

# Histamine Promotes Locomotion Recovery After Spinal Cord Hemisection *via* Inhibiting Astrocytic Scar Formation

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## Keywords

Astrocyte; Histamine; Scar; Spinal cord injury.

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## SUMMARY

**Aim:** This study investigated whether histamine could play a protective role in pathophysiological response of spinal cord injury (SCI) and regulate the glial scar formation. **Methods:** Functional assessment and histological analyses were performed to investigate the effect of histamine after SCI. Histidine decarboxylase knockout (HDC<sup>-/-</sup>) mice were used to confirm the action of histamine. Selective antagonists for H1 and H2 receptors were utilized *in vivo* and *in vitro* to verify the functional properties of histamine on astrogliosis. **Results:** The local administration of histamine significantly attenuated the tissue damage and glial scar formation after SCI. In particular, the astrogliosis and neurocan expression found around the lesion were significantly suppressed by histamine. Immunofluorescent staining for neurofilament showed that histamine promoted axonal growth across the glial scar. The HDC<sup>-/-</sup> mice, lacking in endogenous histamine, showed lower behavior score, increased lesion size and astrogliosis, as compared with the wild types. The effect of histamine on locomotor recovery and reactive astrogliosis is reversed by H1 receptor antagonist but not H2 receptor antagonist. **Conclusions:** Our results indicate that histamine significantly improved the chronic locomotor recovery *via* attenuating astrogliosis after SCI by stimulating histamine H1 receptor. This study highlights a therapeutic potential of histamine and its related drugs for SCI.

## Introduction

Spinal cord injury (SCI) is a serious high disabling disease which results in motor and sensory dysfunction, and even ends people's lives, yet no effective therapeutic approaches have been developed so far. The complicated pathophysiological process in and around the lesion site limits the development of agents. The mechanisms of injury to the spinal cord, including the "primary injury" and "secondary injury", determined the extent of tissue injury and dysfunction of body. The primary injury results in structural disturbance for physical and mechanical trauma [1], followed by secondary injury comprising an inflammatory process and formation of glial scar [2].

Glial cells especially astrocytes are contributed to the glial scar formation around the lesion cavity [3]. Upon the stimulation of inflammatory factors after SCI, astrocytes are activated and proliferated along with the overexpression of glial fibrillary acidic protein (GFAP) and hypertrophied morphology change [4]. Although reactive astrocytes may play a number of beneficial effects by restricting inflammation and promoting wound healing [5,6], glial scar is regarded as one of the major factors blocking spontaneous axon regeneration [7]. The suppression of glial

scar may reduce tissue damage and improve functional recovery [8]. The mice deficient for both GFAP and vimentin recovered substantial locomotor function after a severe SCI [9]. For these reasons, targeting astrogliosis becomes a therapeutic strategy for SCI.

Some studies have shown that astrocytes separated from mammalian brain and spinal cord express histamine receptors [10,11]. Our previous studies revealed that histamine can upregulate expressions of astrocytic glutamine synthetase (GS) and glutamate transporter GLT-1, resulting in neuroprotection against excitotoxicity [12,13]. These data indicate that histamine may be a regulator of astrocyte function during CNS injury. Numerous studies have proved that histamine participated in ischemic brain pathology. The results both *in vivo* and *in vitro* showed that histamine exerts protective effect in brain ischemic pathology [14], probably involving targeting to astrocytes [12]. In SCI, it has been reported that histamine level increases in the traumatized segment at 2 h after injury [15], which may contribute to the increase of the sensitivity of vessels to other vasoactive substances [16]. However, the role of histamine in the functional recovery after SCI, especially its action on glial scar formation remains unclear.

This study is aimed to investigate whether histamine could exert a neuroprotective activity in pathophysiological response of SCI and regulate glial scar formation using two independent approaches including exogenous application of histamine and knockout of endogenous histamine.

## Materials and Methods

### Animals

All experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were in accordance with the Animal Care and Use Committee of School of Medicine, Zhejiang University. Adult female Sprague–Dawley rats weighing 220–250 g were used. Both wild-type (HDC<sup>+/+</sup>) and HDC<sup>-/-</sup> female C57BL/6 mice weighing 22–27 g were used. From 15 day before experiments until sacrifice, HDC<sup>-/-</sup> mice were fed with special chow low in histamine (0.5 µg/g).

### Surgical Procedures

Chloral hydrate (400 mg/kg, intraperitoneally, i.p.) was used to anesthetize animals. The T10 laminectomy was exerted to expose spinal cord. The right hemisection was performed with the tip of an iridectomy scissors (cut twice to make sure complete disconnection), and the injury of dorsal blood vessel should be avoided. This hemisection model was chosen because it created a controlled lesion location. Furthermore, this model facilitates the formation of glial scars in a consistent manner. Immediately after the injury, histamine was applied directly (0.1 nmol or 1 nmol in 10 µL) at the injured site using gelatin sponge (gelfoam, Jinling, Nanjing, China) as a carrier. The animals in vehicle-SCI group were topically treated with saline-soaked gelfoam after SCI. In the antihistamine receptor treatment groups, diphenhydramine (2, 10 mg/kg) or cimetidine (2, 10 mg/kg) was injected 30 min i.p. before operation and once daily for the next 1 weeks. After operation, animals were return to individual cages at 22–25°C and injected with penicillin sodium (80,000 U/day) for up to next 7 days. Manually pressing on bladders was performed twice daily until return of reflexive bladder control.

### Locomotion Recovery Assessment

#### Open-Field Scoring

Hindlimb motor behavior was estimated with the Basso–Beattie–Bresnahan (BBB) [17] scale before the operation and at 1, 7, 10, 14, 21, and 28 days postoperation (dpo) in rats. The Basso mouse scale (BMS) [18] was used to estimate the behavior recovery for all mice at 7, 14 dpo. Briefly, animals were placed individually in an open field so that they can move freely at least for 4 min. Two independent observers scored the performance of ipsilateral hindlimb.

