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HEALTH SCIENCES

Role of the endocannabinoid system on the antihyperalgesic action of gabapentin in animal model of neuropathic pain induced by partial sciatic nerve ligation

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Abstract: Gabapentin has antihyperalgesic action, decreasing central sensitization in neuropathic pain models; this effect depends on the mobilization of endogenous pain control pathways. This study aims to investigate the contribution of the endocannabinoid system to the antihyperalgesic action of gabapentin. *Mus musculus Swiss,* male, were submitted to PSL. On the 7th and 14th days post PSL, different groups were treated with CB1 receptor antagonist, AM281 via i.t. (2 µg/5 µl) or i.pl. (10 µg/20 µl) or CB2, AM630 via i.t. (5 µL i.t.) or (20 µL i.p.) and 15 min after gabapentin (30 mg / kg orally). Mechanical hyperalgesia was measured by the frequency of paw removal by the von Frey monofilament. Gabapentin demonstrated antihypernociceptive action, which was attenuated in animals pretreated with AM281 in both the i.t. and i.pl routes on the 7th and 14th days, differently from animals pretreated with AM630 that did not achieve a significant reduction with administration i.t. only on the 14th day with administration i.pl. The results show that endocannabinoid system contributes to the antihyperalgesic action of gabapetin in neuropathic pain by PSL, suggesting participation in the medullary and peripheral levels of CB1 receptors, and the peripheral performance of CB2 receptors.

Key words: Endocannabinoid, gabapentin, hyperalgesia, nerve ligation.

INTRODUCTION

Hypersensitivity to noxious stimuli is a common symptom in patients suffering from peripheral neuropathic pain in diverse conditions (Bannister et al. 2017) and, for a better understanding of all these phenomena, studies have been carried out at various scales, but mostly using animal models, which are validated to mimic diverse etiologies of neuropathies (Munro et al. 2017).

Gabapentin is a common first or second line treatment in such conditions, promoting antihyperalgesic action through a mechanism that mainly involves its action on the $\alpha_{2\delta}$ subunit of the voltage-dependent Ca^{2+} channels (Lozovaya et al. 2009), decreasing the central sensitization (Colloca et al. 2017) by reducing the release of excitatory neurotransmitters such as glutamate (GLU) and substance P (Moulin et al. 2014). Other mechanisms recorded for its action in cases of pain concern its performance in 9-N-methyl-D-aspartate (NMDA) receptors, in transient receptor potential (TRP) channels, protein kinase-C (PKC), inflammatory cytokines and others (Kukkar et al. 2013). However, this effect still needs to be better elucidated, and the contribution of the endocannabinoid system to the antihyperalgesic activity of gabapentin can be speculated since gabapentin is used to manage cannabis-use disorders (Lile et al. 2016).

The endocannabinoid system is constituted of endogenous lipid-based retrograde neurotransmitters, being the most important the anandamide and 2-arachidonoylglycerol which are degraded, respectively, by the metabolizing enzymes fatty acid amide hydrolase (FAAH), which cleaves anandamide into arachidonic acid and ethanolamine or monoacylglycerol lipase (MAGL), and 2-AG into arachidonic acid and glycerol. Besides of this, after neurotransmitter release, endocannabinoids bind to two main cannabinoid receptors proteins, named CB_1 and CB_2; while the first type is predominantly found in the CNS and peripheral, the second type is mainly found in the immune system and, to a lesser extent scale in the CNS (Burston & Woodhams 2014). CB_1 receptors are widely spread and distributed in the brain, with effects including the control of motor activity, hypothermia, increased hunger and disturbances in memory consolidation, besides psychotropic effects (Dogrul et al. 2012). Meanwhile, the expression of CB_2 receptors was observed in astroglia and microglial cells in the CNS (Burston & Woodhams 2014).

