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P2X7-induced nociception in the temporomandibular joint of rats depends on inflammatory mechanisms and C-fibres sensitization

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Abstract

Background: P2X7 receptors are responsible for triggering inflammatory responses contributing to processes of pain in articular tissues. This study aimed to investigate whether the activation of the P2X7 receptor located in the temporomandibular joint (TMJ) tissues induces nociception through an inflammatory mechanisms and/or the activation of C-fibres (small-diameter primary afferents) of rats' TMJ.

Methods: The TMJ hypernociception induced by the activation of P2X7 receptor was assessed by measuring the behavioural nociceptive responses. After behavioural experiments, the animals were terminally anaesthetized and periarticular tissues were removed and homogenate for enzyme-linked immunosorbent assay, leukocyte infiltration and western blotting analysis.

Results: The nonselective P2X7 receptor agonist BzATP induced a dose-dependent TMJ nociception, which was blocked by the selective P2X7 receptor antagonist A-438079. The co-administration of the selective β2-adrenoceptor antagonist (ICI-118,551) and the pre-treatment with cyclooxygenase inhibitor indomethacin or with the nonspecific selectin inhibitor Fucoidan significantly reduced BzATP-induced TMJ nociception. BzATP also induced an increase of pro-inflammatory cytokines TNFα, IL-1β and CINC-1 levels, as well as leukocyte recruitment in TMJ tissue, effects that were reduced by A-438079. Moreover BzATP-induced TMJ nociception was inhibited in rats neonatal-treated with Capsaicin (depleting C-fibers). Finally, BzATP-induced an increase in TRPV1 expression in TMJ tissue.

Conclusions: These findings suggest that P2X7 receptor activation in TMJ of rats induces nociceptive responses mediated by sympathomimetic amines, prostaglandins, leukocyte migration and increased levels of pro-inflammatory cytokines. Furthermore, the P2X7 receptor activation induces nociceptive responses dependent on the activation of the primary afferent nociceptors of rats' TMJ.

Significance: The activation of P2X7 receptors has an essential role in TMJ nociception and could be an interesting target to control the inflammatory pain in temporomandibular disorders.

1 | INTRODUCTION

Peripheral sensitization of the primary afferent nociceptive fibres may contribute to ongoing pain, hyperalgesia and allodynia, which can occur in many acute or chronic craniofacial pain conditions (Chichorro et al., 2017). Pain is one of the classic signs of an inflammatory process, and in particular, temporomandibular joint (TMJ) pain results from inflammatory episodes involving the release of inflammatory mediators (Kopp, 2001; Rodrigues et al., 2006), which in turn may lead to chronic orofacial pain (Lamana et al., 2017). Furthermore, these inflammatory mediators facilitate the release of pro-nociceptive components such as K^+, leukotriene B4, prostaglandin E2, bradykinin, serotonin, histamine, glutamate and adenosine 5′-triphosphate (ATP), which have been shown to excite and induce pain in TMJ (Alstergren & Kopp, 2000; Cairns et al., 2001; Oliveira et al., 2005; Oliveira-Fusaro et al., 2012; Rodrigues et al., 2006; Teixeira et al., 2010; Ting et al., 2007).

Extracellular ATP level is higher in synovial fluid of patients with arthritis (Ryan et al., 1991). Under normal conditions, extracellular ATP is present only in low concentrations, but after tissue damage, there is an increase in ATP release (Dosch et al., 2018). ATP is the only known physiological activator of the P2X receptors (P2X1–P2X7), a family of ionotropic receptors whose activation leads to membrane depolarization by increasing permeability to Na^+, K^+, and Ca^{2+} (North, 2002).

Specifically, P2X7 receptors are predominantly expressed in cells of immunological origin, type B synoviocytes, and chondrocytes of synovial joints (Caporali et al., 2008; Collo et al., 1997; Kim et al., 2001; Surprenant & North, 2009; Tanigawa et al., 2018). Its activation can trigger membrane permeabilization, inflammasome and caspases activation, cell proliferation and pro-inflammatory cytokine release (Caporali et al., 2008; Franceschini et al., 2015; Kahlenberg & Dubyak, 2004; Verhoef et al., 2003), contributing to processes of pain and hyperalgesia in articular tissues (Broom et al., 2008; Hu et al., 2016; McIlwrath et al., 2017; Teixeira et al., 2017, 2018).

It has been previously shown that while P2X7 receptors do not contribute to carrageenan-induced chemical TMJ inflammatory hyperalgesia, the mRNA of these receptors is expressed in the trigeminal ganglia, and the activation of the P2X7 receptor on rats' TMJ sensitizes primary afferent nociceptors through the release of inflammatory mediators (Teixeira et al., 2010). However, the inflammatory mechanisms underlying P2X7 receptor-induced nociception in TMJ are unknown. Therefore, the current study investigated whether the activation of peripheral P2X7 receptor in the TMJ of rats induces nociception through mechanisms involved in inflammation, such as the previous release of prostaglandins, sympathomimetic amines, pro-inflammatory cytokines tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), chemokine-induced chemoattractant-1 (CINC-1) and by leucocyte infiltration. In addition, it was investigated whether the nociceptive behavioural responses induced by the activation of P2X7 receptors depends on the activation of the primary afferent nociceptive C-fibres and whether the activation of P2X7 receptors modulates TRPV1 expression in the rats' TMJ.

