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Article

Comparative study of cold hyperalgesia and mechanical allodynia in two animal models of neuropathic pain: different etiologies and distinct pathophysiological mechanisms.

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Neuropathic pain (NP) affects more than 8% of the global population. The proposed action of the transient receptor potential ankyrin 1 (TRPA1) as a mechanosensor and the characterization of the transient receptor potential melastatin 8 (TRPM8) as a cold thermosensor raises the question of whether these receptors are implicated in NP. Our study aimed to evaluate the involvement of TRPA1 and TRPM8 in cold and mechanical signal transduction to obtain a comparative view in rat models of streptozotocin-induced diabetes (STZ) and chronic constriction injury of the sciatic nerve (CCI). The electronic von Frey test showed that STZ rats presented mechanical allodynia that was first evidenced on the 14th day after diabetes confirmation, and four days after CCI. This phenomenon was reduced by the intraplantar (ipl) administration of a TRPA1 receptor antagonist (HC-030031; 40 µL/300 µg/paw) in both NP models. Only CCI rats displayed cold hyperalgesia based on the cold plate test. The pharmacological blocking of TRPA1 through the injection of the antagonist attenuated cold hyperalgesia in this NP model. STZ animals showed a reduction in the number of flinches induced by the intraplantar injection of mustard oil (MO; TRPA1 agonist; 0.1%/50 µL/paw), or intraplantar injection of menthol (MT; TRPM8 agonist; 0.5% and 1%/50 µL/paw). The response induced by the ipl administration of MT (1%/50 µL/paw) was significantly different between the CCI and SHAM groups. Together, these data suggest a different pattern in nociceptive behavior associated with different models of NP, suggesting a variant involvement of TRPA1 and TRPM8 in both conditions.

Keywords: Cold hyperalgesia, Mechanical Allodynia, Diabetes, Chronic constriction injury of the sciatic nerve. Transient receptor potential channels.

INTRODUCTION

Transient changes in the nervous system that occur after tissue injury are considered adaptive to evoke acute pain as a warning signal to avoid further lesions. As a natural process of external and internal noxious stimuli, pain helps to maintain individual surveillance because of its unpleasant characteristics (Basbaum *et al.*, 2009). However, when the nervous system is injured, these well-known temporary changes become chronic, resulting in neuropathic pain (NP) syndromes (Basbaum *et al.*, 2009). Under these conditions, the protective role of pain is lost due to disabling and health hampering disorders that affect about 8% of the adult population worldwide (Bouhassira, 2019).

NP may arise as a direct or indirect result of metabolic, traumatic, chemical, and other causes. It commonly manifests as allodynia and hyperalgesia evoked by thermal and/or mechanical stimuli (Bouhassira, 2019). It is important to highlight that even within people suffering from the

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same NP syndrome, a different subset of positive (i.e., cold allodynia) and negative symptoms (i.e., sensory loss) can occur (Clauw *et al.*, 2019). Despite differences in the origin and presentation, NP was previously credited to be caused in a homogenous way, since diagnosis was classically centered around understanding the origin and distribution of the neuropathic cause, and to a lesser extent, finding the mechanisms behind painful sensations. However, we now know that NP syndromes are heterogeneous and multidimensional (Bouhassira, 2019).

Given the need to understand pain as a consequence of neural plasticity mechanisms rather than etiological consequences (Bouhassira, 2019), a family of molecules with potential roles in the development and maintenance of chronic pain have been identified as promising analgesic targets (Moore *et al.*, 2018). Transient receptor potential (TRP) channels are a family of cationic channels with permeability to Ca^{2+} and Na^{+}, as well as for other ions that permit the influx of these ions when activated, leading to depolarization (Jardín *et al.*, 2017).

TRPs play a major role in pain transduction. The transient receptor potential melastatin 8 (TRPM8) and transient receptor potential ankyrin 1 (TRPA1) are thermoreceptors that detect cool and cold temperatures, respectively (Jardín *et al.*, 2017), with additional mechanical sensibility attributed to TRPA1 (Huang *et al.*, 2017). TRPM8 is considered a promising candidate for the treatment of cold hypersensitivity, as it is a major cold transducer (Moore *et al.*, 2018). However, there are conflicting results regarding its analgesic and pro-algesic properties upon activation (Burgos-Vega *et al.*, 2016; Liu *et al.*, 2013). Similarly, results from genetic ablation or pharmacological blockade of TRPA1 are contrasting (Koivisto *et al.*, 2012; Yarmolinsky *et al.*, 2016). TRPA1 and TRPM8 are expressed in different neuronal populations, while TRPA1 is highly expressed in transient receptor potential channel vanilloid 1 (TRPV1)-positive neurons in rodents, TRPM8 is expressed in a small population of nociceptors in the dorsal root ganglia (DRG) that do not express TRPV1 (Dai, 2016). Considering the thermal and mechanical sensorial disturbances in NP patients (Finnerup, Sindrup, Jensen, 2010), this study aimed to evaluate the role and the behavioral pattern of TRPA1 and TRPM8 on cold and mechanical signal transduction as a comparative view in different NP models.

