

## ORIGINAL ARTICLE

# Corticotrophin-releasing hormone (CRH) facilitates axon outgrowth

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**Objective:** To evaluate the role of corticotrophin-releasing hormone (CRH) in facilitating axon outgrowth.

**Background:** Injured neural tissue is difficult to regenerate; the mechanism has not been fully understood.

**Methods:** A rat model of spinal cord transection injury was developed. Levels of BDNF, CRH and oligodendrocyte glycoprotein (OMgp) in injured spinal cord were monitored dynamically after surgery. Cellular interaction among rat dorsal root ganglia (DRG) cells, oligodendrocytes and microglial cells was observed with a coculture model. The axon outgrowth from DRG cells was examined by confocal microscopy.

**Results:** After spinal cord transection, levels of BDNF and CRH increased the next day and decreased afterward, whereas OMgp levels increased from day 3. Administration with BDNF suppressed the levels of OMgp *in vitro*. The results from a coculture model showed that CRH increased microglial cells to release BDNF; BDNF inhibited OMgp levels in oligodendrocytes and enhanced the axon outgrowth from DRG cells.

**Conclusions:** This study shows that CRH has the ability to facilitate the outgrowth of axon in spinal neurons, which has therapeutic potential in the treatment of spinal cord injury.

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**Keywords:** spinal cord injury; brain-derived neurotrophin factor; corticotrophic-releasing hormone; oligodendrocyte glycoprotein; neural regeneration

## Introduction

It is accepted that injured neural tissue is difficult to regenerate. A mechanism is proposed by which some proteins including myelin-associated glycoprotein (MAG), oligodendrocyte glycoprotein (OMgp), Nogo, scar-derived CNS axon growth inhibitors, semaphorins and ephrins in the central nervous system (CNS) myelin function as inhibitors on axon growth.<sup>1–3</sup> The proteins of MAG, OMgp and Nogo bind to the same receptor, the Nogo-66 receptor on neurons, to mediate the inhibitory effect on the outgrowth of new axons. The fact is supported by using an antibody against the Nogo-66 receptor, or knockdown of the Nogo-66 receptor gene that can conceptually attenuate the inhibitory effect on new axon growth, although not completely.<sup>4,5</sup> However, the regulatory mechanism is to be further understood.

The inhibitory proteins of MAG, OMgp and Nogo are derived from oligodendrocytes in the central nervous system. It seems that they are required in maintaining the normal physiological nervous function, such as in the myelination process of nerves in the CNS, which is important in signal propagation. Among the three proteins, OMgp has a critical inhibitory role in axon outgrowth.<sup>1,2</sup> Yet, our knowledge in the regulation of OMgp expression in oligodendrocytes after neural injury is still limited.

It is reported that the brain-derived neurotrophic factor (BDNF) has neuroprotective effects and is beneficial to the functional recovery of injured spinal cord in animal studies.<sup>6,7</sup> BDNF is produced by the glia, such as microglial cells, in the CNS under a physiological environment. The levels of BDNF are suppressed in spinal cord injury.<sup>8</sup> The fact implies that low levels of BDNF may be related to the limitation of new axon outgrowth after injury, but the underlying mechanism needs to be further explored.

Corticotrophin-releasing hormone (CRH) is an active molecule in the regulation of a number of neural activities under both physiological and pathogenic conditions.<sup>9</sup>

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Microglial cells express CRH receptor;<sup>10</sup> activation of microglial cells by CRH releases some bioactive molecules, such as BDNF, which are involved in a number of activities of the body.<sup>11</sup> We thus hypothesized that CRH might drive microglial cells to release BDNF; the latter further promotes axon regeneration in neurons. To test this hypothesis, this study aimed to examine (i) the kinetic changes of CRH and BDNF levels in injured spinal cord; (ii) the effect of BDNF on the expression of OMgp in oligodendrocytes; and (iii) whether CRH has the capability to promote axon regeneration. The results showed that CRH could drive microglial cells to release BDNF, which further facilitates axon outgrowth.

