat motoneurons (76); and 3) NT-3 and BDNF transcripts are localized to different, temporally and spatially separated subpopulations of motoneurons in the spinal cord of the developing chick (34). Although Copray and Kernell (8) reported that TrkB and TrkC are expressed in the motor neurons of both EDL and SOL motor neuron pools, Simon et al. (67) recently reported higher transcript level of TrkC in the adult motor neuron pool of EDL than those innervating SOL. Whether full-length TrkB, TrkC, and CNTFR-α proteins are more abundant in EDL than in SOL motor neurons and muscle fibers remains to be determined.

In summary, neonatal axotomy in rat within the first week of life resulted in extensive lumbar motor neuron loss and muscle atrophy. Muscle fibers were lost in both EDL and SOL, but only the motor neurons innervating EDL were selectively lost. Synergistic effects of CNTF and NT-3 or NT-4 selectively rescued the motor neurons and muscle fibers that are destined to die after neonatal axotomy in EDL. The failure of neonatal axotomy to induce loss of SOL motor neurons suggests that these neurons may have already been rendered stable by exposure to their target-derived NT (possibly NT-4) by P5. The loss of muscle fibers from SOL is more difficult to explain, as is the fact that this loss is not reversed by NT treatment.

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