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Function, Innervation, and Neurotransmitter Signaling in Mice Lacking Type-II Taste Cells

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Title page

TITLE: Function, innervation, and neurotransmitter signaling in mice lacking Type II taste cells

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Conflicts of Interest

The authors declare no conflict of interest.

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ABSTRACT

The *Skn-1a* transcription factor (*Pou2f3*) is required for Type II taste cell differentiation in taste buds. Taste buds in *Skn-1a*$^{-/-}$ mice lack Type II taste cells but have a concomitant expansion of Type III cells, providing an ideal model to determine the relative role of taste cell types in response specificity. We confirmed that chorda tympani responses to sweet, bitter, and umami stimuli were greatly reduced in the knockouts compared to wildtype littermates. *Skn-1a*$^{-/-}$ mice also had reductions to NaCl that were partially amiloride-insensitive, suggesting that both Type II and Type III cells contribute to amiloride-insensitive salt detection in anterior tongue. We also confirmed that responses to sour stimuli are equivalent in the knockouts, despite the large increase in the number of Type III taste cells. To examine their innervation, we crossed the *Htr3a*-GFP (5-HT_{3A}-GFP) reporter mouse with the *Skn-1a*$^{-/-}$ mice and examined geniculate ganglion neurons for GFP expression and responses to 5-HT. We found no change in the number of 5-HT_{3A}-expressing neurons with knockout of *Skn-1a*. Calcium imaging showed that only 5-HT_{3A}-expressing neurons respond to exogenous 5-HT, while most neurons respond to ATP, similar to wildtype mice. Interestingly, despite loss of all Type II cells, the P2X3 antagonist AF353 blocked all chorda tympani responses. These data collectively raise questions pertaining the source of ATP signaling in the absence of Type II taste cells and whether the additional Type III cells are innervated by fibers that would have normally innervated Type II cells.

Significance statement

Despite numerous studies, the role of specific taste cell types in taste responsivity and their connectivity to gustatory nerves is incompletely understood. Here we show that in *Skn-1a* knockout mice where only Type I and III cells exist within taste buds, the number of gustatory ganglion cells innervating Type III cells remains unchanged and these neurons exhibit normal responses to gustatory neurotransmitters. Further, we show that even though ATP release from taste buds is undetectable in *Skn-1a* knockout mice, ATP is still required to drive gustatory neural output via the chorda tympani nerve. The source of the required ATP is unclear and begs further study.

INTRODUCTION

Taste signals generated by the interaction of molecules with receptors located on taste cells are transmitted to gustatory neurons to elicit a sensation of taste. Taste buds comprise 3 elongate taste cell types that are defined by molecular features and physiological properties (for review, Roper and Chaudhari, 2017). Type I cells are generally considered to have primarily a support role, similar to glial cells in the nervous system. Type II cells express the G protein coupled receptors and downstream effectors for bitter, sweet, and umami taste stimuli. These cells when stimulated release ATP via the large conductance CALHM1 channels to activate P2X receptors on gustatory afferent fibers (Taruno et al., 2013). Type III cells respond to acids (sour stimuli) via an apically-located proton channel (Bushman et al., 2015; Ye et al., 2016) and when stimulated, release 5-HT (Huang et al., 2011, 2008) to activate the 5-HT_{3A} receptors on gustatory afferents (Larson et al., 2015). The cell bodies of these neurons form part of the geniculate ganglion (VIIth cranial nerve), petrosal ganglion (IXth cranial nerve) and nodose ganglion (Xth cranial nerve). While 5-HT contributes to only a portion of the nerve response to taste, ATP is required for transmission of all taste modalities, as purinergic receptor antagonism or knockout eliminates all nerve response to taste (Finger et al., 2005; Vandenbeuch et al., 2015). However, the role of ATP in the taste response for non-Type II cell mediated modalities remains elusive as release of ATP has only been detected from Type II cells (Huang et al., 2007; Murata et al., 2010; Romanov et al., 2007).

The development of Type II taste cells requires the transcription factor Skn-1a (*Pou2f3)*. Skn-1a is a transcription factor (POU domain gene) involved in the regulation of gene expression in epidermal keratinocytes (Andersen et al., 1997) and regulates the generation and differentiation of taste cells (Matsumoto et al., 2011). Mice lacking Skn-1a do not express Type II taste cells and show deficient chorda tympani and glossopharyngeal nerve responses to sweet, umami, and bitter stimuli, but interestingly, though the number of Type III cells is increased by about 30% in taste buds of *Skn-1a*$^{-/-}$ mice, acid responses are similar to those observed in wildtype animals (Matsumoto et al., 2011). Recently, Maeda et al. (2017) showed that the absence of Type II taste cells and the overexpression of Type III taste cells in the *Skn-1a*$^{-/-}$ mice do not alter the number

of nodose/petrosal ganglion cells that innervate Type III cells using retrograde transfer of wheat germ agglutinin. These results suggest that connections between taste cells and ganglion cells are not influenced by the type of cells expressed in the tongue but are predetermined. What is not clear, however, is whether the additional Type III cells in the *Skn-1a*$^{-/-}$ mice are innervated by ganglion neurons expressing the serotonin receptor 5-HT$_{3A}$, as in wildtype mice (Stratford et al., 2017). Here, we have crossed the *Skn-1a*$^{-/-}$ animals with mice expressing GFP from the *Htr3a* promoter and examined the progeny for 5-HT$_{3A}$ expression and function in the geniculate ganglion. Further, we examined the dependence of taste signaling on ATP in the *Skn-1a*$^{-/-}$ mice that lack Type II taste cells.

MATERIALS AND METHODS

Animals

Experiments were performed on *Skn-1a*$^{-/-}$ and WT mice obtained from Ichiro Matsumoto at Monell Chemical Senses Center. Mice were crossed for several generation with C57Bl/6. 5HT3A-GFP mice were purchased from MMRRC (catalog # 000273-UNC) and crossed with *Skn-1a*$^{-/-}$ mice. Both males and females aged between 4 and 6 months were used. Mice were housed at the author's institution in ventilated cages with access to water and food ad libitum. All experimental procedures were conducted in accordance with the Animal Care and Use Committee at the [Author University].

