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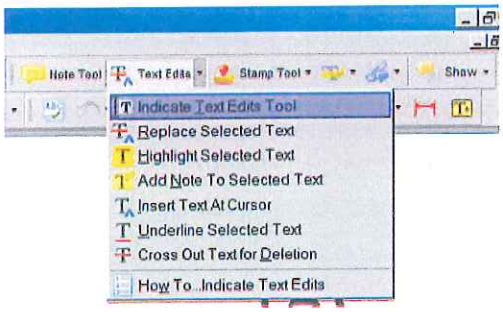

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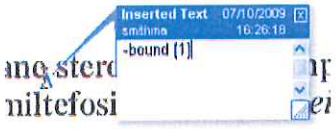

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Expression of GAD67 and Dlx5 in the Taste Buds of Mice Genetically Lacking Mash1

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Abstract

It has been reported that a subset of type III taste cells express glutamate decarboxylase (GAD)67, which is a molecule that synthesizes gamma-aminobutyric acid (GABA), and that Mash1 could be a potential regulator of the development of GABAnergic neurons via Dlx transcription factors in the central nervous system. In this study, we investigated the expression of GAD67 and Dlx in the embryonic taste buds of the soft palate and circumvallate papilla using Mash1 knockout (KO)/GAD67-GFP knock-in mice. In the wild-type animal, a subset of type III taste cells contained GAD67 in the taste buds of the soft palate and the developing circumvallate papilla, whereas GAD67-expressing taste bud cells were missing from Mash1 KO mice. A subset of type III cells expressed mRNA for Dlx5 in the wild-type animals, whereas Dlx5-expressing cells were not evident in the apical part of the circumvallate papilla and taste buds in the soft palate of Mash1 KO mice. Our results suggest that Mash1 is required for the expression of GAD67 and Dlx5 in taste bud cells.

Key words: Dlx, glutamate decarboxylase 67, Mash1, taste bud, type III cell

Introduction

In mammals, most taste buds are observed in the stratified squamous epithelium of the dorsal surface of the tongue where they are concentrated in the circumvallate, foliate, and fungiform papillae. Several elongated cells assemble to form an onion-shaped taste bud, which constantly differentiate from basal stem cells within the taste buds (Beidler and Smallman 1965; Farbman 1980). The gustatory cells (type III cells) observed in taste buds have been identified as paraneurons because they possess the characteristics of both neuronal and epithelial cells (Fujita et al. 1988). Similar to neurons, these cells form synapses with gustatory nerve fibers, store and release transmitters, and are capable of generating action potentials (Roper 1989, 1992). Similar to epithelial cells, taste cells have a limited lifespan; they undergo continuous renewal and are regularly replaced throughout the lifespan of mammals from approximately 10 proliferative basal stem cells per bud (Beidler and Smallman 1965; Farbman 1980; Delay et al. 1986; Stone et al. 2002).

Mammalian homologues of *Drosophila* proneural genes have been identified in the achaete-scute complex. Mash1 is a mammalian achaete-scute homologue of the proneural gene, which encodes basic helix-loop-helix (bHLH) transcription factors (Johnson et al. 1990; Guillemot and Joyner 1993). Mash1 is specifically expressed in subsets of neuronal precursors in both the developing central nervous system and peripheral nervous system (Lo et al. 1991; Guillemot et al. 1993). Disruption of the Mash1 gene in mice results in the elimination of most olfactory and autonomic neurons, suggesting that Mash1 may play a role in determining the cell fate of specific neural lineages (Guillemot et al. 1993; Sommer et al. 1996; Blaugrund et al. 1996). In addition, Mash1 promotes differentiation of the retina, olfactory

epithelium, and neuroendocrine lineages and is essential for the production of the correct ratios of neural cell types (Tomita et al. 1996; Porteus et al. 1994; Gordon et al. 1995).

We demonstrated that the bHLH transcription factor Mash1 is expressed in the taste papillae of mouse embryos. In addition, Mash1 is expressed in subsets of cells within mature taste buds of adult rodents (Seta et al. 1999; Kusakabe et al. 2002; Miura et al. 2003, 2005; Nakayama et al. 2008). Mash1 expression occurs slightly after taste bud cell differentiation in adulthood, implying the continuity of Mash1 expression during cell differentiation from basal cells to elongated cells (Miura et al. 2006; Nakayama et al. 2008). We observed that Mash1 was expressed in some basal cells and in the majority of differentiated type III taste cells but never in type II taste cells (Seta et al. 2006). Furthermore, we demonstrated that Mash1 is required for the expression of aromatic l-amino acid decarboxylase (AADC) in type III cells in the taste buds, which supports the hypothesis that different taste bud cell types have progenitor cells that are specific to each cell type (Seta et al. 2011).

Gamma-aminobutyric acid (GABA) is a known neurotransmitter candidate related to taste signaling in taste buds (Dvoryanchikov et al. 2011, Huang et al. 2011). GABA is the major inhibitory neurotransmitter in the nervous system and it has several roles, including the regulation of proliferation, migration, differentiation, and synapse formation during embryonic development (Barker et al. 1998; Luján et al. 2005; Kwakowsky et al. 2007). GABA is synthesized from glutamate by glutamate decarboxylase (GAD), which has 2 molecular isoforms, GAD65 and GAD67 (Erlander et al. 1991; Martin et al. 2000). Recent studies have demonstrated that GAD67 is expressed in the type III taste cells of mice (DeFazio et al. 2006; Tomchik et al. 2007). In addition, studies have shown that the expression of Dlx genes, which are the vertebrate homologues of the *Drosophila* distal-less (dll) that controls cell differentiation and morphogenesis (Perera et al., 2004), is closely associated with GABAnergic neurons in the central nervous system (Fode et al. 2000). Moreover, the ectopic expression of Dlxs induced the expression of GADs, which are enzymes that synthesize GABA (Stühmer et al. 2002a,b). In the developing forebrain, the GAD67 and GAD65 genes are coexpressed with the homeobox genes Dlx2 and Dlx5, which are sequentially induced and are upstream regulators of GAD (Liu et al. 1997; Eisenstat et al. 1999; Stühmer et al. 2002a,b). Similar to the developing forebrain, Dlx2 expression in the lens is induced prior to Dlx5 according to semiquantitative reverse transcription–polymerase chain reaction (RT-PCR), where these transcription factors overlap with the expression domains of GAD (Kwakowsky et al. 2007). Expression of the Mash1 and Dlx genes overlaps in the mouse forebrain, suggesting that these genes genetically interact during mouse forebrain development (Porteus et al. 1994; Andrews et al. 2003). In addition, Mash1 knockout (KO) mice have defects in the neural specification and in the timing of differentiation in the ventral forebrain, including the altered telencephalic expression of Dlx genes and GAD67 (Casarosa et al. 1999; Horton et al. 1999; Yun et al. 2002; Long et al. 2009). However, the expression of Dlx genes has not been described in taste bud cells.

