ProBDNF inhibits infiltration of ED1+ macrophages after spinal cord injury

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ABSTRACT

The central nervous system (CNS) does not regenerate partly due to the slow clearance of debris from the degenerated myelin sheath by Wallerian degeneration. The mechanism underlying the inefficiency in myelin clearance is not clear. Here we showed that endogenous proBDNF may inhibit the infiltration of ED1+ inflammatory cells after spinal cord injury. After injury, proBDNF and its receptors sortilin and p75NTR are expressed in the spinal cord as determined by Western blots and immunocytochemistry. ProBDNF and mature BDNF were released from macrophages *in vitro*. Macrophages *in vivo* (ED1+) and isolated *in vitro* (CD11b+) express moderate levels of proBDNF, sortilin and p75NTR. ProBDNF suppressed the migration of isolated macrophages *in vitro* and the antibody to proBDNF enhanced the migration. Suppression of proBDNF *in vivo* by administering the antiserum to the prodomain of BDNF after spinal cord injury (SCI) increased the infiltration of macrophages and increased number of neurons in the injured cord. BBB tests showed that the treatment of the antibody to proBDNF improved the functional recovery after spinal cord injury. Our data suggest that proBDNF is a suppressing factor for macrophage migration and infiltration and may play a detrimental role after SCI.

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1. Introduction

Injured peripheral nerves can regenerate and make partial functional connections whereas injured axons in the central nervous system (CNS) do not regenerate. Many factors contribute to the difference in the regeneration between peripheral nervous system (PNS) and CNS. One major difference is that the myelin associated inhibitory factors are more abundant in the CNS than in PNS after injury (Gervasi et al., 2008). The rapid and efficient clearance of myelin debris from Wallerian degenerated nerves in the PNS by macrophages and Schwann cells produces a permissive environment for the regeneration of the peripheral nerve (Barrette et al., 2008; Boivin et al., 2007). Accumulating evidence suggests that one of major obstacles towards the regeneration of the CNS is due to lacking efficiency in macrophage infiltration in the injured spinal cord where myelin debris exists for a longer period of time (Imai et al., 2008; Vargas and Barres, 2007), preventing regeneration of injured nerves and remyelination of axons (Chen and Bisby, 1993). However, the mechanisms underlying the retarded macrophage response and delayed Wallerian degeneration in the injured CNS are not fully understood.

Neurotrophins are family of polypeptides which play critical roles in the neuronal development and in the synaptic plasticity in the adult (Huang and Reichardt, 2003; Davies, 1994). Accumulating evidence suggests that neurotrophins are also involved in the regulation of inflammatory cells such as dendritic cells (Jiang et al., 2008), macrophages (Susaki et al., 1996) and mononuclear lymphocytes (Samah et al., 2008). For example, brain derived neurotrophic factor (BDNF) and its receptor trkB are expressed in macrophages (Artico et al., 2008) and play autocrine and paracrine roles in modulation of regeneration and angiogenesis following nerve injury (Kermani and Hempstead, 2007). Neurotrophins are synthesized as a precursor (pro-form) and subsequently processed intracellularly or extracellularly to form mature neurotrophins (i.e., mature and its unprocessed pro-form) in a physiological setting. After synthesis, proBDNF is sorted by sortilin in the Golgi network and transported to dendrites or nerve terminals where it is released on an activity dependent manner (Chen et al., 2005). It has been well demonstrated that BDNF facilitates neuronal regeneration following CNS injury (Jin et al., 2002; Song et al., 2008). However, the functions of the unprocessed form of neurotrophins are not known. Pro- and mature-forms of neurotrophins have high affinity interaction with different receptors, i.e., p75[NTR] and Trk receptor, respectively (Lee et al., 2001). Pro-neurotrophins may be involved in the promotion of protein folding into its active form and also in regulating intracellular trafficking of the proteins (Heymach et al., 1996; Mowla et al., 2001; Rattenholl et al., 2001).

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Pro-neurotrophins bind to p75[NTR] together with the co-receptor sortilin, to elicit cell death in various cell types such as oligodendrocytes (Beattie et al., 2002), Schwann cells (Teng et al., 2005), motor neurons (Domeniconi et al., 2007) and sensory neurons (Fan et al., 2008) after injury. Thus these studies further enforced the concept that pro-neurotrophins are possible targets for therapy for spinal cord injury (SCI). It is, however, unknown what role proBDNF molecule plays in inflammatory cells.

In the normal and injured spinal cord, proBDNF was present in the dorsal horn and dorsal funiculus (Wang et al., 2006; Zhou et al., 2004). Following injury proBDNF immunoreactivity accumulates in the injured axons and lasts for at least 2 weeks (Wang et al., 2006). In the current study, the effects of proBDNF on inflammatory cells after SCI have been investigated. We found that exogenous and endogenous proBDNF may inhibit the migration and infiltration of ED1+ cells into the spinal cord and play a detrimental role after spinal cord injury. We also found the treatment with the proBDNF antiserum improved functional recovery after SCI.

2. Materials and methods

2.1. Animals

All experiments involving the use of rats were approved by the Animal Welfare Committee of Flinders University, and performed according to the guidelines of the National Health and Medical Research Council, the Flinders Animal Welfare Committees (Ethics no. 623/06). Female adult Sprague–Dawley rats (200–300 g), aged between 6 and 9 weeks, from Flinders Medical Centre Animal House were used in this study. The rats were housed one per cage, exposed to a 12 h light/dark cycle, with constant supply of water and food. Over the course of the experiment, two rats died due to surgical complications and two were sacrificed due to unresolved bladder complications.

2.2. Western blot

Injured rats [day 1 ($n = 3$), day 3 ($n = 3$), day 7 ($n = 3$)] and normal controls ($n = 3$) were sacrificed and the spinal cord was freshly isolated for Western blot analysis. The injured spinal cord tissues were homogenized in 10 mM Tris–HCl buffer (pH 7.4), 10 mM EDTA, 30% Triton-X 100 on ice and then sonicated on ice for three burst, approximately 1 min. Homogenate was then centrifuged at 12,000g for 30 min at 4 °C. The supernatant was phase collected in aliquots and stored at −80 °C. The protein concentration was assayed with BCA protein assay (Pierce). Equal amount of proteins from both homogenates were loaded for Western blots. For cultured macrophage preparations (see below), insoluble membrane fractions of the cell lysate and freeze-dried culture medium fractions were loaded for Western blots. The nitrocellulose membrane was probed with primary antibodies directed to proBDNF (1:200) (Zhou et al., 2004), sortilin (1:500, Abcam) and p75[NTR] (1:500, Promega).

2.3. Primary macrophage isolation and culture

In the present study, we defined macrophages by positive staining for CD11b or Mac-1 in vitro and ED1 in vivo. Primary macrophages from peritoneum and bone marrow were prepared following the described methods of Barouch et al. (2001) and Longbrake et al. (2007), respectively, with some modifications. For peritoneal macrophages, after intraperitoneally (i.p.) injection with 10 ml of sterile 3% Luria–Bertani broth for 3 days in female rats, peritoneal exudate macrophages were harvested by peritoneal lavage after i.p. injection with 10 ml of sterile ice cooled

sucrose solution. The cells were washed and resuspended in RPMI 1640 supplemented with 10% FCS, 2% Pen/Strep and 1% L-glutamine. Following 2 h incubation at 37 °C, nonadherent cells were removed by washing and attached macrophages were cultured overnight in 1640 medium and used for experiments. About 96% of these attached cells were positive for Mac-1, a marker for macrophage types. For macrophages from bone marrow, marrow cores from femurs and tibias were flushed using syringes filled with RPMI 1640 supplement with 10% FBS. Cells were triturated and RBCs were lysed (0.15 M NH$_4$Cl, 10 mM NaHCO$_3$, 0.1 mM Na$_2$EDTA; pH 7.4). Cells were washed once in media, then plated and cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 1% HEPES, 0.001% β-mercaptoethanol, 10% FBS and 9.0×10^3 IU/ml macrophage colony stimulating factors (Sunway Biotech., China). Nonadherent cells were removed 4 days later. Attached macrophages were cultured in RPMI1640/10% FBS used for experiments. About 98% of these attached cells were positive for CD11b, a marker for macrophage types.

