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A_3 receptor agonist modulates IL-1β hippocampus levels in a rat model of neuropathic pain

Stefania Giotti Cioato[1,2,3], Liciane Fernandes Medeiros[1,2,3,4], Bettega Costa Lopes[1,2,3], Helouise Richardt Medeiros[1,2,5], Wolnei Caumo[5], Rafael Roesler[3,5,6], Iraci LS Torres[1,3,5]

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1 Laboratório de Farmacologia da Dor e Neuromodulação: Investigações Pré-clínicas, Centro de Pesquisa Experimental (CPE), Hospital de Clínicas de Porto Alegre (HCPA), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

2 Unidade de Experimentação Animal, Grupo de Pesquisa e Pós-Graduação, HCPA, Porto Alegre, RS, Brazil.

3 Programa de Pós-Graduação em Ciências Biológicas: Farmacologia e Terapêutica, Instituto de Ciências Básicas da Saúde (ICBS), UFRGS, Porto Alegre, RS, Brazil.

4 Programa de Pós-Graduação em Saúde e Desenvolvimento Humano, Universidade La Salle, Canoas, RS, Brazil.

5 Programa de Pós-Graduação em Medicina: Ciências Médicas, UFRGS, Porto Alegre, RS, Brazil.

6 Cancer and Neurobiology Laboratory, Experimental Research Center, HCPA, Porto Alegre, RS, Brazil

Autor correspondente:

Iraci L. S. Torres
iltorres@hcpa.edu.br
Laboratório de Farmacologia da Dor e Neuromodulação: Investigações Pré-clínicas
Centro de Pesquisa Experimental - CPE, Hospital de Clínicas de Porto Alegre (HCPA)
Rua Ramiro Barcelos, 2350.
90035-007 - Porto Alegre, RS, Brazil.
Phone: 00 55 51 3359 8937

ABSTRACT

Introduction: Considering the lack of specific treatments for neuropathic pain, this study aimed to evaluate the effect of a single dose of adenosine A_3 receptor IB-MECA on inflammatory and neurotrophic parameters in rats subjected to a neuropathic pain model.

Methods: 64 adult male Wistar rats were used. Neuropathic pain was induced by chronic constriction injury (CCI) of the sciatic nerve and the treatment consisted of a 0.5 µmol/kg dose of IB-MECA, a selective A_3 adenosine receptor agonist, dissolved in 3% DMSO; vehicle groups received DMSO 3% in saline solution, and morphine groups received 5 mg/kg. Cerebral cortex and hippocampus IL-1β, BDNF, and NGF levels were determined by Enzyme-Linked Immunosorbent assay.

Results: The main outcome was that a single dose of IB-MECA was able to modulate the IL-1β hippocampal levels in neuropathic pain induced by CCI and the DMSO increased IL-1β and NGF hippocampal levels in sham-operated rats. However, we did not observe this effect when the DMSO was used as vehicle for IB-MECA, indicating that IB-MECA was able to prevent the effect of DMSO.

Conclusions: Considering that the IL-1β role in neuropathic pain and the contributions of the hippocampus are well explored, our result corroborates the relationship between the A_3 receptor and the process of chronic pain maintenance.

Keywords: *Adenosine A_3 receptor; Cytokine; DMSO; IB-MECA; Neuropathic pain; Neurotrophin; Rats.*

INTRODUCTION

Neuropathic pain is characterized by pain in the absence of harmful stimuli resulting from an injury or dysfunction of the somatosensory nervous system[1]. This chronic pain leads to several clinical problems since it is generally refractory to current treatments given that many changes in pain pathways are responsible for maintaining symptoms in neuropathic pain[2,3]. In this context, the role of supraspinal circuits in this condition has been investigated , especially the relationship between hippocampal and cortical processes and the dysfunction in neuro-glial communication[4-6].

Pronociceptive factors—such as cytokines and neurotrophic factors—were reported as mediators of the perpetuation of neuropathic pain[3,7]. Interleukin-1 beta (IL-1β), a pro-inflammatory cytokine released from glial cells, has been implicated in the induction and maintenance of neuropathic pain[4,8,9]. Meanwhile, the neurotrophins Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) have been studied as major mediators of neuropathic pain, with an ambiguous role in the cause and maintenance of this condition[10,11].

