Remodeling of cytoskeleton structures, such as microtubule assembly, is believed to be crucial for growth cone initiation and regrowth of injured axons. Autophagy plays important roles in maintaining cellular homeostasis, and its dysfunction causes neuronal degeneration. The role of autophagy in axon regeneration after injury remains speculative. Here we demonstrate a role of autophagy in regulating microtubule dynamics and axon regeneration. We found that autophagy induction promoted neurite outgrowth, attenuated the inhibitory effects of nonpermissive substrate myelin, and decreased the formation of retraction bulbs following axonal injury in cultured cortical neurons. Interestingly, autophagy induction stabilized microtubules by degrading SCG10, a microtubule disassembly protein in neurons. In mice with spinal cord injury, local administration of a specific autophagy-inducing peptide, Tat-beclin1, to lesion sites markedly attenuated axonal retraction of spinal dorsal column axons and cortical spinal tract and promoted regeneration of descending axons following long-term observation. Finally, administration of Tat-beclin1 improved the recovery of motor behaviors of injured mice. These results show a promising effect of an autophagy-inducing reagent on injured axons, providing direct evidence supporting a beneficial role of autophagy in axon regeneration.

Autophagy maintains cellular homeostasis by bulk or selective degradation of cytoplasmic components including organelles or protein aggregates. Its role in axon regeneration remains speculative. Here, we found that boosting autophagy stabilized microtubules by degrading a microtubule destabilizing protein, SCG10 (superior cervical ganglia protein 10), in cultured CNS neurons and promoted axon growth. Furthermore, treatment with a specific autophagy-inducing peptide, Tat-beclin1, attenuated axon retraction, promoted axon regeneration, and improved locomotor functional recovery in mice with spinal cord injury. This study reveals a critical role of autophagy in stabilizing neuronal microtubules and a promising therapeutic effect of an autophagy-inducing reagent on CNS axons following injury.

Significance

Autophagy induction stabilizes microtubules and promotes axon regeneration after spinal cord injury

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Significance

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augmented autophagy caused a down-regulation of SCG10 (superior cervical ganglia protein 10), a MT-stabilizing protein, and stabilized MTs. Remarkably, local administration of Tat-Bec promoted axonal regeneration of descending serotonin neurons, which formed synaptic contact with spinal motor neurons, and improved functional recovery after SCI. Together, this study reveals a promising beneficial effect of an autophagy-inducing agent on injured axons.

**Results**

**Autophagy Induction Following Nerve Injury and the Effects on Neurite Outgrowth.** The disrupted autophagy flux after SCI (43) and the difference in regrowth capability between CNS and PNS axons prompted us to determine autophagy levels in mice subjected to spinal cord hemisection (SCH) and sciatic nerve crush (SNC), which serve as CNS and PNS axonal injury models, respectively. We found that in the SNC sample, the level of MT-associated protein (MAP) light chain-3B II (LC3-II), which is a lipidated form of LC3 associated with autophagosome membranes (44), increased compared with the sham sample (Fig. S1 A and B). This increase was observed at 6 h and was more pronounced 1 d or 7 d after SNC (Fig. S1 A and B). In correspondence, the level of autophagy substrate p62 decreased in SNC samples (Fig. S1 A and C). These results indicate that SNC-induced PNS axonal injury is accompanied by an increased activity of autophagy. By contrast, the increase in LC3-II was not significant and p62 gradually accumulated in SCh samples (Fig. S1 A–C). These results prompted us to investigate the role of autophagy in regulating CNS axons.

First, we determined effects of autophagy-inducing peptide Tat-Bec on cultured cortical neurons. To avoid the deleterious effect of the excessive extent of autophagy, we optimized concentrations of Tat-Bec. As shown in Fig. 1A, 5 μM Tat-Bec treatment for 3 h caused the appearance of a substantial numbers of autophagosome- or autolysosome-like structures in cultured cortical neurons at DIV1 (1 d in vitro) both in soma and neurites. The induction of autophagy activity was also reflected by an increase in LC3-II and a decrease in p62 in Tat-Bec–treated neurons compared with scrambled peptide (Tat-Scr)–treated neurons (Fig. 1B). Interestingly, treatment with Tat-Bec promoted neurite outgrowth (Fig. 1 C and E) and prevented the inhibitory effect of myelin on neurite growth (Fig. 1 D and F). In line with this notion, treatments with rapamycin (Rapa) or spermidine (Spd), two additional widely used autophagy inducers (45), also markedly ameliorated the inhibitory effect of myelin (Fig. 1 D and F). Thus, the induced autophagy activity is associated with the enhanced intrinsic growth potency of CNS axons.

