



Published in final edited form as:

J Comp Neurol. 2013 September 1; 521(13): 2947–2965. doi:10.1002/cne.23324.

Muscle Ciliary Neurotrophic Factor Receptor α Promotes Axonal Regeneration and Functional Recovery Following Peripheral Nerve Lesion

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Abstract

Ciliary neurotrophic factor (CNTF) administration maintains, protects, and promotes the regeneration of both motor neurons (MNs) and skeletal muscle in a wide variety of models. Expression of CNTF receptor α (CNTFR α), an essential CNTF receptor component, is greatly increased in skeletal muscle following neuromuscular insult. Together the data suggest that muscle CNTFR α may contribute to neuromuscular maintenance, protection, and/or regeneration in vivo. To directly address the role of muscle CNTFR α , we selectively-depleted it in vivo by using a “floxed” CNTFR α mouse line and a gene construct (mlc1f-Cre) that drives the expression of Cre specifically in skeletal muscle. The resulting mice were challenged with sciatic nerve crush. Counting of nerve axons and retrograde tracing of MNs indicated that muscle CNTFR α contributes to MN axonal regeneration across the lesion site. Walking track analysis indicated that muscle CNTFR α is also required for normal recovery of motor function. However, the same muscle CNTFR α depletion unexpectedly had no detected effect on the maintenance or regeneration of the muscle itself, even though exogenous CNTF has been shown to affect these functions. Similarly, MN survival and lesion-induced terminal sprouting were unaffected. Therefore, muscle CNTFR α is an interesting new example of a muscle growth factor receptor that, in vivo under physiological conditions, contributes much more to neuronal regeneration than to the maintenance or regeneration of the muscle itself. This novel form of muscle–neuron interaction also has implications in the therapeutic targeting of the neuromuscular system in MN disorders and following nerve injury.

INDEXING TERMS

muscle CNTF receptor α ; sciatic; Cre recombinase; mouse; mlc1f-Cre

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: AJM. Acquisition of data: NL, RPS, KML, RR, DST, COM, RJZ, MKB, RJP, and AJM. Analysis and interpretation of data: NL, RPS, KML, RR, DST, COM, RJZ, MKB, RJP, and AJM. Drafting of the manuscript: AJM. Manuscript review: NL, RPS, KML, RR, DST, COM, RJZ, MKB, and RJP.

Ciliary neurotrophic factor receptor α (CNTFR α) is an essential ligand-binding subunit of the CNTF receptor, which is composed of CNTFR α , leukemia inhibitory factor receptor β (LIFR β) and gp130 (Ip et al., 1992; Davis et al., 1993b). Whereas LIFR β and gp130 are found in other related receptors, CNTFR α is unique to CNTF receptors and is required for all known forms of CNTF receptor signaling (Davis et al., 1993b; Elson et al., 2000; Derouet et al., 2004).

Exogenous CNTF administration has many protective and regenerative neuromuscular effects. It promotes motor neuron (MN) survival following postnatal axotomy (Sendtner et al., 1990), during development (Oppenheim et al., 1991) and in genetic models of MN disease (Sendtner et al., 1992a; Ikeda et al., 1995). It also increases the rate of axonal regrowth (Sahenk et al., 1994) and the size of neuromuscular junctions (Huang et al., 2002) after peripheral nerve lesion, and maintains axons in genetic models of amyotrophic lateral sclerosis (ALS) (Pun et al., 2006). Moreover, the anatomical and functional atrophy of skeletal muscle observed with disuse (Frayssse et al., 2000), aging (Guillet et al., 1999), MN disease models (Mitsumoto et al., 1994), and peripheral nerve lesion (Helgren et al., 1994) are all reduced by exogenous CNTF administration. These data suggest that endogenous CNTF receptor signaling may protect the neuromuscular system, particularly when it is challenged by traumatic or disease-related insult. This possibility is further supported by data indicating that insults, including peripheral nerve lesion and neuromuscular disease, greatly increase CNTFR α expression in skeletal muscle (Davis et al., 1993a; Helgren et al., 1994; Weis et al., 1998; Poea et al., 2001). Together the data suggest that endogenous skeletal muscle CNTFR α may contribute to neuromuscular regeneration in vivo.

However, the exogenous CNTF in the above studies may have induced pharmacological, not physiological, changes. In addition, this CNTF need not be acting on CNTF receptors given that CNTF, at the concentrations employed, can activate LIF receptors (Saggio et al., 1995) which have been implicated in MN survival (Li et al., 1995). Finally, the cellular site of action is not evident in that in vitro data indicate that CNTFR α is released by denervated muscle and can, if bound to ligand, act on cells expressing LIFR β and gp130 (like MNs) to enhance CNTF receptor signaling (Davis et al., 1993a). Therefore, binding of exogenous CNTF to muscle-derived CNTFR α may underlie some of its MN effects.

Manipulation of endogenous CNTF receptors in vivo is required to directly address these issues, and in the process, potentially identify targets that may be exploited to treat neuromuscular disorders. Given that CNTFR α is required for all forms of CNTF receptor signaling but is not involved in other signaling, including LIF receptor signaling (Davis et al., 1993b; Elson et al., 2000; Derouet et al., 2004), disruption of the CNTFR α gene is an effective method to identify in vivo functions of endogenous CNTF receptors.

Unconditional disruption of the CNTFR α gene in mice leads to perinatal death with reduced MN populations (DeChiara et al., 1995). Although these mice reveal an essential in vivo role for CNTF receptors in the development and/or survival of embryonic MNs, their perinatal death precludes their use to study postnatal functions of CNTF receptors. Moreover, the universal CNTFR α gene disruption in these mice does not allow one to address the functions of CNTF receptors expressed in specific cell types. In the studies reported here,

we examine the *in vivo* functions of muscle CNTFR α following peripheral nerve lesion by selectively disrupting the CNTFR α gene in skeletal muscle with *Cre/loxP* techniques.

MATERIALS AND METHODS

Mouse lines

The generation and characterization of the floxed CNTFR α (flxCNTFR α) mice have been described previously (Lee et al., 2008). Briefly, exons 3–5 of the CNTFR α gene (“exon1” containing start codon) were flanked by *loxP* sites (“floxed”) by using previously described methods (Wattler et al., 1999). The *mlc1f-Cre* mice (Bothe et al., 2000) and YFP16 mice (Feng et al., 2000) were generous gifts from Dr. Steven Burden (New York University Medical School, New York, NY) and Dr. J.R. Sanes (Harvard University, Cambridge, MA), respectively. ROSA26 mice (Soriano, 1999) were obtained from Jackson Laboratories (Bar Harbor, ME).

General design and statistical analysis

The flxCNTFR $\alpha^{+/+}$ and flxCNTFR $\alpha^{-/-}$ mice were generated by flxCNTFR $\alpha^{+/-}$ \times flxCNTFR $\alpha^{+/-}$ breeding. The other gene constructs were bred as heterozygote \times wild-type to control for gene dosage. *Mlc1f-Cre⁺/flxCNTFR $\alpha^{-/-}$* mice (i.e., wild-type at the CNTFR α locus) served as primary controls, but were not distinguishable from *mlc1f-Cre⁺/flxCNTFR $\alpha^{+/-}$* mice. All mice (male and female) were backcrossed at least five generations onto a 129SvEvBrd background and genotyped by polymerase chain reaction (PCR) analysis of tail biopsy DNA. All procedures were conducted on 2.5–5-month-old mice. Animal procedures were approved by the University of Cincinnati IACUC committee.

For all procedures, littermate pairs of CNTFR α -depleted and control mice were processed in parallel through the complete procedures including surgery, behavior, and image analysis by individuals kept blind to genotype. This pairing approach controlled for many potential sources of variability including *in utero* and postnatal rearing environments, variation in genetic background and age, and any variability in surgical and/or anatomical reagents. Therefore, statistical analyses consisted of appropriate paired Student's *t*-tests or two-way analysis of variance (ANOVA) tests (depending on the design of the particular experiment). ANOVA tests were followed by Bonferroni post hoc tests of individual group differences. In all cases, results of $P < 0.05$ were considered significant.

General anatomical procedures

Cryostat sections (30 μ m) were stained with either cresyl violet, standard Xgal histology, or previously described immunohistochemistry procedures (MacLennan et al., 1996). Antibodies (Table 1) recognizing muscle fiber types (see below), neurofilament (Millipore), and Fluoro-Gold (Fluorochrome, Englewood, CO) were visualized through either ABC amplification (Vector, Burlingame, CA) and cyanine-3 tyramide (Perkin Elmer, Oak Brook, IL), or Alexa Fluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA).

CNTFR α RT-PCR

Total RNA was extracted with the Micro-to-Midi total RNA purification system (Invitrogen) according to the manufacturer's instructions. The RNA was quantified by optical density, and equal amounts of RNA from control and floxed mice were used to synthesize first-strand cDNAs with the SuperScript III kit (Invitrogen). The cDNAs were amplified with TAQ DNA polymerase (Eppendorf, Hamburg, Germany) and the following CNTFR α -specific primers: 5' GTTCCTGCCTCCATTGAG-CAG 3' and 5' GAGCGGCAGCTGAGCACAG 3'. Reactions with the following glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as an extraction/loading control: 5' AAACCCATCACCATCTTCCA 3' and 5' GTGGTTCACACCCATCACAA 3'. The resulting DNAs were separated on an agarose gel with ethidium bromide. The relative intensity of CNTFR α bands (normalized with the intensity of the corresponding GAPDH bands) was determined with MetaMorph software (Molecular Devices, Sunnyvale, CA).

Unilateral sciatic nerve crush

Mice were anesthetized with ketamine/xylazine (100 mg/kg). Midthigh skin and muscle were opened to expose the nerve, which was freed from surrounding connective tissue where it passes superficial to the tendon of the obturator internus. The nerve was crushed for 10 seconds with Dumont #5 Biologic Tip forceps (Fine Science Tools, San Mateo, CA). The completeness of lesion produced by the crush procedure was assessed by examination of the tibialis anterior and extensor digitorum longus (EDL) muscles of four YFP16+ mice 4–6 days after the crush. This indicated that whereas the muscles ipsilateral to the lesion contain normal α -bungarotoxin (α BTX)-labeled acetylcholine receptor (AChR) clusters, all are devoid of presynaptic terminals. In contrast, the α BTX-labeled AChR clusters in the contralateral tibialis anterior and EDL muscles all contained presynaptic terminals.

