

RESEARCH ARTICLE

Induction of ectopic taste buds by SHH reveals the competency and plasticity of adult lingual epithelium

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ABSTRACT

Taste buds are assemblies of elongated epithelial cells, which are innervated by gustatory nerves that transmit taste information to the brain stem. Taste cells are continuously renewed throughout life via proliferation of epithelial progenitors, but the molecular regulation of this process remains unknown. During embryogenesis, sonic hedgehog (SHH) negatively regulates taste bud patterning, such that inhibition of SHH causes the formation of more and larger taste bud primordia, including in regions of the tongue normally devoid of taste buds. Here, using a Cre-lox system to drive constitutive expression of SHH, we identify the effects of SHH on the lingual epithelium of adult mice. We show that misexpression of SHH transforms lingual epithelial cell fate, such that daughter cells of lingual epithelial progenitors form cell type-replete, onion-shaped taste buds, rather than non-taste, pseudostratified epithelium. These SHH-induced ectopic taste buds are found in regions of the adult tongue previously thought incapable of generating taste organs. The ectopic buds are composed of all taste cell types, including support cells and detectors of sweet, bitter, umami, salt and sour, and recapitulate the molecular differentiation process of endogenous taste buds. In contrast to the well-established nerve dependence of endogenous taste buds, however, ectopic taste buds form independently of both gustatory and somatosensory innervation. As innervation is required for SHH expression by endogenous taste buds, our data suggest that SHH can replace the need for innervation to drive the entire program of taste bud differentiation.

KEY WORDS: Cell lineage, Mouse molecular genetics, Regeneration, Taste

INTRODUCTION

Taste buds are the primary receptor organs of the gustatory system. Each bud houses a heterogeneous collection of 60–100 taste receptor cells, which transduce five basic tastes (sweet, bitter, salt, sour and umami) into electrochemical signals that are transmitted via sensory neurons to the brain (Chaudhari and Roper, 2010). Lingual taste buds

are restricted to taste papillae, which are epithelial appendages distributed on the tongue, and each fungiform taste papilla on the anterior tongue of rodents contains a single taste bud (Mistretta, 1991). Taste bud cells, like cells of the lingual epithelium, are continually renewed from mitotically active, cytokeratin (K) 14⁺ basal keratinocytes (Beidler and Smallman, 1965). This K14⁺ population generates postmitotic cells that enter buds and differentiate into taste receptor cells (Beidler and Smallman, 1965; Farbman, 1980; Miura and Barlow, 2010; Okubo et al., 2009; Sullivan et al., 2010). Type IV cells are ovoid and situated basally in each taste bud, and many if not all of these basal cells express the morphogen sonic hedgehog (*Shh*) (Miura et al., 2006). Using genetic lineage tracing, we have recently demonstrated that *Shh*⁺ basal cells are immediate precursors for all taste cell types within taste buds (Miura et al., 2014).

In embryos, SHH negatively regulates the number, size and distribution of taste bud primordia, termed taste placodes, in the anterior lingual epithelium. In cultured embryonic tongues, exogenous SHH represses taste placode development (Iwatsuki et al., 2007), whereas pharmacological block of SHH signaling results in more and larger taste placodes, as well as their formation in regions of the tongue typically absent of placodes (Hall et al., 2003; Mistretta et al., 2003). By contrast, SHH function in the lingual epithelium of adult mice has remained unexplored. *Shh* is expressed by basal cells inside taste buds, while the SHH target genes *Ptch1* and *Gli1* are expressed by K14⁺ progenitor cells adjacent to buds (Liu et al., 2013; Miura et al., 2001), suggesting that SHH regulates taste bud regeneration. However, the nature of this regulation is unknown. Recently, forced activation of GLI2, a primary transcriptional activator of the SHH pathway (Bai et al., 2002), in K14⁺ progenitors was shown to repress both taste bud and non-taste epithelial cell fates (Liu et al., 2013), indicating that SHH might play a broader role in tongue epithelial maintenance.

Although little is known of the molecular regulation of taste bud renewal, specific steps in taste cell lineage progression are now identifiable via specific gene expression (Miura and Barlow, 2010), including molecular markers for progenitors [K14, SOX2, GLI1, PTCH1 (Okubo et al., 2009)], precursors [SOX2, SHH, SKN-1A (POU2F3) (Matsumoto et al., 2011; Okubo et al., 2006)] and postmitotic taste cells [K8, KCNQ1, SOX2 (Okubo et al., 2006, 2009; Wang et al., 2009)], as well as for differentiated non-taste keratinocytes (K13), which make up the pseudostratified lingual epithelium (Winter et al., 1990).

More than 200 studies, the first from 1877 (Vintschgau and Hönigschmied, 1877), have reported that innervation is required to maintain taste buds in adult mammals (Oakley and Witt, 2004). If gustatory nerves are crushed or severed, taste buds regress within a week of injury, and when nerves regenerate, taste buds reappear in their original locations (Cheal and Oakley, 1977). Thus, gustatory innervation is required for taste bud maintenance and presumably restricts where taste buds form in the tongue surface. Furthermore,

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innervation is tightly correlated with the presence of all differentiated taste cell types, as well as of *Shh*⁺ type IV cells (Miura et al., 2004). *Shh* expression within buds is lost within hours of denervation, as is expression of SHH target genes by the surrounding progenitor cells, both of which precede by several days the disappearance of differentiated taste cells. These findings have led to the proposal that nerve-dependent expression of *Shh* by type IV basal cells may regulate the proliferation of neighboring progenitors (Miura et al., 2006).

Here, we used an *Shh* conditional knock-in allele, SHH-IRES-YFPcKI (referred to here as SHH-YFPcKI; supplementary material Fig. S1), to misexpress SHH in the K14⁺ progenitor cells of the tongue. We find that ectopic SHH transforms lingual epithelial fate by inducing the formation of taste buds in regions of the tongue not thought capable of generating taste buds. Furthermore, SHH triggers the full differentiation program of mammalian taste buds, including the differentiation of support cells and sweet, sour, bitter, salt and umami taste receptor cells, utilizing the same set of genes that is used for the differentiation of endogenous taste buds. Finally, we show that ectopic taste buds, unlike their endogenous counterparts, are completely independent of innervation. We suggest that local misexpression of SHH supersedes any neural requirement in taste bud differentiation, and that SHH expression alone is sufficient to drive differentiation of the full complement of cell types within taste buds.

RESULTS

Ectopic structures with taste bud-like characteristics form in SHH-YFPcKI⁺ non-taste epithelium

The anterior tongue comprises both taste epithelium, i.e. distributed fungiform taste papillae housing individual taste buds, as well as the predominant non-taste epithelium occupied by mechanosensory filiform papillae (Fig. 1A-A'') (Nosrat et al., 2000). Adult mice carrying KRT14-cre/ERT2 (Li et al., 2000) (referred to here as K14CreER) and SHH-YFPcKI (supplementary material Fig. S1) alleles were given a single tamoxifen dose and analyzed between 7 and 28 days later. Activation of the SHH-YFPcKI allele results in SHH production and nuclear YFP expression, which reports the location of the cells expressing SHH (supplementary material Fig. S1). Consistent with a previous report that tamoxifen activation of K14CreER results in variably sized, mosaic patches of reporter-expressing cells in taste and non-taste lingual epithelium (Okubo et al., 2009), SHH-YFPcKI was detected in numerous patches throughout the non-taste lingual epithelium and in some, but not all, fungiform taste buds and papillae (supplementary material Fig. S2).