2 | MATERIALS AND METHODS

2.1 | Subjects

Male Wistar rats (7 weeks old, 200–240 g) were used in this study. The rats were housed in plastic cages with soft bedding (four/cage) on a 12:12 light cycle (lights on at 06:00 a.m.) with food and water available ad libitum. They were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments. Experimental protocols were approved by the Committee on Animal Research of the State University of Campinas (CEUA/UNICAMP no. 2457-1) and were carried out following the guidelines of the National Council for Control of Animal Experimentation (CONCEA) and ARRIVES guidelines (Kilkenny et al., 2010). Each rat was used once and the number of rats per group was kept to a minimum ($n = 6$ per group).

2.2 | Experimental design

To investigate whether the P2X7 receptor activation in the TMJ of rats induces nociception, animals were treated with an intra-TMJ injection of P2X7 receptor agonist BzATP (75, 225, or 675 μg/TMJ, 15 μl) plus 0.9% NaCl (15 μl).

To confirm the nociceptive character of BZATP-induced nociception, QX314 – a lidocaine *N*-ethyl bromide quaternary salt that does not readily diffuse across membranes was used. Animals were treated with an intra-TMJ injection of QX314 (2%, 15 μl) plus BzATP (225 μg/TMJ, 15 μl).

To confirm that BzATP-induced nociception was mediated by P2X7 receptors, animals were treated with an intra-TMJ injection of the selective P2X7 receptor antagonist A-438079 (180, 540 or 1,000 μg/TMJ, 15 μl) plus BzATP (225 μg/TMJ, 15 μl).

The involvement of the sympathomimetic amines in BzATP-induced nociception in the TMJ was assessed by the intra-TMJ of the β1-adrenoceptor antagonist atenolol (6, 18 or 54 μg/TMJ, 15 μl) or the β2-adrenoceptor antagonist ICI-118,551 (1.5, 4.5 or 13.5 μg/TMJ, 15 μl) plus BzATP (225 μg/TMJ, 15 μl).

To verify whether BzATP-induced nociception was mediated by prostaglandins, the inhibition of the cyclooxygenase pathway of arachidonic acid metabolism was performed. Animals were pre-treated with intra-TMJ injection of by

indomethacin (10 or 100 μg/TMJ, 15 μl) 30 min prior of the intra-TMJ injection of BzATP (225 μg/TMJ, 15 μl).

The contribution of neutrophil migration to BzATP-induced nociception was assessed by the pre-treatment with Fucoidan (25 mg/kg, i.v.) 20 min prior the intra-TMJ injection of BzATP (225 μg/TMJ, 15 μl) plus 0.9% NaCl (25 μl). Fucoidan is a polysaccharide that binds to L- and P-selectins and consequently, inhibits neutrophil rolling (Cunha et al., 2008; Shimaoka et al., 1996).

2.3 | Drugs and doses

The following drugs were used: the P2X7 receptor agonist BzATP: 2′(3′)-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate triethylammonium salt (Jacobson et al., 2002) (75, 225 and 675 μg/TMJ; Teixeira et al., 2014); the selective P2X7 receptor antagonist 3-((5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl)methyl pyridine (McGaraughty et al., 2007) (A-438079:180, 540 and 1,000 μg/TMJ; Teixeira et al., 2010); lidocaine *N*-ethyl bromide quaternary salt (2% QX-314; Roveroni et al., 2001); the selective β1 receptor antagonist atenolol (Allibardi et al., 1999; 6, 18 and 54 μg/TMJ; Teixeira et al., 2018); the selective β2 receptor antagonist ICI-118,551 (Yalcin et al., 2009; 1.5, 4.5 and 13.5 μg/TMJ; Teixeira et al., 2018); the cyclooxygenase inhibitor indomethacin (Summ & Evers, 2013; 10 and 100 μg/TMJ; Teixeira et al., 2018); the nonspecific selectin inhibitor Fucoidan (Ley et al., 1993) (25 mg/kg, i.v.; Teixeira et al., 2014) and capsaicin (50 mg/kg, i.p.; Komaki & Esteky, 2005). A-438079 was obtained from Tocris Bioscience, and all other drugs were obtained from Sigma-Aldrich. Each drug was dissolved in sterile saline (0.9% NaCl), except Capsaicin that was dissolved in 0.9% NaCl containing 10% Tween 80 and 10% ethyl alcohol.

2.4 | General procedures

Testing sessions took place during the light phase (between 09:00 a.m. and 05:00 p.m.) in a quiet room maintained at 23°C. Each rat was placed in a mirrored wood test chamber (30 × 30 × 30 cm) with a glass at the front side, for a 15-min habituation period to minimize stress. After this period, each rat was removed from the test chamber and briefly anaesthetized by inhalation of isoflurane (2%) to allow the TMJ injections and/or the intravenous (i.v.) injection of the leukocyte adhesion inhibitor Fucoidan.

2.5 | TMJ injections

A 30-gauge hypodermic needle, connected to a cannula consisting of a polyethylene tube (P20) and to a Hamilton syringe (50 μl), was used in the present study to inject the different drugs or vehicle (BzATP, A-438079, QX-314, atenolol, ICI-118,551, indomethacin and 0.9% NaCl) into the left TMJ of rats. Animals were briefly anaesthetized with inhalation of isoflurane (2%) and the needle was introduced into the left posteroinferior border of the zygomatic arch and advanced in an anterior direction until reaching the posterolateral aspect of the condyle of the TMJ. To keep the volume injected into the TMJ constant in all treatments, each animal received a final total volume of 30 μl. Each rat regained consciousness approximately 30 s after discontinuing the anaesthesia and the nociceptive behavioural responses were assessed.

