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·Original Article·

Valproic acid reduces autophagy and promotes functional recovery after spinal cord injury in rats

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ABSTRACT

Secondary damage is a critical determinant of the functional outcome in patients with spinal cord injury (SCI), and involves multiple mechanisms of which the most important is the loss of nerve cells mediated by multiple factors. Autophagy can result in cell death, and plays a key role in the development of SCI. It has been recognized that valproic acid (VPA) is neuroprotective in certain experimental animal models, however, the levels of autophagic changes in the process of neuroprotection by VPA treatment following SCI are still unknown. In the present study, we determined the extent of autophagy after VPA treatment in a rat model of SCI. We found that both the mRNA and protein levels of Beclin-1 and LC3 were significantly increased at 1, 2, and 6 h after SCI and peaked at 2 h; however, Western blot showed that autophagy was markedly decreased by VPA treatment at 2 h post-injury. Besides, post-SCI treatment with VPA improved the Basso-Beattie-Bresnahan scale, increased the number of ventral horn motoneurons, and reduced myelin sheath damage compared with vehicle-treated animals at 42 days after SCI. Together, our results demonstrated the characteristics of autophagy expression following SCI, and found that VPA reduced autophagy and enhanced motor function.

Keywords: spinal cord injury; autophagy; valproic acid; LC3; Beclin-1

INTRODUCTION

Currently, trauma is the leading cause of mortality in the young population[1]. Trauma-induced spinal cord injury (SCI) drastically impinges on the quality of life in survivors who have residual disability, and causes a healthcare burden nationally[2,3]. In China, the incidence of SCI caused by car crashes, construction and mining accidents has increased ten-fold in the past decade[4]. Injury to the spinal cord causes primary and secondary damage. The secondary damage is often more important in determining the functional outcome and provides a practical target for therapeutic intervention. Although the secondary injury can be regulated, the mechanism is complex[4], and clinical treatment for SCI, especially for recovery of motor function, is not satisfactory[5]. Therefore, it is urgent to further understand the mechanisms underlying the development of SCI and to seek better and more efficient treatments to improve the quality of life of patients.

The post-traumatic systemic stress response is

implicated in the functional loss after SCI[5,6]. The integrated stress response[7] plays a pivotal role in maintaining homeostasis in the body. Autophagy, a central component of this response[7], is influential in the development and progression of damage after injury[8-10]. Autophagy is an intracellular degradation system that delivers proteins and organelles to lysosomes for degradation[11]. It is involved in the development of many diseases[12,13], but both excessive and deficient autophagy are linked to pathophysiological events[14]. Recent studies confirmed that autophagy promotes secondary cell death in traumatic SCI[10,15,16] and affects functional recovery[17]. Therefore, we hypothesized that secondary injury can be controlled and functional outcome can be improved by the interruption of autophagy to some extent after SCI.

In many animal models, histone deacetylase (HDAC) inhibitors have neuroprotective effects in various neurological conditions[18-20]. Valproic acid (VPA) is an HDAC inhibitor, and plays a key role in fundamental cellular activities such as gene transcription[21]. A recent study in glioma cell lines demonstrated that VPA induces autophagy[22]. Therefore, the level of autophagy in spinal cord tissue may be influenced and motor function may be improved by VPA after SCI.

In this study, we used a rat model of SCI to investigate the characteristics of autophagy after SCI as well as the effect of VPA treatment on autophagy. In addition, we assessed the neuroprotective effect of VPA in acute SCI.

MATERIALS AND METHODS

Animals

Adult female Sprague-Dawley rats, 220–250 g, were purchased from the Animal Center of Shanxi Medical University, China. The animals were housed under light-controlled conditions (12/12 h light/dark cycle) at 24°C, with food and water *ad libitum*.

All procedures and experimental manipulations were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University and were in accord with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996), and the Guide for the Care and Use of Laboratory Animals according to the regulation in the People's Republic of China.

