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TRPA1 expression in human lingual nerve neuromas in patients with and without symptoms of dysaesthesia

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ABSTRACT

The TRPA1 receptor is a member of the ankyrin family and is found in both spinal and trigeminal neurones. There is evidence to suggest that this receptor may be a sensor of noxious thermal stimuli in normal animals. After nerve injury, TRPA1 shows increased expression in uninjured axons, and has been implicated in the development and maintenance of hyperalgesia. We examined expression of TRPA1 in lingual nerve neuromas and investigated any potential correlation with the presence or absence of symptoms of dysaesthesia. Thirteen neuroma-in-continuity specimens were obtained from patients undergoing repair of a lingual nerve that had previously been damaged during lower third molar removal. Visual analogue scales (VAS) were used to record the degree of pain, tingling and discomfort. Tissue was processed for indirect immunofluorescence and the percentage area of PGP 9.5-immunoreactive neuronal tissue also labelled for TRPA1 was quantified. No significant difference between levels of TRPA1 in neuromas from patients with or without symptoms of dysaesthesia and no relationship between TRPA1 expression and VAS scores for pain, tingling or discomfort were observed. TRPA1 expression and the time after initial injury that the specimen was obtained also showed no correlation. These data show that TRPA1 is expressed in lingual nerve neuromas, but, it appears that, at this site, TRPA1 does not play a principal role in the development of neuropathic pain.

The TRPA1 receptor is a member of the ankyrin family. It is activated by the pungent ingredients of mustard oil, cinnamon oil, garlic, clove oil and wintergreen oil to induce a painful burning sensation [2,14,4,18]. It has also been suggested that TRPA1 is a sensor of noxious cold and is activated at temperatures below 17 °C [29]; with TRPA1 knockout mice displaying altered cold temperature sensitivity [17]. Whilst this role for TRPA1 as a noxious thermal sensor is still controversial, much interest has focused on this receptor due to its polymodal activation by a variety of nociceptive stimuli.

TRPA1 is expressed on both dorsal root ganglion (DRG) neurones and trigeminal (TG) neurones [29,8], and consistent with its role in nociception, is co-localised with TRPV1 on small-diameter nociceptors [22]. Recent studies have shown that after nerve injury, TRPA1 expression is increased in uninjured axons [15,12,13] and depletion of TRPA1 results in reduced cold hyperalgesia and allodynia [21]. Previous studies in our laboratory, have shown that the vanilloid receptor 1 (TRPV1) is expressed in human lingual nerve neuromas [7]. As it appears that TRPV1 and TRPA1 share a functional co-localisation [10], and TRPA1 expression is altered after nerve injury [15,13], we investigated the possibility that TRPA1 may be involved in the development and/or maintenance of dysaesthesia in patients with damaged lingual nerves.

Peripheral nerve damage in the oro-facial region can lead to the development of neuropathic pain. During third molar extraction the lingual nerve may be damaged, due to its close proximity to the tooth; this leads to sensory loss from the ipsilateral side of the tongue, and some patients develop the abnormal and unpleasant sensations of dysaesthesia [23]. Management of patients with lingual dysaesthesia following nerve injury is difficult but usually starts with microsurgical repair of the nerve [26]. During this procedure, the neuroma at the injury site is excised (thereby resulting in the specimens used in this study) and the nerve is re-approximated using epineurial sutures. Whilst this repair procedure ameliorates symptoms in some patients, it is not always successful and some patients are left with chronic pain that responds poorly to current pharmacological treatments. A better understanding of the aetiology of nerve injury-induced sensory disorders will help in the development of new treatment options and novel analgesics. In this study we evaluated the potential role of TRPA1 by quantifying expression in neuromas obtained from patients with or without symptoms of dysaesthesia.

Ethical approval for the study was obtained from the South Sheffield Research Ethics Committee and all specimens were collected with the patients' informed consent. All patient details

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were kept confidential and each specimen was given a unique code to be used throughout the study. Specimens were obtained from our archive of 84 neuromas obtained from patients referred to our department between 1999 and 2004. Previous published data examining TRPV1 [7] and P2X7 [19] used the majority of the same samples and techniques as the current study. Light and electron microscopical characteristics of these specimens have been described previously [30,31]. Thirteen neuroma-in-continuity specimens were used, each with some element of nerve tissue bridging the gap between the central and distal stumps of the damaged nerve. For a detailed description of the surgical procedures used to excise the neuroma and repair the nerve, see Robinson et al. [24].

Clinical histories and symptoms experienced by the patient were obtained prospectively using a questionnaire and completed by the clinician. Patients were asked whether the affected part of the tongue was painful and if they had tingling either spontaneously or initiated by moving or touching the tongue. VAS scores were also obtained to determine the extent of the patients' pain, tingling and discomfort. We selected patients with a relatively high level of symptoms on the assumption that this would increase the likelihood of revealing differences between the two groups. Thus, in the group of patients showing symptoms of dysaesthesia, each patient scored in excess of 49 mm on a 100 mm VAS for both pain and tingling compared to the group of asymptomatic patients who showed reduced sensation but no unpleasant pain and tingling as shown by very low VAS scores (Table 1).

