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Research report

Blockade of ATP P2X7 receptor enhances ischiatic nerve regeneration in mice following a crush injury

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ABSTRACT

Preventing damage caused by nerve degeneration is a great challenge. There is a growing body of evidence implicating extracellular nucleotides and their P2 receptors in many pathophysiological mechanisms. In this work we aimed to investigate the effects of the administration of Brilliant Blue G (BBG) and Pyridoxalphosphate-6-azophenyl-2′, 4′- disulphonic acid (PPADS), P2X7 and P2 non-selective receptor antagonists, respectively, on sciatic nerve regeneration. Four groups of mice that underwent nerve crush lesion were used: two control groups treated with vehicle (saline), a group treated with BBG and a group treated with PPADS during 28 days. Gastrocnemius muscle weight was evaluated. For functional evaluation we used the Sciatic Functional Index (SFI) and the horizontal ladder walking test. Nerves, dorsal root ganglia and spinal cords were processed for light and electron microscopy. Antinoceptive effects of BBG and PPADS were evaluated through von Frey E, and the levels of IL-1β and TNF-α were analyzed by ELISA. BBG promoted an increase in the number of myelinated fibers and on axon, fiber and myelin areas. BBG and PPADS led to an increase of TNF-α and IL-1β in the nerve on day 1 and PPADS caused a decrease of IL-1β on day 7. Mechanical allodynia was reversed on day 7 in the groups treated with BBG and PPADS. We concluded that BBG promoted a better morphological regeneration after ischiatic crush injury, but this was not followed by anticipation of functional improvement. In addition, both PPADS and BBG presented anti-inflammatory as well as antinociceptive effects.

1. Introduction

Full functional recovery after peripheral nerve lesion remains a challenge, for both clinicians and researchers. A number of experimental strategies has been tested seeking the improvement of functional recovery after peripheral nerve lesion, such as gene (RJ Mason et al., 2011) and cellular therapies (Oliveira et al., 2010), microsurgical techniques and physical therapies (exercise, laser and electrical therapy) (Goulart et al., 2014; Baptista et al., 2008). However, the effectiveness of these therapeutic strategies still falls short of achieving adequate reinnervation and full functional recovery (Lo et al., 2014). Therefore, there is a continuous search for strategies that can, isolated or combined, promote full functional recovery and yet be translated to clinical trials.

Purines, such as adenosine 5′-triphosphate (ATP), play a role as extracellular signaling molecules by acting, for example, as neurotransmitters and co-transmitters of neuropeptides and nitric oxide on the peripheral and central nervous system (Burnstock, 1972). There is a growing body of evidence on the implication of extracellular nucleotides and their P2 receptors in many pathophysiological processes (Burnstock, 2013), such as peripheral nerve lesions (He et al., 2012), neuroinflammation (Beamer et al., 2016) and allodinia (Tsuda and Inoue, 2016).

The consequences of high extracellular ATP concentrations are mainly determined by the activity of ectonucleotidases metabolizing ATP, which account for a rapid removal of extracellular ATP and the formation of adenosine (Zimmermann, 1996). Braun and

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collaborators (1998) showed that during brain ischemia, both ectoapyrase (capable of hydrolyzing nucleoside 5′-tri- and diphosphates) and ecto-5′-nucleotidase (capable of hydrolyzing nucleoside 5′-monophosphates) are upregulated and activated in the hippocampus, and might be part of an innate self-repair neuroprotective mechanism involving purinergic signaling. The leakage of purine nucleotides from damaged cells may reach cytotoxic levels in the extracellular space (Braun et al., 1998; Lämmer et al., 2011) and activate several ATP/ADP- (and UTP/UDP-) sensitive receptor-types and their respective signal transduction cascades (Burnstock, 2013). Purinergic receptors are subdivided into the ligand-gated ion channels P2X and metabotropic P2Y G-protein coupled (Ralevic and Burnstock, 1998). Several nucleotides that act through P2 receptors, such as ATP and 5′-Uridine triphosphate (UTP) or their analogs, are released after a nerve lesion, and may be implicated on the deflagration of neuroinflammation (Davalos et al., 2005; Pineau and Lacroix, 2009; Beamer et al., 2016). In addition, the increase of extracellular ATP, due to nerve lesion, and the subsequent activation of P2 receptors, influence the processes of necrosis and apoptosis as well as neurodegeneration and regeneration (Franke et al., 2006). It is also known that after nerve lesion, extracellular ATP may be involved in the process of demyelination through activation of Schwann cells lysosomal exocytosis (Shin et al., 2014). Among possible therapeutic strategies, the use of antagonists of P2 receptors (receptor for purine nucleotides) has emerged as an alternative for treating nervous system disorders (Lämmer et al., 2011; Ridderström and Ohlsson, 2014). In addition, since ATP is a danger signal in the nervous system (Rodrigues et al., 2015), the use of antagonists of P2 receptors could also be explored as a neuroprotective strategy.

Several drugs including Brilliant Blue G (BBG) interact with the P2X7R and block its channel with high efficacy and good selectivity. Depending on the species, BBG inhibits the P2X7R with IC50 values of 10nM (rat) or 265nM (human), while requiring 100–1000 times higher concentrations to inhibit other P2X receptors (Jiang et al., 2000). The P2X7R can act in concert with the ATP release pannexin 1 (Panx 1) (Pelegrin and Surprenant, 2006; Locovei et al., 2007). In some cellular context, the Panx1 pathway might play a significant role in P2X7 receptor-mediated membrane permeabilization, depending on the expression levels of the two proteins and the duration and extent to which the receptor is activated (Pelegrin and Surprenant, 2006; Locovei et al., 2006). The ATP-binding sites on Panx and P2X7R are similar and several ligands to the receptor, including BBG, inhibit the Panx channel (Wang et al., 2013).

After a brain insult, released ATP fulfills a major role as a danger signal (Rodrigues et al., 2015), and the genetic or pharmacological blockade of P2 receptors has been shown to afford tissue protection (reviewed in Franke et al., 2006; Rodrigues et al., 2015). Previous studies from our group showed that P2X7 receptor antagonist BBG attenuates neurotoxicity and gliosis in a rat model of Parkinsonism (Carmo et al., 2014a). We have also showed the involvement of P2Y1 on ischemic brain damage, and the neuroprotection afforded by PPADS, a broad-spectrum P2R antagonist (Carmo et al., 2014b).