## Materials and methods

### Reagents

Cell culture-related reagents and western blotting reagents were purchased from Invitrogen, Shanghai, China. Enzyme-linked immunosorbent assay (ELISA) kits of CRH, BDNF and OMgp were from R&D System, Shanghai, China. Magnetic cell-sorting reagents were obtained from Myltenyi, Singapore and trkB siRNA and scramble siRNA were from Santa Cruz Biotech, Santa Cruz, CA, USA.

### Rats

Sprague–Dawley rats were purchased from the Shanghai Wanxiang Experimental Animal Institute and housed in pathogen-free cages while being maintained on a 12-h–12-h light/dark cycle (lights on at 0800 AM) and with free access to food and water. All procedures were approved by the Animal Care Committee at the Second Military Medical University.

### ELISA

Levels of CRH, BDNF and OMgp were determined by ELISA with commercial reagent kits, following the manufacturer's instructions.

### Western blotting

Levels of OMgp in oligodendrocytes were determined by western blotting by referring to published procedures.<sup>12</sup> Briefly, protein was extracted from collected oligodendrocytes and separated by the precasted NuPAGE gel system and blotted onto nitrocellulose membrane. The membrane was blotted with primary antibodies (or isotype IgG used as control) ( $1\mu\text{g ml}^{-1}$ ) and then incubated with horseradish peroxidase-conjugated second antibody (1:1000). The enzymatic reaction was detected with enhanced chemiluminescent reagents and recorded with X-ray films.

### Rat spinal cord transection

Grouped rats (Sprague–Dawley, body weight around 300 g) were subjected to the surgical procedure of transection after referring to published papers.<sup>13</sup> Briefly, rats were administered general anesthesia; at the T8/T9 level, the spinal cord was exposed by laminectomy and transected with scissors. The muscle and skin were sutured. Rats were maintained in a

pathogen-free environment and housed under a 12 h light/dark cycle with *ad libitum* access to food and water.

### Dorsal root ganglion (DRG) cell preparation

DRGs were isolated from a 2-day-old Sprague–Dawley rat referred to in a previous study (DRG cells were also isolated from adult rats used as adult control; see Supplementary Figure S1).<sup>14</sup> Briefly, cultures of DRG neurons were established from thoraco-lumbar DRGs excised and freed from their connective tissue sheaths. The DRGs were collected and treated with trypsin (0.25%) for 10 min at 37 °C to remove the ectoblast. Cells were washed with fresh media once and then washed with fetal bovine serum (40%) to neutralize the effect of trypsin. After washing, DRG cells were filtered with a cell strainer and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 50 U ml<sup>-1</sup> penicillin–streptomycin, 2 mM L-glutamine, 25 mM glucose, 25 ng ml<sup>-1</sup> nerve growth factor and 2 ng ml<sup>-1</sup> glial-derived neurotrophic factor.

### Oligodendrocyte and microglial cell preparation

Neonatal rats were killed by cervical dislocation. The cortex was isolated and minced, followed by enzymatic digestion with 0.1% trypsin and 0.001% DNase, and centrifugation for 5 min ( $\times 600\text{ g}$ ). The cell pellet was resuspended in a culture medium (Dulbecco's modified Eagle's medium supplemented with 1% penicillin–streptomycin–fungizone and 10% fetal calf serum). After culture for 15–20 days, cortex-derived single-cell suspension was subjected to magnet cell sorting to isolate oligodendrocytes (byA2B5 antibody) and microglial cells (by CD11c antibody). The purity of the isolated cell population was over 95% as tested by flow cytometry.

### RNA interference

Small interference RNA (siRNA) of trkB transfection was obtained following reported procedures.<sup>12</sup> Briefly, siRNA (30 pMol) was added to the culture of microglial cells ( $10^6$  cells per ml). The efficiency of siRNA transfection was determined by western blotting. The peak inhibitory effect was reached 18 h after transfection, which lasted for another 96 h and declined thereafter. The scramble siRNAs did not affect the target molecule expression. Transfection efficiency was over 90% and inhibition was also over 90% and reproducible in all experiments.

