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DEMYELINATION/REMYELINATION AND EXPRESSION OF INTERLEUKIN-1β, SUBSTANCE P AND NEUROTROPHIC FACTORS IN TRIGEMINAL NEUROPATHIC PAIN IN RATS

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Abstract:	Background: Etiology of trigeminal neuropathic pain is not clear, but there is evidence that demyelination, expression of cytokines, neuropeptides, and neurotrophic factors play a crucial role. In order to elucidate mechanisms underlying trigeminal (cephalic) neuropathic pain, we evaluated the time course of morphological changes in myelinated and unmyelinated fibers of trigeminal nerve, expression of cytokine IL-1β, neuropeptide substance P (SP), and neurotrophic factors nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) in peripheral and ganglion tissues in trigeminal neuropathic pain model in rats. Methods: Wistar rats were submitted to chronic constriction injury (CCI) of infraorbital nerve (IoN) (ION group) or to a sham operation. Mechanical allodynia was evaluated from day 3 to day 15 post surgery. Trigeminal nerves were divided in 2 parts - distal to CCI and ganglion - for morphological analysis, immunohistochemistry (IL-1β, SP), and protein quantification by ELISA (NGF, GDNF). Results: At early postoperative time, decreased mechanical response was observed, in association to demyelination, glial cell proliferation, increased immunoexpression of IL-1 β and SP, and impaired GDNF production. In late postoperative period, mechanical allodynia was present with partial recovery of myelination, glial cell proliferation, and increased immunoreactivity of IL-1β and SP. Conclusions: Our data show that demyelination/remyelination processes are related to development of pain behavior. IL-1β may act both in ganglion and nerve over time, while SP may be an important mediator in nerve endings. GDNF low levels should lead to impairment in signaling which may be involved in generation of pain.
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Belo Horizonte, February 3rd, 2015.

To Editor-in-Chief
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Dr. Herman O. Handwerker

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Dear Editor-in-Chief,

We are pleased to submit our manuscript "Demyelination/remyelination and expression of interleukin-1β, Substance P and neurotrophic factors in trigeminal neuropathic pain in rats" to *European Journal of Pain* and will be honored to have your editorial opinion on our work.

There is evidence that demyelination, proinflammatory cytokines, neuropeptides, and neurotrophic factors play a crucial role in trigeminal neuropathic pain, although time course of these events have not been completed established. In our work we have evaluated the time course of morphological changes in myelinated and unmyelinated fibers of trigeminal nerve, expression of cytokine IL-1β, neuropeptide SP, and neurotrophic factors NGF and GDNF in peripheral and ganglion tissues in rats that underwent infraorbital nerve chronic constriction injury (CCI). We believe that our work contributes to the understanding of mechanism underlying cephalic neuropathic pain development.

We look forward to the opportunity of publishing our manuscript in *Memórias do Instituto Oswaldo Cruz.*

Sincerely,

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ABSTRACT

Background: Etiology of trigeminal neuropathic pain is not clear, but there is evidence that demyelination, expression of cytokines, neuropeptides, and neurotrophic factors play a crucial role. In order to elucidate mechanisms underlying trigeminal (cephalic) neuropathic pain, we evaluated the time course of morphological changes in myelinated and unmyelinated fibers of trigeminal nerve, expression of cytokine IL-1β, neuropeptide substance P (SP), and neurotrophic factors nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) in peripheral and ganglion tissues in trigeminal neuropathic pain model in rats. **Methods:** Wistar rats were submitted to chronic constriction injury (CCI) of infraorbital nerve (IoN) (ION group) or to a sham operation. Mechanical allodynia was evaluated from day 3 to day 15 post surgery. Trigeminal nerves were divided in 2 parts - distal to CCI and ganglion - for morphological analysis, immunohistochemistry (IL-1β, SP), and protein quantification by ELISA (NGF, GDNF). **Results:** At early postoperative time, decreased mechanical response was observed, in association to demyelination, glial cell proliferation, increased immunoexpression of IL-1 β and SP, and impaired GDNF production. In late postoperative period, mechanical allodynia was present with partial recovery of myelination, glial cell proliferation, and increased immunoreactivity of IL-1β and SP. **Conclusions:** Our data show that demyelination/remyelination processes are related to development of pain behavior. IL-1β may act both in ganglion and nerve over time, while SP may be an important mediator in nerve endings. GDNF low levels should lead to impairment in signaling which may be involved in generation of pain.

KEY WORDS: myelin, neuropeptide, cytokine, nerve growth factor, glial derived neurotrophic factor, trigeminal nerve, pain

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What's already known about this topic? There is evidence that demyelination, proinflammatory cytokines, neuropeptides, and neurotrophic factors play a crucial role in trigeminal neuropathic pain. **What does this study add?** Time course evaluation of

morphological changes in myelinated and unmyelinated fibers of trigeminal nerve, expression of cytokine IL-1β, neuropeptide SP, and of neurotrophic factors NGF and GDNF in peripheral and ganglion tissues in rats that underwent infraorbital nerve CCI.

ABSTRACT

Background: Etiology of trigeminal neuropathic pain is not clear, but there is evidence that demyelination, expression of cytokines, neuropeptides, and neurotrophic factors play a crucial role. In order to elucidate mechanisms underlying trigeminal (cephalic) neuropathic pain, we evaluated the time course of morphological changes in myelinated and unmyelinated fibers of trigeminal nerve, expression of cytokine IL-1β, neuropeptide substance P (SP), and neurotrophic factors nerve growth factor (NGF) and glial derived neurotrophic factor (GDNF) in peripheral and ganglion tissues in trigeminal neuropathic pain model in rats. **Methods:** Wistar rats were submitted to chronic constriction injury (CCI) of infraorbital nerve (IoN) (ION group) or to a sham operation. Mechanical allodynia was evaluated from day 3 to day 15 post surgery. Trigeminal nerves were divided in 2 parts - distal to CCI and ganglion - for morphological analysis, immunohistochemistry (IL-1β, SP), and protein quantification by ELISA (NGF, GDNF). **Results:** At early postoperative time, decreased mechanical response was observed, in association to demyelination, glial cell proliferation, increased immunoexpression of IL-1 β and SP, and impaired GDNF production. In late postoperative period, mechanical allodynia was present with partial recovery of myelination, glial cell proliferation, and increased immunoreactivity of IL-1β and SP. **Conclusions:** Our data show that demyelination/remyelination processes are related to development of pain behavior. IL-1β may act both in ganglion and nerve over time, while SP may be an important mediator in nerve endings. GDNF low levels should lead to impairment in signaling which may be involved in generation of pain.

KEY WORDS: myelin, neuropeptide, cytokine, nerve growth factor, glial derived neurotrophic factor, trigeminal nerve, pain

INTRODUCTION

The etiology of trigeminal neuropathic pain is not clear, but there is evidence that demyelination, expression of proinflammatory cytokines, neuropeptides, and neurotrophic factors play a crucial role (Bird *et al.,* 2002; Nagano *et al.,* 2003; Robinson *et al.*, 2004; Vit *et al.,* 2006; Ohara *et al.,* 2008; Takeda *et al.,* 2008; Taylor and Ribeiro-da-Silva, 2011; Donegan *et al.,* 2013), although time course of these events have not been completed established. Great advances in the understanding of physiopathological mechanisms underlying neuropathic pain have been obtained from sciatic neuropathic pain models (Mosconi and Kruger, 1996; Robinson *et al.,* 2004; Gabay and Tal, 2004; Allan *et al.,* 2005; Savastano *et al.,* 2014; Kimura *et al.,* 2015). However, several studies have shown that responses from trigeminal nerve to injury are different when compared to extra cephalic nerves (Latrémolière *et al.,* 2008; Michot *et al.,* 2012, Michot *et al.,* 2013).

Vos *et al.,* (1994) developed an infraorbital (IoN) chronic constriction injury (CCI) model that have been extensively used for study of trigeminal neuropathic pain. It reproduces signs of abnormal spontaneous pain, mechanical allodynia, and heat hyperalgesia (Vos *et al.,* 1994; Imamura *et al.,* 1997; Xu *et al.,* 2008).

In sciatic CCI model, loss of myelinated fibers distal to lesion is a frequent finding (Basbaum *et al.,* 1991; Nuytten *et al.,* 1992; Mosconi and Kruger, 1996), which has also been described in human trigeminal nerves of patients with trigeminal neuralgia (Love and Coakham, 2001; Marinković *et al.,* 2009). Close apposition of demyelinated axons facilitate cross excitation and ectopic impulses, which are important events in pathophysiology of neuropathic pain (Ramon and Moore, 1978; Love and Coakham, 2001). Nerve injury and demyelination activate glial and inflammatory cells to secrete cytokines, growth factors, and other inflammatory mediators which promote neuronal injury or neuronal survival (Tal, 2000; Okamoto *et al.,* 2001; Allan *et al.,* 2005; Austin and Moalem-Taylor, 2010;). Inteleukine-1β (IL-1β) affects myelination, causes a delay in remyelination and is capable of sensitize neurons (Mason *et al.,* 2001; Allan *et al.,* 2005; Bałkowiec-iskra, 2010). Neuropeptide substance P (SP) has been associated to development of ectopic neural activity in sciatic neuropathic pain (Cameron *et al.,* 1997; Bird *et al.,* 2002). Morerover, nerve growth factors are known to contribute to development of hyperalgesia and allodynia after nerve injuries (Nagano *et al.,* 2003; Shi *et al.,* 2011; Taylor and Ribeiro-da-Silva,

2011). Nerve growth factor (NGF) has been associated to hypo- and hyperalgesia (Ren *et al.*, 1995; Anderson and Rao, 2001) and glial derived neutrotrophic factor (GDNF) seems to exert a protective role in neuropathic pain (Nagano *et al.*, 2003; Shi *et al.*, 2011).

There is a lack in knowledge of time course of morphological evaluation of demyelination under light and electron microscopy and expression of mediators and neurotrophic factors in IoN CCI model. In order to contribute to elucidation of mechanisms underlying trigeminal (cephalic) neuropathic pain, we determined the time course of morphological changes and expression of IL-1β, SP, NGF, and GDNF in ganglion and nerve in rats that underwent IoN CCI.

MATERIALS AND METHODS

Animals and Surgery

Adult male Wistar rats (250–350 g) were obtained from Centro de Bioterismo/Universidade Federal de Minas Gerais (Brazil), maintained on a 12-h light/dark cycle, and cared for according to the Ethical and Animal Use Committee on Animal Experimentation (CETEA/UFMG 231/2009). Forty rats received a chronic constriction injury (CCI) to right infraorbital nerve (IoN group) or only a unilateral sham operation (SHM group). All surgery was performed under general anesthesia with 200 mg/kg of ketamin and 10 mg/kg of xilazin (i.m.) and surgery procedures were performed as previously described (Imamura et al., 2007). All operations were performed aseptically; no antibiotics were administered.

Behavioral testing

Rats were tested by a blinded experimenter at 3, 6, 9, 12, and 15 d after the surgery. After 7 min of habituation, von Frey filaments of varying diameters for which the force required to bend each filament was approximately, 1 gm, 2 gm, 4 gm, 9gm, and 16 gm were applied perpendicularly to vibrissae pad. Response score to mechanical facial stimulation consisted of (1) detection, (2) withdrawal reaction, (3) escape/attack, or (4) asymmetric face grooming, as previously described (Vos *et al.*, 1994).

