Clinical study

Burning mouth syndrome as a trigeminal small fibre neuropathy: Increased heat and capsaicin receptor TRPV1 in nerve fibres correlates with pain score

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Abstract

Burning mouth syndrome (BMS) is often an idiopathic chronic and intractable pain condition, affecting 1.5–5.5% of middle-aged and elderly women. We have studied the heat and capsaicin receptor TRPV1, and its regulator nerve growth factor (NGF), in BMS. Patients with BMS ($n = 10$) and controls ($n = 10$) were assessed for baseline and post-topical capsaicin pain scores, and their tongue biopsies immunostained for TRPV1, NGF, and structural nerve markers neurofilament and peripherin. Nerve fibres penetrating the epithelium were less abundant in BMS ($p < 0.0001$), indicating a small fibre neuropathy. TRPV1-positive fibres were overall significantly increased in BMS ($p = 0.0011$), as were NGF fibres ($p < 0.0001$) and basal epithelial cell NGF staining ($p < 0.0147$). There was a significant correlation between the baseline pain score and TRPV1 ($p = 0.0143$) and NGF fibres ($p = 0.0252$). A significant correlation was observed between baseline and post-capsaicin pain ($p = 0.0006$). Selective TRPV1 and NGF blockers may provide a new therapy for BMS.
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1. Introduction

Burning mouth syndrome (BMS) is a chronic and intractable pain condition, which is most common in middle-aged and elderly women, affecting 1.5–5.5% of this population.[1,2] The International Association for the Study of Pain (IASP) has identified BMS as a "distinctive nosological entity" characterized by "unremitting oral burning or similar pain in the absence of detectable oral mucosa changes."[3] The pain is usually of moderate to severe intensity, may vary during the day, and last several years. The onset may be spontaneous, or be associated with drug use, dental treatment or viral infections.

The aetiology of BMS is uncertain, and a number of psychogenic and organic mechanisms have been postulated in idiopathic cases, which include: a disorder of autonomic innervation and of oral blood flow;[4] sensory dysfunction associated with a small, and/or large, fibre sensory neuropathy;[5] disruption in sensory pathways driven by changes in endocrine status at menopause,[6] or a disruption of central sensory and modulatory pathways, including the spinal trigeminal nucleus and the striatum.[7] One study reports small sensory fibre dysfunction in 76% of 46 BMS patients when tested using quantitative sensory testing (QST).[5] Loss of nerve fibres have been reported in lingual mucosa affected by BMS, suggesting a neuropathic process, in at least some patients.[5,8,9]

Recent studies have pointed to dysfunction of several cranial nerves associated with taste sensation as a possible cause of BMS, and have demonstrated significant alterations in heat pain tolerance[10] and elevated sensory and pain thresholds to argon laser stimulation[11] in BMS patients. Alterations in taste occur in as many as two-thirds of patients, and often include complaints of persistent tastes (bitter, metallic or both), or changes in the intensity of taste perception. Dysgeusic tastes accompanying oral burning are often reduced by stimulation with food.[12–14] Some authors[15] state that there is an increased prevalence of so-called 'supertasters' (people with enhanced abilities to detect taste) among patients with BMS. These supertasters may be more likely to be affected by burning pain syndrome because of their higher density of taste buds, each of which is surrounded by a basket-like collection of fibres from the trigeminal nerve.[16]

At present there is no satisfactory treatment for the idiopathic orofacial pain conditions,[17] and attempts are made to manage the intractable pain using a variety of antidepressants, anticonvulsants or other drug combinations, all of which are ineffective and have undesirable side-effects.