Considering the lack of specific and precise treatments for neuropathic pain, different targets have been studied in the last years[12]. The adenosine molecule mediates a huge number of the pathophysiological states via its interaction with four subtypes of G-protein-coupled receptors: A_1, A_{2A}, $A_{2B,}$ and A_3[13-16].

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Regarding A_3 adenosine receptor, studies showed its expression in several structures of the central nervous system (CNS), such as glial cells, peripheral sensory neurons, and hippocampal neurons[17-19]. Some experimental models have reported the antinociceptive role of this receptor in neuropathic pain[20-23]. However, the mechanisms and pathways involved in the antinociceptive effects of A_3 receptor activation are still poorly described. Thus, in this study, we evaluated the effect of a single dose of N6-(3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA), a selective A_3 adenosine receptor agonist, in the inflammatory and neurotrophic parameters of rats subjected to a neuropathic pain model.

METHODS

Animals

A total of 64 adult male Wistar rats (55–65 days old; weight 200–250 g) were used. The number of animals was calculated as eight rats per group to determine a difference between the variables of 1.5 standard deviation and $\alpha = 0.05$[24-26]. The animals were housed in groups of three per polypropylene cage (49 cm x 34 cm x 16 cm) with a sawdust-covered floor in a controlled environment (22 ± 2°C) under a standard 12h light-dark cycle, with water and chow (Nuvital, Porto Alegre, Brazil) *ad libitum*. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (of GPPG-HCPA protocol No. 2018-0377) and performed according to the Guide for the Care and Use of Laboratory Animals, 8th ed. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines[27].

Experimental design

The rats were acclimated to the maintenance room for two weeks before the experiment. Rats were randomized into eight experimental groups: sham-pain; sham-pain + vehicle; sham-pain + morphine; sham-pain + IB-MECA treatment; pain; pain + vehicle; pain + morphine; and pain + IB-MECA treatment. Randomization was performed using weight and paw withdrawal latency measured with the Hot Plate test to ensure that all rats had similar nociceptive behavior. The rats were euthanized around six hours after treatment; cerebral cortices and hippocampi were collected for further analyses of IL-1β, NGF, and BDNF. For all procedures, investigators were blinded to avoid and to prevent bias.

Neuropathic pain model

For neuropathic pain, chronic constriction injury (CCI) of the sciatic nerve previously described by Bennett & Xie and adapted by Cioato et al. was adopted[28,29]. Rats were anesthetized with isoflurane (5% for induction, 2.5% for maintenance); the left thigh was trichotomized and skin antisepsis was performed with iodine alcohol. After skin incision, the common sciatic nerve was exposed, three ligatures separated by a 1-mm interval were tied using Vicryl 4.0 and the skin was sutured using Mononylon 4.0 thread. The same investigator performed the ligatures in all rats to ensure an equal level of constriction, which reduced but did not interrupt epineural circulation. The sham groups rats were anesthetized and the sciatic nerve was similarly exposed to the CCI model, but not ligated. After surgery and anesthetic recovery, the animals were placed into their home cages, where they remained until the day of death. All rats subjected to CCI received tramadol 5 mg/kg intraperitoneally for immediate analgesia, and at every 12 hours for 2 days[30].

Pharmacological treatment

The treatment protocol consisted of a 0.5 µmol/kg intraperitoneal (ip.) dose of a selective A_3 adenosine receptor agonist N6-(3-iodobenzyl) adenosine-5'-methyluronamide (IB-MECA) dissolved in 3% Dimethyl sulfoxide (DMSO) and administered by ip. injection 15 days after the neuropathic pain induction by CCI model[20,31]. The animals in vehicle groups received one dose of DMSO 3% in saline solution intraperitoneally. The gold standard of analgesia used in the positive control group was one dose of morphine 5 mg/kg ip[32,33].

Tissue collection

Rats were euthanized by decapitation six hours after the single dose of IB-MECA. The cerebral cortex and hippocampus were collected and frozen at −80°C until the assays were performed.

