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Neuromuscular Junction Disassembly and Muscle Fatigue in Mice Lacking Neurotrophin-4

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Neurotrophin-4 (NT-4) is produced by slow muscle fibers in an activity-dependent manner and promotes growth and remodeling of adult motorneuron innervation. However, both muscle fibers and motor neurons express NT-4 receptors, suggesting bidirectional NT-4 signaling at the neuromuscular junction. Mice lacking NT-4 displayed enlarged and fragmented neuromuscular junctions with disassembled postsynaptic acetylcholine receptor (AChR) clusters, reduced AChR binding, and acetylcholinesterase activity. Electromyographic responses, posttetanic potentiation, and action potential amplitude were also significantly reduced in muscle fibers from NT-4 knock-out mice. Slow-twitch soleus muscles from these mice fatigued twice as rapidly as those from wild-type mice during repeated tetanic stimulation. Thus, muscle-derived NT-4 is required for maintenance of postsynaptic AChR regions, normal muscular electrophysiological responses, and resistance to muscle fatigue. This neurotrophin may therefore be a key component of an activity-dependent feedback mechanism regulating maintenance of neuromuscular connections and muscular performance.

INTRODUCTION

The neurotrophins are a small group of structurally and functionally related polypeptidic growth factors that regulate cell survival and differentiation in many subpopulations of central and peripheral neurons. Neurotrophins mediate their effects via interaction with two classes of cell surface receptors: the p75 neurotrophin receptor ($p75^{NTR}$), a member of the TNFR superfamily, and the three related receptor tyrosine kinases, TrkA, TrkB, and TrkC (Bibel and Barde, 2000; Friedman and Greene, 1999). Although all neurotrophins can bind to and activate $p75^{NTR}$, their interaction with the Trk receptors is ligand-specific; for example, brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) interact with TrkB, but not with TrkA or TrkC (Ibáñez, 1998). In addition to their effects on cell survival, neurotrophins and Trk receptors regulate plasticity and regeneration responses in both developing and mature central and peripheral neurons (Lewin and Barde, 1996). For several years, the sensory and motor innervation of skeletal muscle fibers has served as one of the most useful models for the study of neurotrophin function in development, plasticity, and regeneration of the nervous system.

The neuromuscular junction is the best studied of all vertebrate synapses. Its development and maturation depend upon the exchange of signals between the nerve terminal and the muscle fiber. The differentiation of the postsynaptic apparatus, including acetylcholine receptor (AChR) synthesis and clustering, is promoted and regulated by nerve-derived molecules such as neuregulin and agrin (Sanes and Lichtman, 1999). On the other hand, muscle-derived signals, including cell adhesion molecules and neurotrophic factors, control the differentiation of presynaptic specializations (Sanes and

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Lichtman, 1999). Several studies indicate that signaling between muscle and nerve may also be required for the ongoing maintenance of neuromuscular junctions during postnatal life and adulthood. Degeneration of muscle fibers is rapidly followed by retraction of nerve terminals from endplates; and blockade of protein synthesis in muscle fibers results in the withdrawal of many terminal branches, indicating that nerve terminals are dependent on the continuous production of trophic proteins by muscle fibers (Sanes and Lichtman, 1999). In contrast, it is still unclear whether extracellular factors are required for the maintenance of the postsynaptic apparatus of neuromuscular junctions (Sanes and Lichtman, 1999).

Neurotrophins and their receptors are expressed by all the cellular components of the neuromuscular system (Funakoshi *et al.*, 1993). Motor neurons express several neurotrophin receptors, including $p75^{NTR}$, TrkB, and TrkC. Neurotrophins promote survival of embryonic motor neurons in culture and *in vivo* after nerve transection (Henderson *et al.*, 1993; Koliatsos *et al.*, 1993, 1994; Oppenheim *et al.*, 1992) and induce maturation and potentiation of developing motor neuron terminals (Wang *et al.*, 1995; Wang and Poo, 1997; Xie *et al.*, 1997). Schwann cells express all members of the neurotrophin family, primarily following lesion-induced activation, and several neurotrophin receptors, including $p75^{NTR}$ and truncated forms of Trk receptors lacking the tyrosine kinase domain (Funakoshi *et al.*, 1993). Finally, muscle fibers also express neurotrophins, as well as $p75^{NTR}$ and a number of truncated Trk receptors (Funakoshi *et al.*, 1993). Intriguingly, localized expression of full-length, catalytically active TrkB receptors in the postsynaptic specializations of neuromuscular junctions has recently been described, suggesting that neurotrophins could also have direct effects on muscle fibers (Gonzalez *et al.*, 1999). Consistent with a trophic role for developing spinal cord motor neurons, muscle-derived neurotrophins are mainly expressed during early stages of muscle development, with decreasing amounts expressed at later times (Funakoshi *et al.*, 1995; Funakoshi *et al.*, 1993). In the adult, nerve transection leads to reexpression of several neurotrophins in muscle fibers, as part of a lesion-induced regeneration response (Funakoshi *et al.*, 1993). An exception to this general rule is NT-4, which, unlike the other neurotrophins, is maximally expressed in adult muscle, where it is enriched in slow-twitch, type I fibers (Funakoshi *et al.*, 1995). Interestingly, NT-4 mRNA levels decrease after blockade of neuromuscular transmission and increase after electrical stimulation and during postnatal development, suggesting a role for this neurotrophin in activity-dependent remodeling and maintenance of adult motor neuron innervation and neuromuscular performance (Funakoshi *et al.*, 1995). In agreement with this notion, intramuscular administration of NT-4 induced sprouting of intact adult motor nerves (Funakoshi *et al.*, 1995).

In this study, we have investigated the effects of the absence of NT-4 on the contractile and electrophysiological properties of adult muscle fibers and the maintenance of adult neuromuscular junctions. Unlike other neurotrophin knock-outs, mice lacking NT-4 develop to adulthood and are fertile, allowing functional studies of this neurotrophin in the adult (Conover *et al.*, 1995). Our results indicate that NT-4 is required for the integrity of postsynaptic AChR regions in the neuromuscular junction, normal muscular electrophysiological responses, and resistance to muscle fatigue, indicating novel roles for this neurotrophin in the maintenance of neuromuscular connections and muscular performance.