Muscle fiber type analysis

EDL and soleus muscles ipsilateral and contralateral to the nerve crush were harvested 3 weeks post lesion, cut in cross section, and immunohistochemically stained with antibodies specific for different muscle fiber types: type I (WB-MHCs; Vector), total type II (MY-32; Sigma, St. Louis, MO) type IIa (A4.74; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), type IIb (10F5; DSHB), and type IIx (6H1; DSHB) fiber types. Images were captured with a Nikon DXM1200 digital camera and analyzed with MetaMorph software.

Motor neuron quantification

Thirty-micron cryostat sections were collected through the complete L5 segment of the lumbar spinal cord, which contains MNs projecting through the sciatic nerve (Janjua and Leong, 1984). The L5 spinal cord segment was located by using the diagrams in Figure 5 of Janjua and Leong (1984). Cresyl violet-stained alpha MNs were counted in every fourth section. Alpha MNs were defined by their characteristic location, large size, irregular shape, heterogeneous cytoplasmic staining, and, in most cases, a visible dark nucleolus in a lighter stained nucleus. To correct for cells potentially split in the Z dimension, all neurons in focus at the top border of the sections were excluded (optical disector; Hyman et al., 1998; Hatton

and Von Bartheld, 1999). Counts were multiplied by 4 to estimate total MNs (“fractionator”).

Muscle contractility analysis

We have previously reported the methods used in detail (He et al., 2001; Crawford et al., 2002). In brief, after the resting length of each muscle was measured in situ, the dissected muscles were mounted vertically in a sealed cylindrical chamber fitted with a magnet stirrer. By means of a small plastic ring tied to each tendon of the EDL with surgical silk, muscles were mounted at one end to a fixed stainless steel post. The other end was fixed to the lever of an isometric force transducer (Harvard Apparatus, Dover, MA), and the muscle length was adjusted to produce a passive tension of 5 mN. Muscles were incubated in sterile Krebs solution (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO₃, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 0.026 EDTA, 11 glucose equilibrated with 95% CO₂/5% O₂ at room temperature (~22°C). Muscles were electrically stimulated via two platinum electrodes positioned along either side of the muscle.

Supramaximal voltage was determined empirically by using a series of short (1.5-second) tetani and subsequently increasing the voltage for each tetani. Similarly, supramaximal frequency of stimulation was determined. Capacitor discharges of equal but alternating polarity (66 Hz at 15 V, 15-ms duration, circuit rate constant, 5 ms) were used for supramaximal stimuli. Experiments consisted of a set (about five) of twitches and a set of tetani with duration of 3–7 seconds. Digital recordings of force were obtained with the BioPac (Goleta, CA) data acquisition system and normalized to cross-sectional area. All recordings were conducted while the observer was blind to genotype. CNTFR α -depleted and control muscles were tested in parallel. The EDL muscle was studied because its smaller dimensions make it unlikely to be oxygen diffusion limited in this in vitro preparation.

Neuromuscular junction analysis

YFP16+ CNTFR α -depleted and YFP16+ control mice were perfused with 4% paraformaldehyde 3 weeks after sciatic nerve crush. Tibialis anterior muscles ipsilateral and contralateral to the nerve crush were dissected, postfixed in 4% paraformaldehyde for 2 hours at 4°C, cryoprotected, and longitudinally sectioned at 100 μ m on a cryostat. The sections were preincubated at room temperature with 0.3% Triton X-100, 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS; pH 7.3) for 1 hour, followed by an overnight 4°C incubation with 1 μ g/ml tetramethylrhodamine-conjugated α BTX to label AChR clusters.

To quantify the number of AChR clusters in a particular muscle, all AChR clusters were counted in each section of the muscle and summed to obtain a total.

To quantify the size of AChR clusters, an image was captured of every 60th AChR cluster encountered while counting along the band of AChR clusters in each section. The images were analyzed with MetaMorph software to determine the length of each AChR cluster along the axis of the muscle fiber. An average AChR cluster size was calculated for each muscle, and these values were normalized relative to the value calculated for the intact (unlesioned) side muscles of the control mice. This index of AChR cluster length along the

axis of the muscle fiber was chosen because in longitudinal muscle sections the length measured along the axis of the muscle fiber reasonably reflects the overall size of the AChR cluster whether it is present on the side or front face of the muscle fiber relative to the surface of the section (see Fig. 7B for examples). In contrast, measures of the AChR cluster area are much more dependent on AChR cluster position on the muscle fiber such that those clusters present along the side of the fiber appear much smaller in area (see Fig. 7B for examples). In addition, AChR clusters in CNTFR α -depleted mice were indistinguishable from those of controls with regard to shape such that measurement of AChR cluster length reflected relative cluster size.

The YFP16 gene construct labels all motor axons and their terminals with yellow fluorescent protein (YFP; Feng et al., 2000). Therefore, to quantify terminal sprouts of regenerated axons (no sprouts were observed for terminals on the unlesioned/intact side), we captured images of the terminals in the same neuromuscular junctions (NMJs) sampled for the AChR cluster size analysis described immediately above. For NMJs with no sprouts, the presynaptic terminals align very closely with the postsynaptic AChR clusters as seen for all NMJs in the unlesioned side muscles (e.g., Fig. 7A–D). Terminal sprouts are seen as additional extensions of the terminal emanating from this structure (e.g., Fig. 7E). For each NMJ analyzed, the number of primary fibers/sprouts emanating directly from the terminal areas aligned with the AChR clusters was counted, and the NMJ was qualified according to the number of sprouts it displayed. For each muscle, we calculated the percent of terminals containing zero, one, two, three, or four sprouts (no terminals were observed with more than four sprouts).

Axon quantification

Nerves were harvested 3 weeks post lesion, and the segment containing the crush site and adjacent nerve was dissected, cut in cross section at 30 μ m, and immunohistochemically stained for neurofilament. Sections immediately distal and immediately proximal to the lesion (i.e., within 200 μ m of the start and end of the lesion) were imaged with a Zeiss 510 LSM confocal microscope and analyzed with MetaMorph software. All axon profiles in all axon bundles of the nerve were counted in three proximal and three distal sections per nerve, and mean proximal and distal values were calculated.

Fluoro-Gold application and analysis

Three weeks after sciatic nerve crush, nerves were transected 7 mm distal to the crush site. The freshly transected proximal stump was immersed in a 5% solution of Fluoro-Gold/saline (Fluorochrome) for 40 minutes prior to religation of the proximal and distal stumps with 9-0 suture. Two weeks later (time for the tracer to be retrogradely transported to MN soma) the mice were perfused, and Fluoro-Gold-positive MNs were immunohistochemically identified and quantified.

Walking track analysis

Mice had nontoxic finger paint smeared on their rear paws and were placed at the end of a 7 (W) \times 13 (H) \times 70-cm (L) walkway whose floor was lined with white paper. A 13 \times 13 \times 20-cm box had been previously positioned at the far end of the walkway such that the mice

walked down the walkway to enter the dark box (a preferred environment for rodents), leaving footprints in the process. The mice were habituated to the apparatus prior to lesioning so that neophobia did not inhibit the walking behavior during testing.

The distance between the marks left by the first and fifth toes (“1–5”) was measured (while the observer was blind to genotype, as for all other procedures in this report) for feet both ipsilateral (lesioned) and contralateral (unlesioned) to the nerve crush. This was done in accordance with the original publication (De Medinaceli et al., 1982), and subsequent work indicating that this measurement of first to fifth toe spread best reflects the motor dysfunction and subsequent recovery from sciatic nerve damage (Hare et al., 1993).

Wild-type rats and mice typically show little if any recovery in walking track scores during the 2 weeks immediately following sciatic nerve crush, but largely recover during the third week post-lesion (Bervar, 2000; Dijkstra et al., 2000). Therefore, the mice were tested 13, 15, 19, and 21 days after the sciatic nerve crush. At least 10 distinct footprints were measured for each leg of each mouse in each session. Walking track analysis scores were calculated for each mouse in each session from the mean toe spread (1–5) values for each leg by comparing the lesioned (“L”) and unlesioned (“UL”) sides in the following equation: $\{([1-5L] - [1-5UL]) \div [1-5UL]\}$. Therefore, as the mice recovered motor function following the lesion, the toe spread values of the lesioned side became closer to the values from the unlesioned side, and the overall walking track analysis scores increased.

Antibody characterization

The 6H1 and 10F5 muscle fiber type antibodies have been previously characterized and shown to be specific to IIx and IIb fibers, respectively, by both western blot and immunohistochemistry (Lucas et al., 2000). The A4.74 antibody has been characterized by western blot (Hughes et al., 1993) and immunohistochemistry (Smerdu and Soukup, 2008) and found to specifically identify IIa fibers. The WB-MHCs antibody has been characterized by western blot (Klover et al., 2009) and immunohistochemistry (Carson et al., 1998; Klover et al., 2009) and shown to specifically label type I fibers. The My-32 antibody has been characterized by western blot (Okuno et al., 2012) and immunohistochemistry (Havenith et al., 1990; Rojiani and Cho, 1998) and shown to specifically label type II fibers. In the above studies of these different muscle fiber type-specific antibodies, western blot characterization demonstrated recognition of an appropriate size protein, and immunohistochemistry characterization demonstrated recognition of the appropriate subset of fibers in different muscle types and frequently also compared results with histochemistry-based assays of fiber types. Our studies also indicated that all these muscle fiber type antibodies labeled the fraction of EDL and soleus fibers expected from previous work.

The neurofilament antibody has been characterized by western blot as recognizing the appropriate size protein (Harris et al., 1991) and produces the same immunohisto-chemical staining pattern in the developing chick retina as the antibody RMO270, a mouse monoclonal against neurofilament (NFM; McCabe et al., 1999). Moreover, in the present study, the antibody produced the expected pattern of labeling for axons in the sciatic nerve.

The Fluoro-Gold antibody has been previously characterized in immunohistochemistry by blocking the signal through preadsorption of the antibody with Fluoro-Gold (Lee et al., 2009). In addition, the labeling reported here was seen only in mice loaded with Fluoro-Gold.

Finally, for all immunohistochemistry procedures reported here with the above antibodies, labeling was not observed without primary antibody.

Microscopy

Images were captured with either a 12-megapixel DXM1200 camera and Nikon E800 microscope or a Zeiss 510 LSM confocal microscope. Image brightness and contrast were optimized with LSM Browser software or Corel Draw software (Mountain View, CA). Images that were compared were identically captured and adjusted.

