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Antiallodynic effect of intrathecal resiniferatoxin on neuropathic pain model of chronic constriction injury

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Introduction: Injuries and/or dysfunctions in the somatosensory system can lead to neuropathic pain. Transient receptor potential vanilloid sub-type 1 (TRPV1) play an important role in the development of allodynia and hyperalgesia following injury and the ensuing inflammatory conditions. Resiniferatoxin (RTX) is an ultrapotent synthetic TRPV1 agonist and many different administration routes are available for different mechanisms and different effects. RTX is used intraperitonially as a model of neuropathic pain or epidurally and topically to produce prolonged analgesic effects. However, the use of RTX is controversial because its neurotoxicity and margin of safety have not been addressed adequately. The present study evaluates the effect of intrathecal RTX on the induction and allodynia behavior of animals submitted to neuropathic pain by chronic constriction injury (CCI). Methods: 160 Swiss mice were randomly distributed into two groups: intrathecal pre-treatment group (PRE) aiming the effect in induction of allodynia and late intrathecal treatment group (POST) to evaluate the antiallodynic effect of the RTX on mechanical nociceptive threshold evaluated by the Von Frey hair filaments. Additionally, we evaluated the expression of TRPV1 in dorsal root ganglia (DRG) by western blotting after PRE- and POST-treatment with RTX. Results: Our results showed that the CCI mice developed prolonged mechanical allodynia-like behavior in ipsilateral paw after surgery up to 24 hours. The PRE- and POST-treatment groups presented significant antiallodynic effects in ipsilateral paw for 24 hours. Only the POST-treatment group showed a significant reduction of expression of the TRPV1 receptor after CCI. Conclusion: The presented data demonstrated that both PRE- and POST-treatment with RTX given intrathecally produced potent antiallodynic activities in CCI mice and that POST-treatment can reduce TRPV1 expression in DRG, suggesting that POST-treatment RTX can revert central sensitization and its associated allodynia.

Key words: neuropathic pain, chronic constriction injury, antiallodynic effect, resiniferatoxin

INTRODUCTION

The TRPV1 receptor is an important nonselective cation channel belonging to the TRP family and expressed at the central and peripheral terminals of the sensory neurons. It is considered to be a key receptor involved in the transmission and modulation of pain signals and is an important transducer of stimuli (Vidal 2004, Latorre et al. 2007). Thus, pharmacological modulation of the TRPV1 receptor has been identified as a promising therapy for pain control (Jara-Oseguera et al. 2008, Wong and Gavva 2009) and the therapeutic potential of many TRPV1 agonists and antagonists are being investigated in preclinical and clinical trials (see Szallasi and Sheta 2012).

Resiniferatoxin (C37H40O9), a diterpene with a molecular mass of 628.71 g/mol, a TRPV1 agonist, isolated from *Euphorbia resinifera*, has an effect similar to capsaicin, such as pungency, inflammation and changes in body temperature (Hergenhahn 1975, Szallasi and Blumberg 1990) however, its desensitizing effect is greater than capsaicin (Szallasi and Blumberg 1990), as well as being the most potent among all the endogenous and synthetic TRPV1 receptor agonists (Brown et al. 2015). It is important to highlight the high selectivity for the sensory nerve terminals expressing TRPV1 receptors, without affecting proprioception and motor function (Neubert et al. 2003, Karai et al. 2004). The intrathecal administration is noteworthy, which has advantages in selective targeting and permanent exclusion of TRPV1 receptors (Karai et al. 2004, Jeffry et al. 2009, Brown et al. 2015), representing a viable and versatile approach to pain control, because it does not present important side effects or tolerance, as the opioid receptors (Iadarola and Mannes 2011).

In the present study, we investigated the therapeutic effect of intrathecal RTX on the development of mechanical allodynia induced by the chronic pain model

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by the constriction of the sciatic nerve. In addition, we examined the level of TRPV1 expression in dorsal root ganglia (DRG).

METHODS

The experiments were conducted using 160 male Swiss mice (25g) from the main animal house of the Universidade Federal de Alfenas (protocol 571/2014, CEUA-UNIFAL-MG). Animals were housed in a cage under controlled temperature (24 ± 2C) and on a 12-hour light- dark cycle (dark cycle beginning at 7 pm) and had free access to food and water. The guidelines of the Committee for Research and Ethical Issues of IASP were followed throughout the experiments. Each mouse was used only once.

