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Neurotrophin 4/5 is required for the normal development of the slow muscle fiber phenotype in the rat soleus

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Summary

During normal postnatal development, rat soleus (SOL) muscle fibers undergo a dramatic fast-to-slow myosin heavy chain (MyHC) isoform transformation. We exploited this phenomenon to evaluate the role of neurotrophin 4/5 (NT-4/5) in the regulation of muscle fiber phenotype. Intramuscular injections of recombinant NT-4/5 into the SOL muscle of rat neonates significantly accelerated the normal fast-to-slow MyHC isoform transformation. Sequestration of endogenous NT-4/5 with TrkB–IgG prevented this transformation from occurring. Administration of the other TrkB ligand, brain-derived neurotrophic factor (BDNF), did not affect the normal course of the MyHC isoform transformation in this muscle, indicating that the observed effect is NT-4/5 specific. Botulinum toxin blockade of synaptic transmission significantly disrupted the normal fast-to-slow MyHC isoform switch. Because administration of NT-4/5 to paralyzed muscles failed to restore the normal course of this MyHC transformation, we believe that the effect of NT-4/5 is not directly on the muscle fibers but that it probably activates or forms a type of retrograde signal to motoneurons. The developmental upregulation of NT-4/5 mRNA in rat SOL muscle fibers occurred earlier than the upregulation of MyHC I/β mRNA associated with muscle fiber transformation. This timing is consistent with the idea that NT-4/5 is involved in early events that lead to the upregulation of the slow MyHC isoform in this muscle.

Key words: neurotrophin, development, skeletal muscle, myosin heavy chain, synaptic transmission, rat.

Introduction

The properties of motoneurons and the properties of the muscle fibers that they innervate are remarkably well matched (Burke, 1981; Mendell et al., 1994; Zengel et al., 1985). Because of this matching, it is believed that the properties of these two types of cells are specified as a consequence of an interaction between them (Mendell et al., 1994). Naturally occurring changes in muscle fiber phenotype could provide useful models to examine the cellular mechanisms involved in this interaction. During the first 12 weeks of postnatal life, nearly half of the fibers in the rat soleus (SOL) muscle change from predominantly expressing the IIA or fast myosin heavy chain (MyHC) isoform to expressing the I/β or slow MyHC isoform (Wigston and English, 1992). The fact that the MyHC transformation is prevented by hind limb suspension or limb immobilization (Asmussen and Soukup, 1991) indicates that this transformation is activity dependent.

It is well known that motoneurons regulate the mechanical and biochemical properties of their muscle fibers by means of their activity (Ausoni et al., 1990; Pette and Vroba, 1985; Salmons and Vroba, 1969; Windisch et al., 1998). Based on the above, it is likely that the MyHC switch in the neonatal rat SOL is a direct consequence of the phasic-to-tonic transformation that occurs in the activity pattern of SOL motoneurons during the first weeks of postnatal development (Brocard et al., 1999; Navarrette and Vrbova, 1993; Vinay et al., 2000b). The patterns of activity of motoneurons are determined both by their synaptic inputs and their intrinsic properties (Binder et al., 1996), and both of these determinants can be regulated by activity-dependent muscle-derived factors (Czéh et al., 1978; Mendell et al., 1994). It is possible, therefore, that the MyHC transformation in the neonatal rat SOL, although directly mediated by the activity-dependent switch in the pattern of activity of SOL motoneurons, might be indirectly determined by factors released by the SOL muscle fibers as a result of the increased activity. The identity of the factor(s) has not yet been determined.

Neurotrophins are well-known retrograde signaling molecules (Curtis et al., 1995; Koliastos et al., 1994) that regulate differentiation and cell survival in many central and peripheral neurons. Three neurotrophins – brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5) – are expressed in skeletal muscle (Funakoshi et al., 1995; Griesbeck et al., 1995). These neurotrophins exert their effect through two classes of cell surface receptors: p^{75NTR} (p^{75}) and a related family of tyrosine protein kinase receptors referred to as Trks (Barbacid, 1994). In addition to their effect on cell survival, neurotrophins play a significant role in the modulation of neuronal plasticity

(Berardi et al., 1994; McAllister et al., 1999; Thoenen, 1995), synaptic efficacy (Wang and Poo, 1998) and neuronal excitability (Kafitz et al., 1999). Although all of these molecules can be considered potential candidates for retrograde specification of motoneuron properties and their synaptic inputs, NT-4/5 is particularly attractive. The mRNA for NT-4/5 increases significantly in muscle during postnatal development, whereas the mRNAs for BDNF and NT-3 are significantly reduced (Funakoshi et al., 1995; Griesbeck et al., 1995). In adult rats, electrically evoked muscle activation significantly increases NT-4/5 mRNA in the SOL in a voltage-dependent manner (Funakoshi et al., 1995).

In the present study, we have exploited the naturally occurring MyHC isoform switch that occurs in the postnatal rat SOL to evaluate the role of NT-4/5 in the regulation of muscle fiber phenotype. One prediction of our hypothesis is that increased levels of NT-4/5 should accelerate the fast-to-slow transformation and a decreased availability of this neurotrophin should block or reverse the MyHC isoform switch. Some of these data have been published in an abstract form (Carrasco and English, 2001).

