

Neurotrophin-3–enhanced Nerve Regeneration Selectively Improves Recovery of Muscle Fibers Expressing Myosin Heavy Chains 2b

G.D. Sterne,* G.R. Coulton,‡ R.A. Brown,§ C.J. Green,* and G. Terenghi*

*Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, West Sussex RH19 3DZ, United Kingdom; ‡Department of Biochemistry, Charing Cross and Westminster Medical School, London W6 8RF, United Kingdom; and §Department of Plastic and Reconstructive Surgery, University College, London W1P 7LD, United Kingdom

Abstract. The purpose of this study was to evaluate the effect of neurotrophin 3 (NT-3) enhanced nerve regeneration on the reinnervation of a target muscle. Muscle fibers can be classified according to their mechanical properties and myosin heavy chain (MHC) isoform composition. MHC1 containing slow-type and MHC2a or 2b fast-type fibers are normally distributed in a mosaic pattern, their phenotype dictated by motor innervation. After denervation, all fibers switch to fast-type MHC2b expression and also undergo atrophy resulting in loss of muscle mass. After regeneration, discrimination between fast and slow fibers returns, but the distribution and fiber size change according to the level of reinnervation.

In this study, rat gastrocnemius muscles (ipsilateral and contralateral to the side of nerve injury) were collected up to 8 mo after nerve repair, with or without local delivery of NT-3. The phenotype changes of MHC1, 2a, and 2b were analyzed by immunohistochemistry, and fiber type proportion, diameter, and grouping were assessed by computerized image analysis. At 8 mo, the

local delivery of NT-3 resulted in significant improvement in gastrocnemius muscle weight compared with controls (NT-3 group 47%, controls 39% weight of contralateral normal muscle; $P < 0.05$). NT-3 delivery resulted in a significant increase in the proportion (NT-3 43.3%, controls 35.7%; $P < 0.05$) and diameter (NT-3 87.8 μm , controls 70.8 μm ; $P < 0.05$) of fast type 2b fibers after reinnervation. This effect was specific to type 2b fibers; no normalization was seen in other fiber types.

This study indicates that NT-3–enhanced axonal regeneration has a beneficial effect on the motor target organ. Also, NT-3 may be specifically affecting a subset of motoneurons that determine type 2b muscle fiber phenotype. As NT-3 was topically applied to cut nerves, our data suggest a discriminating effect of the neurotrophin on neuro–muscular interaction. These results would imply that muscle fibers may be differentially responsive to other neurotrophic factors and indicate the potential clinical role of NT-3 in the prevention of muscle atrophy after nerve injury.

THERE has been much recent interest in the use of growth factors to augment peripheral nerve regeneration. A family of growth factors collectively known as the neurotrophins are now considered critical for the development, maintenance, and regeneration of the nervous system. The neurotrophin family includes NGF, brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3),¹ and neurotrophin-4/5 (NT-4/5) (Lewin and Barde,

1996; Lindsay, 1996). Little is known of their effect on regeneration of the peripheral nervous system.

NT-3 has been shown to act on a subpopulation of muscle sensory neurons innervating muscle spindles and Golgi tendon organs, and there is also evidence of its effect on a subpopulation of cutaneous afferents (Ernfors et al., 1994; Tessarolo et al., 1994; Airaksinen et al., 1996). NT-3 has shown various effects on motor nerve regeneration, including differentiation of motoneurons from avian neural tube progenitor cells (Averbuch-Heller et al., 1994) and survival of neonatal and adult motoneurons in vitro (Hughes et al., 1993) and of neonatal motoneurons in vivo (Li et al., 1994; Vejsada et al., 1995), although the evidence is sometimes contradictory (Eriksson et al., 1994). In cocultures of adult muscle and embryonic motoneurons, NT-3 enhances the number and length of neurite outgrowths, the density of endplates per muscle fiber, and the amount

Address all correspondence to Dr. G. Terenghi, Blond McIndoe Centre, Queen Victoria Hospital, Holtye Road, East Grinstead, West Sussex RH19 3DZ, United Kingdom. Tel.: 01342 313088. Fax: 01342 301701. E-mail: terenghi@dial.pipex.com

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; FN, fibronectin mats; MHC, myosin heavy chain; NG, autologous nerve grafts; NT-3, neurotrophin-3.

of muscle innervation (Braun et al., 1996). NT-3 also plays a role in functional maturation of neuromuscular synapses (Lohoff et al., 1993; Wang et al., 1995) and regulates the cholinergic phenotype of developing motoneurons (Wong et al., 1993; Kato and Lindsay, 1994). NT-3 knockout mice show a loss of all muscle spindle afferent innervation and fusimotor neurons to the muscle but lose only few skeleto-motor nerve fibers (Kucera et al., 1995a). About 80% of adult motoneurons express the NT-3-specific trkC receptor (Henderson et al., 1993; Griesbeck et al., 1995), and NT-3 is the predominant neurotrophin expressed in skeletal muscle (Griesbeck et al., 1995). Furthermore, NT-3 is internalized and retrogradely transported from the periphery to motoneuron cell bodies (Di Stefano et al., 1992). Thus, there is experimental and circumstantial evidence to suggest that NT-3 may play a role in adult motoneurons, although *in vivo* data on the survival effect of NT-3 on adult motoneurons is still lacking. Furthermore, there is no evidence of an NT-3-dependent effect on neuro-muscular interaction.