### Treatment with exogenous CRH

Six SCT rats were treated with CRH (50 ng/rat, *i.p.*) daily for 5 weeks. Six other rats received saline and were used as controls. The general locomotor performance of the animals was assessed using the Basso, Beathic and Bresnahan (BBB) locomotor rating scale with slight modifications.<sup>15</sup> The scores ranged from 0 (for no observable hindlimb movement) to 21 (regular movement). Rats were tested weekly from day 1 after spinal cord transection until they were killed according to this scale.

### Statistical analysis

Data were expressed as mean  $\pm$  s.d. Student's *t*-test was used to compare the difference between the means of two groups. Differences were considered significant if  $P < 0.05$ .

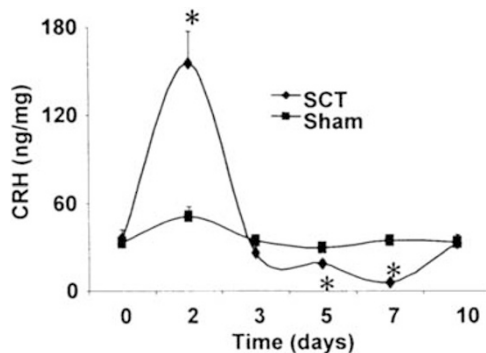
## Results

### Spinal injury modulates CRH levels in the spinal cord

CRH is an important molecule involved in a number of body responses to stress. Neuroinjury is a severe stress factor that is expected to increase the release of CRH in the CNS.<sup>16</sup> To gain an insight into the role of CRH in axon regeneration after spinal injury, our first attempt was to evaluate the levels of CRH in the spinal cord of rats after spinal transection injury. A rat model was developed following our established protocol that was also reported elsewhere.<sup>17</sup> We evaluated the CRH levels in spinal tissue within 1–10 days after surgery. ELISA data showed that a significant increase in CRH levels in the spinal cord of rats was detected on day 2, but decreased sharply from day 3, which did not return to baseline levels when compared with naive rats until day 10 (Figure 1). The sham group shows only a slight increase in CRH levels on day 2. These data show that spinal injury results in an acute enhancement of CRH levels in spinal cord immediately, but the release of CRH is significantly suppressed from day 3 to day 7.

### CRH increases the release of BDNF from microglial cells

Microglial cells are so-called immune cells in the CNS, which are involved in a number of neural activities. As microglial cells express CRH receptors,<sup>11</sup> BDNF is one of the effector mediators released by microglial cells,<sup>18</sup> we hypothesized that spinal injury-derived CRH might activate microglial cells to release BDNF, which is further involved in neuron axon outgrowth. To prove this hypothesis, using the same rat model mentioned above, we assessed BDNF levels in the

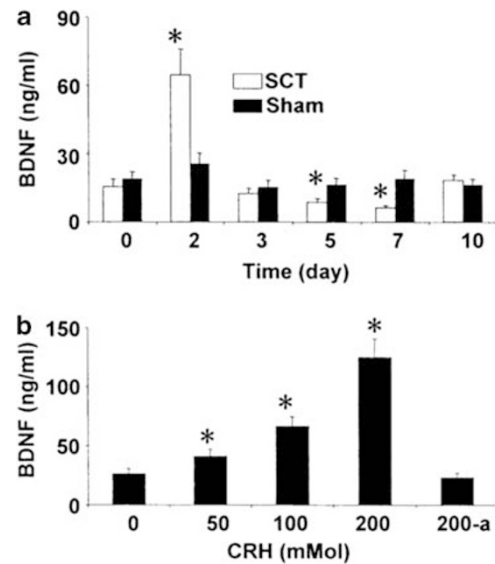


**Figure 1** CRH levels in the spinal cord. Rats were treated with spinal cord transection. The spinal cord was collected 2–10 days after surgery. Protein was extracted from the sampled spinal cords; CRH levels in the extracts were determined by ELISA. The curve indicates the levels of CRH in per mg extracted protein. Data were presented as mean  $\pm$  s.d. (from five rats of each time point). \* $P < 0.05$ , compared with the day 0 group. Isotype IgG was added to control wells instead of primary antibody, which did not show any positive results (data not shown).