Neurochemical assays

The levels of IL-1β, NGF, and BDNF in the cerebral cortex and hippocampus were determined by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies for each cytokine and neurotrophin (R&D Systems, Minneapolis, United States) according to the manufacturer's protocol. Optical density was measured by an ELISA reader at 450 nm wavelength. Total protein was measured by the Bradford method using bovine serum albumin as the standard[34]. Data were expressed in picograms per milligram (pg/mg) of protein.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Significance was established at $p \leq 0.05$. A two-way analysis of variance (ANOVA) was performed, followed by a Bonferroni post-hoc test to compare all groups considering pain and treatment

as independent factors. The Statistical Package for the Social Sciences (SPSS) 26.0 for Windows was used for statistical analysis.

RESULTS

Regarding IL-1β cerebral cortex levels, the pain model induced an increase in this cytokine level in comparison to sham groups (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 10.550$, $p < 0.05$). However, we observed no effects of treatment or interaction between the pain model and treatment in this structure (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 0.146$ and $F_{(3,62)} = 1.852$ respectively, $p > 0.05$) (Figure 1, panel A).

Meanwhile, regarding the IL-1β hippocampal levels, we observed an interaction between pain model and treatment (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 5.595$, $p < 0.05$). We found an increase in the IL-1β hippocampal levels induced by DMSO in the sham group when compared to the pain group, and the IB-MECA treatment reverted this effect. Additionally, the IB-MECA treatment increases the IL-1β levels in the pain group when compared to the sham group. We did not observe the effect of pain or treatment when analyzed separately in this structure (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 0.070$ and $F_{(3,62)} = 2.694$ respectively, $p > 0.05$).

Regarding NGF levels, we found no main effect of pain model or treatment in the cerebral cortex (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 1.333$ and $F_{(3,62)} = 0.503$, respectively, $p > 0.05$). Furthermore, we observed no interaction between the pain model and treatment in this structure (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 0.276$, $p > 0.05$) (Figure 2, panel A).

On the other hand, there were changes in the hippocampal NGF levels with the treatment. We observed an increase in NGF levels in the structure induced by the vehicle (DMSO) administration when compared to the animals that did not receive any treatment (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 4.291$, $p < 0.05$). Furthermore, the IB-MECA was able to revert this increased level in the sham group. We observe no effects of the pain model, and no interaction was found between the pain model and treatment in this structure (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 1.728$ and $F_{(3,62)} = 2.532$, respectively, $p > 0.05$) (Figure 2, panel B).

Regarding BDNF levels, we found no effects of pain model and treatment in both analyzed structures: cerebral cortex (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 0.308$ and $F_{(3,62)} = 1.498$ respectively, $p > 0.05$) and hippocampus (two-way ANOVA/ Bonferroni, $F_{(1,62)} = 1.508$ and $F_{(3,62)} = 1.592$ respectively, $p > 0.05$). Furthermore, there was no interaction between the pain model and treatment neither in the cerebral cortex (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 363$, $p > 0.05$) nor in the hippocampus (two-way ANOVA/ Bonferroni, $F_{(3,62)} = 256$, $p > 0.05$) (Figure 3, panel A and B).

