Axonal maintenance, plasticity, and regeneration are influenced by signals from neighboring cells, in particular Schwann cells of the peripheral nervous system. Schwann cells produce neurotrophic factors, but the mechanisms by which ciliary neurotrophic factor (CNTF) and other neurotrophic molecules modulate the axonal cytoskeleton are not well understood. In this paper, we show that activated signal transducer and activator of transcription-3 (STAT3), an intracellular mediator of the effects of CNTF and other neurotrophic cytokines, acts locally in axons of motoneurons to modify the tubulin cytoskeleton. Specifically, we show that activated STAT3 interacted with stathmin and inhibited its microtubule-destabilizing activity. Thus, ectopic CNTF-mediated activation of STAT3 restored axon elongation and maintenance in motoneurons from progressive motor neuronopathy mutant mice, a mouse model of motoneuron disease. This mechanism could also be relevant for other neurodegenerative diseases and provide a target for new therapies for axonal degeneration.

Introduction

In most neurodegenerative diseases, periods of clinically silent alterations precede the first symptoms. Synaptic dysfunction and loss are thought to occur early but are compensated by sprouting of neighboring axon terminals (Cafferty et al., 2008; Giger et al., 2010). For example, in Smn+/- mice, a model of mild forms of spinal muscular atrophy, >50% of the motoneuron cell bodies are lost, and intensive ciliary neurotrophic factor (CNTF)–dependent sprouting occurs before disease becomes clinically apparent (Simon et al., 2010). Axonal degeneration often starts with alterations in distal axons and presynaptic terminals (Pun et al., 2006), leading to morphological degeneration that normally sets the stage for irreversible alterations that finally lead to neuronal cell death. Axonal degeneration marks the transition from early disease stages when regeneration in principle is possible (Cafferty et al., 2008) and late stages when the pathological alterations are so severe that challenges for effective treatment become insurmountable.

In animal models of motoneuron disease, neuromuscular endplates are lost early (Pun et al., 2006), and pathological alterations in axons normally precede the cell death of spinal motoneurons (Sendtner et al., 1992). Because axons of motoneurons are easily accessible, motoneuron disease models appear as an ideal tool for studying molecular mechanisms of axonal degeneration and disease progression in neurodegeneration. These mechanisms appear important not only for motoneuron disease but for a much broader spectrum of neurodegenerative disorders in which axons degenerate and could guide the development of therapies for such diseases.

A large body of evidence, mainly coming from human genetic experiments and from the analysis of mouse models, points to axonal trafficking and vesicle sorting/transport as critical targets of disease mechanisms in motoneuron diseases (Hafezparast et al., 2003). First, mutations in genes for components of kinesin complexes that are necessary for anterograde axonal transport, i.e., KIF1B-beta and KIF5A, are associated with various forms of motoneuron disease, such as hereditary spastic paraplegy (SPG10) and Charcot-Marie-Tooth type 2A.
Moreover, the underlying gene defect in the wobbler mouse, a classical mouse model of motoneuron disease, inactivates the VPS54 protein, which is important for cellular vesicle sorting (Schmitt-John et al., 2005). Fourth, modulation of neurofilaments (NFLs) and the resulting disturbed stoichiometry of filamentous structures in the axon lead to motoneuron disease in transgenic mouse models (Collard et al., 1995). Moreover, the underlying gene defect in the progressive motor neuronopathy (pmn) mouse, another mouse model of amyotrophic lateral sclerosis, affects the activity of tubulin-specific chaperone E and, by this means, the assembly of microtubules (MTs; Bommel et al., 2002; Martin et al., 2002), which seems to play a key role for axonal trafficking and axonal stability. pmn mutant mice suffer from a severe form of motoneuron disease. First symptoms of weakness appear in the third postnatal week. The mice then die within the following 3 wk. The disease is caused by a point mutation (t1682g) in the Tbce (tubulin-specific chaperone E, cofactor E) gene. This mutation leads to an amino acid exchange of tryptophan to glycine (W524G) at the most C-terminal position of the protein. The mutation destabilizes the TBCE protein (Bommel et al., 2002; Martin et al., 2002), but it does not completely abolish its enzymatic activity because MTs are present in most types of cells, and mitosis that depends on intact MTs for the spindle apparatus is not affected in the developing pmn mutant mice. The same mutation or mutations in the last coding exon of Tbce have not been found in >700 patients with sporadic and familial forms of motoneuron disease (unpublished data). Mutations in other regions of the Tbce gene that abolish the enzymatic activity of the corresponding protein (c.155-166del12; p.del 52–55) have been associated with the hypoparathyroidism–retardation–dysmorphism syndrome (Parvari et al., 2002), a rare autosomal recessive disorder characterized by short stature as a result of growth hormone insufficiency, mental retardation, facial dysplasia, and endocrinological defects, such as hypokutisolemia. In contrast, the W524G mutation found in pmn mutant mice gives rise to a disease in which destabilized Tbce protein results in unstable MTs that causes a neurodegenerative disease that predominantly affects motoneurons (Bommel et al., 2002; Martin et al., 2002).

Cell death of motoneuron cell bodies appears as a consequence of axonal degeneration (Sendtner et al., 1992; Bommel et al., 2002). When bcl-2 is overexpressed in motoneurons of pmn mutant mice, cell death of motoneuron cell bodies is prevented, but this has no influence on disease onset or progression because axon degeneration is not positively affected by the bcl-2 transgene (Sagot et al., 1995). Similarly, treatment with the neurotrophic factor glial-derived neurotrophic factor (GDNF) only prevents loss of motoneuron cell bodies but does not influence axonal degeneration (Sagot et al., 1996) or retrograde axonal transport (Sagot et al., 1998), a functional consequence of the axonal degeneration. In contrast, treatment with the neurotrophic factor CNTF significantly delays disease onset and prolongs survival of pmn mice (Sendtner et al., 1992, 1997). This difference appears surprising because CNTF and GDNF are potent survival factors for motoneurons, both in vitro and in vivo. This observation therefore raises the question on the molecular basis how CNTF, but not GDNF, rescues axons.

Here, we show that CNTF, in contrast to GDNF or brain-derived neurotrophic factor (BDNF), rescues axonal degeneration via a pathway involving signal transducer and activator of transcription-3 (STAT3) and stathmin. Most of the activated STAT3 protein is not transported to the nucleus to activate transcription but interacts locally in axons with stathmin, a protein that destabilizes MTs. This interaction plays a major role in CNTF signaling for MT dynamics in axons. Thus, the STAT3–stathmin pathway could be a potent target for therapy development in motoneuron disease.