2.6 | Measurement of nociceptive behavioural responses

After TMJ treatments, the animal was immediately placed in the test chamber to measure the behavioural response over a 30-min observation period. The nociceptive score was determined by measuring the number of seconds of two types of nociceptive behaviour: rubbing the orofacial region asymmetrically with the ipsilateral fore and hind paw and/or flinching the head in an intermittent and reflexive way characterized by high-frequency shakes of the head. Since head flinches followed a uniform pattern of 1s of duration, each flinch was expressed as 1s. Results are expressed as the duration time of nociceptive behaviour (Roveroni et al., 2001). The sum of these nociceptive behaviours was used as a quantitative measurement of BzATP-induced TMJ nociception. During the tests, the rats had no access to water or food.

Immediately after the analysis of the nociceptive behavioural responses, rats were euthanized by cervical dislocation under deep anaesthesia (80 mg/kg ketamine and 20 mg/kg xylazine, intraperitoneally [i.p.]), and the periarticular tissues were removed by dissection of the temporalis and posterior deep masseter muscles for further analyses. The standard sample size was 1 × 1 × 0.5 cm, as standardized previously (Lamana et al., 2017).

2.7 | Enzyme-linked immunosorbent assay procedure

The enzyme-linked immunosorbent assay (ELISA) assay was used in this study to quantify the concentrations of the pro-inflammatory cytokines (TNF-α, IL-1β, and cytokine-induced neutrophil chemoattractant-1, CINC-1) in periarticular tissues of rats' TMJ. After dissection, the periarticular tissues were weighed and homogenized in the same weight/volume proportion in buffer with protease inhibitors (Ripa Lysis Buffer, Santa Cruz, Biotechnology), followed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants

were stored at −80°C until further analysis. The pro-inflammatory cytokines were quantified by the following Duo Set ELISA kits: TNF-α, Rat TNF-α/TNFSF1A (DY510); IL-1β, Rat IL-1β/IL-1F2 (DY501) and CINC-1, Rat CXCL1/CINC-1 (DY515). All procedures followed the instructions of the manufacturer (R&D Systems). All procedures were repeated twice to guarantee the accuracy of the results.

2.8 | Leukocyte infiltration analysis

Immediately after the nociceptive behavioural responses analysis and periarticular tissues dissection, the TMJ cavity was washed with 10 μl of PBS/EDTA (1 mM) for leukocyte infiltration analysis, as described previously in detail (Silva Quinteiro et al., 2014). Briefly, total leukocyte counts were realized in a Neubauer chamber diluting the exudate in the Türk solution (1:2). The results were expressed as the number of leukocytes $\times 10^4$/cavity.

2.9 | C-fibres depletion

In another set of experiments, to further explore the role of capsaicin-sensitive C fibres (small-diameter primary afferents) in BzATP-induced TMJ nociception, neonatal Wistar rats were deeply anaesthetized with isoflurane (2%) and treated with capsaicin (50 mg/kg, i.p., dissolved in saline containing 10% Tween 80 and 10% ethyl alcohol) within 24 hr of birth. Control neonates were given an equal volume of the vehicle (the capsaicin solvent). Treatment of neonatal rats with capsaicin effectively destroys the majority (95%) of C-fibres (Holzer, 1991; Kiani et al., 2004; Kwan et al., 1996). To verify desensitization (C-fibres depletion) after systemic capsaicin treatment, 7 weeks old rats (capsaicin and vehicle neonatal-treated) were submitted to the corneal chemosensitivity test, as described previously (Hammond & Ruda, 1991). Corneal chemosensitivity is mainly mediated by C-fibres (Holzer, 1991), and its significant reduction is used as a measure of C-fibres depletion. Briefly, 50 μl of 0.01% (w/v) capsaicin was instilled into one eye and the number of wiping motions that occurred in the subsequent 30-s period was counted. One day after the corneal chemosensitivity test, the 7-week-old rats (Capsaicin and vehicle neonatal-treated) received a TMJ injection of BzATP (225 μg/TMJ), or its vehicle (0.9% NaCl), and were submitted to the analysis of the nociceptive behavioural responses.

2.10 | Western blotting

The total protein yield in the periarticular tissues was measured through the BCA protein assay kit (Thermo Scientific). Protein samples (80 μg) of periarticular tissues were separated on SDS/PAGE gel and transferred for nitrocellulose membranes. A molecular mass standard (Bio-Rad) was run in parallel to estimate molecular mass. The blockade of the membranes was performed in TBST containing 5% of milk, at 4°C overnight. The membranes were washed in TBS and then incubated (2 hr) at room temperature with primary antibodies: anti-TRPV1 (VR1) antibody (1:500, #ACC-030, Alomone Labs) or α-tubulin antibody (1:1,000, #sc5286, Santa Cruz Biotechnology), used as internal control. The membranes were then rewashed and incubated (2 hr) with the appropriate secondary antibody conjugated with peroxidase (1:5,000; Sigma-Aldrich). The target protein was visualized in the membrane using a chemiluminescence-based ECL system (Amersham Biosciences, Piscataway) and the digital image was obtained by CCD camera imaging for chemiluminescence (ImageQuant LAS 4000 mini, GE Healthcare Life Sciences). The program Image J (National Institutes of Health) was utilized to measure the optical density of the bands.

