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Triple Knock-Out of CNTF, LIF, and CT-1 Defines Cooperative and Distinct Roles of these Neurotrophic Factors for Motoneuron Maintenance and Function

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Members of the ciliary neurotrophic factor (CNTF)-leukemia inhibitory factor (LIF) gene family play an essential role for survival of developing and postnatal motoneurons. When subunits of the shared receptor complex are inactivated by homologous recombination, the mice die at approximately birth and exhibit reduced numbers of motoneurons in the spinal cord and brainstem nuclei. However, mice in which cntf, lif, or cardiotrophin-1 (ct-1) are inactivated can survive and show less motoneuron cell loss. This suggests cooperative and redundant roles of these ligands. However, their cooperative functions are not well understood. We generated cntf/lif/ct-1 triple-knock-out and combinations of double-knock-out mice to study the individual and combined roles of CNTF, LIF and CT-1 on postnatal motoneuron survival and function. Triple-knock-out mice exhibit increased motoneuron cell loss in the lumbar spinal cord that correlates with muscle weakness during early postnatal development. LIF deficiency leads to pronounced loss of distal axons and motor endplates. Motor neuron survival indicates that developing and postnatal motoneurons depend on a cooperation of these molecules in which CNTF and LIF act together and that LIF, although expressed only at low levels in peripheral nerves, contributes to compensatory mechanisms that prevent functional deficits in CNTF-deficient mice (Sendtner et al., 1996). This at least partial functional overlap is reflected in cntf/lif/ct-1 triple-knock-out mice, various degrees of muscle fiber type grouping are found, indicating that denervation and reinnervation had occurred. We conclude from these findings that CNTF, LIF, and CT-1 have distinct functions for motoneuron survival and function and that LIF plays a more important role for postnatal maintenance of distal axons and motor endplates than CNTF or CT-1.

Key words: CNTF; LIF; CT-1; motoneuron; skeletal muscle; motor endplates

Introduction

The large variety of neurotrophic molecules that can support motoneuron survival in culture indicates that developing and postnatal motoneurons depend on a cooperation of these molecules that is so far not fully understood. Some of these molecules are only expressed postnatally, indicating that the requirement of motoneurons for these factors changes during development and that postnatal survival and function are also controlled by neurotrophic factors. Members of the ciliary neurotrophic factor (CNTF)-leukemia inhibitory factor (LIF) gene family are potent survival factors for motoneurons in vitro and in vivo (Arakawa et al., 1990; Sendtner et al., 1990; Hughes et al., 1993; Banner and Patterson, 1994; Pennica et al., 1996). At least CNTF plays an essential role for long-term maintenance of axons and paranodal networks, structures that are formed by the Schwann cells and axons near the nodes of Ranvier (Gatzinsky et al., 2003). This factor also protects axons under pathophysiological conditions, i.e., in progressive motoneuronopathy mutant mice (Sendtner et al., 1992a) and in experimental autoimmune encephalomyelitis (Linker et al., 2002).

Additional investigations demonstrate that null mutations in the CNTF gene lead to an earlier onset of disease in patients with sporadic and familial amyotrophic lateral sclerosis and in the human superoxide dismutase-1 G93A mouse model of motoneuron disease (Giess et al., 2002).

Gene-targeting experiments show that CNTF and LIF cooperate in maintaining postnatal survival and functional integrity of motoneurons and that LIF, although expressed only at low levels in peripheral nerves, contributes to compensatory mechanisms that prevent functional deficits in CNTF-deficient mice (Sendtner et al., 1996). This at least partial functional overlap is reflected by the fact that they share gp130 and LIF receptor β (LIFRβ) as signal-transducing receptor components in responsible cells (Davis et al., 1993). Cntf/lif double-knock-out mice do not show any alterations in survival of developing embryonic motoneurons. In contrast, a significant loss of motoneurons is observed in cardiotrophin-1 (CT-1)-deficient mice (Oppenheim et al., 2001). Thus, CT-1 function differs significantly from CNTF and LIF.

We generated cntf/lif/ct-1 triple-knock-out mice to study the individual roles of CNTF, LIF, and CT-1 in postnatal maintenance of motoneurons and whether they act together on mo-
toneuron survival and function. In these mice, significant motoneuron cell loss and functional deficits were found very early during postnatal development. In addition, the size of motoneuron cell bodies was significantly reduced in mice lacking \textit{lif} gene expression. It became apparent that LIF plays an important role for the maintenance of distal axons, such as in the plantar nerve. Moreover, LIF deficiency was associated with alterations of motor endplates, whereas lack of CNTF and CT-1 did not lead to significant changes. In \textit{cntf/lif/ct-1} triple-knock-out mice, various degrees of muscle fiber type grouping were found, indicating that denervation and reinnervation had occurred in these mouse mutants.

Altogether, our results suggest that CNTF, LIF, and CT-1 play distinct roles for motoneuron survival and function and that LIF is more potent than CNTF or CT-1 in compensating deficiency of other members of this family of neurotrophic factors.