RESULTS

Accelerated Muscle Fatigue in Muscles from NT-4 Knock-Out Mice

The observation that NT-4 production is dependent upon muscle activity, together with the known effects of activity on muscular performance, prompted us to investigate contractile parameters in muscle fibers of mice lacking NT-4 (NT-4 KO). Soleus muscle was initially chosen for these experiments because of its high proportion of type I slow-twitch fibers expressing NT-4 (Funakoshi *et al.*, 1995). Contractions were induced in isolated muscles stimulated directly with supramaximal current pulses. The force per gram wet weight was not significantly different in the soleus muscle from NT–4 KO compared to wild type (27.4 ± 0.8 mN / g wet wt vs 27.0 ± 1.3 mN / g wet wt, $n = 10$) or in extensor digitorum longus (EDL), a fast-twitch muscle (26.4 ± 1.9 mN / g wet wt vs 28.7 ± 2.1 mN / g wet wt, $n = 5$). The force–frequency relationship was also similar in knock-out and wild-type muscles. The frequency giving half-maximum force (obtained by linear interpolation) was 9.3 ± 0.6 Hz for knock-out and 8.2 ± 0.9 Hz for wild-type soleus muscles; the corresponding values for EDL muscles were 27.0 ± 0.4 and 29.2 ± 1.3 Hz, respectively.

Muscles were fatigued using repetitive tetani until peak force had declined to 40% of the original. Fatigue developed more rapidly in soleus knock-out muscles than in wild-type muscles (Figs. 1A and 1B); the num-

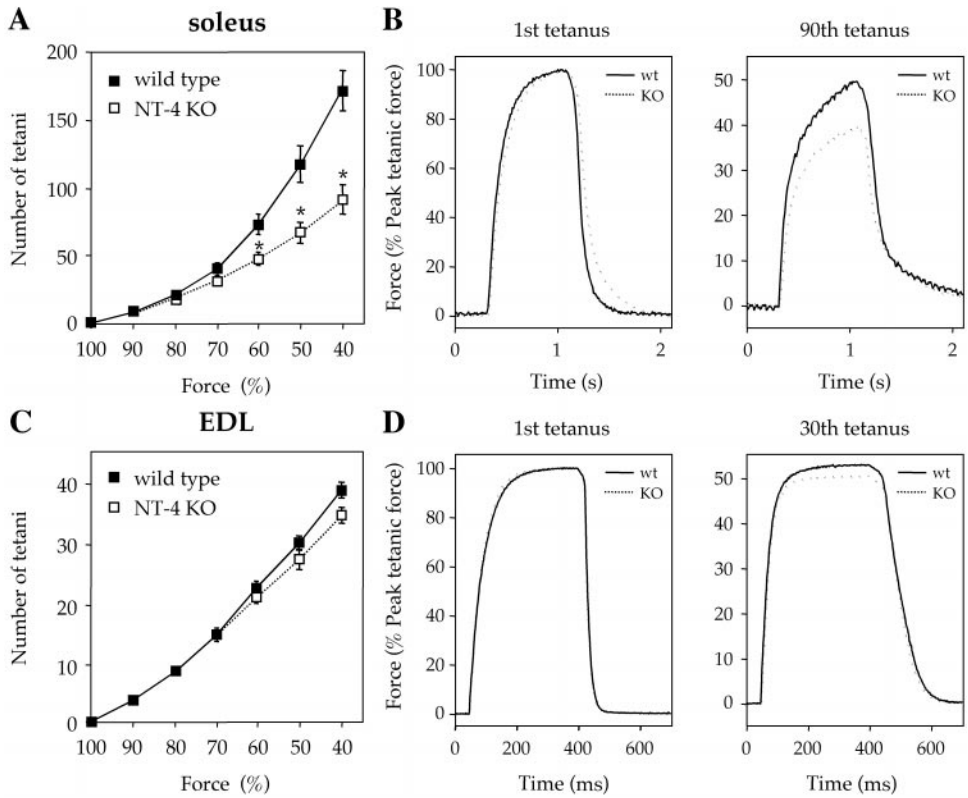

FIG. 1. Development of muscle fatigue in soleus (A and B) and EDL (C and D) muscles from wild-type (solid squares) and NT-4 KO (white squares) mice. (A and C) Number of tetani required to reduce muscle peak force to the percentages indicated in the *x* axes. All values are expressed as means ± SEM; $n = 10$ for soleus, $n = 5$ for EDL. Unpaired *t* tests were used to determine statistical significance. $^*P < 0.05$. (B and D) Typical examples of force transients recorded near the end of fatigue in wild-type (solid) and NT–4 KO (dotted) soleus (B) and EDL (D) muscles.

ber of tetani required to bring force down to 40% of the original was significantly lower in knock-out (92 ± 8) than in wild-type (174 ± 17) muscles. Interestingly, no difference between NT-4 KO and wild type in the fatigue resistance of EDL muscles was observed (Figs. 1C and 1D); here, the number of tetani needed to decrease force to 40% of the original was not significantly different between wild type and knock-out (35 ± 1 vs 39 ± 1). These results indicate that only slow-twitch, NT-4-rich muscle fibers are affected by the disruption of the NT-4 gene.

The rates of recovery after fatigue were very similar in wild-type and knock-out muscles. After 20 min of recovery, knock-out and wild-type soleus muscles displayed almost full recovery and there was no significant difference between the two groups (94.9 ± 2.3% vs 95.6 ± 1.4% of the original). Recovery of EDL muscles was less complete and 30 min after fatigue, knock-out and wild-type muscles produced 82.3 ± 4.1 and 88.1 ± 4.4% of the original force, respectively; the difference between the two groups was not significant. The accelerated fatigue in NT-4 KO soleus muscles was not due to altered oxidative capacity or fiber type composition of mutant soleus muscles, as no changes could be found in the activity of several mitochondrial enzymes (Table 1) or in the myosin chain profile of NT-4 KO soleus muscle (data not shown).