RESULTS

Muscle-specific CNTFR α depletion

In order to selectively disrupt the CNTFR α gene in skeletal muscle *in vivo*, we crossed the previously described floxed CNTFR α (flxCNTFR α) mice (Lee et al., 2008) with mice carrying a Cre recombinase (Cre) gene inserted into the myosin light chain *1f* locus (mlc1f-Cre) (Bothe et al., 2000), a locus expressed very selectively in skeletal muscle cells (Lyons et al., 1990). Previous Southern blot and reporter gene characterization of the mlc1f-Cre mice indicated that the mice express Cre as designed, in that the mlc1f-Cre leads to excision of floxed sequence in the adult in all skeletal muscles tested, but no excision in brain, liver, heart, or stomach (Bothe et al., 2000). To further characterize this line in the context of the present project, we crossed the mice with ROSA26 reporter mice (Soriano, 1999) to examine Cre activity in the EDL and tibialis anterior (TIB) hindlimb muscles, brain, spinal cord, sciatic nerve, and heart of two 4-month-old ROSA26^{+/−}/mlc1f-Cre^{+/−} mice and an age-matched ROSA26^{+/−}/mlc1f-Cre^{−/−} control. As expected, reporter signal was present only in skeletal muscle of the mlc1f-Cre⁺ mice, being absent in all other tissues (Fig. 1 and data not shown). Therefore, in agreement with the very selective skeletal muscle activity of the mlc1f promoter (Lyons et al., 1990) and the previous characterization of this mlc1f-Cre mouse (Bothe et al., 2000), the present data indicate that floxed gene excision is highly restricted to skeletal muscle in mlc1f-Cre⁺ mice, identifying them as a powerful tool for characterizing *in vivo* functions of muscle-derived CNTFR α , independent of the functions of CNTFR α expressed by other cell types.

We chose to focus on the EDL, soleus, and TIB muscles because these well-characterized muscles: 1) were the subject of previous studies showing the effects of exogenous CNTF treatment on muscle atrophy as well as studies reporting increased muscle CNTFR α expression following denervation (see Introduction); and 2) represent muscles with a range of different fiber types.

We crossed mlc1f-Cre^{+/−}/flxCNTFR α ^{+/−} mice with mlc1f-Cre^{−/−}/flxCNTFR α ^{+/−} mice and found that approximately 1/8 of the mice generated are mlc1f-Cre⁺ and homozygous for the floxed CNTFR α gene (mlc1f-Cre^{+/−}/flxCNTFR α ^{+/+}; referred to here simply as CNTFR α -

depleted mice), indicating that the expression of Cre and the depletion of muscle CNTFR α (see below) do not have any detectable effect on survival. In all the present experiments, these CNTFR α -depleted mice were compared to *mlc1f-Cre⁺*, wild-type (*mlc1f-Cre^{+/+}/flxCNTFR α ^{-/-}*) and *mlc1f-Cre⁺*, heterozygous floxed (*mlc1f-Cre^{+/+}/flxCNTFR α ^{+/-}*) littermates, with no differences being found between these two control groups.

We then confirmed that CNTFR α expression is depleted in skeletal muscle of the floxed mice. Real-time (RT)-PCR analysis indicated that CNTFR α mRNA levels in muscle are increased by nerve lesion, as previously reported (Helgren et al., 1994), and substantially decreased in the CNTFR α -depleted mice relative to controls (Fig. 2). As expected from the reporter data, the *mlc1f-Cre* manipulation did not affect spinal cord CNTFR α mRNA expression (floxed spinal cord = $104 \pm 17.7\%$ of controls, $n = 3$). The decrease, but not complete elimination, in muscle is consistent with previous studies using *mlc1f-Cre* (Bothe et al., 2000, Wredenberg et al., 2002) and may at least partially reflect the fact that not all cells expressing CNTFR α in muscle tissue are myofibers.

Muscle CNTFR α and body weight

Although most studies of CNTF receptor signaling and weight regulation have focused on the hypothalamus (Lambert et al., 2001; Kokoeva et al., 2005), other work suggests that skeletal muscle CNTF receptors are involved (Watt et al., 2006). Comparison of the age-matched CNTFR α -depleted and control mice did not detect any difference in weight (Fig. 3), suggesting that endogenous muscle CNTF receptor signaling does not play a significant role in regulating weight.

Muscle CNTFR α depletion does not affect muscle maintenance or peripheral nerve lesion-induced atrophy of muscle

Naive (unlesioned) muscle CNTFR α -depleted mice did not noticeably differ from control mice in terms of gait, balance, or grip strength (data not shown), thereby suggesting that muscle CNTFR α does not play a significant role in the noninjured neuromuscular system. These results are also consistent with the MN quantification and NMJ analysis presented below for the unlesioned control side of muscle CNTFR α -depleted mice.

As described above, CNTFR α expression increases in denervated muscle following peripheral nerve lesion. Moreover, many studies using exogenous CNTF administration suggest that endogenous muscle CNTF receptor signaling may protect the neuromuscular system and promote its regeneration following nerve lesion. Therefore, in order to determine the *in vivo* role(s) played by muscle CNTFR α following nerve lesion, we challenged the muscle CNTFR α -depleted and control mice with unilateral sciatic nerve crush, a well-characterized model in which the lesion produces temporary denervation of muscle followed by regeneration.

The denervated muscle in this model atrophies and then recovers following reinnervation. Exogenous CNTF has been shown to reduce denervation-induced muscle atrophy (Helgren et al., 1994). Comparing muscle weights of CNTFR α -depleted and control mice indicated that CNTFR α -depleted and control muscles are similar in size and atrophy to a similar

extent following denervation (Fig. 4). Like muscle weight, muscle fiber cross-sectional area is similar in CNTFR α -depleted and control muscle (data not shown). The depletion of CNTFR α also did not affect the fiber type composition of the muscles ipsilateral and contralateral to the lesion (Fig. 5) nor their in vitro contractile properties (EDL peak tetanic force, normalized to cross-sectional area; Fig. 6).

Neuromuscular junctions and lesion-induced terminal sprouting are unaffected by muscle CNTFR α depletion

Disruption of critical genes expressed in muscle can lead to changes in postsynaptic AChR clusters (Hosaka et al., 2002). We found no effect of muscle CNTFR α depletion on the number of such structures either ipsilateral or contralateral to the sciatic nerve lesion (Fig. 7F).

Exogenous CNTF administration has been shown to increase the size of AChR clusters following sciatic nerve lesion (Huang et al., 2002) suggesting that endogenous muscle CNTF receptor signaling may regulate AChR cluster size. We found a small, but statistically significant, increase in cluster size following lesion, but no difference between CNTFR α -depleted and control mice, nor any lesion by genotype interaction (Fig. 7G). The CNTFR α depletion also failed to affect the shape of the AChR clusters in that the clusters in CNTFR α -depleted and control mice were indistinguishable and all displayed the normal “pretzel-like” appearance (e.g., Fig. 7B,D).

In order to examine the presynaptic terminals of the NMJs, we utilized the YFP16 gene construct, which drives YFP expression in all MNs and their axons (Feng et al., 2000). This construct was bred into CNTFR α -depleted and control mice that then received the unilateral nerve lesion. NMJs on the intact side of both control and CNTFR α -depleted mice all displayed the normal precise alignment of AChR clusters and terminal arbors with terminal axons corresponding to all parts of the AChR clusters (e.g., Fig. 7C,D). As expected, axons were not observed in ipsilateral muscles following lesion until regenerating axons began to arrive, at approximately 2 weeks post lesion. Although there was variability in the rate of axon return to the AChR clusters, the CNTFR α -depleted and control groups did not differ (data not shown). Moreover, by 3 weeks post lesion, all AChR clusters in both groups were fully reinnervated, in that all parts of all AChR clusters in all mice displayed a matching, fully aligned nerve terminal.

Exogenous CNTF promotes sprouting at axon terminals (Gurney et al., 1992; Kwon and Gurney, 1994; Ikeda et al., 1995; Siegel et al., 2000; Wright and Son, 2007; Wright et al., 2007). Sprouting also occurs from regenerated axon terminals following nerve injury, including sciatic nerve crush (Zhou et al., 2002 and Fig. 7E). Therefore, these data raise the possibility that muscle CNTFR α may regulate the sprouting seen after nerve lesion, either through affecting muscle function and muscle–axon interaction or through the release of muscle CNTFR α (Davis et al., 1993a). We found that the nerve crush led to substantial terminal sprouting in both CNTFR α -depleted and control mice, with most terminals containing at least one sprout and many containing multiple sprouts (Fig. 7H). However, the muscle CNTFR α depletion had no effect on the percentage of terminals with sprouts or the number of sprouts per terminal (Fig. 7H).

Muscle CNTFR α depletion does not decrease motor neuron survival following nerve lesion

Exogenous CNTF administration promotes MN survival during development (Oppenheim et al., 1991), in MN disease models (Sendtner et al., 1992a; Ikeda et al., 1995), and following early postnatal peripheral nerve lesion (Sendtner et al., 1990). Adult peripheral nerve lesion does not lead to MN death, and it is accompanied by an increase in muscle CNTFR α expression (Helgren et al., 1994) and release of soluble CNTFR α that could potentially increase MN CNTF receptor signaling (Davis et al., 1993a). Together, these findings suggest that muscle CNTFR α may be part of an endogenous mechanism that promotes MN survival, most particularly following adult peripheral nerve lesion. Therefore, we quantified sciatic MNs in the muscle CNTFR α -depleted and control mice following sciatic nerve crush. CNTFR α depletion had no effect on MN number on the intact or lesioned sides (Fig. 8).

Muscle CNTFR α plays an essential role in axonal regeneration following peripheral nerve lesion

The increase in muscle CNTFR α expression and release following nerve lesion also raises the possibility that muscle CNTFR α , even though not essential for MN survival, may contribute to axonal regeneration. Moreover, adult CNTF administration increases the rate of axonal regrowth following peripheral nerve lesion (Sahenk et al., 1994) and maintains MN axons in genetic models of ALS (Pun et al., 2006).

Our examination of NMJs (see above) indicated that muscle CNTFR α depletion does not produce a detectable change in the rate or extent of reinnervation following nerve lesion, with complete reinnervation occurring in both CNTFR α -depleted and control mice. However, axonal branching anywhere distal to the lesion could lead to rapid and complete reinnervation of NMJs even if the receptor depletion leads to a reduction in the number of regenerating axons. Therefore, to first determine whether muscle CNTFR α is essential for normal regeneration of axons across the lesion site, we quantified axons immediately proximal and distal to the lesion site in CNTFR α -depleted and control mice 3 weeks after lesion, when essentially all normally regenerating axons should have extended across the lesion site, with most reaching target tissues.