Spinal Cord Contusion Injury

Animals were anesthetized with 10% chloral hydrate (0.3 mL/100 g, i.p.), and SCI was induced using a modified New York University impactor[18,23,24]. In brief, the T10 segment was exposed by laminectomy. Contusion injury was induced by dropping a 10-g rod (tip diameter, 2.5 mm) from a height of 25 mm onto the exposed cord. The sham-operated animals received only a laminectomy to expose the cord, but were not subjected to impact injury. Manual bladder expression was performed on each animal twice daily until the recovery of sphincter control.

VPA Treatment

Rats randomly received VPA (300 mg/kg, i.p., twice daily) or an equal volume of saline vehicle for two weeks, starting immediately after the injury, as reported previously[21,25]. A group of sham-operated rats was included as an additional control.

Western Blot

At different time points (1, 2, 6, 24, 48, and 72 h) after SCI or immediately after the sham operation, the animals were anesthetized and the spinal cord tissue containing the injury epicenter were dissected out, and lysed in radioimmunoprecipitation assay buffer (Beyotime Biotech, China). Protein concentration was determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL; 23228) and 40 μg protein was resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel, electrophoresed (80 V for 30 min followed by 120 V for 90 min), and transferred to a polyvinylidene difluoride membrane (Whatman, Clifton, NJ; 10485289). The membrane was blocked with 0.3% gelatin (A9418; Sigma Aldrich, St. Louis, MO) for 2 h at room temperature, and then incubated with mouse anti-Beclin-1 monoclonal (dilution, 1:1500; Santa Cruz; sc-48381), mouse anti-β-actin monoclonal (1:1 000; Sigma Aldrich; A1978) or rabbit anti-LC3B monoclonal (1:1 000, Cell Signaling, Danves, MA; 2775) antibody overnight at 4°C. After the membranes were washed three times, they were incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG at a dilution of 1:3 000 (Beijing Zhongshan Golden Bridge Biotechnology, China) for 2 h at room temperature. Specific antibody binding was detected by electrochemiluminescence. The density of the scanned protein bands was measured by ImageJ 1.44p

software and the results were presented as a percentage of the loading control.

Quantitative Real-Time Reverse-Transcription PCR

The mRNA expression of Beclin-1 and LC3 was assessed by real-time PCR with SYBR Green (Sigma) detection in the Mx3005 real-time PCR system (Atratagene, CA). Total RNA was extracted from the spinal tissue at different time points using TRIzol reagent (Invitrogen Life Technologies, 15596-026). Total RNA (3 μg) was reverse-transcribed into cDNA. The thermal profile for SYBR Green PCR was 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing/extension at 60°C for 20 s each. The primer sequences were as follows: LC3, sense: 5'-CATGCCGTCCGAGAAGACCT-3' and antisense: 5'-GATGAGCCGGACATCTTCCACT-3' (GenBankTM accession number, NM022867.2); Beclin-1, sense: 5'-TTGGCCAATAAGATGGGTCTGAA-3' and antisense: 5'-TGTCAGGGACTCCAGATACGAGTG-3' (GenBankTM accession number, NM001034117.1). Samples were normalized against GAPDH (Cell Signaling; 2118) expression to ensure equal loading. The specificity of the amplified product was monitored by its dissociation curve. The results, expressed as fold difference in the number of LC3 or Beclin-1 copies relative to the number of GAPDH gene copies, were determined by the relative quantitative $2^{-\Delta\Delta Ct}$ method. ΔΔCt = ΔCt (target gene) − ΔCt (GAPDH); ΔCt (target gene) = Ct (experimental - target) − Ct (control - target); and ΔCt (GAPDH) = Ct (experimental - GAPDH) − Ct (control - GAPDH).

Tissue Preparation

At 2 h and 42 days after SCI, and immediately after sham operation, the animals were anesthetized with 10% chloral hydrate (0.3 mL/100 g, i.p.) and transcardially perfused with 0.1 mol/L PBS, followed by 4% paraformaldehyde (PFA) in 0.1 mol/L PBS (pH 7.4). Then the spinal tissue was removed and post-fixed in 4% PFA for 6–8 h, cryoprotected in 30% sucrose in 0.1 mol/L PBS for 72 h, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and sectioned at 10 μm on a cryostat (CM3050 S, Leica, Deerfield, IL). Every 50th section was collected and placed on microscope slides coated with poly-*L*-Lysine. We collected nine sequential sections, 4 mm in length, centered at the epicenter, at 0.5-mm intervals along the length of the cord. These sections were stored at −20°C until use for immunofluorescence staining, Nissl staining, or myelin sheath staining.