The transient receptor potential (TRP) protein superfamily is a diverse group of voltage-independent calcium-permeable cation channels expressed in mammalian cells. They are structurally related to the transient receptor potential channels first described in Drosophila. Mammalian TRP channels have been organized into six subfamilies based on protein sequence identity. These have been designated C (canonical), V (vanilloid receptor), M (melastatin), A (ANKTM), P (polycystin), and ML (mucolipin). Most mammalian cells express a number of TRP channel family members, since many TRP channels are ubiquitously expressed and most have splice variants. Mammalian isoforms have six putative transmembrane domains similar to the structure of many pore-forming subunits of voltage-gated channels. The cloning of heat-activated TRPV1 (vanilloid receptor-1) was first among a group of six temperature-activated TRP ion channels. There are four heat-activated channels (TRPV1–4) and two cold-activated channels (TRPM8 and TRPA1). The founding member, TRPV1, also known as the capsaicin receptor or vanilloid receptor 1, is the most thoroughly studied channel among TRPV channels. TRPV1 agonists include capsaicin and resiniferatoxin, heat, H^+, endocannabinoid lipids such as anandamide, eicosanoids, and 2-APB. TRPV1 is widely expressed, but its function has been mostly studied in sensory neurons, in which it was first identified.[18,19] TRPV1-null mice lack vanilloid sensitivity in nociceptive, inflammatory, and thermoregulatory models. There is strong evidence for a crucial role for TRPV1 in thermal hyperalgesia.[20,21]

Several recent studies of patients with chronic pain have shown that receptors and ion channels expressed by sensory neurons are up-regulated in skin or mucosa in pain and hypersensitivity states. TRPV1 upregulation is associated with rectal hypersensitivity,[22] inflammation of the bowel,[23] vulvodynia[24] and overactive bladder.[25,26] The increased TRPV1 fibres in rectal tissues correlated with increased thermal and mechanical sensitivity measured clinically before harvesting the tissues.[22] Voltage-gated sodium channels also play key roles in the pathophysiology of pain.[27] The distribution and pathophysiology of these channels, particularly $Na_v1.8$, have been the focus of research in pain mechanisms, and recently antisense treatment blocking expression of this channel reportedly reduced neuropathic pain.[28] We have previously described the temporal and spatial distribution of $Na_v1.8$ in human sensory neurones;[29] TRPV1 and $Na_v1.8$ are expressed in the dental pulp and lingual nerve.[30,31] Both TRPV1 and $Na_v1.8$ are known to be regulated by nerve growth factor (NGF).[32] These potential pathological processes have not yet been explored in the human mucosa in orofacial pain conditions.

The aim of this investigation was to determine whether two key mediators of pain, TRPV1 and $Na_v1.8$, and their regulator NGF, were altered in the tongue in BMS, and if any changes correlated with pain or capsaicin sensitivity. Our hypothesis was that NGF increase in epithelium, or relatively increased NGF uptake in fewer (spared) nerve fibres, may lead to over-expression of TRPV1 and $Na_v1.8$, which in turn may contribute to BMS symptomatology.

2. Methods

2.1. Subjects

Patients attending the Royal London Dental School, Queen Mary University, London for wisdom tooth removal under local analgesia, and patients diagnosed with chronic BMS in accordance with IASP criteria[17] were invited to join this study in accordance with the North East London Ethics Committee guidelines. Prior to the lingual biopsy the patients underwent simple visual analogue score (VAS) estimation of the pain state of their lingual mucosa, followed by evaluation of mucosal hypersensitivity and completion of a questionnaire to assess anxiety and depression scores (HADS, Hospital Anxiety and Depression Score). All tests and biopsies were undertaken on the dorsal lingual mucosa lateral to the midline in the anterior third.

2.2. Pain assessment

Each patient with BMS was asked to describe any altered sensation, including paraesthesia, anaesthesia, hyperaesthesia or dysaesthesia (spontaneous or evoked mechanical or thermal allodynia). All patients, including those in the control group who were undergoing lower wisdom tooth removal under local anaesthesia, were asked to report the degree of pain using a VA) from 0 (no pain) to 10 (worst pain imaginable) at: 1) baseline (on-going or spontaneous) pain, and 2) on stimulation with capsaicin ($\geqslant 95\%$ pure capsicum extract diluted 1:10 000; Sigma-Aldrich, Dorset, UK) on a pledget of cotton wool.