**Materials and Methods**

cntf/lif/ct-1 triple-knock-out, \textit{cntf/lif} double-knock-out, \textit{cntf/ct-1} double-knock-out, and \textit{cntf/lif/ct-1} wild-type mice. Mice with homologous recombination of the \textit{cntf} (Masu et al., 1993), \textit{lif} (Escary et al., 1993), and \textit{ct-1} (Pennica et al., 1996) genes were backcrossed for at least five generations and subsequently at every third generation with C57BL/6 mice (Charles River Wiga, Sulzdorf, Germany). Female mice with homozygous \textit{lif} gene inactivation did not become pregnant (Stewart et al., 1992). Therefore, we crossed \textit{cntf/lif} double-knock-out males with \textit{ct-1} single-knock-out females. Resulting triple-heterozygous mice were crossed, and offspring were analyzed for the individual genotypes by PCR. Mice exhibiting the individual genotypes analyzed in this study were born at a 1.6% ratio. Therefore, mice from the F2 generation were further bred to establish triple-knock-out and corresponding control mouse lines. The number of \textit{cntf/lif/ct-1} triple-knock-out mice could be increased to a 50% ratio by mating triple-knock-out males with \textit{cntf}–/–/\textit{lif}–/–/\textit{ct-1}–/– females. Triple-knock-out mice were compared with \textit{cntf/lif} double-knock-out mice that were generated by crossing \textit{cntf/lif} double-knock-out males with \textit{cntf}–/–/\textit{lif}–/–/\textit{ct-1}–/– females, resulting in 50% offspring devoid of CNTF and LIF. As additional control groups, \textit{cntf/ct-1} double-knock-out and wild-type mice were generated by crossing mice homozygous for each of these gene loci.

**Lumbar spinal cord motoneurons.** We determined the number of motoneuron cell bodies in the lumbar spinal cord of newborn and 4-, 12-, 24-, and 48-week-old \textit{cntf/lif/ct-1} triple-knock-out and control mice, applying established techniques (Oppenheim et al., 2001). Animals were killed by ether overdose and perfused transcardially with 4% paraformaldehyde and 2% glutaraldehyde (PFA) in 0.1M PBS at pH 7.4. The lumbar spinal cord (L1–L6) was dissected, and 15 μm paraffin sections were prepared. After Nissl staining, motoneurons were counted in every 10th section, and the raw counts were corrected for split nuclei (Masu et al., 1993). The diameter of nuclei of at least four mice per group and time point and at least 40 nuclei per animal were calculated using NIH Image software (Scion, Frederick, MD). The size of motoneuron cell bodies (area in square micrometers) was determined in cross sections of the lumbar spinal cord of 12- and 24-week-old \textit{cntf/lif/ct-1} triple-\textit{cntf/lif} double-\textit{cntf/ct-1} double-knock-out, and wild-type mice. At least four animals per group and time point and at least 40 motoneurons per animal were analyzed also using the NIH Image program.

**Axons in plantar, median, and phrenic nerves.** We determined the number of myelinated axons in the plantar nerve of 12-week-old triple-knock-out and control mice. Animals were transcardially perfused with sodium cacodylate buffer containing 4% paraformaldehyde and 2% glutaraldehyde. Plantar and median nerves were carefully removed under a sodium cacodylate buffer containing 4% paraformaldehyde and 2% glu taraldehyde. Axons in plantar, median, and phrenic nerves. Phrenic nerves were removed and postfixed with 2% glutaraldehyde in 0.15 M PBS for 3 h at 4°C. After rinsing in PBS, tissues were incubated with osmium tetroxide (4%) in a buffer containing 4% potassium dichromate and 3.4% NaCl and processed for 3,4-epoxycyclohexylmethyl-3,4-epoxycyclohexylcarboxylate (Serva Feinbiochemica, Heidelberg, Germany) embedding according to standard procedures. Semithin sections (1 μm) were prepared and stained with azur-methyleneblue. Myelinated axons in the proximal and distal part of the phrenic nerve were counted from photographs taken from nerve sections under an inverse Leica (Nussloch, Germany) light microscope equipped with a digital camera (ActionCam; Agfa, Mortsel, Belgium). We measured axonal circumferences in plantar nerves in three triple-knock-out mice (700–800 randomly chosen axons per mouse) and three wild-type littersmates (900–1100 randomly chosen axons per mouse) at the age of 3 months. In the case of phrenic nerves, we scored nerve samples from two triple-knock-out and three wild-type mice.

**Myosin ATPase reaction and muscle fiber typing.** Gastrocnemius and flexor digitorum brevis (FDB) muscles were dissected from mice deeply anesthetized with a lethal dose of Ketanest/Rompun. The muscles were then freshly frozen in nitrogen-cooled isopentane. ATPase staining was performed on 10-μm-thick cryosections under acidic (pH 4.3 and 4.6) and basic (pH 9.4) conditions. Data presented in this study are from preparations at pH 4.3. Type 1 (slow twitch) muscle fibers are resistant to acidic conditions and show ATPase activity and therefore stain dark. Type 2 (A and B) fibers are not resistant to acidic conditions and do not stain. Type 2C fibers show intermediate (gray color) staining.

