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# Early Onset of Severe Familial Amyotrophic Lateral Sclerosis with a *SOD-1* Mutation: Potential Impact of *CNTF* as a Candidate Modifier Gene

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**Mutations in the *copper/zinc superoxide dismutase 1* (*SOD-1*) gene are found in ~20% of patients with familial amyotrophic lateral sclerosis (FALS), or amyotrophic lateral sclerosis 1. Here we describe a 25-year-old male patient who died from FALS after a rapid disease course of 11 mo. Sequencing of the *SOD-1* gene revealed a heterozygous T→G exchange at position 1513 within exon 5, coding for a V→G substitution at position 148 of the mature protein. Genetic analysis of this family revealed the same mutation in both his healthy 35-year-old sister and his mother, who did not develop the disease before age 54 years. Screening for candidate modifier genes that might be responsible for the early onset and severe course of the disease in the 25-year-old patient revealed an additional homozygous mutation of the *CNTF* gene not found in his yet unaffected sister. *hSOD-1G93A* mice were crossbred with *CNTF*$^{-/-}$ mice and were investigated with respect to disease onset and duration, to test the hypothesis that *CNTF* acts as a candidate modifier gene in FALS with mutations in the *SOD-1* gene. Such *hSOD-1G93A/CNTF*-deficient mice develop motoneuron disease at a significantly earlier stage than *hSOD-1G93A/CNTF*-wild-type mice. Linkage analysis revealed that the *SOD-1* gene was solely responsible for the disease. However, disease onset as a quantitative trait was regulated by the allelic constitution at the *CNTF* locus. In addition, patients with sporadic amyotrophic lateral sclerosis who had a homozygous *CNTF* gene defect showed significantly earlier disease onset but did not show a significant difference in disease duration. Thus, we conclude that *CNTF* acts as a modifier gene that leads to early onset of disease in patients with FALS who have *SOD-1* mutations, in patients with sporadic amyotrophic lateral sclerosis, and in the *hSOD-1G93A* mouse model.**

## Introduction

Amyotrophic lateral sclerosis 1 (MIM 105400), or familial amyotrophic lateral sclerosis (FALS), is a fatal progressive disorder caused by selective degeneration of motoneurons in cortex, brainstem, and spinal cord (Munsat et al. 1988). Mutations in the *copper/zinc superoxide dismutase 1* (*SOD-1* [MIM 147450]) gene are found in ~20% of patients with familial ALS (FALS), or 1%–2% of all patients with ALS (Deng et al. 1993; Rosen et al. 1993; Siddique and Deng 1996). In general, the inheritance is autosomal dominant, but homozygous mutations with an autosomal recessive trait have also been reported (Andersen et al. 1996). The *SOD-1* gene is located on chromosome 21q22.1 and consists of five exons encoding a metalloenzyme with 153 amino acids (Levanon et al. 1985). The major function of the SOD1 protein is the detoxification of the superoxide anion radical ($O_2^{\cdot-}$) to form $O_2$ and $H_2O_2$ (Deng et al. 1993). To date, >60 different mutations of the *SOD-1* gene have been reported in >250 families with ALS, involving all five exons and, rarely, noncoding regions of the gene. The majority of the mutations are single-base-pair exonic substitutions (Cookson and Shaw 1999; Orrell 2000). Transgenic mouse models suggest that motoneuron degeneration results from a toxic gain of function of the mutant SOD-1 protein rather than a reduction in superoxide radical scavenging activity (Reaume et al. 1996; Gurney 1997; Bruijn et al. 1998).

Ciliary neurotrophic factor (CNTF [MIM 118945]) is a potent survival factor for motoneurons in cell culture and in vivo (Arakawa et al. 1991; Magal et al. 1991; Oppenheim et al. 1991). Inactivation of the *CNTF* gene leads to loss of 15%–20% of the motoneurons in postnatal mice (Masu et al. 1993). This loss appears well tolerated, and, despite a discrete loss of muscle strength, the mice appear healthy and reach normal age in the absence of any signs of motoneuron disease. In the human *CNTF* gene, a guanine→adenine transition in the splice acceptor site of exon 2 has been