When a skeletal muscle is denervated and subsequently reinnervated, a characteristic sequence of events ensues. The muscle rapidly loses weight as the muscle fibers atrophy (Pellegrino and Franzini, 1963), but after reinnervation, it gradually recovers mass to a variable extent, depending upon the degree of reinnervation (Bertelli and Mira, 1995) and correlating with the maximum force of contraction (Gillespie et al., 1987). The fibers within an individual skeletal muscle do not exist as a homogenous population but can be classified according to their different metabolic and contractile properties (Burke et al., 1971; Peter et al., 1972). Also, they can be identified morphologically according to differential expression of specific myosin heavy chain isoforms. Slow, oxidative type 1 muscle fibers contain myosin heavy chain 1 (MHC 1), fast oxidative glycolytic type 2a fibers contain myosin heavy chain 2a (MHC 2a), while fast glycolytic type 2b fibers contain myosin heavy chain 2b (MHC 2b) (Bar and Pette, 1988). Muscle fiber phenotype is conferred by its innervation, and changes of neuro-muscular interaction lead to alteration of muscle fiber phenotype (Romanul and Van der Meulen, 1966; Fex and Sonneson, 1970; Salmons and Sreter, 1975). The relative proportions of fiber types vary with age, sex, strain, species, and muscle type (Maltin et al., 1989). Generally, there is a high proportion of type 1 fibers in postural muscles (e.g., soleus) and of type 2 fibers in fast muscles (e.g., extensor digitorum longus), while in mixed muscles (e.g., gastrocnemius) there are varying proportions of each type. Muscle fiber type proportion also varies dynamically with physiological and pathological parameters (Jansson et al., 1978; Green et al., 1983; Izumo et al., 1986; Goldspink et al., 1992; Pette and Vrbova, 1992). For example, the distribution of fiber types in normal muscle is dispersed in a "mosaic pattern," but after denervation and reinnervation of the muscle there is a shift to grouping (Karpati and Engel, 1968). Also, there is a change in the proportions of fiber types, and in the rat the majority of fibers become initially fast with denervation, with subsequent fiber specialization being dictated by patterns of reinnervation (Fields and Ellisman, 1986). This plastic nature of muscle makes it an interesting model to investigate reinnervation changes that may occur after NT-3 administration.

We have recently demonstrated that the local delivery of NT-3 to rat sciatic nerve enhances the rate and amount of nerve regeneration, and at 8 mo postoperative, there was a 40% increase in the myelinated fiber count (Sterne et al., 1997). However, enhanced regeneration by itself is not necessarily indicative of a beneficial effect on the target muscles, such as reacquisition of more normal physiological function. Therefore, the aim of this study was to assess whether NT-3-enhanced nerve regeneration resulted in biochemical or morphological changes in a target muscle (gastrocnemius), which would be suggestive of significant improvement of physiological function above that seen after nerve repair without administration of neurotrophin. Immunohistochemistry, in conjunction with computerized quantification and morphometrical analysis, was used to analyze the number, size, and pattern of distribution of the MHC fiber types after denervation and reinnervation of the gastrocnemius muscle.

Materials and Methods

Surgical Procedure

8-wk-old inbred male Lewis rats were anesthetized using 0.3 ml/kg of intramuscular Hypnorm (0.315 mg/ml fentanyl citrate and 10 mg/ml flunixin; Janssen Pharmaceuticals Ltd.) and 2.5 mg/kg of intraperitoneal diazepam (Phoenix Pharmaceuticals Ltd.).

Using an operating microscope (Wild Heerbrugg Ltd.), the left sciatic nerve was exposed 5 mm distal to the sciatic notch via a gluteal muscle splitting incision. A 5-mm length of nerve was removed using sharp microsurgery scissors to produce a gap of 10 mm after retraction of the nerve ends. This gap was grafted with fibronectin impregnated with 500 ng/ml NT-3 (NT) (Sterne et al., 1997); alternatively plain fibronectin mats (FN) or autologous nerve grafts (NG) were inserted as controls.

During insertion, 14-mm-long fibronectin mats were entubulated, the hydroscopic nature of the fibronectin causing them to expand, obliterating the lumen. The proximal and distal nerve stumps were drawn into either end of the entubulated mat to produce a resultant gap of 10 mm and two 10/0 monofilament nylon (Ethicon Ltd., Edinburgh, UK) interrupted sutures were used at each anastomosis. For autologous nerve grafts, a 1-cm length of sciatic nerve was removed, reversed, and reanastomosed to bridge the resulting defect. Animals received postoperative analgesia for 24 h and were kept warm until fully recovered from the anesthetic before their return to the holding room, where they had free access to food and water. On the first postoperative day, the animals were caged in groups of three.

Samples from six animals from each group were harvested at 5, 10, 15, 30, 120, and 240 d postoperatively. The animals were killed and the calf muscles approached by extending the lateral thigh incision beyond the ankle joint. Gastrocnemius muscles were removed bilaterally from each animal. Using an operating microscope, the entire gastrocnemius muscle was carefully cleaned and dissected out, dividing its two tendinous origins and tendinous insertion flush with the bone. Each muscle was then weighed separately to allow calculation of the percentage reduction in muscle mass (denervated muscle weight vs. contralateral muscle weight) after denervation and reinnervation. For the animals harvested at 240 d only, the effects of reinnervation on the gastrocnemius muscle were investigated immunohistochemically. To ensure biopsy of a constant part of the muscle, while holding the knee joint fully extended and the ankle joint flexed to 90°, the midpoint of the gastrocnemius muscle, lying half way between the knee and ankle, was identified and marked with a blue surgical marking pen. The muscles were then harvested as before.

Immunohistochemistry

After weighing, a transverse disc of the whole muscle 5 mm long was cut and carefully orientated on a small piece of cork to allow transverse sections to be made. The muscle specimen, embedded in OCT, was rapidly frozen by rapid inversion and plunging into isopentane cooled in liquid nitrogen for about 30 s.

A panel of mouse monoclonal antisera was used for immunostaining,

and it included BA-F8 antibodies raised against rat MHC 1, SC-71 antibodies raised against rat MHC 2a, and BF-F3 antibodies raised against rat MHC 2b. All primary antibodies were used at a 1:200 dilution in PBS, pH 7.4. Antibodies to the MHCs (Schiaffino et al., 1989) were supplied by Regeneron Pharmaceuticals, USA.