Previous experimental studies have demonstrated positive effects of inhibition of the P2 receptors with BBG after optic nerve injury (Ridderström and Ohlsson, 2014) and Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) after spinal cord injury (Martucci et al., 2008; Wang et al., 2004). However, little is known about these P2 receptor antagonists as a potential treatment strategy for peripheral nerve lesion (Inoue et al., 2007; Peng et al., 2009). In this study we aimed to investigate the role of P2 receptor antagonists, BBG and PPADS, on the regeneration of mouse ischiatic nerve following a crush injury. Our results demonstrate that P2X7 receptor antagonism promoted morphological regeneration after ischiatic crush injury, probably by anti-inflammatory mechanisms, but this was not followed by an anticipation of the functional recovery.

2. Results

2.1. BBG enhances overall morphological nerve regeneration

Four weeks after nerve crush the middle segment of the nerves was harvested and prepared for histological analysis. Semi-thin sections showed an overall better tissue organization in BBG group compared to the other groups. Ultrastructural analysis revealed that the myelinated nerve fibers in BBG group had a more preserved axoplasm and thicker myelin sheath than the other groups. Nerve fibers in PPADS group were more dispersed than in the other groups and presented a general aspect of degenerating tissue (Fig. 1).

Morphometric analyses showed that BBG group had a higher number of myelinated nerve fibers (2206 ± 211.3, $p < 0.05$) compared to Vehicle-BBG (1684 ± 37.23) and PPADS (1192 ± 189.4)

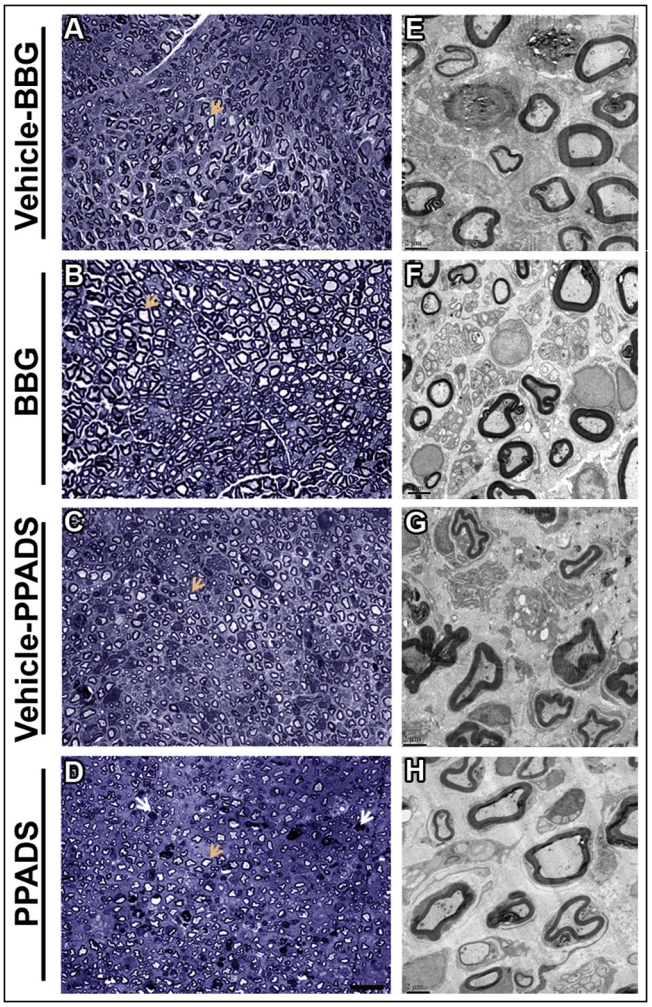

Fig. 1. Light and electron micrographs of cross sections of the medial segment of the regenerating ischiatic nerve of (A, E) Vehicle-BBG, (B, F) BBG, (C, G) Vehicle-PPADS and (D, H) PPADS animals, 4 weeks post-lesion. (A-D) There are regenerated nerve fibers (pink arrows) and myelin ovoids (white arrows). We observe (F) a larger number of myelinated fibers in the BBG group (H) and a smaller number of nonmyelinated fibers in the PPADS group. (H) Degenerating structures can be seen in the PPADS group. Scale bar: (A–D) 20 μm; (E–H) 2 μm.

