A possible role for BDNF, NT-4 and TrkB in the spinal cord and muscle of rat subjected to mechanical overload, bupivacaine injection and axotomy

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Abstract

Neurotrophins play a crucial role in the regulation of survival and the maintenance of specific functions for various populations of neurons. Neurotrophin-4 (NT-4) is most abundant in skeletal muscle, and is thought to promote sciatic nerve sprouting, inhibit agrin-induced acetylcholine receptor (AChR) clustering, evoke postsynaptic potentiation and induce mitochondrial proliferation. Using Western blot analysis, immunoprecipitation and immunohistochemistry, we investigated the distribution of NT-4 in slow- and fast-type muscles. We also tested the adaptive response of this protein in the mechanically overloaded muscle, in the regenerating muscle following bupivacaine injection and in the denervated muscle. Additionally, we investigated whether TrkB phosphorylation in the spinal cord and in the sciatic nerve occurs through the interaction with BDNF or NT-4 when the innervating muscle is damaged. Markedly more NT-4 was expressed in fast-type muscles compared with the slow types. TrkB protein was more frequently observed around the edge of myofibers (neuromuscular junction) of the soleus muscle compared with the gastrocnemius muscle. TrkB tyrosine phosphorylation occurred in the spinal cord but not in the sciatic nerve 24 h after bupivacaine injection of the innervating muscle. At the same time, the amount of TrkB co-precipitating with BDNF was markedly increased in the spinal cord. A rapid activation of TrkB (1–8 h) was also observed in the spinal cord after axotomy, while the amount of TrkB co-precipitating with NT-4 was markedly lower after axotomy. These results indicate that NT-4 is preferentially distributed in fast-type muscles. Furthermore, by interacting with BDNF and NT-4, the TrkB in the spinal cord may be important for the survival of motoneurons and outgrowth of injured peripheral axons following muscle damage. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

The development and maintenance of the vertebrate nervous system requires the activity of a range of polypeptides known as neurotrophic factors. These molecules have been shown to control the generation, survival, differentiation and regeneration of neurons in the peripheral and central nervous system [2,44]. The neurotrophin family consists of six proteins — nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6; they share approximately 50% identity in the amino acids which are clustered in conserved regions intersected by variable regions, the combinations of which define the biological specificity of the neurotrophin [23]. Survival of neurons in the sympathetic and nodose ganglia is promoted by specific neurotrophins, while all four neurotrophins support the survival of embryonic sensory neurons in cell culture [2,21,31].
Recently, research has been performed on the other possible role of NT-4, as an activity-dependent trophic signal for adult motor neurons [14] in skeletal muscle. NT-4 promotes sciatic nerve sprouting [14], induces postsynaptic potentiation [47], inhibits agrin-induced AChR clustering [49], and induces mitochondrial proliferation [46]. Mechanical overloading, bupivacaine injection-induced regeneration and denervation markedly changes the activity and/or volume of muscle fiber. Structural and contractile proteins as well as muscle-specific enzymes needed in hypertrophied muscle may be upregulated on stimulation of activity-dependent postsynaptic potentiation of NT-4. It is possible that NT-4 protein, which inhibits agrin-induced AChR clustering, is lost in the early phase of regeneration due to reconstruction of neuromuscular junctions after damage. However, no study has yet determined the adaptive response of NT-4 protein in hypertrophied and regenerating muscle. Using in situ hybridization and immunohistochemistry, Funakoshi et al. [14] found that NT-4 protein was selectively expressed in the slow-twitch muscle of mouse. In contrast, immunohistochemistry of human muscle demonstrated an equal distribution of NT-4 protein in the slow- and fast-twitch muscle fibers [46]. Therefore, it seems that two different methods, such as immunohistochemistry and Western blotting using whole muscle, are needed to clearly elucidate the distribution of NT-4 in different muscles.

Bupivacaine injection of muscle has been known to damage many types of cells in the muscle tissue (muscle cells, blood vessels, and Schwann cells surrounding branched axons, etc.). Following bupivacaine injection, the muscle fiber is regenerated by co-operative and counteractive efforts of myogenic regulatory factor [36], cell-cycle regulatory protein and various cytokines (e.g., transforming growth factor-β2 [37], leukemia inhibitory factor (LIF) [28,38,39], and insulin-like growth [24]) produced by satellite cells and the many inflammatory cells invading the damaged areas. Although the adaptation following muscle damage has been well documented, the response in the spinal cord to muscle regeneration is still little understood. One recent study [25] showed that muscle contusion induces the upregulation of both LIF and glial cell line-derived neurotrophic factor (GDNF), together with their respective receptors (LIFR, GFRα-1), in the spinal cord. As bupivacaine injection widely destroys muscle fiber and neuromuscular junctions, our hypothesis is that, at least in the spinal cord, BDNF and NT-4 are crucial for inhibiting the cell death of motoneurons and/or for promoting repair to damaged peripheral axons via auto-phosphorylation of the receptor TrkB.