Immunofluorescence Staining

Frozen sections at the epicenter collected 2 h after injury were washed in 0.1 mol/L PBS (pH 7.35) containing 0.3% Triton X-100 for 10 min, blocked with 3% milk and 5% FBS in 0.1 mol/L PBS for 1 h at room temperature, and then incubated with rabbit anti-LC3 antibody (1:400; Cell Signaling) and mouse anti-NeuN (1:100; Millipore, Billerica, MA, MAB377) overnight at 4°C. After rinsing in PBS, the sections were incubated with a mixture of goat anti-rabbit TRITC and goat anti-mouse FITC fluorescence-conjugated secondary antibodies (1:100, Beijing Zhongshan Golden Bridge Biotechnology) for 40 min at room temperature. The ventral horn in these slides was observed under a laser confocal microscope (Olympus, FV1000).

Histological Assessment

Nine sections collected at 42 days after injury were Nissl-stained to detect ventral horn motoneurons. In brief, the sections were placed in 1:1 alcohol/chloroform overnight, then rehydrated with 95% ethanol to distilled water, and stained in 0.1% cresyl violet for 10 min at 37°C. After rinsing in distilled water, sections were differentiated with 95% ethanol for 1 min, dehydrated in 100% ethanol for 2 × 2 min, cleared in xylene for 2 × 2 min and covered by a resinous medium. The area for counting motoneurons was defined by drawing a horizontal line from one side of the transverse section to the other passing through the central canal and the numbers of ipsilateral ventral horn motoneurons were calculated by ImageJ 1.44p software[26].

To evaluate the extent of spared myelin in white matter, the cross-sections were stained with Luxol fast blue (LFB) and examined 42 days after SCI. In brief, the sections were placed in 95% ethanol for 5 min and stained in 0.1% solvent blue 38 (Sigma) in acidified 95% ethanol in a 57°C oven overnight. After rinsing off excess stain with 95% ethanol and distilled water, sections were differentiated with 0.05% lithium carbonate and 70% ethanol several times until the gray matter was largely unstained and white matter sharply defined. Sections were rinsed in distilled water and then counterstained with cresyl violet for 30–40 s. Then the sections were differentiated in 95% ethanol for 1 min

after rinsing in distilled water, dehydrated in 100% ethanol for 2 × 2 min, cleared in xylene for 2 × 2 min and covered by a resinous medium. The images (×40 magnification) were captured using an Olympus digital camera. The LFB-positive area (blue) of spared white matter was analyzed by ImageJ 1.44p[26], and the ratio of LFB-stained residual white matter to normal white matter was calculated.

Behavioral Testing

The motor function of the hindlimbs was evaluated using the Basso-Beattie-Bresnahan (BBB) scale[27] for six weeks following SCI. The BBB locomotor rating scale is widely used to test the behavioral consequences of SCI in rats[28]. The BBB values were determined by three trained observers who were unaware of the experimental procedures, and averaged.

Statistical Analysis

Data are expressed as mean ± SD, and were analyzed with one-way ANOVA with Bonferroni *post hoc* analysis. BBB scores were analyzed with repeated measures ANOVA followed by the Tukey-Kramer test. All statistical values were calculated using SPSS 16.0. P <0.05 was considered to be statistically significant.

RESULTS

Expression of LC3 and Beclin-1 Increased in SCI Rats

To investigate the changes in autophagy at different times after SCI, the mRNA expression and protein levels of Beclin-1 and LC3 were measured by real-time PCR and Western blot analysis respectively. The level of autophagy increased (P <0.05) at 1, 2, and 6 h after injury compared with the sham-operated group. Both the protein and mRNA levels of LC3 and Beclin-1 peaked at 2 h post-surgery *versus* the sham group (P <0.01) (Figs. 1 and 2).