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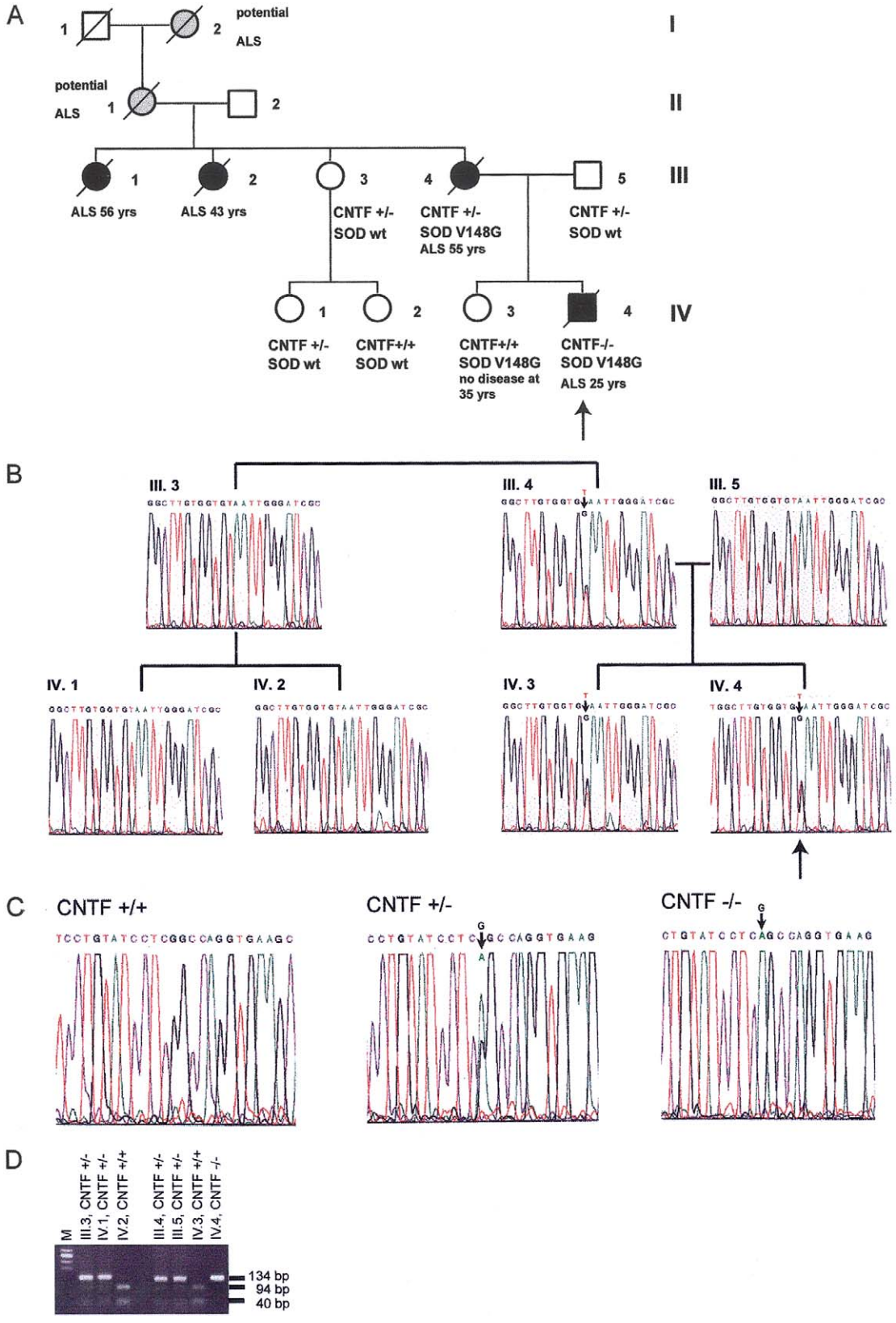

A
potential ALS
potential ALS
ALS 56 yrs
ALS 43 yrs
CNTF +/- SOD wt
CNTF +/- SOD V148G ALS 55 yrs
CNTF +/- SOD wt
CNTF +/- SOD wt
CNTF+/+ SOD wt
CNTF+/+ SOD V148G no disease at 35 yrs
CNTF-/- SOD V148G ALS 25 yrs
I
II
III
IV
B
III. 3
III. 4
III. 5
IV. 1
IV. 2
IV. 3
IV. 4
C
CNTF +/+
CNTF +/-
CNTF -/-
D
M
III.3, CNTF +/-
IV.1, CNTF +/-
IV.2, CNTF +/+
III.4, CNTF +/-
III.5, CNTF +/-
IV.3, CNTF +/+
IV.4, CNTF -/-
134 bp
94 bp
40 bp

described, resulting in a truncated protein without biological activity. This homozygous mutation is found at a similar frequency of ~2% both in healthy individuals and in patients with neurological disorders (Takahashi et al. 1994).

Here we describe a 25-year-old male patient who died from FALS after rapid disease progression of 11 mo. He carried both a V148G mutation of the *SOD-1* gene and the recently reported null mutation in the *CNTF* gene (Takahashi et al. 1994; Giess et al. 1998). His 53-year-old mother and his 35-year-old sister were healthy at the date of diagnosis. Both of them also carry the V148G mutation of the *SOD-1* gene but not the *CNTF* null mutation. His mother was heterozygous for the *CNTF* mutation, and his sister was homozygous wild type for the *CNTF* allele. To test the hypothesis that *CNTF* acts as a candidate modifier gene, we have crossbred mice that overexpress mutated human *SOD-1G93A* with *CNTF*-deficient mice, and we have found an earlier disease onset but not a shortened disease duration in transgenic mice that carry both the human *SOD-1G93A* mutation and an inactive *CNTF* gene in comparison to the disease onset in transgenic mice that carry both *SOD-1G93A* and an intact *CNTF* gene. This finding indicates that *CNTF* acts as a disease-modifying gene in FALS with *SOD-1* mutations.

