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Original Article

ETB receptor activation as a mechanism of modulation of inflammatory pain and neurogenic inflammation in the temporomandibular joint of capsaicin-treated rats

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Key Words
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Abstract

Background: Endothelin (ET), a peptide best known for its vascular effects, also evokes pain and hyperalgesia, independently of its vascular actions. Data suggest that ET can have nociceptive effects, acting directly on receptors expressed in sensory neurons. As such, the aim this study was to investigate the direct effect of ET on hyperalgesia and edema, induced by carrageenan, on the temporomandibular joint (TMJ) of capsaicin-treated rats.

Methods: Capsaicin was administered by subcutaneous injection to newborn, male Wistar rats. Inflammation was induced 60 days later by a single intra-articular injection of carrageenan into the left TMJ (control group received sterile saline). Inflammatory parameters, such as plasma extravasation, leukocyte influx and mechanical allodynia (measured as the head-withdrawal force threshold) were evaluated 4 h after edematogenic stimulus. ET-1 and ET-3, and the ET-B receptor (ETBR) antagonist were administered 3 min before edematogenic stimulus. ET and transient receptor potential vanilloid (TRPV1) mRNA expression was assessed by reverse-transcription polymerase chain reaction (RT-PCR). Edema formation was evaluated by measurement of the extravascular accumulation of injected ^{125}I-human serum albumin into the TMJ soft tissues of anesthetized rats.

Results: Capsaicin neonatal treatment significantly reduced edema formation, leukocyte influx and mechanical allodynia in TMJ, when compared to the control group, while the ETBR antagonist increased plasma extravasation and hyperalgesia in the capsaicin-treated group. ET-1 treatment reduced both plasma extravasation and myeloperoxidase activity. Capsular mRNA for ET-1 was significantly augmented in the TMJ of capsaicin-treated rats, when compared to controls.

Conclusions: Our results suggest, for the first time, that ET-1, via ETBR activation, reduces plasma extravasation, leukocyte influx and inflammatory pain in the temporomandibular joint of capsaicin-treated rats.

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INTRODUCTION

The nervous system contributes to the development of joint inflammation in rats [1]. Increased concentrations of the sensory neurotransmitters, substance P and endothelins (ETs), have been found in the synovial fluid of patients with various forms of inflammatory joint diseases [2, 3]. Endothelin, a peptide best known for its vascular effects, also evokes pain and hyperalgesia independently of its vascular actions. Several arguments suggest that ET, besides its potent involvement in the regulation of vascular tone, is a neurotransmitter/neuromodulator and can have direct, nociceptive effects on the peripheral sensory nervous system [4, 5].

Pharmacological studies have suggested that when ETs are released in peripheral tissues, they could act directly on ET-A receptor (ETAR)-expressing and on ETBR-expressing sensory neurons in dorsal root ganglia (DRG) satellite cells or non-myelinating Schwann cells. Furthermore, in peripheral tissues, ETAR expression may play a role in signaling acute or neuropathic pain, whereas ETBR expression may be involved in the transmission of chronic inflammatory pain [6, 7]. ET provides a strong stimulus for the release of neuropeptides involved in neurogenic inflammation, such as substance P, calcitonin gene-related peptide (CGRP) and catecholamines [7-9]; furthermore ET, acting through ET-B receptors, may play an important role in mediating neurogenic inflammation in the meninges of rats [10]. Moreover, ET-1 potentiation of cholinergic nerve-mediated contraction is mediated by tachykinin release, suggesting that, in addition to nerves and human inflammatory cells, macrophages [11] and T- and B-cells [12], several type of cells, such as airway smooth muscle cell and human dermal microvascular endothelial cells may participate in neuropeptide synthesis and release tachykinins under inflammatory conditions [11, 13, 14]. Furthermore, ET-1 and their ET-B and ET-A receptors have been reported to be present in the rat gastrointestinal tract [15] and ET-1 may act as a potent peptide agonist in the liver [16].

The objective of this study was, for the first time, to investigate the relationship between ETs and primary afferent neurons in neurogenic inflammation and inflammatory pain in the temporomandibular joint (TMJ) in capsaicin-treated rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (250-300 g) were housed in groups of five animals per cage. They received tap water and laboratory chow *ad libitum* and were maintained on a 12/12 h light/dark cycle in a temperature-controlled environment (23 ± 2ºC). All the experimental protocols were approved by the local ethics committee for animal eperimentation and performed in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and adhered to the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals [17]. All the experimental protocols were performed in animals under inhalatory anesthesia with halothane (1.5% v/v in oxygen) or after the intraperitoneal (i.p.) administration of a mixture of ketamine (80 mg/kg) and xylazine (20 mg/kg).