Tissue preparation

Trigeminal nerves form 5 animals per group at 3, 6, 9, 12, and 15 d after the surgery were divided in 2 parts: distal (from infraorbital foramem to vibrissae pad) and proximal

(region where the ganglion is) to lesion. Nerve fragments were fixed, routinely processed and ections of 4 and 7 µm were obtained for hematoxylin and eosin (HE) staining and immunohistochemistry, respectively. Some animals (3 per group at 6 and 15 d after the surgery) were deeply anesthetized, transcardically perfused, and small fragments of distal and proximal portions of trigeminal nerve were processed for electron microscopy. Semi-thin (200 nm) sections were collected on glass slides and stained with 1% toluidine blue. Ultra-thin (60 nm) sections were counterstaining with uranyl acetate.

Morphological analysis and morphometry

Histopathological alterations were evaluated for each time point in IoN and SHM groups. Cell count in nerve fascicles and ganglia was performed in HE sections and myelin area and myelin/axon ratio were quantified in toluidine blue semi-thin sections. Images were obtained with Olympus BX51 microscope and digital images were acquired through Image-Pro Express 4.0 (Media Cybernetics, MD, USA). All morphometric parameters were manually measured using ImageJ 1.45S software (NIH, USA). Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

Ultrastructural analysis and morphometry

Ultrastructural analysis of myelinic and unmyelinic fibers was performed in the Transmission Electron Microscope Tecnai G2-12 - SpiritBiotwin FEI - 120 kV at Center of Microscopy at Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. In unmyelinated fibers, Schwann cell area and number of fibers per cluster were determined. Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

Immunohistochemistry

For immunohistochemistry, antigen retrieval, followed by blockade of endogenous peroxidase activity and nonspecific binding sites. Afterward, slides were incubated with rabbit anti-SP (1:100, AB1566, Millipore), anti-IL-1β (1:200, NBP1-19775, Novus Biologicals), anti GFAP (1:500, Z0334, Dako) and anti S100 (1:400, Z0311, Dako) overnight at 4ºC in a humid chamber. Incubation with secondary biotinylated goat anti-rabbit was followed by incubation with a streptavidin-peroxidase complex (LSAB2 system-HRP, DAKO). The reaction was visualized by incubating the sections with 3,3-

diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), and counterstaining was done with hematoxylin. Negative control was performed by omission of the primary antibody. Immunopositive IL-1β or SP cells were count in nerve fascicles and ganglia. Images were obtained with Olympus BX51 microscope and digital images were acquired through Image-Pro Express 4.0 (Media Cybernetics, MD, USA). All morphometric parameters were manually measured using ImageJ 1.45S software (NIH, USA). Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

NGF and GDNF protein levels

For quantification of NGF and GDNF protein levels, animals (5 per group for each time studied – 6 and 15 d after the surgery) were deeply anesthetized, blood and trigeminal nerve portions were collected, and serum and tissues were frozen at -70 ºC. For GDNF assay, Promega (San Luis Obispo, CA) kit and protocol were used. For NGF, kit and protocol were from the R &D Systems (Minneapolis, MN). In each plate, NGF or GDNF standard curves were obtained along with the samples. The absorbance was read at 450 nm (Versamax microplate reader, Molecular Devices, Sunnyvale, CA). The Bradford (1976) method measured the total protein content of the samples. The neurotrophic factor levels were expressed as pg/ml of serum or pg/mg of total protein in nervous tissue. Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

RESULTS

Behavioral evaluation

Neither SHM nor IoN rats demonstrated any obvious behavioral changes as a result of the surgical procedures, and food nor water intake was unaffected. No significant differences in body weight were observed (data not shown). CCI of the infraorbital nerve resulted in evoked behaviors suggestive of neuropathic pain (**Figure 1).** IoN animals showed an initial period of decreased mechanical sensitivity (seen at days 3 and 6). After 12 days, rats with CCI exhibited a marked increase in responsiveness to von Frey filaments that was suggestive of a mechanical allodynia and of a presumptive transition into an

induced neuropathic pain state. In contrast, sham-injury rats exhibited no changes in behavior on any post-surgical day.

Morphological analysis

Intense nerve lesions were observed as early as 3 days post surgery distal to ligatures. Most intense lesions were detected at day 6, and were maintained up to day 15 post surgery. Loss of tissue organization in nerve fascicles, axonal vacuolization, tissue edema, and intense Wallerian degeneration with axonal and myelin breakdown were present (**Figure 2**).

An increase in cell count inside nerve fascicles was detected (**Figure 3**). Distal to lesion, IoN group showed higher number of cells per area of nerve fascicle in comparison to SHM group at days 9, 12, and 15 post surgery. In contrast, in trigeminal ganglion, the increase in cellularity in IoN group was observed only at day 15. The higher immunopositivity for glial cell markers (GFAP and S100) confirmed that the greater quantity of cells in nerve fascicles was a result of glial cell proliferation (**Figure 3**).

Demyelination analysis

Qualitative analysis of demyelination revealed loss of myelinated axons and intense degeneration of myelinated fibers in trigeminal nerve distal to CCI (**Figure 4**). From day 3 to 9 post surgery, CCI group showed intense loss of myelin in nerve fascicles, with lesion peak at day 6. At days 12 and 15 post surgery, there was reduction in demyelination and myelin degeneration.

By electron microscopy, loss of myelinated axons, degenerating myelinated fibres, and myelin broken up into scroll formations weres observed distal do ligatures (**Figure 5**). Myelin formation of ovoid structures, and invaginations of myelin sheaths were also present. In the endoneurium, there was an increase in collagen fibres and it was observed the presence of Schwann cells and / or macrophages containing phagocytic material, including lipid debris, and myelin fragments. At 6 days post surgery, lesions signs were evident, with partial recovery at 15 days post surgery, when thinly myelinated and remyelinated fibres were observed. Proximal to lesion, demyelination was not observed in any time studied. More discrete alterations were detected in unmyelinated fibers. IoN group showed greater clusters of unmyelinated fibers, which were irregular in shape, in comparison to SHM group at 6 days post surgery (**Figure 5**).

Morphometric analysis revealed reduction in myelin area distal do CCI in IoN group at all time points in comparison to SHM group (**Figure 6**). No difference in myelin area was detected in the trigeminal ganglion. Analysis of axon/myelin ratio was performed in order to determine remyelination process. IoN group had smaller ratio in comparison to SHM group at day 6, confirming disruption of myelin. At day 15, however, no difference was observed between groups (**Figure 6**). Similarly, at trigeminal ganglion, no difference was observed.

Morphometry of unmyelinated fibers was performed to analyze Schwann cell area and number of unmyelinated fibers per cluster. Distal to CCI, Schwann cells area was greater in IoN group at day 15 post surgery (**Figure 7**). Despite qualitative analysis, no difference was observed in number of unmyelinated fibers per cluster between groups for both time points studied (**Figure 7**).

IL-1β and SP immunopositivity

Immunoreactivity of cytokine IL-1β and neuropeptide SP, important mediators in pain physiopathology, was analyzed for 15 days after CCI. Distal to lesion, higher IL-1β immunopositivity was detected inside nerve fascicles of IoN group. Similarly, in trigeminal ganglion, evident expression of IL-1β was observed in neuronal cell bodies of animals that underwent CCI (**Figure 8**). Quantitative analysis showed greater number of immunopositive cells for IL-1β distal to CCI in IoN animals at 9 and15 days post surgery (**Figure 8**). In trigeminal ganglion, IoN group showed higher IL-1β immunoexpression in earlier times (from days 3 to 9 post surgery).

Regarding SP, immunopositivity was higher in nerve fascicles, in addition to a discrete immunoreactivity in neuronal cell bodies of IoN group (**Figure 9**). Morphometric analysis revealed higher amount of immunoreactive cells in distal regions of IoN group at days 6, 9 and 15 post CCI (**Figure 9**). In ganglion, no difference was detected between groups.

NGF and GDNF levels

No differences in seric NGF levels were found between groups (**Figure 10**). In addition, IoN and SHM group produced similar amounts of NGF in both nerve fragment distal to CCI and trigeminal ganglion during the time studied.

GDNF seric levels were lower in IoN group at day 6 post surgery, but no difference was detected between groups at day 15 (**Figure 11**). In both distal region of nerve fragment and in trigeminal ganglion, GDNF levels from IoN and SHM groups were similar, regardless the time point.

DISCUSSION AND CONCLUSIONS

Although there is evidence that demyelination, expression of proinflammatory cytokines, neuropeptides, and neurotrophic factors play a crucial role in neuropathic pain conditions (Bird *et al.*, 2002; Nagano *et al.*, 2003; Robinson *et al.*, 2004; Vit *et al.*, 2006; Ohara *et al.*, 2008; Takeda *et al.*, 2008; Taylor and Ribeiro-da-Silva, 2011; Donegan *et al.*, 2013), etiology of trigeminal neuropathic pain is not clear. There is a lack of morphological and morphometric evaluation of demyelination and neurotrophic factors expression over time in infraorbital CCI model. Here we have evaluated the time course of these changes in order to contribute to elucidation of trigeminal neuropathic pain development.

As previously described by Vos *et al.*, (1994), there was a time dependent change in mechanical sensitivity following infraorbital nerve injury, similar to that observed in the present study. In a late postoperative period, IoN rats became hypersensitive, with increased response to von Frey filaments, demonstrating mechanical allodynia.

Morphological alterations observed here are in accordance to histopathological observations in sciatic (Gautron *et al.*, 1990; Nuytten *et al.*, 1992; Gabay and Tal, 2004) and trigeminal CCI studies (Vit *et al.*, 2006; Ohara *et al.*, 2008). However, other trigeminal CCI studies have focused in glial cell or trigeminal ganglion alterations and the present study describes the time course of nerve histopathological alterations distal to ligatures as it has been done in sciatic CCI (Nuytten *et al.*, 1992). We have also shown that increase in cellularity in nerve fascicles was due to proliferation of glial cells, since immupositivity for glial cell markers was observed. Other studies have shown proliferation of satellite glial cell in trigeminal ganglia (Vit *et al.*, 2006; Ohara *et al.*, 2008; Xu *et al.*, 2008; Donegan *et al.*, 2013) and we found both satellite glial cell and Schwann cell proliferation in nerve fascicles. We believe that peripheral glial cell proliferation in trigeminal nerve is important in pain pathophysiology once they may play pivotal role in secretion of cytokines and

growth factors, besides their role in clearance of myelin, that make then essential for nerve repair success (Defrancesco-Lisowitz *et al.,* 2014).