**Morphometric analysis of muscle fibers.** Morphometric analysis of muscle fibers was performed on ATPase-stained 10-μm-thick cryosections of the FDB muscle from 7- to 9-month-old triple-knock-out, \textit{cntf/lif} double-, \textit{ct-1} single-knock-out, and wild-type mice. Sections were viewed and photographed by using a Zeiss Axioskop microscope in conjugation with a CCD digital camera. The cross-sectional areas of type 1, type 2A and 2B, and type 2C fibers showing evidence of having been sectioned normally to their longitudinal axis were determined planimetrically. The fiber profiles were traced with a cursor on a digitizing tablet of the Vario Vision Docu analysis system at a final 45× magnification. An average of 80 type 1, 40 type 2C, and 200 type 2A and 2B optimally transverse-sectioned muscle fibers were evaluated per animal. A total of three mice per group was investigated.

**Immunostaining of motor endplates.** The FDB muscles from 4-week-old and 6-month-old triple-knock-out and control mice were freshly perfused and postfixed with 2% glutaraldehyde (PFA) in 0.1 M PBS at pH 7.4. The lumbar spinal cord (L1–L6) was dissected, and 15 μm paraffin sections were prepared. After Nissl staining, motoneurons were counted in every 10th section, and the raw counts were corrected for split nuclei (Masu et al., 1993). The diameter of nuclei of at least four mice per group and time point and at least 40 nuclei per animal were calculated using NIH Image software (Scion, Frederick, MD). The size of motoneuron cell bodies (area in square micrometers) was determined in cross sections of the lumbar spinal cord of 12- and 24-week-old \textit{cntf/lif/ct-1} triple-\textit{cntf/lif} double-\textit{cntf/ct-1} double-knock-out, and wild-type mice. At least four animals per group and time point and at least 40 motoneurons per animal were analyzed also using the NIH Image program.

**Motor performance test.** To evaluate functional motor deficits, \textit{cntf/lif/ct-1} triple-knock-out and control mice were subjected to functional motor tests at 4, 12, 24, and 48 weeks of age. The forelimb grip strength (in Newtons) was determined using an automated grip strength meter (Columbus Instruments, Columbus, OH) as described previously (Masu et al., 1993).

Rodent activity wheels (Tecniplast, Hohenpeilenberg, Germany) were used to determine voluntary movement and motor activity over a time period of 7 d. The analysis was done blinded, except for the triple-knock-out mice, which could be identified by their reduced body size.
Statistical analysis. Results are given as mean ± SEM or SD when indicated. Statistical significance of differences was assessed by one-way ANOVA, followed by Bonferroni’s post hoc comparison test using Prism software (GraphPad Software, San Diego, CA). The null hypothesis was rejected on the basis of \( p < 0.05 \).

Statistical analysis of the number of plantar and median nerve axons was performed using a two-tailed \( t \) test, and \( p \) values < 0.05 were considered significant.

Statistical analysis of size distribution of axonal profiles (Kolmogorov–Smirnov test) was performed using Statistica 6.1 software (StatSoft, Tulsa, OK). As statistical significance, we accepted \( p < 0.05 \).

Results

Motoneuron loss in the lumbar spinal cord

Morphological changes associated with functional loss of CNTF, LIF, CT-1, or CNTF/LIF have been described previously (Masu et al., 1993; Banner and Patterson, 1994; Sendtner et al., 1996; Oppenheim et al., 2001). To elucidate how these neurotrophic cytokines act similarly or distinctly on postnatal maintenance of motoneurons, we analyzed \( \text{cntf/lif/ct-1} \) triple-knock-out mice compared with \( \text{cntf/lif} \) double-knock-out, \( \text{cntf/ct-1} \) double-knock-out, and wild-type control mice.

Quantification of lumbar spinal cord motoneurons in newborn mice revealed that the number of motoneurons was already substantially reduced in \( \text{cntf/lif/ct-1} \) triple-knock-out and \( \text{cntf/ct-1} \) double-knock-out mice at birth [2965 ± 47 in triple-knock-out (n = 5) and 3054 ± 39 in \( \text{cntf/ct-1} \) double-knock-out (n = 4) compared with 3622 ± 51 (n = 4) in \( \text{cntf/lif} \) double-knock-out and 3697 ± 87 (n = 4) in wild-type mice] (Fig. 1A). The difference between these groups was statistically significant (\( p < 0.001 \)) and appeared to be attributable to loss of CT-1 function, as reported previously for \( \text{ct-1} \) single-knock-out mice (Oppenheim et al., 2001).

