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Alterations in the von Ebner's gland secretion and implications for taste sensation in diabetic (*db/db*) mice

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Summary. The taste buds and associated glands, known as von Ebner's glands (VEGs), are involved in and augment gustatory function. The obese diabetic *db/db* mouse, which has defects in the leptin receptor, displays enhanced neural responses to, and an elevated behavioral preference for, sweet stimuli. However, the effect of diabetes on the morphology of circumvallate papilla (CVP) taste buds and the role of VEGs have not been investigated. The present study aimed to compare the CVP taste buds and VEGs in wildtype (Wt) and type 2 diabetic (*db/db*) mice. These mice were divided into control and isoproterenol-treated (at 1 h, 2 h, and 4 h after one day of fasting) groups, and were sacrificed for morphometric, immunohistochemical, and ultrastructural analyses. Morphometry revealed no significant difference in papilla size and the number of taste buds in the control and diabetic groups. Detection of PGP 9.5-immunoreactivity revealed nerve fibers in the trench wall of vallate papillae, but no significant differences were detected between groups. α-Amylase immuno-reactivity levels in Wt and *db/db* mice were also similar. However, 1 h after isoproterenol injection, the majority of the VEG secretion of *db/db* mice was discharged, while the level of α-amylase was restored by 2 h after injection. The effect on α-amylase was in line with the quantitative ultrastructural analysis of the secretory granules. Our findings suggest diabetic metabolic disturbances in *db/db* mice do not alter the structure or innervation of CVP taste buds. However, the VEG secretory pattern was altered in *db/db* mice and might disrupt taste sensation.

Key words: *db/db* mouse, von Ebner's gland, Taste bud, α-amylase

Introduction

Diabetes mellitus (DM) is a major health problem associated with excess morbidity and mortality and results in substantial health-care costs (Harris, 1998). Gustation is an important factor for patients with DM in terms of their overall quality of life and the possible dangers in daily life associated with a taste disorder. Changes in food uptake and nutritional behavior as a consequence of a taste disorder might have an influence on blood pressure and metabolic control (Lauer et al., 1976). However, taste disorder is a common observation in DM type 1 (Le Floch et al., 1989) and type 2 (Perros et al., 1996) patients with a significant and somewhat specific impairment in sweet taste detection. Few studies have been performed to determine the possible alteration of gustatory function in DM type 2 and the pathophysiology associated with taste disorders remains unclear.

Taste perception begins when aqueous-based chemicals stimulate the taste buds located in the tongue, which transmit information to the taste centers of the cortex through synapses in the brainstem and thalamus. Tasting normally occurs in the presence of saliva and is mediated by end organs for the sense of taste that are chronically exposed to the fluids produced by the major and minor salivary glands (Pedersen et al., 2002). The von Ebner's gland (VEG), one of the lingual salivary glands, is a tubuloacinar serous gland located at the bottom of the circumvallate papilla (CVP) and drains its serous contents into the deep trench at the periphery of the CVP. The established functions of VEGs are to wash taste substances from the trench, to ready the taste receptors in the walls of the papilla for a new stimulus, modulate the microenvironment at taste reception sites, and affect chemoreception. Thus, the VEG play an important role in taste transduction of CVP taste buds

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Abbreviations. CVP, Circumvallate papilla; DM, Diabetes mellitus; G, Golgi apparatus; M, Mitochondria; Ob-Rb, Long form of the leptin receptor; Ob-Ra, Short form of the leptin receptor; ODs, Optical densities; rER, Rough endoplasmic reticulum; VEG, von Ebner's glands

(Leinonen et al., 2001).

The *db*/*db* mouse, which has a point mutation in the *db* gene, lacks the functional leptin receptor (Ob-Rb), and is known as a genetic model of non-insulin-dependent DM, with symptoms that mimic clinical type-2 diabetic conditions, such as obesity, polydipsia, polyurea, pancreatic β-cell hyperplasia and hypertrophy. A recent study demonstrated that electrophysiological responses of the chorda tympani nerve to sugars in *db*/*db* mice was higher than in control mice (Ninomiya et al., 1995). Analysis of behavioral experiments revealed that the preference for sugars was higher in *db*/*db* mice than in control mice. However, the pathophysiology of diabetic taste disorders remains unclear, and no data have been reported regarding the morphology of taste buds or VEGs in *db*/*db* mice.

In the present study, we aimed to characterize in detail the morphological alterations, if any, in taste buds of *db*/*db* mice relative to those in control mice. Furthermore, we aimed to assess the relative extent of autonomic nervous system control of VEG secretion, in *db*/*db* and Wt mice, by measuring the rate of secretory granule depletion after injection of a parasympathetic agonist drug.

