

# The menthol and cold sensation receptor TRPM8 in normal human nasal mucosa and rhinitis\*

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## SUMMARY

**Background:** Menthol and cold sensation trigger symptoms and reflex responses in the upper airway, but the underlying molecular mechanisms are unknown. We have therefore studied nerve fibres expressing the menthol and cold receptor TRPM8 in normal human mucosa, and in rhinitis. TRPM8 nerve fibres were compared with those expressing other TRP receptors including TRPV1 (capsaicin and heat receptor), and TRPA1 (mechanocold receptor).

**Methods:** Immunohistology and image-analysis were used to study TRP receptors in biopsies of nasal turbinate from control subjects, patients with allergic rhinitis, and non-allergic rhinitis.

**Results:** TRPM8-immunoreactive nerve fibres were observed in the sub-epithelium, and were profuse around blood vessels in deeper regions, where they were markedly greater in number than TRPV1+ fibers. Image analysis of TRPM8 in sub-epithelial and vascular regions showed no significant differences between control and the rhinitis patient groups. TRPA1-immunoreactivity was weak and seen rarely in nerve fibres.

**Conclusion:** We show that TRPM8 nerve fibres are abundant in nasal mucosa particularly around blood vessels, and may mediate neurovascular reflexes. TRPM8 antagonists deserve consideration for therapeutic trial in rhinitis.

**Key words:** rhinitis, menthol, TRPM8, TRPV1, TRPA1

## INTRODUCTION

Sensory nerves monitor the nasal mucosal microenvironment and initiate protective mechanisms through sympathetic and parasympathetic neural pathways. Exaggerated responses to environmental and endogenous stimuli, which may be secondary to an inflammatory process, are regarded as neural hypersensitivity or hyper-responsiveness<sup>(1)</sup>. Rhinitis, both allergic and non-allergic, is associated with this phenomenon, but the underlying molecular mechanisms involved are not well understood. The cold receptors in the upper airway nerve terminals are linked to sensory pathways that may have local and central reflex responses when exposed to cold airflow<sup>(2)</sup>. Transient receptor potential (TRP) ion channels are widely expressed in sensory neurons, and have the ability to function as thermal, chemical, osmo- and mechano-receptors at nerve terminals. However, little is known about their expression and function in normal nasal mucosa, or in patients with allergic and non-allergic rhinitis.

TRPM8 is a member of the TRP channel superfamily and is activated by cooling in the temperature range of 22 to 27°C<sup>(3,4)</sup>. Synthetic 'cooling' compounds such as menthol, icilin and eucalyptol activate the receptor<sup>(3,4)</sup>. Topical menthol produces an innocuous cooling sensation at low concentrations, and a burning sensation at high concentrations. TRPM8 receptors are expressed on two distinct subsets of sensory neurons. One class is sensitive to menthol and features non-nociceptive neuron properties. The other class is also sensitive to menthol but has characteristics of nociceptive neurons including capsaicin-sensitive, ATP-sensitive, and acid (low pH) responses. TRPM8 receptor expressing, sensory nerve fibres can thus have both non-nociceptive and nociceptive functions<sup>(5)</sup>.

TRPV1 is a non-selective cation channel expressed by primary sensory neurons and can be activated by capsaicin, noxious heat (> 43 °C) and protons<sup>(1,6,7)</sup>. We reported recently that the heat-activated receptor TRPV1 was present in nerve fibres in

human nasal turbinate mucosa and epithelium from patients with allergic rhinitis<sup>(8)</sup>. Chronic inflammation may change the phenotype of sensory neurons, and increase expression of TRPV1 and TRPM8<sup>(9)</sup>. TRPA1 is expressed in sensory neurons, activated at temperatures lower than for TRPM8 (< 17°C), and also by cinnamaldehyde and allyl isothiocyanate, the active ingredient found in mustard oil.

In this study, we have used immunohistology with well-characterised antibodies to investigate the expression of TRPM8, TRPV1 and TRPA1 in nasal biopsies from rhinitis patients, and unaffected (control) subjects.

## MATERIALS AND METHODS

### Patients

The study protocol was approved by the Hillingdon and Hounslow Regional Ethics Committee. Informed written consent was obtained from each participant. Patients were consecutively selected from the Charing Cross Hospital Rhinology waiting list.