In 4-week-old mice, motoneuron numbers were reduced by 39\% in \( \text{cntf/lif/ct-1} \) triple-knock-out and \( \text{cntf/ct-1} \) double-knock-out mice (1985 ± 117; n = 4) and by 26\% in \( \text{cntf/ct-1} \) double-knock-out mice [2419 ± 81 (n = 4) compared with 3276 ± 119 (n = 4) in wild-type mice]. Furthermore, a statistically significant reduction of 19\% became apparent in \( \text{cntf/lif} \) double-knock-out mice at this age (2667 ± 108; n = 3). The differences between these groups and wild-type mice were statistically significant (\( p < 0.001 \)) and appeared to be attributable to loss of CT-1 function, as reported previously for \( \text{ct-1} \) single-knock-out mice (Oppenheim et al., 2001).

The number of motoneurons did not sig-
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**Figure 2.** Forelimb grip strength was determined in male wild-type control, cntf/lif double-knock-out, cntf/ct-1 double-knock-out, and cntf/lif/ct-1 triple-knock-out mice using an automated grip strength meter as described previously (Masu et al., 1993). Values shown are mean ± SEM from 10 determinations per animal. Statistical significance of the differences between the groups was tested by one-way ANOVA. Comparison of the individual groups by Bonferroni’s post hoc comparison test gave the following results: *p < 0.05; **p < 0.01; ***p < 0.001. B, Voluntary movement determined in a rodent activity wheel over a time period of 7 d. Results are given in mean ± SEM. The differences between the groups were tested by one-way ANOVA. Additional analysis by Bonferroni’s post hoc comparison test showed that the difference between wild-type and triple-knock-out mice is significant at 12 weeks of age (*p < 0.05) and at 24 and 48 weeks of age (**p < 0.001). Motor function is also significantly reduced in 24-week-old (**p < 0.001) and 48-week-old (*p < 0.05) cntf/lif double-knock-out mice compared with wild-type mice and in 48-week-old triple-knock-out mice compared with cntf/ct-1 double-knock-out mice (*p < 0.05).

Significant differences were observed between cntf/lif and cntf/ct-1 double-knock-out mice but differed between cntf/lif double-knock-out and cntf/lif/ct-1 triple-knock-out mice (*p < 0.01). There was no additional loss of motoneurons in cntf/lif/ct-1 triple-knock-out and cntf/ct-1 double-knock-out mice in 12-, 24-, and 48-week-old animals. In contrast, progressive loss of motoneurons was observed in cntf/lif double-knock-out mice. The number of motoneurons was reduced from 3622 ± 51 (n = 4) at birth to 2372 ± 94 (n = 3) at postnatal week 12, then to 1860 ± 187 (n = 4) at 24 weeks and to 1987 ± 135 (n = 4) at 48 weeks. At 48 weeks of age, the number of motoneurons was similar in cntf/lif double-knock-out and cntf/lif/ct-1 triple-knock-out mice. The difference of motoneuron loss in cntf/lif/ct-1 triple-knock-out mice at this stage was statistically significant when compared with wild-type (*p < 0.001) and cntf/ct-1 double-knock-out (*p < 0.01) mice. This indicates that cntf/lif/ct-1 triple-knock-out mice show an earlier loss of motoneurons than cntf/lif double-knock-out mice during postnatal development. However, after 24 weeks of age, motoneuron loss is not significantly different between triple-knock-out and cntf/lif double-knock-out mice.

To exclude the possibility that differences in the size of nucleoli gave rise to errors in determining the motoneuron cell number, we measured the diameter of nucleoli in the triple and double mutants as well as in wild-type control mice. In 12-week-old animals, the diameter of nucleoli in triple-knock-out mice was 3.32 ± 0.12 μm (n = 182) compared with 3.34 ± 0.12 μm in cntf/lif double-knock-out (n = 162), 3.17 ± 0.08 μm in cntf/ct-1 double-knock-out (n = 160), and 3.31 ± 0.09 μm in wild-type (n = 190) mice. The differences between these groups were not statistically significant (*p > 0.05). Similarly, no differences were found in 24-week-old mice. At this time point, the diameter of nucleoli was 3.31 ± 0.11 μm in triple-knock-out mice (n = 168) compared with 3.41 ± 0.08 μm in cntf/ct-1 double-knock-out (n = 182), and 3.45 ± 0.08 μm in wild-type (n = 186) mice.