In the present study, using Western blot analysis, immunohistochemistry and immunoprecipitation, we examined the normal distribution of NT-4 and the adaptive response of this protein in mechanically overloaded, regenerating and denervated muscles. Moreover, following damage to the innervating muscle we assessed whether TrkB phosphorylation in the spinal cord and sciatic nerve occurs through the interaction with BDNF and NT-4.

2. Materials and methods

2.1. Animals and experimental procedures

2.1.1. Experimental animals

Sixty-seven male (2, 3, 4, 6, 8, 10 and 20 weeks of age) and 67 female (12 weeks of age) Wistar rats were used in the experiments. The rats were housed in a temperature (22±2°C) and humidity (60±5%)-controlled room regulated to provide alternating 12-h periods of light and darkness. They were allowed to feed (commercial rat chow) and drink ad libitum. Rats of both sexes were used in the experiments because no difference between males and females in the level of NT-4, BDNF and TrkB proteins was found in a preliminary study.

2.1.2. Normal adult organs and muscles

Six male Wistar rats (20 weeks of age) were used in this experiment. They were killed with an excess of pentobarbital, and the brain, cerebellum, spinal cord, muscle, heart, liver, kidney, stomach, lung, testis and thymus were rapidly dissected. To compare amounts of NT-4 and TrkB protein among several different muscles, six male Wistar rats (10 weeks of age) with body weights of 300–350 g were used. The rats were killed with an excess of pentobarbital, and the extensor digitorum longus, tibialis anterior, gastrocnemius, soleus and diaphragm muscles were rapidly dissected.

2.1.3. Development

Twelve male Wistar rats (2, 3, 4, 6, 8 and 10 weeks of age) were used in the experiment. The rats were killed with an excess of pentobarbital and the tibialis anterior, gastrocnemius, soleus and diaphragm muscles were rapidly dissected.

2.1.4. Overloading

Twenty-seven adult female Wistar rats (12 weeks of age) with body weights of 200–250 g were used in this experiment. Compensatory enlargement of the plantaris muscle was induced in one leg of each rat by surgical removal of the ipsilateral soleus and gastrocnemius muscles as described previously [39]. The rats were killed with an excess of pentobarbital, and dissected of the plantaris muscle of both legs in groups of three at 1, 2, 3, 4, 6, 8 and 10 days and 2 and 4 weeks postsurgery.

2.1.5. Bupivacaine treatment

Forty-three adult male Wistar rats (10 weeks of age) with body weights of 270–360 g were used in this experiment. Regeneration of the tibialis anterior muscle was induced in one leg of each rat by an intramuscular
injection of 0.5 ml of 0.5% bupivacaine hydrochloride prepared in a 0.9% saline solution as described previously [37]. The rats were killed with an excess of pentobarbital and dissected of the tibialis anterior muscles, the sciatic nerves of both legs and the spinal cord in groups of three at the same time points as for the overloading experiment. To determine whether the phosphorylation of TrkB protein has an earlier time course (15 min to 12 h postsurgery) after muscle damage, rats were killed and dissected of the spinal cord and the sciatic nerve of the operated leg in groups of two at 0, 15, 30 and 60 min, and 2, 4, 8 and 12 h after bupivacaine injection.

2.1.6. Denervation

Forty adult female Wistar rats (12 weeks of age) with body weights of 200–240 g were used in the experiment. The left sciatic nerve was cut at mid-thigh and a 1-cm length of the nerve resected as described previously [35]. The rats were killed with an excess of pentobarbital in groups of three at 1, 2, 3, 4, 7 and 10 days and 2 and 4 weeks postsurgery. The soleus, plantaris and gastrocnemius muscles, the sciatic nerves of both legs and the spinal cord were dissected. To determine whether the phosphorylation of TrkB protein has an earlier time course after axotomy, rats were killed and dissected of the spinal cord and the sciatic nerve of the operated leg in groups of two at 0, 15, 30 and 60 min, and 2, 4, 8 and 12 h after axotomy.