## Subjects and Methods

### *Subjects*

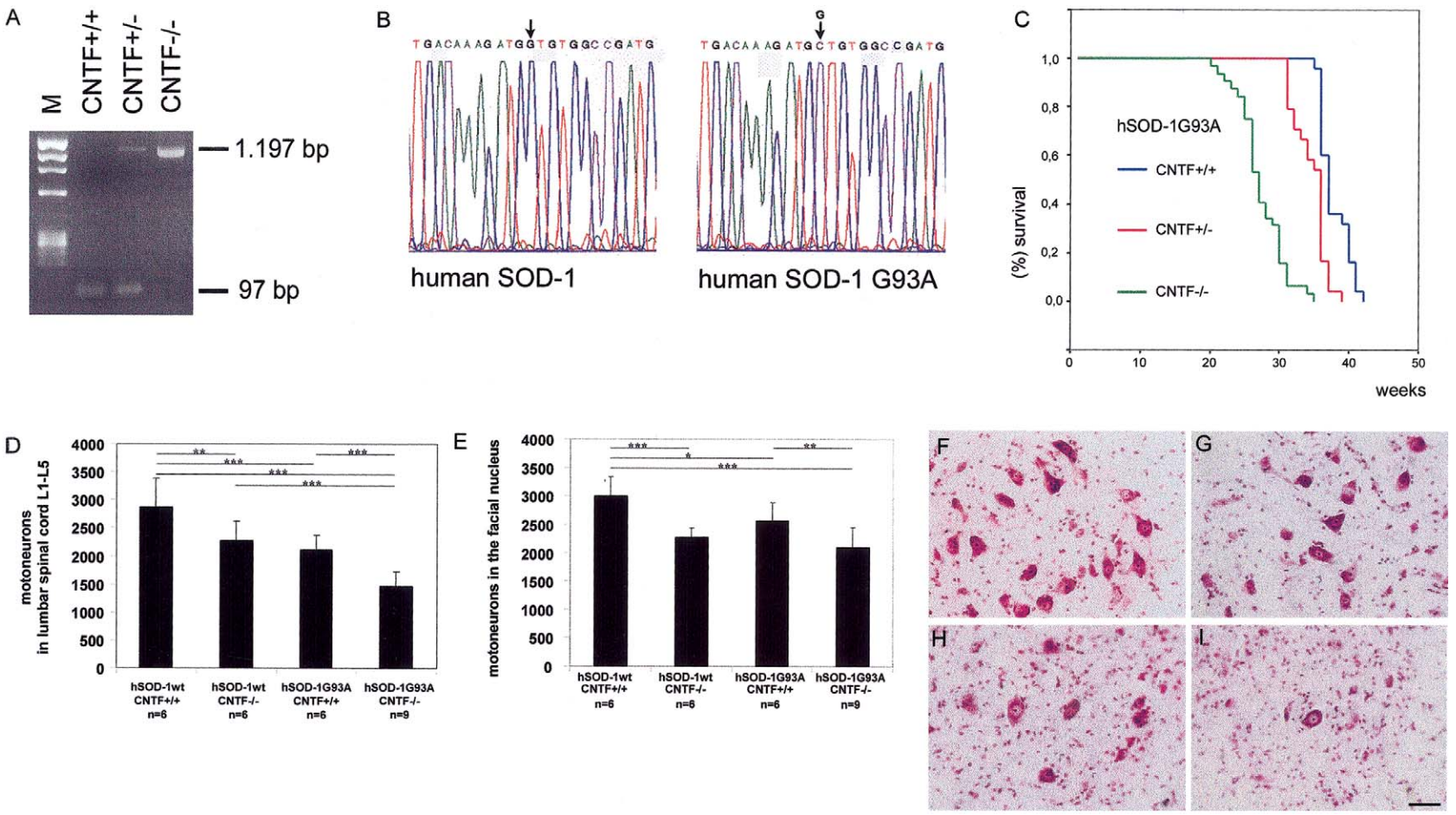
*Case report and family history.*—A 24-year-old male (IV.4; fig. 1*A*) came to our attention with progressive muscular atrophy of bulbar predominance typical of ALS (Brooks 1994). The first signs of the disease were dysarthria and dysphagia, which had developed 5 mo earlier. The tongue was atrophic, and alternating movements were extremely slow. Head and proximal arm muscles were weak. Fasciculations were seen in the tongue, masseter, platysma, deltoid, pectoral, and abdominal muscles. Reflexes of masseter and triceps surae muscles were hyperactive, but no other signs of involvement of the upper motoneurons were found. Sensory abnormalities were not detectable. Needle electromyography confirmed systemic denervation. Nerve conduction velocity and F-wave studies excluded conduction block. Examination of the cerebrospinal fluid obtained by lumbar puncture revealed no pleocytosis or oligoclonal bands. Cranial and cervical magnetic-resonance–imaging investigations showed no abnormalities. The patient rapidly progressed to respiratory failure and died 11 mo after disease onset, at age 25 years. Autopsy was denied.

His mother (III.4) developed ALS at age 54 years. First signs of the disease were dyspnea caused by paralysis of the right diaphragm and weakness of the shoulder muscles. Dysarthria and dysphagia developed 6 mo later. Clinical examination 12 mo after disease onset revealed an atrophic tongue, alternating movements were slow, and speech was slurred. Fasciculations were seen in the tongue and in the proximal arm muscles. Reflexes of the masseter muscles and deep tendon reflexes of arms and legs were hyperactive, and Babinski sign was present. Head, arm, and leg muscles were weak. Sensory abnormalities were not found. Needle electromyography revealed systemic denervation. Nerve conduction studies and analysis of F-waves were performed to exclude multifocal neuropathy. The patient progressed to respiratory failure and died 16 mo after disease onset, at age 55 years. A postmortem biopsy was denied.

In this family (fig. 1*A*), two of the mother's sisters had died from ALS at 56 (III.1) and 43 (III.2) years of age, respectively. Diagnosis of ALS was confirmed in subject III.2 by autopsy. One of his grandmothers (II.1) and one of his great-grandmothers (I.2) had died from a progressive muscle weakness and atrophy at ages 62 and <50 years, respectively.

*Patient samples.*—Genomic DNA was extracted from EDTA blood samples from seven family members (fig. 1*A*) by use of standard methods (Miller et al. 1988). All