#### Footprint Analysis

Footprint analysis was performed following the procedure of Karimi-Abdolrezaee *et al.* [19]. Briefly, the forepaws of the

animals were inked with red color, and their hindpaws were inked with dark-blue color. The animals were allowed to walk along a runway (7 cm width, 100 cm length) which was previously covered with blank article. Hindlimb footprints were used to determine the aberrant gait by calculating the angle rotation (AR) and interlimb coordination (ILC). The AR was assessed by testing the angle formed by two lines, one connecting the third toe and center of the paw pad; the other one connecting the stride line and center of the paw pad. The ILC was assessed by measuring the distance between the central pads of the forelimb and hindlimb on ipsilateral side of the body.

### Histology and Immunohistochemistry

At different time point, animals were killed and perfused with 4% paraformaldehyde (PFA). A 1.5-cm-long segment containing lesion epicenter was separated, postfixed overnight and then cryoprotected in 30% sucrose for processing into 16 µm longitudinally frozen sections by acryostat (SM2000R, LEICA, Wetzlar, Germany). For HE stain, the sections were stained with hematoxylin solution (2 min) and followed by eosin (10–20 second). For Luxol fast blue (LFB) stain, the sections were stained with LFB solution (Solvent blue 38, Sigma-Aldrich, St. Louis, MO, USA) at 56°C overnight. The stained images were used to assess the preserved myelinated area using the Image J software. For immunofluorescence, primary antibodies were added onto sections at 4°C overnight as follows: mouse anti-GFAP (1:300, EMD Millipore, Billerica, MA, USA), rabbit anti-GFAP (1:300, Boster, Wuhan, China), rabbit antineurocan (1:200, Boster), and mouse anti-NF200 (1:100, Abcam, Cambridge, UK). After washing with phosphate-buffered saline repeatedly, sections were incubated sequentially in Alexa 488 anti-rabbit IgG (1:400, Invitrogen, Eugene, OR, USA) or Alexa 594 anti-mouse IgG (1:400, Invitrogen) for 2 h. Images were acquired under fluorescent microscopy (Olympus BX61, Tokyo, Japan). Using Image-Pro Plus program, the mean fluorescence intensity of images (four areas per section) was assessed (corrected by cell number) and normalized to obtain relative ratios that were compared between experimental groups. To estimate the thickness and volume of glial scar, 3, 3'-diaminobenzidine (DAB) histochemistry staining of GFAP was performed in serial sections spaced 200 µm apart using a SABC kit (Boster), and DAB was used as a chromogenic agent. Mean thickness of glial scar was calculated from fixed four areas per section. The scar volume was estimated according to the Cavalieri principle [20].

### Western Blot Analysis

To quantitate the GFAP protein levels, animals were killed and perfused with ice-cold saline at 2 week after surgery. Five millimeter length of spinal cord segments containing lesion epicenter was quickly taken out and then lysed with homogenization buffer. Equal amount (40 µg) of tissue lysates was separated by 10% gels and transferred onto a nitrocellulose membrane. The membranes were incubated with primary antibodies (mouse anti-GAPDH or rabbit anti-GFAP) at 4°C overnight followed by IRDye 800 anti-rabbit or IRDye 700 anti-mouse antibodies for

2 h. Images were captured by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

### Cell Scratch Injury

Primary astrocyte cultures were separated from cerebral cortex of 1 day postnatal Sprague–Dawley rats as described [21]. The GFAP-positive cells in these cultures were more than 95% (data not shown). Astrocytes were plated on PLL-coated slides in 24-well plates. After the cells were grown to confluence, mechanical injury was performed using a sterile pipette tip (200  $\mu$ L).

### Proliferation Assay

For labeling the proliferation cells, 5-bromo-2-deoxyuridine (BrdU) incorporation assay was performed as described [22]. After scratching injury, different concentrations of histamine ( $10^{-7}$ – $10^{-5}$  M) were added into astrocyte cultures for 24 h. And 10  $\mu$ M BrdU (Sigma-Aldrich) was added for the last 18 h before the experiment was finished. After fixed with 4% PFA, astrocytes were incubated in 1 M HCl and then neutralized using 0.1 M sodium borate (pH 8.5). Then, the slices were incubated with anti-BrdU antibody (1:600, Sigma-Aldrich) and sequentially secondary antibody (Alexa 594 anti-mouse IgG, 1:400, Invitrogen). Images were captured under fluorescent microscopy. Cell proliferation was estimated as ratio of BrdU<sup>+</sup> cells to DAPI<sup>+</sup> cells. To investigate the antiproliferation effect of histamine, histamine H1-specific antagonist diphenhydramine ( $10^{-6}$ – $10^{-4}$  M), and H2 specific antagonist cimetidine ( $10^{-6}$ – $10^{-4}$  M) were added 30 min before histamine. After treatment with or without histamine for 24 h, cell proliferation index was determined as above.

### Statistical Analysis

The Student *t*-test was used for single comparison, one-way ANOVA followed by Tukey's *post hoc* tests was used for multiple comparisons, and repeated measures two-way ANOVA followed by least-significant difference's *post hoc* tests was used to analyze the locomotor behavioral data. All values are expressed as means  $\pm$  SEM.

