Cell, Vol. 83, 313–322, October 20, 1995, Copyright © 1995 by Cell Press

Mice Lacking the CNTF Receptor, Unlike Mice Lacking CNTF, Exhibit Profound Motor Neuron Deficits at Birth

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Summary

Ciliary neurotrophic factor (CNTF) supports motor neuron survival in vitro and in mouse models of motor neuron degeneration and was considered a candidate for the muscle-derived neurotrophic activity that regulates motor neuron survival during development. However, CNTF expression is very low in the embryo, and *CNTF* gene mutations in mice or human do not result in notable abnormalities of the developing nervous system. We have generated and directly compared mice containing null mutations in the genes encoding CNTF or its receptor (CNTFRα). Unlike mice lacking CNTF, mice lacking CNTFRα die perinatally and display severe motor neuron deficits. Thus, CNTFRα is critical for the developing nervous system, most likely by serving as a receptor for a second, developmentally important, CNTF-like ligand.

Introduction

Ciliary neurotrophic factor (CNTF) was originally identified, and named, on the basis of its ability to promote the survival of parasympathetic motor neurons found in the embryonic chick ciliary ganglion (Adler et al., 1979; Lin et al., 1989; Stockli et al., 1989). CNTF also acts on a variety of other embryonic neurons, including peripheral sensory neurons (Barbin et al., 1984), sympathetic neurons (Barbin et al., 1984; Ernsberger et al., 1989; Saadat et al., 1989), hippocampal neurons (Ip et al., 1991), preganglionic sympathetic neurons (Blottner et al., 1989), and cranial and spinal motor neurons (Arakawa et al., 1990; Sendtner et al., 1990; Wewetzer et al., 1990; Oppenheim et al., 1991; Martinou et al., 1992; Vejsada et al., 1995). Of all the actions of CNTF, those on motor neurons have attracted the most attention. In addition to its survival and differentiative effects on cultured embryonic motor neurons in vitro (Arakawa et al., 1990; Martinou et al., 1992), exogenously provided CNTF can rescue motor neurons from ontogenetic cell death in the embryo (Wewetzer et al., 1990; Oppenheim et al., 1991) and can support motor neurons following axotomy in newborns (Sendtner et al., 1990; Vejsada et al., 1995). Exogenously provided CNTF can also blunt progression in several mouse models of motor neuron disease (Sendtner et al., 1992a; Mitsumoto et al., 1994; Sagot et al., 1995), raising the possibility that CNTF may be of therapeutic value for motor neuron diseases in humans. The ability of CNTF to maintain motor neurons led to consideration of CNTF as a candidate for the long sought-after nerve- or muscle-derived neurotrophic activity that seemingly regulates motor neuron survival in the embryo (Hamburger, 1975; Oppenheim, 1989; Wewetzer et al., 1990; Oppenheim et al., 1991). This neurotrophic activity is thought to be present in limiting amounts during the developmental period when motor neurons contact their target and compete for neurotrophic support and thus apparently ensures survival of only those motor neurons making appropriate connections.

Despite the impressive effects of ectopically administered CNTF on embryonic and adult motor neurons, as well as on other neuronal populations, recent analyses of mice and humans containing mutated *CNTF* genes suggest that endogenous CNTF does not, in fact, play a critical role in the development of motor neurons or other neuronal populations. Mice homozygous for null mutations in the *CNTF* gene appear remarkably normal (Masu et al., 1993). They are viable and initially thrive, and only later in adulthood do they exhibit a very mild loss of motor neurons with resulting minor muscle weakness. Even more astonishingly, a study of the Japanese population has revealed that a significant fraction, approximately 2.5%, are homozygous for mutations that inactivate the *CNTF* gene (Takahashi et al., 1994). These individuals lacking CNTF are seemingly not adversely affected in any way and have not yet been shown to have any associated neurologic abnormalities. Consistent with this genetic evidence that CNTF does not play a major role during development, it has been shown that CNTF is normally expressed only at very low levels in the embryo (Stockli et al., 1991; Ip et al., 1993). Furthermore, CNTF lacks a signal peptide and is found stored inside of adult glial cells, perhaps awaiting release by some mechanism induced by injury (Sendtner et al., 1992b; Rende et al., 1992; Friedman et al., 1992). These studies of CNTF expression, together with the findings that mice and humans lacking CNTF develop and mature quite normally, have led to the suggestion that CNTF is not essential during development or for life but instead acts in response to injury or other stresses.

CNTF is a member of a family of distantly related cytokines that includes leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), and cardiotrophin-1 (Bazan, 1991; Rose and Bruce, 1991; Pennica et al., 1995). CNTF utilizes a three-component receptor system consisting of a CNTF-specific binding component, known as CNTFRα (Davis et al., 1991), as well as two signal-transducing β receptor subunits, gp130 and LIFRβ (Taga et al., 1989; Hibi et al., 1990; Gearing et al., 1991), which it shares with its cytokine relatives (Ip et al., 1992;