In view of the above mentioned facts: 1) the high prevalence/incidence of neuropathic pain in patients around the world, 2) the need of better understanding endogenous mechanisms for analgesia promoted by gabapentin in painful conditions, 3) the fact that gabapentin is used to manage cannabis-use disorders, as well as that endocannabinoids modulate glutamate release, which is involved in the effects of gabapentin, the present study aims to investigate the participation of the endocannabinoid system in the antihyperalgesic effect of gabapentin in the model of partial sciatic nerve ligation in mice.

MATERIALS AND METHODS

Animals

Mus musculus Swiss mice, weighing between 25-35g were used. The animals were housed at a temperature of 22 ± 2 °C, under a cyclic regime of 12 h of light and 12 h of darkness (lights on at 6:00 p.m. and off at 6:00 p.m. by a timer) and with free access to food and water. The animals were acclimatized to the laboratory for at least 1 h before the behavioral tests, which were carried out between 8:00 a.m. and 12:00 a.m. The number of animals per group was calculated in n= 8, using the formula for comparison of two means, among independent samples, considering the power of the test of 80%, the significance level of 5%, the standard deviation of 12.5% for the hyperalgesic response from previous study records in our laboratory (Martins et al. 2015a) and the difference value to be detected equal to 18%. The animals were used once during the experiment and animal care and experiments were carried out in accordance with the provisions of National Institutes of Animal Health Care (NIH publications 80-23), after approval by the CEUA-UNISUL Animal Ethics Committee under protocol number 16.027.5.01. IV. The number of animals and the intensity of the noxious stimulus used were the minimum necessary to demonstrate the consistency of the treatments.

Induction of neuropathic pain

To assess the neuropathic pain behavior, the procedure used was similar to that described in a previous study (Martins et al. 2015a). Briefly, the mice were anesthetized with 2% isoflurane in 100% oxygen, with surgery performed by 1/3-1/2 ligation of the medial portion of the sciatic nerve with 8-0 silk thread. In the control group, the nerve was exposed but not ligated.

Influence of gabapentin on the hyperalgesia observed in the partial sciatic nerve ligation model (PSL)

To confirm the antihyperalgesic effect of gabapentin, different animals were submitted to the PSL procedure as described above and treated on days 7 and 14 after the PSL procedure with intraperitoneal (i.p.) vehicle administration (saline, 10 ml/kg) or gabapentin at the dose of 30 mg/kg, which is the dose that induced the most efficacious antihyperalgesic effect in the previous study model performed in our laboratory, and these periods of observation were chosen considering the minimum time required for the installation of the neuropathic process in the model, also according to an earlier study (Martins et al. 2015a). During these days, the animals were evaluated in relation to mechanical hyperalgesia at different time points after administration (0.5 to 4 h after), in order to select the best treatment time with this drug.

Influence of intrathecal (I.T.) or intraplantar (I.PL.) treatment with CB_1 receptor antagonists on the antineuropathic effect of gabapentin

To evaluate the contribution of CB_1 receptors at central or peripheral level on the effect of gabapentin, the mice were submitted to the PSL procedure and, on the 14th day after, they were treated with AM281 by i.t. (2 µg/5 µl) or i.pl. (10 µg/20 µl) and 15 min later they were given oral gabapentin (30 mg/kg). After 1 h of this last treatment, the animals were evaluated for mechanical hyperalgesia. These doses of antagonists were selected from the study by Martins et al. 2013.

Influence of treatment by i.t. or i.pl. with CB_2 receptor antagonists on the antineuropathic effect of gabapentin

To evaluate the contribution of CB_2 receptors at the central or peripheral level on the effect of gabapentin, the mice were submitted to the PSL procedure and after the 14th day, they were treated by i.t. (5 µL) or i.pl. (20 µL) with AM630 and 15 min later they received oral gabapentin (30 mg/kg). After 1 h of this last treatment, the animals were evaluated for mechanical hyperalgesia. These doses of antagonists were selected from the study by Martins et al. 2013.