MATERIAL AND METHODS

Animals

Adult male Wistar rats (n=432), provided by the vivarium of the Federal University of Paraná and weighing between 180 and 220 g were used. All the animals were housed in plastic cages (41 x 32 x 16.5 cm) with a maximum of four rats per cage, with food and water *ad libitum*. The room conditions were maintained in order to have a standard controlled environment with appropriate temperature (23 ± 2 ºC) and illumination (under 12h/12h light/dark cycle). All procedures were conducted in accordance with the National Institutes of Health for the Care and Use of Laboratory Animals (NIH Publication # 8023, revised in 1978) and approved by the local Ethics Committee (CEUA/BIO-UFPR: #685).

Drugs and solutions

Streptozotocin (STZ) from Santa Cruz Biotechnology Inc., Santa Cruz, California, USA and sodium citrate from Merck S.A. Indústrias Farmacêuticas (Brazil) were used. The anesthetics applied were ketamine from Vetecia Laboratórios de Produtos Veterinários (Brazil) and xylazine from Rhobifarma Indústrias Farmacêuticas (Brazil). Mustard oil (MO) and menthol (MT) from Santa Cruz Biotechnology Inc., were used as TRPA1 and TRPM8 agonists, respectively, and were diluted in corn oil. HC-030031 (Sigma Aldrich) and AMTB (Santa Cruz Biotechnology Inc.) were used as TRPA1 and TRPM8 antagonists, respectively, and were freshly diluted in a 1:17 of dimethyl sulfoxide and saline solution.

Induction of neuropathic pain models

Experimental diabetes was induced, after an overnight fast, by a single intraperitoneal (ip) injection of STZ, dissolved in citrate buffer (10 mM, pH 4,5), at the dose of 60 mg/kg. Confirmation of diabetes was performed three days after STZ injection (Day 0 of diabetes condition) and also at the end of the experiments (Day 28), through the application of a small volume of peripheral blood, obtained through a small prick in the

tail, on the test strips impregnated with glucose oxidase (Accu-Check Active™, Roche). Only animals with non-fasting blood glucose levels of 250 mg/dL or greater were included in the diabetic group and proceeded in the study (STZ groups). Only citrate buffer was injected in the normoglycemic control group (NGL groups).

The chronic constriction injury (CCI) model was performed according to Bennett and Xie study (1988). Briefly, each rat was anesthetized with ketamine (60 mg/kg, ip) and xylazine (7,5 mg/kg, ip), had the left thigh shaved with a razor and moistened with iodine solution. The left sciatic nerve was exposed and four ligatures of sterilized 4.0 silk threads were placed with 1 mm space between them (CCI groups). Sham operated animals (SHAM groups) had the nerve only exposed, and no ligatures were made. Anesthesia recovery was accompanied during 1-2 h after surgery.

Measurement of mechanical allodynia

Mechanical allodynia was assessed through electronic von Frey equipment. After an acclimation of 15 min in acrylic cages (12 × 20 × 17 cm) with a wire-grid floor (5 mm^2), the stimulus of increasing pressure was applied to the hind paw and ceased when the animal showed paw withdrawal. The mechanical threshold, defined as the pressure responsible for paw withdrawal, was calculated using the average of three measurements taken from each paw (Yamamoto *et al*., 2009). Mechanical threshold was evaluated before and 7, 14, 21 and 28 days after the first diabetes confirmation (day 0), as well as 4, 7, 10, and 14 days after the surgical procedure (CCI), as well as in different times after antagonist treatment (HC-030031).

Assessment of cold hyperalgesia

The cold plate (Ugo Basile, Comerio, Italy) test was used to determine cold hyperalgesia in CCI and diabetic rats. The cold plate was kept in a temperature of 4 ± 0,5ºC, the animals were placed in the center of the plate and the latency to the first response (shaking, licking and/or withdrawing the paw) was assessed, and an average of 2 measurements used as a score of cold sensitivity. A cutoff time of 30 s was used to avoid tissue damage (Kostich *et al*., 2016). Cold hyperalgesia was assessed before and 7, 14, 21 and 28 days after diabetes confirmation (day 0), as well as 4, 7, 10, and 14 days after the surgical procedure (CCI), and in different times after antagonists treatment (HC-030031 or AMTB). Diabetic animals, 7, 14, 21 and 28 days after diabetes confirmation, went through the acetone test (evaporation-evoked cooling), as an additional measurement of cold hypersensitivity. The animals received an instillation of 100 µL of acetone in one of the hind paws and the number of nociceptive behaviors such as licking and flinching of the paw was counted for 2 min (Werner *et al*., 2007).

Effect of selective TRPA1 and TRPM8 antagonists on mechanical allodynia and cold hyperalgesia

In this set of experiments, after mechanical threshold and cold plate (4 ± 0.5º C) latency assessment, CCI rats (14 days after surgery) and diabetic rats (28 days after diabetes confirmation) were treated with HC-030031, a selective TRPA1 antagonist. Treatment consisted of a single intraplantar (ipl) injection of HC-030031 (300 µg/40 µL/paw) or vehicle (VEH; DMSO and saline solution 40 µL/paw) (da Costa *et al*., 2010) in the ipsilateral paw for CCI rats and in the right paw for the diabetic rats. Mechanical threshold was reassessed 60, 120, 180 and 240 min after HC-030031 treatment. Cold plate latency was reassessed 60 and 120 min after HC-030031 treatment.