Gearing et al., 1992; Davis et al., 1993; Stahl et al., 1993; Stahl and Yancopoulos, 1993; Baumann et al., 1993; Stahl and Yancopoulos, 1994). These three components are all initially unassociated on the cell surface, but form a complex in response to CNTF. The first step in complex formation involves the binding of CNTF to its α receptor component, followed by recruitment of the β components to form the complete complex; the β components do not bind to CNTF in the absence of CNTFRα (Ip et al., 1992; Davis et al., 1993). Complex formation, in particular, β component dimerization, initiates the signaling process by activating cytoplasmic tyrosine kinases (members of the Jak/Tyk family) that are constitutively preassociated with the cytoplasmic domains of each of the β components (Stahl and Yancopoulos, 1993; Stahl et al., 1994; Lutticken et al., 1994; Stahl et al., 1995). In contrast with the β components, CNTFRα lacks a cytoplasmic domain (it is linked to the surface via a glycosylphosphatidylinositol linkage) and plays no role in signaling (Davis et al., 1991; Stahl and Yancopoulos, 1994). The sole function of CNTFRα seems to involve specificity determination; i.e., since CNTF cannot bind to or activate its β components in the absence of CNTFRα, the expression of CNTFRα determines which cells can respond to CNTF (Ip et al., 1993; Stahl and Yancopoulos, 1994). Thus, because the CNTFRα subunit is generally restricted to the nervous system in its expression, the actions of CNTF are largely limited to neurons and glia (Ip et al., 1993; Stahl and Yancopoulos, 1994). It is this specific expression of CNTFRα that underlies the role of CNTF as a neurotrophic factor, and which distinguishes CNTF from its more generally acting cytokine relatives. Other members of the CNTF cytokine subfamily, such as IL-6 and IL-11, also have their own specificity-determining α components, which are widely expressed outside of the nervous system, for example in hematopoietic cells, explaining the more general actions of these cytokines (Kishimoto et al., 1992; Hilton et al., 1994).

CNTFRα is expressed on all cells that are known to respond to exogenously provided CNTF and is also expressed on many additional embryonic and adult neuronal populations that have not yet been evaluated for their CNTF responsiveness (Ip et al., 1993). The widespread expression of CNTFRα in the developing nervous system of the embryo is consistent with the observations that exogenously provided CNTF can act on many embryonic neurons, but seems perplexing if CNTFRα merely acts as a receptor for a factor that only acts later in life (Ip et al., 1993). This apparent paradox has led to speculation that a ligand other than CNTF may also use CNTFRα and that this ligand might play a much more critical role during early development, and perhaps even in the adult, than does CNTF (Ip et al., 1993). To explore this possibility, we have generated and compared mice containing null mutations in either the gene encoding CNTF or that encoding CNTFRα. In contrast with mice lacking CNTF, mice lacking CNTFRα die shortly after birth and exhibit profound deficits in all motor neuron populations examined. These mice provide compelling genetic evidence that CNTFRα does indeed play a critical role in the developing nervous system, most likely by serving as the receptor for a second, developmentally important, CNTF-like factor.

Results

Contrasting Phenotypes Result from *CNTF* and *CNTFRα* Genetic Mutations

Replacement vectors were constructed and employed in positive–negative selection protocols, involving successive G418 and gancyclovir selections (Mansour et al., 1988), intended to disrupt either the *CNTF* or *CNTFRα* genes by homologous recombination in embryonic stem (ES) cells (Figures 1 and 2). Successful targeting of the *CNTF* gene was achieved in 4 of 82 transfected clones surviving the double selection, while succesful targeting of the *CNTFRα* gene was achieved in 2 of 180 ES clones screened. Male chimeras derived from each of these clones were bred with C57BL/6 females. All four of the *CNTF* gene–targeted clones transmitted the mutant allele to the F1 generation, while one of the *CNTFRα* gene–targeted clones transmitted the mutant allele through the

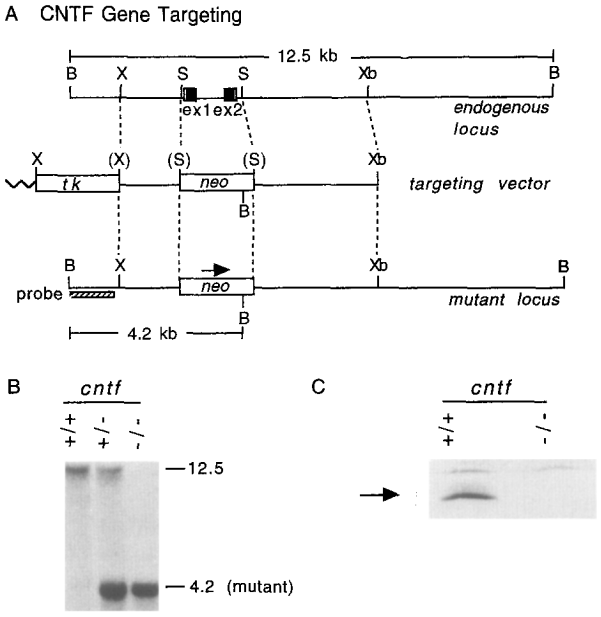

Figure 1. Targeting of the Murine *CNTF* Gene

(A) Schematic representation of the 12.5 kb BamHI genomic DNA fragment encompassing the endogenous *CNTF* gene, of the targeting vector constructed, and of a mutant locus following successful targeting. The two exons of the *CNTF* gene are indicated as boxes, with coding portions in black and associated noncoding sequences in white. The PGK–*neo* expression cassettes (from pKJ1) and MC1–*tk* cassettes are also indicated as boxes, and the novel 4.2 kb BamHI (B) fragment generated following successful targeting is also marked; note that the probe used to detect this fragment was derived from genomic DNA not included in the targeting construct. Restriction sites are indicated with a parenthesis when the site has been ablated during the construction of the targeting vector. X, XhoI; Xb, XbaI; S, SpeI.

(B) A representative Southern blot of tail DNA from wild-type, heterozygous, and homozygous F2 progeny, showing the endogenous and mutant BamHI fragments.

(C) Western blot analysis of adult sciatic nerve extracts for CNTF protein (arrow). Each lane contains 200 μg of protein.