## Results

### Exogenous Delivery of Histamine Reduced Lesion Size and Improved Hindlimb Functions after SCI

To investigate whether histamine plays a relevant role in the pathophysiology following SCI, we first evaluated the morphological changes and recovery of motor function after topical delivery of histamine following SCI (Figure 1). It has been reported that following incomplete SCI a large cyst with amorphous material, trabeculae and liquid surrounded by a glial scar has formed at 4 weeks, and remained stable even at 8 and 16 weeks after SCI [23]. Moreover, many studies have shown a strong relationship between the severity of SCI and the recovery of motor functions based on behavioral assessments [24]. So, the histological outcomes and functional recovery were assessed at 4 weeks after SCI. The lesion cavitation measured from HE staining and GFAP im-

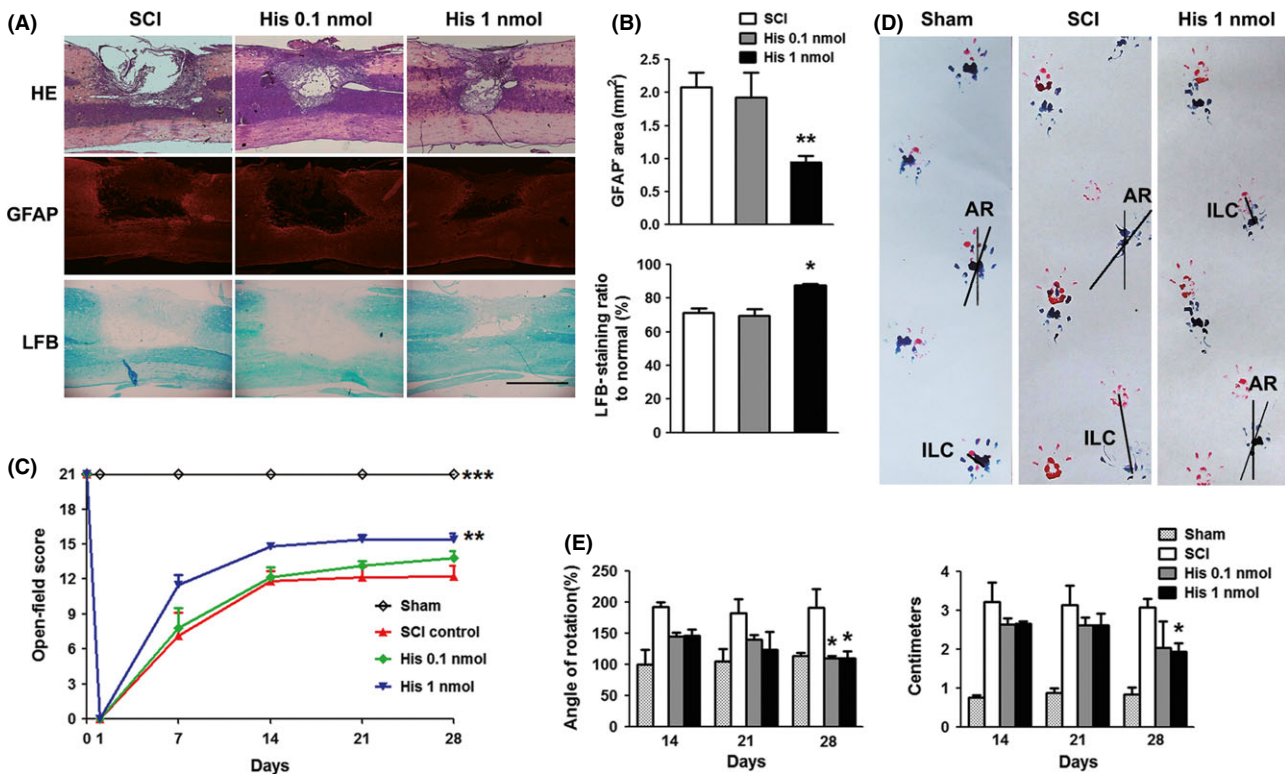
munostaining was significantly smaller in histamine treatment (1 nmol) group compared with SCI group at 4 weeks after SCI ( $P < 0.01$ ). Moreover, histamine delivery markedly increased the LFB-stained myelinated area which was probably related to increased preservation of myelin and/or increased remyelination ( $P < 0.05$ , Figure 1A,B).

To determine whether histamine improved functional recovery after SCI, two independent behavioral tasks, including 21-point BBB locomotor rating scale and footprint analysis, were used. Functional recovery was evaluated before operation and 1, 7, 14, 21, 28 dpo. Before operation, all the animals showed normal locomotion, corresponding to a BBB scale of 21. On the 1 dpo, the animals dragged the injured hindlimb, and their behavior score was 0. During the first 14 dpo, recovery of locomotion proceeded rapidly and then maintained at a relatively slower pace thereafter. Compared with SCI group, animals from two histamine groups recovered faster during the entire experimental period. One nmol dose of histamine was more effective than 0.1 nmol dose. It significantly improved the functional recovery compared with SCI group ( $P < 0.01$ , two-way ANOVA, Figure 1C).

To analyze the animals' gait patterns after injury, AR and ILC were estimated in the footprint test. During the first week, animals in all the groups dragged their right hindpaw, so we examined the footprint test from 14 dpo. Relative to the SCI animals, rats which have received histamine (1 nmol) revealed a markedly behavior improvement in AR and ILC at 28 dpo after injury ( $P < 0.05$ , Figure 1D,E), suggesting that histamine can greatly improve the gait and coordination of forelimb and hindlimb foot placements.

### Exogenous Histamine Delivery Attenuated the Reactive Astroglia after SCI

GFAP is regarded as a sensitive and reliable marker of astrocyte activation after CNS injury [8], so immunohistochemistry analysis of GFAP expression was performed. It has been reported that astrocytic glial scar is fully established by 14 dpo [25], and our results indicated that locomotion recovery reached a plateau around 2 weeks by BBB score after operation (Figure 1C), so we took 14 dpo as a time point for investigating the extent of astroglia. Severe spinal cord trauma resulted in excessively reactive astroglia around the lesion site as shown in Figure 2A. Compared with that in 1 mm proximal area, the GFAP signal intensity was much stronger and the number of GFAP-labeled astrocytes in perilesion dramatically increased (Figure 2A). Histamine markedly reduced the number of reactive astrocytes, downregulated GFAP expression in immunofluorescent staining, and reversed their morphological alteration compared with the SCI group (Figure 2A,B). Furthermore, the expression of GFAP was confirmed by Western blot analysis. We found that histamine markedly attenuated the GFAP expression induced by SCI ( $P < 0.05$ , Figure 2C). To test whether histamine impacts the glial scar formation, the thickness of scar was measured by DAB staining of GFAP. The results showed that the histamine treatment could significantly reduce the mean thickness of scar ( $P < 0.01$ ) compared with SCI group. As it has been reported that the volume of the glial scar negatively correlates with functional recovery and neurogenesis in