In this study, we investigated the altered expression of GAD67 in the type III cells in mouse taste buds using Mash1 KO mice, which expressed green fluorescent protein (GFP) under the control of an endogenous GAD67 promoter. Furthermore, we examined the expression of Dlx5 in the taste buds of Mash1 KO mice.

Materials and methods

Animals

All of the animals used in this study were maintained and handled according to protocols approved by Kyushu Dental University Animal Care. The adult animals used in this study were Mash1 heterozygous mutant ($Mash1^{+/-}$) mice (Guillemot et al. 1993) and GAD67-GFP knock-in mice (Tamamaki et al. 2003). All of the embryos used in this study were obtained from timed pregnant heterozygous Mash1 mutant ($Mash1^{+/-}$) mice with heterozygous GAD67-GFP knock-in mice. $Mash1^{+/-}$; GAD67-GFP heterozygous parents were obtained by crossing $Mash1^{+/-}$ transgenic males with heterozygous GAD67-GFP females and crossed with $Mash1^{+/-}$; GAD67-GFP mice to obtain Mash1 KO; GAD67-GFP embryos. The genotyping of Mash1 KO mice used PCR with the following primers: Mash1 KO sense, 5′-ACGACTTGAACTCTATGGCGGGTTCTC-3′; Mash1 wild-type antisense, 5′-GCCACTCTCAGGGGCCA AGACTGAAGTTAA-3′; Mash1 KO antisense, 5′-AAATT AAGGGCCAGCTCATTCCTCCACTCA-3′. This PCR-based technique enabled the discrimination of $Mash1^{+/-}$, $Mash1^{-/-}$, and wild-type $Mash1^{+/+}$ mice. GAD67-GFP mice were genotypes by PCR using the following primers: GAD67-GFP sense, 5′-GGCACAGCTCTCCCTTCTGTTTGC-3′; GAD67-GFP mutant antisense, 5′-CTGCTTGTCGGCCAT GATATAGACG-3′. In this study, we used Mash1 KO; GAD 67-GFP ($Mash1^{-/-}$; GAD67-GFP), Mash1 KO ($Mash1^{-/-}$), wild-type; GAD67-GFP ($Mash1^{+/+}$; GAD67-GFP), and wild-type ($Mash1^{+/+}$) mice.

The day of vaginal plug detection was considered to be embryonic day 0.5 (E0.5). Pregnant mice were sacrificed on E18.5 by administering an overdose of sodium pentobarbital, and the embryos were surgically removed. Adult mice (6–8 weeks) were anesthetized by administering an intraperitoneal injection of pentobarbital (50 mg/kg) and perfused via the left ventricle with 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4. The heads and tongues of the embryos and adults were fixed overnight in 4% PFA and embedded in OCT compound (Sakura). Cryosections (6–8 μm) were mounted on Superfrost slides (Matsunami) and stored in airtight boxes at −80°C.

In situ hybridization

Tissue sections were processed for *in situ* hybridization as previously described (Seta et al. 2006). In brief, rehydrated sections were treated for 10 min with 0.2 N HCl and for 5 min with proteinase K (1 μg/mL in Tris-EDTA). The sections were subsequently washed in phosphate-buffered saline (PBS), refixed for 20 min in 4% PFA, and treated twice for 15 min with glycine (2 mg/mL in PBS). The sections were prehybridized for 1 h at room temperature in hybridization buffer. Digoxigenin-labeled antisense and sense riboprobes were generated from plasmids containing Dlx5. Hybridization was performed overnight at 68°C in hybridization buffer containing 0.5–1.0 μg/mL of the riboprobe. Excess probe was removed by sequential washes, and the sections were then blocked for 1 h in 1% blocking reagent in maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl). The sections were incubated for 2 h with anti-digoxigenin antibody conjugated with alkaline phosphatase in a 1:250 dilution in blocking solution. The sections were rinsed thrice with PBS, and the bound antibody was visualized using the 4-nitro blue tetrazolinium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) blue color reaction. The sections were refixed in 4% PFA for subsequent imaging or were subjected to further immunohistochemical experiments.

Immunohistochemistry

After in situ hybridization, some sections were analyzed to determine the presence of taste receptor cells using antibodies against AADC and gustducin. The sections were rinsed in PBS, blocked for 2 h in 5% goat serum in PBS, and incubated overnight with primary rabbit anti-gustducin (1:200; sc-395, Santa Cruz) and anti-AADC (1:200; AZ1030, Enzo Life Sciences) overnight at 4°C in a humidified chamber. After rinsing with PBS, the sections were incubated overnight at 4°C with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:1000; A11034, Invitrogen). After rinsing with PBS, the sections were incubated with 2 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; MERCK) and mounted in Vectashield (Vector Laboratories) under a coverslip.

Immunofluorescent and in situ hybridization images were collected using an Olympus DP72 CCD camera mounted onto an Olympus BX50 microscope. Digital images were acquired using the DP2-BSW software, converted into TIFF format, and contrast- and color-adjusted using Adobe Photoshop CS3 for Macintosh. In addition, overlays of fluorescence and in situ hybridization images were generated in Photoshop as follows: the in situ hybridization image was initially inverted and the blue and green channels were deleted to black, leaving an inverted, pseudocolored red image. Furthermore, the green fluorescence image of the same field of view was pasted into the green channel to produce the overlay.

