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SHORT COMMUNICATION

The Gene for Ciliary Neurotrophic Factor (CNTF) Maps to Murine Chromosome 19 and its Expression is Not Affected in the Hereditary Motoneuron Disease 'Wobbler' of the Mouse

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Abstract

The cDNA for ciliary neurotrophic factor (CNTF), a polypeptide involved in the survival of motoneurons in mammals, has recently been cloned (Stöckli *et al.*, *Nature*, **342**, 920–923, 1989; Lin *et al.*, *Science*, **246**, 1023–1025, 1989). We have now localized the corresponding gene *Cntf* to chromosome 19 in the mouse, using an interspecific cross between *Mus spretus* and *Mus musculus domesticus*. The latter was carrying the gene wobbler (*wr*) for spinal muscular atrophy. DNA was prepared from backcross individuals and typed for the segregation of species-specific *Cntf* restriction fragments in relation to DNA markers of known chromosomal location. The *M.spretus* allele of *Cntf* cosegregated with chromosome 19 markers and mapped closely to *Ly-1*, to a region of mouse chromosome 19 with conserved synteny to human chromosome 11q. *Cntf* is not linked to *wr*, and the expression of CNTF mRNA and protein appears close to normal in facial and sciatic nerves of affected (*wr/wr*) mice, suggesting that motoneuron degeneration of wobbler mice has its origin in defects other than reduced CNTF expression.

Introduction

Ciliary neurotrophic factor (CNTF) has been shown to support the survival of a variety of neuronal cell types from embryonic chicks in culture (Barbin *et al.*, 1984) and to promote the differentiation of neuronal and glial cell types (Hughes *et al.*, 1988; Ernsberger *et al.*, 1989; Saadat *et al.*, 1989). Recently it has been found that CNTF is also a potent survival factor for cultured spinal motoneurons from chick embryos (Arakawa *et al.*, 1990) and that it is able to prevent the death of motoneurons in newborn rats after axotomy (Sendtner *et al.*, 1990).

Cell death of motoneurons can be observed in a variety of sporadic and hereditary diseases in humans and other mammalian species. The autosomal recessive mutation wobbler (Duchen and Strich, 1968) is thus far the best studied model for hereditary motoneuron disease in the mouse. Wobbler (phenotype WR, genotype *wr/wr*) mice are characterized by a loss of motoneurons in the spinal cord and in brainstem motor nuclei (Duchen and Strich, 1968; LaVail *et al.*, 1987) and an astrogliosis (Laage *et al.*, 1988). Reduced axonal regenerative capacity and reduced protein synthesis of cervical anterior horn cells have been reported (Murakami *et al.*, 1980; Mitsumoto, 1985). The high levels of CNTF found in peripheral nerves of adult rodents (Williams *et al.*, 1984; Lin *et al.*, 1989) are reached at postnatal week 3 (Stöckli *et al.*, 1989), which coincides with the appearance of the symptoms of motoneuron disease in WR mice. Therefore, by genetic mapping studies and by analysis of CNTF mRNA and protein expression, we have investigated whether motoneuron degeneration in the WR mouse might be caused by either a disruption of the CNTF gene or other genetic defects preventing CNTF expression in peripheral nerves of WR mice.

Here we show that the gene for CNTF is not linked to *wr* and that the expression levels of CNTF mRNA and protein are at most slightly reduced in peripheral nerves during the course of the disease, suggesting

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that the motoneuron degeneration of WR mice has its origin in defects other than a disturbance of CNTF structure or expression. A preliminary report of part of this work has been given previously (Kaupmann *et al.*, 1991b).

Materials and methods

Mice

The wobbler (*wr*) mutation was maintained on a C57BL/6J background. Homozygous wobbler mice (*wr/wr*) were distinguishable from unaffected littermates (+/+ or *wr*/+, phenotype WT, wild-type) from 4 weeks of age by their reduced body weight, wobbly gait and difficulties in using their forelimbs. The *Mus spretus* stock (strain SEG/1) was obtained in 1987 from Dr O. von Deimling, Freiburg, FRG. Females heterozygous for the wobbler gene (*wr*/+) were mated with wild-type *M.spretus* (+/+). From the F_1 generation, carrier females for the wobbler gene were identified by the production of mutant progeny in the backcross (BC) to heterozygous wobbler (*wr*/+) males.