**Figure 1** Protective effect of histamine on spinal cord injury. (A) Representative photographs from HE, GFAP, and LFB staining at 28 days postoperation (dpo). Scale bars: 1 mm. (B) The GFAP<sup>+</sup> area and LFB-positive ratio in both groups. n = 5. \**P* < 0.05; \*\**P* < 0.01 versus the SCI group. (C) The Basso–Beattie–Bresnahan (BBB) scores at 1, 7, 14, 21, and 28 dpo. n = 4 for sham group, n = 6 for other groups. \*\**P* < 0.01; \*\*\**P* < 0.001 versus the SCI group. (D) Representative photographs of footprints from sham, SCI, and histamine rats on 28 dpo. (E) Quantitative analyses of the hindlimb angle rotation (AR) and interlimb coordination (ILC) at 28 dpo in both groups. n = 4 for sham group, n = 6 for other groups. \**P* < 0.05 versus the SCI group.

the SCI [26], the volume of glial scar was also estimated. The data showed that histamine treatment reduced the scar volume (*P* < 0.05, Figure 2D,E) compared with SCI group.

The CSPGs secreted from reactive astrocytes are the main inhibitory constituents for axon regeneration. Neurocan, one of the main scar-associated CSPGs, was highly expressed within the lesion core after injury (Figure 2F,G). Compared with SCI group, the mean fluorescence intensity of neurocan was significantly downregulated by 33.9% after histamine treatment (*P* < 0.05), indicating that histamine can inhibit the expression of neurocan.

We then asked whether histamine can promote axonal growth through reducing glial scar. In the SCI group, neurofilament (NF-200)-labeled axons were lost in the epicenter of injury. By contrast, in histamine-treated group a number of NF-200-labeled fibers were present around the lesion site and grown beyond the glial front (dashed lines in Figure 2H). The density of axons was highly increased adjacent to the lesion site compared with SCI group. These data indicated that histamine can inhibit the formation of glial scar, which probably facilitates axonal regeneration.

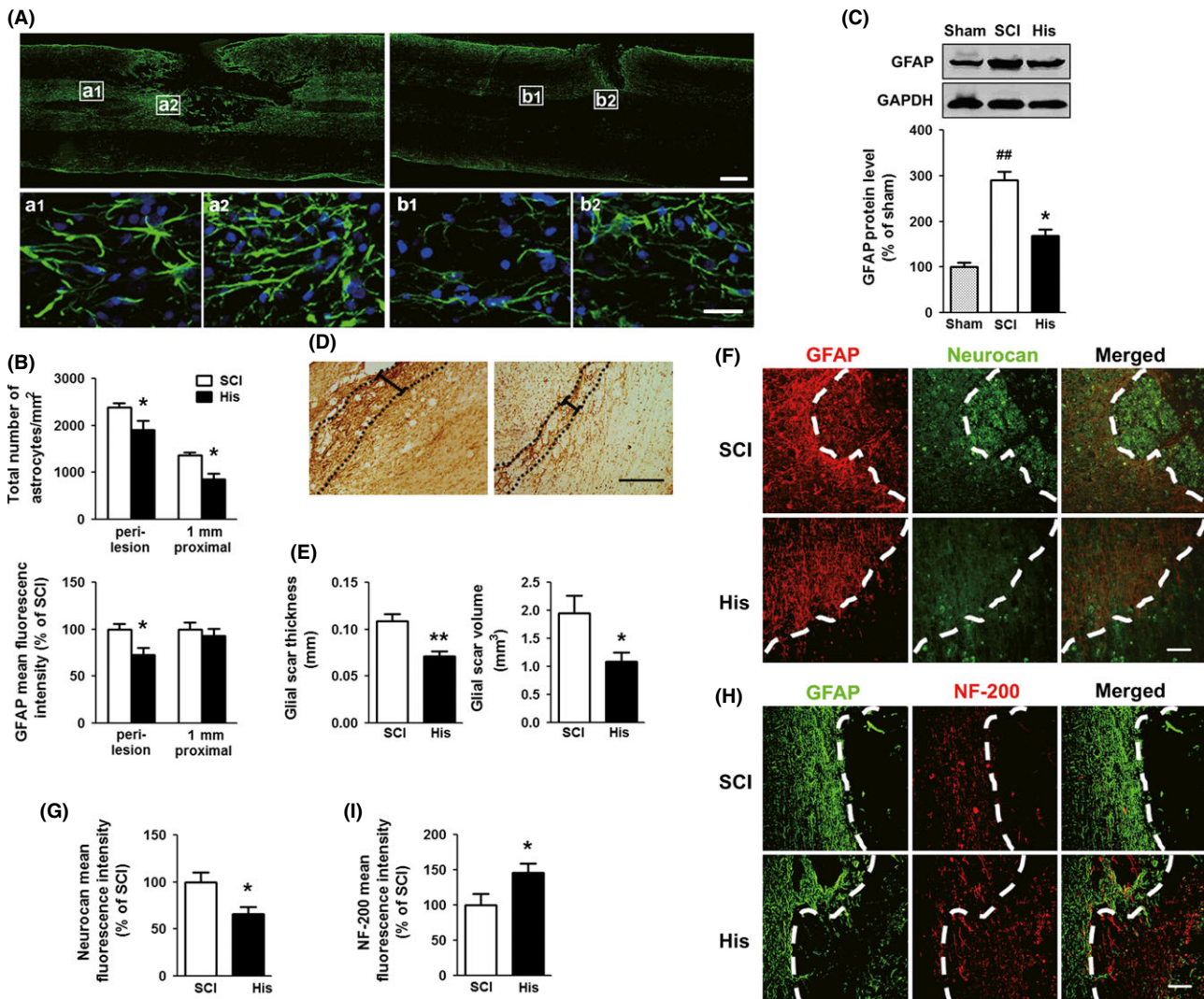
### Functional Recovery and Reactive Astroglia were Aggravated in HDC<sup>-/-</sup> Mice after SCI