VPA Reduced the Levels of LC3 and Beclin-1

Western blot analysis showed that VPA treatment decreased the Beclin-1 and LC3-II protein levels at 2 h after SCI compared with the vehicle-treated rats (P <0.05, P <0.01; Fig. 3A–C). Immunofluorescence staining of the epicenter sections also showed that at 2 h after the injury, LC3 was increased compared with the sham operation, and displayed a punctate pattern in the cell soma. VPA decreased the LC3 staining, as revealed by decreased numbers of LC3 puncta under confocal microscopy (Fig. 3D).

VPA Promoted Neuroprotection Following SCI

At 42 days after injury, there was a trend of increase in the number of motoneurons near the injury epicenter in VPA-treated rats (relative to the vehicle control). VPA increased the number of motoneurons at 2 mm both rostral and caudal to the epicenter (P <0.05 *vs* vehicle control) (Fig. 4C). Representative photomicrographs of motoneurons from VPA-treated and vehicle-treated rats are shown in Fig. 4A and B.

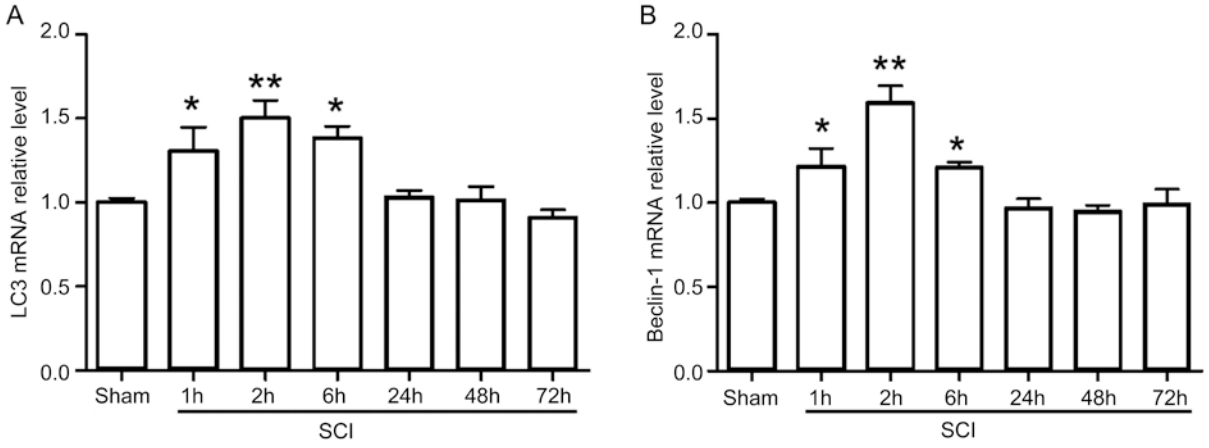
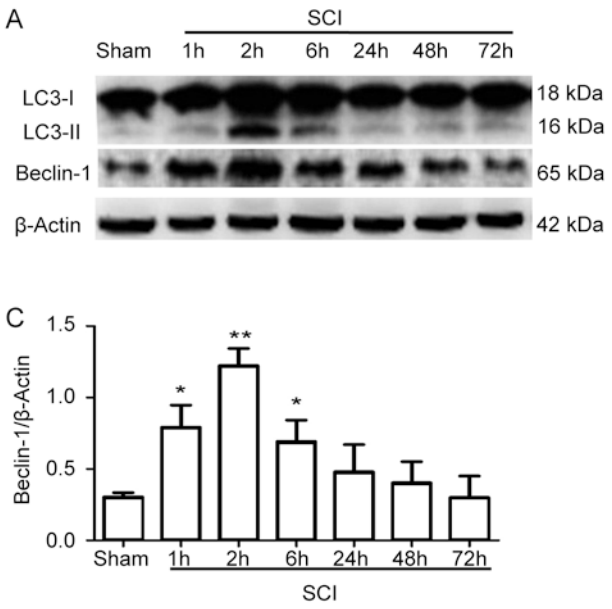


Fig. 1. Spinal cord injury (SCI) increased LC3 and Beclin-1 mRNA levels. The levels of LC3-II and Beclin-1 mRNA were detected at various times after SCI by real-time PCR. mRNA expression of LC3-II (A) and Beclin-1 (B) was significantly increased at 1, 2, and 6 h, peaking at 2 h after SCI, compared with the sham-operated group. Mean ± SD (n = 6/group). *P <0.05, **P <0.01 *vs* sham control.



