

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

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**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 | microtubule stabilization | axon regeneration

It is generally believed that the inability of adult central nervous system (CNS) neurons to regenerate their axons following injury is due to the presence of abundant inhibitory factors in extrinsic milieu and the lack of intrinsic growth ability (1–5). A number of extrinsic growth-inhibitory factors have been identified, including oligodendrocyte-derived myelin-associated glycoprotein (MAG), Nogo, OMgp, or astrocyte-derived chondroitin sulfate proteoglycans (CSPGs), which act through their respective receptors to suppress axon growth and regeneration (6–11). However, genetic ablation or elimination of these inhibitory receptors does not promote axon regeneration (12, 13) or shows marginal effects (14, 15).

Many inhibitory factors act through signaling cascades to modulate cytoskeletal dynamics (16, 17). Indeed, it has been observed that CNS axons form numerous retraction bulbs (RBs) with a disorganized array of microtubules (MTs), whereas peripheral nervous system (PNS) axons rapidly form a growth cone with stable, well-organized bundling of MTs following injury (18). In line with this notion, pharmacological stabilization of MTs promotes axon regeneration after spinal cord injury (SCI) (19, 20). In addition, analyses of gene-targeted mice have led to identification of several intrinsic inhibitors of axon regeneration in the adult CNS, including phosphatase and tensin homolog (PTEN) and the suppressor of cytokine signaling 3 (SOCS3) (21, 22). However, manipulating individual proteins or in combinations allows limited axonal regeneration or sprouting, which is usually associated with temporary improvement in functional recovery. Moreover, achieving axonal regeneration through manipulating certain genes still remains therapeutically unattainable. Thus, development of other effective approaches to promote axonal regeneration is still a challenging task.

A lesion triggers drastic responses in soma and axon, including chromatolysis, axonal membrane sealing, growth cone RB formation, and Wallerian degeneration (23). The growth cone initiation and axon regeneration require an extensive remodeling of cytoplasmic compartment and axon structures, which involves the synthesis and degradation of local proteins (24, 25). Autophagy is one of the major pathways for bulk cytosolic degradation and efficient turnover under stress (26). It plays an important role in maintaining cellular homeostasis by degrading damaged organelles or pathological proteins through a lysosomal degradative process (27–29). In the nervous system, autophagosomes have been observed in cultured neurons or in vivo (30–35). Inhibition of autophagy by genetic elimination of autophagy-determination proteins has been shown to cause neuronal degeneration in mouse cerebellum Purkinje cells or other neurons in various brain regions (36, 37). By contrast, pharmacological inhibition of autophagy attenuates acute degeneration of retinal ganglion cell (RGC) axons (38, 39). Nevertheless, these seemingly controversial results suggest a critical role of autophagy in maintaining axonal homeostasis. It remains speculative that modulation of autophagy activity may promote axon regeneration in the adult CNS.

Augmenting autophagy to see the effect on injured axons is a direct approach, but most compounds have uncontrollable pleiotropic effects. In this study, we took advantage of Tat-beclin1 (Tat-Bec), a specific autophagy-inducing peptide (40), which has been used to enhance autophagy both in vitro and in vivo (41, 42). We found that treatment with Tat-Bec with optimized protocol caused an increase in autophagy activity, promoted axonal growth of CNS neurons cultured on a nonpermissive substrate, and prevented axonal degeneration following injury. Mechanistically,