Demyelination analysis revealed severe loss of myelin in trigeminal nerve distal to CCI at all time points and discrete alterations in unmyelinated fibers. Our observations of disruption of myelin are in accordance to sciatic CCI studies and to trigeminal neuralgia human studies (Basbaum *et al.,* 1991; Nuytten *et al.,* 1992; Hilton *et al.,* 1994; Mosconi and Kruger, 1996; Love and Coakham, 2001; Marinković *et al.,* 2009). However, evaluation of demyelination over time in trigeminal CCI is first described here. Our data showed that higher demyelination occurred at early postoperative time, and partial recovery was detected later. This is particularly important as hypo-or anesthetic responses are mostly due to conduction block caused by edema and degeneration of myelinated axons, as shown for CCI of other peripheral nerves (Basbaum *et al.,* 1991; Nuytten *et al.,* 1992; Mosconi and Kruger, 1996). In the other hand, mechanical allodynia may be secondary to unpaired remyelination processes. Mosconi and Kruger (1996) have suggested demyelination and remyelination in sciatic CCI and it seems that this is the case for trigeminal CCI as well. These phenomena favor ectopic generation of spontaneous nerve impulses (Love and Coakham, 2001) and close apposition of demyelinated axons facilitates ephaptic transmission of nerve impulses (Ramon and Moore, 1978; Rasminsky, 1978). Impaired remyelination between fibers that mediate light touch and those involved in the generation of pain favor ephaptic cross-talk which is relevant for mechanical allodynia observed in trigeminal neuralgia (Love and Coakham, 2001).

Peripheral nerve injury activates glial and inflammatory cells to produce cytokines and other molecules which mediate inflammation and pain (Takeda *et al.,* 2007). We have found higher IL-1β immunopositivity distal to CCI in late postoperative period and in trigeminal ganglion in early postoperative period. IL-1β expression is also observed in dorsal root ganglion neurons (Copray *et al.,* 2001) and represents an early response of trigeminal neurons to peripheral lesion following CCI, as it has been demonstrated for other models of nerve injury (Boutin *et al.,* 2003; Allan *et al.,* 2005). Recent studies have shown that cytokines, including IL-1β, play a very important role in the pathogenesis of the immune response within peripheral endings of trigeminal neurons and are capable of sensitizing nociceptive neurons (Bałkowiec-iskra, 2010). Moreover, activation of glial cells

modulates excitability of trigeminal neurons via IL-1β which may contribute to inflammatory hyperalgesia (Takeda *et al.*, 2007; Takeda *et al.*, 2008). IL-1 has numerous effects on glial cells, as stimulation of proliferation and release of several mediators that can be neurotoxic or potentially beneficial (Aloisi , 2001; Basu *et al.*, 2004). IL-1β might also affect myelination and cause a delay in remyelination (Mason *et al.*, 2001). Hence, we believe that increase in IL-1β shown here may mediate neuron hyperexcitability and lesion, glial cell proliferation and demyelination / remyelination processes in trigeminal neuropathic pain.

Regarding SP, we observed higher expression distal to lesion in IoN group, but no evident immunopositivity in the trigeminal ganglion. In contrast, Bird *et al.*, (2002) have found higher immunoexpresion of SP in trigeminal ganglion up to 15 days in another model of trigeminal CCI (tight ligation of the inferior alveolar nerve). We believe that this response was triggered by the injury blockade generated by the tight ligation, in contrast to lose ligation in our CCI model. Some studies are in accordance to our findings of low expression of SP in ganglion after CCI (Garrison *et al.*, 1993; Kajander and Xu, 1995; Abbadie *et al.*, 1996; Xu and Yaksh, 2008). We found a significant relationship between allodynia and SP expression levels in nerve fascicles, with higher levels at late postoperative period. This finding is in agreement to strong SP immunopositivity observed in trigeminal nerve specimens obtained from patients with trigeminal neuralgia (Marinković *et al.*, 2009). Hence, it is possible that nerve injury-induced allodynia is associated with neurochemical reorganization in primary afferents and that SP accumulation may be linked to the development of ectopic neural activity, as demonstrated for sciatic CCI and for another model of trigeminal pain (Cameron *et al.*, 1997; Bird *et al.*, 2002).

Nerve growth factors are postulated to contribute to the development of hyperalgesia and allodynia after sciatic and trigeminal nerve injuries (Anderson and Rao, 2001; Nagano *et al.*, 2003; Shi *et al.*, 2011; Taylor and Ribeiro-da-Silva, 2011). We have found no differences in NGF seric or tissue levels between IoN and SHM groups. Measurement of NGF levels at trigeminal ganglion at 15 days had not been done as we describe here, although we could not correlate NGF levels to pain behavior. Recently, it has been shown that NGF expression was not correlated to thermal hyperalgesia after CCI

(Evans *et al.*, 2014). It seems that effects of NGF following nerve injury are quite complex, if not paradoxical, and dependent on time and concentration (Anderson and Rao, 2001).

In our study, we detected higher levels of GDNF only in the serum 6 days after surgery in IoN group, although an upregulation of GDNF in nervous tissue was expected considering glial cell proliferation observed. In sciatic CCI-treated rats, GDNF contents in dorsal root ganglia were markedly decreased at day 7 and 14 (Nagano *et al.*, 2003). Behavioral changes were correlated with loss of GDNF in the distal stump of the injured sciatic nerve and involvement of GDNF loss in pathogenesis of CCI-induced neuropathic pain was suggested (Shi *et al.*, 2011). In our study, SHM group showed higher serum levels of GDNF and we could presume that a dysfunction in GDNF synthesis and signaling in IoN group may contribute to the development and/or maintenance of trigeminal neuropathic pain, showing a protective effect of this neurotrophic factor. As mentioned for NGF, GDNF may play a pivotal role in neuron-glial cell interactions and neurotrophic factor support and its loss may to contribute to the development of peripheral neuropathies.

Early postoperative time, characterized by decreased mechanical response, courses with disruption of myelinated fibers, glial cell proliferation in nerve fascicles, increased IL-1β immunoexpression in trigeminal ganglion, SP immunoreactivity distal to CCI, and impaired GDNF production. In late postoperative period, when mechanical allodynia is observed, we have observed partial recovery of myelination, glial cell proliferation both in nerve fascicles and ganglia, and increased immunopositivity of IL-1β and SP in nerve fascicles. Hence, we could conclude that demyelination/remyelination processes are related to development of pain behavior, IL-1β may act both in nerve and ganglion over time, while SP may be an important mediator for trigeminal neuropathic pain mainly in nerve endings. GDNF lower production should lead to impairment in signaling which may be involved in generation of pain. Further studies involving blockade of these mediators are warranted to better understanding of their roles in development of mechanical allodynia after trigeminal nerve injury.

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AUTHOR CONTRIBUTIONS

Costa GMF and Oliveria AP performed the experiments. Martinelli PM and Camargos ERS contributed to the experimental design and the interpretation of the results. Almeida-Leite CM and Arantes RME conceived the idea for the study and wrote the manuscript. All authors discussed the results and commented on the manuscript

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FIGURE LEGENDS

Figure 1

Behavioral characterization of evoked mechanical thresholds with von Frey filaments. using response score previously described by Vos et al. (1994). Von Frey monofilaments of bending forces of 0.05g (a), 0.2g (b), 2g (c), 4 g (d) and 10 g (e) were used in IoN and SHM rats. At first week, IoN rats were hypo- or anesthetic, with decreased response to von Frey filaments. In a late postoperative period (days 12-15), IoN rats became hypersensitive, with increased response to von Frey filaments. 40 animals (day 3), 35 animals (day 6), 30 animals (day 9), 17 animals (day 12), 12 animals (day 15), *p <0.05, CCI versus sham-injury. Mann-Whitney or unpaired t test. Error bars represent SEM.

Figure 2

Nerve lesions distal to ligatures at days 6 (a) and 15 (c) post surgery and in trigeminal ganglion at days 6 (b) and 15 (d) post surgery in IoN rats (a-d) in comparison to SHM rats (e,f). Axonal vacuolization (a,c, thick arrows) and increased cellularity in nerve fascicles in distal region (c, arrow heads) and in trigeminal ganglion (b,d, thick arrows). Preserved nerve fascicles in SHM group (e,f). HE staining. Bars indicate 50 µm (a, c, e) and 30 µm (b, d, f).

Figure 3

Glial cell proliferation in trigeminal nerve and ganglion in IoN rats. Morphometric evaluation of cellularity distal to CCI (a) and in trigeminal ganglion (b) expressed as number of cells per nerve fascicle area (μm^2) in IoN and SHM rats over time. Distal to CCI, greater cellularity from day 9 to 15 post surgery in IoN group. In trigeminal ganglion, increased cellularity at day 15 post CCI. Five animals per group, *p <0.05, CCI versus sham-injury. Mann-Whitney or t test. GFAP (c, arrows) and S100 (d, arrows) immunopositivity in IoN animals 15 days post surgery. Bars indicate 50 µm.

Figure 4

Demyelination distal to CCI in IoN rats (a,b) in comparison to SHM rats (c, d) at days 6 e 15 days post surgery. Focal demyelination (arrows) and presence of degenerated myelin (*) in IoN group at days 6 (a) and 15 post CCI (b). Preserved myelin fibers (c, d, arrow heads) in SHM animals at days 6 (c) and 15 post CCI (d). Semi thins sections. Toluidine blue staining. Bars represent 50 µm.

Figure 5

Transmission electron microscopy distal to CCI in IoN rats (a-d) in comparison to SHM rats (e,f) at days 6 e 15 days post surgery. Severe demyelination (a, arrow head), invagination of the myelin sheath (a, arrow), myelin breakdown (b, arrow head) and loss of myelin density (b, arrow) at days 6 (a) and 15 (b) post surgery. Increase in number of unmyelinated fibers per cluster (arrow heads) in IoN rats at days 6 (c) and 15 (d) post surgery. SHM animals showed preserved myelinic fibers (e, arrow) and unmyelinated fiber cluster (f, arrowhead).

Figure 6

Demyelination in IoN group in comparison to SHM group. Morphometric evaluation of demyelination (a,b) and demyelination / remyelination (c,d) distal to CCI and in trigeminal ganglion. a-b: Demyelination is expressed as area of myelin per total area of nerve fascicle. Distal to CCI, intense reduction in myelin area in IoN group over time (a). In trigeminal ganglion, no difference was observed between IoN and SHM groups (b) c-d: Demyelination / remyelination is expressed as ratio of axon area per myelin area. IoN group had smaller ratio in comparison to SHM group at day 6 (c). In trigeminal ganglion, no difference was observed (d). Three animals per group, *p <0.05, CCI versus sham-injury. Unpaired *t* test.

Figure 7

Unmyelinated fiber morphometric evaluation. Schwann cell area (a) and size of unmyelinated fiber clusters (b) distal to CCI in IoN and SHM groups. Distal to CCI, Schwann cells area was greater in IoN group at day 15 post surgery (a). No difference was observed in number of unmyelinated fibers per cluster between groups (b). Two animals per group, *p <0.05, CCI versus sham-injury. Mann-Whitney or unpaired *t* test.

Figure 8

Immunopositivity for IL-1β in IoN animals. IL-1β immunopositivity (arrows) in nerve fascicles at days 6 (a) and 15 (b) and in trigeminal neurons at day 3 (e) in IoN group in contrast to SHM animals at same time points (c, d, f). Higher number of immunopositive cells at 9 and 15 days post surgery distal to CCI (g) and higher IL-1β immunoexpression in trigeminal ganglion of I IoN rats at earlier times (h). Bars represent 50 µm. Five animals per group, *p <0.05, CCI versus sham-injury. Unpaired *t* test.

Figure 9

Immunopositivity for SP in IoN animals. SP immunopositivity (arrows) in nerve fascicles at days 9 (a) and 15 (b) and in a few trigeminal neurons at day 15 (e) in IoN group in contrast to SHM animals at same time points (c, d, f). Higher number of immunopositive cells at 6, 9, and 15 days post surgery distal to CCI in IoN rats (g). No diffenrence between groups in trigeminal ganglion (h). Bars represent 50 µm. Five animals per group, *p <0.05, CCI versus sham-injury. Unpaired *t* test.