Materials and methods

Animals and procedures

Fisher 344 rat (*Rattus norvegicus* L.) neonates were used. All experiments were carried out in accordance with the Institutional Animal Care and Use Committee of Emory University. For the NT-4/5 experiments, rats were injected with 10 μl of 0.9% NaCl (saline) containing 1.5 μg of human recombinant NT-4/5 (a generous gift of Regeneron Pharmaceuticals Inc., Terrytown, NY, USA). Before each injection, the rats were placed in a chamber containing a mixture of room air and halothane. This procedure provided a light plane of anesthesia in the animal that allowed the time necessary to perform the injections without any animal distress. The rats did not show any sign of harm after the injections. Injections were made through the skin, along the dorsolateral aspect of the left hind limb, at the line of demarcation between the lateral gastrocnemius and the soleus muscle. Small aliquots of the 10 μl volume were deposited at three different locations as the needle was withdrawn. To decrease the availability of endogenous TrkB ligands, rats were injected with 10 μl of saline containing 15 μg of TrkB–IgG (Regeneron Pharmaceuticals Inc). Control animals were injected with 10 μl of saline. Injections were made into the left triceps surae (medial + lateral gastrocnemius, soleus) twice a week starting at postnatal day 2. Rats in the NT-4/5-treated and control groups were euthanized at the end of the second, fourth and sixth weeks of postnatal life. Rats in the TrkB–IgG-treated group were euthanized at the end of the fourth and sixth weeks of postnatal life. To block neuromuscular signaling, rats were injected with 10 μl of saline containing 8 ng of botulinum toxin A (BTX; Sigma, St Louis, MO, USA), either alone or with 1.5 μg of human recombinant NT-4/5. In both cases, BTX injections were made into the left triceps surae on postnatal day 8. In some animals, a second injection of BTX was performed on postnatal day 10 in order to obtain full paralysis of the limb. When NT-4/5 was administered, injections were performed as described for the NT-4/5 experiments above, starting at postnatal day 8. Rats in the BTX and BTX + NT-4/5 experiments were euthanized at the end of the fourth week of postnatal life. The biological activity of the neurotrophins and TrkB–IgG was determined by Regeneron Pharmaceuticals Inc. The triceps surae muscles from all the animals were harvested by dissecting the muscles from their tendons of origin and insertion, arranging them on a thin piece of polystyrene foam and quick-freezing them in isopentane that had been cooled to its freezing point in a liquid nitrogen bath. The muscles were stored at –50°C until processed for immunohistochemistry.

RNA isolation and RT-PCR

Total RNA was extracted from SOL muscles of two groups of untreated rats at postnatal ages of 4 days, 7 days, 14 days, 21 days and 28 days with Trizol® reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was dissolved in 20 μl of diethyl pyrocarbonate (Sigma)-treated H_2O. Because SOL muscles from 4-day-old rats were so small, the muscles from the animals at this age were pooled. RNA concentration was quantified by determining absorbance at a wavelength of 260 nm and using an OD_{260} unit equivalent to 40 μg ml^{-1}. 2 μg of total RNA was reverse transcribed into complementary DNA (cDNA) with the SuperScript™ Preamplification Sytem kit (Life Technologies) according to the manufacturer's instructions. Negative controls were performed in the absence of reverse transcription. 10 μl of the cDNA solution was amplified by means of polymerase chain reaction (PCR) in the presence of oligonucleotide primers specific to either NT-4/5, MyHC I/β or MyHC IIA in conjunction with 18S rRNA primers and competimers (Ambion, Austin, TX, USA). The 18S rRNA and competimers were used as an internal control for each sample. The NT-4/5 cDNA fragment (248 bp) corresponding to nucleotides 467–715 of the rat cDNA sequence (GenBank M86742) was synthesized with the following primers: sense, 5′-CCCTGCGTCAGTACTTC-TTCGAGAC-3′; antisense, 5′-CTGGACGTCAGGCACGGC-CTGTTC-3′. The MyHC I/β and MyHC IIA fragments (288 bp and 310 bp, respectively) were obtained using primers previously described in the literature (Jaschinski et al., 1998). MyHC I/β: sense, 5′-ACAGAGGAAGACAGGAAGAACC-TAC-3′; antisense, 5′-GGGCTTCACAGGCATCCTTAG-3′. MyHC IIA: sense, 5′-TATCCTCAGGCTTCAAGATTTG-3′; antisense, 5′-TAAATAGAATCCATGGGGACA-3′. The linear portion of the amplification curve for each transcript was defined and then utilized to determine the appropriate number of cycles for their amplification. Amplification conditions for the NT-4/5-specific primer pair were as follows: 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, followed by extension at 72°C for 10 min. Amplification conditions for the MyCH I/β- and MyHC IIA-specific primer pairs were as follows: 3 min at 94°C followed by 27 cycles of 94°C for 45 s, 55°C for 1 min and 72°C for

1 min, followed by extension at 72°C for 10 min. PCR reactions were performed three times on each sample. To quantify amplicons, PCR products were resolved in 1.5% agarose–ethidium bromide gel, visualized and photographically recorded with a UV documentation station (FOTO/Phoresis®; Fotodyne® Inc., Hartland, WI, USA). The optical density of the amplicons for NT-4/5, MyHC I/β, MyHC IIA and 18S rRNA were determined using the Scion Image software (Frederick, MD, USA). Relative mRNA expression was determined by dividing the optical density of each amplicon by the optical density of their corresponding 18S rRNA amplicon.

Immunohistochemistry

Serial 10 μm cross-sections from each triceps surae, which included the widest portion (medial) of the soleus muscle, were cut in a cryostat and placed onto subbed microscope slides. At least eight fields of view were taken from each cryosection and used for the data analysis. The fields were taken from the most lateral to the most medial portion of the cryosection following a zigzag pattern (Fig. 1). Thus, they covered very different areas of the entire cryosection. Initially, sections were incubated for one hour in a 0.1 mol l^{-1} phosphate-buffered saline (PBS) solution containing 2% normal goat serum and 0.03% Triton X-100 and then in PBS containing primary antibody BA-D5 overnight at 4°C. Antibody BA-D5 is specific for the I/β or slow MyHC isoform (Schiaffino et al., 1989). Sections were washed in PBS and incubated in a peroxidase-conjugated goat anti-mouse secondary antibody (Cappel Research Products, Aurora, OH, USA) for 1 h at room temperature, followed by a standard diaminobenzidine reaction for the demonstration of peroxidase. To better characterize the fast-to-slow MyHC transition, sections were double-stained with antibody A4.840 and MY-32. Antibody A4.840 is specific for I/β or slow MyHC isoform (Hughes et al., 1993), and antibody MY-32 recognizes the neonatal and all of the adult fast MyHC isoforms (Harris et al., 1989). After an overnight incubation at 4°C in PBS containing both antibodies, sections were washed in PBS and incubated in a biotin-conjugated goat anti-mouse IgG secondary antibody (Cappel Research Products) for 1 h at room temperature. Following a wash in PBS, sections then were incubated with Texas Red®-conjugated streptavidin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA) for 1 h. After a wash in PBS, sections were incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM secondary antibody (Jackson Immunoresearch Laboratories, Inc.) for 1 h at room temperature. By using the double-staining protocol, we were able to differentiate between fibers containing the slow MyHC only (slow; FITC-labelled) or fast MyHC only (fast; Texas Red-labelled) from fibers containing both isoforms (hybrid). Images of double-stained sections were acquired using appropriate epifluorescence illumination *via* a cooled CCD camera (Optronics, Goleta, CA, USA) and captured using the Scion Image software.