In contrast, we found that, in 12- and 24-week-old triple- and double-knock-out mice, persisting motoneurons showed significant changes in cell body size (Fig. 1B). In particular, LIF deficiency was associated with a pronounced reduction in motoneuron cell body size. In 12-week-old mice, the size of motoneuron cell bodies was reduced by >20% in cntf/lif/ct-1
triple- and cntf/lif double-knock-out mice [721 ± 16.49 μm² (n = 186) and 730 ± 22.3 μm² (n = 162)] and by 16% in cntf/ct-1 double-knock-out mice [818 ± 24.45 μm² (n = 172) compared with 974 ± 24.6 μm² (n = 192) in wild-type mice]. The differences between these groups and wild-type mice were statistically significant (p < 0.001). Furthermore, a statistically significant difference was also observed between triple- and cntf/ct-1 double-knock-out mice (p < 0.05). In 24-week-old animals, a similar reduction was found. In cntf/lif/ct-1 triple-knock-out mice (n = 175), motoneuron cell body size was 721 ± 23.05 μm², in cntf/lif double-knock-out mice 688 ± 18.7 μm² (n = 183), and in cntf/ct-1 double-knock-out mice 829 ± 25.78 μm² (n = 159) compared with 819 ± 26.53 μm² in wild-type mice (n = 162). The differences were found statistically significant when triple- and cntf/lif double-knock-out mice were compared with cntf/ct-1 double-knock-out and wild-type mice (Fig. 1B).

However, the size of motoneuron cell bodies was at least 688 μm² so that they could be clearly separated from other neurons in the ventral horn of the lumbar spinal cord.

Changes in motor performance
To evaluate whether the loss of motoneurons in cntf/lif/ct-1 triple-knock-out mice correlates with reduced motor function, we measured grip strength in single-, double-, and triple-knock-out mice at different time points (Fig. 2A). Triple-knock-out mice showed a reduction in grip strength by ~42% at 4 weeks of age [0.34 ± 0.03 N (n = 5)] compared with 0.59 ± 0.04 N (n = 8) in wild-type mice. Muscle strength increased when the mice became older but was significantly lower than in wild-type mice at each time point analyzed. At 12 weeks, average grip strength in triple-knock-out mice (n = 9) was 0.57 ± 0.02 N compared with 0.93 ± 0.05 N in wild-type controls (n = 8), 0.74 ± 0.04 N (n = 12) compared with 0.97 ± 0.03 N (n = 13) at 24 weeks, and 0.61 ± 0.04 N (n = 6) compared with 0.94 ± 0.05 N (n = 9) at 48 weeks. The difference between these groups was statistically significant at each time point (p < 0.01).

Measurement of grip strength in 4-week-old cntf/lif double-knock-out mice revealed a slight but not significant reduction of muscle strength compared with wild-type control mice. At 12-, 24-, and 48 weeks, a reduction of >20% was observed in accordance with previous reports (Sendtner et al., 1996). The differences in cntf/lif double-knock-out mice were statistically significant from 12 weeks on when compared with wild-type mice (p < 0.05) but not when compared with cntf/ct-1 triple-knock-out mice. In addition, 12-week-old cntf/lif double-knock-out mice also showed significantly lower grip strength when compared with cntf/ct-1 double-knock-out mice (p < 0.05). In contrast, mice lacking bothCNTF and CT-1 did not show impaired motor function before 24 weeks of age. At that time, muscle strength was reduced by ~18% [0.8 ± 0.07 N (n = 7)] compared with 0.97 ± 0.03 N (n = 13) in wild-type mice and by ~24% in 48-week-old mice [0.71 ± 0.07 N (n = 7)] compared with 0.94 ± 0.05 N (n = 9) in wild-type mice. The differences between these groups were statistically significant when compared with wild-type mice (p < 0.05).

We also determined voluntary motor activity of cntf/lif/ct-1 triple-knock-out and control mice by counting the number of turns in cages with an activity wheel over a time period of 7 d (Fig. 2B). A significant reduction was observed in cntf/lif/ct-1 triple-knock-out mice already at 12 weeks of age. At this stage, the number of turns was 30% lower (39,290 ± 5631; n = 5) compared with age-matched controls (56,230 ± 3988; n = 12). An additional reduction was observed at 24 (25,830 ± 4591; n = 7) and 48 (17,830 ± 5931; n = 7) weeks compared with wild-type control mice [53,390 ± 2773 (n = 9) and 49,360 ± 5708 (n = 13), respectively]. Cntf/lif double-knock-out mice also showed reduced motor activity in the running wheels at 24 and 48 weeks of age [24,390 ± 8882 (n = 6) and 26,680 ± 4434 (n = 6)]. The differences between cntf/lif double-knock-out and wild-type mice were statistically significant (p < 0.05). These data show that cntf/lif/ct-1 triple-knock-out mice become weak at an earlier time point but that their motor activity does not differ at later developmental periods from that in cntf/lif double-knock-out mice. Moreover, mice lacking bothCNTF and CT-1 do not show impaired motor function, indicating thatLIF plays a more important role for postnatal maintenance of motor function than CT-1.

Axon loss in the plantar and median nerve
Because cntf/lif/ct-1 triple-knock-out mice showed reduced muscle strength, we investigated the morphology of peripheral nerves and determined the number of myelinated axons in the plantar and median nerves of mutant and control mice. At 12 weeks, the number of myelinated axons in the plantar nerve of triple-knock-out mice (1431 ± 37; n = 3) was significantly lower than that observed in wild-type (1763 ± 22; n = 3) andcntf single-knock-out (1671 ± 105; n = 3) mice. Reduced axon numbers were also found in cntf/lif double-knock-out mice at this age (1487 ± 103; n = 3). The difference was statistically significant when compared with wild-type controls (Fig. 3).