2.11 | Statistical analysis

To determine if there were significant differences ($p < 0.05$) between treatment groups, one-way ANOVA or *T*-test was performed. If there was a significant between-subjects main effect of treatment group following one-way ANOVA, post hoc contrasts using the Tukey test were used to determine the basis of the significant difference. Statistical analysis was performed using Prism v5 (GraphPad). Data are presented as means ± SEM.

3 | RESULTS

3.1 | BzATP-induced nociception in TMJ of rats

BzATP administered into the TMJ (225 and 675 μg/TMJ, but not 75 μg/TMJ) induced significantly higher nociceptive behavioural responses than that induced by its vehicle (0.9% NaCl, Figure 1a, $p < 0.05$, one-way ANOVA, post hoc Tukey test). The dose of 225 μg/TMJ was used in all the subsequent experiments. The co-administration of QX-314 (2%) with BzATP (225 μg/TMJ) blocked the BzATP-induced nociception (Figure 1a, $p < 0.05$, one-way ANOVA, post hoc Tukey test), confirming its nociceptive character.

The co-administration of the A-438079 (1,000 μg/TMJ, but not 180 and 540 μg/TMJ) blocked the BzATP-induced nociception (Figure 1b, $p < 0.05$, one-way ANOVA, post hoc Tukey test) and did not affect the nociceptive behavioural responses when administered on the contralateral (ct) TMJ,

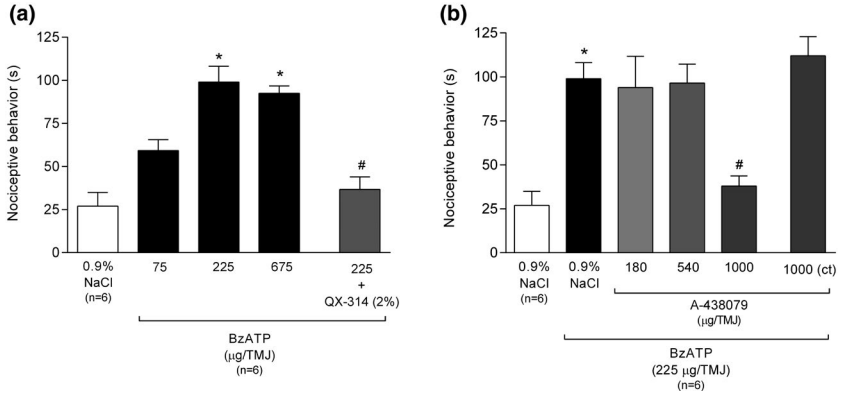

FIGURE 1 TMJ nociception induced by the agonist of P2X7 receptor BzATP. (a) The administration of BzATP (75, 225 and 675 μg/TMJ) into the TMJ of rats induced a dose-dependent TMJ nociception, as indicated by the symbol '*' ($p < 0.05$, Tukey test). Co-administration of QX-314 (2%) with BzATP (225 μg/TMJ) blocked the BzATP-induced TMJ nociception, as indicated by the symbol "#" ($p < 0.05$, Tukey test). (b) The BzATP-induced TMJ nociception (225 μg/TMJ) was blocked by the co-administration of A-438079 (1,000 μg/TMJ, $p < 0.05$, Tukey test), as indicated by the symbol '#'. A-438079 (1,000 μg/TMJ) administered in the contralateral TMJ (ct) did not affect the BzATP-induced TMJ nociception ($p > 0.05$, Tukey test). The symbol '*' indicates responses significantly greater than that induced by 0.9% NaCl group ($p < 0.05$, Tukey test). Results are expressed as the mean ± SEM of six animals per group. In this and subsequent figures, the number of rats or samples per group (n) is shown in parentheses