**Figure 1** Characterization of mutations in the genes that encode *SOD-1* and *CNTF* in a family with autosomal dominant ALS. *A,* Family pedigree with FALS. Squares denote male family members, circles denote female family members, black symbols denote affected family members, gray symbols denote affected members who are possibly affected on the basis on family history, and slash marks (/) through symbols denote deceased family members. The arrow indicates the 25-year-old male index patient who carried both a V148G mutation in *SOD-1* and a null mutation in *CNTF. B*, Heterozygous T→G transition at position 1513 within exon 5 of the *SOD-1* gene in genotyped family members. Arrows indicate the T→G transition in family members III.4, IV.3, and IV.4, as revealed by automated DNA sequencing of the PCR product of exon 5 of the *SOD-1* gene. Family members III.3, III.5, IV.1, and IV.2 do not show the T→G transition. The large arrow indicates index patient IV.4. *C, CNTF* genotyping in family members. Demonstration of the G→A transition within the intron-exon boundary of the second exon of the *CNTF* gene, leading to a truncated, biologically inactive CNTF protein. $CNTF^{+/+}$ (i.e., no mutation) was found in subjects IV.2 and IV.3; $CNTF^{+/-}$ (i.e., a heterozygous G→A transition) was found in subjects III.3, III.4, III.5, and IV.1; and $CNTF^{-/-}$ (i.e., a homozygous G→A transition) was found in index patient IV.4. *D,* Identification of the *CNTF* null mutation by *Hae*III-restriction-polymorphism analysis of PCR-amplified genomic DNA. The three different genotypes were identified by characteristic digestion patterns: $CNTF^{+/+}$ showed two bands of 94 and 40 bp (in subjects IV.2 and IV.3); $CNTF^{+/-}$ showed three bands of 134, 94, and 40 bp (in subjects III.3, III.4, III.5, and IV.1); and $CNTF^{-/-}$ showed one band of 134 bp (in index patient IV.4). "M" denotes the Phi-x-174/*Hae*III marker.


A
M
CNTF+/+
CNTF+/-
CNTF-/-
1.197 bp
97 bp
B
human SOD-1
human SOD-1 G93A
C
hSOD-1G93A
CNTF+/+
CNTF+/-
CNTF-/-
(%) survival
weeks
D
motoneurons in lumbar spinal cord L1-L5
hSOD-1wt CNTF+/+ n=6
hSOD-1wt CNTF-/- n=6
hSOD-1G93A CNTF+/+ n=6
hSOD-1G93A CNTF-/- n=9
E
motoneurons in the facial nucleus
F
G
H
I

family members in the investigation gave their informed consent, and the study was approved by the local ethics committee.

### Molecular Genetic Analyses

*Analysis of the* SOD-1 *gene in human subjects.*—In all individuals tested, PCR amplification of all five exons of the *SOD-1* gene was performed by procedures described elsewhere (Deng et al. 1993; Rosen et al. 1993; Jackson et al. 1997). We sequenced PCR products of all five exons by use of a 373A DNA sequencer (Perkin Elmer). To exclude PCR errors, all PCRs were repeated at least once in independent experiments with patient DNA. For confirmation of mutations, sense and antisense strands were sequenced.

*Identification of the* CNTF *null mutation in human subjects.*—The *CNTF* null mutation was identified by a *Hae*III-restriction-polymorphism assay of PCR-amplified genomic DNA, as described elsewhere (Takahashi et al. 1994) (fig. 1*D*). Automated DNA sequencing was applied for confirmation of the underlying G→A point mutation at the intron-exon boundary of exon 2 of the *CNTF* gene, leading to a frameshift and subsequent truncation of the resultant protein, which lacks biological activity (fig. 1*C*). Each analysis was repeated at least once from patient DNA, to confirm the genotypes.

*Transgenic human* SOD-1G93A *and human wild-type* SOD-1 *and* CNTF-*deficient and* CNTF-*wild-type mice.*—Mice with homologous recombination of the *CNTF* gene (Masu et al. 1993) were crossbred with transgenic mice carrying a low copy number of the human mutated *SOD-1G93A* gene (*hSOD-1G93A*) (Gurney et al. 1994). These mice were obtained from Jackson Laboratories (stock 002300). The offspring were genotyped by PCR. Tail DNA was used as a template. Mice of the F2–F5 generations that were *hSOD-1G93A* transgenic and $CNTF^{-/-}$ were compared with *hSOD-1G93A/* $CNTF^{+/+}$ mice generated from *hSOD-1G93A/* $CNTF^{+/-}$ mice in the F1 generation. As an additional control group, we generated *hSOD-1wt/* $CNTF^{+/+}$ and *hSOD-1wt/* $CNTF^{-/-}$ mice by crossbreeding transgenic mice obtained from Jackson Laboratory (stock 002297) that overexpress the human wild-type *SOD-1* gene (*hSOD-1wt*) and $CNTF^{-/-}$ mice.

CNTF *genotyping in mice.*—For identification of the disrupted *CNTF* gene, a primer pair (5′-GAGCAATCACCTCTGACCCTT-3′ and 5′-ATTCCATAAGAGCAGTCAGGTC-3′) was designed to distinguish the wild-type from the mutated allele. PCR amplification was performed using a *Gene Amp* 2400 thermal cycler (Perkin Elmer). Amplification was performed under the following conditions: 5 min at 95°C initial denaturation; 30 s at 95°C denaturation, 45 s at 55°C annealing, 3 min at 72°C extension, entrained for 34 cycles; and 10 min at 72°C final extension. The expected product sizes were 97 bp for the wild-type allele and 1.197 bp for the mutated allele. PCR products were visualized with an ethidium bromide–stained 2% agarose gel (fig. 2*A*).

*SOD-1 genotyping of transgenic mice.*—The "set b" primer sequences for human exon 4 (Rosen et al. 1993) of the transgenic human *SOD-1* gene (Gurney et al. 1994) were used for identification of transgenic mice by PCR, as recommended by Jackson Laboratories. The expected size of the PCR product was 236 bp. Automated DNA sequencing was applied for confirmation of the wild-type sequence of the human *SOD-1* gene at position 1087 (Levanon et al. 1985) or the underlying guanine→cytosine transition leading to an amino acid exchange from glycine to alanine at position 93 of the mature SOD-1 protein (*hSOD-1G93A*) (fig. 2*B*).

