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10. For this analysis, SSU rDNA sequences were obtained from nine fungi, including four Ascomycetes [*Arthonia radiata* (GenBank accession number U23537), *Dendrographa leucophaea* (U23538), *Lecanactis abietina* (U23539), and *Schismatomma pericleum* (U23540)] and five Basidiomycetes [*Agaricus bisporus* (U23724), *Dictyonema pavonia* (U23541), *Multiclavula mucida* (U23542), *Omphalina umbellifera* (U23543), and *Pleurotus ostreatus* (U23544)]. DNA was isolated and SSU rDNA was amplified by the polymerase chain reaction (PCR) from fungus-specific oligonucleotide primers as described in (*9*). Double-stranded PCR products were sequenced as described (*9*) or by the PRIZM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with detection on a 373A automatic sequencing apparatus (Applied Biosystems). Sequence fragments were assembled manually. These DNA sequences were aligned to 8 SSU rDNA sequences from the first author's previous studies—*Lecanora dispersa* (L37535), *Leotia lubrica* (L37536), *Morchella elata* (L37537), *Mycocalicium albonigrum* (L37538), *Peziza badia* (L37539), *Porpidia crustulata* (L37540), *Sclerotinia sclerotiorum* (L37541), and *Sphaerophorus globosus* (L37532)—and to 58 sequences available from GenBank: *Alternaria alternata* (U05194), *Athelia bombacina* (M55638), *Auricularia auricula* (L22254), *Auricularia polytricha* (L22255), *Boletus satanas* (M94337), *Bullera alba* (X60179), *Calocera cornea* (L22256), *Candida albicans* (X53497), *Chaetomium elatum* (M83257), *Coprinus cinereus* (M92991), *Cronartium ribicola* (M94338), *Cudonia confusa* (Z30240), *Dacrymyces chrysospermus* (L22257), *Dacrymyces stillatus* (L22258), *Endogone pisiformis* (X58724), *Eremascus albus* (M83258), *Eurotium rubrum* (U00970), *Filobasidiella neoformans* (L05427), *Gigaspora albida* (Z14009), *Gyromitra esculenta* (Z30238), *Heterotextus alpinus* (L22259), *Histoplasma capsulatum* (X58572, S45469), *Inermisia aggregata* (Z30241), *Kluyveromyces lactis* (X51830), *Leptosphaeria bicolor* (U04202), *Leucosporidium scottii* (X53499), *Leucostoma persoonii* (M83259), *Malbranchea gypsea* (L28066), *Neolecta vitellina* (Z27393), *Neurospora crassa* (X04971), *Ophiostoma ulmi* (M83258), *Peridermium harknessii* (M94339), *Plectania nigrella* (Z27408), *Pleospora herbarum* (U05201), *Pleospora rudis* (U00975), *Pneumocystis carinii* (X12707), *Podospora anserina* (X54864), *Protomyces inouyei* (D11377), *Pseudohydnum gelatinosum* (L22260), *Rhodosporidium toruloides* (X60180), *Saccharomyces cerevisiae* (J01353, M27607), *Schizophyllum commune* (X54865), *Schizosaccharomyces pombe* (X54866), *Septoria nodorum* (U04236), *Spathularia flavida* (Z30239), *Spongipellis unicolor* (M59760), *Sporidiobolous johnsonii* (L22261), *Sporobolomyces roseus* (X60181), *Taphrina wiesneri* (D12531, D01175), *Thanatephorus cucumeris* (M92990), *Thermoascus crustaceus* (M83263), *Tilletia caries* (U00972), *Tremella foliacea* (L22262), *Trichosporon cutaneum* (X60182), *Ustilago hordei* (U00973), *Xerocomus chrysenteron* (M94340), *Zoophthora culistaea* (D29949), and *Zygosaccharomyces rouxii* (X58057). The 75 sequences were aligned with the program PileUp (Program Manual for the Wisconsin Package, Version 8, September 1994; Genetics Computer Group, Madison, WI); gaps were reduced by manual adjustment. An alignment of 1927 nucleotides from 75 taxa was used for parsimony analysis with the program PAUP 3.1 (*20*). No characters were excluded, invariant characters were ignored, all characters were equally weighted, and branch lengths equal to 0 were collapsed to polytomies. The analysis produced unrooted networks that were rooted with three zygomycetous fungi as outgroups. Two equally parsimonious trees were produced, with tree lengths of 3491, consistency indices of 0.3893, and retention indices of 0.6941; the trees differed only in sister taxa relations within one clade of three fungi (Fig. 1). Bootstrap percentages (*21*) to assess support for each branch were determined for 200 resamplings of the data set. Bootstrap values of >80% provided strong support for the monophyly of groups such as Dicaryomycotina, Ascomycetes, and Basidiomycetes.
11. This phylogenetic hypothesis supports the monophyly of most groups recognized in traditional classifications, and the addition of taxa may resolve finer relations. For example, in Basidiomycetes, Auriculariales (represented by *Auricularia auricula*, *A. polytricha*, and *Pseudohydnum gelatinosum*) is recognized as monophyletic, yet its placement within the Homobasidiomycetes clade (defined by *Multiclavula mucida*, *Thanatephorus cucumeris*, *Spongipellis unicolor*, and *Athelia bombacina*) is problematic. In Ascomycetes, only the order Pezizales (represented by *Peziza badia*, *Gyromitra esculenta*, *Morchella elata*, *Inermisia aggregata*, and *Plectania nigrella*), here recognized as paraphyletic, differs from the first author's previous molecular phylogenies (*9*).
12. In Ascomycetes, the hypothesis of two independent gains of the lichen symbiosis is more parsimonious than that of a single origin with three subsequent losses. In Basidiomycetes, the hypothesis of three independent gains of the lichen symbiosis is more parsimonious, because a single origin would require one gain and eight losses. Although gain and loss can be variably weighted so that the loss of the lichen symbiosis is easier than its gain, this strategy ignores the necessary replacement of the lost lichen symbiosis with a gained saprobic, symbiotic, or parasitic habit.
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14. We predict other independent gains of the lichen symbiosis in Ascomycetes lineages not represented in this analysis, especially the orders Dothideales, Graphidales, Ostropales, and Pyrenulales, which include taxa that are saprobes, parasites, and lichen symbionts. Finer resolution may detect lichen loss and gain within each of these orders as well as within Arthoniales and Lecanorales.
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23. We thank V. Wirth, C. Scheidegger, and K. Rasbach for color photographs; the Laboratory of Molecular Systematics of the Smithsonian Institution for DNA sequencing facilities; D. L. Swofford and P. O. Lewis for support of phylogenetic analyses; and D. J. Futuyma, D. L. Hawksworth, F. Oberwinkler, R. H. Petersen, A. Y. Rossman, M. L. Sogin, A. R. Hornbuckle, and three anonymous reviewers for manuscript comments. A.G. and P.T.D. thank M. A. Faust, V. A. Funk, M. D. Kane, W. J. Kress, H. E. Robinson, and especially J. W. Taylor for support and encouragement. Supported by a Smithsonian Institution postdoctoral fellowship (A.G.), a Smithsonian Institution Scholarly Studies grant and a National Museum of Natural History Research Initiatives award (P.T.D. and A.G.), a Smithsonian Institution Visiting Scientist fellowship and Fonds zur Förderung der wissenschaftlichen Forschung (M.G.), and the Swedish National Research Council (A.T.).