**Results**

**CNTF, but not BDNF or GDNF, rescues axonal pathology in pmn mutant motoneurons**

CNTF influences synaptic and axonal degeneration in a variety of mouse models (Sendtner et al., 1992; Mitsumoto et al., 1994; Pun et al., 2006). In superoxide dismutase G93A mice, it prevents pruning and loss of synaptic vesicles at neuromuscular endplates (Pun et al., 2006), and in pmn mutant mice, the severe paralysis and respiratory failure can be significantly delayed by systemic CNTF treatment (Sendtner et al., 1992). Interestingly, GDNF, another potent survival factor for motoneurons (Henderson et al., 1994), cannot prevent synaptic pruning and axon degeneration in the superoxide dismutase G93A and the pmn mouse models (Sagot et al., 1996). To investigate the underlying mechanism for this difference, we compared the effects of BDNF, GDNF, and CNTF in cultured embryonic motoneurons from control and pmn mutant mice. Although these three neurotrophic factors were equally potent in supporting motoneuron survival (Fig. S1), both in wild-type and pmn mutant motoneurons, they differed significantly with respect to their effects on axon growth (Fig. 1 A and B). After 7 d in culture, each of these factors promoted axon elongation in wild-type motoneurons to ~1,000–1,300 μm. However, axons of pmn mutant motoneurons were significantly shorter with BDNF or GDNF but not with CNTF (Fig. 1 B). Furthermore, CNTF, but not BDNF or GDNF, reduced the number of axonal swellings that are characteristic for pmn mutant motoneurons (Fig. 1 C; Bommel et al., 2002). Ultrastructural analysis also revealed reduced MT density in axons of pmn mutant mice (Fig. 1 D), confirming a previous study (Schaefer et al., 2007). However, the reduction of MT density was less than expected in distal regions and reached statistical significance only in the proximal axons (Fig. S2). The axonal swellings in axons in pmn
The effects of CNTF on enhancing rate and speed of axonal transport were specific for pmn mutant motoneurons. No differences in mitochondrial transport were observed with BDNF and CNTF in wild-type motoneurons. STAT3 is necessary for CNTF effects on axons in pmn mutant motoneurons. BDNF and GDNF mediate their effects on motoneuron survival and axon growth through the transmembrane tyrosine kinase receptors TrkB and c-ret, respectively. In contrast, CNTF modulates these functions through a cytokine receptor involving the STAT3 signaling pathway.

**Figure 1.** CNTF rescues axon elongation and mitochondrial transport in pmn mutant motoneurons in vitro. (A) Representative images of pmn mutant and wild-type motoneurons cultured for 7 DIV in the presence of BDNF or CNTF and stained against MAP2 (green) and against tau (red). Bars, 100 µm. (B and C) Differential effect of CNTF on axon length and reduction in axonal swellings. n = 3 independent experiments. At least 50 cells were measured per condition and experiment. (D) Electron micrographs of axonal segments (left) and swellings (right) of wild-type and pmn mutant motoneurons cultured in presence of BDNF for 7 DIV, showing swellings filled with organelles in pmn mutant motoneurons. Bars: (left) 500 nm; (right) 1,000 nm. (E and F) Representative kymographs of axonal mitochondria labeled with Rhodamine 123 in wild-type and pmn mutant motoneurons cultured for 5 DIV in the presence of BDNF or BDNF and CNTF. Bars, 25 µm. (G and H) CNTF normalizes axonal transport of mitochondria in pmn mutant (54 cells for 5 ng/ml BDNF and 19 cells for 10 ng/ml BDNF + CNTF) motoneurons to levels in wild-type motoneurons (28 cells for BDNF and 12 cells for BDNF + CNTF). n = 6 independent experiments. Statistical analysis: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ANOVA with Bonferroni posthoc test. wt, wild type. Data shown represent means ± SEM.
In the presence of CNTF, axon length of STAT3fl/KO;NFL-Cretg;pmn−/− motoneurons was reduced by >50% when compared with wild-type and pmn−/− mutant motoneurons that were cultured with CNTF. One functional copy of STAT3 was sufficient to rescue axon length (STAT3 fl/wt;NFL-Cre tg;pmn−/−) in the presence of CNTF.

We then investigated how much of the activated STAT3 moves to the nucleus when CNTF was added as a pulse for 5, 15, and 30 min, or continuously for 5 d, by fractionating motoneuron cell extracts. Surprisingly, STAT3 phosphorylated at Y705 remained almost quantitatively in the cytoplasm, and very little STAT3 could be detected in the nucleus at any time after CNTF treatment (Fig. 2, A and B) in motoneurons, indicating that the effects of CNTF-activated STAT3 on axons of pmn−/− mutant motoneurons could involve a local effect in the axonal cytoplasm rather than a mechanism involving nuclear transcription.

glycosylphosphatidylinositol-linked CNTFR-α and LIFR-β and gp130 as transmembrane receptor subunits (Davis and Yancopoulos, 1993). Binding of CNTF to this receptor complex results in activation of cytosolic tyrosine kinases of the Janus kinase family and activation of STAT3 through phosphorylation at tyrosine 705 (Y705). CNTF addition as a pulse to cultured motoneurons results in rapid phosphorylation of STAT3 at Y705, and also continuous exposure to CNTF for 5 d in vitro (DIV) results in STAT3 activation (Fig. 2, A and B). Therefore, we investigated whether STAT3 is responsible for CNTF-mediated effects on pmn−/− mutant motoneurons. We generated STAT3 KO/wt;NFL-Cretg/tg;pmn+/− and STAT3fl/fl;pmn+/− mice and crossbred them to delete STAT3 in pmn−/− mutant motoneurons, using the same breeding scheme as described previously (Schweizer et al., 2002). CNTF-mediated rescue of axonal elongation was abolished when STAT3 was depleted from pmn−/− motoneurons (Fig. 2 C).