*Linkage analysis in mouse families carrying* SOD-1G93A *as a transgene and a* CNTF *mutation.*—The primary binary phenotype (i.e., affected/not affected) was modeled as an autosomal dominant trait, corresponding to the genotype-disease correlation for the *SOD-1G93A*

**Figure 2** Motoneuron disease in mice with combined *CNTF* and *SOD-1* gene defects. *A, CNTF* genotyping of mice. Ethidium bromide–stained 2% agarose gel reveals bands of 97 bp for the wild-type allele ($CNTF^{+/+}$) and 1.197 bp for the mutated allele ($CNTF^{-/-}$). Heterozygous mice ($CNTF^{+/-}$) show both bands. "M" denotes the Phi-x-174/*Hae*III marker. *B,* Automated DNA sequencing of PCR products of exon 4 of the human *SOD-1* gene, showing either the wild-type sequence of the human *SOD-1* gene in transgenic *hSOD-1wt* mice (*arrow*) or the guanine→cytosine transition at position 1087 (*arrow*) that leads to an amino acid exchange from glycine to alanine at position 93 of the SOD-1 protein in transgenic *hSOD-1 G93A* mice. C, Kaplan-Meier curves, showing survival of *hSOD-1G93A/* $CNTF^{+/+}$ ($n = 25$), *hSOD-1G93A/* $CNTF^{+/-}$ ($n = 24$), and *hSOD-1G93A/* $CNTF^{-/-}$ mice ($n = 32$) ($P < .001$ by log-rank test). *D* and *E,* Motoneuron numbers in lumbar spinal cord and facial nuclei. Values shown are mean ± SD of motoneuron numbers in lumbar spinal cord (L1–L5; *D*) and facial nuclei (*E*) corrected for split nuclei as described (Oppenheim et al. 2001). The differences between the four groups of mice were tested by one-way ANOVA; at the levels for which $P < .0001$, differences were significant. *D,* Motoneuron counts of the lumbar spinal cord. Comparison of individual groups by Bonferroni's multiple-comparison test gave the following results: $P < .01$ (**) and $P < .001$ (***). *E*, Motoneuron counts of the facial nucleus. Comparison of individual groups by Bonferroni's multiple comparison test gave the following results: $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***). *F–I,* Morphology of degenerating motoneurons in the lumbar spinal cord of mice with combined *SOD-1* and *CNTF* gene mutations. In *hSOD-1wt/* $CNTF^{+/+}$ mice (*F*), the spinal cord appeared to be histologically normal. In *hSOD-1wt/* $CNTF^{-/-}$ (*G*) and *hSOD-1G93A/* $CNTF^{+/+}$ (*H*) mice, evident reduction in motoneuron number is detectable. In mice carrying the double mutation, *hSOD-1G93A/* $CNTF^{-/-}$ (*I*), the spinal cord appeared to be nearly void of motoneurons. The more severe reduction in motoneuron number of the spinal cord compared to the facial nucleus and the obvious gliosis suggests a caudorostral progression of the disorder, confirming the clinical phenotype. Bar = 50 μm.

mutation. Parametric linkage analysis was performed using FASTLINK, version 4.1 (Cottingham et al. 1993). We also considered disease onset in affected animals as a quantitative trait. This was tested using the orthogonal method of Abecasis et al. (2000) as implemented in QTDT. We report both asymptotic and empirical *P* values.

*Histological analysis.*—At onset of disease, *hSOD-1G93A/CNTF*$^{-/-}$ mice ($n = 9$; age 19–35 wk) and *hSOD-1G93A/CNTF*$^{+/+}$ mice ($n = 6$; age 41 wk) were anesthetized with ether and were transcardially perfused with 0.1 M phosphate buffer, pH 7.4, and, subsequently, with freshly prepared 4% paraformaldehyde in the same phosphate buffer. The *hSOD-1wt/CNTF*$^{+/+}$ ($n = 6$) and *hSOD-1wt/CNTF*$^{-/-}$ control mice ($n = 6$) were analyzed at age 41 wk. The brainstem and the lumbar spinal cord (L1–L5) were dissected and were embedded in paraffin, and serial sections (7 $\mu$m for brainstem and 15 $\mu$m for lumbar spinal cord) were prepared. After Nissl staining, lumbar spinal cord and facial motoneurons were counted at both sides, and the raw counts were corrected for split nuclei as described elsewhere (Oppenheim et al. 2001).

*Statistical analysis.*—All data are expressed as mean ± SD. Survival of mice (fig. 2*C*) was tested by Kaplan-Meier and log-rank test through use of the SPSS software program (SPSS). Statistical analysis of disease onset and duration in patients with ALS (table 2) was performed using two-tailed unpaired Student's *t* test. *hSOD-1G93A/CNTF*$^{+/+}$, *hSOD-1G93A/CNTF*$^{+/-}$, and *hSOD-1G93A/CNTF*$^{-/-}$ mice (table 1) were compared by one-way analysis of variance (ANOVA) followed by Bonferroni's test with aid of the GraphPad Prism software (GraphPad). The null hypothesis was rejected at $P < .05$. The same test was used for analysis of the morphometric data shown in figs. 2*D* and 2*E*.

## Results

### *Genotyping of the* SOD-1 *and* CNTF *Genes in a Family with Autosomal Dominant ALS with Variations in Disease Onset*

In the index patient (IV.4; fig. 1*A*), a heterozygous T→G transition was identified at position 1513 within exon 5 of the *SOD-1* gene (Levanon et al. 1985), which leads to an amino acid exchange from valine to glycine at position 148 (V148G) of the mature SOD-1 protein (fig. 1*B*). This patient also carried a homozygous mutation of the *CNTF* gene (*CNTF*$^{-/-}$) (fig. 1*C*). At the time when diagnosis was made for this patient, his 35-year-old healthy sister (IV.3) and 53-year-old mother (III.4) also exhibited the T→G transition in the *SOD-1* gene, but they carried at least one wild-type *CNTF* allele (*CNTF*$^{+/+}$ and *CNTF*$^{+/-}$, respectively). His 57-year-old father (III.5) did not show the *SOD-1* mutation but was heterozygous for the *CNTF* mutation (*CNTF*$^{+/-}$). The healthy aunt (III.3) and the two healthy cousins (IV.1 and IV.2) did not carry the *SOD-1* mutation and were *CNTF*$^{+/-}$ (III.3 and IV.1) and *CNTF*$^{+/+}$ (IV.2), respectively (figs. 1*B* and 1*C*).