The neuropathic animals were divided into two groups, subdivided into four groups: previous treatment group (PRE-group; pretreated with vehicle or RTX and sham operated; pretreated with vehicle or RTX and neuropathic) and late treatment group (POST-group; sham operated and treated with vehicle or RTX, neuropathic and treated with vehicle or RTX).

Model of neuropathic pain

We used the experimental model of chronic constriction injury of the sciatic nerve (CCI) (Malmberg and Basbaum 1998). After inhalation anesthesia (2% isoflurane), the lateral surface of the right hind thigh was shaved, and we performed antisepsis of the skin of animals followed by the incision of the femoral muscle, exposing the three branches of the sciatic nerve. This model consists of moorings 1/3 to 1/2 of the dorsal portion of the sciatic nerve with 5.0 suture. The muscle and skin were sutured after the procedure. Animals were considered to be in neuropathic pain when they exhibited mechanical allodynia i.e., paw flinching behavior response to the application of a bending force of less than 2 g after 72 hours of CCI.

The control experimental group consisted of sham operated animals (SHAM), the incision was made and sciatic nerve was exposed similarly to the CCI group, but without tying this nerve.

Drugs and intrathecal administration

RTX (Sigma - Aldrich, St. Louis, MO) 1 mg was dissolved in 0.1 ml of 95% ethanol and 0.9 ml of saline to a concentration of 1 µg/µL and stored at -20°C (Lee et al. 2012). The concentration of 2 µg in 5 µL (2 µl solution added 3 µl of saline) was intrathecally administered. The control group received the same volume of saline. The route of administration used was intrathecal (Papir-Kricheli et al. 1997). The animals were anesthetized with inhaled isoflurane 2% and shaved in the lower back. With the spinal column arched, a 25-gauge hypodermic needle was inserted into the subarachnoid space at the level of the lower sacral spinal, at midline, with an angle of 45 o between L4 and L5 vertebrae.

The PRE-group received RTX or vehicle 1 hour before CCI or SHAM. The POST-group received RTX or vehicle after 72 hours of CCI or SHAM (Fig. 1).

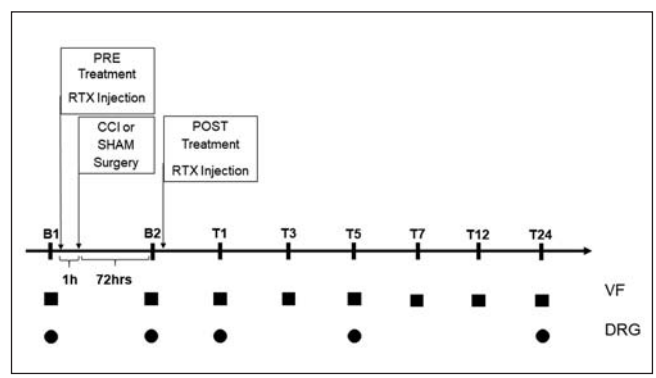

Fig. 1. Intrathecal Resiniferatoxin experimental protocols. Sham (SHAM); chronic constriction injury (CCI); PRE- and POST-treatment with intrathecal RTX or VEHICLE; Times for Von Frey evaluation (VF); Times of dorsal root ganglia extraction (DRG); Baseline 1 (B1); Baseline 2 (B2); 1 hour (T1); 3 hours (T3); 5 hours (T5); 7 hours (T7); 12 hours (T12) and 24 hours (T24).

Behavioral tests

Mechanical stimulation test was performed by the same researcher. The mechanical allodynia thresholds of the animals were evaluated according to the up-and-down method (Chaplan et al. 1994) using the Von Frey filaments test (North Coast Medical, Inc. Morgan Hill, CA). Each mouse was placed under a transparent plastic dome on a metal mesh floor for 15min. The Von Frey filaments was applied to the plantar surface of the right hind paw for 4 seconds or until the animals showed nociceptive behavior, characterized by the paw withdrawal, licking of the same and/or "flinch". We started with the filament of 0.4 g and a maximum of 6 assessments per animal were made, with 10 second intervals.