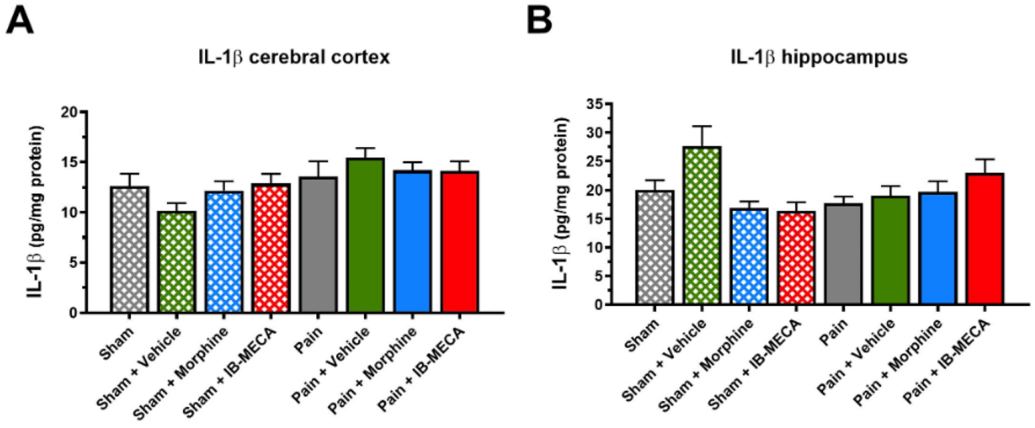

Figure 1: Effect of a single administration of IB-MECA on IL-1β levels in the cerebral cortex and hippocampus of rats subjected to neuropathic pain model. Panel A: IL-1β levels in the cerebral cortex (pain effect, two-way ANOVA/Bonferroni, $p < 0.05$). Panel B: IL-1β levels in the hippocampus (pain and treatment interaction, two-way ANOVA/Bonferroni, $p < 0.05$) Data are expressed as mean ± standard error of the mean (SEM) of pg of IL-1β/mg protein; n = 7–8 animals per group.

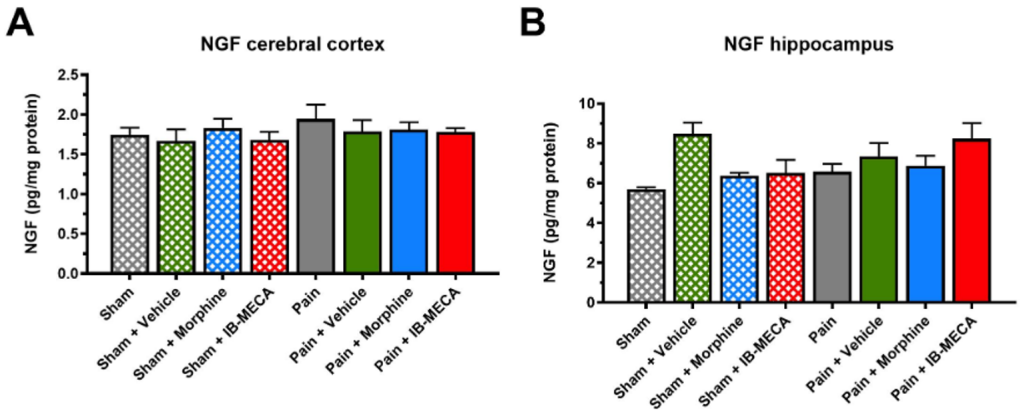

Figure 2: Effect of a single administration of IB-MECA on NGF levels in cerebral cortex and hippocampus of rats subjected to neuropathic pain model. Panel A: NGF levels in the cerebral cortex (no effects, two-way ANOVA/SNK, $p > 0.05$). Panel B: NGF levels in the hippocampus (treatment effect, two-way ANOVA/Bonferroni, $p < 0.05$).
Data are expressed as mean ± standard error of the mean (SEM) of NGF levels (pg/mg protein); n = 7–8 animals per group.