In tongue epithelium, K8 marks differentiated taste bud cells (Knapp et al., 1995) and is expressed by fungiform taste buds in wild-type and mutant mice (Fig. 2A,C,J,L). Unexpectedly, K8⁺ taste bud-like structures were present in non-taste epithelium, but only where SHH-YFPcKI was expressed (Fig. 1B-E). Ectopic K8⁺ cell clusters were never encountered in SHH-YFPcKI⁻ epithelium in mutant mice, nor in tongues of control mice. We found ectopic K8⁺ structures both nearby and distant from endogenous fungiform taste papillae, as well as scattered laterally and dorsally in the anterior tongue (Fig. 1C-E). However, we discerned no clear pattern of SHH-YFPcKI⁺ or ectopic cluster formation across mutant mice (data not shown), supporting the hypothesis that the random and mosaic induction of SHH-YFPcKI under K14CreER by tamoxifen (Okubo et al., 2009) dictates the location of K8⁺ cell cluster formation. Importantly, not all SHH-YFPcKI⁺ patches contained K8⁺ cell clusters (Fig. 1C-E, arrows). Rather, SHH-YFPcKI⁺ cells

in these patches appeared to develop normally, as they appropriately turned off K14, transitioned through a squamous stage, and ultimately contributed to the cornified barrier of the tongue surface (data not shown). These data thus suggest that additional regional factors are required for the formation of K8⁺ clusters in response to SHH misexpression.

Ectopic K8⁺ clusters were morphologically similar to endogenous taste buds, as they were composed of onion-shaped aggregates of elongated cells (Fig. 1B-E and Fig. 2D-I, arrowheads in Fig. 2J,L). To confirm that these ectopic cell clusters were indeed taste buds, we assayed for expression of claudin 4 (CLDN4), a protein that marks

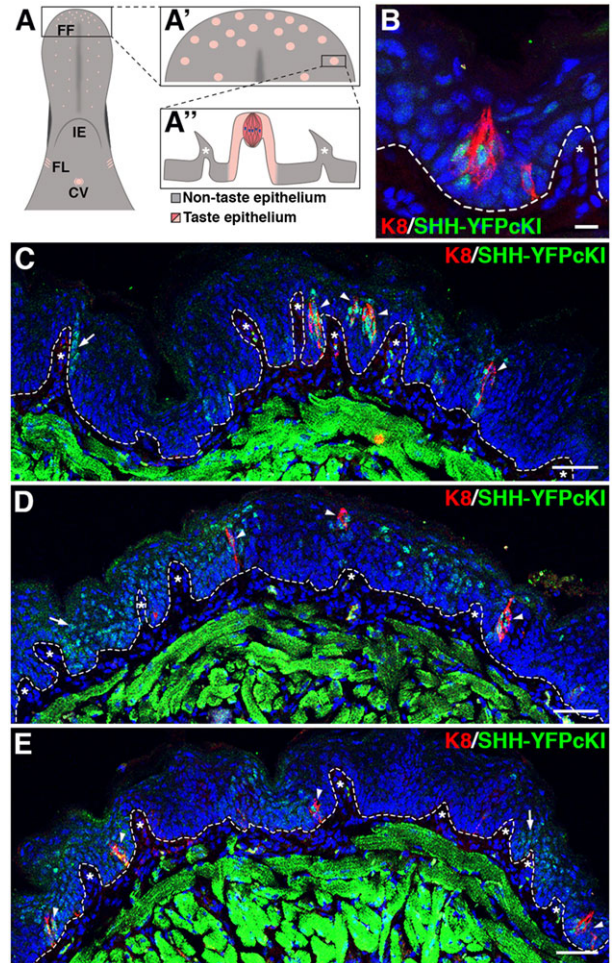


Fig. 1. Ectopic K8⁺ clusters form in SHH-YFPcKI⁺ non-taste epithelium. (A) Lingual taste buds occur in three types of taste papillae: FF, fungiform; CV, circumvallate; and FL, foliate. IE, intermolar eminence. (A') The anterior 1.5 mm of the tongue, with fungiform papillae (pink) distributed in non-taste epithelium (gray), was analyzed in this study. (A'') Taste epithelium of the anterior tongue comprises fungiform taste buds and papilla epithelium (dark and light pink, respectively); the remainder of the tongue surface is non-taste epithelium (gray) with dense mechanosensory filiform papillae (asterisks). (B) Clusters of K8⁺ cells (red) form within SHH-YFPcKI⁺ domains (green), often near filiform papillae (asterisk). (C-E) Low-magnification images of ectopic K8⁺ cells (red, arrowheads) within SHH-YFPcKI⁺ patches (green) of non-taste lingual epithelium of three different mice show the distribution of K8⁺ ectopic clusters; not all SHH-YFPcKI⁺ patches contain ectopic taste bud-like structures (arrows). Note that the green signal in the lingual muscle is non-specific background. Nuclei are counterstained with Draq5 (blue). White dashed lines delimit the basement membrane; asterisks mark the mesenchymal core of filiform papillae. All images are confocal z-stacks. Scale bars: 10 μm in B; 50 μm in C-E.