Response to von Frey filament-induced hind paw removal

For the evaluation of mechanical hyperalgesia, the mice were individually placed in transparent acrylic boxes (9 X 7 X 11 cm) on a wire mesh screen, elevated 30 cm from the stand, to allow access to the ventral surface of the right hind paw. The hyperalgesic response was recorded as the frequency of paw removal for 10 applications of the von Frey 10 filament. Data were presented as a percentage of each animal's response to 10 stimulus applications. A significant increase in the number of this response in the animals with PSL in relation to the animals of the sham group, in the different time periods mentioned previously, was interpreted as a response of mechanical hyperalgesia.

Statistical analysis

Results are expressed as media ± standard mean (S.M.) and a $p \leq 0.05$ value was considered statistically significant. The comparison between and within groups were assessed using one-way or two-way ANOVA, when appropriated, followed by Bonferroni's test. The GraphPad InStat® software was used for data analysis.

RESULTS

Contribution of peripheral and spinal CB_1 receptors to the antihyperalgesic effect of gabapentin

In the present study we reproduced data from previous registers of our laboratory (Martins et al. 2015a) demonstrating the efficacy of the PSL model to cause a state-dependent effect, since operated animals presented higher frequency of response to mechanical stimulus when compared to sham animals (Figure 1).

The results presented in Figure 2a demonstrate that CB_1 peripheral receptors are important for the antihyperalgesic activity of gabapentin, since the treatment by i.pl. with the antagonist of these receptors, AM281 (10 µg/20 µl, 15 min before) partially reversed this effect of the drug on the mechanical hyperalgesia promoted by PSL. Although this effect was not observed on the 7th day after the nerve injury procedure, when this route of administration was used, AM281, which have no isolated effect (92.5 ± 3.7% response) on hyperalgesia due to PSL (85.0 ± 5.0% response), it reversed the antihyperalgesic effect for gabapentin (75.0 ± 11.8% response) on the 14th day after this procedure (47.5 ± 10.6% response). Important to note still that, at the dose of 30 mg/kg, gabapentin did not influence animals' locomotor activity in the Rota Rod test, demonstrating the specificity of its antyhiperalgesic action (Martins et al. 2015a).

Detailed information from the statistical analyses of these data are following described. From 7th day: for pre-treatment factor F= 35.58/ DF= 1/ p= < 0.0001; for treatment factor F= 0.02905/ DF= 1/ p= 0.8659 and for interaction F= 0.2614/ DF= 1/ p= 0.6132. From 14th day: for pre-treatment factor F= 9.358/ DF= 1/ p= 0.0051; for treatment factor F= 10.09/ DF= 1/ p= 0.0038 and for interaction F= 3.810/ DF= 1/ p= 0.0618.

Unlike what happened with the i.pl. treatment, the results presented in Figure 2b show that CB_1 spinal receptors have already contributed to the antihyperalgesic activity of gabapentin since the first observation