Tissue samples were collected from unaffected subjects as controls (n = 17; age range 20 – 74 y; 11 male) and rhinitis subjects (allergic rhinitis n = 16; age range 23 – 64 y; 9 male; non-allergic rhinitis n = 15; age range 23 – 60 y; 8 male). The existence of two of the three symptoms of nasal discharge, blockage, sneeze/itch for more than one hour in most days was diagnostic of rhinitis and sub-divided as either non-allergic or allergic on the basis of skin prick test. The diagnosis of allergic rhinitis was made after taking nasal and allergy history, nasal examination, nasendoscopy and positive skin-prick test to mixed tree pollen, mixed grass pollen, house dust mite, cat, dog, horse, rabbit, *Alternaria*, *Aspergillus* and *Cladosporium*. No patient was taking antihistamines at the time of the study. Patients with negative skin prick test but symptom score for nasal congestion and rhinorrhea of more than 5 out of 10 were diagnosed as non-allergic rhinitis. Serum total IgE tests were not performed on control subjects or patients. Subjects were included in the control group only if there was no evidence of rhinitis on history and examination. All of the subjects in the control group had indications for surgery for non-allergic reasons such as nasal cosmetic appearance or nasal septal deviation and were negative to skin prick test.

### Biopsy sampling

The nose was prepared using a modified Moffet's solution (3 ml of 10% cocaine and 7 ml of sodium bicarbonate solution). A 3 mm-punch biopsy (Stiefel, Woodburn Green, UK) was used and a biopsy taken from both of the inferior turbinates at 1 cm from the anterior border of the inferior turbinate to avoid extracting tissue demonstrating squamous metaplasia. Biopsies were snap frozen on dry ice and stored at -70°C until use or fixed by immersion in Zamboni's fluid for 2 hours before being transferred to PBS containing 15% w/v sucrose cryoprotectant and 0.01% w/v sodium azide until use. All participating patients received routine post-operative care and no post-operative complications were noted.

### Immunohistochemistry

Frozen sections were immunostained (ABC immunoperoxidase) with antibodies to TRPM8, TRPV1 or TRPA1 (Table 1), as described previously<sup>(1,6,7)</sup>. Sites of attachment of primary antibodies were detected using nickel-enhanced, avidin-biotin immunoperoxidase (ABC-Vector Labs) and nuclei counterstained with aqueous neutral red. Nerve fibres immunoreactive for TRPM8 were quantified in 5 fields (x 40 objective magnification) per tissue section by computerized image analysis (Olympus Analysis Five DP Soft, UK). Analogue images were captured via video link to an Olympus BX50 microscope and converted into digital monochrome images by the computer. The grey-shade detection threshold was set at a constant level to allow detection of positive immunostaining and the area of highlighted immunoreactivity obtained as a percentage (% area) of the field scanned. Intra-epithelial nerve fibres immunoreactive for TRPV1 were counted along the entire length of up to four tissue sections per biopsy. The length of epithelium was measured using a calibrated microscope eyepiece graticule and values expressed as fibres per mm length of epithelium.

Table 1. Antibody characteristics.

Antibodies to	Source/Ref#	Titre
TRPM8	GSK Stevenage, Herts, UK/ #SEL-2 1323	100-200
TRPV1	GSK Stevenage, Herts, UK/#C22	10000
TRPA1	GSK Stevenage, Herts, UK/#1962	100-500

## RESULTS

### Human nasal turbinate

TRPM8 immunoreactivity was present as a mixture of single fine and dense thick fibres throughout the sub-epithelium, with profuse fibres surrounding blood vessels in deeper glandular/vascular regions (Figure 1A - D). In contrast, TRPV1-immunoreactive fibres were detected principally in the epithelium and sub-epithelium in rhinitis (Figure 1E - G). There were very few TRPV1-immunoreactive fibres in deeper vascular regions. TRPA1-immunoreactivity of variable intensity was observed in epithelial cells (Figure 1H) and detected in a few, fine calibre fibres within sub-epithelial (Figure 1H - arrows) and deeper nerve fascicles (Figure 1I - arrows).

### Quantification and image analysis

Image analysis was used to quantify immunoreactivity in biopsies of nasal turbinate from control subjects (n = 17), patients with allergic rhinitis (n = 16), and non-allergic rhinitis (n = 15). In some smaller biopsies fewer sections were available hence the reduced number of subjects for analysis of TRPV1 and TRPA1, as indicated.