Altered Electrophysiological Responses in Muscle Fibers Lacking NT-4

In line with a role for NT-4 as a regulator of muscle function, several electrophysiological responses were found reduced or impaired in muscle fibers from NT-4 KO mice. A significant reduction in the amplitude of intracellularly recorded action potentials elicited by direct fiber stimulation was observed in the soleus muscle of NT-4 KO mice compared to wild type (90.0 ± 1.7 mV, $n = 25$ fibers from four muscles, vs 99.0 ± 1.3 mV, $n =$

TABLE 1

Enzymatic Activities (in nmol/min/mg Protein) of Mitochondrial Enzymes in Soleus and Gastrocnemius Muscles of Wild-Type and NT-4 KO Mice

	Soleus		Gastrocnemius	
Enzyme	Wild type	NT-4 KO	Wild type	NT-4 KO
NADH–ubiquinone oxidoreductase	30.3 ± 7.7	27.7 ± 3.5	31.0 ± 4.0	43.0 ± 8.8
NADH–cytochrome c reductase	51.4 ± 11.4	54.3 ± 8.3	30.8 ± 6.0	33.1 ± 7.0
Citrate synthase	253.8 ± 20.0	246.2 ± 15.0	214.3 ± 19.0	204.7 ± 24.3
Succinate dehydrogenase	88.4 ± 16.5	86.0 ± 20.0	123.1 ± 10.0	134.6 ± 33.0
Cytochrome oxidase	369 ± 57.0	385.7 ± 61.3	455.5 ± 55.0	467.2 ± 89.1

Note. Values are expressed as means ± SEM. The ANOVA test was used for statistical analysis.

18 fibers from four muscles, $P < 0.05$, unpaired t test, see Fig. 2A for action potential traces), suggesting alterations downstream of AChR function, possibly in the function or distribution of Na^+ channels. Resting membrane potential was not different from control in muscles of NT–4 KO mice (−72.0 ± 0.7 mV vs −71.4 ± 1.0

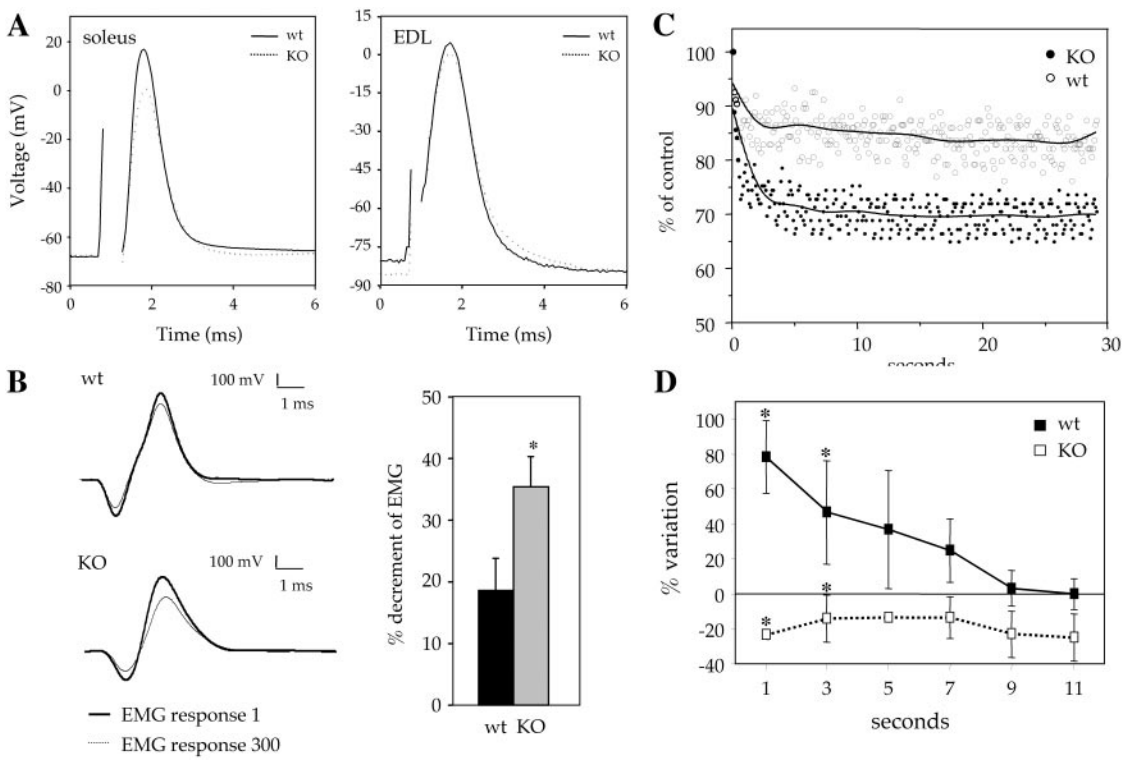

FIG. 2. Reduced EMG responses and absent PTP in soleus muscle fibers of NT–4 KO mice. (A) Intracellular recordings of typical action potentials from wild-type (solid) and NT–4 KO (dotted) soleus (left) and EDL (right) muscles. The stimulus at the beginning of each trace was removed for clarity. (B) EMG responses after 1 (solid lines) or 300 (dotted line) stimulations at 10 Hz in wild-type (wt) and NT–4 KO soleus muscles. The histogram shows the quantification of the decrement of the EMG response after 300 stimulations relative to the first stimulus. The data represent results from three independent experiments (3 wild-type and 3 NT–4 KO mice in each experiment). *$P < 0.01$. (C) Time course of percentage amplitude decrease of compound soleus muscle action potential during repetitive sciatic nerve stimulation at 10 Hz. For each series the points represent peak-to-peak amplitude normalized to the amplitude values measured after the first stimulus (100% on vertical scale). The curve indicates the prevalent trend of the data. (D) Posttetanic potentiation (PTP) induced after a 2 s tetani. The percentage variation of the EMG response is plotted at different times after the tetanus. PTP is observed for wild-type muscle fibers 1 and 3 s following a 2 s tetanus treatment. The data represent results from three independent experiments (3 wild-type and 3 NT–4 KO mice in each experiment). *$P < 0.001$.