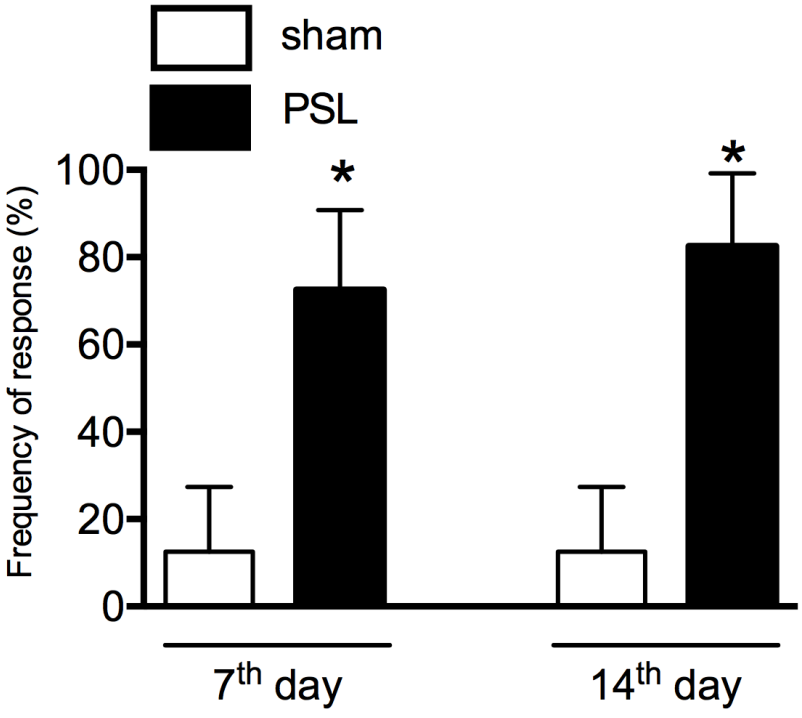

Figure 1. Efficacy of the PSL model to cause a neurophatic pain state. Hyperalgesia to mechanical stimulus was assessed in non-operated (sham) animals or in animals submitted to the PSL induction on days 7 and 14 after procedure. Data are expressed as the mean ± standard error of the mean (S.M.), n= 8 animals. (*) represents the comparison with the sham group. One-way ANOVA followed by Bonferroni, p≤ 0.05.

period of this study. The i.t. treatment with the antagonist of these receptors, AM281 (2 µg/5 µl, 15 min before) partially reversed the antihyperalgesic effect of gabapentin on the mechanical hyperalgesia promoted by PSL on the 7th day after PSL (saline-gabapentin: 35.0 ± 8.2% response; AM281-gabapentin: 70.0 ± 5.3% response), and this participation continued to be observed on the 14th day after this procedure (saline-gabapentin: 47.5 ± 10.6% response; AM281-gabapentin: 82.5 ± 5.9% response). Once more, when administered under the same conditions by this route, the antagonist did not have an effect on PSL hyperalgesia alone nor on the 7th, nor on the 14th day after the nerve injury. Detailed information from the statistical analyses of these data are following described. From 7th day: for pre-treatment factor F= 16.77/ DF= 1/ p= 0.0003; for treatment factor F= 4.990/ DF= 1/ p= 0.0337 and for interaction F= 8.871/ DF= 1/ p= 0.0059. From 14th day: for pre-treatment factor F= 10.98/ DF= 1/ p= 0.0026; for treatment factor F= 9.449/ DF= 1/ p= 0.0072 and for interaction F= 2.842/ DF= 1/ p= 0.1033.

Contribution of peripheral and spinal CB_2 receptors to the antihyperalgesic effect of gabapentin

Results similar to that observed for CB_1 receptors were observed for CB_2 receptors assessed peripherally. The data presented in Figure 3a show that the i.pl. treatment with the antagonist of these receptors, AM630 (4 µg/20 µl, 15 min before), only on the 14th day after the PSL partially reversed this effect of gabapentin (67.5 ± 5.3% response) on the mechanical hyperalgesia promoted by the procedure (40.0 ± 5.3% response). Furthermore, AM630 also had no effect in isolation (95.0 ± 3.3% response) on hyperalgesia due to PSL (88.7 ± 4.4% response). Detailed information from the statistical analyses of these data are following described. From 7th day: for pre-treatment factor F= 26.20/ DF= 1/ p< 0.0001; for treatment factor F= 0.3772/ DF= 1/ p= 0.5440 and for interaction F= 0.3772/ DF= 1/ p= 0.5440. From 14th day: for pre-treatment factor F= 67.30/ DF= 1/ p= <0.0001; for treatment factor F= 13.19/ DF= 1/ p= 0.0011 and for interaction F= 5.227/ DF= 1/ p= 0.0300.