2.2. Primary antibodies

The antibodies employed in the present study were as follows: affinity-purified rabbit polyclonal antibody to NT-4 [1:400, N-20, Santa Cruz Biotechnology (SCB), Santa Cruz, CA, USA]; affinity-purified rabbit polyclonal antibody to BDNF (1:260, N-20, SCB); affinity-purified rabbit polyclonal antibody to TrkB (1:260, H-181, SCB); and affinity-purified mouse monoclonal antibody to phospho-tyrosine (1:2000, Upstate Biotechnology).

2.3. Tissue preparation, gel electrophoresis and immunoblots

Tissues of each muscle were homogenized in 10–20 volumes of 50 mM tris (hydroxymethyl) aminomethane (Tris)–HCl, pH 7.4, 5 mM EDTA, 10 µg/ml phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 0.2 µg/ml aprotinin, 0.2% Nonidet P-40, 0.1% Triton X-100, 0.05% mercaptoethanol, and 1 mM Na3VO4 using a polytron for 30 s. The homogenized tissues were centrifuged for 25 min at 15 000×g at 4°C, and the protein concentration of the supernatant was determined colorimetrically (Bio-Rad protein determination kit, Bio-Rad Laboratories, Richmond, CA, USA). Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8% acrylamide for TrkB and 12.5% acrylamide for NT-4 and BDNF) was performed according to the method of Laemmli [29]. Proteins separated by SDS–PAGE were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL Western, Amersham, Arlington Heights, IL, USA). The blots were incubated with a blocking buffer of 0.1% Tween-20 and 1% gelatin in 10 mM Tris-buffered saline (TBS, 10 mM Tris, 135 mM NaCl, 1 mM KCl, 0.02% Na3VO4, pH 7.4) in a cold room overnight. The blots were incubated with the primary antibodies for 1 h, and with biotinylated anti-rabbit IgG (1:300, BA-1000, Vector Laboratories) for 1 h. They were then incubated for 30 min with peroxidase streptavidin conjugate (Vector), and visualized with diaminobenzidine (DAB) and H2O2.

2.4. Immunoprecipitation

Protein extracts (100 µg) were incubated with protein A-Sepharose beads (10 µl; Pharmacia Biotech, Uppsala, Sweden) and antibodies against TrkB receptors (anti-TrkB, 1 µg), BDNF (1 µg) and NT-4 (1 µg) in lysis buffer [20 mM tris (hydroxymethyl) aminomethane (Tris)–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1% Nonidet P-40, 0.04% mercaptoethanol, and 1 mM Na3VO4] for 1 h at 4°C. The beads were washed four times with lysis buffer. Washed beads were resuspended in reducing sample buffer and boiled for 2 min before being size-fractionated on 8% SDS–polyacrylamide gels.

2.5. Immunohistochemistry

Serial 8-µm transverse sections made with a cryostat (Bright 5030 Microtome, Bright Instrument, Huntingdon Cambridgeshire, UK) were mounted on silanized slides (Dako Japan, Tokyo, Japan). All subsequent steps were as described previously [37]. Briefly, the sections were incubated with primary antibodies diluted in phosphate-buffered saline (PBS) for 60 min at room temperature (RT). Sections were subsequently rinsed well with PBS, and incubated with biotinylated anti-rabbit IgG (1:500 dilution, Vector) for 30 min at RT. After extensive rinsing, sections were incubated for 30 min with peroxidase streptavidin conjugate (Vector), and visualized with DAB and H2O2. The sections were mounted in a glycerol-based medium containing p-phenylenediamine. A negative control, omission of the primary antibodies, was included in the immunostaining procedures in each instance.