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To obtain additional evidence that STAT3 acts locally after activation in axons, we developed lentiviral vectors for transduction of EYFP-labeled STAT3 into cultured motoneurons. Motoneurons were transduced with the STAT3-EYFP construct, an area of 100 μm length within the axon was bleached, and the rate of anterograde and retrograde movement of labeled STAT3 into the bleached area was measured after CNTF addition (Fig. 2, D and E). Lentivirus expressing EGF was that was not coupled to any other protein served as a control (Fig. 2 F). FRAP was used to determine the mobility and direction of movement of EGF and STAT3-EYFP fusion protein into 20-μm segments proximal and distal within the bleached axon region. There was no significant increase of movement of STAT3-EYFP from the distal part of the axon into the bleached area, indicating that activation of STAT3 did not result in quantitative retrograde movement of this signaling molecule toward the cell body and the nucleus. Mobility of EGF used as a control (Fig. 2 F) was generally higher than that of the STAT3-EYFP fusion protein, most likely because of the higher molecular mass of the fusion protein. Interestingly, CNTF enhanced mobility of EGF in the axon (Fig. 2 F), both in the proximal and distal segment. Treatment with nocodazole reduced enhanced mobility, both of EGF and also of mitochondria (Fig. 2, G and H), indicating that the effect of CNTF depends on intact MTs.

**STAT3-dependent rescue of axon growth in pmn mutant motoneurons is transcription independent**

Because most of the activated STAT3 is not retrogradely transported after CNTF treatment, we analyzed whether CNTF-mediated transcriptional activity is required to rescue axon growth in pmn mutant motoneurons. Therefore, we blocked transcription in pmn mutant motoneurons using actinomycin D at a concentration of 5 nM to test whether CNTF could still rescue axonal elongation under such conditions. Cultured wild-type and pmn mutant motoneurons were treated with actinomycin D at 4 DIV, and axon elongation at 5 DIV was measured (Fig. 3 A). Treatment with actinomycin D ≤10 nM between day 3 and 5 in culture did not affect the survival of motoneurons (Fig. S4). Wild-type motoneurons showed reduced axon elongation with actinomycin D. In pmn mutant motoneurons, the CNTF-mediated rescue of axon elongation was not abolished by actinomycin D, providing further evidence that CNTF signaling for effects on axon pathology in pmn mutant motoneurons does not rely on the transcriptional effects of STAT3.

We then generated a lentiviral construct for mutant STAT3EE434–435AA-EYFP by site-directed mutagenesis. This mutation abolishes the DNA binding activity but can be activated and phosphorylated at Y705 (Horvath et al., 1995). STAT3EE434–435AA-EYFP, which cannot be phosphorylated by CNTF stimulation, was used as a control. Lentiviral transduction of these mutants in primary motoneurons showed that STAT3EE434–435AA-EYFP completely abolishes phosphorylation at Y705. Both STAT3EE434–435AA-EYFP and STAT3EE434–435AA-EYFP can be activated by CNTF and phosphorylated at Y705 (Fig. 3 B). To test whether STAT3EE434–435AA-EYFP abolishes STAT3-dependent transcription, we exploited leukemia inhibitory factor (LIF)–mediated GFAP expression in embryonic day 12 (E12) mouse forebrain neural stem cells. Transcriptional activity of STAT3 is necessary for generating GFAP-positive astrocyte-like cells from these cultures (Rajan and McKay, 1998). As expected, STAT3EE434–435AA-EYFP repressed GFAP expression in cultured neural stem cells (Fig. 3 C). As a control, galiellalactone, a transcriptional inhibitor of STAT3, was used at 10 μM, and this treatment also blocked GFAP induction in these cells. To investigate whether STAT3 transcriptional activity is required for axon extension, we analyzed STAT3-deficient pmn mutant motoneurons (STAT3<sup>−/−</sup>–NFL-Cre<sup>+</sup>–pmn<sup>−/−</sup>) transduced with STAT3EE434–435AA-EYFP, STAT3<sup>−/−</sup>–EYFP, and STAT3<sup>−/−</sup>–EYFP lentiviruses. STAT3-deficient pmn mutant motoneurons (STAT3<sup>−/−</sup>–NFL-Cre<sup>+</sup>–pmn<sup>−/−</sup>) cultured with BDNF alone showed shorter axons when compared with wild-type littermates. Overexpression of STAT3<sup>−/−</sup>–EYFP (control) and STAT3EE434–435AA-EYFP completely rescued CNTF-dependent axon growth in STAT3<sup>−/−</sup>–NFL-Cre<sup>+</sup>–pmn<sup>−/−</sup>–mutant motoneurons, showing that transcriptional activity is not required for this effect. Overexpression of STAT3<sup>−/−</sup>–EYFP was not capable of rescuing axon outgrowth, thus providing further evidence that STAT3 activation is necessary, but not its transcriptional activity, for CNTF-mediated rescue of axon growth in pmn mutant motoneurons (Fig. 3, D and E).

**STAT3-stathmin interaction mediates axonal CNTF effects in pmn mutant motoneurons**

Stathmin is a MT-destabilizing protein that binds to free α/β-tubulin heterodimers and thereby reduces the pool of available tubulin subunits for MT elongation (Amayed et al., 2002). STAT3 has previously been shown in nonneuronal cells to interact with the C terminus of stathmin (Verma et al., 2009), thereby antagonizing its MT-destabilizing activity (Ng et al., 2006). This interaction is enhanced upon STAT3 phosphorylation at Y705 (Verma et al., 2009). Therefore, we investigated whether stathmin is involved in CNTF-STAT3–mediated rescue of axonal pathology in cultured pmn mutant motoneurons. We first investigated the localization of stathmin and STAT3 in cultured motoneurons and found that these two proteins are colocalized in axons (Fig. 4 A), in cell bodies, and dendrites (Fig. S5 A). To test whether CNTF-dependent activation of STAT3 results in enhanced binding to stathmin, we cultured motoneurons for 4 DIV in the presence of 5 ng/ml BDNF, deprived the cultures overnight of serum, and stimulated them the next day with 10 ng/ml CNTF for 30 min. We then immunoprecipitated stathmin and tested the levels of coprecipitated STAT3. STAT3 interaction with stathmin increased more than twofold in CNTF-treated cultures when compared with controls treated with BDNF only (Fig. 4, B and C). We also tested whether stathmin interaction with tyrosinated α-tubulin is altered by CNTF application. This interaction was reduced after CNTF treatment (Fig. 4, B and C), indicating that CNTF-dependent interaction of STAT3 with stathmin leads to a release of stathmin-bound α/β-tubulin heterodimers.

