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The Neural Differentiation Gene *Mash-1* has a Distinct Pattern of Expression from the Taste Reception-related Genes *gustducin* and *T1R2* in the Taste Buds

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Abstract

Taste bud cells have a limited lifespan and are continuously replaced just like other epithelial cells. Although there is some evidence that taste buds may arise from the local epithelium, taste receptor cells have neuronal properties. This implies that there must be a critical stage at which the epithelial precursor cells for taste receptor cells start to exhibit neural properties during the differentiation of the taste receptor cells. The expression of the neural-specific transcription factors *Mash-1* and *Prox-1* in the nervous system is transient and precedes neuronal differentiation. Therefore, we examined the expression of *Mash-1* and *Prox-1* in the epithelium of circumvallate papillae of the tongue in order to clarify the localization of the precursor cells with neural properties and observed that both expressions are restricted to the taste buds. Two-colour *in situ* hybridization showed that the signals for *Mash-1* did not overlap those for taste receptor cell-specific genes such as *gustducin* and *T1R2*. In the process of development and regeneration of the taste buds, the expression of *Mash-1* preceded that of *gustducin* and *T1R2*. These observations suggest that *Mash-1* could be a candidate for a marker of immature taste receptor cells, including the cells that express *gustducin* and/or *T1R2* at a later stage.

Introduction

Taste buds are sensory end organs that are composed of 50–150 cells and the maintenance of their morphology is dependent on innervation by gustatory fibres of the chorda tympani (VIIth) and glossopharyngea (IXth). It is known that taste bud cells are endodermal in origin (Barlow and Northcutt, 1995), that they have a limited lifespan and that they are continuously replaced in the manner of other epithelial cells (Beidler and Smallman, 1965; Farbman, 1980; Delay *et al*., 1986). In particular, a study on the tongues of X chromosome-inactivation mosaic mice suggested that the taste bud cells and epithelial cells around the taste buds arose from a common progenitor and that the taste bud cells originated from local tissue elements (Stone *et al*., 1995). Our previous study showed that Patched 1, a conserved target of sonic hedgehog, is expressed in the proliferating cells around the taste buds and that denervation causes the loss of Patched 1 expression, thereby providing the first example of a denervation-induced change in gene expression in the proliferating zone around the taste buds (Miura *et al*., 2001).

A taste bud might contain the cells in various stages of maturation. It is assumed that, in aged taste bud cells, there might be an apoptotic cell death pathway that mediates the apoptotic death factors P53, Bax and Caspase-2 (Zeng and Oakley, 1999; Zeng *et al*., 2000). On the other hand, the existence of undifferentiated taste bud cells has been suggested by morphological and physiological studies (Delay *et al*., 1986; Mackay-Sim *et al*., 1996), although no molecular marker has yet been found. In this study we hypothesized that a phase in which the precursor for taste receptor cells acquires the features of neural precursor cells might be found in the differentiation process of taste receptor cells from epithelial precursor cells, since mature taste receptor cells show the characteristics of neuronal cells that form synapses and release neurotransmitters and which are capable of generating action potentials.

In order to identify the location of the neural precursor-like cells for taste receptor cells in the epithelium of the tongue, we here focus on the two genes *Mash-1* and *Prox-1*, which are related to the development of the central nervous system (CNS). *Mash-1* is a mammalian homologue of the proneural genes of the *Drosophila achaete–scute* complex

