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Immune activation is required for NT-3-induced axonal plasticity in chronic spinal cord injury

Qin Chen^{1,2}, George M. Smith⁵, and H. David Shine^{1,2,3,4,*}

¹ Department of Neurosurgery, Baylor College of Medicine, Houston, Texas, 77030

² Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas, 77030

³ Department of Neuroscience, Baylor College of Medicine, Houston, Texas, 77030

⁴ Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, 77030

⁵ Department of Physiology and Spinal Cord and Brain Injury Research Center (SCoBIRC), University of Kentucky, Lexington, KY 40536, USA

Abstract

After an unilateral lesion of the corticospinal tract (CST) at the level of the medulla over-expression of Neurotrophin-3 (NT-3) in lumbar spinal cord motoneurons induced axonal sprouting of the intact CST in the acutely injured but not uninjured or chronically injured spinal cord in rats. This suggested that processes associated with immune-mediated wound healing may act with NT-3 to induce neuroplasticity. To test whether immune processes were involved we measured NT-3 induced axonal sprouting in immunosuppressed compared to immunocompetent rats. Rats were immunosuppressed with anti-leukocyte antibodies 1 day before receiving a CST lesion and then 2 weeks later NT-3 was over-expressed in the lumbar spinal motoneurons with an adenoviral vector carrying the NT-3 gene targeted to the motoneurons by retrograde transport. At 35 days post-lesion no axonal sprouting was measured in immunosuppressed rats whereas axonal sprouting was measured in the immunocompetent rats. We then tested whether re-evoking an immune response in chronically lesioned rats would induce neuroplasticity. Rats received CST lesions and then 4 months later were treated with systemic injections of lipopolysaccharide (LPS) 7 days before NT-3 was over-expressed in the lumbar spinal motoneurons. Axonal sprouting was observed in the LPS treated rats but not in control animals that were not treated with LPS. Further studies showed that lesioning the CST activated and LPS re-activated microglia and CD4⁺ T-cells in the acutely lesioned and chronically lesioned rats, respectively. However, immunosuppression only decreased the number of activated CD4⁺ T-cells suggesting they were responsible for the support of axonal growth. These observations demonstrate that processes associated with immune-mediated wound healing play a role in NT-3 induced neuroplasticity after injury.

Keywords

neuroplasticity; spinal cord injury; neuroinflammation; Neurotrophin-3; microglia; CD4⁺ T-cells; corticospinal tract

Corresponding author: *H. David Shine Ph.D. (corresponding author), Center for Cell and Gene Therapy and Department of Neurosurgery, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA; Tel: 713-798-3828, Fax: 713-798-1230, E-mail: E-mail: hshine@bcm.edu.

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Introduction

The CNS's failure to regenerate has been attributed to the presence of inhibitory molecules, the presence of cellular barriers, the absence of supportive molecules, and the inability of adult neurons to support long axonal growth (Maier, I. and Schwab, M. 06; Silver, J. and Miller, J. H. 04). Experimental manipulations designed to overcome these impediments have been successful in inducing axonal growth and, in some cases, improving function after injury. However, these manipulations are mostly successful only in the acute phase after injury (Houle, J. D. and Tessler, A. 03). This lack of regeneration following a delay of 1 month suggests that either the neuron loses its ability to mount a regenerative response or that the environment changes to a more inhospitable state or both.

Previously, we reported that when Neurotrophin-3 (NT-3) was over-expressed in motoneurons of the lumbar spinal cord in rats 2 weeks after they had received an unilateral corticospinal tract (CST) lesion (CSTL), the unlesioned CST would grow towards the source of NT-3 (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03; Zhou, L. and Shine, H. D. 03; Chen, Q., Zhou, L., and Shine, H. D. 06). However, we also reported that if the CST was not lesioned or if we delayed the NT-3 over-expression by 4 months after a lesion we did not measure a significant growth response (Chen, Q., Zhou, L., and Shine, H. D. 06). These observations suggested that processes associated with Wallerian Degeneration (WD) of the distal axon segments in the lumbar region of the spinal cord with the injury were required to induce axonal growth in the presence of NT-3. WD induces a rapid activation of microglia, the CNS resident macrophages (Koshinaga, M. and Whitemore, S. R. 95; Popovich, P. G., Wei, P., and Stokes, B. T. 97) and subsequently an influx of peripheral macrophages (Perry, V. H., Brown, M. C., and Gordon, S. 87). Besides phagocytosing cellular debris, these activated immune cells may have neuroprotective and neuro-reparative functions through their release of molecules such as cytokines, chemokines, and growth factors (Profyris, C., Cheema, S. S., Zang, D., Azari, M. F., Boyle, K., and Petratos, S. 04).

We surmised that immune related cellular events associated with the acute WD processes could act with the elevated levels of NT-3 to induce the axonal growth in our model of acute CSTL. If this were the case then modification of the immune response to the CST lesion in our model would modify the axonal growth response to elevated NT-3 concentrations. We report here that immunosuppression of rats with acute lesions of their CST blocked the axonal growth promoting effects of NT-3 over-expression. Reactivation of the immune response associated with WD enabled NT-3-induced axonal growth in animals with chronic (4 month) CST lesions. Unilateral lesions of the CST at the level of the medulla resulted in the activation of microglia and CD4⁺ T-cells in the lumbar region of the spinal cord. Microglia and CD4⁺ T-cells were reactivated by LPS but immunosuppression only affected the activation of CD4⁺ T-cells suggesting that CD4⁺ T-cells are associated with processes that support axonal growth in the presence of supraphysiological levels of NT-3 in both the acutely and chronically lesioned models of spinal cord injury (SCI).

Materials and Methods

Animals and pyramidotomy

Adult female Sprague Dawley rats (250–300gm) were used in this study. All surgical procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Unilateral CST lesions were performed at the level of the pyramids above the decussation of the CST as described previously (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03; Zhou, L. and Shine, H. D. 03). Rats were

anesthetized with continuous isoflurane using a vaporizing system (Vip 3000; Matrx Medical Inc., Orchard Park, NY). A midline incision was made in the ventral neck region and the basiooccipital portion of the skull was exposed by blunt dissection. A 2 mm craniotomy was performed with a drill burr just lateral to the midline ridge to reveal the left pyramid. Taking the basal artery as the landmark of the midline, a 1.5-mm wide and 0.5-mm deep incision was made into the pyramid followed by aspiration the incision site with a fine-tipped glass suction pipette to ensure that all the CST was completely transected. The exposed brain tissue was covered with gel foam and the skin was closed with wound clips. Buprenorphine (0.1 – 0.5 mg/kg) or ketoprofen (5mg/kg) was administrated by subcutaneous injection for post-operative analgesia.

