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RESEARCH ARTICLE

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Histological and immunohistochemical studies of the fungiform and the circumvallate papillae through the life stages from 6- to 72-week-old Sprague-Dawley male rats

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Abstract

Taste sensitivity decreases with age. Therefore, we investigated the histological and immunohistochemical changes in the receptive fields circumvallate papilla (CvP) and fungiform papilla (FfP) to explore the mechanism underlying age-related changes in taste sensitivity in 6- to 72-week-old rats. We analyzed papilla size, the thickness of the keratin layer of the papilla and stratified squamous epithelium, taste bud size, the keratin layer around the taste pores in the CvP and FfP, and the number and distribution of taste buds in the CvP coronal section. We further assessed the expression of marker proteins for Type II and III cells, phospholipase C subtype beta 2 (PLCβ2), and synaptosomal-associated protein 25 (SNAP-25). The cellular activity of these taste cells was examined through co-localization with the senescence cell marker protein-30 (SMP30). There were no differences in the number of taste bud sections in the CvP among the age groups. However, the size of the CvP increased and the density of the taste bud area in the CvP area decreased with increasing age. In contrast, the number of cells with co-expression of SMP30, PLCβ2, and SNAP-25 decreased with age. Furthermore, the morphological structures of the CvP, FfP, and taste buds in these regions changed with age, but not the overall taste bud number in the CvP coronal section. The decrease in cell count with co-expression of SMP30 and PLCβ2, or SNAP-25 may indicate reduced cellular functions of taste cells with aging.

KEYWORDS

circumvallate papillae, fungiform papillae, life stage, PLCβ2, SNAP-25, senescence cell

1 | INTRODUCTION

Food preferences have been shown to change with age, shifting to foods of higher intensities, which include higher concentrations of salt or a higher density of calories, especially in older people. Many studies on humans have reported that taste sensitivity declines with age (Barragán et al., 2018; Bartoshuk, 1989; Cohen & Gitman, 1959; Cooper et al., 1959; Mojet et al., 2001; Schiffman et al., 1979, 1994; Weiffenbach et al., 1982, 1986; Yoshinaka et al., 2016). The decline in taste sensitivity leads to a

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preference for higher-concentration foods and drinks among older people. As such, they would not be satisfied with lower- or normal-concentration foods. The deviation of food preferences may cause lifestyle-related diseases such as diabetes and hypertension (Stenkamp-Strahm et al., 2013; Zhao et al., 2022). Therefore, it is important to elucidate the mechanisms underlying age-induced changes in food preferences and to improve their mechanisms. Rodent studies have demonstrated that taste sensitivity and preference change with age (Inui-Yamamoto et al., 2017; Miura et al., 2014; Smith & Wilson, 1989; Thaw, 1996). Therefore, it has been hypothesized that aging-induced changes in food preference may be attributed to alterations in the taste-receptive system in humans and animals.

The taste bud number of circumvallate papilla (CvP) or the number of fungiform papilla (FfP) that receive chemical substances contained in food and drinks decreases with age (Arey et al., 1935; Karikkineth et al., 2021). However, the number of FfPs and taste buds in the papilla does not differ among age groups (Arvidson, 1979; Saito et al., 2016). The number of fungiform, circumvallate, and foliate papillae did not differ with age among five age groups between 4 and 31 years in rhesus monkeys (Bradley et al., 1985). There were no age-related differences in the number of taste buds in the three gustatory papilla types, and the taste bud diameter did not change with age. In rodents, similar numbers of taste buds on CvP and FfP with taste buds were observed between 5- to 7- and 23- to 24-month-old Wistar rats (Mistretta & Baum, 1984).

In a previous study, 18-month-old B6C3F1/J background mice showed reduced taste bud size and number of taste cells per taste bud in the CvP compared to 2- and 10-month-old mice (Shin et al., 2012). The taste bud number of FfP in Fischer rats also shows some reduction with age among the 4- to 6-, 20- to 24-, and 30- to 37-month-old groups (Mistretta & Oakley, 1986). As the average lifespan of rats and mice (approximately 2–3 years in rats, but less than that in mice) was markedly different from that of rhesus monkeys (26–30 years), these seemingly inconsistent findings may be attributed to differences in lifespan across species. The age groups used for the comparison of age-related changes in the number of taste buds may be controversial. It is essential to occasionally track the morphological and histological changes in the peripheral taste-receptive site because age-related changes do not occur rapidly. Therefore, we investigated the histological characteristics of the FfP and CvP in the peripheral taste-receptive system of rats of different ages to clarify the beginning of age-induced changes. We compared the histology of the CvP and FfP in male Sprague-Dawley rats across a wide range of ages (6, 11, 22, 37, and 72 weeks) to elucidate the differences in the characteristics of the CvP and FfP as a function of age. Estradiol treatment may change neurophysiological responses to taste stimuli by influencing factors that determine taste receptor cell fate (Curtis & Contreras, 2006). Therefore, this study focused on the taste-related peripheral system, not including female hormones. Examination of changes among these age groups provided evidence for the occurrence of changes.

TABLE 1 The list of primary antibodies for taste and senescence cell markers.

Taste cell type	Taste quality	Marker
Type II	Sweet, umami, bitter	Phospholipase C β2 (PLCβ2)
Type III	Sour	Synapse-associated protein of 25 kDa (SNAP-25)
Senescence cell	–	Senescence cell marker protein-30 (SMP30)

We compared the immunohistochemical changes in the peripheral taste system, including the expression of taste-related and cell senescence proteins, among different age groups to address our research question at the protein level. Taste buds contained spindle-shaped cells and proliferative round basal cells. Morphologically, the spindle-shaped taste cells are classified as dark or light. Light cells are divided into Types II and III based on the presence or absence of synaptic connections with the gustatory nerves. Type II cells are associated with sweet, bitter, and umami taste receptors as well as transduction components. In contrast, Type III cells express putative sour taste receptors and respond to multiple taste stimuli. Type II cells are immunoreactive for α-gustducin (a taste-specific G protein), phospholipase C β2 (PLCβ2), and inositol 1,4,5-triphosphate receptor (IP3R3) (Clapp et al., 2001, 2004; Yang, Tabata, et al., 2000). These proteins are all involved in the transduction cascade downstream of taste stimuli. Type III cells show neuronal features and are immunoreactive for a synapse-associated protein of 25 kDa (SNAP-25), protein gene product 9.5, neural cell adhesion molecule, and serotonin (5HT) (Kanazawa & Yoshie, 1996; Nelson & Finger, 1993; Smith et al., 1993; Yang, Crowley, et al., 2000; Yee et al., 2001). We assessed the expression of marker proteins for Type II, PLCβ2, and that for III cells, SNAP-25 (Table 1). We compared the percentages of taste cells (PLCβ2 or SNAP-25 positive) per taste bud section and the expression of senescence cell marker protein-30 (SMP30) using a double-staining technique. SMP30 is localized in the cytosol of hepatocytes and renal tubular epithelial (Fujita et al., 1992). It is also a Ca^{2+}-binding protein in the cytosol of hepatocytes (Shimokawa & Yamaguchi, 1993). SMP30 expression increases during tissue maturation and decreases with age