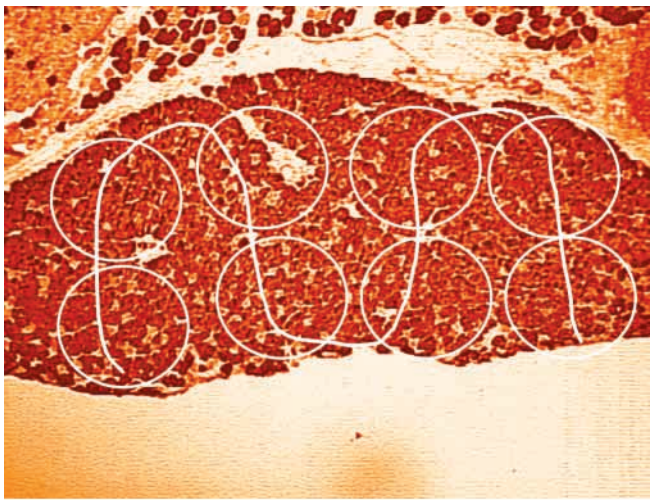

Fig. 1. Sampling scheme used to acquire the fields for data analysis (see Materials and methods). Figure shows a cryosection incubated in antibody BA-D5 from a neurotrophin 4/5 (NT-4/5)-treated soleus muscle of a 4-week-old pup.

Data analysis

The number of fibers that react positively with each antibody as well as those stained positively with both antibodies (double stained) were counted and expressed as a percentage of the total number of fibers in the field. A minimum of 250 fibers was analyzed from each muscle. Two-way (treatment *X* phenotype) contingency tables, using the original counts, were used to evaluate whether differences in fiber type proportions observed between groups were the result of the different treatment conditions rather than differences in sample sizes. Except for the 2-week data, all the contingency table analyses provided significantly greater values than 9.21, which is the critical χ^2 value for α=0.01 and d.f.=2. Thus, the differences in fiber type proportions observed between the groups are indeed due to the different treatments and not due to sampling error. Differences in fiber phenotype proportions between SOL muscles from treated and control groups were determined using a two-way (treatment *X* phenotype) analysis of variance (ANOVA). *Post hoc* comparisons were performed using the Fisher least significant difference (LSD) test. The α level of significance $P<0.05$ was used for all comparisons.

Results

Effect of exogenous NT-4/5 on MyHC isoform content

The main purpose of this study was to determine whether NT-4/5 is involved in the regulation of muscle fiber phenotype in the rat SOL. Rats were treated by intramuscular injections of recombinant NT-4/5 twice a week starting at postnatal day 2. In a first set of experiments, the untreated contralateral SOL muscle of each rat was used as a control. In the second set of experiments, the SOL from saline-treated rats served as controls. In all groups, rats were euthanized at the end of the second, fourth and sixth weeks of postnatal life. The

proportions of slow fibers in untreated rat SOL muscles increased with age. At 6 weeks of age, SOL muscles contained significantly more slow fibers than at 4 weeks of age, and at 4 weeks of age, SOL muscles contained significantly more slow fibers than at 2 weeks of age (Fig. 2A). This finding is similar to that previously reported by Wigston and English (1992). At 2 weeks of age, the differences in the proportions of slow fibers and fast fibers between the NT-4/5-treated and contralateral untreated SOL were not statistically significant (Fig. 2A). By 4 weeks and 6 weeks of age, however, the proportion of slow fibers was significantly greater and the proportion of fast fibers was significantly lower in the NT-4/5-treated SOL compared with the contralateral untreated SOL (Fig. 2A–E). Based on the analysis of the double-stained muscle sections, we find that at 2 weeks of age the proportion of fast fibers, slow fibers and hybrid fibers was not significantly different between the NT4/5-treated and control animals (data not shown). By 4 weeks of age, however, SOL muscles from NT-4/5-treated animals contained significantly more hybrid fibers and fewer fast fibers than SOL muscles from control animals (Figs 3, 4A). At 6 weeks of age, the proportion of slow fibers was significantly greater and the proportion of fast fibers was significantly lower in the NT-4/5-treated SOL than in the saline-treated SOL (Fig. 4B). Thus, the increase in the number of slow fibers, described above based on the immunoperoxidase reaction, in the NT-4/5-treated SOL at 4 weeks, is due primarily to an increase in the number of hybrid fibers. At 6 weeks of age, this upregulation is mainly due to an increase in the number of slow fibers. At both ages, the lower number of fibers observed with the NT-4/5 treatment that did not react with the anti-slow antibody is due primarily to a reduction in the number of fast fibers.