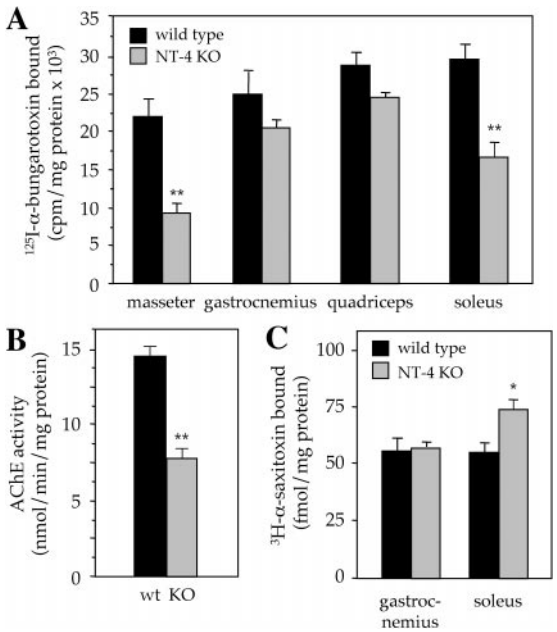

FIG. 3. Reduced ^{125}I-α-bungarotoxin binding and AChE activity and increased number of Na^+ channels in slow-twitch muscle fibers of NT–4 KO mice. (A) Binding of ^{125}I-α-bungarotoxin to extracts from the indicated muscles from wild type (solid bars) and NT–4 KO (hatched bars). Results are expressed as means ± SEM of triplicate determinations. $^{**}P < 0.001$. (B) AChE activity in soleus muscle from wild type (solid bars) and NT–4 KO (hatched bars). Results are expressed as means ± SD of triplicate determinations. $^{**}P < 0.001$. (C) Binding of [^{3}H]saxitoxin to extracts from the indicated muscles from wild type (solid bars) and NT–4 KO (hatched bars). Results are expressed as means ± SEM of two independent determinations, $n = 6$. $^{*}P < 0.02$.

mV). Interestingly, no differences in either membrane potential (−78.3 ± 2.0 mV, $n = 17$ fibers from three muscles, vs −81.0 ± 2.8 mV, $n = 25$ fibers from four muscles, $P > 0.05$ unpaired t test) or action potential (89.7 ± 3.2 mV vs 95.5 ± 2.5 mV, $P > 0.05$) amplitudes were observed in the EDL muscle (see also Fig. 2A).

Electromyographic (EMG) responses in soleus muscle of wild-type and NT-4 KO mice were measured after repetitive stimulation of the sciatic nerve. The amplitude of the EMG response was significantly reduced in muscles of NT-4 KO mice compared to wild type following 300 repetitive stimuli at 10 Hz (Fig. 2B). Analysis of the time course of EMG decay after repetitive stimulations showed a sharp decrease within the first 30 stimulations followed by a much slower decay in both wild type and NT-4 KO (Fig. 2C). The two groups differed in the first sharp decay of the EMG, which was twice as pronounced in NT-4 KO as in wild type, but not in the subsequent slower phase (Fig. 2C). We also examined the ability of neuromuscular synapses in the soleus muscle to undergo posttetanic potentiation (PTP). Following a tetanus (the intensity of which had been set to produce maximal stimulation), the EMG response of wild-type soleus muscle was greatly potentiated, returning back to pretetanus levels between 7 and 9 s later (Fig. 2D). No PTP could be observed in muscles of NT-4 KO mice after a similar tetanus (Fig. 2D). In fact, a significant 24% decrease was observed 1 and 3 s after the tetanus, while the EMG response of the wild-type muscle at the same time point was potentiated by 180% (Fig. 2D).

Reduced AChR and AChE Levels in Slow Muscle Fibers in the Absence of NT-4

The alterations observed in the electrophysiological responses of muscles from NT-4 KO prompted us to quantitatively assess AChR levels in muscles from NT–4 KO and wild-type mice. To this purpose, we performed binding of ^{125}I-α-bungarotoxin to membranes prepared from soleus and masseter, two muscles rich in type I slow fibers, and from gastrocnemius and quadricep, two muscles which predominantly contain fast fibers and express low levels of NT-4 (Funakoshi *et al.*, 1995). A 50% reduction in ^{125}I-α-bungarotoxin binding was observed in soleus and masseter of NT-4 KO compared to wild-type mice (Fig. 3A), indicating reduced AChR numbers in slow muscle fibers in the absence of NT-4. Image analysis of neuromuscular junctions from mutant and wild-type soleus muscles affinity labeled with ^{125}I-α-bungarotoxin also confirmed the decrease in AChR levels at the junction (data not shown). Interestingly, no significant reduction was observed in gastrocnemius and quadriceps (Fig. 3A), which produce much lower levels of NT-4 (Funakoshi *et al.*, 1995), supporting the role of muscle-derived NT-4 in the maintenance of postsynaptic AChR levels. The level of muscle-derived acetylcholinesterase (AChE) activity, a component of the junction synaptic basal lamina, was also reduced by about 50% in the soleus muscle of NT-4 KO compared to wild-type mice (Fig. 3B), indicating that NT-4 regulates the levels of several postsynaptic components of the neuromuscular junction. Intriguingly, the Na^+ channel number, assessed by [^{3}H]saxitoxin binding, was increased by 34% ($P < 0.02$, $n = 12$) in soleus muscle fibers from NT-4 KO compared to wild type (Fig. 3C). No changes in saxitoxin binding were detected in gastrocnemius muscle (Fig. 3C).

Disassembly of Postsynaptic Receptor Clusters and Enlargement of Neuromuscular Junctions in Soleus Muscle of NT-4 KO Mice

The alterations in the levels of postsynaptic AChR in slow muscle fibers from NT-4 KO mice suggested that NT-4 may also be involved in the maintenance of the structural organization of the postsynaptic apparatus of neuromuscular junctions. In order to address this possibility, we performed a histological analysis of neuromuscular junctions in mutant mice lacking NT-4. Postsynaptic AChR-rich regions were visualized with rhodamine-conjugated α-bungarotoxin (RαBTX) in soleus muscle. AChR clusters in soleus muscle of wild-type mice displayed the typical pretzel-like morphology, with a majority of junctions showing one or two contiguous branches of postsynaptic AChR as labeled by RαBTX (Fig. 4A). In contrast, a great proportion of neuromuscular junctions in soleus muscle of NT-4 KO showed fragmented clusters of RαBTX staining with many small, discrete AChR regions (Fig. 4A), suggesting disassembly of postsynaptic AChR regions in muscles lacking NT-4. The disassembly of postsynaptic AChR clusters was evaluated by counting the number of discrete AChR regions in neuromuscular junctions of muscle fibers of NT-4 KO and wild type. Junctions from NT-4 KO soleus muscle contained a threefold higher number of small, discrete AChR regions compared to wild-type mice ($P < 0.0001$; $n = 77$ (wt), $n = 88$ (NT-4 KO), Mann–Whitney test) (Fig. 5A). Analysis of the frequency distribution of junctions with different numbers of AChR areas revealed a greater spreading in the distribution of junctions from NT-4 KO mice, with many junctions containing 10 or more AChR areas compared to wild-type muscles (Fig. 5A). Despite their fragmentation, most AChR regions in neuromuscular junctions of NT-4 KO mice appeared well innervated (Fig. 4B), as assessed by staining of axons and terminals with anti-neurofilament and SV2 antibodies. Disorganization of AChR clusters was accompanied by a 50% increase in the major diameter of junctions from NT-4 KO compared to wild type ($P < 0.0001$; $n = 96$ (wt), $n = 104$ (NT-4 KO), Mann–Whitney test) (Fig. 5B), indicating dispersion of the AChR region in mutant mice. In agreement with this, image analysis of RαBTX-stained muscle preparations indicated a significant 50% increase in the area occupied by AChRs in the neuromuscular junctions of NT-4 KO mice compared to controls ($P < 0.001$). Together with our data demonstrating a reduced number of AChRs in the mutant junctions, these results suggest a reduced density of AChRs in NT-4 KO muscles, indicating that NT-4 is required for the maintenance of postsynaptic AChR regions in the adult soleus muscle.

