Missense mutation in the *tubulin-specific chaperone E* (*Tbce*) gene in the mouse mutant *progressive motor neuronopathy*, a model of human motoneuron disease

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Progressive motor neuronopathy (*pmn*) mutant mice have been widely used as a model for human motoneuron disease. Mice that are homozygous for the *pmn* gene defect appear healthy at birth but develop progressive motoneuron disease, resulting in severe skeletal muscle weakness and respiratory failure by postnatal week 3. The disease starts at the motor endplates, and then leads to axonal loss and finally to apoptosis of the corresponding cell bodies. We localized the genetic defect in *pmn* mice to a missense mutation in the *tubulin-specific chaperone E* (*Tbce*) gene on mouse chromosome 13. The human orthologue maps to chromosome 1q42.3. The *Tbce* gene encodes a protein (cofactor E) that is essential for the formation of primary α-tubulin and β-tubulin heterodimeric complexes. Isolated motoneurons from *pmn* mutant mice exhibit shorter axons and axonal swelling with irregularly structured β-tubulin and tau immunoreactivity. Thus, the *pmn* gene mutation provides the first genetic evidence that alterations in tubulin assembly lead to retrograde degeneration of motor axons, ultimately resulting in motoneuron cell death.

Introduction

Autosomal recessive traits of motoneuron disease are observed in classical spinal muscular atrophy (SMA;* Lefebvre et al., 1995), in SMA with respiratory distress type 1 (SMARD1; Grohmann et al., 2001), and in a rare form of juvenile amyotrophic lateral sclerosis (ALS2; Hadano et al., 2001; Yang et al., 2001). The responsible gene defects have been characterized; SMA and SMARD1 are caused by defects in RNA processing. The discovery of gene defects in ALSIN/ALS2, a GTPase with structural similarities to RanGEF as well as RhoGEF in ALS2 (Hadano et al., 2001; Yang et al., 2001), has given a first hint from human genetic studies that signaling mechanisms regulating cytoskeletal turnover might also be disturbed in motoneuron disease. Motoneurons differ from other types of neurons within the spinal cord by the length of their axons, which can reach a length of >1 m in adult human. Structural and functional maintenance of these large cells are dependent on the axonal transport of organelles and cytoskeletal proteins. Deficiency or interruption of axonal transport has been previously reported as a cause of motoneuron disease (Cote et al., 1993; Xu et al., 1993; LaMonte et al., 2002). However, in a significant number of SMA and sporadic ALS cases, the underlying gene defects have not yet been identified. Therefore, the characterization of additional disease genes is expected to provide further information on the pathophysiology of motoneuron disease.

The autosomal recessive *progressive motor neuronopathy* (*pmn*) mouse mutant (Schmalbruch et al., 1991) resembles SMA by progressive degeneration of motoneurons in the early postnatal period. Genetic mapping has identified the *pmn* locus on mouse chromosome 13 in a region defined by markers D13Mit172 and D13Mit207 (Martin et al., 2001). We localized the genetic defect to a point mutation in the *tubulin-specific chaperone E* (*Tbce*) gene affecting an evolu-
tionary conserved amino acid residue. The identification of this gene defect may help to define new candidate genes that are mutated and thus might be involved in the pathophysiology underlying human motoneuron disease.

Results and discussion

To identify the corresponding gene defect in pmn mice, we have performed detailed genetic mapping by genotyping 229 pmn/pmnm F2 progeny from one male NMRI founder that was heterozygous for the pmn gene defect and two female AKR mice. 29 microsatellite markers spanning a region of 31 cM were used and 34 recombination events were observed (Fig. 1 A). Our results refined the pmn locus to a 2-cM region flanked by markers D13Mit173 and D13Mit207.