We then determined whether STAT3 phosphorylation at Y705 is required for interaction with stathmin. Stathmin was
immunoprecipitated from motoneurons overexpressing STAT3wt-EYFP and STAT3Y705F-EYFP and then tested for STAT3 interaction. Stathmin interaction with STAT3Y705F-EYFP was reduced when compared with STAT3wt-EYFP, indicating that phosphorylation of STAT3 at Y705 by CNTF is required for binding of stathmin to STAT3 (Fig. 4D).
The release of α/β-tubulin heterodimers from inactivated stathmin could increase the availability of α/β-tubulin heterodimers for polymerization and thus lead to enhanced axon elongation in CNTF-treated pmm mutant motoneurons. To test this hypothesis, we generated a short hairpin RNA (shRNA) lentivirus for knockdown of stathmin. Using this lentivirus, stathmin levels decreased to <20% of control or control mismatch virus-treated cultures (Fig. 4 E). As expected, axon growth in pmm mutant motoneurons recovered to wild-type levels after stathmin knockdown (Figs. 4, F and G; and S5 B). CNTF addition did not lead to any further increase in axon growth, indicating that stathmin inhibition is the major pathway in which CNTF rescues axon growth in pmm mutant motoneurons. Interestingly, wild-type motoneurons did not show any enhanced axon elongation when treated with the stathmin shRNA virus (Fig. 4, F and H), and this was not caused by altered expression of stathmin.
in wild-type versus pmn mutant motoneurons (Fig. 4 H). This observation indicates that local effects of STAT3 in the axon, involving stathmin, mediate the rescue effects of CNTF observed in pmn mutant motoneurons.

**CNTF enhances MT stability in cultured motoneurons**

Based on the finding that the interaction of stathmin with tyrosinated tubulin was reduced, we then tested MT dynamics in wild-type and pmn mutant motoneurons. Tyrosination of MTs has been shown as a characteristic feature of highly dynamic MTs, i.e., the MTs close to a moving growth cone (Witte et al., 2008). In contrast, acetylation occurs predominately in stable, long-living MTs. Confirming a previous study with other neuronal cell types (Witte et al., 2008), we found acetylated tubulin in all parts of the axon but relatively excluded from axonal growth cones and dendrites (Fig. 5 A), where most MTs are thought to be more stable than in distal parts. Tyrosinated tubulin was in all regions of the neuron, including regions close to the axonal growth cone and in dendrites (Fig. 5 A). Surprisingly, levels of tyrosinated tubulin were significantly increased in axons of pmn mutant motoneurons in comparison to wild-type motoneurons when cultured with BDNF (Fig. 5 B), indicating that there are more highly dynamic MTs in the pmn mutant motoneurons under conditions when axons are shorter and axonal transport is reduced. Addition of CNTF and stathmin knockdown (Fig. 5 B, right bar) significantly reduced the levels of tyrosinated tubulin (Fig. 5 B), indicating that CNTF influences the dynamics of MTs and stabilizes them, presumably by deactivating the MT-destabilizing activity of stathmin. The levels of acetylated stable MTs were not different in pmn and wild-type motoneurons and not affected by CNTF or by stathmin knockdown (Fig. 5 C). We then tested whether stabilization of MTs is sufficient to restore axon elongation in pmn motoneurons. For this experiment, pmn and wild-type motoneurons were cultured in the presence of 10 nM taxol for 5 DIV, an MT-stabilizing drug reported previously to promote axon elongation via stabilization of MTs at these concentrations (Witte et al., 2008). Taxol treatment promoted increased axon growth (Fig. 5, D and E) comparable with the axon length observed in pmn motoneurons that were cultured with CNTF.

**CNTF enhances MT polymerization in cultured motoneurons**

Previous investigations with isolated pmn mutant motoneurons showed that MT polymerization is impaired because of the mutation in the TbcE gene (Schaefer et al., 2007). To test whether CNTF could influence MT dynamics and polymerization, we assessed MT reassembly after nocodazole treatment (Schaefer et al., 2007). Wild-type and pmn mutant motoneurons were plated on coverslips, and 10 µM nocodazole was added for 6 h to completely depolymerize the MT network (Fig. 6 A). MT regrowth after nocodazole washout was observed within 5 min. The polymerized MTs were extracted and subsequently fixed with 2% PFA. Cells were stained with antibodies for γ- and α-tubulin to localize the MT-organizing center (MTOC) and polymerized MTs, respectively. Sholl analysis was performed with radius increments of 0.25 µm to quantify the extent of MT polymerization emanating from the MTOC (Fig. 6 B). pmn mutant motoneurons showed lower numbers of intersections when compared with wild-type motoneurons (Fig. 6 C), corresponding to lower rates of MT polymerization. Application of CNTF significantly increased MT formation in pmn mutant motoneurons (Fig. 6 D). Also the mean length of MTs was lower when compared with wild-type motoneurons, and this was restored after CNTF application (Fig. 6 E). STAT3 deficiency abolished the effect of CNTF on the elongation of MTs in motoneurons (Fig. 6 F). Interestingly, CNTF treatment also enhanced MT reassembly in wild-type motoneurons (Fig. 6 G).

**Discussion**

Here, we show that CNTF, in contrast to BDNF or GDNF, stabilizes the axonal cytoskeleton of motoneurons by activation of STAT3. STAT3 stimulates MT regrowth and rescues MT destabilization in pmn mutant motoneurons that suffer from a mutation in the TbcE gene, coding for a chaperone that interacts with α-tubulin and stimulates the formation of αβ-tubulin dimers. The rescue effect of activated STAT3 involves stathmin, an 18-KD MT-interacting protein that binds αβ-tubulin heterodimers (Ng et al., 2006), thus reducing their availability for the assembly of MTs.