2.4. Immunocytochemistry on isolated macrophages

Macrophages from peritoneum and bone marrows were washed with PBS, fixed with 4% paraformaldehyde, blocked with 10% normal goat serum in PBS, and incubated with primary antibody in 10% normal goat serum at 4 °C overnight followed with Cy2 or Cy3 labelled secondary antibody (1:1000, Beyotime, China). OX42 (1:100, Serotec), rabbit anti-p75[NTR] (Ab 9650, a gift from Dr. M. Chao, New York University School of Medicine, New York) (Massa et al., 2006) and anti-sortilin (1:500, Abcam) were used for double staining macrophages, p75[NTR] and sortilin, respectively. Nuclei were labelled with Hoechst33342 (10 μg/ml, Molecular Probes). Controls without using primary antibodies and pre-immune normal rabbit IgG were used for antibody specificity controls. After mounting in fluorescent mounting medium, cells were visualized with an Olympus fluorescence microscope. At least one thousand cells from 10 to 15 viewing fields per group were used to calculate percentages of cells.

2.5. Cell migration assay

Costar Transwell polycarbonate filters (8.0 μm pore size) were used in a migration assay to examine the ability of macrophage migration (Liao et al., 2005). After primary macrophages were treated in the absence or presence of LPS (10 ng/ml for bone marrow cells and 1 μg/ml for peritoneal cells) for 2 h, the cells were harvested and plated in serum-free culture medium into the upper chamber and allowed to migrate through the pores onto the under surface at 37 °C in a CO$_2$ incubator in the absence or presence of LPS. Different protein (BDNF or proBDNF) was added into upper chamber medium for treating macrophages in the presence or absence specific antibody BDNF antibody (5 μg/ml), proBDNF antibody (10 μg/ml) or rabbit or sheep IgG (10 μg/ml) for 24 h. BDNF antibodies well characterized in our previous studies and can specifically block the biological activity of mature BDNF on neurite growth from DRG neurons (Deng et al., 2000). The mature BDNF was a gift from Regeneron and proBDNF for neutralization was produced within our laboratory and well characterized in our previous studies (Fan et al., 2008). After 24 h, cells from the inner surface of the insert were gently wiped out with cotton-tipped swabs, and the inserts were fixed with 2.5%, 4% paraformaldehyde and stained with cresyl violet solution (0.2%). After a final PBS washing, the cells from five viewing fields per group were used to calculate percentages of cells under a microscope. For quantification of peritoneal macrophage migration, the dye was extracted with 10% acetic acid and the absorbance was measured at 570 nm using a

microplate reader. The dye levels are directly proportional to the number of cells. The data are presented as means ± SE.

2.6. Surgery procedures and postoperative care

The SCI model was produced following the procedures reported previously (Feng et al., 2008). In brief, isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) was used as a general inhalation anaesthesia to perform SCI on rats. Laminectomy of T9 was performed to expose the spinal cord. A pair of clamps was used to hold vertebrae to stabilize the spinal column and the impact rod was centred on the spinal cord at T9. The cord was then contused with a weight drop device similar to the New York University (NYU) device (Basso et al., 1996a,b). The extent of the injury was made consistent by dropping a 10 g (grams) rod at the height of 25 mm. The wound was closed by sutures on muscle and skin for the animals which were used for gene expression analysis ($n = 24$). In animals which were treated with sera, the dura matter on the injured cord was punctured and removed with a 27 gauge needle. A piece of gel foam ($5 \times 5 \times 5$ mm^3) soaked in either sheep antibody to proBDNF, or normal sheep serum (NSS) was placed directly onto the contusion injured area. After closure of the wound the rats were i.p. injected with antibodies or NSS. For animals that were killed at 1 and 3 days, the antibodies to proBDNF ($n = 18$) or normal sheep serum (NSS) ($n = 18$) were injected i.p. once, immediately after surgery at 10 µl/g body weight as described (Deng et al., 2000). For animals that were killed at 1 week, the antibodies ($n = 9$) or NSS ($n = 9$) were injected i.p. at day 1 (10 µl/g body weight) and day 4 (5 µl/g body weight). For the behavioral tests ($n = 20$, 10 in each group), the antibodies were injected 4 times at day 1, day 4, day 8 and day 12 (10 µl/g, first injection and 5 µl/g, rest injections). The antibodies to proBDNF were well characterized in our previous studies and can specifically block the biological activity of proBDNF (Fan et al., 2008).

Postoperative nursing care was provided to the rats, which included bladder expression three times per day until the subjects had regained the bladder function. The rats were treated with Tetravet 100 (Bomac laboratories), a soluble antibiotic powder containing oxytetracycline base in drinking water. Buprenorphine (32 mg/ml, dose dependent on weight; 30 µl/200 g) was injected intraperitoneally post-surgery (once a day for 3 days) to provide long lasting analgesic effect.

2.7. Sampling

All rats except for those used for Western blots underwent perfusion with sodium nitrate (NaNO$_2$) (sigma, 1%, 50 ml) to flush out blood from the circulatory system and then with paraformaldehyde (PFA) (sigma, 4% in 0.1 M phosphate buffer, pH 7.4, perfused with 1 ml/g). The injured spinal cord was harvested 1.5 cm rostral and caudal to the injury centre. The rostral end was made distinguishable by an angular cut. The harvested tissue was preserved in 4% PFA at 4 °C for up to 2 weeks and then placed in 30% sucrose solution (APS chemicals) 1 day prior to cryosection.

2.8. Immunostaining procedure for ED1+ macrophages in the spinal cord

The spinal cord was cryosectioned, parasagittally at 35 µm, and placed in free floating, in phosphate buffer solution (PBS), consisting of 12 wells (24 tissue culture plate, Sarstedt). To ensure the tissue was sagittally sectioned in parallel with the midline of the spinal cord, a sectioning mould was developed (for details, please see Supplement, Fig. S1). Ten tissue sections, which fall within the central part of the cord containing dorsal and ventral funicular white matter and central grey matter, were used for immunostaining analysis.

To reduce the variability of the staining in a large number of samples and ensure consistency between staining, we developed a staining apparatus where 24 samples can be processed at the same time with the same length of time (for details please see Supplement, Fig. S2). DAB staining was performed to observe and quantify macrophages present. Following cryosection, the free-floating tissue sections were incubated in mouse monoclonal antibody to ED1 (1:200, Serotec) and biotinylated secondary antibody (1:1000, Vectorstain). After washing the sections were incubated in ABC developed in DAB until colour developed (approximately 1–10 min) as described (Zhou et al., 2004). The sections were rinsed with distilled water to stop reactions and transferred to glass slides. Finally the slides were coverslipped (cover glasses, HD scientific supplies Pty. Ltd.) with DPEX (BDH chemicals), a xylene-based medium. To examine the survival of neurons we also stained spinal cord sections with NeuN antibody (1:500, Millipore) with an immunofluorescence method. To define demyelination and injury severity, sections from the central part of the cord containing dorsal and ventral funicular white matter and central grey matter were used for Fast Blue myelin stain following a standard method.

2.9. Behavioral tests

In a separate group of animals, rats ($n = 20$) with contused SCI were treated with NSS ($n = 10$) or anti-proBDNF antiserum ($n = 10$) as described above. The BBB test was conducted based on the method (Basso et al., 1996a) on the following days; 0 (immediately following surgery), 1, 3, 7, 14, 21 and 28. Briefly, rats were carefully removed from their cages and placed in an open space and hind limb movements viewed based on the open field BBB score sheet and scored via the BBB locomotor rating scale. BBB scores were recorded by two independent researchers in a blind manner. After completion of the test, the scores from individual animals recorded from two researchers were averaged, and the data from two different groups of animals were analysed and compared by ANOVA and Turkey's tests. Only animals at day 0 scored zero or one were included in the study.