## Significance

**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.**

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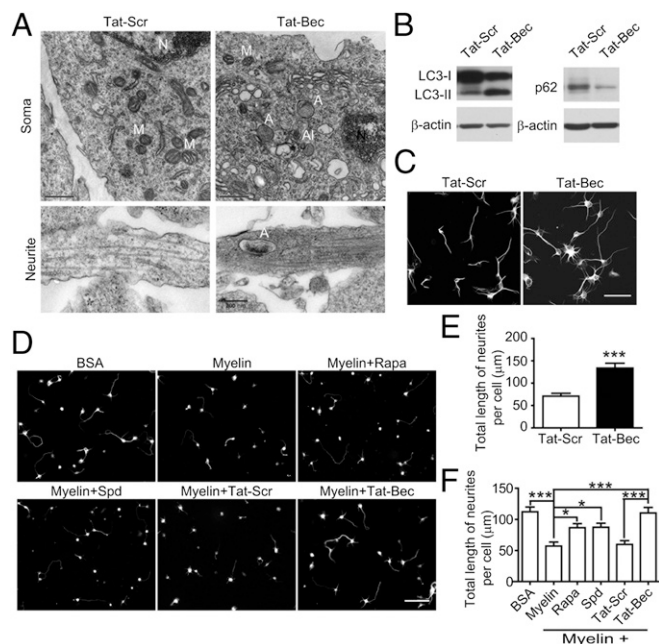
augmented autophagy caused a down-regulation of SCG10 (superior cervical ganglia protein 10), a MT-destabilizing protein, and stabilized MTs. Remarkably, local administration of Tat-Bec promoted axonal regeneration of descending serotonin neurons, which formed synaptic contact with spinal motor neurons, 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. 1*A*, 5  $\mu$ M 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. 1*B*). 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 underlies 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  $\alpha$ -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 (NCDZ) (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. S2*B*). 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,

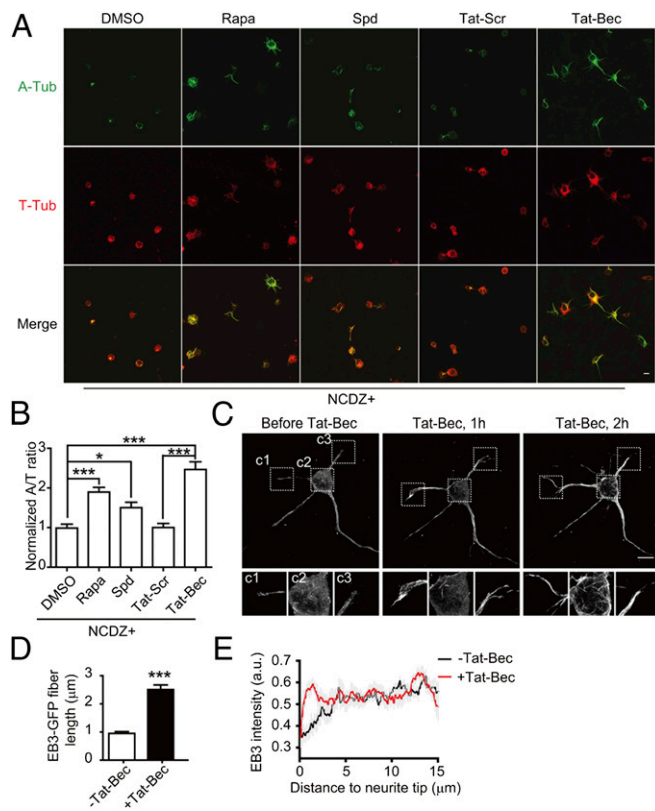


**Fig. 1.** Effects of autophagy induction on neurite outgrowth of cultured cortical neurons. (*A*) Representative electron microscopic images of cultured DIV1 cortical neurons treated with the indicated peptide (5  $\mu$ M, 3 h). A, autophagosome; Al, autolysosome; M, mitochondria; N, nucleus. (Scale bars, 0.5  $\mu$ m in soma and 0.2  $\mu$ m in neurite.) (*B*) Immunoblots of LC3 and p62 in cultured DIV1 cortical neurons treated with Tat-Bec or Tat-Scr (5  $\mu$ M, 3 h).  $\beta$ -actin was probed as a loading control. (*C*) Immunostaining for  $\beta$ -tubulin III (Tuj1) in DIV1 cortical neurons treated with Tat-Bec or Tat-Scr (5  $\mu$ M, 3 h). (Scale bar, 50  $\mu$ m.) (*D*) Immunostaining for Tuj1 in DIV2 cortical neurons cultured on BSA or myelin substrate with the indicated treatments (10 nM Rapa, 1  $\mu$ M Spd, or 2.5  $\mu$ M Tat-Scr or Tat-Bec) for 24 h. (Scale bar, 100  $\mu$ m.) (*E* and *F*) Quantification of neurite length. Data are presented as means  $\pm$  SEM from three experiments with 70–80 cells in each group. \* $P$  < 0.05, \*\*\* $P$  < 0.001, Student's *t* test (*E*) and ANOVA with Tukey's post hoc tests (*F*).