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# Muscle-Derived Neurotrophin-4 as an Activity-Dependent Trophic Signal for Adult Motor Neurons

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**The production of neurotrophin-4 (NT-4) in rat skeletal muscle was found to depend on muscle activity. The amounts of NT-4 messenger RNA present decreased after blockade of neuromuscular transmission with α-bungarotoxin and increased during postnatal development and after electrical stimulation in a dose-dependent manner. NT-4 immunoreactivity was detected in slow, type I muscle fibers. Intramuscular administration of NT-4 induced sprouting of intact adult motor nerves. Thus, muscle-derived NT-4 acted as an activity-dependent neurotrophic signal for growth and remodeling of adult motor neuron innervation. NT-4 may thus be partly responsible for the effects of exercise and electrical stimulation on neuromuscular performance.**

Intact adult motor neurons grow nerve processes or sprouts when muscles are partially denervated or in response to blockage of transmitter release and paralysis (*1*, *2*). Candidate muscle-derived signaling factors whose expression is up-regulated by muscle inactivation include insulin-like growth factors (IGFs) (*2*) and some neurotrophic factors of the neurotrophin family (*3*, *4*). However, motor nerve sprouting also occurs in normal vertebrate muscle under physiological conditions (*5*). In the rat, qualitative changes in nerve terminal structure occur particularly during the first half year of life, when nerve terminal branches become organized in distinct groups with increased terminal length (*6*, *7*). Muscle activity can directly influence the formation and maintenance of synaptic sites (*5*, *8*, *9*); in particular, exercise training has been shown to influence neuromuscular junction morphology and to induce axonal sprouting

(*10, 11*). A muscle-derived retrograde factor would be a likely candidate to mediate remodeling and activity-induced changes in neuromuscular connections (*8*).

The neurotrophins are a family of structurally and functionally related polypeptides that control the differentiation, survival, and maintenance of vertebrate neurons (*12*). The neurotrophin family consists of four proteins—nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4—that share approximately 50% amino acid sequence identity. Neurotrophins and their receptors are expressed in muscle and motor neurons, respectively (*3, 4, 13–15*), and some members of this family promote survival of embryonic motor neurons in culture and in vivo after nerve transection (*4, 14, 16, 17*). Neurotrophin mRNAs are maximally expressed early in embryonic skeletal muscle development, with amounts decreasing at later times (*15*), which is consistent with a target-derived trophic role for developing spinal cord motor neurons. Although innervation of skeletal muscle in the rat occurs during the last week of embryonic development, neuromuscular connections do not achieve their mature state until the second or third postnatal week (*18*). Thus, we examined the pattern of neurotrophin mRNA expression during postnatal development of rat gastrocnemius muscle by ribonuclase protection analysis (RPA) (*19*). NGF mRNA was expressed in very small amounts throughout postnatal muscle development without appreciable change (*20*). BDNF and NT-3 mRNA were maximal during the first 1 to 3 weeks of postnatal development (17 and 350 fg per microgram of total RNA, respectively) but decreased thereafter (Fig. 1, A and B). Levels of BDNF and NT-3 mRNA in adult muscle were six to seven times lower than in early postnatal development (3 and 50 fg per microgram of total RNA, respectively). In contrast, expression of NT-4 mRNA increased progressively after birth and during the first 5 weeks (approximately 10-fold), reaching maximal amounts in adult muscle (20 fg per microgram of total RNA) (Fig. 1, A and B). Thus, unlike the other neurotrophins, the temporal pattern of NT-4 mRNA expression during postnatal development of skeletal muscle correlated with growth and functional maturation of neuromuscular connections.