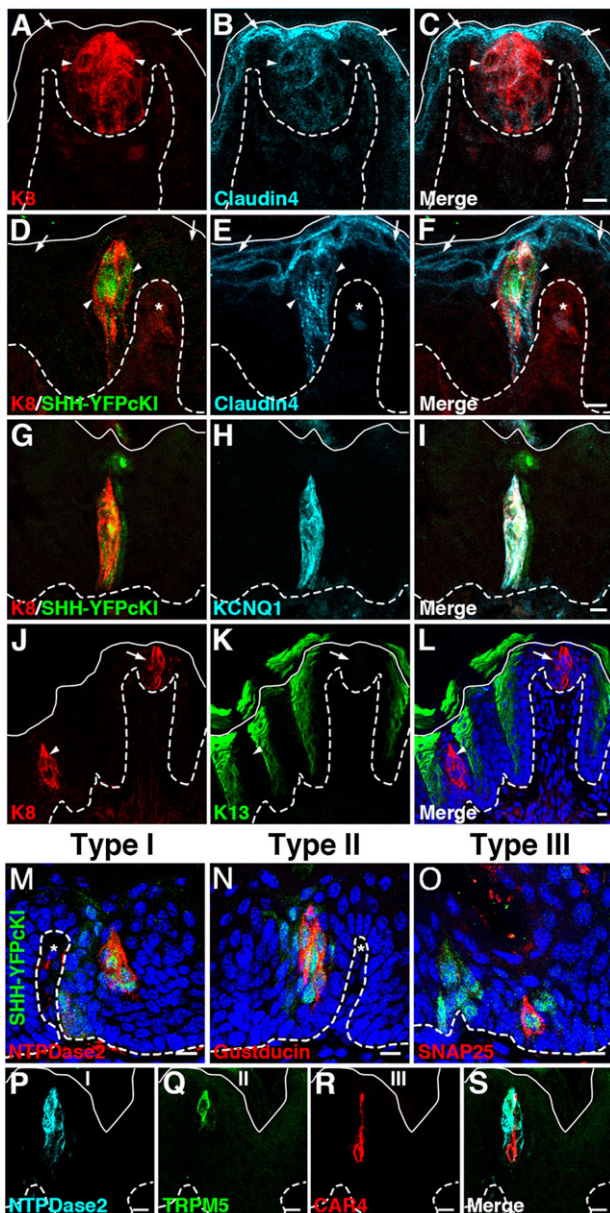


Fig. 2. The ectopic $K8^+$ clusters that form in SHH-YFPcKI⁺ non-taste epithelium have taste bud-like characteristics and comprise all three differentiated taste cell types. (A-F) Like endogenous taste buds (A-C), ectopic $K8^+$ cells express claudin 4 (D-F, cyan, arrowheads), which marks taste receptor cells and the cornified epithelial layer in an endogenous fungiform papilla (B,C, arrows) and non-taste epithelium (E,F, arrows). (G-I) Ectopic $K8^+$ cells (red) within SHH-YFPcKI⁺ domains (green) co-express KCNQ1 (cyan). (J-L) Ectopic $K8^+$ cells (red, arrowhead) adjacent to a $K8^+$ endogenous taste bud (red, arrow) do not express K13 (green). (M-O) Groups of ectopic type I (M, NTPDase2⁺, red) and II (N, gustducin, red) taste cells reside in SHH-YFPcKI⁺ lingual epithelium (green); type III cells (O, SNAP25, red) generally occur singly. (P-S) All differentiated taste cell types (I, NTPDase2⁺, cyan; II, TRPM5, green; and III, CAR4, red) are present within an ectopic taste bud. Nuclei are counterstained with Draq5 (blue); white dashed lines delimit the basement membrane; white solid lines delimit the epithelial surface; asterisks mark the mesenchymal core of filiform papillae. All images are confocal z-stacks. Scale bars: 10 μ m.

differentiated taste cells in fungiform taste buds (Michlig et al., 2007) (Fig. 2A-C, arrowheads) and cells in the superficial cornified layer of the tongue (Michlig et al., 2007) (Fig. 2A-C, arrows), but neither basal keratinocytes nor suprabasal epithelial cells adjacent to taste

buds. Like endogenous taste buds, 100% of ectopic $K8^+$ clusters were claudin 4⁺ (Fig. 2D-F, arrowheads; ectopic $K8^+$ clusters, $n=30$; mice, $N=2$; supplementary material Table S1), as was the superficial cornified layer (Fig. 2D-F, arrows), whereas neighboring basal and suprabasal epithelial cells were claudin 4⁻. Another general marker of taste cells is KCNQ1, a voltage-gated potassium channel (Wang et al., 2009), and like endogenous taste buds, 100% of ectopic $K8^+$ cell clusters were KCNQ1⁺ (Fig. 2G-I; ectopic $K8^+$ clusters, $n=30$; mice, $N=2$; supplementary material Table S1). In adult non-taste epithelium, the suprabasal layers of the lingual epithelium are K13⁺ (Iwasaki et al., 2006; Winter et al., 1990), whereas taste buds are K13⁻ (Fig. 2J-L, arrow). Like endogenous taste buds, all ectopic $K8^+$ clusters were devoid of K13 expression (Fig. 2J-L, arrowhead; ectopic $K8^+$ clusters, $n=30$; mice, $N=2$; supplementary material Table S1). Thus, analysis of general markers of lingual epithelial cell fates was consistent with a taste bud identity for ectopic $K8^+$ clusters in SHH-YFPcKI⁺ epithelium.

Ectopic taste buds comprise all three differentiated taste cell types

To determine if ectopic $K8^+$ clusters contain the normal complement of taste bud cells, we used an extensive panel of markers for specific taste cell types, including type I glial cells [NTPDase2 (ENTPD2) (Bartel et al., 2006)], type II sweet/umami/bitter receptor cells [gustducin (GNAT3), PLC β 2 and TRPM5 (Huang et al., 1999; Pérez et al., 2002; Yang et al., 2000b)] and type III sour-sensing cells [SNAP25, NCAM and CAR4 (Chandrashekar et al., 2009; Nelson and Finger, 1993; Yang et al., 2000a)]. NTPDase2⁺ type I, gustducin⁺, TRPM5⁺ or PLC β 2⁺ type II, and SNAP25⁺, NCAM⁺ or CAR4⁺ type III cells were each evident in SHH-YFPcKI⁺ patches (Fig. 2M-O; supplementary material Fig. S3), indicating that all principal cell types characteristic of mature, fully differentiated taste buds were present in ectopic taste bud-like structures. By triple immunostaining (type I NTPDase2, type II TRPM5, type III CAR4), we found that all taste cell types co-occurred within single ectopic clusters (Fig. 2P-S). In fact, 73% of ectopic taste buds, compared with 83% of endogenous taste buds, contained all three taste cell types (ectopic $K8^+$ clusters, $n=30$; mice $N=2$; endogenous taste buds, $n=18$; mice, $N=1$; supplementary material Table S1). In summary, our data indicate that the $K8^+$ cell clusters are indeed ectopic taste buds.

Ectopic taste buds increase in number and become positioned progressively more superficially in the lingual epithelium

Overall, the number of ectopic $K8^+$ taste buds increased with time post-tamoxifen, so that by 28 days more than 100 ectopic taste buds were found in the anteriormost 1.5 mm of mutant tongues (Fig. 1A and Fig. 3A). Likewise, the number of ectopic taste buds positive for type I and II taste cell markers steadily increased after tamoxifen treatment (Fig. 3B,C). Ectopic buds harboring type III cells detected via SNAP25 or CAR4 immunofluorescence were less frequent, and did not increase significantly over the several weeks following tamoxifen induction (Fig. 3D; note that SNAP25 and CAR4 are co-expressed in 97% of type III cells, supplementary material Table S1).

Taste cells within fungiform papillae are slender, fusiform cells reaching the dorsal tongue surface, where they extend specialized processes apically to contact the oral cavity. We examined whether ectopic taste cells exhibited a similar tendency to extend to the oral cavity. $K8^+$ ectopic buds were situated in a variety of locations, including: (1) immediately adjacent to the basement membrane;

(2) embedded within the epithelium; and (3) close to the epithelial surface, some with apical processes appearing to extend into the oral cavity (supplementary material Fig. S4). When we systematically mapped the position of $K8^+$ ectopic buds at intervals after tamoxifen induction, $K8^+$ cell clusters increasingly approached the surface, with the proportion of apically situated clusters, many with apical processes accessing the tongue surface, increasing to 50% at 28 days post-tamoxifen (Fig. 3E).

Ectopic taste buds arise from $K14^+$ progenitors and express genes required for the differentiation of endogenous taste cells

Because the ectopic taste buds arise from regions of the tongue that do not generate taste buds in control mice, we investigated whether they develop via a novel process or differentiate according to the lineage progression of endogenous taste buds. $K14^+$ basal keratinocytes represent the stem cell population for endogenous taste buds (Okubo et al., 2009) and, as taste cells differentiate, they downregulate $K14$ and become $K8^+$ (Asano-Miyoshi et al., 2008). Likewise, all ectopic taste buds were derived from $K14^+$ progenitor cells, as evidenced by the expression of the reporter component of SHH-YFPcKI, i.e. nuclear YFP (Fig. 1B-E, Fig. 2D-I, M-O and Fig. 4). Furthermore, differentiated ectopic taste cells ceased expression of $K14$ and expressed $K8$ (Fig. 4A-C).

The transcriptional target of SHH, *Gli1*, is widely used to identify SHH-responsive cells. A small number of SHH⁺ basal cells

reside in each fungiform taste bud (Miura et al., 2004), with SHH-responsive $GLI1^+$ cells surrounding taste buds (supplementary material Fig. S5, nicked arrowheads); non-taste epithelial cells remote from fungiform taste buds are *Gli1*⁻ (Liu et al., 2013). Like endogenous taste buds, ectopic $K8^+$ taste buds were surrounded by *Gli1-lacZ*⁺ cells (Fig. 4D-F, nicked arrowheads). This result also validates that expression of the SHH-YFPcKI allele results in local secretion of SHH, which turns on *Gli1* in adjacent cells.