2.3. Tongue biopsies

Segments of snap-frozen human tongue (lingual dorsal mucosa 1–2 cm from the tip of the tongue) were obtained from control subjects ($n = 10$) and patients with BMS ($n = 10$), with ethical approval (North East London), at the Royal London Hospital. All BMS patients had pain at the site of the biopsy. This was an additional procedure in the control subjects undergoing planned wisdom tooth surgery, requiring no additional local analgesia. All the subjects attending for wisdom tooth removal were in no pain as they were in between-episodes of pain due to pericoronitis. 'Control' patients in pain were excluded from the study. A punch biopsy was used (disposable punch 3×3 mm Steifel CE 0120, Steifel Laboratories Ltd., Bucks, UK) under local analgesia (routinely used 4.4 mL of xylocaine for inferior alveolar block and buccal infiltration). Suturing of the biopsy site was not necessary. The mucosal biopsies were immediately placed on a sterile suture card, then in liquid nitrogen, and subsequently transferred to −70 °C storage until used for immunohistochemical analysis.

2.4. Antibodies

A list of primary antibodies used in this study is shown in Table 1, and were used as described previously.[33] Because peripherin and neurofilament antibodies label distinct subpopulations of neurons in dorsal root ganglia (DRG)[34] in order to maximise detection of "total large and small sensory fibres" in our frozen sections, the antibodies to the light (peripherin) and heavy subunits of neurofilaments were combined in a cocktail.

2.5. Immunohistochemistry

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 mol phosphate buffered saline (PBS) for 30 min. Endogenous peroxidase was blocked by incubation in industrial methylated spirits containing 0.3% w/v hydrogen peroxide for 20 min. After rehydration, four successive sections were incubated overnight with primary antibody

(Table 1). Sites of primary antibody attachment were revealed using nickel-enhanced, avidin-biotin peroxidase (ABC – Vector Laboratories, Peterborough, UK) as previously described.[35] For specificity studies (TRPV1, NGF and $Na_v1.8$), experiments were performed with the primary antibody incubated with antigenic peptide for 2 h prior to incubation with the sections. Omission of primary antibodies and sequential dilution of antibodies gave appropriate results. Peptide was not available for immunoabsorption specificity studies for the neurofilament/peripherin cocktail, but these are well characterised; for other antibodies immunoabsorption controls were successfully performed. 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. Epithelial neurofilaments/peripherin fibres were counted for each section and results expressed as epithelial nerve fibres/papilla.

2.6. Image 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 (V5.0) 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. For the nerve markers (neurofilaments/periperin cocktail, TRPV1, NGF or $Na_v1.8$), the entire sub-epithelial region of the section was analysed, excluding muscle; up to five fields per tissue section were scanned at the same magnification of ×40, depending on the size of the section. The section values were used to calculate the mean value. The latter was used in subsequent statistical group analyses, and results presented as mean ± standard error of the mean (SEM). For the NGF in basal epithelial cells, the region was highlighted and scanned. The Mann-Whitney test was used for statistical analysis (p-values <0.05 were considered statistically significant). The ratio between TRPV1 and neurofilaments/periperin cocktail in serial tissue sections was calculated.

The results of immunohistochemistry were correlated with baseline (on-going) VAS pain score, and VAS pain score following topical capsaicin application using Spearman's correlation, including all specimens studied.

Table 1
List of primary antibodies and the dilutions used in this study

Antibody	Host	Source: Ref. no.	Titre
NGF	Rabbit	Genentech Inc., San Francisco, CA, USA, 12756/71	1:2000
$Na_v1.8$	Rabbit	GSK, Harlow, UK/K107	1:200
TRPV1	Rabbit	GSK/C22	1:10 000
Neurofilament Phosphorylated and non-phosphorylated 200 kDa subunit	Mouse	Dako Cytomation, Ely, UK: Clone N52	1:100 000
Neurofilament Phosphorylated 200 kDa and 70 kDa subunits	Mouse	Dako Cytomation, Ely, UK: Clone 2F11	1:10 000
Peripherin	Mouse	Novacastra, Newcastle upon Tyne, UK: Clone PJM50	1:500

NGF, nerve growth factor; $Na_v1.8$, voltage-gated sodium channel 1.8; TRPV, transient receptor potential (vallinoid 1).