Anterograde tracing unlesioned CST fibers with biotinylated dextran amine

Biotinylated dextran amine (BDA; lysine fixable, MW 10,000; Molecular Probes, Eugene, OR) was used to label axons of unlesioned CST as described previously (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03; Zhou, L. and Shine, H. D. 03; Chen, Q, Zhou, L., and Shine, H. D. 06). The rats were anesthetized with isoflurane, positioned in a stereotaxic frame, and the sensorimotor cortices were exposed. Using a Nanoliter Injector (World Precision Instruments, Sarasota, FL) fitted with a glass pipette with a 40 μ m tip, a solution of BDA (10% in phosphate buffered saline (PBS), pH 7.4) was injected into 12 sites (147 nl per site) at a depth of 1.2 μ m in the sensorimotor cortex (Paxinos, G. and Watson, C. 86; Grill, R. J., Murai, K., Blesch, A., Gage, F. H., and Tuszynski, M. H. 97). The skin was closed with wound clips and ketoprofen (5mg/kg) or buprenorphin (0.1–0.5 mg/kg) was administered as an analgesic.

Retrograde delivery of adenoviral vectors to motoneurons of the rat lumbar spinal cord

Replication-defective adenoviral vectors (Adv) carrying the DNA sequences for rat NT-3 (Adv. NT-3) or LacZ gene (Adv. LacZ) under control of the mammalian EF1 α promoter (Baumgartner, B. J. and Shine, H. D. 97; Baumgartner, B. J. and Shine, H. D. 98) were delivered into the rat lumbar spinal cord using the same method previously reported (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03; Zhou, L. and Shine, H. D. 03; Chen, Q, Zhou, L., and Shine, H. D. 06). Rats were anesthetized and an incision was made posterior and parallel to the femur on the right leg to expose the sciatic nerve. The sciatic nerve was transected approximately 2 mm proximal to the bifurcation of the common peroneal and tibial branches. The proximal end of the cut nerve was placed into a small polyethylene chamber filled with 1×10^9 infectious units of either Adv. NT-3 or Adv. LacZ, fixed with cyanoacrylate glue, and the incision was closed with sutures and wound clips.

Immunosuppression with anti-leukocytic antibodies and lipopolysaccharide treatment

Purified monoclonal antibodies against the rat CD4 (W3/25) and CD45 (MRC OX-22) was used to transiently suppress the immune system (Romero, M. I. and Smith, G. M. 98; Caballero, F., Pelegri, C., Castell, M., Franch, A., and Castellote, C. 98). One day before CST lesion, rats received an intraperitoneal injection (i.p.) of a combination of 50 μ g of each antibody. To reactivate the immune response to WD, rats were given intraperitoneal injections of LPS (250 μ g/kg prepared in sterile saline; from *Escherichia coli*, serotype 055:B5, Sigma, L-2880). This dose was selected based upon discussions with V. H. Perry to reactivate WD in the lesioned CST without causing a significant elevation in fever. Rectal temperatures were measured before and 1, 5, 18, and 24 hours after LPS injection.

Isolation of CNS mononuclear cells from the spinal cord and flow cytometry analysis

A modified method based on methods described by others (Campanella, M., Sciorati, C., Tarozzo, G., and Beltramo, M. 02; Mack, C. L., Vanderlugt-Castaneda, C. L., Neville, K. L.,

and Miller, S. D. 03) and avoids the alteration of surface antigens caused by enzymatic digestions (Ford, A. L., Foulcher, E., Goodsall, A. L., and Sedgwick, J. D. 96) was used to isolate mononuclear cells from the dorsal columns. Rats were anesthetized with isoflurane and perfused through the left ventricle with cold PBS until the effluent ran clear. Spinal cords were removed and placed in cold Hank's Basic Salt Solution (HBSS). The dorsal columns were dissected from the spinal cords and forced through nylon mesh (100 μ m, Falcon) into cold HBSS with 10% FBS to dissociate the tissue. The homogenate was resuspended in 30% Percoll solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and underlaid with 70% Percoll solution. The gradients were centrifuged at $1000 \times g$ at 24°C for 20 minutes. CNS mononuclear cells were collected from the Percoll interface, washed once with HBSS supplemented with 10% FBS and centrifuged at $200 \times g$ at room temperature for 10 minutes. The pellets were resuspended in FACS buffer (PBS with 0.08% NaN_3) and incubated with FITC-conjugated anti-rat CD 45 (1:100, clone OX-1, BD Biosciences Pharmingen, San Jose, CA) and PE-conjugated anti-rat CD 11b/c (1:100, clone OX-42, BD Biosciences Pharmingen) antibodies for 40 minutes at 4°C in FACS buffer with 2% FBS, washed twice in FACS buffer, resuspended in FACS buffer, and analyzed with BD FACS Aria (BD Biosciences Pharmingen). Debris was excluded from analysis by using forward and side scatter parameters.

Histochemistry and immunohistochemistry

At the end of observation time points, rats were transcardially perfused with cold 4% paraformaldehyde in PBS. A portion of the lumbar spinal cord (L3 to L6) was removed and postfixed in the same fixation buffer for 6 hours and then infiltrated with 21% sucrose in PBS for cryoprotection. Cross sections of spinal cord 40 μ m thick were cut on a cryostat and stored in cryoprotectant solution at -20°C until processed for histochemistry and immunohistochemistry study. Sections representing different levels of the lumbar spinal cord were picked randomly for BDA staining. The cryosections were washed in PBS and incubated with 0.3% H_2O_2 in PBS for 30 minutes at room temperature to remove endogenous peroxide activity. After 3 washes in PBST (PBS containing 0.1% Triton X-100), sections were incubated with ABC reagents (Vector Laboratories, Burlingame, CA) overnight at 4°C. Following 2 washes in PBST and one wash in PBS, the CST axons were incubated in freshly prepared diaminobenzidine solution (0.7mg/ml) containing 0.06% nickel chloride and 0.015% hydrogen peroxide until the neurites that were labeled with BDA developed a dark-brown reaction product. The sections were subsequently dried on glass slides, dehydrated in graded ethanol, cleared in xylene and coverslipped.