Neurotrophic factors from several gene families, including BDNF, GDNF, and CNTF, have originally been identified as potent survival factors for embryonic motoneurons (Sendtner et al., 1996). Similarly, these factors rescue motoneuron cell bodies after nerve lesion at early stages after birth (Sendtner et al., 1990, 1992). It remained open from these studies whether the requirements of adult neurons for survival are similar and whether the maintenance of axons and neuromuscular endplates depends on the same signals and signaling cascades as the maintenance of cell bodies during embryonic development and early postnatal stages. The observation that GDNF and CNTF differ in their capacity to prolong survival in mouse models of motoneuron disease, in particular the pmn mutant (Sendtner et al., 1992; Sagot et al., 1996) that suffers from a mutation in the TbcE gene (Bommel et al., 2002; Martin et al., 2002)—despite similar survival effects on motoneuron cell bodies—indicates that signaling pathways for axon and synapse maintenance are different from those that support survival. Furthermore, cell type–specific depletion of STAT3 in motoneurons of embryonic mice does not influence survival of developing motoneurons (Schweizer et al., 2002). In the adult, STAT3 is necessary for survival (Schweizer et al., 2002) and axon regeneration (Bareyre et al., 2011). From these previous studies, it remained open whether STAT3 promotes axon regeneration via transcriptional effects in the nucleus, such as up-regulated expression of bcl-xl or reg-2 (Nishimune et al., 2000; Schweizer et al., 2002; Ben-Yaakov et al., 2012) or local effects in axons that directly modify the cytoskeleton and thus axon stability and regeneration. Neither inhibition of transcription nor overexpression of a DNA binding–deficient STAT3 abolishes the effects of CNTF on axon growth in pmn mutant motoneurons, and most of the axonal STAT3 remains local in axons and is not
This finding indicates that local regulatory mechanisms modifying the turnover and stability of MTs could play a central role under conditions when axons regenerate and possibly also under conditions of synapse pruning and axonal degeneration, such as in motoneuron disease. Stathmin, also named Op18 (oncoprotein-18) is a member of a family of MT interaction proteins that also includes the SCG10 (superior cervical ganglion protein 10), the SCLIP (SCG10-like protein), retrogradely transported upon CNTF treatment, indicating that local signaling pathways could be involved in STAT3 effects on axon stability and maintenance.

Stabilization of MTs by taxol has a major effect on axon regeneration after spinal cord injury (Hellal et al., 2011). It increases total polymerized tubulin and decreases tyrosinated tubulin after axonal lesion and thus promotes regeneration of dorsal root sensory nerve fibers after spinal cord lesion (Hellal et al., 2011). This finding indicates that local regulatory mechanisms modifying the turnover and stability of MTs could play a central role under conditions when axons regenerate and possibly also under conditions of synapse pruning and axonal degeneration, such as in motoneuron disease.

**Figure 5. MT stability is altered in pmn mutant motoneurons.** (A) Wild-type motoneuron stained with antibodies against acetylated and tyrosinated α-tubulin. Acetylated (Ac) tubulin (Cy2) labels stabilized MTs and is enriched in the axons but relatively excluded from dendrites and axonal growth cones (arrowheads). Tyrosinated (Tyr) tubulin (Cy3) labels dynamic and unstable MTs, including those in dendrites and axonal tips as shown by arrowheads. Bars, 20 µm. (B and C) Levels of tyrosinated and acetylated tubulin in pmn mutant motoneurons. (B) Levels of tyrosinated tubulin were increased in pmn mutant motoneurons when compared with wild-type motoneurons. 10 ng/ml CNTF treatment or stathmin knockdown in pmn mutant motoneurons reduced tyrosinated tubulin levels to wild-type levels. (C) Levels of acetylated tubulin were unchanged under conditions investigated. Numbers in bars indicate number of cells analyzed. BDNF and CNTF are indicated by B and C, respectively. Statistical analysis: ***, P < 0.001; ANOVA with Bonferroni posthoc test. (D) Representative pictures of pmn mutant motoneurons cultured with BDNF, 10 nM taxol, and BDNF and CNTF showing increased axon length upon stabilization of MTs. Bars, 100 µm. (E) Stabilization of MTs in pmn mutant motoneurons in the presence of 10 nM taxol increased axon length in pmn motoneurons to wild-type levels. Numbers in bars represent cells measured. Statistical analysis: ***, P < 0.001; ANOVA with Bonferroni posthoc test. wt, wild type. Error bars shown represent means ± SEM from three independent experiments.
mutant motoneurons. MT regrowth in isolated motoneurons after nocodazole treatment also showed that the effect of CNTF on MT polymerization depends on STAT3. shRNA-mediated knockdown of stathmin has similar effects as STAT3 activation. This raises the question why other members of the stathmin family could not rescue. SCG10, SCLIP, and RB3 are palmitoylated at domain A and thus targeted to Golgi- and vesicle-like structures, in contrast to stathmin, which is mainly found in the cytosol (Chauvin et al., 2008). This indicates that stathmin could be more abundant in axons and axon terminals in comparison to SCLIP and the other members of this family. Indeed, SCLIP has been shown to be a major regulator of dendrite growth in Purkinje cells (Poulain et al., 2008), indicating that differences in subcellular distribution and binding to the Golgi compartment could contribute to such differential effects.

A recent study has shown that TBCE and the related TBCE-like protein can also cause degradation of α/β-tubulin heterodimers, in particular when these proteins are overexpressed (Sellin et al., 2008), and that stathmin counteracts the tubulin-disrupting activity of these proteins. Stathmin depletion thus shifts the balance between preservation of α/β-tubulin and RB3, all sharing a conserved tubulin-binding domain in their C terminus (Charbaut et al., 2001). There are two mechanisms by which stathmin and related family members modulate MT turnover and stability. They directly induce catastrophe-promoting MT depolymerization, and they sequester tubulin heterodimers and thus could have an indirect effect on polymerization of new MTs. Our observation that CNTF restores regrowth of MTs after nocodazole treatment suggests that the second mechanism could play a central role, in particular under the specific condition in pmn mice in which a mutation of TBCE leads to reduced assembly of tubulin heterodimers.

The p-STAT3Y705–stathmin interaction has been observed upon cytokine stimulation in several cell types, including T lymphocytes (Verma et al., 2009) and various cell lines (Ng et al., 2006). These studies showed that STAT3, when overexpressed together with stathmin, binds to its C terminus, the same region that also interacts with tubulin heterodimers (Ng et al., 2006). Our data suggest that this interaction plays a major role for the maintenance of motor axons in the pmn mutant mouse, a model for motoneuron disease. Depletion of STAT3 by genetic ablation abolishes the rescue effect of CNTF on axon elongation in pmn mutant motoneurons. MT regrowth in isolated motoneurons after nocodazole treatment also showed that the effect of CNTF on MT polymerization depends on STAT3. shRNA-mediated knockdown of stathmin has similar effects as STAT3 activation. This raises the question why other members of the stathmin family could not rescue. SCG10, SCLIP, and RB3 are palmitoylated at domain A and thus targeted to Golgi- and vesicle-like structures, in contrast to stathmin, which is mainly found in the cytosol (Chauvin et al., 2008). This indicates that stathmin could be more abundant in axons and axon terminals in comparison to SCLIP and the other members of this family. Indeed, SCLIP has been shown to be a major regulator of dendrite growth in Purkinje cells (Poulain et al., 2008), indicating that differences in subcellular distribution and binding to the Golgi compartment could contribute to such differential effects.