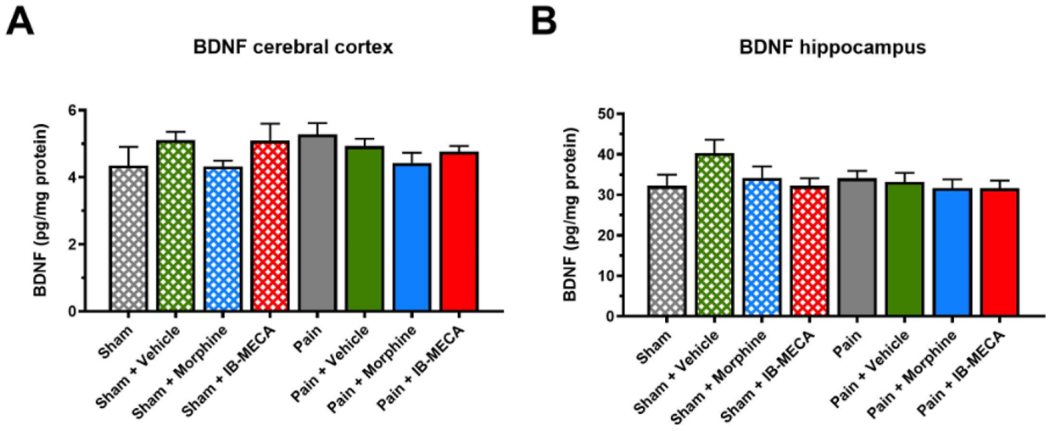

Figure 3: Effect of a single administration of IB-MECA on BDNF levels in cerebral cortex and hippocampus subjected to neuropathic pain model. Panel A: BDNF levels in the cerebral cortex (no effects, two-way ANOVA/Bonferroni, $p > 0.05$). Panel B: BDNF levels in the hippocampus (no effects, two-way ANOVA/Bonferroni, $p > 0.05$).
Data are expressed as the mean ± standard error of the mean (S.E.M.) of BDNF levels (pg/mg of protein); n = 7–8 animals per group.

DISCUSSION

Our key finding was that a single dose of A3 adenosine receptor agonist—IB-MECA—was able to modulate the IL-1β hippocampus levels in neuropathic pain induced by chronic constriction injury (CCI) in rats. Furthermore, the DMSO alone increased IL-1β and NGF hippocampus levels in sham animals; however, this effect was not observed when DMSO was associated as vehicle to IB-MECA, indicating that IB-MECA prevented DMSO effect. Note that, this study used only a single dose of IB-MECA, which may be insufficient to provide substantial changes in the IL-1β and NGF cerebral cortex levels, and NGF hippocampus levels. Regarding neuropathic pain model, the CCI model increased the IL-1β cerebral cortex levels. Several studies have been approaching the mechanisms of CCI neuropathic pain concerning pro-inflammatory cytokines, like IL-1β, IL-6, and TNF-α[3,24,25]. In our study, we found that the IL-1β cerebral cortex levels were increased by the pain model in comparison with the sham groups. Two studies using the same CCI model employed in our study showed increased IL-1β cerebral cortex levels in animals subjected to neuropathic pain at distinct time points of evaluation[26,28]. On the other hand, a study conducted by Apkarian et al. did not show endogenous modifications in supraspinal sites (brainstem and prefrontal cortex) induced by the CCI model over the IL-1β levels evaluated 10 and 24 days after the injury. However, the same study verified changes in IL-1β levels induced by the spared nerve injury model, suggesting the involvement of

this cytokine in the maintenance of neuropathic pain across supraspinal brain regions. Therefore, the causal and temporal relationship between the brain and the IL-1β levels seems to be dynamic and dependent on the primary nerve injury[9].

Based on the wide range of mechanisms and biomarkers linked to neuropathic pain maintenance, the A_3 receptor emerges as a proper target for antinociceptive outcomes, since it is present in supraspinal structures modulating chemokine release activity in astrocytes and microglial cells, as well as decreasing the neuronal excitability, thus reducing neuronal inflammation[22,35,36]. Therefore, our study demonstrates that a single dose of IB-MECA can modulate hippocampal IL-1β level s in the neuropathic pain state, which could induce different CNS responses to the neuropathic pain maintenance process. To our knowledge, few studies characterize the intrinsic mechanism of IB-MECA as anti-inflammatory. A chloride analogue of IB-MECA (Cl-IB-MECA) showed inhibition of pro-inflammatory cytokine expression by modulating Phosphoinositide 3-kinases/ Protein kinase B (PI3K/Akt) and Nuclear Factor – Kappa B (NF-κB) signaling pathways in macrophage cells[37]. Despite contradictory effects, the role of IL-1β at the peripheral site is well understood; however, studies have highlighted the limitation of the inflammatory paradigm and suggested non-immunological functions of IL-1 in the CNS, for example in Alzheimer's disease and chronic unpredictable depressive-like behavior induced by stress[38].

