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Progressive Motor Neuronopathy: A Critical Role of the Tubulin Chaperone TBCE in Axonal Tubulin Routing from the Golgi Apparatus

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Axonal degeneration represents one of the earliest pathological features in motor neuron diseases. We here studied the underlying molecular mechanisms in progressive motor neuronopathy (pmn) mice mutated in the tubulin-specific chaperone TBCE. We demonstrate that TBCE is a peripheral membrane-associated protein that accumulates at the Golgi apparatus. In pmn mice, TBCE is destabilized and disappears from the Golgi apparatus of motor neurons, and microtubules are lost in distal axons. The axonal microtubule loss proceeds retrogradely in parallel with the axonal dying back process. These degenerative changes are inhibited in a dose-dependent manner by transgenic TBCE complementation that restores TBCE expression at the Golgi apparatus. In cultured motor neurons, the pmn mutation, interference RNA-mediated TBCE depletion, and brefeldin A-mediated Golgi disruption all compromise axonal tubulin routing. We conclude that motor axons critically depend on axonal tubulin routing from the Golgi apparatus, a process that involves TBCE and possibly other tubulin chaperones.

Key words: motor neuron disease; ALS; axon degeneration; tubulin chaperone; microtubules; Golgi apparatus

Introduction

Human motor neuron diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) are incurable and fatal disorders characterized by loss of motor neuron cell bodies, axonal degeneration, and skeletal muscle denervation (for review, see Boillee et al., 2006; Pasinelli and Brown, 2006). In several ALS and SMA mouse models, motor axonal degeneration occurs weeks to months before cell body loss and displays numerous features of a retrograde “dying back” process. In transgenic mutant superoxide dismutase 1 G93A mice, a model of familial ALS1, denervation of endplates appears at presymptomatic stage, loss of ventral root axons at disease onset, and loss of spinal motor neuron cell bodies only at end stage (Fischer et al., 2004) (see also Kennel et al., 1996a; Frey et al., 2000; Pun et al., 2006). SMA model mice deficient in neuronal SMN (survival motor neuron) gene express microtubule and display extensive muscle denervation and motor axon loss at a moment when the number of spinal motor neuron cell bodies is only modestly reduced (Cifuentes-Diaz et al., 2002). Alsin/ALS2 knock-out mice show retrograde axonal degeneration in the dorsolateral spinal cord tract but no loss of cell bodies in the motor cortex (Devon et al., 2006; Yamanaka et al., 2006). It remains unclear however whether axonal dying back is triggered in the axon itself or whether it is a consequence of degenerative changes originating in the cell body (Fischer et al., 2004; Conforti et al., 2007).

We here studied this question in mice with progressive motor neuronopathy (pmn). Homozygous pmn mice suffer from a severe motor neuron disease characterized by axonal dying back (Schmalbruch et al., 1991) and progressive loss of motor units (Kennel et al., 1996b). Axonal degeneration and clinical disease course in pmn mice can be attenuated by neurotrophic factor gene therapy (Sendtner et al., 1992; Sagot et al., 1995a; Haase et al., 1997) or expression of the axonoprotective WldS protein (Ferri et al., 2003) but not by overexpression of the antiapoptotic protein Bcl-2 (Sagot et al., 1995b). We and others showed that pmn mice are mutated in TBCE (Bömmel et al., 2002; Martin et al., 2002), one of five tubulin-specific chaperones (TBCA–TBCE) involved in tubulin folding and dimerization (Tian et al., 1996, 1997). The pmn mutation, a tryptophan to glycine exchange at the C terminus of TBCE, causes axonal microtubule loss in vivo (Martin et al., 2002) and, according to Bömmel et al. (2002), also impedes motor axon growth in vitro.

We identify TBCE as a tubulin chaperone that accumulates at the cis-Golgi apparatus and demonstrate its requirement for axonal tubulin routing. In spinal motor neurons of early symptom-
atic pmn mice, the TBCE protein is destabilized, leading to a drastic reduction in tubulin levels and microtubule densities in distal axons. Axonal tubulolin loss progresses from distal to proximal, correlates with axonal degeneration, and is inhibited by transgenic TBCE protein expression. These data help to explain the axonal dying back process in pmn mice and provide a mechanistic link between motor axon maintenance and TBCE function at the Golgi apparatus.

Materials and Methods

Antibodies and reagents. Antiserum against TBCE (SA53) was generated by immunizing a rabbit with a mixture of two peptides corresponding to amino acids 87–102 and 389–402 of mouse TBCE (Eurogentec, Liege, Belgium). A second anti-TBCE antiserum (GP52) was generated against the same peptides. In control experiments, antiserum was preabsorbed with antigenic peptides or recombinant green fluorescent protein (GF–)–TBCE protein (G. Haase and A. Elmarjou, unpublished observation). Antibodies and their dilutions in immunocytochemistry were as follows: TBCE (1:300), α-tubulin (1:1000; Sigma, St. Louis, MO), βIII-tubulin (TuJ1; 1:2000; Babco, Richmond, CA), GFP (monoclonal antibody, 1:1000; Roche, Indianapolis, IN), myelin basic protein (1:500; Chemicon, Temecula, CA), neurofilament medium chain (NF18, 1:2000; Sigma), S-100 (SH-B1, 1:1000; Sigma), and GM130, p115, Vti1a, and Vti1b (1:250; Becton Dickinson, Walton, KY). Fluorochrome– or base radish peroxidase-conjugated secondary antibodies were from Invitrogen (Carlsbad, CA) or Jackson ImmunoResearch (West Grove, PA). Reagents were from the following suppliers: PBS, HBSS, HAM F-10, trypsin, culture media, and supplements (Invitrogen); Hibernate E gen (Carlsbad, CA) or Jackson ImmunoResearch (West Grove, PA).