2.10. Quantitative analysis

Labelled cells that used DAB for detection were quantified at 1, 3, and 5 mm rostral and caudal to the contusion site, where images of white matter and gray matter were collected at 20× magnification with light microscope (Olympus BX50, Japan) using constant bulb temperature and exposure, with all images acquired in the same session. The area of the white matter and gray matter were measured using ImagJ and the number of macrophages in the corresponding area was counted in a blinded manner. The density of macrophage was calculated as number of macrophages per mm^2.

Western blot results on the films were scanned as grey scale TIFF files. The intensity of the band was quantified and then normalized with beta-actin intensity in a blind manner.

2.11. Statistical analysis

For histology, quantitative analysis was done at both randomly selected white matter and grey matter; two white matters (top and bottom) and one grey matter at specific distances away from the contusion site. To reduce the possibility of variability, three sections from each subject were quantified and the mean of this value was taken. For statistical evaluation, a one-way ANOVA test was used to evaluate the cells counts between groups and the data were expressed as mean ± standard error of the mean (SEM).

For Western blot, as different amount of proteins were loaded, the data for the result expressed as OD value of the particular protein divided by the corresponding OD value of β-actin for each sample (mean ± SEM).

3. Results

3.1. Expression of proBDNF and its receptors (p75[NTR] and sortilin) in the injured spinal cord

Western blot analysis was used to examine the expression of endogenous proBDNF and its receptor in the contusion injured spinal cord. As shown in Fig. 1, in the normal spinal cord, p75[NTR] and sortilin are easily detectable but proBDNF is also detectable (Fig. 1). Following spinal cord injury, a band corresponding to proBDNF at 35 kDa was present in the spinal cord 1 day and 3 days after injury but reduced at day 7. The p75[NTR] receptor protein (75 kDa) was significantly upregulated in the injured spinal cord (Fig. 1). Sortilin receptor protein (110 kDa) was expressed in normal and injured spinal cord but without significant change after injury. β-Actin (42 kDa) protein level was used as an internal control for protein loading in the Western blotting. These data suggest that proBDNF may play a role in the spinal cord injury.

3.2. Macrophages express p75[NTR] and sortilin

The isolated macrophages from both peritoneum and bone marrows were used for immunocytochemistry. There was no specific staining if the primary antibodies were omitted or pre-immune normal IgG was used. As shown in Fig. 2, almost all cells from bone marrow are OX42 positive, indicating the high purity of macro-

phage preparation. Most isolated OX42+ macrophages expressed both sortilin and p75[NTR] (Fig. 2). Over 95% of macrophages isolated from the peritoneum were also OX42+ and expressed both sortilin and p75NTR (data not shown). The immunohistochemical data were further confirmed by Western blots. Sortilin and p75[NTR] are easily detectable in the cell membrane fraction of both peritoneal and bone marrow macrophages but not in the culture medium fractions (Fig. 3). Lipopolysaccharides (LPS) reduced the level of p75[NTR] but had no effect on the level of sortilin in these cells.

3.3. Release of BDNF and proBDNF from macrophages

To test whether mature BDNF and proBDNF are released from macrophages we examined their release with Western blot methods. As shown in Fig. 3A, proBDNF was present in the culture medium but not in the unsoluble macrophage membrane fractions, suggesting proBDNF was secreted from macrophages in the presence and absence of LPS. LPS increased secretion of proBDNF from peritoneal macrophages but not bone marrow macrophages. ProBDNF and mature BDNF were also detected in the culture medium but barely detectable in the cell lysates, however, LPS had no significant effects on the release of proBDNF and mature BDNF. The ratio of proBDNF and mature BDNF was also not affected by LPS treatment (Fig. 3B).

3.3. The effect of proBDNF and BDNF on migration of activated macrophage stimulated by LPS

Transwell assay was conducted to examine the effects of proBDNF on the migrating ability of macrophage induced by LPS. The results showed the migration of peritoneal macrophage was significantly increased by BDNF and inhibited markedly by proBDNF. Notably, the effect exerted by either the BDNF or the proBDNF occurred in a dose-dependent manner (Fig. 4A–C). Antibodies to BDNF or proBDNF could reverse the effects on migration induced by either BDNF or proBDNF. The irrelevant antibody rabbit IgG controls exhibited values very similar to PBS controls (Fig. 4A and D).

As bone marrow macrophages may have different properties to those of peritoneal macrophages (Longbrake et al., 2007), we also tested effects of proBDNF on the migration and BDNF secretion of bone marrow macrophages. The results showed that the migration of bone marrow macrophage was also significantly increased by BDNF and inhibited markedly by proBDNF in the absence (Fig. 5A and B) or presence of LPS (10 ng/ml) (Fig. 5C–F). Antibody to BDNF or proBDNF could reverse the effects on migration induced by either BDNF or proBDNF. The irrelevant antibody rabbit IgG controls exhibited values very similar to PBS controls.

3.5. Effects of proBDNF on the macrophage infiltration in injured spinal cord

One day after spinal cord injury, there were significant number of macrophages in the injured spinal cord. The number of macrophages was maintained at 3 days after injury and reduced at 7 days. In the centre of injury, the number of macrophages was maximal and reduced gradually with the distance from the injury sites. Macrophages were present in both grey and white matter of the cord. The treatment with the antiserum to proBDNF increased infiltration of macrophage into the injured spinal cord, in particular into the white matter of the cord (Fig. 6A). The increase in macrophages occurred in all regions examined, in particular, the regions close to the injury centre. Quantitation data showed that the proBDNF antiserum mainly affected the infiltration of macrophages at 1 and 3 days after injury (Fig. 6B and C). Seven days after injury, the number in proBDNF antiserum treated group was not different from that treated by NSS (Fig. 6D). There were also the trend in
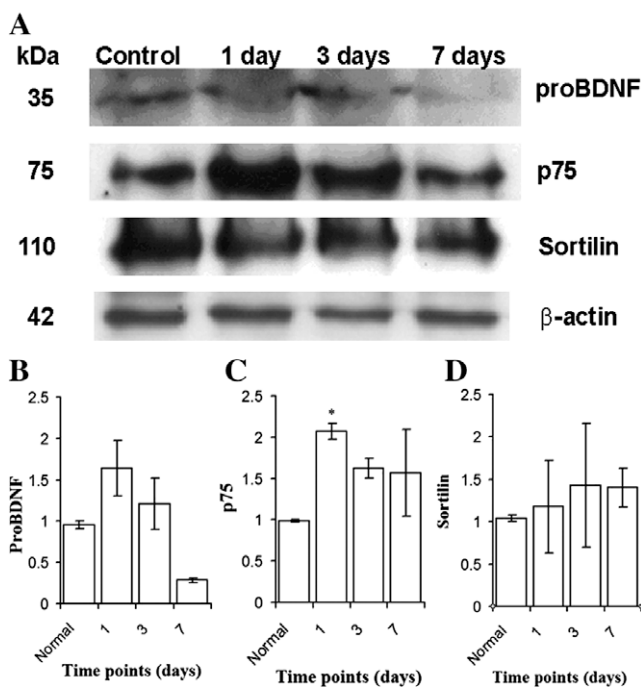