(AS–C), which encodes a basic helix–loop–helix (bHLH)-type transcription factor (Johnson *et al.*, 1990). *Mash-1* has been found to be expressed in the developing peripheral nervous system (PNS) and CNS (Lo *et al.*, 1991; Guillemot *et al.*, 1993). *Prox-1*, which is also expressed in the developing brain, encodes a homeobox protein that is structurally homologous to *Drosophila prospero* (Oliver *et al.*, 1993). Both *Mash-1* and *Prox-1* are defined as molecular markers for transient precursors in the CNS (Torii *et al.*, 1999). *Mash-1* expression was recently investigated in round cells in the basal compartment of rat taste buds using *in situ* reverse transcriptase polymerase chain reaction (*in situ* RT-PCR) (Seta *et al.*, 1999) and, in cavefish, *Prox-1* was found in the developing taste buds, as well as the lens and retina (Jeffery *et al.*, 2000). However, the precise localization of *Mash-1* and *Prox-1* expression in the epithelium of the tongue is still poorly understood. Here we report that the expression of *Mash-1* and *Prox-1* was restricted to the taste buds and that the signals for *Mash-1* did not overlap those for *gustducin* (McLaughlin *et al.*, 1992) and *T1R2* (Hoon *et al.*, 1999), which are the molecules related to taste signal transduction.

Materials and methods

Experimental animals

The adult animals used in this study were C57BL/6N mice of 8–20 weeks old. The animals were initially purchased from Charles River Japan Inc. (Yokohama, Japan) and were bred at National Food Research Institute. Timed pregnant female mice were also purchased from Charles River Japan Inc. Embryonic day 0.5 ($E_{0.5}$) was defined as the day on which the vaginal plug was found and postnatal day 0 (P_0) was designated as the day of birth. The surgery for the glossopharyngeal (IXth) nerve crushes was performed as described previously (Miura *et al.*, 2001). Adult mice were intraperitoneally anaesthetized with Nembutal (50 mg/kg body weight) (Dainabot Co., Ltd, Osaka, Japan) and we followed the guidelines of our institute for the care and use of experimental animals.

Anti-sense RNA probes for *in situ* hybridization

cDNA fragments of *Mash-1*, *Prox-1*, *gustducin* and *T1R2* were cloned by RT-PCR using the total RNA extracted from the epithelium of the circumvallate papillae and E13.5 brain and then used for synthesis of cRNA probes. The sequences of the primers were 5′-CGACAGTTTGGCCCGGCATGGAGA-3′ (+59 to +82) and 5′-CCTGGCAGGTCCTCAGAACCAGTT-3′ (+783 to +760) (Genbank M95603) for Mash-1 and 5′-ATGGGAAGTGGAATTAGTTC-3′ (+114 to +133) and 5′-TCAGAAGAGCCCACAGTCTT-3′ (+1178 to +1159) (Genbank X65747) for gustducin. *Prox-1* and *T1R2* were cloned using nested PCR. The primer sequences for the first PCR were as follows: Prox-1 5′-ATGCCTGACCATGACAGCACA-3′ (+68 to +88) and 5′-CTACTCGTGAAGGAGTTCTTG-3′ (+2281 to +2261) (Genbank AF061576) and T1R2 5′-TTCGCCGTGGAGGAAATCAA-3′ (269+ to +288) and 5′-GAAGGAGAAGGTCATGCTGA-3′ (2353+ to +2334) (Genbank NM_031873). The primer sequences for the second PCR were as follows: Prox-1 5′-TCTTAAGCCGGCAAACCAAGA-3′ (+93 to +113) and 5′-TAGGCAGTTAGGGGATTTGAA-3′ (+2260 to +2240) and T1R2 5′-AACTGTAGCTCTCTGCTGCC-3′ (290+ to +309) and 5′-GTGATGAACTTGGCTTCGTT-3′ (2331+ to +2312). The PCR was carried out for 40 cycles under the following conditions: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Each resulting fragment was cloned into a pGEM-T easy vector (Promega Co., Madison, WI) and sequenced. Digoxygenin-UTP-labelled or fluorescein-UTP-labelled RNA probes were synthesized using an RNA transcription kit (Roche Diagnostics, Mannheim, Germany).

