Expression of *Mash1* in basal cells of rat circumvallate taste buds is dependent upon gustatory innervation

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Abstract ***Mash1*, a mammalian homologue of the *Drosophila achaete-scute* proneural gene complex, plays an essential role in differentiation of subsets of peripheral neurons. In this study, using RT-PCR and in situ RT-PCR, we investigated if *Mash1* gene expression occurs in rat taste buds. Further, we examined dynamics of *Mash1* expression in the process of degeneration and regeneration in denervated rat taste buds. In rat tongue epithelium, *Mash1* gene expression is confined to circumvallate, foliate, and fungiform papilla epithelia that include taste buds. In taste buds, *Mash1*-expressing cells are round cells in the basal compartment. In contrast, the mature taste bud cells do not express the *Mash1* gene. Denervation and regeneration experiments show that the expression of *Mash1* requires gustatory innervation. We conclude that *Mash1* is expressed in cells of the taste bud lineage, and that the expression of *Mash1* in rat taste buds is dependent upon gustatory innervation.**

Key words: Taste bud; *Mash1*; Denervation; Regeneration; Rat

1. Introduction

Taste buds are chemoreceptor end-organs for taste. In mammals, most are concentrated in the stratified squamous epithelium of the dorsal surface of the tongue that covers the circumvallate, foliate, and fungiform papillae. Mammalian taste bud cells are not true neurons and do not derive from embryonic neural tissue, but they do have some properties of neurons in their ability to develop voltage changes when stimulated appropriately [1,2]. Taste buds cells have a limited life span and are regularly replaced throughout life by a proliferative basal cell population [3,4]. The gustatory cells in taste buds have been identified as the most typical sensory paraneurons; they have an endocrine or paracrine function in addition to their role in the synaptic transmission of taste [5,6]. Furthermore, it has been recognized for more than a century that mammalian taste buds are nerve-dependent: denervated taste buds degenerate, yet readily re-form if circumvallate, foliate, and fungiform papillae are subsequently re-innervated.

All receptor cells other than those in taste buds, including the neural tube, neural crest, or ectodermal placodes, are known to arise from neurogenic ectoderm. They share the same progenitor population as neurons [7,8]. In contrast, taste bud cells arise from local epithelium, not from neurogenic ectoderm [9,10].

Recently, mammalian homologues of *Drosophila* proneural genes of the achaete-scute complex have been identified. *Mash1* (mammalian *achaete-scute* homologue 1) encodes basic helix-loop-helix (bHLH) transcription factors [11,12]. *Mash1* is expressed in subsets of neuronal precursors in both the central nervous system and the peripheral nervous system [13–15]. Disruption of the *Mash1* gene in mice results in the elimination of most olfactory and autonomic neurons, showing a role for *Mash1* in the development of particular neural lineages [13,15,16]. In addition, *Mash1* promotes differentiation during retinal development and is essential for proper ratios of neural cell types [17].

There have been no published reports describing the genes controlling taste bud cell lineage. To investigate if *Mash1* gene expression occurs in rat taste buds, we used the reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ RT-PCR. In addition, we examined the dynamics of *Mash1* expression in degeneration and regeneration in denervated rat taste buds.

2. Materials and methods

2.1. Animals and surgery

Adult male and female Sprague-Dawley rats were utilized for this study. They were intraperitoneally anesthetized with Nembutal (Dainabot, Osaka, Japan; 50 mg/kg body weight). An incision was made in the skin of the ventral neck, and the submandibular and sublingual glands were retracted downward to expose the digastric muscle. After the posterior belly of the digastric muscle had been retracted and the stylohyoid muscle had been pulled aside, the underlying glossopharyngeal nerves were cut bilaterally. The rats were killed on one of the following post-operative days: 2, 5, 8, 10, 14, 28, and 48. Untreated rats were used as a control.