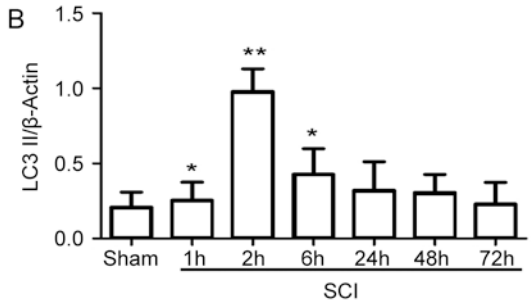


Fig. 2. Spinal cord injury (SCI) increased LC3 and Beclin-1 protein levels. A: Western blots of LC3 and Beclin-1 at different times after SCI. B and C: Quantitative analysis. The levels of LC3-II and Beclin-1 were upregulated at 1, 2 and 6 h *versus* the sham-operated group and peaked at 2 h after SCI. Mean ± SD (*n* = 6/group). **P* <0.05, ***P* <0.01 *vs* sham control.

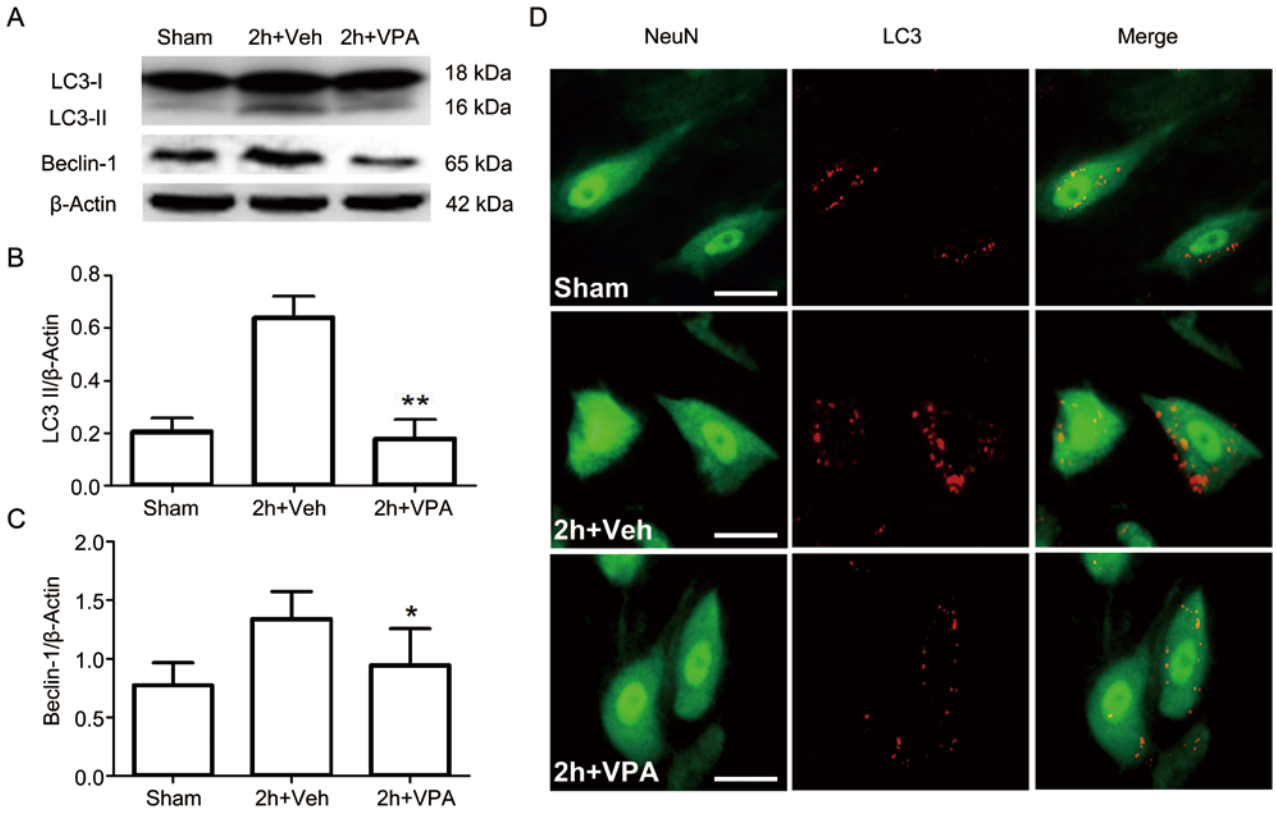


Fig. 3. VPA reduced autophagy activation. A: Western blots of LC3 and Beclin-1 proteins in the spinal cord at 2 h after SCI. B and C: Quantitative analysis of Western blots revealed that VPA reduced the protein levels of LC3-II and Beclin-1 at 2 h after SCI. Mean ± SD (*n* = 6/group). **P* <0.05, ***P* <0.01 compared to the vehicle group. D: Representative confocal