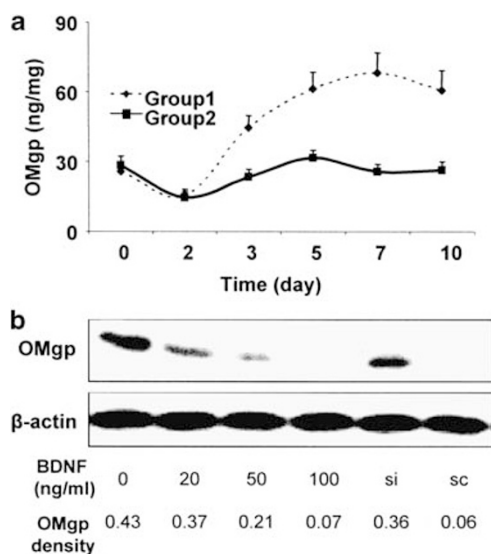
spinal cord. As shown by ELISA data, a marked increase in BDNF was detected in injured spinal cord tissue on day 2 in the SCT group (the sham group also showed a slight increase, but was much lower as compared with the SCT group); but gradually decreased from day 3 to day 7, and returned to baseline on day 10 (Figure 2a). The changes in BDNF in spinal tissue were positively correlated with the changes in CRH in spinal tissue ( $P < 0.05$ ). To obtain further evidence by which CRH induces BDNF release from microglial cells, we generated microglial cells with rat spinal cells. Microglial cells were treated with CRH in culture at graded doses for 48 h. As shown by ELISA data, BDNF levels in culture media were increased significantly in a CRH dose-dependent manner, which could be blocked by pretreatment with CRH antagonist  $\alpha$ -helical CRH (Figure 2b). The results indicate that CRH has the capacity to induce microglial cells to release BDNF.

### BDNF inhibits the expression of oligodendrocyte myelin glycoprotein in oligodendrocytes

It is reported that OMgp is one of the inhibitory molecules of neuron axon regeneration.<sup>1,2</sup> To confirm whether our rat model of spinal injury also had an increase in OMgp in its spinal cord tissue, we measured OMgp levels in protein extracts from the spinal cord using the same rat model mentioned above. Indeed, high levels of OMgp were detected in the spinal cord of the rat after spinal injury from



**Figure 2** BDNF levels in the spinal cord and in microglial cells. (a) Rats were treated with the same procedures in Figure 1. (b) Microglial cells were generated and cultured in the presence of CRH for 48 h. BDNF levels in extracted spinal protein (a) or culture media (b) were determined by ELISA. Data were presented as mean  $\pm$  s.d. (from five rats of each time point in panel A, or from three independent experiments in panel B). \* $P < 0.05$ , compared with the day 0 (a) or dose 0 (b) group. 200-a: microglial cells were pretreated with CRH antagonist  $\alpha$ -helical CRH (200 mMol). Isotype IgG was added to control wells instead of primary antibody, which did not show any positive results (data not shown).



**Figure 3** BDNF modulates OMgp expression. Rats were treated with spinal transection (group 1) or with BDNF i.p. and spinal transection (group 2). (a) OMgp levels in the spinal cord were determined by ELISA. (b) Oligodendrocytes were generated and cultured with or without the presence of BDNF ( $50 \text{ ng ml}^{-1}$ ) for 48 h. OMgp in cellular extracts were measured by western blotting. si and sc: cells were pretransfected with siRNA (or scramble siRNA) of BDNF receptor siRNA, then exposed to BDNF ( $100 \text{ ng ml}^{-1}$ ). BDNF(-): cells were not treated with BDNF. BDNF(+): cells were treated with BDNF. Immune blots were quantified using densitometry analysis and presented as the folds of  $\beta$ -actin. Samples treated with isotype IgG did not show any positive results (data not shown).

day 3 to day 7, but not on day 2 (Figure 3a). To determine whether there was a connection between BDNF release and an increase in OMgp in the spinal cord, a group of rats were treated with BDNF i.p. As expected, OMgp levels did not increase in the spinal cord from day 3 to day 7 (Figure 3a). Next, we generated oligodendrocytes *in vitro*; cells were cultured in the presence or absence of BDNF for 1–5 days. In an environment without BDNF, OMgp levels increased in oligodendrocytes, which were not observed in the presence of BDNF (Figure 3b). The results indicate that BDNF has the capacity to suppress the expression of OMgp in oligodendrocytes.