To evaluate the effect of a TRPM8 antagonist on cold hyperalgesia, an independent group of CCI rats (14 days after surgery) had their cold plate latency assessed and received an ipl injection of AMTB (300 µg/40 µL/paw), a selective TRPM8 antagonist, or vehicle (VEH; DMSO and saline solution 40 µL/paw)(Sałat, Filipek, 2015). Cold plate latency was reassessed 60 and 120 min after AMTB treatment.

Nociceptive response to intraplantar injection of TRPA1 and TRPM8 agonists

The TRPA1 and TRPM8 agonists MO and MT, respectively, were used for the evaluation of direct activation of these receptors in different models of NP.

MO (50 µL/paw; 0,1%, 0,5% or 1%) or vehicle (VEH; corn oil, 50 µL/paw) (Hamity, White, Hammond, 2010) was injected (ipl) in the ipsilateral paw for CCI and sham rats 14 days after surgery, and in the right paw for the diabetic (STZ group) and normoglycemic rats (NGL group) 28 days after diabetes confirmation. Direct nociceptive responses (flinches) were counted for 20 min. The same procedure was performed for menthol (MT, 50 µL/paw; 0,1%, 0,5% or 1%) or vehicle (VEH; corn oil) (Liu *et al.*, 2013).

Statistical Analysis

The data consists of mean ± standard error of the mean (SEM). Two-way ANOVA with repeated measures was used to establish differences among experimental groups in the time course of cold and mechanical alterations evaluated on the cold plate and electronic von Frey, respectively. The independent factors used were treatment and time. When appropriate, the post-hoc analysis of Bonferroni was applied. One-way ANOVA followed by Newman-Keuls post-hoc test was used to establish differences among experimental groups in the nociceptive response (flinches) triggered by TRPA1 and TRPM8 agonists injection. The level of significance was established as $p < 0.05$. All the tests were carried out using GraphPad Prism version 7.0 for Mac (GraphPad Software, San Diego, CA, USA).

RESULTS

Development of mechanical allodynia in diabetic and CCI rats

As shown in the Figure 1 (panel A), two-way ANOVA with repeated measures showed significant main effects on experimental groups [$F_{(1,14)}$=10.85; p=0.0053], time (days) [$F_{(4,56)}$=8.40; p<0.0001], and also interaction between these two factors [$F_{(4,56)}$=7.04; p=0.0001]. Bonferroni's multiple comparison test indicated that the mechanical threshold was reduced in STZ animals 14 days after diabetes confirmation and remained lower for 28 days when compared to the NGL control group, indicating the development of mechanical allodynia. In the Figure 1 (panel B), two-way ANOVA with repeated measures showed effects on experimental groups [$F_{(1,14)}$=164.8; p<0.0001], time (days) [$F_{(4,56)}$=28.10; p<0.0001], and also an interaction [$F_{(4,56)}$=28.38; p<0.0001]. Bonferroni's multiple comparison test indicated a reduced mechanical threshold in CCI animals, when compared to the SHAM group, also indicating mechanical allodynia. Mechanical allodynia was observed at 4, 7, 10 and 14 days after the surgical procedure.

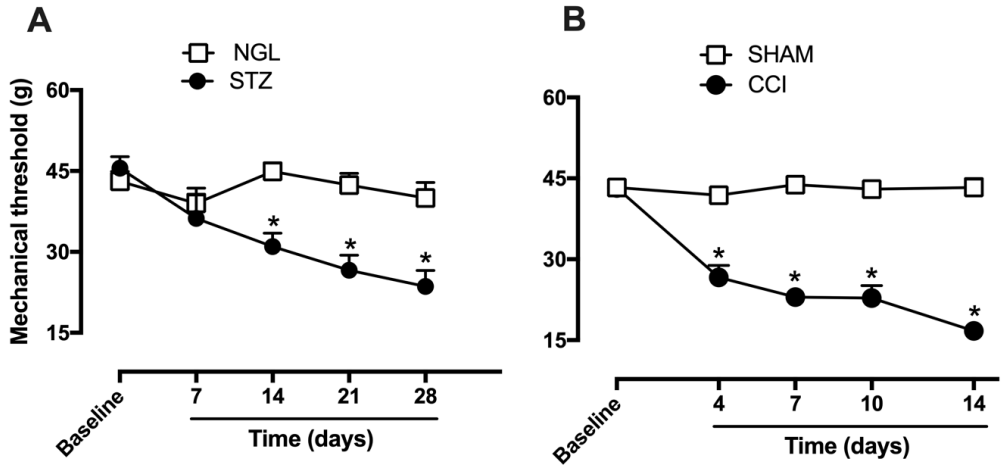

FIGURE 1 - Evaluation of mechanical allodynia, using electronic von Frey test, in different time points after (A) diabetes confirmation (STZ) and (B) chronic constriction of the sciatic nerve (CCI). Values represent mean ± SEM and * indicates p<0.05 when in comparison to their respective control groups (NGL or SHAM). Repeated-measures two-way ANOVA followed by Bonferroni's *post hoc* test.