The transcription factor SOX2 is required for the development of taste buds and is expressed in lingual epithelia of embryos and adult mice (Okubo et al., 2006). In adults, $K14^+$ basal cells throughout the lingual epithelium express SOX2 at low levels, whereas $K14^+$ progenitors adjacent to taste buds strongly express SOX2, as does a subset of cells within taste buds (Okubo et al., 2006, 2009; Suzuki, 2008). A similar pattern of expression was evident for all ectopic taste buds examined: SOX2 was expressed at high levels by epithelial cells surrounding (Fig. 4G-I, nicked arrowheads) and within (Fig. 4G-I, arrowheads) ectopic buds, whereas SOX2 expression was low in non-taste basal keratinocytes where SHH-YFPcKI was absent (Fig. 4G-I, arrows; data not shown).

SKN-1A, a POU domain transcription factor, is required for type II taste cell fate, and is expressed by type II cells as well as their presumed precursors within taste buds (Matsumoto et al., 2011). Similarly, SKN-1A expression was restricted to SHH-YFPcKI⁺ cells within all ectopic buds examined (Fig. 4J-L; data not shown).

In summary, SHH misexpression in non-taste epithelium drives the differentiation of taste cells from the $K14^+$ stem population, and results in patterns of regulatory gene expression that mirror those of endogenous taste buds.

By contrast, the expression of SHH in ectopic taste buds differed from that in endogenous buds. In the latter, *Shh* expression is transient and limited to type IV basal cells, while differentiated type I, II and III taste cells turn off *Shh* (Miura et al., 2004; Miura et al., 2006). Interestingly, ectopic taste cells constitutively express SHH-YFPcKI, yet still differentiate, indicating that the differentiation of all taste cell types can occur despite continued SHH expression.

SHH induction of ectopic taste buds is independent of taste and somatosensory innervation

As the maintenance of adult taste buds depends upon innervation (Oakley and Witt, 2004), we examined if the differentiation of ectopic taste buds was likewise nerve dependent. SHH functions as an axon guidance factor during early brain development (Sánchez-Camacho and Bovolenta, 2009), and we reasoned that it might play a similar role in taste innervation. Although gustatory fibers are restricted to fungiform taste buds in adult tongue, we hypothesized that ectopic SHH might attract gustatory nerves to non-taste epithelium, and thus ectopic bud differentiation would be secondary to *de novo*, ectopic gustatory innervation. Using antiserum against the purinergic receptor P2X2 (P2RX2), which marks taste fibers (Finger et al., 2005), we found that, whereas endogenous taste buds were amply innervated by P2X2⁺ fibers (Fig. 5A), ectopic taste buds were not (Fig. 5B). P2X2⁺ fibers did not stray from endogenous fungiform papillae and in no instance, in any animal or at any time point, were ectopic P2X2⁺ fibers observed in or near $K8^+$ ectopic taste buds.

The mechanosensory filiform papillae of the anterior tongue, the epithelial cells of fungiform papillae, as well as taste buds per se are innervated by somatosensory fibers emanating from the trigeminal ganglia (Finger and Simon, 2000), and trigeminal fibers support

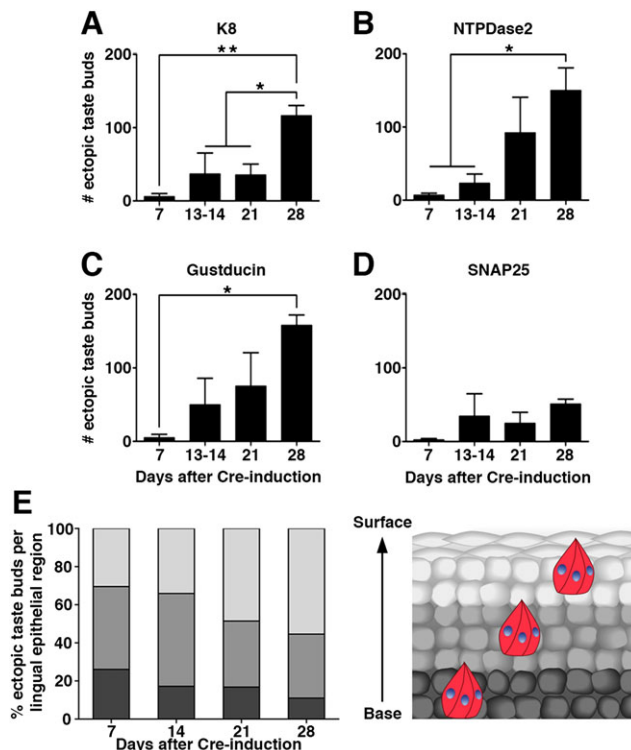


Fig. 3. Ectopic taste buds increase in number and are located progressively more superficially in the lingual epithelium. (A) Ectopic $K8^+$ taste buds are evident at 7 days post-tamoxifen and increase over time. (B-D) Over time, more ectopic taste buds containing type I (B), II (C) and III (D) cells are detected. (E) Progressively more ectopic $K8^+$ clusters are found in the surface layers of the epithelium at later times post-induction. Three mice per time point; error bars indicate s.e.m. * $P < 0.05$, ** $P < 0.01$; one-way ANOVA with Tukey–Kramer post-hoc test.

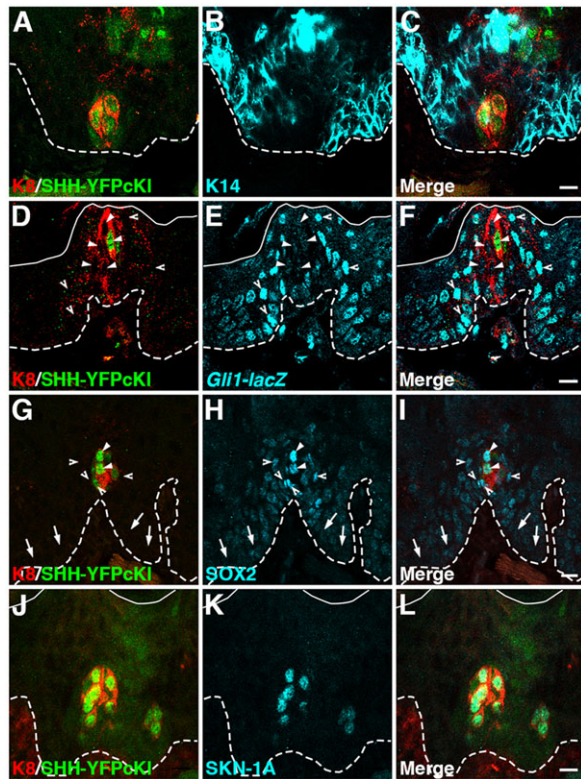


Fig. 4. Ectopic taste buds recapitulate the differentiation program of endogenous taste buds. (A-C) An ectopic $K8^+$ taste bud (red) lacks $K14$ expression (cyan), whereas adjacent progenitors are $K14^+$. (D-F) *Gli1-lacZ*⁺ cells, revealed with anti- β -gal (cyan, nicked arrowheads), surround an ectopic $K8^+$ taste bud (red, arrowheads), which is $SHH-YFPcKI^+$ (green) in the non-taste epithelium of a $K14CreER;SHH-YFPcKI;Gli1-lacZ$ mouse. $K8^+$ cells are dimly β -gal⁺. (G-I) $SOX2$ (cyan) is highly expressed by ectopic $K8^+$ cells (red, arrowheads) and by epithelial cells immediately adjacent (cyan, nicked arrowheads). $SOX2$ expression is low (cyan, arrows) in epithelial cells distant from $SHH-YFPcKI^+$ cells (green). (J-L) $SKN-1A$ (cyan) is expressed solely within $K8^+$ ectopic taste buds (red). White dashed lines delimit the basement membrane; white solid lines delimit the epithelial surface (in A-C, G-I the surface is up, and out of the field of view). All images are confocal z-stacks except D-F, which show a single optical section ($0.76 \mu\text{m}$). Scale bars: $10 \mu\text{m}$.