As expected, we found that in control mice the number of distal axons was equivalent to the number of proximal axons (distal =97.0 \pm 6.0% of proximal; n =10). In contrast, the number of distal axons was only 81.1 \pm 7.3% of proximal axons in CNTFR α -depleted mice (n =10; Fig. 9). Comparing the CNTFR α -depleted mice with littermate controls that were lesioned, assayed, and analyzed in parallel confirmed that the ratio of distal to proximal axons was significantly lower in the CNTFR α -depleted mice (P =0.02; 10 pair; t =2.81; paired t -test).

Muscle CNTFR α contributes to motor neuron axon regeneration following peripheral nerve lesion

The above result indicates that muscle CNTFR α is required for normal axon regeneration following the lesion, in that without it there is a decreased/abnormal amount of axons extending across the lesion site. The reduction in distal axons in the CNTFR α -depleted mice could result from a decrease in the number of lesioned neurons that respond by extending a

regenerating axon at least across the crush site by 3 weeks post lesion. However, the result is also theoretically consistent with a reduction in axonal branching at the lesion site, if any such branching were muscle CNTFR α -dependent. To distinguish between these possibilities, we loaded axons 7 mm distal to the lesion site with the retrograde tracer Fluoro-Gold and quantified the number of subsequently labeled MNs. The spinal cords of the muscle CNTFR α -depleted mice contained $81.5 \pm 4.5\%$ as many labeled MNs as their paired littermate controls run in parallel ($P < 0.015$; 6 pair; $t = 3.65$; paired t -test; Fig. 10). Therefore, at 3 weeks post lesion, when MNs have normally regenerated axons back to the muscle, approximately 20% fewer of the MNs in the muscle CNTFR α -depleted mice have regenerated axons even as far as 7 mm distal to the lesion site.

Muscle CNTFR α is essential for normal recovery of motor function following peripheral nerve lesion

In order to assess the potential contribution of muscle CNTFR α to motor function recovery following nerve lesion, we compared the muscle CNTFR α -depleted and control mice with “walking track,” or “footprint” analysis, the most widely used behavioral method to quantify the recovery of motor function following sciatic nerve lesion (De Medinaceli et al., 1982; Bain et al., 1988; Wang et al., 1997) (see Materials and Methods section). Wild-type rats and mice typically show little if any recovery in walking track scores during the 2 weeks immediately following sciatic nerve crush, but largely recover during the third week post lesion (Bervar, 2000; Dijkstra et al., 2000). As animals regain motor function, the footprints left by their foot ipsilateral to the lesion progressively take on the characteristics of the normal footprints of the contralateral control side. In particular, recovery from sciatic nerve lesion has been shown to be best reflected by the “toe spread” between the first and fifth toes such that the lesion reduces this value ipsilateral to the lesion and with recovery the toe spread increases with time to more closely match that of the uninjured side (Hare et al., 1993).

In our study the control mice displayed a recovery consistent with this literature (Fig. 11). In significant contrast, the muscle CNTFR α -depleted mice recovered function to a substantially lesser extent (Fig. 11; $P < 0.003$; two-way ANOVA; main effect of CNTFR α depletion). Less powerful post hoc individual group comparisons, appropriately designed to protect against cumulative type I error, were unable to identify differences at individual time points. Consequently, the data do not allow for more refined conclusions regarding the relative time courses of motor function recovery in the muscle CNTFR α -depleted and control mice. However, given the highly significant overall effect of CNTFR α depletion on motor function recovery (ANOVA; see above), it is clear that endogenous muscle CNTFR α is required for normal motor function recovery following sciatic nerve crush because with CNTFR α depletion motor function is clearly impaired.

DISCUSSION

We report that selective in vivo depletion of skeletal muscle CNTFR α decreases MN axonal regeneration and the recovery of motor function following sciatic nerve crush. Surprisingly, the same manipulation has no detectable effect on the aspects of muscle maintenance and

regeneration previously shown to respond to exogenous CNTF administration. These results suggest that either the exogenous CNTF in the previous studies acted on sites other than muscle CNTF receptors or it elicited pharmacological effects not seen under physiological conditions.

The depletion of skeletal muscle CNTFR α produced a significant but modest effect on axon regeneration, indicating that, not surprisingly, other proteins participate in axon regeneration, and that the system likely has some redundancy *in vivo*. Some of this redundancy may well involve CNTFR α expressed by the regenerating MNs themselves. Future work combining MN and muscle-specific CNTFR α gene disruption *in vivo* will be required to definitively address this issue. Regardless, 3 weeks after sciatic nerve crush, when most normal MNs have regenerated axons back to the muscle, the muscle CNTFR α -depleted mice displayed a decrease of approximately 20% relative to controls in the number of MNs regenerating axons even as far as the Fluoro-Gold loading site, just 7 mm distal to the crush site. Therefore, the data clearly indicate that muscle CNTFR α is required for normal axonal regeneration, in that without it the regeneration is decreased/abnormal. However, the present data do not distinguish between the possibility that a distinct subclass of MNs, which constitute approximately 20% of all MNs, absolutely require muscle CNTFR α for axonal regeneration and the possibility of redundancy in the system such that multiple proteins contribute to axonal regeneration and partially compensate for the loss of a single protein from muscle.

Muscle-specific manipulation studies have identified muscle-derived factors that regulate the differentiation and stabilization of motor nerve terminals (Nguyen et al., 2000; Yumoto et al., 2012). There is also evidence that as yet unidentified muscle-derived factors may influence axon growth in terminal branching decisions following nerve lesion (Robinson and Madison, 2009). However, to the best of our knowledge, the present data are the first to identify a specific endogenous muscle protein, muscle CNTFR α , that contributes to motor axon regeneration across the nerve lesion site. Application of exogenous MDP77, an unrelated protein largely, but not exclusively, expressed in skeletal muscle, has been shown to increase motor axon regeneration across the site of sciatic nerve lesion (Itoh et al., 2005). Along with the present results, these findings suggest that the interaction of skeletal muscle and regenerating motor axons at the lesion site may involve a variety of proteins. With the advent of genetic methods to selectively disrupt genes in skeletal muscle, exploration for additional muscle proteins involved in this interaction *in vivo* should become straightforward.

The muscle CNTFR α -depleted mice also display a decrease in motor recovery as determined by walking track analysis. The decrease in axonal regeneration may be at least partially responsible for the impaired motor recovery detected by this sensitive test. The decrease in motor recovery could reflect the reduced number of MN soma that successfully regenerate axons back to the muscle. It could also reflect the detrimental effects of the apparent collateral branching/sprouting that occurs to occupy the NMJs that would otherwise have been innervated by the nonregenerating MNs. However, other as yet unidentified mechanisms may also contribute. Regardless, the present data reveal an

essential *in vivo* role for muscle CNTFR α in the normal recovery of motor function following peripheral nerve lesion.

There are several possible mechanisms by which muscle CNTFR α could play its role in axonal regeneration following nerve lesion. *In vitro* data indicate that soluble CNTFR α is released from muscle following denervation (Davis et al., 1993a). If it combines with a CNTF receptor ligand, such as the CNTF that has been shown to be released at the lesion site from Schwann cells following the same injury (Sendtner et al., 1992b), it can act on other cell types that express the other receptor components, LIFR β and gp130 (such as MNs), to enhance CNTF receptor signaling (Davis et al., 1993a). Therefore, if the increase in muscle CNTFR α expression seen following sciatic nerve lesion (Helgren et al., 1994) leads to muscle CNTFR α reaching the regenerating axons at the lesion site and/or cell bodies of the MNs, it would be expected to enhance MN CNTF receptor signaling, and potentially increase MN axonal regeneration. Alternatively, or in addition, the increase in muscle CNTFR α may promote CNTF receptor signaling in denervated muscle, which could lead to an increased release of one or more unidentified factors that act on MNs to promote their regeneration. Although the separation between the midthigh nerve lesion site and the denervated muscles of the lower limb would at first seem to argue against the above possible mechanisms, the rapid and dramatic increase in CNTFR α mRNA expression in denervated muscle that is sustained for at least 10 days post lesion (Helgren et al., 1994) suggests that CNTFR α and/or CNTFR α -dependent factors released from the denervated muscles could be involved. In addition, evidence suggests that, although denervation increases the release of muscle CNTFR α , the CNTFR α is also released from intact muscle at a slower rate (Davis et al., 1993a). Therefore, muscles adjacent to the nerve may continuously supply the nerve with this soluble CNTFR α , even in the absence of nerve injury, such that sufficient quantities are immediately available to contribute to axonal regeneration following nerve injury. Finally, the Schwann cell CNTF released at the injury site may act on CNTF receptors in this adjacent muscle to induce the local release of a factor or factors that promote axonal regeneration.

As noted above, the number of retrogradely labeled MNs was decreased by approximately 20% in the muscle CNTFR α -depleted mice. The number of total sciatic nerve axons distal to the lesion site was similarly affected. Given that MN axons make up a small minority of total sciatic nerve axons at the lesion site (Swett et al., 1991), a decrease of approximately 20% in MN axons cannot account for the approximate 20% decrease in total axons. Therefore, the results suggest that muscle CNTFR α also contributes to the regeneration of at least some sensory axons. Unfortunately, we were unable to obtain reliable, quantifiable retrograde labeling of dorsal root ganglion neurons or identify workable markers for sensory axons in order to confirm this.

