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

Michael K. E. Schaefer,1,2 Henning Schmalbruch,3 Emmanuelle Buhler,1,2 Catherine Lopez,1,2 Natalia Martin,4 Jean-Louis Guénet,4 and Georg Haase1,2

1Inserm, Unité 29, Equipe Avenir, 13273 Marseille, France, 2Aix Marseille Université, Institut de Neurobiologie de la Méditerranée, 13284 Marseille, France, 3Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark, and 4Institut Pasteur, 75015 Paris, France

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 Boillée 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) protein Bcl-2 (Sagot et al., 1995b). We and others showed that expression of the axonoprotective WldS protein (Ferri et al., 2003) but not by overexpression of the antiapoptotic gene therapy (Sendtner et al., 1992; Sagot et al., 1995a; Haase et al., 1997) or expression of the axonoprotective Wld5 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) or expression of the axonoprotective Wld5 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 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-
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, antisera were preabsorbed with antigenic peptides or recombinant green fluorescent protein (GFP)–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 light chain (NF78, 1:2000; Sigma), G-50 (SH-B1, 1:1000; Sigma), and GM130, p115, Vt1a, and Vt1b (1:250; Becton Dickinson, Walton, KY). Fluorochrome- or biotin 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); Hiberne E (BrainBits, Springfield, IL); DNase I, polymyxin, lamin, glial cell line-derived neurotrophic factor (GDNF), taxol, and nocodazole (Sigma), CNTF and BDNF (R & D Systems, Minneapolis, MN), Vectashield–4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA); complete protease inhibitors and Dextran Blue (Roche); Hynorm (Jansen, Beerse, Belgium); bisbenzimide and brefeldin A (BFA) (Fluka, Saint Quentin Fallavier, France); and Fluoromount (Southern Biotechnology, Birmingham, AL).

Mouse lines and genotyping. Mice were anesthetized with Hypnorm/Dormicum (fentanyl at 0.5 mg/ml, fluanisone at 2.5 mg/ml, and midazolam at 1.25 mg/ml; 6 ml/kg body weight, s.c.). Under a stereomicroscope, a ventral incision was made on the right side of the neck. The cervical plexus was isolated, and the phrenic nerve was identified. The phrenic nerve was cut without opening the pleural cavity, and a piece of dextran tracers (Spongostan; Ferrosan, Soeborg, Denmark) 1 mm thick was soaked in 10% Fluoro-royal blue (Chemicon) in Ringer’s solution with 2% DMSO, and applied to the proximal stump. Labeling by dextran tracers is restricted to cut axons (Richmond et al., 1994), and care was taken not to injure other branches of the cervical plexus or muscles innervated by it. The wound was closed in two layers with 9-0 and 6-0 sutures.

Fluorescent, light, and electron microscopy. Confocal microscopy was performed with upright or inverted LSM 510 microscopes (Zeiss, Oberkochen, Germany) using 63×, 40×, or 10× objectives. For light and electron microscopy, glutaraldehyde-fixed and roots were postfixed with osmic tetroxide and embedded in epoxy resin. For light microscopy, cross sections 3 μm thick were stained with 1% uranyl acetate and lead citrate. To facilitate identifying microtubules, the sections were tilted in the electron microscope (CM100; Philips, Eindhoven, The Netherlands) by means of a goniometer to obtain exact cross sections. The magnification of the microscope was calibrated against a replica of a diffraction grating (2160 lines/mm). With the aid of a digitizer tablet, all apparently intact myelinated axons were counted on light micrographs of the phrenic nerves, axonal areas were measured, and microtubules were counted on electron micrographs printed to ~60,000 times. The investigator (H.S.) was blinded with respect to the genotype of the animals.

Expression plasmids and small interference RNAs. Expression vectors for GFP–TBCE, hemagglutinin–TBCE, and FLAG–TBCE were generated by subcloning mouse wild-type TBCD cDNA using pCAGGS–GFP (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 (Raoul et al., 2002; Jacquier et al., 2006). For immunocytochemistry, cells were fixed by adding an equal volume of 8% Healthcare, Little Chalfont, UK). Western blots were scanned and analyzed by densitometry (NIH Image). For immunohistochemistry, deeply anesthetized mice were perfused with 4% paraformaldehyde, and spinal cords, sciatic nerves, and phrenic nerves were dissected, postfixed overnight, and cryoprotected in 30% sucrose for 48 h. After tissue embedding, 14 μm transverse or 20 μm frontal sections of the spinal cord and 10 μm cross-sections of nerves were cut on a cryostat and collected on glass slides. Sections were blocked, incubated overnight with primary antibodies, washed, incubated with fluoroconjugated secondary antibodies, reacted with 0.1 μg/ml bisbenzimide, and mounted in Fluoromount.