A recent study has shown that TBCE and the related TBCE-like protein can also cause degradation of α/β-tubulin heterodimers, in particular when these proteins are overexpressed (Sellin et al., 2008), and that stathmin counteracts the tubulin-disrupting activity of these proteins. Stathmin depletion thus shifts the balance between preservation of α/β-tubulin
heterodimers to degradation, and this could explain why stathmin depletion is less active in promoting axon growth in wild-type motoneurons than in pmn mutant motoneurons in which TBCE levels are reduced.

CNTF reduces the levels of tyrosinated MTs in distal parts of axons in pmn mutant motoneurons and thus resembles the effects observed with taxol after axonal lesion (Hellal et al., 2011). The stabilization of MTs in the distal axons could play a major effect for the improved clinical phenotype in CNTF-treated mice (Sendtner et al., 1992), and it will be interesting to know whether the effect of CNTF on pruning of neuromuscular endplates (Pun et al., 2006) depends on this effect on MTs. Proximal axons are generally more stable, and this correlates with acetylation of MTs. Interestingly, CNTF effects on MT acetylation were low, although the number of proximal MTs was reduced. This indicates that the dynamics of MTs that is higher in distal axons is influenced by CNTF via STAT3–stathmin signaling.

Previous studies have shown that stathmin and other members of this family are inactivated by phosphorylation. These proteins are substrates of JNK kinases, and the effects of JNK on the MT cytoskeleton seem to be mediated by this interaction (Tarakuk et al., 2006; Ng et al., 2011). Inactivation of stathmin has also been shown to occur via phosphorylation in a Rac-dependent manner via DOCK7 for axon initiation in developing neurons (Watabe-Uchida et al., 2006). Once axons are initiated, a dramatic influx of tubulin heterodimers into the nascent axon contributes to axon elongation (Yu et al., 2001).

The phosphorylation of stathmin, which inactivates this protein in its MT-binding activity in a similar way as interaction with STAT3, could in principle occur in two modes that need to be distinguished. For example, BDNF stimulates transcriptions of MKP-1, a phosphatase that dephosphorylates JNK in the context of axon guidance. Similar effects were observed downstream of Kidins220/ARMS, which associates with ephrins and neurotrophin receptors (Higueru et al., 2010). Stabilization of axon terminals at synapses and axons thus could represent a specific target for local signals at neuromuscular junctions and motor axons that stabilize neuromuscular endplates and distal axons. In line with this hypothesis, increase in acetylated tubulin, which marks stable MTs in enlarged axonal growth cones, was identified as a specific effect of agrin signaling in cultured hippocampal neurons (Bergstrom et al., 2007). In other mouse models of motoneuron disease, i.e., SOD-1 mutant mice, local application of CNTF, but not GDNF, significantly delays the depletion of synaptic vesicles from presynaptic zones during the process of pruning of neuromuscular endplates (Pun et al., 2006). This difference suggests that similar mechanisms could play a role for axonal maintenance and synaptic vesicle stalling.

Collectively, these findings indicate that local signals that stabilize MTs also stabilize and maintain neuromuscular endplates and axonal projections in postnatal motoneurons. Thus, inhibition of stathmin could be a target for therapy in neurodegenerative disorders, such as motoneuron disease, in which destabilization of synapses and distal axons reflect a major step in the pathophysiological cascade.

Materials and methods

Animals

All mouse lines used in this study were maintained on a Naval Medical Research Institute (NMRI) genetic background. pmn mutant mice were maintained as a heterozygote line by backcrossing with wild-type NMRI mice, and homozygous pmn mutant embryos were obtained by crossing heterozygous mice. Conditional STAT3 knockout (KO) was achieved by crossing a mouse line homozogyous transgenic forNFL-Cre and carrying a STAT3-null allele ([STAT3<sup>fl/</sup>;NFL-Cre<sup>tg/tg</sup>] with mice homozygous for flox<sup>+</sup>-flanked STAT3 ([STAT3<sup>fl/</sup>;NFL-Cre<sup>tg/tg</sup>] as previously described (Schweizer et al., 2002). To obtain the STAT3-deficient pmn mutant ([STAT3<sup>fl/</sup>;NFL-Cre<sup>tg/</sup>;pmn<sup>−/−</sup>]) embryos for motoneuron culture experiments, STAT3<sup>fl/</sup>;NFL-Cre<sup>tg/</sup> and STAT3<sup>fl/</sup> mice were crossed with pmn<sup>−/−</sup> mice to generate STAT3<sup>fl/</sup>;NFL-Cre<sup>tg/</sup>;pmn<sup>−/−</sup> mice. These two lines served as the parental generation for litters that contain STAT3-deficient pmn mutant embryos.

Antibodies

Antibodies against MAP2 (clone AP20; M1406), tau (T6402), acetylated α-tubulin (clone 6-11B1; T7451), and α-tubulin (clone B5-1-2; 15168) were purchased from Sigma-Aldrich. Antibodies against tyrosinated α-tubulin (clone YL1/2; ab6160), stathmin (clone EP1573Y; ab52630), histone 3 (ab1791), and chicken anti-GFP (ab13970) were obtained from Abcam. Antibodies against STAT3 and pSTAT3<sup>705</sup> were supplied from Cell Signaling Technology, anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from EMD Millipore, rabbit anti-GFP (sc 8334) and rabbit anti-Frb (sc 8316) were obtained from Santa Cruz Biotechnology, Inc., and chicken anti-NFL heavy (AB5539) was purchased from EMD Millipore.

Primary motoneuron culture

Lumbar spinal motoneurons were isolated from E13.5 mouse embryos as previously described (Wiese et al., 2010) with minor modifications. The spinal cord was dissected, and the ventrolateral parts of the lumbar segments were collected in HBSS. The tissues were trypsinized (0.1%) for 15 min at 37°C, and motoneurons were enriched by immunopanning using the p75NTR antibody. The p75NTR antibody, clone MR2 (a gift from R. Rush, Flinders University, Adelaide, Australia; Rogers et al., 2006), also commercially available through Biosensis (catalog no. M-009-100) and Abcam (catalog no. ab61425), was used at 5 ng/ml to precoat Nunclon Δ surface culture dishes for immunopanning. Enriched motoneurons were plated on glass coverslips (Marienfeld) from a pretested batch in culture dishes (Greiner Bio-One) or µ-dishes (Ibidi) precoated with polyornithine and laminin-111 (catalog no. 23017–015, lot no. 1347084; Invitrogen). The same batch of laminin was used for all experiments with cultured motoneurons shown in this paper. Motoneurons were grown at 37°C and 5% CO2 in neurobasal medium (Invitrogen) containing 500 µM GlutaMAX (Invitrogen), 2% horse serum (Flinn), 2% B27 supplement (Invitrogen), and neurotrophic factors BDNF (1–5 ng/ml), CNTF (1 or 5 ng/ml) or GDNF (5 ng/ml) as indicated. 50% of culture medium was exchanged on day 1 and then every second day. For axon length measurement, motoneurons were plated at a density of 2,000 cells/cm2, and for electron microscopy and live imaging, they were plated at densities of 5,000 or 10,000 cells/cm2.