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Regulation of NT-4 mRNA in skeletal muscle differs from that of other neurotrophins. The amount of NT-4 mRNA in gastrocnemius muscle decreases markedly 24 hours after sciatic nerve transection, whereas those of other neurotrophin mRNAs increase (BDNF) or do not change (NGF and NT-3) (*3*). We hypothesized that expression of NT-4 mRNA in skeletal muscle may be dependent on neuronal stimulation. Thus, we analyzed the amount of NT-4 mRNA in soleus muscle after local blockade of neuromuscular transmission with α-bungarotoxin (BTX), a competitive antagonist of the neuromuscular neurotransmitter acetylcholine (*21*). Maximal muscle paralysis (80%) was seen 12 hours after BTX application (Fig. 1C). Activity recovered progressively thereafter, with 40% paralysis remaining 48 hours after the beginning of the treatment (Fig. 1C). The levels of NT-4 mRNA were significantly reduced 33 hours after BTX application, and at 48 hours, NT-4 mRNA levels were decreased by more than 20-fold as compared with the control (Fig. 1D). Thus, as previously reported after denervation (*3*), neuromuscular transmission blockade down-regulated NT-4 mRNA expression 24 hours after the onset of muscle paralysis, which indicates that NT-4 expression in skeletal muscle is controlled by the postsynaptic action of acetylcholine.

To investigate the effects of increased activity on the expression of NT-4 mRNA, we analyzed the levels of different neurotrophin mRNAs in soleus and gastrocnemius muscles after electrical stimulation of the sciatic nerve (*22*). An increase in expression of NT-4 mRNA in soleus and gastrocnemius muscles was already detected 3 hours after a 1-hour electrical stimulation of the sciatic nerve (Fig. 2, A and B). Expression of NT-4 mRNA reached maximal levels (a five- to sevenfold increase as compared with the control) 12 hours after stimulation and decreased thereafter, reaching control levels 48 hours after treatment (Fig. 2, A and B). A greater increase was observed in soleus than in gastrocnemius muscle (Fig. 2A). A small increase in NT-4 mRNA expression was also observed in muscle on the nonstimulated side (Fig. 2, A