in an androgen-independent manner, indicating that SMP30 is a marker of aging regardless of sex (Feng et al., 2004; Fujita et al., 1999). Thus, a reduction in SMP30 expression indicates cell senescence with age. We further examined differences in the activity of each taste cell line based on the expression of SMP30 at different ages.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue preparation

Male Sprague-Dawley rats (CLEA Japan, Inc.) aged 6, 11, 20, 37, and 72 weeks (bodyweight, 85–1050 g) served as subjects for at least a week before beginning the experiment. All rats had ad libitum access to food pellets (MF; Oriental Yeast) and distilled water. The animals were individually housed in plastic cages suitable for their body mass: 225 × 338 × 140 mm for rats 6–11 weeks old and 345 × 403 × 177 mm for rats 20–72 weeks old. The ambient temperature was maintained at 23°C in a 12:12 h light/dark cycle (lights on between 08:00 a.m. and 08:00 p.m.).

At 6, 11, 20, 37, and 72 weeks old (each group, $n = 5$), the animals were anesthetized with thiopental sodium (40 mg/kg, intraperitoneal) and perfused with 0.02 M phosphate-buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde. Blood was collected from the heart immediately before perfusion, stored cold, and sent to a testing company for analysis. The posterior and anterior parts of the tongue containing the CvP and FfP, respectively, were removed and soaked in 4% paraformaldehyde. After 2–3 days, tissues were dehydrated and embedded in paraffin using a standard procedure. Each anterior part of the FfP and the caudal part of the CvP were sectioned in a coronal plane perpendicular to the long axis of the tongue. They were serially cut at 7 μm thickness from the dorsal to the ventral surfaces. The FfPs were observed mainly on the anterior side of the tongue. As the tip of the tongue was round and the epithelial tissue was not flat, the tip of the tongue (1 mm) was cut off and the residual anterior part of the tongue was coronally sectioned. Half of the slices were stained with hematoxylin and eosin (H&E), and the other half was used for immunofluorescence staining. In the blood specimen biochemical examination, all rats were within the limits of the biochemical blood levels and did not exhibit severe and noticeable disease during the experiments.

2.2 | Histochemistry

Serial sections were stained with H&E following the standard procedure (Feldman & Wolfe, 2014; Takahashi & Ohkubo, 2018). H&E-stained sections were photographed using a microscope (BX50; Olympus Corporation) equipped with a digital camera (DP25; Olympus Corporation) and cellSens Standard Software (Olympus Corporation). Histological features were analyzed using ImageJ software (ImageJ 1.50b; NIH). The CvP- and FfP-recorded images were analyzed using the morphometric parameters described below.

2.2.1 | Image analysis for the CvP

The maximum CvP width was measured for each rat. The section containing the widest CvP was considered the middle section. Images of the middle section and 7–10 sections rostral and caudal to the middle section were obtained. Taste buds with pores were counted in each section to calculate the number of taste buds per CvP section. The width and depth (horizontal and dorsoventral lengths) of the CvP were defined as shown in Figure 1a. The borders of the taste buds and the trench wall of the CvP are shown in Figure 1b. To estimate the distribution of taste buds observed in the trench walls of the CvP, the density ratio was calculated by dividing the sum of the areas of all taste buds by the area of the trench walls in the CvP for each section. The area of the trench wall was considered as the summed area, including taste buds. However, this did not include the stratified squamous epithelium at the top of the CvP. The thicknesses of KL (Figure 1c) and stratified squamous epithelium (Figure 1c) were measured. To analyze the size of the taste buds, 3–5 sections were selected from 10 coronal sections from the anterior to the posterior of the CvP, and the lengths of the short and long axes of the taste buds were measured (Figure 1d). The thickness of KL around the taste pores (Figure 1d) was measured. Taste buds chosen randomly from the top, middle, and bottom of the trench wall with the largest taste pore sizes were analyzed. These histological features were compared among age groups.

2.2.2 | Image analysis for the FfP

We measured at least 3–5 FfPs for which the taste pore was confirmed clearly and the largest between sections after investigating all FfPs from the 10 coronal sections. The width and depth (Figure 1e) were used to assess the size of the FfP. The lengths of the short and long axes of the selected taste buds were measured (Figure 1f) to analyze the size of taste buds in the FfP. The thickness of KL around the taste pores (Figure 1f) was measured.