Unlike what happened with the peripheral pathway treatment, the results presented in Figure 3b demonstrate that the spinal CB_2 receptors have not contributed to the antihyperalgesic activity of gabapentin in any of the observation periods evaluated in this study. The i.t. treatment with the antagonist of these receptors, AM630 (2 µg/5 µl, 15 min before), did not reverse the antihyperalgesic effect of gabapentin on the mechanical hyperalgesia promoted by PSL on the 7th day after PSL (saline-gabapentin: 35.0 ± 8.2% response; AM281-gabapentin: 45.7 ± 7.2% response), and this absence of influence was also observed on the 14th day after this procedure (saline-gabapentin: 47.5 ± 10.6% response; AM281-gabapentin: 82.5 ± 5.9% response). Here again, when administered under the same conditions by this route, the antagonist has not had an effect either alone on hyperalgesia resulting from PSL nor on the 7th day, nor on the 14th day after the nerve injury. Detailed information from the statistical analyses of these data are following described. From 7th day: for pre-treatment factor F= 30.71/ DF= 1/ p< 0.0001; for treatment factor F= 0.1595/ DF= 1/ p= 0.6927 and for interaction F= 1.206/ DF= 1/ p= 0.2817. From 14th day: for pre-treatment factor F= 50.11/ DF= 1/ p= <0.0001; for treatment factor F= 0.04682/ DF= 1/ p= 0.8304 and for interaction F= 1.308/ DF= 1/ p= 0.2631.