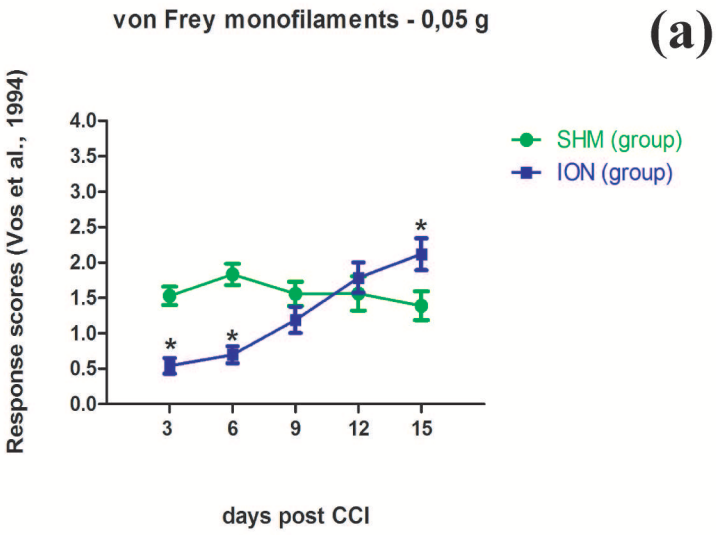
Figure 10

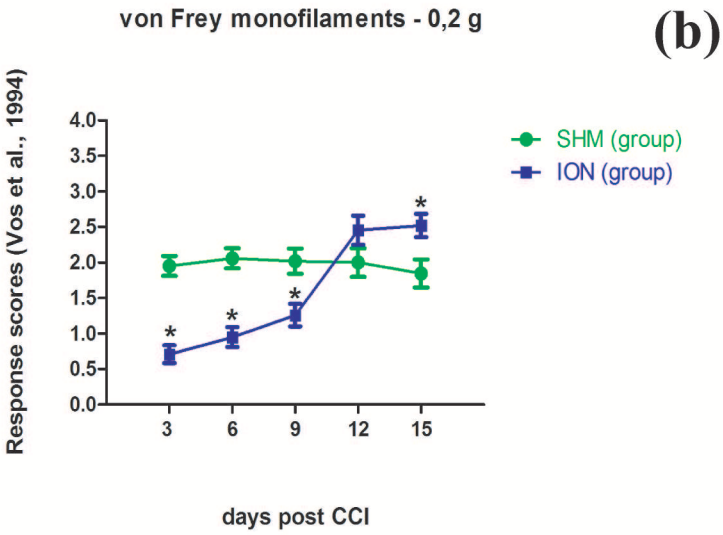
NGF levels in serum (a, pg of NGF per ml of serum) and in nervous tissue (pg or NGF per mg of total protein): distal to CCI (b) and in trigeminal ganglion (c). No differences were observed between IoN and SHM groups. Ten animals per group, unpaired *t* test.

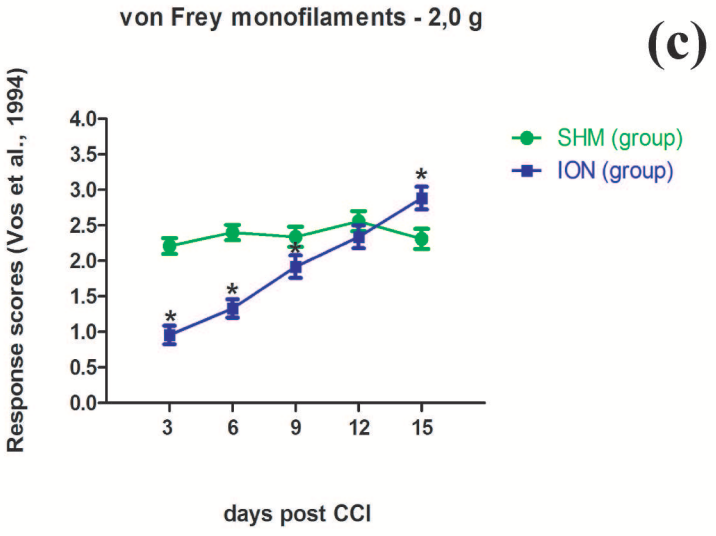
Figure 11

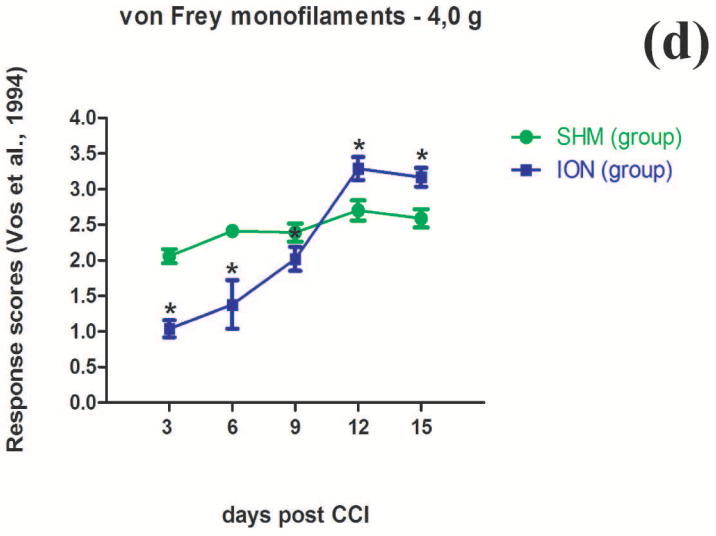
GDNF levels in serum (a, pg of GDNF per ml of serum) and in nervous tissue (pg or GDNF per mg of total protein): distal to CCI (b) and in trigeminal ganglion (c). GDNF seric levels were lower in IoN group at day 6 post surgery (a). No differences in tissue levels were observed between IoN and SHM groups (b,c). Five animals per group, *p <0.05, CCI versus sham-injury. Unpaired *t* test.

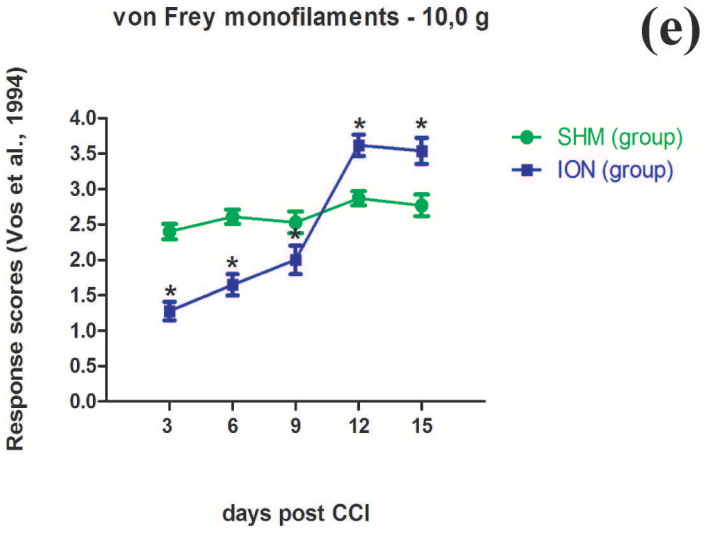
Figure


von Frey monofilaments - 0,05 g
(a)
Response scores (Vos et al., 1994)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)

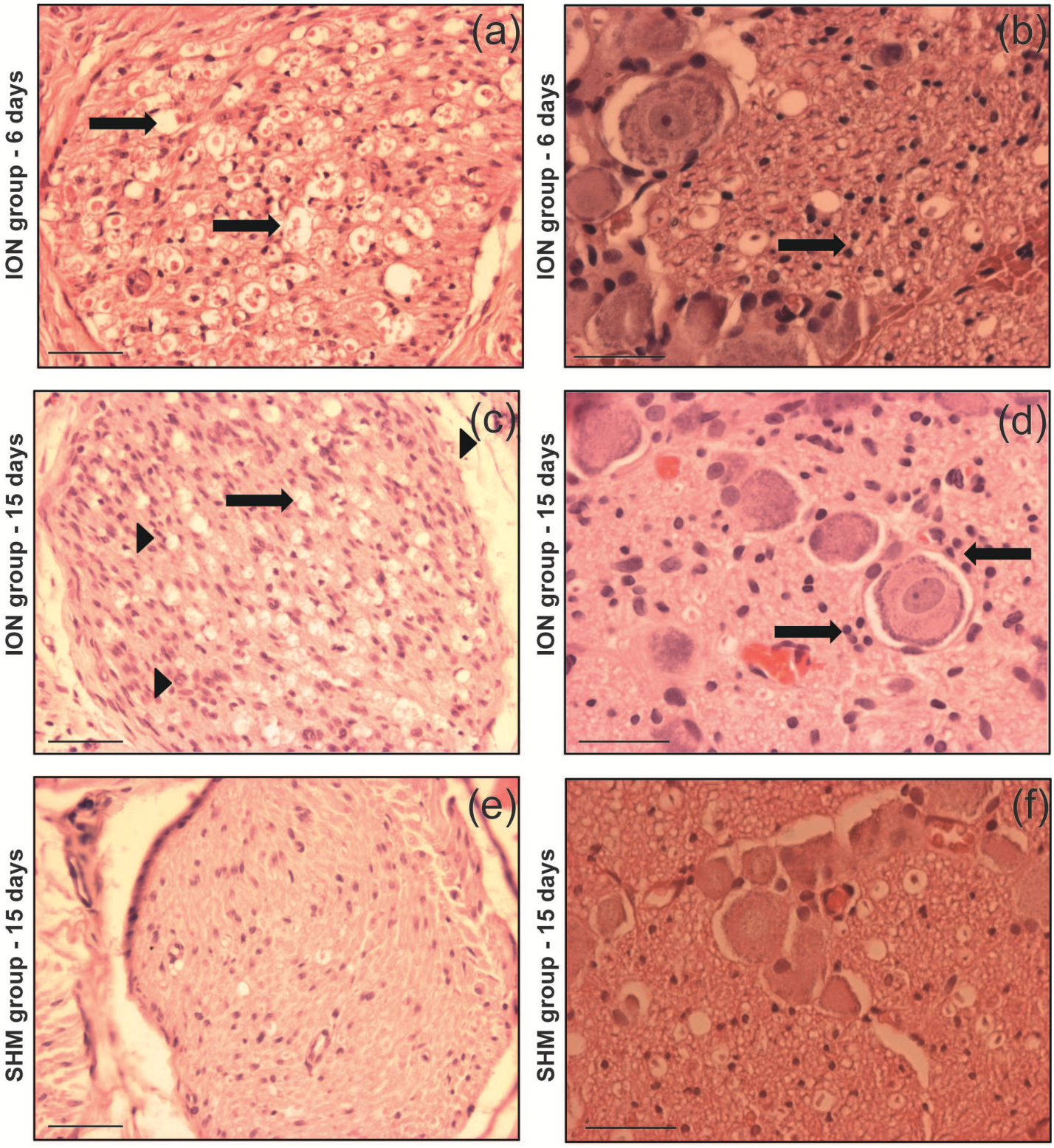

von Frey monofilaments - 0,2 g
(b)
Response scores (Vos et al., 1994)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)


von Frey monofilaments - 2,0 g
(c)
Response scores (Vos et al., 1994)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)