Microglia in the lumbar spinal cord were assessed by immunohistochemistry using OX-42 raised against the complement receptor type 3 (CR3) that is expressed on ramified, amoeboid, and reactivated microglia and on macrophages (Aldskogius, H. and Kozlova, E. N. 98). Sections were washed in PBS and incubated in a blocking solution of 3% goat serum in PBS for 1 hour at room temperature followed by 3 washes in PBST. Then they were sections were incubated with OX-42 (monoclonal mouse anti-rat CD11b; MRC OX-42, Serotec, Oxford, UK, 1:200 in PBST) overnight at 4°C. Following 2 washes in PBST, the sections were incubated for 2 hours in 1:200 Cy2 conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) prepared in PBST with 1.5% goat serum. After 2 PBS washes, the sections were incubated 15 minutes with TO-PRO-3 (1:1000 in PBS, Molecular Probes, Eugene, OR) to stain the nuclei, and then washed in PBS for 2 times. The sections were mounted onto slides and coverslipped with Airvol 205 (Air Products Chemical Inc., Allentown, PA).

The W3/25 antibody reacts with CD4 molecule expressed on helper T-cells (Clark, S. J., Jefferies, W. A., Barclay, A. N., Gagnon, J., and Williams, A. F. 87) as well as some microglia and macrophages (Sroga, J. M., Jones, T. B., Kigerl, K. A., McGaughy, V. M., and Popovich,

P. G. 03). After incubating with 0.3% H₂O₂ in PBS for 30 minutes, the cryosections were washed in PBS and incubated in a blocking solution of 5% horse serum in PBS for 1 hour at room temperature and then washed 3 times in PBST. The sections were then incubated with W3/25 (1:200 in PBST) overnight at 4 °C. Following 3 washes in PBST, the sections were incubated with biotin conjugated horse anti-mouse secondly antibody (1:200) and avidin-biotin complex (ABC) reagents (Vector Laboratories) according to the procedures recommended by the manufacturer. The W3/25 positive cells developed the dark brown reaction product after 4 minutes of incubation in freshly prepared diaminobenzidine solution (0.7mg/ml) containing 0.03% H₂O₂ and 0.06% nickel chloride. The sections were subsequently dried on glass slides, dehydrated in graded ethanol, cleared in xylene and coverslipped.

Morphometric analysis and quantification of axonal plasticity

Slides were coded so that axon densities and number of inflammatory cells were determined by an investigator unaware of the treatment of each animal. Quantification of the amount of axonal crossing from the intact CST to the region of motoneurons over-expressing NT-3 was measured from photomicrographs using image analysis software as described previously (Chen, Q, Zhou, L, and Shine, H. D. 06; Grider, M. H. and Shine, H. D. 06). Dark-field photomicrographs of the spinal cord sections were taken with a digital camera (SPOT RT Color-2000, Diagnostic Instruments, USA) at 100X magnification. The photomicrographs were then analyzed using Image J software and the Feature J software plug-in (Sato, Y., Nakajima, S., Shiraga, N., Atsumi, H., Yoshida, S., Koller, T., Gerig, G., and Kikinis, R. 98) to automatically highlight linear figures representing BDA-labeled axons against a white background. The number of axons in the midline region was measured as the number of pixels in a region 26 µm wide and running from the bottom of the dorsal column to the top of the ventral column. To avoid error resulting from variable BDA labeling efficiencies among animals, the number of pixels in a region adjacent to the midline area and ipsilateral to the intact CST was measured. The value of axons crossing the midline was expressed as the ratio of pixels in the midline region to the number of pixels in the lateral region normalized to values of normal animals.

Quantification of microglia and CD4⁺ T-cells

Quantification of the amount of activation of microglia was measured from digital photomicrographs taken with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany) at 20x magnification. A series of photos were compiled using the Z-stack program in the LSM 510 software suite. Morphology of microglia change from ramified morphology to amoeboid and rounded (macrophage) morphology after activation (Hossain-Ibrahim, M. K., Rezajooi, K., MacNally, J. K., Mason, M. R., Lieberman, A. R., and Anderson, P. N. 06). Only microglia containing identifiable nuclei were counted. T-lymphocytes were counted from digital photomicrographs at 200x magnification. Since CNS T-cells are typically uniform in shape and size and are sparse in CNS tissue they were easily identified in the spinal cord sections. Only cells that had a clearly defined pattern of membrane staining were counted. For both quantitative analyses, the cells were counted in the dorsal columns of the lumbar spinal cords in 3 cross sections cut from each animal at each of the observation time points. To avoid the possibility of counting the same cells in adjacent sections the sections were spaced 600 µm apart.

Statistical analyses

All data are reported as group means ± standard error of the mean (SEM). A one-way ANOVA test, followed by the Student–Newman–Keul's or Dunn's test, was used to assess variances. All values with a $p < 0.05$ were accepted as significant.