Cortical precursor cell culture

Cortical precursor cell cultures were prepared as previously described (Götz et al., 2005). In brief, embryonic forebrains were dissected at E12, trypsinized (0.1%), and cultured as neurospheres in neurobasal medium containing 500 µM GlutaMAX, 50 U/ml penicillin G sodium, 50 U/ml streptomycin sulfate (Invitrogen), 2% B27 supplement, basic FGF (bFGF), and EGF (Cell Concepts) at final concentrations of 20 ng/ml each. The cells were grown at 37°C in a 5% CO2 humidified atmosphere. Cells were dissociated cultured on poly-ornithine- and laminin-coated 12-well cell culture dishes at a density of 100,000 cells per well in culture medium with...
bFGF and EGF. Appropriate lentiviral infections were performed before plating of the cells. bFGF and EGF were withdrawn after 48 h, and cells were then treated with 10 ng/ml EGF for 24 h.

**Electron microscopy**

Primary motoneurons ([E13.5] were cultured for 7 DIV and fixed for 5 min at 37°C with 2% glutaraldehyde in 0.05 M phosphate buffer, pH 6.8. Cells were then treated with osmium tetroxide, subjected to series of alcohol dehydration, and embedded in Epon resin. After polymerization, the resin were stained with methylene blue. Ultrathin sections of 80 nm were prepared, transferred to Formvar-coated nickel grids, and contrasted with uranyl acetate and lead citrate. Electron micrographs were obtained with a transmission electron microscope (LEO 912 AB; Carl Zeiss). MT density in longitudinal axonal sections was assessed following a method described previously (Daniels, 1973). 12 regularly spaced (173-nm distance) parallel lines perpendicular to the long axis of the axon were superimposed upon micrographs taken from proximal parts of the axons (within ~50 µm of the cell body), distal parts (less than ~100 µm from the intermediate parts (in between). For each line, the distance across the axon and the number of MT intersections were recorded, and the mean number of MT intersections was divided by the mean distance across the axon to yield an estimate of MT density [given as intersections per 500 µm].

**Lentiviral production**

STAT3-EYFP, STAT3EE434–435AA-EYFP, and STAT3Y705F were subcloned into the FUGW (Lois et al., 2002) vector through the following mutagenesis primers as follows: STAT3Y705F forward, 5′-GCCTCTAGTTCCCTGGAGAACCCGAC-AAGG-3′; STAT3Y705F reverse, 5′-TCGAGAAAAAAGGGGAGAAAGTGAAAGTG-3′; STAT3EE434–435AA forward, 5′-CCCTCCGTGATCTCAGCCGGGCGCGCCGTACCAGTACA-3′; and STAT3EE434–435AA reverse, 5′-GGTGATACGGGTAGCGGCTGTACGAGCCAGACCA-3′. The knockdown vector for stathmin was generated cloning stathmin shRNA sequence [5′-TGCAAGAAAAAGGGGAAAGTCTGTGTTCTAACCA-3′] and mismatch shRNA sequence (5′-GAAAAGGTTAGAACTTAAAGTGTTCTCTTGAAACACTTTAAGT-3′) into pL3.7 Lentilx vector. Lentiviruses were generated and stored at -80°C until use.

**Immunocytochemistry**

Cells were cultured for either 5 or 7 DIV and depending on the experiment, washed once with PBS to remove serum components, and fixed with 4% PFA for 20 min at room temperature. Cells were washed three times with PBS and blocked for 30 min at room temperature with blocking buffer containing 1% BSA and 0.5% Triton X-100 in TBST (TBS-Tween). Primary antibodies diluted in blocking buffer were then added and incubated overnight at 4°C. Cells were then washed three times with TBST, and corresponding secondary antibodies coupled with fluorophores were added and incubated at room temperature for 1 h. Coverslips were washed and mounted on glass slides with Mowiol or aqua polymount. Imaging was performed with either an ECL or ECL Advance systems (GE Healthcare). Western blots were scanned and quantified by densitometry analysis with ImageJ (National Institutes of Health).

**Immunoprecipitation**

Primary motoneurons (approximately three million cells derived from ~300 mouse embryos) were cultured for 5 DIV. Nuclear and cytoplasmic proteins were extracted as described in the Nuclear fractionation section. CNTF was applied for 30 min on day 5 before fractionation. Cell lysates from 5 ng/ml BDNF- and 10 ng/ml BDNF + CNTF–treated cultured motoneurons were incubated with 5 µl rabbit anti-stathmin antibody overnight at 4°C. IgG control was included by incubating the lysate with irrelevant rabbit antibody. Proteins were incubated with PBS and equilibrated with lysis buffer. Protein lysate and antibody were incubated with equilibrated beads for 1 h under rotary agitation at 4°C. After incubation, supernatant was removed by centrifugation at 1,000 g, and beads were washed three times with lysis buffer. Proteins were eluted by boiling the beads with 2x Laemmli buffer. Immunoblotting was performed for STAT3, stathmin, tyrosinated tubulin, and GGF to confirm coimmunoprecipitation. Densitometry analysis was performed using ImageJ software to quantify band intensities.

**MT degrowth**

An established MT degrowth assay was performed in cultured motoneurons (Schaefer et al., 2007). In brief, motoneurons were plated on laminin-coated coverslips, and 1 h after attachment, the cells were treated with 10 µM nocodazole and incubated for 6 h at 37°C to depolymerize the MT network. Cells were washed with neurobasal medium and treated with 10 ng/ml CNTF for 5 min. Cells were rinsed with MT-stabilizing buffer PHEM (50 mM NaCl, 1% NP-40, 2 mM EDTA, pH 8.0, protease inhibitor (Roche), 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 mM okadaic acid for 10 min at 4°C. Cells were scrapped off and centrifuged at 1,000 g for 15 min at 4°C. The supernatant was removed and used as a cytoplasmic fraction. The pellet was washed once with PBS and centrifuged again at 20,000 g for 10 min at 4°C. The pellet was used as a nuclear fraction. Cytoplasmic and nuclear fractions were controlled using GAPDH (clone 6C5; CB 1001) and histone 3 antibodies as respective markers.