To verify the role of histamine in the pathophysiological response after SCI, we investigated the effect of the absence of histamine on

SCI using HDC knockout mice. Wild-type and HDC<sup>-/-</sup> mice underwent a right hemisection after T10 laminectomy, and the lesion area and functional recovery were evaluated for 2 weeks. After hemisection, the injured spinal cord showed structural disturbance from HE staining in both groups. We found that GFAP<sup>+</sup> area and the loss of white matter were significantly less in wild-type mice than that in HDC<sup>-/-</sup> mice (Figure 3A–C). We next determined the reactive astroglia in HDC knockout mice by GFAP immunostaining. It showed that endogenous absence of histamine increased the number of astrocytes (*P* < 0.05) and GFAP expression (*P* < 0.05) around the lesion compared with wild types (Figure 3D,E). The thickness and the volume of glial scar were increased in HDC<sup>-/-</sup> group (Figure 3F,G). Although no discernible differences in BMS behavior performances were found between wild-type and HDC<sup>-/-</sup> mice before operation, HDC<sup>-/-</sup> mice had significantly lower scores in BMS compared with that of wild-type mice after SCI (Figure 3H).

### Histamine Inhibits Reactive Astroglia via Stimulating Histamine H1 Receptor *in vivo* and *in vitro*

Histamine action is believed to be mediated by binding to its specific cell-surface receptors. H1 receptor antagonist diphenhydramine (10 mg/kg, i.p.) reversed the inhibitory properties of