DNA isolation and Southern blot analysis

DNA was prepared from frozen mouse tissues according to Maniatis *et al.* (1982). Ten μg DNA was digested with a restriction endonuclease, electrophoresed on 0.8% (w/v) agarose gels and transferred to Biodyne B (Pall, Dreieich, FRG) nylon membranes (Southern, 1975). Blots were treated in 7% sodium dodecyl sulphate ($NaDodSO_4$), 1% bovine serum albumin, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2 (Church and Gilbert, 1984) for 10 min at 65°C, and hybridized with an [α-^{32}P]dATP-labelled probe (Feinberg and Vogelstein, 1983) in the same solution for 1 day. Blots were washed with 2×SSC, 0.1% $NaDodSO_4$ (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 30 min at 37°C and for 60 min with 0.1×SSC, 0.1% $NaDodSO_4$ at 65°C. Kodak X-OMAT AR films were exposed to the blots for 1−3 days at −70°C with intensifying screens.

RNA isolation and Northern blot analysis

Total RNAs from sciatic nerves of WR and WT mice were isolated according to Chomczynski and Sacchi (1987). Twenty pg of a shortened synthetic CNTF RNA standard [0.6 kilobases (kb)], corresponding to the coding region for CNTF, was added prior to the extraction to each tissue sample to assess the recovery. Following electrophoresis in a 1.4% (w/v) agarose gel containing 2.0 M formaldehyde (Lehrach *et al.*, 1977), the RNA was vacuum blotted to nylon membranes (Hybond-N, Amersham Corp.). CNTF RNA standards (0.6 and 0.34 kb) were coelectrophoresed in separate lanes, permitting the determination of the absolute quantities of CNTF mRNA in samples (Heumann and Thoenen, 1986).

Hybridization was performed at 65°C in 10 ml 5×SSC, 50% formamide, 5×Denhardt's solution, 20 mM $NaPO_4$, pH 7.0, 5 mM EDTA, 1% $NaDodSO_4$, 350 μg/ml denatured salmon sperm DNA with a [^{32}P]cRNA CNTF probe (5×10^6 c.p.m./ml). After washing in 0.1×SSC containing 0.5% $NaDodSO_4$ at 70°C, an X-ray film (Fuji) was exposed to the blots for 24 h at −70°C.

The cRNA probe was prepared from a Bluescript SK+ vector containing the entire coding region of the rat CNTF cDNA (Stöckli *et al.*, 1989). The vector was linearized with *Eco*RI, and a single-stranded RNA probe was transcribed using the Promega *in vitro* transcription system with T3 RNA polymerase.

Probes

The CNTF clone pCMV6 was a 606-base pair (bp) rat cDNA (Stöckli *et al.*, 1989). The pro-opiomelanocortin (*Pomc-2*) probe ME-150 was a 140-bp mouse cDNA, purchased from the American Type Culture Collection (Rockville, MD, USA). The lymphocyte antigen (*Ly-1*) probe pMD-10 was a 2.1 kb mouse cDNA (Huang *et al.*, 1987).

Immunoblotting of nerve extracts

Facial and sciatic nerves were dissected from WR and WT mice and homogenized in 200 μl phosphate-buffered saline in a glass−glass homogenizer. After ultracentrifugation for 30 min at 100 000 *g* in a Beckman TL100 ultracentrifuge, the clear supernatants were removed, the protein content was determined using the Coomassie blue-based BioRad protein assay (BioRad, Munich, FRG), and 30 μg protein of each supernatant was loaded on a 15% polyacrylamide gel for electrophoresis under reducing conditions. After electrophoresis, the gels were blotted onto a nitrocellulose membrane (BA 83, Schleicher and Schüll), and a parallel lane with the molecular mass markers was separated from the blot and stained with Amidoblack (0.1% in 7% acetic acid). The blot was blocked with 5% horse serum in Tris-buffered saline (TBS) for 10 min and incubated overnight at 4°C with anti-CNTF monoclonal antibody 4−68 (Stöckli *et al.*, 1991) hybridoma supernatant diluted 1:1 with TBS containing 5% horse serum. After four wash cycles with TBS, the blot was blocked again with 5% horse serum and incubated with an affinity-purified goat anti-mouse IgG horseradish peroxidase conjugate (BioRad, # 172-1011), diluted 1:1000 in TBS with 5% horse serum. After four wash cycles with TBS, the CNTF-immunoreactive bands were visualized with chloronaphthol.

Bioassay with embryonic chick ciliary neurons

Protein extracts from nerves were prepared and analysed for their ability to support the survival of chick embryonic day 8 ciliary neurons in culture, as described by (Saadat *et al.* 1989; Stöckli *et al.*, 1991).