DISCUSSION

Although many of the signals regulating the development of the postsynaptic specializations of neuromuscular junctions have been well characterized, it is still unclear whether extracellular factors are required for the maintenance of the postsynaptic apparatus in adult neuromuscular junctions (Sanes and Lichtman, 1999). Distinct postsynaptic specializations remain for long periods following axotomy, even after degeneration of the nerve terminal and retraction of Schwann cells from the synaptic site (Frank *et al.*, 1976), indicating that the postsynaptic apparatus can be maintained in the absence of other synaptic cells. However, these observations do not rule out the participation of autocrine, muscle-derived signals in the maintenance of postsynaptic sites, and several studies indicate a role for components of the synaptic basal lamina in the induction and maintenance of postsynaptic specializations in the absence of nerves (Sanes and Lichtman, 1999). To our knowledge, NT-4 is the first signal shown to be required for maintenance of the postsynaptic apparatus of adult neuromuscular junctions *in vivo,* extending considerably the repertoire of biological actions currently attributed to members of the neurotrophin family. The fact that postsynaptic maintenance can be regulated by a muscle-derived signal, as opposed to a nerve-derived anterograde factor, may at first be surprising. However, the importance of bidirectional signaling for neuromuscular junction maintenance is preserved by the fact that NT-4 production by muscle is activity-dependent (Funakoshi *et al.*, 1995).

Are the effects of NT-4 on neuromuscular junction organization mediated by muscle TrkB receptors? In a recent study, Gonzalez *et al.* (1999) found full-length TrkB receptors localized to the postsynaptic membrane of adult neuromuscular junctions. Moreover, disruption of TrkB signaling in adult muscle fibers using a dominant negative approach resulted in the disassembly of AChR clusters at neuromuscular junctions (Gonzalez *et al.*, 1999). Similar defects were also observed in neuromuscular junctions from *trkB*$^{+/-}$ mutant mice and after disruption of TrkB signaling in cultured myotubes stimulated with agrin in the absence of motor nerve terminals or Schwann cells (Gonzalez *et al.*, 1999), indicating that TrkB-mediated autocrine signaling in muscle fibers is required for maintenance of AChR clusters. The similarity of the neuromuscular junction

