Int. J. Oral Maxillofac. Surg. 2010; 39: 815–819
doi:10.1016/j.ijom.2010.03.013, available online at http://www.sciencedirect.com

International Journal of Oral & Maxillofacial Surgery

Research Paper
Oral Medicine/Facial Pain

Sensory purinergic receptor $P2X_3$ is elevated in burning mouth syndrome

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Abstract. Recent studies show that $P2X_3$ may play a role in neuropathic pain, including orofacial pain. Burning mouth syndrome (BMS) is a chronic neuropathic pain condition affecting 0.6–12% of post-menopausal women in the Western world. This study evaluates, for the first time, $P2X_3$ immunoreactivity levels in lingual mucosa in BMS patients. Patients diagnosed with BMS ($n = 9$) in accordance with International Association for the Study of Pain criteria and patients attending for wisdom tooth removal ($n = 10$, controls), were involved in this study. A pain history and score was recorded on a visual analogue scale (VAS) prior to obtaining a lingual biopsy. Immunohistochemistry and image analysis were used to quantify submucosal nerve fibres expressing $P2X_3$ and the structural marker neurofilaments. $P2X_3$ positive fibres were significantly increased in BMS compared with controls ($p = 0.024$). In contrast, neurofilament-staining fibres were reduced in BMS, and when expressed as a ratio of the neurofilament percentage area, there was a trend for an increase of $P2X_3$ positive fibres in the BMS group. Increased $P2X_3$ immunoreactivity in the trigeminal sensory system may play a role in the symptoms observed in BMS. $P2X_3$ may therefore be a therapeutic target for treating BMS and trigeminal neuropathic pain.

Keywords: $P2X_3$; burning mouth syndrome; purinergic receptors; neuropathic pain; facial pain.

Accepted for publication 16 March 2010
Available online 24 April 2010

Burning mouth syndrome (BMS) is a chronic and intractable pain condition which predominantly affects post-menopausal women in their fifth to seventh decade. Of the middle aged to elderly female group, 0.6–12% of that population seems to be affected[1], and up to a million people in the USA suffer from BMS[26]. The International Association for the Study of Pain (IASP) has identified BMS as a distinct neuropathic orofacial pain condition characterized by bilateral burning oral mucosal pain, usually affecting the anterior two-thirds of the tongue that may comply with the anatomy of peripheral nerves, lacking any visible signs of mucosal pathology, and usually lasting for more than 6 months[6]. The pain intensity ranges from moderate to severe throughout the day, and may last several years[18,19]. The onset of symptoms can be spontaneous, and sometimes associated with systemic factors such as diabetes, nutritional deficiencies, hormonal changes, psychological disorders, and local causes including oral infections, allergies, salivary gland dysfunction, salivary component changes and dental treatment[17]. Previously, BMS was thought to be psychogenic in origin, but there is growing evidence to show that it is a neuropathic pain disorder. There is little understanding of the underlying molecular mechanisms in BMS: in this study the authors have investigated the potential role of the $P2X_3$ receptor.

P2X receptors belong to a large family of purinergic receptors. These purinoceptors can be classified into two groups: the

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ligand-gated ion channels known as P2X receptors and the G-protein-coupled receptors called P2Y receptors[30]. Six of the seven receptors of the P2X family are located on primary sensory neurons[11,23]. Of the seven currently cloned members of the P2X family, one receptor, the $P2X_3$ receptor, is expressed in a subset of predominantly small neurons in sensory ganglia[8,25].

Previous studies have reported P2X family channels in the trigeminal system. $P2X_2$ receptors have been detected in sensory ganglia and their central nerve terminals, in some areas co-localizing with $P2X_3$ receptors[37]. In a study of nociceptive versus non-nociceptive neurons in the rat dorsal root ganglion (DRG), Ueno et al.[36] found that capsaicin-sensitive (nociceptive) small-sized DRG neurons expressed mainly the homomeric $P2X_3$ subunit, but that capsaicin-insensitive (non-nociceptive) medium-sized DRG neurons expressed the heteromultimeric $P2X_2$ and $P2X_3$ receptor. The rodent pulp contains both capsaicin-sensitive[22] and capsaicin-insensitive neurons[15]. Within the trigeminal system, $P2X_3$ receptor immunoreactivity has been shown in rat dental pulp[12]. In human tissues, this receptor has been reported in the innervation of the heart, intestine, bladder and DRG[16,39]. In the human trigeminal system, $P2X_3$ immunoreactivity has been demonstrated in dental pulp[31]. To the authors' knowledge, the presence of $P2X_3$ in human oral mucosa has not been shown previously.