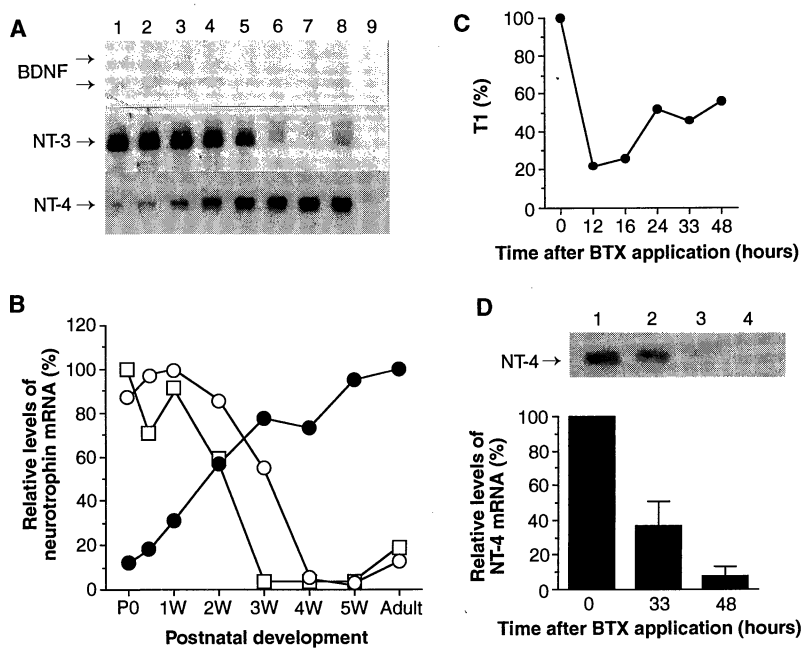


**Fig. 1.** (A) and (B) show different patterns of expression of neurotrophin mRNAs during postnatal development of rat gastrocnemius muscle. Total RNA was extracted from gastrocnemius muscle at different times during postnatal development and equal amounts (10 μg) were analyzed by RPA with the use of riboprobes specific for rat BDNF, NT-3, and NT-4 (*19*). (**A**) Lanes in autoradiograms contain RNA samples from: 1, postnatal day 0 (P0); 2, P4; 3, P7; 4, P14; 5, postnatal week 3 (3W); 6, 4W; 7, 5W; 8, adult; and 9, yeast tRNA. (**B**) The increase in the relative levels of expression of NT-4 mRNA (●) during muscle postnatal development and the concomitant decrease in the levels of BDNF (□) and NT-3 (○) mRNAs are shown. The maximal levels of expression of BDNF, NT-3, and NT-4 mRNAs (17, 350, and 20 fg per microgram of total RNA, respectively) are defined here as 100%. (C) and (D) show down-regulation of NT-4 mRNA after neuromuscular transmission blockade. (**C**) Time course of neurotransmission (T1%) blockade after local application of BTX in adult rat soleus muscle (*21*). (**D**) Time course of NT-4 mRNA expression in soleus muscle after local application of BTX. Muscle NT-4 mRNA levels in soleus muscle 0 hours (lane 1), 33 hours (lane 2), and 48 hours (lane 3) after BTX application. Lane 4, yeast tRNA. Solid bars below lanes illustrate the decrease in the relative levels of NT-4 mRNA after BTX treatment. The level of expression of NT-4 mRNA in untreated animals is defined here as 100%. Values correspond to the average of two to four independent experiments ± SE.

and D). No increase was seen in sham-operated animals that received no electrical stimulation (*20*). Levels of NT-4 mRNA were increased further after a second stimulation at a 6-hour interval (Fig. 2A), which indicates that NT-4 mRNA up-regulation after muscle activation was not refractory to repeated stimulation. In contrast to NT-4, levels of NGF mRNA were not changed by the treatment (*20*), whereas those of BDNF and NT-3 decreased (approximately 50%) between 3 and 12 hours after stimulation, returning to normal levels 24 to 48 hours after treatment (Fig. 2A). Thus, after electrical stimulation, the absolute level of NT-4 mRNA in soleus muscle (135 fg per microgram of total RNA) was 90- and 6.5-fold higher than those of BDNF (1.5 fg per microgram of total RNA) and NT-3 (20 fg per microgram of total RNA), respectively. The increase in muscle NT-4 mRNA expression after electrical stimulation was dose dependent: Higher levels of NT-4 mRNA were seen with increasing voltage or stimulation time (Fig. 2, C and D). An increase was also observed 6 hours after direct electrical stimulation of denervated muscle (*23*) (Fig. 2E), which suggests that NT-4 mRNA induction was linked to excitation-contraction coupling in muscle and that synaptic activity or release of other transmitters or factors by motor neurons was not a prerequisite for NT-4 mRNA up-regulation. A comparable increase in NT-4 mRNA was never seen after mechanical compression, passive stretching, or muscle injury, which indicates that the increase in NT-4 mRNA was a direct consequence of muscle activation and not due to muscular mechanical stress. Thus, the pattern of NT-4 mRNA expression after denervation, blockade of neuromuscular transmission, electrical stimulation, and during postnatal development indicated that the amount of NT-4 mRNA in skeletal muscle was controlled by muscle activity.

Next, we investigated the cellular localization of NT-4 mRNA and protein in soleus and gastrocnemius muscles by in situ hybridization and immunohistochemistry, respectively (*24*). NT-4 mRNA was localized to sparse muscle fibers in transverse sections of gastrocnemius muscle (Fig. 3A). A larger proportion of fibers was found to express NT-4 mRNA in soleus than in gastrocnemius muscle (*20*), which is in agreement with the greater amount of expression of NT-4 mRNA in this muscle after electrical stimulation. Electrical stimulation markedly increased NT-4 mRNA expression (Fig. 3B), although it did not augment the proportion of fibers expressing NT-4 mRNA, which suggests that NT-4 expression in muscle was restricted to a specific type of muscle fiber. Like NT-4 mRNA, immunoreactivity corresponding to NT-4 was localized to sparse muscle fibers in gastrocnemius muscle (Fig. 3C), and to a larger proportion of fibers in soleus muscle (Fig. 3D). As predicted (*3*), denervation markedly decreased NT-4 immunoreactivity (Fig. 3E), demonstrating a correspondence between the regulation of NT-4 mRNA and protein in skeletal muscle. Electrical stimulation, on the other hand, increased the intensity of NT-4 immunoreactivity but did not change the number of fibers expressing NT-4 (*20*). Because of the larger proportion of NT-4–immunoreactive fibers in soleus than in gastrocnemius muscle, we speculated that slow-twitch, type I fibers may have been the origin of NT-4 immunoreactivity in these muscles. Acid-resistant adenosine triphosphatase (ATPase) staining of adjacent sections showed a similar pattern to that obtained after NT-4 immunohistochemistry (Fig. 3F), which demonstrates that type I muscle fibers were the primary source of NT-4 in adult skeletal muscle. Interestingly, prolonged exercise training (*11*), as well as chronic electrical stimulation in humans (*25*), increase the proportion of type I muscle fibers.