2.6. Histochemistry

To clarify each fiber as slow-twitch or fast-twitch, successive cryosections were stained for myofibrillar actomyosin adenosine triphosphatase (mATPase) after preincubation at pH 10.3 [18].
Fig. 1. Western blot analysis of NT-4, BDNF and their receptor TrkB in extracts of various organs from normal adult rats (20 weeks old). (A) NT-4 is expressed in brain, cerebellum, spinal cord, skeletal muscle, heart and kidney. Immunoblotting with antibodies to NT-4 revealed prominent bands consistent with a molecular size of 46–47 kDa. (C) TrkB was abundant in the brain, spinal cord, liver and kidney, but was scarce in the skeletal muscle. Proteins (30 μg in 20-μl aliquots) were subjected to SDS–PAGE, followed by Western blotting. Proteins transferred to a nitrocellulose sheet were stained with primary antibodies and visualized with diaminobenzidine. (B and D) Values are expressed as percentages of the immunostaining intensity of brain, which is taken as 100%, and are given as mean±S.E.M. (n = 6/organ). The amount of NT-4 protein in the skeletal muscle is significantly greater than that in brain, cerebellum, spinal cord, liver, etc. Brain has significantly more TrkB protein than skeletal muscle, kidney, stomach and testis. (E) BDNF protein was expressed only in the brain, cerebellum and kidney.
Fig. 1. (continued)
2.7. Statistical analysis

All values are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was used for determining the main statistical effects. A Scheffé's post-hoc test was conducted if the ANOVA indicated a significant difference. $P < 0.05$ was considered statistically significant.

3. Results

As shown in Fig. 1A, immunoblotting with antibodies to NT-4 revealed prominent bands consistent with a molecular size of 46–47 kDa. NT-4 protein was detected in the brain, cerebellum, spinal cord, skeletal muscle, heart, liver, kidney and testis, but not thymus or lung, which is a similar distribution to that found for NT-4 mRNA [45]. The NT-4 protein level was significantly higher in the skeletal muscle than in the brain, cerebellum and spinal cord (Fig. 1B). The brain, cerebellum, spinal cord, heart, liver and kidney all possess abundant amounts of TrkB protein (Fig. 1C and D). Although to date the data regarding the presence of the full-length TrkB receptor in skeletal muscle are conflicting [15,41], we did observe a low level expression of TrkB protein in skeletal muscle of adult rat. As shown in Fig. 1E, immunoblotting with antibodies to BDNF revealed prominent bands consistent with a molecular size of 35–36 kDa. In the adult rat, BDNF protein was detected in the brain, cerebellum and kidney, but not in any other organ.

The postnatal change in the level of NT-4 protein in hindlimb and diaphragm muscles is shown in Fig. 2. As demonstrated by Funakoshi et al. [14], NT-4 protein content was markedly increased in the soleus and gastrocnemius muscles at 2–4 and 2–10 weeks of age, respectively. In contrast, the tibialis anterior and diaphragm muscle showed no such postnatal change. The distribution patterns of NT-4 and TrkB proteins in different muscles are unknown. We determined whether, in normal adult rats, the NT-4 protein content differs between slow-type (soleus) and fast-type (extensor digitorum longus, tibialis anterior, gastrocnemius and diaphragm) muscles. Western blot and densitometric analyses showed that the NT-4 level was significantly higher in the fast-type muscles than the soleus muscle (Fig. 3A and B). In contrast to fast-type phasic muscles, a minor expression of TrkB protein was observed in the soleus and diaphragm muscles. To understand more clearly the cellular localization of NT-4, we performed immunohistochemistry on transverse sections of the soleus and tibialis anterior muscles of normal adult rats. In the tibialis anterior muscle, NT-4 was diffusely expressed in the cytosol of both slow-twitch and fast-twitch fibers (Fig. 4A and C). Slight expression of NT-4 protein was observed around the edge of soleus muscle fibers (Fig. 4D). Immunoreactivity of TrkB protein was more frequently observed around the edge of myofibers (neuromuscular junction) in the soleus muscle (Fig. 4E) compared with the gastrocnemius muscle (Fig. 4F).

Overloading induced marked increases in the weight of the plantaris muscles during the postsurgical period (1 day to 4 weeks). The weight after 1 day showed a 56.0% increase over the control value. The percentages of hypertrophy after 3, 6 and 10 days were 40.9, 31.3 and 44.8%, respectively, and after 2 and 4 weeks were 46.8 and 76.2%, respectively. At 1 and 2 days post-injection, the tibialis anterior muscle had increased in wet weight by 49.3 and 20.4%, respectively, over that of the control. Six, 8 and 10 days after bupivacaine injection, the tibialis anterior wet weight decreased 29.7, 20.2 and 13.8% under the control value, respectively. Complete restoration of wet weight was achieved by 28 days in tibialis anterior muscles subjected to bupivacaine. The functional significance of
Fig. 3. NT-4 and TrkB are preferentially distributed in the fast-type and slow-type muscles of normal adult rats, respectively. (A) The amount of NT-4 peptide in fast-type muscles, such as EDL, TA and gastrocnemius, was markedly greater than that in slow-type soleus muscle. (B) Values are expressed as percentages of the immunostaining intensity of TA muscle (mean ± S.E.M.; n = 6/muscle). (C) A little TrkB protein was detected in the soleus and diaphragm muscles, while it was completely absent from fast-type muscles (EDL, extensor digitorum longus; TA, tibialis anterior).