2.2. Light microscopy

Rats were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg), and perfused through the left ventricle with cold, half-strength Karnovsky's [18] fixative buffered with phosphate. The posterior part of the tongue containing the circumvallate papilla was excised, kept in the same fixative overnight, and postfixed in 1% osmium tetroxide in the same buffer for 2 h. The tissues were then dehydrated in ascending concentrations of ethanol, passed through propylene oxide, and embedded in Epon 812. Semithin sections were stained with toluidine blue.

2.3. RT-PCR

For RT-PCR, the tissues containing circumvallate papilla, fungiform papillae, and indifferent lingual epithelium were incubated for 60 min at 37°C with 2% collagenase, type IV (Sigma) in supplemented α-MEM (Cosmo Biol. Co.). After incubation, the papillary epithelium was manually separated from the underlying connective tissue with fine forceps. The total RNAs were isolated from the epithelium of fungiform and foliate papillae, from both control and denervated circumvallate papillae after 2 and 4 weeks, and from tongue epithelium without taste buds; all RNAs were incubated with DNase I. The resultant 1 μg of RNA was reverse transcribed using oligo-dT primer and avian myeloblastosis virus (AMV) reverse transcriptase at 42°C for 4 h, yielding first-strand cDNAs. Following denaturation at 94°C

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for 120 s, PCR amplification was performed under the following conditions: 94°C for 1 min, 53°C for 1 min, 72°C for 2 min for a total of 35 cycles. After the 15 min elongation step at 72°C, the reaction was terminated. The reverse transcriptase step was omitted in controls to confirm removal of all genomic DNA. Amplification products were analyzed on 2% agarose gels and visualized with ethidium bromide. Amplification products were subcloned and sequenced to confirm identities. *Mash1*: 5′-TCACAAGTCAGCGGCCAAGC-3′ (forward) and 5′-TGTCAAGAAACACTGAAGAC-3′ (reverse).

2.4. In situ RT-PCR

In situ RT-PCR was done essentially as described previously[19]. Frozen tissue sections (6–8 μm) of control and denervated circumvallate papillae after 2 and 4 weeks were placed on glass slides. Slides containing tissue sections were fixed in 4% paraformaldehyde for 10 minutes, then washed in PBS and in autoclaved demineralized distilled water (ddH_2O). They were reverse transcribed using reverse *Mash1* primer and AMV reverse transcriptase at 42°C for 1 h. PCR solution containing buffer (final concentration 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$); 50 μM dNTP; 1 mM DTT, 10 U Taq DNA polymerase and 2.5 μM Dig-11-dUTP was applied to the tissue section. In situ PCR amplification was performed under the following conditions: 94°C for 1 min, 53°C for 1 min, 72°C for 2 min for a total of 10 cycles. The digoxigenin-labeled cDNA segments were detected by immunological detection using antibody against digoxigenin. The reverse transcriptase step was omitted in controls. These sections were lightly counterstained with hematoxylin.

3. Results

To determine *Mash1* gene expression in rat taste buds, we performed RT-PCR experiments (Fig. 1). When the RT-PCR was carried out using mRNA prepared from epithelium of circumvallate, foliate, and fungiform papillae, amplification products of the expected size (556 bp) were obtained with primer sets specific for rat *Mash1* and sequenced to confirm identities. However, an amplification product was not obtained using mRNA prepared from tongue epithelium that did not include taste buds.

We used in situ RT-PCR to examine the expression patterns of the *Mash1* gene in taste buds of circumvallate papillae. As shown in Fig. 4a, *Mash1*-expressing cells were detectable in taste buds, whereas they were not observed in the other papillary epithelial elements. In taste buds, *Mash1*-expressing cells were round cells in the basal compartment. But the spindle-shaped cells, which are regarded as mature taste bud cells, did not express the *Mash1* gene.