Targets of NT-4 action must express

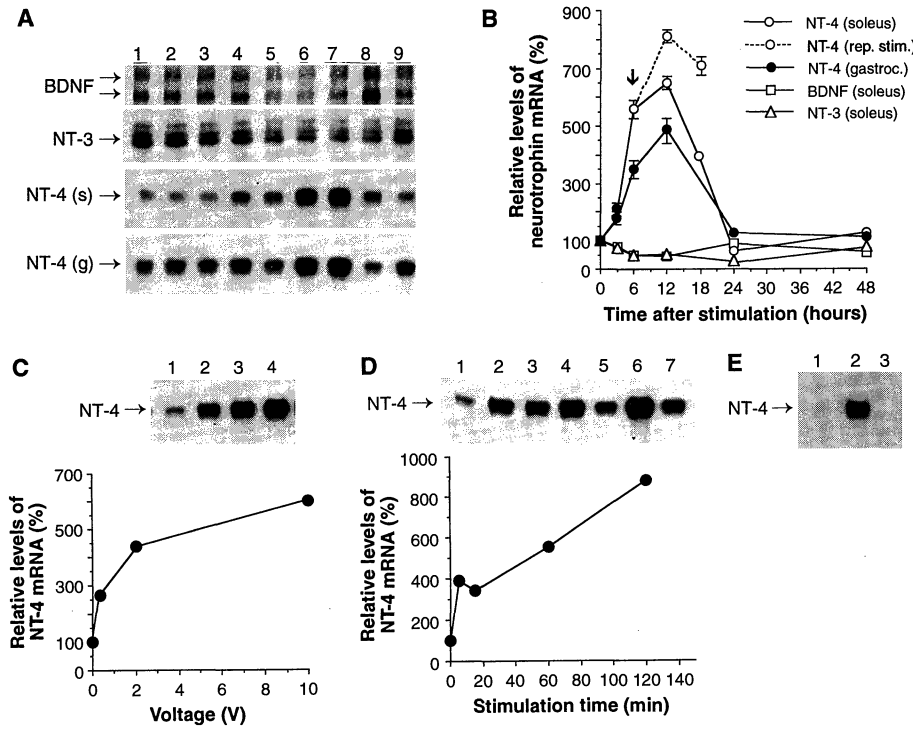


**Fig. 2.** Different patterns of expression of neurotrophin mRNAs in skeletal muscle after electrical stimulation. Total RNA was extracted from adult rat soleus or gastrocnemius muscles after electrical stimulation, and equal amounts of RNA (10 μg) were analyzed by RPA with the use of riboprobes specific for rat BDNF, NT-3, and NT-4. (**A**) Time course of neurotrophin mRNA expression after a 1-hour unilateral electrical stimulation of the sciatic nerve. RNA samples from muscle from untreated animals (lane 1); from ipsilateral muscle from treated animals 0 hours (lane 2), 3 hours (lane 4), 6 hours (lane 6), 12 hours (lane 7), 24 hours (lane 8), or 48 hours (lane 9) after stimulation; and from contralateral muscle from treated animals 0 hours (lane 3) or 3 hours (lane 5) after stimulation (s, soleus; g, gastrocnemius). (**B**) The increase in the relative levels of expression of NT-4 mRNA in gastrocnemius (●) and soleus (○) muscles after nerve electrical stimulation and the concomitant decrease in the levels of BDNF (□) and NT-3 (△) mRNAs in soleus muscle are shown. A second stimulation at 6 hours (arrow) further increased the expression of NT-4 mRNA in soleus muscle (broken line). The level of expression for each neurotrophin before stimulation is defined here as 100%. Values correspond to the average of two to four independent experiments ± SE. The absolute levels of expression of BDNF, NT-3, and NT-4 mRNAs in soleus muscle 12 hours after stimulation were 1.5, 20, and 135 fg per microgram of total RNA, respectively. (C) and (D) show dose response of NT-4 mRNA levels in soleus muscle to increasing voltage or time of unilateral electrical stimulation of the sciatic nerve. (**C**) Lanes correspond to RNA extracted from muscle of untreated animals (lane 1) and ipsilateral muscle from treated animals 6 hours after a 1-hour stimulation of 0.1 V (lane 2), 2 V (lane 3), or 10 V (lane 4). The plot below illustrates the increase in the relative levels of NT-4 mRNA with increasing stimulation voltage. The level of expression of NT-4 mRNA in untreated animals is defined here as 100%. (**D**) Lanes correspond to RNA extracted from normal rat soleus muscle (lane 1); from ipsilateral muscle from treated animals 6 hours after a 5-min (lane 2), 15-min (lane 3), 60-min (lane 4), or 120-min (lane 6) stimulation; and from contralateral muscle from treated animals after a 60-min (lane 5) or 120-min (lane 7) stimulation. The plot below illustrates the increase in the relative levels of NT-4 mRNA with increasing stimulation times. The level of expression of NT-4 mRNA in untreated animals is defined here as 100%. (**E**) NT-4 mRNA levels in denervated gastrocnemius muscle (lane 1) increased dramatically after direct electrical stimulation of the muscle (lane 2). Lane 3, yeast tRNA.

the NT-4 tyrosine kinase receptor (TrkB) and the low-affinity neurotrophin receptor ($p75^{LNGFR}$). The two NT-4 receptor mRNAs were detected in skeletal muscle during the first 3 weeks of postnatal development (*26*). However, their levels decreased markedly during later postnatal development and were almost undetectable after the fourth to fifth postnatal weeks (*26*). In addition, the TrkB transcripts detected in skeletal muscle only corresponded to truncated TrkB isoforms (that is, lacking the tyrosine kinase domain), and no TrkB mRNA corresponding to full-length catalytic receptors was seen at any stage of muscle postnatal development (*26*). In contrast, adult motor neurons do express full-length TrkB mRNA (*16, 27*) and could therefore respond to muscle-derived NT-4.