NT-4 in fiber hypertrophy and regeneration of fully differentiated muscle is not yet known. Compared to the unoperated plantaris muscle, the muscle subjected to mechanical overloading had a slightly decreased level of NT-4 protein (Fig. 5A). Densitometric analysis (n = 3/time point) showed after 1 day of mechanical overloading the NT-4 amount to be 70% that of the control and this had still not recovered by 28 days postsurgery (Fig. 5B). On the other hand, the amount of NT-4 decreased markedly in the regenerating muscle following bupivacaine injection (Fig. 5A). At 1 day postsurgery, the NT-4 protein content rapidly decreased, reaching only 15% of the control value.
The level was gradually restored and it returned to the control level 10 days after the bupivacaine injection. Immunohistochemical data also showed a marked decrease of NT-4 in the regenerating muscles (Fig. 5E) compared to contralateral muscle (Fig. 5C). However, this marked decrease was not observed in the overloaded muscle (Fig. 5D and F).

Since bupivacaine injection widely destroys neuromuscular junctions and promotes the necrosis of muscle fibers, it seems likely that various growth factors and neurotrophin act to inhibit the cell death of spinal motoneurons that could not contact muscle and receive a retrograde signal. Therefore, following damage to the innervating muscle caused by bupivacaine injection we checked the spinal cord and sciatic nerve for changes in BDNF, NT-4 and TrkB protein expression. In the spinal
Fig. 5. Mechanical overloading and bupivacaine injection diminishes the expression of NT-4 protein. (A) Western blot analysis of NT-4 protein in the plantaris muscle subjected to mechanical overloading (upper lane) and in the TA muscle subjected to bupivacaine injection (lower lane). (B) Values are expressed as percentages of the immunostaining intensity of NT-4 in control muscle (mean±S.E.M.; n = 3/plot). (C–F) Immunohistochemical staining of NT-4 in sections from the control TA (C) and plantaris (E), regenerating TA (D) and mechanically overloaded plantaris (F) muscles of rats. In the regenerating TA muscle at 6 days postsurgery (D), the NT-4 immunoreactivity of muscle fibers was markedly reduced compared to the control (C). Three days of overloading for the plantaris muscle (F) did not alter the NT-4 expression pattern of muscle fibers from those in the normal plantaris (E). Primary antibody was visualized by a standard avidin–biotin peroxidase method. Bar 50 μm (TA, tibialis anterior).

In the spinal cord, the levels of NT-4 and TrkB protein did not change during damage or regeneration (Fig. 6A). In contrast, those of NT-4 and TrkB protein decreased markedly in the sciatic nerve after 2 days (Fig. 6B). No BDNF protein was found in spinal cord (Fig. 6C), sciatic nerve (data not shown) or muscle (data not shown) during the postsurgical period of the regenerating tibialis anterior muscle. Additionally, we investigated whether TrkB tyrosine phosphorylation occurs in the spinal cord and sciatic nerve after muscle damage. As found in previous studies [3,13], we
could not detect a band showing TrkB phosphorylation in non-operated spinal cord (Fig. 7A and C). TrkB protein was markedly phosphorylated 1 day postsurgery in the spinal cord, but not in the sciatic nerve. At 15 min to 12 h after muscle damage, no phosphorylated TrkB could be detected in the spinal cord (Fig. 7C) or sciatic nerve (data not shown).

Next we determined the changes in BDNF and NT-4 expression after axotomy. In the spinal cord and the sciatic nerve, BDNF expression increased 2–7 and 4–28 days after denervation, respectively (Fig. 8A). The NT-4 protein level was markedly increased in the axotomized spinal cord at 7 days postsurgery (Fig. 8B) while, in contrast, it was markedly decreased in the sciatic nerve after 2 days (Fig. 8B). The data obtained by immunoprecipitation showed that TrkB was phosphorylated in the spinal cord at 2–8 h after axotomy (Fig. 8C). To confirm that the phosphorylation was induced by the interaction with NT-4 and/or BDNF, we measured the amount of TrkB co-precipitating with BDNF and NT-4. Following muscle damage (after 24 h), the amount of TrkB in spinal cord co-precipitating with BDNF was increased compared to the control (Fig. 9A and C). The amount of co-precipitation with NT-4 peaked when TrkB was maximally phosphorylated at 2 h axotomy (Fig. 9B and C).