taste buds in cross-innervation studies (Kinnman and Aldskogius, 1988). To test if somatosensory fibers innervate ectopic taste buds in $SHH-YFPcKI^+$ non-taste epithelium, we used $PGP9.5$ (*Uchl1*), a general marker of somatosensory and gustatory nerve fibers (Kanazawa and Yoshie, 1996), to examine both intragemmal (within buds) and perigemmal (surrounding buds) innervation (Fig. 5C) of endogenous and ectopic taste buds. As expected, $PGP9.5^+$ fibers were present in and around endogenous taste buds (Fig. 5D). $PGP9.5^+$ nerve fibers traveled along the basal lamina and invaded both $SHH-YFPcKI^+$ and $SHH-YFPcKI^-$ non-taste epithelium (Fig. 5E). Fine $PGP9.5^+$ fibers were found near some ectopic taste buds (Fig. 5E, arrow), whereas other ectopic buds were situated in epithelium entirely lacking $PGP9.5^+$ neurites (Fig. 5F).

To determine in an unbiased fashion if and to what extent ectopic buds were innervated, we developed an automated quantitative analysis using MATLAB (see Materials and Methods and supplementary material Fig. S6 for details). To avoid any experimental bias, innervation density was analyzed for randomly selected endogenous and ectopic taste buds, which represented subsets of 10 and 15 buds per mouse tongue, respectively. $PGP9.5^+$ nerve fibers associated with each randomly selected bud were then

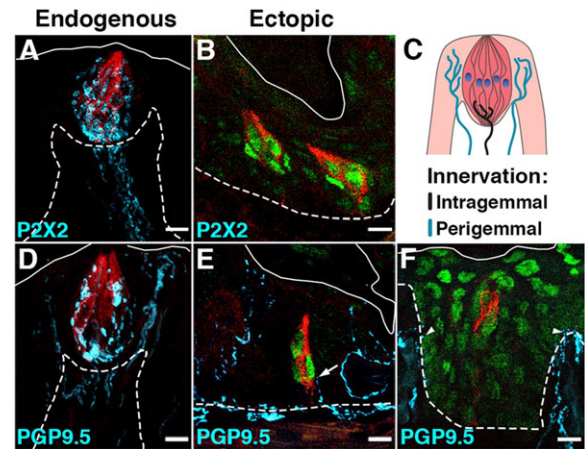


Fig. 5. Fungiform taste buds are innervated by $P2X2^+$ and $PGP9.5^+$ nerve fibers, whereas ectopic taste buds form in lingual epithelium innervated only by $PGP9.5^+$ fibers. (A) A fungiform taste bud is innervated by $P2X2^+$ taste fibers, whereas ectopic taste buds (B) lack $P2X2^+$ fibers ($P2X2^+$, cyan; $K8^+$, red; $SHH-YFPcKI^+$, green). (C) Diagram of intragemmal versus perigemmal innervation of an endogenous fungiform taste bud. The taste bud (dark pink) is innervated by $P2X2^+$ and $PGP9.5^+$ intragemmal fibers (black); the papilla epithelium (light pink) is innervated by $PGP9.5^+$ perigemmal fibers only (blue). (D) $PGP9.5^+$ neurites innervate endogenous taste buds ($PGP9.5^+$, cyan; $K8^+$, red). (E, F) $PGP9.5^+$ neurites are found near an ectopic taste bud with $SHH-YFPcKI^+$ expression (arrow in E), while another ectopic bud lies in $SHH-YFPcKI^-$ epithelium remote from sparse $PGP9.5^+$ fibers (F, arrowheads) ($PGP9.5^+$, cyan; $K8^+$, red; $SHH-YFPcKI^+$, green). White dashed lines delimit the basement membrane; white solid lines delimit the epithelial surface. All images are confocal z-stacks. Scale bars: $10 \mu\text{m}$.

quantified and categorized as intragemmal or perigemmal (Fig. 5C; Materials and Methods; supplementary material Fig. S6).

Ectopic taste buds had significantly fewer intragemmal nerve fibers than endogenous buds at all time points examined (Fig. 6A-D). Remarkably, at every time point, 35-40% of ectopic buds were completely devoid of $PGP9.5^+$ fibers, in contrast to the extensive $PGP9.5^+$ innervation of endogenous taste buds (Fig. 5D and Fig. 6). An identical outcome was observed for perigemmal innervation, i.e. $PGP9.5^+$ fibers immediately adjacent to taste buds (Materials and Methods; supplementary material Figs S6, S7). Whereas perigemmal innervation of endogenous taste buds was robust, that of ectopic taste buds was extremely sparse; $\sim 10\%$ of ectopic buds across all time points completely lacked perigemmal fibers.

In rodents, the size of endogenous taste buds is positively correlated with the number of gustatory neurons providing afferent innervation (Krimm and Hill, 2000). We examined whether $PGP9.5^+$ intragemmal neurite density also correlated with taste bud size. We tallied $K8^+$ pixels per taste bud as a proxy for taste bud size (see Materials and Methods) and compared this value with intragemmal $PGP9.5^+$ innervation. Whereas intragemmal innervation density was strongly correlated with endogenous bud size, no such relationship was evident for ectopic taste buds (Fig. 6E). Furthermore, comparing endogenous and ectopic taste buds of similar size (Fig. 6E, e.g. $\sim 20,000$ $K8^+$ pixels) revealed a clear difference in intragemmal neurite density, with up to 18,000 $PGP9.5^+$ pixels per endogenous bud versus 0 pixels for several ectopic taste buds of the same size.

Finally, we compared the sizes of ectopic and endogenous taste buds over time post-tamoxifen. At day 7 post-tamoxifen, ectopic taste buds were much smaller than endogenous buds (4403 ± 654 $K8^+$ pixels). Ectopic taste buds enlarged significantly by 14 days, yet were still significantly smaller than endogenous buds (ectopic,