**Autophagy Induction Stabilizes Neuronal MTs by Down-Regulating SCG10.** Cytoskeleton remodeling undergoes axonal growth and repair (23, 46). Injured CNS axons have been found to contain many RBs, which are associated with disorganized MTs and autophagy-associated proteins (18, 47). We determined the relevance of autophagy with MT stability in neurons. The ratio of acetylated to tyrosinated α-tubulin (A/T ratio) was used to measure the relative ratio of stable to dynamic MTs (48). We found that treatment with Tat-Bec caused a marked increase in the A/T ratio compared with the Tat-Scr–treated group (Fig. S2 A and C), even under conditions of treatment with MT depolymerization agent Nocodazole (NCD22) (Fig. 2 A and B). The A/T ratio was also increased in neurons treated with Rapa or Spd (Fig. 2 A and B). These results indicate that autophagy induction stabilizes MTs. In line with this notion, Tat-Bec–treated neurons exhibited more bundled MTs, which accumulated in the cell periphery and neurites (Fig. S2B). Time-lapse analysis for MT assembly in neurons expressing GFP-tagged MT plus-end binding protein EB3 (EB3-GFP) indicates that Tat-Bec treatment promoted MT polymerization toward the cell periphery and elongation into the distal regions of neurites (Fig. 2 C–E and Movies S1 and S2). The increased MT stabilization was not observed in neurons treated with Tat-Bec270S or Tat-Bec274S, two mutated forms of Tat-Bec with the phenylalanine changed to serine, which have been shown to lose autophagy-inducing activity (40) (Fig. S2 D and E). Together, autophagy induction increases MT stabilization in neurons.

The MT stability and dynamics are regulated by combined actions of several classes of accessory proteins, including MAPs that bind along tubulin sides to stabilize MTs and proteins that destabilize MTs (49), including stathmin family proteins that bind free tubulin subunits to prevent MT assembly (50, 51) and kinesin-13 family proteins that insulate tubulin dimers from the ends of MT filaments (52). In a screening using mass spectrometry analysis for changed proteins upon Tat-Bec treatment of cultured DIV1 cortical neurons treated with Tat-Bec or Tat-Scr (5 μM, 3 h), the kinesin-13 family proteins, and SCG10, also named Stathmin-2, a neuron-specific member of the stathmin family proteins (49). Next, we verified these changes by immunoblot and found that SCG10 exhibited a marked decrease in Tat-Bec–treated cells (Fig. 3 A and B). However, there was no significant change in MAP2 and tau, two major neuronal MAPs, as well as stathmin or KIF2A (Fig. 3 A and B), suggesting that SCG10 is prone to Tat-Bec-mediated autophagic degradation. In line with this notion, treatments with Rapa or Spd also caused down-regulation of SCG10 (Fig. S3 A and B). Analysis for the localization of SCG10 with transmission electron microscopy (TEM) showed recruitment of SCG10 into autophagosomes or autolysosomes in Tat-Bec–treated neurons, accompanied by a decrease of SCG10 in cytoplasm (Fig. 3 C and D).
This result suggests that SCG10 is a selective autophagy substrate in neurons. The role of SCG10 in regulating dynamics of neuronal MTs was determined by manipulating levels of SCG10. Down-regulation of SCG10 by small interference RNAs (54) increased the A/T ratio in DIV1 cortical neurons treated with NCDZ (Fig. S3 C and D), and overexpression of SCG10 caused the opposite effect (Fig. 3 E and F). These results suggest that SCG10 is a critical modulator for MT dynamics in neurons. Remarkably, the decreased MT stability in SCG10-overexpressing neurons was rescued by treatment with Tat-Bec (Fig. 3 E and F), suggesting the strong capability of induced autophagy in clearing exogenous obsolet proteins in neurons.