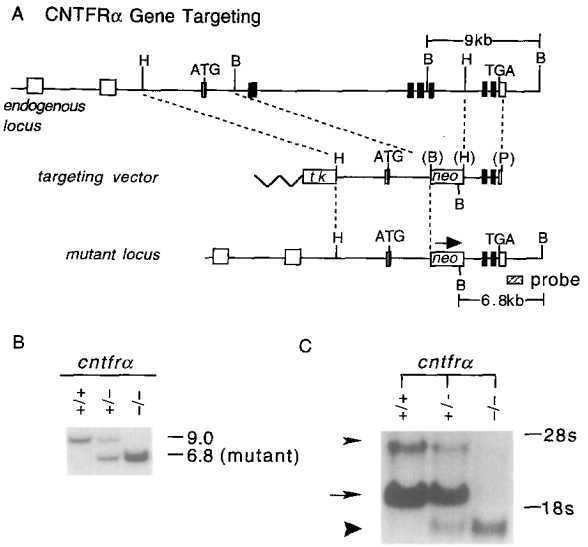

Figure 2. Targeting of the Murine *CNTFRα* Gene

(A) Schematic representation of >35 kb of genomic DNA encompassing the 10 exons of endogenous *CNTFRα* gene (Valenzuela et al., 1995), of the targeting vector constructed, and of a mutant locus following successful targeting; exons are indicated as boxes, with coding portions in black and noncoding sequences as open boxes. The PGK–*neo* (replacing about 18 kb of genomic DNA, including exons 2–5) and MC1–*tk* cassettes are also indicated as boxes, and the novel 6.8 kb BamHI fragment generated following successful targeting is also marked; the probe used to detect this fragment was derived from genomic DNA not included in the targeting construct. Restriction sites are indicated with a parenthesis when the site has been ablated during the construction of the targeting vector. H, HindIII; P, PstI; B, BamHI.

(B) A representative Southern blot of tail DNA from wild-type, heterozygous, and homozygous F2 progeny, showing the endogenous and mutant BamHI fragments.

(C) Northern blot analysis of 20 μg of total brain RNA from newborn *CNTFRα*$^{+/+}$, *CNTFRα*$^{+/-}$, and *CNTFRα*$^{-/-}$ pups, using a rat *CNTFRα* cDNA probe. Indicated are *CNTFRα* precursor (small arrowhead), normal coding (arrow), and truncated transcripts (from the mutant allele; large arrowhead).

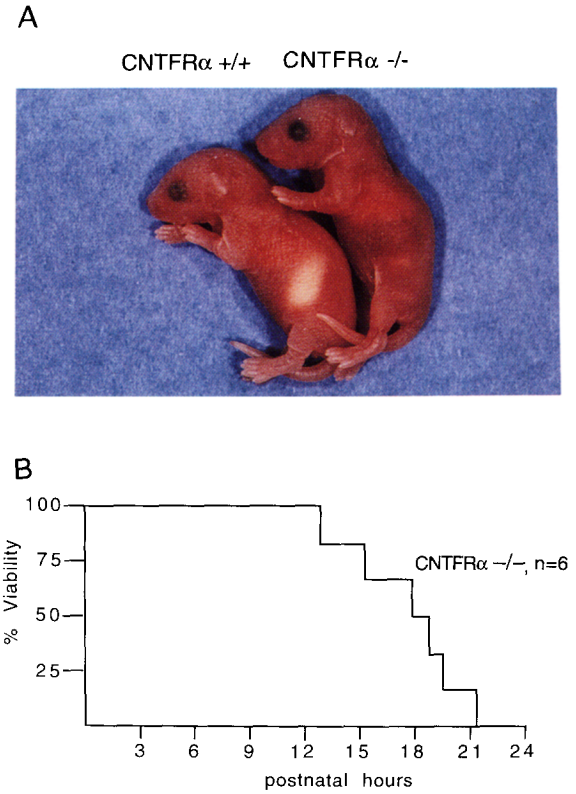

Figure 3. Appearance and Viability of Mice Carring a *CNTFRα* Gene Disruption

(A) Newborn F2 littermates from an F1 heterozygous cross showing that *CNTFRα*$^{-/-}$ pups, unlike wild-type pups, do not contain milk in their stomachs, owing to lack of feeding.

(B) Time course demonstrating postnatal longevity of *CNTFRα*$^{-/-}$ newborn pups, observed since time of birth.

germline at high frequency. The F1 progeny heterozygous for either the *CNTF* mutation or the *CNTFRα* mutation were viable and appeared overtly normal and fertile and were bred to generate mice homozygous for either the null mutation in the *CNTF* gene (designated *CNTF*$^{-/-}$) or the null mutation in the *CNTFRα* gene (designated *CNTFRα*$^{-/-}$) (Figures 2B and 3B, respectively). Consistent with previous findings (Masu et al., 1993), mice we generated that were lacking CNTF were viable and exhibited no overt abnormalities. Conversely, in litters intended to generate mice homozygous for the null mutation in *CNTFRα*, approximately 25% (45 of 169) of the newborn pups did not feed, as evidenced by a lack of milk in their stomachs, and died during the first postnatal day (Figure 3). Genotyping by Southern blot analysis revealed that most of the pups exhibiting perinatal lethality (90 of 99 genotyped) were homozygous for the disrupted *CNTFRα* allele. In contrast, all of the surviving pups were either wild-type or heterozygous for this allele; not a single newborn mouse homozygous for the disrupted *CNTFRα* allele survived longer than 24 hr (Figure 3B).

Like the *CNTF*$^{-/-}$ pups, the overt appearance of the *CNTFRα*$^{-/-}$ mutant pups was normal aside from the lack of milk in their stomachs (Figure 3A), and their weights at birth did not significantly differ from those of their wild-type littermates. However, a behavioral analysis performed within the first 6–8 hr after birth revealed that the mice homozygous for the disrupted *CNTFRα* allele did not move their jaws as notably as feeding littermates, qualitatively assessed by their ability to open their mouths in response to a tail pinch and to vibrissal pin pricks. In addition, these mice were unable to suckle. The dramatic viability differences between mice lacking CNTFRα and CNTF are consistent with speculation that CNTFRα serves a critical role during development that does not involve CNTF, most likely by serving as a receptor for a second, developmentally important, CNTF-like ligand.