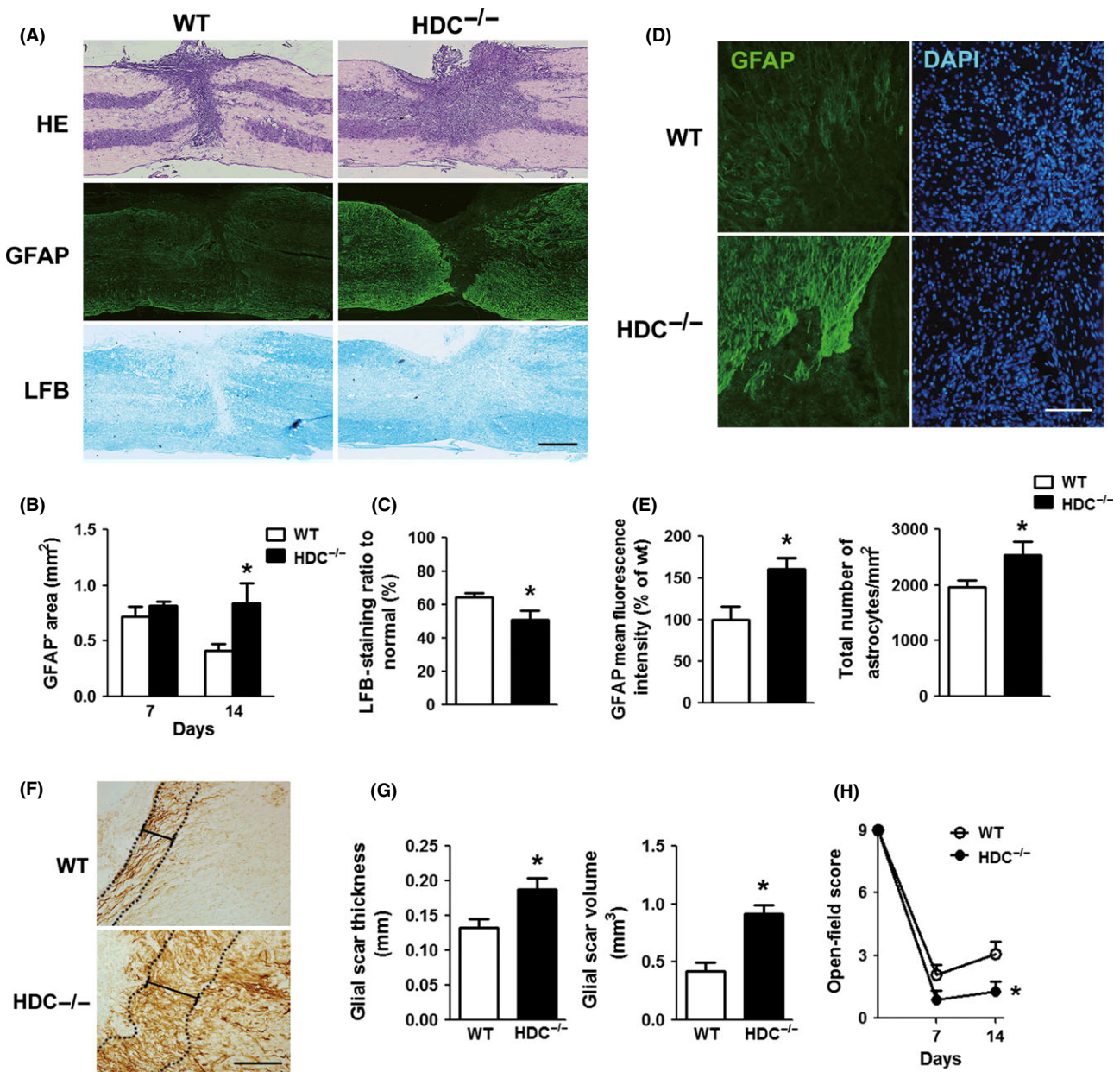


**Figure 2** Effect of histamine on reactive astrogliosis in the damaged spinal cord. The astrocytic gliosis of the rats under the treatment of normal saline or histamine (1 nmol) using gelfoam was analyzed by immunohistochemistry and Western blot examination of GFAP at 14 dpo. **(A)** Representative photographs from GFAP immunostaining. The areas at 1 mm proximal (a1, b1) and close to lesion site (a2, b2) in upper panel are enlarged in the lower panel. Scale bars: 500  $\mu\text{m}$  in upper panel; 25  $\mu\text{m}$  in lower panel. **(B)** Quantitative analyses of reactive astrogliosis around the lesion site and 1 mm proximal to the epicenter, including the number of astrocytes, and the GFAP mean fluorescence intensity which has been corrected by cell number.  $n = 5$ . \* $P < 0.05$  versus SCI group. **(C)** Western blot analysis of GFAP expression.  $n = 3$ . ## $P < 0.01$  versus sham group, \* $P < 0.05$  versus SCI group. **(D)** Representative photographs from DAB staining of GFAP which contain glial scar (dotted lines). The black calibration bar indicates glial scar thickness. Scale bars: 200  $\mu\text{m}$ . **(E)** Quantitative analyses of thickness and volume glial scar.  $n = 5$ . \*\* $P < 0.01$  versus SCI group, \* $P < 0.05$  versus SCI group. **(F)** Representative photographs containing astrocytic fronts (dashed lines) from neurocan immunostaining. Scale bars: 100  $\mu\text{m}$ . **(G)** Quantitative analysis of neurocan mean fluorescence intensity.  $n = 5$ . \* $P < 0.05$  versus SCI group. **(H)** Representative photographs containing astrocytic fronts (dashed lines) from neurofilament (NF-200) immunofluorescence on spinal cord sections. Scale bars: 100  $\mu\text{m}$ . **(I)** Quantitative analysis of NF-200 staining intensity.  $n = 5$ . \* $P < 0.05$  versus SCI group.

histamine on astrogliosis assessed by the number of astrocytes and GFAP expression, and partly reversed the neuroprotective effect of histamine on locomotor recovery measured by open-field score ( $P < 0.01$ , two-way ANOVA). The thickness and volume of scar were significantly increased in rats treated with diphenhydramine, compared with histamine treatment (Figure 4E,F). However, cimetidine, a selective H2 antagonist, had no effect on the

inhibition of glial scar formation induced by histamine even at a high dose of 10 mg/kg (Figure 4), indicating that histamine impedes astrogliosis through activating histamine H1 receptor but not H2 receptor.

To further confirm the effect of histamine on astrogliosis *via* H1 receptor, we used *in vitro* "scratch-wound" model. Treatment of  $10^{-5}$  M histamine resulted in a suppression of proliferation after