Denervation induced marked decreases in the weight of the soleus, plantaris and gastrocnemius muscles. At 7 days postsurgery, all the muscles showed a wet weight of about 73–80% of the control value. The weight of the denervated muscles had decreased to 58–63% of the control value at 14 days, and to 30–45% at 4 weeks postsurgery. In contrast to the soleus muscle, the amount of NT-4 protein in the plantaris and gastrocnemius muscles gradually decreased after 7 days of denervation, reaching 30–35% of the control value at 4 weeks after axotomy (Fig. 10A and B). As demonstrated by Funakoshi et al. [15] using Northern blot analysis, denervation of fast-type but not slow-type muscles clearly induces the expression of BDNF protein (Fig. 10C). Denervation of gastrocnemius muscle clearly induced an atrophy and loss of myofibers and increased the connective tissues of extracellular spaces (Fig. 10D–I). The muscle fibers of normal gastrocnemius does not possess BDNF immunoreactivity (Fig. 10D). Similar to Western blot analysis, denervation markedly increased BDNF protein in the cell cytosol and sarclemma of many fibers of gastrocnemius muscle (Fig. 10E). In normal gastrocnemius muscle, TrkB protein was observed at the edge of myofibers (Fig. 10F), but some fibers possessed more marked TrkB immunoreactivity after denervation (Fig. 10G). In contrast, no difference in the distribution pattern of NT-4 was detected between normal and denervated gastrocnemius muscles (Fig. 10H and I).
4. Discussion

Two recent studies [14,46] differed in their findings on the distribution of NT-4 in slow-type and fast-type muscles. One found NT-4 to be equally distributed in both muscle types of humans [46], while the other, using in situ hybridization and immunohistochemistry, found NT-4 to be selectively expressed in the slow-twitch fibers of mice [14]. Using Western blot analysis and immunohistochemistry we found that NT-4 protein was distributed in both the slow-twitch and fast-twitch muscle fibers of the adult rat, although they were more abundant in the latter. It is possible that the larger AChR clusters found in fast-twitch fibers may require more NT-4 protein. In Xenopus nerve-muscle cultures, postsynaptic NT-4 positively modulated presynaptic ACh release and enhanced the postsynaptic response to ACh in an activity-dependent manner [47]. Fast-type intermittent high-frequency electrical stimulation enlarged AChR clusters in the slow-twitch fibers of ALD muscle [11]. The abundance of NT-4 protein in fast-type muscles may be important for nitric oxide (NO) synthase. In spinal cord cultures, BDNF, NT-3, and NT-4 markedly upregulated nicotinamide adenine dinucleotide phosphate-diaphorase, a histochemical marker for NO synthase in motoneurons [22]. Selective expression of NO synthase I in sarcolemma [27] and neuromuscular junctions [16] of fast-twitch fibers has also been demonstrated.

To our knowledge, this study is the first to show that NT-4 was decreased in both mechanically hypertrophied and regenerating muscles with the changes in the latter
being marked. The overloading-induced decrease in NT-4 would be due to the change in the activity pattern of the plantaris muscle. In particular, the function of the soleus muscle in rat is to maintain posture and almost all the fibers are chronically activated [34]. Following removal of the soleus muscle, the activity pattern of the plantaris muscle becomes more tonic and continuous, but not phasic or intermittent. Chronic activation of muscle may decrease the NT-4 level partially via fast-to-slow transformations in the plantaris muscle during mechanical overloading [40]. One interpretation of the reduction in NT-4 during muscle regeneration is that it promotes the remodeling of damaged neuromuscular junctions. Brenner et al. [4] demonstrated that, in the damaged rat soleus muscle, the α- and ε-subunits of AChR were upregulated near the synaptic site 4 days after venom injection and this remained even at 15 days. Thus, similar to myogenesis, the formation of neuromuscular junctions requires the neo-formation and clustering of AChR at synaptic sites in the regenerating muscle. Agrin, which is released from motor nerve terminals, activates a muscle-specific receptor tyrosine kinase (MuSK) in muscle cells [17]. In addition, it inhibits neurite outgrowth but promotes attachment of embryonic motor neurons to muscle fiber [7]. Furthermore, in cultured
Fig. 8. Western blot analysis of NT-4 and BDNF in extracts of the spinal cord and sciatic nerve after axotomy. The amount of BDNF protein was markedly increased in both regions after transection of the sciatic nerve (A). The NT-4 protein level was markedly decreased in the sciatic nerve at 3 days postsurgery (B), although it was increased in the spinal cord after 7 days of axotomy. TrkB was phosphorylated in the spinal cord from 60 min to 8 h after axotomy (C). Protein (100 μg) extracted from the spinal cord was immunoprecipitated with a TrkB antibody (1 μg) and the resulting immunocomplexes were analyzed by 8% SDA–PAGE, blotted, and probed with the anti-phosphotyrosine antibody (IP, immunoprecipitation; IB, immunoblotting; Ptyr, phosphotyrosine).