Results and conclusions

The structural gene for CNTF, termed *Cntf*, was chromosomally mapped using a rat CNTF cDNA as a probe. The segregation of species-

TABLE 1. Restriction fragments of marker genes in the *Mus musculus domesticus* (C57BL/6J)/*Mus spretus* backcross

Gene			Restriction fragments[a] (sizes in kb)		
Symbol	Designation	Probe	Nuclease	*Mus spretus*	C57BL/6J
Cntf	Ciliary neurotrophic factor	pCMV6	*Pvu*II	≈ *13.0*	≈ 6.3, 5.9
Ly-1	Lymphocyte antigen-1	pMD-10	*Bam*HI	≈ 12.0, 5.0, *3.2*	≈ 18.0, 7.8, 5.0
Pomc-2	Pro-opiomelanocortin-2	ME-150	*Hind*III	≈ *5.2*, 2.0	≈ 12.0, 5.6

[a]The *Mus spretus* restriction fragments in italics were followed in the backcross.

specific restriction fragments of *Cntf* was followed in an interspecific backcross (BC) between *M.spretus* and *M.m.domesticus* (review: Avner *et al.*, 1988), the latter carrying the wobbler (*wr*) gene for spinal muscular atrophy. A screening for restriction fragment length variants in *M.spretus* as compared to C57BL/6J was done by digesting the genomic DNAs with restriction endonucleases *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Pst*I, *Pvu*II, *Sst*I, *Taq*I, *Xba*I and subsequent Southern hybridization with the CNTF probe pCMV6. Polymorphism at the *Cntf* locus was found only with *Pvu*II-digested DNA (Table 1). *M.spretus* genomic DNA yielded a fragment at 13.0 kb, and C57BL/6J a band at 6.3 kb and a minor band at 5.9 kb (Fig. 1).

The segregation of the *M.spretus Cntf* fragment was followed in 60 individuals of a (*M.spretus* +/+ × C57BL/6J *wr*/+) *wr*/+ × C57BL/6J *wr*/+ backcross panel. Forty-four mice used in this study were homozygous (*wr/wr*) wobbler mice, and an additional 16 (out of a total of 148) wild-type (+/+ or *wr*/+) BC individuals were analysed for control purposes. We compared the segregation pattern for *Cntf* fragments to that of 50 mapped molecular probes that cover all autosomes and have previously been used to characterize this backcross panel. Linkage of *Cntf* was found with mouse chromosome 19 markers lymphocyte antigen-1 (*Ly-1*) and pro-opiomelanocortin-2 (*Pomc-2*) (Table 2). *Ly-1* has been mapped close to the centromere of mouse chromosome 19 (Hillyard *et al.*, 1991), and *Pomc-2* has been assigned to chromosome 19 using somatic cell hybrid analysis (Uhler *et al.*, 1983). Both loci have also been mapped using an interspecific backcross (Glaser *et al.*, 1989). The gene order inferred from our data is:

chromosome 19: centromere−(*Cntf*/*Ly-1*)−*Pomc-2*.

A region of conserved synteny between mouse chromosome 19 and human chromosome 11q has been identified on the basis of the genes for ferritin heavy chain (*Fth*), myophosphorylase (*Pygm*), *Ly-1* (*CD5* in humans) and oxysterol binding protein (*Osbp*) (Nadeau *et al.*, 1990). If this synteny group includes *Cntf*, the human CNTF gene would be expected to be located on chromosome 11.

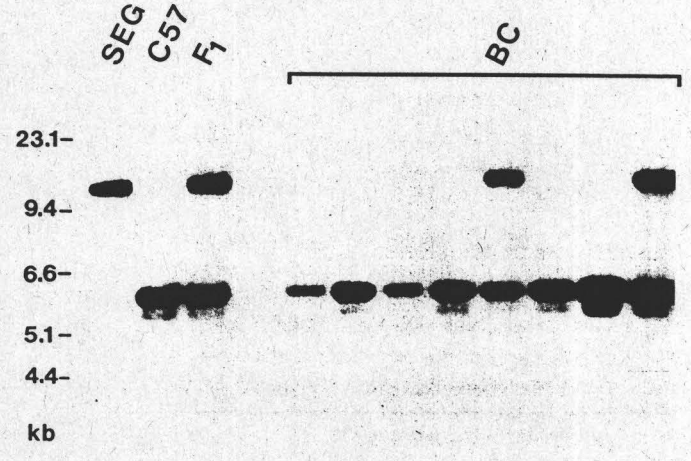

FIG. 1. Segregation of species-specific *Cntf* restriction fragments in an interspecific backcross. Southern hybridization of the CNTF probe pCMV6 to *Pvu*II-restricted DNA (10 μg per lane) from *Mus spretus* SEG/1, an interspecific F_1 hybrid (SEG/1×C57BL/6J), *Mus musculus* C57BL/6J (C57) and eight backcross (BC) individuals. Chain length markers (left scale) are phage lambda DNA restriction fragments.