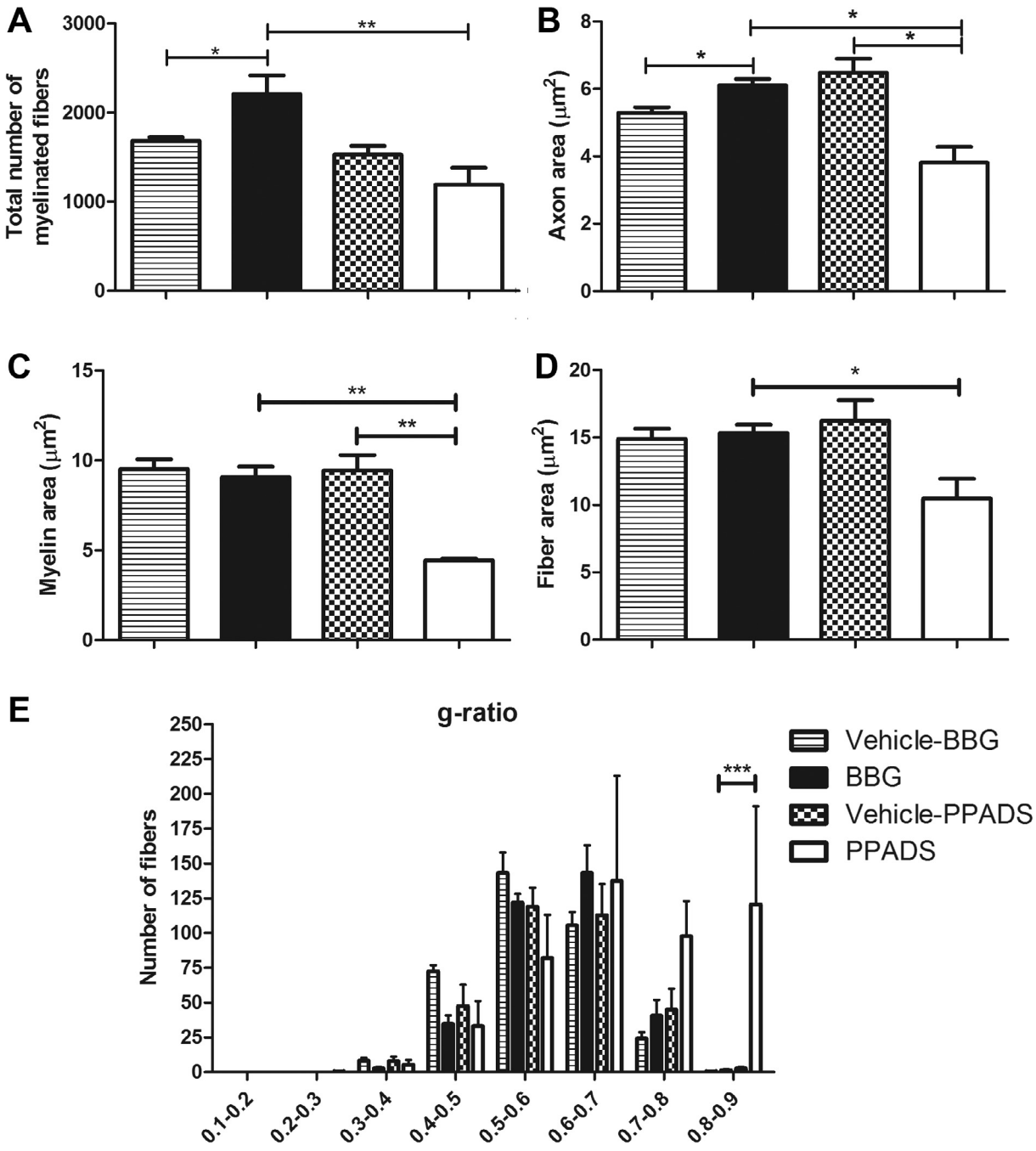

Fig. 2. Morphometric analyses of myelinated fibers of ischiatic nerve 4 weeks post-lesion. (A) Total number of myelinated fibers, (B) axon area, (C) myelin area, (D) fiber area and (E) G-ratio. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}P < 0.001$.

groups (Fig. 2A). Sectional area of axons was larger in BBG group (6.111 ± 0.1862, $p < 0.05$) than Vehicle-BBG (5.293 ± 0.1647) and PPADS (3.809 ± 0.4724) groups, which in turn showed smaller sectional area of axons compared to Vehicle-PPADS group (6.474 ± 0.4197) (Fig. 2B). Myelin area was larger in BBG group (9.071 ± 0.5845) group as compared to PPADS group (4.438 ± 0.1117) (Fig. 2C). Nerve fiber area was larger in BBG group (15.34 ± 0.6310, $p < 0.05$) compared to PPADS group (10.48 ± 1.463), which, in turn, presented lower nerve fiber area (10.48 ± 1.463, $p < 0.05$) than Vehicle-PPADS group (16.24 ± 1.463) (Fig. 2D).

Regarding G-ratio analysis, BBG group presented the highest number of fibers in the optimal G-ratio range (Fig. 2E). PPADS, however, presented a high number of myelinated fibers in the highest ranges.

2.2. BBG and PPADS have no effect on the number of sensory and motor neurons

Four weeks after nerve crush, the L4 spinal cords and the L4 DRGs were harvested and prepared for histological analyses. The primary sensory neurons and the motor neurons were quantified. There was no difference between all groups regarding the number of DRG (Vehicle-BBG: 92.82 ± 9.18; BBG: 102.72 ± 12.46; Vehicle-PPADS: 114.91 ± 7.03; PPADS: 110.72 ± 11.96 and motor neurons

(Vehicle-BBG: 13.9 ± 1.57; BBG: 14.3 ± 2.87; Vehicle-PPADS: 12.9 ± 1.45; PPADS: 14.3 ± 1.33) (Fig. 3).

2.3. BBG preserves muscle trophism

Four weeks after nerve crush the gastrocnemius muscles from both limbs were harvested and immediately weighed for trophism analysis. Muscles from BBG group (0.8720 ± 0.0436, $p < 0.05$) were heavier than those from PPADS group (0.7180 ± 0.0389) (Fig. 4).

2.4. BBG and PPADS have no effects on locomotor function

The Ladder Walking Test and SFI were performed prior to surgery and weekly after it until the 28th day after surgery. There was no difference between the groups regarding these two behavioral tests (Fig. 5).

2.5. Mechanical allodynia was reverted by BBG and PPADS treatment on day 7

The mechanical allodynia test was performed prior to surgery and on days 1, 3 and 7 after surgery. We observed that the withdrawal threshold of the ipsilateral paw was lower in Vehicle-BBG, BBG, Vehicle-PPADS and PPADS groups compared with the Sham group on days 1 and 3 (Fig. 6A). These results indicate the presence of mechanical allodynia, suggesting neuropathic pain.

On day 7 the withdrawal threshold of the ipsilateral paw was not different between Sham, BBG, PPADS and Vehicle-PPADS groups, suggesting that mechanical allodynia in these groups was reverted. The ipsilateral paw presented lower withdrawal threshold in the Vehicle-BBG group as compared to the Sham group, on day 7 (Fig. 6A).

There was no difference between the groups regarding the withdrawal threshold of the contralateral paw. (Fig. 6B).