Burnstock[7] demonstrated the release of ATP from peripheral nerves, and its action as a neurotransmitter. It is present in many different classes of neurons, and on release, produces receptor mediated post-synaptic effects[5]. Earlier studies showed that ATP, when applied intradermally to humans, causes pain[2]. Based on these findings, it was proposed that ATP can initiate pain by acting on purinoceptors expressed by nociceptive nerve terminals[7]. Since then, the presence of $P2X_3$ in sensory ganglia has been confirmed by immunocytochemical studies[27,37], and a growing body of evidence has shown that ATP can activate peripheral nociceptors[12]. Recent studies have reinforced the association of ATP release and hyperalgesic states in human skin[20].

The potential mechanisms underlying the symptoms of BMS have been studied in intraoral biopsies[24]. The authors evaluated local nociceptor expression in tongue biopsy samples from patients with BMS, and showed a significant increase in sub-epithelial transient receptor potential Vanilloid-1 (TRPV1) positive nerve fibres, whereas intra-epithelial nerve fibres were decreased, indicating a neuropathic process[40]. This has led to an interest in other key ion channels involved in pain perception: this study aimed to investigate $P2X_3$ immunoreactive nerve fibres in the lingual mucosa of patients with BMS.

Methods

Patients diagnosed with chronic BMS ($n = 9$) in accordance with IASP criteria[41] and those attending for routine lower wisdom teeth removal under local anaesthesia (control, $n = 10$) were invited to join this study in accordance with the North East London Ethics Committee guidelines. An effort was made to age- and sex-match the BMS patients with controls, but although the age ranges overlapped, BMS subjects were older than the controls. The mean ages for the controls and the BMS patients were 40.8 years (M:F = 6:4) and 62.4 years (M:F = 3:6), respectively.

Each patient was asked to describe any altered sensation, including paraesthesia, anaesthesia, hyperaesthesia or dysaesthesia (spontaneous or evoked mechanical or thermal allodynia). A visual analogue scale (VAS) estimation of the patients' pain state of their lingual mucosa was recorded at rest. All patients were asked to report the degree of pain using a VAS from 0 (no pain) to 10 (worst pain imaginable).

For the tongue biopsies, a punch biopsy technique was used (disposable punch 3 mm × 3 mm Steifel CE 0120, Steifel Laboratories Ltd., Bucks, UK) under local anaesthesia, and all specimens were obtained from the dorsal lingual mucosa, lateral to the midline in the anterior third. All BMS patients had pain at the site of the biopsy. Control patients had a lingual biopsy taken with no additional local anaesthesia other than their requirement for planned lower wisdom teeth surgery. None of the patients attending for wisdom tooth removal were in any pain as they were between episodes of symptoms due to pericoronitis.

The mucosal biopsies were immediately placed in liquid nitrogen, and subsequently transferred to −70 °C storage until used for immunohistochemical analysis.

A commercial polyclonal antibody to $P2X_3$ (RA10109, Neuromics Antibodies, MN, USA) was used in this study.

Tissues were supported in optimum cutting tissue (OCT) medium (RALamb Ltd., Eastbourne, UK) to allow for best orientation. Frozen sections (12 μm) were collected onto poly-L-lysine (Sigma, Poole, UK) coated glass slides and post-fixed in freshly prepared, 4% (w/v) paraformaldehyde in 0.15 M phosphate buffered saline (PBS) for 30 min. Endogenous peroxidase was blocked by incubation in industrial methylated spirit containing 0.3% (w/v) hydrogen peroxide for 20 min. After rehydration, four successive sections were incubated overnight with primary antibody. Sites of primary antibody attachment were revealed using nickel-enhanced, avidin-biotin peroxidase (ABC Vector Laboratories, Peterborough, UK). Omission of primary antibodies and sequential dilution of antibodies gave appropriate results. Sections were counter-stained for nuclei in 0.1% (w/v) aqueous neutral red and mounted in xylene-based mountant (DPX; BDH/Merck, Poole, UK), prior to analysis.