In situ hybridization

The mouse tongues were dissected, embedded in OCT compound (Sakura Finetech. U.S.A., Inc., LosAngeles, CA), frozen in liquid nitrogen and sectioned into 5 μm slices. *In situ* hybridization was performed as described previously (Asano-Miyoshi *et al.*, 1998) and two-colour *in situ* hybridization experiments were carried out under the same conditions with the following modifications. The sections of circumvallate papillae were hybridized to both digoxigenin-labelled and fluorescein-labelled cRNA probes. Following hybridization and washing with 0.2 × SSC, the sections were incubated with alkaline phosphatase-conjugated anti-fluorescein or anti-digoxigenin antibodies (1:400 for 1 h at room temperature) (Roche Diagnostics), washed with TBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) and treated with 2-hydroxy-3-naphthoic acid-2′-phenylanilidephosphate (HNPP)/Fast Red alkaline phosphatase substrate (Roche Diagnostics). The signals were observed by fluorescence microscopy. After treatment with 0.1 M glycine, pH 2.2, with 0.1% Tween-20 for inactivating alkaline phosphatase activity, the sections were re-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) and incubated with alkaline phosphatase-conjugated anti-fluorescein or anti-digoxigenin antibodies (1:400) (Roche Diagnostics) overnight at 4°C. After washing the sections with TBS, the colour reaction was performed with 1.5 mg/ml nitoroblue tetrazolium (NBT) and 0.75 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) overnight at room temperature. The HNPP/Fast Red signals were removed using an alcohol dehydration step and then the NBT/BCIP signals were observed by light microscopy. The images for HNPP/Fast Red and NBT/BCIP were overlaid by mean of the program Photoshop® (Adobe Systems Inc., San Jose, CA). The numbers of *Mash-1*+ cells

and *gustducin and T1R2* (*Ggust/T1R2*)+ cells were counted in the same section, averaged and represented graphically. Statistical analysis was performed using paired *t*-tests.

Results

Expression of *Mash-1* and *Prox-1* is restricted to the taste buds in adult mice

In order to determine whether the neural precursor-like cells are located in the taste buds, we carried out *in situ* hybridization in the mouse circumvallate papillae with the *Mash-1* and *Prox-1* probes. A taste bud consists of elongated cells and round-shaped cells and it is known that localization of the round-shaped cells is restricted to the basal side of a taste bud. The signals for *Mash-1* were mainly observed in a subset of the elongated taste bud cells (Figure 1A). Strong signals for *Prox-1* were observed in a limited number of the round-shaped basal cells of the taste buds. On the other hand, moderate and diffuse signals were observed in the elongated cells (Figure 1B). The taste buds appeared to contain a greater number of *Prox-1*+ cells than *Mash-1*+ cells. We next used two-colour *in situ* hybridization for determining whether or not *Mash-1* was expressed in the taste receptor cells. We used a mixed cRNA probe of *T1R2* and *gustducin* (*Ggust/T1R2*) in order to detect the mature taste receptor cells. Two-colour *in situ* hybridization showed that both *Mash-1* and *Ggust/T1R2* cRNA probes were detected in a subset of the taste bud cells but, interestingly, *Mash-1*+ cells did not overlap *Ggust/T1R2*+ cells (Figure 2). The intensity of the signals in the elongated taste bud cells against *Prox-1* was not sufficiently pronounced for detection with HNPP/Fast Red for two-colour *in situ* hybridization.

Mash-1 and *Prox-1* expression precedes *Ggust/T1R2* expression during taste bud development

In order to ascertain whether *Mash-1* was expressed earlier than taste receptors in the taste bud development, two-colour *in situ* hybridization with *Mash-1* and *Ggust/T1R2* probes was carried out in $P_{0.5}$ adult circumvallate papillae. At $P_{0.5}$, when discernible taste buds were rarely observed, *Mash-1*+ cells were distributed in the centre of the circumvallate papillary epithelium and circumvallate trench (Figure 3A). The number of *Mash-1*+ cells was significantly larger than that of *Ggust/T1R2*+ cells from $P_{0.5}$ to $P_{4.5}$ (Figures 3C,G,K and 4). The number of *Ggust/T1R2*+ cells was more rapidly increased than that of *Mash-1*+ cells. The numbers of *Mash-1*+ cells and *Ggust/T1R2*+ cells were approximately equal at $P_{10.5}$ and the number of *Mash-1*+ cells was smaller than that of *Ggust/T1R2*+ cells at adulthood (Figure 4). *Mash-1*+ cells did not overlap *Ggust/T1R2*+ cells at any stage (Figure 3 C,G,K,O,R).