Mouse lines and genotyping. The pmn and wild-type alleles were detected using primers 5′-GTCTTACTGCCTCCATCTGT (C45F) and 5′-GTGAAACAGAAAAGGCAGG (C45R) and 35 cycles of amplification (95°C, 40 s; 58°C, 40 s; 72°C, 40 s), followed by DNA purification (Qiagreen; Qiagen, Hilden, Germany), 2 h incubation with the restriction enzyme MseI (New England Biolabs, Ipswich, MA), and gel electrophoresis (3% Metaphor agarose; Tebu, Le Perray en Yvelines, France). The NSETBCE transgene was detected by PCR using primers 5′-CAACAGGATGACTCTAGAG (NSEG–7F), 5′-AAAGGACGTTCTCATTTC (TB-7R), 5′-GATCATGACCCGCTAG (X1), and 5′-CATGAACTTGCACCAGTT (X2), 35 cycles (92°C, 40 s; 58°C, 40 s; 72°C, 1 min) and 2% agarose gel electrophoresis.

Mouse genotyping was done as follows. The pmn mice and wild-type alleles were detected using primers 5′-GTCTTACTGCCTCCATCTGT (C45F) and 5′-GTGAAACAGAAAAGGCAGG (C45R) and 35 cycles of amplification (95°C, 40 s; 58°C, 40 s; 72°C, 40 s), followed by DNA purification (Qiagreen; Qiagen, Hilden, Germany), 2 h incubation with the restriction enzyme MseI (New England Biolabs, Ipswich, MA), and gel electrophoresis (3% Metaphor agarose; Tebu, Le Perray en Yvelines, France). The NSETBCE transgene was detected by PCR using primers 5′-CAACAGGATGACTCTAGAG (NSEG–7F), 5′-AAAGGACGTTCTCATTTC (TB-7R), 5′-GATCATGACCCGCTAG (X1), and 5′-CATGAACTTGCACCAGTT (X2), 35 cycles (92°C, 40 s; 58°C, 40 s; 72°C, 1 min) and 2% agarose gel electrophoresis.

Expression plasmids and small interference RNAs. Expression vectors for GFP–TBCE, hemagglutinin–TBCE, and FLAG–TBCE were generated by subcloning mouse wild-type TBCE cDNA using pCAGGS–GF (Jacquier et al., 2006) or pCMV–Tag1 vectors (Stratagene, La Jolla, CA) as backbone. The GFP–α-tubulin expression vector was generated by subcloning a fragment from pAGFP1–tubulin (Clontech) into pCAGGS (Jacquier et al., 2006). Small interference RNAs (siRNAs) against luciferase or TBCE were from Dharmacon (Chicago, IL). Targeted regions of mouse TBCE (AY082332) were as follows: siTBCE 1, nucleotides 118–137; siTBCE 2, nucleotides 242–262; siTBCE 3, nucleotides 792–812; siTBCE 4, nucleotides 1108–1126; and siTBCE pool, equimolar mix of siTBCE 1–4. Cell cultures and in vitro assays. Motor neurons were prepared from embryonic day 12 spinal cords, electroporated with DNA plasmids and/or siRNAs, and cultured in the presence of the neurotrophic factors BDNF, CNTF, and GDNF (Rao et al., 2002; Jacquier et al., 2006). For immunocytochemistry, cells were fixed by adding an equal volume of 8%
formaldehyde for 20 min at room temperature, blocked for 30 min in PBS containing 5% goat serum, 1% BSA, and 0.5% Triton X-100, and immunostained. NSC34 cells (Cashman et al., 1992) were cultured in DMEM supplemented with 10% fetal calf serum and transfected with DNA plasmids and lipofectamine (Invitrogen). To induce Golgi disassembly, cells were treated with 10 μM brefeldin A for the indicated times, and reassembly was induced by washout for 30 min in culture medium.

For quantification of GFP-α-tubulin and Discosoma red (DsRed) fluorescence, images were acquired in electroporated motor neurons cultured for 2 or 3 DIV (DIV) from wild-type and pmn embryos or transduced with siRNAs, respectively. Images from cell bodies or distal axons were obtained by confocal microscopy in optical sections of 2.8 μm using identical acquisition parameters in each experiment and analyzed by MetaMorph (Universal Imaging, Downingtown, PA). Ratios of GFP-α-tubulin to DsRed were calculated from mean fluorescent intensities of each condition and experiment.

Microtubule growth assays were performed as described by Ahmad and Baas (1995) with the following modifications. Primary motor neurons were allowed to attach for 45 min. Microtubules were depolymerized by adding 10 μM nocodazole and incubated for 6 h at 37°C. Nocodazole was washed out with warm culture medium, and neurons were further incubated for 0, 1, or 30 min. Cultures were rinsed in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, and 1% formaldehyde, pH 6.9) and extracted for 3 min in PHEM containing 0.2% Triton X-100, and 20 μM taxol, fixed, blocked, and immunostained for β₃ tubulin and α-tubulin to visualize microtubules and centrosomes, respectively. Images were obtained by confocal microscopy in sections of 2.5 μm optical thickness covering the centrosome using identical acquisition parameters. Mean β₃ tubulin fluorescence and the length of microtubules emanating from the centrosome were measured using MetaMorph and NIH ImageJ software, respectively.