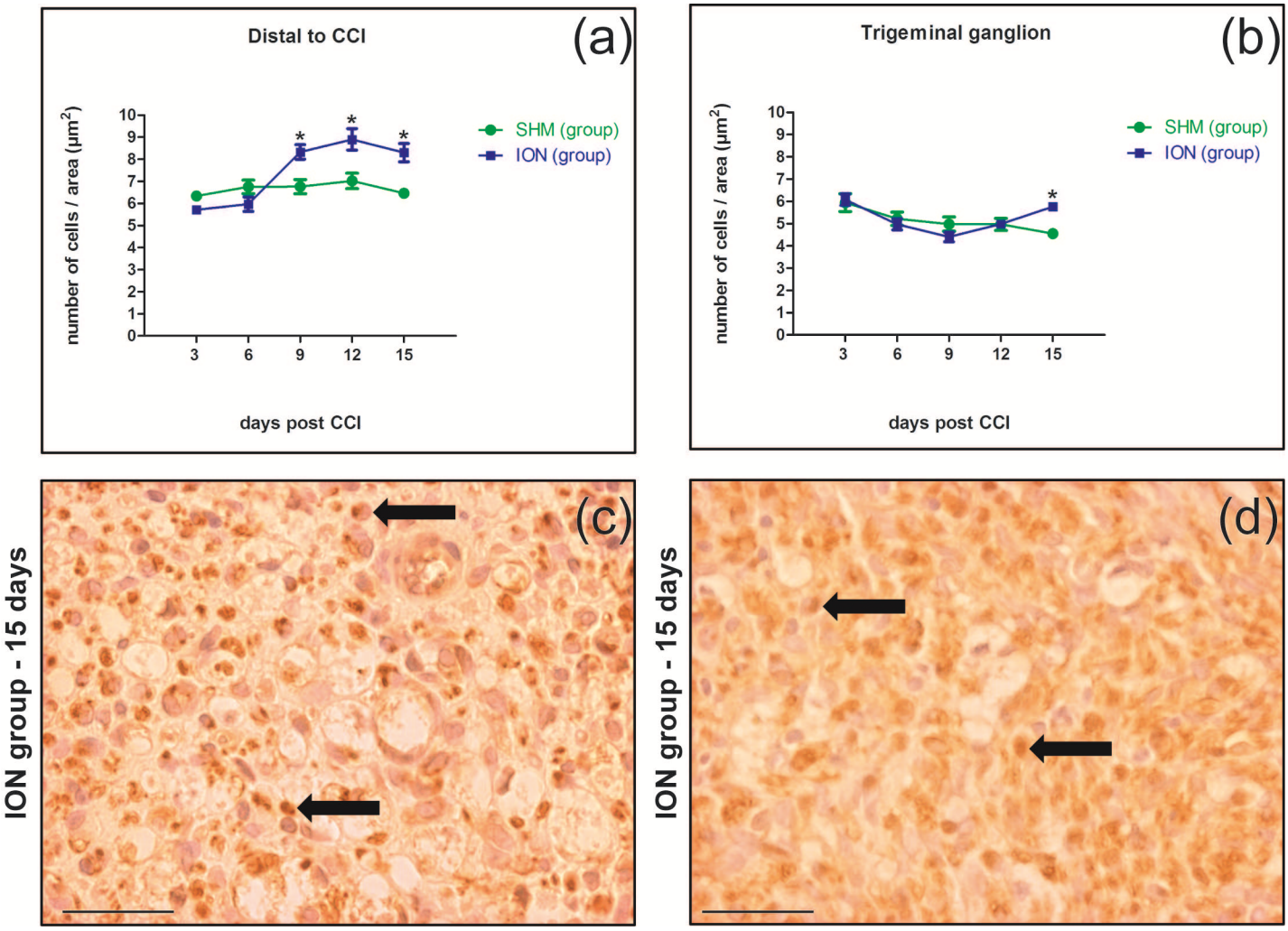

von Frey monofilaments - 4,0 g
(d)
Response scores (Vos et al., 1994)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)


von Frey monofilaments - 10,0 g
(e)
Response scores (Vos et al., 1994)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)

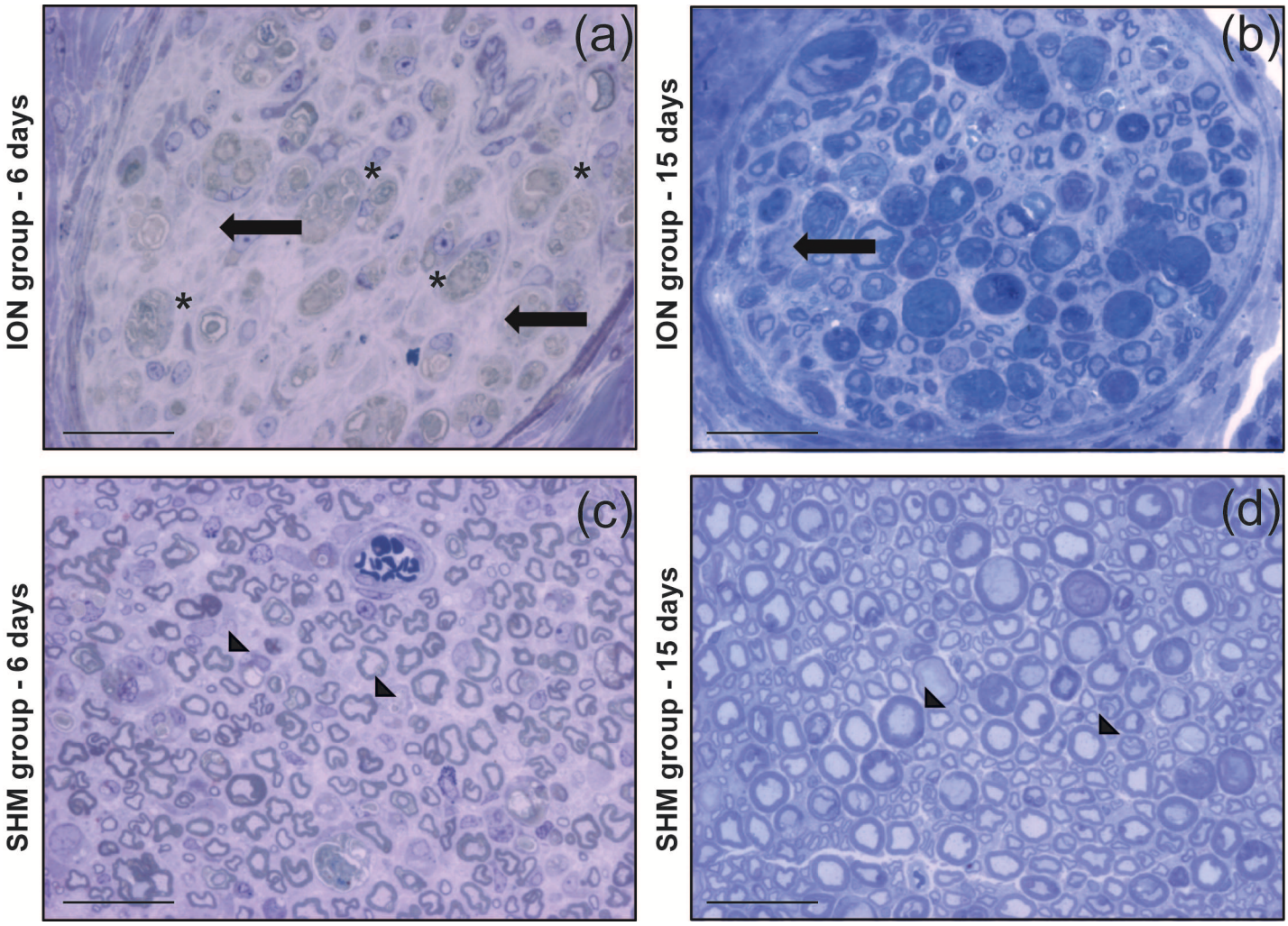
Figure


ION group - 6 days
(a)
ION group - 6 days
(b)
ION group - 15 days
(c)
ION group - 15 days
(d)
SHM group - 15 days
(e)
SHM group - 15 days
(f)

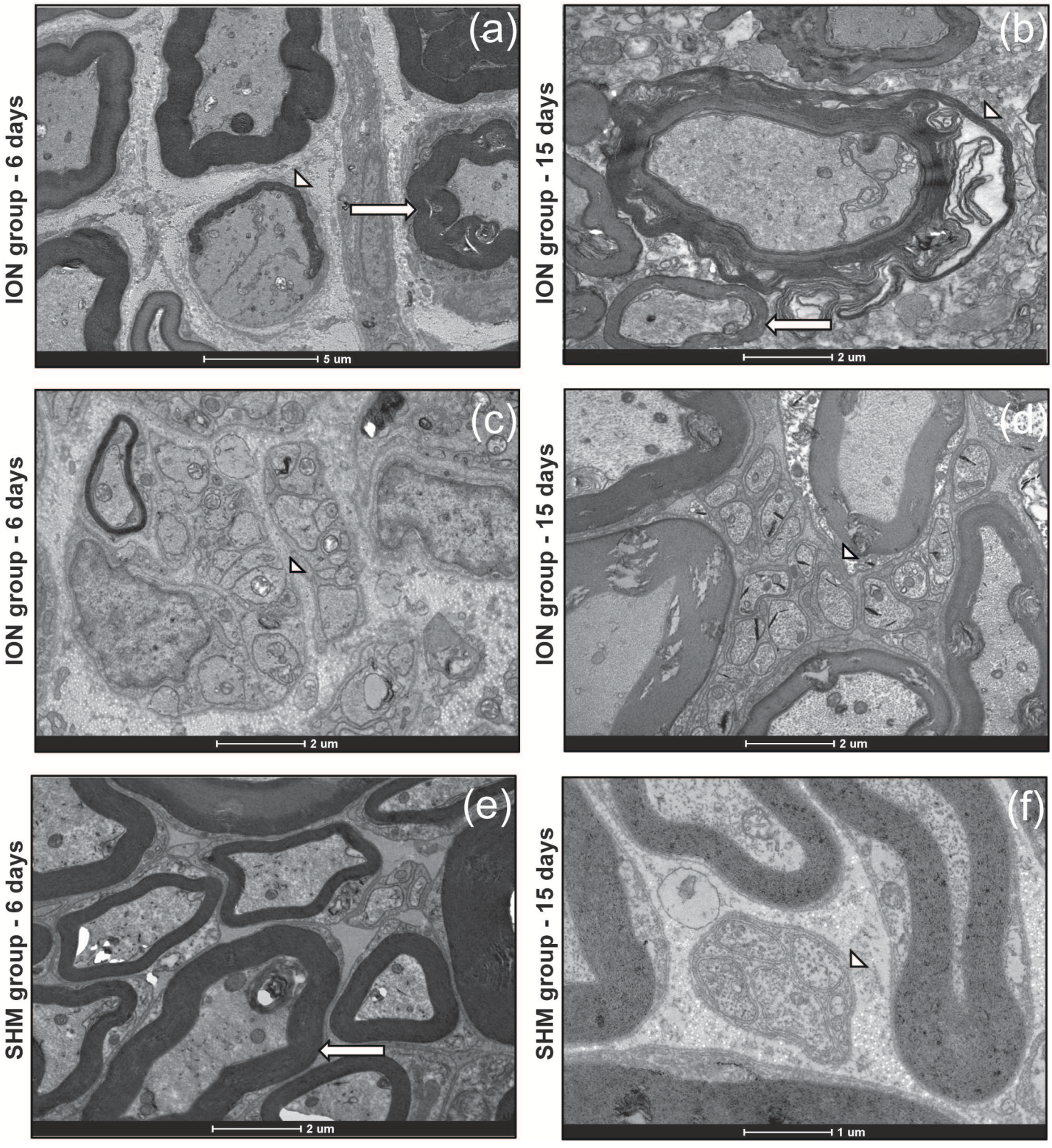
Figure


Distal to CCI
(a)
number of cells / area (µm²)
SHM (group)
ION (group)
days post CCI
Trigeminal ganglion
(b)
number of cells / area (µm²)
SHM (group)
ION (group)
days post CCI
ION group - 15 days
(c)
ION group - 15 days
(d)