Fig. 1. Effects of spinal cord injury on the expression of proBDNF, sortilin and p75[NTR] in the injured cord. (A) Representative Western blots of endogenous proBDNF, sortilin and p75[NTR]. The blots were performed using injured spinal cord from the following groups of rat: no injury (lane 1), 1 day post-contusion injury at T9 (lane 2), 3 day after injury (lane 3), and 7 day after injury (lane 4). Rabbit anti β-actin blot was added simultaneously as internal protein loading controls and used as standard control to valuate the levels of proBDNF, sortilin and p75[NTR]. (B–D) Group data from three experiments (*n* = 3) from each time points. *p < 0.05 compared with the uninjured group.
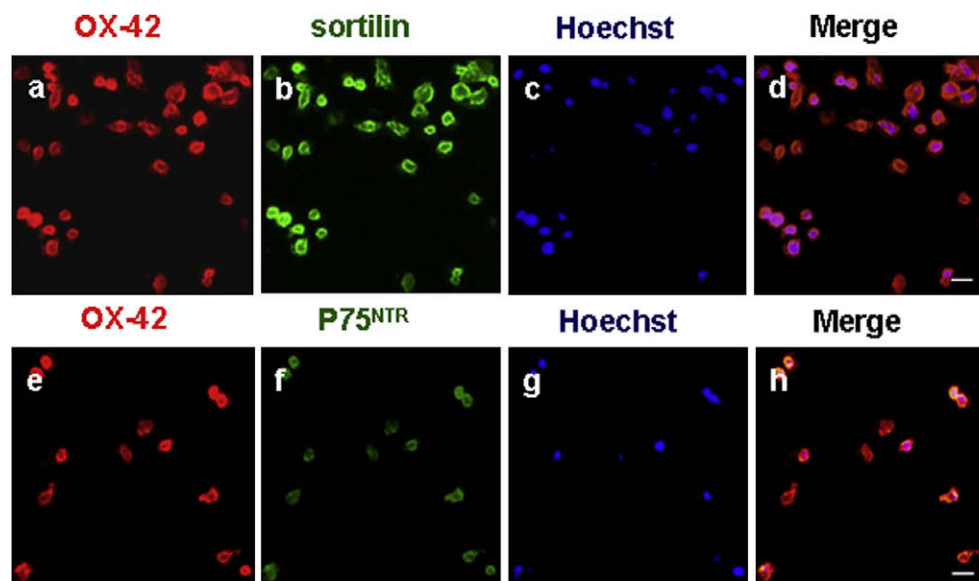
Fig. 2. Expression of p75[NTR] and sortilin in bone marrow derived macrophage *in vitro*. Immunolocalization was employed to detect p75[NTR] and sortilin expression on the primary macrophage. Primary antibody: OX42 (mouse, 1:100, Serotec), p75[NTR] (MC192) or sortilin (rabbit, 1:500, Abcam). The primary antibodies were used to incubate cover slips overnight at 4 °C, followed by Cy2 or Cy3 labelled secondary antibody (1:1000, Amersham Bioscience). After mounting in fluorescent mounting medium, cells were visualized with an Olympus fluorescence microscope. Scar bar = 10 μm.

the increase of macrophages in grey matter after proBDNF antiserum treatment overall but the effect is not as obvious as on white matter (Fig. 6E–G).

3.5. Effects of anti-proBDNF on neuroprotection after spinal cord injury

It is known that after spinal cord injury, most of neurons will be lost around the epicentre of the injury site. Primary injury usually causes cavity and secondary neuronal loss and demyelination. To see whether proBDNF had detrimental effects on neurons and myelin we stained injured spinal sections for total neurons and for myelin. Choosing central sections for neuronal staining, we found that the number of neurons in the epicentre and 3 mm away from the epicentre in anti-proBDNF treated animals was significantly increased compared with that in NSS treated rats at 7 days after injury (Fig. 7), suggesting the anti-proBDNF treatment had a neuroprotective effect after spinal cord injury.

We also stained myelin in the central sections of rats at 7 days after spinal cord injury. As shown in Fig. S3, there was a significant myelin loss in the epicentre of the injury site from both NSS- and ant-proBDNF-treated rats. Quantitation results showed that demyelination area appeared reduced in rats treated with anti-proBDNF compared with rats treated with NSS. The reduction in demyelination in the antibody treated rats may not be associated with the increased macrophages in these animals but it may be related to the neuroprotective effect of anti-proBDNF.

3.6. Effect of anti-proBDNF on the functional recovery after contusion spinal cord injury

To see whether the anti-proBDNF treatment has any therapeutic effects on the functional recovery after SCI, we performed open field behavioral test using the BBB scaling method. Following SCI almost all animals were paralyzed immediately after injury and gradually recovered on the following days (Fig. 8). One day after injury the BBB score from the anti-proBDNF treated rats did not recover, whereas animals in the control group recovered significantly. However, from day 3 on, the locomotor functions in

anti-proBDNF treated rats improved significantly in comparison with the NSS treated group. BBB scores from day 3 to day 28 after injury in anti-proBDNF treated group were significantly higher than the NSS controls. These data suggest that anti-proBDNF can improve the functional recovery possibly via promoting the infiltration of macrophages.

4. Discussion

Following nerve injury Wallerian degeneration is an essential step towards successful regeneration (Bisby and Chen, 1990; Brown et al., 1992). The key component of Wallerian degeneration is the clearance of myelin debris by phagocytes such as macrophages and microglia (Brown et al., 1991). In the present study, we found that the BDNF precursor, proBDNF, is involved in the regulation of macrophage migration and infiltration into the spinal cord. We have demonstrated that injured spinal cord express proBDNF, p75[NTR] and sortilin. The levels of p75[NTR] are upregulated after spinal cord injury. We have also demonstrated that peritoneal and bone marrow macrophages secrete proBDNF and express sortilin and p75[NTR], suggesting that proBDNF may have its function on macrophages by autocrine regulation. Blocking endogenous proBDNF with specific antibodies to proBDNF increased infiltration of macrophages in the injured spinal cord whereas the treatment of isolated macrophages *in vitro* with proBDNF reduced the migration of macrophages. The anti-proBDNF treatment preserved myelin and neurons and improved functional recovery after SCI. Our data suggest that endogenous proBDNF may play a detrimental role in SCI by reducing the infiltration of macrophages.