**Tat-Bec Prevents Axons from Injury-Induced Degeneration.** It has been shown previously that injured CNS axons form RBs that contain dispersed and disorganized MTs (18, 23), and autophagy-related proteins are associated with RBs (55). The regulation of MT stability by induced autophagy observed here prompted us to determine its effects on injured axons. First, cultured DIV3 cortical neurons pretreated with Tat peptides were subject to laser injury at a distance of 100 μm from the soma for 20 s (Fig. S4A), followed by live imaging for axonal behavior (Fig. S4B and Movies S3 and S4). As shown in time-lapse images of straightened axons, laser injury caused marked axonal retractions with intermittent bulbs formed in axonal shafts proximal to the injury site (Fig. S4B). Notably, pretreatment with Tat-Bec attenuated axonal retractions (Fig. S4 B and C and Movies S3 and S4), suggesting a protective role of autophagy for axons following injury.

The effect of Tat-Bec was further investigated in a mouse model of SCI. First, we used adult Thy1-YFP-M transgenic mice (56) to visualize the dynamic behavior of the central axons of dorsal root ganglion (DRG) neurons coursing in the dorsal columns of the spinal cord following unilateral lesion at a segment between cervical 4 and 5 (C4–C5). The lesion sites were treated immediately with Tat peptides (3 μM in ACSF) for 1 h, washed with ACSF, and observed for 5 h after injury (Fig. 4A). In agreement with the previous observation (18), evident RBs were induced in many axons shortly after lesion, and subsequently the injured axons retracted gradually (see Fig. 4A, Tat-Scr). Interestingly, Tat-Bec treatment caused a marked decrease in the percentage of axons with RBs (Fig. 4B) and attenuated axon retraction distance (Fig. 4C) compared with the control peptides. Next, we analyzed the effects of Tat-Bec on cortical spinal tracts.
(CSTs) of Emx1-Cre; Ai9 mice, which expressed tdTomato in corticospinal neurons and the descending CST in the spinal cord (57), following SCH at C4–C5. We found that Tat-Bec also markedly attenuated axonal retraction at 3, 7, or 28 d postinjury (Fig. 4D and E and Fig. S5 A–D). Thus, Tat-Bec treatment markedly prevents axons from injury-induced degeneration.

We also analyzed MTs of CST following lesion and peptide administration (Fig. 4 F and G). Immunostaining showed that the detyrosinated α-tubulin (Glu-tub), which marks polymerized MTs (58), was better preserved in lesioned CSTs of Tat-Bec–treated mice compared with the Tat-Scr control group (Fig. 4G and H). TEM analysis showed that, in contrast to randomly arrayed MTs in the Tat-Scr group, MTs mostly aligned in parallel after Tat-Bec treatment (Fig. 4F), with much smaller deviation angles relative to the axonal axis (Fig. 4I). Notably, many autophagosomes accumulated in axons of Tat-Bec–treated animals (Fig. 4F). These results further support the conclusion that Tat-Bec–induced autophagy maintains the stability of MTs in injured axons.

**Tat-Bec Promotes Axonal Regeneration of Monoaminergic Neurons and Improves Motor Behavior Recovery After SCI.** In addition to CSTs, other descending axonal pathways, including raphespinal and rubrospinal fiber tracts, also contribute to locomotor recovery after CSTs, other descending axonal pathways, including raphespinal and rubrospinal fiber tracts, also contribute to locomotor recovery after SCI (15, 59). Thus, we determined regeneration of serotonergic and dopaminergic axon fibers by staining serotonin (5-HT) and tyrosine hydroxylase (TH), respectively, in adult mice after dorsal bilateral SCH at C4–C5 for days to weeks (Fig. 5A). We found that Tat-Bec administration immediately after SCH significantly promoted regrowth of 5-HT or TH-positive fibers caudal to the injury sites, which were delimited by the GFAP-labeled glia scar (Fig. 5B and E and Figs. S6 and S7). Remarkably, the regenerated 5-HT fibers formed synaptic contacts with ventral horn motor neurons marked by choline acetyltransferase (ChAT) in the caudal spinal cord (Fig. 5D). Notably, the Tat-Bec–induced regeneration of 5-HT or TH-positive fibers was observed as early as 3 or 7 d after injury (Figs. S6 and S7). At 8 wk after injury, we also noticed subtle but significantly more regenerated CST axons, which were labeled by BDA (biotinylated dextran amine) injected into layer 4/5 of the mouse sensorimotor cortex, in Tat-Bec–treated animals following SCI (Fig. S8 A and B). Thus, Tat-Bec treatment promotes regrowth of descending spinal axons after injury.