Mice Lacking CNTFRα Lack Responsiveness to CNTF, Confirming That CNTFRα Is a Required CNTF Receptor Component

Before further analyzing the phenotype of the *CNTFRα*$^{-/-}$ mice and comparing them with the *CNTF*$^{-/-}$ mutant mice, we confirmed that the gene disruptions resulted in null mutations. Immunoblot analysis for CNTF protein in the adult sciatic nerve revealed that while wild-type nerve ex-

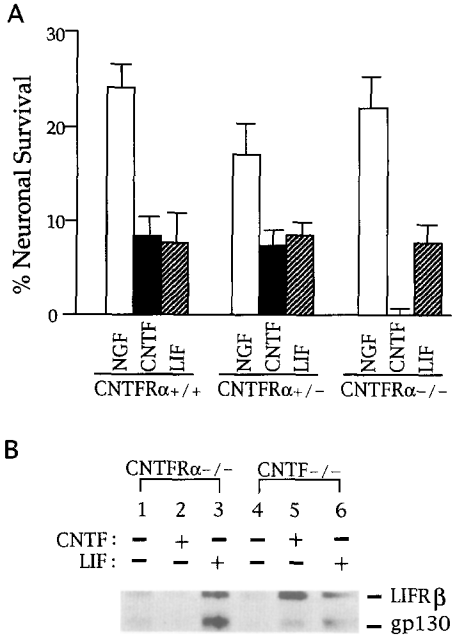

Figure 4. The CNTFRα Is the Only Functional CNTF Receptor Used by DRG Neurons and in the Brain

(A) Survival of DRG neurons cultured in the presence of either NGF, LIF, or CNTF at 10 ng/ml. Data are the average ± SEM of results from three indpendent experiments from *CNTFRα*$^{+/+}$ (n = 2), *CNTFRα*$^{+/-}$ (n = 3), and *CNTFRα*$^{-/-}$ (n = 3) mice.

(B) LIF (50 ng/ml), but not CNTF (50 ng/ml), can induce tyrosine phosphorylation of their shared β subunits (LIFRβ and gp130) in brains isolated from *CNTFRα*$^{-/-}$ mice; brains from *CNTF*$^{-/-}$ mice were used as control.

tracts contained abundant amounts of CNTF protein, CNTF was undetectable in nerve extracts of *CNTF*$^{-/-}$ mice (see Figure 1C). Similarly, Northern blot analysis of CNTFRα brain RNA levels revealed a novel truncated RNA species (large arrowhead) with reduced normal transcripts (arrow) in *CNTFRα*$^{+/-}$ mice, and complete absence of the normal CNTFRα transcripts in *CNTFRα*$^{-/-}$ mice (see Figure 2C).

To determine whether CNTFRα is the major in vivo receptor mediating CNTF responses, we tested whether neurons in the *CNTFRα*$^{-/-}$ mutant mice had lost the ability to respond to exogenously provided CNTF. Sensory neurons from the dorsal root ganglia (DRGs) of newborn *CNTFRα*$^{-/-}$ mutant mice and wild-type controls were isolated and cultured in the presence of CNTF, LIF, or nerve growth factor (NGF), which normally mediate in vitro survival of these neurons. As seen in Figure 4A, neurons cultured from the *CNTFRα*$^{-/-}$ mice were comparable to their wild-type and *CNTFRα*$^{+/-}$ littermates in their responsiveness to both NGF and LIF, but differed in their responsiveness to CNTF. The complete lack of survival in the presence of CNTF demonstrates that for these peripheral sensory neurons, CNTFRα is a required CNTF receptor component, and that there is not an alternate CNTF receptor.

We then confirmed that CNTFRα is the major CNTF receptor not just for peripheral neurons but for those in the central nervous system as well. Membrane fractions were prepared from *CNTFRα*$^{-/-}$ newborn brains, as well as from *CNTF*$^{-/-}$ brains to serve as controls, and used to compare the ability of LIF and CNTF to induce tyrosine phosphorylation of their shared β subunits, gp130 and LIFRβ. In the absence of added factor, there was a low basal level of β subunit phosphorylation in both the *CNTFRα*$^{-/-}$ and *CNTF*$^{-/-}$ brains (Figure 4B, lanes 1 and 4). Following the addition of LIF, the β subunit phosphorylation levels in both the *CNTFRα*$^{-/-}$ and *CNTF*$^{-/-}$ samples increased as expected (lanes 3 and 6). However, the addition of CNTF induced a phosphorylation increase in the *CNTF*$^{-/-}$ sample (lane 5) but not in the sample from *CNTFRα*$^{-/-}$ mice (lane 2). These observations demonstrate that, as was the case for peripheral sensory neurons, central nervous system neurons do not possess a major alternative CNTF receptor component that can be used by CNTF to activate its β subunits.

Motor Neuron Number Is Dramatically Reduced in Brainstem Motor Nuclei and in the Spinal Cord of Mice Lacking CNTFRα but Not in Mice Lacking CNTF

Because of the dramatic phenotypic differences between the mice lacking CNTFRα and those lacking CNTF, we decided to examine specific neuronal populations in which deficits could account for the phenotypic differences. Motor neuron populations were obvious candidates, since embryonic motor neurons are known to respond to CNTF but are reportedly not affected in young mice lacking CNTF, raising the possibility that the putative second ligand for CNTFRα could play a critical role in the normal development of these neurons. Because the motor neurons most likely to be involved in the feeding and suckling process would be found in brain stem motor neuron nuclei, we first counted neurons in the facial motor nucleus (FMN), trigeminal motor nucleus (TMN), and hypoglossal motor nucleus (HMN), which provide motor innervation to the face, jaw, and tongue musculature, respectively (Kandel et al., 1991). Cell counts of these motor nuclei in newborn pups revealed that each exhibited significant losses in *CNTFRα*$^{-/-}$ mice as compared with littermate controls, amounting to reductions of 41% in the FMN, 27% in the TMN, and 51% in the HMN (Table 1; e.g., Figure 5A); reductions were not seen in mice heterozygous for the *CNTFRα* mutation. As previously noted for the FMN in young mice lacking CNTF (Masu et al., 1993), we did not notice significant reductions in either the FMN or TMN in newborn mice lacking CNTF (Table 1).