We generated a yeast artificial chromosome (YAC)– and a bacterial artificial chromosome (BAC)–based physical map of the genomic region encompassing nearly the entire pmn candidate region (Fig. S1, available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1). Sequences from the ends of YAC and BAC clones were subsequently integrated into the complete sequence of the region flanked by markers D13Mit173 and D13Mit207 when this information became available in public and Celera databases. The characterized region had a size of ~2.19 Mb. Together with previously published data (Martin et al., 2001), the critical region could be refined between D13Mit173 and 378J7-F, which is located between Pmn14 and Mrpl32 (Fig. 1 B; Table S1, available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1).

Comparative analysis of mouse and human homology maps resulted in a transcript map of the pmn critical region (Fig. 1 B; Table S1). Synteny with segments located on human chromosomes 1q42–q43 and 7p14.1–p13 was found, as described earlier (Martin et al., 2001). We mapped 15 transcriptional units between D13Mit173 and 378J7-F and 23 between D13Mit173 and D13Mit237, including 11 previously characterized genes and 12 transcripts that all had been identified before as EST in mouse and human. cDNA
were screened. In none of these mice, the T
NMRI, AKR, C57BL/6, or 129SvJ genetic background
according to Mendelian rules (Fig. 1 D). In addition, mice with
al., 1999) to mammals (Tian et al., 1997). During transla-
species ranging from yeast (Hoyt et al., 1997; Radcliffe et
(Tian et al., 1996). This specific function is conserved between
both alleles in the
brain and spinal cord. In addition, genomic DNA was inves-
tigated to rule out that the nucleotide exchange is due to
obtained from our breeding colony. They were not related

The nucleotide sequences of CofE were taken from public
databases, translated to the corresponding amino acid sequence,
and then aligned using ExPasy (www2.ebi.ac.uk/clustal). The amino
numbers correspond to murine CofE. Conserved substitutions
and semiconserved substitutions are indicated in gray; the remaining
black amino acids are identical. Amino acids without similarity are
shown in light gray. The arrow indicates the amino acid mutated
in pmn mice.

from brain of pmn/pmn and NMRI wild-type (wt) mice
was sequenced for all 11 previously characterized genes and
12 new transcripts to identify the possible mutation. Among
the 23 genes surveyed, only one gene was found to carry a
specific change in sequence, which was subsequently con-
confirmed by sequencing genomic DNA from pmn mice. A
T→G transition occurred at nucleotide position 1682 of the
Tbce gene (Fig. 1 C). This results in a missense mutation ex-
changing tryptophan for glycine at position 524 of the mu-
ine cofactor E (CofE) protein. Sequence comparison re-
vealed that this amino acid is strictly conserved among verte-
brate species (Fig. 2). In nonvertebrate orthologues such as
Arabidopsis thaliana and Caenorhabditis elegans, glutamate
and histidine are found instead of tryptophan at this posi-
tion. It is not clear whether this alteration correlates with
specific differences in chaperone function in tubulin assem-
lization between vertebrate and nonvertebrate species. At least
for the homologue of vertebrate cofactor A, such differences
have been proposed from genetic studies with Schizosaccha-
romyces pombe (Radcliffe et al., 2000).

To correlate this mutation with the pmn phenotype, we
analyzed 60 pmn/pmn and 147 wt mice. These mice were
obtained from our breeding colony. They were not related
to the heterozygous male used for the initial backcross with
AKR. RT-PCR was performed with RNA from both the
homologue for the pmn/pmn mouse embryos at day 13.5 and to distinguish them from wt and heterozygous littermates. After a culture period of 7 d in the presence of 1 ng/ml BDNF, the motoneurons were fixed, stained with antibodies against tau and β III tubulin, and the length of axonal processes was measured by observ-
ers who were blinded with respect to the genotype. In pmn

mutant motoneurons, both the length of the longest axon and the total length of the axon including all its branches
(Fig. 3, A and B; Fig. 4, A and B) were significantly reduced,
by 58.77 ± 1.25% and 64.76 ± 3.66%, respectively (mean ±
SEM, three independent experiments from nine pmn/pmn
and seven wt embryos).