#### CRH has a role in axon outgrowth

Inhibition of axon outgrowth is one of the mechanisms interfering in neuron axon regeneration.<sup>1–3</sup> As we observed that CRH-induced BDNF could suppress the inhibitory protein OMgp expression in oligodendrocytes (which is proposed to inhibit the outgrowth of new axons), we postulated that CRH might have the capacity to maintain axon outgrowth indirectly. To prove this hypothesis, a DRG cell culture model was developed. DRG cells were cocultured with generated oligodendrocytes or/and microglial cells for 5 days in the presence or absence of CRH. The results showed that coculture with oligodendrocytes and microglial cells induced mild outgrowth of axon from DRG cells, which could be dramatically promoted by the addition of CRH. Considering the fact that axon outgrowth was not

significant in DRG cells and oligodendrocytes, whereas axon outgrowth increased in neurons in the presence of microglial cells, we inferred that the microglial cell-derived BDNF might have a critical role in axon outgrowth. To test this hypothesis, a batch of DRG cells was pretransfected with BDNF receptor *trkb* siRNA; the cells were then cocultured with oligodendrocytes and microglial cells in the presence of CRH. As expected, the coculture with oligodendrocytes and microglial cells in the presence of CRH did not result in axon outgrowth in DRG neurons (Figure 4). To further prove the effect of BDNF on the induction of axon outgrowth, another batch of DRG cells was cultured in the presence of BDNF at graded doses for 5 days. Indeed, axon outgrowth was induced in a BDNF dose-dependent manner (data not shown). As the CNS naturally has axon growth inhibitory molecules such as those we mentioned in the introduction section, we added CNS protein extract to the culture system. The results showed that the added CNS protein extracts did not affect CRH-facilitated axon outgrowth (Figure 4, panel 4). Results indicate that CRH-induced microglial cell-derived BDNF has a critical role in the promotion of axon outgrowth.

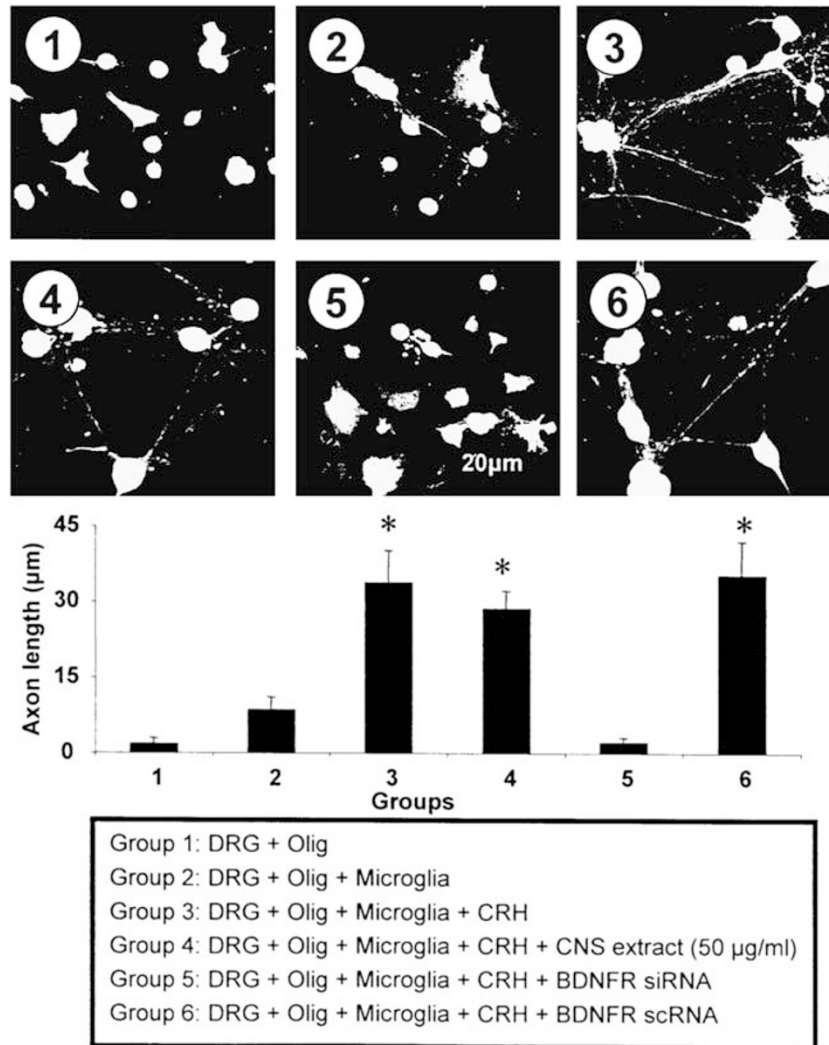
#### Exogenous CRH facilitates SCT recovery

