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Gabapentin decreases microglial cells and reverses bilateral hyperalgesia and allodynia in rats with chronic myositis

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ABSTRACT

In the present work, we investigated the antinociceptive effect of gabapentin in a chronic myositis model and its interference in spinal glial cells. Chronic myositis was induced by injection of Complete Freund Adjuvant (CFA) into the right gastrocnemius (GS) muscle of rats and tests for evaluating mechanical hyperalgesia, thermal hyperalgesia and tactile allodynia were performed. Pharmacological treatment with gabapentin was administrated intrathecally and 100 µg and 200 µg doses were tested. For analyzing astrocytes and microglia in the spinal cord, immunochemistry assay was performed. It was found that gabapentin 200 µg reverted CFA-induced chronic muscle pain bilaterally, in all applied tests and it was able to attenuate microglial but not astrocytes activation in the dorsal horn of spinal cord. In conclusion, gabapentin was able to inhibit hyperalgesia and allodynia in chronic myositis and also to attenuate spinal microglial activation. Therefore, gabapentin could be used as treatment for targeting chronic muscle pain.

1. Introduction

Researchers have pointed musculoskeletal pain as one of the most prevalent types of pain in the population and one of the hardest types of pain to treat (Magni et al., 1990; Teixeira et al., 2001; Miranda et al., 2012). Muscle pain can arise from disorders such myofascial pain, fibromyalgia, myositis and strain. The muscle injury then leads to sensitization of peripheral nociceptors and dorsal horn neurons, transmitting nociceptive information to higher brain centers and results in hyperalgesia and allodynia (Hoheisel et al., 1994, 1998; Sluka et al., 2001; Schafers et al., 2003).

Studies have demonstrated that glia cells are involved in nociception, especially in chronic conditions (Watkins and Maier, 2003a; Tsuda et al. (2005); Milligan and Watkins (2009) by releasing pro-inflammatory substances, playing a relevant role in the transmission of nociceptive information (Watkins and Maier, 2003a; Watkins et al., 2003b; Marchand et al., 2005) and leading to allodynia and hyperalgesia (Sweitzer et al., 1999; Milligan et al., 2000). Studies also demonstrated that chronic myositis induced into the muscle tissue leads to morphological and functional alterations of astrocytes and microglia (Tenschert et al., 2004; Chacur et al., 2009).

Gabapentin, an anticonvulsant with few side effects, has been reported to have antinociceptive effect in chronic pain states, such as diabetes, neuropathic pain and arthritis (Yang et al., 2012; Ali et al., 2015; Park et al., 2015; Zvejniece et al., 2015). It is thought that its mechanism of action is by reducing neural hyperexcitability by binding to the $\alpha2/\delta{-}1$ subunit of dependent voltage calcium channel present in neurons (Cheng and Chiou, 2006; Tsukumo et al., 2011; Zhou and Luo, 2014; Morioka et al., 2015). However, its use in chronic musculoskeletal pain remains unclear. In the present study we induce chronic myositis to investigate whether gabapentin is able to reverse chronic muscle pain and interfere in astrocytes and microglial cells.

2. Materials and methods

2.1. Animals

Male Wistar rats (60–80 days old; 200–220 g; Institute of Biomedical Sciences Central Biotery, University of Sao Paulo) were housed with food and water available ad libitum at room temperature of 22 ± 2 °C.All rats were maintained on a 12:12 h light/dark cycle (lights on at 07 h) and were given two days to adapt to the experimentation room. All experimental procedures were conducted in accordance with the University of São Paulo Institutional Animal Care Committee (certification 47/2013). Animals were handled considering the principles and animal laboratory guide involving pain and nociception (Zimmermann, 1983).

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2.2. Induction of chronic myositis

Chronic myositis was induced by one single injection of Complete Freund's Adjuvant (CFA) (*M. butyricum,* Difco) into the animals right gastrocnemius (GS) muscle (150 μg /300 μl) and the analyses were performed 12 days after the injection. Previous studies have shown that the following doses and time point chosen were able to induce a chronic muscle pain model in animals. Naive animals were used as control (Chacur et al., 2009).

2.3. Pharmacological intervention

Single Gabapentin (GBP - Neurontin®/Ache) injection was administered intrathecally 13 days after the CFA-induced injury. Gabapentin injection doses used were 100 μg and 200 μg into 50μLsterile saline 0.9% (vehicle). A control group received equivolume injection of vehicle intrathecally (Chacur et al., 2009; Yamama et al., 2010; Dias et al., 2012; Cheng and Chiou, 2006). Animals were anesthetized through inhalation with isoflurane via loose-fitting cone-shaped masks and puncture of the spinal subarachnoid space was performed. A 29-gauge needle was introduced through the shaved skin into the L5-L6 intervertebral space. The correct positioning of the needle was assured by a tail flick reaction. Then the drug was administered and the needle was carefully removed (Mestre et al., 1994).

2.4. Behavioral tests

Blind and randomized behavioral tests were applied before any procedures (Baseline - BL) and repeated on the 6thand 12thdays after CFA injection. In order to analyze the drug action, behavioral tests were repeated 1, 3, 5, 7 and 24 h after gabapentin administration. All behavioral tests were measured in both ipsilateral and contralateral hindpaw.