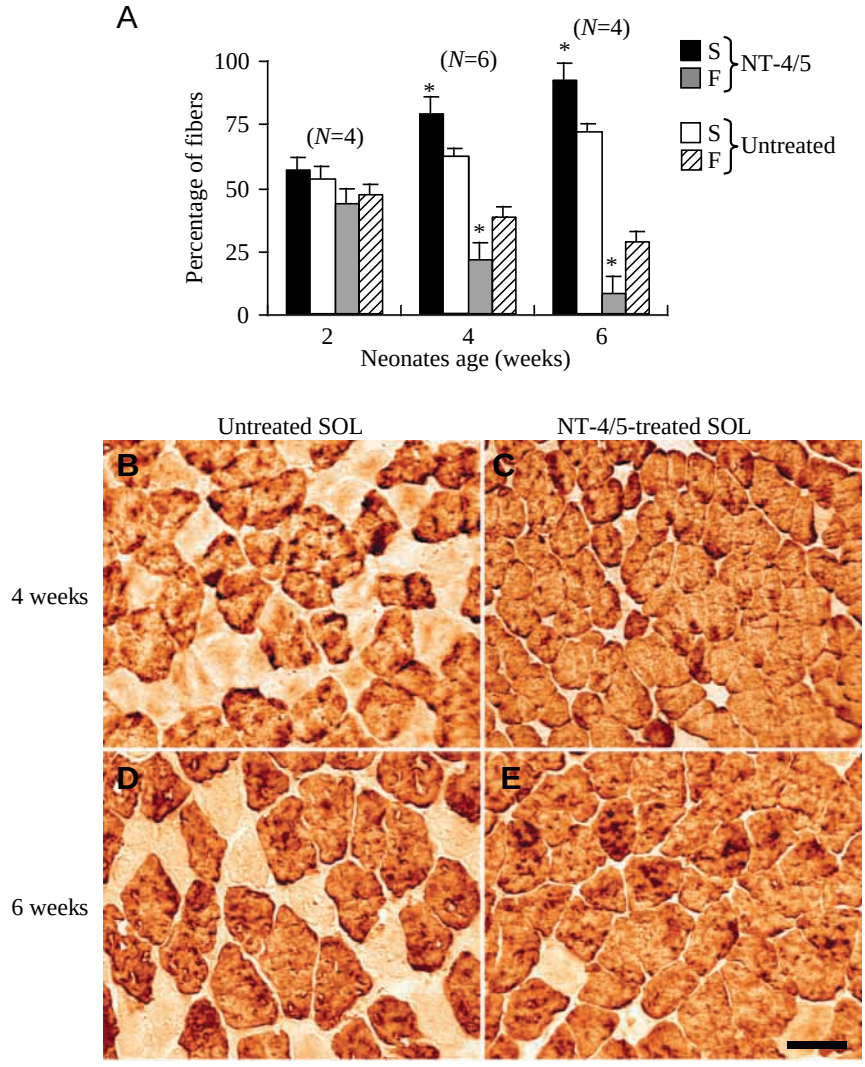

Fig. 2. (A) Percentage of BA-D5-immunopositive fibers (slow) and unstained fibers (fast) is shown for 2-, 4- and 6-week-old neurotrophin 4/5 (NT-4/5)-treated soleus and untreated contralateral soleus muscle. Values represent means ± S.E.M. *=P<0.05 *vs* untreated. (B–E) Representative images taken from histological sections of rat soleus muscle obtained at 4 weeks and 6 weeks of age: untreated contralateral soleus (B,D); NT-4/5-treated soleus (C,E). Each section was stained with monoclonal antibody BA-D5, which is specific to the slow or I/β myosin heavy chain (MyHC) isoform. Immunopositive fibers were visualized with peroxidase-conjugated goat anti-mouse secondary antibody. Scale bar, 50 μm.

Effect of reducing availability of endogenous NT-4/5 on MyHC isoform content

The simplest explanation of these findings might be to hypothesize that the fast-to-slow MyHC isoform transformation normally observed in rat SOL during development might be mediated by endogenous NT-4/5. To evaluate this hypothesis, we decreased the availability of endogenous TrkB ligands to the SOL muscle by injecting rats with TrkB–IgG, a recombinant protein that binds to both NT-4/5 and BDNF. Injections followed the same time course as that used for NT-4/5. Since the MyHC content in SOL muscles from NT-4/5- and saline-treated rats was not different at 2 weeks of age, animals in the TrkB–IgG groups were euthanized only at the end of the fourth and sixth weeks of postnatal life. Treatment with TrkB–IgG blocked or attenuated the fast-to-slow MyHC transformation normally observed in the SOL of rat neonates. At 4 weeks of age, SOL muscles from TrkB–IgG-treated animals contained significantly less slow fibers and significantly more fast fibers than SOL muscles from control animals (Figs 3, 4C). At 6 weeks of age, SOL muscles from TrkB–IgG-treated animals contained significantly fewer fast and slow fibers and significantly more hybrid fibers than SOL muscles from controls animals (Fig. 4D). The fact that the percentage of slow fibers was