3. Results

3.1. Neurofilaments/peripherin

The distribution of nerve fibres were as described previously in normal tongue biopsies, with nerve bundles observed in deeper layers, numerous fibres in the sub-epithelial region, and a few fibres in the epithelium. Strong staining of nerve fibres was apparent with the cocktail of neurofilaments/peripherin antibodies in all the samples examined (Fig. 1), particularly in the sub-epithelial region. Some fibres penetrated the epithelium and these were significantly reduced in BMS compared to controls. The mean ± SEM of the epithelial fibres/papilla were: control, 0.92 ± 0.19; and BMS, 0.27 ± 0.04 (Fig. 1 insets and bottom panel). Neurofilaments/peripherin fibres were often observed within the papilla: these were not significantly changed in BMS tissue. The mean ± SEM of the sub-epithelial fibres were: control, 7.27 ± 0.63; and BMS, 8.47 ± 0.63).

3.2. TRPV1

Numerous TRPV1 nerve fibres were seen in all the specimens examined and these entered and traversed the papillae, but were not seen in epithelium (Fig. 2). The percentage immunoreactive area of these TRPV1 fibres was significantly increased in the BMS tongue compared to controls. The mean ± SEM of the percentage area was: control, 0.54 ± 0.13; and BMS, 1.61 ± 0.24 (Fig. 2, bottom panel). There was also a significant increase when the results were expressed as a ratio of the neurofilament/peripherin percentage area ($p = 0.0047$). TRPV1 fibres were relatively sparse in deeper muscle layers, which were not included in the image analysis. The TRPV1 fibres were abolished when the primary antibody was incubated with TRPV antigenic peptide.

3.3. Na_v1.8

Like the other nerve markers, strongly immunoreactive $Na_v1.8$ fibres were seen in bundles and as scattered fibres in the sub-epithelial region. There was a tendency for an increase of these fibres in BMS compared to controls, but this was not statistically significant. The mean ± SEM of the percentage area was: control, 3.03 ± 0.72; and BMS, 4.52 ± 0.84 ($p = 0.077$). The $Na_v1.8$ fibre staining was abolished when the primary antibody was incubated with $Na_v1.8$ antigenic peptide.

3.4. Nerve growth factor

Strongly immunoreactive NGF fibres were seen in bundles, and occasionally as scattered fibres in the sub-epithelial region (Fig. 3). There was a marked significant increase of these fibres in BMS compared to control tissues. The mean ± SEM of the percentage area was: control,
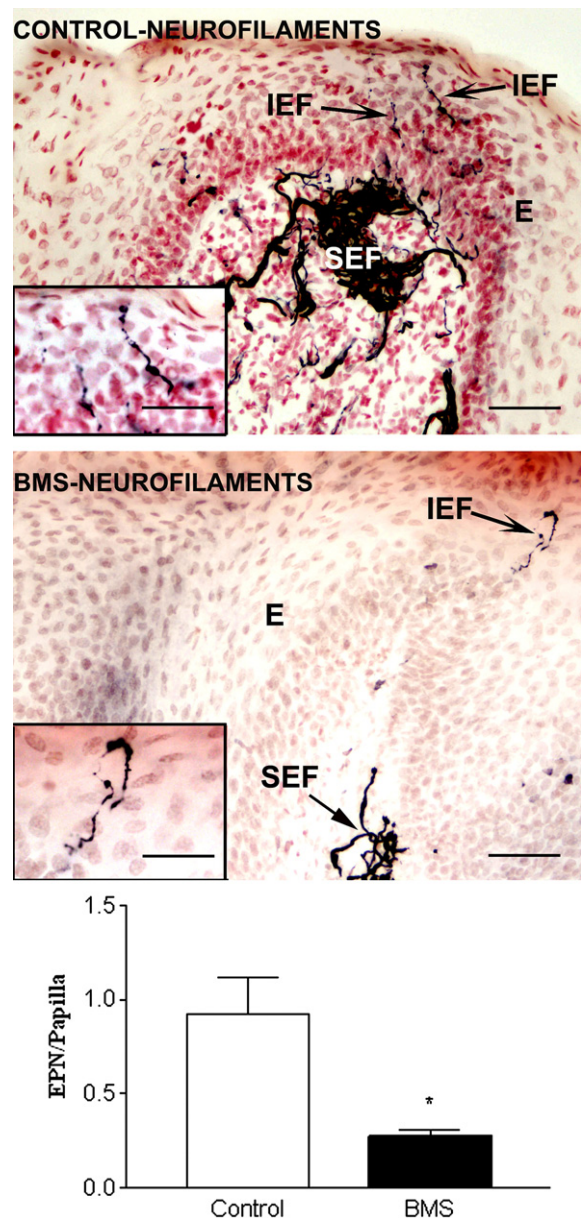