To test the role of CRH in facilitating neural axon outgrowth *in vivo*, six rats that underwent SCT received exogenous CRH 50 ng/rat daily, i.p. Results showed that BBB scores were significantly increased after a 4-week treatment, whereas the rats that received saline showed no significant changes in their BBB scores (Figure 5). Results indicate that exogenous CRH treatment facilitates SCT recovery.

## Discussion

Spinal cord transection injury usually results in paralysis in patients. Currently, recovery is very poor, as determined on the basis of the poor outgrowth of neuron axons and nerve regeneration. This study sheds new light on this area by providing evidence that CRH has the capacity to induce release of BDNF from microglial cells; BDNF has a critical role in nerve regeneration by a mechanism of inhibition of OMgp in oligodendrocytes.

Oligodendrocytes have a critical role in nerve physiology by providing myelin to form the myelin layer for neurons and axons in the central nervous system. OMgp is a membrane glycoprotein expressed by oligodendrocytes that appears in the human central nervous system at the time of myelination. OMgp has the potential to function as an adhesion molecule and may contribute to the interactions between the plasma membranes of oligodendrocytes and axons required for myelination. However, it is reported that OMgp and two other molecules, MAG and Nogo-A, inhibit axon regeneration after injury in the adult mammalian central nervous system.<sup>19</sup> Therefore, different antagonists against these three molecules have been developed trying to block their inhibitory effect on axon regeneration.<sup>20</sup> In line with these pioneer studies, this study shows that microglial cell-derived BDNF shows a promising capability in antagonizing the inhibitory effect of OMgp on the axon



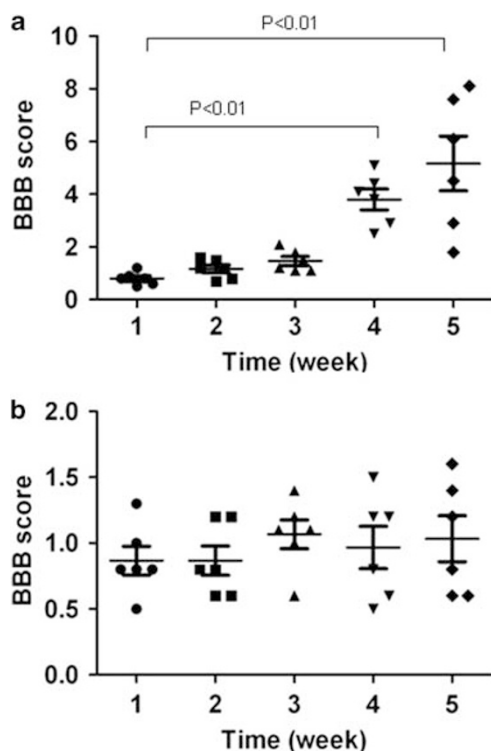
**Figure 4** CRH facilitates axon outgrowth. DRG cells (cells appear round, and are smaller) were prepared and cultured with oligodendrocytes (Olig), or together with microglial cells (Microglia) in the presence or absence of CRH. Another group was cultured with addition of CNS extract ( $50 \mu\text{g ml}^{-1}$ ). The cultured cells were photographed under a confocal microscope ( $\times 200$ ). Axon length was measured with a ruler on photomicrographs at 100 DRG cells per group; the measurements were converted to the real size based on the enlargement. The measurements are presented in the bar graph. Data are presented as mean  $\pm$  s.d. from three separate experiments. \* $P < 0.001$ , compared with group 1. BDNFR siRNA: Some DRG cells were pretransfected with BDNF receptor siRNA. scRNA is the scramble RNA used as control.