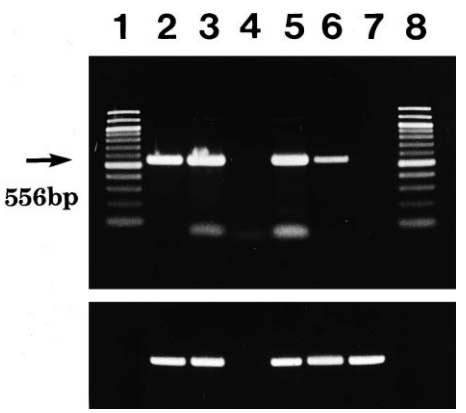

Fig. 1. Analysis of *Mash1* expression in rat tongue. When the RT-PCR is performed using mRNA prepared from epithelium of circumvallate, foliate, and fungiform papillae, amplification products of the expected size (556 bp) are obtained with primer sets specific for rat *Mash1*. Lanes 1, 8: 100 bp DNA ladder marker; lane 2: brain of E14; lane 3: epithelium of circumvallate papilla including taste buds; lane 4: epithelium of circumvallate papilla (no-RT control); lane 5: epithelium of foliate papilla including taste buds; lane 6: epithelium of fungiform papilla including taste buds; lane 7: indifferent lingual epithelium. β-Actin (bottom) is used as a control.

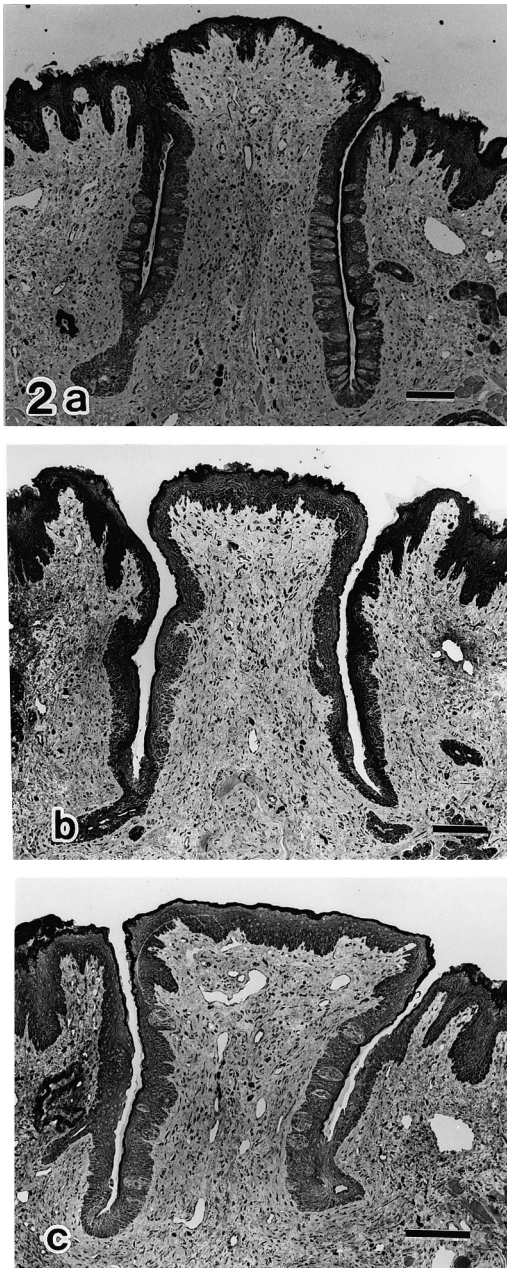

Fig. 2. Semithin section of circumvallate papillae from control and denervated rats. a: In the control rat, numerous taste buds are situated in the inner and outer trench walls (×63). b: Two weeks after denervation, taste buds have completely disappeared (×72). c: Four weeks after denervation. taste buds begin to appear on the lateral surface of the papilla, but not in the outer wall of the groove surrounding the papilla (×81). Toluidine blue. Bars = 100 μm.