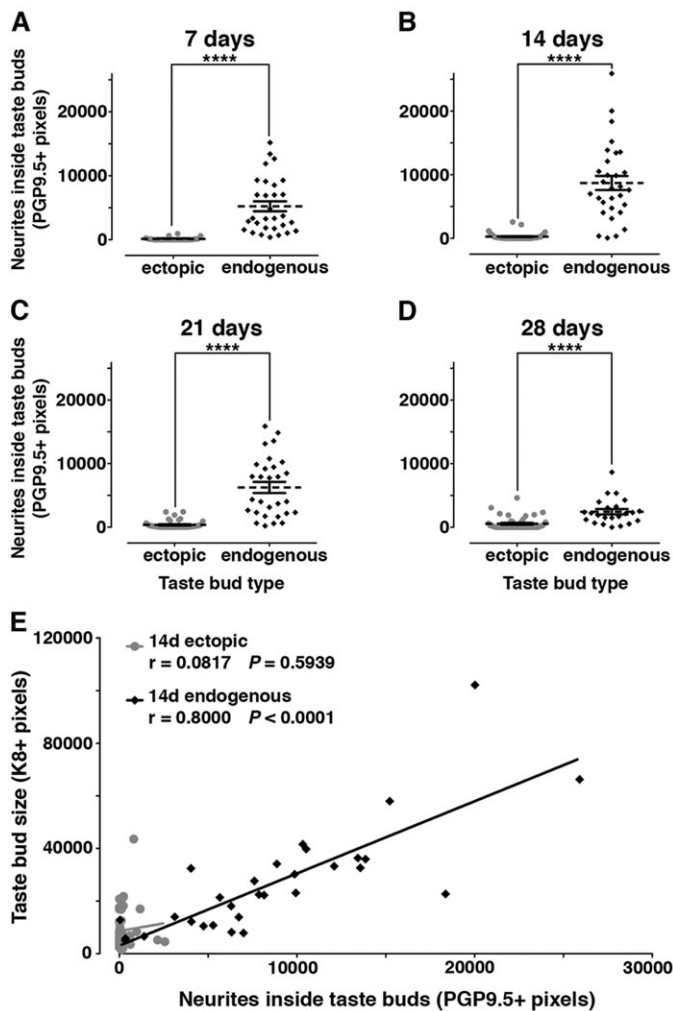


Fig. 6. Ectopic taste buds are independent of intragemmal innervation. Significantly fewer PGP9.5⁺ neurites are found in ectopic taste buds (gray symbols) than in endogenous taste buds (black symbols) at all time points examined. Two-tailed Mann–Whitney U-test: (A) 7 days, U=5.0; (B) 14 days, U=43.0; (C) 21 days, U=54.0; (D) 28 days, U=139.0; 22–45 ectopic and 23–30 endogenous taste buds per time point, randomly selected from all taste buds in three mice per time point; *****P*<0.0001. (E) At 14 days post-tamoxifen, endogenous taste bud size and PGP9.5⁺ innervation are strongly correlated (black; 30 endogenous taste buds from three mice), whereas ectopic taste bud size and PGP9.5⁺ innervation are not (gray; 45 ectopic taste buds from three mice).

8887±1159 K8⁺ pixels; endogenous, 45,586±4985 K8⁺ pixels; *P*<0.0001, two-tailed Student's *t*-test). Ectopic taste bud size did not grow beyond the average reached at 2 weeks (21 days, 8840±1144 K8⁺ pixels; 28 days, 9968±1202 K8⁺ pixels), suggesting that although SHH drives the differentiation and maintenance of ectopic taste buds, neural support, which these ectopic taste buds never receive, might be required to obtain mature bud size (Krimm and Hill, 1998).

DISCUSSION

In adult mammals, lingual taste buds are restricted to taste papillae, where they undergo continual renewal. Here, we reveal an unrecognized competency of adult tongue epithelium to generate taste buds *de novo* in response to ectopic SHH expression. These ectopic taste buds contain the full complement of functional taste cell types and differentiate via the same lineage steps as endogenous buds.

Thus, ectopic SHH expression causes a transformation of lingual epithelial cell fate, as non-taste progenitor cells give rise to cell type-replete taste buds rather than produce K13⁺ pseudostratified epithelium. Unlike endogenous taste buds, however, we find that ectopic taste buds differentiate in the absence of innervation, as neither contact by gustatory nor somatosensory nerve fibers is required. We propose that SHH misexpression supersedes the requirement for innervation that is essential for the renewal of endogenous taste buds, and suggest a pro-differentiation function for SHH in adult taste buds.

Ectopic SHH expression in non-taste lingual epithelium induces ectopic taste buds

To date, despite over 100 years of study, taste buds have never been reported outside of taste papillae in any mammalian tongue. Moreover, attempts to generate taste buds from lingual keratinocytes *in vitro* have failed (Hisha et al., 2013; Luo et al., 2009). Thus, we show for the first time not only that the tongue is broadly competent to make taste buds, but also that SHH is a key inducer and positive regulator of taste bud cell differentiation.

During embryogenesis, taste placodes [embryonic precursors of the first postnatal taste buds (Thirumangalathu et al., 2009)] arise in distinct bilateral rows in the anterior tongue at E12.0–12.5, and manipulation of a number of signaling pathways has revealed that this early pattern is plastic. For example, activated Wnt/β-catenin signaling (Liu et al., 2007) or loss of follistatin (Beites et al., 2009) results in excess and enlarged ectopic taste placodes. Like follistatin, SHH is also a key negative regulator of embryonic taste placode pattern in that pharmacological inhibition of the pathway results in expanded and ectopic taste placodes (Hall et al., 2003; Mistretta et al., 2003), whereas placode formation is blocked in embryonic tongue cultures treated with SHH ligand (Iwatsuki et al., 2007). This repressive function of SHH in embryos contrasts starkly with its function in adults, in which, as we have shown, SHH promotes taste cell differentiation. Our data thus indicate different mechanisms of action for SHH in embryonic pattern formation versus regulation of taste cell differentiation during adult taste bud homeostasis.

Importantly, not all SHH-YFPcKI⁺ patches in non-taste epithelium differentiate taste buds, indicating that additional components render specific areas of non-taste epithelium competent to generate ectopic taste buds in response to SHH. The tongue epithelium has a diversity of cellular compartments responsible for homeostasis (Feng et al., 2013; Miura and Barlow, 2010; Tanaka et al., 2013) and ectopic taste bud formation might coincide with an as yet unidentified epithelial compartment.

Recently, hyperactivation of an effector of SHH signaling, GLI2, in lingual epithelium has been shown to cause loss of endogenous taste buds, while ectopic taste buds were not observed (Liu et al., 2013). However, broad misexpression of GLI2 caused severe disruption of all of the lingual epithelium, such that the specific function of GLI2 in taste bud differentiation remains to be elucidated.

SHH-induced ectopic taste buds resemble endogenous taste buds in morphology, cell complement and differentiation program

Here, we discovered that SHH misexpression induces numerous clusters of elongated cells that are morphologically distinct from the surrounding non-taste epithelium and are made up of all three differentiated taste bud cell types (I, II and III). Type I support cells are most prevalent in taste buds, followed by type II, then the least abundant type III cells (Chaudhari and Roper, 2010; Ma et al.,

2007). The relative proportions of these three cell types in ectopic taste buds reflect those of endogenous buds (I>II>III). The limited increase in ectopic type III cells is consistent with previous studies showing that type III cells are least common, representing only 6% of cells in each fungiform taste bud (Ohtubo and Yoshii, 2011), are generated less frequently than type I and II cells (Miura et al., 2014), and are also significantly longer lived than types I and II (Perea-Martinez et al., 2013). These factors are likely to contribute to the less frequent genesis of type III cells in ectopic taste buds, although we cannot rule out the possibility that a full type III cell complement might not differentiate in taste buds that lack innervation (and see below).

Adult taste buds follow a sequential process of differentiation, in which bipotential K14⁺ progenitors adjacent to taste buds generate either K13⁺ keratinocytes or taste cells (Okubo et al., 2009). Like their endogenous counterparts, ectopic taste buds: (1) descend from K14⁺ progenitor cells, since ectopic taste cells were always SHH-YFPcKI⁺; (2) were devoid of K13 immunoreactivity, demonstrating their non-keratinocyte fate; and (3) expressed general markers of taste buds [K8, claudin 4 and KCNQ1 (Asano-Miyoshi et al., 2008; Knapp et al., 1995; Michlig et al., 2007; Wang et al., 2009)]. Ectopic taste buds also exhibited the appropriate expression of transcription factors known to regulate taste bud development and renewal, i.e. SOX2 and SKN-1A (Matsumoto et al., 2011; Okubo et al., 2006; Suzuki, 2008), further supporting that differentiation of ectopic taste buds mirrors that of endogenous taste buds.