Whole mount observation of GAD67 in the soft palate of mouse embryos

To visualize the localization of GAD67, we performed whole mount observations of soft palates obtained from Mash1 KO; GAD67-GFP (Mash1$^{-/-}$; GAD67-GFP) and wild-type; GAD67-GFP (Mash1$^{+/+}$; GAD67-GFP) mice. The soft palates were dissected from E18.5 embryos and incubated for 60 min at 37°C in α-MEM (Invitrogen), which was supplemented with 2% collagenase, type IV (Sigma). After incubation, the epithelium of the soft palate was manually separated from the underlying connective tissue using fine forceps. The epithelia were fixed in 4% PFA in phosphate buffer for 60 min at 4°C and washed with PBS. Furthermore, the epithelia were incubated with 2 μg/mL DAPI. Fluorescence images were captured using a CCD camera (Olympus).

Reverse transcription–polymerase chain reaction

For RT-PCR, the circumvallate papillae were dissected from adult mouse tongues and incubated for 60 min at 37°C in α-MEM (Invitrogen) containing 2% collagenase, type IV (Sigma). After incubation, the epithelia of the circumvallate papillae were manually separated from the underlying connective tissue using fine forceps. The total RNA was isolated from the epithelia of the circumvallate papillae, and the RNA was incubated with DNase I. Single-stranded cDNA was produced from the total RNA via reverse transcription using an oligo-dT primer and avian myeloblastosis virus (AMV) reverse transcriptase at 42°C for 4 h. Following denaturation at 94°C for 120 s, PCR amplification was performed under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for a total of 35 cycles, followed by a final elongation for 15 min at 72°C. The reverse transcriptase step was omitted for the negative control samples to confirm the removal of all the genomic DNA. The amplification products were analyzed on 2% agarose gels and visualized using ethidium bromide. The sequences of the primers used were as follows:

Dlx2: 5′-CGGGGACGATTTTCTAACCT-3′ (forward) and 5′-CTGCTGAGGTCACTGCTACG-3′ (reverse); Dlx5: 5′-CAGAAGAGTCCCAAGCATCC-3′ (forward) and 5′-CTGGTGACTGTGGCGAGTTA-3′ (reverse); β-actin: 5′-cacctgtgctgctcacc-3′ (forward) and 5′-gcacgatttccctctcag-3′ (reverse).

Results

Expression of GAD67 in the type III cells of the taste buds

To determine the cell types that express GAD67 in the taste buds, we performed immunohistochemistry with an antibody against AADC using the taste buds of GAD67-GFP knock-in mice (adult). Previous studies demonstrated that

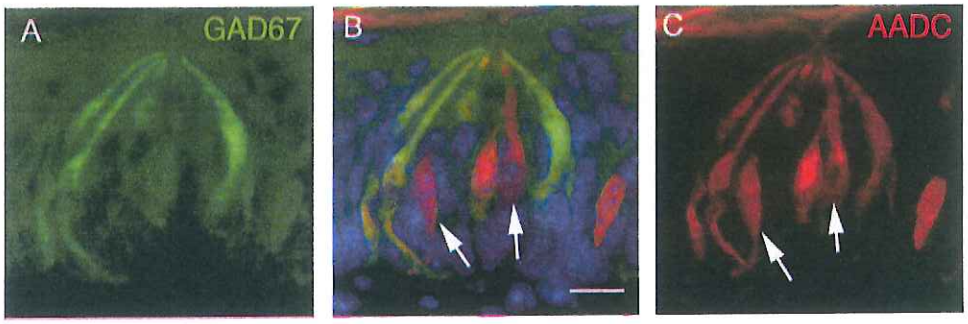

Figure 1 Expression of GAD67 in the circumvallate papillae of adult mice. GFP-positive GAD67-expressing cells (green, A), AADC (red, C), and their overlay (B) in a longitudinal section. A small number of AADC-positive taste cells lacking GFP (GAD67; arrows). Nuclei are labeled with DAPI (blue, B). Scale bar in B = 20 μm.