Subcellular fractionation. Spinal cords or NSC34 cells were homogenized in buffer (50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 5% sucrose, 0.1 mM DTT, and protease inhibitors) and centrifuged at 900 × g for 10 min at 4°C to remove nuclei and cell debris. The postnuclear supernatant was centrifuged for 1.5 h at 4°C and 60,000 rpm in a TLA-100 rotor (Beckman Coulter, Fullerton, CA) to obtain cytosol and crude membrane fractions. The membrane pellet was dissolved in homogenization buffer containing 0.5% Triton X-100 and incubated for 1 h on ice. Equal volumes of subcellular fractions were subjected to SDS-PAGE and processed as outlined above.

Retrograde labeling of motor neurons. Mice were anesthetized with Hypnorm/Dormicum (fentanyl at 0.5 mg/ml, fluanisone at 2.5 mg/ml, and midazolam at 1.25 mg/ml; 6 ml/kg body weight, s.c.). Under a stereomicroscope, a ventral incision was made on the right side of the neck, the cervical plexus was isolated, and the phrenic nerve was identified. The phrenic nerve was cut without opening the pleural cavity, and a piece of dextran tracers (Spongostan; Ferrosan, Herreschof, Denmark) 1 mm thick was soaked in 10% Fluoro-royal blue (Chemicon) in Ringer’s solution with 2% DMSO, and applied to the proximal stump. Labeling by dextran tracers is restricted to cut axons (Richmond et al., 1994), and care was taken not to injure other branches of the cervical plexus or muscles innervated by it. The wound was closed in two layers with 9-0 and 6-0 sutures.

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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 taxol, fixed, blocked, and immunostained for βIII-tubulin and γ-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 βIII-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. 1J). These findings were further supported by Western blot analysis of neuronal βIII-tubulin levels in

![Image](52x408 to 382x729)

Figure 1. Axonal microtubules and tubulin levels in pmn mice. A, B, Electron micrographs showing entire cross sections through distal phrenic nerves from 15-d-old early symptomatic pmn mice and wild-type (wt) littermate mice. C, D, High-power electron micrographs from distal phrenic nerve axons at day 15. Microtubules (arrows) in pmn phrenic nerve axons are rarefied, whereas intermediate filaments are preserved. E, F, High-power electron micrographs from C4 ventral roots at day 15. G, H, High-power electron micrographs from C4 ventral roots at day 28. Scale bars: A, B, 1 μm; C–H, 0.5 μm. I, Diagram showing that axonal microtubule densities in pmn phrenic nerves mice are reduced by 61 and 56%, respectively, at the distal and intermediate level compared with those in wild-type nerves (***p < 0.0001, Student’s t test). No significant microtubule loss is observed in cervical and lumbar ventral root motor axons of 15-d-old pmn mice. A total of 378 individual axon cross sections were analyzed. J, Diagram showing reduction of microtubule densities in C4 ventral roots of 28-d-old pmn mice (**p < 0.0001, Student’s t test). K, Western blot analysis showing protein levels of βIII-tubulin and β-actin in lysates from lumbar spinal cord and proximal and distal sciatic nerves at day 15. Note the reduction of βIII-tubulin in distal sciatic nerves of pmn mice.
distal and proximal sciatic nerves and lumbar spinal cord of 15-d-old mice. In \textit{pmn} mice, the \( \beta_{38} \)-tubulin levels were significantly reduced in distal sciatic nerves but normal in proximal nerves and spinal cord (Fig. 1K).

To analyze the temporal progression of microtubule loss in \textit{pmn} mice, we studied C4 cervical ventral roots of 28-d-old \textit{pmn} mice (Fig. 1G, H). At this advanced disease stage, microtubule densities were reduced to 40 \( \pm \) 13\% of normal (Fig. 1J). Axonal microtubule loss in \textit{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 \textit{pmn} and wild-type mice but also two lines of transgenic \textit{pmn} mice complemented with wild-type TBCE (Martin et al., 2002), termed TBCE\textsubscript{PA} \textit{pmn} and TBCE\textsubscript{PC} \textit{pmn}. In TBCE\textsubscript{PA} \textit{pmn} mice, disease onset is retarded by 2–3 weeks and survival is prolonged; in TBCE\textsubscript{PC} \textit{pmn} mice, disease is completely prevented. Using electron microscopy, we compared axonal pathology and microtubule density in distal phrenic nerves of \textit{pmn}, wild-type and transgenic TBCE \textit{pmn} mice (Fig. 2A–D). In phrenic nerves of 15-d-old \textit{pmn} mice, we observed numerous axons with irregular contours (Fig. 2A) and a loss of 25\% of myelinated axons (195 \( \pm \) 25, mean \( \pm \) SD) compared with wild-type littermate mice (260 \( \pm \) 20, mean \( \pm \) SD) (Fig. 2C). In TBCE\textsubscript{PA} \textit{pmn} mice, axon contour irregularities were present but less prominent than in \textit{pmn} mice (Fig. 2A), and microtubule loss was observed in only one of four mice (Fig. 2B, D). In TBCE\textsubscript{PC} \textit{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 \((r = 0.939; \alpha = 0.01; p < 0.001)\) (Fig. 2D). Microtubule densities below 10/\( \mu \)m\(^2\), corresponding to a microtubule loss of \( \sim \)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 expressed 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 \textit{Thy1–YFP line 16 mice} (Feng et al., 2000). In phrenic and sciatic 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 \textit{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. 4A) and end stage (Fig. 4B). 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. 4C–E) and P35 (data not shown). Similar observations were made in Schwann cells in the sciatic nerve (supplemental Fig. 1B–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 TBCEPA pmn mice and supranormal in TBCEPC pmn mice compared with pmn and wild-type mice (Fig. 4A, B). Immunostainings of cervical and lumbar spinal cord motor neurons showed that TBCE expression at the Golgi apparatus was partially restored in TBCEPA pmn mice and normal in TBCEPC pmn mice (Fig. 4D, 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 αβ-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. 5A). TBCE depletion had no effect on total levels of α- or β1-tubulin (Fig. 5A). TBCE-depleted cells, how-
ever, contained more soluble and less precipitable, microtubule-incorporated, α-tubulin than control cells (Fig. 5B,C), suggesting that some α-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 pmm 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–α-tubulin expression vector along with a DsRed vector and siRNAs. In si-luciferase-transduced control motor neurons, newly synthesized GFP–α-tubulin and DsRed were evenly distributed in cell bodies and axons (Fig. 5D). In TBCE-depleted motor neurons, however, the amount of GFP–α-tubulin was reduced in distal axons (Fig. 5D). We therefore compared the fluorescence ratios of GFP–α-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–α-tubulin/βIII-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 pmm motor neurons