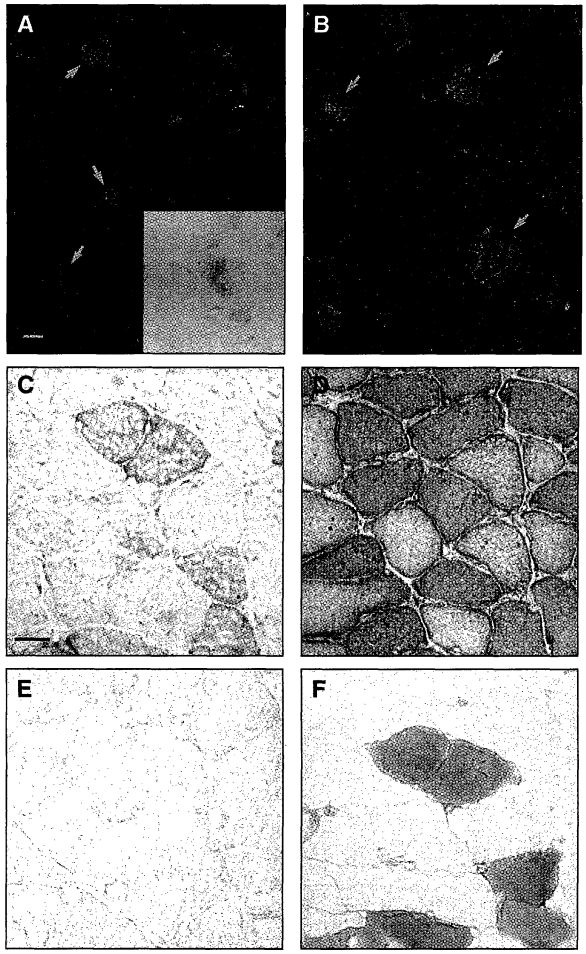


**Fig. 3.** Localization of NT-4 mRNA (A) and (B) and NT-4 immunoreactivity (C) through (E) in adult skeletal muscle. (**A**) NT-4 mRNA was localized to sparse fibers (arrows) in normal gastrocnemius muscle by in situ hybridization. Scale bar, 20 μm. (**Inset**) High magnification bright-field micrograph of a muscle fiber expressing NT-4 mRNA. (**B**) Up-regulation of NT-4 mRNA expression in gastrocnemius muscle fibers (arrows) after electrical stimulation. Same magnification as (A). (**C**) NT-4 immunoreactivity in transverse sections of normal gastrocnemius muscle was localized to sparse fibers. Scale bar, 20 μm. (**D**) A larger proportion of fibers showed NT-4 immunoreactivity in normal soleus muscle. (**E**) Six days after sciatic nerve transection, NT-4 immunoreactivity is no longer detected in gastocnemius muscle. (**F**) ATPase staining at low pH of a section adjacent to (C) demonstrated that type I muscle fibers were the primary source of NT-4 in adult skeletal muscle.

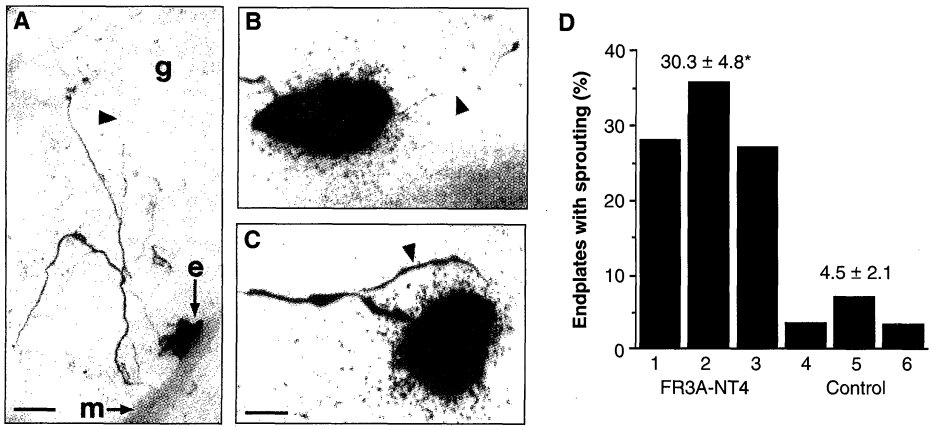


**Fig. 4.** NT-4 induced sprouting of intact adult motor nerves in skeletal muscle. (A), (B), and (C) show combined silver and cholinesterase staining of sections of gastrocnemius muscle 10 days after grafting of NT-4–expressing FR3T3 fibroblasts. (**A**) Sprouts (arrowhead) are seen growing into the graft (g); m, muscle fiber; e, endplate. Scale bar, 22.5 μm. (**B**) Sprout (arrowhead) emanating from an endplate. (**C**) Sprout (arrowhead) emanating from a node. Scale bar, 11.2 μm. (**D**) Proportion of sprouts observed in neurites and endplates associated with or in close proximity to the transplant in three animals that received grafts of NT-4–expressing fibroblasts (FR3A-NT4; animals 1, 2, and 3) and in three animals that received grafts of mock-transfected cells (control; animals 4, 5, and 6). Asterisk, $P < 0.005$ (Student $t$ test).