We also found a reduced number of myelinated axons in the median nerve in 12-week-old triple-knock-out mice compared with wild-type control mice [1034 ± 21 (n = 3) compared with1178 ± 93 (n = 3)] (data not shown). However, this difference was smaller than that in plantar nerves. Interestingly, the reduction of myelinated axons (19% in the plantar nerves and 12% in the median nerves) appeared smaller than the loss of motoneurons in the cervical spinal cord of ct-1 single-knock-out mice (Oppenheim et al., 2001), suggesting that a formation of axonal collaterals might compensate for the loss of motoneurons, at least in part.

We could not find any loss of either proximal or distal phrenic nerve axons in triple-knock-out, cntf/lif, and cntf/ct-1 double-knock-out mice at each time point analyzed (data not shown).

To investigate whether axonal sizes have been changed in the plantar nerves of the mutants, we determined the size distribu-
fasciculating, extensively folded processes reminiscent of regrowing sprouts (Fig. 4B, C, arrowheads). Occasionally, we found fiber bundles containing one or two large-caliber axons (Fig. 4D, asterisks). These fiber bundles were collectively enveloped by a thin myelin sheath, possibly induced by the large-caliber axons (Fig. 4D). In some cases, we found large-caliber axons having achieved a 1:1 ratio with a pro-myelinating Schwann cell (Fig. 4B, arrow). This indicates that collaterals had formed in the triple-knock-out mice and that compensatory mechanisms took place even when all three members of the cntf/lif/ct-1 gene family were deficient.

We then investigated the morphology of phrenic nerves of three wild-type and two triple-knock-out mice ~3 mm proximal to the diaphragm. Neither at the light nor at the electron microscopic level could the nerves be discriminated from each other based on morphological criteria, and there were no hints for axonal degeneration, axonal sprouting, or axon collateralization. In the triple mutants, nonmyelinated axon–Schwann cell units appeared normal and did not contain unusually large axons, fasciculating and extensively folded sprouts, or ectopic myelin sheaths, as observed in the plantar nerves of the triple mutants.

Alterations at motor endplates
We then investigated motor endplate morphology and size in the FDB muscles of 4- and 24-week-old triple-knock-out and control mice. The morphology of endplates appeared grossly normal in all mice investigated. However, quantitative analysis revealed a significant decrease of motor endplate size in mice lacking lif gene expression. In 4-week-old mice, motor endplate areas were reduced by 27% in the lif single-knock-out, by 16% in the cntf/lif double-knock-out, and by 23% in the cntf/lif/ct-1 triple-knock-out mice [159 ± 7.37 μm² (n = 22), 182 ± 7.35 μm² (n = 33), and 168 ± 12.33 μm² (n = 18) compared with 217 ± 8.7 μm² (n = 28) in wild-type mice]. The differences between these groups and wild-type mice were statistically significant (Fig. 5A). This reduction was even more notable in 24-week-old mice (Fig. 5B, F–H) when compared with wild-type mice (Fig. 5C–E). We found a reduction of 37% in the triple-knock-out (153 ± 6.04 μm²; n = 84), 32% in the cntf/lif double-knock-out (165 ± 11.67 μm²; n = 27), and 24% in the lif single-knock-out (183 ± 13.1 μm²; n = 19) mice when compared with wild-type control mice (241 ± 14.91 μm²; n = 48). The differences between these groups and wild-type mice were statistically significant (p < 0.001 for the triple- and cntf/lif double-knock-out mice and p < 0.05 for the lif single-knock-out mice).

Fiber type grouping in flexor digitorum brevis and gastrocnemius muscle
We also investigated the ATPase activity in skeletal muscle fibers of two different muscles, the FDB and the gastrocnemius muscle.
We chose these muscles because, in many neuromuscular disorders in which degeneration of motoneurons occurs, distal muscles groups are usually more prone to pathological changes than proximal muscles. Using ATPase activity (pH 4.3), we stained the FDB and gastrocnemius muscle of 3-month-old and 7- to 9-month-old wild-type, ct-1 single-knock-out, cntf/lif double-knock-out, and cntf/lif/ct-1 triple-knock-out mice. Type 1 (slow twitch) muscle fibers are resistant to acidic conditions and show ATPase activity and stain dark (Fig. 6A–E, black asterisks). Type 2C muscle fibers show intermediate (gray color) staining (Fig. 6A–E, white asterisks). In the FDB muscle, the cross-sectional area of type 1 fibers was not significantly different in 7- to 9-month-old triple-knock-out (506 ± 153 μm², mean ± SD; n = 234) compared with cntf/lif double-knock-out (401 ± 55 μm², mean ± SD; n = 119), ct-1 single-knock-out (490 ± 72 μm², mean ± SD; n = 219), and wild-type (440 ± 67 μm², mean ± SD; n = 241) mice. The type 2 (A and B) fibers are not resistant to acidic conditions and do not stain (Fig. 6A–E, black asterisks). Type 2C muscle fibers show intermediate (gray color) staining (Fig. 6A–E, arrows).