Finally, we determined the effects of Tat-Bec on locomotor behavior of lesioned mice. We found that Tat-Bec treatment markedly increased the time mice stayed on Rotarod treadmillss beginning from 1 wk postinjury (Fig. 5F) and decreased the number of foot fall errors in the grid walk test as early as 3 d postinjury (Fig. 5G). In the CatWalk analysis for motor coordination of mice...
8 wk after SCI, Tat-Bec treatment markedly elevated the Regularity Index (RI) and increased the stride length of both hind paws and fore paws (Fig. S5 H and I). Together, Tat-Bec administration promotes functional recovery after SCI.

Discussion

It has been speculated that autophagy-related proteins might act as promising therapeutic targets for curing axonal injuries following traumatic lesion. However, because of unavoidable pleiotropic effects of most autophagy-inducing substances, the role of autophagy in axonal homoeostasis still remains unclear. Furthermore, the extent and duration of autophagy are crucial to cell health, and acute or chronic manipulations of autophagy have led to, in several instances, controversial conclusions. Here, we report a marked therapeutic effect of a specific autophagy-inducing peptide Tat-Bec on SCI. We found that administration of Tat-Bec at optimized doses causes autophagy in neurons and promotes axonal growth in neurons exposed to inhibitory substrate myelin. Interestingly, induced autophagy stabilizes MTs by down-regulating SGC10. Notably, local and temporal Tat-Bec administration attenuates axonal retraction within the critical window that has been shown to be crucial for the regeneration potential of injured axons (60). In line with this notion, Tat-Bec administration also promotes axon regeneration and consequently improves locomotor ability after SCI. The limited intrinsic axon growth capacity, the presence of extracellular inhibitory factors, and the lack of neurotrophic factors are major obstacles limiting regeneration of CNS axons after injury (61). Interestingly, several combinatory approaches have been shown to be able to promote axon regeneration after injury (62, 63). However, interventions for multiple targets make clinical applications difficult. Because several signaling pathways converge on the regulation of cytoskeleton dynamics (23, 46), it is probable to probe the effects of most autophagy-inducing substances, the role of autophagy in axonal homoeostasis still remains unclear. Furthermore, the extent and duration of autophagy are crucial to cell health, and acute or chronic manipulations of autophagy have led to, in several instances, controversial conclusions. Here, we report a marked therapeutic effect of a specific autophagy-inducing peptide Tat-Bec on SCI. We found that administration of Tat-Bec at optimized doses causes autophagy in neurons and promotes axonal growth in neurons exposed to inhibitory substrate myelin. Interestingly, induced autophagy stabilizes MTs by down-regulating SGC10. Notably, local and temporal Tat-Bec administration attenuates axonal retraction within the critical window that has been shown to be crucial for the regeneration potential of injured axons (60). In line with this notion, Tat-Bec administration also promotes axon regeneration and consequently improves locomotor ability after SCI.