myotubes, exogeneous injection of BDNF, NT-4, and their receptor TrkB, but not NGF or NT-3, inhibits agrin-induced AChR clustering [49]. Therefore, agrin-induced remodeling of damaged neuromuscular junctions would require a rapid and marked decrease in NT-4 (Fig. 5) and the absence of BDNF (Fig. 7C).

The evidence for a full-length TrkB being present in skeletal muscle is conflicting [15,41]. Funakoshi et al. [15] reported that, in normal adult rats, the skeletal muscle (gastrocnemius) does not contain either truncated or full-length TrkB mRNA. In contrast, Shelton et al. [41] demonstrated the existence of an 8.1-kb transcript of TrkB containing the tyrosine kinase domain in human muscle. The data from our investigation proves the existence of a full-length TrkB protein in the soleus and diaphragm muscles of normal adult rats, but not in fast-type muscles such as the EDL, TA and gastrocnemius. Additionally, immunohistochemical analysis clearly showed that TrkB protein was detected in the edge of myofibers corresponding to the position of neuromuscular junctions.

The expression of TrkB protein in several muscles may have resulted from anterograde and retrograde transport of BDNF and/or NT-4 in combination with the receptor TrkB. Neuronal injury increases retrograde and antero-
Fig. 9. Amount of TrkB co-precipitating with BDNF and NT-4 antibodies in the spinal cord when the innervating muscle was damaged by bupivacaine injection or at axotomy. Protein extracts (100 μg) were immunoprecipitated with BDNF (1 μg) and NT-4 (1 μg) antibodies and the resulting immunocomplexes were analyzed by 8% SDA–PAGE, blotted, and probed with the anti-TrkB antibody. (A and B) Lane 1, the spinal cord of un-operated TA muscle; lane 2, the spinal cord of regenerating TA muscle when TrkB was phosphorylated at 24 h after bupivacaine injection; lane 3, the spinal cord of normal hindlimb muscle; lane 4, the spinal cord after axotomy for 2 h when TrkB was maximally activated. (C) Values are expressed as percentages of the immunostaining intensity of TrkB co-precipitating with BDNF and NT-4 in each control spinal cord. TrkB co-precipitating with BDNF was markedly increased in the spinal cord following bupivacaine injection compared to unoperated spinal cord (A and C). In contrast, TrkB co-precipitating with NT-4 was markedly decreased in the spinal cord after axotomy (B and C) (IP, immunoprecipitation; IB, immunoblotting; TA, tibialis anterior).
Fig. 10. Western blot analysis of NT-4, BDNF and TrkB in extracts of the spinal cord, sciatic nerve and muscle after axotomy. (A) Denervation gradually decreased the amount of NT-4 in the fast-type muscles, but not in the slow-type soleus muscle. (B) Values are expressed as percentages of immunostaining intensity of NT-4 in control muscle (mean ± S.E.M.; n = 3/plot). (C) The expression of BDNF protein was detected in the plantaris and gastrocnemius muscles at 7 and 4 days after denervation, respectively. Compared to the normal gastrocnemius muscle (D), denervation for 2 weeks markedly increased the amount of BDNF protein in the cell cytosol and sarcolemma of many gastrocnemius muscle fibers (E). After denervation, some fibers possessed intense TrkB immunoreactivity at the edge of myofibers (F and G). The distribution pattern of NT-4 protein did not change in the gastrocnemius muscle after denervation (H and I). White arrows denote the position of TrkB immunostaining in myofibers. Primary antibody was visualized by a standard avidin–biotin peroxidase method. Bar 100 μm (TA, tibialis anterior).
grade axonal transport of NGF, BDNF, and NT-3 by inducing binding to the low-affinity neurotrophin receptor (p75NTR) or Trk receptors [10]. Another explanation for the conflicting results regarding the presence of full-length TrkB in skeletal muscle could be differences in species, muscle-types (slow or fast), and/or age. There is limited information on the reactions in nerve and motoneurons following muscle damage. One recent study [25] showed that LIF, and GDNF together with respective receptors were upregulated in the motoneurons and intramuscular nerves when innervating muscle was subjected to contusion. We have clearly demonstrated that TrkB phosphorylation occurs in the spinal cord 1 day after bupivacaine injection. Additionally, when innervating muscle was damaged, the amount of TrkB co-precipitating with BDNF increased as compared with intact spinal cord. Null mutation of TrkB induces severe motoneuron death [26,42]. Axotomized hippocampal and motor neurons of TrkB (−/−) mutant mice have significantly lower survival rates than those of wild-type littermates [1]. Furthermore, in adult rat spinal cord, direct injection of BDNF and NT-4 postponed naturally occurring motoneuron death following
axotomy [12, 50]. Therefore, we propose that, by interacting with NT-4 and the more abundant BDNF, TrkB in the spinal cord plays a crucial role in inhibiting the death of motoneurons and in promoting the outgrowth of injured peripheral axons when innervating muscle is damaged by bupivacaine injection as well as at axotomy.