Results

Anti-leukocyte immunosuppression blocked NT-3-induced axonal sprouting in acutely CSTL rats

Previously, we found that NT-3 induced axonal sprouting of the intact CST in the acutely injured but not in the uninjured or chronically injured spinal cord suggesting that processes associated with immune-mediated wound healing participated to induce neuroplasticity. To test whether an immune response mediated axonal sprouting we measured the effect of NT-3 expression on sprouting in rats that were immunosuppressed with antibodies against the CD4 and CD45 receptors. One day before CST lesion, 10 rats (immunosuppressed group) received intraperitoneal injections (i.p.) of purified monoclonal antibodies against the rat CD4 receptor (W3/25; 50 µg) and the CD45 receptor (MRC OX-22; 50 µg) to transiently suppress the immune system (Caballero, F., Pelegri, C., Castell, M., Franch, A., and Castellote, C. 98; Pelegri, C., Castell, M., Serra, M., Rabanal, M., Rodriguez-Palmero, M., Castellote, C., and Franch, A. 01; Romero, M. I. and Smith, G. M. 98). Six rats received saline i.p. as control (immunocompetent group). Ten days later after CST lesion, all rats received multiple injections of BDA into the somatosensory region of the cortex that innervated the intact CST in order to label the unlesioned CST fibers (Zhou et al., 2003). At 2 weeks following CSTL, adenoviral vectors carrying genes for NT-3 or LacZ under the control of the EF1α promoter were delivered by retrograde transport through the sciatic nerve to the lumbar spinal motoneurons on the side of the lesioned CST. Five rats that were immunosuppressed with anti-leukocyte antibodies received Adv. NT-3 (immunosuppressed, CSTL+Adv. NT-3, n=5) and 5 received Adv. LacZ (immunosuppressed, CSTL+Adv. LacZ, n=5). Three rats that were not immunosuppressed received Adv. NT-3 (immunocompetent, CSTL+Adv. NT-3, n=3) and 3 received Adv. LacZ (immunocompetent, CSTL+Adv. LacZ, n=3). Another 3 intact rats served as normal controls. At 35 days after CSTL the amount of axonal growth from the intact CST to the contralateral side was assessed.

Photomicrographs of cross sections taken from the lumbar spinal cord showed that few axons projected from the intact CST to the lesioned side of the lumbar spinal cord in either the immunocompetent rats that received Adv. LacZ (Fig. 1D) or the immunosuppressed rats that received Adv. NT-3 (Fig. 1A) or Adv. LacZ (Fig. 1C). In contrast, considerably more axons projected across the midline into the lesioned side of the spinal cord in the immunocompetent rat that received Adv. NT3 rats (Fig. 1B) as has been reported previously (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03; Zhou, L. and Shine, H. D. 03; Chen, Q., Zhou, L., and Shine, H. D. 06).

Quantification of sprouting (Fig. 1E) showed that the amount of axonal sprouting in immunosuppressed CSTL+Adv. NT-3 group was significantly less than that of immunocompetent CSTL+Adv. NT-3 group (ANOVA followed by the Student-Newman-Keuls test; $P < 0.001$; $F = 9.85$; $df = 18$). There was no difference in the number of sprouting axons among the immunocompetent CSTL+Adv. LacZ group and the immunosuppressed groups treated with Adv. NT3 or Adv. LacZ. These data show that over-expression of NT-3 failed to induce axonal sprouting in the acutely lesioned rats when their leukocyte population was transiently depleted and suggests that an immune-related process is required for axonal growth in the presence of supraphysiological levels of NT-3.

NT-3 induced axonal sprouting in chronic spinal cord lesioned rats when treated with LPS

Previously, we reported that if NT-3 over-expression was delayed for 4 months after a CST lesion no axonal sprouting was observed (Chen, Q., Zhou, L., and Shine, H. D. 06). One explanation for this observation is that processes associated with WD that were required for axonal growth in the acute situation had resolved by 4 months in the chronic situation so that

NT-3 could not induce axonal growth. We surmised that a likely process was the acute immune response to WD and that re-evoking the response in the chronically lesioned animal may enable the NT-3-induced axonal growth. To test whether reactivating the immune response associated with WD would permit NT-3-induced axonal growth we reactivated the immune reaction associated with WD in rats with chronic CST lesions using LPS, an endotoxin derived from gram-negative bacteria that stimulates macrophages and microglia cells. Systemic injection of LPS triggers cellular responses such as phagocytosis (Vallieres, N., Berard, J. L., David, S., and Lacroix, S. 06) and induces expression of the major histocompatibility class II antigen on microglia in the CNS (Ng, Y. K. and Ling, E. A. 97). Rats with chronic (4 month) CST lesions were given a single systemic (250 µg/kg; i.p.) injection of LPS and four days later, BDA was injected into cortex to label intact CTL fibers. At 1 week after LPS injection, rats were treated with Adv. NT-3 (n=8) or Adv. LacZ (n=8) in the same manner as described above. Another 8 rats without CST lesions of same age as the chronically injured rats were used as unlesioned controls. All animals were treated with LPS, their CST was labeled with BDA, and Adv. NT-3 (n=4) or Adv. LacZ (n=4) was delivered to the lumbar region by retrograde transport from sciatic nerve 7 days later after LPS injection. All animals were killed at 28 days after LPS administration to assess the axonal sprouting.

Photomicrographs of cross sections of lumbar spinal cord showed that more axons grew from the unlesioned CST and projected across the midline to lesioned side in the rats that had been treated with LPS and Adv. NT-3 (Fig. 2A) compared to rats that had been treated with LPS and Adv. LacZ (Fig. 2C) or unlesioned rats treated with LPS and Adv. NT-3 (Fig. 2B) or LPS and Adv. LacZ (Fig. 2D).

Quantification of axonal sprouting demonstrated a significantly greater number of axons crossing the midline in the rats treated with LPS and Adv. NT3 than other groups (ANOVA followed by the Student–Newman–Keuls test; $p < 0.001$, $F = 10$, $df = 26$). There was no difference in the amount of sprouting among rats treated with LPS and Adv. LacZ, and unlesioned rats that treated with LPS and Adv. NT3 or LPS and Adv. LacZ (Fig. 2E). In contrast to our previous findings that delaying NT-3 over-expression 4 months after CSTL resulted in no axonal sprouting (Chen, Q, Zhou, L, and Shine, H. D. 06) these data demonstrated that re-activating the immune response associated with WD enables NT-3-induced axonal sprouting in the chronically lesioned rats. Additionally, these data suggest that an immune-related process is required for post-traumatic neuroplasticity and may be the explanation why others have not observed axonal growth in their experimental models at times greater than 1 month after injury (Houle, J. D. and Tessler, A. 03).