2.6. PPADS induces an increase of TNF-α tissue levels on days 1, 3 and 7 and of IL-1β on day 1

After the survival periods, the middle segment of the nerves were harvested and used for cytokines analyses. BBG group presented higher levels of TNF-α compared to Sham group on days 3 (30.36 ± 2.33 and 28.85 ± 7.64, respectively, $p < 0.05$) and 7 (34.68 ± 4.18 and 22.36 ± 5.71, respectively, $p < 0.05$) (Fig. 6C). PPADS group presented higher levels of TNF-α compared to Vehicle-PPADS and Sham groups on days 1 (118 ± 29.14; 38.27 ± 5.24 and 22.09 ± 5.72, respectively, $p < 0.05$), 3 (104 ± 16.37; 37.2 ± 6.28 and 25.85±, respectively, $p < 0.05$) and 7 (94.37 ± 14.79; 32.11 ± 3.67 and 22.36 ± 5.71, respectively, $p < 0.05$) (Fig. 6C). PPADS group presented lower levels of IL-1β than BBG group on days 3 (73.82 ± 18.58 and50.06 ± 6.69, respectively, $p = 0.001$) and 7 (41.12 ± 7.07 and 39 ± 3.86, respectively, $p < 0.05$), but higher levels than Sham and BBG groups on day 1 (87.79 ± 36.88 and 94.06 ± 26.42, $p < 0.05$ and $p < 0.01$, respectively) (Fig. 6D).

Table 1 displays a summary of the results obtained in this study, according to the analyzed parameters.

3. Discussion

Peripheral nerve lesion triggers a sustained release of ATP from Schwann cells and the nerve fiber itself (Shin et al., 2012, 2014). ATP acts on P2 receptors, playing a role on several processes, such as apoptosis, necrosis and regeneration (Franke et al., 2006). Thus, ATP releasing in several pathological conditions can yield either protection or toxic effects, depending mainly on the type of P2X receptors. P2X receptors are a family of ligand-gated ion channels; specifically the P2X7R is a ligand-operated ion channel with high permeability to small cations (North, 2002), but after being prolonged activated it can form a large pore, which allows the flux of larger molecules (Pelegrin and Surprenant, 2006; Locovei et al., 2007). After nerve injury, P2X7 activation leads to an increase in calcium influx (Bao et al., 2004; Dahl, 2015) and this is believed to play a role in the initiation of Wallerian degeneration (Lee and Wolfe, 2000; Yoo et al., 2003).

In this work we have used two P2 receptor antagonists, PPADS and BBG, in the mouse ischiatic nerve subjected to crush, as a potential treatment strategy, since it is known that peripheral nerve injury is not always prone to functional recovery, particularly in humans. Our findings show that BBG treatment improved nerve regeneration, as observed by a higher total number of myelinated nerve fibers as well as a higher axon area compared to Vehicle-BBG. Rodriguez-Feo and collaborators (2013) used Brilliant Blue FCF (BB FCF), a P2X7 receptor blocker, which is structurally similar to BBG (Wang et al., 2013), in the rat ischiatic nerve transection model, and found a trend toward improvement in axon counting. They suggested that this improvement was most likely due to the prevention of P2X7 receptor-mediated calcium influx after axonal injury. Calcium influx is one of the first events that trigger Wallerian degeneration by activation of proteases such as calpains which are responsible for axonal disintegration (Martinez and Ribeiro, 1998; Park et al., 2013; Rodriguez-Feo et al., 2013). Thus, we hypothesize that, in our work, BBG might also dampen P2X7 receptor-mediated calcium influx into neurons and glia cells.

PPADS treatment, however, presented poorer results on nerve regeneration when compared to BBG, as observed by the total number of myelinated nerve fibers and the areas of axon, myelin and nerve fiber. In fact, PPADS results were even poorer in comparison to its control (Vehicle-PPADS). It is known that PPADS is a broad spectrum antagonist of P2 receptors, promoting the blockade of several different purinergic receptors (Ralevic and Burnstock, 1998; Lambrecht, 2000), such as P2Y1. The activation of P2Y1 receptors in the central nervous system has been related to a decrease in the inflammatory response and also to neuroprotection (Zheng et al., 2013). Thus, in this work it is possible that by blocking P2Y1 with PPADS, neuroprotection was impaired leading to a poorer nerve regeneration. Several studies have shown that the pharmacological or genetic P2Y1R blockade affords neuroprotection in ischemic conditions (Sun et al., 2008; Kuboyama et al., 2011; Carmo et al., 2014b) or trauma (Choo et al., 2013). The pathological role of P2Y1R has been predominantly associated with reactive astrocytes since P2Y1R play a key role in entraining the propagation of calcium waves throughout the astrocyte network (Fam et al., 2003; Neary et al., 2003; Bowser and Khakh, 2007) resulting in astrocytic hyperactivity and astrogliosis, after mechanical injury (Franke et al., 2001) and ischemic conditions (Sun et al., 2008), which are known to interfere with neuronal repair and regeneration (Burnstock, 1972; Tian et al., 2006). Thus, the blockade or the stimulation of P2Y1R in astrocytes can cause paradoxical effects (see Rodrigues et al., 2015, for review). This receptor mediates trophic effects (Franke et al., 2009) but also facilitates the endogenous release of glutamate inhibited by PPADS (Krügel et al., 2004). The PPADS pretreatment prevented not only the P2Y1 receptor mediated raise of the apoptotic pERK1/2 but also the anti-apoptotic pAkt (Franke et al., 2009). These finding can, at least partially, explain the less effective action of PPADS on neuronal regeneration observed in this study.

Behavioral tests (SFI and ladder walking tests) showed no significant difference between the groups studied. Nerve crush injury is known to lead to full functional recovery by three weeks after injury. Thus, as expected, animals treated with BBG and PPADS