### *Combination of* SOD-1 *and* CNTF *Gene Defects in Mice Leads to Enhanced Loss of Motoneurons and Earlier Onset of Disease*

Transgenic mice carrying a low copy number of the *hSOD-1G93A* mutated gene (Jackson Laboratories, stock 002300) develop a classical motoneuron disease that becomes apparent as weakness of hind limbs between 37 and 41 wk of age. In contrast, the *CNTF*$^{-/-}$ mice, despite a loss of 15%–20% motoneurons at an age of 6 mo (Masu et al. 1993) never exhibit any signs of disease such as limb paralysis, respiratory defects, and difficulties in either feeding or reproduction. Thus, homozygous *CNTF* gene defects are not associated with severe neurological disease in mouse and human (Takahashi et al. 1994). To test whether the *CNTF* gene defect could modify disease onset in *hSOD-1G93A* mice, we established two independent families of *hSOD-1G93A* and *CNTF* mutant mice. The binary phenotype (motoneuron disease) was found to be linked to the *SOD-1* locus ($z_{max} = 5$4.08 at recombination fraction [$\theta$] 0). The *CNTF* locus was found to be unlinked to both the binary phenotype ($z = -\infty$ at $\theta = 0$; exclusion of linkage to a region of >20 cM around *CNTF* [$z \leqslant -2$ criterion]) and the *SOD-1* locus ($z = -\infty$ at $\theta = 0$; exclusion of linkage to a region of >10 cM around *CNTF* [$z \leqslant -2$ criterion]). However, very clear-cut evidence by use of QTDT was obtained for an association between disease onset and alleles at the *CNTF* locus (asymptotic *P* value $6 \times 10^{-18}$; empirical *P* value estimated at 0 [0 of 1,000,000 permutations giving equally extreme or more-extreme evidence than the original data]). The estimated proportion of variance explained is 57%. For *SOD-1* the corresponding asymptotic *P* value is >90% and thus is clearly nonsignificant.

*hSOD-1G93A/CNTF*$^{-/-}$ ($n = 32$), *hSOD-1G93A/CNTF*$^{+/-}$ ($n = 24$), and *hSOD-1G93A/CNTF*$^{+/+}$ ($n = 25$) mice were investigated with respect to disease onset and duration. The onset of neurological symptoms occurred much earlier in *hSOD-1G93A/CNTF*$^{-/-}$ mice compared to *hSOD-1G93A/CNTF*$^{+/+}$ mice (on average at 26.72 ± 3.36 wk in comparison to 37.08 ± 2.08 weeks, respectively) (table 1). *hSOD-1G93A/CNTF*$^{+/-}$ mice developed disease earlier than *hSOD-1G93A/CNTF*$^{+/+}$ mice but later than *hSOD-1G93A/CNTF*$^{-/-}$ mice ($P < .001$ by ANOVA and Bonferroni's test for comparison of individual groups). Kaplan-Meier curves showed significant differences in survival of *hSOD-1G93A/CNTF*$^{-/-}$, *hSOD-1G93A/CNTF*$^{+/-}$, and *hSOD-1G93A/CNTF*$^{+/+}$ mice (fig. 2*C*).

**Table 1**

**Disease Onset and Duration in *hSOD-1G93A/CNTF*$^{+/+}$, *hSOD-1G93A/CNTF*$^{+/-}$, and *hSOD-1G93A/CNTF*$^{-/-}$ Mice**

| | Mean ± SD | |
|---|---|---|
| Mice | Age at Onset[a] (wk) | Duration[b] (d) |
| CNTF$^{+/+}$ ($n$ = 25) | 37.08 ± 2.08 | 3.96 ± .61 |
| CNTF$^{+/-}$ ($n$ = 24) | 33.67 ± 2.26 | 4.08 ± .83 |
| CNTF$^{-/-}$ ($n$ = 32) | 26.72 ± 3.36 | 3.91 ± .64 |

[a] $P < .001$ by ANOVA.
[b] $P$ value was not significant by ANOVA.

### *Histological Analysis*

To characterize morphological changes associated with early onset of motoneuron disease in mice, we compared motoneuron numbers in lumbar spinal cord and facial nucleus of *hSOD-1G93A/CNTF*$^{-/-}$ mice at onset of disease (age 19–23 [$n$ = 3], 32 [$n$ = 3], and 35 [$n$ = 3] wk) with *hSOD-1G93A/CNTF*$^{+/+}$ ($n$ = 6, age 41 wk), *hSOD-1wt/CNTF*$^{+/+}$ ($n$ = 6, age 41 wk), and *hSOD-1wt/CNTF*$^{-/-}$ mice ($n$ = 6, age 41 wk). Numbers and morphology of motoneurons in the lumbar spinal cord of 41-wk-old *hSOD-1wt/CNTF*$^{+/+}$ mice appeared normal (fig. 2*D*, 2*F*). In *hSOD-1wt/CNTF*$^{-/-}$ mice, a slight but significant reduction in motoneuron number was detectable (20.9% ± 12.5%). Similar reduction was observed in 41-week-old *hSOD-1G93A/CNTF*$^{+/+}$ mice (26.5% ± 8.5%). *hSOD-1G93A/CNTF*$^{-/-}$ mice showed a 48.9% ± 9.2% reduction in motoneuron number, which was highly significant compared with *hSOD-1wt/CNTF*$^{+/+}$ mice and *hSOD-1G93A/CNTF*$^{+/+}$ mice (fig. 2*D*). Surviving motoneurons in *hSOD-1G93A/CNTF*$^{-/-}$ mice showed disintegration of Nissl structure and reduced size. In some sections, very few surviving motoneurons could be detected that were surrounded by reactive glia (fig. 2*I*).