images of LC3 and NeuN staining in sham control, vehicle-treated, and VPA-treated animals. Vehicle-treated rats exhibited increased staining of LC3 compared with sham control at 2 h after SCI. VPA decreased the staining *versus* vehicle control (*n* = 3/group; scale bars, 50 μm).

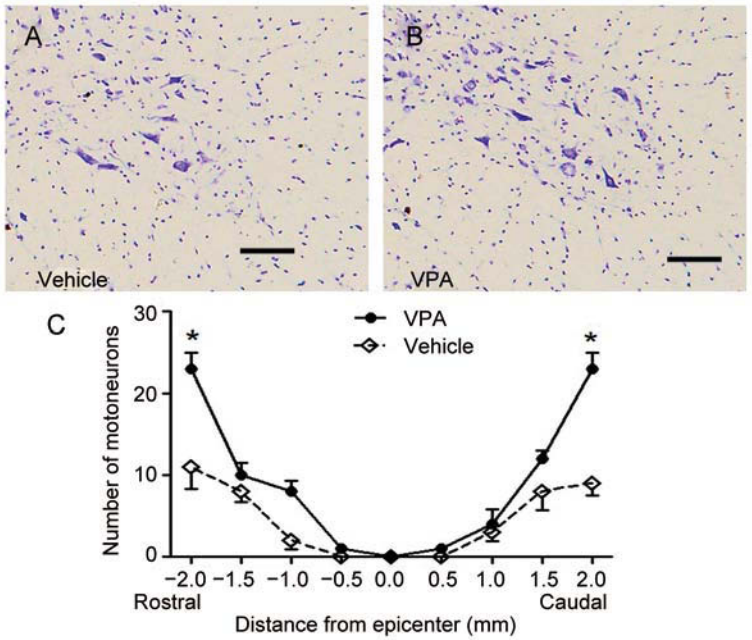


Fig. 4. Valproic acid (VPA) reduced motoneuron loss after spinal cord injury (SCI). A and B: Representative microphotographs showing Nissl-stained ventral horns at 42 days after SCI. Sections located 2 mm rostral to the epicenter revealed that VPA increased the number of motoneurons. C: The numbers of motoneurons in the VPA-treated animals were higher than those in the vehicle-treated animals at 2 mm both rostral and caudal to the injury epicenter (*P <0.05, n = 5 per group, scale bars, 200 μm).