Serial 8- μ m transverse cryostat sections were cut from each muscle block, collected on Vectabond-coated slides (Vector, UK), and air dried for 30 min at room temperature before immunofluorescent staining. The sections were permeabilized by immersion in 0.2% Triton X-100 in PBS (Merck/BDH, UK) for one hour, rinsed three times in PBS, and incubated in 3% goat serum (TCS Biologicals, UK) in PBS to block any background binding from the secondary labeled antibody. Sections were then incubated with the primary antibodies for 24 h at 4°C in a humid chamber, washed in PBS/Tween (0.02%) to remove nonspecifically bound antibody, rinsed three times in PBS, and incubated with cyanine-3-conjugated goat anti-mouse serum (Affinity, UK, diluted 1:100 in PBS). Nonspecifically bound second layer antibodies were again removed by washing with PBS/Tween, and the sections were then rinsed in PBS and mounted with PBS/glycerol containing 2.5% (wt/vol) 1,4-diazabicyclo [2.2.2] octane (Aldrich Chemical, Gillingham, UK) as an antifading agent.

Quantification and Morphometric Analysis

All morphometric measurements were performed on coded sections without knowledge of their source. The sections were viewed using an Olympus (Lake Success, NY) microscope connected to a computerized image analysis system (Seescan Analytical Services, Cambridge, UK) appropriately calibrated for each objective magnification. Six fields (10 \times objective) were chosen at random from each muscle section providing a minimum of 100 positive fibers from each section. After image capture, background subtraction, image enhancement, thresholding, and final editing, automatic counting and measurement of the diameter of each positive fiber was performed using a dedicated calibrated software program.

An assessment of the degree of fiber type distribution was performed by placing a randomly orientated line from an eyepiece graticule across the entire muscle section, and the number of fibers in each group of positive and negative fibers along this line were recorded. 10 random linear transects were made on each muscle, counting a minimum of 900 fibers. These counts were used as a representative indication of the total number of muscle fibers in each whole muscle section and allowed to quantify the proportion, diameter, and size distribution of each fiber type in each experimental group.

Statistical Analysis

Statistical analysis was carried out using the STATA (STATA Corporation, College Station, TX) and SIGMASTAT (Jandel Scientific, Erkrath, Germany) software packages. A one-way analysis of variance (ANOVA) was used to analyze the results of the muscle masses, fiber type distributions, and diameters. The normality assumption of ANOVA was checked using Shapiro-Francia's *W'* test and Bartlett's test was used to check the equal variance assumption. If data violated the assumptions of normality and variance they were compared using a Kruskal-Wallis one-way ANOVA on ranks, and data was then expressed as the median values (25th–75th centiles). Differences between individual groups were subsequently isolated by the use of an appropriate all pair-wise multiple comparison procedure. To statistically analyze the size distribution profiles, the 5th and 95th centiles of each fiber type in normal muscle were calculated and the frequencies and percentages of muscle fibers in each group that were smaller, intermediate, or larger than these centiles were then compared using Fisher's exact test, introducing a Bonferroni adjustment to allow for the number of comparisons made.

Results

Effect of NT-3-enhanced Nerve Regeneration on Muscle Atrophy

The mass of both gastrocnemius muscles in each animal was recorded to allow calculation of the percentage atrophy (mass of denervated muscle/mass of normal contralateral muscle \times 100) in each group at each time point (Fig. 1). After denervation, initially there was a rapid loss of mus-

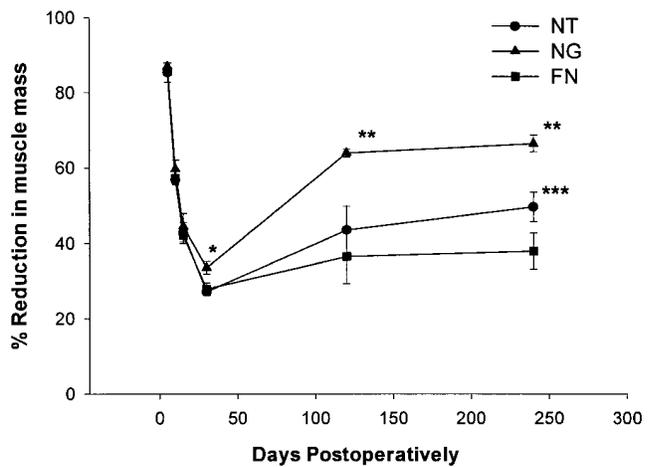


Figure 1. The percentage reduction in gastrocnemius muscle mass at various times after sciatic nerve division and repair. Each data point represents the mean of six animals. Error bars show the standard error of the mean. * $P < 0.05$ for NG vs. NT and FN, ANOVA. ** $P < 0.001$ for NG vs. NT and FN, ANOVA. *** $P < 0.05$ for NT vs. FN, ANOVA. NG, autologous nerve graft repair; NT, repair with NT-3-impregnated fibronectin mats (500 ng/ml); FN, repair with plain fibronectin mats.

cle mass equally in each group up to day 15. By day 30, the muscles in the groups repaired with fibronectin conduits continued to lose muscle mass at an equal rate; however, in the animals repaired with autologous nerve grafts, the reduction in muscle mass was significantly less ($P < 0.05$). At subsequent time points, the NT group showed steady recovery of the muscle mass, which reached 47% of the weight of contralateral normal muscle at 8 mo. The degree of recovery of muscle mass was higher after NT-3 administration than in the FN group, which reached only 39% normal muscle weight at the final time point. This difference failed to reach significance at 120 d but was significant at 240 d ($P < 0.05$). The muscle in the NG group returned to 71% of their original mass by day 120 but remained stable at this level by day 240 ($P < 0.001$ NG vs. NT and FN).