Immediately following surgical removal of the neuroma, a 9/0 Ethilon suture (Ethicon, UK) was used to mark the central end of the nerve and the specimen placed in 2% Zamboni's fixative (0.1 M phosphate buffer, pH 7.4, containing 4% paraformaldehyde and 0.2% picric acid) for 24 h at 4 °C. Specimens were placed in a 30% sucrose solution for 6 h to cryoprotect the tissue and then embedded longitudinally in Tissue-Tek® O.C.T compound (Sakura, Europe) and frozen. Serial sections (14 μm) were cut on a cryostat and collected onto poly-D-lysine (Sigma–Aldrich, UK) coated glass slides in 20 sets so that each section was 280 μm from the adjacent section on the same slide. One slide from each specimen was washed in PBS containing 0.2% Triton (PBST) for 2× 10 min and sections incubated with 10% normal donkey serum (NDS, Jackson ImmunoResearch Inc., USA) for 1 h in a moisture chamber at room temperature to reduce any non-specific background staining. Sections were double labelled with a mixture of a monoclonal human PGP 9.5 antibody raised in mouse (1:1000, Ultraclone, UK) and a polyclonal human TRPA1 antibody raised in rabbit (1:50, GlaxoSmithKline, UK). Primary antisera were diluted in PBST containing 5% NDS and slides were left to incubate in the primary antisera solution for 24 h in a moisture chamber at 4 °C.

Following overnight incubation with the primary antisera, the sections were washed (2× 10 min, PBST) and incubated for 90 min in the dark at room temperature with the fluorescent secondary antibodies, which comprised of donkey anti-rabbit IgG conjugated to indocarbocyanine (Cy3, 1:200, Jackson ImmunoResearch, USA) and donkey anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, 1:20, Jackson ImmunoResearch, USA) diluted in 1.5% NDS in PBST solution. Sections were washed for a final time (2× 10 min, PBST), mounted in Vectashield (Vector Laboratories) and coverslipped.

Immunohistochemical controls were carried out by replacing the primary antibody with normal serum and incubating with the secondary alone. Furthermore, validation of the specificity of the TRPA1 antibody was assessed by immunocytochemistry and western blotting performed using stable hTRPA1 HEK293 cells versus non-transfected cells. Only cells transfected with hTRPA1 resulted in a positive signal [personal communication, Coelho, GlaxoSmithKline, UK, 2007].

All analysis was performed with the investigator blind to the symptom group. Sections were viewed using a Zeiss Axioplan fluorescent microscope and digital images captured and stored onto a PC via a QImaging Retiga 1300R digital camera. Immunoreactive labelling was quantified using computer assisted image analysis Image-Pro Plus (Media Cybernetics). Thresholding of positive immunolabelling was performed on digital monochrome images. Highlighted areas representing the positive staining were automatically measured by the computer software and a percentage area of staining (PAS) was calculated. To assess reproducibility of this technique and the investigator, all specimens were re-examined and the PAS determined for a second time. All counts were found to differ from the original counts by less than 5%.

One section from approximately the middle of each neuroma was selected for analysis to ensure that tissue across the entire width of the nerve was sampled. The very central and distal portions of the neuroma were eliminated from analysis as these regions have often been manipulated during the surgical procedure. The section to be analysed was divided into quarters transversely with analysis starting in the second quarter from the distal end and continuing proximally along a length of at least 1800 μm of the neuroma, with a minimum of 30,000 μm^2 of PGP 9.5 positively labelled nerve tissue included. To determine the proportion of nerve tissue expressing TRPA1, each field of interest was initially viewed through the FITC filter and the area of positively labelled neuronal tissue was measured. The same field was then viewed using the Cy3 filter to determine the percentage of nerve tissue expressing TRPA1. This method was necessary to allow the neural tissue in the neuromas to be distinguished from the extensive scar tissue found in some specimens, and to determine a value for 'intra-neural' TRPA1.

Table 1
Summary of patients included in the study, including presence of symptoms, TRPA1 expression, age, gender and VAS scores for pain, tingling and discomfort.

Neuroma specimen	Dysaesthesia	% TRPA1	Spontaneous pain	Spontaneous tingling	Gender	Age	Pain VAS	Tingling VAS	Discomfort VAS	Time following initial injury (months)
1	Yes	2.74	Yes	Yes	M	33	58	99	69	27
2	No	18.78	No	No	F	22	0	0	44	18
3	No	19.31	No	No	F	26	1	2	10	21
4	No	34.63	No	No	F	29	1	0	81	16
5	Yes	16.69	Yes	Yes	M	29	65	81	50	41
6	Yes	14.82	Yes	Yes	F	37	29	97	70	14
7	No	14.83	No	No	F	40	1	2	39	26
8	Yes	19.96	Yes	Yes	F	23	34	99	42	15
9	Yes	9.42	Yes	No	M	28	66	8	50	7
10	No	1.38	No	No	F	32	1	1	48	11
11	No	1.68	No	No	F	31	1	1	55	8
12	Yes	8.24	Yes	Yes	F	35	79	95	95	9
13	Yes	23.70	Yes	Yes	F	32	81	50	99	17