2.4.1. Mechanical hyperalgesia

For assessing the animals' mechanical threshold the Electronic Von Frey (IITCInc.) was used, according to the modified method described by Chacur et al. (Chacur et al., 2009). In this test, a tip was pressed with increasing force to the medial head of the GS muscle in the medio-lateral direction until the limb withdrawal response was observed. Therefore, the applied force in grams (g) was considered as nociceptive threshold.

2.4.2. Thermal hyperalgesia

For determining the animals thermal sensitivity the plantar test described by Hargreaves was used (Hargreaves et al., 1988). The animals were placed in enclosure compartments and positioned on a platform under an infrared heat source (Ugo Basile). Latency to paw withdrawal and infrared intensity were recorded automatically in seconds (s) and was defined as the paw withdrawal latency (PWL) and nociceptive threshold. A cutoff time of 30 s was defined as time limit to the heat exposure.

2.4.3. Mechanical allodynia

In order to assess the animals tactile sensitivity, the Von Frey Hairs test described by Chaplan et al. was used (Chaplan et al., 1994). A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (Ugo Basile) were applied to the plantar surface of the animals hind paw. Log stiffness of the hairs was determined and ranged from manufacturer designated 3.61 (0.407 g) to 5.18 (15.136 g) filaments, all applied at maximum 10 s to standardize the time. The thinner filament (0.407 g) was primarily applied to find the threshold stimulus intensity required to elicit a paw withdrawal response. The experiment was carried out, increasing the filament thickness in case the animal did not respond twice to the previous filament on the scale. Failure to respond to the thicker filament, corresponding to the strongest stimulus (15.136 g) was considered to be the cutoff value.

2.5. Immunohistochemistry

On the 14th day (24 h after gabapentin injection), animals were deeply anesthetized with injection of ketamine and xylazine (5 mg/100 g; i.p). After animals were completely unresponsive, they were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The L4-L6segment of spinal cord was rapidly collected and postfixed for 4 h and transferred to 30% sucrose in PB for 24 h. The spinal cord was horizontally sectioned using a freezing microtome at 30 μm thickness.

For the detection of astrocytes and microglia in spinal cord, floating sections were incubated with GFAP (Monoclonal Anti-Glial Fibrillary Acidic Protein, Clone G-A-5 - Sigma-Aldrich)or OX-42 (Purified mouse anti-rat Cd11b/c monoclonal antibody -BD Biosciences Pharmigen) diluted 1:1000 in PB containing 0.3% Triton X-100% and 5% normal goat serum per overnight at 24 °C. Following washing, sections were incubated with biotinylated secondary antibody (1:200, Jackson ImmunoResearch) diluted in 0.3% triton X-100 and washed in PB. Then, incubated with avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame) for 1 h, labeling with peroxidase using the chromogen 3, 3′-diaminobenzidine (DAB - Sigma-Aldrich) and hydrogen peroxide solution 0.01% in PB.

The immunoreactivity of both sides of lumbar spinal cord, specifically lamina I and II of the dorsal horn were examined by light microscopy (Nikon Eclipse; ACT-1 Program). The specific lamina were choose based in previous articles showing that fibers from gastrocnemius muscle were conducting to lamina I and lamina II % (Ling et al., 2003; Panneton et al., 2005). Images were subjected to quantitative analysis involving ten sections from each lumbar spinal cord processed from each experimental and control animal. All images were quantified with the identical software and imaging parameters (Image J Program). Data were reported as the numbers of glial-positive cells. For the comparative analyses, the controls were considered as 100% (Chacur et al., 2009).

2.6. Statistical analyses

All data were presented as mean ± SEM. Statistical analyses consisted of Two-way ANOVA for behavioral tests and One-way ANOVA for immunohistochemistry assays. Both analyzes were followed by Bonferroni test, using Prism 5.01 (GraphPad Software Inc., CA, USA). In all cases, differences between group means were considered statistically significant if $p < 0.05$ (Sokal and Rohlf, 1981).

3. Results

3.1. Standardization of treatment with gabapentin

For determining the dose to be used in the animals' treatment, we started with two different gabapentin doses. Therefore, the initial doses tested were 100 μg and 200 μg of gabapentin, injected intrathecally. After its administration, the electronic Von Frey test was performed to assess the animals'nociceptive threshold. The behavior test was conducted before (BL), 6 and 12 days after CFA injection and once again after intrathecal application of gabapentin (1–24 h after). The results showed that the 200 μg dose was more effective in reversing the nociceptive behavior compared to lower dose ($p < 0.001$ for the 1st and 3rd hour compared to the Inflamed GS muscle group). The higher dose of gabapentin increased the nociceptive threshold in the first and third hours after administration. Later, in the 5th and 7th hour, the antinociceptive effect gradually decreases. At 24th hour time point, the drug was ineffective (Fig. 1). No statistical difference was observed within 100 μg of gabapentin during the analyzed time. Furthermore,

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