Signals for *Prox-1* were also observed by *in situ* hybridization before taste bud formation (Figure 3D,H,L). *Prox-1* was expressed in the centre of the circumvallate papillary epithelium and circumvallate trench cells at $P_{0.5}$ (Figure 3D) and in the trench cells between $P_{2.5}$ and $P_{4.5}$ (Figure 3H,L).

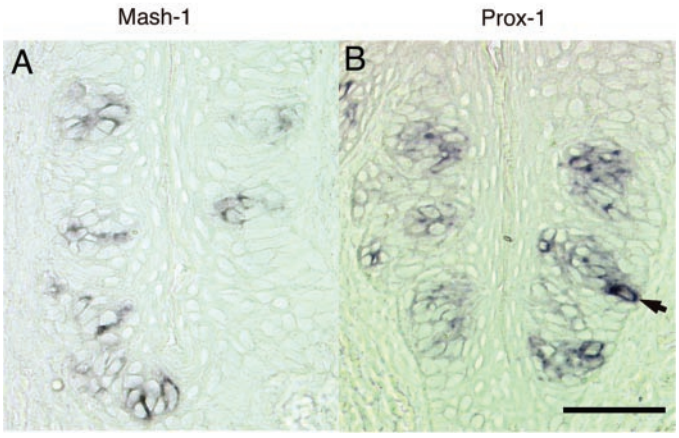

Figure 1 Expression of *Mash-1* and *Prox-1* in adult circumvallate papillae. *In situ* hybridization was used for detecting the expressions of *Mash-1* and *Prox-1*. Sections were prepared from the circumvallate papillae of a male mouse of 8 weeks old. Each section was hybridized with a digoxigenin-labelled cRNA probe for **(A)** *Mash-1* or **(B)** *Prox-1*. The arrow in (B) indicates the location of a positively stained round cell in the basal side of a taste bud. The scale bar indicates 50 μm.

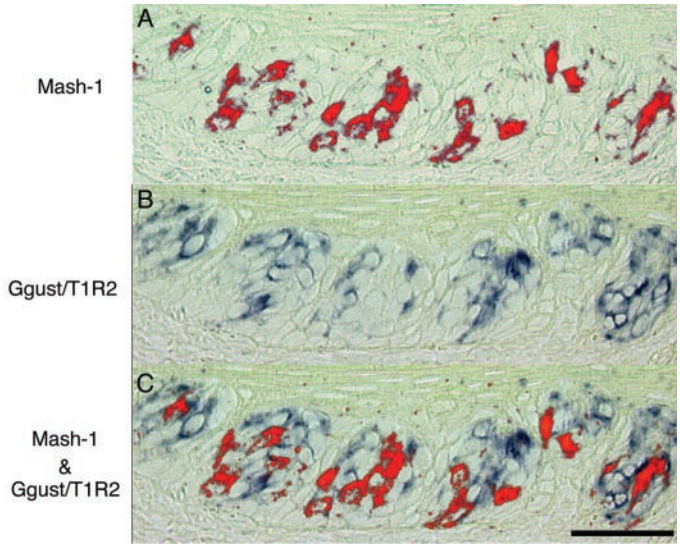

Figure 2 The expression pattern of *Mash-1* differs from that of *gustducin* and *T1R2*. Two-colour *in situ* hybridization was used for examining the overap in cellular expression of *Mash-1* and *gustducin/T1R2*. The section was hybridized with a digoxigenin-labelled *Mash-1* probe and a fluorescein-labelled *gustducin* and *T1R2* mixed probe (*Ggust/T1R2*). **(A)** The *Mash-1* probe was visualized with HNPP/Fast Red. **(B)** The *Ggust/T1R2* probe was visualized with NBT/BCIP (purple). Columns A and B show *Mash-1* or *Prox-1* signals obtained in the same field. An overlaid image is shown in column C. The scale bar indicates 50 μm.