RT-PCR

Separate pools of circumvallate and fungiform taste tissues were isolated from three *Skn-1a*$^{-/-}$ and WT littermates. RNA was extracted in a QIAcube according to manufacturer's instructions using reagents from Qiagen (Valencia, CA). A 30 minute DNase I treatment at room temperature to remove genomic DNA was included. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit which included an additional step for removal of genomic DNA. Two microliters of cDNA were added to the PCR reaction (Tas PCR Core). PCR conditions included an initial 5 minute denaturation step followed by 40 cycles of 30s

denaturation at 95°C, 30s annealing at 60°C and 45s extension at 72°C and a final 7 minute extension step. PCR primer sequences can be found in Table 1. We included fungiform cDNA from wildtype mice as a positive control and a no template negative control where water was added in place of cDNA. Amplified sequences were visualized by gel electrophoresis in 2% agarose gels stained with GelRed (Biotium, Hayward, CA).

Taste bud immunohistochemistry

Intraperitoneal injections of 5-hydroxy-L-tryptphan (5-HTP; 50mg/kg; Sigma Aldrich) were performed on animals to increase 5-HT levels. After 1 hour, mice were deeply anesthetized with urethane and transcardially perfused using 4% paraformaldehyde. For lingual tissues, after 4 hours postfix and cryoprotection in 20% sucrose overnight, 12-16 µm cryostat sections were cut and mounted onto slides. After buffer washes with PBS, specific primary antibodies for each cell type were applied to the sections and immunoreacted overnight at 4°C. Secondary antibodies were reacted for 2 hours at room temperature and mounted with Fluoromount (Southern Biotech). Z-stack images were collected on a Leica SP5 or SP8 confocal microscope. Antibodies and their sources are listed in Table 2.

Geniculate ganglion immunohistochemistry

For geniculate ganglion imaging, geniculate ganglia were removed from paraformaldehyde-perfused heads and post-fixed for 1 hour. Ganglia were optically cleared with PACT (Cronan et al., 2015) and labeled with primary antibodies against GFP, P2X3, and SNAP25 and imaged on a Leica SP8 confocal microscope.

Geniculate ganglion image quantification

Cleared whole mount ganglia were imaged in a single field of view with 1 µm axial spacing. Maximal z-projections of 10 µm substacks were created with ImageJ. Regions of interest were drawn around individual

cells as identified by SNAP25 and DAPI and the average fluorescence value of each channel was measured for each region of interest. Fluorescence intensity values were background subtracted and normalized for each image. Cells were scored as positive or negative for each channel using a threshold intensity value (Larson et al., 2015). Thresholds for each channel were determined by fitting intensity value histograms with Gaussian functions (two peak if bimodal). For bimodal distributions, threshold was determined by any value > 2 SD above the median of the lower peak.

Taste receptor cell quantification

Type III cells were counted from images of lingual slices labeled with antibodies against SNAP25. We chose SNAP25 to identify Type III cells as it has been previously shown as the broadest marker of Type III cells in the anterior tongue (Wilson et al., 2017). From each 3 dimensional image collected, a plane from the middle, upper, and lower quadrants was extracted, and cell profiles with a clear nuclear region were counted per taste bud. This method minimized counting cells that persisted through multiple optical sections. Counts were parsed and plotted using a custom 'R' script. Count data were analyzed using a Kruskal-Wallis test followed by Dunn Test for multiple comparisons in 'R' with the *dunn.test* and *FSA* packages (Dinno, 2017; Ogle et al., 2019).

Taste bud innervation quantification

Image stacks of different taste fields were analyzed using ImageJ. Stacks were processed using 'Subtract Background' (rolling ball radius 50 px), 'Median' (radius 2), and 'Auto Threshold' (Otsu method, stack histogram) to create multichannel binary images. Regions of interest were drawn around individual taste buds and the area, mean fluorescence, integrated density, and voxel size/volume were measured for each optical section. Using a custom 'R' script, the total analyzed volume and the total labeled volume were calculated for each region of interest. Innervation density was plotted as labeled volume / total volume.

5HT3A-GFP, P2X3 nerve fiber quantification

Lingual sections were labeled with antibodies against GFP and P2X3. High resolution 3D images were acquired on a Leica SP8 of all taste fields. Images were subject to a custom analysis pipeline to quantify the proportion of P2X3 immunoreactivity that overlapped with GFP immunoreactivity. In ImageJ, ROIs pertaining to individual taste buds were extracted and saved as new images for further processing which included 'Subtract Background' (rolling ball radius 50 px), 'Median' (radius 2), and 'Auto Threshold' (Otsu method, stack histogram) to create multichannel binary images. Images and image metadata were imported to R using *raster*, *rgdal* and *sp* packages (Bivand et al., 2019, 2013; Hijmans, 2019; Pebesma and Bivand, 2005). A custom script was used to calculate the volume of each taste bud ROI that was occupied by a P2X3+ and/or GFP+ voxel. Data is displayed as GFP:P2X3+ volume divided by P2X3+ volume using *ggplot2*. Statistical comparison was performed using a nonparametric Kruskal-Wallis test followed by Dunn Test for multiple comparisons.