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 spectra analysis for changed proteins upon Tat-Bec treatment of cultured DIV1 cortical neurons, we found that 219 among 2,232 proteins with validated peptide sequences exhibited a decrease in Tat-Bec-treated samples (Dataset S1). Among them, 19 proteins were found to be related with MT regulation after functional relevance analysis using the Gene Ontology database (53), including KIF2A, a member of 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 electronic 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*).





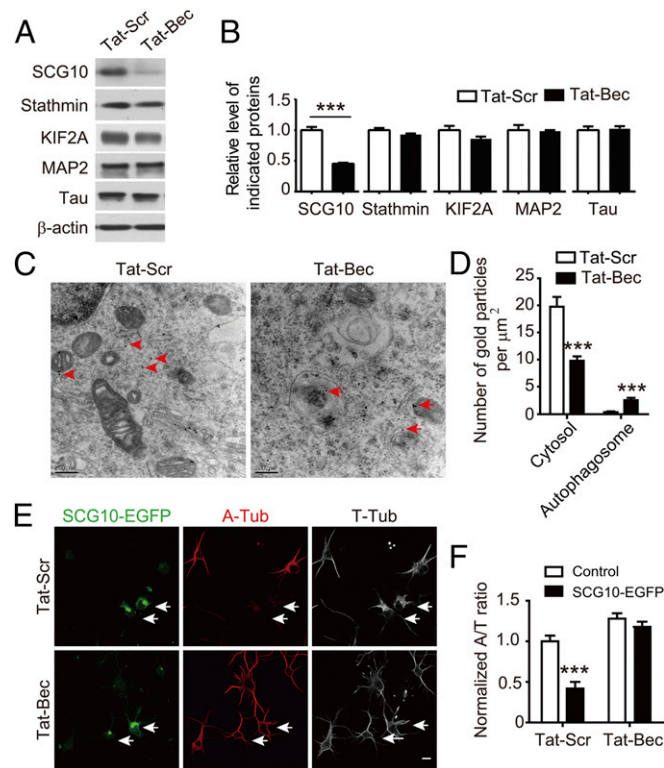
**Fig. 2.** Enhanced autophagy stabilizes MTs. (A) Immunostaining of acetylated  $\alpha$ -tubulin (A-Tub) and tyrosinated  $\alpha$ -tubulin (T-Tub) in DIV1 cortical neurons treated with NCDZ (1  $\mu$ g/mL) plus the indicated reagents (40 nM Rapa, 2  $\mu$ M Spd, or 5  $\mu$ M Tat-Scr or Tat-Bec) for 3 h. Shown are the representative images. (Scale bar, 10  $\mu$ m.) (B) Quantification for the A/T ratio. Data are shown as means  $\pm$  SEM from three experiments with 70–80 cells in each group.  $*P < 0.05$ ,  $***P < 0.001$ , ANOVA with Tukey's post hoc tests. (C) Time-lapse images of EB3-GFP expressed in DIV2 cortical neurons before and after 5  $\mu$ M Tat-Bec treatment. Bottom images show EB3-GFP signals in neurites (c1 and c3) and soma (c2). (Scale bar, 10  $\mu$ m.) (See also [Movies S1](#) and [S2](#).) (D) Quantification of EB3-GFP fiber length before and 2 h after Tat-Bec treatment. Data are shown as means  $\pm$  SEM from three experiments with 18–20 cells in each group.  $***P < 0.001$ . (E) EB3-GFP fluorescence intensity in neurites under the indicated conditions.

These results suggest 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 obsolete 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  $\mu$ m 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  $\mu$ M 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