Effect of TRPA1 antagonist on mechanical allodynia in diabetic and CCI rats

Independent groups of STZ rats (28 days after diabetes confirmation) and CCI rats (14 days after surgery), had their mechanical threshold evaluated before and after an ipl injection of HC-030031 (300 µg/40 µL/paw), a TRPA1 antagonist, or vehicle. As shown in the Figure 2 (panel A), two-way ANOVA with repeated measures showed significant effects on treatment [$F_{(2,150)}$=75.53; p<0.0001], time (min) [$F_{(5,150)}$=12.60; p<0.0001] and also interaction between these two factors [$F_{(10,150)}$=7.04; p<0.0001]. Bonferroni's multiple comparison test showed that STZ animals treated with HC-030031 (STZ+HC-030031) showed an increase in the mechanical threshold only 60 min after treatment, when compared to the control group treated with vehicle (STZ+VEH).

In the Figure 2 (panel B), two-way ANOVA with repeated measures showed effects on treatment [$F_{(1,60)}$=19.80; p<0.0001], time (min) [$F_{(5,60)}$=120.4; p<0.0001], in addition to an interaction between these two factors [$F_{(5,60)}$=10.48; p<0.0001]. Similar to what was found in diabetic animals, Bonferroni's post-test indicated that CCI animals had an increase in the mechanical threshold at 60 min after HC-030031 treatment (CCI+HC-030031), when compared to the vehicle-treated group (CCI+VEH).

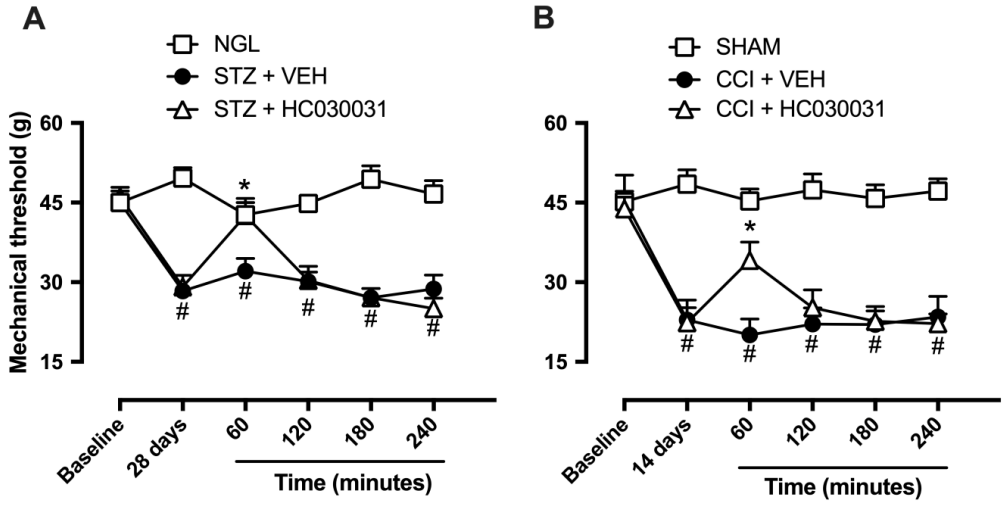

FIGURE 2 - Effect of the intraplantar (ipl) injection of a TRPA1 agonist, on mechanical allodynia in (A) diabetic (STZ) and (B) CCI rats. Diabetic (28 days after confirmation) and CCI (14 days after surgery) animals received ipl injection of HC-030031 (300 µg/40 µL/paw) and had their mechanical threshold reassessed 60, 120, 180 and 240 min after treatment. Values represent mean ± SEM. # indicates p<0.05 when compared to control groups (NGL or SHAM) and * indicates p<0.05 when in comparison to the diabetic or CCI group treated with vehicle (STZ+VEH or CCI+VEH). Repeated-measures two-way ANOVA followed by Bonferroni's *post hoc* test.

Development of cold hyperalgesia in CCI rats

In regards to cold sensibility, no difference was observed between the STZ and NGL groups on the latency to response evaluated on either the cold plate or the acetone test in any of the time periods (data not shown). In the Figure 3 (panel A) two-way ANOVA with repeated measures revealed significant effects on experimental groups [$F_{(1,14)}$=20.75; p<0.0004], time (days) [$F_{(4,56)}$=10.22; p<0.0001], and also an interaction between these factors [$F_{(4,56)}$=4.68; p=0.0025]. Bonferroni's post test showed that, when compared to the SHAM group, the CCI rats presented a decrease in the withdrawal latency, this response was detected in the 4th day and persisted until the 14th day after surgery.