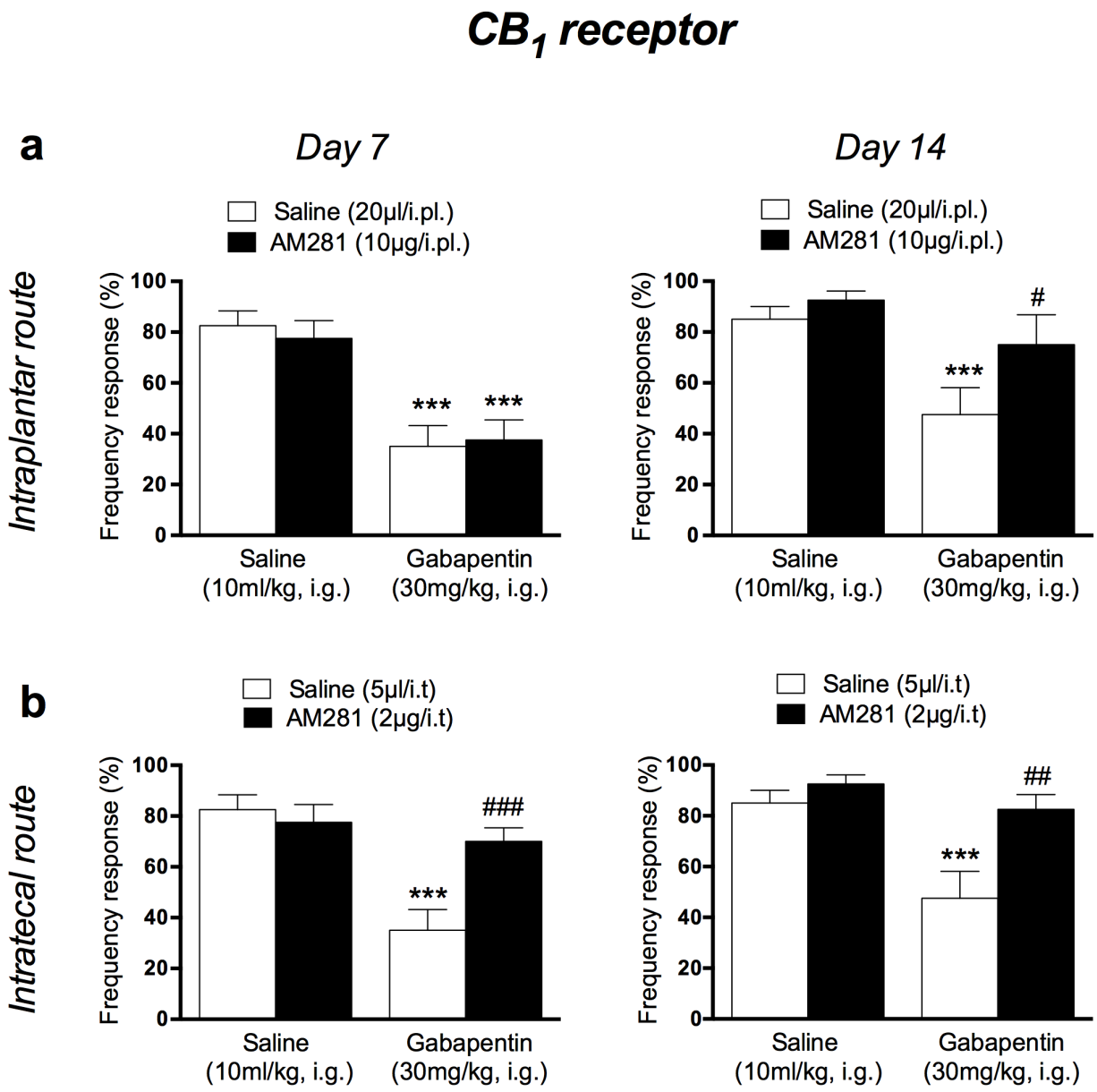

Figure 2. Influence of the CB_1 antagonist, via i.pl. (panel A) or i.t. (panel B), on the antihyperalgesic effect of gabapentin on the 7th day and on the 14th day after nerve injury induction in the PSL model in mice. Data are expressed as the mean ± standard error of the mean (SEM), n= 8 animals. (*) represents the comparison with the saline-saline group; (#) denotes the comparison with the salt-gabapentin group. Two-way ANOVA followed by Bonferroni, $p \leq 0.05$.