CST lesion induced activation of microglia in the spinal cord

To verify that a CST lesion at the level of the medulla would lead to a cellular immune response in the CST we determined the amount of leukocyte activation after CST lesion by flow cytometry analysis in the dorsal columns of rats that had received a CST lesion 14 days previously compared to unlesioned rats. FITC-conjugated anti-rat CD 45 and PE-conjugated anti-rat CD 11b/c were used to characterize cell population in the suspension. CD11b/c antibody recognizes monocytes/macrophages, granulocytes, and microglia whereas the CD 45 antibody identifies the rat leukocyte common antigen expressed in all leukocytes (van den Berg, T. K., Puklavec, M. J., Barclay, A. N., and Dijkstra, C. D. 01). In using these 2 antibodies we could identify and measure 3 different cell populations (Fig. 3). The $CD45^{lo}CD11b/c^{+}$ cell population (P4; Fig. 3) represents the quiescent microglia, the $CD45^{hi}/CD11b/c^{+}$ cell population (P3; Fig. 3) represents activated microglia and macrophage-like cells, and the $CD45^{hi}CD11b/c^{-}$ cell population (P2; Fig. 3) represents lymphocytes (Campanella, M., Sciorati, C., Tarozzo, G., and Beltramo, M. 02; Badie, B., Schartner, J. M., Paul, J., Bartley, B. A., Vorpahl, J., and Preston, J. K. 00). Flow cytometry analysis revealed that the $CD45^{hi}/$

CD11b/c⁺ population was increased in the spinal cords of rats that had CST lesions 14 days previously compared to unlesioned rats (P3 population = $1.55 \pm 0.35\%$ rats lesioned 14 days earlier compared to $0.15 \pm 0.05\%$ in unlesioned rats; Fig. 3). Additionally, the percentage of CD45^{hi}/CD11b/c⁻ cells in lesioned rats was also higher than that in the unlesioned rats (P2 population = $1.8 \pm 0.40\%$ in lesioned rats compared to $0.65 \pm 0.35\%$ in unlesioned rats; Fig. 3). These data verified that WD associated with a CST lesion stimulated activation of microglia and macrophages in the dorsal column of spinal cords.

To further identify the localization and amount of activation of microglia after CSTL, we measured the activation of microglia in cross sections of the lumbar spinal cord using morphological criteria with OX-42 that labels the CD11b receptor to label activated microglia and macrophages. Activated microglia appear more amoeboid in shape (Fig. 4A, 3d*, arrow) than quiescent microglia that are more ramified (Fig. 4A; 3d*, asterisk) (Hossain-Ibrahim, M. K., Rezajooi, K., MacNally, J. K., Mason, M. R., Lieberman, A. R., and Anderson, P. N. 06). Rats received unilateral lesions of the CST and then killed at different observation time points: 0d (before injury), 1, 3, 7, 14 and 120 days after CSTL. Photomicrographs of cross sections at the level of the lumbar spinal cord showed that more amoeboid and round OX-42 positive cells in the lumbar dorsal column on the lesioned side within the CST than in the dorsal column on the intact side after CSTL (Fig. 4A). There were also many OX-42 positive cells with high fluorescence signal in grey matter adjacent to lesioned CST (Data not show).

The number of activated microglia in the lesioned side showed a marked increase at 3 days after the injury and reached a peak at 7 days, then dropped slightly at 14 days but remained relatively higher ($P < 0.01$) than unlesioned CST at 4 months after injury (the last time point tested; Fig. 4B). The number of activated microglia in lesioned side CST was significantly higher than that in intact CST side area at 3, 7, 14 and 120 days after injury ($p < 0.01$). However, there was no difference in the number of activated microglia in intact CST area among different time points. These data verify that a unilateral CST lesion at the level of the medulla and the subsequent WD of the tract induced activation of microglia in the lumbar region that is approximately 8 cm from the injury site and that these morphological changes persist for at least 4 months.

Systemic injection of LPS re-activated microglia in the rats with chronic injury

To verify that systemic endotoxin challenge reactivates an immune response in the lumbar region of the spinal cord in rats with chronic (4 month) CST lesions we measured microglial activation in rats that were treated with LPS 4 months after CSTL and compared them to chronically lesioned animals that did not receive LPS. LPS (250 mg/kg) was injected systemically (i.p.) into rats with chronic CST lesions and then killed 3, 7 and 14 days later. The amount of microglial activation was determined morphologically as described above. There were many amoeboid or round reactive microglia showed in the region of the lesioned CST region compared to the unlesioned CST (Fig. 5A). The number of activated microglia in the region of the lesioned CST in rats that received LPS was increased at 3 days after LPS injection, peaked at 7 days and then decreased at 14 days after LPS injection. Compared to the chronically lesioned rats that did not receive LPS injections, the number of activated microglia was significantly greater at 7 days after LPS injection ($P < 0.05$; Fig. 5B). However, the number of activated microglia in the region of the intact CST did not change after LPS injection within the observation period (Fig. 5B). These data demonstrate that the systemic administration of LPS was sufficient to re-activate a cellular immune response in the lumbar region within and adjacent to the previously lesioned CST.

Activation of microglia after immunosuppression in the rats with acute injury

The observations that axonal sprouting coincided with microglial activation after CSTL and with LPS microglial reactivation suggested that microglial activation was responsible for NT-3 induced neuroplasticity in our model. We predicted then that the inhibitory effect of anti-leukocytic immunosuppression on NT-3-induced axonal sprouting was due to an effect on microglial activation after CSTL. To test whether systemic immunosuppression with anti-leukocyte antibodies would alter activation of microglia after CSTL by WD rats (n=8) received i.p. injections of anti-leukocyte antibodies 1 day before CSTL and then the amount of microglial activation in the lumbar dorsal columns was assessed at 1, 3, 7, and 14 days later in the same manner as above. In a similar fashion to immunocompetent rats the number of activated microglia in immunosuppressed rats were significantly higher at 3, 7, and 14 days after CST lesion ($p<0.01$) than before lesion (0 day; Fig. 6B). This lack of difference in the number of activated microglia between the immunosuppressed and immunocompetent rats demonstrated that immunosuppression had no effect on the number of activated microglia after CSTL and suggested that NT-3-induced axonal growth can not simply be attributed to microglial activation as measured by morphological criteria.