Statistical analyses. Experiments were performed in duplicate or triplicate and repeated at least once. Data were analyzed with Excel (Microsoft, Seattle, WA); statistical testing and linear regression analysis were performed with SigmaStat 3.1 (Systat, Evanston, IL). When data showed a Gaussian distribution, they were analyzed with Student’s t test (two-tailed, unpaired); otherwise the Mann–Whitney U test was used.

Results

Retrograde progression of axonal microtubule loss in pmn mice

Homozygous pmn mice develop first signs of muscle atrophy and paresis at 2 weeks of age and die by respiratory failure 4 to 5 weeks later (Schmalbruch et al., 1991). Motor neuron degeneration in affected mice starts at endplates and distal axons and progresses retrogradely (Schmalbruch et al., 1991). Proximal motor axons in ventral roots, however, show only modest degeneration (Schmalbruch et al., 1991), and spinal motor neuron cell bodies are preserved until end stage (Haase et al., 1997). To assess the role of microtubules in the axonal dying back process, we selected the phrenic nerve that contains mainly motor axons (Langford and Schmidt, 1983) and analyzed it at a distal level, close to the diaphragm, and at an intermediate level corresponding to the thoracic inlet. We also investigated C4 ventral root nerves because they contain the most proximal phrenic axons. As time points, we chose day 15 (disease onset) and day 28 (advanced disease stage). Using electron microscopy, we determined microtubule number and axonal surface in at least 10 entire cross-sectional axon profiles per nerve in four wild-type and four pmn littermate mice (Fig. 1A, B).

In normal phrenic nerves, we counted a mean of 15.2 ± 2.3 microtubules/μm² cross-sectional area of the distal axon (means of mean ± SD). In distal phrenic nerves of pmn mice, axonal microtubule densities were reduced to 6.0 ± 1.6 microtubules/μm², corresponding to 39 ± 10% of normal (p < 0.0001) (Fig. 1C, D, I). A similar reduction in axonal microtubule density (44 ± 9% of normal; p < 0.0001) was found at the intermediate level (Fig. 1J). Interestingly however, C4 ventral roots from pmn mice displayed no significant reduction in axonal microtubule density (89 ± 5% of wild type) (Fig. 1E, F, I). Lumbar ventral roots that supply the most severely affected territories in pmn mice also showed normal axonal microtubule densities (97 ± 22% of wild type) (Fig. 1I). These findings were further supported by Western blot analysis of neuronal β₃ tubulin levels in...
distal and proximal sciatric nerves and lumbar spinal cord of 15-d-old mice. In pmn mice, the βIII-tubulin levels were significantly reduced in distal sciatric nerves but normal in proximal nerves and spinal cord (Fig. 1K).

To analyze the temporal progression of microtubule loss in pmn mice, we studied C4 cervical ventral roots of 28-d-old pmn mice (Fig. 1G, H). At this advanced disease stage, microtubule densities were reduced to 40 ± 13% of normal (Fig. 1J). Axonal microtubule loss in pmn motor nerves thus first manifests distally and then progresses from distal to proximal, in parallel with the axonal dying back neuropathy.

Consequences of microtubule loss on axon degeneration
We then investigated the requirement of microtubules for motor axon maintenance. To this purpose, we analyzed not only pmn and wild-type mice but also two lines of transgenic pmn mice complemented with wild-type TBCE (Martin et al., 2002), termed TBCEPA pmn and TBCEPC pmn. In TBCEPA pmn mice, disease onset is retarded by 2–3 weeks and survival is prolonged; in TBCEPC pmn mice, disease is completely prevented. Using electron microscopy, we compared axonal pathology and microtubule density in distal phrenic nerves of pmn, wild-type and transgenic TBCE pmn mice (Fig. 2A–D). In phrenic nerves of 15-d-old pmn mice, we observed numerous axons with irregular contours (Fig. 2A) and a loss of 25% of myelinated axons (195 ± 25, mean ± SD) compared with wild-type littermate mice (260 ± 20, mean ± SD) (Fig. 2C). In TBCEPA pmn mice, axon contour irregularities were present but less prominent than in pmn mice (Fig. 2A), and microtubule loss was observed only in four of many mice (Fig. 2B, D). In TBCEPC pmn mice, axon contour irregularities were completely absent, and axonal microtubule densities and numbers in phrenic nerves were normal (Fig. 2A–C). Linear regression analysis revealed a highly significant correlation between microtubule density and axon numbers (r = 0.939; α = 0.01; p < 0.001) (Fig. 2D). Microtubule densities below 10/μm², corresponding to a microtubule loss of ~40% were associated with beginning axon loss. Together, the clinical and EM data thus indicate a close correlation between axonal microtubule density, motor axon maintenance, and clinical disease course.