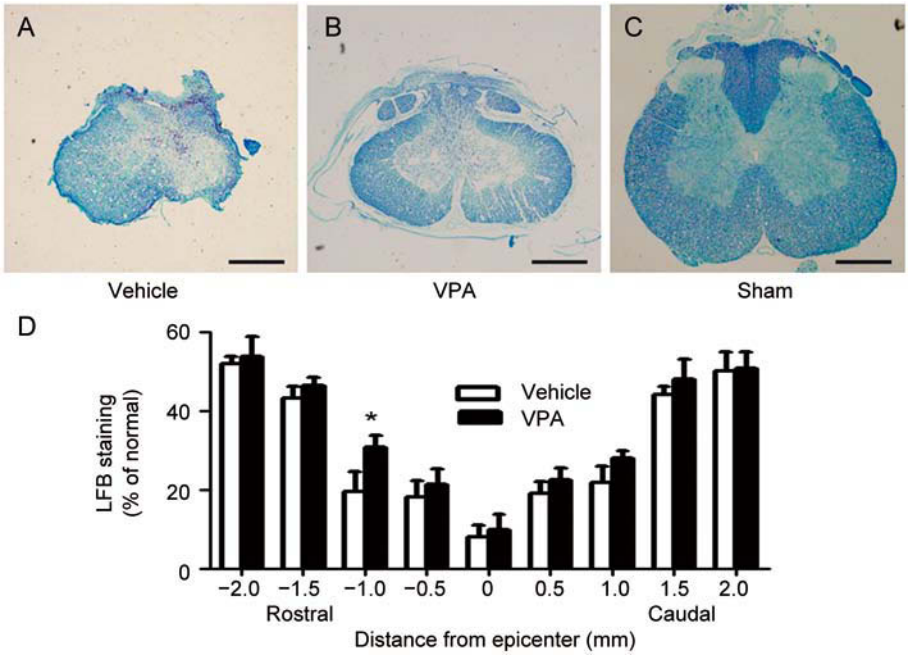


Fig. 5. Valproic acid (VPA) reduced myelin sheath damage following spinal cord injury (SCI). A–C: Compared with the vehicle-treated group, the luxol fast blue (LFB) staining of sections 1 mm rostral to the epicenter at 42 days after SCI showed a remarkable reduction in the area of demyelination in the VPA-treated group. The total areas of tissue preserved in the VPA-treated and vehicle-treated groups apparently declined compared with the sham-operated group. D: Quantification of LFB-positive spared myelin. The area of spared myelin sheath at 1 mm rostral to the epicenter in the VPA-treated animals was greater than that in the vehicle-treated animals. *P <0.05, n = 5/group. Scale bars, 500 μm.

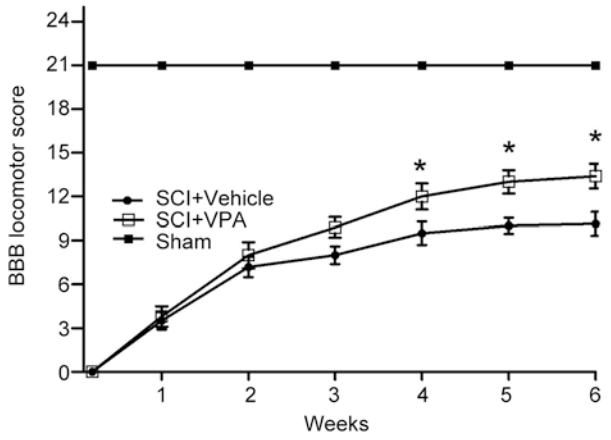


Fig. 6. Valproic acid (VPA) promoted functional recovery after SCI. The BBB score was evaluated once a week for six weeks after surgery. The scores were significantly higher in the VPA-treated group than in the vehicle-treated group from weeks 4 to 6. Mean ± SD (P* <0.05 *vs* vehicle treatment, *n* = 5/group).**

VPA also reduced myelin sheath damage 1 mm rostral to the epicenter at 42 days after SCI (Fig. 5B), as reflected by LFB staining compared with the vehicle control (Fig. 5A). The total area of tissue preserved in injured rats receiving VPA or vehicle was apparently smaller than that in the sham control (Fig. 5C). VPA increased the LFB-positive spared myelin sheath ratio (30.96 ± 0.58% *vs* 19.67 ± 0.83% in vehicle control, P <0.05, Fig. 5D) at 1 mm rostral to the epicenter at 42 days after SCI.

VPA Promoted Functional Recovery Following SCI

To assess the effect of VPA on hindlimb recovery from SCI, the BBB score was measured once a week for six weeks (Fig. 6). From weeks 4 to 6 after injury, the scores in the VPA-treated animals were consistently higher than those in the vehicle-treated animals. At 42 days, the scores declined in the VPA-treated and vehicle-treated groups compared with the sham-operated group, but the VPA-treated animals had higher scores than the vehicle-treated animals (BBB scores: Sham, 21; VPA, 13.39 ± 0.85; Vehicle, 10.13 ± 0.81, P <0.05 for both comparisons; Fig. 6).