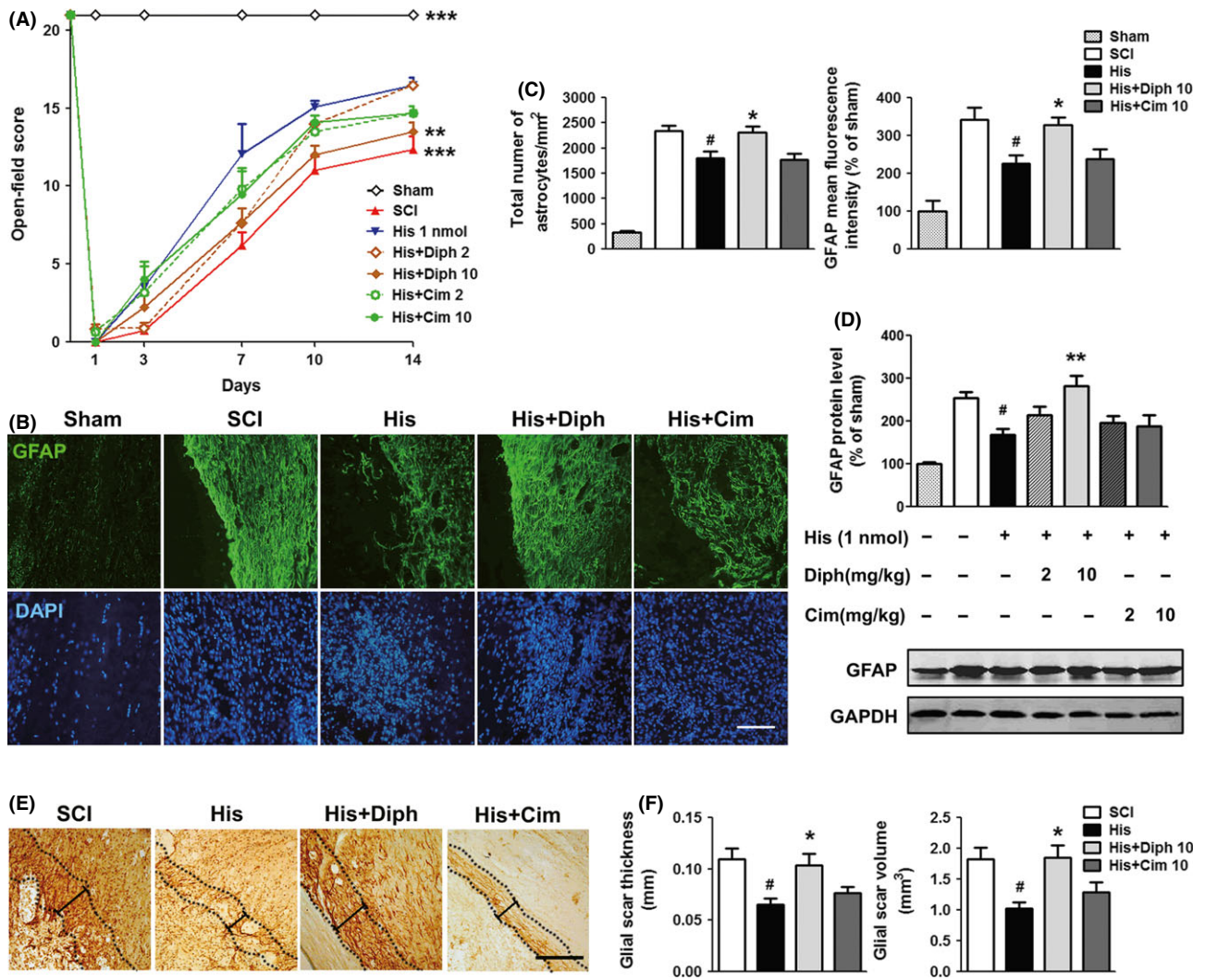


**Figure 3** Aggravation of spinal cord damage in HDC<sup>-/-</sup> mice after SCI. **(A)** Representative photographs from HE, GFAP, and LFB staining at 14 days. Scale bars: 1 mm. **(B, C)** Analyses of GFAP<sup>+</sup> area and LFB-positive ratio in both groups. n = 4. \*P < 0.05 versus the wild type. **(D)** Representative images from GFAP immunostaining at the lesion site on 14 dpo. Scale bars: 100 μm. **(E)** Quantitative analyses of the number of astrocytes and GFAP fluorescence mean intensity (corrected by cell number). n = 4. \*P < 0.05 versus the wild type. **(F)** Representative images containing glial scar from DAB staining of GFAP at 14 dpo. Scale bars: 200 μm. **(G)** Quantitative analyses of thickness and volume of glial scar. n = 4. \*P < 0.05 versus the wild type. **(H)** The Basso mouse scale (BMS) of SCI model mice at 7, 14 dpo. n = 8 for wild type, n = 7 for HDC<sup>-/-</sup>. \*P < 0.05 versus the wild type.

scratched by a plastic pipette tip, showing 23% less BrdU<sup>+</sup>/DAPI<sup>+</sup> cells than controls (P < 0.05; Figure 5A,B). The antiproliferation effect of histamine was reversed by anti-H1 antagonist, but not anti-H2 antagonist, further suggesting that histamine possessed the ability to inhibit astrocyte proliferation via stimulation of H1 receptor (Figure 5C). Moreover, both the anti-H1 antagonist and anti-H2 antagonist had no effect on the astrocyte proliferation in controls.

## Discussion

So far, the role of histamine in pathophysiological response of SCI is still unclear. In this study, we investigated the effect of histamine on long-term recovery after SCI using several methods, including histological examination and behavioral evaluation. Our results showed that histamine markedly decreased lesion area, inhibited glial scar formation, and improved functional



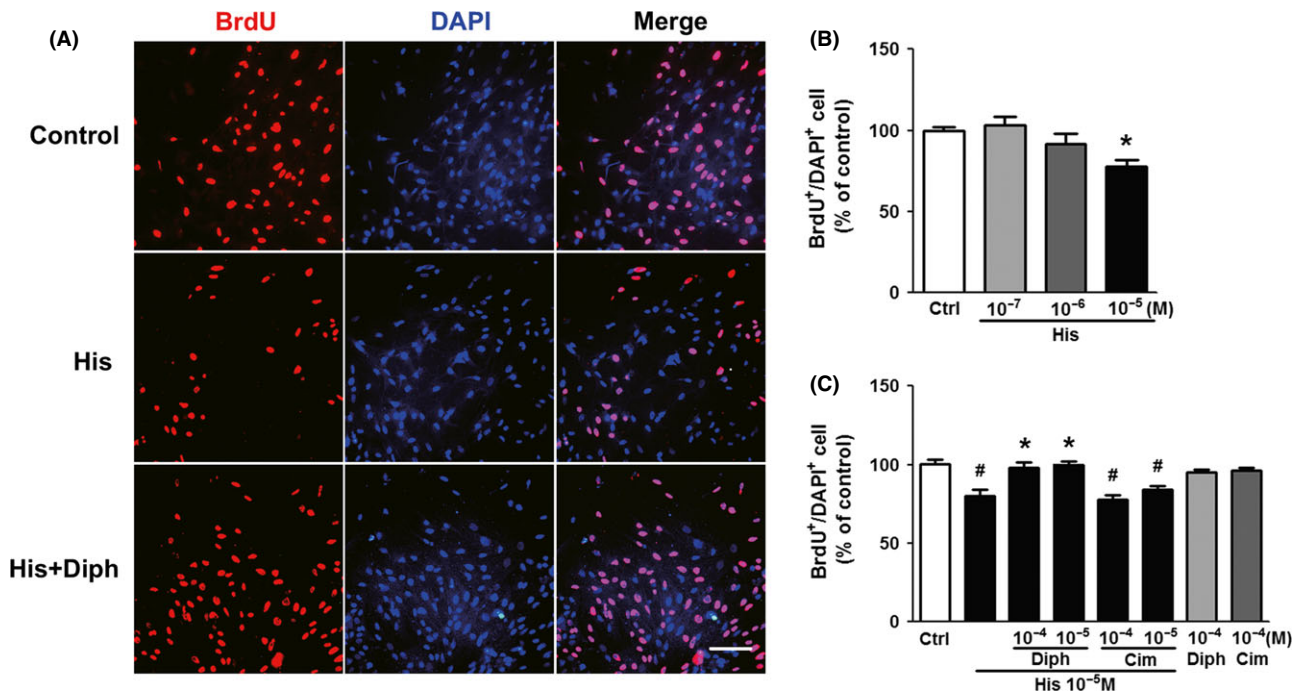
**Figure 4** Inhibitory effect of histamine on reactive astrogliosis via stimulating histamine H1 receptor in SCI. (A) The BBB scores at 1, 3, 7, 10, and 14 dpo. n = 6. \*\*P < 0.01; \*\*\*P < 0.001 versus the histamine group. (B) Representative photographs from GFAP immunostaining of the lesion site at 14 dpo. (C) Quantitative assessments of the total number of astrocytes and GFAP fluorescence mean intensity. n = 4. #P < 0.05 versus SCI group, \*P < 0.05 versus histamine group. (D) Western blot analysis of GFAP level in spinal cord. n = 3. #P < 0.05 versus SCI group, \*\*P < 0.01 versus histamine group. (E) Representative photographs containing glial scar from DAB staining of GFAP. Scale bars: 200 μm. (F) Quantitative assessments of thickness and volume of glial scar. n = 4. #P < 0.05 versus SCI group, \*P < 0.05 versus histamine group.

recovery even 28 day after SCI. It is likely that exogenous delivery of histamine may be a therapeutic strategy for functional recovery following SCI.