Western Blotting

The animals were anaesthetized (Isoflurane 2%, inhalation), in times B1, B2, T1, T5 and T24, in order to

evaluate the expression of the TRPV1 receptors. They were then trichotomized in the dorsal-lumbar region, and a posterior-medial incision was performed, followed by the opening of the plans until the bone blade and a laminectomy was performed in order to view the DRG. DRG of the L5-L4-L6 segments were dissected and posteriorly homogenized and lysed with buffer containing 50 mmol/l of Tris (pH 7.4), 250 mmol/l of sodium chloride (NaCl), 10 mmol/l of sodium ethylenediaminetetraacetate (EDTA) (pH 8.0), 0.5% Nonideto P-40 (NP40) (Sigma, USA), phenylmethylsulfonyl fluoride (PMSF) 1 mmol/l, 10 µg/l of leupeptin, 4 mmol/l of sodium fluoride (NaF). Supernatants were centrifuged at 1000 g for 10 min at 4°C. The protein content was determined with a protein quantification kit by the Bradford method (Pierce, USA). Protein samples (20 µg/well) were separated through electrophoresis in acrylamide gel SDS-PAGE 10% and transferred to nitrocellulose membranes. The membranes were blocked by milk 5% for 60 min at room temperature and incubated with anti-TRPV1 primary antibody (1: 500, Calbiochem, Oncogene, USA, 35 kDa) for 48 hours at 4°C with blocking buffer [PBS 5% (p/v) of skim milk and 0.1% Tween 20]. After washing, the membranes were incubated with anti-rabbit conjugated secondary antibody HRP (1: 2000, Jackson, USA) and washed again. Membranes were then developed with the chemiluminescence kit (ECL, Amershan Pharmacia Biotech, Little Chalfont, U.K.) as per the manufacturer's instructions. The optical density ratio TRPV1/β-actin was used to calculate the expression of the TRPV1 receptors.

Statistical analysis

Behavioral results were expressed as mean ± standard error of the mean (SEM) of 5-7 animals per group. The analysis was done by analysis of variance (ANOVA) and comparisons were performed by *post-hoc* test Bonferroni using the software Statistical Package for Social Sciences (SPSS) (IBM, Chicago, USA) version 15.0; the level of significance was set at $p<0.05$.

RESULTS

Neuropathic pain model

The results of the evaluation by the Von Frey test in the PRE-treatment are expressed in Fig. 2. B1 represents the pre-operative period (Baseline 1, before CCI surgery), RTX was administered shortly after this first evaluation. CCI or SHAM surgery were performed and B2 (Baseline 2, 72 hours after CCI) was evaluated. The mechanical allodynia was them evaluated at T1 (1 hour), T3 (3 hours), T5 (5 hours), T7 (7 hours), T12 (12 hours) and T24 (24 hours) after B2.

In the B2 period, it was possible to observe the decreased threshold for the CCI/VEHICLE group, whereas for the SHAM/RTX and SHAM/VEHICLE groups the thresholds were not altered. For the CCI/RTX PRE-treatment group, a significant increase in nociceptive threshold was observed shortly after administration of RTX, with this increase remaining throughout all evaluations, demonstrating the antiallodynic effect of RTX for PRE-treatment in CCI-neuropathic pain.

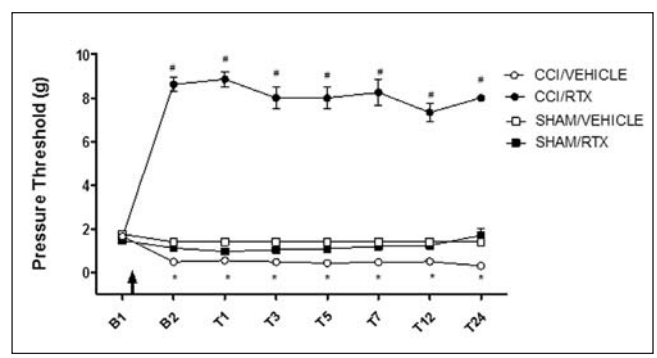

Fig. 2. Effects of the previous treatment (PRE-group) with vehicle or RTX (2 µg, i.t), agonist for the TRVP1 receptor, on the nociceptive threshold evaluated by the Von Frey filaments in animals submitted to sham (SHAM) or chronic constriction injury (CCI) surgery. Data represent the mean ± SEM of the average nociceptive threshold (g) performed before CCI or SHAM surgery (B1) and after 72 hours (B2), 1 hour (T1), 3 hours (T3), 5 hours (T5), 7 hours (T7), 12 hours (T12) and 24 hours (T24) after B2. The curves were significantly different with regard to treatment ($F_{3,286}$=220.41, $p<0.05$) and time ($F_{7,28}$=18.38, $p<0.05$) and show a significant treatment X time interaction ($F_{21,286}$=36.32, $p<0.05$). $P<0.05$ compared to SHAM/VEHICLE (*) or any other group (#) using the Bonferroni *post hoc* test.