What could be the function of muscle-derived NT-4 in the adult neuromuscular system? Neuromuscular junctions in adult vertebrates are highly modifiable, undergoing remodeling throughout life (*6, 7*) [but see also (*28*)]. It has been proposed that growing and active muscles release signals that influence remodeling of neuromuscular junctions by stimulating nerve sprouting and synapse formation (*5, 8, 9, 11*). Because production of NT-4 in the muscle correlated with neuromuscular growth and muscle activity, we hypothesized that muscle-derived NT-4, unlike other neurotrophins whose production in muscle depends on nerve lesion, may have effects on intact adult motor nerves. In order to investigate possible functions of muscle-derived NT-4, we administered NT-4 protein within normal adult gastrocnemius muscle using transplants of genetically engineered fibroblasts expressing large amounts of NT-4 (*29*). As a negative control, a mock-transfected cell line was grafted into a second group of animals. Ten days after transplantation, muscles were sectioned and double-stained by silver and cholinesterase histochemistry, and motor nerve sprouting was quantified (*30*). A larger number of branching neurites was observed in endplates and terminals associated with the NT-4–expressing graft than with control grafts, which indicates that NT-4 mediated an increase in motor nerve sprouting (Fig. 4). Three different animals that received NT-4–expressing grafts showed sprouting indexes of 35.9, 28.2, and 27.1%, respectively (30.3 ± 4.8%), as compared with 3.2, 6.9, and 3.4% (4.5 ± 2.1%) observed in three animals receiving control grafts (Fig. 4). In a similar paradigm, optimal doses of ciliary neurotrophic factor (CNTF), a lesion factor produced by injured nerves that has trophic effects on regenerating motor neurons, induces an increase in motor nerve sprouting of 12% as compared with vehicle-treated muscle (*31*). In combination with fibroblast growth factor (FGF), CNTF-induced sprouting can reach up to 32% (*31*). Our results indicate that NT-4 is at least as potent as an optimal combination of CNTF and FGF in stimulating sprouting of motor nerves in skeletal muscle and demonstrate that this neurotrophin is able to elicit trophic responses on adult motor neurons.

We propose that muscle-derived NT-4 is an activity-dependent retrograde signal involved in maintenance and remodeling of

neuromuscular connections. The regulation of NT-4 expression in muscle contrasts with that of IGF-1, which is up-regulated after denervation and has been proposed to mediate the reestablishment of the neuromuscular junction after axotomy by inducing axonal sprouting (*2*). Our findings suggest that skeletal muscle uses different molecules to promote axonal sprouting after paralysis (such as IGF-1) and after activity (such as NT-4), which probably reflects distinct underlying mechanisms involved in repair and maintenance, respectively. Results of recent studies have shown how activity may regulate the production of signals that can either eliminate or maintain neuromuscular contacts (*32*). The ability of NT-4 to induce sprouting of adult motor nerves in vivo, the expression of functional NT-4 receptors by adult spinal motor neurons, and the production of NT-4 by skeletal muscle in situations of increased neuromuscular growth and activity, suggest that NT-4 is a physiological factor influencing activity-dependent changes in neuromuscular junctions. The fact that the increase in muscle NT-4 production was proportional to the intensity of electrical stimulation and that it could be maintained with repeated treatment agrees with other reports that demonstrate a correlation between the extent of nerve sprouting and the intensity of exercise training (*5*, *10*), suggesting that NT-4 could be partly responsible for the beneficial effects of exercise on neuromuscular performance. Because of its ability to promote sprouting and survival of motor neurons, NT-4 may have therapeutic benefits in motor neuron diseases, such as amyotrophic lateral sclerosis, in which distal axonal loss precedes or even leads to cell loss (*33*). Thus, NT-4 could prevent degeneration of axons that have been damaged by the disease, and it could induce sprouting and reinnervation by motor neurons not affected by the disease process. Chronic stimulation of paralyzed muscles of patients suffering from upper motor neuron lesions leads to an increase in the population of type I muscle fibers (*25*) and to clinical improvement. The expression of NT-4 by adult type I muscle fibers and the demonstration of an effect of NT-4 on adult motor neurons raises the possibility of the therapeutic use of this protein.