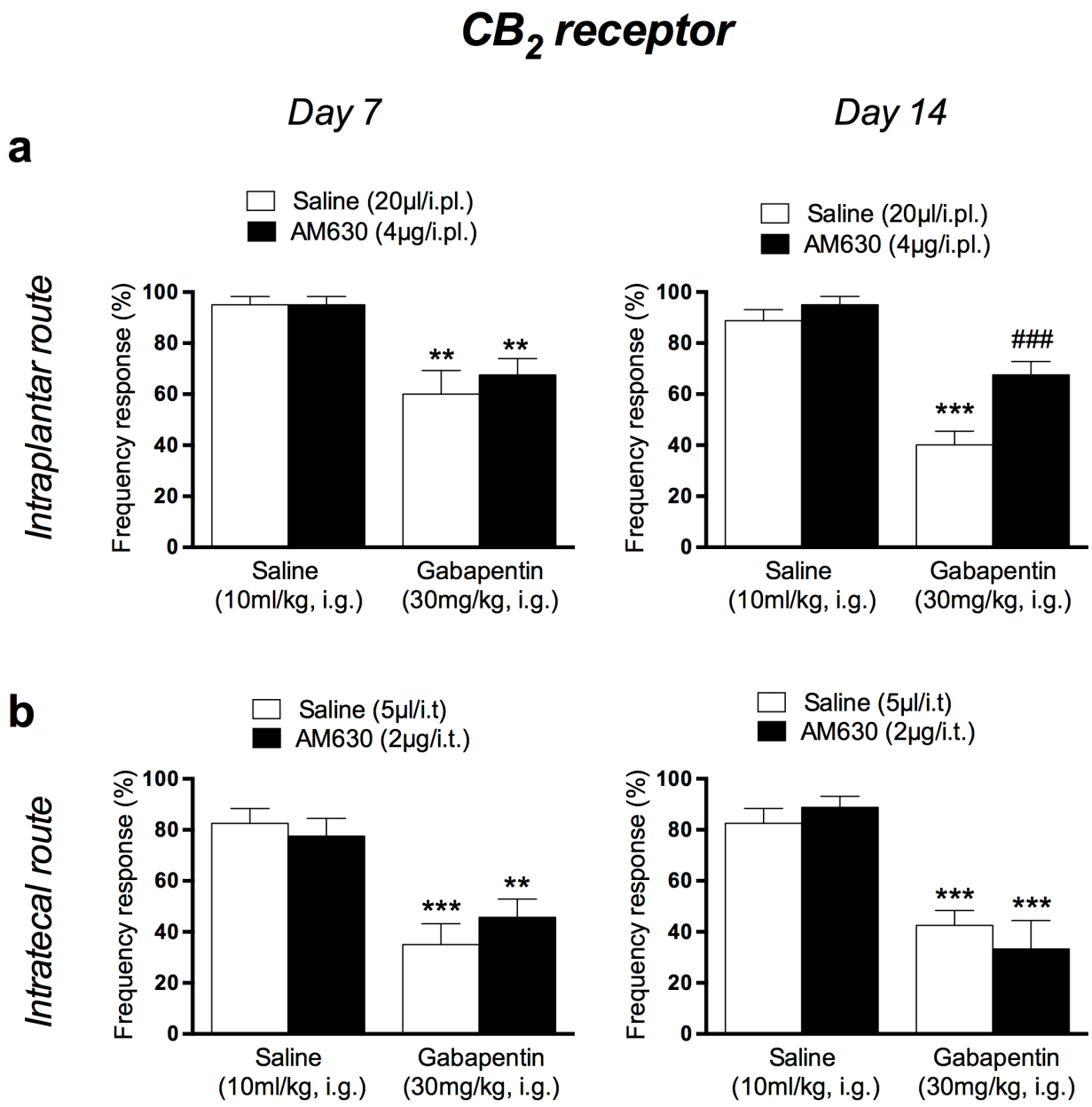

Figure 3. Influence of the CB_2 antagonist, via i.pl. (panel A) or i.t. (panel B), on the antihyperalgesic effect of gabapentin on the 7th day and on the 14th day after nerve injury induction in the PSL model in mice. Data are expressed as the mean ± standard error of the mean (SEM), n= 8 animals. (*) represents the comparison with the saline-saline group; (#) denotes the comparison with the salt-gabapentin group. Two-way ANOVA followed by Bonferroni, $p \leq 0.05$.

DISCUSSION

A large number of preclinical studies have demonstrated the beneficial role of gabapentin in different models of neuropathic pain. These studies demonstrate the action of gabapentin on the reduction of pain behaviors in several models, via i.p. or i.t (Kukkar et al. 2013). In this same sense, a previous study carried out in our laboratory demonstrated the antineuropathic activity of gabapentin, at a dose of 30 mg/kg orally, in the model of partial sciatic ligation (PSL); moreover, the participation of the adenosinergic system for this action of gabapentin (Martins et al. 2015a) was recorded. The present study expands these findings by noting that the endocannabinoid system also contributes to this action of gabapentin in the same model, both at the central (spinal, i.t.) and peripheral (i.pl) levels.

In the present study, the CB_1 receptor antagonist (AM281) when administered i.pl or i.t. partially reversed the effect promoted by gabapentin on mechanical hyperalgesia at days 7 and 14 after PSL. This data shows that antagonising CB_1 receptors can reverse the loss of hypersensitivity induced by gabapentin, from what we could suggest that this subtype of receptors contribute to the anihyperalgesic activity of the gabapentinoid; this point, however, only could be clarified by the use of a convincing method to demonstrate an interaction between the drugs, such as an isobologram analysis using different drug gradients.

Besides this is a limitation of the present study, suggestions on the contribution of this receptor in the medullary and peripheral level for a specific antihyperalgesic effect are found in other studies in literature, strengthening this idea. Recently, evaluating the antineuropathic effect of spinal electrical stimulation in rats following PSL-induced neuropathy, Sun et al. (2017) demonstrated that the antihyperalgesic effect (mechanical hyperalgesia) induced by therapeutic approach is amplified by the administration of the endocannabinoid receptor inhibitor, LY2183240, and the systemic administration of the CB_1 antagonist, AM251, reverses this amplification. In the publication of Hama et al. (2014), the model of neuropathy induced by spinal cord injury in rats was used to evaluate the possible acute or chronic effect of different classes of drugs on mechanical hyperalgesia. The authors reported that a previous systemic treatment of animals with a specific CB_1 receptor antagonist, rimonabant, blocked the antinociceptive effect of CP 55,940, an analog of the non- selective endocannabinoid receptor agonist. Similar effects were also observed after central or systemic administration of the CB_1 or CB_2 receptor antagonists (Yang et al. 2016, Munawar et al. 2017). Another approach to investigate contribution of CB_1 or CB_2 receptors in the peripheral antinociceptive action of ANA and 2-AG was the use of knock out animals. Knocking out for one or both receptors affected the antihyperalgesic effect of peripheral administration of ANA, 2-AG, URB597 and URB602 (Desroches et al. 2014).