Fig. 3 demonstrates the nociceptive thresholds evaluated in the POST-treatment group. For this group, B1 represents the pre-operative period (Baseline 1, before CCI surgery), B2 (Baseline 2, 72 hours after CCI) and RTX was administered shortly after this second evaluation. After RTX injection the mechanical allodynia was evaluated at T1 (1 hour), T3 (3 hours), T5 (5 hours), T7 (7 hours), T12 (12 hours) and T24 (24 hours). In the CCI/RTX group, neuropathic pain was reversed after 1 hour (T1) of the receptor agonist TRVP1, RTX persisting up to 24 hours (T24). In the CCI/VEHICLE group, the nociceptive threshold decreased from B2 when compared to B1, indicating mechanical allodynia. In the SHAM/VEHICLE and SHAM/RTX groups the nociception thresholds were not altered.

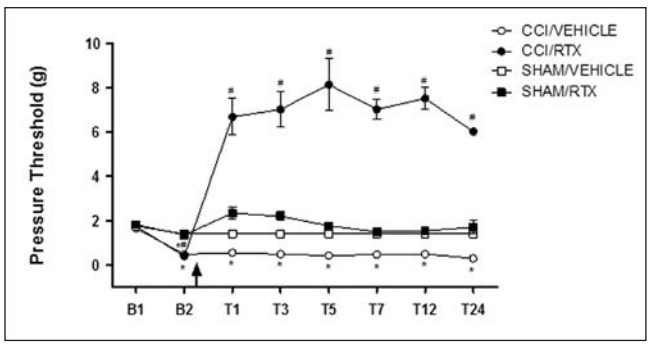

Fig. 3. Effects of the late treatment (POST-group) with the vehicle and RTX (2 μg, i.t.), an agonist of the TRVP1 receptor, on the nociceptive threshold by the Von Frey filaments test in animal submitted to sham (SHAM) or chronic constriction injury (CCI) surgery. Data represent the mean ± SEM of the average nociceptive threshold (g) performed before CCI or SHAM surgery (B1) and after 72 hours (B2), 1 hour (T1), 3 hours (T3), 5 hours (T5), 7 hours (T7), 12 hours (T12) and 24 hours (T24) after the administration of the RTX or VEHICLE. The curves were significantly different with regard to treatment ($F_{3,286}$=4.21, p<0.01) and time ($F_{7,28}$=8.83, p<0.05) and show a significant treatment X time interaction ($F_{21,286}$=22.28, p<0.01). P<0.05 compared to SHAM/VEHICLE (*) or any other group (#) using the Bonferroni *post hoc* test.

TRPV1 Expression

Fig. 4 represents Western Blotting analysis of TRPV1 receptor expression compared to B-actin found in DRG of animals submitted to the experimental model of neuropathic pain, CCI and intrathecal administration of RTX at times B1 (before CCI), B2 after 72 hours (CCI or SHAM), T1 (1hr), T5 (5hr) and T24 (24hr). TRPV1 receptor expression decreased significantly at T1, T5 and T24 times for the POST-treatment RTX group when compared to the vehicle group. PRE-treatment not changed the TRPV1 expression.