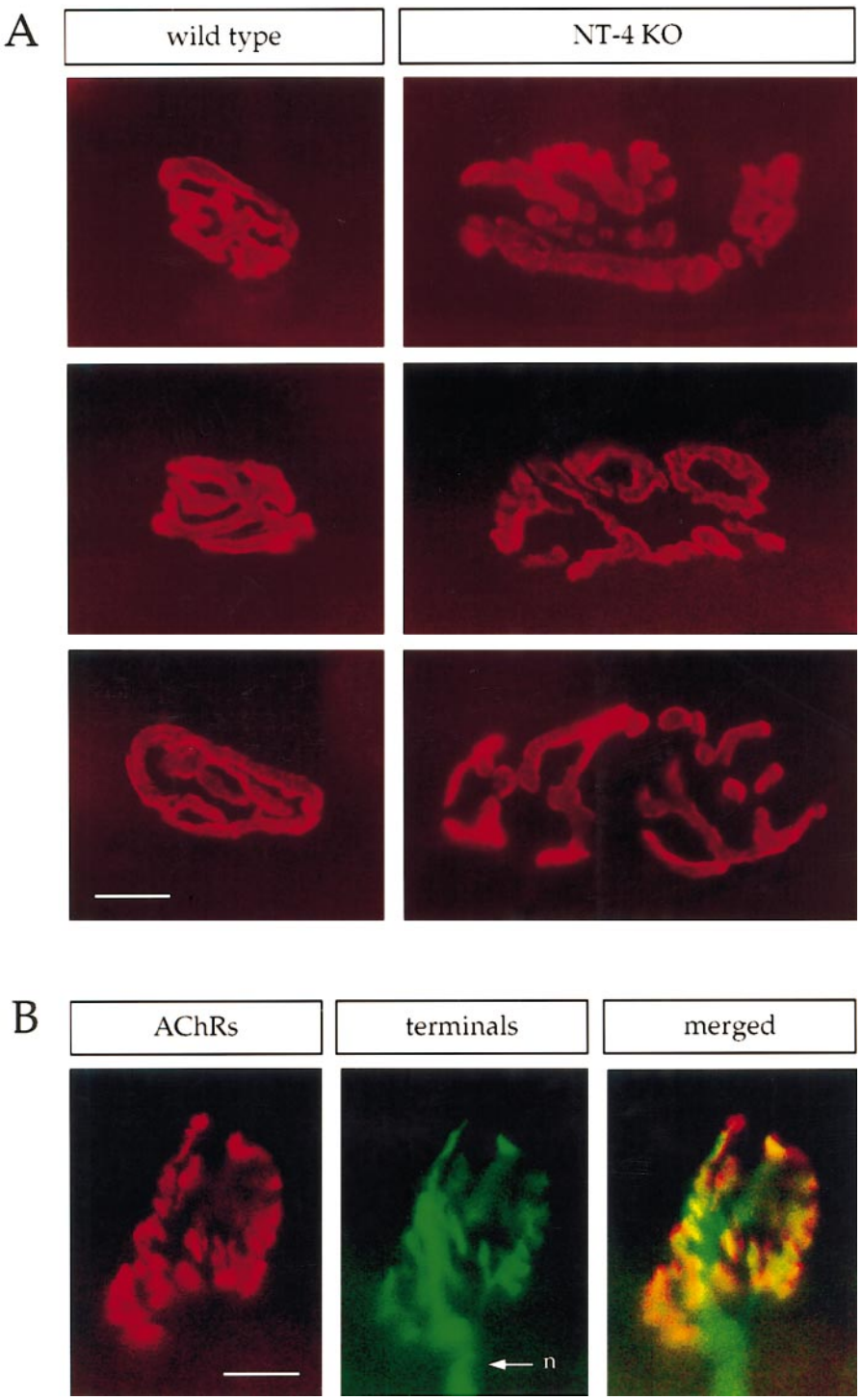

FIG. 4. Disassembly of postsynaptic receptor clusters and enlargement of neuromuscular junctions in soleus muscle of NT–4 KO mice. (A) Representative examples of soleus neuromuscular junctions from wild-type and NT–4 KO mice stained with RαBTX. The numbers of discrete AChR regions in the neuromuscular junctions shown are 1 (top), 2 (middle), and 2 (bottom) for wild type and 13 (top), 7 (middle), and 9 (bottom) for NT–4 KO. Scale bar, 12.5 μm. (B) A representative neuromuscular junction from NT–4 KO soleus muscle stained with RαBTX (AChRs, red) and anti-neurofilament and SV2 antibodies (terminals, green). The right panel (merged) shows the overlay of the two images. Pre- and postsynaptic alignment was similar to that observed in wild-type mice (not shown). n, nerve. Scale bar, 12.5 μm.

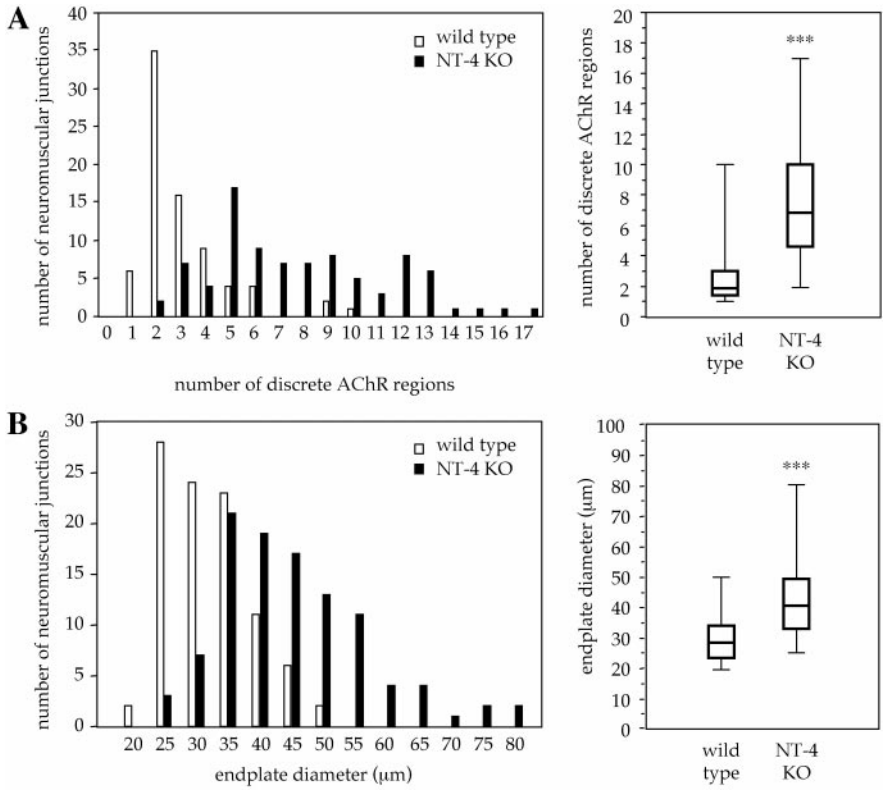

FIG. 5. Quantification of postsynaptic neuromuscular junction disassembly and enlargement. (A) The left panel shows a frequency histogram of the number of discrete AChR regions per junction in wild-type (white bars) and NT–4 KO (solid bars) soleus muscles. The results are derived from three independent experiments and represent 77 junctions from wild type (5 mice) and 88 junctions from NT–4 KO (5 mice). The right panel shows the statistical distribution of the data. Boxes comprise the 25 and 75 percentiles, horizontal lines within boxes indicate the median, and smaller horizontal lines denote the highest and lowest values. Statistical comparisons between medians were made with an unpaired nonparametric test (Mann–Whitney). ***$P < 0.0001$. (B) Quantification of postsynaptic neuromuscular junction enlargement. Frequency histogram (left) and statistical data distribution (right) of the large diameter of neuromuscular junctions in soleus muscles of wild-type and NT–4 KO mice. The results are derived from three independent experiments and represent 96 junctions from wild type (5 mice) and 104 junctions from NT–4 KO (5 mice). ***$P < 0.0001$.

phenotypes of NT-4 KO and *trkB*$^{+/-}$ mice, together with the presence of functional NT-4 receptors in the postsynaptic neuromuscular junction, suggests that muscle-derived NT-4 exerts some of its effects directly on muscle fibers in an autocrine manner. Thus, our results suggest that muscle-derived NT-4 is an activity-dependent bidirectional signal regulating maintenance of neuromuscular connections, motor innervation, and muscular performance. Fragmentation of postsynaptic AChR clusters similar to that observed in NT-4 KO and *trkB*$^{+/-}$ mice has been described previously in several myopathic disorders such as the human disease Duchenne muscular dystrophy and mdx mouse dystrophy, caused by mutations in the cytoskeletal protein dystrophin (Kong and Anderson, 1999; Lyons and Slater, 1991), suggesting that dystrophin could play a role in the localization or function of TrkB receptors in the postsynaptic neuromuscular junction.