Changes in Distribution of Muscle Fiber Types after Denervation and Reinnervation

Type 1 Fibers. Fibers staining positive for MHC 1 were confined to the deeper third of the contralateral normal muscle, distributed in a mosaic pattern, and were rarely seen in the peripheral superficial two thirds of the gastrocnemius muscle. After reinnervation, the distribution of these groups was much more scattered throughout the muscle: although MHC 1 staining fibers predominated in the deeper parts of the muscle, there were considerable numbers of groups in more superficial regions. There was no obvious difference between experimental groups in the redistribution patterns of type 1 fibers after reinnervation.

Type 2a Fibers. In normal muscles, MHC 2a staining fibers had a similar distribution to that of MHC 1 fibers, namely a mosaic distribution within the deep third of the muscle with few fibers in the superficial two thirds. After reinnervation, this pattern was disrupted with groups present throughout the muscle but with a slight prepon-

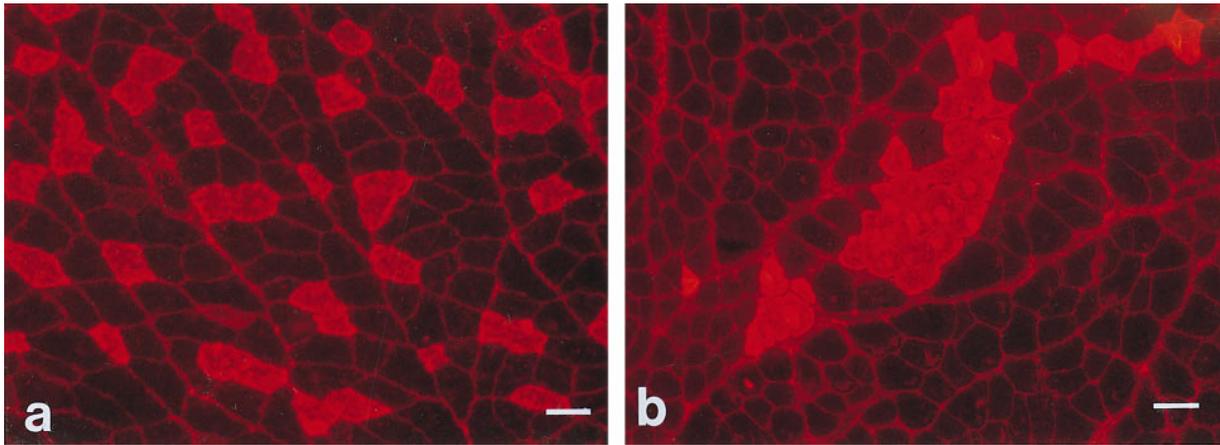


Figure 2. (a) Transverse immunofluorescent sections of normal gastrocnemius muscle stained for MHC 2b, demonstrating the typical size and mosaic pattern of distribution. (b) 8 mo after repair of a 1-cm-long sciatic nerve defect using a plain fibronectin mat, the MHC 2b immunoreactive fibers are generally smaller, and there is a loss of the mosaic pattern of fiber type distribution. Bar, 50 μ m.

derance for the deeper parts remaining; again, no obvious intergroup differences were noted.

Type 2b Fibers. Staining for MHC 2b shows a fiber distribution mainly in the superficial two thirds of the gastrocnemius muscle and to a lesser extent in the deep muscle regions. The usual mosaic distribution pattern was evident in all areas of the muscle (Fig. 2 a). Reinnervation resulted in significant disruption of the mosaic pattern (Fig. 2 b), but the general preponderance of MHC 2b stained fibers within the superficial two thirds of the muscle persisted; no difference was evident between experimental groups.

Preferential Effect of NT-3-mediated Reinnervation on MHC Fiber Type

From 10 transects across the entire section of each muscle, a minimum of 900 fibers were counted, allowing a comparison of the estimated number of muscle fibers (Table I) and of the percentage distribution of each fiber type in each group (Table II). From these counts it was evident that there was no statistical difference between mean fiber counts from different groups. An interesting difference in fiber proportions was noted for type 2b fibers (Table II). After reinnervation, the percentage of type 2b fibers in the NT group was similar to that in normal muscle and significantly greater than in either of the NG or FN groups ($P < 0.05$). In contrast, the type 2b proportion fell significantly in the NG and FN groups compared with normal muscle ($P < 0.01$).

The proportion of type 1 muscle fibers fell significantly from normal levels following denervation and reinnervation ($P < 0.02$), with no differences between experimental groups in the reinnervated muscles. The proportion of

type 2a muscle fibers in all experimental groups rose significantly compared with normal after reinnervation ($P < 0.01$), without differences between the three groups.

NT-3-dependent Changes of Muscle Fiber Morphometry

Consistent with the results of percentage distribution of fiber types, morphometric analysis of muscle fiber diameters showed that type 2b fibers regained normal average size after NT-3-mediated reinnervation (Table III). Conversely, in the two control groups the diameter of type 2b fibers was significantly smaller than in muscle of normal and NT groups ($P < 0.01$). The diameters of type 2a fibers in reinnervated muscles of all groups were significantly smaller than in normal muscles ($P < 0.01$). This was also the case for type 1 fibers ($P < 0.01$), with the exception of the NG group, where they recovered normal size.