Comparison of levels (% area) of TRPM8- and TRPV1-immunoreactive fibres in control tissues showed highly sig-

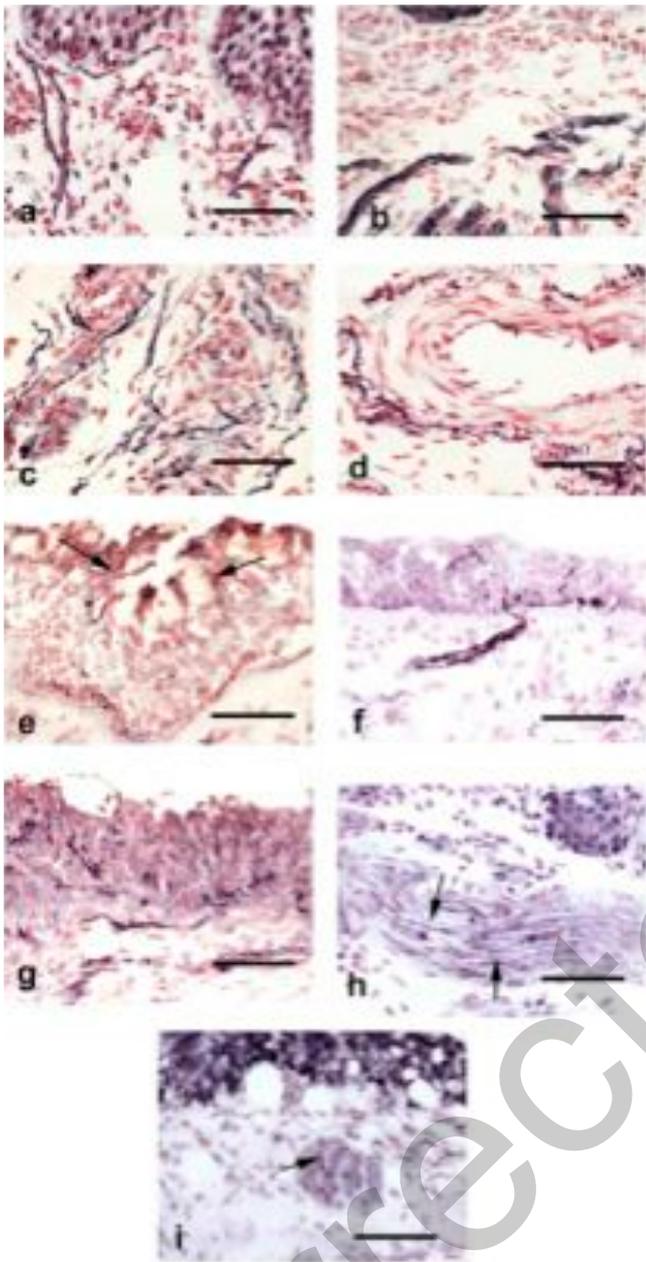


Figure 1. Receptor immunoreactivity in nasal turbinate. TRPM8 (a - d), TRPV1 (e – arrows indicate intra-epithelial fibres, f, g) and TRPA1 (h, i) -immunoreactive nerve fibres in human nasal turbinate. Scale bar = 50  $\mu$ m.

nificantly more TRPM8-fibres ( $p < 0.0001$ ;  $n = 12$  for TRPV1) in vascular (Figure 2) than sub-epithelial regions. However, measurement of TRPM8-immunoreactivity by image analysis (% area) in the vascular (Figure 3A) and sub-epithelial (Figure 3B) regions showed no significant difference between control and either rhinitis patient group. TRPM8 and TRPV1 sub-epithelial % area analyses did not show significant differences from each other or controls, and while a trend was observed for increased counts of intra-epithelial TRPV1 fibres per mm length of epithelium in the disease groups, the difference was not statistically significant (Mean TRPV1 fibres/mm  $\pm$  SEM: controls  $0.20 \pm 0.08$ ; rhinitis  $0.72 \pm 0.20$ ,  $p = 0.08$ ; non-allergic

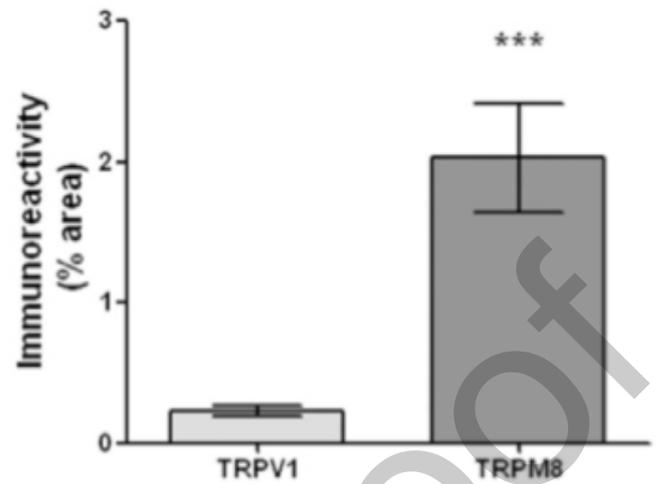


Figure 2. Quantification of receptor immunoreactivity.