SCI results in loss of nerve connectivity between the brain and the peripheral nerves leading to permanent paralysis. The failure in nerve regeneration after SCI has attributed to the detrimental environment within the injured tissues which prevents the regeneration of injured neurons. Abundant myelin associated proteins such as myelin associated glycoproteins (MAG), Nogo, and oligodendrocyte myelin glycoprotein (OMGP) are recently identified as inhibitory factors that are highly expressed in the spinal cord and released from myelin after injury and suppress neurite growth (Yiu and He, 2006). Thus, the promotion of myelin debris clearance
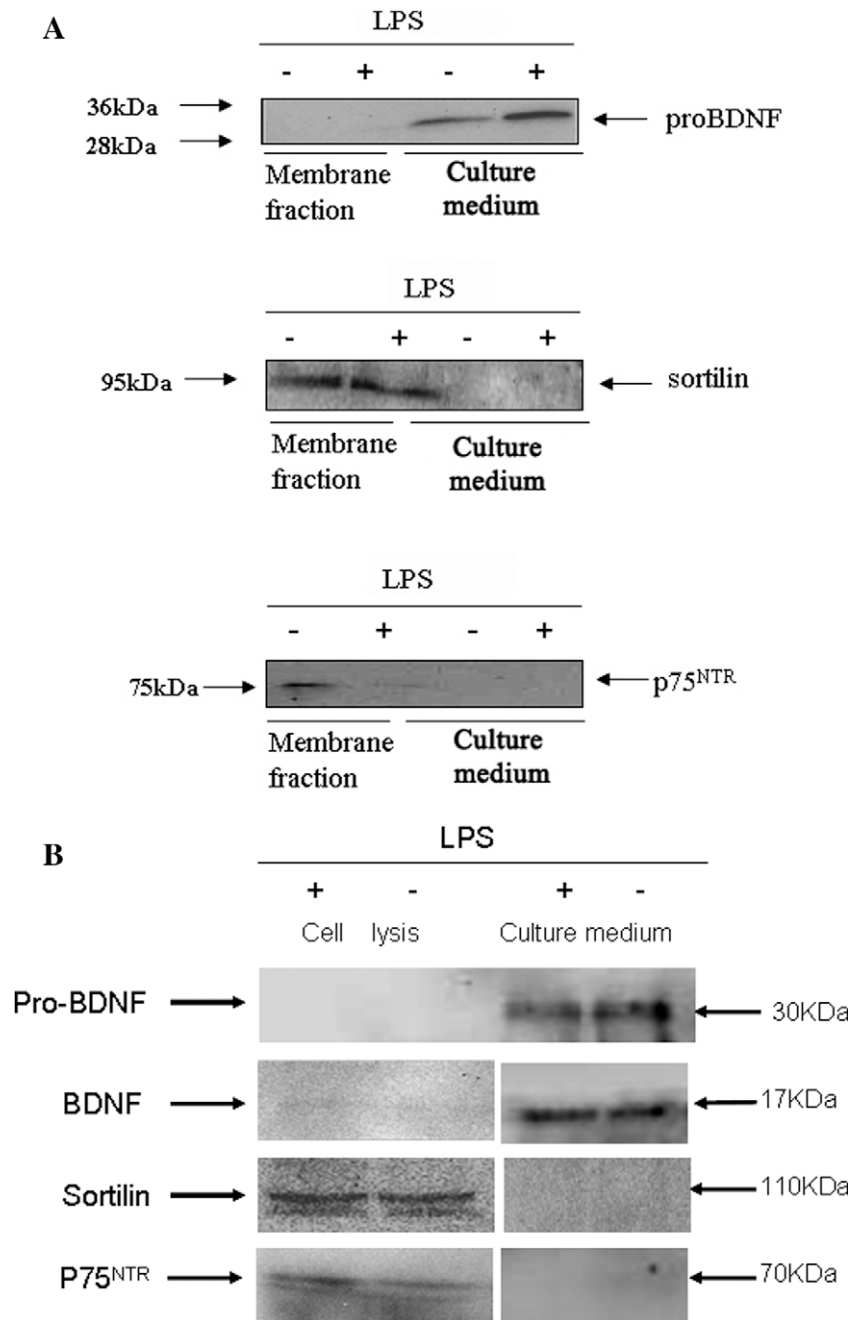
Fig. 3. Western blot analysis demonstrated that p75[NTR] and sortilin expression and proBDNF secretion by macrophages in the presence and absence of LPS. (A) Western blot analysis on peritoneal macrophages. (B) Western blot analysis on bone marrow macrophages. Unsoluble membrane fractions and freeze-dried culture medium were used for Western blots. Sortilin and p75[NTR] expressed on the membrane of macrophages and proBDNF is in the culture medium. Each sample was mixed with $2\times$ SDS loading buffer and separated by 8% SDS–PAGE, blotted onto nitrocellulose membranes and immunoblotted with the respective primary antibodies: rabbit anti-proBDNF (1:1000) (Zhou et al., 2004), sheep anti-mature BDNF (1:1000), rabbit anti-sortilin (1:400, Abcam), mouse anti-p75[NTR] (1:500, MAC192) , mouse anti-β-actin (1:4000, R&D) and visualized with the Pierce chemiluminescent detection reagents.

(Barrette et al., 2008; Boivin et al., 2007) by macrophages and the suppression of the inhibitory factors become important approaches to promote the regeneration of the injured axon and improve the functional recovery after SCI (Yu et al., 2008). The infiltration of blood borne macrophages is a critical factor involved in the regeneration of neurons and is required for regeneration of nerves in PNS and CNS (Barrette et al., 2008; Boivin et al., 2007). Studies made on macrophages have shown that macrophage infiltration into CNS following injury is restricted only to the injured site and the rate of infiltration is slower compared to PNS (Bartholdi and Schwab, 1997; Popovich and Hickey, 2001). This differ-

ence could possibly indicate why degenerating myelin is cleared faster in PNS than in CNS following injury. However, the mechanisms in regulating macrophages infiltration and migration after spinal cord injury are not fully understood.

In the present study, we found that the introduction of the antiserum to proBDNF improved the functional recovery after contused SCI, suggesting that endogenous proBDNF is detrimental to injured spinal cord. However, we found that the antiserum treatment at the early stage (day 1 after injury) caused significant delay in the functional recovery, being consistent with the time course of increased ED1+ macrophage activation by the anti-proBDNF treat-
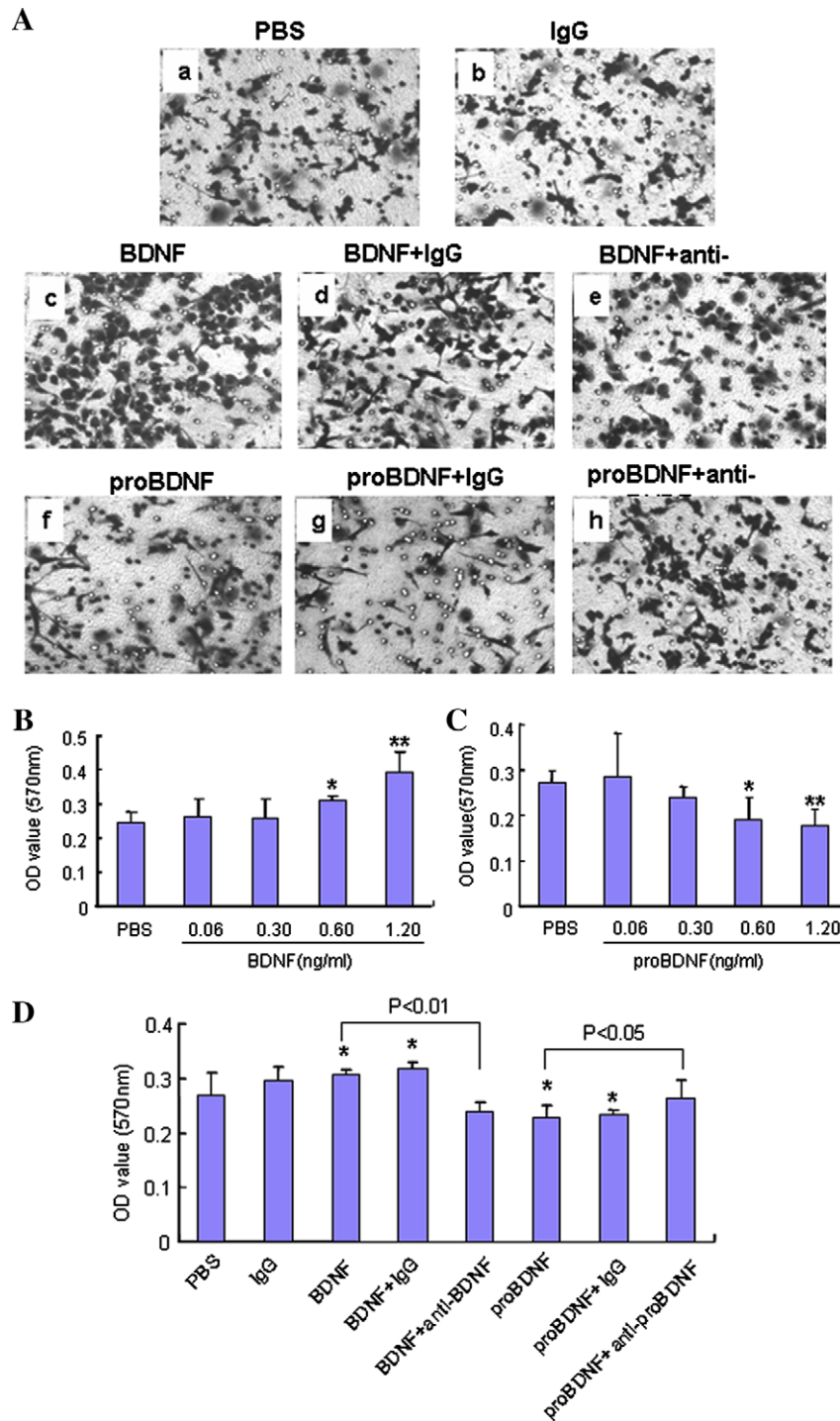
Fig. 4. Effects of proBDNF and BDNF on migration of peritoneal macrophage stimulated by LPS (1 µg/ml). (A) Photomicrograph of cultured macrophage that have migrated through transwell membranes. Cultures were treated by different proteins (BDNF, 0–1.2 ng/ml; proBDNF, 0–1.2 ng/ml) or pre-treated with BDNF antibody (5 µg/ml), proBDNF antibody (10 µg/ml) or sheep IgG (10 µg/ml) for 24 h. (B–D) Quantitative assessment of cells transmigrated through the transwell membranes (B, treated with BDNF; C, treated with proBDNF; D, treated with BDNF antibody or proBDNF antibody by cresyl violet staining of the cells followed by cell lysis and measurement of absorbance at 570 nm (see Section 2). Data are presented as mean ± SEM. **$p < 0.01$ and *$p < 0.05$ compared with PBS control group.