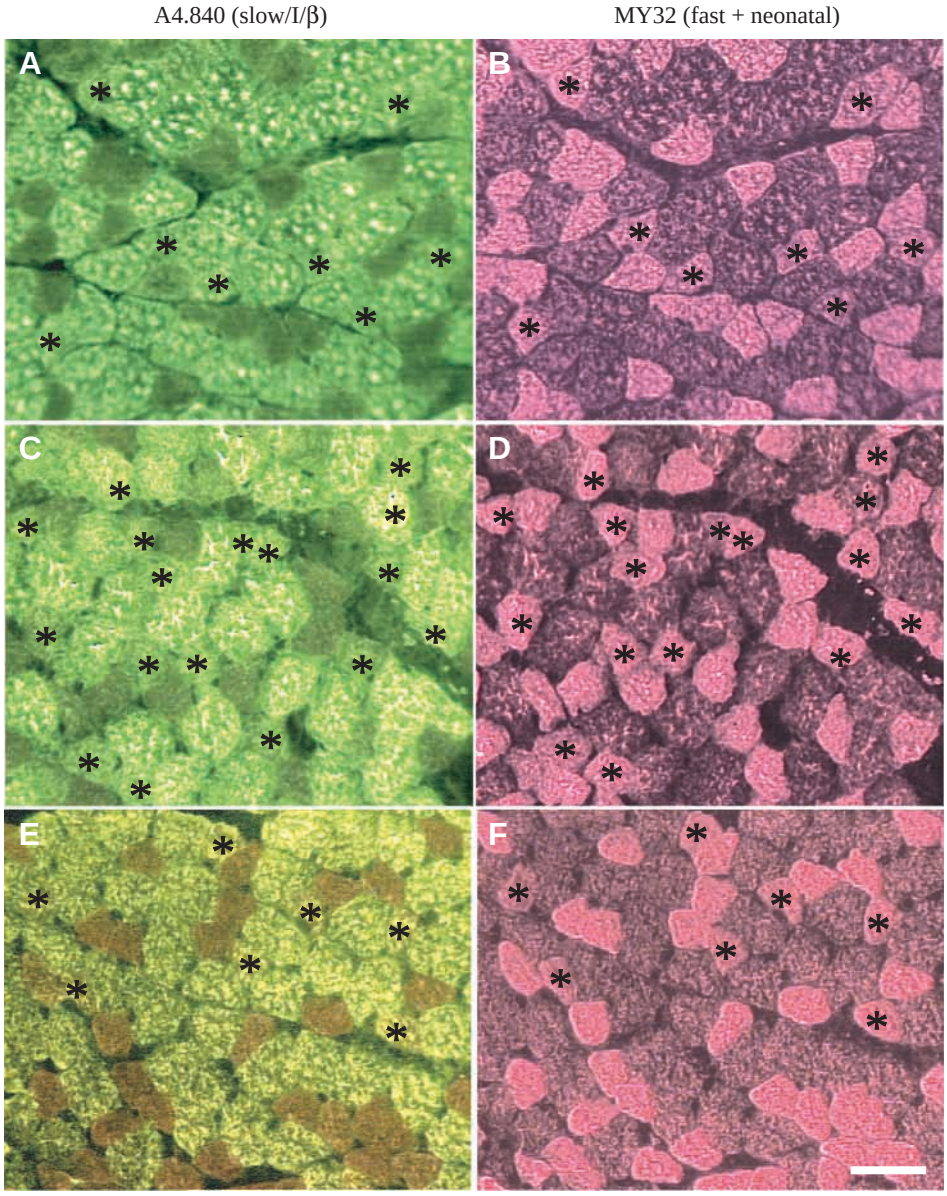

Fig. 3. Representative images taken from histological sections of soleus muscle obtained at 4 weeks of age from rats injected with (A,B) saline, (C,D) neurotrophin 4/5 (NT-4/5) and (E,F) TrkB–IgG. Each section was double-stained with monoclonal antibodies A4.840 (I/β) and MY-32 (fast and neonatal). The A4.840-immunopositive fibers were visualized with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (A,C,E), and the MY-32-immunopositive fibers were visualized with a Texas Red®-conjugated streptavidin secondary antibody (B,D,F). Asterisks mark double-stained fibers. Scale bar, 50 μm.

reduced below 50%, which is lower than the proportion of slow fibers normally found in the rat soleus at 1 week of age (Wigston and English, 1992), clearly indicates that endogenous NT-4/5 is required by SOL muscle fibers to express the slow phenotype. The increase in the population of hybrid fibers, therefore, comes at the expense of a significant number of slow fibers, which, due to the reduced levels of endogenous NT-4/5, are now also expressing the fast MyHC.

Effect of exogenous BDNF on MyHC isoform content

To determine whether the acceleration of fast-to-slow MyHC isoform switch in developing SOL muscle observed with NT-4/5 treatment is specific to NT-4/5 and is not a general effect of the TrkB ligands, five animals were injected with 15 μg of BDNF, as described above, and euthanized at the end of the fourth week of postnatal life. We think that this dose is appropriate because a similar concentration of BDNF injected into the gastrocnemius muscles of adult rats was shown to increase both the catalytic activity and the phosphorylation state of Trk receptors (Bhattacharyya et al., 1997). The number of fast, hybrid and slow fibers was not significantly different between BDNF- and saline-treated rats (Fig. 5).

Analysis of NT-4/5 and MyHC isoform mRNA

If NT-4/5 is indeed involved in the fast-to-slow MyHC transformation of the SOL muscle, one would predict that the upregulation of this neurotrophin should occur before the upregulation of the slow MyHC isoform. To test this possibility, RNA extracted from SOL muscles of rats of different postnatal ages was reverse transcribed and submitted to PCR using oligonucleotide primers specific to NT-4/5, MyHC I/β and MyHC IIA. Consistent with the prediction, the upregulation of NT-4/5 mRNA occurred significantly earlier (approximately 2 weeks earlier) during postnatal development than did the upregulation of MyHC I/β mRNA (Fig. 6A,C,D). MyHC IIA mRNA was significantly upregulated by postnatal day 14 and was followed by a continuous downregulation during the rest of the study period (Fig. 6B,D). The patterns of expression of both MyHC mRNAs are consistent with the normal developmental MyHC isoform transitions that occur in the rat SOL during this period of time. During the first week of life, approximately half of the fibers in the rat SOL contain embryonic and neonatal MyHCs. After that, the majority of these fibers start to express only MyHC IIA, and a subset of these fibers begin to express MyHC I/β by 4 weeks of age (Butler-Brown and Whalen, 1984).

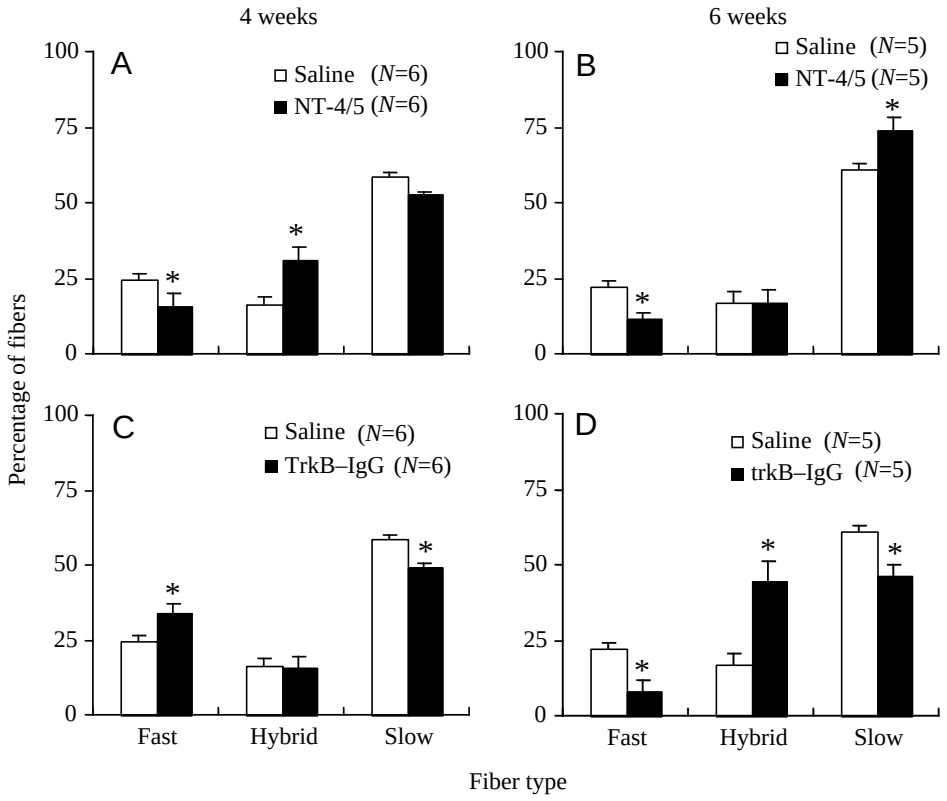

Fig. 4. Neurotrophin 4/5 (NT-4/5) accelerates the fast-to-slow myosin heavy chain (MyHC) isoform switch in neonatal rat soleus and its signaling is required for the normal development of the soleus muscle fiber phenotype. The percentage of fibers expressing fast MyHC only (fast), both fast and slow MyHC simultaneously (hybrid) and slow MyHC only (slow) are shown for 4-week-old (A,C) and 6-week-old (B,D) rat soleus muscles treated with saline, NT-4/5 or TrkB–IgG. Values represent means ± S.E.M. *=P<0.05 *vs* saline.