| Homo sapiens | QREIILELQFQYVSVEGDKCLVRW |
| Rattus norvegicus | QREIILELQFQYVSVEGDKCLVRW |
| Bos taurus | QREIILELQFQYVSVEGDKCLVRW |
| Xenopus laevis | QREIILELQFQYVSVEGDKCLVRW |
| Arabidopsis thaliana | -PFTALDES-TTLQDVE-DEG-LLV- |
| Caenorhabditis elegans | QREIILELQFQYVSVEGDKCLVRW |

Figure 2. Multiple amino acid sequence alignment of the COOH-
terminal region of CofE from different vertebrate and nonvertebrate
species. The nucleotide sequences of CofE were taken from public
databases, translated to the corresponding amino acid sequence,
and then aligned using ExPasy (www2.ebi.ac.uk/clustal). The amino
code numbers correspond to murine CofE. Conserved substitutions
and semiconserved substitutions are indicated in gray; the remaining
black amino acids are identical. Amino acids without similarity are
shown in light gray. The arrow indicates the amino acid mutated
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Figure 3. Axonal growth and survival in isolated wt and pmn
mutant motoneurons. (A and B) After 7 d in culture with 1 ng/ml
BDNF, the length of the longest axon and the total length of the
axon including all its branches from pmn mutant motoneurons
(Tbce\textsuperscript{W}/Tbce\textsuperscript{W}; open bar) are significantly (P < 0.0001) shorter than from wt motoneurons (Tbce\textsuperscript{W}/Tbce\textsuperscript{W}; closed bar). (C) Survival of
motoneurons isolated from wt (Tbce\textsuperscript{W}/Tbce\textsuperscript{W}; •) and pmn mice (Tbce\textsuperscript{W}/Tbce\textsuperscript{W}; ■) in the presence (closed symbols) and absence
(open symbol) of 1 ng/ml BDNF.
Moreover, pmn mutant motoneurons exhibited axonal swellings that were irregularly stained with antibodies against β III tubulin and tau, whereas axonal swellings were only observed in very few wt motoneurons (Fig. 4; Fig. 5, A–H). The percentage of motoneurons with prominent axonal swellings (more than threefold increase in axon diameter) was determined by a blinded observer, revealing a 20-fold increase in this specific pathological feature in Tbce<sup>+</sup>/Tbce<sup>−/−</sup> mutant motoneurons (Fig. 4 C). The growth cones that are mostly devoid of microtubular structures were morphologically indistinguishable in wt and pmn mutant motoneurons (Fig. 5, I–L).

Survival of wt and pmn mutant motoneurons was not different (Fig. 3 C). This finding indicates that the mutation in the Tbce gene does not primarily affect motoneuron survival, but disrupts axon growth and integrity. These data are in agreement with previous observations that crossing of a bel-2 transgene into pmn mice prevented the death of the motoneuron cell bodies but had no detectable effect on axon degeneration, progressive muscle weakness, or time of death (Sagot et al., 1995b).

To test whether the selectivity of the disease process is caused by specific expression of Tbce in motoneurons, we determined levels for Tbce mRNA by semiquantitative RT-PCR in spinal cord, several brain regions, and other organs of 3-wk-old mice. This time point was chosen because it reflects the most active stage of the disease process in pmn mutant mice. Tbce seems ubiquitously expressed in the nervous system and other organs, with no selectivity for spinal cord (Fig. S2 A, available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1). Similar observations were made when isolated embryonic motoneurons (E13.5) were compared with cortical neurons (E13.5) or neural stem cells derived from E11.5 mouse embryos (Fig. S2 B). Thus, the disease process is not caused by specific expression of Tbce in motoneurons, and the pathomechanism underlying the motoneuron disease in pmn mutant mice apparently is caused by other means than specific Tbce gene expression. This finding resembles observations in other forms of motoneuron disease, such as familial ALS and SMA. Neither Cu/Zn superoxid dismutase nor Smn is specifically expressed in motoneurons; therefore, the selectivity of the disease process for motoneurons is still elusive.