After axotomy, retrograde transport of BDNF, NT-3, NT-4, ciliary neurotrophic factor (CNTF) and LIF was increased [8–10]. Additionally, following axotomy, re-expression of p75NTR by adult motor neurons is triggered by retrograde transport of a positive signal (e.g., neurotrophin) from axons regrowing through damaged or denervated peripheral nerve tissue [5]. The earlier activation of TrkB seen after axotomy (2–8 h) as opposed to that after muscle contusion (24 h) seems attributable to the shorter distance from the lesioned area of the nerve to the motoneuron and/or to a more severe destruction of the nerve. It is unknown whether a positive or negative retrograde signal from damaged neuromuscular junctions contributes to the TrkB activation during muscle regeneration. Unlike the changes in BDNF after axotomy, the elevation in BDNF after bupivacaine injection was not detectable in the spinal cord, sciatic nerve, and muscle by Western blot analysis. Thus, it is questionable whether BDNF acts as a retrograde positive signal from peripheral nerve after muscle contusion. Interestingly, both in the case of muscle regeneration and denervation, the NT-4 protein in sciatic nerve disappeared.

Induction of BDNF in peripheral nerve and muscle would be important to stimulate Schwann cell dedifferentiation and proliferation, a prerequisite to axonal regeneration. In vitro, the neuregulin family, which activate AChR genes by inducing tyrosine phosphorylation on their receptors erbB2, erbB3, and erbB4 [6] at neuromuscular junctions, has a potent effect on Schwann cell mitogens and differentiation. In vitro, BDNF, NT-3, NT-4 and GDNF, but not other neurotrophic factors such as CNTF or LIF, increased neuregulin mRNA and protein levels in the spinal cord neurons [30]. Both in vitro [3, 20] and in vivo [12, 33, 50], the application of BDNF, NT-3 and NT-4 are equally effective for motoneuron survival, although why is not yet known. In the rat transected sciatic nerve, BDNF and NT-3 mRNA are markedly upregulated in the gastrocnemius muscle, the sciatic nerve and in the spinal cord, but NT-4 protein and mRNA levels are rapidly decreased. We speculate that NT-4 in spinal cord interacts inefficiently with TrkB under denervated conditions, even if NT-4 is abundant, as little TrkB was co-precipitated with NT-4 even when TrkB autophosphorylation was maximally activated after axotomy.

Neurotrophin is a potential target of therapeutic agents for muscular disorders such as amyotrophic lateral sclerosis (ALS) [19, 32]. Uregulation of CNTF receptor alpha in the muscle of ALS patients [48] may be indicative of a higher sensitivity to CNTF. On the other hand, the injection of NT-3 is thought to induce selective survival and sprouting of nerve-connecting-type IIB fibers [43]. We do not know whether NT-4 selectively acts on the nerve and/or motoneurons innervating type I, IIA, IID and IIB fibers. However, as suggested in this study, injection of NT-4 may act negatively on the repair of damaged neuromuscular junctions. Whether such a selective effect of NT-4 occurs in muscle fibers remains to be elucidated.

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