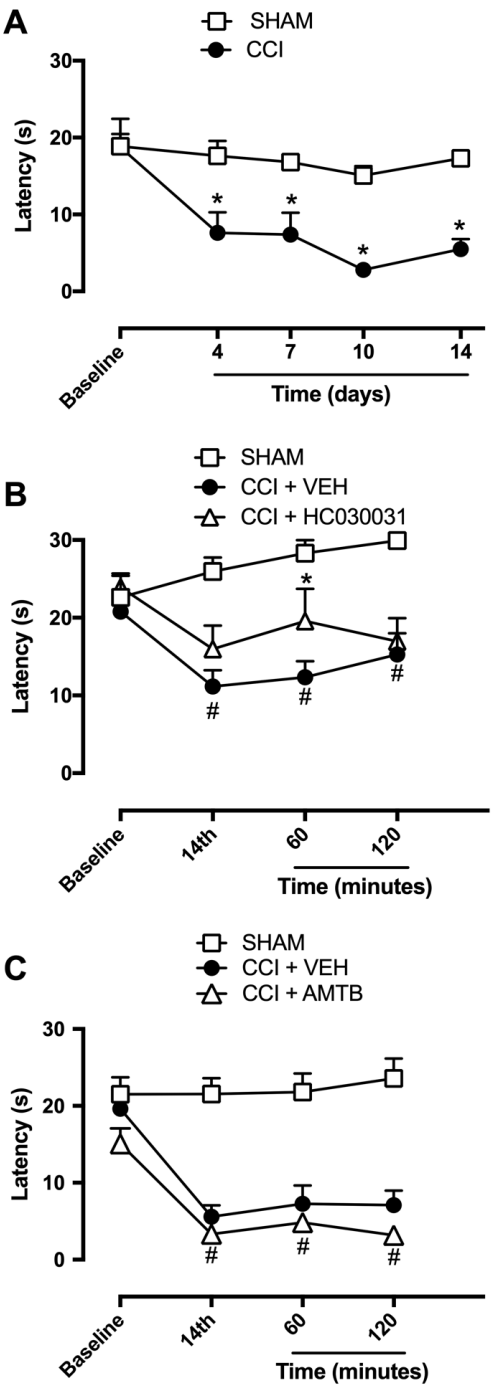

FIGURE 3 - Time course of (A) cold hyperalgesia (cold plate test) in CCI and SHAM rats, and effect of intraplantar injection with (B) TRPA1 and (C) TRPM8 antagonists, HC-030031 and AMTB, respectively, on cold hyperalgesia in CCI animals, 60 and 120 min after treatment (300 µg/40 µL/paw). Values represent mean ± SEM. # indicates $p<0.05$ when compared to the SHAM group and * indicates $p<0.05$ when in comparison to the CCI group treated with vehicle (CCI+VEH). Repeated-measures two-way ANOVA followed by Bonferroni's *post hoc* test.

Effect of TRPA1 and TRPM8 antagonists on cold hyperalgesia in CCI rats

In the Figure 3 (panel B) two-way ANOVA with repeated measures revealed effects on treatment [$F_{(2,84)}$=22.46; $p<0.0001$], but not time (min) [$F_{(3,84}$)=1.81; p=0.1510]. A significant interaction between these factor was also observed [$F_{(6,84)}$=2.30; p=0.0413]. Bonferroni's post-test showed that the i.pl. injection of HC-030031 (TRPA1 antagonist) induced an increase on the withdrawal latency of CCI animals (CCI+HC-030031) on the cold plate test 60 min after treatment, when compared to the group treated with vehicle (CCI+VEH). In the Figure 3 (panel C) two-way ANOVA with repeated measures showed significant effects on treatment [$F_{(2,72)}$=144.7; $p<0.0001$] and time (min) [$F_{(3,72)}$=9.33; $p<0.0001$], and an interaction between these two factors was observed [$F_{(6,72)}$=8.49; $p<0.0001$]. However, Bonferroni's multiple comparison test showed that the ipl injection of AMTB (TRPM8 antagonist) was not able to increase the latency of CCI rats (CCI+AMTB) in any time point evaluated, when compared to the control group treated with vehicle (CCI+VEH).

Nociceptive response of diabetic and CCI rats to $TRPA_1$ and $TRPM_8$ agonist injection

In Figure 4 (panel A), one-way ANOVA showed that the MO (TRPA1 agonist) treatment had significant effect on the total number of flinches [$F(7,67) = 25.40$; $p < 0.0001$]. All rats that received ipl injection of MO (all concentrations) showed a higher number of flinches, when compared to their respective vehicle (VEH) groups (STZ or NGL). Neuman-Keuls multiple comparison test showed that the nociceptive response to the TRPA1 agonist at the concentration of 0.1% was lower in STZ rats, compared to the NGL group. No difference in the number of flinches was observed for the concentrations of 0.5% and 1%, between STZ and NGL groups.

In the Figure 3 (panel C), one-way ANOVA showed that the MT (TRPM8 agonist) treatment had significant impact on the total number of flinches [$F_{(7,62)}$ ***=17.76; p < 0.0001***]. All STZ and NGL rats showed a higher number of flinches when compared to their respective vehicle-treated groups. Neuman-Keuls post-test showed that the

concentration of 0.1% of MT did not induce any significant difference in the nociceptive response between the STZ and NGL groups. However, STZ rats showed a lower total number of flinches at the MT concentrations of 0.5% and 1%, when compared to their respective NGL groups.

As shown in Figure 3 (panel B), one-way ANOVA revealed that the MO treatment had significant effect on the total number of flinches [$F_{(7,58)}$ ***=22.5; p < 0.0001]. All*** concentrations of MO injected in CCI and SHAM animals induced a higher total number of flinches, when compared to their respective vehicle-treated groups. Neuman-Keuls multiple comparison test showed that the concentrations of 0.5% and 1% of MO triggered a more intense direct nociceptive response in the CCI animals compared to the SHAM group. The injection of MO 0.1% did not induce any difference between these groups.