In adult taste buds, type IV basal cells are *Shh*⁺ and are immediate postmitotic precursors of all three taste cell types (Miura et al., 2014). However, SHH is only transiently expressed in taste precursors, as taste receptor cells turn off SHH as part of the endogenous differentiation program (Miura et al., 2006). By contrast, owing to the nature of the SHH-YFPcKI allele, SHH is constitutively expressed in ectopic taste buds, yet this persistent expression does not block taste cell differentiation. In fact, our findings indicate that cessation of SHH expression is not required for taste cell differentiation and, moreover, are consistent with the observation that cells within taste buds do not express SHH target genes (Liu et al., 2013) (Fig. 4D-F; supplementary material Fig. S5) and are therefore not influenced by local SHH signaling.

Our expression analysis is not an exhaustive survey of regulatory genes expressed in adult taste buds, which include members of the Notch, TGF β and Wnt pathways (Gaillard and Barlow, 2011; Nakamura et al., 2010; Nguyen and Barlow, 2010; Seta et al., 2003), as well as numerous transcription factor genes, such as *Six1*, *Six4*, *Ascl1*, *Hes6* and *Hes1* (Ota et al., 2009; Seta et al., 2003, 2011; Suzuki et al., 2010). The degree to which these genes are similarly or differentially expressed by endogenous versus ectopic taste buds might shed light on the genes that are required for taste cell differentiation regardless of location versus those required for interactions with sensory afferents or perhaps for taste papilla genesis.

SHH induces taste bud differentiation in non-taste epithelium without taste papillae formation

Adult fungiform taste buds reside within taste papillae. By contrast, the ectopic buds reported here lack taste papillae and are interspersed among filiform papillae. Thus, in adult tongue, the differentiation of ectopic taste buds is distinct from taste papilla morphogenesis. This morphology is similar to that of taste buds in aquatic salamanders (Barlow, 1999), fishes (Caprio, 1987) and mammalian soft palate (Cleaton-Jones, 1971), where buds lack papillae and are embedded directly in the epithelium. Additionally, during mouse embryogenesis *Shh*⁺ taste placodes contribute

exclusively to taste buds, but not to taste papillae, revealing that, once patterned, taste buds and papillae descend from distinct cell populations (Thirumangalathu et al., 2009). Our results further support the hypothesis that taste bud genesis and papillary morphogenesis are separable events in development and evolution (Krimm and Barlow, 2007).

Does SHH bypass the requirement for innervation in taste bud differentiation?

In contrast to classical observations that the maintenance of adult taste buds depends upon intact innervation (Oakley and Witt, 2004), ectopic taste buds induced by SHH do not. Previous studies have revealed both axon chemoattraction and chemorepulsion by SHH during development of the central nervous system (Avilés et al., 2013; Charron and Tessier-Lavigne, 2005; Sánchez-Camacho and Bovolenta, 2009). Furthermore, in the peripheral nervous system, HH signaling plays a role in the maintenance and regeneration of peripheral nerves (Angeloni et al., 2011), including facial motor neurons (Akazawa et al., 2004) and parasympathetic innervation of the submandibular glands (Hai et al., 2013). Thus, it is possible that misexpression of SHH in lingual epithelium attracted gustatory and/or somatosensory neurites, and hence ectopic taste bud formation would be secondary to SHH-directed innervation. This was decidedly not the case for gustatory innervation, as these nerves did not stray from their taste bud targets, consistent with the highly targeted development of taste bud innervation (Farbman and Mbiene, 1991; Lopez and Krimm, 2006).

The anterior tongue is densely innervated by trigeminal somatosensory fibers, which in cross-innervation studies support taste buds *in situ* (Kinnman and Aldskogius, 1988). Additionally, dorsal root ganglion sensory neurons paired *in oculo* with tongue explants also maintain taste buds (Zalewski, 1973). However, our quantitative analysis revealed that ectopic taste buds were not contacted consistently by somatosensory fibers; almost half of ectopic taste buds analyzed had no innervation whatsoever, such that the spatial relationship between ectopic taste buds and somatosensory neurites appeared random and incidental. Despite these findings, we cannot rule out the possibility that transient contact of SHH⁺ epithelial cells by somatosensory fibers may be sufficient to trigger taste bud differentiation in this genetic model. However, we consider this unlikely, as taste bud maintenance in all experimental contexts requires persistent innervation (Oakley and Witt, 2004).

Shh is expressed by basal cells within taste buds, and this expression is exquisitely nerve dependent (Miura et al., 2004). Upon denervation, *Shh* is rapidly lost, as is expression of SHH target genes by surrounding progenitor cells, and this loss precedes the loss of differentiated taste cells (Miura et al., 2004). Likewise, upon reinnervation, *Shh* and target gene expression rapidly resume before differentiated taste buds reappear. Although SHH has been proposed to regulate taste progenitor proliferation (Miura et al., 2006), our data support a pro-differentiation role for SHH, which can drive the entire lineage program from progenitor specification to differentiated taste cells. Thus, we hypothesize that transgenic SHH expression supersedes any neural requirement for *Shh* expression and its subsequent function in taste bud differentiation, and might account for the differentiation of ectopic taste buds in the absence of innervation. Importantly, basal cell carcinoma patients receiving chemotherapeutics targeting the SHH pathway often experience a loss or distorted sense of taste (Ng and Curran, 2011). Our results suggest that these drugs might affect the differentiation of taste cells, providing new insight for possible mitigation of taste dysfunction in patients.

MATERIALS AND METHODS

Mice

A pROSA26PA vector containing a lox-STOP-lox cassette followed by cDNA encoding human SHH, an internal ribosomal entry site (IRES) and three NLS sequences driving nuclear expression of Venus yellow fluorescent protein (YFP) was used to target the *Rosa26* locus (Soriano, 1999) in C57BL/6 embryonic stem cells (ESCs) (supplementary material Fig. S1). Correctly targeted ESCs were identified by Southern blotting and injected into blastocysts using standard techniques. Germline transmission was obtained after crossing the resulting chimaeras with C57BL/6N females. Mice carrying the KRT14-cre/ERT2 (K14CreER) (Li et al., 2000) and SHH-YFPcKI alleles were maintained at UCSF and genotyped using standard primers for detection of the *Cre* recombinase gene, presence of a Neo cassette and presence of a wild-type *Rosa* allele. Mice carrying the *Gli1-lacZ* allele were maintained and genotyped as previously described (Bai et al., 2002). All rodent work was undertaken in the UCSF vivarium according to approved protocols.

Tamoxifen induction

A single dose of 5 mg tamoxifen (Sigma, T5648) dissolved in corn oil was given via oral gavage to K14CreER;SHH-YFPcKI mice (5-10 weeks of age). Mice were sacrificed 7, 14, 21 or 28 days post-tamoxifen induction, following protocols approved by the Animal Care and Use Committee at UCSF. Data for this study were gathered from three to four mice per time point.

Tissue preparation

Tongues were harvested and fixed by immersion in 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) overnight at 4°C. Processing of tissue was restricted to the anterior tongue with a high density of fungiform papillae (Fig. 1A,A'). Tissue was cryoprotected in 20% sucrose in 0.1 M PBS overnight at 4°C, embedded in Tissue-Tek O.C.T. Compound (Sakura, 4583), frozen, and stored at -80°C. Eight sets of serial cryosections (12 µm) per tongue were collected on Superfrost Plus Slides (Fisher Scientific, 12-550-15).