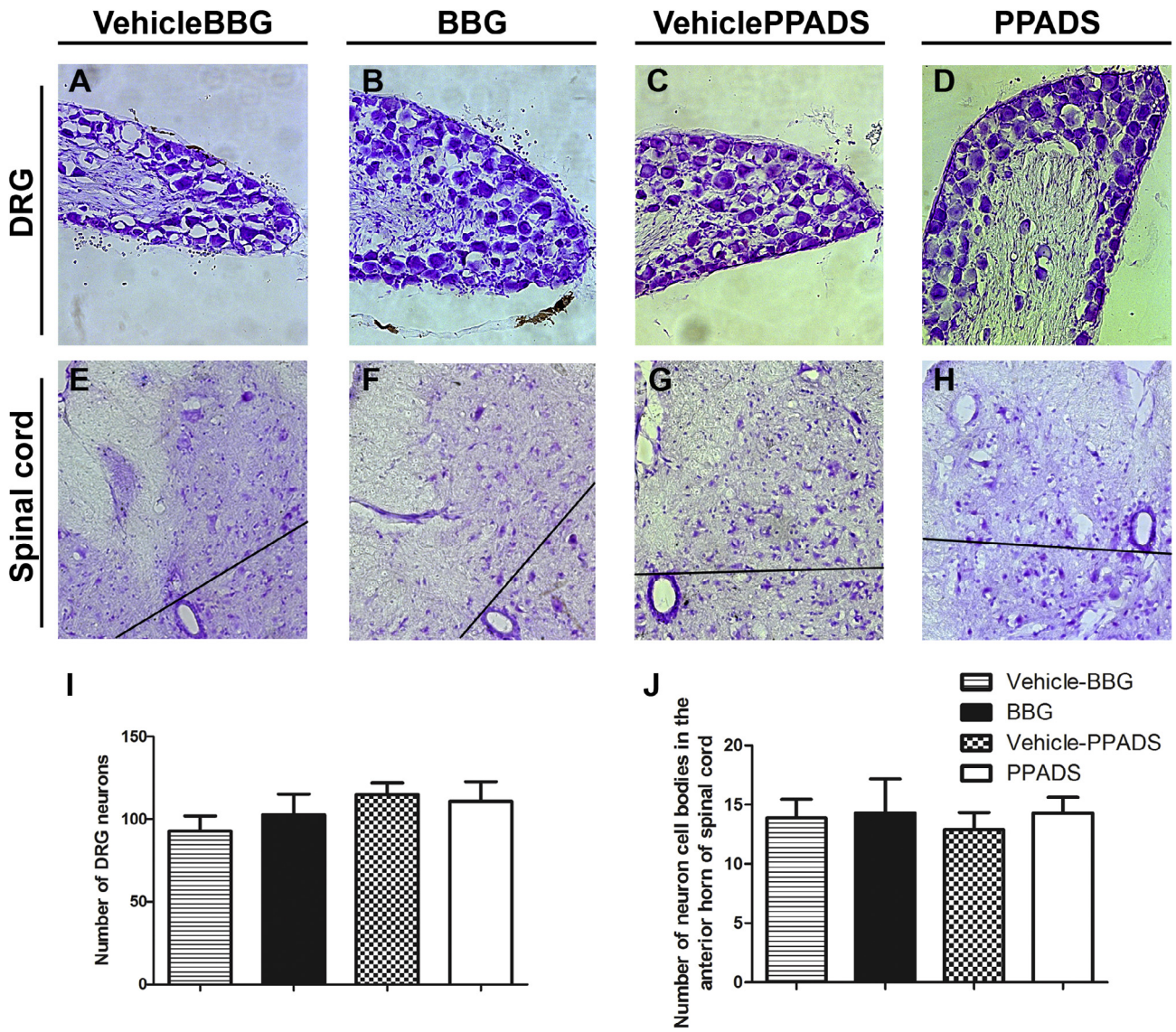

Fig. 3. Light micrographs of longitudinal sections of (A-D) dorsal root ganglia (DRG) and cross sections of (E-H) L4 spinal cord segments stained by Nissl 4 weeks post-lesion. (A-D) Neuron cell bodies can be seen in the cortical area of DRGs and (E-H) in the anterior horn of the spinal cord (area ahead the central canal delimited by a black line). (I,J) Quantification of DRG and spinal cord motor neurons . Scale bar: 50 μm.

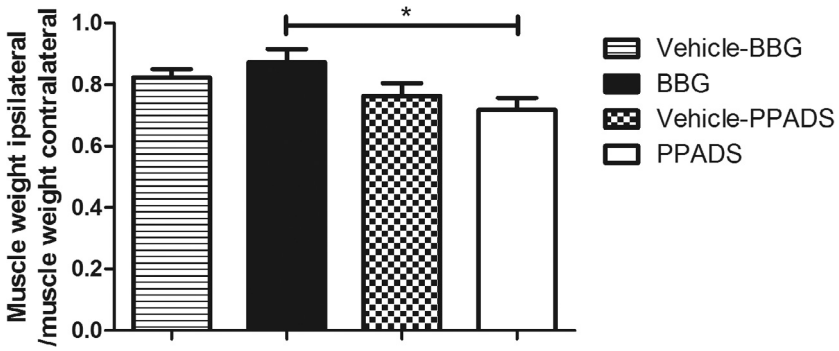

Fig. 4. Muscle weight analysis measured 4 weeks post-lesion. Ipsilateral muscle weight normalized by the contralateral muscle weight in the Vehicle-BBG, BBG, Vehicle-PPADS and PPADS groups. *p<0.05.

showed functional recovery but these treatments did not anticipate it. Our results are in accordance with Marcillo and coworkers (2012) who performed two experiments in a contusive rat spinal cord injury model (Marcillo et al., 2012). In the first experiment, the authors administered intra-spinal injection of P2X7 receptor antagonists, PPADS or BBG, or their vehicles, and