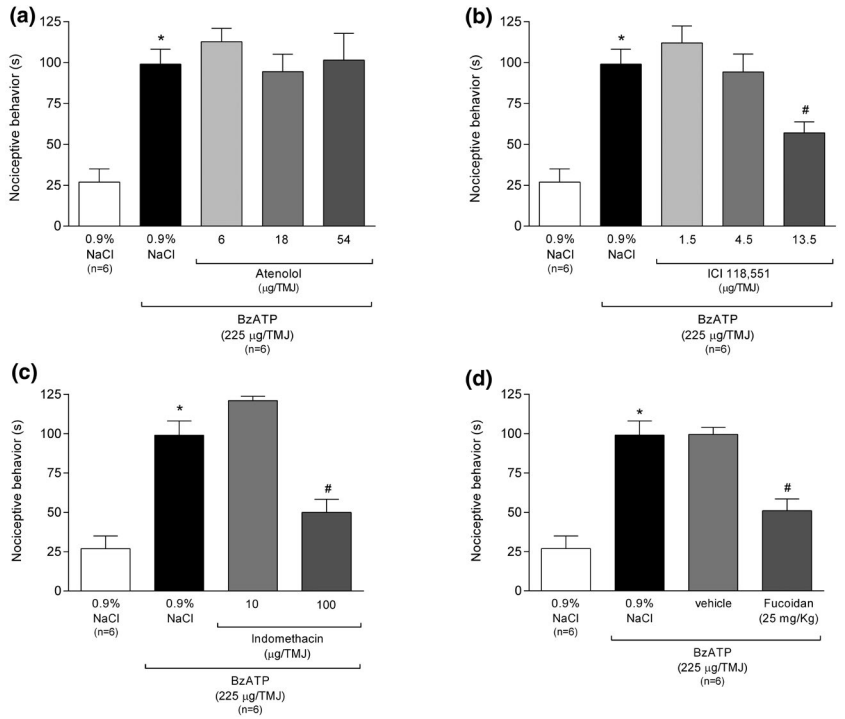

FIGURE 2 Effect of β1- and β2-adrenoceptor antagonists, indomethacin, and Fucoidan on BzATP-induced TMJ nociception. (a and b) Co-administration of ICI-118551 (13.5 μg/TMJ, b), but not atenolol (6, 18 and 54 μg/TMJ, a, $p > 0.05$, Tukey test), significantly reduced the BzATP-induced TMJ nociception in a dose-related manner, as indicated by the symbol '#' ($p < 0.05$, Tukey test). (c) Local treatment with indomethacin (100 μg/TMJ) 30 min before BzATP administration significantly reduced the BzATP-induced TMJ nociception in a dose-related manner, as indicated by the symbol '#' ($p < 0.05$, Tukey test). (d) Pre-treatment with Fucoidan (25 mg/kg, i.v.), but not with its vehicle, 20 min before BzATP administration significantly reduced the BzATP-induced TMJ nociception, as indicated by the symbol '#' ($p < 0.05$, Tukey test). The symbol '*' indicates a nociceptive behavioral response significantly greater than that induced by 0.9% NaCl ($p < 0.05$, Tukey test). Results are expressed as the mean ± *SEM* of 6 animals per group

confirming its local action (Figure 1b, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

3.2 | BzATP-induced TMJ nociception depends on the release of sympathomimetic amines, PGE_2 and neutrophil migration

The intra-TMJ injection of ICI-118,551 (13.5 μg/TMJ, but not 1.5 and 4.5 μg/TMJ, Figure 2b,$p < 0.05$, one-way ANOVA, post hoc Tukey test), but not atenolol (6, 18 and 54 μg/TMJ, Figure 2a,$p > 0.05$, one-way ANOVA, post hoc Tukey test) significantly reduced the BzATP-induced nociception (225 μg/TMJ).

The local pre-treatment with indomethacin (100 μg/TMJ, but not 10 μg/TMJ), 30 min before BzATP (225 μg/TMJ) administration, significantly reduced the BzATP-induced nociception (Figure 2c, $p < 0.05$, one-way ANOVA, post hoc Tukey test).

The pre-treatment with Fucoidan (25 mg/kg, i.v.), but not with its vehicle, 20 min before BzATP (225 μg/TMJ) administration, significantly reduced the BzATP-induced nociception (Figure 2d, $p < 0.05$, one-way ANOVA, post hoc Tukey test).

3.3 | BzATP-induced increase of pro-inflammatory cytokines concentration and leukocyte infiltration in the TMJ of rats

The administration of BzATP (225 μg/TMJ) significantly increased the protein level of the pro-inflammatory cytokinesf TNF-α (Figure 3a), IL-1β (Figure 3b) and CINC-1 (Figure 3c) when compared with 0.9% NaCl group ($p < 0.05$, one-way ANOVA, post hoc Tukey test) in the periarticular tissues. The intra-TMJ injection of the P2X7 receptor antagonist A-438079 (1,000 μg/TMJ) abrogated the protein level of the pro-inflammatory cytokines TNFα (Figure 3a), IL-1β (Figure 3b), and CINC-1 (Figure 3c) induced by BZATP ($p < 0.05$, one-way ANOVA, post hoc Tukey test). The TMJ administration of A-438079 (1,000 μg/TMJ) alone did not induce an increase of protein levels of TNF-α, IL-1β, and CINC-1 by itself (Figure 3a–c, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

The administration of BzATP (225 μg/TMJ) significantly increased the leukocyte infiltration when compared with 0.9% NaCl group (Figure 3d, $p < 0.05$, one-way ANOVA, post hoc Tukey test) in the periarticular tissues. The intra-TMJ injection of A-438079 (1,000 μg/TMJ) blocked the leukocyte migration induced by BzATP (Figure 3d, $p < 0.05$, one-way ANOVA, post hoc Tukey test). The TMJ administration of