Analysis of distribution profiles of individual muscle fiber diameters (Fig. 3, a-c) showed that the distribution profile of type 2b fibers for the reinnervated NT group closely resembled that seen in control muscle ($P = 1$, NT vs. control). The distribution profiles for the NG and FN groups contained more small fibers and fewer large fibers, with a pattern different from both control and NT groups. The difference was particularly evident after reinnervation without NT-3 administration (FN group), where there was

Table II. The Percentage Distribution of Fiber Types in Each Group ($n = 6$)

	Percentage \pm SEM			
	Normal	NG	NT	FN
MHC 1	21.4 \pm 3.2	13.3 \pm 1.9**	12.3 \pm 1.7**	11.9 \pm 1.5**
MHC 2a	15.8 \pm 0.6	21.8 \pm 1.7***	25.9 \pm 1.1***	29.8 \pm 1.7***
MHC 2b	44.3 \pm 3.5	33.8 \pm 0.9***	43.3 \pm 1.3*	35.7 \pm 1.1***

$P < 0.05$, NT vs. NG and FN (MHC 2b); ** $P < 0.02$, normal vs. reinnervated muscle (MHC 1); *** $P < 0.01$, normal vs. reinnervated muscles (MHC 2a and 2b). ANOVA, Dunns method. NG, autologous nerve graft repair; NT, repair with NT-3-impregnated fibronectin mats; FN, repair with plain fibronectin mats. In all experimental groups, there is a percentage of undefined fibers not represented in the table. These fibers are derived from the inability to label specific MHC IIx/d.

Table I. The Mean Number of Fibers Counted in 10 Transects of the Whole Muscle in Each Group

	Normal	NG	NT	FN
Mean fiber count \pm SEM	1,353 \pm 66	1,347 \pm 99	1,179 \pm 42	1,185 \pm 87

NG, autologous nerve graft repair; NT, repair with NT-3-impregnated fibronectin mats; FN, repair with plain fibronectin mats.

Table III. Diameters of the Different Fiber Types in Each Group

	Diameters			
	Normal	NG	NT	FN
	μm			
MHC 1	67.8 (60.9–76.8)	65 (50.4–77.5)	54.7* (34.2–73.9)	58.4* (49.7–65.4)
MHC 2a	64.8 (52.9–75)	53.2* (45.8–59.8)	54.5* (47.6–63.9)	49.5* (43.7–57.6)
MHC 2b	86.7 (77.8–97.3)	81* (71.3–92.8)	87.8 (80.7–98.3)	70.8* (61.2–82.7)

Measurements are given in microns as the median values (25th–75th centiles) of each group ($n = 6$). * $P < 0.01$ for normal vs. reinnervated muscles, ANOVA on ranks, Dunns method. NG, autologous nerve graft repair; NT, repair with NT-3-impregnated fibronectin mats; FN, repair with plain fibronectin mats.

a statistically significant difference from both control and NT groups ($P < 0.005$).

Reinnervated muscle in all experimental groups demonstrated a shift toward small type 1 fibers compared with normal muscle, though a few large fibers were still present. Instead, type 2a fibers showed a shift toward intermediate size fibers, with fewer large and small diameter fibers than normal muscle. For both type 1 and 2a fibers, statistical analysis confirmed a significant shift toward smaller sizes ($P < 0.005$ for each group vs. normal).

Discussion

Here we present evidence that NT-3 improves motor target organ reinnervation in terms of recovery of both normal proportions of type 2b muscle fibers and their return to normal diameter. These results are consistent with our previous report that the local delivery of NT-3 to injured sciatic nerves in rats enhances both the rate and amount of axonal regeneration (Sterne et al., 1997).

After denervation, the gastrocnemius muscle was found to lose weight rapidly, falling to $\sim 30\%$ of its original weight by day 30, consistent with similar results reported previously (Sunderland, 1978). At day 30, muscles in the NG group were significantly less atrophic than in both fibronectin groups. It is likely from the results of the regeneration rate in the NG group (Sterne, G.D., unpublished results) that by day 30 the muscle was already becoming reinnervated. By day 120, the recovery of muscle mass seen after NT-3 administration was improved compared with control FN group, although it was still less than after nerve grafts. The muscle recovery of the NT-3 group was maintained and actually improved with statistical significance by 8 mo. This correlates well with our previously reported myelinated fiber counts distal to the nerve repair in which NT-3-impregnated mats supported a significant improvement in myelinated fibers at 8 mo after repair