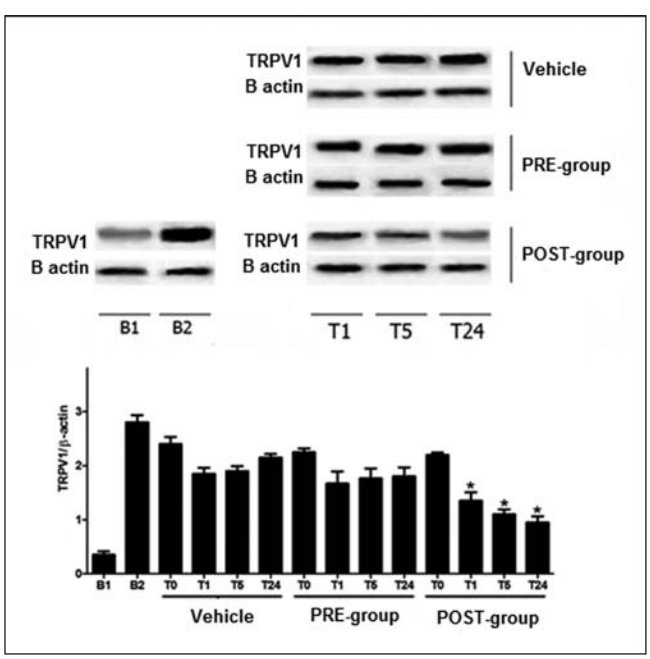

Fig. 4. Expression of TRPV1 receptors compared to B-actin found in the DRG of times B1, B2, T1, T5 and T24 after CCI in the VEHICLE or PRE- and POST-treatment with intrathecal RTX. At least six slices from three mice were examined, and the data represent the mean ± SEM. P<0,05 in t test regarding the relationship to the treatment group when compared to the CCI/VEHICLE group (*).

DISCUSSION

The results obtained in this work demonstrate that the intrathecal administration of RTX, as a previous and late treatment, after the model of neuropathic pain induced by CCI, significantly induces antiallodynia in this model. This idea is supported by experimental data demonstrating that chronic hyperalgesia induced by nerve damage releases several inflammatory mediators, which sensitize TRPV1 receptors in a constant manner (Kissin et al. 2007). This sensitization induces an increase in TRPV1 receptor expression in the primary afferent terminals, a fact that demonstrates that this receptor is involved in the process of induction and maintenance of neuropathic pain (Kanai et al. 2005). Thus, using a TRPV1 receptor antagonist, such as capsazepine, would be a possible treatment for inflammatory and neuropathic hyperalgesia (Caterina et al. 1997, Kawao et al 2002, Honore et al. 2005). However, blocking these receptors can lead to changes in body temperature (Caterina et al. 1997), therefore, the use of an agonist to treat neuropathic hyperalgesia induced by increased expression and number of receptors seems to be a proposal to increase pain sensation (Haanpää and Treede 2012). Therefore, the particularity of the potency of the action of RTX (Lee et al. 2012, Brown et al. 2015) leads to a constant depolarization and sustained inactivation of the receptor reducing hyperalgesia (Szallasi and Blumberg 1990). One of the main findings of this work was to demonstrate, for the first time in this model, that RTX causes a persistent nociceptive threshold increase.

Kanai et al. (2005) demonstrated in a study with rats with neuropathic hyperalgesia through the CCI model that the levels of TRPV1 expression in the spinal cord after CCI, quantified by Western blotting analysis were significantly increased on the ipsilateral side of the lumbar spine within 14 days after CCI surgery, but not on the contralateral side, results evidencing increased TRPV1 sensitization in the development and/or maintenance of mechanical allodynia in the CCl model. The results of this study show that mice submitted to CCI model presented hyperalgesia and mechanical allodynia evaluated by Von Frey filaments test, as well as the increased expression of TRPV1 receptors, by Western blotting analysis.

In contrast to the present study, Zhang et al. (2014) evaluated the antiallodynic effect of capsaicin administered intrathecally 3 days after the neuropathic pain model (CCI) in rats that no significant effect on the mechanical response threshold was observed, only thermal hyperalgesia, and can be justified by the fact that RTX is a more potent analogue than capsaicin (Szallasi and Blumberg, 1990). However, one study evaluated the systemic performance of capsaicin, and may act punctually when administered orally (Caterina and Julius 2001). Systemic effects are not observed with topical administration. Vidal (2004) concluded in a systematic review, on the efficacy of topical administration of capsaicin to 0.075% for the treatment of neuropathic pain, as a viable therapeutic alternative. Distinctly, RTX provides a long-lasting local action with no major systemic effects (Kissin et al. 2005).

Although there is a long way to go and many studies are still needed to evaluate the therapeutic potential of TRPV1 agonist drugs, a new approach to pain management arises from detailed research on capsaicin, RTX and other vanyloids in its physiological actions and its molecular binding to TRPV1 receptor (Iadarola and Mannes 2011). Therefore, studies that administered RTX intrathecally demonstrate the advantages in selective targeting and permanent exclusion of TRPV1 receptors, with a long-acting antiallodynic effect (Lee et al. 2012, Brown et al. 2015) These observations support the route of administration adopted by the present study in which the increased expression of TRPV1 after CCI was prevented by intrathecal RTX treatment.