Materials and methods

Animals

Male, wildtype (Wt) C57BL mice (18-20 g/mouse, n=10) and C57BL/KsJ-lepr^{db}/lepr^{db} diabetic (*db*/*db*) mice (40-45 g/ mouse, n=16) were purchased from the National Taiwan University Animal Center. All mice used in this study were males between 8-10 weeks of age, when plasma levels of insulin are at peak values with concomitant hyperglycemia. Mice were housed in groups of four to five per cage in a room maintained at 23±1°C and 55±5% humidity, with a 12-h light/dark cycle, and were given *ad libitum* access to food and water. All mice were treated according to the guidelines on accommodation and care of animals formulated by the Laboratory Animals Committee, College of Medicine, National Taiwan University. For the VEG study, mice were starved overnight to cause accumulation of secretory granules in the VEG acini and were injected i.p. with 20 mg/kg isoproterenol (to block potential postganglionic β-adrenergic effects) or saline at 08:30 on the following morning. All mice were sacrificed at 0.5, 1, 2, or 4 h after injection and the tongue was rapidly removed for the following studies.

Electron microscopy

Tongue specimens were obtained from the mice perfused with fixative containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C, and cut into 50-μm-thick slices containing taste buds of CVP and VEG using a vibratome (Vibratome VT1200s, Leica Microsystems, Nussloch, Germany). Following post-fixation in 1% osmium tetroxide (in 0.1 M phosphate buffer for 30 minutes), the sections were dehydrated with gradient ethanol and flat-embedded in Polybed-Araldite mixture (Embed 812, EMS, Hatfield, PA, USA). Thin sections (70 nm) were cut in an ultramicrotome (Reichert-Jung Ultracut E, Reichert-Jung, Buffalo, NY, USA) and stained with uranyl acetate and lead citrate before examination using a Hitachi H-7100 transmission electron microscope (Tokyo, Japan), equipped with a Gatan 832 digital camera (Gatan Inc., Pleasanton, CA, USA).

Immunohistochemistry

After perfusion fixation, the tissues containing VEGs and/or CVP were excised from the individual tongues and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 12 h at 4°C. For immunocytochemistry, fixed specimens were cryoprotected in 30% sucrose at 4°C overnight and rapidly frozen in isopentane precooled to -35°C. Frozen sections (7 μm) were cut on a cryostat (CM1900; Leica, Wetzlar, Germany), mounted on APS (aminopropyl-silane)-coated glass slides (Matsunami, Osaka, Japan), and processed for immunostaining.

Immunostaining was performed using the avidin-biotin complex (ABC) technique. Briefly, endogenous peroxidase was quenched by immersion of sections in a solution of 0.3% H_2O_2 in PBS for 30 min. After washing in PBS, the sections were blocked in 0.1% Triton X-100, 1% bovine serum albumin (BSA), and 1% normal goat serum in 0.1 M PBS (blocking solution) for 1 h. Subsequently, sections were incubated with rabbit anti-protein gene product 9.5 (PGP 9.5) (1:300; Chemicon, Temecula, CA, USA) or rabbit anti-α-amylase (1:1000; Sigma-Aldrich, St. Louis, MO, USA) diluted in blocking solution overnight at 4°C. After 3 washes, sections were then reacted with biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) (1:200) for 1 h at room temperature. The immunoreactivity was detected using an avidin-biotin-peroxidase complex kit (ABC reagent, Vector Laboratories) (1:100)) and visualized by incubation in 3, 3'-aminobenzidine (DAB, Sigma-Aldrich) for 2-5 minutes. Sections were then counterstained with hematoxylin (Merck and Co., San Jose, CA, USA) for 5 min, dehydrated and mounted (Leica, Heidelberg, Germany). Digital images were taken using a light microscope (Leica DM 1000 LED), digital compact camera (Leica DFC 420C), and an image processor (Leica Application Suite v3.3.0).

Analysis - number of taste buds

The number of taste buds was counted using a method similar to that of Bradley and Peng (Bradley et al., 1980; Peng et al., 2016). Each thick section (1 μm) stained with toluidine blue was examined under a light microscope and every series of cross sections of a taste

bud was counted. Twelve taste buds from each CVP were then selected at random and the number of sections in which each taste bud appeared was determined. The average number of sections required to transverse a taste bud was calculated. This average was then divided into the total number of taste bud sections for that CVP, to yield a total taste bud count. Thus, for each CVP, the total number of taste bud cross sections was counted, the average number of times a single taste bud appeared in sections was calculated, and the total count of sectioned taste buds was divided by this average, to yield the numbers of taste buds per papillae.