TBCE accumulates at the Golgi apparatus of motor neurons
To investigate the role of TBCE in the maintenance of axonal microtubules, we studied its protein expression profile in the peripheral nervous system. In immunoblots of adult spinal cord extracts, our polyclonal antibody detected a single band of 59 kDa, corresponding to recombinant TBCE (Fig. 3A). On cervical spinal cord sections, TBCE was mainly expressed in ventral horn motor neurons and in neurons of superficial dorsal horn layers (Fig. 3B). To verify that TBCE is expressed in phrenic motor neurons in the cervical spinal cord, we used retrograde rhodamine–dextran labeling. We found that all of them express high levels of TBCE in their soma (Fig. 3C). Surprisingly, however, TBCE was barely detectable in axons, identified by neurofilament labeling (Fig. 3D) or genetic labeling (Fig. 3E) in Thy1–YFP line 16 mice (Feng et al., 2000). In phrenic and sciatric nerves and also in cervical and lumbar ventral roots, TBCE expression predominated in Schwann cell bodies (Fig. 3D, E and supplemental Fig. 1, available at www.j-neurosci.org as supplemental material).

In motor neurons, TBCE accumulated at discrete tubulovesicular structures surrounding the nucleus that were reminiscent of the Golgi apparatus (Fig. 3F–I). Double immunolabeling and confocal microscopic analysis confirmed a striking overlap (Fig. 3F, G) between TBCE and the two cis–Golgi markers GM130 (Nakamura et al., 1995) and p115 (Sapperstein et al., 1995). The TBCE-positive structures were often found juxtaposed to Vti1a–stained trans–Golgi compartments (Fig. 3H) but clearly distinct from Vti1b–stained post–Golgi compartments (Fig. 3I). TBCE accumulation at the Golgi was also observed in Schwann cells, as shown by TBCE and GM130 double immunolabeling (supplemental Fig. 1D, E, available at www.j-neurosci.org as supplemental material). Golgi localization of TBCE was confirmed with a second antibody against TBCE and specificity by antibody preabsorption on TBCE peptides or on recombinant TBCE proteins (data not shown).

Previous TBCE overexpression studies in HeLa cells had detected TBCE only in the cytoplasm (Bhamidipati et al., 2000; Tian et al., 2006). To test this, we overexpressed GFP- and FLAG-tagged TBCE in the NSC34 motor neuron cell line (Cashman et al., 1992). When expressed at a low level, tagged TBCE could be clearly detected at Golgi membranes (supplemental Fig. 2A,
TBCE is destabilized at the Golgi apparatus in pmn mice and is restored by transgenic TBCE complementation

To explore the molecular causes of microtubule loss in pmn mice, we analyzed whether the protein levels or subcellular localization of TBCE were modified in pmn motor neurons in vivo. Our previous in vitro studies had indicated that the pmn mutation alters the conformation and reduces the half-life of the TBCE protein (Martin et al., 2002). Western blot analyses now showed that TBCE protein levels in pmn spinal cords were drastically lower than in wild-type spinal cords at both disease onset (Fig. 4 A) and end stage (Fig. 4 B). Immunofluorescence analyses confirmed that TBCE expression at the Golgi apparatus was reduced in cervical and lumbar pmn motor neurons at postnatal day 15 (P15) (Fig. 4 C–E) and P35 (data not shown). Similar observations were made in Schwann cells in the sciatic nerve (supplemental Fig. 1 B–E, available at www.jneurosci.org as supplemental material). In conclusion, the pmn mutation destabilizes TBCE and reduces its steady-state levels at the Golgi apparatus in vivo.

To determine the levels of TBCE protein required for motor axon maintenance, we analyzed the two lines of transgenic TBCE pmn mice. In these mice, wild-type TBCE is expressed under control of the neuron-specific NSE promoter (Forss-Petter et al., 1990). Western blot analyses showed that spinal cord levels of TBCE protein were intermediate in TBCE PA pmn mice and supranormal in TBCE PC pmn mice compared with pmn and wild-type mice (Fig. 4 A, B). Immunostainings of cervical and lumbar spinal cord motor neurons showed that TBCE expression at the Golgi apparatus was partially restored in TBCE PA pmn mice and normal in TBCE PC pmn mice (Fig. 4 D, E). Together, the biochemical, electron microscopic, and clinical data thus demonstrate that TBCE protein levels in spinal motor neurons determine the histopathological and phenotypic severity of progressive motor neuronopathy.

Effects of TBCE depletion in motor neuron cultures

Microtubules are made of α/β-tubulin dimers that are generated in a complex biological process requiring the sequential action of prefoldin, CCT (cytosolic chaperonin complex), and five tubulin-specific chaperones. During this process, TBCE assists in the folding of α-tubulin and the formation of α/β-tubulin heterodimers (Tian et al., 1996, 1997). To investigate the function of TBCE in motor neurons, we performed RNA interference-mediated TBCE depletion experiments in NSC34 cells. Western blot analysis showed that endogenous TBCE was efficiently depleted by siRNAs (Fig. 5 A). TBCE depletion had no effect on total levels of α- or β-tubulin (Fig. 5 A). TBCE-depleted cells, how-

available at www.jneurosci.org as supplemental material). After high-level expression, however, tagged TBCE was found exclusively in the cytoplasm. This discrepancy is probably due to the fact that high-level TBCE overexpression disrupts the cellular microtubule network that is required for maintenance of the Golgi apparatus (supplemental Fig. 2 A–C, available at www.jneurosci.org as supplemental material).