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19. Total muscle RNA was purified and analyzed by RPA as previously described (*3*). Riboprobes for rat neurotrophins were as previously described (*3*, *15*). The recovery of RNA was measured spectrophotometrically, and the quality and quantity of RNA was confirmed by ethidium bromide staining after electrophoresis in agarose gels. Quantitation of absolute amounts of neurotrophin mRNA was done as previously described (*15*), comparing the signals obtained with a standard curve generated with in vitro synthesized unlabeled sense strand neurotrophin RNA.
20. H. Funakoshi; *et al.*, data not shown.
21. A strip of silicon rubber containing 100 μg of BTX (Sigma), prepared as previously described [A. L. Connold *et al.*, *Dev. Brain Res.* **28**, 99 (1986)], was implanted under sodium pentobarbital anesthesia between soleus and flexor hallucis longus muscles in adult rats. To determine the extent of neurotransmission blockade, soleus twitch was elicited by sciatic nerve stimulation with a supramaximal 2-Hz train-of-four square wave impulse (0.2 ms duration). The extent of neuromuscular transmission after BTX application (T1%) was expressed as the percentage of the height of the first twitch (T1) at different times after BTX application compared with T1 before treatment. At the indicated times, soleus muscle was dissected and immediately frozen for RNA preparation.
22. For electrical stimulation, the rat sciatic nerve was exposed under anesthesia and a bipolar silver electrode was positioned beneath the nerve at the mid-thigh portion. The nerve was then stimulated with an electrical stimulator (Grass, Waltham, MA). Stimulation was for 2.5 s per 10 s and had duration 5 ms and frequency 50 Hz. Stimulation time and voltage were 1 hour and 10 V, respectively, except where indicated. This treatment produced massive muscle fiber depolarization and tetanic contraction.
23. To assess the effect of direct muscle stimulation, rat sciatic nerves were bilaterally transected 2 days before electrical stimulation. Gastrocnemius muscles were exposed under anesthesia, and three wire electrodes were positioned in the proximal part of the right muscle; another was inserted in the distal part. The left muscle was used as a control. Parameters for electrical stimulation were as described (*22*).
24. In situ hybridization was done on transversal sections (8 μm) of freshly frozen muscles, with the use of a 48-mer oligonucleotide probe complementary to rat NT-4 mRNA as previously described [C. F. Ibáñez *et al.*, *Development* **117**, 1345 (1993)]. For immunohistochemistry, soleus and gastrocnemius muscles were rapidly removed from hindlimbs of $CO_2$-asphyxiated rats and quickly frozen in isopentane cooled with dry ice. Cryostat cross-sections (5 μm) were cut, thawed, and placed on siliconized glass slides. Sections were rinsed in phosphate-buffered saline (PBS) and then incubated for 48 hours at 4°C with a rabbit polyclonal antiserum raised against a peptide corresponding to amino acid residues 42 to 52 from a variable loop region of rat NT-4 diluted 1:250 in PBS containing 0.3% Triton X-100. This antiserum reacts specifically with NT-4 and does not recognize any of the other three neurotrophins in protein immunoblots (D. Kaplan, personal communication). After incubation in primary antiserum, sections were rinsed in PBS and incubated for 1 hour at room temperature (RT) with blocking solution containing PBS and 5% goat serum, then incubated for 1 hour at RT with biotinylated secondary antibody (goat antibody to rabbit, Vector Labs, Burlingame, CA) diluted 1:500 in PBS. After washing with PBS, sections were incubated with ABC reagent (Vector) for 1 hour at RT, rinsed in PBS and tris-HCl buffer (pH 7.4), and incubated with 0.01% peroxidase and 0.05% diaminobenzidine in 0.1 M tris-HCl (pH 7.4). The specificity of the staining was assessed by (i) preadsorption of the primary antiserum with excess purified recombinant NT-4, (ii) omission of the primary antiserum, or (iii) replacement of primary antiserum with preimmune rabbit serum. No specific staining was detected in any of these three control experiments. To identify specific muscle fiber types, acid-resistant myosin ATPase staining was done as previously described [A. Lind and D. Kernell, *J. Histochem. Cytochem.* **39**, 589 (1991)].
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29. Genetically modified Fisher rat 3T3 fibroblasts (FR3T3) expressing rat NT-4 (FR3A-NT4) produce 100 ng of NT-4 per day per $1 \times 10^6$ cells and have been shown to retain the ability to produce NT-4 after transplantation [E. Arenas and H. Persson, *Nature* **367**, 368 (1994)]. Exponentially growing FR3A-NT4 or mock-transfected FR3T3 cells ($1.5 \times 10^6$) were resuspended in 10 μl of serum-free Dulbecco's modified Eagle's medium and injected with a Hamilton syringe into the right gastrocnemius muscle of adult Fisher 344 rats under anesthesia.
30. Gastrocnemius muscles were dissected 10 days after transplantation and quickly frozen in isopentane cooled with dry ice. Cryostat cross-sections (40 μm) were cut, thawed, placed on siliconized glass slides, and stored at −20°C until processed for staining. Sections were thawed at RT in a formamide-saturated atmosphere, fixed, and stained by combined silver and cholinesterase histochemistry as previously described [W. G. Hopkins and J. R. Slack, *J. Neurocytol.* **10**, 537 (1981)]. Motor nerve sprouting was defined as thin, unmyelinated axon growth emanating from endplates or from neurites associated with endplates. The sprouting index was calculated as the proportion of endplates or neurites associated with endplates with one or more sprouts in an area of 1 mm around the graft. Typical graft size was 8 to 9 $mm^3$. Sprouts were counted on consecutive sections across the total extension of the graft or until the number of total endplates counted reached 500.
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34. H. Persson passed away 16 May 1993, during the initial part of this study; we dedicate this paper to his memory. We thank G. Mudó and W. Friedman for help with immunohistochemistry, D. Kaplan for the antiserum to NT-4, H. Otsuka and K. Takita for help with electrical stimulation, and W. Friedman and I. Black for critical reading of the manuscript. Supported by the Swedish Medical Research Council, the Swedish Cancer Society, the International Research Institute for Paraplegia, and funds from the Karolinska Institute. H.F. was supported by a long-term fellowship from the Human Frontier Science Program Organization, N.B. was supported by a combined program from the Swedish Medical Research Council and the Italian National Research Council, and E.A. and C.F.I. were supported by the Swedish Medical Research Council.

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