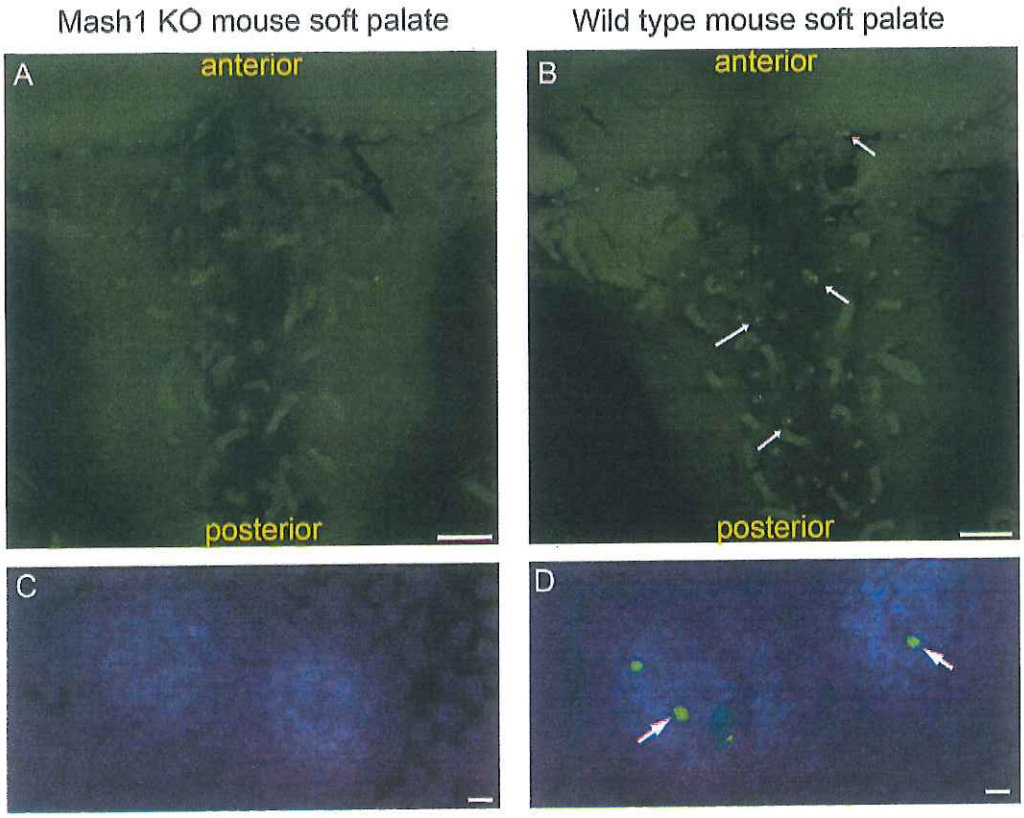

Figure 2 Whole mount observation of GAD67 in the soft palate taste buds of Mash1 KO (A, C) and wild-type mice (B, D) at E18.5. At E18.5, GAD67-positive cells were expressed in the epithelium of the soft palate in wild-type mice (B, D; arrows). In contrast, GAD67-positive cells were absent from the Mash1 KO soft palate epithelia (A, C). Nuclei are labeled with DAPI (blue, C, D). Scale bars in A, B = 200 μm; scale bars in C, D = 10 μm.

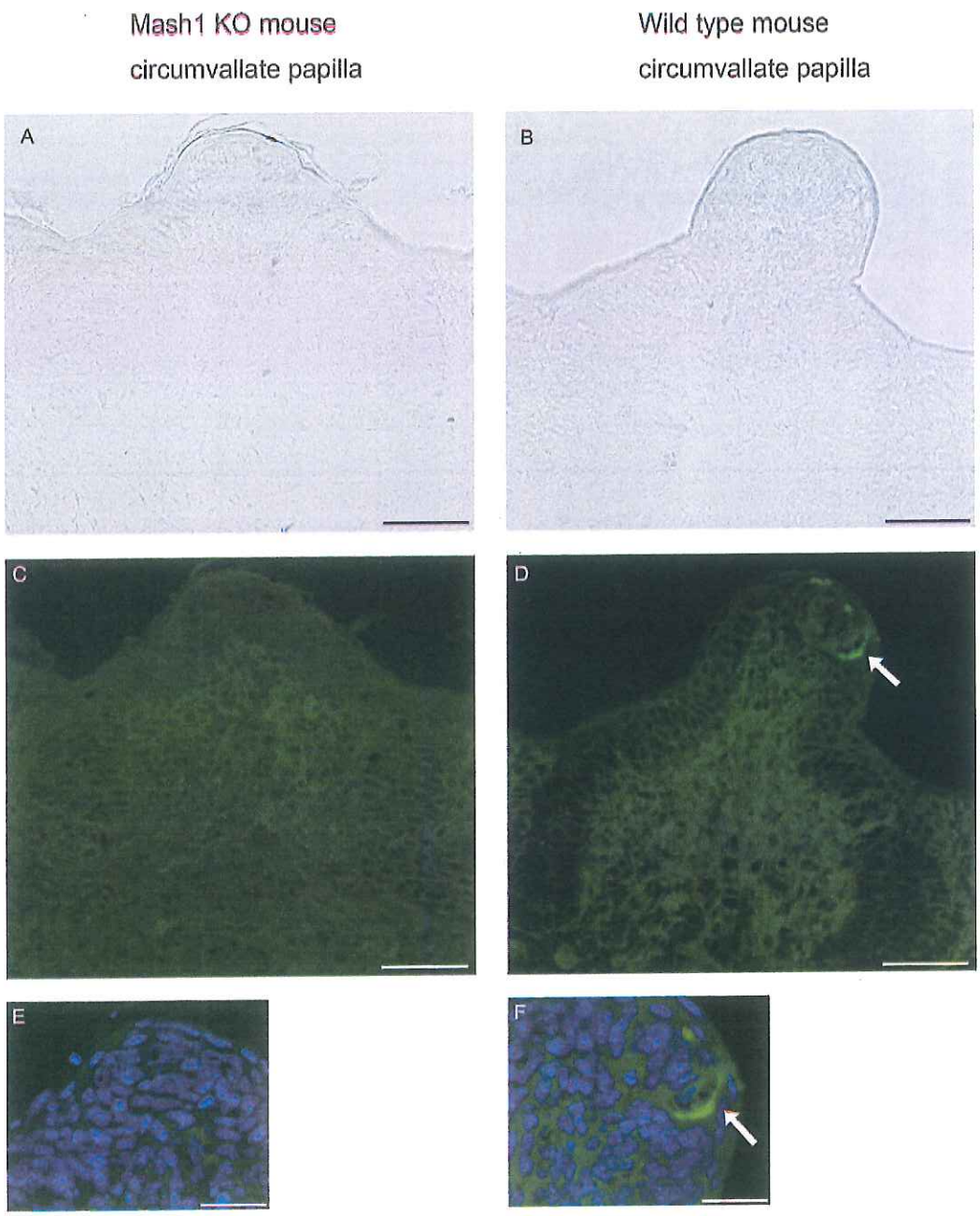

Figure 3 Expression of GAD67 in the circumvallate papillae of Mash1 KO mice (A, C, E) and wild-type mice (B, D, F) at E18.5. GAD67 was expressed in the apical epithelia of the circumvallate papillae in wild-type mice (D, F; arrows). In contrast, GAD67-positive cells were missing from the epithelia of the circumvallate papillae in Mash1 KO mice(C, E). Nuclei are labeled with DAPI (blue, E, F). Scale bars in A, B, C, D = 50 μm; scale bars in E, F = 25 μm.

both GAD67 and AADC are markers of a subset of type III taste cells (Murata et al. 2010; Seta et al. 2011). GFP-positive GAD67-expressing cells were observed in a small population of taste cells. All of the GAD67-expressing taste cells exhibited immunoreactivity with AADC (100%; 107/107). However, approximately 56.9% of the AADC-positive taste cells also expressed GAD67 (56.9%; 107/188) (Figure 1).