Induction of inflammation

Inflammation was induced by the unilateral intra-articular (i.art.) injection of 500 µg of carrageenan (10 µL of a 5% carrageenan solution in sterile saline) into the supra-discal space of the left TMJ (ipsilateral), using a microsyringe (Hamilton model 702RN; Hamilton Co, Reno, NV, USA) coupled to a 30G gingival needle (BD, Franklin Lakes, NJ, USA). As a control procedure, the same volume of vehicle was injected into the contralateral TMJ, with the exception of the mechanical allodynia experiments, where different experimental groups of animals were submitted to the administration of either carrageenan or vehicle [18].

Evaluation of mechanical allodynia

Mechanical allodynia in the TMJ was evaluated by measuring the threshold of force intensity (in g) needed to be applied to the TMJ region, until the occurrence of the reflex response of the animal (*e.g.* head-withdrawal). The measurements were performed by an examiner unaware of the treatments, making use of a digital device (Insight, Ribeirao Preto, SP, Brazil). Four to five animals were put into individual plastic cages 30 min before the beginning of the tests, and were submitted to a conditioning session of head-withdrawal threshold measurements in the test room during 2 consecutive days under controlled temperature and low illumination [18]. On the third day, the basal force threshold value was recorded before the i.art. injections of either carrageenan or vehicle. Measurement of force thresholds were performed (in triplicate) from both ipsi- and contralateral TMJs at 0, 1, 4 and 24 h, and the obtained values at each time-point were averaged. Occasional petting by the investigator ensured that the rat kept its position, thus allowing the uninterrupted determination of the responses in unrestrained animals.

Measurement of plasma extravasation

The amount of plasma extravasation was estimated by the extravascular accumulation of intravenously (i.v.) injected ^{125}I-BSA (0.0925 MBq/rat), 1 h prior to the end of the experimental period, as previously described [18, 19]. Rats were killed by cervical dislocation at the end of the period, and the amount of ^{125}I-BSA present in the dissected TMJs was determined by gamma counting (Cobra II, Packard BioScience, Dreieich, Germany). The recorded dpm values were related to the radioactivity present in a 0.1 ml plasma aliquot and divided by the corresponding TMJ weight. Plasma extravasation values obtained from the ipsilateral TMJ (carrageenan) were expressed as percentage increase, compared to the contralateral TMJ (vehicle).

Measurement of myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined in the cavity lavages as a marker of neutrophil accumulation. Rats were killed by cervical dislocation, the facial skin was excised and the temporal muscle overlying the TMJ was carefully dissected. A 30G needle (BD Ultra-

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Fine II insulin syringe, 0.3 ml) was inserted through the posterior membrane and the synovial cavity was washed by injecting and immediately aspirating 50 µl of heparinized saline solution (5 U/ml; Liquemine, Roche, Basel, Switzerland). The washing procedure was repeated, the collected fluids were pooled and immediately kept at –70°C until analysis. The pooled lavage fluids were diluted with phosphate buffer (pH 6) containing hexadecyl trimethylammonium bromide (HTAB; Sigma) and heated at 60°C for 2 h (in order to inactivate endogenous catalase). After centrifugation (12,000g for 2 min), MPO activity was measured in the supernatants according to a method previously described by Bradley *et al* [20]. Results are expressed as MPO units (U) per joint (1 U of MPO is defined as the amount of enzyme responsible for the degradation of 1 µmol of hydrogen peroxide/min at 25°C).

Ablation of sensory afferent neurons by neonatal capsaicin treatment and the effect of ET receptor agonism and antagonism on the susceptibility of TMJ plasma extravasation

Animals were treated on the 2nd day of life with capsaicin (30 mg/kg s.c.) or vehicle [10% ethanol and 10% Tween 80 in 0.9% (w/v) NaCl solution], as previously described [21, 22]. At adulthood (60 days), vehicle and capsaicin-pretreated rats were submitted to carrageenan injection. The effects of systemic administration of the endothelin ET-A and ET-B receptor agonist were achieved using ET-1 or ET-3 (0.5 nmol/kg, i.p.) and receptor blockade was achieved using selective ET-B receptor antagonist BQ-788 (0.1 mg/kg, i.v., 3 min prior to TMJs experiments, n = 5) [23].