Golgi proteins can be membrane bound, like the Golgi matrix protein GM130 (Nakamura et al., 1995), or peripheral membrane-associated, like the vesicle-tethering protein p115, which shuttles between membrane and cytosolic pools (Brandon et al., 2006). To distinguish between these possibilities for TBCE, we prepared subcellular fractions from spinal cord. TBCE was present in both crude membrane and cytosolic fractions, in a ratio similar to p115 (Fig. 3 F). GM130 was found exclusively in the membrane fraction, as expected (Fig. 3 F). Collectively, our immunohistochemical and biochemical data thus identify TBCE as the first tubulin-specific chaperone that accumulates at the Golgi apparatus.

Figure 3. TBCE accumulation at the Golgi apparatus of spinal motor neurons. A, Western blot analysis shows that a polyclonal antibody directed against murine TBCE detects a band of ~59 kDa in adult spinal cord protein extracts and in COS cells overexpressing TBCE. B, Immunolabeling for TBCE in an adult cervical spinal cord cross section. C, TBCE immunostaining in retrogradely labeled phrenic motor neurons on a frontal section of adult cervical spinal cord. D, Immunolabeling for TBCE and neurofilament medium chain (NF) in a ventral root shows that TBCE is barely detectable in axons. E, Triple labeling for TBCE (in red), S-100 (in blue), and the axon (in green) in the sciatic nerve of a 16-month-old line 16 mouse shows TBCE expression in the cell body of a Schwann cell. F, G, Immunolabeling for TBCE and the cis-Golgi markers GM130 (F) or p115 (G) demonstrates that TBCE decorates cis-Golgi membranes in motor neurons of adult spinal cord. H, I, Immunolabeling for TBCE and Vti1a (H), a trans-Golgi marker, or Vti1b (I), a marker of post-Golgi compartments. Insets show magnified areas depicted by arrows. Scale bars: B–D, 100 μm; E, 5 μm; F–I, 20 μm. J, Western blot analysis showing distribution of TBCE, p115, and GM130 in postnuclear, cytosolic, and crude membrane fractions of spinal cord.
ever, contained more soluble and less precipitable, microtubule-incorporated, \( \alpha \)-tubulin than control cells (Fig. 5B,C), suggesting that some \( \alpha \)-tubulin was not correctly folded and polymerization incompetent.

In neurons, tubulins are mainly synthesized in the cell body (Eng et al., 1999), and most of them are rapidly routed into the axon (Campenot et al., 1996). Our in vivo observations in \( pmn \) mice showed that microtubule loss starts in the distal axon and progresses retrogradely. We therefore wondered whether TBCE is required for axonal tubulin routing. To address this question, we used embryonic motor neurons in primary culture. To monitor their axonal tubulin routing, we electroporated them with a GFP–\( \alpha \)-tubulin expression vector along with a DsRed vector and siRNAs. In si-luciferase-transduced control motor neurons, newly synthesized GFP–\( \alpha \)-tubulin and DsRed were evenly distributed in cell bodies and axons (Fig. 5D). In TBCE-depleted motor neurons, however, the amount of GFP–\( \alpha \)-tubulin was reduced in distal axons (Fig. 5D). We therefore compared the fluorescence ratios of GFP–\( \alpha \)-tubulin with DsRed in motor neuron cell bodies and axons by confocal microscopy and quantitative image analysis (Fig. 5D–G). In cell bodies of TBCE-depleted cells, the mean GFP–\( \alpha \)-tubulin/\( \beta \)_III-tubulin fluorescence ratio was reduced to 74.3 ± 1.7% of the ratio in control cells (mean ± SD; \( p < 0.01 \)) (Fig. 5G). In distal axons of TBCE-depleted cells, this ratio was reduced to 41.9 ± 1.6% of the control value (mean ± SD; \( p < 0.01 \)) (Fig. 5G), which is significantly lower than the reduction in the cell bodies. These results indicate that TBCE in motor neurons is not only required for proper tubulin folding but also for its axonal routing.

Microtubule polymerization and axonal routing in \( pmn \) motor neurons
To test the consequences of the \( pmn \) mutation on microtubule polymerization, we examined \( pmn \) and wild-type motor neurons in culture. Motor neurons were purified from individual embryos, seeded, and incubated for 6 h with 10 \( \mu \)M nocodazole to depolymerize microtubules (Ahmad and Baas, 1995). In wild-type motor neurons, microtubules began to emanate from the centrosome as early as 1 min after nocodazole washout and formed extended microtubule asters at 30 min (Fig. 6A). In \( pmn \) motor neurons, microtubule polymerization was initially normal but severely affected at 30 min (Fig. 6B). At this time, the immunoreactivity of microtubule-incorporated \( \beta \)_II-tubulin was reduced to 43.9 ± 8.1% of wild type (mean ± SD; \( p < 0.0001 \)) (Fig. 6C), and microtubules were significantly shorter than in wild-type motor neurons (Fig. 6D). These results indicate that the \( pmn \) mutation in TBCE impairs microtubule polymerization.

We next analyzed axonal tubulin routing in \( pmn \) motor neurons. Motor neurons were electroporated with GFP–\( \alpha \)-tubulin and DsRed plasmids and cultured for 2 DIV. In cell bodies, the mean GFP–\( \alpha \)-tubulin/\( \beta \)_III-tubulin fluorescence ratios were not significantly different between \( pmn \) and wild-type motor neurons (Fig. 6F). In distal axons of \( pmn \) motor neurons, however, the mean GFP–\( \alpha \)-tubulin/DsRed ratio was reduced to 54.2 ± 9.6% of wild type (\( p < 0.05 \)) (Fig. 6E,F). The reduction of tubulin levels in distal axons of \( pmn \) motor neurons indicates that the \( pmn \) mutation in TBCE compromises axonal tubulin routing.