Mash-1 is expressed at the early stage of taste bud regeneration

We also examined the expression of *Mash-1* and taste recep-

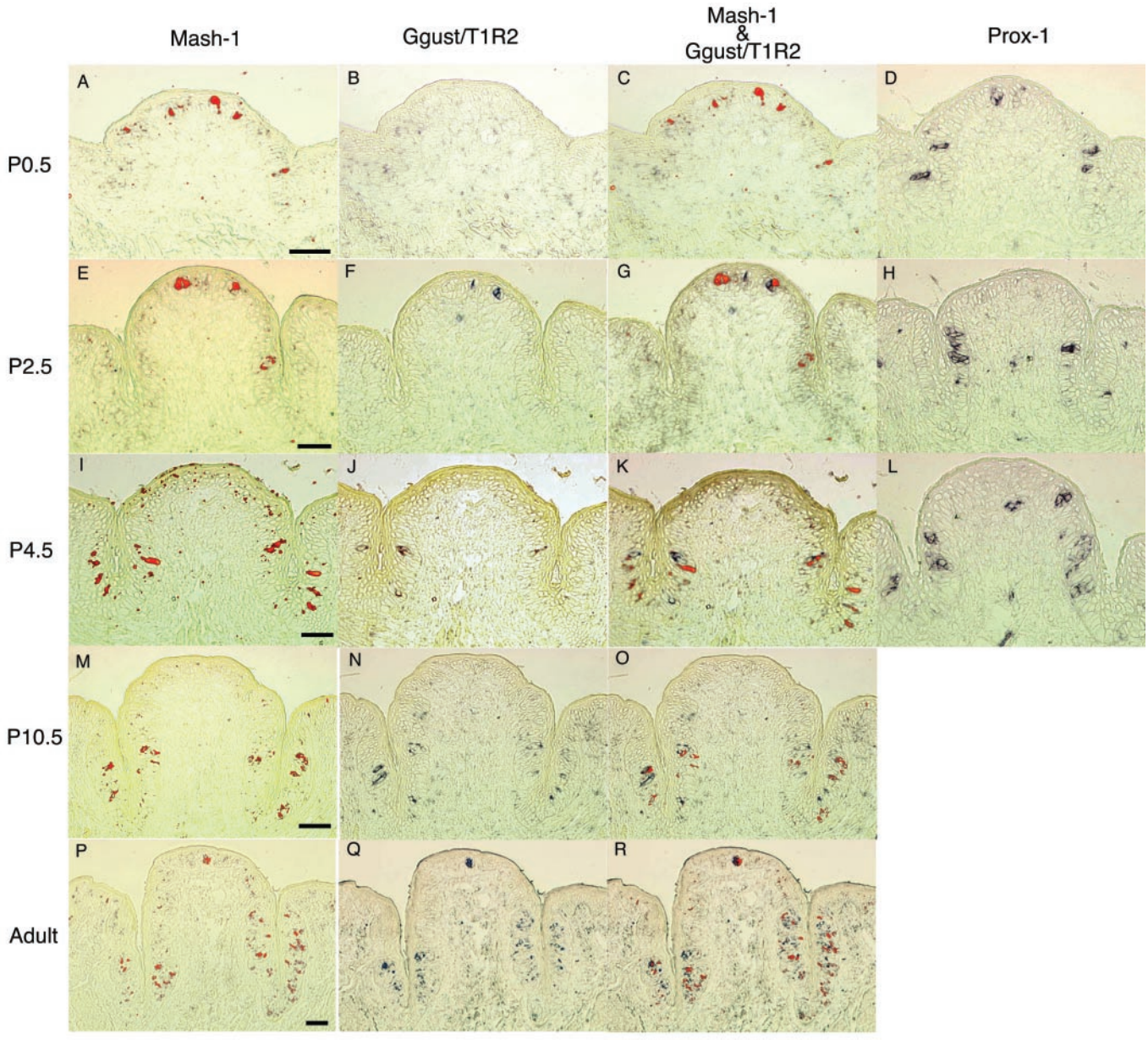

Figure 3 Expression of *Mash-1*, *Ggust/T1R2* and *Prox-1* during postnatal development. **(A–C, E–G, I–K, M–O, P–R)** Two-colour *in situ* hybridization was used for examining the change in the expression of *Mash-1* and *Ggust/T1R2* during postnatal development. The sections were observed as Figure 2. **(D, H, L)** *In situ* hybridization was used for examining the change in the expression of *Prox-1* during early postnatal development. Each scale bar indicates 50 μm.

tors during the regeneration of taste buds. In a previous study the expression of neural cell adhesion molecule (NCAM) after the crash of bilateral IXth nerves showed that regeneration of the taste buds proceeds from the 10–12 days after the nerve crush and that the taste buds are nearly normal in appearance by 20 days (Smith *et al.