TRPM8 and TRPV1-immunoreactive nerves in the vascular regions of control (unaffected subjects) nasal turbinates by image analysis \*\*\*  $p < 0.0001$ .

rhinitis  $0.44 \pm 0.27$ ,  $p = 0.18$ ). The scarcity of TRPA1 fibres precluded quantitative analysis. Image analysis of TRPA1 immunoreactive epithelial cells showed no significant change between groups (Mean TRPA1 % area  $\pm$  SEM: controls  $15.98 \pm 2.93$ ; rhinitis  $9.33 \pm 1.79$ ,  $p = 0.09$ ; non-allergic rhinitis  $10.17 \pm 2.09$ ,  $p = 0.25$ ;  $n = 6$ ).

## DISCUSSION

We have demonstrated the presence of TRPM8, the menthol and cool receptor, in nerve fibres of human nasal mucosa. TRPM8 nerve fibres were observed throughout the sub-epithelium, with profuse fibres surrounding blood vessels in deeper vascular regions in the nasal biopsies. Furthermore, comparison of TRPM8 and TRPV1 nerve fibres showed TRPM8 nerve fibres were vastly more abundant in the vascular region. In accord, we have previously shown the presence of TRPM8 in a sub-set of small sensory neurons in human dorsal root ganglia and nerve terminals in skin, using the same methods and antibodies <sup>(10)</sup>. TRPM8 receptors expressed by sensory nerves within the nasal mucosa may play a role in airflow and symptom perception. While rhinitis biopsies failed to show significant difference of TRPM8 innervation from controls, TRPM8 activation may contribute to symptoms and reflexes, including vasomotor effects.

Nasal congestion is one of the main presentations of rhinitis, which is frequently associated with increased nasal airway resistance. Previous studies have shown that the objective measurement of nasal airway resistance does not always correlate with the subjective perception of the degree of nasal obstruction <sup>(11,12)</sup>. It has been demonstrated that nasal inhalation of menthol exerted a stimulant or sensitizing effect on nasal cold receptors supplied by the trigeminal nerve, which induced a sensation of increased patency even though there was no change in objective assessment of nasal resistance to airflow <sup>(13,14)</sup>. The reduction in respiratory discomfort may