To test the consequences of the pmm mutation on microtubule polymerization, we examined pmm and wild-type motor neurons in culture. Motor neurons were purified from individual embryos, seeded, and incubated for 6 h with 10 μM 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 pmm motor neurons, microtubule polymerization was initially normal but severely affected at 30 min (Fig. 6B). At this time, the immunoreactivity of microtubule-incorporated βIII-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 pmm mutation in TBCE impairs microtubule polymerization.

We next analyzed axonal tubulin routing in pmm motor neurons. Motor neurons were electroporated with GFP–α-tubulin and DsRed plasmids and cultured for 2 DIV. In cell bodies, the mean GFP–α-tubulin/βIII-tubulin fluorescence ratios were not significantly different between pmm and wild-type motor neurons (Fig. 6F). In distal axons of pmm motor neurons, however, the mean GFP–α-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 pmm motor neurons indicates that the pmm 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
total cellular amounts of GM130, α-tubulin, or β-tubulin (Fig. 7B) under these experimental conditions.

To test the effects of Golgi disruption on axonal tubulin routing, we electroporated motor neurons with GFP–α-tubulin or GFP plasmids, treated them with BFA or mock (Fig. 7C), and counterstained them for βIII-tubulin. BFA had no obvious effect on the levels or intracellular distribution of GFP (data not shown) or endogenous βIII-tubulin (Fig. 7C). BFA, however, modified the intracellular distribution of GFP–α-tubulin. In BFA-treated motor neurons, GFP–α-tubulin was predominantly found in cell bodies and strikingly diminished in axons (Fig. 7C,D). Quantitative confocal analyses in distal axons of BFA-treated motor neurons confirmed that the ratio of GFP–α-tubulin fluorescence to βIII-tubulin fluorescence was reduced to 33.3 ± 4.8% (mean ± SD) of the ratio in mock-treated motoneurons (p < 0.05 as analyzed per Student’s t test; n = 30 cells per condition). BFA-induced Golgi disruption and TBC4 depletion, albeit differing in their molecular mode of action, thus have very similar effects on axonal tubulin routing. These data indicate that axonal tubulin routing in motor neurons is controlled at the Golgi apparatus and implicate TBC4 in this process.

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: Vincristine, 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 topic application of Vincristine 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 dynemin/dynactin for example cause progressive motor neuron degeneration in mice (LaMonte et al., 2002; Haf ezparast et al., 2003) and humans (Puls et al., 2003).

Retrograde microtubule loss in pmn mice

We here investigated how a missense mutation in TBC4 causes axonal dying back in pmn mice. We show that in normal mice, TBC4 is expressed in motor neuron cell bodies in which it accumulates at the cis-Golgi apparatus. In early symptomatic pmn mice, TBC4 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 TBC4 complementation restores TBC4 levels in spinal motor neurons of pmn mice and inhibits microtubule loss, axonal degeneration, and clinical disease in a dose-dependent manner. These data indicate that destabilization of TBC4 in motor neurons is responsible for the axonal dying back process in pmn mice.

How can a defective tubulin chaperone cause axonal dying back?

Using cultured pmn motor neurons, we show that the pmn 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 pmn 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 Hof berg (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 \(A_\alpha\)-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 axonoprotective strategies. Crossing of pmn mice with Wld<sup>S</sup> mice reduced axonal degeneration and extended the lifespan of pmn mice (Ferri et al., 2003). The mechanisms remained enigmatic because Wld<sup>S</sup> is a nuclear protein and undetectable in axons. Recently, Simonin et al. (2007) reported an upregulation of kinesin-1 in pmn × Wld<sup>S</sup> 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 Wld<sup>S</sup> 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 signaling 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**


