

Research Paper
Oral Medicine/Facial Pain

Sensory purinergic receptor P2X₃ is elevated in burning mouth syndrome

Kiran Beneng¹, Zehra Yilmaz¹,
Yiangos Yiangou², Helen McParland¹,
Praveen Anand², Tara Renton¹

¹Kings College London, Dental Institute, Oral Surgery Department, Guy's Hospital, London, UK; ²Peripheral Neuropathy Unit, Hammersmith Hospital, Imperial College London, London, UK

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Abstract. Recent studies show that P2X₃ 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, P2X₃ 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 P2X₃ and the structural marker neurofilaments. P2X₃ 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 P2X₃ positive fibres in the BMS group. Increased P2X₃ immunoreactivity in the trigeminal sensory system may play a role in the symptoms observed in BMS. P2X₃ may therefore be a therapeutic target for treating BMS and trigeminal neuropathic pain.

Keywords: P2X₃; burning mouth syndrome; purinergic receptors; neuropathic pain; facial pain.

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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 affected¹, and up to a million people in the USA suffer from BMS²⁶. 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 affect-

ing 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 months⁶. 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 treatment¹⁷. 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 P2X₃ receptor.

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

ligand-gated ion channels known as P2X receptors and the G-protein-coupled receptors called P2Y receptors³⁰. 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 P2X₃ 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. P2X₂ receptors have been detected in sensory ganglia and their central nerve terminals, in some areas co-localizing with P2X₃ receptors³⁷. 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 P2X₃ subunit, but that capsaicin-insensitive (non-nociceptive) medium-sized DRG neurons expressed the heteromultimeric P2X₂ and P2X₃ receptor. The rodent pulp contains both capsaicin-sensitive²² and capsaicin-insensitive neurons¹⁵. Within the trigeminal system, P2X₃ receptor immunoreactivity has been shown in rat dental pulp¹². 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, P2X₃ immunoreactivity has been demonstrated in dental pulp³¹. To the authors' knowledge, the presence of P2X₃ in human oral mucosa has not been shown previously.

BURNSTOCK⁷ 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 effects⁵. Earlier studies showed that ATP, when applied intradermally to humans, causes pain². Based on these findings, it was proposed that ATP can initiate pain by acting on purinoceptors expressed by nociceptive nerve terminals⁷. Since then, the presence of P2X₃ in sensory ganglia has been confirmed by immunocytochemical studies^{27,37}, and a growing body of evidence has shown that ATP can activate peripheral nociceptors¹². Recent studies have reinforced the association of ATP release and hyperalgesic states in human skin²⁰.

The potential mechanisms underlying the symptoms of BMS have been studied in intraoral biopsies²⁴. The authors evaluated local nociceptor expression in tongue biopsy samples from patients with BMS, and showed a significant increase in sub-epithelial transient receptor poten-

tial Vanilloid-1 (TRPV1) positive nerve fibres, whereas intra-epithelial nerve fibres were decreased, indicating a neuropathic process⁴⁰. This has led to an interest in other key ion channels involved in pain perception: this study aimed to investigate P2X₃ immunoreactive nerve fibres in the lingual mucosa of patients with BMS.

Methods

Patients diagnosed with chronic BMS ($n = 9$) in accordance with IASP criteria⁴¹ 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 P2X₃ (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 col-

lected 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 $\times 40$. Results are presented as mean \pm 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 % P2X₃ immunoreactive area.

Results

Numerous P2X₃ 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 P2X₃ fibres was significantly increased in the BMS tongue (Fig. 1B) compared with the controls (Fig. 1A). The mean \pm SEM of the percentage area was, control 0.96 ± 0.30 and BMS 2.51 ± 0.61 (Fig. 2). P2X₃ 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 P2X₃ immunoreactive area and VAS scores ($R^2 = 0.0313$).

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