ETs and transient receptor potential vanilloid (TRPV1) mRNA expression

Capsular ETs and TRPV1 mRNA expression was examined by reverse-transcription polymerase chain reaction (RT-PCR). Samples were collected from the corpus at the end of chamber experiments. The RNA was isolated using the TRIZOL method and extracted with chloroform after tissue homogenization (Gibco BRL, Gaithesburg, MD, USA). It was then recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in DEPC-treated water. cDNA was synthesized from 10 µg of total RNA using 1 µl of reverse transcriptase (M-MLV, Gibco BRL). cDNA samples were stored at –20°C until use. The nucleotide sequence of the primers for ET-1 and ET-3 were those previously reported in the literature [13, 24]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was the internal control for the PCR reaction. Forty cycles of PCR amplification for ET isoforms, and 33 for GAPDH were chosen following pilot experiments to define amplification conditions. PCR reactions were performed in a final volume of 25 µl containing 2.5 µl cDNA, 2.5 µl 10x Taq buffer, 0.75 µl $MgCl_2$ (1.5 mM), 0.5 µl dNTPs (0.2 mM), 1.5 µl (0.5 µM) of each oligonucleotide pair (**ET-1:** GCT CCT GCT CCT CCT TGA TG-sense, CTC GCT CTA TGT AAG TCA TGG-antisense; **ET-3:** GCT GGT GGA CTT TAT CTG TCC-sense, TTC TCG GGC TCA CAG TGA CC-antisense; and **TRPV1:** TCA TGG GTG AGA CCG TCA ACA AG-sense, TGG CTT AAG GGA TCC CGT ATA AT-antisense), 15.5 µl H_2O Milli-Q and 0.25 µl Taq DNA polymerase (1.25 U). The amplification cycle was carried out with denaturation for 1 min at 94°C, annealing for 45 sec at 57°C and extension for 1.5 min at 72°C for ET-1/ET-3 isoforms, respectively, 15.5 µl H_2O Milli-Q and 0.25 µl Taq DNA polymerase (1.25 U). The amplification cycle was performed with denaturation for 1 min at 94°C, annealing for 45 sec at 57°C and extension for 1.5 min at 72°C for ET-1/ET-3 isoforms and annealing for 45 sec at 60°C and extension for 1.5 min at 72°C for TRPV1. The PCR products (10 µl), previously normalized to provide equivalent amounts of the GAPDH control, were separated on 1.5% agarose gel containing 10% ethidium bromide. Gels were visualized under UV light and images captured (Chemimager 5500, Alpha Inotech, San Leandro, CA, USA). The band sizes for ET-1, ET-3 and TRPV1 were 471 and 477, 428 bp, respectively. Band densitometry was determined to compare the expression of each isoform relative to GAPDH.

Drugs/chemicals

All drugs were of analytical grade or obtained from Sigma (St. Louis, MO, USA), or Calbiochem (San Diego, CA, USA). Sodium pentobarbital (Hypnol®; Cristalia, Itapira, SP, Brazil), xylazine (Rompun®, Bayer) and ketamine (Ketolar®, Parke-Davis) were used as clinically available preparations. ^{125}I-BSA (18 MBq/ml in sterile saline solution) was supplied by IPEN (Sao Paulo, SP, Brazil).

Statistical analysis

Data are expressed as means ± SEM and comparisons among groups analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Statistical significance was considered for $P < 0.05$.

RESULTS

Protein plasma extravasation

Fig.1a shows a significant increase of plasma extravasation in the TMJ (ipsi) of the control group after i.art. injection of carrageenan ($P < 0.05$) at 4 h. In contrast, a significant decrease in plasma extravasation was observed in the ET-1-treated group, but not in the ET-3-treated group. Moreover, protein plasma

extravasation in the (ipsi) capsaicin-injected TMJ was significantly reduced to levels similar to those of the control, while no change was observed in the BQ-788-treated group (Fig.1b).

Leukocyte influx in the TMJ of capsaicin and ET-treated rats

MPO activity in the TMJ lavage fluids was significantly reduced in the capsaicin, ET-1- and ET-3-treated groups, when compared with the control group (Fig.2ab, respectively).