Computerised image analysis was performed to quantify immunoreactivity. Images were captured using an Olympus DP70 camera mounted to an Olympus BX50 microscope and analysed using analySIS (version 5.0) software. Positive immunostaining was highlighted by setting the grey-level detection limits to threshold and the area of highlighted immunoreactivity obtained as percentage area of the field scanned. The entire sub-epithelial region of the section was analysed at the same magnification of ×40. Results are presented as mean ± standard error of the mean (SEM). The Mann–Whitney test was used for statistical analysis (p-values < 0.05 were considered statistically significant). Linear regression analysis was carried out to assess the relationship between VAS scores and the % $P2X_3$ immunoreactive area.

Results

Numerous $P2X_3$ nerve fibres were seen in all the specimens examined, they entered and traversed the papillae, but were not seen in epithelium. The percentage immunoreactive area of these $P2X_3$ fibres was significantly increased in the BMS tongue (Fig. 1B) compared with the controls (Fig. 1A). The mean ± SEM of the percentage area was, control 0.96 ± 0.30 and BMS 2.51 ± 0.61 (Fig. 2). $P2X_3$ fibres were also sometimes seen in deeper muscle layers, but were not included in the image analysis. The range of VAS scores at rest in the BMS group was 2–10, with a mean of 6.27. There was no significant correlation between percentage $P2X_3$ immunoreactive area and VAS scores ($R^2 = 0.0313$).

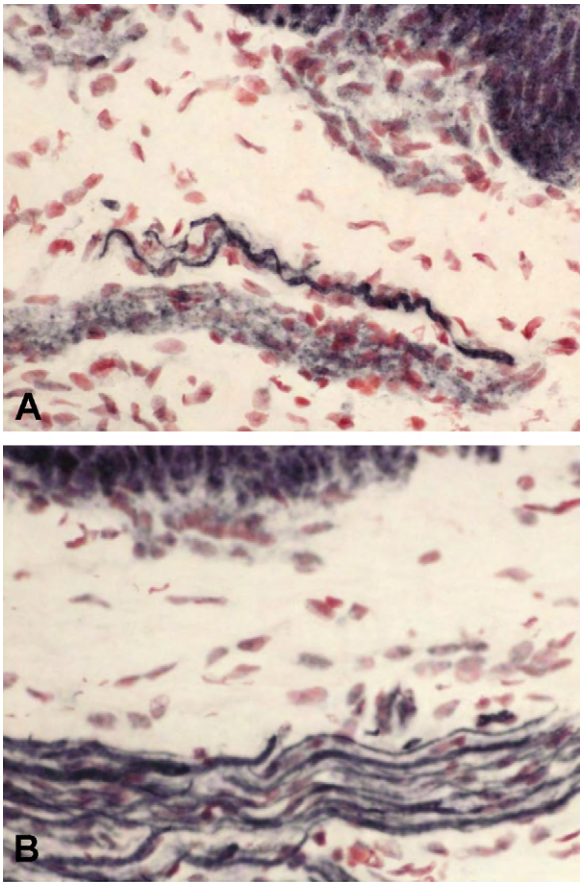

Fig. 1. P2X$_3$ immunoreactive fibre staining in a control tongue biopsy (A) and from a patient with BMS (B), magnification ×40.

Discussion

Burning mouth syndrome (BMS) is a relatively common chronic pain condition, but there is little understanding of the underlying molecular mechanisms. In this study the authors report, for the first time, an increase in P2X$_3$ immunoreactive nerve fibres in lingual biopsies taken from BMS patients. The authors have previously reported similar findings with respect to the heat and capsaicin receptor, transient receptor potential Vanilloid-1 (TRPV1)[40].