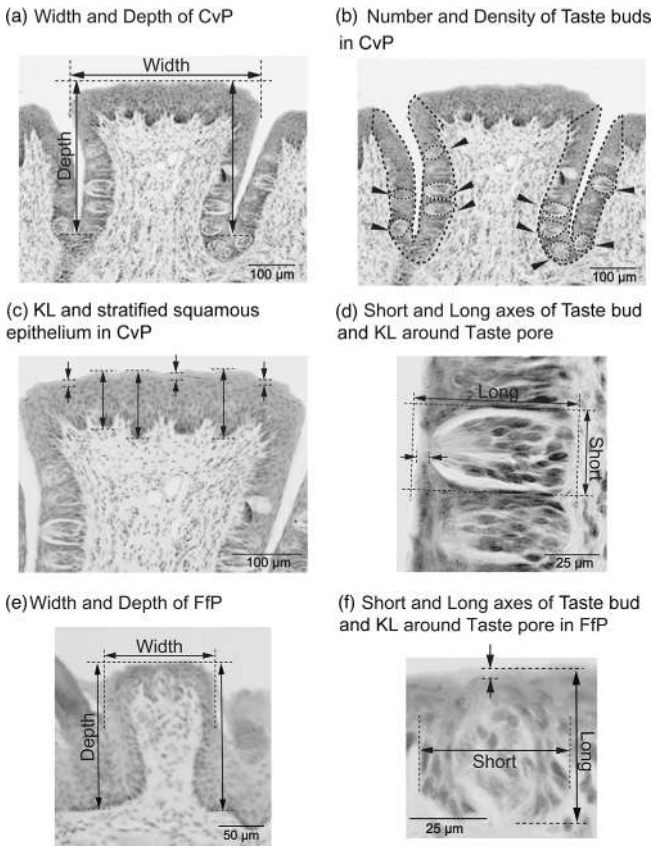

FIGURE 1 Measurement methods. (a–d) The representative picture shows the coronal section of the circumvallate papilla (CvP). (e, f) The representative picture of the coronal section of the fungiform papilla (FfP). (a) The width is considered the horizontal length between the most lateral lines. The depth is the dorsoventral length of the CvP. The value is calculated by taking the mean values of the length of two sulci. (b) The number of taste buds (arrowheads) and the density ratio of all taste bud areas (circle) to both sides of the CvP trench walls (dotted lines). Each whole taste bud showing a taste pore was counted (arrow). Density was calculated as a ratio of the taste bud area (total amount of circle of taste bud marked by arrows) to both sides of the trench wall area (dot lines). (c) The thickness of the keratin layer (KL) (gaps between short arrows) and the stratified squamous epithelium (length of bidirectional arrows). (d) The length of the short and long axes of a taste bud and the thickness of the KL around the taste pore (between arrows). (e) The width is considered the horizontal length between the most lateral lines. The depth is the dorsoventral length of the FfP. The width is considered the distance between the most lateral lines of the FfP. The depth is considered the distance between the surface of the FfP and the bottom of the stratified squamous epithelium of the FfP. (f) The size of a taste bud of FfP indicates the short and long axes of a taste bud (between arrows) and the thickness of the KL around the taste pore of the FfP.

2.3 | Double-labeling immunofluorescence

Paraffin sections were dewaxed, rehydrated in ethanol in a descending series, and washed with PBS. The antigen was activated by L.A.B Solutions (Polysciences, Inc.) for 20 min at 60°C. The sections were incubated in a blocking solution (PBS containing 3% normal goat serum), 1% bovine serum albumin (Roche Diagnostics K.K.), and 0.3% Triton X-100 at room temperature (23 ± 2°C) for 2 h. The sections were incubated for 45–48 h with SMP30 antibody (mouse monoclonal, Sc-374019; Santa Cruz Biotechnology, Inc.) and either PLCβ2 (rabbit polyclonal, Sc-206; Santa Cruz) or SNAP-25 (rabbit polyclonal, 111002; Synaptic Systems GmbH) diluted at 1:200 in the blocking solution. Sections were washed in PBS (3 × 10 min) and incubated for 2 h in the dark with the appropriate secondary antibody: FITC-conjugated anti-mouse IgG (1:500, AP308F; Sigma-Aldrich Japan K.K.) or Cy3-conjugated anti-rabbit IgG (1:100, AP132C; Sigma-Aldrich Japan K.K.). The two types of secondary antibodies were mixed and mounted on slides. Finally, the sections were washed (3 × 5 min each) in 1X PBS and mounted with DAPI (Fluoroshield Mounting Medium with DAP, ab104139; Abcam).

The slides were viewed under a Carl Zeiss immunofluorescence microscope (AXIO Imager M1; AxioCaMR3 camera). All images were acquired at the same resolution and size using the AxioVs40 program (v4.6.3.0; Carl Zeiss AG). Image analysis was performed using ImageJ software (ImageJ 1.50b; NIH). The number of PLCβ2, SNAP-25, or SMP30-positive cells and the total number of nuclei labeled with DAPI were counted in each taste bud section. The number of SMP30-positive cells outside the taste bud area was not included, although SMP30 expression was observed in the epithelial tissues of the stratified squamous epithelium. At least 10 taste buds were used per subject, and the number of immunoreactive cells were averaged. Cell counts were performed by observers who were blinded to the experimental conditions. The slides were taken out every other slide and half of them were used for immunohistochemistry (IHC) and the rest for H&E stains. However, the data were removed from the group when the number of sections, including taste buds, was less than five or the expression in the IHC was not equally confirmed on the section.

2.4 | Statistical analyses

All obtained data were compared across age groups using one-way analysis of variance (ANOVA), and post hoc analysis was performed using Šídák's multiple comparison test. All statistical tests were performed using the data analysis software Prism 9 version 9.5.1 (GraphPad Software). Statistical significance was set at $p < 0.05$.