**Western blotting**

Primary motoneurons were lysed with nuclear fractionation buffer or directly with Laemmli buffer (125 mM Tris, pH 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.004% bromophenol blue) and boiled for 10 min at 99°C. Proteins were then subjected to SDS-PAGE, blotted onto nitrocellulose membrane, incubated with corresponding antibodies, and developed with either ECL or ECL Advance systems (GE Healthcare). Western blots were scanned and quantified by densitometry analysis with ImageJ (National Institutes of Health).
Live imaging of mitochondria
Mitochondria were stained with 0.2 µg/ml Rhodamine 123 (Invitrogen) or 100 nM MitoFluor red 594 (Invitrogen) in culture medium containing 10 nM β-mercaptoethanol. Motoneurons were incubated for 15 min at 37°C and 5% CO2 in the presence of dye and subsequently washed three times with neurobasal medium. Imaging was performed using a microscope (Eclipse TE2000; Nikon) with a 60x Plan Apochromat, NA 1.4 immersion objective combined with Perfect Focus System (Nikon) and a top stage incubation chamber and objective heater (Tokai Hit). Mitochondrial dye was excited with a xenon arc lamp using a 470/22-nm or 556/20-nm band pass filter for Rhodamine 123 or MitoFluor red 594, respectively. Emission was filtered by a 512/630-nm dual-band filter through a 493/574-nm dual-band beam splitter and recorded with a charge-coupled device camera (Hamamatsu). For quantification of mitochondrial movement, image sequences were analyzed using the ImageJ plugins manual tracking and multiple kymographs.

FRAP
Lentivirally transduced motoneurons expressing STAT3-EYFP fusion protein or EGFP under the human ubiquitin C promoter as a control were cultured for 6 DIV. FRAP experiments were performed with a confocal laser-scanning microscope (SP5; Leica) using LAS AF imaging software with FRAP wizard. Motoneurons grown on coverslips (see Primary motoneuron culture method) were placed into an imaging chamber and perfused with prewarmed (37°C) carbogen-gased culture medium. Images were acquired in 12-bit mode with a 20x glycerol immersion objective, NA 0.7, and an optical zoom factor of 2.0. EGFP or EYFP were excited using a 488- or 514-nm laser line, respectively, with 80% initial power of a 2.5-mW krypton/argon laser. Imaging speed was set to 400 Hz with a resolution of 512 x 512 pixels. To avoid photobleaching during prebleach as well as postbleach acquisition, laser intensity was set to 6% for 488-nm or 15% for 514-nm laser line of the preset 80% power. To achieve maximal bleaching during the bleaching phase, the laser beam was focused on the region of interest using the zoom-in mode (FRAP wizard; Leica) together with 100% laser intensity. Bleaching was achieved with 10 frames at one frame/0.662 s.

Imaging conditions were as follows: Application of 10 ng/ml CNTF was achieved by changing the perfusion supply from culture medium containing only 5 ng/ml BDNF to culture medium containing 10 ng/ml BDNF and 10 ng/ml CNTF. Motoneurons were incubated with factors for 30 min before the FRAP experiments and kept under continuous perfusion. To inhibit MT-based transport, motoneurons were transferred to the imaging chamber 15 min before imaging and perfused with prewarmed (37°C) carbogen-gased culture medium containing 10 µM nocodazole without or with CNTF. To block local translation, motoneurons were incubated at 37°C and 5% CO2 in culture medium containing 100 µg/ml cycloheximide 1 h before FRAP experiments.

Data analysis
Intensity of fluorescence was read out from bleached areas proximal and distal within the bleached axonal segment. FRAP data were exported from LAS-AF imaging software (Leica) to Excel [2003; Microsoft]. Fluorescence intensities in proximal and distal segments were background corrected and normalized as indicated: \( F_{\text{norm}} = \frac{F - F_{\text{background}}}{F_{\text{max}} - F_{\text{background}}} \), in which \( F_{\text{norm}}, F \) is the normalized fluorescence intensity at time point \( t \), \( F \), is the fluorescence intensity at given time point \( t \), \( F_{\text{background}} \) is the background signal at time point \( t \), \( F_{\text{max}} \) is mean fluorescence intensity of 10 consecutive images taken before bleaching, and \( F_{\text{background}} \) is the background signal during the prebleach phase. Normalized and background-corrected FRAP data were exported to Prism 4 (GraphPad Software). Single experiments were fitted using one-phase decay function and least-square fit to obtain rate constants of different conditions.

For better visualization of the representative FRAP image in Fig. 2 D, images from the time-lapse image series were processed with the image enhancement plugin of ImageJ using the following settings: background subtraction radius = 50, filter to reduce noise = median, percentage saturation = 90%, and value adjustment = 0.6. Subsequently, mitochondrial morphology was altered using the ImageJ plugin straighten for better representation of the montage shown in Fig. 2 D.

Actinomycin D treatment
Primary pmn mutant and corresponding wild-type motoneurons of the same litter were cultured with 5 ng/ml BDNF or 10 ng/ml BDNF and CNTF. Afterward, 4 DIV wild-type and pmn mutant motoneurons cultures were treated with actinomycin D (Sigma-Aldrich) at a final concentration of 5 nM. Actinomycin D-treated and control cultures were fixed 24 h later and stained for tyrosinated α-tubulin and MAP2 and subsequently imaged.

Statistical analysis
Statistical analyses were performed with the Prism 4 software. All data are expressed as means ± SEM. Student’s t test was used to compare two groups and analysis of variance (ANOVA) test with Bonferroni post-hoc test, or nonparametric Kruskal-Wallis test with Dunn’s multiple comparison test was used if there were more than two groups. Two-way ANOVA was used to test for significant differences between two categories and two conditions.

Online supplemental material
Fig. S1 shows the survival of motoneurons with different neurotrophic factors. Fig. S2 shows the quantification of axon diameter and MT density in cultured wild-type and pmn mutant motoneurons. Fig. S3 shows that CNTF restores reduced anterograde mitochondrial transport in pmn mutant motoneurons. Fig. S4 shows survival of motoneurons on different concentrations of actinomycin D. Fig. S5 demonstrates that STAT3 colocalizes with stathmin in the cell body and dendrites of cultured motoneurons and the specific function of the shRNA against stathmin.

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