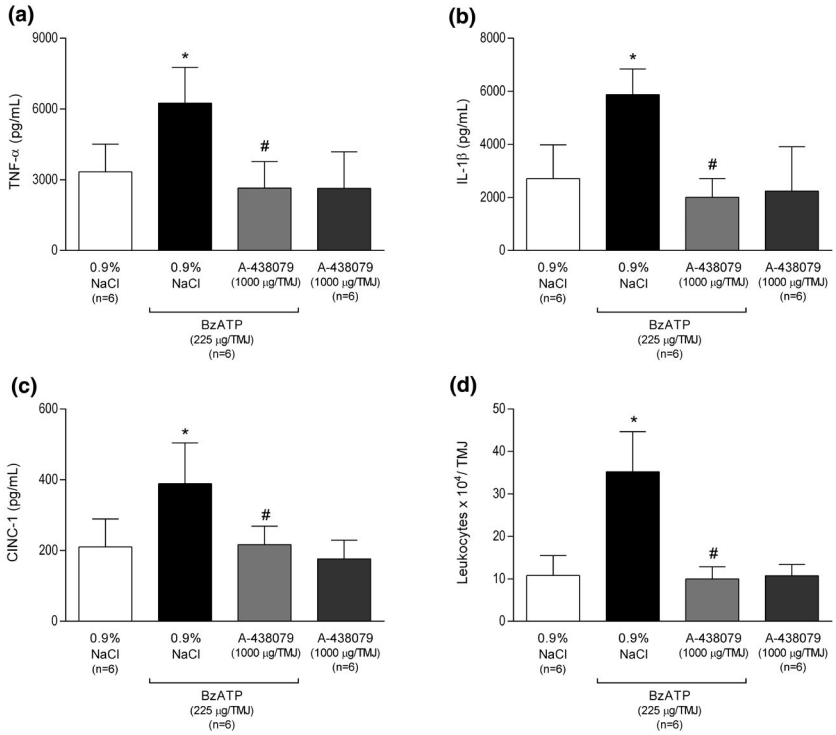

FIGURE 3 Effect of the agonist of P2X7 receptor BzATP on pro-inflammatory cytokines release. The TMJ administration of BzATP (225 μg/TMJ) induced a local increase of TNFα (a), IL-1β (b) and CINC-1 (c) concentrations, when compared with 0.9% NaCl group, as indicated by the symbol '*' ($p < 0.05$, Tukey test). The co-administration of A-438079 (1,000 μg/TMJ) with BzATP blocked the BzATP-induced increase of TNFα (a), IL-1β (b) and CINC-1 (c) concentrations, as indicated by the symbol '#' ($p < 0.05$, Tukey test). The TMJ administration of A-438079 (1,000 μg/TMJ) did not induce the increase of TNFα (a), IL-1β (b) and CINC-1 (c) concentrations by itself, when compared with 0.9% NaCl group ($p > 0.05$, Tukey test). Results are expressed as the mean ± *SEM* of six animals per group

A-438079 (1,000 μg/TMJ) alone did not induce an increase of leukocyte infiltration by itself (Figure 3d, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

3.4 | Effect of Capsaicin treatment in the rats' corneal chemosensitivity

The corneal chemosensitivity was significantly reduced in the adult rats neonatal-treated with Capsaicin (50 mg/kg, i.p.) (1.3 number of wipes ± 0.9) compared with the adult rats neonatal-treated with the vehicle of Capsaicin (8.6 number of wipes ± 1.2) (*t*-test, $p < 0.001$).

3.5 | Effect of C-fibre depletion on the BzATP-induced TMJ nociception

The administration of BzATP (225 μg/TMJ) into the TMJ of adult rats neonatal-treated with Capsaicin (50 mg/kg, i.p.) induced a significantly lower behavioural nociceptive behavioural responses than that induced by the administration of BzATP (225 μg/TMJ) in the TMJ of adult rats neonatal-treated with the vehicle of capsaicin (Figure 4a, $p < 0.05$, one-way ANOVA, post hoc Tukey test). The nociceptive behavioural response induced by the administration of BzATP (225 μg/TMJ) in the TMJ of adult rats neonatal-treated with capsaicin did not significantly differ from the nociceptive behavioural response induced by the injection of 0.9% NaCl in the TMJ of adult rats neonatal-treated with capsaicin (50 mg/kg, i.p.) (Figure 4a, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

The nociceptive behavioural responses induced by the administration of BzATP (225 μg/TMJ) in the TMJ of adult rats neonatal-treated with the vehicle of capsaicin did not significantly differ from the nociceptive behavioural response induced by the administration of BzATP (225 μg/TMJ) in the TMJ of naïve rats (Figure 4a, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

3.6 | BzATP-induced increase of TRPV1 expression in the TMJ of rats

The administration of BzATP (225 μg/TMJ) into the TMJ of rats significantly increase the protein level of the TRPV1 when compared with 0.9% NaCl group (Figure 4b, $p < 0.05$, one-way ANOVA, post hoc Tukey test) in the TMJ tissue. The intra-TMJ injection of A-438079 (1,000 μg/TMJ) significantly reduced the protein level of TRPV1 induced by BzATP in periarticular tissues (Figure 4b, $p < 0.05$, one-way ANOVA, post hoc Tukey test). The TMJ administration of A-438079 (1,000 μg/TMJ) alone did not induce alterations on the protein level of TRPV1 in the TMJ tissue by itself (Figure 4b, $p > 0.05$, one-way ANOVA, post hoc Tukey test).

4 | DISCUSSION AND CONCLUSIONS

This study demonstrated that the activation of P2X7 receptors in the TMJ of rats induces nociception via an inflammatory response mediated by the sensitization of primary