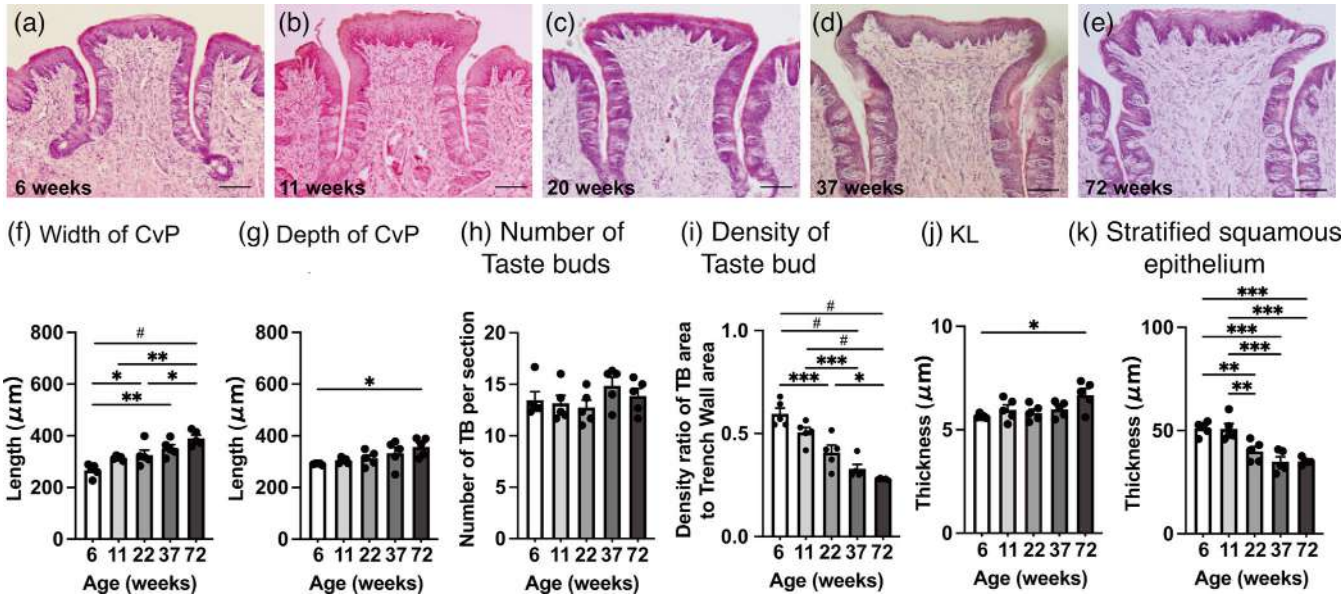

FIGURE 2 Representative photomicrographs of the coronal sections in the circumvallate papilla (CvP) of 6- to 72-week-old rats (a–e). (f) The width of the CvP. (g) The depth of the CvP. (h) The number of taste buds per section. (i) The density ratio of all taste bud areas to the trench wall areas of the CvP. (j) The thickness of the KL. (k) The thickness of the stratified squamous epithelium. Data are presented as the mean ± standard error of the mean (SEM) of five rats from each group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$. Scale bar = 100 μm.

3 | RESULTS

3.1 | Age-related histological changes in the CvP, taste bud, and epithelial tissue

H&E-stained sections revealed that CvP size increased with age (Figure 2a–e). One-way ANOVA showed significant main effects of age for the following measures: the width of the CvP (Figure 2f; F [4, 20] = 12.06, $p < 0.0001$), the depth of the CvP (Figure 2g; F[4, 20] = 3.46, $p < 0.05$), the number of taste buds per section (Figure 2h; F[4, 20] = 1.10, $p = 0.38$), the density ratio of the taste bud area to the trench wall area (Figure 2i; F[4, 20] = 27.32, $p < 0.0001$), the thickness of the KL (Figure 2j; F[4, 20] = 3.70, $p < 0.05$), and the thickness of the stratified squamous epithelium (Figure 2k; F[4, 20] = 16.26, $p < 0.0001$). Post hoc analysis revealed that the CvP width of the 72-week-old group was significantly greater than that of the 6-, 11-, and 22-week-old groups (Figure 2f; $p < 0.0001$; $p < 0.01$; $p < 0.05$, respectively). The CvP width in the 37-week-old group was significantly greater than that in the youngest (6 weeks old) group ($p < 0.01$), and that in the 22-week-old group was significantly wider than that in the youngest group ($p < 0.05$). Similarly, the CvP depth in the 72-week-old group was greater than that in the 6-week-old group (Figure 2g; $p < 0.05$). These results indicate that the size of the CvP increased with age, whereas the number of taste buds per section did not change (Figure 2h). The density ratio of the taste bud area to the trench wall area was lower in the older groups (Figure 2i; 6- vs. 22-week-old group and 11- vs. 37-week-old group, $p < 0.001$; 22- vs. 72-week-old group, $p < 0.05$; 6- vs. 37- or 72-week-old group, 11- vs. 72-week-old group, $p < 0.0001$), indicating that taste bud density in the CvP decreased with age. This result indicates that the number of taste buds in the older age group was relatively smaller than the size of the entire trench wall area because the trench wall became larger with age.

The KL of the 72-week-old group was thicker than that of the 6-week-old group (Figure 2j; $p < 0.05$), whereas the stratified squamous epithelium on the surface of the CvP was thinner in the older group. Post hoc analyses revealed a significant difference between the younger (6- or 11-week-old) and older (22-, 37-, or 72-week-old) groups (Figure 2k; 6- vs. 37- or 72-week-old group, $p < 0.001$; 6- vs. 22-week-old group, $p < 0.01$; 11- vs. 37- or 72-week-old group, $p < 0.001$; and 11- vs. 22-week-old group, $p < 0.01$).

H&E-stained sections revealed that the taste bud size of the CvP slightly decreased with age (Figure 3a–e). One-way ANOVA revealed significant main effects of age, as well as post hoc analysis for the following measures: the short axis of the taste bud did not differ among groups (Figure 3f; F[4, 20] = 0.93, $p = 0.47$), whereas the long axis of the 6-week-old group was significantly smaller than that of the 72-week group (Figure 3g; F[4, 20] = 6.44, $p < 0.01$; post hoc analysis revealed $p < 0.01$). A similar decrease in the thickness of the stratified squamous epithelium was observed. However, the difference