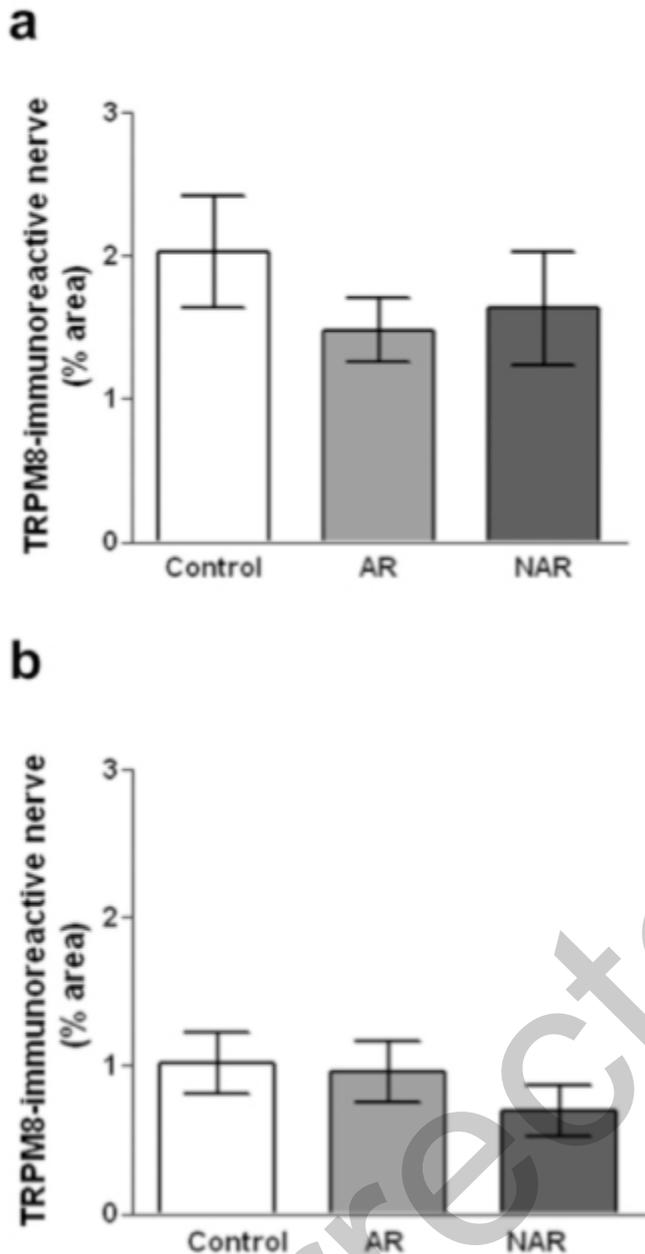


Figure 3. Quantification of receptor immunoreactivity. Image analysis of TRPM8- immunoreactive nerves in the vascular (a) and sub-epithelial (b) regions of control, allergic (AR) and non-allergic (NAR) rhinitis patients.

be the effects of L-menthol on cold receptors during loaded breathing <sup>(12)</sup>. Our study has shown that these cold receptors are likely to be TRPM8. It might be speculated that while TRPM8 antagonists may ameliorate pathophysiological mechanisms, they might increase sensation of nasal stuffiness, unless their efficacy overrides the latter phenomenon. The relationship of TRPM8 levels and coolness of menthol concentrations would be of interest and included in future studies. TRPA1 has been shown to be an important neuronal mediator of noxious including very cold stimuli in sensory neurons <sup>(15)</sup>. However, in the present study, immunostaining for TRPA1 in human turbinate revealed only few, weak fibres, suggesting that there

may be a low expression of TRPA1 in nerves supplying nasal turbinate, and little contribution to cold hypersensitivity. Chemical compounds in tobacco smoke and air pollutants <sup>(16)</sup> may activate TRPA1 directly, leading to activation of nasal sensory fibres <sup>(15)</sup>. Until further antibodies to TRPA1 have been tested and the relationship between TRPA1 levels and cool sensation is established the interpretation of our results with TRPA1 should be treated with caution.

Inflammation leads to increased trophic factors such as Nerve Growth Factor (NGF), which in turn sensitises and increases the expression of TRPV1, TRPM8, and promotes nerve sprouting, thus contributing to hypersensitivity <sup>(17,18)</sup>. We have shown previously that NGF levels are increased in the nasal mucosa in allergic rhinitis <sup>(8)</sup>, and proposed that this may account for the increased sensory sodium channel expression in rhinitis <sup>(19)</sup>. NGF can increase sensitivity of menthol sensitive neurons cold via sensory neurons expressing TRPM8, and also induce expression of this receptor in sensory neurons, which do not normally express TRPM8 <sup>(20)</sup>.

There was a trend for increased TRPV1 intra-epithelial fibres in allergic rhinitis as shown by us previously <sup>(8)</sup> and now also in non-allergic rhinitis, although the data fail to be statistically significant. This could be due to the collection of majority of the nasal biopsy out of season, and further studies are needed to study the seasonal variation in levels and function of TRPV1 and TRPM8. Patients with seasonal allergic rhinitis appeared to be more sensitive to TRPV1 agonist during hay fever season, which suggests a seasonal variation of TRPV1 expression <sup>(21,22)</sup>.

In conclusion, TRPM8, TRPV1 and TRPA1 are expressed by sensory nerves, which innervate the human nasal mucosa. Although there were no significant differences between control and rhinitis subjects, the abundance of TRPM8 nerve fibres around blood vessels suggest that the clinical correlations with function should be investigated further prior to consideration of therapeutic trials.

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#### AUTHORS' CONTRIBUTIONS

SK and AY collected the human nasal biopsies and prepared the manuscript; PF performed the immunocytochemical studies, performed the image analysis and prepared part of the manuscript; GS and HAS initiated and supervised the clinical aspects of the study and the manuscript, PA initiated and supervised the tissue aspects of the study, and helped with interpretation of the data.

#### CONFLICT OF INTEREST

None.

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