We targeted CNTFR α because CNTFR α disruption is the most comprehensive approach to determining the *in vivo* functions of endogenous CNTF receptor signaling. Thus, CNTFR α is essential for all known forms of CNTF receptor signaling, regardless of which ligands and signaling pathways are involved (Davis et al., 1993b; Elson et al., 2000; Derouet et al., 2004). Consequently, the present data do not address which individual ligand(s) or pathways participate in the muscle CNTFR α -dependent signaling that promotes MN regeneration and

recovery of motor function. As noted above, CNTF may contribute to MN regeneration given that it is released from Schwann cells at the site of peripheral nerve lesion (Sendtner et al., 1992b) and may combine with CNTFR α released from the denervated muscle (Davis et al., 1993a). A previous study showing decreased motor recovery following sciatic nerve crush in unconditional CNTF knockout mice (Yao et al., 1999) suggests that endogenous CNTF may be involved, but it does not address the source of the CNTF or the CNTFR α . The cardiotrophin-like cytokine/cytokine-like factor 1 (CLC/CLF) CNTF receptor ligand, which plays a substantial role in embryonic MN survival (Forger et al., 2003; Zou et al., 2009), may also participate. Exogenous CLC has been found to protect retinal ganglion cells after optic nerve crush (Schuettauf et al., 2005). Although this neuroprotection was observed with a central nervous system lesion, the study raises the possibility that endogenous CLC may be involved in the muscle CNTFR α -dependent signaling underlying the effects reported here. Studies are needed to determine whether CLC is expressed in the adult neuromuscular system. Of course, the ligands may act together. Regardless, conditional disruption of individual ligands will be necessary to definitively answer these questions.

The conditional gene disruption technique utilized here produced a substantial but not total depletion of muscle CNTFR α . This result is consistent with other work done with the same *mlc1f-Cre* gene construct (Bothe et al., 2000, Wredenberg et al., 2002), and it has two important implications for interpretation of the present data. First, it is still theoretically possible that muscle CNTFR α contributes to muscle regeneration, such that even a small amount of muscle CNTFR α is sufficient to provide maximal effect. Similarly, muscle CNTFR α may play a role in the other functions that were unaffected in the present studies. However, it seems most likely that if muscle CNTFR α were to play a significant role in any of these processes, its substantial depletion would be expected to produce a detectable effect. The second important implication regarding the degree of muscle CNTFR α depletion pertains to the interpretation of the effects seen on neuronal regeneration. It should be noted that muscle CNTFR α may play a larger role in these functions than that detected here, in that total removal of all muscle CNTFR α may produce larger effects. Regardless, the present results surprisingly demonstrate that muscle CNTFR α plays a more important role in the regeneration of neurons than the regeneration of the muscle itself.

Exogenous CNTF administration has been shown by several groups to elicit terminal sprouting of MN axons (Gurney et al., 1992; Kwon and Gurney, 1994; Ikeda et al., 1995; Tarabal et al., 1996; Siegel et al., 2000; Wright and Son, 2007; Wright et al., 2007). Conflicting reports from CNTF knockout studies suggest that endogenous CNTF is (Siegel et al., 2000) or is not (Wright and Son, 2007) involved in MN terminal sprouting observed after nerve lesion. The present results suggest that muscle CNTFR α does not significantly contribute to terminal sprouting following sciatic nerve crush. However, the data do not rule out the possibility that MN CNTFR α is essential for this response, through an interaction with either CNTF or CLC/CLF.

Work with genetic models of ALS indicates that exogenous CNTF administration can protect MN axons from this genetic insult (Pun et al., 2006). Several lines of evidence suggest that loss of MN axons is a critical event leading to ALS symptoms (Gould et al., 2006; Pun et al., 2006; Dadon-Nachum et al., 2011). However, clinical ALS trials with

systemic CNTF injections were stopped due to unacceptable side effects (ALS CNTF Treatment Study Group, 1995, 1996; Miller et al., 1996), indicating that any therapeutic manipulation of CNTF signaling will need to be more specifically targeted. Therefore, the present data indicating that endogenous muscle CNTFR α -dependent signaling contributes to MN axon regeneration following a different insult (nerve lesion) raises the possibility that muscle CNTFR α should be considered as a potential specific target in the treatment of ALS, whether this involves interventions designed to increase muscle CNTFR α expression or other approaches. Similarly, the present data suggest that targeting muscle CNTFR α may prove beneficial in the treatment of peripheral nerve injury.

Acknowledgments

Grant sponsor: National Institutes of Health; Grant numbers: NS35224 and NS052700 (to A.J.M.).

We thank Dr. S. Burden for the *mlc1f-Cre* mice and for helpful comments on the manuscript, Dr. J.R. Sanes for the YFP16 mice, Peggy Bowman, Tatiana Radzyukevich, and Dan Shelly for technical assistance, and Glenn Doerman for assistance with the figures.

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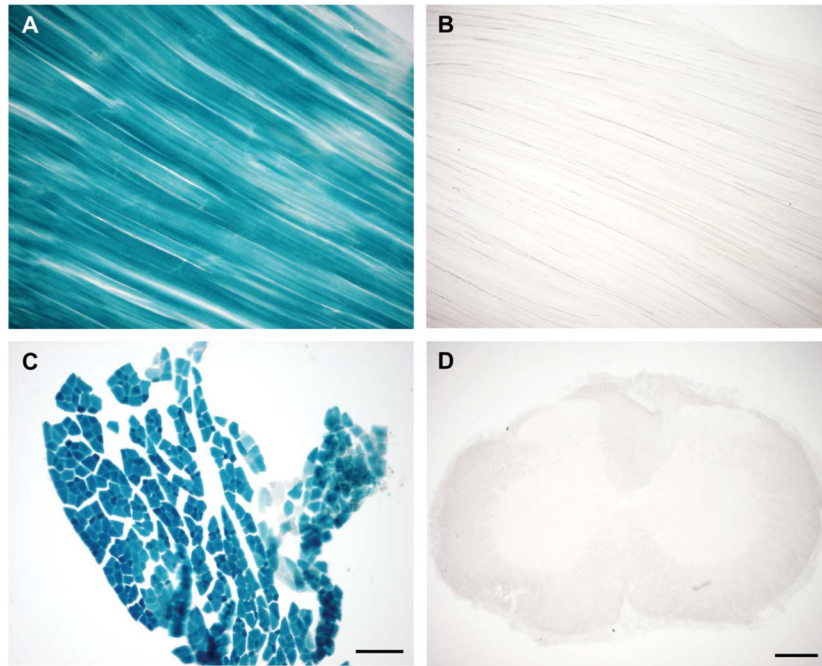


Figure 1.

The *mlc1f-Cre* gene construct leads to selective excision of floxed gene sequence in skeletal muscle. Xgal histology of: (A) longitudinal section of tibialis anterior muscle from a ROSA26 reporter+; *mlc1f-Cre*+ mouse with blue Xgal reaction product indicating *mlc1f-Cre*-dependent floxed gene excision; and (B) a ROSA26 reporter+ *mlc1f-Cre*- control; (C) cross section of EDL muscle from a ROSA26 reporter+; *mlc1f-Cre*+ mouse; (D) transverse spinal cord section from a ROSA26 reporter+; *mlc1f-Cre*+ mouse showing lack of MN floxed gene excision Scale bar =200 μ m in D (applies to A,B,D); 100 μ m in C.

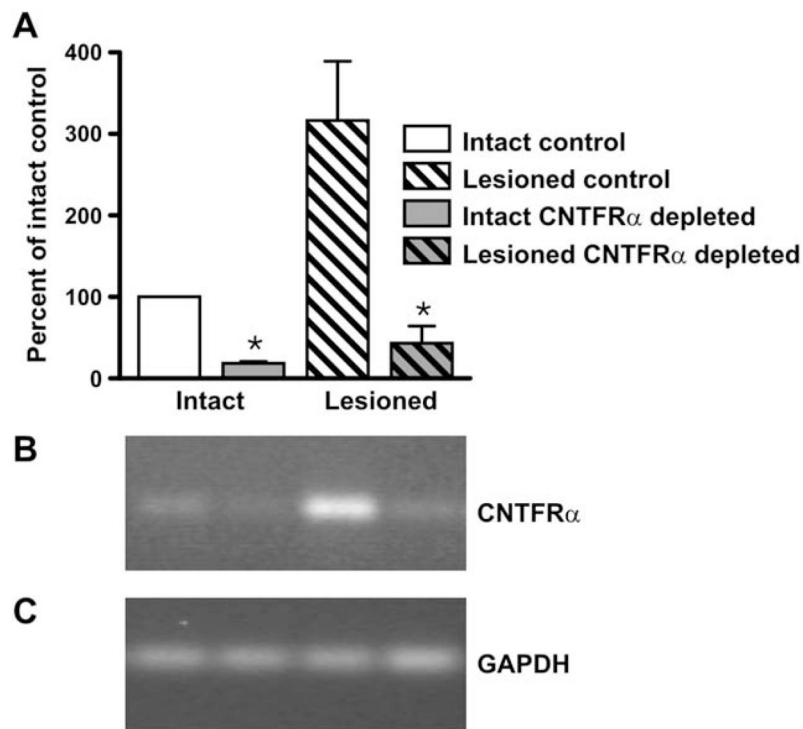


Figure 2.

Combining the *mlc1f-Cre* gene construct with the floxed ciliary neurotrophic factor receptor α (CNTFR α) gene leads to CNTFR α mRNA depletion in skeletal muscle. CNTFR α mRNA was measured by RT-PCR in EDL muscles ipsilateral (“Lesioned”) and contralateral (“Intact”) to sciatic nerve crush. The muscles were dissected 24 hours post lesion from floxed CNTFR α and litter-mate control mice, all containing the *mlc1f-Cre* gene construct. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured and used to normalize values. **A**: Summarized quantitative data. **B,C**: Representative gels from CNTFR α (B) and GAPDH (C) RT-PCR. As expected from past studies, the lesion led to an increase in muscle CNTFR α mRNA expression ($P < 0.015$; $F = 10.09$; two-way ANOVA). As designed, muscles from floxed CNTFR α mice contained less CNTFR α mRNA than controls ($* P < 0.002$; $F = 21.90$; two-way ANOVA). $n = 3$ mice per condition. The same pattern of results was observed with TIB and soleus muscles (data not shown).