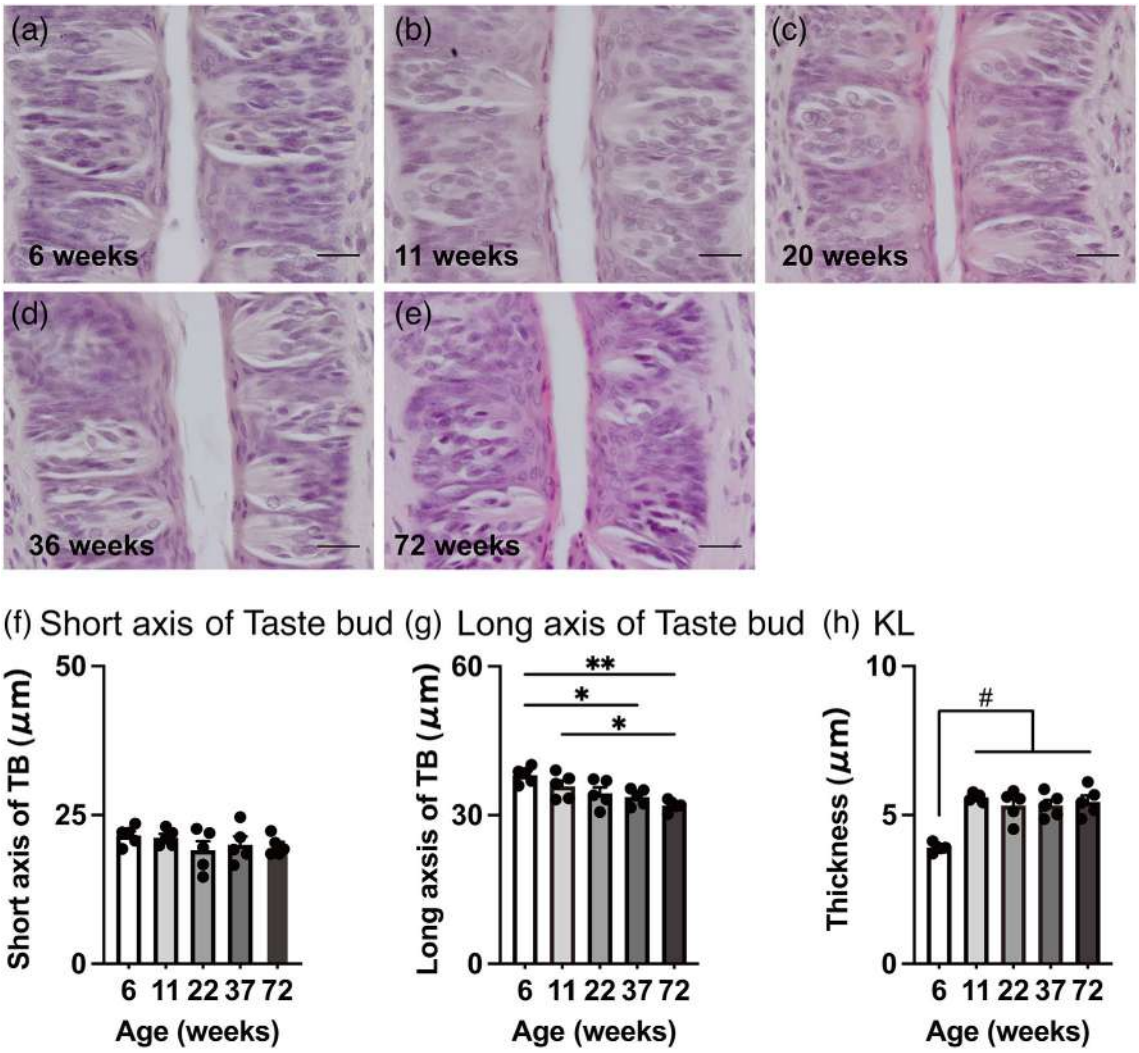

FIGURE 3 Representative photomicrographs of the trench wall of the circumvallate papilla (CvP) of 6- to 72-week-old rats (a–e). (f) The short axis of the taste bud in the CvP. (g) The long axis of the taste bud in the CvP. (h) The thickness of the KL around the taste pore. Data are presented as the mean ± SEM of five rats from each group. *$p < 0.05$; **$p < 0.01$; #$p < 0.0001$. Scale bar = 25 μm.

was not significant (not shown). The 6-week-old group also had a thinner KL around the taste pores than the other groups (Figure 3h; $F[4, 20] = 17.19$, $p < 0.001$; post hoc analysis revealed $p < 0.001$).

3.2 | Age-related histological changes in the FfP, taste bud, and epithelial tissue

The H&E-stained sections showed a difference in the size of the FfP among the age groups (Figure 4a–e). The size of the FfP appeared to increase in an age-dependent manner. However, the widths and depths of the papilla (Figure 4f,g; $F[4, 20] = 2.76$, $p = 0.056$; $F[4, 20] = 2.72$, $p = 0.058$) were not significantly different. The short axis of the taste bud (Figure 4h; $F[4, 20] = 0.04$, $p = 0.10$) was not significantly affected, whereas the long axis was significantly affected (Figure 4i; $F[4, 20] = 2.95$, $p < 0.05$). However, there were no significant differences among the groups. The depth of the papilla (Figure 4g) and the thickness of the KL around the taste pores (Figure 4j; $F[4, 20] = 4.28$, $p < 0.05$) increased. Post hoc analysis demonstrated significant differences in the depth of the FfP and the thickness of the KL around the taste pores between the 6- and 37- or 72-week-old groups ($p < 0.05$).

3.3 | Expression of SMP30, PLCβ2, and SNAP-25 in the CvP

Figures 5a–e and 6a–e show representative immunofluorescent images of double-staining for PLCβ2 and SMP30, as well as SNAP-25 and SMP30, in the CvP of each age

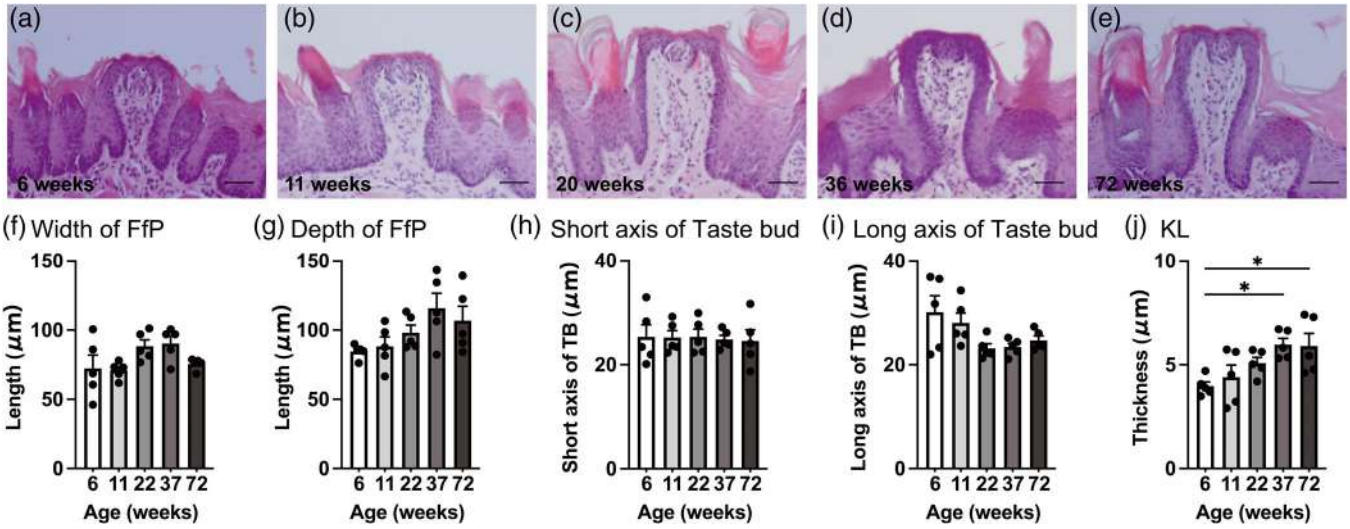

FIGURE 4 Representative photomicrographs of the coronal sections of the fungiform papilla (FfP) of the 6- to 72-week-old group (above, a–e). (f) The width of the FfP. (g) The depth of the FfP. (h) The short axis of the taste bud. (i) The long axis of the taste bud. (j) The thickness of the KL around the taste pore. Data are presented as the mean ± SEM of five rats from each group. *$p < 0.05$. Scale bar = 50 μm.

group. FITC-labeled SMP30 (green) indicates mature cells, whereas Cy3-labeled PLCβ2 or SNAP-25 (red) marks Type II and III cells. DAPI-labeled cells (blue, not shown in the images) were counted to estimate the number of cells per taste bud section.