*, 1994). We therefore carried out two-colour *in situ* hybridization with the *Mash-1* and *Ggust/T1R2* probes using the sections of circumvallate papillae at 11 days after the nerve crash for the early stage and those at 16 days for the late stage of regeneration (Figure 5A). Although there was no discernible taste bud at 11 days after the nerve crush, *Mash-1*+ cells were seen in the epithelium while *Ggust/T1R2*+ cells were not observed. At 16 days after the operation, when a few taste buds had become detectable, both *Mash-1* and *Ggust/T1R2* were expressed in the taste buds and the number of *Mash-1*+ cells was larger than the number of *Ggust/T1R2*+ cells (Figure 5B).

Discussion

In this study we found that *Mash-1* was expressed in a subset of taste bud cells. Based on this finding, we will now discuss

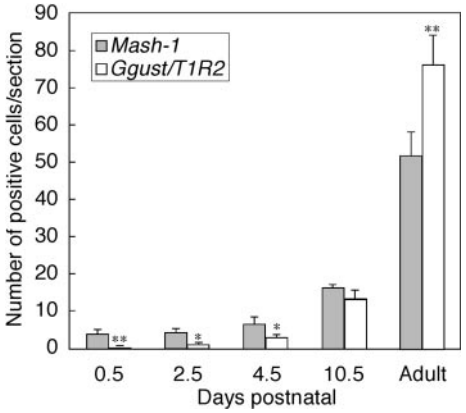

Figure 4 Temporal changes in the number of *Mash-1*+ or *Ggust/T1R2*+ cells in circumvallate papillae. *Mash-1*+ and *Ggust/T1R2*+ cells in the same sections were counted. Each column represents the means ± SEMs from four sections. Paired *t*-test: $^{**}P < 0.05$ and $^{*}P < 0.01$ versus *Mash-1*.