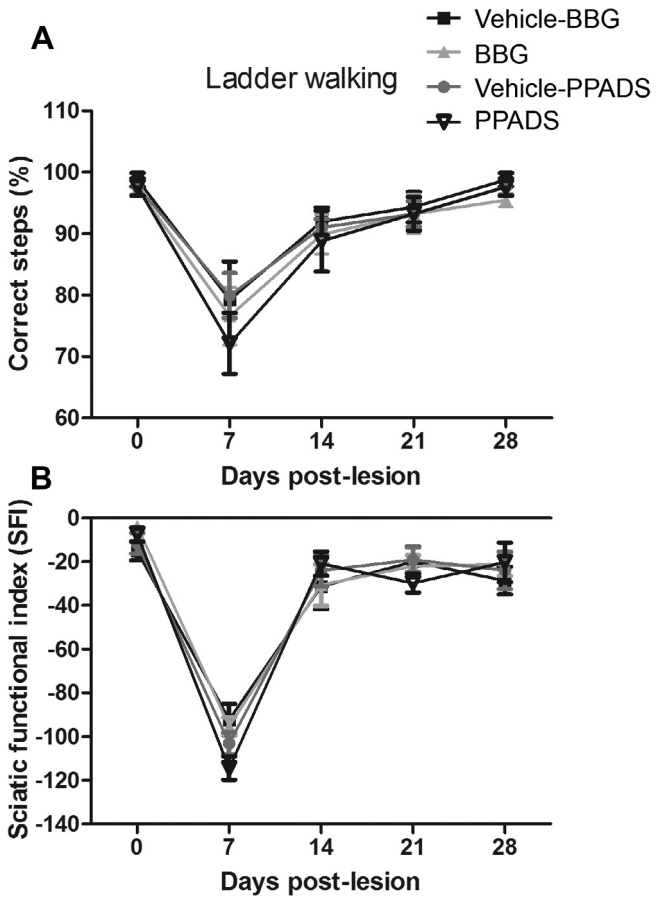

Fig. 5. Functional analyses of the ischiatic nerve 4 weeks post-lesion of the Vehicle-BBG, BBG, Vehicle-PPADS and PPADS groups. (A) Sciatic Functional Index (SFI) and (B) percentage of the number of correct steps of the hindpaw analyzed by Ladder Walking Test.

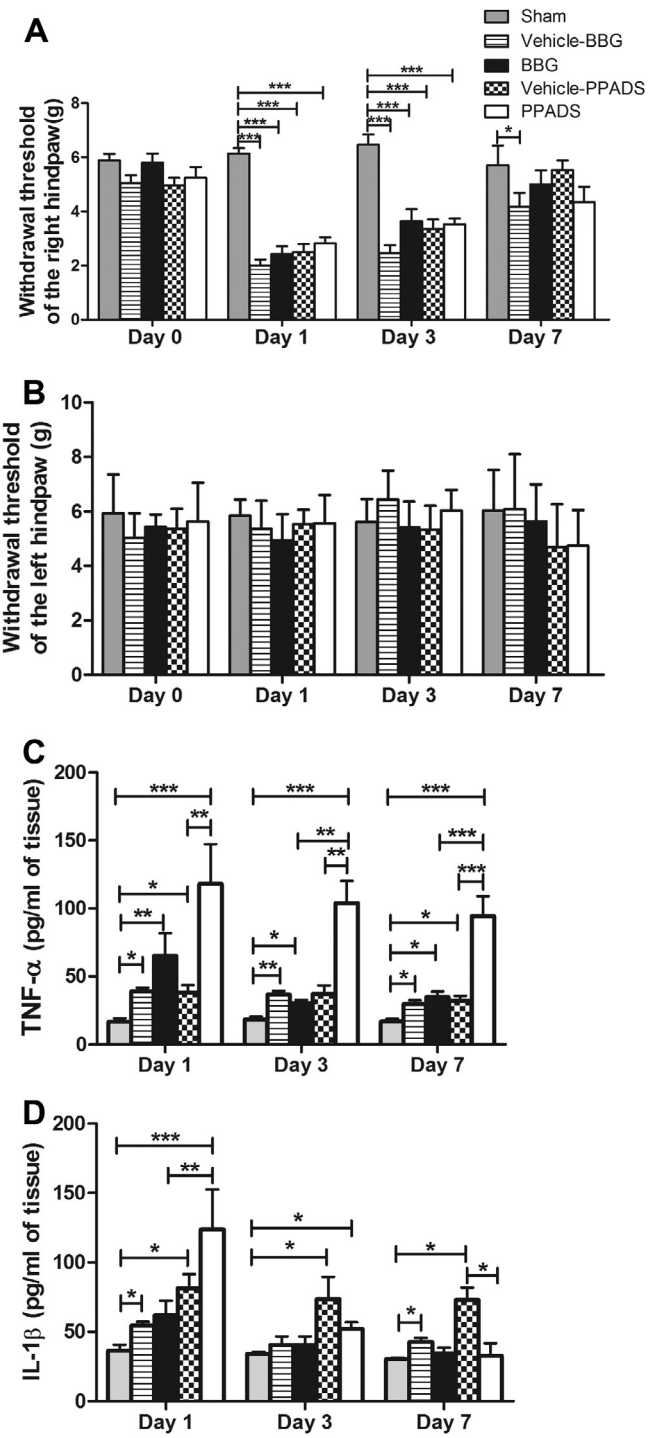

Fig. 6. Effects of BBG and PPADS treatments in the (A, B) mechanical sensitivity analyzed by von Frey and in the (C, D) proinflammatory cytokine levels in the ischiatic nerve in the Sham, Vehicle-BBG, BBG, Vehicle-PPADS and PPADS groups. (A) Withdrawal threshold of the right hindpaw that underwent crush lesion and (B) of the contralateral hindpaw that did not undergo crush lesion on days 0, 1, 3 and 7 of surgical procedure. (C) Dosage of TNF-α and (D) IL-β, measured by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}P < 0.001$.

in the second experiment BBG or its vehicle was systemically administered. The authors did not observe any difference between the groups regarding motor functional recovery by the BBB rating scale. Although the behavioral test they used was the BBB rating scale, and in our study we used the SFI, a high correlation between these two tests has been described (Basso et al., 1995).

Although the P2X4 receptor plays an important role in neuropathic pain, the participation of other P2X receptors cannot be ruled out; the P2X3 receptor, for example, is important in the initiation of pain (Barclay et al., 2002; Honore et al., 2002) and the P2X4 (Tsuda et al., 2003) and P2X7 (Chessell et al., 2005) receptors act through microglial activation, possibly by different mechanisms (Chessell et al., 2006). P2X receptor activation in the spinal cord may elicit allodynia, with P2X4 receptor upregulation on spinal cord microglia playing a predominant role (Tsuda et al., 2003), but it has been shown that P2X7 receptors in microglia are also involved in neuropathic pain (Inoue, 2006; Trang et al., 2006). The P2X7 receptor, via regulation of IL-1β production, plays an important role in the development of neuropathic and inflammatory pain (Chessell et al., 2005). The immunoreactivity for P2X7 receptors was detected in oligodendrocytes from the optic nerve and the spinal cord (Matute et al., 2007a; Matute, 2008). In oligodendrocytes P2X7 may operate in pathological conditions, since in experimental autoimmune encephalomyelitis (an animal model for multiple sclerosis) treatment with BBG ameliorated demyelination and restored nerve conduction velocity (Matute et al., 2007b; Matute, 2008).