Calcium imaging

Calcium imaging of isolated geniculate ganglion neurons was performed as previously described (Larson et al., 2015). Briefly, geniculate ganglia were extracted from euthanized animals, washed briefly in minimum essential medium with Earle's balanced salts (MEM/EBSS; Hyclone) and placed in MEM/EBSS supplemented with 1.25 mg/ml trypsin (Sigma-Aldrich) and 2.5 mg/ml collagenase A (Roche Diagnostics) for 30 minutes. Ganglia were washed with MEM/EBSS, gently triturated with a fire-polished glass pipette, and resuspended in HEPES buffer (in mM: 136 NaCl, 5.6 KCl, 1 $MgCl_2$, 2.2 $CaCl_2$, 11 glucose, 10 HEPES; pH 7.4). Cells were plated on poly-D-lysine (0.02 mg/ml) coated coverslips before loading with 2 µM Fura-2-AM (Invitrogen) with 0.01% Pluronic F-127 (Invitrogen). Cells were continually perfused with HEPES buffer or HEPES buffer plus 10 µM ATP, 10 µM 5-HT, or HEPES buffer with 55 mM KCl. Cells were imaged using an inverted microscope equipped with a 40x oil immersion lens, Lamba 10-3 filter wheel (with 340 and 380 excitation filters; Sutter), and a QImaging Retiga R3 CCD camera. Acquisition was controlled using Imaging Workbench 6.1 (Indec Biosystems). Baseline emission ratio (340_{ex} and 380_{ex}) values were determined as the first 10-15 seconds of recording and

were subtracted from the peak ratio during stimulus perfusion. In the absence of a visible response, the value was taken 30s after onset of stimulus perfusion. Data are plotted as baseline subtracted values divided by the peak values.

Nerve recording

Skn-1a$^{-/-}$ and WT littermates were anesthetized with an intraperitoneal injection of urethane (2mg/kg; Sigma Aldrich) and placed in a head holder. The trachea was cannulated to facilitate breathing. Using a ventral approach, the chorda tympani was exposed, cut near the tympanic bulla and placed on a platinum-iridium wire. A reference electrode was placed in a nearby muscle. The signal was fed to an amplifier (P511; Grass Instruments), integrated and recorded using AcqKnowledge software (Biopac). The anterior part of the tongue containing the fungiform papillae was continuously stimulated with NH_4Cl 100mM, sucrose 500mM, quinine 10mM, MSG 100mM, MSG 100mM + IMP 0.5mM, HCl 10mM, citric acid 10mM, NaCl (30, 100 and 300mM). In some experiments, amiloride (100μM), an epithelial Na^+ channel (ENaC) blocker was added to NaCl. In some experiments, the P2X3 antagonist, AF353, was diluted in water (1mM) and applied on the tongue for 10 minutes. Each stimulus was applied for 30s and rinsed with water for 40s. Since all responses could potentially be affected in the knockout mice, each response was normalized to the baseline (Vandenbeuch et al., 2013; Larson et al., 2015; Vandenbeuch et al., 2015). Though we drew similar conclusions when data were normalized to NH_4Cl responses (not shown) we present data as baseline-normalized as the cell type involved in the NH_4Cl response is likely to be Type III cells (Oka et al., 2013). To analyze the data, the baseline integrated response was averaged over 10s prior to stimulation of each tastant and subtracted from the average amplitude of each integrated response (over 30s). The baseline subtracted responses were then divided by the respective baseline.

ATP release

An enzymatic cocktail containing Dispase II (3mg/ml; Roche) and Elastase (2.5mg/ml; Worthington) diluted in Tyrode's was injected under the circumvallate papillae. The epithelium was peeled after 20 minutes incubation in Tyrode's and placed on a modified Ussing chamber (42µl). The basolateral part of the papilla was bathed in Tyrode's while the apical part was stimulated with 5µl of artificial saliva, citric acid (20mM), or NaCl (500mM) diluted in artificial saliva. Only one stimulus was applied on each preparation and could not be rinsed because of the high sensitivity to mechanical stimulation. The Tyrode's containing the releasate from taste buds was collected after each stimulation, transferred to a 96-well plate and placed in a plate reader (Synergy HT, Biotek). 42µl of luciferase (ATP bioluminescence kit HS II; Roche) was automatically injected into each well and the luminescence reading was performed. Known concentrations of ATP solutions were also read to obtain a standard ATP curve and convert relative light units into ATP concentrations (nM). The Tyrode's solution contained (in mM): 140 NaCl, 5 KCl, 4 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 1 Na-Pyruvate, 10 Hepes, pH adjusted to 7.4 with NaOH. Artificial saliva contained (in mM): 2 NaCl, 5 KCl, 3 $NaHCO_3$, 3 $KHCO_3$, 1.8 HCl, 0.25 $CaCl_2$, 0.25 $MgCl_2$, 0.12 K_2HPO_4, 0.12 KH_2PO_4.

Statistics

All statistical calculations were performed in Sigmaplot (V12.5, Systat Software, SCR_003210). Statistical tests within each figure are described in Table 3. $P < 0.05$ was considered significant.

RESULTS

Skn-1a$^{-/-}$ mice do not express canonical Type II taste cell markers

RT-PCR confirmed that fungiform and circumvallate taste buds of *Skn-1a*$^{-/-}$ mice do not express *Pou2f3* (Skn-1a) or the Type II cell marker (*Gnat3*), however all taste buds expressed the Type III cell marker *Snap25* (Figure 1). *Skn-1a*$^{-/-}$ mice do not express the ATP release channel *Calhm1*. We also used immunohistochemistry

to confirm that no Type II cell markers were present in *Skn-1a*$^{-/-}$ mice. As shown in Figure 2, neither GNAT3 nor PLCβ2 immunoreactivity was observed in *Skn-1a*$^{-/-}$ mice. However, the number of Type III cells labelled with 5-HT and SNAP25 is increased in *Skn-1a*$^{-/-}$ compared to WT as observed previously in Matsumoto et al. (2011). Cell counting of SNAP25 immunoreactive cell profiles confirms previous reports that the number of Type III cells is increased in CV taste buds. Additionally, we show that there is a significant, commensurate increase in Type III cells in the anterior taste fields as well (Figure 2B). In mice pre-injected with the 5-HT precursor 5-HTP, the majority of Type III cells in *Skn-1a*$^{-/-}$ mice were immunoreactive for 5-HT (not shown).