We do not know yet how the Trp→Gly mutation at position 524 of the CoFΕ protein interferes with its cellular function. This mutation could destabilize the protein or lead to either reduced or even enhanced chaperone activity, but it may also change its subcellular distribution or alter its interaction with other cellular binding partners. Dysregulation of CoFΕ could interfere with tubulin assembly in several ways. Loss of the CoFΕ homologue Alp21 in S. pombe leads to severe microtubule disintegration and reduced viability (Radcliffe et al., 1999). Genetic inactivation of PFI, the orthologue of the vertebrate Tbce in Arabidopsis, leads to defects in microtubule organization, mitotic division, and cytokinesis, but does not primarily interfere with cellular survival (Mayer et al., 1999; Steinborn et al., 2002).

Forced overexpression of CoFΕ in Hela cells sequesters α-tubulin, thus inhibiting dimer formation with β-tubulin and destabilizing free α- and β-tubulin subunits (Bhamidipati et al., 2000). Moreover, CoFΕ, together with other tubulin-specific cofactors, can act as GTPase, converting GTP-tubulin to GDP-tubulin. Thus, the capacity of polymerization into microtubules is lost (Tian et al., 1999) either by overexpression, altered protein interactions, or loss of CoFΕ.
Microtubules play an important role in axonal transport (Ishihara et al., 1999). The pmn mouse has been used in many studies as an animal model for motoneuron disease. Treatment of these mice with CNTF or other neurotrophic factors delays axon degeneration and motoneuron cell death (Sendtner et al., 1992; Haase et al., 1997). Neurotrophic factors enhance axonal transport both in retrograde (Sagot et al., 1998) and anterograde directions (Sahenk et al., 1994), and thus, at least in part, could functionally compensate for defects in microtubule structure in pmn mice (Sendtner et al., 1992; Sagot et al., 1995a).

The pmn mice appear healthy during embryonic development when axons grow out and make first contacts with skeletal muscle, resembling the clinical phenotype of human SMA. Thus, the function of CofE may become clinically relevant after birth. However, we observe a significant reduction in axonal growth in isolated embryonic pmn motoneurons. Nevertheless, the motoneurons still grow out axonal processes, suggesting that mechanisms exist that can at least partially compensate. This observation could reflect differential requirements of embryonic tubulin isoforms for CofE. It will be interesting to find out whether the class III β-tubulin isotype, which is highly expressed during development but not in the adult (Ferreira and Caceres, 1992), does not or to a lower degree depend on CofE.

Defects in microtubule function are associated with many forms of neurodegenerative disease. Deletion of kinesin, a microtubule-associated motor protein leads to severe motoneuron dysfunction in Drosophila (Hurd and Saxton, 1996). In human, Charcot Marie Tooth disease type 2A is caused by a mutation in the microtubule motor KIF1Bβ (Zhao et al., 2001). Recently, it was shown that overexpression of dynamin leads to disturbed retrograde axonal transport and thus causes motoneuron disease in mice (LaMonte et al., 2002). The symptoms are similar to those observed in mice overexpressing neurofilament genes (Cote et al., 1993; Xu et al., 1993). Moreover, mutations in tau, another microtubule binding and stabilizing protein, are not only associated with

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**Figure 5. Morphology of spinal motoneurons isolated from 13.5-d-old wt (Tbce<sup>W</sup>/Tbce<sup>W</sup>) and pmn mutant (Tbce<sup>G</sup>/Tbce<sup>G</sup>) embryos.**

Motoneurons were cultured for 7 d with 1 ng/ml BDNF, fixed, and stained against β III tubulin (red) and tau (green). (A and B) Motoneurons derived from pmn mutant embryos (B) exhibit shorter axons compared with axons from control cells (A). In pmn mutant motoneurons axonal varicosities are detectable (B, arrowhead), which were rarely observed in wt motoneurons. (C–H) Whereas β III tubulin and tau immunoreactivity is regularly organized and evenly distributed in axonal branch points in motoneurons derived from wt embryos (C), immunoreactivity for β III tubulin and tau in pmn mutants (D–H) is irregularly distributed in aggregate-like structures, and both proteins do not colocalize. (I–L) In contrast, growth cones that are mostly devoid of microtubular structures are morphologically indistinguishable in wt (I and J) and pmn mutant motoneurons (K and L). Bars: (A and B) 25 μm; (C, E, F, H) 5 μm; (D and G) 25 μm; (I and L) 5 μm; (J and K) 25 μm.
various forms of frontotemporal dementia (Garcia and Cleveland, 2001; Goedert and Spillantini, 2001) but also with the ALS/parkinsonism-dementia complex (Ishihara et al., 1999). Thus dysfunction of cellular mechanisms regulating tubulin assembly plays an essential role in motoneuron function and maintenance.