Both types of muscles showed the typical chessboard staining pattern of wild-type mice. In 3-month-old mutant mice, there was no detectable difference in fiber type distribution compared with age-matched wild-type mice (data not shown). Furthermore, in 7- to 9-month-old ct-1 single (Fig. 6B) and cntf/lif double (Fig. 6C) mutants investigated, the morphological appearance and the distribution of fiber types was very similar to that seen in age-matched wild-type mice (Fig. 6A). Interestingly, the size of type 2C fibers was significantly smaller in cntf/lif double-knock-out (367 ± 33 μm², mean ± SD; n = 110; p < 0.05) and cntf/lif/ct-1 triple-knock-out (434 ± 150 μm², mean ± SD; n = 81; p < 0.05) mice compared with wild-type control mice (610 ± 44 μm², mean ± SD; n = 78). Moreover, we saw various degrees of grouping of muscle fiber types in triple-knock-out mice (Fig. 6D, E).

The gastrocnemius muscles of single- and double-mutant mice did not show any morphological changes or alterations in fiber type distribution at any stage investigated when compared with wild-type mice (data not shown). In the same muscle from 3-month-old cntf/lif/ct-1 triple-mutant mice, more intermediately stained fibers were seen than in wild-type mice. These subtle changes were not detectable in older animals (data not shown).

Discussion

Here we show that CNTF, LIF, and CT-1 exhibit overlapping but also distinct effects on motoneuron survival during development and in the adult. Whereas CNTF and/or LIF deficiency only leads to postnatal loss of motoneurons, lack of CT-1 causes a significant reduction of lumbar spinal motoneurons during prenatal development. In 48-week-old mice, CNTF/LIF deficiency causes higher losses of motoneurons than CNTF/CT-1 deficiency, and lack of LIF in combination with either CNTF or CNTF and CT-1 deficiency appears predominantly responsible for functional motor deficits. This correlates with reduced motor endplate areas, which are detectable in both young (4-week-old) and adult (24-week-old) mice. Interestingly, reduced motor endplate areas are not found in CNTF- or CT-1-deficient mice, and CNTF/CT-1-deficient mice do not show reduction in motor endplate size.

CNTF, LIF, and CT-1 share the signal transducing receptor units gp130 and LIFRβ in the formation of their respective receptor complexes (Stahl and Yancopoulos, 1994). In addition, CNTF binds to a nontransducing α chain (CNTFRα) that acts as a third
binding component (Stahl and Yancopoulos, 1994). Genetic deletion of LIFR
binding component (Stahl and Yancopoulos, 1994). Genetic de-

Figure 6. Muscle fiber type distribution in the FDB muscle of wild-type (A), ct-1 single-knock-out (B), cntf/lif double-knock-
out (C), and triple-knock-out (D, E) mice. Type 1 (slow twitch) muscle fibers are resistant to acidic conditions and show ATPase
activity and therefore stain dark (A–E, white asterisks). Type 2A and 2B fibers are not resistant and do not stain (A–E, black
wild-type mouse show the typical chessboard staining pattern (white asterisk, type 1 fiber; black asterisk, type 2A and 2B fibers;
arrows, type 2C fiber). B, C, Muscle of 7- to 9-month-old ct-1 single-knock-out (B) and cntf/lif double-knock-out (C) mice.
The morphological appearance and the distribution of fiber types is very similar to that found in age-matched wild-type mice (white
asterisks, type 1 fibers; black asterisks, type 2A and 2B fibers; arrows, type 2C fibers). Large structures of darker appearance at
the top right corner of C are obliquely sectioned type 1 fibers. D, E, Seven- to 9-month-old triple-knock-out mice show various degrees
of muscle fiber type grouping (white asterisks, type 1 fibers; black asterisks, type 2A and 2B fibers; arrows, type 2C fibers). ko,
Ko, Knock-out. Scale bar: A–E, 100 μm.

in intact postnatal nerves, but a rapid and
dramatic increase occurs when nerves are
lesioned (Banner and Patterson, 1994; Cortis et al., 1994; Sun et al., 1996). LIF
acts as an autocrine survival factor for
Schwann cells (Dowsing et al., 1999) and
may play a role as a regulator of macro-
phage attraction to the lesioned nerve (To-
faris et al., 2002). Third, LIF can be re-
leased from Schwann cells via the classical
secretory pathway, in contrast to the
CNTF protein, which lacks a conventional
leader sequence. Therefore, only a small
part of the cytosolic pool of CNTF in dif-
ferentiated Schwann cells might be avail-
able for the motoneurons under physio-
logical conditions. CT-1 and also CLC and
CLF, in contrast to CNTF, are produced at
relatively high levels in the embryonic limb
bud (Pennica et al., 1996; Forger et al.,
2003), and thus these factors could act as
target-derived neurotrophic factors on re-
sponsive motoneurons.