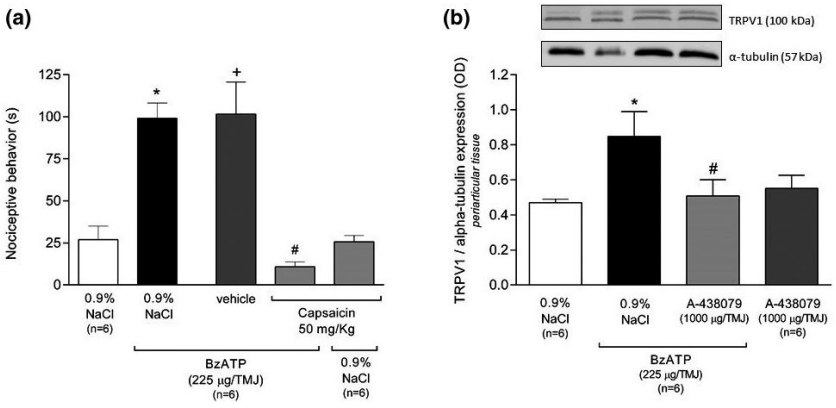

FIGURE 4 BzATP induces nociception in TMJ through nociceptive C-fibres activation and increases the TRPV1 expression. (a) The BzATP-induced TMJ nociception (225 μg/TMJ) was significantly inhibited by pre-treatment with Capsaicin (50 mg/kg, i.p., neonatal), as indicated by the symbol '#' ($p < 0.05$, Tukey test), but not by its vehicle (80% 0.9% NaCl plus 10% Tween 80 plus 10% ethyl alcohol, i.p., neonatal), as indicated by the symbol '+' ($p > 0.05$, Tukey test). (b) The TMJ administration of BzATP (225 μg/TMJ) induced an increase of TRPV1 protein levels in the periarticular tissue of TMJ. The co-administration of A-438079 (1,000 μg/TMJ) with BzATP reduced the BzATP-induced increase of TRPV1 protein levels, as indicated by the symbol '#' ($p < 0.05$, Tukey test). The TMJ administration of A-438079 (1,000 μg/TMJ) did not induce the increase of TRPV1 protein levels by itself when compared with 0.9% NaCl group ($p > 0.05$, Tukey test). The symbol '*' indicates significantly greater optical density than that in 0.9% NaCl group ($p < 0.05$, Tukey test). Representative TPRV1 and α-tubulin bands of each group are displayed above the graph. Results are expressed as the mean ± *SEM* of six animals per group

afferent nociceptor (C-fibres). Once activated in the TMJ tissues, P2X7 receptors increased the protein level of TRPV-1, a vital ion channels that mediate nociceptive signalling (Julius, 2013). The current results demonstrated that the administration of BzATP in the TMJ of rats induced nociceptive behavioural responses, which was reversed by the quaternary lidocaine derivative 2% QX-314, a neuronal action analgesic drug due to its ability to modulate sodium channels (Stueber et al., 2016). This data suggest that BzATP-induced TMJ nociception is a specific response mediated by the activation of P2X7 receptors located in the TMJ region.

Although BzATP is a non-selective P2X7 receptor agonist, once it also binds to P2X1 and P2X3 receptor (Bianchi et al., 1999; Jacobson et al., 2002), it is the most potent agonist for the P2X7 receptor available. The present study demonstrated that the TMJ nociception, pro-inflammatory cytokines release, leukocyte infiltration, and the increase of TRPV-1 expression induced by BzATP was prevented by the selective P2X7 receptor antagonist A-438079, suggesting that those effects in the TMJ of rats were mediated by P2X7 receptor activation. Taken together, the current results corroborate with previous which have demonstrated that the activation of P2X7 receptors can trigger nociceptive and hyperalgesic behaviours in different tissues, such as the spinal cord (Ito et al., 2013; Munoz et al., 2017), subcutaneous tissue (Teixeira et al., 2014), dermal tissue (Wismer et al., 2003), knee joint (Teixeira et al., 2018), as well as TMJ of rats (Teixeira et al., 2010).

It is well established that important events involved in inflammatory pain processes consist of two pathways: the local production of prostaglandins and the release of sympathomimetic amines, which directly sensitize the primary afferent nociceptor (Gold et al., 1996; Khasar et al., 1999; Rush & Waxman, 2004). Studies have already shown that prostaglandin E_2 and sympathomimetic amines contribute to the development of TMJ nociception (Alstergren & Kopp, 2000; Favaro-Moreira et al., 2012; Rodrigues et al., 2006). TMJ is densely innervated by sensory and sympathetic fibres arising from cells of the trigeminal ganglia and superior cervical ganglion, respectively (Widenfalk & Wiberg, 1990). Similarly, in the subcutaneous tissue (Teixeira et al., 2014) and knee joint (Teixeira et al., 2018) of rats, in the present study it was also demonstrated that the activation of the P2X7 receptors induced TMJ nociception, which depends on both pathways, one mediated by prostaglandins and other by sympathomimetic amines. Specifically, the blockage of just one pathway significantly prevented the BzATP-induced TMJ nociception. Therefore, the current study can suggest that the TMJ nociception induced by P2X7 activation may involve more than one type of receptor activation (prostaglandins receptors and β2-adrenoceptor) and, consequently, more than one signaling pathway activation in primary afferent nociceptor.

The present results demonstrated that P2X7 receptors activation increases the concentration of pro-inflammatory cytokines TNF-α, IL-1β and CINC-1 in the TMJ tissue. These findings are also in agreement with in vitro (Ferrari et al., 2006; Rampe et al., 2004; Sanz & Di Virgilio, 2000) and in vivo studies (Colomar et al., 2003; Gourine et al., 2005; Mingam et al., 2008; Teixeira et al., 2014, 2018), showing that P2X7 receptor activation triggers the release of pro-inflammatory cytokines. Studies have systematically demonstrated that the P2X7 receptor is selectively expressed in peripheral macrophages, mast cells, lymphocytes, fibroblasts, erythrocytes, monocytes, FLS cells and chondrocytes (Caporali et al., 2008; Collo et al., 1997; Kim et al., 2001; Surprenant & North, 2009; Tanigawa et al., 2018), which produce and secrete pro-inflammatory cytokines (Aida et al., 2006; Caporali et al., 2008; Hayashida et al., 2001; Mor et al., 2005; Shakoory et al., 2004). Pro-inflammatory cytokines contribute to the development and maintenance of TMJ hyperalgesia (Ohtani et al., 2012). Taken together, the current results could suggest that the release of pro-inflammatory cytokines mediated by the activation of P2X7 receptors expressed in resident cells, such as synovial macrophages, FLS cells and chondrocytes of TMJ tissue, can contribute to BzATP-induced TMJ nociception.