Time course of mechanical allodynia

The i.art. injection of carrageenan resulted in a time-dependent and long-lasting mechanical allodynia in the control group, as measured by a clear decrease in the mechanical threshold for head withdrawal; however, no significant changes in mechanical withdrawal thresholds were observed at any time-point in the capsaicin group (Fig.3ab, respectively). However, the BQ-788 intravenous injection restored the mean basal force thresholds for head withdrawal after i.art. injection of carrageenan in the capsaicin-treated group at the 1 and 4 time-points (Fig.3c).

Capsular ETs and TRPV1 mRNA expression

ET-1 mRNA expression was slightly augmented in capsular samples collected from the ipsi- and contralateral TMJs of capsaicin-treated rats, while ET-3 and TRPV1 mRNA expressions were reduced in comparison to control animals (Fig.4abc, respectively). Curiously, TRPV1 mRNA expression was increased in the contralateral capsular samples in vehicle-treated controls (Figure 4b).

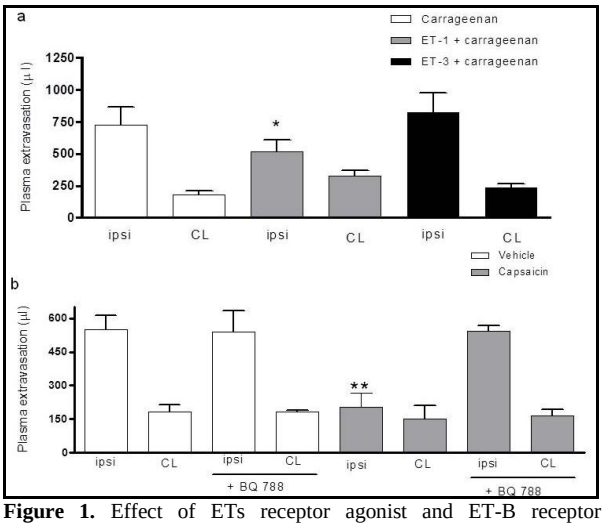

Figure 1. Effect of ETs receptor agonist and ET-B receptor antagonist and capsaicin neonatal treatments on TMJ inflammation induced by carrageenan. **(a)** At the 4 h time-point, ET-1 (0.5 nmol/kg, i.v.; n = 5) treatment caused a significant decrease ($P < 0.05$) in plasma extravasation in the TMJ (ipsi) of the control group after i.art. injection of carrageenan. This did not occur with ET-3 (0.5 nmol/kg, i.v; n = 5) treatment. **(b)** Capsaicin newborn treatment (50 mg/kg; n = 5) also significantly ($P < 0.01$) reduced protein plasma extravasation in the TMJ (ipsi) after i.art. injection of carrageenan. Asterisks denote significant difference, compared to vehicle-treated control rats (*****$P < 0.05$ and ******$P < 0.01$).

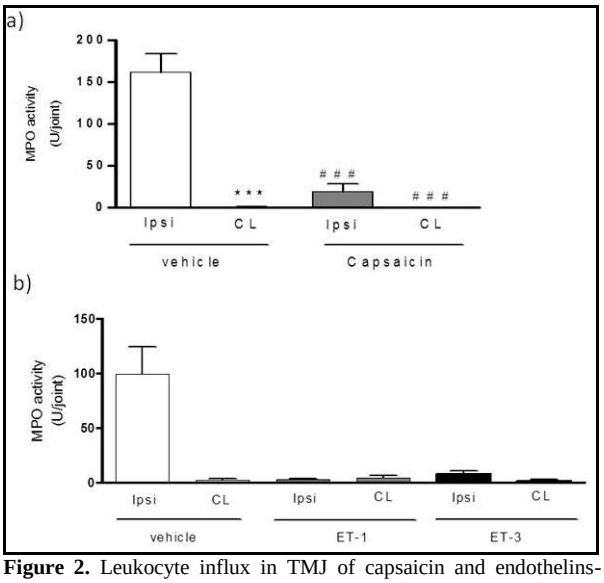

Figure 2. Leukocyte influx in TMJ of capsaicin and endothelins-treated rats. MPO activity in the TMJ lavage fluids was significantly reduced in **(a)** capsaicin and **(b)** ET-1 and ET-3-treated groups (n = 5) when compared to the vehicle-treated group. Number sign denote significant difference, compared to vehicle-treated control rats ($^{###}P < 0.001$).