Interestingly, the hippocampus composes the limbic system, providing spatial and contextual memory, and is activated in the presence of persistent nociceptive input, such as neuropathic pain. The limbic circuitry can modulate the cortex by producing functional and anatomical changes, and IL-1β partially regulates synaptic plasticity in the hippocampus[39-42]. Thus, del Rey et al. demonstrated that neuropathic pain could interfere with cytokine interactions in the hippocampus and showed the correlation between hippocampal IL-1β expression and nociceptive behavior[4]. Therefore, this IL-1β modulation induced by IB-MECA in the CCI model may correlate with disruptions of hippocampal function; however, this conclusion is limited, requiring further studies to clarify the hypothesis.

Previously, our research group reported that an IB-MECA single dose promotes antinociceptive effects in chronic inflammatory pain and neuropathic pain and these results may not be related to the cytokine expression in the spinal cord and brainstem. On the other hand, the same study showed an increase in brainstem BDNF levels in the CCI model, which was reversed by IB-MECA[23]. The BDNF role in the functional and structural synaptic plasticity and, consequently, the microglia, may be implicated in synaptic remodeling induced by neuropathic pain[43]. Also, Terayama et al. showed that the A_3 receptor activation could attenuate the microglial activation, decreasing the BDNF release[44]. Regardless of the presumed involvement of hippocampal and cortical BDNF between this pain model and the A_3 receptor agonism, our study failed to show the association between the process by this neurotrophin and the hippocampal and cortical NGF levels, at least in the two specific structures assessed. Thus, considering the complexity linked to neuropathic pain and its potential relationship with adenosine A_3 receptors, it is essential to investigate the wide-ranging structures and biochemical markers involved in this course.

Finally, our data showed that an IB-MECA single dose prevented DMSO-induced increases in hippocampal NGF and IL-1-β levels. Several studies have used DMSO as an IB-MECA or Cl-IB-MECA vehicle for *in vitro* and *in vivo* administration without effects of this substance[20,45-47]. Regarding our data, it is noteworthy that the DMSO-induced changes were state-dependent since this was observed only in the sham group, which may be related to the potential effects of DMSO on biochemical pathways involved in inflammation, even in naive animals, despite that its effects depend on the administration route, such as orally, at the injury site, or intraperitoneally[48]. Considering that these changes were observed only in the sham groups, we can suggest that the concentration used in this study may not promote an expressive effect on the inflammation process induced by the CCI model, ruling out the vehicle-induced bias in the pain groups.

The effects of IB-MECA may be linked to A_3 receptor expression, but its influence on inflammation is unclear. The presence of A_3 receptors in the rat's hippocampal neurons, astrocytes, and microglia has been previously reported[19,49]. In this context, the effects of A_3 receptor agonism by modulation of supraspinal neuroinflammatory processes is a strong hypothesis to explain the reversion of DMSO-induced effects in sham groups[44,50]. Notably, although DMSO is a frequently used organic solvent, the immunomodulatory effects induced by this substance have not been thoroughly studied[27].

In brief, our study showed the modulation of IL-1β hippocampal levels induced by a single dose of IB-MECA in chronic neuropathic pain. Considering that the literature broadly explores the role of IL-1β in neuropathic pain and the contributions of the hippocampus, our result corroborates the relationship between the A_3 receptor and the chronic pain maintenance process, mainly in the CCI of the sciatic nerve. However, we failed to demonstrate the effects of A_3 receptor agonism in this neuropathic pain model regarding neurotrophins expression. It is noteworthy that this study used only one dose of IB-MECA in neuropathic pain, which may be

insufficient to provide substantial changes in the levels of cytokine and neurotrophin, especially in CNS structures. Nevertheless, we considered this to be the first step before repeated administration. Also, we ruled out hormonal interference, since we only assessed the outcomes in male rats, which can be considered a limitation of this study. Therefore, our results reinforce that the development of treatments for neuropathic pain requires a better understanding of the cascade of phenomena involved in nerve injury.

Conflict of Interest

All authors declare no financial or commercial interests in the study outcome.

Availability of Data and Material

The authors will provide the raw data supporting this manuscript conclusions to any qualified researcher, without undue reservation.

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