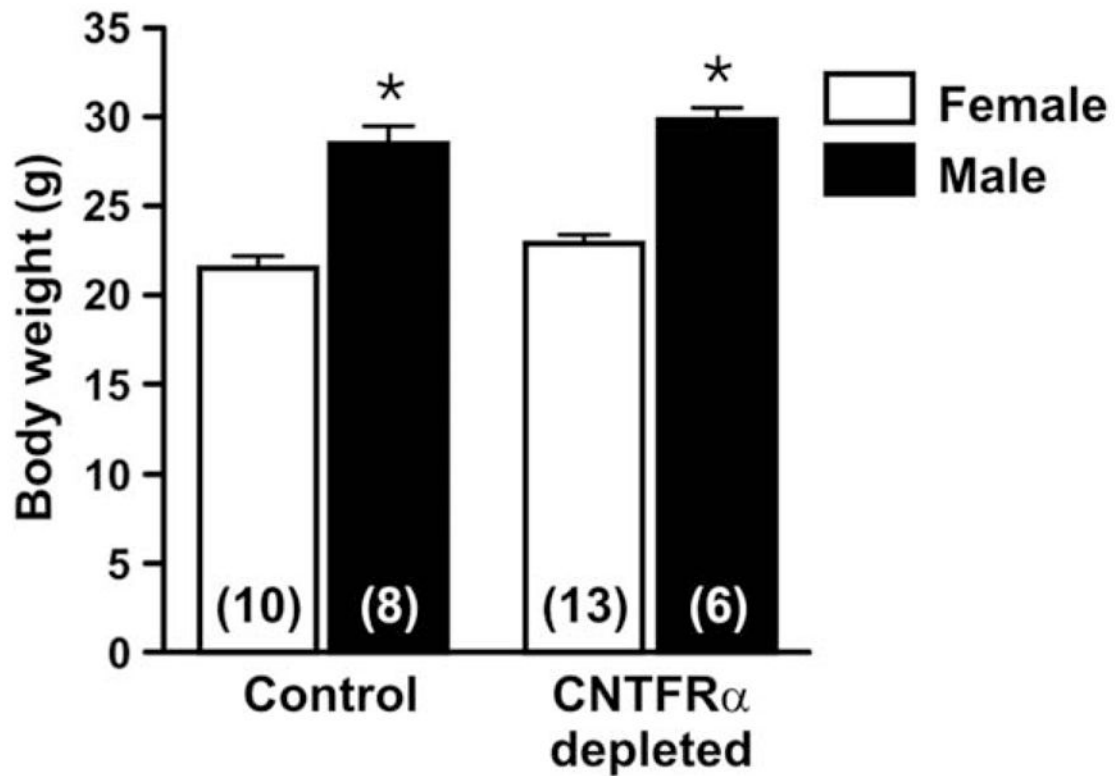


Figure 3.

Body weight is not affected by depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α). As expected, male mice weighed significantly more than female mice ($*P < 0.001$; $F = 92.14$; two-way ANOVA). However, CNTFR α -depleted and control mice (age-matched; 3–5 months of age) did not differ ($P > 0.05$; $F = 3.54$; two-way ANOVA). Means with SEM. are presented. Numbers in bars indicate numbers of mice.

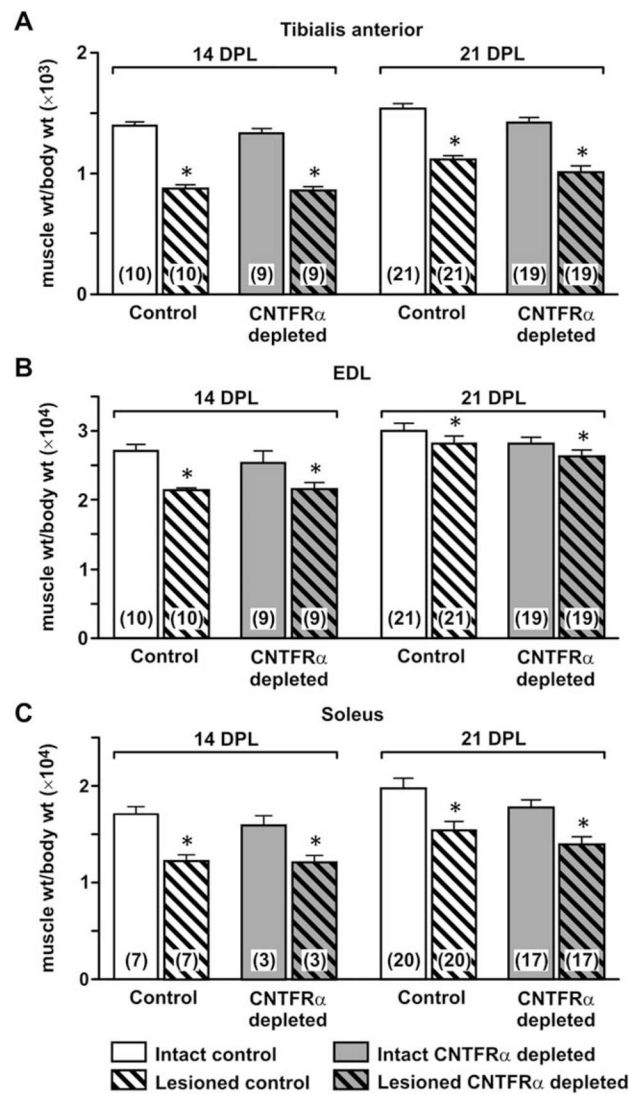


Figure 4.

Depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α) does not affect muscle weight or nerve lesion induced muscle weight loss. As expected, sciatic nerve crush led to decreased weight of tibialis anterior (A), extensor digitorum longus (EDL; B), and soleus (C) muscles at both 14 and 21 days post lesion (DPL) (* $P < 0.002$; main effect of lesion in two-way ANOVAs with side relative to lesion treated as a within-measure). However, muscle CNTFR α depletion had no effect on muscle weight or the lesion-induced atrophy in any of the muscles at either postlesion interval (all comparisons $P > 0.05$; main effect of CNTFR α depletion and interaction in two-way ANOVAs described above). Means with SEM are presented. Numbers in bars indicate number of mice. Legend in C applies to all panels.

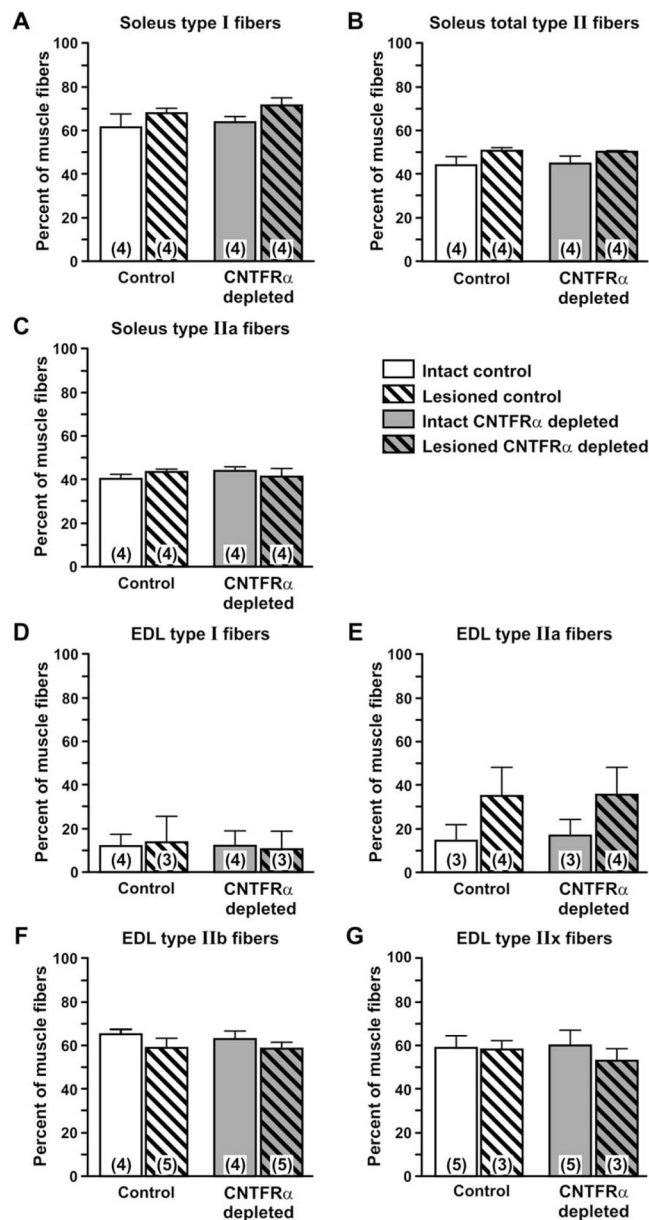


Figure 5.

Depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α) does not affect muscle fiber type composition. Immunohistochemical analysis of muscle fiber types in soleus (A–C) and extensor digitorum longus (EDL; D–G) muscles harvested from CNTFR α -depleted and control mice 3 weeks after nerve crush did not detect any effect of CNTFR α depletion on fiber type composition in muscles either ipsilateral (“Lesioned”) or contralateral (“Intact”) to nerve crush (all comparisons $P > 0.05$; two-way ANOVA terms for main effect of CNTFR α depletion). IIb and IIx fibers were not detected in soleus muscle. Means with SEM are presented. Numbers in bars indicate numbers of mice. Legend applies to all graphs.