Immunofluorescence

Immunofluorescence was performed on 12 µm cryosections as described (Nguyen and Barlow, 2010). Primary antisera and dilutions: rat anti-K8 (Troma) (1:200; Developmental Studies Hybridoma Bank, University of Iowa, USA), rabbit anti-claudin 4 (1:250; 364800, Invitrogen), rabbit anti-NTPDase2 (1:3000; mN2-36L, CHUQ), rabbit anti-α-gustducin (1:1000; sc-395, Santa Cruz), rabbit anti-SNAP25 (1:8000; S9684, Sigma), rabbit anti-PLCβ2 (1:1000; sc-206, Santa Cruz), rabbit anti-NCAM (1:1000; AB5032, Millipore), goat anti-KCNQ1 (1:500; sc-10646, Santa Cruz), guinea pig anti-TRPM5 (1:1000; from Dr Emily Liman at USC), goat anti-CAR4 (1:1000; AF2414, R&D Systems), rabbit anti-K14 (1:3500; PRB-155P, Covance), guinea pig anti-K13 (1:500; BP5076, Acris Antibodies), goat anti-SOX2 (1:500; sc-17320, Santa Cruz), rabbit anti-SKN-1A (POU2F3) (1:500; sc-330, Santa Cruz), rabbit anti-P2X2 (1:500; APR-003, Alomone Labs), rabbit anti-PGP9.5 (1:1000; 7863-0504, AbD Serotec), rabbit anti-β-galactosidase (1:1000; 55976, MP Biomedicals) and chicken anti-GFP (1:1000; GFP-1020, Aves Labs). Appropriate secondary antisera from Invitrogen (A11081, A11010, A11039, A11073, A21208, A31573, A11055) or Jackson ImmunoResearch (703-545-155, 706-486-148) were used at 1:1000 or 1:800, respectively. Sections were counterstained with Draq5 (1:8000; 108410, AbCam) or TO-PRO-3 (1:1000; T3605, Invitrogen), and coverslipped with Fluormount G (0100-01, SouthernBiotech) or ProLong Gold Antifade (P36930, Invitrogen).

Image acquisition and analysis

Initially, taste buds were counted in immunostained sections using a Zeiss Axioplan II microscope, AxioCam CCD camera and Axiovision software. Eight serial sets of 12 sections were prepared from the most anterior 1.5 mm of each tongue; a single series was used per taste cell type marker. Thus, sections on each slide were separated by 7×12 µm=84 µm. Each endogenous taste bud is ~35 µm in diameter (data not shown), so this eliminated double counting of ectopic or endogenous taste buds. For each

immunomarker, endogenous and ectopic buds were tallied and assigned a number. Endogenous taste buds were distinguishable from ectopic buds, as the former were always located apically within fungiform papillae (e.g. Fig. 2A-C,J-L, arrows). Ectopic taste buds were embedded in the lingual epithelium and lacked papillae (e.g. Fig. 1B-E).

To determine the percentages of ectopic taste buds co-expressing specific molecular markers, a subset of 15 ectopic taste buds was randomly selected from the total enumerated taste buds per mouse tongue using the Rand() function in Excel and taste buds immunoreactive for each marker were counted, e.g. for K8 and KCNQ1 (supplementary material Table S1). The number and proportion of ectopic and endogenous taste bud profiles housing all three taste cell types were determined in a randomly selected subset of taste buds identified with a type III cell marker (supplementary material Table S1).

The size and innervation density of endogenous and ectopic taste buds were quantified in tissue double immunostained with rabbit anti-PGP9.5 (Alexa 647 secondary) and rat anti-K8 (Alexa 547 secondary). For each mouse tongue, subsets of 15 ectopic and 10 endogenous taste buds were randomly selected from the total tallied taste buds per mouse tongue as described above (supplementary material Table S2). Confocal images of each taste bud were obtained as a z-stack of 0.76 µm optical sections acquired sequentially in the red (K8) and long red (PGP9.5) channels using a Leica TCS SP5 II confocal microscope and LASAF software. z-stacks were subjected to MATLAB (MathWorks) analysis, which we developed to quantify the innervation density of taste buds. This analysis included the following steps. (Step 1) For each taste bud z-stack, a K8⁺ taste bud (endogenous or ectopic) was outlined in the red channel and defined as the region-of-interest (ROI) (dashed line, supplementary material Fig. S6). (Step 2) PGP9.5⁺ nerve fiber density within a taste bud ROI (long red channel) was quantified in each optical section of the z-stack. (Step 3) The total PGP9.5⁺ pixels per taste bud ROI (intragemmal innervation) was determined using a MATLAB script, which summed PGP9.5⁺ pixels in the taste bud ROI across all optical sections of the z-stack. To correct for background fluorescence, mean fluorescence intensity of a user-designated ROI drawn in a PGP9.5-immunonegative or K8-immunonegative region of the tissue was subtracted from each optical section of a confocal z-stack. The MATLAB script then calculated a threshold for fluorescence intensity in each optical section using Otsu's method (Otsu, 1979), where all pixels with an intensity above threshold were designated as signal. Only PGP9.5⁺ pixels that (1) fell within the K8⁺ taste bud ROI and (2) were clustered in groups of ≥5 pixels were tallied as intragemmal PGP9.5⁺ pixels. Importantly, although some taste cells are PGP9.5⁺ (Kanazawa and Yoshie, 1996), these cells were rare and significantly dimmer, in terms of immunolabeling, than nerve fibers. Therefore, MATLAB thresholding eliminated the taste cell signal (confirmed by double labeling with K8; data not shown), allowing us to isolate and analyze the brighter PGP9.5⁺ neurites.

To determine PGP9.5⁺ fiber density in epithelium adjacent to taste buds, i.e. perigemmal innervation, the same randomly selected taste buds as above were used, and PGP9.5⁺ pixels were counted within a 17.6 µm-wide ring of cells encircling the K8⁺ taste bud ROI (solid line, supplementary material Fig. S6); 17.6 µm is the mean radius of the ROI for endogenous K8⁺ taste buds in our study. To quantify taste bud size, we used K8⁺ pixel density within the taste bud ROI, and background correction was automated as above.

Categorization of ectopic taste bud position in the lingual epithelium

K8⁺ ectopic taste buds were assigned to categories 1, 2 or 3 based on the following criteria. Category 1: situated within the basal layer and/or immediate suprabasal layer of the epithelium. Category 2: located in layers between the two most basal layers and uppermost two layers. Category 3: found in the uppermost two layers and/or with apical processes extending into the uppermost layers or lingual surface (supplementary material Fig. S4).

Statistics

A one-way ANOVA with Tukey-Kramer post-hoc test was used to compare taste bud number and size across groups. To compare the number of PGP9.5⁺ pixels between endogenous and ectopic buds, the non-parametric Mann-Whitney U-test was used, as the data did not fit a normal distribution.

Correlation and linear regression analyses were used to compare taste bud size with intragemmal neurite density. A two-tailed Student's *t*-test was used to compare ectopic and endogenous taste bud size. Significance was taken as $P < 0.05$ with a confidence interval of 95%. Data are presented as mean \pm s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Experimental design was developed by D.C., K.S., O.D.K. and L.A.B. K.S. generated bigenic mice, treated them with tamoxifen and harvested tongue tissue. D.C. performed the immunofluorescence experiments and all quantitative and statistical analyses, D.C. and E.S. developed and implemented the MATLAB analysis, C.A. and F.J.d.S. developed the SHH-YFPc1 mouse line, and D.C., K.S., O.D.K. and L.A.B. wrote the manuscript. All authors edited drafts of the manuscript.

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Supplementary material

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