It has been demonstrated that TNF-α level increases after nerve injury with the peak occurring at about 12 h and is maintained up to 7 days after lesion (George et al., 2004). In our study, the expression of TNF-α was increased in PPADS treated groups on days 1, 3 and 7. PPADS blocks P2X7 but in a less effective way than BBG does. Thus, the blockage of P2X7 by PPADS is little or ineffective

Table 1
Overall effects of BBG and PPADS treatments on nerve function and regeneration.

Parameters of analysis	Effects of BBG treatment	Effects of PPADS treatment
Morphology	-Higher number of myelinated fibers compared to PPADS and Vehicle-BBG - Larger sectional area of axons compared to PPADS and Vehicle-BBG - Larger myelin area	- Smaller sectional area of axons compared to vehicle-PPADS
# of neurons	-None	-None
Muscle weight	-None	-None
Locomotor function	-None	-None
Mechanical allodynia	-Reverted on Day 7	-Reverted on Day 7
Cytokines assay	-Higher levels of TNF-α compared to Sham group on days 3 and 7	-Higher levels of TNF-α compared to Vehicle-PPADS and Sham on days 1, 3 and 7 - Lower levels of IL-1β compared to BBG group on days 3 and 7 - Higher levels of IL-1β compared to Sham and BBG on day 1

in terms of TNF-α expression (Communi et al., 1999). We also observed by the electronic von Frey test that allodynia was not reverted in the animals treated with PPADS. Martucci and coworkers (2008) showed that a single dose of PPADS (25 mg/kg) injected 3 days after ischiatic nerve chronic constriction reverted mechanical allodynia. It is possible that a single dose instead of multiple doses of PPADS can be more beneficial in terms of nociception. It is known that TNF-α can induce the production of other cytokines, such as IL-1 (Dinarello et al., 1986). Macrophages and Schwann cells express IL-1β (Shamash et al., 2002). The high levels of IL-1β RNAm detected after nerve injury are correlated with larger number of macrophages in the injured microenvironment (Leskovar et al., 2000). In accordance with our results Martucci and collaborators (2008) showed that PPADS decreased both tactile allodynia and thermal hyperalgesia after injury induced by sciatic nerve chronic constriction; PPADS (25 mg/kg) completely reversed nociceptive hypersensitivity and simultaneously reduced the increased NO/NOS and IL-1 β in both peripheral (injured ischiatic nerve and L4-L6 ipsilateral dorsal root ganglia) and central nervous system (L4-L6 spinal cord and thalamus), and IL-6 only in sciatic nerve (Martucci et al., 2008). Sacerdote and colleagues (2013) also demonstrated that neuropathic animals treated with PPADS showed reduction of the elevated IL-1 levels in the sciatic nerve, DRG and spinal cord, and improved morphological parameters, with the animals showing an increased number of fibers with large axons and reduction of fiber alterations (Sacerdote et al., 2013). Our findings showed that PPADS led to an increase of IL-1 expression in the animals treated on day 1, as we observed for TNF-α tissue levels at the same time. We also detected a decrease in the IL-1β expression in PPADS group as compared to Vehicle-PPADS, only on day 7, which correlated with the reversion of the mechanical allodynia at the same time.

4. Conclusions

We concluded that the use of selective P2X7 receptor antagonists like BBG (50 mg/kg) has advantages in comparison to the non-selective P2 receptor antagonist PPADS (25 mg/kg) as pharmacological tools for the treatment of mouse ischiatic crush injury. BBG (50 mg/kg) promoted a better morphological regeneration after ischiatic crush injury, but this was not followed by an anticipation of the functional recovery. In addition, both PPADS and BBG presented anti-inflammatory as well as anti-nociceptive effects. This is the first study investigating the role of two P2 receptor antagonists, BBG and PPADS, on the peripheral nerve regeneration after a crush injury. We hope that our results can encourage further studies investigating the role of these receptors at different parameters such as time, doses and administration via, and hopefully in the future it will open up new avenues for the treatment of peripheral nerve injuries.

5. Experimental procedures

5.1. Drugs

Pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), P2 receptor antagonist and Brilliant Blue G (BBG) P2X7 specific antagonist were purchased from Sigma-Aldrich, USA. Ketamine hydrochloride (Ketanest©, 100 mg/kg) and xylazine hydrochloride (Rompum®, 50 mg/kg) were used for anaesthesia.

5.2. Animals and experimental groups

Female adult C57BL/6 mice weighing 19–25 g were randomly assigned to BBG (animals that received intraperitoneal injection of BBG (50 mg/kg) in saline solution on alternate days, starting at one hour after crush injury, n = 5), Vehicle-BBG (animals that received intraperitoneal injection of saline solution on alternate days, starting at one hour after crush injury, n = 5), PPADS (animals that received intraperitoneal injection of PPADS (25 mg/kg) in saline solution on alternate days, starting at one hour after crush injury, n = 5), Vehicle-PPADS (animals that received intraperitoneal injection of saline solution on alternate days, starting one hour after crush injury, n = 5) or Sham group (animals that had their ischiatic nerve exposed but not crushed, n = 5), during 28 days.

After surgery the animals were housed in cages with food and water ad libitum and 12/12-h light/dark cycle. All procedures conformed to the ethical guidelines regarding the care and use of animals and were approved by the Animal Care Commission of the Universidade Federal do Rio de Janeiro (Protocol Number DAHEICB003).

5.3. Surgical procedure

Animals were deeply anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). The right ischiatic nerve was exposed and a fine forceps was used to crush the nerve at mid-thigh level for 30 s. Four weeks later, the animals were anesthetized with ketamine and xylazine, and then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for morphological analysis. For cytokines analysis 8 mm long nerves segments were dissected, weighted, and processed for ELISA assay.

The middle segment of the regenerated right ischiatic nerve, the L4 spinal cord and the L4 dorsal root ganglion (DRG) were harvested and prepared for light microscopy and transmission electron microscopy.