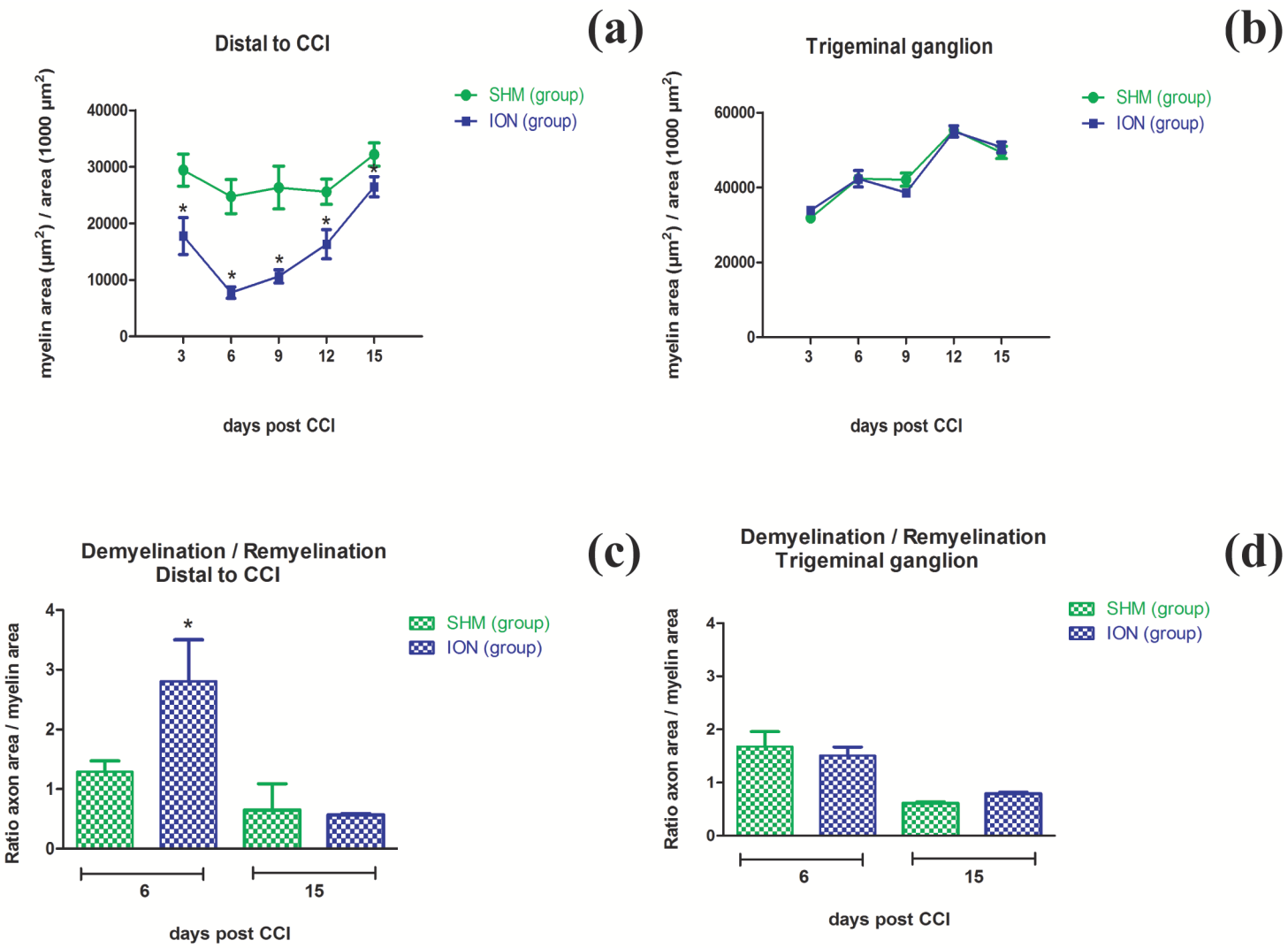
Figure


(a)
(b)
(c)
(d)
ION group - 6 days
ION group - 15 days
SHM group - 6 days
SHM group - 15 days

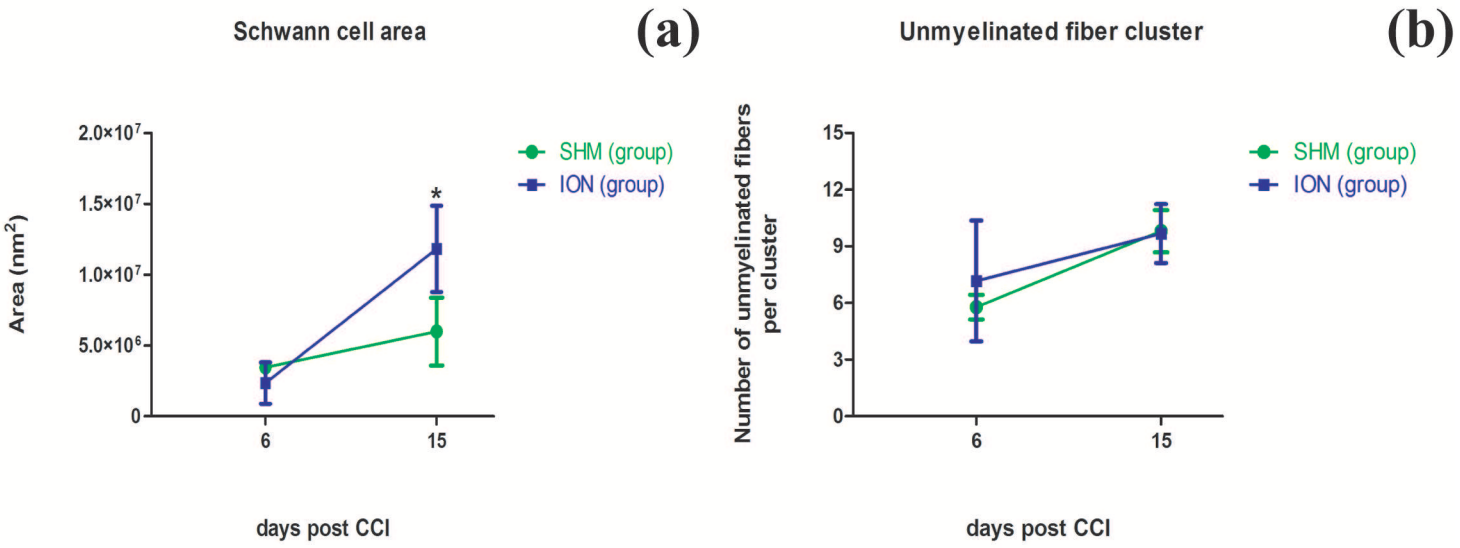
Figure


ION group - 6 days
(a)
5 um
ION group - 15 days
(b)
2 um
ION group - 6 days
(c)
2 um
ION group - 15 days
(d)
2 um
SHM group - 6 days
(e)
2 um
SHM group - 15 days
(f)
1 um

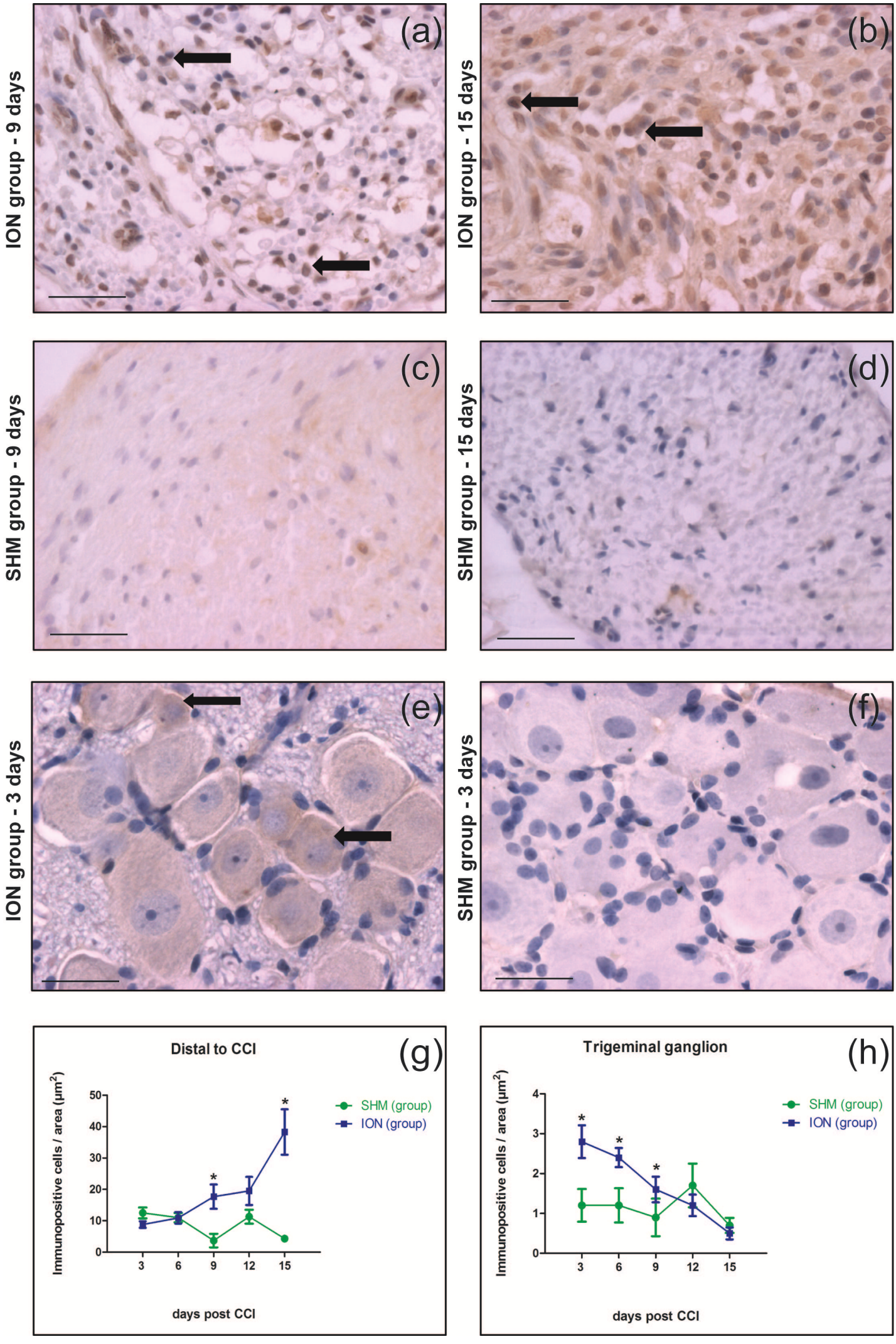
Figure


(a)
Distal to CCI
myelin area (µm²) / area (1000 µm²)
40000
30000
20000
10000
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)
(b)
Trigeminal ganglion
myelin area (µm²) / area (1000 µm²)
60000
40000
20000
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)
(c)
Demyelination / Remyelination
Distal to CCI
Ratio axon area / myelin area
4
3
2
1
0
6
15
days post CCI
SHM (group)
ION (group)
(d)
Demyelination / Remyelination
Trigeminal ganglion
Ratio axon area / myelin area
4
3
2
1
0
6
15
days post CCI
SHM (group)
ION (group)