ment. The mechanism underlying the detrimental effect of the antiserum at the early stage is not clear but it is likely due to the detrimental effects of a subpopulation (M1) of early activated resident macrophages (Blight, 1992; Kigerl et al., 2009; Longbrake et al., 2007; Popovich et al., 1997). A large body of evidence suggests that activation of macrophage after SCI exerts detrimental as well as beneficial effects (Bethea and Dietrich, 2002; Popovich

et al., 1999; Schwartz and Yoles, 2005). Part of the macrophage controversy is due to its complexity in expression of the plethora of chemokines and cytokines in the spinal cord parenchyma and in the circulation after SCI (Kigerl et al., 2009; Kostyk et al., 2008; McTigue et al., 2000). Secretion of neurotoxic factors or cytokines such as TNF-α and/or neuroprotective cytokines such as GM-CSF and interleukin-10 and neurotrophic factors from mac-
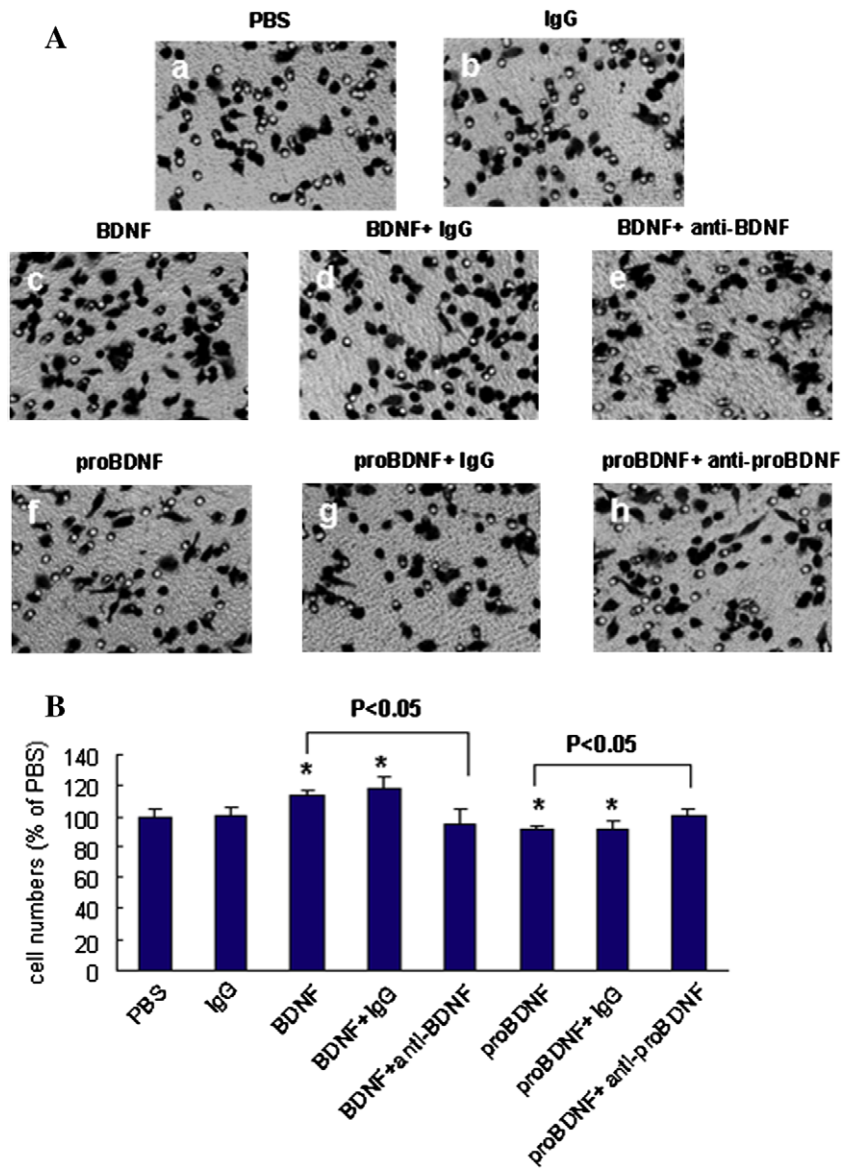
Fig. 5. Effects of proBDNF and BDNF on migration of bone marrow derived macrophage in the absence or presence of LPS. (A and C) Photomicrographs of cultured macrophage that have migrated through transwell membranes in the absence (A) or presence of LPS (10 ng/ml, C). Cultures were treated by different protein (BDNF, 2 ng/ml; proBDNF, 2 ng/ml) or pre-treated with BDNF antibody (5 μg/ml), proBDNF antibody (10 μg/ml) or rabbit IgG (10 μg/ml) for 24 h. (B and D) Quantitative assessment of cells transmigrated through the transwell membranes. The cells from five viewing fields per group were used to calculate percentages of cells under a microscope. Data are presented as mean ± SEM. $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with PBS control group.

rophages may underline their double-sword effects after spinal cord injury (Bethea and Dietrich, 2002; Bouhy et al., 2006; Giulian et al., 1993). It is likely that early activated infiltrated ED1+ macrophages secrete more detrimental and pro-inflammatory cytokines and factors which delays the functional recovery by interfering with the neural plasticity (Giulian et al., 1993; Gris et al., 2004) and causing axon dieback (Busch et al., 2009; Horn et al., 2008), whereas the infiltration of bone marrow-derived ED1+ macrophages and residential microglia, 3 days after injury, may be more beneficial for the functional recovery, as seen in the present studies. It is known that macrophages infiltrated 3 days after injury are mostly derived from bone marrow and express a number of cytokines including anti-inflammatory interleukin-10 (Longbrake et al., 2007; Shechter et al., 2009). Further studies are required to identify the phenotypes of ED1+ cells at different stages after the treatment of the anti-proBDNF serum in SCI in order to identify the window of optimal time for the intervention. Based on our

current behavioral data and histological data, we propose that it would be more appropriate and beneficial to treat injured subjects with anti-proBDNF serum 3 days after injury.

