Depletion of bitter taste transduction leads to massive spermatid loss in transgenic mice

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Submitted on November 17, 2011; resubmitted on January 3, 2012; accepted on January 13, 2012

ABSTRACT: Bitter taste perception is an important sensory input warning against the ingestion of toxic and noxious substances. Bitter receptors, a family of ∼30 highly divergent G-protein-coupled receptors, are exclusively expressed in taste receptor cells that contain the G-protein α-subunit gustducin, bind to α-gustducin in vitro, and respond to bitter tastes in functional expression assays. We generated a taste receptor type 2 member 5 (T2R5)-Cre/green fluorescent protein reporter transgenic mouse to investigate the tissue distribution of T2R5. Our results showed that Cre gene expression in these mice was faithful to the expression of T2R5 in taste tissue. More surprisingly, immunostaining and X-gal staining revealed T2R5 expression in the testis. Ablation of T2R5 + cells led to a smaller testis and removed the spermatid phase from most of the seminiferous tubules. The entire taste transduction cascade (α-gustducin, Gγ13, phospholipase Cβ2) was detected in spermatogenesis, whereas transient receptor potential, cation channel subfamily M member 5 (Trpm5), was observed only in the later spermatid phase. In short, our results indicate that the taste transduction cascade may be involved in spermatogenesis.

Key words: bitter receptors / taste transduction / spermatogenesis / mouse / α-gustducin

Introduction

Taste receptor type 2 (T2R) is a family of ∼30 highly divergent G-protein-coupled receptors initially identified in taste tissues. They are selectively expressed in subsets of taste receptor cells distinct from those containing sweet and umami receptors. T2R genes are linked to the bitter taste in humans and mice, and are clustered in regions of the genome (Adler et al., 2000; Chandrashekar et al., 2000; Mueller et al., 2005). Heterologous expression assays have shown that many T2Rs function as bitter taste receptors (Chandrashekar et al., 2000; Bufe et al., 2002). Knockout and misexpression studies have further confirmed that T2Rs are necessary and sufficient for bitter taste perception. Mice lacking mT2R5 (the candidate cycloheximide receptor) markedly and selectively lose their ability to taste the cognate bitter compound (Mueller et al., 2005). Therefore, T2Rs are currently considered to be the bitter taste receptors in mammals.

More recent evidence shows that taste transduction-related signaling is found in various cell types from the stomach to the large intestine (Kaske et al., 2007). Furthermore, it has been proposed that solitary chemosensory cells are present throughout the upper respiratory system and express the entire suite of taste-related signaling molecules, including T2R receptors, phospholipase Cβ2 (PLCβ2), α-gustducin and the transduction channel Trpm5 (Tizzano et al., 2010, 2011). Interestingly, cultured human airway epithelium expresses some T2Rs along with associated downstream elements, and the T2Rs are present on the cilia of the ciliated epithelial cells (Shah et al., 2009). Another report shows that the smooth muscle cells of human airways express T2R (bitter) taste receptors along with gustducin and some components of the taste-associated PLCβ2 signaling cascade (Deshpande et al., 2010). Expression of both T1R3 and T1R2 has been detected in gut (Margolskee et al., 2007), pancreatic beta cells (Nakagawa et al., 2009) and testis (Max et al., 2001; Iwatsuki et al., 2010); thus, the T1R3/T1R2 receptor complex is thought to detect nutrients such as sugar and amino acid in non-taste tissues.

Although taste receptors and their associated downstream signaling components are widely distributed in diverse organ systems, the function of the receptors in many tissues remains unclear. In addition, limited availability of reliable antibodies against T2Rs makes their study more complicated and confusing. Transgenesis with bacterial artificial chromosomes (BACs) has been used in various biological, medical and biotechnological applications and is considered a major breakthrough in the generation of transgenic animals (Giraldo and Montoliu, 2001). In the present study, the use of BACs in transgenesis is likely to ensure the position-independence of the expression of the transgenes, and precisely mark T2R5-expressing cells in vivo.
Materials and Methods