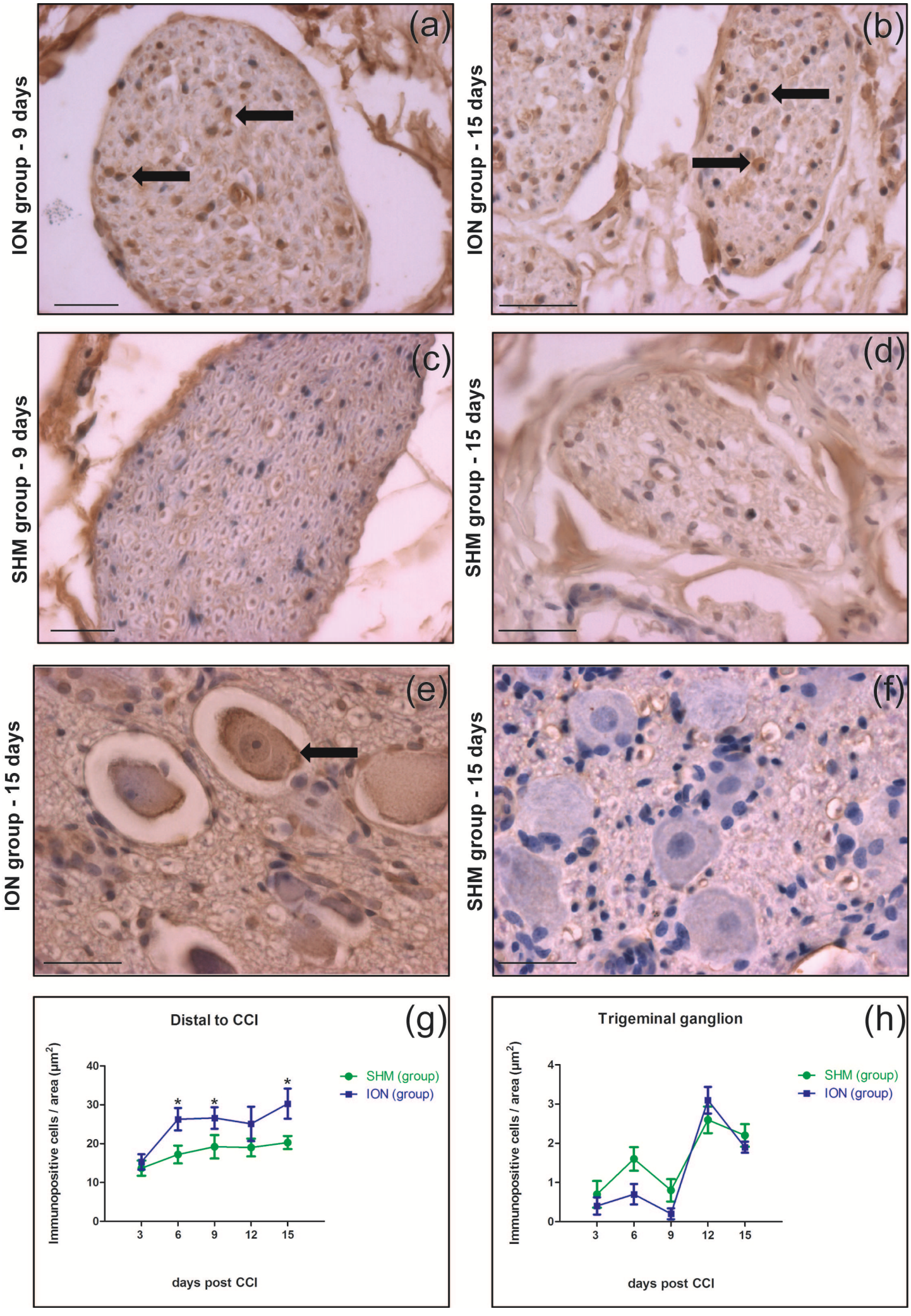
Figure


Schwann cell area
(a)
Area (nm²)
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
6
15
*
days post CCI
SHM (group)
ION (group)
Unmyelinated fiber cluster
(b)
Number of unmyelinated fibers per cluster
15
12
9
6
3
0
6
15
days post CCI
SHM (group)
ION (group)

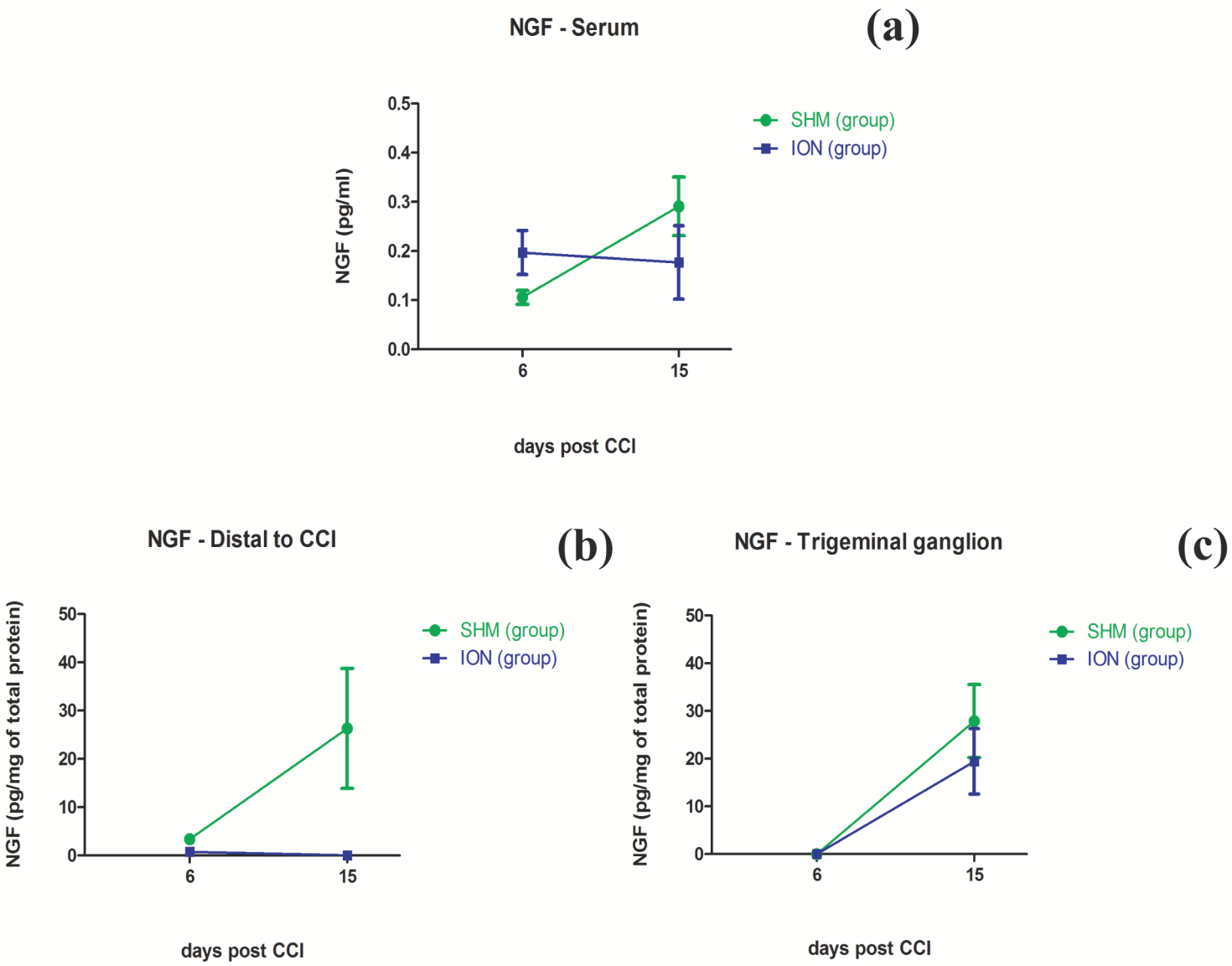
Figure


ION group - 9 days
(a)
ION group - 15 days
(b)
SHM group - 9 days
(c)
SHM group - 15 days
(d)
ION group - 3 days
(e)
SHM group - 3 days
(f)
Distal to CCI
(g)
Immunopositive cells / area (µm²)
50
40
30
20
10
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)
Trigeminal ganglion
(h)
Immunopositive cells / area (µm²)
4
3
2
1
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)