To determine whether the dramatic reduction in motor neuron number seen in mice lacking CNTFRα was restricted to brainstem motor nuclei or instead generally true of motor neuron populations, we quantitated motor neuron numbers in the lumbar region of the spinal cord. This motor neuron population exhibited about a 33% reduction in cell number in *CNTFRα*$^{-/-}$ mice as compared with wild-type littermate controls (Table 1; Figure 5B). Furthermore, morphometric measurements showed that the cross-sectional area of spinal cord motor neurons of *CNTFRα*$^{-/-}$ mice was significantly reduced by 12% ($P < 0.0001$, according to

Table 1. Quantitative Analysis of Motor Neuron Populations in *CNTFRα*$^{-/-}$ and *CNTF*$^{-/-}$ Mice Compared with Controls

Population	*CNTFRα*$^{+/+}$	*CNTFRα*$^{-/-}$	Percent of (+/+)	*CNTF*$^{+/+}$	*CNTF*$^{-/-}$	Percent of (+/+)
Facial nucleus	2515 ± 195 (n = 5)	1475 ± 64 (n = 6)	59	2490 ± 117 (n = 3)	2468 ± 138 (n = 4)	99
Trigeminal nucleus	935 ± 42 (n = 4)	685 ± 14 (n = 5)	73	819 ± 80 (n = 3)	852 ± 46 (n = 4)	104
Hypoglossal nucleus	1005 ± 68 (n = 3)	437 ± 52 (n = 3)	44			
Spinal cord, lumbar	1983 ± 46 (n = 3)	1333 ± 135 (n = 3)	67			

Numbers provided are the average ± SEM; n refers to the number of animals examined. Approximately 16 sections were examined for each specimen of the facial nucleus, 12 sections for the trigeminal nucleus, 16 sections for the hypoglossal nucleus, and 200 sections for the lumbar spinal cord; the counts were not corrected for split nucleoli.

the two-tailed t test controlled for Leven's test for equality of variance) compared with the area of those from wild-type controls (the reduction was from a mean area of 364.7 ± 8.4 μm² in 100 cells counted from three wild-type animals to an area of 321.2 ± 8.1 μm² in 97 cells counted from four *CNTFRα*$^{-/-}$ mice). In contrast with mice lacking CNTFRα, it has been previously noted that mice lacking CNTF do not exhibit an effect on spinal cord motor neurons until 8 weeks of age (Masu et al., 1993).

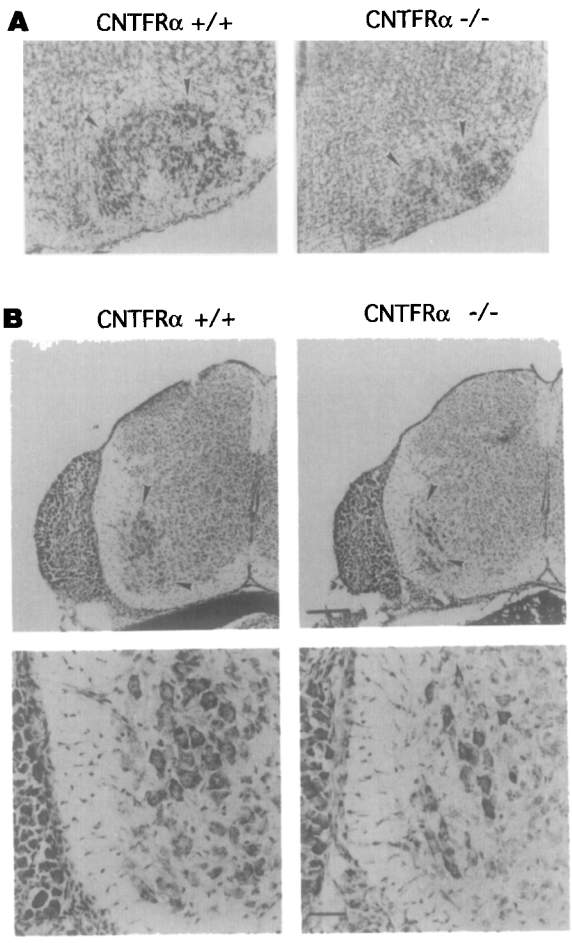

Figure 5. Comparison of the Facial Motor Nucleus and Lumbar Spinal Motor Neurons in *CNTFRα* Mutant and Control Mice

Histological comparison of the facial motor nucleus (A) and lumbar cord spinal motor neurons (B) in *CNTFRα* mutant and control mice, depicting reduction in size of both motor neuron populations (bordered by arrows in top two panels), as quantitated in Table 1. DRGs adjacent to the spinal cord are also depicted; volume of DRGs did not vary significantly between *CNTFRα* mutant and control mice, as noted in Table 2. In (B), upper scale bar is 150 μm, and lower bar is 40 μm.