BAC-mT2R5-IRES-Cre construction and generation of transgenic mouse lines

BAC RP23-342N5 containing the entire mT2R5 gene was purchased from BACPAC Resources, Oakland, USA. The IRES-Cre/green fluorescent protein (GFP)-frt-KanR-frt fragment was obtained from plasmid pICGN21 by PCR. Two homologous arms of 48 bp from the mT2R5 gene were synthesized into both sides of the primers used to amplify the IRES-eGFPCre-frt-KanR-frt cassette in the pICGN21 plasmid. The IRES-eGFPcre-frt-KanR-frt cassette was introduced into the T2R5 gene downstream of the start codon (93 bp) and upstream of its stop codon (60 bp) by Red-mediated homologous recombination, followed by flp-mediated removal of the KanR selectable marker from the BAC-mT2R5-IRESCre/GFP construct. Final construct was verified by pulse field gel electrophoresis analysis and sequence. P1/P2 and P3/P4 primers are used for genotyping (A). In order to verify the function of Cre, we crossed the T2R5-Cre/GFP transgenic mouse with RosA-LoxP-LacZ mouse and got double transgenic mouse. After X-gal staining of sections of mouse tongue, we found positive cells in circumvallate papillae (B) and foliate papillae (C). Scale bar 12.5 μm.

Histology and immunostaining procedure

For immunocytochemistry, mice were perfused transcardially with 2–4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; pH 7.2–7.4). The testis tissues were dissected, post-fixed in PFA for 2–12 h and cryoprotected in 30% sucrose in PBS at 4°C overnight. After sectioning on a cryostat, 10–12 μm

Figure 1 T2R5-Cre/GFP construction. BAC DNA (RP23-342N5) contains more than 70 kb 3′ and 5′ sequence of T2R5 gene. The IRES-GFP/Cre-frt-KanR-frt fragment is obtained from plasmid pICGN21 by PCR. Two homologous arms of 48 bp from the mT2R5 gene are synthesized into both sides of the primers used to amplify the IRES-eGFPcre-frt-KanR-frt cassette in the pICGN21 plasmid. The IRES-eGFPcre-frt-KanR-frt cassette is introduced into the T2R5 gene downstream of the start codon (93 bp) and upstream of its stop codon (60 bp) by Red-mediated homologous recombination, followed by flp-mediated removal of the KanR selectable marker from the BAC-mT2R5-IRESCre/GFP construct. Final construct was verified by pulse field gel electrophoresis analysis and sequence. P1/P2 and P3/P4 primers are used for genotyping (A). In order to verify the function of Cre, we crossed the T2R5-Cre/GFP transgenic mouse with RosA-LoxP-LacZ mouse and got double transgenic mouse. After X-gal staining of sections of mouse tongue, we found positive cells in circumvallate papillae (B) and foliate papillae (C). Scale bar 12.5 μm.
sections were collected onto Superfrost Plus Microscope slides (Fisher Scientific, Loughborough, UK). The polyclonal primary antibodies used were specific for GFP (goat ab-5450, rabbit ab-6556; Abcam, Cambridge, UK), PLCb2 (rabbit sc-206; Santa Cruz Biotechnology, Santa Cruz, USA), Ggamma13 (goat sc-26781; Santa Cruz Biotechnology) and α-gustducin (rabbit sc-395; Santa Cruz Biotechnology) T1R3 (Goat sc-22458; Santa Cruz Biotechnology). The rabbit polyclonal antibody against Trpm5 (1028–1049 amino acids) was as described by Perez et al. (2002). Staining was performed using the Tyramide signal amplification Plus (modified horse-radish peroxidase) system from Perkin Elmer, Waltham, USA according to the manufacturer’s instructions. Fluorescent images were captured using a Leica TCS SP2 Spectral Confocal Microscope (Leica Microsystems Inc., Mannheim, Germany). Staining against GFP was performed using the standard immunocytochemical procedure according to the manufacturer’s instructions (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, USA).