There are some reports regarding histamine's action at early stage after SCI, which suggested that histamine can mediate the edema formation within 5 h [27, 28]. In contrast to that, our data indicated that exogenous histamine provided prominent neuroprotection in the long-term injury of SCI. To confirm the role of histamine, we used HDC knockout mice to reveal the role of endogenous histamine in SCI. We found that HDC<sup>-/-</sup> mice, which are absent of histamine, displayed increased astrocyte activation, larger lesion size, impaired myelination, and behavior recovery compared with wild-type mice. Therefore, these results at least indicated that the endogenous histamine

also plays a protective role in the pathophysiological response of SCI.

It is well accepted that excessive scar tissue formation is a major impediment for axonal regeneration both physically and chemically [3], while ablation of the proliferation of scar-forming astrocytes or removing inhibitory CSPGs secreted from reactive astrocytes have been proved to ameliorate functional deficit after injury [29,30]. So, manipulation of astrocytic glial scar may facilitate the axon regeneration and locomotor recovery after SCI. In this study, we demonstrated that physical barriers, including astroglial proliferation, GFAP upregulation, and glial scar formation, were markedly reduced after topical administration with histamine in the hemisection model. In the "scratch injury model", we also found that histamine



**Figure 5** Inhibitory effect of histamine on the proliferation of reactive astrocytes in scratch injury model *in vitro*. (A) Representative photographs from BrdU immunostaining around the lesion site. Scale bar: 100  $\mu\text{m}$ . (B) Quantitative assessments of BrdU-positive astrocytes administrated with different concentrations of histamine at 24 h after scratch. \* $P < 0.05$  versus control. (C) Quantitative assessments of BrdU-positive astrocytes treated with histamine ( $10^{-5}$  M) and its receptor antagonists (Diph  $10^{-4}$ ,  $10^{-5}$  M, Cim  $10^{-4}$ ,  $10^{-5}$  M) for 24 h. # $P < 0.05$  versus control, \* $P < 0.05$  versus histamine ( $10^{-5}$  M) group. Experiments were repeated three times independently.

inhibited the proliferation of astrocytes. Furthermore, we evaluated the expressions of neurocan and phosphacan which are known to be secreted by reactive astrocytes and contribute to the glial scar formation. The topical treatment of histamine markedly inhibited expression of neurocan around the lesion site, but had no significant effect on expression of phosphacan (data not shown). This is similar to other studies both *in vitro* [21] and *in vivo* [31]. We speculate that phosphacan may not be a sensitive indicator of glial scar. In addition, more NF-200-positive fibers and myelination were found at the lesion site after SCI (Figure 1A, 2D). Taken together, our results suggest that exogenous delivery of histamine impairs the physical and chemical barrier induced by glial scar which can subsequently support axonal regrowth beyond the glial scars. Recent studies have shown that reactive astrogliosis limited the inflammatory response [5,32], and conditional ablation of scar-forming astrocytes induced widespread infiltration of inflammatory cells [6]. However, our result showed that histamine treatment reduced the area of Iba1<sup>+</sup> (a marker of microglia/macrophages) cells (Figure S1). It suggested that here the inhibition of glial scar by histamine did not favor the spread of inflammation and even restricted the inflammatory area, although the detailed mechanism is still unclear.

In addition, histamine H1- and H2-specific antagonists (diphenhydramine and cimetidine) were used to verify the participation of histamine in SCI. Diphenhydramine reversed the neuroprotective effect of histamine in BBB scores, while cimetidine had no effect on the protection of histamine. Pretreatment

of diphenhydramine, but not cimetidine, can reverse the inhibitory effect of histamine on astrogliosis. It suggests that the suppression of astrogliosis and glial scar formation by H1 receptor stimulation may account for the alleviation of histological outcome. Although we focused on astrogliosis and glial scar formation in the current research, histamine may provide a multitude of neuroprotective effects relying on its different receptors. It has been reported that histamine could upregulate astrocytic GLT-1 and GS expression *via* stimulating histamine H1 receptor in astrocytes, leading to neuroprotection against excitotoxicity [12,13]. On the other hand, a number of studies showed that the stimulation of H2 receptor in neurons may be protective. Treatment of H2 agonist alleviates the neuronal damage after MCAO, which is aggravated by the H2 antagonists [33,34]. In cultured neuron, histamine protects against NMDA-induced excitotoxicity through stimulation of H2 receptors [35]. Therefore, we raise a notion that the protection of histamine may be accounted for by different receptors in different cell types.

Histamine increases in the brain tissue, spinal cord lesion site, brain blood, and cerebrospinal fluid in various forms of CNS injury [15,16]. In our study, we found that histamine content increased in the lesion segment from 2 h, peaked at 24 h, and returned to normal at 3 dpo in the experimental female rats (data not shown). We speculated that the gradual decrease of histamine boosts the glial scar formation. An observation that astrocyte proliferation usually occurs within 7 dpo [5] indicates that first 7 day may be an appropriate therapeutic time window to intervene against glial scar formation. For this purpose, we



administered histamine topically using gelfoam on the lesion site after surgery. Gelfoam is an absorbable medical device used as a hemostatic and as a carrier to deliver some agents such as thrombin in the clinic [36]. Topical treatment of gelfoam soaked with histamine makes it more convenient for administration. Also, a high concentration and long application term can be achieved without systemic side effect. Indeed, we found that this early topical treatment leads to a persistent increase of histamine content until 7 dpo (data not shown) and is sufficient to attenuate the astrogliosis and improve the functional recovery at 4 weeks after SCI.

In conclusion, our current data are the first to provide direct evidence that histamine significantly improved the chronic locomotor recovery *via* attenuating astrogliosis after SCI by stimulating

histamine H1 receptor. This study highlights a therapeutic potential of histamine and its related drugs for SCI.

## Acknowledgments

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## Conflict of Interest

The authors declare no conflict of interest.

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## Supporting Information

The following supplementary material is available for this article:

**Figure S1.** Effect of histamine on reactive microglia and macrophages in the damaged spinal cord.