The Golgi apparatus controls axonal tubulin routing in motor neurons
To test whether the Golgi apparatus is involved in axonal tubulin routing, we pharmacologically disrupted this organelle with BFA. BFA inhibits activation of ADP-ribosylation factors (Donaldson et al., 1992), which causes Golgi disassembly and redistribution of its components into the endoplasmic reticulum (Lippincott-Schwartz et al., 1990), the cytosol (Klausner et al., 1992), or into so-called Golgi remnants (Seemann et al., 2000). In mock-treated NSC34 motor neurons (Fig. 7A), we found TBCE to be associated with GM130-stained cis-Golgi membranes, as in vivo. In BFA-treated motor neurons, GM130 localized to dispersed Golgi remnants, as expected, but TBCE was redistributed into the cytosol (Fig. 7A). The BFA-induced cytosolic TBCE redistribution was reversible on BFA washout (Fig. 7A) and not accompanied by a modification in cellular TBCE levels (Fig. 7B). Immunoblot analyses further confirmed that BFA had no significant effect on the

Figure 4. Loss of TBCE expression at the Golgi apparatus of \( pmn \) mice and its restoration by transgenic TBCE complementation. A, B, Western blot analysis showing protein levels of TBCE and neurofilament-M (NF-M) in spinal cord lysates of wild-type (wt), \( pmn \), TBCE\(^{PA} \) \( pmn \), and TBCE\(^{PC} \) \( pmn \) mice at P15 (A) and P35 (B). C, Immunolabeling showing reduced TBCE expression in spinal cord motor neurons of \( pmn \) mice at P15. D, E, Immunolabeling showing TBCE protein expression in cervical (D) and lumbar (E) spinal cord motor neurons of wild type, \( pmn \), TBCE\(^{PA} \) \( pmn \), and TBCE\(^{PC} \) \( pmn \) mice at P15. Scale bars: C, 100 \( \mu \)m; D, 10 \( \mu \)m.
Figure 5. Role of TBCE in tubulin formation and axonal routing. A, TBCE depletion in NSC34 cells. Western blots showing total levels of TBCE, α- and βIII-tubulin, and β-actin in cells 3 d after transfection of si-luciferase, si-TBCE 1, or si-TBCE pool. B, Western blot showing increased amounts of soluble α-tubulin (S) and reduced amounts of precipitable α-tubulin (P) in subcellular fractions of TBCE-depleted NSC34 cells compared with control cells. C, Total tubulin in postnuclear supernatant. D-F, Images of primary motor neurons at 3 DIV after electroporation with GFP–α-tubulin and DsRed expression vectors and siRNAs. Shown are low-power magnifications of neurons (D) and high-power magnifications of cell bodies (E) and distal axons (F). Note diminished GFP–α-tubulin fluorescence in the distal axons of TBCE-depleted motor neurons. G, Diagram showing the ratio of GFP–α-tubulin to DsRed fluorescence in cell bodies and distal axons of electroporated motor neurons. After TBCE depletion, this ratio is significantly lower in distal axons than in cell bodies. Scale bars: D, 50 μm; E, F, 10 μm. Statistical significance: *p < 0.01, Student’s t test. Data represent means of means ± SD from three independent experiments (n = 75 neurons per condition).

Discussion

Subcellular origin of axonal degeneration

Axonal degeneration, whether of traumatic, toxic, or genetic origin, often manifests as axonal dying back: distal axons are affected first, and degeneration apparently progresses from distal to proximal (Cavanagh, 1964; Coleman, 2005). The subcellular origin of such dying back processes remains debated (Conforti et al., 2007). "Centroneuronal" hypotheses have attributed the axonal dying back to dysfunction of the neuronal perikaryon and progressive withdrawal of metabolic support to the axon (Cavanagh, 1964) or to defects in the proximal axonal cytoskeleton and blockade of anterograde axonal transport (Griffin and Watson, 1988). "Axonal" hypotheses postulated that the degenerative process is triggered in the axon itself: Vinca alkaloid, a chemotherapeutic agent used in human cancer therapy, causes a peripheral neuropathy with prominent distal axonal degeneration (Bradley et al., 1970). The axonal dying back process can be mimicked in neuronal cultures (Ravula et al., 2007) in which topical application of Vinca alkaloid at clinically relevant concentrations injures the axon but not the neuronal soma (Silva et al., 2006). Several genetic studies have also linked impaired retrograde axonal transport to motor neuron degeneration. Mutation or disruption of the motor protein complex dynein/dynactin for example cause progressive motor neuron degeneration in mice (LaMonte et al., 2002; Hafezparast et al., 2003) and humans (Puls et al., 2003).