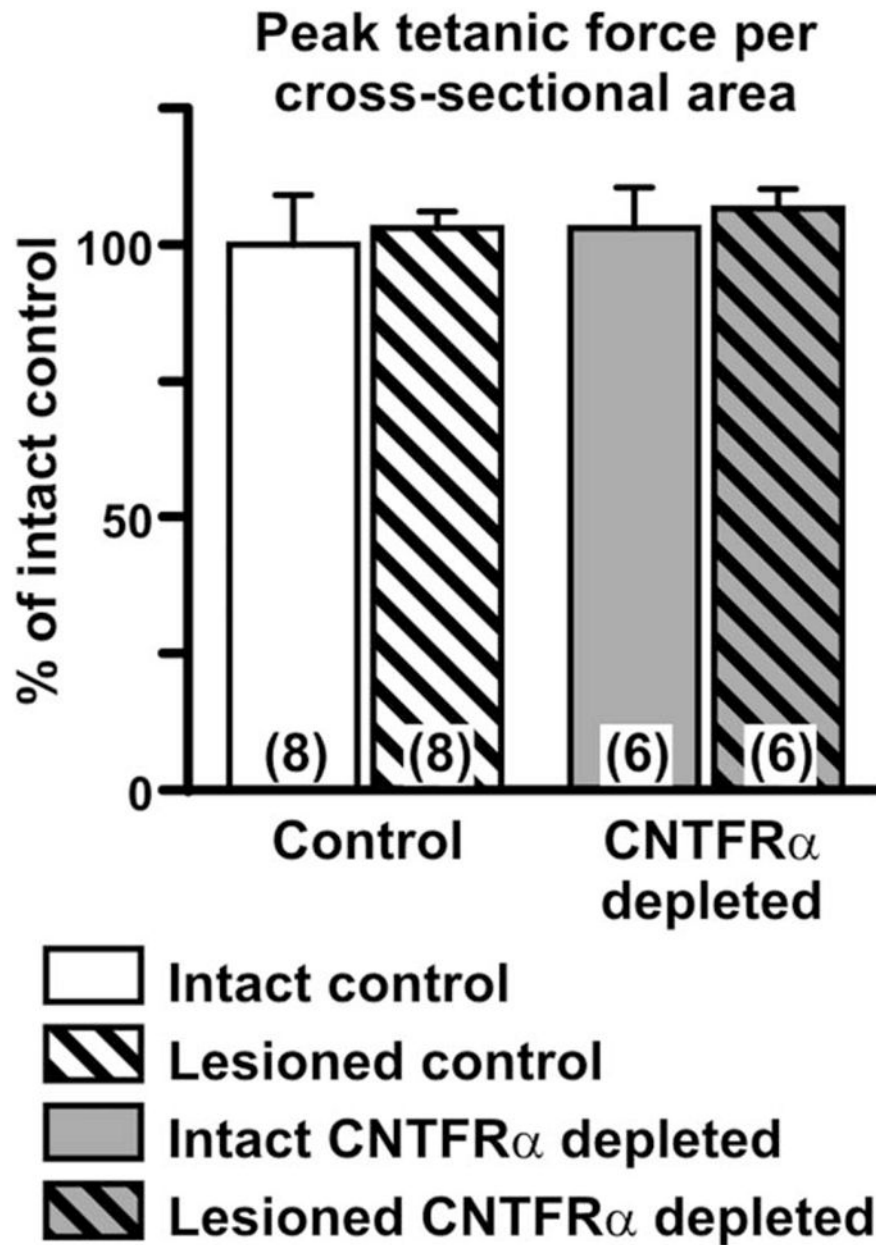


Figure 6.

Depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α) does not affect in vitro contractility. EDL muscles ipsilateral (“Lesioned”) and contralateral (“Intact”) to nerve crush were harvested 3 weeks post lesion, and their contractility (peak tetanic force per cross-sectional area) was determined in vitro, as described in the Materials and Methods section. Muscle CNTFR α depletion had no effect on contractility ($P > 0.05$; two-way ANOVA). Numbers in bars indicate numbers of mice.

Mean value of the intact controls = 438.1 mN/mm².

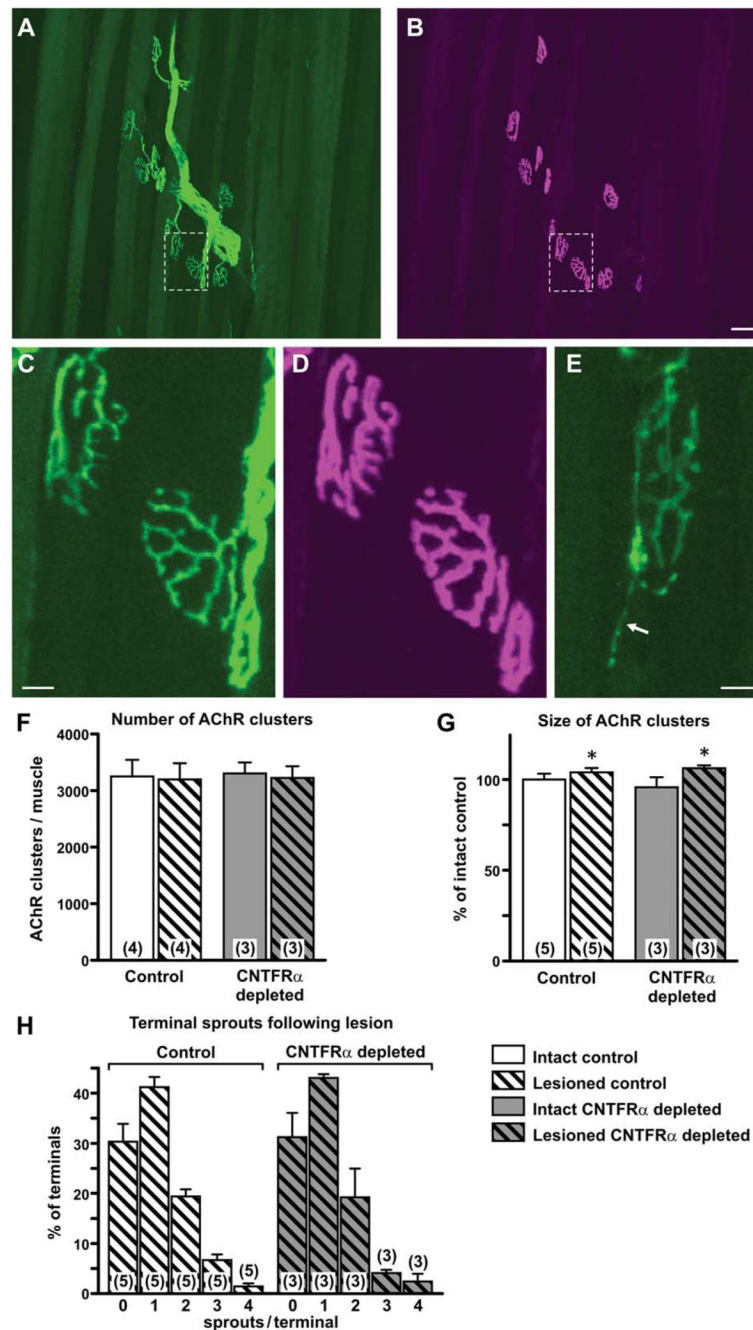


Figure 7.

Neuromuscular junctions and lesion-induced terminal sprouting are not affected by depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α). Neuromuscular junctions were visualized in tibialis anterior muscles of muscle CNTFR α -depleted and control mice 3 weeks post lesion through use of the YFP16 gene construct (which drives YFP expression in all motor axons) and rhodamine-conjugated α -bungarotoxin (α BTX) binding (which labels postsynaptic acetylcholine receptor [AChR] clusters). The YFP-labeled axons and terminals (A,C,E) and the corresponding α BTX-labeled, postsynaptic AChR clusters (B,D) were visualized at high resolution with confocal microscopy. Regions boxed in A and B are digitally “magnified” and presented again in C and D respectively. A–D are from muscle contra-lateral to the lesion. E is from muscle ipsilateral to the

lesion and illustrates a terminal sprout (arrow) typical of regenerated axons. **F:** Quantification indicated that both the lesion and CNTFR α depletion had no effect on the total number of AChR clusters per muscle ($P > 0.05$; two-way ANOVA). **G:** The lesion produced a small but statistically significant overall increase in the average size of the AChR clusters ($*P < 0.05$; $F = 6.95$; main effect of lesion in two-way ANOVA [with side relative to lesion treated as a within measure]; Bonferroni post hoc comparisons did not reveal any individual group differences), but CNTFR α depletion did not affect this index ($P > 0.05$ for CNTFR α depletion main effect and interaction terms of same two-way ANOVA described above). **H:** Finally, the number and distribution of lesioned-induced terminal sprouts were unaffected by CNTFR α depletion ($P > 0.05$ for CNTFR α depletion main effect and interaction terms of two-way ANOVA). Means with SEM are presented. Numbers in or above bars indicate numbers of mice.

Legend applies to all graphs. Scale bar = 50 μm in B (applies to A,B); 10 μm in C (applies to C,D); 10 μm in E.

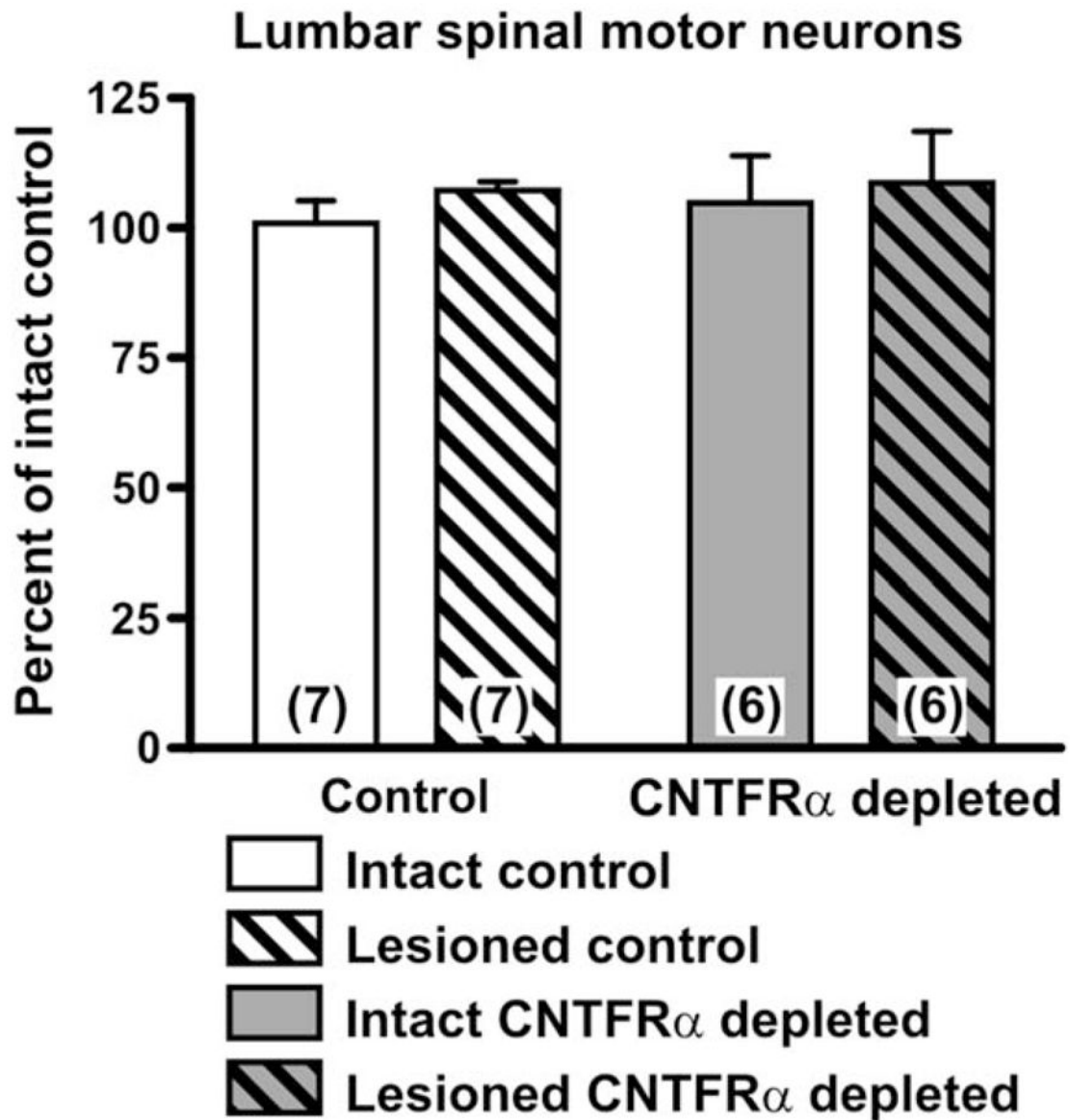


Figure 8.