The examination of lingual biopsies from BMS patients followed previous studies of skin biopsies in human sensory neuropathies, which showed a reduction in epidermal nerves in a variety of painful or small nerve fibre neuropathies[9,28,29,32]. The authors and others have shown similarly reduced intra-epithelial neurofilament fibres in BMS tongue, indicating that it is a neuropathic disorder[40].

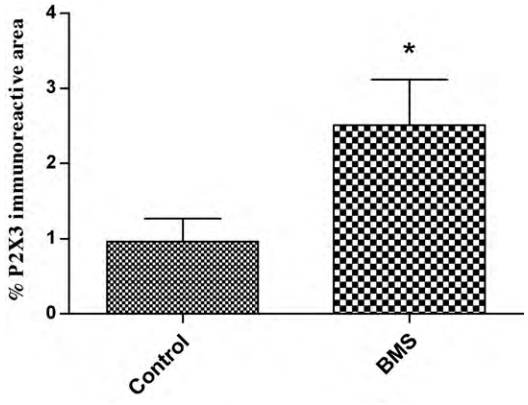

Fig. 2. The mean percentage P2X$_3$ immunoreactive area as a result of image analysis, $^{*}p = 0.024$.

Small-diameter nociceptive neurons have been divided into two categories on the basis of: the growth factors to which they respond; the neural markers they express; and their central projections. One group responds to nerve growth factor (NGF), has the NGF receptor tyrosine kinase TrkA, expresses the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), and terminates in laminae I and outer II of the spinal dorsal horn. The other 'non-peptide' population responds to glial cell-line-derived neurotrophic factor (GDNF), contains the enzyme fluoride-resistant acid phosphatase (FRAP), binds the isolectin B4 (IB4) of the plant lectin *Griffonia simplicifolia*, and terminates in the inner part of dorsal horn lamina II. These two groups of nociceptors are thought to play different roles[34]. They may be activated by different stimuli, may be responsible for different types of hyperalgesia, and may ultimately respond to different therapeutic drugs. In rat DRG neurons most (94–98%) P2X$_3$ immunoreactive (P2X$_3$-IR) nerves are IB4-binding, respond to GDNF, and terminate in the inner lamina II of the spinal cord, indicating that most P2X$_3$ containing neurons belong to the second 'non-peptide' population of nociceptors[4]. These findings have been confirmed in a study of postmortem human DRG neurons[39]. Other studies have shown an overlap of up to 59% between peptidergic calcitonin gene-related peptide immunoreactive (CGRP-IR) and non-peptidergic (IB4-binding) neurons in the DRG[38]. It was reported recently that up to 30% of IB4-binding cells in the trigeminal ganglion are not P2X$_3$-IR, indicating that there may be differences between the trigeminal and DRG neurons[13]. TRPV1 is expressed by the majority of nociceptors, and is increased in BMS[40]. Further studies of ATP receptor expression and co-localisation are needed in the human trigeminal system to elucidate the specific role played in the mediation of symptoms in BMS.

A useful model for studying the molecular mechanisms involves culturing trigeminal neurons. In this model, CGRP was found persistently and selectively to up-regulate the membrane expression and activity of P2X$_3$ receptors[14]. These P2X$_3$ receptors are gated by extracellular ATP and transmit nociceptive stimuli to brainstem trigeminal nuclei on activation[10,35]. Continuous activation of the

$P2X_3$ receptors can result in long-term sensitisation of $P2X_3$ receptor function via increased receptor trafficking and neosynthesis.

To conclude, increased $P2X_3$ immunoreactivity in the trigeminal sensory system may play a pivotal role in developing and maintaining symptoms in BMS, and may be a therapeutic target for treating trigeminal neuropathic pain.

Funding

AstraZeneca and Central London University Fund.

Competing interests

None declared.

Ethical approval

All work in this study has been approved by the North East London Ethics Committee. All subjects gave informed consent to the work involved in this study.

Acknowledgements. The authors gratefully thank all the patients involved in this study, and thank AstraZeneca for supporting the tissue studies.

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