Figure 3 (panel D) shows that all concentrations of MT injected in SHAM and CCI rats induced a higher nociceptive response, when compared to their respective vehicle-treated group. Indeed, one-way ANOVA showed that MT treatment significantly altered the total number of flinches [$F_{(7,55)}$=7.39; p <0.0001]. Only the MT concentration of 1% was statistically different between SHAM and CCI rats. The number of flinches in this concentration was lower in CCI rats when compared to the SHAM group.

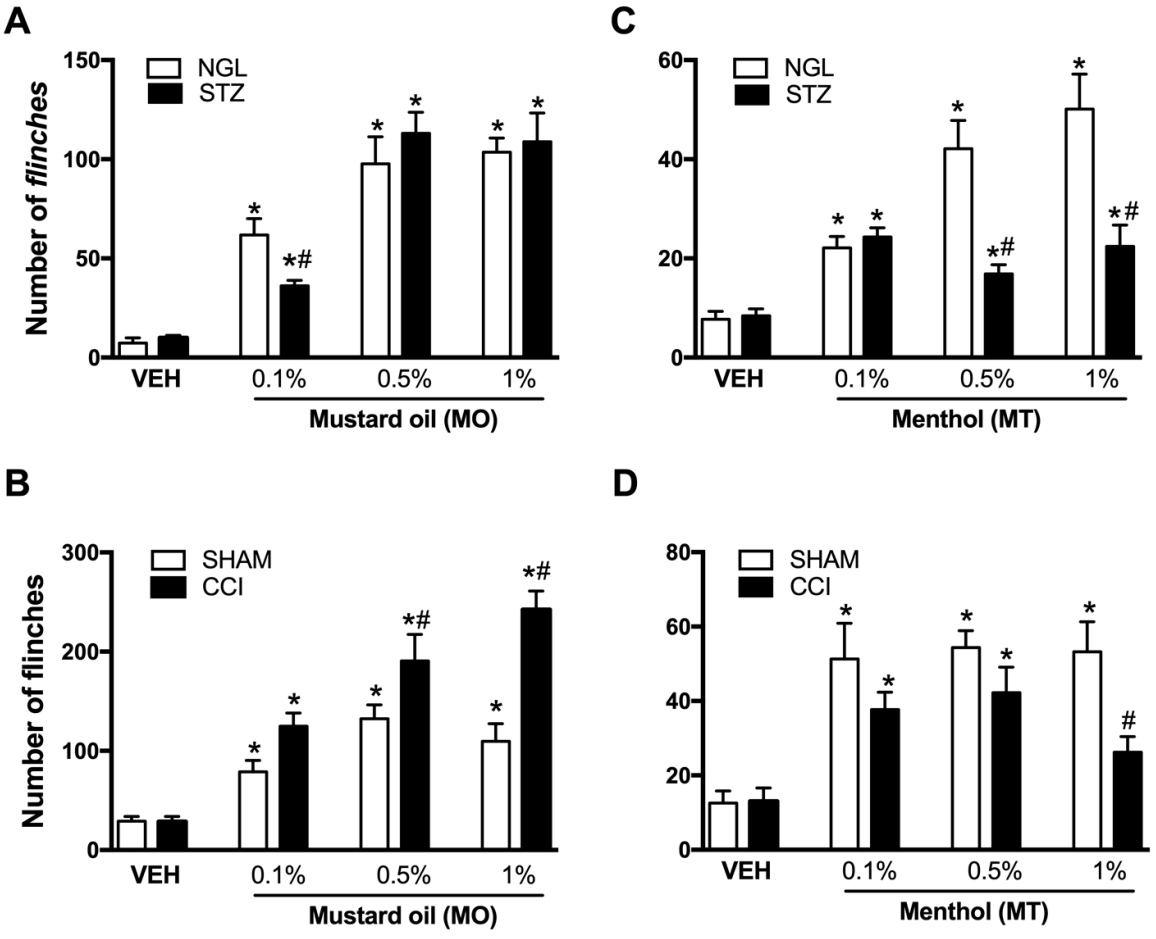

FIGURE 4 - Direct nociceptive response to intraplantar (ipl) injection of TRPA1 and TRPM8 agonists, mustard oil (MO) and menthol (MT), respectively, in (A, C) diabetic and (B, D) CCI rats. Bars represent the total number of flinches evoked after ipl injection of 50µL of MO or MT (0.1, 0.5 or 1%) in STZ or CCI rats, as well as their control and vehicle-treated groups. Values represent mean ± SEM. *indicates p<0.05 when compared to the respective group treated with vehicle (VEH; corn oil). # indicates p<0.05 when STZ or CCI groups were compared with their control groups, NGL or SHAM, treated with the same concentration of either MT or MO. One-way ANOVA followed by Newman-Keuls' *post hoc* test.

DISCUSSION

The present study demonstrated that depending on its etiology, NP may have different symptoms and underlying mechanisms. Although both diabetes and CCI showed a notable change in the mechanical threshold, cold hypersensitivity, using this specific protocol on the cold plate test, was only observed in CCI animals. Diabetic animals showed no significant response in this test when compared to the normoglycemic group (cold plate data not shown). Moreover, two proteins potentially related to the transduction of nociceptive stimuli, TRPM8 and TRPA1, seem to be distinctly affected in these two models of NP.