The number of cells per taste bud section did not differ among groups in both the PLCβ2/SMP30 and SNAP-25/SMP30 double staining (Figures 5f and 6f; $F[4, 17] = 1.78$, $p = 0.18$ and $F[4, 17] = 0.45$, $p = 0.77$), suggesting that the cell number in a taste bud remains the same from 6- to 72-week-old.

Approximately 40% of the cells co-expressed SMP30 and PLCβ2. However, the percentage of the double-labeled cells reduced by 10%–20% in the older group. One-way ANOVA comparing the percentages of SMP30- and PLCβ2-positive cells in the total cell number of a taste bud revealed a main effect of age (Figure 5g; $F[4, 17] = 3.71$, $p < 0.05$; Figure 5h; $F[4, 17] = 4.14$, $p < 0.05$). Post hoc analysis revealed smaller percentages of SMP30 and PLCβ2 cells in the 72-week-old group than in the 11-week-old group ($p < 0.05$). A significant effect of age was also found for the percentage of the double-labeled cells (merge) (Figure 5i; $F[4, 17] = 5.82$, $p < 0.01$). Furthermore, the double-labeled cell percentage of the 72-week-old group was lower than the 11- and 22-week-old groups ($p < 0.05$).

The percentage of SMP30- and SNAP-25-positive cells was approximately 40%, but the percentage of the double-labeled cells decreased by less than 20%, especially in the older age groups. One-way ANOVA for the double-labeling of SNAP-25 and SMP30 revealed a significant effect of age on the percentage of the SMP30-positive cells to the total number of cells per taste bud section (Figure 6g; $F[4, 17] = 6.28$, $p < 0.01$). The older (37- and 72-week-old) groups showed lower percentages than the youngest group. The differences in the SMP30 cells between the 6-, 11-, 37-, and 72-week-old groups were significant (6- vs. 37-week-old group, $p < 0.01$; 6- vs. 72-week-old group, $p < 0.05$; 11- vs. 37-week-old group, $p < 0.05$). Age had a significant effect on the percentage of SNAP-25-positive cells in all cells of the taste bud (Figure 6h; $F[4, 17] = 9.87$, $p < 0.01$). The older group had a lower percentage of SNAP-25-positive cells (11- vs. 37- or 72-week-old groups, $p < 0.05$ or 0.01). The percentage of double-labeled SNAP-25 and SMP30 was low in all groups, and that in the older groups tended to decrease as age increased (Figure 6i; $F[4, 17] = 3.19$, $p < 0.05$). The 72-week-old group showed a lower percentage than the 11-week-old group ($p < 0.05$).

4 | DISCUSSION

This study investigated histological differences in the CvP, FfP, and mucosal epithelium around the papillae in 6- to 72-week-old rats. Moreover, the ratio of Type II and III taste cells of the CvP and cellular activity inferred by the expression of senescence-related markers were compared across age groups. Overall, the number of taste buds in the CvP was maintained from ages 6 to 72 weeks. However, the density ratio of the taste bud area to the trench wall area of the CvP decreased because of an increase in the size of the papilla after the age of 22 weeks, indicating that aging reduces the distribution of taste buds. The thickness of the papillae KL increased