Motor neuron survival is not affected by muscle ciliary neurotrophic factor receptor α (CNTFR α) depletion. Lumbar spinal motor neurons were counted in spinal cords harvested from muscle CNTFR α -depleted and control mice 3 weeks after sciatic nerve crush. Neither the lesion nor the CNTFR α depletion influenced the number of motor neurons, nor was there any interaction ($P > 0.05$ for all two-way ANOVA terms). Means with SEM are presented. Numbers in bars indicate numbers of mice.

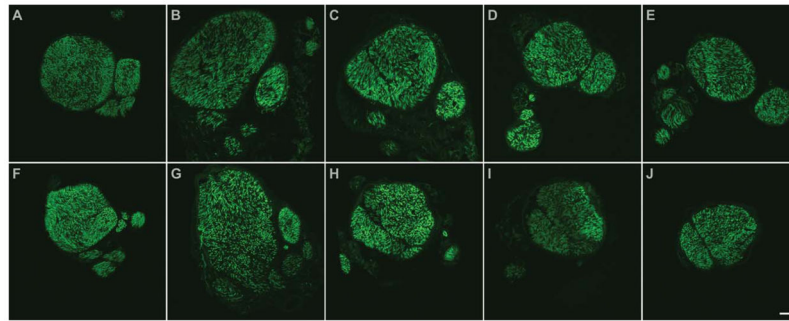


Figure 9.

Axonal regeneration across the lesion site is impaired by muscle CNTFR α depletion. Examples of cross sections from nerve segments harvested 3 weeks post lesion from a control mouse (A–E) and a littermate muscle CNTFR α -depleted mouse (F–J), which were lesioned and processed in parallel. Neurofilament-stained (to label axons) sections are presented proximal to distal (left to right) showing sections proximal to the lesion site (A,F), sections from the lesion site (B,C,G,H) with the typical expansion of axon growth in the proximal part of the lesion (B,G), and sections distal to the lesion site (D,E,I,J). Distal to the lesion the nerves from the muscle CNTFR α -depleted mice displayed fewer axons, and in some cases, like the one shown here, appeared “collapsed” relative to controls with less clearly separated axon bundles. Sections presented above each other originate from the same position relative to respective crush sites. Quantification of the data indicated that while controls displayed the same number of distal and proximal axons by this postlesion interval (distal =97.0 \pm 6.0% of proximal; n =10), this ratio was significantly decreased in muscle CNTFR α -depleted littermates that were lesioned, assayed, and analyzed in parallel (distal =81.1 \pm 7.3% of proximal; n =10; P =0.02; 10 pair; t =2.81; paired t -test). Scale bar =75 μ m in J (applies to A–J).

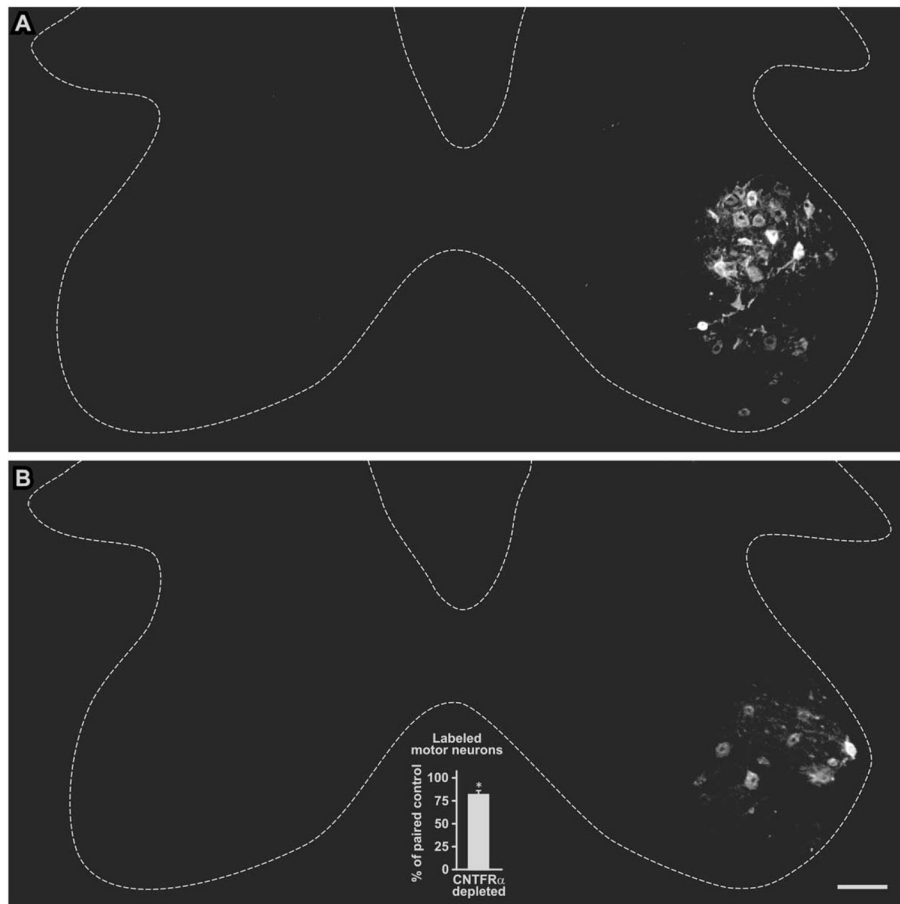


Figure 10.

Depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α) reduces motor neuron axonal regeneration following nerve lesion. Axons distal to the crush site in CNTFR α -depleted and control mice were loaded with the retrograde tracer Fluoro-

Gold 3 weeks post lesion. Spinal cords harvested 2 weeks later were processed for Fluoro-Gold immunohistochemistry to identify motor neurons with axons that regenerated to the loading site. Control (A) and muscle CNTFR α -depleted (B) examples are shown here with the lesioned and tracer loaded sides on the right. A graph summarizing all the data is presented as an insert in B. The muscle CNTFR α -depleted mice displayed significantly fewer labeled motor neurons than their littermate control pairs processed in parallel (* $P < 0.015$; 6 pair; $t = 3.65$; paired t -test). Broken lines designate approximate borders of spinal cord gray matter. Scale bar = 100 μ m in B (applies to A,B).

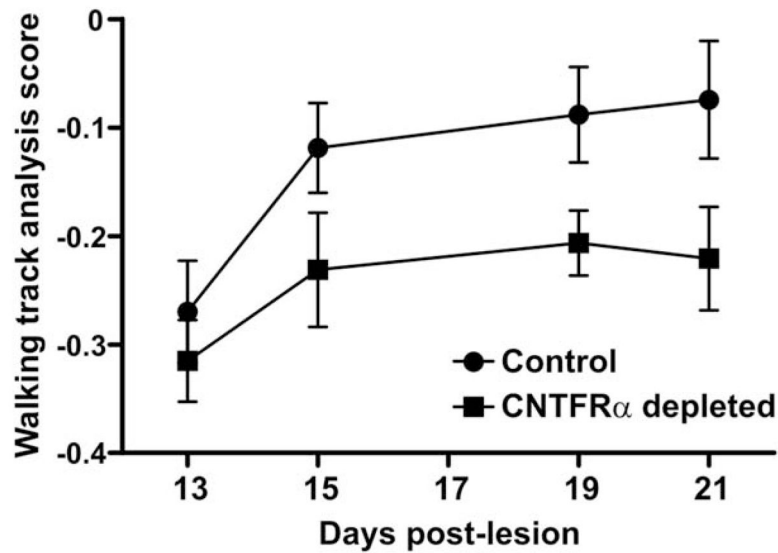


Figure 11.

Depletion of muscle ciliary neurotrophic factor receptor α (CNTFR α) impairs recovery of motor function following nerve lesion. Walking track analysis revealed that the muscle CNTFR α -depleted mice display impaired motor function recovery following sciatic nerve crush, relative to controls ($P < 0.003$; $F = 9.71$; main effect of muscle CNTFR α depletion in two-way ANOVA; less powerful post hoc individual group comparisons, appropriately designed to protect against cumulative type I error, were unable to detect differences on individual days). “Walking track analysis scores” compare lesioned and control sides within animals such that values increase as the lesioned side leg recovers function to more closely resemble the function of the unlesioned side (see Materials and Methods). Animals were tested in parallel by investigators blind to genotype. Means with SEM bars are presented. Numbers of control mice tested: 13 days post lesion (DPL) 9; 15DPL, 11; 19DPL, 11; 21DPL, 9. Number of muscle CNTFR α -depleted mice tested: 13DPL, 7; 15DPL, 7; 19DPL, 6; 21DPL, 6.

TABLE 1

Primary Antibodies

Antibody	Host isotype	Recognizes	Immunogen	Source	Cat. no.	Lot no.
A4.74	Mouse IgG1	Type IIa muscle fibers	Human MyHC	Developmental Studies Hybridoma Bank	—	2D3
MY-32	Mouse IgG1	All type II muscle fibers classes	Rabbit muscle myosin	Sigma	M 4276	121K4813
6H1	Mouse IgM, kappa light chain	Type IIx muscle fibers	Rabbit muscle myosin	Developmental Studies Hybridoma Bank	—	—
10F5	Mouse IgM, kappa light chain	Type IIb muscle fibers	Rabbit muscle myosin	Developmental Studies Hybridoma Bank	—	14J4
WB-MHCs	Mouse IgG1	Type I muscle fibers	Rabbit muscle myosin	Vector	VP-M667	—
Fluoro-Gold	Rabbit polyclonal	Fluoro-Gold	Fluoro-Gold	Fluorochrome	—	—
Neurofilament	Rabbit polyclonal	Neurofilament (NF-M)	Recombinant fusion protein with C-terminal 168 amino acid residues of rat NF-M	Millipore	AB1987	19030930