Mechanical allodynia is among the most frequent symptoms of NP (Basbaum *et al.*, 2009), as evidenced by its manifestation in both models. However, in the CCI model, early onset of this condition was observed when compared to the diabetic model. This is likely considering that the CCI model mimics a direct traumatic injury of the somatosensory system, which might have immediate consequences than an injury due to long-term metabolic disease such as diabetes (Sewell, 2018). Corroborating our results, Su et al. (2011) also found that mechanical allodynia developed early in CCI animals, while a previous study from our group provided evidence that animals developed mechanical allodynia more than two weeks after diabetes induction (Cunha *et al.*, 2009).

The role of TRPA1 in mechanical transduction has been reported in some studies. For example, the knockdown of TRPA1 expression in mechanosensitive hair cells of the inner ear of mammals was able to abolish the mechanically induced currents in these cells, indicating that TRPA1 plays a crucial role in signal transduction (Corey *et al.*, 2004). In the somatosensory system, this receptor is expressed in a subpopulation of nociceptive afferent primary nerve fibers (Dai, 2016). In a study using skin-nerve preparations from TRPA1 deficient mice, the cutaneous fibers demonstrated a reduction in firing rate in response to mechanical stimuli (Kwan *et al.*, 2009). Furthermore, TRPA1 has also been proposed to mediate chemotherapy-induced mechanical allodynia in rats by the synthesis of reactive oxygen and nitrogen species (Huang *et al.*, 2017), which are well-known TRPA1 activators (Jardín *et al.*, 2017). Here, we provide further evidence that TRPA1 is involved in the detection of mechanical nociceptive stimuli since the intraplantar injection of a TRPA1 antagonist, HC-030031 was able to reduce mechanical allodynia in both STZ and CCI animals 60 min after treatment. These results corroborate other studies in which TRPA1 antagonist treatment reduced mechanical nociception in NP models (Huang *et al.*, 2017; Liu *et al.*, 2019).

Cold hypersensitivity is an important feature of chronic pain, and the central processing of cold stimuli is altered in NP states. It was not surprising that the responses to cold stimuli were exacerbated in CCI animals even a few days after the constriction. In our study, treatment with HC-030031, a TRPA1 antagonist, reduced cold hyperalgesia in CCI animals 60 min after treatment. Corroborating our findings, Katsura et al. (2006) showed that after one day of chronic constriction of the sciatic nerve, the animals already manifested cold allodynia. Furthermore, studies have demonstrated that systemic treatment with HC-030031 reduced cold allodynia and cold hyperalgesia in acute and NP models, respectively (del Camino *et al.*, 2010; Pinheiro *et al.*, 2015). In contrast, in our study, treatment with a TRPM8 antagonist (AMTB) did not alter the latency of CCI animals in the cold plate test. Similar results were found in a previous study (Katsura *et al.*, 2006) in which the knockdown of TRPM8 in rats with NP induced by spinal nerve ligation was unable to prevent or reduce cold hyperalgesia. However, these findings are conflicting because other studies have reported that treatment with TRPM8 antagonists attenuates cold hypersensitivity in NP models (Dai, 2016). Together, these results indicate that TRPM8 may not be directly involved in the manifestation of cold hypersensitivity in the model of chronic constriction injury, which might be explained by the reduced expression of this receptor in the animal model (Caspani *et al.*, 2009).

Other studies using STZ-induced diabetic rats and applying different protocols have previously shown reduced withdrawal latency in the cold plate test and a higher number of nociceptive behaviors after acetone application (Kim, Kim, 2013; Nam *et al.*, 2014). However, during this study (weekly measurements, up to 4 weeks;

data not shown), we were unable to detect differences in the response of diabetic animals in the cold plate or acetone test. These results represent a limitation of the present study. The cold plate test is particularly difficult to apply in diabetic animals because of the sudden rise in plate temperature caused by urine release, which is a common characteristic since diabetic animals in this model develop polyuria (Fathollahi *et al.*, 2015). Consequently, most animals did not show any signs of paw withdrawal until the cut-off time was reached. It must also be mentioned that studies have shown that streptozotocin-induced diabetic animals may develop thermal hypoalgesia and loss of sensitivity. However, these sensorial disturbances are known to develop in later stages (4 to 8 weeks after diabetes induction), after the development of hyperalgesia and allodynia, for both mechanical and thermal stimuli (Beiswenger, Calcutt, Mizisin, 2008; Calcutt, Freshwater, Mizisin, 2004; Christianson *et al.*, 2003).

Diabetic neuropathy may cause demyelination and axonal degeneration of the small and large nerve fibers. Clinical studies indicate that these anatomical disturbances can be found in diabetic neuropathy (Dobretsov et al., 2007). In addition, the loss of warm and cold stimuli may or may not be accompanied by these anatomical impairments (Maser *et al.*, 1989; Young *et al.*, 1986). Non-clinical studies have also demonstrated demyelination and loss of nerve fibers (Aghanoori *et al.*, 2019; Jolivalt *et al.*, 2016). Therefore, demyelination may also have a direct impact on the absence of response to cold stimuli, such as the cold plate, which was found in our study. The use of different protocols to evaluate cold hypersensitivity will be useful to clarify the onset of cold-evoked pain in diabetic animals, as well as the impact of treatment with TRPA1 and TRPM8 antagonists, which remain to be explored.