regeneration of DRG neurons. This fact shows that although difficult, axon regeneration is practical.

It is suggested that neurotrophic factors have a role in promoting axon regeneration.<sup>21</sup> BDNF is a neurotrophic factor derived from microglial cells in response to appropriate stimuli.<sup>18</sup> Spinal transection injury is a severe stimulus that can regulate the expression of BDNF in the spinal cord somehow. Our data proved this inference. Other investigators also found that BDNF levels were elevated in the lumbar spinal cord of rats with spinal cord injury.<sup>22</sup> BDNF is a powerful synaptic facilitator and likely has a key role in motor and sensory functions and in shaping synaptic plasticity and in defining the level of recovery of locomotor performance after a spinal cord injury.<sup>23</sup> Our data have enriched the understanding of BDNF function in neural recovery after injury by providing evidence that BDNF has

the ability to block the expression of OMgp in oligodendrocytes.

Previous studies indicate that microglial cells express CRH receptors.<sup>11</sup> Exogenous CRH can induce microglial cells to release inflammatory mediators and certain neural tissue responses such as inflammation.<sup>10,11</sup> Our results have extended these findings by showing that CRH has the capacity to drive microglial cells to release BDNF in response to spinal cord injury. Apart from inducing the undesired response, such as in stress-related disorders,<sup>24</sup> CRH can modulate behavior by enhancing the behavioral responses to stressors.<sup>25</sup> We regard the increase in CRH levels in the spinal cord a day after injury as an active response trying to minimize the impact of injury on the body. Indeed, further results indicate that CRH can induce the release of BDNF from microglial cells; BDNF reduces the expression of OMgp



**Figure 5** CRH treatment increases the BBB score. Six SCT rats were treated with CRH (50 ng/rat, i.p.) daily for 5 weeks; six other SCT rats received saline and were used as control. The scattered dot plots indicate the BBB score in CRH-treated rats (a) and saline-treated rats (b). Each dot represents a score from one rat.

in oligodendrocytes. As OMgp is one of the inhibitory molecules on axon regeneration,<sup>1,2</sup> the fact implies that CRH and BDNF have a role in promoting the recovery of the spinal cord from injury. Supporting data were provided by the *in vivo* data (Figure 5) in this study.

However, from day 3 after injury, levels of CRH and BDNF decreased dramatically in the spinal cord and levels of OMgp increased. The data presented here do not reveal the mechanism for these changes, but provide a novel notion that exogenous CRH or/and BDNF may be beneficial in preventing further neural damage and in promoting axon regeneration. Our subsequent data support this notion by providing evidence that CRH facilitates axon outgrowth from DRG cells.

In summary, this study provided a set of novel data that showed that spinal cord injury resulted in increases in CRH and BDNF the next day, which profoundly decreased later. CRH increased the expression of BDNF from microglial cells. BDNF had an inhibitory effect on OMgp expression in oligodendrocytes and promoted axon outgrowth in DRG neurons. The results implicate that supplementing with exogenous CRH and BDNF may be beneficial for spinal cord recovery after injury.

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgements

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Supplementary Information accompanies the paper on the Spinal Cord website (<http://www.nature.com/sc>)