Skn-1a$^{-/-}$ mice have diminished taste responses conveyed by Type II cells

Chorda tympani responses to various tastants were compared between *Skn-1a*$^{-/-}$ and WT (Figure 3A). While responses to acids (citric acid and HCl) and NH_4Cl were similar in both genotypes, responses to bitter (quinine), sweet (sucrose), umami (MSG with or without IMP) and salty (NaCl) stimuli were significantly decreased. NaCl responses were significantly decreased indicating that salt taste is also partially conveyed by Type II taste cells. When amiloride was added to NaCl at different concentrations, responses were diminished in the *Skn1a*$^{-/-}$ mice but not abolished suggesting that Type III cells participate in the transduction of the amiloride-insensitive salt response (Figure 3B).

Skn-1a$^{-/-}$ mice have the same number of 5-HT_{3A}-expressing geniculate ganglion neurons as the WT littermates

Maeda et al. (2017) showed, via retrograde transfer of wheat germ agglutinin from Type III cells, that the total number of nodose/petrosal ganglion neurons that innervate Type III cells remains unchanged in *Skn-1a*$^{-/-}$ mice, despite the increased number of Type III cells. All gustatory neurons express the P2X-family purinergic receptors and a subset expresses 5-HT_3 receptors which preferentially contacts Type III cells (Larson et al., 2015; Stratford et al., 2017). We tested the hypothesis that additional geniculate ganglia express 5-HT_3 receptors to compensate for the additional Type III cells in *Skn-1a*$^{-/-}$ mice, and that this change would be reflected when the 5-HT_{3A}-GFP reporter mouse is crossed with *Skn-1a*$^{-/-}$ mice. We showed that the total number

of geniculate ganglion neurons expressing SNAP25, P2X3, or 5-HT3A was not significantly different between genotype (Figure 4C). Additionally, we showed that the number of geniculate ganglion neurons expressing both P2X3 (gustatory neuron marker; Bo et al., 1999; Dvoryanchikov et al., 2017; Ishida et al., 2009) and 5-HT_{3A}-driven GFP is similar to the number in wildtype littermates (Figure 4D).

Skn-1a$^{-/-}$ mice show normal calcium response to ATP and 5-HT in geniculate ganglion neurons

We next isolated geniculate ganglion neurons from *Skn-1a*$^{-/-}$ mice crossed with 5-HT_{3A}-GFP mice and examined response profiles to ATP and 5-HT. We observed similar response profiles in both KO and WT neurons. That is, GFP-expressing ganglion neurons responded to exogenous 5-HT and ATP while GFP-negative neurons responded only to ATP, similar to observations in WT neurons (Figure 5C,D). Thus, in agreement with Maeda et al. (2017) we conclude that there is no change at the level of the geniculate ganglion to compensate for the increased number of Type III taste cells in *Skn-1a*$^{-/-}$ mice. 5-HT signaling is not upregulated in *Skn-1a*$^{-/-}$ mice.

Skn-1a$^{-/-}$ mice show normal innervation of taste buds

To determine if the taste buds of *Skn-1a*$^{-/-}$ mice have similar numbers of nerve fibers compared to wildtype, we used an antibody against P2X3, since all gustatory nerve fibers express P2X3 (Bo et al., 1999; Dvoryanchikov et al., 2017; Ishida et al., 2009). The density of P2X3-labeled gustatory nerve fibers was compared in taste fields of KO and WT animals (Figure 6). Total innervation density was calculated as percent labeled voxels divided by total number of voxels per imaged taste bud. No appreciable differences were observed between KO and WT in any taste field examined.

5-HT_{3A}-GFP innervation of taste buds in *Skn-1a*$^{-/-}$ mice

We have now shown that the overall level of P2X3+ innervation is unchanged and the overall number of 5-HT_{3A}-GFP (Type III cell innervating) ganglion cells remains unchanged in *Skn-1a*$^{-/-}$ mice. This raises the possibility that the increased Type III cells in *Skn-1a*$^{-/-}$ mice are innervated by either 1) non 5-HT_{3A} neurons, 2)

increased branches of 5-HT_{3A} neurons, or 3) no neurons at all. The most former seems unlikely, as if the new Type III taste cells were innervated by a new population of neurons, we would have expected to see increased chorda tympani responses to sour and salty as more fibers would be activated. In fact, we observed decreased salty responses and unchanged sour responses in *Skn-1a*$^{-/-}$ mice. Thus, a more plausible explanation is that either the "new" Type III cells of *Skn-1a*$^{-/-}$ mice are not innervated or are innervated by increased branching of neurons. To address this, we imaged taste buds of *Skn-1a*$^{-/-}$ mice (and wildtype littermates) crossed with 5-HT_{3A}-GFP mice. Quantitative analysis of these images showed a subtle increase in the proportion of P2X3-immunoreactive fibers that were also 5-HT_{3A}-GFP+ when Skn-1a was knocked out. While significance was only achieved in more posterior taste fields, a positive trend was observed in the anterior fields (Figure 7).

Skn-1a$^{-/-}$ mice require ATP to transmit gustatory neural responses

ATP is required for the transmission of all taste qualities (Finger et al., 2005; Vandenbeuch et al., 2015) and the P2X3 antagonist AF353 blocks all taste responses (Vandenbeuch et al., 2015). When applying AF353 for 10 min on the anterior part of *Skn-1a*$^{-/-}$ tongues, chorda tympani responses to all taste stimuli were totally abolished (Figure 8). These results suggest that ATP is required to transmit the signal from taste cells to nerve fibers.

Skn-1a$^{-/-}$ mice do not release ATP to any tastants

To clarify the source of ATP required for the taste signal transmission in *Skn-1a*$^{-/-}$ mice, the amount of ATP released to different tastants was measured. Using a luciferase assay, no ATP was released from taste buds of the *Skn-1a*$^{-/-}$ mice following NaCl or citric acid stimulation. Conversely, ATP was released in the WT animals in response to the same stimuli (Figure 9). Although we cannot rule out the possibility that the technique used was not sensitive enough, it seems unlikely that the source of ATP required for transmission comes from Type III cells in the *Skn-1a*$^{-/-}$ mice.