The Effect of the *CNTFRα* Mutation on Sympathetic and Sensory Ganglia

The CNTFRα is expressed on sensory neurons in embryonic DRGs and trigeminal sensory ganglia (TGs), as well as on sympathetic neurons in the superior cervical ganglia (SCGs); CNTF is known to elicit survival and differentiative responses from DRG and SCG neurons. To begin to determine whether the putative second ligand for the CNTFRα might be as critical for the normal development of these neurons, as it appears to be for the normal development of motor neuron populations, we performed a preliminary histological analysis of these ganglia. Volumetric measurements revealed that the size of L5 DRGs, TGs, and SCGs were unaffected by the *CNTFRα* gene disruption (Table 2). Histological examination of these ganglia did not reveal substantial morphological differences in their neuronal populations in *CNTFRα*$^{-/-}$ and control mice (e.g., Figure 5B, upper panels). More extensive analysis will be required to determine whether these ganglia are affected in a more subtle manner in *CNTFRα*$^{-/-}$ mice, prior to ruling out a role of the second ligand for CNTFRα on these neuronal populations.

Discussion

Evidence for a Second Ligand Using the CNTF Receptor

The widespread expression of CNTFRα in the developing nervous system, together with the well-known survival and differentiative effects of CNTF on many different types of embryonic neurons, seemed to be at odds with findings that CNTF itself was not prominently expressed in the em-

Table 2. Volumetric Analysis of Peripheral Ganglia in *CNTFRα*$^{-/-}$ Mice Compared with Controls

Population	*CNTFRα*$^{+/+}$	*CNTFRα*$^{-/-}$	Percent of (+/+)
L5 DRG	26.8 ± 2.9 (n = 4)	26.3 ± 6.2 (n = 5)	98
TG	106.3 ± 15.3 (n = 3)	101.3 ± 14.7 (n = 3)	95
SCG	45.3 ± 7.6 (n = 3)	47.3 ± 10.8 (n = 3)	104

Numbers are the average volumes × 10⁶ μm³ ± SEM. DRG, dorsal root ganglia; TG, trigeminal ganglia; SCG, superior cervical ganglia.

bryo, and that mice and humans lacking CNTF did not exhibit any notable problems with the development of their nervous systems. This apparent paradox led to speculation first that CNTFRα might bind a second CNTF-like ligand; second, that this alternative ligand might be much more critical during normal development than CNTF itself; and third, that the actions of exogenously provided CNTF on embryonic neurons might simply reflect the more physiologically relevant actions of this CNTF-like factor (Ip et al., 1993). We now provide compelling genetic evidence supporting the notion that CNTFRα is indeed utilized by a ligand other than CNTF, that this factor is critical for normal development and postnatal viability, and that it is particularly required for the normal development of all motor neuron populations examined. This genetic evidence comes from the comparative analysis of mutant mice carrying either *CNTF* or *CNTFRα* gene disruptions. While mice lacking CNTF are viable, initially thrive, and only late in life develop a mild loss of motor neurons as previously described (Masu et al., 1993), mice lacking CNTFRα are much more severely affected; they cannot feed, suffer from profound reductions in their motor neuron numbers, and die on the day of birth.

Until the putative second ligand for CNTFRα is identified, it cannot be excluded that the discrepancy in phenotypes between the *CNTFRα*$^{-/-}$ and *CNTF*$^{-/-}$ mice results from a ligand-independent role of CNTFRα that is critical for proper neuronal development. However, discrepancy between the phenotype resulting from the lack of a ligand as compared with the phenotype stemming from the lack of its cognate receptor has been seen in other systems. In all these cases, the discrepancy has been explained by the fact that the receptor does indeed prove to be utilized by multiple closely related ligands. For example, humans lacking interleukin-2 are mildly affected as compared with individuals mutant in the interleukin-2 γ receptor component, who suffer from severe combined immunodeficiency, as this receptor component is also utilized by interleukin-4, interleukin-7, interleukin-13, and interleukin-15/T (Noguchi et al., 1993; Nowak, 1993). Likewise, mice lacking the insulin-like growth factor I receptor (Liu et al., 1993) are more severely compromised than mice lacking insulin-like growth factor I (Baker et al., 1993), because this receptor is also utilized by the developmentally important insulin-like growth factor II (DeChiara et al., 1990). Similarly, some neuronal populations are much more dramatically affected in mice lacking TrkB than in mice lacking brain-derived neurotrophic factor (BDNF) (Klein et al., 1993; Ernfors et al., 1994; Jones et al., 1994), presumably because TrkB serves as the in vivo receptor for other neurotrophins as well (Ip et al., 1993; Snider, 1994). Supporting this notion, particular neuronal populations (i.e., nodose sensory neurons) are similarly reduced in mice lacking both BDNF and neurotrophin-4, as they are in mice lacking TrkB (Conover et al., 1995). Thus, CNTFRα resembles not only other growth factor receptors, but more specifically other neurotrophic factor receptors, in that it appears to be utilized by multiple ligands in vivo. Interestingly, CNTFRα is somewhat unusual as compared with these and other promiscuous receptor components, in that it functions solely as a specificity-determining subunit, and not as a signal transducer, making it the first example of a specificity-determining subunit that is shared by multiple ligands. It remains to be determined whether the presumptive alternative ligand for CNTFRα utilizes the same signal-transducing β components as does CNTF.