The activation of TRPA1 by its agonist, MO, evoked a greater direct nociceptive response in CCI animals when compared to the SHAM group at all concentrations used. Corroborating our results, Pinheiro et al. (2015) demonstrated an exacerbated nociceptive response to the injection of TRPA1 agonist allyl isothiocyanate in the CCI mice model. However, this was not observed in the diabetic animals in our study. Interestingly, one of the concentrations of MO (0.1%) caused a diminished nociceptive response in the STZ animals compared to the NGL group. Although a previous study presented contrasting results in which STZ-induced diabetic rats showed increased nociceptive behavior after allyl isothiocyanate injection (Hiyama *et al.*, 2018), it is well known that TRPA1 is activated and desensitized by endogenous ligands such as 4-hydroxynonenal, methylglyoxal, and reactive oxygen species (Jardín *et al.*, 2017). The production of these metabolites is enhanced in diabetic animal models (Barrire *et al.*, 2012; Hiyama *et al.*, 2018), as well as in other models of chronic pain, such as trigeminal neuralgia and gout (Jardín *et al.*, 2017). Therefore, the reduced nociceptive response to MO injection observed in our study in diabetic animals might be related to established receptor desensitization caused by the products of glucose metabolism.

There are several conflicting studies on the effects of TRPM8 activation by its agonists. Liu et al. (2013) have shown that TRPM8 is the principal mediator of menthol-induced analgesia in models of inflammatory and acute pain. In contrast, a study has shown that cutaneous application of MT, in areas where NP patients did not report allodynia, was able to evoke allodynia in these patients (Wasner *et al.*, 2008). Furthermore, the dural application of icilin, another TRPM8 agonist, produced cutaneous facial and hind paw allodynia in rats (Burgos-Vega *et al.*, 2016). In the present study, the activation of TRPM8 by its agonist MT at all concentrations induced a greater nociceptive response in all groups from both models of NP, when compared to the same groups receiving vehicle injection. However, in diabetic animals, MT injection at concentrations of 0.5% and 1% induced a lower nociceptive response when compared to the NGL group. In addition, CCI animals that received MT injection at 1% concentration also showed a lower number of flinches in comparison to the SHAM group receiving the same injection.

MT has been linked to a regulatory activity in different studies. Zhang et al. (2008) have shown that MT can exert a suppressive effect on hippocampal neurons through the activation of $GABA_A$ receptors. Similarly, Gaudioso et al. (2012) demonstrated that at low concentrations, MT can also inhibit sodium channels

Nav. 1.8 and Nav. 1.9, important for repetitive firing under conditions of excitability and pain-related hyperexcitability. Furthermore, as a primary target for MT, TRPM8 can be modulated by endogenous lipids (Moore *et al.*, 2018). TRPM8 activation by either cold or agonists such as MT requires the presence of phosphatidylinositol-4,5-bisphosphate (PIP2), which interacts with the channel and functions as an activator (Moore *et al.*, 2018; Yudin, Rohacs, 2012). Hence, increased amounts of this molecule lead to higher activation of this receptor. In STZ-induced diabetes, there is an increased rate of PIP2 hydrolysis by phospholipase C (PLC) and a consequent decrease in the level of this phospholipid (Kamada et al., 1992; Yudin et al., 2012). Moreover, there is evidence of increased PLC activity after sciatic nerve injury in rodents (Kusuda *et al.*, 2013). Together, these findings might explain the decreased nociceptive response found in both models of NP after TRPM8 agonist injection.

In our study, the animals were kept in a standard controlled environment with a temperature of 23 ± 2 °C, and TRPM8 has an activation threshold of ~ 28 °C (McKemy et al., 2002). Studies have provided conclusive evidence that the temperature threshold for the activation of TRPM8 can be changed by varying the ambient temperature. Fujita et al. (2013) demonstrated that at higher than usual ambient temperatures (30 and 40 °C), the threshold for TRPM8 activation decreases significantly. Considering that our ambient temperature is lower than the TRPM8 activation threshold, further studies are needed to investigate the effects of lower ambient temperatures, such as 23 °C, on the TRPM8 activation threshold, as well as the impact of this potential mechanism in models of neuropathic pain and cold hypersensitivity.

In conclusion, our results show a different pattern in nociceptive behavior within different models of NP, suggesting the varying involvement of TRPM8 and TRPA1 in both chronic constriction and diabetes-induced NP. Compared to diabetes, chronic constriction injury induces alterations in both cold and mechanical stimuli. Further studies are necessary to elucidate the onset of cold-evoked nociception in diabetic rats and the impact of both TRPA1 and TRPM8 on this development. Our study shows that sensory alterations to a cold stimulus after chronic constriction of the sciatic nerve are dependent on the participation of TRPA1, whereas TPRM8 might be involved in response alterations to higher temperature thresholds. The role of TRPA1 in mechanical stimulus transduction in both NP models was reinforced. Further studies are necessary to elucidate the mechanisms involved in the nociceptive responses observed in our study.

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