Several key players have been implicated in the induction of neuronal death, including sortilin and p75^{NTR} receptors and the death inducing ligands, proBDNF and proNGF (Beattie et al., 2002). ProBDNF and proNGF bind both sortilin and p75^{NTR} receptors with a high affinity to induce apoptosis (Teng et al., 2005). We found that proBDNF and its receptor, sortilin and p75^{NTR}, are all present in both the spinal cord following SCI and the uninjured spinal cord. Previous studies show that the levels of neurotrophins are increased after injury (Donovan et al., 1995; Dougherty et al., 2000; Batchelor et al., 1999) and recent findings have also noted an increase in proNGF after SCI (Beattie et al., 2002). Our Western blot and immunohistochemical data showed that sortilin was also present in the spinal cord both before and after injury. It is interesting to note that sortilin expression did not change significantly
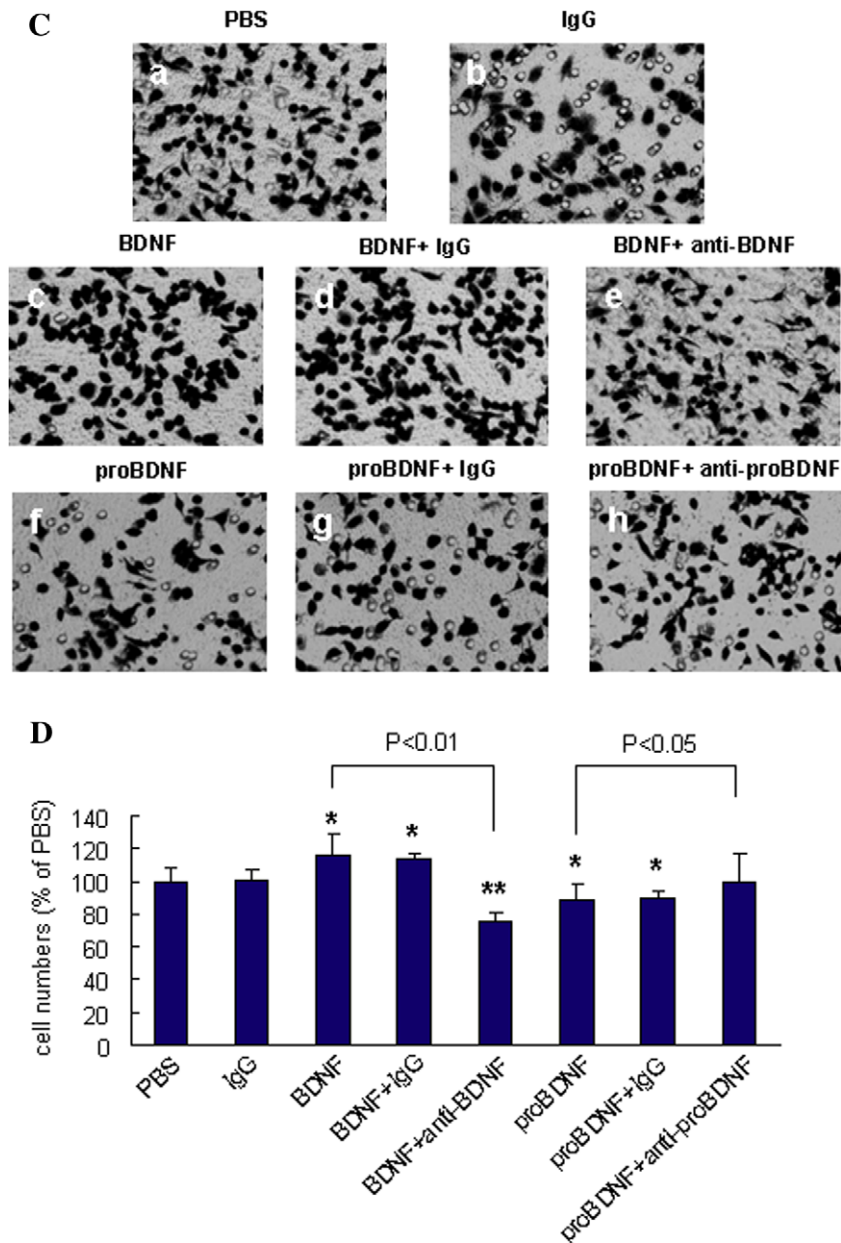
Fig. 5 (*continued*)

after SCI, whereas the pan-neurotrophin receptor, p75[NTR], a member of the tumour necrosis factor (TNF) receptor superfamily (Roux and Barker, 2002), appeared significantly increased after SCI. This result is consistent with other studies demonstrating that p75[NTR] is induced after injuries in the CNS (Beattie et al., 2002; Byers et al., 1992; Calza et al., 1997). P75[NTR] is required for pro-neurotrophins such as proNGF and proBDNF to initiate neuronal death (Nykjaer et al., 2004; Teng et al., 2005). Induction of p75[NTR] could be the first step for apoptotic cascade after injury or it may suggest regenerative responses initiated by the injured system (Beattie et al., 2002). Our current studies found that the anti-proBDNF serum treatment significantly protected spinal cord from neuronal loss, preserved spinal cord tissues and reduced demyelinated tissues, suggesting that endogenous proBDNF may have direct effects on neurons which express p75[NTR] and sortilin. However, we cannot eliminate the possibility that the neuronal protection was due to the indirect effects of enhanced activation and infiltration of macrophages.

It is well known that macrophages express BDNF and its receptors in a number of inflammatory conditions and tissues including spinal cord injury (Bouhy et al., 2006; Ikeda et al., 2001), lingual tonsils (Artico et al., 2008), respiratory allergic inflammation and pulmonary sarcoidosis (Braun et al., 1999, 2004; Ricci et al., 2005), brain injury (Batchelor et al., 1999) and peripheral nerve injuries (Meyer et al., 1992). Accumulating evidence indicates that phagocytic activity of macrophages depends on BDNF synthesis and/or TrkB (FL) expression and BDNF participates in the activation processes of macrophages by acting in an autocrine manner (Asami et al., 2006). Activated macrophages which secret BDNF and other neurotrophic factors are likely beneficial for the axonal sprouting and regeneration after nerve injury (Batchelor et al., 2002). As BDNF is synthesized as a precursor and is present as a major gene product (Zhou et al., 2004; Peng et al., 2005), it is essential to understand the functions of proBDNF in macrophages, in particular in the setting of the spinal cord injury. Our data showed that after applying an antibody to proBDNF, the number of macrophages in
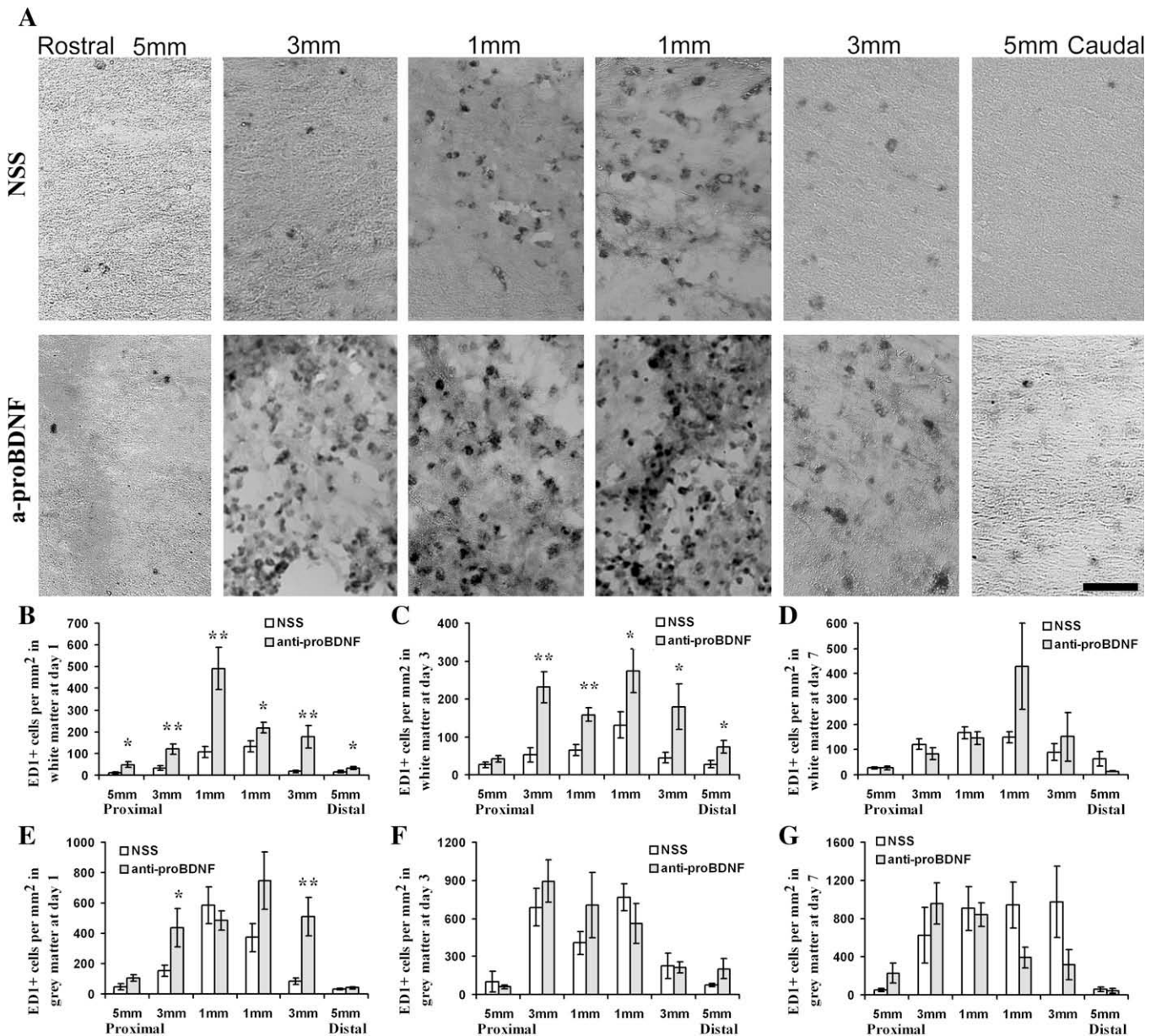
Fig. 6. Effects of proBDNF antibody treatment on the infiltration of ED1+ macrophages in the injured spinal cord. (A) Representative microphotographs of the white matter of injured spinal cord tissues stained for ED1+ macrophages at different distance from the central injury point 3 days after spinal cord injury. Top panels were taken from normal sheep serum treated control mice and bottom panels were taken from the proBDNF antiserum treated mice. (B–D) Group data on the number of macrophages counted at different regions of the white matter 1, 3, and 7 days after spinal cord injury. More ED1 positive macrophages are detected in the injured white matter of the injured spinal cord in proBDNF antiserum treated mice than that in the normal sheep serum treated mice. (E–G) Group data on the number of macrophages counted at different regions of the grey matter 1, 3, and 7 days after spinal cord injury, $n = 9$ in each group. Scale bar: 50 μm. $*p < 0.05$, $**p < 0.01$ compared with normal sheep serum treated mice.