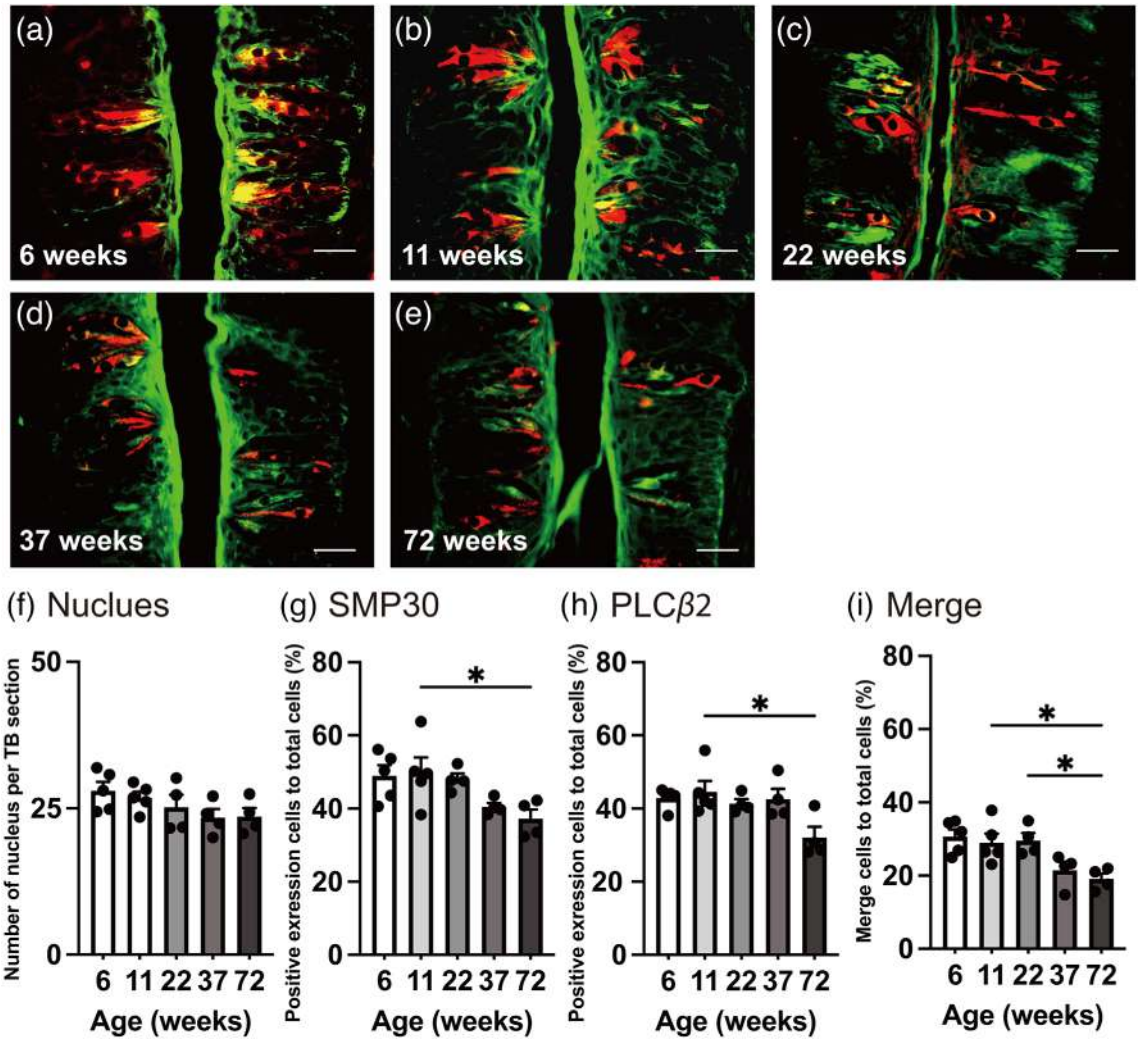

FIGURE 5 Representative immunofluorescent images of the double-staining for PLCβ2 and SMP30 in each group (a–e). SMP30 stains mature cells (FITC, bright green), and PLCβ2 stains Type II cells (Cy3, bright red) in the circumvallate papilla. (f) The whole-cell number per taste bud in each group. Immunofluorescence for DAPI was detected in the nuclei of intragemmal cells in the taste bud section. (g) The percentage of SMP30-positive cells per taste bud section. (h) The percentage of PLCβ2-positive per taste bud section. (i) The percentage of double-labeled PLCβ2 and SMP30 cells per taste bud section. Data are presented as the mean ± SEM of four to five rats from each group. $^*p < 0.05$. Scale bar = 25 μm.

with age and was different from that of the gustatory papilla compared to the 6-week-old group (CvP and FfP). Moreover, the percentage of Type II and Type III taste cells identified by PLCβ2 and SNAP-25 gradually decreased with age. The co-expression of SMP30, an age-related marker, was further reduced. The components of taste cells also changed with histological alterations in the papillae and around them.

Histological changes provide evidence of the underlying mechanisms of functional alterations. Here, the width and depth of the CvP increased with age and reached their highest values in the 72-week-old group. However, the number of taste buds in the CvP did not differ. The number of taste buds was consistent with previous findings (Mistretta & Baum, 1984) that reported no difference in the number of taste buds in the CvP between 5- to 7 (20–28 weeks) and 23- to 24-month-old (92–96 weeks) Wistar-derived rats. Our results for the size of the CvP and FfP differ from those in previous reports stating that the papilla sizes of the CvP were not different between 5- to 7 (20–28 weeks)- and 23- to 24 (92–96 weeks)-month-old rats. However, this is a reason why different ages were compared. As rats mature at approximately 8 weeks of age, the papilla size in premature rats may be smaller than that in mature rats. The size of the FfP in the 37-week-old group was larger than that in the 6-week-old group. However, the size of the FfP in the 72-week-old group did not differ. The size of the papillae seems to increase with development, although this differs because of the regions of the papilla.