Still reinforcing the data of the present study for the CB_2 receptor antagonist, Martins et al. (2015b) shows that the antihyperalgesic effect (mechanical hyperalgesia) promoted by immersion of the paw of mice in heated water in the model of pain induced by i.pl. of CFA (Complete Freund's Adjuvant) was antagonized by i.pl. of AM630, a CB_2 receptor antagonist. On the other hand, despite this peripheral action, CB_2 spinal receptors did not influenced the reversion of mechanical hyperalgesia promoted by gabapentin in the present study. In contrast to the present results, however, Fu & Taylor (2015) reported that i.t. administration of JWH-133, a specific CB_2 receptor agonist, dose-dependently

reduced mechanical and thermal hyperalgesia observed in an experimental model of autoimmune encephalomyelitis, this effect being reversed by the same CB_2 receptor antagonist employed in the present study at similar doses and also via i.t., AM630.

Our results regarding that CB_1 antagonist reverted the loss of hypersensitivity induced by gabapentin at the central level are consistent with findings that show the expression of these receptors physiologically at the central (dorsal horn and dorsal root ganglion) and peripheral (Fu & Taylor 2015) levels, which may have their expression increased after nerve injury (Maldonado et al. 2016). On the other hand, the fact that this same effect for the CB_2 antagonist has been observed peripherally can be explained since in the periphery, besides being present in nerve endings this receptor also occurs in immune cells and in keratinocytes, from which they may reduce the release of nociceptive agents (Maldonado et al. 2016). Furthermore, since receptor expression is a time dependent process, this could explain why in our study the AM630 antagonist had effect only on the 14^{th} day after the PSL. Despite this, other authors have suggested the participation of this receptor in the spinal cord and dorsal root ganglion levels in the antihyperalgesic action after sciatic nerve injury (Hsieh et al. 2011). Such differences may be justified by factors such as 1) the sensory modality evaluated, which means that different results could be acquired for thermal hyperalgesia, for example, 2) the level of endocannabinoid produced by the injury, 3) the species and genus of animals used in the studies, among others.

Regarding the mechanisms for the reversion of the antihyperalgesic effect induced by gabapentin observed in the present study for the CB_1 antagonist at the spinal level, it could involve a potentiating effect of inhibition of the nociceptive ascending pathway or in retrograde inhibitory modulation of GLU release with consequent depression of presynaptic activity (Alger et al. 2012). From this on, it can be hypothesized that the contribution of EC, acting on CB_1 receptors in the dorsal horn of the spinal cord, to the action of gabapentin in the PSL model (mechanical stimulus), could result from a potentiation of the latter's action to inhibit the nociceptive stimulus ascension through the ascending nociceptive pathway by activating the pain inhibitory descending pathway.

In addition, regarding the effect found in the present study for i.pl. of the CB_2 antagonist, one could think of a summation effect between the central action of gabapentin with the action of endocannabinoid on neuronal cells (CB_1, as discussed above) and immune ones at the periphery. This is because it is known that CB_2 receptors located in immune cells and keratinocytes reduce the release of pronociceptive agents or even increase the release of antinociceptive agents (opioids), according to a recent review by Maldonado et al. (2016).

In conclusion, the present study demonstrated the participation of the endocannabinoid system in the effect of gabapentin reducing mechanical hyperalgesic in the model of partial sciatic nerve ligation in mice, through participation of CB_1 and CB_2 receptors. These results suggest that agonists of these receptors could be better investigated as potential target in the adjuvant treatment of neuropathic pain in humans.

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Author contributions

Alexandre C. Buffon and Anna P. Piovezan: participated in the study design, data collection, data analysis and interpretation, manuscript drawing, critical review of the paper submitted. Marcelo A. Javornik, Daniel F. Martins, Ana C. Heymanns, Daiana C. Salm and Verônica V. Horewicz: participated in the analysis