Expression of GAD67 in the soft palate, circumvallate papillae, and fungiform papillae of Mash1 KO mice

To determine the effects of the loss of Mash1 on the differentiation of taste cells, we examined the expression of GAD67 in the soft palate, circumvallate papillae, and fungiform papillae of Mash1 KO mice at E18.5 because Mash1 KO mice die within 24 h of birth. At E18.5, GAD67-positive cells were expressed in the epithelium of the soft palate in wild-type mice (Figures 2B,D). In contrast, GAD67-positive cells were absent from the Mash1 KO soft palate epithelia (Figures 2A,C). Similarly, GAD67 expression was present in both the circumvallate papillae and fungiform papillae of E18.5 of wild-type mice (Figures 3B,D,F and 4B,D). However, GAD67 expression was absent from Mash1 KO mice at E18.5 (Figures 3A,C,E and 4A,C). At E18.5, the taste buds could be visualized in the epithelium of the soft palate, circumvallate papillae, and fungiform papillae in the wild-type and Mash1 KO mice. These results indicate that Mash1 was involved in the promotion of GAD67-GFP-labeled type III taste cell differentiation although Mash1 did not play a role in taste bud development.

RT-PCR analysis

We performed RT-PCR experiments to assess the expression of the Dlx2 and Dlx5 genes in the mouse circumvallate papillae epithelium. RT-PCR using RNA prepared

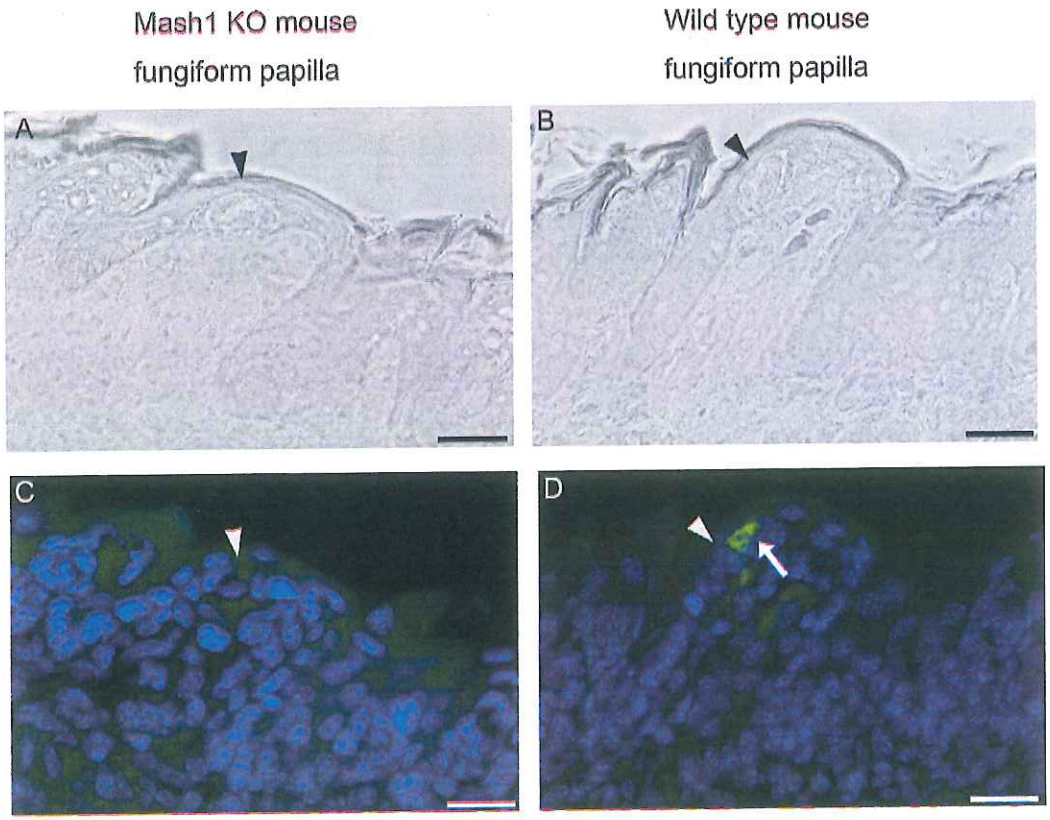

Figure 4 Expression of GAD67 in the fungiform papillae of Mash1 KO mice (A, C) and wild-type mice (B, D) at E18.5. GAD67 was expressed in the apical epithelia of the fungiform papillae in wild-type mice (B, D; arrows). In contrast, GAD67-positive cells were missing from the epithelia of the fungiform papillae in Mash1 KO mice (A, C). Arrowheads indicate taste buds. Nuclei are labeled with DAPI (blue in C, D). Scale bars = 20 μm.

from the epithelium of the circumvallate papillae and brain detected amplification products of the expected size (Dlx2: 931 bp, Dlx5: 647 bp), which were obtained using primer sets specific for mouse Dlx2 and Dlx5 (Figure 5), and they were sequenced to confirm their identities. Moreover, an amplification product was not obtained using RNA prepared from the epithelium of circumvallate papillae in the absence of reverse transcription.