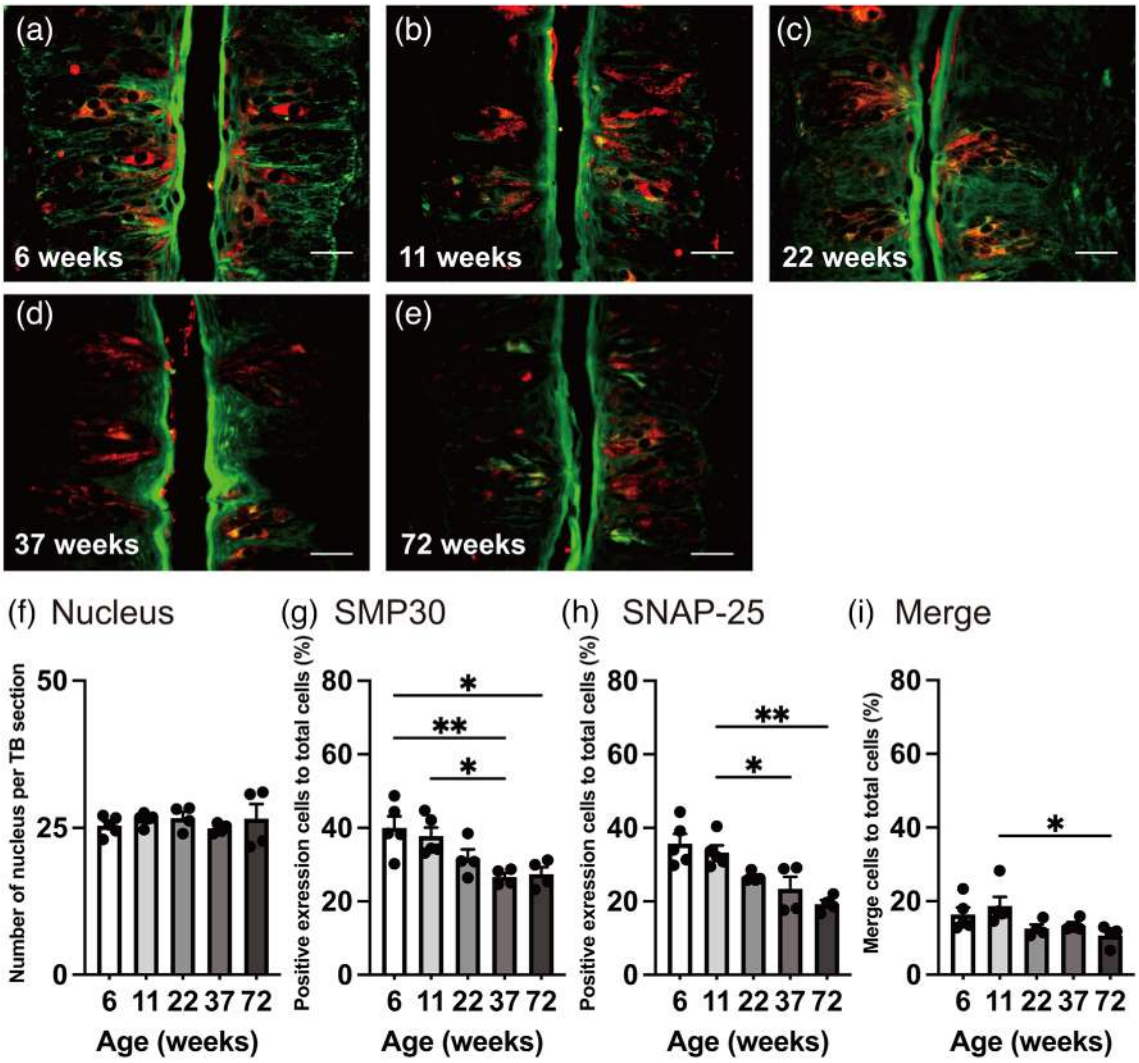

FIGURE 6 Representative immunofluorescent images of the double-staining for SNAP-25 and SMP30 in each group (a–e). SMP30 stains mature cells (FITC, bright green), and SNAP-25 stains Type II cells (Cy3, bright red) in the circumvallate papilla. (f) The total cell number per taste bud section in each group. Immunofluorescence for DAPI was detected in the nuclei of intragemmal cells in the taste bud section. (g) The percentage of SMP30-positive cells per taste bud section. (h) The percentage of SNAP-25-positive cells per taste bud section. (i) The percentage of cells with double-labeled SNAP-25 and SMP30 per taste bud section. Data are presented as the mean ± SEM of four to five rats from each group. *$p < 0.05$; **$p < 0.01$. Scale bar = 25 μm.

Taste bud density in the 72-week-old group was approximately half of that in the 6-week-old group. The decreased density of taste buds in the trench wall of the CvP suggests that fewer chemical substances enter the taste pores and bind to receptors in older animals. Thus, the sparse distribution of taste buds in the CvP of the 72-week-old rats may have decreased the amount of chemical substances entering the taste pores. As the number of taste buds did not change with age, the size of the papilla increased. Therefore, the density of taste buds in the CvP gradually decreased with age.

The long axis of taste buds in the CvP was smaller in the older groups, demonstrating that aging causes a decrease in the size of taste buds in the CvP. This is consistent with the data from a previous study (Shin et al., 2012) reporting that the taste bud size of 18-month-old B6C3F1/J male mice was significantly smaller than that of 2- and 10-month-old mice. Shin et al. (2012) also showed a decreased cell number in the taste buds of 18-month-old mice compared to 2- and 10-month-old mice. However, our results did not reveal a significant decrease in the number of cells in the taste buds of CvP (Figure 5f). This may be attributed to differences in the lifespan of the species. The 18-month-old mice were considered older during their lifespan. The present results were not congruent with those of other reports, which showed that aging can gradually alter the histology of the peripheral taste system.

The conditions surrounding the papilla and taste buds are crucial for the prompt reception of receptive substances. Assessment of the histology around taste-receptive areas by observing the surface and areas around the taste pores of the CvP and FfP showed that KL thickness of the CvP and FfP increased with age. The KL of the CvP and FfP groups was thickest in the 72- and 37-week-old groups, respectively. A previous study showed that the KL of the tongue dorsum increased in 8-, 14-, and 20-month-old mice compared to 2-month-old female mice (Carrard et al., 2008). Thus, KL thickness appears to increase with age. The KL thickness of the CvP and FfP in zinc-deficient rats was greater than that in control rats (Kinomoto et al., 2010). This suggests that chemical substances are prevented from contacting taste receptors by thick KL in zinc-deficient rats, which is a taste disorder animal model. As we previously showed no changes in serum zinc levels in 72-week-old rats (Inui-Yamamoto, 2015), the zinc level is unlikely to contribute to KL thickness in the older group. However, we cannot rule out the possibility that the thick KL caused by aging is one of the reasons underlying the age-related reduction in taste function, as a thick KL can prevent the chemical substances contained in food and drink from reaching the taste pores.

Consistent with the mounting evidence for the deterioration of taste function with age, the present study showed that the percentage of cells positive for PLCβ2 decreased in the older group (Figure 5h). These findings are in line with previous reports that the expression of PLCβ2 mRNA in the CvP of old (120–139 weeks) B6 male mice is lower than that of younger (8–24 weeks) mice (Narukawa et al., 2017). Shin et al. (2012) reported a reduction in the number of cells expressing the protein gene product 9.5, sonic hedgehog, T1R3, and glucagon-like peptide-1 in the CvP of 18-month-old mice compared with 10-month-old mice. These genes may be associated with age-related reduction in taste function. In the present study, the percentage of SNAP-25-positive cells in the CvP group was lower in the older group. SNAP-25b-deficient mice demonstrate alterations in synaptic transmission and increased insulin secretion, which progress toward pronounced metabolic diseases in a time-dependent manner (Irfan et al., 2019). SNAP-25 is a soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor complex that contributes to cell metabolism. Therefore, a decrease in the number of SNAP-25-positive cells suggests a reduction in cellular metabolism. To examine whether the reduction of PLCβ2- and SNAP-25-positive cells was induced by the senescence of taste cells, we conducted double-labeling with a senescence marker, SMP30. An age-related decline in the number of positive cells was also observed in slices stained for SMP30 (Figures 5g and 6g). Cells double-labeled for PLCβ2/SMP30 and SNAP-25/SMP30 also decreased in the older age groups (Figure 5i and 6i). These results demonstrate that the senescence of taste cells progresses, which suggests that decreased taste sensitivity in older animals is caused by a decrease in the function of taste cells.

Our findings demonstrate that the histology of the papillae, the peripheral taste structures, and the components of taste cells change with age, thereby inducing a decline in taste sensitivity. However, the timeline for these changes in Sprague-Dawley male rats began at approximately 37 weeks of age.

5 | CONCLUSIONS

In this study, the histological structures of the CvP, FfP, and taste buds in these regions, not the overall taste bud number, changed with age. The data also revealed an age-related reduction in the number of cells co-expressing an age-related marker, SMP30, and taste cell markers, PLCβ2 and SNAP-25. Cellular senescence has also been associated with a decrease in the proportion of taste cells at 72 weeks of age.

AUTHOR CONTRIBUTIONS

Fatma M. Abdel-maksoud: Investigation; writing – original draft; formal analysis; validation; methodology. **Chizuko Inui-Yamamoto:** Conceptualization; funding acquisition; writing – original draft; methodology; software; formal analysis; writing – review and editing; investigation; validation; visualization; data curation; resources. **Akiyo Kawano:** Methodology; visualization; writing – review and editing. **Shiho Honma:** Methodology; supervision; writing – review and editing; resources. **Naoya Saeki:** Methodology; investigation. **Makoto Abe:** Methodology; data curation; resources. **Moe Kuraki:** Methodology; validation; data curation. **Shinsuke Ohba:** Writing – review and editing. **Satoshi Wakisaka:** Conceptualization; supervision; funding acquisition; writing – review and editing; resources; project administration; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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