The first effect of LIF identified in neu-
rons was alteration of the neurotransmit-
ter phenotype of sympathetic neurons. In
vitro, it induces a switch from noradrener-
gic to cholinergic transmitter phenotype
(Yamamori et al., 1989; Rao et al., 1992) and
influences neu-
ropetide expression, including the induction of vasoactive in-
testinal peptide (VIP), substance P, and somatostatin (Nawa et
al., 1991). However, in vivo, it has been shown that LIF is not
required for the target-directed change in neurotransmitter
expression from noradrenaline to acetylcholine and VIP that occurs
in developing sympathetic neurons innervating sweat glands
(Rao et al., 1993; Francis et al., 1997). LIF induces cholinergic
function in spinal cord motoneurons and supports survival of
embryonic motoneurons and sensory neurons in culture (Mar-
tinou et al., 1992). Furthermore, it can induce several neuropep-
tides through alteration of neuronal gene expression (Patterson
and Nawa, 1993) known to be involved in the response of neural
tissue to injury (Sun et al., 1996). LIF is retrogradely transported
to the cell body of spinal motoneurons and rapidly upregulated
when nerve injury occurs (Curtis et al., 1994; Dowsing et al.,
2001). LIF also protects facial motoneurons from axotomy-
directed cell death (Hughes et al., 1993) and promotes regenera-
tion after nerve injury (Tham et al., 1997; Cafferty et al., 2001).
However, if gene knock-out does not lead to enhanced mo-
toneuron loss but only enhances motoneuron loss when it occurs
in combination with CNTF or CNTF/CT-1 deficiency.

In this paper, we show that LIF deficiency leads to significantly
reduced motor endplate size, which correlates with reduced muscle
strength. In particular, cntf/ct-1 double-knock-out mice, which
show motoneuron loss to the same extent as cntf/lif
double-knock-out mice, show significantly better grip strength
performance at 12 weeks than cntf/lif double mutants. This sug-
ests that the reduced endplate size in LIF-deficient mice con-
tributes to the phenotype, in addition to the loss of motoneurons
detectable in the spinal cord of these mutants. It appears interest-
ing that the presence of CNTF from Schwann cells and/or CT-1
from muscle cannot compensate for the deficiency of LIF in con-
trolling motor endplate area. Indeed, the reduction in motor
endplate area in lif single-knock-out mice is not higher than the
reduction in the cntf/lif/ct-1 triple-knock-out mice, thus pointing to a specific role of LIF.

The development and maturation of the motor endplate is regulated by interactions between presynaptic (nerve terminal and Schwann cells) and postsynaptic (muscle fiber) components (Sanes and Lichtman, 2001). The differentiation of the postsynaptic apparatus, including AchR synthesis and clustering, is promoted and regulated by nerve-derived molecules such as neuregulin and agrin (Gautam et al., 1996; Rosenbaum et al., 1997; Sanes and Lichtman, 2001). In addition, muscle-derived signals, including cell adhesion molecules and neurotrophic factors, control formation of the presynaptic part (Sanes and Lichtman, 1999). LIF has been shown to interfere with maturation of motor units by transiently delaying the onset of synapse withdrawal (Kwon et al., 1995). On the other side, in mice that lack Schwann cells as a result of mutations in neuregulin receptors, motoneurons only transiently form neuromuscular synapses with derailed patterns of innervation (Riethmacher et al., 1997; Lin et al., 2000). This also demonstrates that Schwann cells and trophic factors from Schwann cells are necessary for motor endplate formation.

We also observed muscle fiber type grouping in 7- to 9-month-old cntf/lif/ct-1 triple-knock-out mice. Although the degree of muscle fiber type grouping varies, it seems as if LIF deficiency does not play such a major role here compared with the other ligands. The distribution of fast fibers in the flexor digitorum brevis muscle appears unchanged in cntf/lif double-knock-out mice compared with wild-type mice, and also ct-1 single-knock-out mice do not show major alterations. In contrast, triple-knock-out mice show a higher degree of fiber type grouping, which also correlates with significantly reduced voluntary movement, as detected by determining the number of turns in a rodent activity wheel over 1 week. In this case, CT-1 seems to add on the detectable deficits that are observed in cntf/lif double-knock-out mice.

In conclusion, our data suggest that CT-1 is particularly important for developing motoneurones, whereas CNTF and LIF are essential for postnatal maintenance of motoneurons. LIF seems important for developing motoneurons, whereas CNTF and LIF are knock-out mice.

add on the detectable deficits that are observed in triple-knock-out mice, thus pointing to a specific role of LIF.

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