The neutrophil is the first immune cell to enter into inflamed tissues and it has been associated with the development and chronicity of inflammatory diseases, such as TMD (Mikami et al., 2014). Also, leukocytes are present in the joint fluid of approximately 50% of patients with TMD (Mikami et al., 2014). Moreover it has been shown that ATP released in an inflamed joint, via P2X7 receptor activation, induces articular hyperalgesia that depends on neutrophil migration (Teixeira et al., 2017). Based on that, the current study investigated whether leukocytes infiltration contributes to the TMJ nociception induced by P2X7 receptors activation.

Likewise in the subcutaneous tissue (Teixeira et al., 2014), the present study also demonstrated that the activation of the P2X7 receptor induces TMJ nociception that depends on neutrophil migration to the rat's TMJ, since the pre-treatment with the nonspecific selectin inhibitor Fucoidan prevented the BzATP-induced TMJ nociception. It is important to explain that although P2X7 receptor activation is involved in nociceptive signaling of articular tissue (Hu et al., 2016; Teixeira et al., 2010, 2018), the mechanisms by which this receptor contributes to pain process are not necessarily the same. For instance, it has been previously demonstrated that neutrophil migration does not contribute to the development of BzATP-induced articular hyperalgesia in the rats' knee joint (Teixeira et al., 2018) as it does in the subcutaneous tissue (Teixeira et al., 2014) and in the TMJ of rats (present data). Also, the present study demonstrated that the activation of P2X7 induces leukocyte infiltration to the TMJ site. The leukocyte influx induced by P2X7 receptors agonist probably results from its ability to induce the release of the pro-inflammatory cytokines, especially the chemokine CINC-1,

well known to induce chemotaxis and leukocytes activation (Ramos et al., 2003). It is important to point out that neutrophils, the most abundant circulating leukocyte subtype (Fiset et al., 2003), may also contribute to prostaglandin release (Cunha et al., 2008) and consequently, to the development of TMJ nociception induced by P2X7 receptors activation.

The TMJ is innervated with nerve endings, including unmyelinated C-fibres and thinly myelinated A-δ axons (Kido et al., 1995). The activation of C-fibres and A-δ nerve endings has been associated with nociceptive responses in the TMJ (Bellinger et al., 2007). Capsaicin given to neonatal rats or mice eliminates most C-fibres, but a majority of the A-δ fibres remain after capsaicin treatment (Nagy & van der Kooy, 1983). The present results demonstrated that BzATP was not able to induce TMJ nociception in rats neonatal-treated with Capsaicin. Therefore, the current study can suggest that the activation of peripheral P2X7 receptors induced TMJ nociception, which depends on the C-fibres activation in the TMJ tissue. Corroborating with this idea, the present study has shown that the activation of peripheral P2X7 receptors can induce the release of pro-inflammatory cytokines, which in turn can induce synthesis of PGE_2 and the release of sympathomimetic amines (Cunha et al., 1991, 1992; Ferreira et al., 1993), which can directly sensitize the unmyelinated C-fibres (Gold et al., 1996; Khasar et al., 1999; Rush & Waxman, 2004).

TRPV1 is a nonselective cation channel activated by Capsaicin, protons and heat (>43°C). It has been predominantly detected on the nerves terminals of unmyelinated C-fibres (Caterina & Julius, 2001) of the TMJ (Ioi et al., 2006) as well as in the synovial lining cells in both rat and human TMJs (Ioi et al., 2006; Sato et al., 2005). The activation of TRPV1 in sensory neurons induces the release of inflammatory neuropeptides that cause neurogenic inflammation and pain (Lin et al., 2007). The current study demonstrated that the BzATP-induced TMJ nociception depends on C-fibres activation. Therefore, it was investigated whether the activation of P2X7 receptors can modulate the TRPV1 expression in the rats' TMJ. The results demonstrate that P2X7 receptors activation induces an increase of TRPV1 expression in the periarticular tissue of TMJ, which was inhibited by the P2X7 receptor antagonist. Considering that TRPV1 has been strongly implicated in nociception and pain of TMJ (Park, 2015; Urtado et al., 2007; Wu et al., 2015), the present findings suggest that the increase of TRPV1 mediated by the activation of P2X7 receptors can contribute, at least in part, to BzATP-induced TMJ nociception.

In conclusion, the findings of the present study suggest that P2X7 receptors activation mediates TMJ nociception by an inflammatory mechanism that involves the release of pro-inflammatory cytokines and the subsequent prostaglandins and sympathomimetic amines, which ultimately sensitize the primary afferent nociceptors, such as C-fibres, in the TMJ tissue. Besides, after the P2X7 receptor activation, the expression of TRPV1 is increased in the TMJ, suggesting that its activation may contribute to the BzATP-induced TMJ nociception process. Therefore, the P2X7 receptors could be an important target for drug development for the treatment of the inflammatory pain symptoms in temporomandibular disorders.

CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

J.M.T., H.B.A, M.H.N, C.H.T, M.C.G.O.F and J.T.C.N contributed to the conception and designed the research study. J.M.T., R.M.P, H.B.A, H.M.X.S and C.G.M performed the experiments. J.M.T., H.B.A, M.H.N, C.H.T, M.C.G.O.F and J.T.C.N analysed and interpreted the data. J.M.T. and H.B.A wrote the manuscript. J.T.C.N revised the study critically for important intellectual content and approved the version to be published.

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