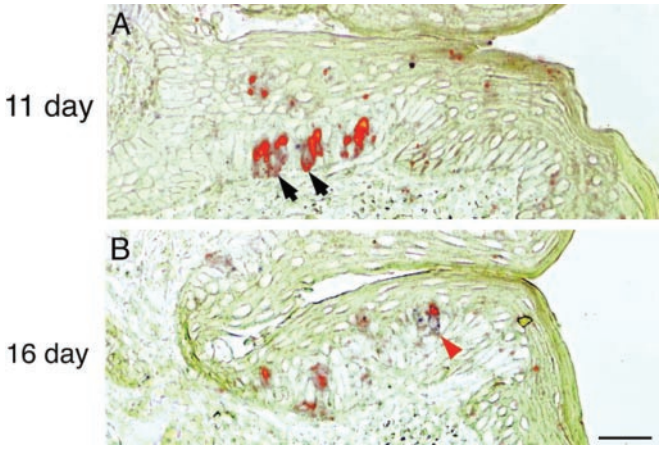

Figure 5 Expression of *Mash-1* and *Ggust/T1R2* in circumvallate papillae at 11 and 16 days after bilateral IXth nerve crash. The sections were treated as in Figure 2. **(A)** Arrows indicate individual *Mash-1*+ cells in the papilla epithelium before the appearance of taste buds. **(B)** The arrowhead indicates *Ggust/T1R2*+ cells in a taste bud. The scale bar indicates 50 μm.

the characteristics of *Mash-1*+ cells. *Mash-1*+ cells in the CNS are defined as a transient proliferating population that is molecularly distinct from self-renewing stem cells and induction of *Mash-1* might be one of the critical molecular events that control early development in the CNS (Torii *et al*., 1999). Our results showed that, during the generation and regeneration of taste buds, the expression of *Mash-1* in non-elongated cells was observed in the epithelium of circumvallate papillae prior to the formation of taste buds and the expression of *gustducin* and *T1R2*. The morphology of the non-elongated cells is similar to that of the cells expressing Protein gene-product 9.5, neuron-specific enolase and NCAM in the apical epithelium during early development of the taste buds (Takeda *et al*., 1992; Wakisaka *et al*., 1996). Although morphological studies are needed for determining the property of the non-elongated cells that express *Mash-1* and/or these proteins, the non-elongated cells are expected to be confined to the elongated taste bud cells since the expression of *Mash-1* and these proteins is restricted to the taste buds in adult circumvallate papillae.

We also showed that *Mash-1*+ cells consistently failed to overlap *Ggust/T1R2*+ cells from $P_{0.5}$. It has been reported that there are two taste receptor families in the mammalian tongue, namely T1Rs and T2Rs and that the members of T1Rs, namely T1R2 and T1R3 and T2Rs are expressed in a subset of the taste bud cells of circumvallate papillae (Hoon *et al*., 1999; Adler *et al*., 2000; Kitagawa *et al*., 2001; Max *et al*., 2001; Montmayeur *et al*., 2001; Nelson *et al*., 2001; Sainz *et al*., 2001). *T1R2*+ cells in circumvallate papillae express *T1R3* (Montmayeur *et al*., 2001) and *T2Rs*+ cells express *gustducin*, which is a taste bud-specific G protein related to sweet and bitter taste signal transduction (Adler *et al*., 2000). In addition, *T1R2*+ cells and *gustducin*+ cells represent separate populations (Hoon *et al*., 1999). Taking these data from recent studies into consideration, *Ggust/T1R2*+ cells express the taste receptors *T1R2*, *T1R3* or *T2Rs* and are regarded as mature taste receptor cells that receive taste stimuli and form synapses with gustatory nerve fibres and are electrically excitable as neural cells, thereby indicating the possibility that *Mash-1*+ cells are not mature taste receptor cells. If the role of *Mash-1* in the turnover of adult taste bud cells is not different from that in the development and regeneration of taste buds, our results raise the possibility that *Mash-1* might be involved in the differentiation of taste receptor cells and expressed transiently before the expression of taste receptors.

The cellular localization of *Mash-1*+ cells in the epithelium of circumvallate papillae might reveal the point at which taste bud cells arisen from the local epithelium start to display their neural characteristics in the taste receptor cell lineage. The previous finding that [^{3}H]thymidine or Brd-U is incorporated during cell division indicates that proliferating cells might not exist in the taste buds but in the region around the taste buds. In this case a part of the proliferation cells would migrate into the taste buds and finally gave rise to elongated taste receptor cells (Beidler and Smallman, 1965; Cho *et al*., 1998). Our previous study suggested that the proliferating cells around the taste buds expressed *Patched 1* depending on innervation and that *Patched 1*+ cells were not present in taste buds (Miura *et al*., 2001). In the present study we showed that *Mash-1* was expressed only in taste bud cells, but not expressed in proliferation cells around the taste buds, while in a previous study a fraction of *Mash-1*+ cells was mainly observed in proliferating cells in the developing forebrain neuroepithelium (Torii *et al*., 1999). Our results lead us to a speculate that, when the property of the precursor cells around the taste buds is switched from epithelial to neuronal, the cells might stop their proliferation, enter the taste buds and become transient neuronal precursor cells that are defined by the expression of *Mash-1*.