Fig. 1. Epithelial nerve fibres in human tongue. Top panel, representative neurofilament/peripherin fibres in a control; middle panel, BMS tongue section. Scale bars 100 μm, and insets epithelial nerve fibres (arrowed) scale bars 50 μm. IEF, intra-epithelial nerve fibre; SEF, sub-epithelial nerve fibre. Bottom panel, bar charts of the mean ± SEM of epithelial nerve (EPN) fibres per papilla in control and burning mouth syndrome (BMS) tongue. *$p < 0.0001$.

0.39 ± 0.11; and BMS, 3.11 ± 0.89 (Fig. 3, bottom panel). NGF also stained basal epithelial cells (Fig. 4) in all tongue samples; there was a significant increase in the BMS group compared to controls. The mean ± SEM of the percentage area was; controls, 2.84 ± 0.44; and BMS, 5.51 ± 0.74 (Fig. 4, bottom panel). The NGF fibre and basal epithelial cell staining was abolished when the primary antibody was incubated with recombinant human NGF antigenic peptide.
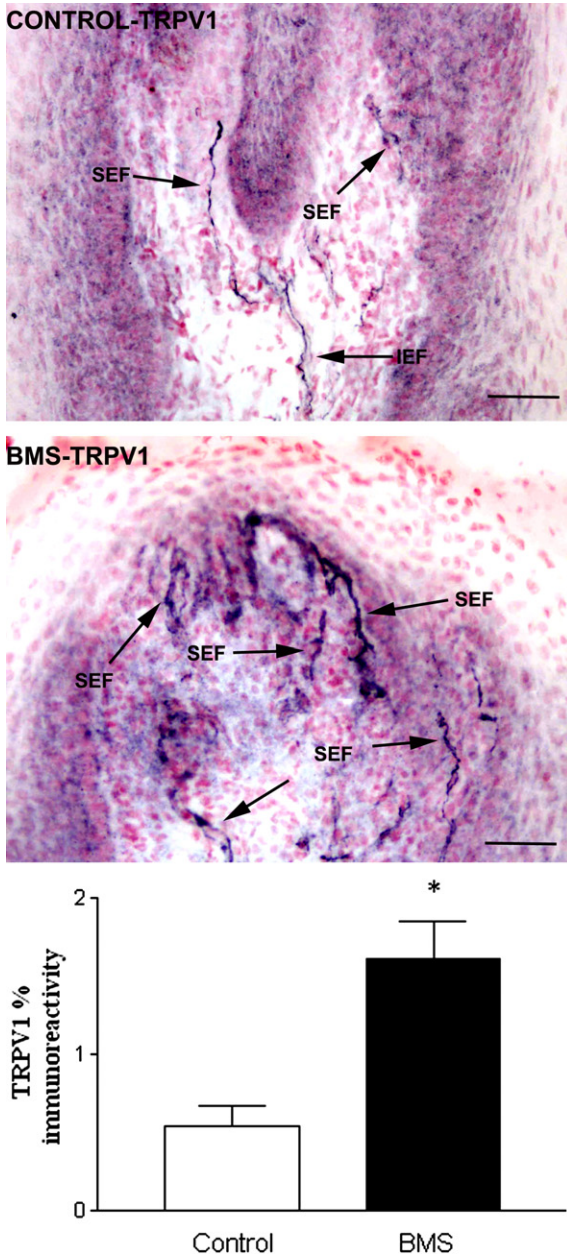
Fig. 2. Sub-epithelial transient receptor potential vanilloid receptor-1 (TRPV1) fibres in human tongue. TRPV1 fibres (arrows) within papillae staining in control (top panel) and in burning mouth syndrome (BMS) tongue (middle panel), scale bars 100 μm. SEF, sub-epithelial nerve fibres. Bottom panel, bar charts of the mean ± SEM of percentage area of TRPV1 fibres in control and BMS tongue. *p = 0.0011.