In *hSOD-1wt/CNTF*$^{-/-}$ mice, a pronounced loss of facial motoneurons was detectable (24.5% ± 5.7%), thus confirming earlier reports (Masu et al. 1993) describing that this population of motoneurons is significantly affected in these mice. In *hSOD-1G93A/CNTF*$^{+/+}$ mice, loss of facial motoneurons was only 14.4% ± 10.5%. This points out a specific difference in disease phenotype between the two mouse mutants. Interestingly, animals carrying the double mutation *hSOD-1G93A/CNTF*$^{-/-}$ did not demonstrate significantly enhanced motoneuron loss in comparison to *hSOD-1wt/ CNTF*$^{-/-}$ mice (fig. 2*E*).

### *Early Onset of Disease in Patients with Sporadic ALS Who Have the* CNTF$^{-/-}$ *Genotype*

We have previously reported that three patients with motoneuron disease and the *CNTF*$^{-/-}$ genotype showed significantly earlier disease onset in comparison to patients with *CNTF*$^{+/+}$ genotype (Giess et al. 1998). Because of the low number of patients and on the basis of the heterogeneity of the various forms of motoneuron disease investigated in the previous study, no conclusion could be drawn about a potential effect that CNTF has on disease duration. In the meantime, eight patients with ALS with the *CNTF*$^{-/-}$ genotype came to our attention. Disease onset among this group was at 48.6 ± 15.0 years, in a control group of 30 patients with ALS with two intact alleles of the *CNTF* gene at 58.4 ± 9.0 years. Interestingly, disease duration was not different in these two groups (table 2), indicating that the *CNTF*$^{-/-}$ genotype modulates disease onset but not disease duration in ALS.

## Discussion

Here we have described the coincidence of mutations in the *SOD-1* and the *CNTF* genes leading to earlier disease onset in a family with autosomal dominant FALS. Similar observations were made in an animal model: Offspring of human *SOD-1G93A* transgenic mice crossbred with *CNTF*$^{-/-}$ mice show a shift toward earlier onset of disease, from 37.08 ± 2.08 wk in *hSOD-1G93A/CNTF*$^{+/+}$ mice to 26.72 ± 3.36 wk in *hSOD-1G93A/CNTF*$^{-/-}$ mice. Furthermore, *hSOD-1G93A/CNTF*$^{-/-}$ mice show higher rates of motoneuron loss in lumbar spinal cord compared to *hSOD-1wt/CNTF*$^{+/+}$, *hSOD-1wt/CNTF*$^{-/-}$, and *hSOD-1G93A/CNTF*$^{+/+}$ mice.

On the basis of clinical observations in patients with sporadic ALS, we hypothesize that homozygous mutations in the *CNTF* gene could be a modifying factor in this disorder (Giess et al. 1998, 2000). Approximately 2% of the population in Japan and Europe are homozygous for a *CNTF* null mutation (Takahashi et al. 1994; Giess et al. 1998). The *CNTF* mutation is not associated with any neurological disease (Takahashi et al. 1994). A similar distribution of the *CNTF* mutation was also found in patients with motoneuron disease (Giess et al. 1998). Sporadic degenerative disorders of the motoneuron may be based on a combination of gene defects and epigenetic influences which individually do not result in disease. In this respect, mice in which both the *CNTF* and *leukemia inhibitory factor* (*LIF* [MIM 159540]) genes are mutated serve as a model (Sendtner

**Table 2**

**Disease Onset and Duration in *CNTF*-Deficient and *CNTF*-Wild-Type Patients with Sporadic ALS**

| | Mean ± SD | |
|---|---|---|
| Patients | Age at Onset[a] (years) | Duration[b] (mo) |
| CNTF$^{+/+}$ ($n$ = 30) | 58.40 ± 9.00 | 39.00 ± 16.40 |
| CNTF$^{-/-}$ ($n$ = 8) | 48.60 ± 15.00 | 34.00 ± 13.80 |

[a] $P < .05$ by Student's $t$ test.
[b] $P$ value was not significant by Student's $t$ test.

et al. 1996). These mice show significant postnatal motoneuron death and reduction of muscle strength by ~30%, corresponding to functionally relevant motor deficits and clinical disease. In addition, transection of the facial nerve at age 4–6 wk leads to significantly enhanced loss of motoneuron cell bodies in the *CNTF/LIF* double knockout mice in comparison to $CNTF^{-/-}$ and wild-type mice (Sendtner et al. 1996). Both CNTF and LIF are expressed in myelinating Schwann cells (Sendtner et al. 1992; Curtis et al. 1994). Whereas CNTF is present at relatively high amounts within the cytoplasm of these glial cells, LIF production is low under physiological conditions but rapidly increases after nerve lesion (Banner and Patterson 1994; Curtis et al. 1994). This observation has led to the hypothesis that these molecules serve as lesion factors, acting on motoneurons under pathological situations by promoting their survival and axonal regeneration.