DISCUSSION

In this manuscript we confirm earlier studies showing that the transcription factor Skn-1a is required for generation of Type II taste cells (Matsumoto et al., 2011) and that the resulting expansion of Type III cells in the *Skn-1a*$^{-/-}$ mice does not alter the number of innervating gustatory neurons (Maeda et al., 2017). Here, we extend these earlier studies to show that despite the large increase in the number of serotonergic Type III taste cells in the *Skn-1a*$^{-/-}$ mice, the ratio of geniculate ganglion neurons expressing the serotoninergic target receptor 5-HT_{3A} is unchanged with respect to wildtype mice. Further, we show that *Skn-1a*$^{-/-}$ mice still require ATP for the transmission of sour and salty tastes to the nervous system despite the absence of Type II taste cells, which are the only taste cells known to release ATP.

We first confirmed using RT-PCR that the transcription factor *Skn-1a,* the G protein *Gnat3,* and ATP release channel *Calhm1* are indeed missing in the *Skn-1a*$^{-/-}$ mice. In addition, we confirmed that the Type III cell marker *Snap25* is present in both WT and KO mice, ensuring the integrity of the mRNA in the KO mice. We then used immunohistochemistry to show that the *Skn-1a*$^{-/-}$ mice lack expression of the canonical Type II taste cell marker PLCβ2 and that SNAP25 expressing Type III taste cells were expanded in both anterior and posterior taste fields to compensate for the loss of Type II taste cells. We also confirmed, using chorda tympani nerve recording of *Skn-1a*$^{-/-}$ mice, that responses to stimuli normally transduced by Type II cells, i.e., bitter, sweet, and umami, were largely missing in the knockouts compared to wildtype littermates. Also, in agreement with Matsumoto et al., (2011), we showed that responses to sour (acidic) stimuli were similar in knockout and control mice, despite the large expansion of Type III cells. However, Matsumoto et al., (2011) showed a non-statistically significant decrease to NaCl, while we found responses to NaCl were significantly reduced, although not eliminated in the knockouts, and that other sodium containing stimuli (e.g. MSG) showed small residual responses in the knockout. We hypothesize that this difference is mainly due to experimental conditions: we used urethane anesthesia and baseline normalization while Matsumoto et al used pentobarbital plus urethane anesthesia and NH4Cl normalization. Larson et al (2015) have shown that sodium pentobarbital

interferes with accurate measurement of NaCl responses. Our data suggest that Type II cells transduce a portion of the responses to NaCl, as others have suggested, although this point is still debated (Lewandowski et al., 2016; Oka et al., 2013; Roebber et al., 2019). Two distinct mechanisms are responsible for transduction of NaCl, an amiloride-sensitive mechanism, mediated by the epithelial Na channel ENaC and limited to anterior tongue (Chandrashekar et al., 2010; Kretz et al., 1999), and an unidentified amiloride-insensitive mechanism, found in all taste fields. Our results show that the addition of amiloride to NaCl does not completely abolish the salt response in the *Skn-1a*$^{-/-}$ mice suggesting that a portion of the amiloride-insensitive response is conveyed by Type III cells. Both Type II and Type III cells were previously thought to be responsible for amiloride-insensitive salt taste (Lewandowski et al., 2016; Oka et al., 2013). However, a recent study using Pirt-GCamp6 mice showed NaCl responses in fungiform taste buds were present only in Type II taste cells, and that these responses were entirely amiloride-insensitive (Roebber et al., 2019). These latter data are inconsistent with our data, which show a significant amiloride-insensitive response in mice lacking Type II taste cells. The amiloride-sensitive salt taste is believed to be transduced by a cell type independent of Type II and Type III cells in mice (Chandrashekar et al., 2010; Vandenbeuch et al., 2008), although whether it is a unique cell type or a subset of Type I cells is still unclear.

PKD1L3 is expressed in Type III cells of posterior tongue, and transgenic mice expressing wheat germ agglutinin (WGA) from the *Pkd1l3* promoter show WGA in Type III cells of circumvallate and foliate papillae as well as in petrosal ganglion neurons (Yamamoto et al., 2011). Maeda et al. (2017) crossed these PKD1L3-WGA expressing mice into the *Skn-1a*$^{-/-}$ mice and examined the circumvallate taste buds and petrosal ganglion neurons for evidence of WGA protein. WGA protein was much higher in the taste buds of the knockout mice than wildtype, but the number of ganglion cells containing WGA was not different from control mice, leading them to conclude that ganglion cell innervation is not regulated by taste cell type. We have expanded on this observation to examine the geniculate ganglion using mice expressing GFP from the serotonin receptor *Htr3a* promoter. Geniculate neurons expressing 5-HT$_{3A}$-GFP preferentially innervate the serotonergic Type III taste