Quantification of taste bud innervation

Measurements of taste bud innervation were obtained from both Wt and *db*/*db* mice in which axons and cells were visualized by immunohisto-chemical detection of PGP 9.5 using the method of Pai (Pai et al., 2007). Twenty profiles of the taste bud from each section that passed longitudinally through the basal lamina and extended to the free surface of the trench were then randomly selected. The selected sections were viewed using a 20× objective in a Leica DM 1000 LED microscope. The computerized image analysis system Image-Pro Plus 5.1 for Windows (Media Cybernetics, Rockville, MD, USA) was used to quantify the immunoreactive cells and nerve fibers in the taste bud.

Quantification of von Ebner's gland α-amylase immunoreactivity

α-Amylase immunoreactivity in mouse VEGs was examined using a Zeiss microscope equipped with a Nikon D1X digital camera and quantitative analysis was conducted with Image-Pro Plus software using a method similar to that used by Yin et al. (2010). Under a fixed level of illumination, the immunostained VEG was photographed through a 40× objective and the images were converted to gray-levels. The staining intensity (optical densities, ODs) of α-amylase immunoreactive granules was determined using an OD of 0.15 as a threshold for immunopositive, after applying a gray-level thresholding procedure. Measurement was done on 40× fields under the microscope on each of 10 VEG sections from each mouse (n=6) of the respective experimental groups. The analysis was performed by the same investigator blinded to the study groups.

Table 1. Summary of quantitative morphological analysis of Wt and *db/db* mice.

	Wt	*db/db*
Thickness of CVP (μm)(n=6)	330±19.25	343±11.43
No. of taste buds (n=6)	95.27±10.92	98.69±5.06
Dense granules (per μm^2) (n=10)	4.12±1.2	3.40±1.14
Density of PGP 9.5 positive fibers (%) (n=5)	50±2.84	48±8.79

Quantification of acinar cell granules

The ultrastructure of acinar cell fields was photographed at 5000× and electron micrographs were selected for quantification if an acinar nuclear profile was visible. Quantification of the number of granules in VEGs was measured in at least 20 cells for each animal (n=6), using the Image-Pro Plus 5.1 software package.

Statistics

All quantitative data were presented as mean ± SEM and analyzed by Student's *t* test. A p value less than 0.05 was considered as statistically significant.

Results

Papilla size and taste bud quantification

We first determined whether the deficiency of the *leptin* receptor in *db*/*db* mice caused any alterations in papilla thickness and the number of taste buds per papilla (Table 1). CVP were located toward the back of the tongue and averaged 467 μm in diameter and 488 μm in height. Among the CVP assessed, thicker ones generally contained more taste buds. Analysis revealed that there was no difference in the thickness of CVP in *db*/*db* and Wt mice (*db*/*db* 343±11.43 μm, Wt 330±19.25 μm; Table 1). Additionally, there was no difference in the number of taste buds in CVP of *db*/*db* and Wt mice (*db*/*db* 98.69±5.06, Wt 95.27±10.92; $p>0.05$). Thus, at the light microscopic level, the number of taste buds and the thickness of papilla were not altered in *db*/*db* mice.

Ultrastructure features of taste cells in db/db mice

Electron microscopy revealed that in longitudinal section the taste buds were oval shaped and comprised a number of flask-shaped taste cells, including type I (dark cells), type II (light cells), and type III (intermediate cells) taste cells in Wt mice (Fig. 1A) and *db*/*db* mice (Fig. 1B), which were classified on the basis of nuclear morphology, characteristic subcellular organelles and different cytoplasmic electron densities, which were described in our previously published article (Peng et al., 2016).

We carefully observed the ultrastructure of various types of taste bud cells in *db*/*db* mice (Fig. 1C-F). At low magnification, epithelial cells were observed surrounding the taste bud. In the apical region of taste buds in *db*/*db* mice, numerous microvilli were identified projecting into the taste pore, providing an increased surface area in the pore for chemosensitivity (Fig. 1C), while some of the cytoplasm contained small granules (Fig. 1D). These granules could be classified into two groups: dense granules (small vesicles with a dark center, Fig. 1D arrows), and double-ring granules (small vesicles with double-ring membrane, Fig. 1D arrowheads). Notably, these different granules were