To investigate whether *Cntf* is linked to the *wr* gene, we compared the segregation pattern of *Cntf* to the expression of the WR phenotype (Table 2). No linkage of *wr* with *Cntf* was found. Using this backcross panel, we have recently mapped the *wr* gene to mouse chromosome 11 (Kaupmann *et al.*, 1991a). Thus, the gene locus *Cntf* is distinct from the *wr* locus.

To determine whether the *wr* gene affects CNTF expression, we measured the levels of CNTF mRNA and protein in nerves of 9-week-old WR mice. Northern blots with total RNA from the sciatic nerves of 9-week-old WR mice and unaffected controls (+/+ or *wr*/+) showed comparable signal intensities (as related to the internal recovery standard) after hybridization with a specific CNTF probe (Fig. 2a).

Immunoblotting with a monoclonal antibody against CNTF (Stöckli *et al.*, 1991) of extracts from sciatic, facial (Fig. 2b) and brachial (not shown) nerves yielded doublet bands at a position corresponding to 22 and 24 kD (cf. Lin *et al.*, 1989). The signals were of similar or somewhat lower intensities when comparing WR to WT nerve extracts. The moderate reduction in WR nerves is probably secondary to the reduced number of nerve fibres (Duchen and Strich, 1968; LaVail *et al.*, 1987). Similarly, comparable survival activities for cultured embryonic day-8 chick ciliary neurons were found with facial (Fig. 2c) and sciatic (not shown) nerve extracts from WR and WT mice, indicating that the CNTF protein in WR mice is functional and that its level in facial nerve Schwann cells of WR mice is at the most slightly reduced. In frozen sections of the facial nerve of WR mice, the immunoreactivity for CNTF was not reduced within the remaining Schwann cells (not shown).

In conclusion, our data demonstrate that neither the CNTF gene nor its expression is directly affected in WR mice. This result is consistent with the cytopathology of affected motoneurons of WR mice being distinct from that of motoneurons undergoing cell death in response to CNTF deprivation. Cell enlargement by vacuoles within the cell bodies is specific for WR mice, and has recently been described for neuronal degeneration in mutants of *Caenorhabditis elegans* (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991). On the other hand, facial motoneurons after axotomy in newborn rats appear shrunken, without discernible Nissl structure and without vacuoles (Sendtner *et al.*, 1990). This appearance resembles more closely the morphology of affected motoneurons in human amyotrophic lateral sclerosis (ALS): The atrophy of the motoneurons observed in these patients is also characterized by shrunken, dark, basophilic cytoplasm and pycnosis of the nucleus (Hirano and Iwata, 1979). Therefore, it will be interesting to look for changes occurring in the CNTF gene and its expression, either in animal models with similar morphological characteristics to those observed in ALS or in ALS patients themselves.

TABLE 2. Recombination fractions and genetic distances between *Cntf*, chromosome 19 markers and *wr*

	Cntf		
	Recombinants/total	*P*	Map distance (centimorgans)
Ly-1	0/60	< 0.001	4.9[a]
Pomc-2	3/60	< 0.001	5.0±2.8
wr	17/44	> 0.1	

[a]Upper 95% confidence limit for distance between the two loci.