cells and these neurons respond to both 5-HT and ATP, while neurons lacking GFP expression respond only to ATP (Larson et al., 2015; Stratford et al., 2017). A recent study confirms that most 5-HT_{3A} fibers innervate Type III cells. Secondary analysis of single cell RNA-sequencing data shows that geniculate ganglion neurons that respond to sour lingual stimuli are enriched with *Htr3a* (5-HT_{3A}; not shown (Dvoryanchikov et al., 2017; Zhang et al., 2019)). We crossed the 5-HT_{3A}-GFP mice into the *Skn-1a*$^{-/-}$ mice and asked whether the ratio of GFP expressing neurons differed between knockout and wildtype mice. Our results suggest that while the Type III taste cells of the knockout are expanded, the 5-HT_{3A} expressing ganglion cells do not undergo a similar expansion, in general agreement with Maeda et al. (2017). Further, we showed that only the GFP-labeled ganglion neurons responded to exogenous 5-HT, while the majority of neurons responded to ATP, as with the wildtype animals. These results beg the question of whether the "extra" Type III cells in the knockout mice are actually innervated. To assess this we compared the innervation density of P2X3-expressing nerve fibers surrounding taste buds of circumvallate, foliate, fungiform, and palatal taste buds in knockout and wildtype mice. All gustatory geniculate ganglion neurons express P2X3 (Bo et al., 1999; Dvoryanchikov et al., 2017; Ishida et al., 2009) and thus P2X3 is a reliable marker for taste bud innervation density. However, we found no difference in the overall innervation density between taste buds of the knockout and wildtype mice, suggesting that all taste cells are likely to be innervated. It still remains possible that either 5-HT_{3A} expressing ganglion neurons have increased nerve fiber branching to innervate the expanded population of Type III cells in the knockout, or the ganglion neurons expressing only P2X3 are now innervating the Type III cells. To address this, we quantified the proportion of P2X3-immunoreactive nerve fibers that were also 5-HT_{3A}-GFP+ in Skn-1a$^{-/-}$ and Skn-1aWT mice crossed with 5HT3A-GFP mice (Figure 7). We observed slight increases in the proportion of GFP+ gustatory fibers, though significance was only reached in the circumvallate and foliate papillae. This suggests that increased branching of GFP+ fibers could be occurring Skn-1a$^{-/-}$ mice. This would be consistent with observations that chorda tympani response to sour and salty stimuli are not increased, as one would expect much larger nerve signals if more neurons are being recruited from the increased number of Type III cells. However, further studies will be required to explore this question in more detail with enhanced resolution.

Previous studies have shown that ATP is required for the transmission of all taste qualities (Finger et al., 2005; Vandenbeuch et al., 2015), although only Type II cells are known to release ATP (Huang et al., 2007; Romanov et al., 2018, 2007; Taruno et al., 2013). The source of ATP required for transmission from Type III cells to gustatory afferents has remained an enigma, although it has been assumed to require the presence of Type II cells. Thus it was of interest to examine the role of ATP in *Skn-1a*$^{-/-}$ mice which lack Type II cells. Using a luciferin/luciferase assay, we found that taste buds of wildtype mice release detectible amounts of ATP to sour and salty stimuli applied to the apical membrane, but the *Skn-1a*$^{-/-}$ mice failed to release ATP over background levels in the tissue. The finding that ATP release, presumably from Type II cells, is elicited by sour and salty was surprising and consistent with a model where signaling between taste receptor cells occurs (Huang et al., 2009). In this situation, activation of Type III cells by sour or salty stimuli causes indirect activation of Type II cells to release ATP. ATP release to sour and salty stimuli is specific to Type II cells as it is absent in *Skn-1a*$^{-/-}$ mice.

We found that the purinergic receptor antagonist AF353 blocked transmission of sour and salty tastes in the knockout as well as in the wildtype littermates. Thus, purinergic signaling is still required in these mice that lack Type II taste cells. But what is the source of the ATP, if not Type II cells? Our best explanation is that small amounts of ATP may be released from Type III cells that are undetectable by our assay and others' attempts to measure ATP release from Type III cells (Huang et al., 2007; Romanov et al., 2007). One possibility is that ATP is co-released with serotonin (or other transmitters) from synaptic vesicles, thus it may be very focal and difficult to detect in this type of assay. Indeed, ATP is very commonly co-packaged in synaptic vesicles and often co-released with transmitters such as norepinephrine and acetylcholine (as reviewed in Borges, 2013 and Burnstock, 2006). Additionally, analysis of data from Qin et al., 2018 shows that using single cell RNA-sequencing *Slc17a9* (VNUT) is detectable in both Type II and III cells. While Iwatsuki et al., 2009 showed immunoreactivity to VNUT only in Type II cells the discrepancy could arise from the sensitivity of the assays

used. These findings support the hypothesis that ATP could be released from vesicles of Type III cells at low levels sufficient for synaptic communication but insufficient for detection by conventional methods.

We also propose that pannexin channels could be a source of ATP release from Type III cells. While knockout of pannexin-1 shows no phenotype, it is possible that in the absence of Type II cells and CALHM1 channels, pannexin-1 could take over a role for ATP release. Indeed, analysis of single cell RNA-sequencing data from Qin et al., 2018 shows detectable levels of *Panx1* in the majority of Type III cells (not shown). A final possibility is that background levels of ATP in the tissues serve to keep gustatory afferents in a slightly depolarized state, so that release of serotonin or other transmitters will be sufficient to generate action potentials in the ganglion cells. Further studies will be required to resolve this issue.

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493 **LEGENDS**

494 Figure 1. RT-PCR confirms lack of Type II cell markers. RNA was extracted from fungiform and circumvallate
495 taste buds of wildtype and Skn-1a$^{-/-}$ mice. After reverse transcription, cDNA was interrogated for the presence
496 of *Skn1a*, *Gnat3*, *Calhm1*, and *Snap25.* Arrowheads denote ladder bands: *Skn1a*- 200bp and 100bp, *Gnat3*-
497 300bp and 200bp, *Calhm1*- 200bp and 100bp, *Snap25*- 300bp and 200bp. Data are representative of RNA
498 extracted from 3 mice of each genotype. – is no template negative control, FF lanes in *Skn1a* and *Gnat3* gel are
499 RNA from C57bl/6j fungiform taste buds.