Role of neuromuscular signaling in the effect of exogenous NT-4/5

To evaluate whether neuromuscular signaling is required for the effects of NT-4/5, in one group of rats we blocked synaptic transmission using botulinum toxin (BTX) and in another group of rats, in addition to blocking synaptic transmission, we injected recombinant NT-4/5. Both groups received intramuscular injections of BTX on postnatal day 8. In the group receiving NT-4/5, injections started on postnatal day 8 and were continued twice a week as described above. Rats in both groups were euthanized at the end of the fourth week of postnatal life. Blockade of neuromuscular synaptic transmission with BTX significantly decreased the number of slow and fast fibers and significantly increased the number of hybrid fibers. Treatment of BTX-blocked muscles with NT-4/5 did not significantly alter the changes produced by neuromuscular paralysis (Fig. 7).

Discussion

In the present study, we show that intramuscular injections of recombinant NT-4/5 into the SOL muscle of rat neonates significantly accelerates the fast-to-slow MyHC isoform transformation that normally takes place in this muscle during development. The acceleration of the MyHC switch is not due to a systemic effect of NT-4/5 because it occurs in the treated but not in the contralateral SOL. The observed effect is NT-4/5 specific, since BDNF, the other TrkB ligand, does not affect the normal course of this transformation. More importantly, because sequestration of endogenous NT-4/5 with TrkB–IgG prevented the transformation from occurring, we believe that muscle-derived NT-4/5 is required for the normal development of rat SOL muscle fiber phenotypes.

It is well known that motoneurons regulate the mechanical and biochemical properties of their muscle fibers by means of their activity (Ausoni et al., 1990; Pette and Vroba, 1985; Salmons and Vroba, 1969; Windisch et al., 1998). Early in rat postnatal development, approximately 50% of the

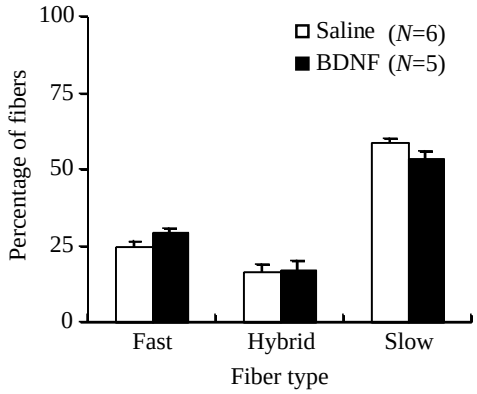

Fig. 5. Brain-derived neurotrophic factor (BDNF) does not affect the fast-to-slow myosin isoform switch in neonatal rat soleus. The percentage of fast, hybrid and slow fibers is shown for 4-week-old rat soleus muscles treated with saline (open bars) and BDNF (filled bars). Values represent means ± S.E.M. Differences are not statistically significant at P<0.05.