**Materials and methods**

**Mouse strains**

Two female AKR mice (Jackson ImmunoResearch Laboratories) and one male NMRI founder heterozygous for the pmn gene defect were bred and the F1 generation intercrossed for an F2 generation that could be used for genetic mapping of the pmn locus. The pmn mutation has been first detected at the Panum Institute (Kopenhagen, Denmark), where it spontaneously arose in a colony of NMRI outbred mice (Schmalbruch et al., 1991). This line was continuously backcrossed to NMRI (Jackson ImmunoResearch Laboratories) at our animal facility, and mice from this colony were used for confirmation of the Tbce gene mutation in pmn mice.

**Genetic mapping of the pmn locus**

Radioactive PCR with primers specific for the microsatellite markers was performed. Recombination events were investigated by size polymorphisms of the resulting fragments. A total of 229 pmn/pmn F2 offspring were genotyped with 29 microsatellite markers corresponding to a region spanning 31 cm from D13Mit11 to D13Mit10. 34 crossovers were identified, allowing us to map the pmn locus in a 2-cM region of chromosome 13 between D13Mit173 and D13Mit207.

**Physical mapping of the pmn locus**

All YAC clones were obtained from Research Genetics. The BAC clones were identified by screening of BAC mouse (release I and II) high-density filters with probes corresponding to the genes for nidogen and Gli3. All BAC clones were obtained from Genome Systems Inc., except BAC clone 369D23, which was supplied by Research Genetics. The physical map was validated by testing of markers listed on the physical map and design of new sequence-tagged sites (STSs) generated by YAC and BAC clone end sequences. The novel STS sequences are listed in Table S2, available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1.

**Used databases**

A Cela genomic scaffold covering ~2.19 Mb (nucleotides 9,285,719–11,297,173) located on mouse chromosome 13 was used to derive the genomic structure of 23 putative and previously identified transcriptional units.

Open reading frames were additionally confirmed using information deposited in public EST databases (www.ncbi.nlm.nih.gov/; Altschul et al., 1997). Only sequences of expressed genes were analyzed. Homology searches and sequence alignment were performed with BLAST (www.ncbi.nlm.nih.gov/; Altschul et al., 1997).

**Sequence analysis of candidate genes**

Total RNA from tissue of pmn and wt mice was prepared by the Trizol procedure (Invitrogen) and was reverse transcribed with oligo dt primers according to the manufacturer’s instructions (Invitrogen). Primers used for PCR reactions and the specific PCR conditions are shown in Table S3, available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1.

Double-stranded DNA was subjected to automated sequencing on an ABI 373 sequencer by dideoxy sequencing using a commercial standard sequencing kit (ABI). Both strands were sequenced with respective primers. Resulting DNA sequences obtained from PCR products from pmn and NMRI wt mice were aligned to corresponding sequence information in databases in order to identify nucleotide variations.

**Embryonic mouse motoneuron cultures**

Spinal motoneurons from 13.5-d-old mouse embryos were isolated, enriched, and plated at a density of 3,000 cells/well on Greiner 4-well Culture dishes as previously described (Wiese et al., 2001). Cells were grown in neurobasal medium, B27 supplement, 10% horse serum, 500 μM glutamine (Invitrogen), and 1 ng/ml BDNF. Medium was first replaced at day 1 and every other day after.

Surviving cells were counted under phase-contrast microscopy every second day. Initial counting of plated cells was done when all cells were attached to the culture dish at 4 h after isolation.