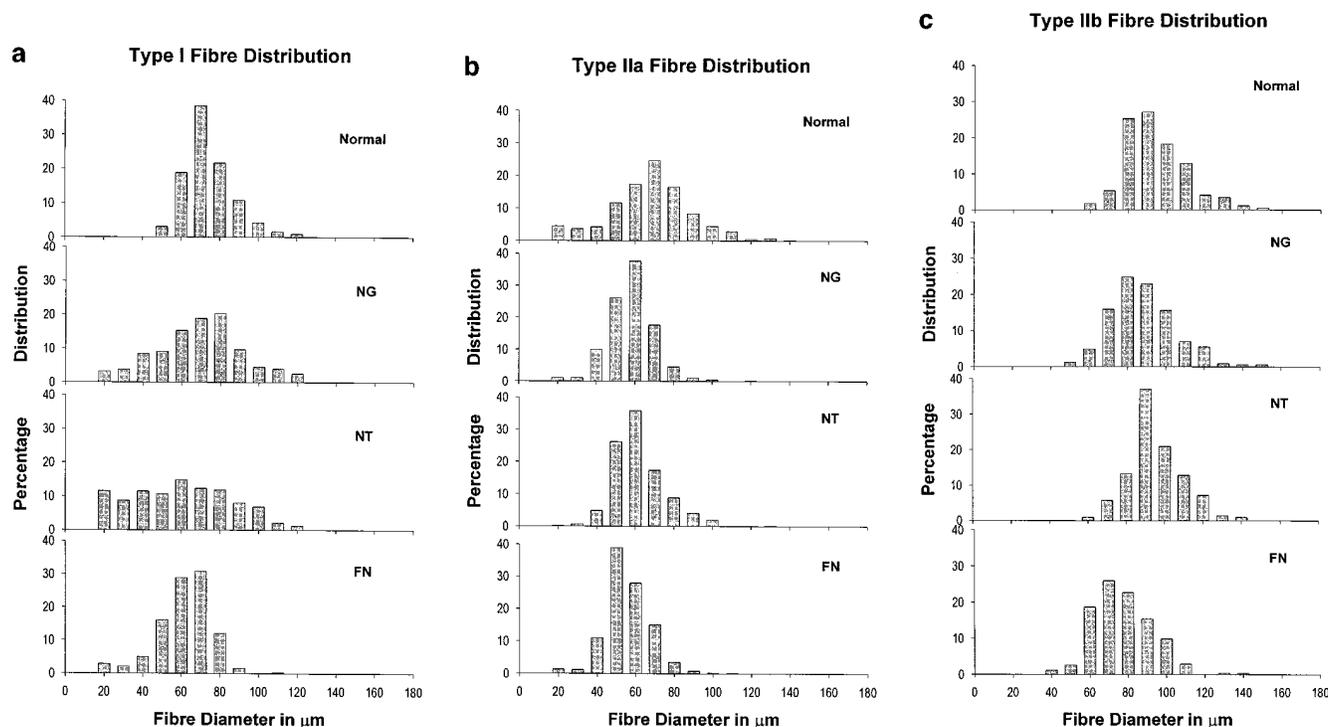


Figure 3. (a) The size distribution of type I muscle fibers in each group. Note how after regeneration there is a general reduction in fiber diameter, with each of the reinnervated groups being significantly different from normal ($P < 0.005$; Fisher's exact test, Bonferroni adjustment). However, some muscle fibers in each group regain normal diameter. (b) Size distribution profiles of type IIa muscle fibers by group. There is a significant loss of large fibers in all groups but significantly fewer small diameter muscle fibers ($P < 0.005$; Fisher's exact test, Bonferroni adjustment). Overall, there is a tendency toward slightly smaller muscle fiber diameters in the reinnervated muscle compared with normal fibers. (c) Size distribution profiles of type IIb muscle fibers by group. The distribution profiles of the reinnervated NT group resembled the profile seen in normal muscle ($P = 1$ for NT vs. normal; Fisher's exact test, Bonferroni adjustment), but the profile for muscle fibers in the FN group was significantly different from both normal and the NT group ($P < 0.005$; Fisher's exact test, Bonferroni adjustment). Normal, normal gastrocnemius muscle; NG, autologous nerve graft repair; NT, repair with NT-3-impregnated fibronectin mats (500 ng/ml); FN, repair with plain fibronectin mats.

(Sterne et al., 1997). By comparison, the muscles in the NG group had regained some 70% of their original mass at 4 mo. However, the benefit did not progress, remaining unchanged up to 8 mo postoperatively. This level of recovery is similar to that reported after nerve transection and muscle reinnervation (Gillespie et al., 1987; Bertelli et al., 1995), though nerve crush injury, with the preservation of Schwann cell basal lamina tubes, results in greater muscle recovery (Bertelli et al., 1995).

The mechanism by which NT-3 exerts its effect on muscle is uncertain. NT-3 may act directly on motoneurons, for example by enhancing survival (Eriksson et al., 1994; Lefebvre et al., 1994) or stimulating axonal branching (Schnell et al., 1994; Isaacson et al., 1996). This might explain the increased number of myelinated axons previously observed in NT-3-treated animals (Sterne et al., 1997), which in turn may lead to reinnervation of greater numbers of muscle fibers. Alternatively, NT-3 may be acting via muscle afferent innervation. Recent studies have shown that in transgenic mice, NT-3 overexpression in muscle influences the survival of proprioceptive sensory neurons (Ozaki et al., 1996; Henderson et al., 1996) and increases the number of muscle spindles in gastrocnemius muscle without an overall increase in motoneuron numbers (Wright et al., 1996). Consistently, deletion of NT-3 during development results in a decreased survival of muscle sensory afferents during development (Ernfors et al., 1994; Kucera et al., 1995a,b; Oakley et al., 1995). It may be possible that after nerve injury, the survival or enhanced regeneration effect of NT-3 on this subpopulation of neurons leads to a greater reinnervation of muscle spindles and Golgi tendon organs, consequently resulting in improved sensory feedback from the muscle. This in turn would result in greater use of the muscle and hence lead to improvement in muscle mass.

Our data show that there are specific and highly significant effects of both nerve graft and NT-3 treatment upon type 1 and 2b muscle fibers, respectively. Nerve graft, by the nature of it, offers the regenerating nerve an almost ideal environment because of the presence of activated Schwann cells and a scaffolding of basal lamina to facilitate the progression of regenerating axons. However, treatment by nerve graft normalized type 1 fiber size but did not bring their proportion back to normal, while NT-3 treatment normalized both size and proportion. It should be remembered that we were unable to analyze MHCx/d, and therefore we cannot say if the increase in muscle mass seen in the NG group was also the result of the presence of large and numerous IIX/d fibers.

After reinnervation, morphometric analysis showed that the diameter distribution of type 2b fibers showed a pattern similar to normal only in the NT group. Furthermore, the general trend for the other fiber types in all experimental groups showed a shift to smaller diameter sizes. Analysis of the proportion of different muscle fiber types in each group at 8 mo postoperatively revealed a generalized reduction of type 1 fibers and a significant increase of type 2a fibers. By contrast, the proportion of type 2b muscle fibers was restored to normal only in the NT group. This result is consistent with the enhanced diameters of type 2b fibers in NT-3-treated animals, which uniquely return to the same size as in normal muscle. This result is strongly indicative that NT-3 applied to the cut nerve may

influence the regeneration of a physiologically distinct subset of motor neurons supplying type 2b muscle fibers. While functional assessment of NT-3-enhanced reinnervated muscles has yet to be performed, it is known that for reinnervated muscle, as in normal muscle, force generated is proportional to cross-sectional area of muscle fibers (Schantz et al., 1983; Davis et al., 1988; Totosty de Zepetnek et al., 1992) and increase in diameter and proportion of 2b fibers seen in the NT group is likely to result in increased force generation.