Are any of the known members of the CNTF cytokine family likely candidates for the alternative ligand utilizing CNTFRα? Examination of available data argues that each of these ligands is an improbable choice. LIF, OSM, IL-6, and IL-11 neither require nor seem to bind CNTFRα in vitro (Stahl and Yancopoulos, 1994); the recently identified cardiotrophin 1 acts on cells that do not express CNTFRα and thus also does not appear to require this receptor component (Pennica et al., 1995). Furthermore, disruption of the genes for LIF or IL-6 does not yield phenotypes resembling that seen in mice lacking CNTFRα (Stewart et al., 1992; Escary et al., 1993; Kopf et al., 1994). Growth-promoting activity (GPA) is a CNTF relative recently cloned from chicken (Leung et al., 1992). Because we have not been able to clone another CNTF homolog from chick following extensive PCR-based and low stringency homology cloning efforts (N. Y. I., P. Masiakowski, and G. D. Y., unpublished data), GPA appears to be the closest chicken counterpart to CNTF rather than a new family member. Thus, it seems that our genetic evidence points toward the existence of a heretofore undescribed CNTF relative that shares the CNTF specificity-determining component, CNTFRα; this ligand may share only limited homology with CNTF, on the basis of our inability to clone it via homology-based approaches. In addition to this novel and neurally acting CNTF-like relative, it is possible that other members of the CNTF/LIF/IL-6/IL-11/OSM/cardiotrophin 1 family, which do not bind CNTFRα, remain to be discovered. Recent analyses of mice lacking LIFRβ reveal that they die at birth, as we have described for mice lacking CNTFRα, but also that they exhibit much more widespread problems, in the development of bone, glycogen metabolism, placenta, and glia, than we have observed in mice lacking CNTFRα (Ware et al., 1995; T. D., C. S., C. Ware, and G. D. Y., unpublished data). Because these widespread abnormalities have also not been found in mice lacking LIF, it seems likely that LIFRβ is shared in vivo not only by the LIF and CNTF receptor systems, but by yet another widely acting cytokine; OSM and cardiotrophin 1 may correspond to this cytokine.

Role of the Alternative CNTFRα Ligand during Motor Neuron Development

On the basis of our analysis, it remains unclear as to precisely why motor neuron numbers are reduced in the mice lacking CNTFRα. If the presumptive new ligand for CNTFRα acts as a classic target-derived neurotrophic factor during embryonic development, it may well be regulating survival of nascent motor neurons during the period

of naturally occurring cell death that takes place just after these neurons contact their target, the developing muscle; for facial and lumbar cord motor neurons, this process is complete by late gestation in the mouse (Lance-Jones, 1982; Ashwell and Watson, 1983). Alternatively, the new CNTFRα ligand may be involved in the generation of motor neurons, by modulating the proliferation or differentiation of precursors, and thus the lack of CNTFRα expression may result in reduced production of motor neurons; recent findings that CNTF can collaborate with other factors to promote the differentiation of neuronal precursors is consistent with this possibility (Ip et al., 1994). Clearly, a detailed analysis of motor neuron development in embryos lacking CNTFRα is required to understand precisely the actions of the new CNTFRα ligand on these neurons.

Many neurotrophic agents have been shown to act on motor neurons in vitro or when artificially supplied in vivo. The physiological relevance of most of these agents for motor neuron development remains unclear. It had been reported that newborn mice lacking TrkB tyrosine kinase receptors displayed motor neuron deficits and consistently died within the first postnatal week (Klein et al., 1993). More recently, and upon continuous breeding into a C57BL/6 background, the TrkB-defective mice survive up to 3 weeks of age and do not display significant motor neuron deficits (I. Silos-Santiago and M. Barbacid, personal communication). These observations resemble recent results obtained with mice lacking both BDNF and NT-4 (Conover et al., 1995; Liu et al., 1995) and suggest that signaling through TrkB receptors is not required for the generation of normal numbers of motor neurons during development. Analysis of mice disrupted for the *CNTFRα* gene appears to have defined a neurotrophic factor receptor system essential for normal motor neuron development in vivo. It should be noted, however, that substantial numbers of motor neurons, albeit somewhat atrophic, remain in newborn mice lacking CNTFRα. Therefore, it remains quite possible that motor neurons depend on additional classes of neurotrophic factors. It will be interesting to determine whether such ligands function independently or cooperatively with ligands using the CNTF receptor system, since collaborative and synergistic interactions between CNTF and other classes of factors have been demonstrated (e.g., Ip et al., 1994; Mitsumoto et al., 1994). It also will be necessary to determine if such factors act on distinct motor neuron subpopulations. Just as distinct motor neuron subsets can be defined on the basis of their target specificities and their patterns of LIM homeobox gene expression (Lewin, 1994; Tsuchida et al., 1994), it may be found that different motor neuron subsets display distinct neurotrophic requirements in vivo; such subsets have been defined for sensory neuron populations in the dorsal root ganglia (Snider, 1994) or nodose ganglia (Conover et al., 1995).

Experimental Procedures

Targeting Vectors and ES Cells

Targeting vectors were constructed from mouse genomic DNA fragments isolated from an EMBL3 λ phage library prepared with BALB/c strain DNA (Clontech). For the *CNTF* replacement vector, a 5.4 kb genomic XhoI–XbaI DNA fragment containing the two coding exons of the *CNTF* gene and flanking sequences was subcloned into the corresponding sites of the polylinker of the pKS Bluescript (Promega) plasmid (Figure 1A); an XhoI–SalI 1.85 kb DNA fragment containing the MC1–thymidine kinase (*tk*) expression cassette was cloned into the unique XhoI site in this plasmid, and the resulting plasmid was digested with SpeI to release a 1.5 kb DNA fragment (containing coding exons 1 and 2 of the *CNTF* gene) that was then replaced with the phosphoglycerate kinase (PGK)–*neo* expression cassette flanked with XbaI linkers to allow cloning into the compatible SpeI site (Figure 1A). For the *CNTFRα* replacement vector, an 8 kb HindIII–BamHI DNA fragment including exon 1 from the 5′ end of the *CNTFRα* gene was cloned into pKS plasmid (Figure 2A). A 1.8 kb XhoI–HindIII DNA fragment containing the MC1–*tk* expression cassette was cloned into the unique XhoI–HindIII sites of this plasmid, while a 1.8 kb BglII DNA fragment containing the PGK–*neo* expression cassette was cloned into the unique BamHI site. A 3 kb HindIII–PstI DNA fragment from the 3′ end of the *CNTFRα* gene, containing exons 8, 9, and 10, was blunt-ended, flanked with XbaI linkers, and cloned into the unique XbaI site to complete the targeting vector (Figure 2A). Both targeting vectors were linearized by digestion with NotI and then electroporated into E14.1 ES cells, which were grown in 200 μg/ml G418 (GIBCO) and 2 μM gancyclovir; gancyclovir addition resulted in a 5- to 10-fold enrichment compared with selection in G418 alone.