500

501 Figure 2. IHC confirms lack of GNAT3- and PLCB2-expressing Type II cells in *Skn-1a*$^{-/-}$ mice. A. Lingual
502 sections from *Skn-1a*$^{-/-}$ and wildtype littermates were labeled with antibodies against SNAP25 (magenta) and
503 GNAT3 (green) or PLCB2 (green). In *Skn-1a*$^{-/-}$*,* there was no detectable levels of Type II cell markers GNAT3
504 or PLCB2. B. Semi-quantitative IHC reveals increased numbers of Type III cells in circumvallate, fungiform,
505 and soft palate taste buds. Each dot represents a single taste bud. Box plots are summaries of all datapoints.
506 Acquisition settings were equalized between wildtype and knockout for all channels. Images are maximal z-
507 projections of 12-16 µm image stacks. CV = circumvallate, FF = fungiform. * shows $p < 0.05$ by Kruskal-
508 Wallis test followed by Dunn Test for multiple comparisons.

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510 Figure 3. *Skn-1a*$^{-/-}$ mice have suppressed responses to Type II mediated taste modalities. A. Chorda tympani
511 nerve activity of *Skn-1a*$^{-/-}$ and wildtype littermates was monitored in response to lingually applied taste
512 solutions (100 mM NH_4Cl, 500 mM sucrose, 10 mM quinine-HCl, 100 mM mono-sodium glutamate, 100 mM
513 mono-sodium glutamate plus 0.5 mM inosine monophosphate, 100 mM NaCl, 10 mM HCl, 10 mM citric acid).
514 Integrated nerve activity over 30 seconds of stimulation was normalized to baseline. N= 6 mice for each
515 genotype. B. Baseline normalized chorda tympani activity of *Skn-1a*$^{-/-}$ mice in response to NaCl (30, 100, and
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300 mM) with pre- and concurrent application of 100µM amiloride. No significant differences were detected between wildtype and *Skn-1a*$^{-/-}$. Bar charts indicate sample mean, error bars represent SEM, and superimposed dot plot represents each individual animal. ns = no significance. * = $p < 0.05$ by two-way repeated measures ANOVA with Holm-Sidak post-hoc test.

Figure 4. No change in the number of 5-HT$_{3A}$-GFP geniculate ganglion neurons in *Skn-1a*$^{-/-}$ mice. *Skn-1a*$^{-/-}$ mice were crossed to 5-HT$_{3A}$-GFP mice. A,B. Geniculate ganglia of *Skn-1a*$^{-/-}$/5-HT$_{3A}$-GFP and wildtype littermates (still expressing 5-HT$_{3A}$-GFP) were labeled with antibodies against GFP (green), P2X3 (magenta), and SNAP25 (blue). The number of labeled cells was counted for each label. C. Percent of total cells showing GFP, P2X3, or SNAP25 immunoreactivity. Knockout of Skn-1a had no effect. D. Percent of total cells showing each combination of labels. Knockout of Skn-1a had no effect. Data is a summary of from 3 wildtype and 4 knockout mice. Bar chart depicts sample mean and error bars show SEM.

Figure 5. Knockout of Skn-1a does not affect responsiveness of geniculate ganglion neurons to ATP or 5-HT. *Skn-1a*$^{-/-}$ mice were crossed to 5-HT$_{3A}$-GFP mice. A,B. Raw calcium imaging traces of isolated, individual GFP+ (green) or GFP- (black) geniculate ganglion neurons in response to 10 µM ATP, 10 µM 5-HT or 55 mM KCl. C,D. Summary of responses to exogenous stimuli. N=7 GFP+, 4 GFP- from 3 *Skn-1a*$^{-/-}$/5-HT$_{3A}$-GFP, and 6 GFP+ and 6 GFP- from 3 *Skn-1a*WT/5-HT$_{3A}$-GFP. Bar charts indicate sample mean, error bars represent SEM, and superimposed dot plot represents each individual cell. * = $p < 0.05$ by Two-way ANOVA with Holm-Sidak post hoc test.

Figure 6. No change in the innervation density of taste buds in *Skn-1a*$^{-/-}$ mice. A,B. Lingual slices of *Skn-1a*$^{-/-}$ and wildtype littermates were labeled with antibodies against SNAP25 and P2X3. C. P2X3 immunoreactivity density was calculated for each taste bud. Dots represent individual taste buds and the box plot demonstrates distribution of data. CV- circumvallate, Fol- foliate, FF- fungiform, SP- soft palate. Data are from 6 wildtype and 5 *Skn-1a*$^{-/-}$ mice. No significant interactions were found by Kruskal-Wallis test.

Figure 7. 5-HT$_{3A}$-GFP innervation in *Skn-1a*$^{-/-}$ mice. 5-HT$_{3A}$-GFP mice were crossed with *Skn-1a*$^{-/-}$. *Skn-1a*$^{-/-}$ mice that had the GFP transgene and wildtype littermates that also had the GFP transgene were used for experiments. A, B. Maximal Z-projections of fungiform taste buds from wildtype and knockout mice labeled with antibodies against GFP (green) and P2X3 (magenta). A',B'. Same image as A,B but after prepressing which included Otsu thresholding. C. Analysis was performed on each optical plan of individual taste buds to quantify the relative immunoreactivity of P2X3 and GFP. * indicates $p < 0.05$ by Kruskal-Wallis test followed by Dunn Test for multiple comparisons. 3-4 mice from each genotype were used and 228 taste buds were quantified.

Figure 8. Purinergic receptor antagonism eliminates residual chorda tympani responses to lingually applied taste solutions. The chorda tympani response to taste solutions was measured in *Skn-1a*$^{-/-}$ before and after application of 1 mM AF353. Data are representative integrated responses to each stimulus. The y-axis scale is an order of magnitude smaller on the responses after AF353. Responses are representative of 4 animals.

Figure 9. No detectable ATP release from taste buds of *Skn-1a*$^{-/-}$ mice. ATP release was measured from peeled circumvallate epithelium of *Skn-1a*$^{-/-}$ and wildtype littermates via luciferase assay. Stimuli were 500 mM NaCl, 20 mM citric acid, and artificial saliva. Data are presented relative to artificial saliva. Bar charts indicate sample mean relative to artificial saliva, error bars represent SEM, and superimposed dot plot represents each

individual trial. * = $p < 0.05$ compared to artificial saliva. ns = not significant compared to artificial saliva. N= 14 wildtype and 8 *Skn-1a*$^{-/-}$ mice.