Several reports have pointed at a presynaptic locus for PTP (Woodson *et al.*, 1978). High-frequency stimulation of motor nerves produces increases in evoked and spontaneous neurotransmitter release and PTP appears to be related to these changes (Gage and Hubbard, 1966). In addition, several studies have shown an important role of Ca^{2+} in PTP, as its accumulation in nerve terminals during repetitive stimulation can result in an increase in neurotransmitter release (Gage and Hubbard, 1966; Magleby, 1973; Miledi and Thies, 1971; Rosenthal, 1969; Weinreich, 1971). Postsynaptic release of NT-4 has been shown to potentiate neuromuscular synapses in *Xenopus* nerve–muscle cultures (Wang and Poo, 1997), suggesting that PTP could be mediated by

activity-dependent production and release of muscle-derived NT-4. The effects of NT-4 in *Xenopus* neuromuscular synapses have been shown to involve a potentiation of presynaptic transmitter secretion and a lengthening of the mean burst duration of postsynaptic low-conductance acetylcholine (ACh) channels (Wang and Poo, 1997), both of which could contribute to PTP. In particular, channel conductance kinetics are acutely modulated by NT-4 released from muscle fibers as a consequence of synaptic activity (Wang and Poo, 1997). Moreover, it is known that AChR density has a major influence on the conductance change that occurs in response to a single quantum of transmitter (Salpeter, 1987). Thus, the decay in EMG responses and impaired PTP observed in soleus muscles of NT-4 KO mice could result from an acute failure to potentiate presynaptic neurotransmitter secretion or a failure to modulate postsynaptic ACh channel conductance or to a reduced postsynaptic AChR density and neuromuscular junction disorganization in the absence of NT-4.

Although acute defects in NT-4 signaling could underlie some of the impairments observed in electrophysiological responses, the alterations in contractile properties and action potential, observed after direct supramaximal stimulation of muscle fibers, were most likely due to chronic impairments in one or several components of muscle fibers directly or indirectly regulated by NT-4. Previous work has shown that fragmented AChR regions have lower AChR density (Balice-Gordon *et al.*, 1993), and junctions in which TrkB signaling has been disrupted contain many regions of faint AChR intensity (Gonzalez *et al.*, 1999). We have found a reduction in AChR numbers and an increase in the junction area, indicating reduced AChR density at the neuromuscular junctions of NT-4 KO mice. Disorganized AChR clusters and a reduced AChR number could induce secondary or adaptive changes in muscle fibers, including, for example, impairments in the activation of contractile proteins or in Na^+ channel distribution or function. The increase in the Na^+ channel number observed in soleus muscle of NT-4 KO may be a compensatory alteration produced by the absence of NT-4. Increase in Na^+ channel number is also seen after muscle denervation (Lupa *et al.*, 1995; Yang *et al.*, 1991), although in that case a population of TTX-resistant channels is expressed. In NT-4 KO mice, however, no TTX-resistant action potentials could be detected in muscle fibers (H.W., unpublished observations), indicating that the alterations observed in these mice cannot be explained by the same type of transformation that occurs in denervated muscle. In addition to its direct effects on postsynaptic TrkB receptors, NT-4 could conceivably act indirectly on muscle by inducing the production of a motor neuron-derived myotrophic factor required for normal muscle function and neuromuscular junction maintenance (Loeb and Fischbach, 1997). We were, however, unable to find changes in the expression levels of several candidate factors for such a role in the spinal cord or of the corresponding receptors in muscle of NT-4 KO mice, including neuregulin-1 and the agrin receptor component Musk (unpublished observations).

The observation that soleus muscles from NT-4 KO mice fatigue twice as rapidly as those from wild type is intriguing and offers a novel and evolutionarily selectable *raison d'être* for this neurotrophin. NT-4 KO mice are viable and fertile and the apparent lack of an overt phenotype in these mice had made it difficult to find an explanation for the evolutionary conservation of this protein in vertebrates (Ibáñez, 1996). Slow-twitch muscle fibers are fatigue-resistant fibers responsible for extended muscle performance after prolonged activity. Soleus and other muscles rich in slow fibers play a crucial role in body weight support and posture. Thus, a higher fatigue rate in slow muscle fibers may have drastic consequences in the feeding and mating performance of an animal in the wild and could thereby result in a considerable reduction in fitness.

In conclusion, we show that absence of NT-4 leads to accelerated muscle fatigue, altered electromyographic responses, reduced AChR levels, and disassembly of neuromuscular junctions in slow muscle fibers, indicating a novel role for this neurotrophin in the maintenance of adult neuromuscular connections and muscle performance.

EXPERIMENTAL METHODS

Histological Analyses

Soleus muscles were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min, washed in 50 mM Tris–HCl buffer (pH 7.4) for 5 min, and incubated for 1 h in α-bungarotoxin conjugated to tetramethylrhodamine (RαBTX, Molecular Probes, Eugene, OR) at 5 μg/ml together with bovine serum albumin (BSA) at 1 mg/ml. Muscles were then washed, permeabilized in methanol at -20°C for 7 min, washed, and observed in a fluorescence microscope. The number of discrete AChR regions was counted by three independent observers who were blind to the genotype of the animals with identical results. Endplate diameter measurements were performed on a personal

computer using the PC version of the NIH IMAGE program (http://rsb.info.nih.gov/nih-image). Because the number of AChR regions and the large diameter of neuromuscular junctions do not follow a normal distribution (see Figs. 2A and 2B), an unpaired nonparametric test (Mann–Whitney) was used to compare the median value of the number of regions in the two groups (representing 77 to 88 junctions in each group).

Soleus muscles were processed as described for RαBTX except that after two washes in PBS they were incubated for 2 h at room temperature with mouse monoclonal anti-neurofilament SMI31 antibody (Stemberger Monoclonals) diluted 1:200 and mouse monoclonal anti-SV2 antibody (Developmental Studies Hybridoma Bank, IA) diluted 1:20 in PBS with 1 mg/ml of BSA and 0.3% Triton X-100. Following three washings in PBS, the muscles were incubated for 1 h with appropriate secondary antibodies diluted 1:100.