Fig. 5. Tat-Bec promotes regrowth of monoaminergic axons and improves motor function recovery after SCI. (A) Schematic representation of the spinal cord bilateral hemisection model. Boxed areas indicate the longitudinal (C4/C5) and transverse plane of the ventral horn at C6. (B) Immunostaining of serotonin (5-HT) and GFAP in the longitudinal section of level C4/C5 spinal cord 8 wk after injury. Boxed areas indicate caudal regions to the injury sites (dashed lines). (Scale bar, 100 μm.) (C) Quantification for the ratio of 5-HT+ fibers caudal to rostral sides of the injury sites. Shown are means ± SEM from four mice in each group. (D) Immunostaining of 5-HT, ChAT, and synaptophysin (Syn) in cross-sections of the spinal cord level C6. Arrowheads in boxed areas indicate ChAT-positive motor neurons innervated by 5-HT+ fibers. (Scale bars, 100 μm (Left) and 50 μm (boxed areas).) (E) Quantification for the area of 5-HT+ fibers in the ventral horn. Data are shown as means ± SEM from four mice in each group. (F) Quantification for the Recovery Index in the rotarod test at the indicated time postinjury and peptide administration (see Materials and Methods). Data are shown as means ± SEM from 14 mice in each group. (G) Number of foot falls on the grid in 5 min at the indicated time postinjury and peptide administration. Data are shown as means ± SEM from 12 mice in each group. (H and I) Quantification for RI (H) and stride length (I) in gait analysis. Data are shown as means ± SEM from 12–15 mice in each group. (J) Proposed model for a role of Tat-Bec-induced autophagy in axonal regrowth after injury.

Materials and Methods

Mice, Axon Injury, and Peptide Application. The use of animals was approved by the Institutional Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences. Adult mice (8–10 wk) expressing fluorescent proteins (Thy1-YFP and Emx1-Cre; A9) were subject to SCI, followed by observation at different times after lesion. For live imaging of axoniasia status through i.p. injection with a mixture of tribromoethanol and tert-amyl alcohol every 2 h during the imaging session.

To determine the effects of peptides on regeneration of CSTs or descending, monoaminergic axons and the motor behavior of the animal, adult mice (8–10 wk) were subjected to bilateral spinal hemisection followed by peptide administration. Briefly, mice were intraperitoneally anesthetized with a mixture of tribromoethanol and tert-amyl alcohol, followed by a shear along the dorsal midline to expose the segments of spinal cord C4-C8 and a laminectomy to cut off the dura matter. The bilateral halves of the C4 segment were hemisectioned with straight cornea scissors at a depth of 2 mm from the dorsal surface. After hemostasis with a gelatin sponge, Tat peptides were injected into incision gaps with a microsyringe. After muscle and skin reposition and suture, mice were placed at a warm cage until recovered. Axonal repair and animal motor behavior were observed at different times after injury.

For SCI, the sciatic nerves below the ischial tuberosity were exposed and crushed with dissecting forceps three times, with each lasting for 15 s, until the crushed trunk became transparent with the myelin sheath.

Neuroscience, Chinese Academy of Sciences. Adult mice (8–10 wk) expressing Thy1-YFP were subject to SCI, followed by washes with ACSF. Axons were subjected to bilateral spinal hemisection followed by peptide administration. Briefly, mice were intraperitoneally anesthetized with a mixture of tribromoethanol and tert-amyl alcohol, followed by a shear along the dorsal midline to expose the segments of spinal cord C4-C8 and a laminectomy to cut off the dura matter. The bilateral halves of the C4 segment were hemisectioned with straight cornea scissors at a depth of 2 mm from the dorsal surface. After hemostasis with a gelatin sponge, Tat peptides (500 μM, 5 μL) were injected into incision gaps with a microsyringe. After muscle and skin reposition and suture, mice were placed at a warm cage until recovered. Axonal repair and animal motor behavior were observed at different times after injury.

For SNC, the sciatic nerves below the ischial tuberosity were exposed and crushed with dissecting forceps three times, with each lasting for 15 s, until the crushed trunk became transparent with the myelin sheath.
significant differences between two groups was performed using two-tailed Statistical Analysis. 


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Behavior Analysis. Motor coordination was assessed using the rotator and grid walk test at different days after injury. The CatWalk system (Noldus) was used for gait analysis. See SI Materials and Methods for detailed information.

Statistical Analysis. All results are expressed as means ± SEM. Analysis for significant differences between two groups was performed using two-tailed unpaired Student’s t test. One-way ANOVA followed by post hoc Tukey’s test was used for comparisons among multiple groups. All statistical analyses were conducted using GraphPad Prism5 for Windows.