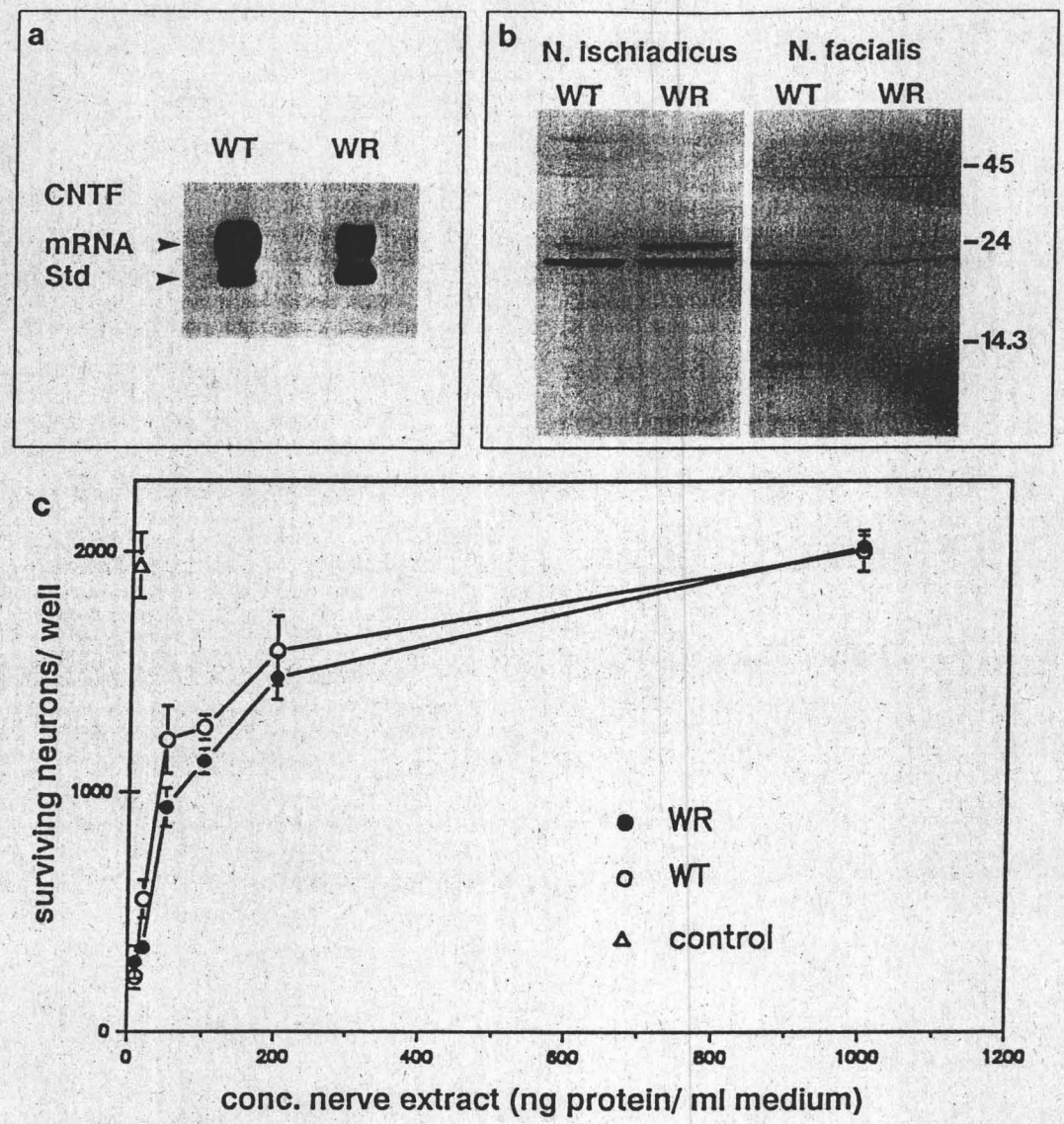

FIG. 2. (a) Northern blot analysis of CNTF mRNA in sciatic nerves of wild-type (WT) and wobbler (WR) mice. One of three independently obtained results, in which the ratio of CNTF mRNA from WR nerves to the added recovery standard (Std) was similar to or somewhat lower than the ratio in wild-type controls. (b) Immunoblot of extracts from sciatic and facial nerves of WT and WR mice. Doublet bands (22 and 24 kD) were also found with purified CNTF (cf. Lin *et al.*, 1989). Marker polypeptides for the molecular masses given in kD were ovalbumin, trypsinogen and lysozyme. (c) Effect of protein extracts from facial nerves of WT and WR mice on the survival of ciliary neurons. Survival of embryonic day 8 chick ciliary neurons plotted against concentration of extract protein in the medium. Data show the mean ± SEM of individual extracts from six WR mice and three age-matched WT mice. Control: maximal survival of ciliary neurons in the presence of 5 ng/ml of purified rat CNTF.

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Abbreviations

ALS	amyotrophic lateral sclerosis
BC	backcross
bp	base pair
CNTF	ciliary neurotrophic factor
Cntf	structural gene for CNTF
EDTA	ethylenediamine tetraacetic acid
kb	kilobase
kDa	kilodalton
Ly-1	lymphocyte antigen-1 gene
$NaDodSO_4$	sodium dodecyl sulphate
Pomc-2	pro-opiomelanocortin-2 gene
TBS	Tris-buffered saline
wr	wobbler (allele)
WR	wobbler (phenotype)
WT	wild-type (phenotype)

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