the injured spinal cord increased, and the difference was obvious particularly in the white matter in the first 3 days, suggesting that endogenous proBDNF may inhibit the infiltration of macrophages. This assumption was further supported by our *in vitro* data showing that proBDNF inhibited migration of isolated macrophages in a dose-dependent manner. The inhibitory effect was reversed when the specific neutralization antibodies were present. Our data also show that mature BDNF promotes the migration of OX42+ macrophages, which is consistent with previous studies (Asami et al., 2006; Pernet and Di Polo, 2006). We showed that the proBDNF receptors sortilin and p75[NTR] are expressed by macrophages and proBDNF is secreted by macrophages and the secretion appeared increased in the presence of LPS, suggesting that proBDNF plays an autocrine role regulating the migration and infiltration after

nerve injury. The opposite effects of mature and proBDNF on macrophage functions is analogous to their effects on neuronal survival, where mature BDNF increases the survival via activation of TrkB whereas proBDNF induces the death of neurons via p75[NTR] (Teng et al., 2005). The opposing effect of proBDNF is also seen in the modulation of hippocampal synaptic plasticity where proBDNF mediates long term depression via p75[NTR] signalling pathway and mature BDNF mediates long term potentiation via TrkB (Woo et al., 2005) and in the neuromuscular synapse (Yang et al., 2009).

Our data also suggest that the functions of BDNF gene products are complicated and different sets of signalling pathways are possibly involved. The proBDNF/sortilin/p75[NTR] pathway may elicit opposite signals from that of the BDNF/TrkB pathway (Woo et al., 2005), which oppose to each other to cause activation or deactiva-
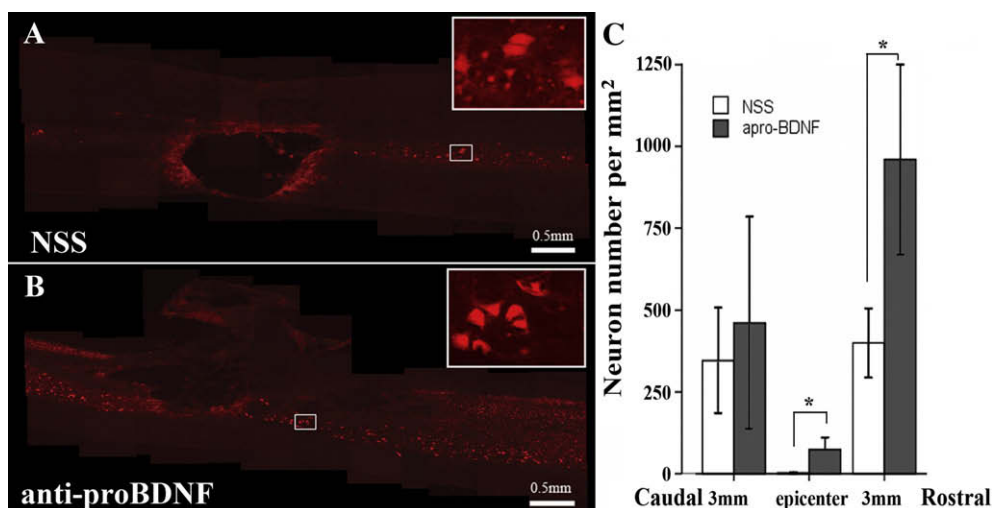
Fig. 7. Effects of proBDNF antiserum treatment on the number of neurons in the centrally located sections of injured spinal cord. Spinal cord sections from rats with SCI 7 days post-injury were stained for NeuN. (A) Examples of NeuN stained sections from a NSS-treated rat and (B) from an antibody treated rat. (C) Quantitation data on neuron numbers counted at the epicentre and 3 mm away from the centre, $n = 6$ in each group. $*p < 0.05$.
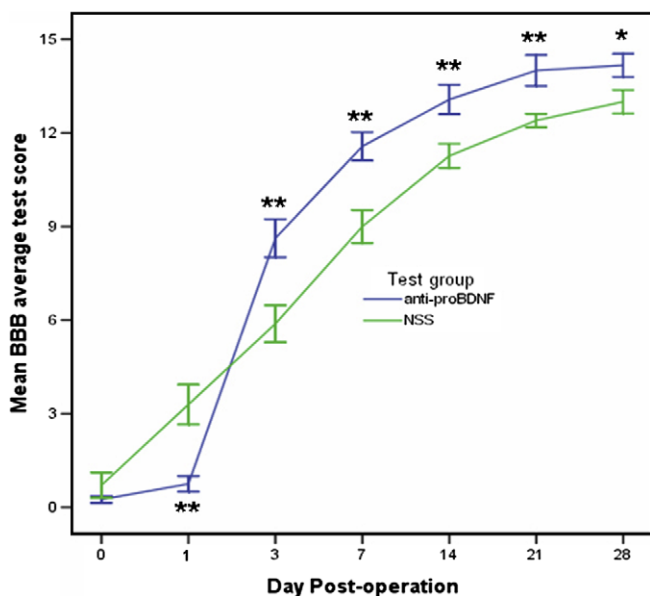

Fig. 8. Effects of proBDNF antiserum treatment on the recovery of locomotor functions. After the contusion injury on the spinal cord, rats were treated either by normal sheep serum or by antiserum to the prodomain of BDNF. After injury, BBB scores were recorded by two independent researchers in a blind manner. After completion of the test, the scores from individual animals recorded from two researchers are averaged, and average scores from two different groups of animals were analysed and compared by ANOVA and Turkey's test. $*p < 0.05$, $**p < 0.01$ compared with normal sheep serum group.

tion of macrophages. As macrophages are important players in the innate immune response and inflammation, understanding the signalling mechanisms underlying regulation of macrophage functions by mature BDNF and proBDNF would allow us to harness the inflammation in many clinical settings (Yong and Rivest, 2009). Our data showed that the functional recovery was much improved in rats with contused spinal cord injury after the treatment with the antibodies to proBDNF. One likely mechanism underlying this beneficial effect of anti-proBDNF is to improve the function of macrophages (Bouhy et al., 2006). Whether proBDNF antibody treatment acts to protect neurons from cell death and/or to enhance axonal regeneration awaits further analysis. Nevertheless,

our study suggests that proBDNF may be a therapeutic target for the acute spinal cord injury.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.01.001.

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