We observed that the expression of *Mash-1* in adult taste buds was mainly in elongated cells, suggesting that elongated taste bud cells also include immature taste receptor cells that are at the stage at which the cells undergo their final division and might begin to differentiate into taste receptor cells with the properties of neurons. On the other hand, Seta *et al*. (1999) showed that *Mash-1* was only expressed in round-shaped cells in the basal compartment, which had previously appeared to be basal cells in the taste buds. We cannot deny the possibility that *Mash-1* is also expressed in the basal cells since they are considered to give rise to mature cells (Naga *et al*., 1970), but our result showed that the expression in round cells was not predominant. More morphological studies are required in order to determine whether *Mash-1* is or is not expressed in round-shaped cells.

Both *Mash-1* and *Prox-1* were expressed at $P_{0.5}$–$P_{4.5}$ and were expressed earlier than *Ggust/T1R2*. *Prox-1* is regarded as a molecular marker for transient precursors in the developing CNS (Torii *et al*., 1999), supporting the idea that not only *Mash-1* but also *Prox-1* might play a role in the differentiation of taste receptor cells. The strong signal for *Prox-1* in adult taste buds in the present study was observed in round-shaped cells and the weak one was observed in elongated cells. The number of *Prox-1*+ elongated cells appeared to be much larger than the number of *Mash-1*+ cells, thereby increasing the probability that *Prox-1*+ elongated cells could overlap both *Mash-1*+ cells and *Ggust/T1R2*+ cells. Two-colour *in situ* hybridization using *Prox-1*, *Mash-1* and *Ggust/T1R2* probes could determine whether *Prox-1*+ cells overlap with *Mash-1*+ cells and/or *Ggust/T1R2*+ cells, but the signal for *Prox-1* in this study was too weak for determining cellular localization by two-colour *in situ* hybridization. In a recent study, forced expression of *Mash-1* in a stem cell-derived cell line suggested that *Mash-1* functions upstream of *Prox-1*, which is consistent with results on spatial and temporal expression in the developing CNS (Torii *et al*., 1999). In the case that *Mash-1* leads to the expression of *Prox-1* in taste bud cells, our results raise the possibility that *Prox-1* might be expressed from the *Mash-1*+ stage to the *Ggust/T1R2*+ stage during taste receptor cell differentiation. Culturing of stem cells for taste bud cells will need to be performed in order to confirm this idea. The possibility that *Prox-1*+ round-shaped cells could be the basal cells differentiated into taste receptor cells and share the cell lineage with *Prox-1*+ elongated cells should be tested in the same way.

Taste receptor cells are classified into different groups depending on the expression of the taste receptors, e.g. *T2Rs*+ cells, *T1R2*+ cells and so on. Is *Mash-1* expressed in precursor cells for all groups of taste receptor cells? In our study no cells showed co-expression of *Mash-1* and *Ggust/T1R2*. This result leaves the question of whether or not *Mash-1*+ cells mature to express *gustducin* and/or *T1R2* unanswered. However, in the olfactory receptor system, despite the fact that individual olfactory receptor neurons are thought to express one of the 1000 odorant receptor genes, *Mash-1* is essential for the differentiation of almost all receptor neurons (Guillemot *et al*., 1993), thereby supporting our speculation that *Mash-1* might play a role in taste receptor cell differentiation and might be defined as a marker for immature taste receptor cells.

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