**Fig. 3.** SCG10 as the substrate of autophagy. (A) Immunoblots of indicated proteins in DIV1 cortical neurons treated with 5  $\mu$ M Tat-Scr or Tat-Bec for 3 h. Shown is a representative blot from three independent experiments with similar results.  $\beta$ -actin was probed as a loading control. (B) Quantification for levels of indicated proteins relative to  $\beta$ -actin. Data are presented as means  $\pm$  SEM ( $n = 3$ ).  $***P < 0.001$  (Tat-Bec vs. Tat-Scr), Student's *t* test. (C) Immunocytochemistry analysis for the distribution of SCG10 in DIV1 cortical neurons treated with Tat-Scr or Tat-Bec. Note the SCG10 signals in the cytosol of the Tat-Scr group (red arrowheads) and autophagosomes in the Tat-Bec group (red arrows). (Scale bar, 200 nm.) (D) Quantification for numbers of SCG10-positive gold particles in cytosol and autophagosomes. Data are shown as means  $\pm$  SEM of 30 cells from three experiments. (E) Immunostaining for A-Tub and T-Tub in SCG10-EGFP-transfected cortical neurons treated with Tat-Scr or Tat-Bec (5  $\mu$ M, 3 h) at DIV2. Note the difference in A-Tub signals in SCG10-EGFP-expressing neurons (arrows) between the two groups. (Scale bar, 10  $\mu$ m.) (F) Quantification for the A/T ratio. Data are shown as means  $\pm$  SEM of 70–80 cells from three experiments.  $***P < 0.001$ , SCG10-EGFP vs. control, Student's *t* test.

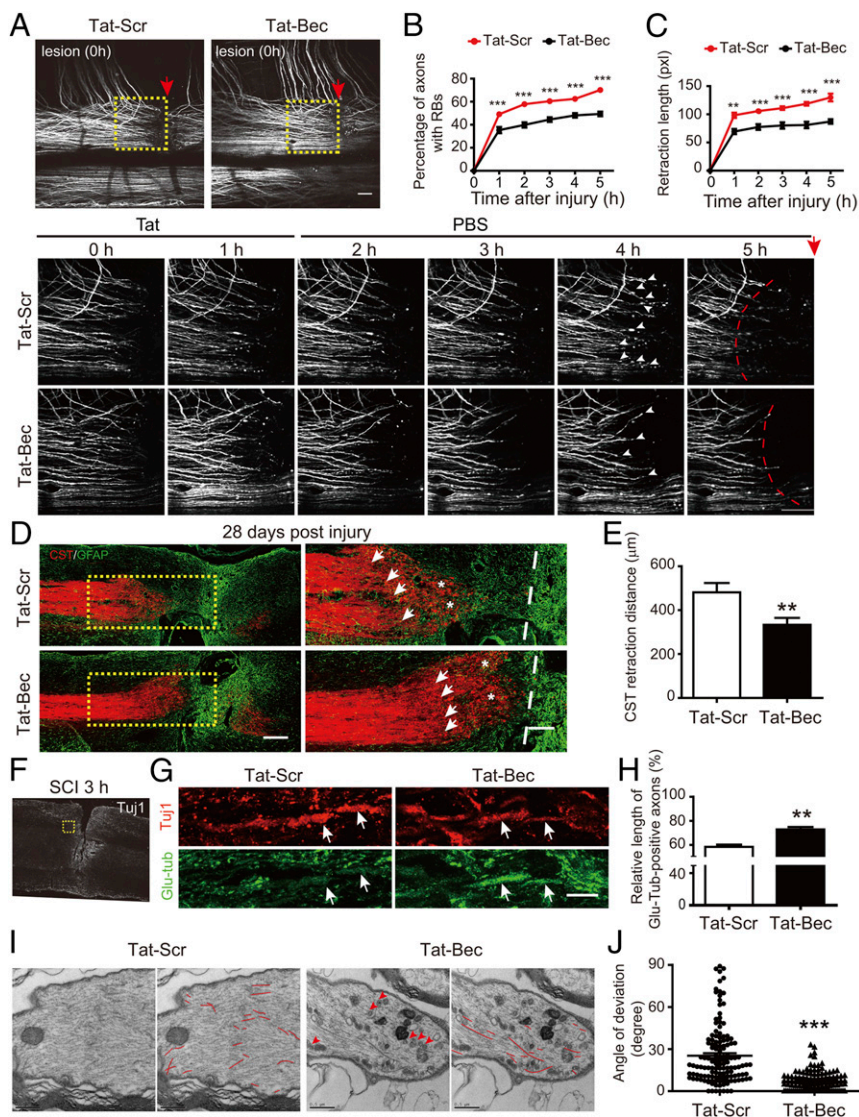
(CSTs) of *Emx1-Cre; Ai9* mice, which expressed tdTomato in cortical neurons and the descending CST in the spinal cord (57), following SCH at C4–C5. We found that Tat-Bec also markedly attenuated axonal retraction at 3, 7, or 28 d postinjury (Fig. 4 *D* and *E* and Fig. S5 *A–D*). Thus, Tat-Bec treatment 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  $\alpha$ -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. 4 *G* 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. 4*I*), with much smaller deviation angles relative to the axonal axis (Fig. 4*J*). Notably, many autophagosomes accumulated in axons of Tat-Bec-treated animals (Fig. 4*I*). 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 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. 5*A*). 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. 5*B* and *E* and Figs. S6 and S7). Remarkably, the regenerated 5-HT axons formed synaptic contacts with ventral horn motor neurons marked by choline acetyltransferase (ChAT) in the caudal spinal cord (Fig. 5*D*). 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 treadmills beginning from 1 wk postinjury (Fig. 5*F*) and decreased the number of foot fall errors in the grid walk test as early as 3 d postinjury (Fig. 5*G*). In the CatWalk analysis for motor coordination of mice