Standard hematoxylin and eosin staining was conducted and bright field images of the sections were captured digitally. Biometric morphometric data were analyzed quantitatively using ImagePro Plus (Media Cybernetics, Inc., Silver Spring, MD, USA) in serial sections of testis (at least seven sections) as described by Franca et al. (2006). The length density of the seminiferous tubule was obtained by dividing the tubule volume by the squared tubule radius (R^2) multiplied by π. Morphometric data are presented as the mean ± SEM. These data were analyzed by one way analysis of variance using SPSS11.0 software. Differences were considered to be significant when P < 0.05.

**LacZ staining**

Animals were perfused with 2% PFA in PBS. Testis tissue was then fixed in 2% PFA for 1 h, after which it was cryoprotected in 30% sucrose in PBS at 4°C overnight. The following morning, tissue was cryosectioned at 20 μm thickness. The sections were washed three times for 20 min in PBS and stained in X-gal solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2, 0.02% detergent NP-40, 0.01% Na deoxycholate, 1 mg/ml X-gal) at 37°C overnight. Stained sections were washed three times for 20 min in PBS and counterstained with nuclear fast red. Bright field images were captured using a SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, USA) attached to a Nikon SA Microphot microscope and minimally processed using ImagePro Plus image analysis software (Media Cybernetics, Inc.).

**Results**

**Generation and identification of T2R5-Cre/GFP transgenic mice**

It is well known that T2R5 is expressed in taste buds and thought to be a bitter receptor (Chandrashekar et al., 2000; Zhang et al., 2003; Mueller et al., 2005). Recently, it was reported that T2R is expressed in nose (Tizzano et al., 2010) and lung (Deshpande et al., 2010) tissues. To further study the expression of T2R, we replaced the T2R5 sequence with the IRES-Cre/GFP sequence using the Red recombinase system (Fig. 1A). After modified BAC DNA had been microinjected into C57BL/6 oocytes, we obtained three transgenic founders. To confirm the expression of Cre/GFP, we crossed these transgenic mice with R26:loxP-Stop-loxP LacZbpA transgenic mice (Supplementary data, Fig. S1A). β-Gal staining showed that LacZ
was expressed as expected in circumvallate and foliate papillae (Fig. 1B and C). However, we failed to observe LacZ expression in fungiform papillae, suggesting selective expression in taste buds as reported by Adler et al. (2000; Chandrashekar et al., 2000). We then crossed the transgenic mice with R26:lacZbpAflox DTA (Supplementary data, Fig. S1B). A two-bottle taste preference test was performed to determine whether ablation of T2R5 + cells (co-expression of the DTA gene leads to cell death) resulted in the tasteless phenotype. As expected, the double transgenic mice did not show any preference for normal water over a bitter compound (data not shown), indicating the successful generation of T2R5-Cre/GFP/DTA double transgenic mice.

**T2R5 expression in testis**

When we crossed T2R5-Cre/GFP transgenic mice with R26:lacZbpAflox DTA (Supplementary data, Fig. S1B), it was observed that the male double transgenic mice are infertile. After dissection, their testes were observed to be smaller than those of control mice (Fig. 3A). The testis:body weight ratio differed significantly from that of the controls (n = 5, 0.0071 ± 0.0003, > 0.0024 ± 0.0007) (B). Compared with control mice (R26:lacZbpAflox DTA transgenic mice), the seminiferous tubule diameter was smaller and there were more seminiferous tubules. The length densities of the seminiferous tubules were similar. We observed fewer tubules containing spermatids (3–4/37.89) (C).

**Figure 3** To confirm the expression of T2R5 in testis, we crossed T2R5-GFP/Cre transgenic mice with R26:lacZbpAflox DTA transgenic mice. These male double transgenic mice are infertile. After dissection, their testes were observed to be smaller than those of control mice (A). The testis:body weight ratio differed significantly from that of the controls (n = 5, 0.0071 ± 0.0003, > 0.0024 ± 0.0007) (B). Compared with control mice (R26:lacZbpAflox DTA transgenic mice), the seminiferous tubule diameter was smaller and there were more seminiferous tubules. The length densities of the seminiferous tubules were similar. We observed fewer tubules containing spermatids (3–4/37.89) (C).