DISCUSSION

In the present study, we demonstrated that the characteristics of autophagic expression in the thoracic spinal cord increased at 1, 2, and 6 h after SCI, and administration of VPA markedly decreased the Beclin-1 and LC3 protein levels at 2 h after SCI. As expected, VPA significantly reduced neuronal damage and improved locomotor function after SCI. To our knowledge, these are the first findings on a link between VPA-mediated neuroprotection and autophagy following SCI.

All forms of cell death (autophagy, apoptosis, and necrosis) can occur in neurons after cerebral ischemia[13]. Although autophagy and apoptosis spread into the lesion penumbra later than the necrosis that occurs at the injury epicenter after SCI, they can promote secondary cell death[9,15].

Autophagy is a physiological phenomenon that plays an important role in maintaining cellular homeostasis. Upon starvation or injury, autophagy is initiated to engulf damaged organelles and proteins to provide additional energy; however, in pathological conditions, autophagy may promote secondary cell death and become another form of cell death[9,29]. Previous research has demonstrated that Beclin-1 and LC3, biological markers of autophagy, can be detected in spinal tissue after SCI[9,16,30]. Beclin-1 is a unique autophagy-related protein, thought to be important in mediating the localization of other autophagy proteins to pre-autophagosomal structures[31]. Therefore, it is commonly used for autophagy detection. LC3, a mammalian homolog of yeast Atg8, is the most important and reliable marker in autophagy research[32]. In the formation of the autophagy membrane, the conversion of LC3-I to LC3-II is considered to be one of the symbols of autophagic induction, so LC3-II is often used as a marker for autophagosomes[16]. A recent study found that the expression of Beclin-1 protein increases and autophagic cell death is promoted after SCI in a mouse model of spinal cord hemisection injury[9]. Similar to previous studies[9,30], here the expression of Beclin-1 increased sharply after contusion injury. The level of LC3-II was raised at 1 h after SCI, peaked at 2 h, and then decreased. The time-course of LC3-II change in our study is consistent with the finding by Chen[16], but not with another report showing that the rise of LC3-II is relatively late after SCI[15]. This discrepancy may reflect differences in the model.

To minimize the secondary cell death after SCI, it is imperative that drug therapy is performed promptly. VPA,

a commonly-used drug for treating epilepsy and bipolar disorder, has been used for many years and there is no doubt about its safety. It is one of the HDAC inhibitors, enhances histone acetylation, and this results in chromatin structuring and gene regulation[21,33]. In the present study, we showed that autophagic activity was markedly inhibited by VPA, possibly by lowering the Beclin-1 and LC3-II protein levels. Immunofluorescence and laser confocal microscopy also demonstrated that the LC3 fluorescence that was located in the cytoplasm was markedly decreased at the epicenter in the VPA group. These data indicate that autophagic cell death is reduced by inhibiting autophagic activity by VPA.

Previous studies showed that VPA promotes the repair of sciatic nerve injury[18], has anti-inflammatory and neuroprotective effects[25] and increases the expression of Bcl-2 and Hsp70 to inhibit apoptosis[21]. Combined with our results, these observations show that VPA has multiple roles in SCI. In the present study, VPA improved functional recovery after injury, significantly decreased the demyelinated area, and increased the number of spared motor neurons. Therefore, we suggest that VPA-induced neuroprotection and functional recovery following SCI may be, at least in part, associated with the inhibition of SCI-induced autophagy activation.

Further research is warranted to verify the specific role of autophagy in VPA-mediated neuroprotection after SCI. Besides, although Fu *et al.*[22] showed that the ERK pathway is involved in VPA-induced autophagy in glioma cells, the specific signaling pathways for VPA inhibition of autophagy after injury remain to be determined and need further studies. In conclusion, our results suggest that VPA inhibits injury-induced autophagy. Treatment using VPA for SCI may provide a new strategy to reduce secondary damage.

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