**Fig. 4.** Local administration of Tat-Bec protects axons and stabilizes MTs after nerve injury in mice. (*A*) Live-imaging observation of dorsal column axons in adult *Thy1-YFP* mice immediately after lesion (0 h) and 1–5 h after Tat peptide treatment. (*Bottom*) High magnification of boxed areas caudal to lesion sites in *Tat-Scr* and *Tat-Bec* groups from 1 to 5 h after injury. Red arrows indicate the injury sites; white arrowheads indicate RBs; dashed lines indicate the ends of intact axons. (Scale bar, 100  $\mu$ m.) (*B*) The percentage of axons with RBs from 1 to 5 h after injury. Data are shown as means  $\pm$  SEM from 10 mice in each group. (*C*) Quantification of axon retraction length 1 to 5 h after injury. (*D*) Adult *Emx1-Cre; Ai9* mice were subjected to bilateral hemisection of the spinal cord at the C4/C5 level, followed by immediate application of Tat-Bec or Tat-Scr peptides. After 4 wk, longitudinal sections of injured regions were analyzed for tdTomato-labeled CST and GFAP-labeled glial scar. Dashed lines, incision border; arrows, ends of intact axons; asterisks, RBs. (Scale bar, 200  $\mu$ m.) (*E*) Quantification of CST retraction distance from the incision border of the spinal cord. Data are shown as means  $\pm$  SEM from 12 mice in each group.  $**P < 0.01$ . (*F* and *G*) Immunostaining for Glu-tub and Tuj1 in longitudinal sections of the spinal cord 3 h after SCI. Boxed area, regions rostral to the injury site; arrows, axonal tracts stained with Tuj1. (Scale bar, 10  $\mu$ m.) (*H*) Quantification of the relative length of Glu-tub-positive fragments to Tuj1-positive axons in CST 3 h after SCI and peptide application. Data are shown as means  $\pm$  SEM from six to eight mice in each group.  $**P < 0.01$ . (*I*) EM images of axonal stumps after injury with red lines tracing MTs manually. Samples were from *Emx1-Cre; Ai9* mice treated with peptides 3 h after SCI. Arrowheads indicate autophagosomes. (Scale bar, 0.5  $\mu$ m.) (*J*) Quantification for the MT deviation angles relative to axonal axis. Shown are distribution and means  $\pm$  SEM of MT degrees in at least 15 fields from three mice in each group.  $***P < 0.001$ .



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. 5 *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 homeostasis 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 SCG10. 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 promote axon regeneration by manipulating axonal cytoskeleton directly. Interestingly, moderate MT stabilization by treatment with Taxol or epothilone B, two clinically approved drugs, promotes axon regeneration after SCI (19, 20). However, these reagents cause MT stabilization globally and affect many other cellular functions, such as cell proliferation, secretion, or migration. Thus, more precise control of cytoskeletal dynamics merits further investigation.

Accumulating evidence suggests that MTs play important roles in the regulation of autophagosome formation and transportation in axons (33, 64). Whether autophagy in turn modulates MT dynamics is not known. In this study, we found that induced autophagy increases MT stability through the degradation of SCG10, a MT-destabilization protein, and promotes axon regeneration after injury (Fig. 5*J*). These results are in agreement with previous findings that administration of MT stabilizers can promote axon regeneration and improve locomotor activity (19, 20). A drug design based on Tat-Bec regulation of SCG10 has great potential for precise control of MT dynamics that is crucial for axon repair. This study does not exclude the possibility for the effect of autophagy on other substrates that restrain the intrinsic axonal regrowth ability of CNS neurons.