DISCUSSION

The results of the present study show, for the first time, the involvement of neuropeptides, ETs and the ET-B receptor (ETBR) in mediating a reduced susceptibility to inflammation and neuropathic pain in the temporomandibular joint of capsaicin-treated rats.

Capsaicin is the active ingredient of the pungent *Capsicum* peppers and, in acute doses, activates the primary afferent nerves, whereas in higher doses given to neonatal animals it permanently ablates these nerves. This protein has proven remarkably useful over the past three decades as a pharmacological tool to explore the physiology of primary afferent nerves [25]. In a rat model, pretreatment with capsaicin and surgical denervation decreased the neuropeptide content in the trigeminal ganglia and the TMJ [3]. Moreover, our previous results strongly suggest the involvment of neuropeptides in control hemodynamic parameters [23, 26].

Our data show that capsaicin newborn treatment and ET-1 administration reduce protein plasma extravasation, MPO activity and mechanical allodynia, while increasing ETBR blockade. All three endothelin isoforms have similar affinities for the ETBR [27]. As such, we suggest that ET-1 via ETBR activation reduces plasma extravasation, leukocyte influx and inflammatory pain in the temporomandibular joint in capsaicin-treated rats.

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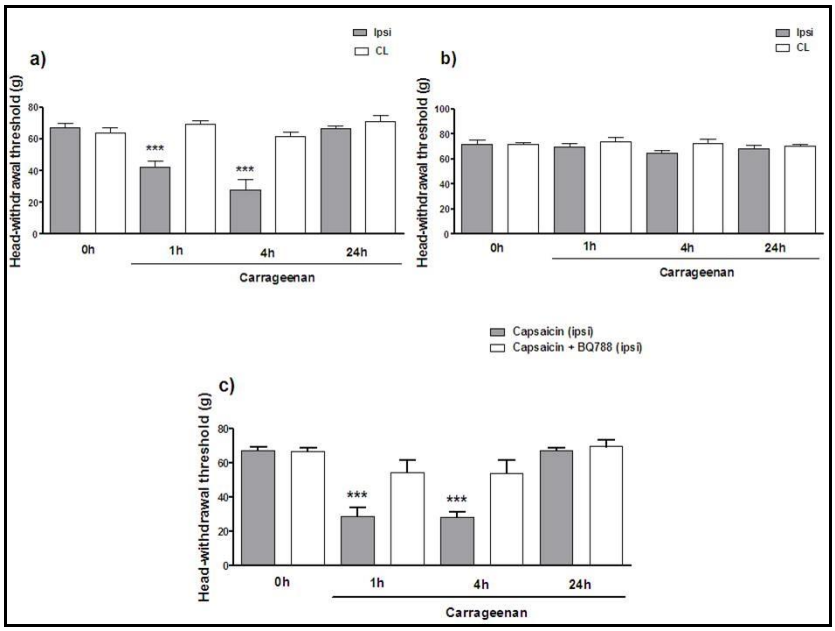

Figure 3.
Time course of mechanical allodynia.
(a) The i.art. injection of carrageenan resulted in a time-dependent and long-lasting mechanical allodynia in the control group (n = 5).
(b) No significant changes in mechanical withdrawal thresholds were observed at any time-point in the capsaicin group (n = 5).
(c) BQ-788 (0.1 mg/kg, i.v.; n = 5) restored the mean basal force thresholds for head withdrawal by i.art. injection of carrageenan in the capsaicin-treated group at the 1 and 4 h time-points.
Asterisks denote significant difference, compared to vehicle-treated control rats (***P < 0.001).