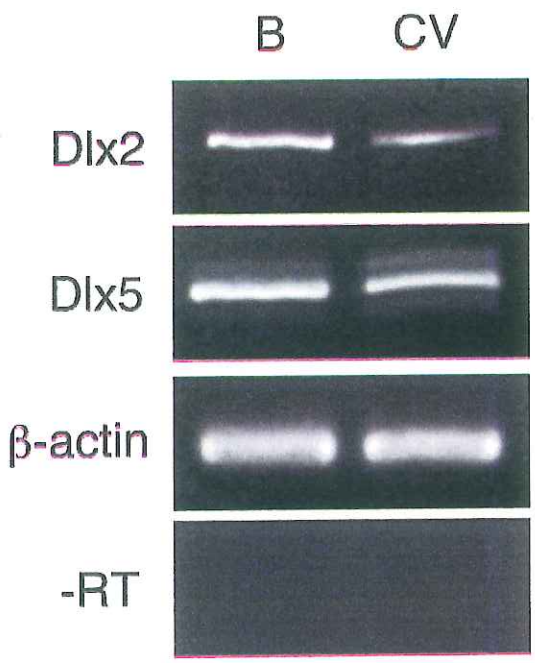

Figure 5 Analysis of Dlx2 and Dlx5 expression in the mouse tongue. RT-PCR was performed using mRNA prepared from the epithelium of circumvallate papillae (CV) and brain (B). Amplification products of the expected sizes (Dlx2: 931 bp, Dlx5: 647 bp) were obtained using primer sets specific for mouse Dlx2 and Dlx5. Expression of β-actin mRNA was used as a control. The reverse transcriptase step was omitted for the negative controls to confirm the removal of all the genomic DNA.

Expression of Dlx5 in the circumvallate papillae of adult mice

To examine whether Dlx5-expressing cells are located in the taste buds, we performed in situ hybridization in the mouse circumvallate papillae using a Dlx5 probe. Signals for Dlx5 were observed in a subset of the elongated taste bud cells and epithelial cells that surrounded the taste buds (Figure 6). To further assess the cells that expressed Dlx5 in the taste buds, we performed double labeling using in situ hybridization for Dlx5 and immunohistochemistry for the taste cell markers (AADC and gustducin). Immunofluorescence indicated that Dlx5 was expressed with a staining pattern that did not overlap with gustducin-immunoreactive (IR) cells (Figure 7C; Table 2). Approximately 35% of the Dlx5-expressing cells exhibited immunoreactivity for AADC (35.1%, 39/111, Figure 7F; Table 2), whereas 16% of the AADC-positive cells also expressed Dlx5 mRNA (15.9%, 39/245, Figure 7F; Table 2).

Expression of Dlx5 in both the circumvallate papillae epithelium and soft palate at E18.5

To investigate the effect of the loss of Mash1 on the expression of Dlx5, we examined the expression of Dlx5 in both the circumvallate papillae and soft palate in Mash1 KO mice. At E18.5, Dlx5-expressing cells were observed in the circumvallate papillae in abundance in the epithelium of the deep portion of the trenches. In addition, Dlx5 expression was detected in a small number of cells in the apical

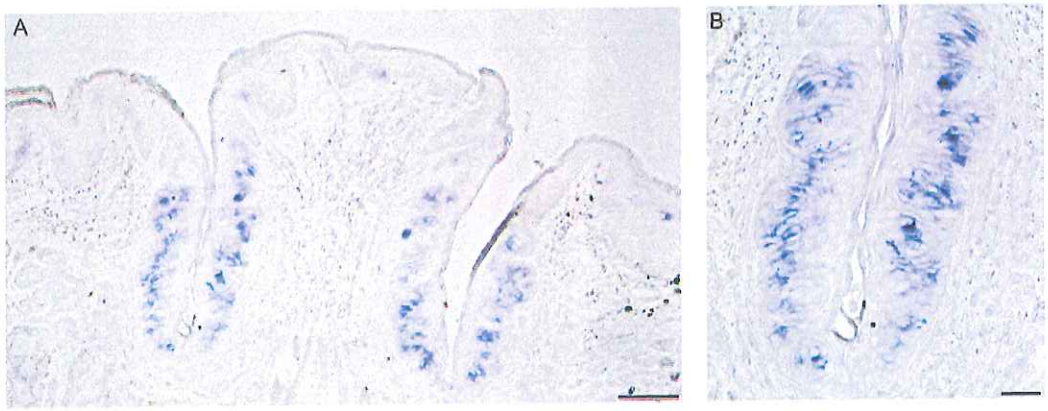

Figure 6 Expression of Dlx5 mRNA in the circumvallate papilla of adult mice (A, B). Dlx5 mRNA was detected in a subset of taste bud cells and epithelial cells that surrounded the taste buds but were not observed in other papillary epithelial elements. Scale bar in A = 200 μm; scale bar in B = 50 μm.