## Materials and Methods

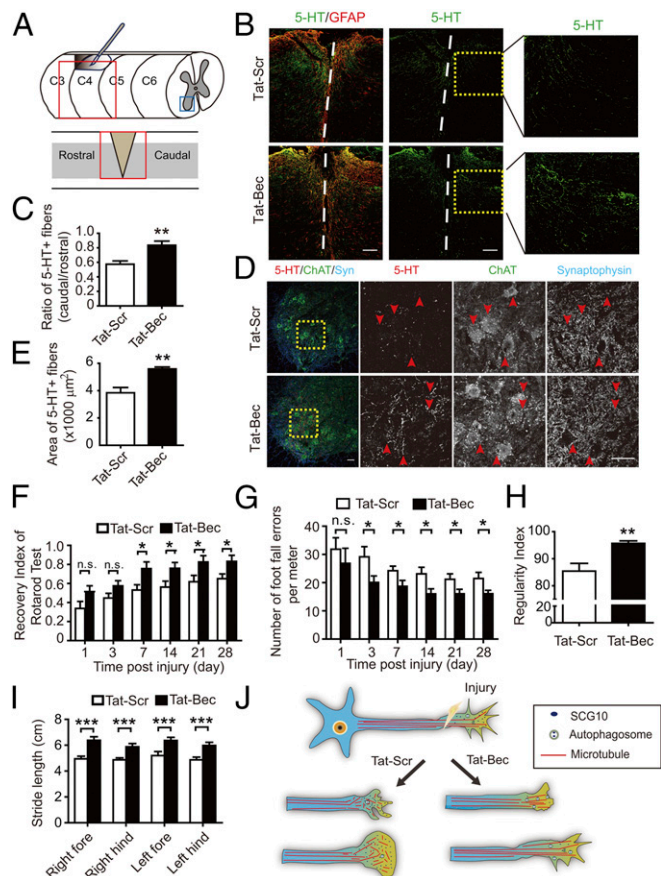
**Mice, Axon Injury, and Peptide Application.** The use of animals was approved by the Institutional Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Sciences. Adult mice (8–10 wk) expressing fluorescent proteins (*Thy1-YFP* and *Emx1-Cre; Ai9*) were subject to SCI, followed by observation at different times after lesion.

For live imaging of axons after injury in vivo, a small unilateral lesion was produced in *Thy1-YFP* mice by transecting only the superficial axons of the dorsal columns at the C4/C5 level with Vannas spring scissors after removing the lamina. Immediately after lesioning, injury sites were bathed in ACSF containing 3  $\mu$ M Tat peptides for 1 h, followed by washes with ACSF. Axons before and after peptide treatment were observed under a Prairie two-photon microscopy every 1 h for a total of 5 h. The animals were kept on a heating pad under anesthesia 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 hemisected with straight cornea scissors at a depth of 2 mm from the dorsal surface. After hemostasis with a gelatin sponge, Tat peptides (500  $\mu$ M, 5  $\mu$ L) were injected into incision gaps with a microsyringe. After muscle and skin reposition and suture, mice were placed at a worm 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.



**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 in C6. (B) Immunostaining of serotonin (5-HT) and GFAP in the longitudinal section of level C4/5 spinal cord 8 wk after injury. Boxed areas indicate caudal regions to the injury sites (dashed lines). (Scale bar, 100  $\mu$ m.) (C) Quantification for the ratio of 5-HT+ fibers caudal to rostral sides of the injury sites. Shown are means  $\pm$  SEM from seven 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  $\mu$ m (Left) and 50  $\mu$ m (boxed areas).] (E) Quantification for the area of 5-HT+ fibers in the ventral horn. Data are shown as means  $\pm$  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  $\pm$  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  $\pm$  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  $\pm$  SEM from 12–15 mice in each group. (J) Proposed model for a role of Tat-Bec-induced autophagy in axonal regrowth after injury.

**Behavior Analysis.** Motor coordination was assessed using the rotarod test 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  $\pm$  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.

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