Retrograde microtubule loss in pmm mice

We here investigated how a missense mutation in TBCE causes axonal dying back in pmm mice. We show that in normal mice, TBCE is expressed in motor neuron cell bodies in which it accumulates at the cis-Golgi apparatus. In early symptomatic pmm mice, TBCE is destabilized and lost from this organelle. Microtubules are first lost in distal motor axons and only at end stage in proximal motor axons. This retrograde microtubule loss parallels the axonal dying back: at disease onset, the extent of microtubule loss in distal phrenic nerves correlates with the severity of axonal degeneration and the axonal loss. At end stage of disease, microtubules (this study) and axonal diameters (Schmalbruch et al., 1991) are also reduced in ventral roots. We further show that neuron-specific TBCE complementation restores TBCE levels in spinal motor neurons of pmm mice and inhibits microtubule loss, axonal degeneration, and clinical disease in a dose-dependent manner. These data indicate that destabilization of TBCE in motor neurons is responsible for the axonal dying back process in pmm mice.

How can a defective tubulin chaperone cause axonal dying back?

Using cultured motor neurons, we show that the pmm mutation compromises the routing of newly synthesized tubulin from the cell body to the distal axon and impedes the incorporation of tubulin into growing microtubules. In vivo analyses of early symptomatic pmm mice further demonstrate that neuronal βIII-tubulin levels are reduced in distal sciatic nerves but normal in proximal nerves and corresponding lumbar spinal cord segments. Hoffman et al. (1992) reported that tubulins are anterogradely transported and continuously deposited into stationary axonal microtubules, which are stabilized when axons mature (Watson et al., 1990). Zenker and Hohberg (1973) emphasized...
that the proximal motor axon has a much smaller cross-sectional area and contains many fewer microtubules than all of its distal branches taken together. They found that the combined cross sections through all terminal axons of a typical A-motor neuron contain 11 times as many microtubule profiles as the stem axon. These data suggest that defective axonal tubulin routing leads to reduced tubulin supply to distal axons and thereby causes microtubule rarefaction. Motor neurons might be particularly vulnerable to defective tubulin routing because of their extremely long axons and their extended axonal arborizations.

What is the role of the Golgi apparatus in axonal tubulin routing?

Studies in cultured neurons have shown that axonal tubulins are mainly synthesized in the cell soma (Eng et al., 1999) and transported into the distal axon within 2 d after synthesis (Campenot et al., 1996). Metabolic labeling experiments in compartmentalized cultures had provided evidence that this process depends on the Golgi apparatus (Campenot et al., 2003). Using immunohistochemical and biochemical experiments, we clearly identify TBCE as a peripheral membrane-associated protein of cis-Golgi membranes in vivo. In vitro data in cultured NSC34 and primary motor neurons further confirm the accumulation of tagged and endogenous TBCE at this organelle. Functional TBCE depletion assays indicate that TBCE is required for axonal routing of tubulins, and BFA-mediated Golgi disruption experiments indicate that this process depends on an intact Golgi apparatus. TBCE is thus related to a growing number of Golgi proteins that control microtubule formation or dynamics, such as GMAP-210 (Golgi microtubule-associated protein of 210 kDa) (Infante et al., 1999; Rios et al., 2004), AKAP350 (A kinase anchor protein 350) (Larocca et al., 2006), and CLASPs 1/2 (cytoplasmic linker associated proteins 1/2) (Mimori-Kiyosue et al., 2005).

Are defects in tubulin chaperones also implicated in other forms of axonal degeneration?

Human TBCE deletions have been found in children with HRD (hypoparathyroidism, growth retardation, dysmorphism) syndrome (Parvari et al., 2002) that eventually develop motor neuron disease symptoms when becoming elder (Parvari et al., 2007). The TBCE-related tubulin chaperone TBCB has been reported to be
dysregulated in gigaxonin knock-out mice, a model of human giant axonal neuropathy (Wang et al., 2005). Gigaxonin binds TBCB, promotes its poly-ubiquitination and thereby targets it to the proteasome for degradation. The absence of gigaxonin leads to an accumulation of TBCB and a loss of microtubules in neurons (Wang et al., 2005). Interestingly, gigaxonin, like TBCE, localizes at the Golgi apparatus (Cullen et al., 2004). Wobbler mice, a model of progressive motor neuron degeneration, are mutated in VPSS4 (vacuolar protein sorting 54), a protein of the Golgi-associated retrograde complex (Schmitt-John et al., 2005). In these mice, defective axonal transport (Mitsumoto and Gambetti, 1986) and progressive axonal degeneration (Mitsumoto and Bradley, 1982) are associated with downregulation of the tubulin chaperone TBCA and of α3-tubulin (Perrin et al., 2006). The role of tubulin chaperones and their interactors at the Golgi apparatus thus warrants additional investigations.

Therapeutic implications

In the past, pmn mice have been instrumental in developing new axonoptrope strategies. Crossing of pmn mice with WldΔ mice reduced axonal degeneration and extended the lifespan of pmn mice (Ferri et al., 2003). The mechanisms remained enigmatic because WldΔ is a nuclear protein and undetectable in axons. Recently, Simonin et al. (2007) reported an upregulation of kinesin-1 in pmn × WldΔ motor neurons. Together, these data, the known role of kinesins in axonal tubulin transport (Terada et al., 2000; Kimura et al., 2005), and our findings raise the possibility that WldΔ protects pmn motor neurons by recovering their deficit in axonal tubulin supply. Various studies have also shown that gene therapy with neurotrophic factors can provide substantial therapeutic benefit to pmn mice (Sendtner et al., 1992; Sagot et al., 1995a; Haase et al., 1997; Bordet et al., 1999). It has been shown that neurotrophic factors can be internalized into signal-endosomes and retrogradely transported toward the Golgi apparatus (Howe and Mobley, 2005). This suggests that neurotrophic factors exert some of their therapeutic effects by modulating Golgi function and axonal tubulin routing.