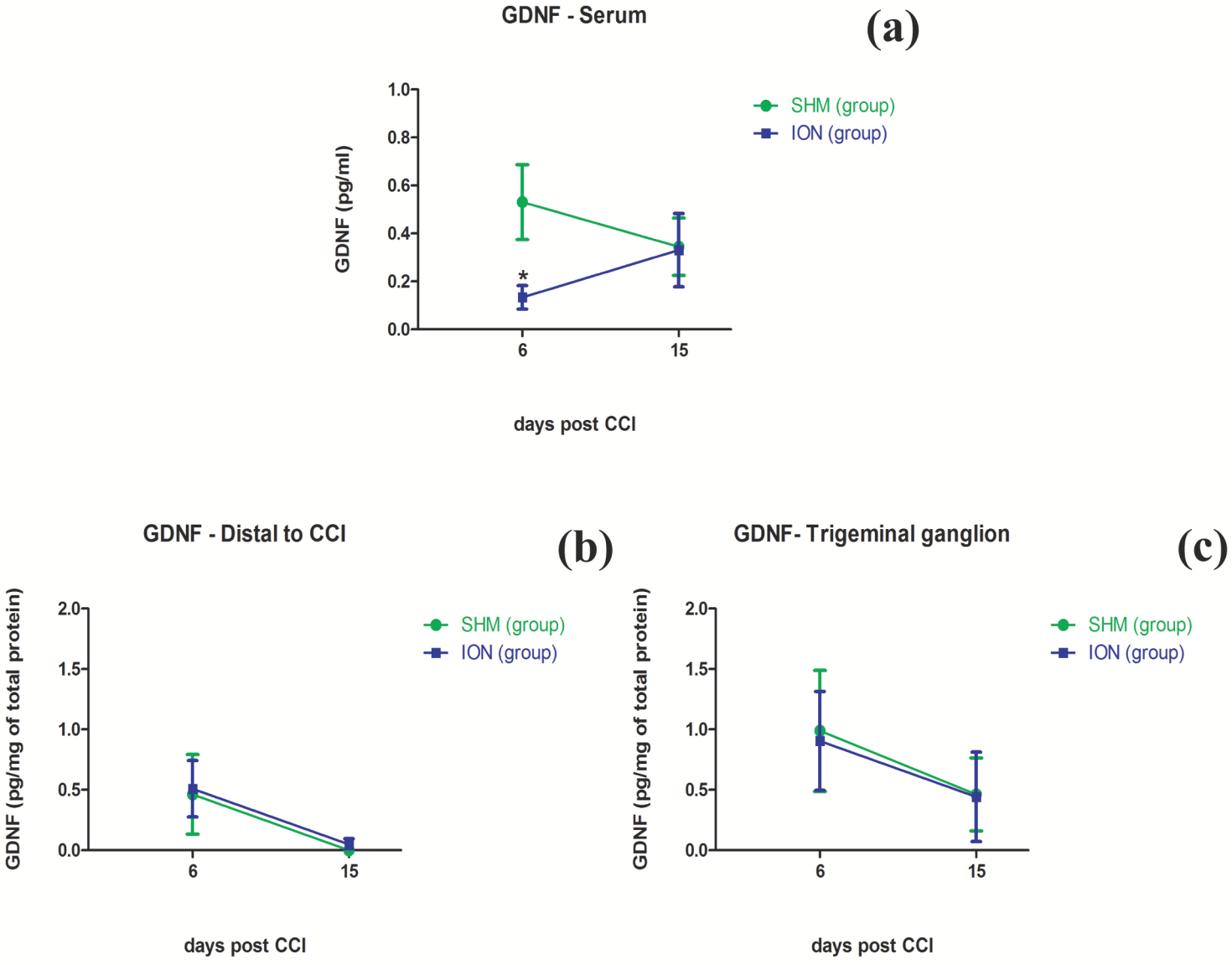
Figure


ION group - 9 days
(a)
ION group - 15 days
(b)
SHM group - 9 days
(c)
SHM group - 15 days
(d)
ION group - 15 days
(e)
SHM group - 15 days
(f)
Distal to CCI
(g)
Immunopositive cells / area (µm²)
40
30
20
10
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)
Trigeminal ganglion
(h)
Immunopositive cells / area (µm²)
4
3
2
1
0
3
6
9
12
15
days post CCI
SHM (group)
ION (group)

Figure


NGF - Serum
(a)
NGF (pg/ml)
0.5
0.4
0.3
0.2
0.1
0.0
6
15
days post CCI
SHM (group)
ION (group)
NGF - Distal to CCI
(b)
NGF (pg/mg of total protein)
50
40
30
20
10
0
6
15
days post CCI
SHM (group)
ION (group)
NGF - Trigeminal ganglion
(c)
NGF (pg/mg of total protein)
50
40
30
20
10
0
6
15
days post CCI
SHM (group)
ION (group)

Figure


GDNF - Serum
(a)
GDNF (pg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
*
6
15
days post CCI
SHM (group)
ION (group)
GDNF - Distal to CCI
(b)
GDNF (pg/mg of total protein)
2.0
1.5
1.0
0.5
0.0
6
15
days post CCI
SHM (group)
ION (group)
GDNF- Trigeminal ganglion
(c)
GDNF (pg/mg of total protein)
2.0
1.5
1.0
0.5
0.0
6
15
days post CCI
SHM (group)
ION (group)

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–350 g) were obtained from Centro de Bioterismo/Universidade Federal de Minas Gerais (Brazil) and maintained on a 12-h light/dark cycle. Standard rat chow and water were available ad libitum. Animals were treated and cared for according to the Ethical and Animal Use Committee on Animal Experimentation (CETEA/UFMG 231/2009) and the ethical standards and guidelines for investigations of experimental pain in animals prescribed by the International Association for the Study of Pain (Zimmermann, 1983).

Surgery

Forty rats received a chronic constriction injury (CCI) to right infraorbital nerve (IoN group) or only a unilateral sham operation (SHM group). All surgery was performed under general anesthesia with 200 mg/kg of ketamin and 10 mg/kg of xilazin (i.m.). Surgery procedures were performed as previously described (Imamura et al., 2007). Briefly, 1 cm long incision was made intraorally along the gingivobuccal margin. The incision begun proximal to the first molar and about 0.5 cm of the IoN was freed of adhering tissue. Two ligatures (4.0 chromic gut) were tied loosely around it, separated by approximately 2 mm. The incision was sutured at two points using 4.0 silk, and animals were allowed to recover. The sham operation was identical except that the nerve was not ligated. All operations were performed aseptically; no antibiotics were administered. Animals were monitored daily; they exhibited normal feeding and drinking behavior.

Behavioral testing

Rats were tested by a blinded experimenter at 3, 6, 9, 12, and 15 d after the surgery. Rats were placed in transparent cages and allowed to acclimate to their surroundings. After 7 min of habituation, von Frey filaments of varying diameters for which the force required to bend each filament was, respectively, approximately, 1 gm, 2 gm, 4 gm, 9gm, and 16 gm were applied perpendicularly to vibrissae pad to determine rat's response to mechanical facial stimulation. Response score was established as (1) detection, (2) withdrawal reaction, (3) escape/attack, or (4) asymmetric face grooming, as previously described (Vos et al., 1994).

Tissue preparation

After behavioral testing, animals (5 per group for each time studied – 3, 6, 9, 12, and 15 d after the surgery) were deeply anesthetized with 200 mg/kg of ketamin and 10 mg/kg

of xilazin (i.m.), decapitated and trigeminal nerves were dissected and divided in 2 parts: distal (from infraorbital foramem to vibrissae pad) and proximal to lesion (region where the ganglion is). Nerve fragments were fixed in 4% formaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4, and routinely processed for paraffin embedding. Sections of 4 and 7 µm were obtained for hematoxylin and eosin (HE) staining and immunohistochemistry, respectively. Some other animals (3 per group at 6 and 15 d after the surgery) were deeply anesthetized, transcardically perfused with 4% paraformaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer, and small fragments of distal and proximal portions of trigeminal nerve were dissected and immersed in the same fixative for 12h. After this time, tissues were immersed in 0.1M cacodylate buffer for 12 h at 4°C and then postfixed in 1% 0s04 and 1.6% K_4 [$Fe(CN)_6$] for 90 min at room temperature. Tissue blocks were dehydrated in graded ethanol and embedded in epoxy resin (*Poly/Bed® 812)*. Semi-thin (200 nm) and ultra-thin (60 nm) cross-sections were cut on an ultramicrotome. Semi-thin sections were collected on glass slides and stained with 1% toluidine blue. Ultra-thin sections were counterstaining with uranyl acetate and lead citrate and analyzed in the Transmission Electron Microscope Tecnai G2-12 - SpiritBiotwin FEI - 120 kV located at the Center of Microscopy at the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Semi-thin and ultra-thin were used for morphological evaluation and morphometry measurements.

Morphological analysis and morphometry

Histopathological alterations, such as presence of inflammatory infiltrate, edema, degeneration of glial cell or neurons, and axonal swelling were evaluated for each time point in IoN and SHM groups. Cell count in nerve fascicles and ganglia was performed in HE sections and nerves of at least 5 IoN or SHM animals were used at each time point. Myelin area and myelin/axon ratio were quantified in toluidine blue semi-thin sections and nerves of at least 3 IoN or SHM animals were used at each time point. Images were obtained with Olympus BX51 microscope and digital images were acquired for documentation through Image-Pro Express 4.0 (Media Cybernetics, MD, USA). All morphometric parameters were manually measured using ImageJ 1.45S software (NIH, USA). Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

Ultrastructural analysis and morphometry

Ultrastructural analysis of myelinic and unmyelinic fibers was performed. In myelinated fibers, demyelination, myelin breakdown and density, and invagination of myelin sheath were analysed. In unmyelinated ones, Schwann cell area and number of fibers per cluster were determined. All morphometric parameters were manually measured using ImageJ 1.45S software (NIH, USA). Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

Immunohistochemistry

For immunohistochemistry, antigen retrieval was performed in deparaffinized and hydrated sections using Target Retrieval Solution (S1700, Dako Corporation) for 30 min at 98ºC. Endogenous peroxidase activity was abolished by incubation with 3.5% H2O2, and a 1:20 dilution of normal goat serum and 2% bovine serum albumin solution PBS was used to block nonspecific binding sites. Both blockages were during 30 min at room temperature. Afterward, slides were incubated with rabbit anti-SP (1:100, AB1566, Millipore), anti-IL-1β (1:200, NBP1-19775, Novus Biologicals), anti GFAP (1:500, Z0334,Dako) and anti S100 (1:400, Z0311,Dako) overnight at 4ºC in a humid chamber. Incubation with secondary biotinylated goat anti-rabbit was followed by incubation with a streptavidin-peroxidase complex (LSAB2 system-HRP, DAKO), each for 30 min at room temperature. The reaction was visualized by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), and counterstaining was done with hematoxylin. Negative control was performed by omission of the primary antibody. Sections were examined, images were obtained with Olympus BX51 microscope and digital images were acquired for documentation through Image-Pro Express 4.0 (Media Cybernetics, MD, USA). Immunopositive IL-1β or SP cells were count in nerve fascicles and ganglia of at least 5 of IoN or SHM animals at each time point. All morphometric parameters were manually measured using ImageJ 1.45S software (NIH, USA). Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

NGF and GDNF protein levels

For quantification of NGF and GDNF protein levels, animals (5 per group for each time studied – 6 and 15 d after the surgery) were deeply anesthetized with 200 mg/kg of ketamin and 10 mg/kg of xilazin (i.m.), and blood was collected from right atrium. After this procedure, trigeminal nerves were dissected, divided in distal and proximal

portions, as previously described, and frozen at -70 ºC. Blood was maintained at room temperature and at 10 ºC for 30 minutes each and centrifuged at 14.000 rpm for 10 min at 4 °C. Serum was collected and frozen at -70 °C. For tissue quantification, frozen samples were sonicated in a cold extraction 20 mM Tris–HCl buffer at Ph 8.0 with 137 mM NaCl, 1% NP40 detergent, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin A, 10 mM EDTA, 10 μM E-64 and 0.5 mM sodium vanadate (Sigma products, St. Louis, MO). The homogenates were centrifuged for 20 min at 14000 g (Bennett et al., 1999, using different proteases inhibitors). For GDNF assay, Promega (San Luis Obispo, CA) kit and protocol were used. For NGF, kit and protocol were from the R&D Systems (Minneapolis, MN). In each plate, NGF or GDNF standard curves were obtained along with the samples. The absorbance was read at 450 nm (Versamax microplate reader, Molecular Devices, Sunnyvale, CA). The Bradford (1976) method measured the total protein content of the samples. The neurotrophic factor levels were expressed as pg/ml of serum or pg/mg of total protein in nervous tissue. Data were compared through Mann-Whitney or unpaired t test (GraphPad Prism software, San Diego, USA) and probability values of 0.05 or less were considered significant.

Statistical analysis

All comparisons between groups were made by Mann-Whitney (non parametric data) or unpaired t test (parametric data) using GraphPad Instat (GraphPad Software, San Diego, CA, USA). A p value of less than 0.05 was considered statistically significant. Data were expressed as mean+/-SEM.