Similarly, Brown et al. (2015) investigated the electrophysiological effects of intrathecal administration of RTX, demonstrating that the drug leads to the prolonged selective opening of the TRPV1 receptor ion channel potential, located primarily in the C-fibers of the primary afferent nociceptive neurons, promoting the desensitization of this receptor and consequently analgesia and anesthesia, which decreases the nociceptive threshold of the animals submitted to CCI. These researchers also reported a sequence of animal studies that explored the use of intrathecal RTX to control the spontaneous pain of bone cancer in dogs. Behavioral tests were performed to establish paw withdrawal latency, and subsequent general anesthesia was induced for the administration of RTX intrathecally, with hemodynamic parameters being monitored, the authors concluded that intrathecal RTX causes transient hemodynamic effects, and produces a prolonged antiallodynic response.

Lee et al. (2015) demonstrated the antiallodynic effect of RTX on the experimental model of neuropathic pain by spinal nerve ligation model in rats. The nociceptive paw withdrawal threshold was assessed through Von Frey filaments test and the animals were treated with epidural RTX at a concentration of 1 μg/μl. Among their findings, a significant increase in the nociceptive threshold was observed from the treatment, demonstrating RTX antiallodynic effect. These results support the present study, even though it was performed with another experimental model and another route of administration of RTX. Similarly Brown et al. (2015) demonstrated that the administration of RTX, guided by computed tomography, in DRG, is able to reduce the nociceptive transmission in pigs and after four weeks, the reduction of TRPV1 expression and reduction of nociception was verified. Also, no side effects were observed, such as impairment in motor function. Similarly, Lee et al. (2012) presented a study that evaluated the action of RTX in the control of neuropathic pain in rats, using the technique of Von Frey filaments test for the evaluation of mechanical allodynia, reporting that after 15 minutes of the RTX application (≥1 μg), an increase in the nociceptive threshold was observed, similar to our study, which used the same form of evaluation, applied concentrations close to RTX with similar effects.

It is important to emphasize that the use of the RTX concentration should be carefully administered because some studies have shown that high doses (≥10 μg) may not be beneficial (Jeffry at al. 2009, Lee et al. 2012), and it is also believed that the intrathecal form of administration is more advantageous, since this form presents a selective segmentation and permanent exclusion of TRPV1 receptors with antiallodynic effects of long duration (Karai et al. 2004, Brown et al. 2015).

The choice of previous RTX treatment was based on studies demonstrating this TRPV1 agonist in pain management (Kissin et al. 2005, Lee et al. 2012). Thus, Meller et al. (1992) demonstrated the preventive effect on thermal hyperalgesia, neonates were treated with capsaicin, and this prevented the development of thermal hyperalgesia produced by constrictive ligatures around the sciatic nerve 18 weeks later (Shir and Seltzer 1990), similarly to studies by Gaus et al. (2003), in which capsaicin administered intraperitoneally before CCI prevented the development of thermal hyperalgesia. In analogy, Kissin et al. (2005) verified the induction of short thermal and mechanical hypoalgesia after RTX application. In his work the application was performed via perineural as a treatment prior to incisional pain. Corroborating with the results found in the present study, in which the RTX obtained antiallodynic effect in the previous treatment evaluated in a short period of time, without altering the expression of TRPV1 receptors in GDR.

To date, there are no data in the literature demonstrating the administration of RTX in clinical trials. Iadarola and colleagues (2011) encouraged the search for clinical trials in humans, initiating research on the use of RTX to demonstrate its efficacy and safety.

Although the participation of the TRPV1 receptor in the nociceptive mechanisms has been widely demonstrated (Julius 2001, Neubert et al. 2003, Karai et al. 2004, Kissin et al. 2005, Jeffry et al. 2009, Iadarola and Mannes 2011, Lee et al. 2012, Brown et al. 2015) the present study significantly

amplifies the understanding of the function of this receptor in the neuropathic pain model by CCI, besides evidencing the increase of the nociceptive threshold through prior and late treatment of RTX intrathecally.

Through the presented findings, it is possible to show that RTX works effectively in the promotion of analgesia; however, we suggest new studies with a longer time of observation in an attempt to clarify the mechanisms, duration and effect of the therapy.

CONCLUSION

The presented data demonstrated that both PRE- and POST-treatment with RTX given intrathecally produced potent antiallodynic activities in CCI mice and that POST-treatment can reduce TRPV1 expression in DRG, suggesting that POST-treatment RTX can revert central sensitization and its associated allodynia.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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