References

lation at the motor endplate and lack of axonal sprouting in a spinal 
Coleman M (2005) Axon degeneration mechanisms: commonality amid di-
the end begin? Trends Neurosci 30:159–166.
Cullen VC, Bowles N, Banner S, Anderton BH, Leigh PN, Shaw CE, Miller 
CC (2004) Gigantoxin is associated with the Golgi and dimerises with 
Devon RS, Orban PC, Gerrow K, Barbieri MA, Schwab C, Cao LP, Helm JR, 
Bissada N, Cruz-Aquado R, Davidson TL, Witmer J, Metzler M, Lam CK, 
Tetzlaff W, Simpson EM, McCaffery JM, El-Husseine AE, Leavitt BR, 
trafficking associated with motor behavioral abnormalities. Proc Nat 
Acad Sci USA 103:9595–9600.
membrane-catalysed exchange of guanine nucleotide onto ARF protein. 
Eng H, Lund K, Camenot RB (1999) Synthesis of β-tubulin, actin, and 
other proteins in axons of sympathetic neurons in compartmented cul-
Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, 
in transgenic mice expressing multiple spectral variants of GFP. Neuron 
30:41–51.
Ferri A, Sanes JR, Coleman MP, Cunningham JM, Kato AC (2003) Inhibit-
ing axon degeneration and synapse loss attenuates apoptosis and disease 
Fischer LR, Culver DG, Tennant P, Davis AA, Wang M, Castellano-Sanchez 
Forss-Petter S, Danielson PE, Catsicas S, Battenberg E, Price J, Nerenberg M, 
end begin? Trends Neurosci 30:159–166.
of class II and III beta-tubulin: evidence that the slow component 
represents the movement of only a small fraction of the tubulin in 
Howe CL, Mobley WC (2005) Long-distance retrograde neurotrophic sig-
nal from the motor endplate and lack of axonal sprouting in a spinal 
Klausner RD, Donaldson JG, Lippincott-Schwartz J (1992) Brefeldin A: in-
sights into the control of membrane traffic and organelle structure. J Cell 
LaMonte BH, Wallace KE, Holloway BA, Shelly SS, Ascano J, Tokito M, Van 
Winkle T, Howland DS, Holzbaur EL (2002) Disruption of dynein/dy-
nin inhibitor axonal transport in motor neurons causing late-onset pro-
Langford LA, Schmidt RF (1983) An electron microscopic analysis of the left 
Larocca MC, Jin M, Goldenring JR (2006) AKAP350 modulates mitotuba-
Lippincott-Schwartz J, Donaldson JG, Schweizer A, Berger EG, Hauri HP, 
Yuan LC, Klausner RD (1990) Microtubule-dependent retrograde transport 
of proteins into the ER in the presence of brefeldin A suggests an ER 
Martin N, Jaubert J, Gouzon P, Salido E, Haase G, Szatmank, Guenet JL, 
Martin E, Schmidt JM, Azzouz M, Borg J, Guenet JL, Rios RM (1999) A 
mis sense mutation in Tbec causes progressive motor neuro-
Mimori-Kiyosue Y, Grigoriev I, Lansbergen G, Sasaki H, Matsu C, Severin F, 
CLASPL1 and CLASPL2 bind to EB1 and regulate microtubule plus-end 
Mitsumoto H, Bradley WG (1982) Muscine motor neuron disease (the wob-
bler mouse): degeneration and regeneration of the lower motor neuron. 
Brain 105:811–834.
Mitsumoto H, Gambari P (1986) Impaired slow axonal transport in wob-
Mortimer A, Kjaersgard IJ, Schiødt F, Gomi K, Sehested M, Westergaard J, 
Srinivas R, Hansen PD, Westergaard N, Hedegaard H, Gormsen NW, 
into the ER in the presence of brefeldin A suggests an ER recycling 
pathway. EMBO J 7:2299–2307.
Parvari R, Hershkovitz E, Grossman N, Gorodischer R, Loeyts B, Zecia A, 
Aqel AI, Al Humaidan AK, Al Zanhrani F, Al Swayd A, Al Othman J, Diaz 
causes hyperparathyroidism-retardation-dysmorphosis and autosomal 
Parvari R, Diaz GA, Hershkovitz E (2007) Parathyroid development and the 
role of tubulin chaperone E. Horm Res 67:12–21.
differ in several mouse models with motoneurone disease: analysis of pure 
Pels P, Jonnacuty K, LaMonte BH, Holzbaur EL, Tokito M, Mann E, Fleoter 
MK, Bidus K, Drayna D, Oh SJ, Brown Jr RH, Ludlow CL, Fiskech KB 
Pun S, Santos AF, Saxena S, Xu L, Caroni P (2006) Selective vulnerability and 
pruning of phasic motoneuron axons in motoneuron disease allevi-
ated by CNTF. Nat Neurosci 9:408–419.
Raoal C, Estevez AG, Nitshume H, Cleveland DW, de Lapeyriere O, Hender-
Spatiotemporal localization of injury potentials in DRG neurons during 
Richmond FJ, Gladry R, Creasy JL, Kitamura S, Smits E, Thomson DB 
(1994) Efficacy of seven retrograde tracers, compared in multiple-
and motor performance studies in the pmn mouse model of neurodegen-