3.5. Clinical correlation (pain scores)

Mean age for the controls ($n = 10$) and the BMS patients ($n = 10$) were 40 years (range 16–79, M : F = 6 : 4) and 62 years (range 48–82, M : F = 5 : 5), respectively. The average duration of symptoms for the BMS group was 37.6 months.

The mean pain scores (mean ± SEM [and range]) for the control group were: at rest 0.5 ± 0.20 9 (range 0–2); pre-capsaicin 0.5 ± 0.20 9 (range 0–2); and post-capsaicin
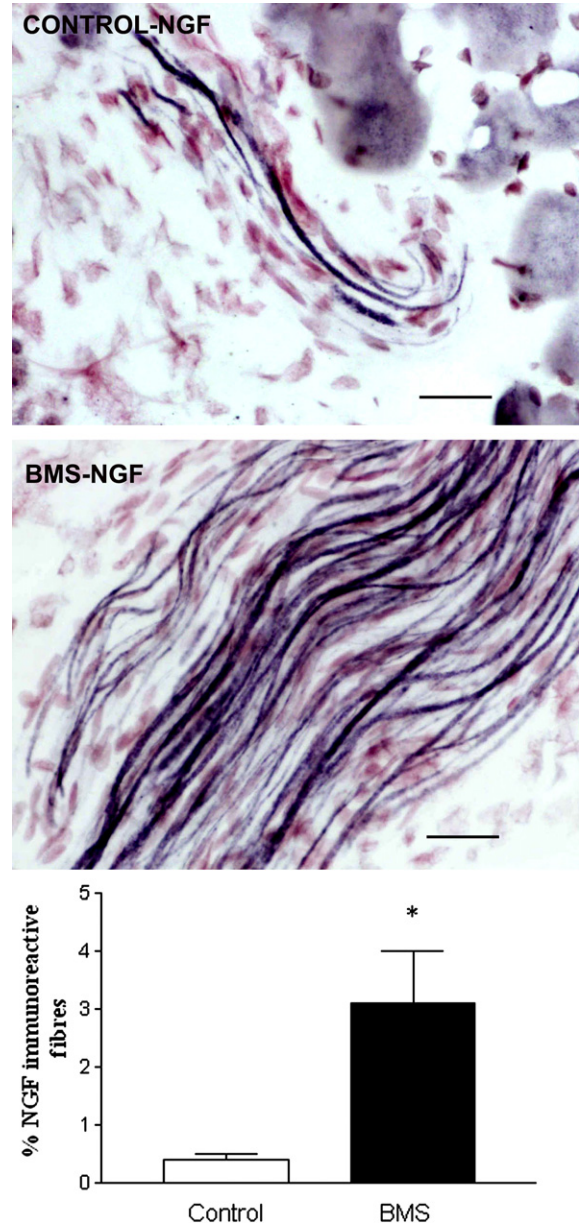

Fig. 3. Sub-epithelial nerve growth factor (NGF) fibres in human tongue. Top panel, NGF immunoreactive fibre staining of a control tongue; middle panel of a burning mouth syndrome (BMS) tongue, scale bars 50 μm. Bottom panel bar charts of the mean ± SEM of percentage area of NGF nerve fibres in control and BMS tongue. *p < 0.0001.