In conclusion, we have demonstrated that NT-3 enhancement of peripheral nerve regeneration does have a beneficial biochemical and morphological effect on the reinnervation of motor target organs, supporting the hypothesis of a specific phenotypic effect of growth factor-enhanced nerve regeneration. This is the first indication of a differential effect of NT-3 on neuromuscular interaction in normal adult animals in vivo. It seems doubtful that muscle reinnervation alone may be able to provoke a selective response from the muscle fibers, and it is likely that a more complex signaling system, possibly involving neurotrophic factors, may be involved. This still awaits clarification, but these results also suggest the remarkable possibility that other subpopulations of motoneurons, as classified by the phenotype they confer on muscle fibers, may be differentially responsive to other neurotrophic factors, and this leads us to believe that single or combined application of trophic factors might prove of future therapeutic value.

The authors are grateful to The Geoffrey Burton Trust, Smith's Charity, and Glaxo Holdings for financial support.

Received for publication 17 April 1997 and in revised form 14 August 1997.

References

- Airaksinen, M.S., M. Koltzenburg, G.R. Lewin, Y. Masu, C. Helbig, E. Wolf, G. Brem, K.V. Toyka, H. Thoenen, and M. Meyer. 1996. Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron*. 16:287-295.
- Averbuch-Heller, L., M. Pruginin, N. Kahane, T. Pantelis, L. Parada, A. Rosenthal, and C. Kalcheim. 1994. Neurotrophin 3 stimulates the differentiation of motoneurons from avian neural tube progenitor cells. *Proc. Natl. Acad. Sci. USA*. 91:3247-3251.
- Bar, A., and D. Pette. 1988. Three fast myosin heavy chains in adult rat skeletal muscle. *FEBS Lett.* 235:153-155.
- Bertelli, J.A., and J.C. Mira. 1995. The grasping test: a simple behavioral method for objective quantitative assessment of peripheral nerve regeneration in the rat. *J. Neurosci. Methods*. 58:151-155.
- Braun, S., B. Croizat, M.C. Lagrange, J.M. Warter, and P. Poindron. 1996. Neurotrophins increase motoneurons ability to innervate skeletal muscle fibers in rat spinal cord-human muscle cocultures. *J. Neurol. Sci.* 136:17-23.
- Burke, R.E., D.E. Levine, F.E. Zajac, P. Tsairis, and W.K. Engel. 1971. Mammalian motor units: physiological histochemical correlation in three types in cat gastrocnemius. *Science (Wash. DC)*. 174:709-712.
- Davis, H.L., B.H. Bressler, and L.G. Jasch. 1988. Myotrophic effects on denervated fast-twitch muscles of mice: correlation of physiologic, biochemical, and morphologic findings. *Exp. Neurol.* 99:474-489.
- DiStefano, P.S., B. Friedman, C. Radziejewski, C. Alexander, P. Boland, C.M. Schick, R.M. Lindsay, and S.J. Wiegand. 1992. The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron*. 8:983-993.
- Eriksson, N.P., R.M. Lindsay, and H. Aldskogius. 1994. BDNF and NT-3 rescue sensory but not motoneurons following axotomy in the neonate. *Neuroreport*. 5:1445-1448.
- Ernfors, P., K.F. Lee, J. Kucera, and R. Jaenisch. 1994. Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell*. 77:503-512.
- Fex, S., and B. Sonneson. 1970. Histochemical observations after implantation of a 'fast' nerve into an innervated mammalian 'slow' skeletal muscle. *Acta Anat.* 77:1-5.
- Fields, R.D., and M.H. Ellisman. 1986. Axons regenerating through silicone tube splines 2. Functional morphology. *Exp. Neurol.* 92:61-74.