566 **TABLES**

567 *Table 1. PCR primers*

Gene	Accession number	Primer sequence (5' to 3')	Product size
Calhm1	NM_001081271	*F: GTGCTTTCTCTGTGCCTTCT* *R: CGTACCACGAACGCTAGTAATG*	240
Skn1a *(Pou2f3)*	NM_011139	*F: GGCGATGGGAAAGCTGTAT* *R: CTCCAAAGTCAGGCGTATGT*	249
Gustducin (*Gnat3*)	NM_001081143	*F: GCAACCACCTCCATTGTTCT* *R: AGAAGAGCCCACAGTCTTTGAG*	285
Snap25	NM_001355254	*F: GGCAATAATCAGGATGGAGTAG* *R: AGATTTAACCACTTCCCAGCA*	307

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569 *Table 2. List of primary and secondary antisera*

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Antiserum	Company	Catalog Number	Dilution	RRID
Chicken Polyclonal Anti-GFP	Aves Lab; Trigard, OR	GFP-1020	1:2000	AB_10000240
Rabbit polyclonal anti-PLCB2	Santa Cruz Biotechnology, Dallas, TX	SC-206	1:1000	AB_632197
Goat polyclonal anti-GNAT3	Aviva Systems Bio; San Diego, CA	NC9510598	1:500	AB_10882823
Rabbit polyclonal anti-P2X3	Alomone, Jerusalem, Israel	APR-016	1:500	AB_2313760
Goat polyclonal anti SNAP25	Genetex, Irvine, CA	GTX89577	1:200	AB_10724125
Donkey anti-Chicken 488	Jackson Immuno Research; West Grove PA	703-546-155	1:1000	AB_2340375
Donkey anti-Rabbit 568	Invitrogen; Carlsbad, CA	A10042	1:1000	AB_2534017
Donkey anti-Goat 647	Invitrogen; Carlsbad, CA	A1157	1:1000	AB_2758603
DAPI	Invitrogen; Carlsbad, CA	62248	1:10,000	AB_2307445

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572 *Table 3. Statistical table*

Figure	Data structure	Type of test	Sample size	Statistical data
Fig. 2B	Two-factors (genotype and taste field). Data are counts	Kruskal-Wallis test followed by Dunn Test for multiple comparisons	WT mice: 6 mice CV: 110 buds FF: 19 buds SP: 15 buds KO mice: 6 mice CV: 125 buds FF: 16 buds SP: 17 buds	$\chi^2(5, N = 302) = 219.74$, $p < 0.0001$ CV_WT vs CV_KO: p < 0.0001 FF_WT vs FF_KO: p = 0.0395 SP_WT vs SP_KO: p = 0.00331
Fig. 3A	Two-factors (genotype and tastants). Data are continuous	Two-way ANOVA	WT mice: n = 6 *Skn-1a*$^{-/-}$ mice: n =6	Genotype: $F(1,91) = 12.755$, $p < 0.001$ Interaction: $F(7,91) = 3.315$, $p = 0.004$ Holm-Sidak's multiple comparisons test: KO/WT, NH4CL: $p = 0.282$ KO/WT, Suc: $p = 0.008$ KO/WT, Qui: $p = 0.034$ KO/WT, Msg: $p = 0.039$ KO/WT, NaCl: $p = 0.008$ KO/WT, Msg+Imp: $p = 0.007$ KO/WT, Hcl: $p = 0.127$ KO/WT, Ca: $p = 0.975$
Fig. 3B	Two-factors (genotype and tastants)	Two-way ANOVA	WT mice: n = 5 KO mice: n = 9	Genotype: $F(1,41) = 6.726$, $p = 0.014$ Interaction: $F(2,41) = 0.976$, $p = 0.386$
Fig. 4C	Categorical	Chi-squared	WT cells: 3262 from 3 mice KO cells: 4236 from 4 mice	$\chi^2(2, N = 7498) = 5.312$, $p = .070$
Fig. 4D	Categorical	Chi-squared	WT cells: 3262 from 3 mice KO cells: 4236 from 4 mice	$\chi^2(3, N = 7498) = 5.064$, $p = .167$
Fig. 5C	Two-factors (genotype and stimulus)	Two-way ANOVA	WT cells: 7 KO cells: 6	Genotype: $F(1, 38) = 0.0670$, $p = 0.797$ Interaction: $F(2,38) = 0.0272$, $p = 0.973$
Fig. 5D	Two-factors (genotype and stimulus)	Two-way ANVOA	WT cells: 4 KO cells: 6	Genotype $F(1,29) = 0.0156$, $p = 0.902$ Interaction: $F(2,29) = 0.409$, $p = 0.669$
Fig. 6C	Two-factors (genotype and	Kruskal-Wallis test	WT taste buds: 166 KO taste buds: 207	$\chi^2(7, N = 302) = 7.0057$, $p = 0.4283$

	taste field)			
Fig. 7C	Two-factors (genotype and taste field).	Kruskal-Wallis test followed by Dunn Test for multiple comparisons	WT taste buds: 74 KO taste buds: 154	$\chi^2(7, N = 302) = 57.047$, $p < 0.0001$ CV_WT vs CV_KO: p = 0.0181 FOL_CV vs FOL_KO: p <0.0135 FF_WT vs FF_KO: p > 0.05 SP_WT vs SP_KO: p > 0.05
Fig. 9	Normal distribution	Paired t-test vs artificial saliva	WT NaCl: 10 WT CA: 9 KO NaCl: 5 KO CA: 8	WT NaCl: t(9) = 2.363, p = 0.0424 WT CA: t(8) = 2.697, p = 0.027 KO NaCl: t(4) = 1.262, p = 0.275 KO CA: t(7) = 0.473, p = 0.650

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