5.4. Transmission electron microscopy

The middle segment of the regenerated nerves was immersed for 2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed in 0.1 M cacodylate buffer (pH 7.4), and postfixed for 90 min in 1% osmium tetroxide containing 0.8% potassium ferrocyanide and 5 nM calcium chloride in 0.1 M cacodylate buffer (pH 7.4). The segments were washed in 0.1 M cacodylate buffer (pH 7.4) and stained in 1% uranyl acetate overnight in the dark, dehydrated in increasing concentration of acetone, infiltrated with Poly/Bed 812 resin (Polysciences, Inc.), and polymerized at 60 °C for 48 h. Semi-thin (500ηm) and ultra-thin (70 ηm) cross sections were obtained with an ultramicrotome (MT-6000-XL; RMC, Inc.). The semi-thin sections were stained with toluidine blue and examined on a light microscope (Zeiss Axioskop 2 plus), while the ultra-thin sections were collected on copper grids and contrasted in 5% uranyl acetate and 1% lead citrate and analyzed on a transmission electron microscope operated at 80 kV (Zeiss EM 900).

5.5. Light microscopy

After perfusion and dissection, the L4 DRGs and L4 spinal cord segments were removed, washed in 0.1 M phosphate buffer (pH 7.4), cryoprotected with 10%, 20%, and 30% sucrose for 24 h each, and mounted in Tissue-Tek O.C.T. Compound (Sakura Fine Technical). Frozen longitudinal serial sections of DRGs (12 mm) and transverse serial spinal cord sections (20 mm) were cut on a cryostat (Leica CM, 1850) and mounted onto gelatin-coated slides.

5.6. Morphological analysis

Images of the semi-thin cross sections were captured under the light microscope at a 63x magnification and used for quantitative analyses. The total number of myelinated nerve fibers in each regenerated nerve was counted using ImageJ Software (1.42q, USA).

The parameters of axonal area, fiber area, myelin area and G-ratio were calculated using five samples per nerve; pictures were taken on a light microscope, using a 100x magnification. Myelin area was obtained by subtracting the area of the axon from the area of the nerve fiber. The G-ratio was calculated by dividing the axon diameter by the fiber diameter, and the results were stratified in ranges of 0.0–0.4, 0.4–0.5, 0.5–0.6, 0.6–0.7, 0.7–0.8 and 0.8–0.9. For each range, the lowest value was included and the highest one excluded (e.g., the 0.0–0.4 range includes 0.0 through 0.399, excluding 0.4). We used the ImageJ Software (1.42q, USA) for the quantification of the above parameters.

5.7. Neuronal quantification

Slides with DRG and spinal cord were stained with 0.1% cresyl violet and used for quantification of primary sensory neurons and motor neurons, respectively. Sections (12 μm thickness) were collected in series, totalizing 8 slides per sample. Thus, each slide contained 8–10 sections, 96 μm apart from each other. This methodology prevented the same neuron to be counted twice. Sections were photographed on a Zeiss Axioskop 2 plus microscope at a 20x magnification. Nucleoli of neurons in the DRGs sections and of neurons in the spinal cord ventral horn sections were counted using ImageJ Software (1.42q).

5.8. Analysis of gastrocnemius muscle weight

Just before perfusion, the gastrocnemius muscles from both limbs were dissected and immediately weighed to analyze its trophism as an indirect measurement of nerve regeneration and muscle reinnervation.

5.9. Behavioral tests

5.9.1. Ischiatic functional index (SFI)

Motor function was evaluated with use of the SFI, based on a protocol described by (Inserra et al., 1998). The animals' pawprints were registered weekly and two measurements were taken: the print length (PL), corresponding to the distance from the heel to the third toe, and the toe spread (TS), corresponding to the distance from the first to the fifth toe. Both measurements were taken from injured (E, from experimental) as well as uninjured (N, from normal) sides and the SFI was calculated according to the following equation: SFI = 118.9 (ETS – NTS/NTS) – 51.2 (EPL – NPL/NPL) – 7.5. A SFI around zero corresponds to normal nerve function and a SFI around -100 represents total loss of ischiatic nerve function.

5.9.2. Horizontal ladder walking test

Prior to surgery, animals were trained to walk along the ladder. The ladder rung walking test apparatus consisted of two clear Perspex sidewalls (100 cm in length × 20 cm height) and a floor with removable metal rungs spaced 1 cm apart. The position of the metal rungs were placed 1 to 5 cm apart from each other and modified every training session depending on the desired degree of difficulty and to prevent the animals from learning the task. Tests were accomplished before surgical procedure and weekly after it. A video camera (Lifecam vx-800, Microsoft) was positioned to record the lateral portion of the ladder when animals were allowed to walk unidirectionally through it. Each animal had 3 runs per session and a mean of the number of correct steps of the hindlimbs was obtained (Metz and Whishaw, 2009).

5.9.3. Mechanical allodynia test (electronic von Frey)

A digital Von Frey (Insight®, Ribeirão Preto, Brasil) was used for hind paw withdrawal in response to graded mechanical stimulation. This test was only performed in the animals used for cytokines analysis and it was carried out before lesion and 1, 3 or 7 days post-lesion. Animals were placed on a wire mesh plate and the plantar surface of the hindpaws was stimulated by a transducer with a 1.00 mm sensor pin at the tip, when a gradative and linear force was applied by the investigator. The threshold was determined as the mean of the lowest force that evoked a withdrawal response to three stimuli.

5.10. Cytokines analysis

Eight mm long injured nerves segments were dissected from animals belonging to all analyzed groups, weighted, and processed by ELISA assay. Briefly, the muscles were homogenized with PBS and centrifuged (3500 rpm/min at 4 °C). Then the supernatant was purified by new centrifugation, frozen in liquid nitrogen and stored in a −80 °C freezer until analysis by two-sandwich ELISA kit (BD, Biosciences Reagents, USA). The assay was then performed as recommended by the manufacturer. Sensitivity of the assay procedure was ensured by addition of known amounts of TNF-alpha.

5.11. Statistical analyses

Mechanical hipersensibility data were analyzed by Two-way ANOVA followed by Bonferroni post test. Paired inferences were made by Student's *t*-test (between two groups) or one-way ANOVA (among three groups), followed by Tukey post hoc analysis when necessary. The confidence interval was 95%, with an accepted alpha value of 5% ($P < 0.05$). The analyses were carried out using the Prism Graph Pad 5.01 statistical software.

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