Several reports have provided evidence that motoneuron cell death in ALS—in particular, ALS caused by mutations of the *SOD-1* gene—occurs by apoptosis (Pasinelli et al. 1998; Li et al. 2000). This indicates that signaling pathways that counteract apoptosis could reduce or at least delay cell death in familial ALS. Alternatively, reduced activation of these pathways—that is, by lack of neurotrophic factors such as CNTF—could lead to more rapid progression of the disease, as observed in mice with disruption of the genes for CNTF, LIF, and cardiotrophin-1 (CTF1 [MIM 600435]) (Sendtner et al. 1996; Oppenheim et al. 2001), or in mice in which specific antiapoptotic genes such as *bcl-2* (*BCL2* [B-cell chronic lymphatic leukemia/lymphoma 2] [MIM 109565]) are disrupted (Michaelidis et al. 1996).

Mutations in the *SOD-1* gene are found in ~20% of patients with autosomal dominant familial ALS (Deng et al. 1993; Rosen et al. 1993; Siddique and Deng 1996). The pathomechanisms of specific motoneuron degeneration in these patients are less clear. Several hypotheses—including the formation of hydroxyl radicals (Wiedau-Pazos et al. 1996); the abnormal cytoplasmic aggregation of mutated SOD-1 in motoneurons (Shibata et al. 1996; Durham et al. 1997; Bruijn et al. 1998); and the formation of peroxynitrite, which yields tyrosine nitration (Beckman et al. 1993; Estevez et al. 1998)—have been proposed to underly the toxic gain of function of mutant *SOD-1*. Furthermore, neurofilament disaggregation may have a primary role in ALS pathogenesis, since mutant transgenic *SOD-1* mice, as well as patients with ALS who have *SOD-1* mutations, develop aberrant neurofilament accumulations in the motoneurons (Rouleau et al. 1996; Tu et al. 1996). Recent reports show that calcineurin, a protein phosphatase involved in calcium-regulated pathways, may be a target in the pathogenesis of ALS. Overexpression of mutant *SOD-1* impairs calcineurin activity both in transfected human neuroblastoma cell lines and in the motor cortex of *hSOD-1G93A* transgenic mice, leading to a dysregulation of intracellular calcium levels (Ferri et al. 2000). This could lead to proapoptotic signaling mechanisms that are normally counteracted by neurotrophic factors such as CNTF.

The *SOD-1V148G* mutation, which was found in the family investigated, was one of the first mutations that has been described in patients with ALS (Deng et al. 1993). The mutation is thought to destabilize the subunit fold or dimerization of the SOD-1 protein. A specific correlation between this mutation and clinical features is not known, and the mutation has not yet been found in a European pedigree (Radunovic and Leigh 1996; Orrell 2000). Several mutations of the *SOD-1* gene show a wide variation, within and between families, in duration (1–20 years) and onset (25 to ~75 years) of the disease. For example, the I113T mutation leads to highly variable disease course that can differ 2–20 years in the same family. Furthermore, age at disease onset is reported to vary up to 16 years in one family that carries the same *SOD-1* mutation (Cleveland et al. 1995; Orrell et al. 1995; Radunovic and Leigh 1996). Hence, additional gene defects or epigenetic factors are highly likely to modulate the clinical presentation of the *SOD-1* mutation.

Our results represent a first report on a modifier gene in a patient with a *SOD-1* mutation that influences onset of the disease. This finding may not be restricted to FALS, since a significantly earlier onset was also observed in patients with sporadic ALS with the $CNTF^{-/-}$ genotype. In the family described in this study, we observed three patients with ALS in generation III with disease onset at age 41–54 years and one patient with ALS in generation IV with disease onset at age 24 years (fig. 1*A*). The sister of patient IV.4 carries the same *SOD-1V148G* mutation but is still healthy at age 35 years. She differs from her brother by two intact alleles of the *CNTF* gene ($CNTF^{+/+}$). Her mother (III.4) with the *SOD-1V148G* mutation carried one intact allele of the *CNTF* gene ($CNTF^{+/-}$). This constellation strongly suggests that the absence of both *CNTF* alleles modulates disease onset in familial ALS with mutations of the *SOD-1* gene.

Mice overexpressing mutated SOD-1 (*hSOD-1G93A*) at a low copy number show a relatively uniform clinical phenotype. Disease onset in these mice occurs at age 42 wk, and all mice become severely sick during the following weeks. (Dal Canto and Gurney 1997). The relevance of *CNTF* deficiency as a modifier for motoneuron disease is supported by the animal model. Linkage analysis clearly demonstrates that disease is regulated by the *SOD-1* genotype. However, disease onset as a quanti-

tative trait in affected animals is not regulated by the *SOD-1* genotype but by the allelic constitution at the *CNTF* locus. Loss of motoneurons in either $CNTF^{-/-}$ or *hSOD-1G93A* mice is similar in a range of 15%–25% both in the lumbar spinal cord and facial nucleus. In *hSOD-1G93A*/$CNTF^{-/-}$ mice, onset of disease evolves earlier and loss of motoneurons is significantly enhanced in the lumbar spinal cord, which corresponds to the observation that the hind limbs become paraplegic at an early stage of the disease. In addition, patients with sporadic ALS who have the $CNTF^{-/-}$ genotype show significantly earlier onset of disease but similar disease duration compared to patients with ALS who have the $CNTF^{+/+}$ genotype.

In summary, our data indicate that *CNTF* acts as a modifier gene in patients with ALS. Our finding that onset of disease is earlier and neuronal loss is enhanced in mice with combined *SOD-1* and *CNTF* gene mutations indicates that the coincidence of gene mutations has a major influence on the pathophysiological dysfunction and loss of motoneurons in motoneuron disease.

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## Electronic-Database Information

Accession numbers and the URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for FALS [MIM 105400], *SOD-1* [MIM 147450], *CNTF* [MIM 118945], *LIF* [MIM 159540], CTF1 [MIM 600435], and *BCL2* [MIM 109565])

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