Lymphocyte infiltration was affected by administration of neutralization antibodies and LPS

To examine whether the CNS lymphocyte profiles were affected by anti-leukocyte antibody immunosuppression or LPS immune activation, CD4⁺ T-cells were visualized by immunocytochemistry with the W3/25 antibody in sections of lumbar spinal cords. Paralleling the activation of microglia and macrophages reported above, the number of T-lymphocytes progressively increased in the lesioned lumbar CST within 7 days post-injury, dropped slightly at 14 days after injury, and remained elevated at 120 days post-injury in immunocompetent rats (Fig. 7B). The numbers of CD4⁺T-lymphocytes in spinal cords of immunosuppressed rats were dramatically less in the first week after injury but, at 14 days after injury, the numbers of CD4⁺ T-cells were similar in both immunosuppressed rats and immunocompetent rats (Fig. 7B). These data suggested that administration of neutralization antibodies transiently blocked the T-cell infiltration that was triggered by WD.

In the chronically injured rats administration of systemic LPS increased the number of CD 4⁺T-cells within 7 days after LPS injection compared to rats that were not treated with LPS (Fig. 7C) These data indicated that the processes of infiltration of T-cells were re-stimulated by systemic injection of LPS in chronic lesioned rats.

Discussion

Previously, we reported the failure to induce axonal growth when an experimental manipulation, that was successful in the acute phase, was applied to an animal model of chronic injury (Zhou, L., Baumgartner, B. J., Hill-Felberg, S. J., McGowen, L. R., and Shine, H. D. 03), suggesting that processes associated with the acute injury and subsequent wound healing acted to initiate or support new axonal growth in the presence of supraphysiological concentrations of NT-3. We predicted that if an immune-related process associated with WD was involved then altering these processes would affect the influence of NT-3 over-expression on axonal growth after CST lesion. In testing this prediction we found that immunosuppressing the rats during an acute phase (14 days) of WD would block NT-3-induced axonal growth and re-activating cellular immune processes in a chronic phase (4 months) of WD would support NT-3-induced axonal growth. These observations support the hypothesis that processes associated with immune-associated wound healing can support axonal growth after neural injury and, perhaps more importantly, these supportive processes can be reactivated months after the initial injury.

Axotomy of CST fibers in the medulla induces persistent WD over long distances in the spinal cord

Cellular responses associated with WD result from the degeneration of axons distal to a point where they are cut from their soma that, in turn, leads to the fragmentation of intact myelin and induces the activation of microglia and astrocytes and, in some instances, recruitment of peripheral leukocytes. Several laboratories have reported the alteration of immune cells after WD but only at short distances from the lesion site raising the question as to whether the cellular activation was due to processes in the wound area or only from the degeneration of axons (Vallieres, N., Berard, J. L., David, S., and Lacroix, S. 06; George, Rachel and Griffin, John W. 94; Koshinaga, M. and Whittemore, S. R. 95). In the present studies we measured cellular activation at a distance of approximately 8 cm from the lesion site where factors associated with the wound would have little or no influence on the cellular activation. We found that WD alone induced significant microglial and T-cell activation that persisted for up to 4 months after the lesion supporting the findings of others.

Microglia and T-cells and NT-3 induced axonal growth

The temporal and spatial associations of activated microglia with NT-3-induced axonal growth suggests that they are involved in initiating or supporting axonal growth in the presence of supraphysiological amounts of NT-3 in our model of acute and chronic injury. As expected, lesioning the CST quickly resulted in an activation of microglia within and adjacent to the lesioned tract and, as predicted by V.H. Perry (personnel communication), a single intraperitoneal injection of LPS re-activated microglia in the spinal cords of animals with chronic CST lesions. Microglia are pluripotent immune cells in CNS that, on one hand, mediate secondary injury after SCI by releasing neurotoxic factors, including nitric oxide, TNF- α and IL-1 (Jones, T. B., McDaniel, E. E., and Popovich, P. G. 05) but on the other hand, may have neuroprotective and pro-regenerative functions (Jones, T. B., McDaniel, E. E., and Popovich, P. G. 05; Nakajima, K. and Kohsaka, S. 01; Streit, W. J. 02; Donnelly, D. J. and Popovich, P. G. 07). Activated microglia induced by SCI display several phenotypes, such as phagocytes and antigen-presenting cells (Shaked, I., Porat, Z., Gersner, R., Kipnis, J., and Schwartz, M. 04), have multiple functions including phagocytosis of cellular debris, presenting antigens to helper T-cells, and secreting neurotrophins (Nakajima, K., Honda, S., Tohyama, Y., Imai, Y., Kohsaka, S., and Kurihara, T. 01) and glutamate transporters that may protect neurons from excitatory transmitter death (Nakajima, K., Tohyama, Y., Kohsaka, S., and Kurihara, T. 01; van Landeghem, F. K., Stover, J. F., Bechmann, I., Bruck, W., Unterberg, A., Buhner, C., and von, Deimling A. 01). When grafts of activated microglia were placed into a spinal cord lesion axons grew into the graft (Rabchevsky, A. G. and Streit, W. J. 97). Hence, our observations are consistent with the hypothesis that microglia activated by the initial injury and subsequently re-activated by LPS participated in the NT-3 induced axonal growth. However, our observations that anti-leukocyte immunosuppression blocked NT-3 induced axonal growth but the immunosuppression did not reduce the number of activated microglia in the lesioned CST contradict this simple explanation. Either microglia do not directly participate in the NT-3 induced axonal growth and another arm of the cellular immune response is involved, or the immunosuppression effects the characteristics of the microglia in a way that blocks their ability to support axonal growth but are not measurable with the techniques that we employed.

An explanation for the discrepancy between the amount of microglial activation and axonal growth in the immunosuppressed rats is the possibility that peripheral T-cells are involved in a cellular cascade that supports NT-3 induced axonal growth. Our finding that there was an association with the number of CD4⁺ T-cells in the lumbar spinal cord and the amount of axonal growth in rats with acutely and chronically lesioned CST in the presence of supraphysiological levels of NT-3 suggest that they play a necessary role in neuroplasticity after injury. This

interpretation of our results is consistent with the findings of Popovich and colleague that SCI induced a progressively increasing number of T- cells, mostly CD4⁺, accompanied by the activation of microglia and influx of peripheral macrophages (Popovich, P. G., Wei, P., and Stokes, B. T. 97). CD4⁺ T-cells have been shown to protect neurons from axotomy-induced apoptosis (Jones, Kathryn J., Serpe, Craig J., Byram, Susanna C., DeBoy, Cynthia A., and Sanders, Virginia M. 05; Streit, W. J. 02). Microglia, activated by WD, may express MCP-1 that could, in turn attract CD4⁺ T-cells from the peripheral blood supply that may act with NT-3 to support axonal growth. Immunosuppression with anti-leukocytic antibodies would remove the CD4⁺ T-cells from the cascade but would not block WD induced activation of phagocytic microglia.