<table>
<thead>
<tr>
<th>Parameter</th>
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<th>T2R5-DTA</th>
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<tr>
<td>Seminiferous tubule diameter (um)</td>
<td>221.03±23.62</td>
<td>149.8±20.22</td>
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<td>Seminiferous tubule volume density (%)</td>
<td>87.12±0.9</td>
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<td>Seminiferous tubule length density (cm/cm³)</td>
<td>2271±12.3</td>
<td>2255±15.08</td>
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<tr>
<td>Seminiferous tubule numerical density (tubule/mm²)</td>
<td>18.46±1.13</td>
<td>37.89±2.53</td>
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* Statistically significant (P < 0.05 or less). ** Seminiferous tubule containing spermatid per mm²

**Taste signal transduction is expressed in spermatogenesis**

It is well known that the transduction of bitter, sweet and amino acid tastes uses elements of a common pathway (Margolskee, 2002; Zhang et al., 2000). In summary, our data suggested that T2R5 was expressed in the spermatid phase, not all spermatids were T2R5 positive, and ablation of T2R5 + spermatids did not completely block spermatogenesis.
et al., 2003). It has been reported that T1R3 (Max et al., 2001) and T1R2 (Iwatsuki et al., 2010) are expressed in testis. Interestingly, α-gustducin is also expressed in testis and spermatozoa (Fehr et al., 2007). Our results also showed the expression of T2R5 in testis. The next question is whether the common elements of taste transduction are found in the testis.

First, we investigated α-gustducin expression by confocal analysis. Immunostaining with anti-α-gustducin showed that α-gustducin was expressed in spermatogenesis, in the spermatogonial, spermatocyte and spermatid phases (Fig. 5A–D). DTA expression in T2R5 + cells ablated the α-gustducin + spermatid phase in most of the seminiferous tubules (Fig. 5E). However, we still observed the α-gustducin + spermatid phase in few seminiferous tubules (Fig. 5F), matching the results of H&E staining. It should be noted that α-gustducin expression was still observed in the spermatogonial and spermatocyte phases after ablation of T2R5 + cells (Fig. 6D). We also determined the expression of Trpm5, which is thought to be a component of the channel that mediates taste transduction (Liu and Liman, 2003; Zhang et al., 2003). Confocal analysis showed that Trpm5 was expressed only in the later spermatid phase (Fig. 6E and F), which differed from the pattern of expression of the above components.

It has previously been reported that T1R3 is expressed in the testis (Max et al., 2001), but its histological distribution is unclear. Our results show T1R3 expression in spermatogenesis (Fig. 7A–D) and in interstitial tissues (Fig. 7B and C; arrow). After ablation of T2R5 + cells, we still observed the expression of T1R3 (Fig. 7E and F). Ggamma13 is thought to be an important gamma subunit of α-gustducin and mediates IP3 responses to bitter compounds.
As expected, $\gamma_13$ expression was detected in spermatogenesis (Fig. 8A and B). $\gamma_13$ expression was also observed in the later spermatid phase.

Our current data collectively suggest that three important components of taste transduction ($\alpha$-gustducin, $\gamma_13$, PLC-$\beta_2$) were present throughout spermatogenesis, whereas Trpm5 was observed only in the later spermatid phase. T1R3 expression was also observed in spermatogenesis.

**Discussion**

The present study suggests that T2R5 is expressed in the spermatid phase, but not all spermatids express T2R5. We detected the expression of three important components of taste signal transduction ($\alpha$-gustducin, $\gamma_13$, PLC-$\beta_2$) in spermatogenesis. However, Trpm5 expression was found only in the later spermatid phase, indicating that spermatogenesis involves another transient receptor potential cation channel. It is well known that the T2R family comprises $\approx 30$ highly divergent G-protein-coupled receptors (Adler et al., 2000; Chandrashekhar et al., 2000). Thus, it is possible that other T2Rs may be expressed in spermatogenesis. However, there are few available antibodies for T2Rs, which makes it difficult to localize their in the body. To detect protein expression reflecting endogenous T2R promoter activity, we need to generate more knock-in mice in the future.