Binding Assays

Muscles were dissected and homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4), then centrifuged for 10 min at 25,000*g*. The pellets were washed and resuspended at 10 mg tissue wet wt/ml in ice-cold 50 mM Tris–HCl (pH 7.4) containing 2 mg/ml BSA. Aliquots (400 μl) of tissue suspension were preincubated with 50 μl of buffer alone or with nonradioactive α-BTX (at 100 μM) as competitor for 10 min in polypropylene culture tubes. Binding reaction was initiated by adding 50 μl ^{125}I-α-BTX (NEN, Boston, MA) at a saturating concentration of 100 nM, as defined by saturation experiments. Reactions were incubated at 37°C for 1 h and then terminated by dilution with 4 ml of cold buffer containing 0.1 mg/ml of BSA followed by filtration onto Whatman GF/C filters. After three washes, filters were counted in a gamma counter (Beckman). Triplicate determinations were made for each experimental condition. The binding of ^{125}I-α-BTX was completely displaced by 100 mM unlabeled α-BTX.

Quantitative changes in Na^+ channels were assessed by [^{3}H]saxitoxin (Amersham) binding assay, using the same procedure as described above for α-BTX with buffer (pH 7.4) containing (in mM) 130 choline chloride, 5.4 KCl, 5.5 glucose, 0.8 $MgCl_{reset,\ 1.8\ CaCl_2}$, 50 Hepes, 2 benzamidine, 0.1 phenylmethylsulfonyl fluoride, and 0.5 mg/ml bacitracin. The binding reaction was performed at 4°C at a saturating concentration of 50 nM [^{3}H]saxitoxin. Nonspecific binding was determined in the presence of 10 mM unlabeled TTX (Latroxan) and was subtracted from the total binding to determine specific binding.

Enzymatic Activities

For AChE activity, soleus muscles were homogenized in 10 mM Tris–HCl buffer (pH 7) which contained 10 mM EDTA, 1 M NaCl, 1% Triton X-100, 1 mg/ml bacitracin, and 25 U/ml aprotinin then centrifuged at 20,000*g* for 15 min at 4°C. Aliquots were evaluated for AChE activity according to Ellman *et al.* (1961).

For evaluation of tricarboxylic acid cycle and electron transfer chain enzyme activities, tissue was homogenized in 0.25 M sucrose and 1 mM EDTA, diluted with 0.25 M sucrose–EDTA, and centrifuged, and the sediment was rehomogenized and recentrifuged. The two supernatants obtained were centrifuged at 14,000*g*, and the mitochondrial sediment was resuspended at 100 mg/ml.

The V_{max} values of citrate synthase, NADH–ubiquinone oxidoreductase, succinate dehydrogenase, NADH, cytochrome c reductase, rotenone insensible, and cytochrome oxidase were determined. Enzyme activities were recorded graphically for at least 3 min with a double-beam recording spectrophotometer (Beckman 35) and each value was calculated from two blind determinations on the same sample. Enzyme specific activities were expressed as n mol of substrate transformed/min/mg of protein.

Electrophysiological Measurements

For measurements of membrane potential, an individual fiber was impaled by a microelectrode filled with 3 M KCl and the membrane potential recorded. An action potential was produced by activating the impaled fiber with a single current pulse delivered via a blunt microelectrode placed over the fiber. After the resting membrane potential and action potentials were recorded under control conditions, muscles were exposed to 1 μM TTX and the above procedure was repeated. Application of TTX had no significant effect on the resting membrane potential and completely blocked action potentials in both NT-4 KO and wild-type groups.

The sciatic nerve was exposed under anesthesia, and electrical stimulation was delivered by bipolar silver electrodes positioned beneath the nerve at the midthigh portion. Electromyographic recordings were made via a bipolar concentric needle electrode inserted in the soleus muscle. The EMG signals were filtered (30- to 1000-Hz bandpass), amplified, displayed, and stored on a personal computer for subsequent offline analysis. Paired *t* test analysis ($P < 0.01$) was used to compare NT-4 KO with wild-type muscle responses. For repeti-

tive nerve stimulation, stimulus trains of 300 pulses were applied at a frequency of 10 Hz. Each single pulse had a duration of 100 μs and an intensity equivalent to that required for an EMG response corresponding to 50% of maximal muscle contraction. For each series and for each animal, EMG responses were collected and peak-to-peak amplitudes measured. For PTP, the tetanic stimulus train consisted of pulses delivered at a frequency of 90 Hz with a duration of 2 s and an intensity equivalent to that required for a maximal EMG response. After cessation of the train, the time course of posttetanic potentiation was tested. EMG responses to single pulses were taken at 1, 3, 5, 7, 9, and 11 s, respectively, after the end of the tetanus.

Analyses of Contractile Properties

Soleus muscles were isolated, the tendons were gripped by aluminum clips, and the muscle was placed in a stimulation chamber filled with Tyrode solution of the following composition (in mM): 121 NaCl, 5 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 0.4 NaH_2PO_4, 0.1 NaEDTA, 24 $NaHCO_3$, and 5.5 glucose. Fetal calf serum (0.2%) was added to the solution to improve viability of the muscles. The solution was continuously bubbled with 95% O_2–5% CO_2 which gives a pH of 7.4. Experiments were performed at room temperature (22–24°C). The muscles were allowed to rest for at least 30 min after being mounted; while one muscle was being studied, the other muscle was maintained in Tyrode solution. At the end of each experiment, the muscle was weighed. In the stimulation chamber muscles were mounted between a movable stainless steel hook and a force transducer. The muscle length was adjusted so that tetanic force was maximal. The muscle was stimulated with supramaximal current pulses delivered via two plate electrodes placed on each side and extending the whole length of the muscle. Contractions with a duration of 760 ms were used throughout. A force–frequency relationship was obtained using contractions elicited at 2-min intervals and measuring the maximum force. Frequencies studied were 1, 10, 15, 20, 50, and 70 Hz. Force is expressed relative to the force at 70 Hz, which was set to 100%. Muscles were fatigued using 70-Hz tetani repeated at 3-s intervals until peak force had declined to 40% of the original. The number of tetani required to bring force down to 90–40% (in steps of 10%) of the original force was measured in each fatigue run. Force recovery from fatigue was studied by giving 70-Hz tetani at 2, 5, 10, 15, and 20 min after the end of fatiguing stimulation. Experiments on EDL muscles were performed as described for soleus muscles except that the tetanus duration was 350 ms and frequencies tested ranged between 1 and 120 Hz. EDL muscles were fatigued at 350 ms by tetani at 100 Hz given at 3-s intervals. All values are expressed as means ± SEM. Unpaired *t* tests were used to determine statistical significance and the significance level was set to $P = 0.05$.

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