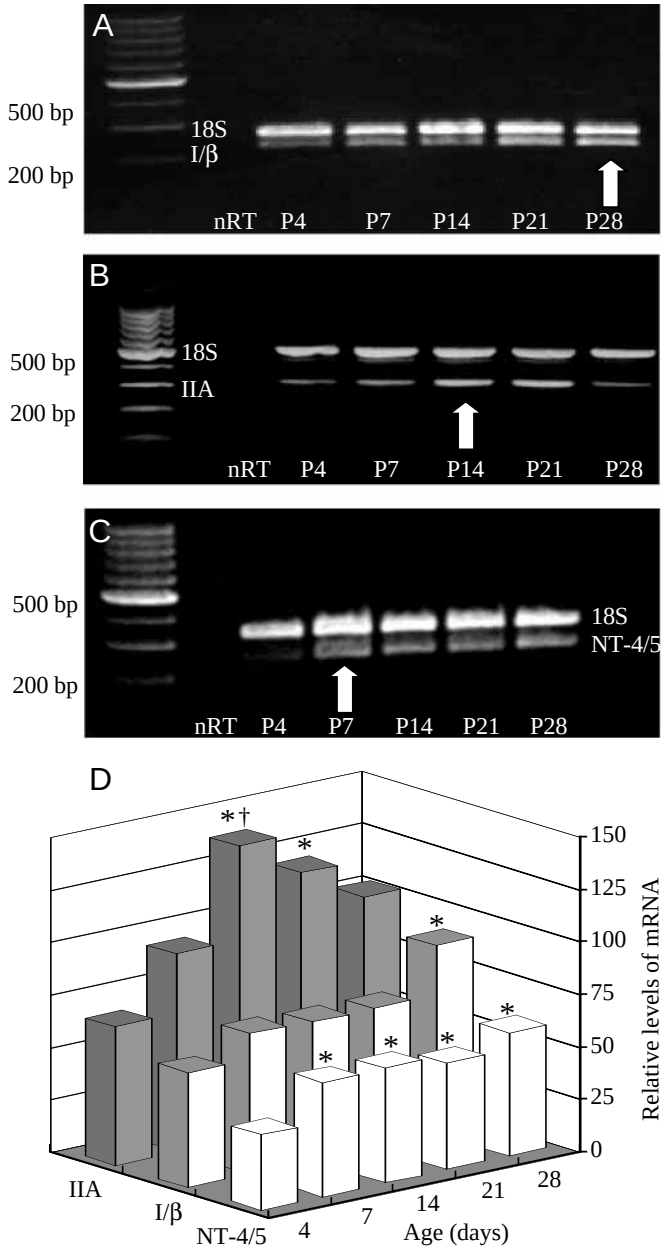

Fig. 6. Neurotrophin 4/5 (NT-4/5) mRNA in rat soleus becomes upregulated earlier during postnatal development than do the myosin heavy chain (MyHC) I/β (slow) and MyHC IIA (fast) mRNAs. PCR products of reverse-transcribed RNA extracted from soleus muscles (four at each age) using primer pairs for the 18S rRNA and competimers in conjunction with primer pairs for either (A) MyHC I/β, (B) MyHC IIA or (C) NT-4/5 at postnatal age 4 days (P4), 7 days (P7), 14 days (P14), 21 days (P21) and 28 days (P28). Arrows show the postnatal ages at which increases in MyHC I/β, MyHC IIA and NT-4/5 mRNA synthesis occur. Note that a different set of primer pairs for 18S rRNA and competimers (product size 489 bp) was used to visualize the MyHC IIA product (310 bp). nRT = no reverse transcription. (D) The relative levels of MyHC I/β, MyHC IIA and NT-4/5 mRNAs. Values represent means ± S.E.M. *=P<0.05 *vs* day 4; †=P<0.5 *vs* day 7.

motoneurons innervating the triceps surae muscle are unable to generate more than a single action potential. By the second week of life, however, the majority of these motoneurons are able to fire repetitively for prolonged periods of time (Brocard et al., 1999; Navarrette and Vrbova, 1993; Pflieger et al., 2002; Vinay et al., 2000a). Based on the above, we believe that the changes in MyHC isoform expression that occur in the neonatal rat SOL are a direct consequence of the change in pattern of activity of the motoneurons that occurs during this developmental period.

The gradual acquisition of the repetitive pattern of discharge of developing motoneurons has been attributed to the development of synaptic inputs onto them and the maturation of their membrane intrinsic properties (Brocard et al., 1999; Navarrette and Vrbova, 1993). Since both the synaptic and intrinsic properties of motoneurons can be regulated retrogradely by activity-dependent muscle-derived factors (Czéh et al., 1978; Mendell et al., 1994), all the muscle-derived neurotrophins could influence motoneuron activity and thereby muscle fiber phenotype. We decided to focus on the role of NT-4/5 on the determination of SOL muscle fiber phenotype for the following reasons. First, the mRNA for NT-4/5, but not for BDNF or NT-3, is upregulated in the muscle during postnatal development (Funakoshi et al., 1995; Griesbeck et al., 1995). Second, this upregulation occurs during the first weeks of postnatal development (Funakoshi et al., 1995) and, as demonstrated in the present study, it occurs significantly earlier than the upregulation of MyHC I/β mRNA associated with muscle fiber transformation. This difference in gene transcription timing is consistent with the idea that NT-4/5 is involved in early events that lead to the upregulation of the slow MyHC isoform in this muscle. Third, NT-4/5 synthesis by muscle fibers is modulated by electrically evoked muscle activity in a dose-dependent manner (Funakoshi et al., 1995). Finally, the fact that treatment with ciliary neurotrophic factor

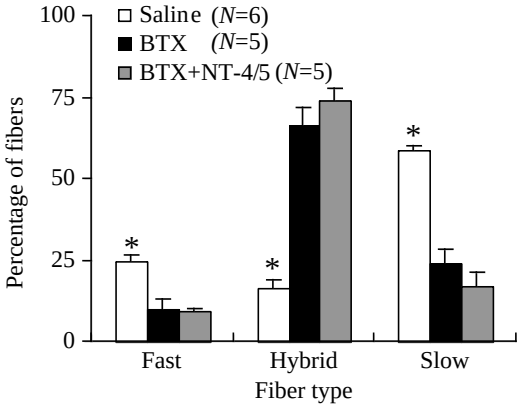

Fig. 7. Neurotrophin 4/5 (NT-4/5) requires normal neuromuscular synaptic transmission to exert its effect on the neonatal rat soleus. The percentage of fast, hybrid and slow fibers is shown for 4-week-old rat soleus treated with saline, botulinum toxin (BTX) alone and BTX+NT-4/5. Values represent means ± S.E.M. *=P<0.05 *vs* BTX and BTX+NT-4/5.

(CNTF) + NT-4/5, but not with CNTF + NT-3, attenuates the reduction of the proportion of slow fibers that occurs in the rat SOL after sciatic nerve crush (Mousavi et al., 2002) provides additional support that this neurotrophin is involved in the determination of the slow muscle phenotype in the rat SOL.

In the present study, the normal fast-to-slow MyHC isoform switch was disrupted in BTX-treated muscles, and exogenous administration of NT-4/5 failed to restore the normal course of this transformation. Based on this finding, and the fact that NT-4/5 is retrogradely transported by developing motoneurons and adult sensory neurons (Curtis et al., 1995; Koliastos et al., 1994), we believe that the effect of NT-4/5 is not directly on the muscle fibers but that it forms or activates a type of retrograde signal to motoneurons. It is possible that our NT-4/5 treatment enhanced the change in synaptic strength occurring at the Ia/motoneuron synapses during this period (Seebach and Mendell, 1996) and, *via* this mechanism, accelerated the acquisition of the repetitive pattern of discharge of SOL motoneurons and, consequently, the fast-to-slow MyHC transformation in the SOL muscle (Fig. 8A). However, we believe that this is unlikely. Seebach et al. (1999) show that treatment with BDNF depresses and TrkB–IgG increases excitatory postsynaptic potential (EPSP) amplitude at Ia/motoneuron synapses during postnatal development. If changes in the pattern of activity of SOL motoneurons leading to the MyHC isoform transformation in rat SOL muscle fibers during postnatal development were produced by an increase in synaptic strength, then one would have predicted that BDNF would prevent and TrkB–IgG would have accelerated the transformation. Instead, BDNF did not affect its normal course and TrkB–IgG prevented or even reversed it. Therefore, we believe that NT-4/5 exerts its effect indirectly on SOL muscle fiber MyHC isoform expression by mechanisms other than increasing synaptic strength. In the study of Seebach et al. (1999), neither input resistance nor rehobase were altered after the treatment with BDNF or NT-3. However, the fact that treatment with TrkB–IgG in the same rats significantly increased rehobase indicates that this property is influenced by NT-4/5, the other TrkB ligand. Based on the above, it is possible that our NT-4/5 treatment influenced some of the membrane intrinsic properties of SOL motoneurons and, *via* this mechanism, accelerated the acquisition of the repetitive