Increasing evidence suggests that taste receptors and components of taste transduction are widely distributed in non-taste tissues (Finger and Kinnamon, 2011). Taste transduction for umami, sweet and bitter compounds, including receptors, has been observed in the gastrointestinal tract (Rozengurt, 2006; Margolskee et al., 2007). The T1R3/T1R2 receptor complex is further suggested to function in sensing sugar and maintaining glucose homeostasis through secretion of incretin (Margolskee et al., 2007). In addition, several studies have shown that T1R3/T1R2 is expressed in testis (Max et al., 2001; Iwatsuki et al., 2010). Interestingly, a segmental distribution of $\alpha$-gustducin along the flagellum is observed in the sperm of different mammalian species, suggesting a functional role for this G-protein either in signal transduction controlling sperm chemotaxis, or in sperm motility (Fehr et al., 2007). The current study found most elements of the taste transduction cascade, from receptor (T2R5/T1R3) to transduction channel, in spermatogenesis. In short, our findings indicate the taste transduction cascade may be involved in spermatogenesis. The taste signaling cascade may be used to detect chemical elements in the lumen of the seminiferous tubule or the physical compartments established by Sertoli cell-to-Sertoli cell junctions.

Recent studies have demonstrated the expression of T2Rs in the airways (Shah et al., 2009; Deshpande et al., 2010; Tizzano et al., 2011), suggesting that taste transduction may also act by autocrine signaling; that is, the chemical binds to taste receptors on a cell, leading to changes in that cell. Surprisingly, T2R agonists cause airway smooth...
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Figure 6 PLC-β2 expression in testis. PLC-β2 expression was observed in spermatogenesis (A–D) in control mice (R26: lacZbpAlloxDTA transgenic mice). DTA expression in T2R5 + cells ablated the spermatid phase from most of the seminiferous tubules (E and F). Trpm5 expression was observed in the later spermatid phase. PLC-β2 and Trpm5 expression were not detected in interstitial tissues. (A and E) Low magnification. (B–D and F) High magnification. Scale bars: (A and E) 80 μm; (D) 30 μm; (B, C and F) 50 μm.

Figure 7 T1R3 expression in testis. T1R3 expression was observed in spermatogenesis (A–D) and in interstitial tissues (B, C; arrow) in control mice (R26: lacZbpAlloxDTA transgenic mice). After ablation of T2R5 + cells, we still observed the expression of T1R3 (E and F). (A and E) Low magnification. (B–D and F) High magnification. Scale bars: (A and E) 80 μm; (B–D, F) 50 μm.
muscle relaxation in the trachea (Deshpande et al., 2010). However, it is not known how a bitter substance might interact with the smooth muscle cells of the trachea under physiological conditions, as suggested by the relatively tight airway epithelium covering the smooth muscle of the trachea, and it is doubted whether an inhaled bitter substance could rapidly penetrate the epithelium to approach T2R receptors on the muscle (Finger and Kinnamon, 2011). In the present study, the entire bitter taste transduction cascade was detected in spermatogenesis. The Sertoli cell-to-Sertoli cell junctional complex not only provides physical compartmentalization by dividing the seminiferous epithelium into basal and luminal compartments, but also creates a permeability barrier called the blood–testis barrier (Ross et al., 1989). Thus, it is almost impossible that a sweet/bitter substance in the environment (lumen of seminiferous tubule or blood vessel) could penetrate and influence spermatogenesis. A further study of the taste transduction cascade in spermatogenesis will help us to comprehend the role of these receptors in the body.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

Acknowledgements

We thank Liquan Huang and Robert Margolskee (Monell Chemical Senses Center) for his valuable advice and discussions. We also thank Transgenic and Chimeric Mouse Facility (University of Pennsylvania) for generating transgenic mice.

Authors’ roles

F.L. designed the study, carried out and analyzed the experiments and wrote the manuscript. M.Z. screened transgenic mice and performed two-bottle taste preference test.

Funding

This work was supported by grants from the National Institutes of Health (R01 DC007487), the National Science Foundation (DBJ-0216310), Shanghai Municipal Education Commission (Technological Innovation Projects 08YZ42) and Shanghai leading Academic Discipline Project (S30201).

Conflict of interest

There is no financial or other potential conflict of interest.

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