LPS and axonal growth after spinal cord injury

It is interesting to note that this is not the first report on the effects of LPS in the study of axonal growth after spinal cord lesions. In the 1950s Windle and colleagues reported an improved motor and sensory outcome in cats with spinal cord injuries after treatment with a lipopolysaccharide (Windle, W. F. and Chambers, W. W. 50). They reported that LPS treatment reduced astroglial scar formation at the lesion site and increased the number of macrophages at the site. These early reports resulted in clinical trials with equivocal results (Windle, W. F. 56). In 1994 Guth and colleagues reported that LPS and indomethacin significantly reduced cavity formation and improved functional recovery in rats with moderate contusions (Guth, L., Zhang, Z., DiProspero, N. A., Joubin, K., and Fitch, M. T. 94). They concluded that treatments improved outcome by promoting, “secretory activities of non-neuronal microglial cells” (Guth, L., Zhang, Z., DiProspero, N. A., Joubin, K., and Fitch, M. T. 94). David and colleagues attributed LPS induced microglial activation in decreasing the amount of cellular debris after CNS lesion although they did not report a benefit on functional outcome (Vallieres, N., Berard, J. L., David, S., and Lacroix, S. 06).

Immune-mediated wound healing and axonal growth in the chronically injured CNS

Our observation that immune activation was necessary to induce axonal growth in the mammalian nervous system with a chronic (> 1 month) injury provides evidence that immune-related healing processes have a beneficial effect on axonal growth. Additionally, it is intriguing to speculate that axonal growth induced in acutely lesioned animals by other experimental manipulations were a consequence, in part, of an immune response associated with the experimental lesion and that the inability to induce axonal growth in chronic injuries with these experimental manipulations was a consequence of a failure to maintain an adequate level of immune activation in the CNS.

Neuroplasticity and recovery after SCI

These observations are fundamental to the central problems of devising a repair for neural injury in general and SCI in particular. A major impediment to the treatment of neural injury is the intrinsic inability of the CNS to regenerate axons for long distances (centimeters) after injury due to a combination of the presence of an inhibitory environment, the lack of a suitable supportive environment, and the inability of neurons to effectively grow for long distances (Maier, I. and Schwab, M. 06; Silver, J. and Miller, J. H. 04). Hence, long distance axonal regeneration may not be readily amenable to therapeutic intervention. However, short distance growth of surviving axons, and subsequent formation of new connections, to sites denervated by the injury termed “compensatory reorganization”, “compensatory collateral sprouting” or “remodeling” may permit functional recovery after injury (Iarikov, Dmitri E., Kim, Byung G., Dai, Hai Ning, McAtee, Marietta, Kuhn, Penelope L., and Bregman, Barbara S. 07; Weidner,

N. and Tuszynski, M. H. 02; Bareyre, F. M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T. C., Weinmann, O., and Schwab, M. E. 04).

Another critical aspect for the development of a treatment of SCI is the appropriate time for a therapeutic intervention. The optimal window of opportunity for treatment of SCI is months after injury. Early interventions, within weeks after the initial injury, are not clinically feasible since the patient must be stabilized, there are often additional injuries that must be addressed first, and some function can be regained in the early days after injury. Hence, the use of novel therapies such as those based upon gene transfer would be impracticable to treat neural injuries during the acute phase of the injury process. Optimally, a treatment should be effective in patients with chronic injuries. The observation that immune-related processes may be required for successful re-growth of axons during the acute phase after injury and the observation that re-activation of these immune-related processes will re-evoke a neurotrophin-induced compensatory response suggests that treatment of chronic CNS injuries is possible.

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Figure 1.

Detection and quantification of axonal sprouting in acute spinal cord injury in immunosuppressed and immunocompetent rats. **A–D.** Photomicrographs the lumbar region of the spinal cord showing the axons of the intact CST labeled with BDA in immunosuppressed and immunocompetent animals treated with Adv. NT-3 or Adv. LacZ. One day before CSTL rats were treated with anti-leukocyte antibodies raised against the rat CD4 and CD45 lymphocytic receptors (immunosuppressed rats). Control rats received injections of saline (immunocompetent rats). After CST injury, the rats were treated with either Adv. NT-3 or Adv. LacZ. A, C, sections from immunosuppressed rats treated with Adv. NT-3 or Adv. LacZ. B, D sections from immunocompetent rats treated with Adv. NT-3 or Adv. LacZ. A greater number of BDA-labeled CST neurites was observed arising from the intact CST, crossing the midline, and growing into the gray matter on the lesioned side of the spinal cord in immunocompetent rats that received Adv. NT-3 (B). These axons were present at long

distances from the intact CST in layers V, VI, VII, and VIII of the grey matter on the lesioned side (arrowheads in B). Scale bar = 100 μ m. Rats that were immunosuppressed and treated with Adv. NT-3 or Adv. LacZ, and the rats that were immunocompetent and received Adv. LacZ did not have equivalent numbers of crossing neurites compared to the rats that were immunocompetent and received Adv. NT-3. **E.** Quantification of CST axons crossing the midline in response to the local expression of NT-3 in immunosuppressed and immunocompetent rats with CSTL. As in our previous reports the immunocompetent rats treated with Adv. NT-3 had a significantly greater number of axons that crossed the midline compared to normal animals and controls that received Adv. LacZ. The immunosuppressed rats treated with Adv. NT-3 exhibited significantly lower axonal sprouting compared to the immunocompetent rats receiving Adv. NT-3 and were similar to control rats. Values are means $\pm$ SEM; ***, $P < 0.001$ (ANOVA followed by the Student–Newman–Keuls test).

Figure 2.