In control rats, taste buds are barrel-shaped structures embedded in the stratified squamous epithelium covering the lateral walls of the circumvallate papilla (Fig. 2a). The taste buds consist of approximately 60–100 spindle-shaped cells that end apically in a taste pore. Two days after the sectioning of the bilateral glossopharyngeal nerves, many dense bodies

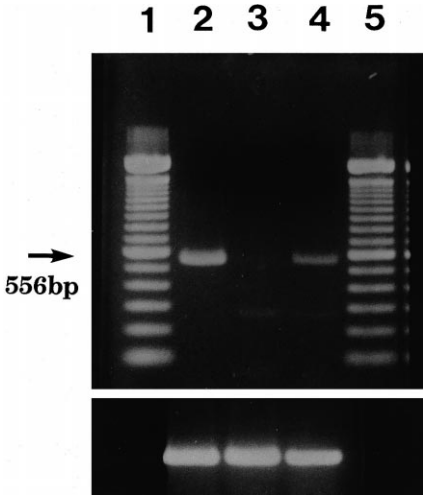

Fig. 3. The dynamics of *Mash1* expression in denervation and regeneration in denervated rat. Amplification products are not obtained using mRNA prepared from denervated epithelium of circumvallate papillae after 2 weeks. But the expression of *Mash1* becomes detectable 4 weeks post surgery. Lanes 1, 5: 100 bp DNA ladder marker; lane 2: epithelium of circumvallate papilla including taste buds (control); lane 3: denervated epithelium of circumvallate papillae after 2 weeks; lane 4: denervated epithelium of circumvallate papillae including taste buds after 4 weeks. β-Actin (bottom) is used as a control.

were found in the cytoplasm of the taste bud cells. Decreases in size and number of taste buds on circumvallate papillae continued until about the 10th post-operative day. Two weeks after the operation, the taste buds had completely disappeared (Fig. 2b). Furthermore, in denervated circumvallate papilla there was a thinning of the lateral wall epithelium. Four weeks after the operation, regenerated taste buds began to appear near the base of the nascent papilla, but not in the outer wall of the groove surrounding the papilla (Fig. 2c). The new taste buds were fewer in number and smaller in size than in controls. The thickness of the formerly atrophied epithelium of the denervated papilla reached that of control.

When RT-PCR was performed using mRNA prepared from denervated epithelium of circumvallate papillae after 2 weeks, amplification products of the expected size (556 bp) were not obtained with primer sets specific for rat *Mash1*. But the expression of *Mash1* became detectable at 4 weeks after the operation in the epithelium of circumvallate papilla, at which time regenerated taste buds appeared (Fig. 3).

Fig. 4 shows the expression of *Mash1* in denervation and regeneration in denervated rat. Two weeks after denervation, *Mash1*-expressing cells had completely disappeared (Fig. 4c). Four weeks after the operation, *Mash1*-expressing cells were detectable in the basal compartment of regenerated taste buds (Fig. 4d).

4. Discussion

Mash1 appears to control the neurogenesis of both central and peripheral neurons [12,14]. Indeed, disruption of the *Mash1* gene in mice results in a profound reduction in the number of several types of neurons including autonomic, enteric, and olfactory receptor neurons [13,16,20]. Changes in the numbers and proliferative states of *Mash1*-expressing cells correlate with induced changes in overall neurogenesis, indi-