0.9 ± 0.61 9 (range 0–5) ($p > 0.05$). The mean pain scores for the BMS group were: at rest 5.3 ± 0.99 (range 0–10); pre-capsaicin 5.3 ± 0.99 (range 0–10); and post-capsaicin 7.0 ± 0.84 (range 4–9) ($p > 0.05$). There was a significant correlation between the baseline (on-going) VAS pain score and TRPV1 fibre area (Spearman's $r = 0.55$, $p = 0.014$) and NGF fibre area (Spearman's $r = 0.53$, $p = 0.025$) by image analysis, but not with neurofilaments/peripherin cocktail fibre area (Spearman's $r = 0.37$, $p = 0.12$). A significant correlation was also observed between the baseline (on-going) VAS Pain score and the pain evoked pain by topical capsaicin challenge (Spearman's $r = 0.82$, $p = 0.0006$) was also noted. Correlations between TRPV1
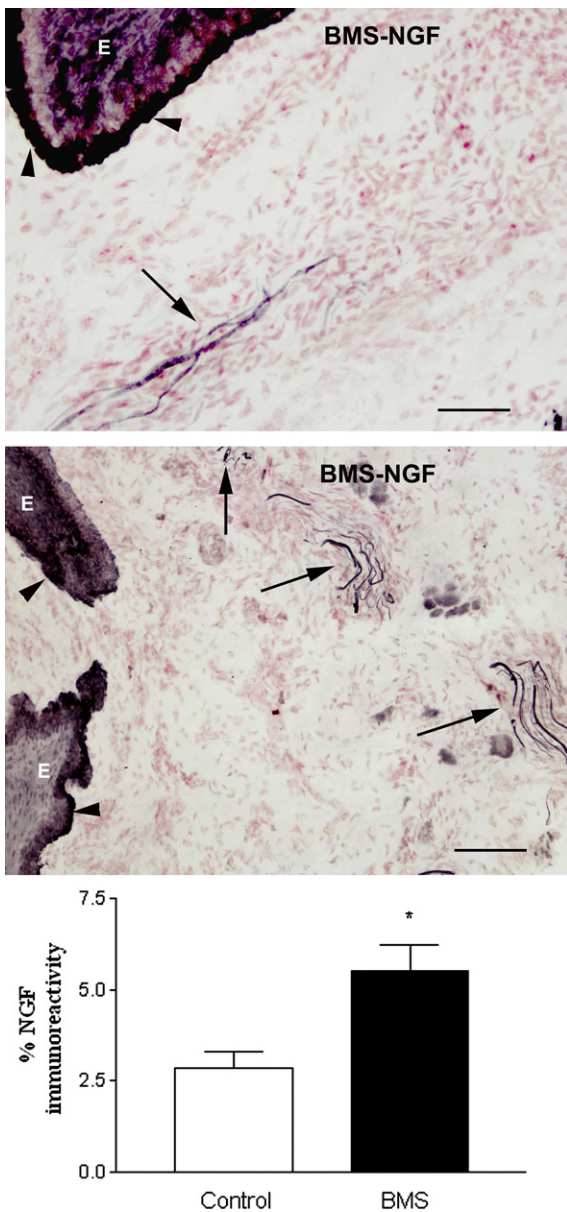
Fig. 4. Nerve growth factor (NGF) immunoreactivity in epithelial basal cells. Top panel, scale bar 100 μm; middle panel, 200 μm. Nerve fibres are indicated by arrows and epithelial basal cells by arrowheads. Bottom panel, bar charts of the mean ± SEM of percentage area of NGF epithelial basal cell staining in control and burning mouth syndrome (BMS) tongue. *$p < 0.0147$.

(Spearman's $r = 0.46$, $p = 0.36$) or NGF-fibres (Spearman $r = 0.32$, $p = 0.68$) and capsaicin-evoked pain were not statistically significant. There were no correlations between age and any of the nerve markers or between groups.

The HADS score demonstrated that only two of the 10 BMS patients had scores of mild anxiety, and one patient of mild depression.

4. Discussion

While BMS is a relatively common condition, its causation and molecular mechanisms underlying the symptoms,

are poorly understood. Our study focused on NGF and the expression of TRPV1 and $Na_v1.8$ in nociceptor fibres, as these are key molecules in pain and hypersensitivity. Our finding that TRPV1 and NGF-positive nerve fibres were significantly increased in patients with BMS, and correlated with on-going pain, is both novel and important. There was a trend toward increase of $Na_v1.8$ fibres, and more subjects may be needed to show a significant increase. Increased NGF levels are known to up-regulate the expression TRPV1 and $Na_v1.8$ by nociceptor fibres, and to sensitise nociceptors. However, large fibre dysfunction has been reported in BMS, and so the changes we describe may contribute, but may not be exclusively responsible for, the symptomatology.[5]