Detection and quantification of axonal sprouting in rats with chronic spinal cord injuries and treated with LPS. **A–D.** Photomicrographs of the lumbar region of the spinal cord showing the axons of the intact CST labeled with BDA in rats treated with Adv. NT-3 or Adv. LacZ and treated with LPS. Rats with chronic (4 months post-lesion) CST lesions were treated with LPS. Uninjured rats at the same age of the chronic lesioned groups received saline injections and served as control groups. Seven days after LPS injection, the rats were treated with Adv. NT-3 or Adv. LacZ. **A, C,** sections from chronic injured rats treated with LPS and Adv. NT-3 or Adv. LacZ. **B, D** sections from uninjured control rats treated with LPS and Adv. NT-3 or Adv. LacZ. A greater number of BDA-labeled CST neurites arose from the intact CST, crossed the

midline, and grew into the gray matter of the lesioned side of the spinal cord in the rats that received LPS and Adv. NT-3 (A) compared to the other groups (B–D). These axons were visible for long distances in layers V, VI, and VII of the grey matter on the lesioned side of the spinal cord (arrowheads in A). Scale bar=100 μ m. **E.** Quantification of CST axons crossing the midline in response to the local expression of NT-3 in the lesioned and unlesioned rats treated with LPS.

Chronically injured rats treated with LPS and Adv. NT-3 exhibited a greater value of axonal sprouting than that of the other groups. There was no difference in the amount of axonal sprouting in unlesioned rats treated with LPS and received either Adv. NT-3 or Adv. LacZ. Values are means $\pm$ SEM; ***, $P < 0.001$ (ANOVA followed by the Student–Newman–Keuls test).

Figure 3.

Flow cytometric analysis of immune cells in the dorsal column after CSTL lesion. Cells isolated from the dorsal columns of spinal cords from unlesioned (**A**) and 14 day post-CSTL rats (**B**) were analyzed using FITC-conjugated anti-rat CD 45 and PE-conjugated anti-rat CD 11b/c antibodies. The CD45^{lo}CD11b/c⁺ cells (P4) represents the quiescent microglia, the CD45^{hi}/CD11b/c⁺ cells (P3) represents activated microglia and macrophages and the CD45^{hi}CD11b/c⁻ cells (P2) represents infiltrating lymphocytes. There were more CD45^{hi}/CD11b/c⁺ cells in the dorsal columns of rats with CSTL than in unlesioned rats. Concomitantly with an increase in activated microglia after CSTL the population of lymphocytes increased in the dorsal columns. These data indicate that WD induced by remote CSTL caused an activation of microglia and infiltration of lymphocytes in the dorsal columns.

Figure 4.

CSTL induced activation of microglia/macrophages in the lumbar dorsal column. **A.** Sections (40 μ m) taken from the lumbar spinal cord were immunostained for microglia and macrophages with OX-42 antisera and anti-mouse IgG labeled with Cy2. A series of photomicrographs were taken of the dorsal column of lumbar spinal cord at 1, 3, 7 and 14 days after CSTL using confocal microscopy and combined using software to construct 3D stacked images (Scale bars=50 μ m). (3d* denotes a high magnification image of OX-42 positive cells in 3d; scale bar = 20 μ m.) In all of photographs the left CST is the intact CST and the right is the lesioned one. After injury the morphology of microglia changed from ramified (* in 3d*) to amoeboid (arrowhead in 3d*), representing resting microglia and activated microglia/macrophages,

respectively. **B.** The numbers of activated microglia/macrophages were counted in the intact and injured CST region, respectively. Data are shown as the mean $\pm$ SEM. The number of activated microglia/macrophage in the injured CST area were significantly increased at 3 days post-lesion, peaked at 7days, and then dropped slightly at 14 days compared with that of unlesioned group (day 0) (**, $P<0.01$). There was no difference in the number of activated microglia/macrophage in unlesioned sides of all groups.

Figure 5.

Activation of microglia in chronic CST injury rats after injection of LPS. **A.** Cross sections of lumbar spinal cord stained with OX-42 in chronic lesioned rats 0 d (before injection), 3, 7, and 14 days after LPS injection. In all sections, right side is lesioned CST area, left side is intact CST area. Scale bar = 50 μ m. **B.** Quantification of activated microglia in both the intact and the lesioned CST area. The number of activated microglia in the lesioned CST area was increased after LPS injection. Compared to the chronic CST injured rat without LPS injection, the number of activated microglia was significant higher at 7 days post LPS injection (*, $P < 0.05$). There was no difference in the number of activated microglia in the intact CST area.

Figure 6.

Activation of microglia in immunosuppressed rats with acute CST injury. **A.** Cross sections from immunosuppressed acute injury rats at 1, 3, 7, and 14 days after CST lesion were stained with OX-42 to recognize microglia and macrophage like-cells. In all sections, right side is lesioned CST area, left side is intact CST area. Scale bar=50μm. **B.** Quantification of activated microglia in the intact and lesioned CST. The number of activated microglia in the lesioned CST area of immunosuppressed rats was significantly increased at 3, 7, 14 days after injury compared to uninjured rats (**, $P < 0.01$). There was no increase in the number of activated microglia in the intact side CST area.

Figure 7.

Examination and quantification of CD4+ T-cells in CST lesioned spinal cords. **A.** Photograph taken from the cross section of the lumbar spinal cord of the injured spinal cord showed that T-lymphocytes visualized with antibodies against CD4 were round and small. Several CD4+ T-lymphocytes distributed in the lesioned CST region (arrow). CD4 was also expressed on a subset of activated microglia and macrophages that had a different morphology from the T-lymphocytes (arrowhead). Scale bar=40 μ m; **B.** Quantitative comparisons of W3/25 labeling T-lymphocytes in the lesioned CST regions in the lumbar spinal cords of immunocompetent (Acute; white bar) rats and that of immunosuppressed (Acute + Ab; black bar) rats. (***, $P<0.001$; immunocompetent rats compared to immunosuppressed rats) **C.** Quantitative comparisons of W3/25 labeling of T-lymphocytes in the lesioned CST region in the lumbar

spinal cords of rats with chronically injured spinal cord. Day 0 represents untreated controls. (*, $P < 0.05$; rats treated with LPS compared to control rats without injection of LPS).