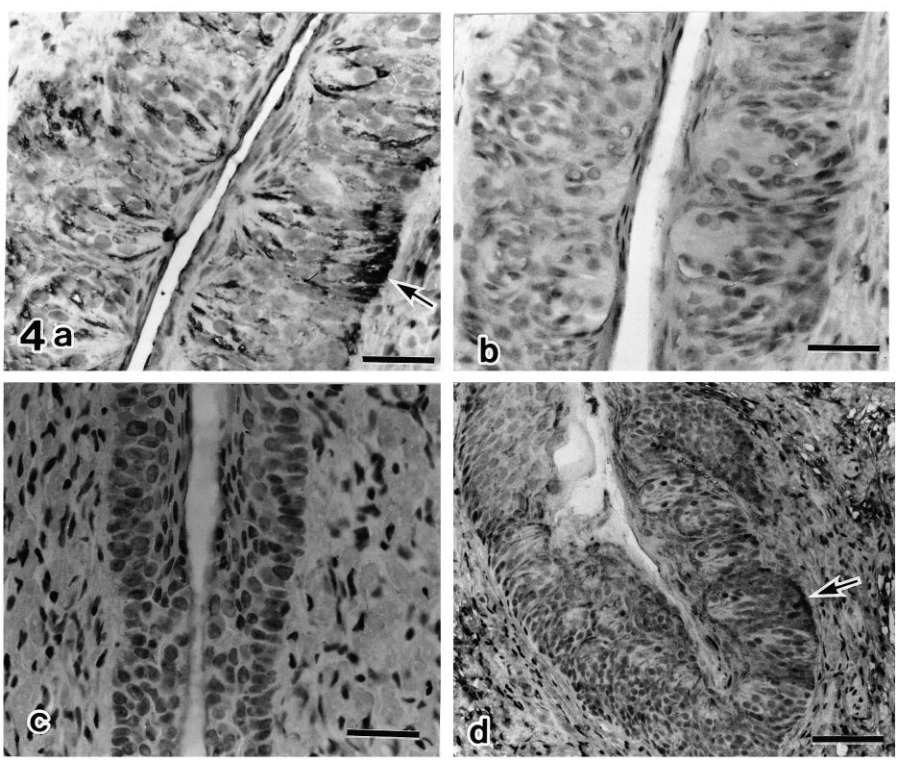

Fig. 4. Sections of circumvallate papillae from control and denervated rats treated with in situ RT-PCR for Mash1 mRNA. a: In the control rat, *Mash1*-expressing cells (arrow) are detectable in basal cells in the taste bud (×300). b: No signals are detectable in rat circumvallate taste bud cells when the reverse transcriptase step is omitted (×300). c: Two weeks after denervation, *Mash1*-expressing cells have completely disappeared (×300). d. Four weeks after denervation, *Mash1*-expressing cells (arrow) are detectable in basal cells in taste bud (×300). Bars = 30 μm.

cating that *Mash1*-expressing cells give rise to the immediate precursors of olfactory receptor neurons, but are not the self-renewing stem cells of the olfactory epithelium [21].

In our RT-PCR analysis, expression of *Mash1* could be detected in the epithelium of fungiform, foliate, and circumvallate papillae, all of which contain taste buds. In contrast, we failed to amplify transcript for *Mash1* from tongue epithelium lacking taste buds. These results indicate that in rat lingual epithelium *Mash1* is expressed exclusively in cells of the taste bud lineage.

In our observations of in situ hybridization in rat taste buds, *Mash1*-expressing cells are round cells that lie in the basolateral portion of these structures. The morphology and location of *Mash1*-expressing cells fit the description of basal cells, which are thought to give rise to mature taste bud cells [22]. Furthermore, the expression of *Mash1* became undetectable in the epithelium of circumvallate papilla at 2 weeks after the operation, at which time the taste buds have completely disappeared. This result implies that *Mash1*-expressing cells are postmitotic precursors to mature taste cells or are an early stage of progenitor cells (transit amplifying cells) that are committed to terminal (neuronal) differentiation, but which are not self-renewing stem cells of the taste buds as they are in the olfactory receptor neuron lineage [21].

Mammalian taste buds consist of several distinct cell types [5,23–26]. These cells have been classified as dark cells and light cells by both light and electron microscopy and are considered to have either gustatory or supporting functions [27–29]. The present study raises the question as to whether *Mash1*-expressing cells give rise to all cell types or specific cell types in taste buds. This study leaves unanswered the question as to whether or not stem cells actually exist in taste buds. To further verify and elucidate the exact timing of expression of Mash1 mRNA in the rat taste bud cell lineage, more studies are required.

Despite the fact that taste buds differentiate from local lingual epithelium rather than from neural crest cells, *Mash1* appears to play a role in taste bud cell differentiation as it does in other receptor neurons. The dynamics of *Mash1* expression in degeneration and regeneration of denervated rat tongue indicate that the expression of *Mash1* in rat taste buds requires gustatory innervation.

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