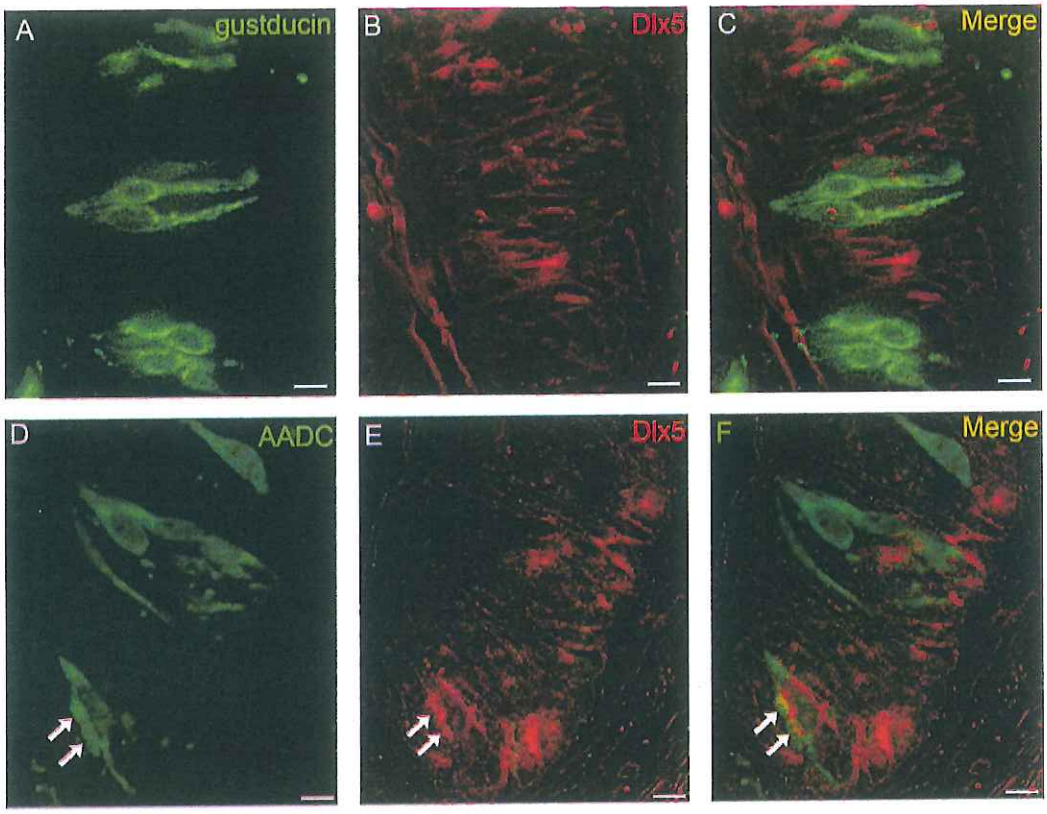

Figure 7 Comparison of Dlx5 mRNA expression based on the immunofluorescent detection of gustducin and AADC in the circumvallate papillae of adult mice. The immunofluorescence of the taste cell markers is shown in the left column (green; A: gustducin, D: AADC), Dlx5 mRNA expression is shown in the middle column (red), and computer-generated overlays are shown in the right column (C: gustducin, F: AADC). Several AADC-positive taste cells coexpressed with Dlx5 mRNA (arrows). Scale bars in A–F = 10 μm.

Table 1 Expression of GAD67 and Dlx5 in Mash1 KO and wild-type mice at E18.5

GAD67	Soft palate		Circumvallate papilla		Fungiform papilla	
	KO	WT	KO	WT	KO	WT
Taste bud	(–)	(+)	(–)	(+)	(–)	(+)
Epithelium	(–)	(–)	(–)	(–)	(–)	(–)
Dlx5	**Soft palate**		**Circumvallate papilla**			
	KO	WT	KO	WT		
Taste bud	(–)	(+)	(–)	(+)		
Epithelium	(+)	(+)	(+)	(+)		

(–), not detectable; (+), detectable.

papillary epithelium of wild-type mice (Figure 8B, arrow). In Mash1 KO mice, Dlx5 expression was also detected in cells in the deep trench epithelium of the circumvallate papillae, which was similar to the wild-type mice. However, Dlx5-expressing cell clusters were not observed in the apical circumvallate papillae in Mash1 KO mice (Figure 8A). Dlx5-expressing cells were observed in taste bud cells and in the epithelial ridge of the soft palate in

wild-type mice (Figure 9B). However, Dlx5-expressing cells were not observed in the taste bud cells in Mash1 KO mice (Figure 9A).

Table 2 Extent of the overlap of taste bud cells that expressed Dlx5 mRNA with cells that were immunopositive for taste cell markers

	Total labeled cells			
In situ hybridization markers	Dlx5	$n = 22$	Dlx5	$n = 111$
	Gustducin	$n = 61$	AADC	$n = 245$
	Coexpression	$n = 0$	Coexpression	$n = 39$

Discussion

In this study, we demonstrated that GAD67-positive cells were absent from both the circumvallate papillae and taste buds of the soft palate in Mash1 KO mice. Moreover, Dlx5 was expressed in a subset of the type III cells of the adult taste buds, whereas Dlx5-expressing cell clusters were not observed in the apical circumvallate papillae and taste buds of the soft palate in Mash1 KO embryos.

Our results indicate that GAD67-positive cells and AADC–IR cells colocalized within the taste buds. We previously showed that AADC–IR cells were absent from the taste papillae of Mash1 KO mice (Seta et al. 2011). In this study,

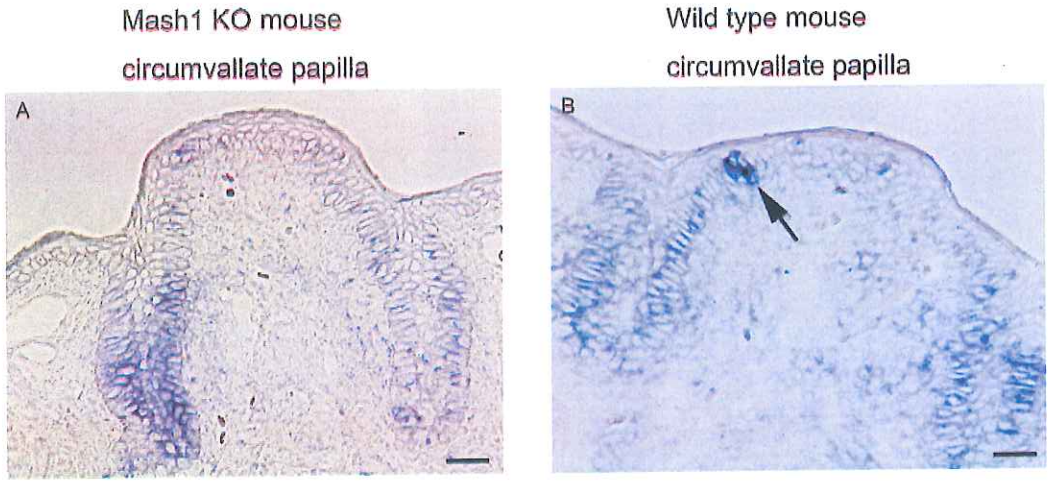

Figure 8 Expression of Dlx5 mRNA in the circumvallate papillae of Mash1 KO (A) and wild-type (B) mice at E18.5. Dlx5 was expressed at E18.5 in the epithelial cells in the deeper portion of the trenches in the circumvallate papilla. Dlx5 was also observed in small cell clusters in the apical papilla epithelium (B; arrow) of wild-type mice, whereas these cells were missing from Mash1 KO mice (A). Scale bars = 25 μm.