In accord with some previous studies, decreased intra-epidermal fibres were found in BMS tissues using a structural marker, suggesting that in some patients BMS may represent a peripheral sensory small-fibre neuropathy.[8,9,36] This suggests over-expression of TRPV1 by surviving fibres or nerve terminals, which may be the result of the relatively increased uptake of NGF per fibre, analogous to increased expression seen in undamaged nerve fibres after partial nerve injury in rodents.[37] NGF is not produced by nerve fibres; hence the increased number of NGF-positive fibres indicates detection of fibres with greater NGF uptake from target tissues, here mainly from basal epithelial cells, as discussed below. There was no correlation between pain and neurofilament/peripherin cocktail-positive nerve fibres, but the TRPV1 to neurofilament/peripherin cocktail fibre ratio was significantly increased, suggesting a relationship of symptoms to TRPV1 over-expression rather than loss or "dying-back" of intra-epithelial fibres. TRPV1 and $Na_v1.8$ intra-epithelial fibres were rare or absent compared to deeper layers of the tongue. The use of thicker sections and free-floating method may be required to detect them. In these tissues NGF levels in basal epidermal cells appeared increased, which may be the result of partial denervation, as shown in rodent skin,[38] or a persistent increase following an inflammatory or other tissue insult.[32] The cause of decrease in intra-epithelial fibres is not known, and other mechanisms may be responsible for the aetiology and symptomatology of this condition; however, at the very least, our findings may provide surrogate markers. The underlying pathology may have damaged nerve fibres, which at different stages may regenerate into abnormal tissues (e.g. with uptake of relatively excessive NGF), which in turn may lead to persistent phenotypic change. A recent study of idiopathic breast pain and tenderness showed increased TRPV1 and upregulation of NGF-immunostaining intensity.[33] We have proposed a hypothesis relating these tissue mechanisms to hypersensitivity.[22,24,33]

We demonstrate in this study that hypersensitivity to capsaicin application and on-going (spontaneous) pain scores were significantly correlated. This would support a role for TRPV1 in symptomatology. However, TRPV1 fibres were not significantly correlated with capsaicin-evoked symptoms, and post-capsaicin application pain scores were

also not significantly increased when compared with the baseline scores, possibly because the numbers of subjects/patients studied needs to be greater, or a range of capsaicin concentrations needs to be applied. Further, both central and peripheral hypersensitivity may account for the increased response to capsaicin. Studies are in progress to address these points, and to correlate changes in TRPV1 nerve fibres with quantitative sensory perception thermal thresholds, using previously described methods of quantitative sensory testing (QST).[39] A study reported warm allodynia in only two of 52 BMS patients studied;[5] demonstration of a role for TRPV1 may require suprathreshold and incremental heat-as-pain stimuli, that is, heat hyperalgesia.

Topical capsaicin has been anecdotally reported to be useful as treatment for some BMS patients, but it is unlikely to help substantially, as some of the symptoms are likely to arise from deeper fibres. Oral selective TRPV1 antagonists thus deserve consideration for clinical trials: both mechanical and thermal hyperalgesia may be reversed by capsazepine, a TRPV1 antagonist[40] suggesting an effect on polymodal nociceptors.

Another TRP receptor (TRPM8) which responds to menthol and cool stimuli has been reported to be co-localized with TRPV1 and calcitonin gene-related peptide (CGRP) fibres in the lingual nerve sensory fibres in rats, within the trigeminal ganglion, but not within the fungiform or filiform taste buds. The role of other temperature and mechanosensory channels, including TRPs, and their correlations with other modes of topical stimulation, may be of relevance in BMS, and may lead to other novel treatment strategies.

In this study the BMS patients were selected in accordance with IASP criteria to minimise any heterogeneity.[41] These patients reported moderate to severe pain intensity (VAS scores of 4–10) similar to previous studies.[10,14] The HADS score demonstrated that only two of the 10 BMS patients illustrated signs of mild anxiety with one patient displaying mild depression. These results indicate that these BMS patients were not overly depressed or anxious despite their ongoing disability, in contrast to patients in some previous studies.[42] This may reflect differences in longevity of symptoms between the cohorts of patients with BMS, and possibly the criteria for selection of patients.

In conclusion, burning mouth syndrome appears to be associated with increased levels of NGF in nerve fibres, and expression of TRPV1. While further studies are necessary to establish functional links between the TRPV1, NGF immunohistological changes and pain symptoms in BMS, our findings indicate a path for increasing understanding and treatment of BMS.

Acknowledgement

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