Culture of Dissociated Neurons Isolated from DRGs

DRGs were dissected from P1 mice, desheathed, trypsinized, and mechanically dissociated, then preplated on a polyornithine substrate for 2 hr to allow nonneuronal cells to attach. Neurons, which remained unattached, were harvested and then plated onto 48-well dishes coated with polyornithine–laminin (approximately 2000 cells per well) and counted. Either NGF, LIF, or CNTF was added, and 24 hr later, the number of surviving neurons was assessed in each well by counting. Data are expressed as percent neuronal survival, i.e., the number of surviving neurons at the end of 24 hr relative to the number of neurons initially plated.

Phophorylation Assays, Immunoblotting, and Northern Blotting

To determine CNTF or LIF responsivity in newborn brains, the dissected P1 mouse brains were triturated on ice in 0.75 ml of PBS into a fine suspension. Each sample was then equally divided and incubated with either control solution, CNTF (50 ng/ml), or LIF (50 ng/ml) for 15 min at room temperature with gentle agitation. Receptor complex activation was determined by LIFRβ immunoprecipitation and phosphotyrosine immunoblotting as described (Stahl et al., 1993, 1994). For CNTF detection in sciatic nerves from adult *CNTF*$^{-/-}$ or *CNTF*$^{+/+}$ mice, the nerves were homogenized in buffer containing 20 mM Tris (pH 7.6), 1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF and spun at 13,000 × g for 20 min. The supernatants were then concentrated with a Centricon-10 concentrator (Amicon) and quantified by using the BCA reagents (Pierce), and ~200 μg of protein was loaded in each lane of a 12% polyacrylamide gel, which was then immunoblotted with the CNTF-specific polyclonal antibody RG0036. Northern blotting for *CNTFRα* RNA was performed as previously described (Ip et al., 1993).

Motor Neuron Cell Counting and Morphometric Analysis

Newborns were anesthetized and perfused with 3 ml of 0.9% NaCl containing 5 U/ml of heparin followed by 3 ml of 4% paraformaldehyde in PBS (pH 7.4–7.6) and postfixed in 4% paraformaldehyde at 4°C. The brain and spinal cords were dissected out and cryoprotected by incubation in successive solutions of 17% and 30% sucrose in PBS at 4°C. Tissues were embedded in OCT compound (Miles Laboratory, Incorporated) and frozen at −58°C (spinal cord) or −30°C (brains) in isopentane cooled on dry ice. Cryostat serial sections of 20 μm (spinal cord) or 30 μm (brain) were mounted on gelatin-coated glass coverslips, air-dried, and stained with 0.1% cresyl violet. In some cases, the entire vertebral column including the cord was mounted in the coronal plane and sectioned. Large cells in the ventral horns with abundant cytoplasm and a prominent nucleolus were counted on both

sides; in the facial, trigeminal, and hypoglossal motor nuclei, large cells with a distinct nucleus were counted. Cross-section area of motor neurons was measured in sections 30 μm thick stained with cresyl violet, by using a PC-assisted image analysis system (Software Qwin of the Leica Quantimat 500). Only cell profiles containing a distinct nucleus with nucleolus were included. Spinal motor neurons were measured in the L4-L5 segments.

Volumetric Analyses of Peripheral Ganglia

Ganglia were removed from perfused (DRGs and TGs) or nonperfused (SCGs) pups, postfixed as above, dehydrated in graded alcohols, and then paraffin-embedded. Serial sections of 6 μm were cut and mounted on ProbeOn Plus slides (Fisher) and stained with cresyl violet. Each section was microscopically examined to identify all sections that contained neuronal somata. These sections were parcellated, from the beginning to the end of the ganglia, into 12 groups, and by using systematic random sampling methods, one section from each group was chosen for further analysis (Pover and Coggeshall, 1991). Analysis was performed on a digitized image of the section by using a computer-assisted planimeter to measure the area occupied by neuronal cell bodies (Jandel Image Analysis Systems). The volume of the ganglia, expressed in μm^3, was calculated from the product of the average mean area (μm^2) determined from the 12 sections, the section thickness (6 μm), and the total number of sections per ganglion.

Acknowledgments

Correspondence should be addressed to T. M. D. or G. D. Y. We would like to thank Drs. Len S. Schleifer and Roy Vagelos, and the rest of the Regeneron community, for support and insightful comments. We thank Drs. Frederick W. Alt for the PGK-*neo* plasmid, Kirk Thomas for the MC1-*tk* plasmid, and Richard Murray for the 129/*Ola* ES cells (E14.1). We thank Dr. Paolo Meda for his valuable discussion on morphometric analysis, and Jean Jacquet for performing statistical analysis, Mary Simmons, Michelle Russel and Nathalie Flores for expert technical assistance, Monique Gisser and Jennifer Griffiths for DNA sequencing and oligonucleotide synthesis, Pat Boland for help with tissue preparation and volumetric determinations, and Claudia Murphy and Eric Hubel for expert assistance with graphics. Gancyclovir was generously provided by Syntex Corporation (Palo Alto, California). R. V. is permanently at the Institute of Physiology, Academy of Sciences of the Czech Republic, Prague.

Received January 31, 1995; revised April 12, 1995.

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Note Added in Proof

We are grateful to Drs. Ronald W. Oppenheim and Linxi Li for independently verifying the motor neuron losses we have noted in mice lacking the CNTF receptor.