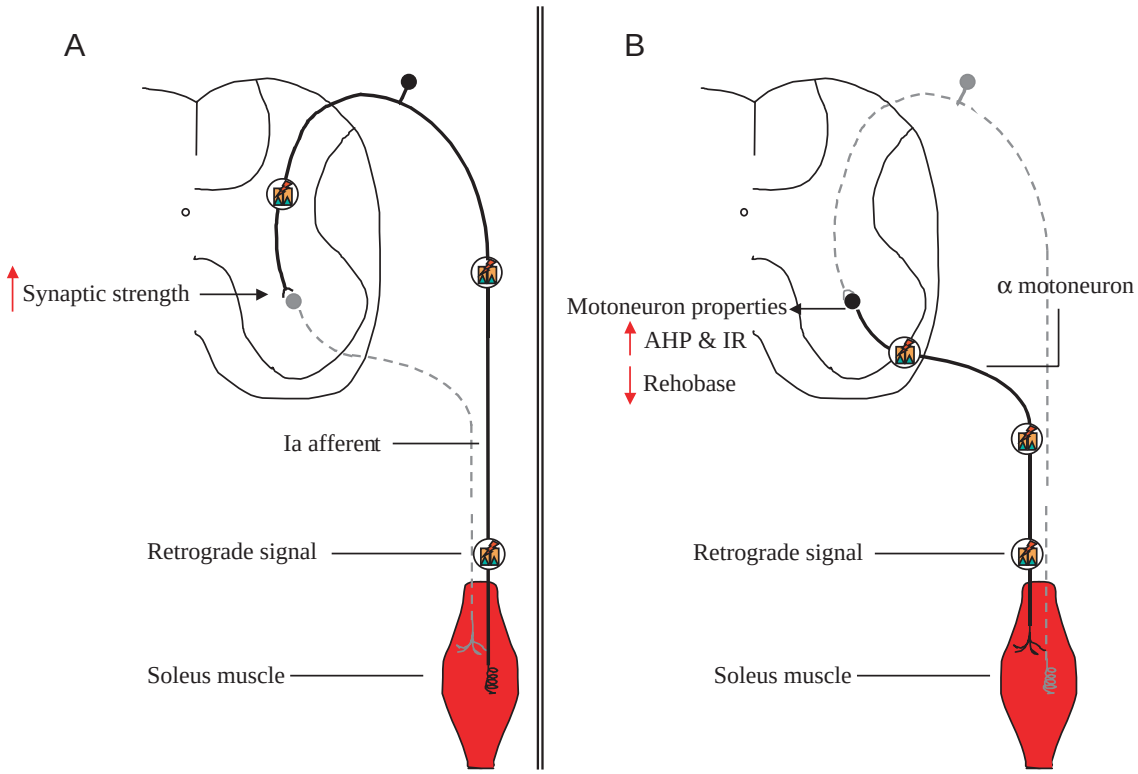

Fig. 8. Hypothetical models of retrograde regulation of soleus (SOL) motoneuron properties by neurotrophin 4/5 (NT-4/5). (A) Following injection in the muscle, NT-4/5 forms or activates a retrograde signal that is transported by primary afferent (Ia) neurons from muscle spindles. This signal influences cellular mechanisms involved in the determination of the properties of the synapses of these afferent neurons onto SOL motoneurons and, *via* this mechanism, accelerates the acquisition of the repetitive pattern of discharge of SOL motoneurons and, consequently, the fast-to-slow myosin heavy chain (MyHC) transformation in the SOL muscle. (B) Alternatively, following injection in the muscle, NT-4/5 forms or activates a retrograde signal that is transported by SOL motoneurons. This signal influences cellular mechanisms involved in the maturation of intrinsic SOL motoneuron properties, i.e. rehobase, afterhyperpolarization (AHP) or input resistance (IR), and, *via* this mechanism, accelerates the acquisition of the repetitive pattern of discharge of SOL motoneurons and, consequently, the fast-to-slow MyHC transformation in this muscle. ↑ = increase; ↓ = decrease.

pattern of discharge of SOL motoneurons and, consequently, the fast-to-slow MyHC transformation in this muscle (Fig. 8B).

Administration of NT-4/5 and BDNF into the rat SOL produced very different effects. When compared with controls, NT-4/5 accelerated the fast-to-slow MyHC isoform transformation whereas BDNF did not alter the course of this transformation. Several mechanisms could be responsible for the different effects produced by NT-4/5 and BDNF. The binding of these two neurotrophins might promote different conformational changes in the receptor and different downstream signaling. Point mutation of the Shc-binding site of the TrkB receptor mainly affects NT-4/5 signaling *in vivo* and *in vitro* without producing major effects on BDNF signaling (Fan et al., 2000; Minichiello et al., 1998). The differential effectiveness of NT-4/5 and BDNF could also be related to the subcellular location at which they bind and activate the TrkB receptor. Signaling pathways activated by the internalization and activation of the Trk receptor at the nerve terminals differ from those activated at the cell bodies (Watson et al., 2001). Finally, the fact that NT-4/5- and BDNF-mutant mice exhibit significantly different phenotypes (Conover, 1995) is a clear demonstration that endogenous NT-4/5 and BDNF perform different functions *in vivo*.

In conclusion, we have provided evidence demonstrating that NT-4/5 is required for the normal development of the slow muscle fiber phenotype of the rat SOL. Based on the results from previous studies and the present study, we believe that muscle-derived NT-4/5 probably forms or activates a retrograde signal that influences the pattern of activity of SOL motoneurons and, *via* this mechanism, leads to changes that promote the upregulation of the slow MyHC isoform in the SOL muscle. We cannot entirely rule out a role of NT-4/5 of motoneuronal origin (Buck, 2000) as a source of developmental changes in motoneuron properties or synaptic inputs. However, the fact that sequestration of muscle-derived NT-4/5 with TrkB–IgG prevented the SOL MyHC isoform transformation from occurring makes this possibility unlikely.

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