After 7 d in culture, motoneurons were fixed with 4% paraformaldehyde for 15 min at 4°C. The cells were washed with TBS (25 mM Tris/HCl, pH 7.4, 0.8% NaCl, 0.2% KCl). Nonspecific binding sites were blocked for 1 h with TBS containing 1% goat serum and 0.1% Tween-20. Cells were incubated for 24 h at 4°C with antibodies against β III tubulin (1:500; RDI) and tau (1:200; Sigma-Aldrich), and then washed three times with TBS. β III tubulin was visualized by goat anti–mouse Cy3 (5 μg/ml; Jackson ImmunoResearch Laboratories) and tau with goat anti–rabbit Cy2 (10 μg/ml; Jackson ImmunoResearch Laboratories) for 1 h. Cells were washed again three times with TBS, and then covered with Mowiol in 50% glycerol/PBS (vol/vol) and observed under a Leica confocal microscope (TCS; Leica). Controls were done in the absence of the first antibodies (not depicted).

Digital photographs of motoneurons were taken using a Leica confocal microscope. As previously described (Wiese et al., 1999), axons are at least three times longer than dendrites after 7 d in culture and thus can be distinguished. Axon length was determined by applying a morphometric system (ScionImage; Scion Corporation, Inc.). These analyses of mutant and wt motoneurons were performed blindly. The investigator did not know the genotype of the cultured cells. Values from independent experiments were pooled, and the results were expressed as the mean ± SEM. Statistical significance of differences were assessed by t test using the GraphPad Prism software.

**Neural stem cells**

Neural stem cells of neural stem cell neurospheres from forebrain of embryonic day 11.5 mice were prepared and transferred to 100 μl HBSS. After treatment with trypsin (0.05%, 10 min; Worthington), cell suspensions were generated by trituration. Trypsin was inactivated with trypsin inhibitor from egg yolk sarc (0.05%; Sigma-Aldrich) and the cells plated on 75-cm2 culture dishes (Greiner) in 5 ml neurobasal medium containing 500 μM glutamax, B27 supplement, and bFGF and EGF (Cell Concepts) at final concentrations of 10 ng/ml each. Medium was changed every second day. Cells from the supernatant were centrifuged at 400 g, trituated, and cultured in fresh medium. Cells were passaged at least six times to enrich neurospheres. These neurospheres were centrifuged and RNA was isolated.

**Accession nos.**

The GenBank/EMBL/DBJ accession numbers for Mus musculus are as follows: NM031999 (Tmn5f1), NM010917 (Nid), NM010747 (Lyst), NM010317 (Gg34), NM010282 (Gg35), Ak018194 (Rbp1-like), Ak002753 (Mbp3), NM008944 (Psma2), and X092255 (Gli3). The following accession numbers are for Homo sapiens: NM 003193 (TBCE), NM 003272 (TM7SF1), NM 000081 (CHS1), NM004485 (GNG4), NM 004837 (GPGS), NM 016374 (RBPI-like), NM 045232 (LOC94980), NM 031903 (MRPL32), NM 002787 (PSMA2), NM 024054 (MGCG282), and BC021686 (EFL-1a). The accession number for Tbce is AV605313 for Bos taurus, B1314293 for Xenopus laevis, AF486851 for A. italiana, and NP 501395 for C. elegans. The accession numbers from OMIM are as follows: 25350 (SMA), 604320 (SMARD1), 205100 (ALSD2), and 118210 (Charcot Marie Tooth disease type 2A).

**Online supplemental material**

Figs. S1 and S2 and Tables S1, S2, and S3 are available online at http://www.jcb.org/cgi/content/full/jcb.200208001/DC1. Fig. S1 shows the pmn candidate region. Fig. S2 shows the expression profile of Tbce mRNA in neurons and nonneuronal tissue. Table S1 shows the transcript map of the pmn region, including the sytény region of the human genome. Table S2 shows the novel STSs isolated from the pmn candidate region. Table S3 shows a list of primers used for PCR reactions.

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