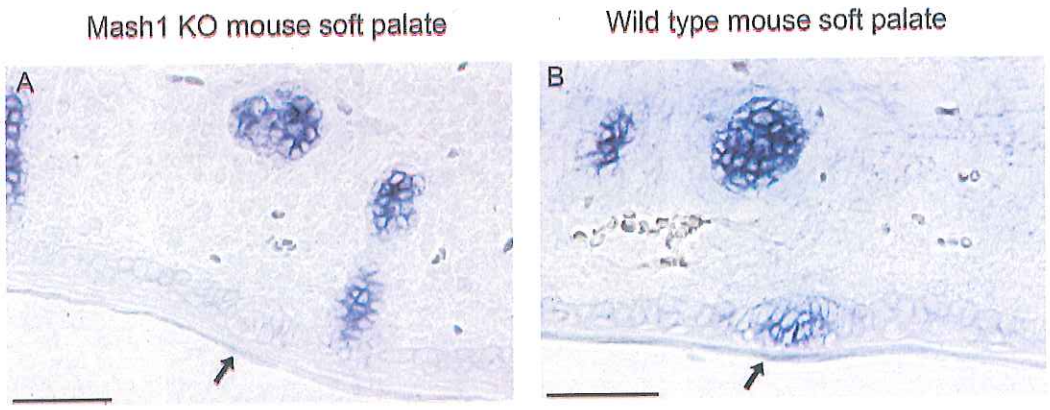

Figure 9 Expression of Dlx5 mRNA in the soft palate of Mash1 KO (A) and wild-type (B) mice at E18.5. Dlx5-expressing cells were observed in taste buds and the epithelial ridge in wild-type mice (B). However, Dlx5-expressing cell clusters were not observed in the taste bud cells of Mash1 KO mice(A). The arrows indicate taste buds. Scale bars = 50 μm.

we observed that GAD67-positive cells were absent from the taste papillae of Mash1 KO mice. These results suggest that the expression of both GAD67 and AADC may be regulated by Mash1 during type III taste bud cell differentiation. We previously showed that the type III cell markers NCAM and SNAP25 were expressed in the soft palate epithelia of Mash1 KO mice using RT-PCR, and NCAM-immunopositive cells were observed in the soft palate taste buds of Mash1 KO mice (Seta et al. 2011). Two hypotheses may explain our results obtained using Mash1 KO mice: 1) AADC- and GAD67-expressing type III cells are absent from Mash1 KO mice but other type III cells (NCAM- and SNAP25-expressing) are not affected or 2) the differentiation of type III cells is not affected by the loss of Mash1, but Mash1 is required for the expression of AADC and GAD67 in a subset of type III cells. Because Mash1 KO mice die prior to taste bud formation in the taste papillae, it was not possible to determine whether Mash1 affected the differentiation of type III cells. Thus, it remains to be determined whether Mash1 is required for the differentiation of type III cells in the taste buds.

The vertebrate Dlx homeobox gene family consists of 6 known murine members. Among these genes, Dlx1, Dlx2, Dlx5, and Dlx6 are expressed in the subcortical forebrain, mainly in those areas where GABAnergic neuron differentiation occurs and in precursors of the GABAnergic lineage. In general, these genes are expressed in the following temporal sequence: Dlx2, Dlx1, Dlx5, and Dlx6. Targeted inactivation of Dlx1 and Dlx2 in the mouse resulted in abnormal differentiation in the embryonic subcortical forebrain, which was associated with the loss of Dlx5 and Dlx6 expression (Perera et al., 2004). As a result, GABAnergic interneurons were depleted in the cerebral cortex, olfactory bulb, and hippocampus. Ectopic expression of Dlx2 or Dlx5 in cortical neurons could induce the expression of GADs, which are the enzymes that synthesize GABA. Using RT-PCR, our present results indicated that Dlx2 and Dlx5 were expressed in the circumvallate papillae epithelium, whereas Dlx5 mRNA was expressed in a subset of type III cells in the taste buds. Taken together, these results suggest that GAD expression in the taste buds may be regulated by Dlxs in a manner similar to that in the central nervous system.

Previous studies have shown that GABAnergic neuronal differentiation is controlled by Mash1 (Roybon et al. 2010). Ectopic expression of Mash1 results in the misspecification of a subpopulation of early-born cortical neurons, which ectopically express Dlx1, Dlx2, Dlx5, and GAD67 (Fode et al. 2000). These studies indicate that Mash1 appears to function upstream of Dlxs and GAD67 in the central nervous system. We observed that both Dlx5-expressing cell clusters and GAD67-expressing cells were absent from the apical region of circumvallate papillae in Mash1 KO mice. In addition, these results suggest that Mash1 may result in the expression of GAD67 via Dlxs in the type III cells of the taste buds, which is consistent with the results on their spatial and temporal expression in the central nervous system (Fode et al. 2000).

In this study, the loss of Mash1 affected the expression of Dlx5 mRNA in the apical epithelium of the circumvallate papillae and taste buds of the soft palate at E18.5. However, the Dlx5 mRNA in Mash1 KO mice was not affected in the trench epithelium of circumvallate papillae and epithelial ridges, which extend into the underlying connective tissue in soft palate. We previously demonstrated that Mash1 is expressed in a small number of epithelial cells of the apical circumvallate papillae but not in the deep trench epithelia at E18.5 (Seta et al. 2003). Taken together, these results suggest that Mash1 may be required for the upregulation of Dlx5 in the taste buds, but not in the trench epithelium of the circumvallate papillae or the epithelial ridge near soft palate papillae.

Our present and previous results indicate that Mash1 is required to regulate the expression of GAD67, AADC, and Dlx5 within the developing taste bud. In this study, we did not investigate whether the loss of Mash1 affected the expression of Dlx2 mRNA in the developing papilla epithelium and taste buds. Therefore, further studies will be required to investigate the changes in Dlx2 expression in the taste buds of Mash1 KO mice.

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