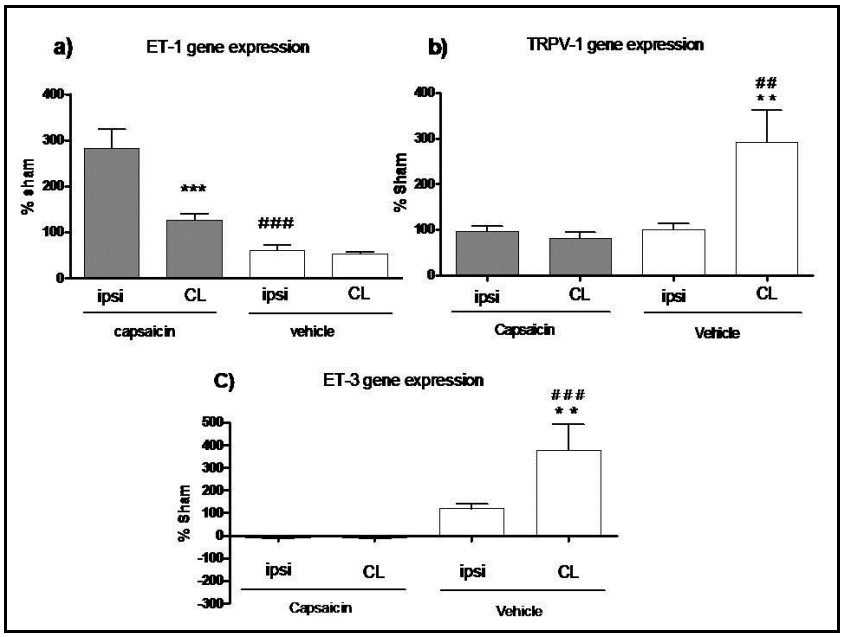

Figure 4.
Capsular ETs and TRPV1 mRNA expression.
(a) ET-1 mRNA expression was slightly (P < 0.001) augmented in capsular samples collected from ipsi- and contralateral TMJ of capsaicin-treated group (n = 5) compared to control animals (n = 5).
(b) TRPV1 mRNA expression was significantly (P < 0.01) increased in the contralateral capsular samples, compared to ipsi capsular samples in the vehicle-treated group (n = 5).
(c) ET-3 mRNA expression was significantly (P < 0.001) reduced in the capsaicin-treated group, in comparison to the control group (n = 5).

RT-PCR analysis showed a decreased level for TRPV1 and ET-3 mRNA in capsular homogenates obtained from capsaicinized rats compared to vehicle rats, while ET-1 mRNA was elevated in capsular homogenates of capsaicinized rats.

Taken together, these results suggest that the increased resistance of capsaicin-treated rats to inflammatory pain and injury is associated with increased ET-1 expression and a reduction in TRPV1 mRNA expression, which could enhance the defense mechanisms against injury, including reducing leukocyte influx responses to carrageenan, as observed in our experiments.

A previous study showed that a selective antagonist of ETBR, Ro 46-8443, but not a selective antagonist of ET-A receptors, BQ-123, was able to prevent plasma protein leakage in the dura mater. Furthermore, the effect of sarafoxin S6c was prevented by spantide, a selective antagonist of tachykinin receptors, suggesting that ETBR activation induces plasma protein extravasation, at least in part through the release of substance P from perivascular fibers [10].

ET provides a strong stimulus for the release of neuropeptides involved in neurogenic inflammation, such as substance P, CGRP and others [5, 10]. Furthermore, ET may play a role in the repair of

damaged neurons [14], and several pharmacological studies have suggested that ET-A and ET-B receptors are expressed in sensory neurons [11, 21, 28]. Moreover, the ET-1 isoform predominates in gastrointestinal systems [23, 29, 30], while the ET-3 isoform seems to predominate over ET-1 in neurons of the brain [14].

Clinical studies should evaluate the potential efficacy of an ETBR agonist and neurokinin 1 (NK1) receptor antagonist in the treatment of arthritis in the TMJ. Drugs that inhibit the release of multiple trigeminal neuropeptides (*e.g.* substance P, neurokinin A, ET-3, and CGRP) block both the tachykinin-induced plasma protein extravasation and CGRP-induced neurogenic vasodilatation components of neurogenic inflammation [1, 13, 21].

ET-1 promotes tachykinin release from nociceptive neurons, whereas vasodilatation is mainly caused by binding of released tachykinins (substance P) to their NK1 receptors on the endothelial cell of blood vessels in the TMJ [13, 21].

The availability of this family of drugs, able to prevent neurogenic inflammation without possessing vasoconstrictor activity, may help to reveal the relative contribution of neurogenic and vascular mechanisms in arthritis in the TMJ.

Our data suggest, for the first time, that the blockage of the ET-B receptor restores plasma extravasation and hyperalgesia, induced by carrageenan in the TMJ of capsaicin newborn treated rats. As such, we can suggest that interplay of neuropeptides and ETs, particularly ET-1, may participate in the regulatory mechanisms of inflammation and neurophatic pain.

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CONFLICT OF INTEREST

We declare that all financial and material support received for research and development work which resulted in the drafting of the manuscript in the text are clearly informed of the same.

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