- Gillespie, M.J., T. Gordon, and P.R. Murphy. 1987. Motor units and histochemistry in rat lateral gastrocnemius and soleus muscles: evidence for dissociation of physiological and histochemical properties after reinnervation. *J. Neurophysiol.* 57:921–937.
- Goldspink, G., A. Scutt, P.T. Loughna, D.J. Wells, T. Jaenicke, and G.F. Gerlach. 1992. Gene expression in skeletal muscle in response to stretch and force generation. *Am. J. Physiol.* 262:R356–363.
- Green, H.J., H. Reichmann, and D. Pette. 1983. Fibre type specific transformations in the enzyme activity pattern of rat vastus lateralis muscle by prolonged endurance training. *Pflüg. Arch. Eur. J. Physiol.* 399:216–222.
- Griesbeck, O., A.S. Parsadanian, M. Sendtner, and H. Thoenen. 1995. Expression of neurotrophins in skeletal muscle: quantitative comparison and significance for motoneuron survival and maintenance of function. *J. Neurosci. Res.* 42:21–33.
- Henderson, C.E., W. Camu, C. Mettling, A. Gouin, K. Poulsen, M. Karihaloo, J. Rullamas, T. Evans, S.B. McMahon, M.P. Armanini, et al. 1993. Neurotrophins promote motor neuron survival and are present in embryonic limb buds. *Nature (Lond.)* 363:266–270.
- Henderson, T.A., D.E. Wright, W.D. Snider, and M.F. Jacquin. 1996. Effects of NT-3 overexpression in muscle on the trigeminal mesencephalic nucleus. *Soc. Neurosci.* 22:999.
- Hughes, R.A., M. Sendtner, and H. Thoenen. 1993. Members of several gene families influence survival of rat motoneurons in vitro and in vivo. *J. Neurosci. Res.* 36:663–671.
- Isaacson, L.G., B.N. Saffran, and K.A. Crutcher. 1996. Nerve growth factor-induced sprouting of mature uninjured sympathetic axons. *J. Comp. Neurol.* 326:327–336.
- Izumo, S., B. Nadal-Ginard, and B. Mahdavi. 1986. All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science (Wash. DC)* 231:597–600.
- Jansson, E., B. Sjödin, and P. Tesch. 1978. Changes in muscle fibre type distribution in man after physical training. A sign of fibre type transformation? *Acta Physiol. Scand.* 104:235–237.
- Karpati, G., and W.K. Engel. 1968. 'Type grouping' in skeletal muscles after experimental reinnervation. *Neurology.* 18:447–449.
- Kato, A.C., and R.M. Lindsay. 1994. Overlapping and additive effects of neurotrophins and CNTF on cultured human spinal cord neurons. *Exp. Neurol.* 130:196–201.
- Kucera, J., P. Ernfors, J. Walro, and R. Jaenisch. 1995a. Reduction in the number of spinal motor neurons in neurotrophin-3-deficient mice. *Neuroscience.* 69:321–330.
- Kucera, J., G.P. Fan, R. Jaenisch, S. Linnarsson, and P. Ernfors. 1995b. Dependence of developing group Ia afferents on neurotrophin-3. *J. Comp. Neurol.* 363:307–320.
- Lefebvre, P.P., B. Malgrange, H. Staeker, M. Moghadass, T.R. Van de Water, and G. Moonen. 1994. Neurotrophins affect survival and neuritegenesis by adult injured auditory neurons in vitro. *Neuroreport.* 5:865–858.
- Lewin, G.R., and Y.A. Barde. 1996. Physiology of the neurotrophins. *Annu. Rev. Neurosci.* 19: 289–317.
- Li, L., R.W. Oppenheim, M. Lei, and L.J. Houenou. 1994. Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. *J. Neurobiol.* 25:759–766.
- Lindsay, R.M. 1996. Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons: An overview. *Philos. Trans. R. Soc. Lond. Biol. Sci.* 351:365–73.
- Lohof, A.M., N.Y. Ip, and M. Poo. 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature (Lond.)* 363:350–353.
- Maltin, C.A., M.I. Delday, G.S. Baillie, D.A. Grubb, and P.J. Garlick. 1989. Fibre type composition of nine rat muscles I. Changes in the first year of life. *Am. J. Physiol.* 257:E823–827.
- Oakley, R.A., A.S. Garner, T.H. Large, and E. Frank. 1995. Muscle sensory neurons require neurotrophin-3 from peripheral tissues during the period of normal cell death. *Development (Camb.)* 121:1341–1350.
- Ozaki, S., D.E. Wright, L. Zhou, and W.D. Snider. 1996. Influences of NT-3 on proprioceptive axon projections to spinal motoneuron pools. *Soc. Neurosci.* 22:1000.
- Pellegrino, C., and C. Franzini. 1963. An electron microscope study of denervation atrophy in red and white skeletal muscle fibers. *J. Cell Biol.* 17:327–332.
- Peter, J.B., R.J. Barnard, V.R. Edgerton, C.A. Gillespie, and K.E. Stemper. 1972. Metabolic profiles of three fibre types of skeletal muscle in guinea pigs and rabbits. *Biochemistry.* 11:2627–2633.
- Pette, D., and G. Vrbova. 1992. Adaptation of mammalian skeletal muscle fibres to chronic electrical stimulation. *Rev. Physiol. Biochem. Pharmacol.* 120:115–120.
- Romanul, F.C.A., and J.P. Van der Meulen. 1966. Reversal of the enzyme profiles in fast and slow muscles by cross innervation. *Nature (Lond.)* 212:1369–1371.
- Salmons, S., and F.A. Sreter. 1975. The role of impulse activity in the transformation of skeletal muscle by cross innervation. *J. Anat.* 120:412–415.
- Schantz, P., E. Randall-Fox, W. Hutchison, A. Tyden, and P.O. Astrand. 1983. Muscle fibre type distribution, muscle cross-sectional area and maximal voluntary strength in humans. *Acta Physiol. Scand.* 117:219–226.
- Schiaffino, S., L. Gorza, S. Sartore, L. Saggini, S. Ausoni, M. Vianello, K. Gundersen, and T. Lomo. 1989. Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J. Muscle. Res. Cell Motil.* 10:197–205.
- Schnell, L., R. Schneider, R. Kolbeck, Y. Barde, and M.E. Schwab. 1994. Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature (Lond.)* 367:170–173.
- Sterne, G.D., R.A. Brown, C.J. Green, and G. Terenghi. 1997. Neurotrophin-3 delivered locally via fibronectin mats enhances peripheral nerve regeneration. *Eur. J. Neurosci.* 9:1388–1396.
- Sunderland, S. 1978. Morphological changes in striated muscle due to denervation. Atrophy. In *Nerves and Nerve Injuries*. 2nd ed. Churchill Livingstone, Edinburgh, Scotland. 229–245.
- Tessarollo, L., K.S. Vogel, M.E. Palko, S.W. Reid, and L.F. Parada. 1994. Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proc. Natl. Acad. Sci. USA.* 91:11844–11848.
- Totosy de Zepetnek, J.E., H.V. Zung, S. Erdebil, and T. Gordon. 1992. Innervation ratio is an important determinant of force in normal and reinnervated rat tibialis anterior muscles. *J. Neurophysiol.* 67:1385–1403.
- Vejsada, R., Y. Sagot, and A.C. Kato. 1995. Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomized motoneurons in vivo. *Eur. J. Neurosci.* 7:108–115.
- Wang, T., K. Xie, and B. Lu. 1995. Neurotrophins promote maturation of developing neuromuscular synapses. *J. Neurosci.* 15:4796–4805.
- Wong, V., R. Arriaga, N.Y. Ip, and R.M. Lindsay. 1993. The neurotrophins BDNF, NT-3 and NT-4/5, but not NGF, up-regulate the cholinergic phenotype of developing motor neurons. *Eur. J. Neurosci.* 5:466–474.
- Wright, D.E., L. Zhou, J. Harding, and W.D. Snider. 1996. Overexpression of NT-3 in muscle stimulates the formation of supernumerary muscle spindles. *Soc. Neurosci.* 22:512.