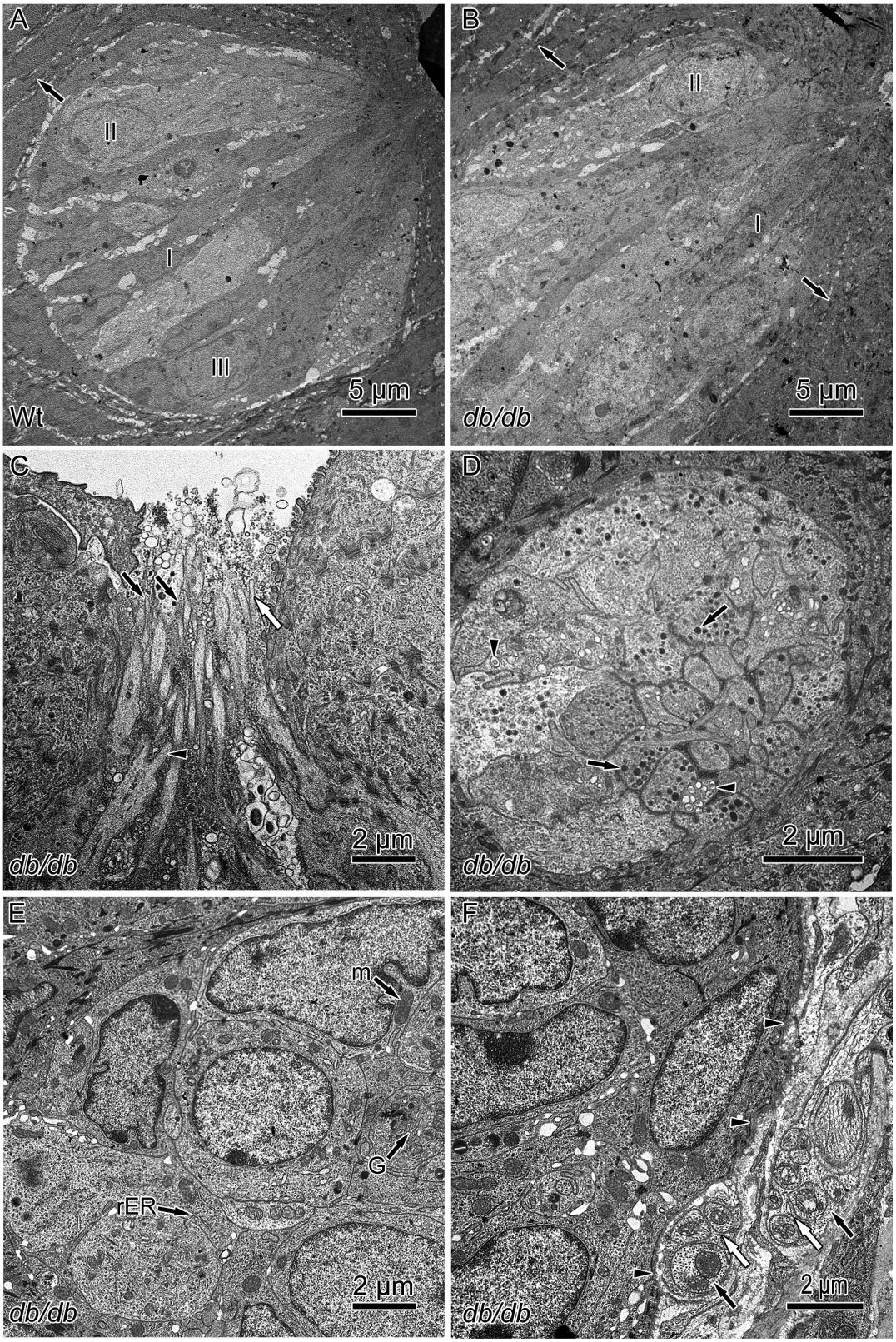


Fig. 1. Ultrastructural analysis of taste bud cells in Wt **(A)** and *db/db* **(B–F)** mice. Low magnification electron micrograph of a taste bud in Wt **(A)** and db/db **(B)** mice. Taste buds contained both dark and light cells with a long, thin shape and a round or invaginated nucleus. Type I cell (I) displays dark cytoplasm. Type II (II) cell contains a pale cytoplasm with a round nucleus. The nuclei of type III cells are slender and possess prominent invaginations. The taste bud is surrounded by epithelial cells containing numerous bundles of cytokeratin filaments (arrows). **C.** Longitudinal section through the taste pore region from a *db/db* mouse circumvallate taste bud. Microvilli project into the taste pore and provide a greatly increased surface area for chemoreception. Type I cells (I) have long, slender microvilli (black arrows), type II cells have brush-like microvilli (white arrow), whereas type III cells contain a single, large, blunt microvillus that extends onto the taste pore (black arrowhead). **D.** Electron micrograph of a transverse section through the apical region of the circumvallate taste bud in a db/db mouse. Type I cells (I) are characterized by the presence of apical dense granules (arrows), whereas the apical granules of the type II cells (II) are double-ring granules (arrowheads). **E.** High magnification transmission electron microscopy revealed a maintained structural organization of organelles (m, mitochondria; G, Golgi apparatus; rER, rough endoplasmic reticulum), and nuclear membrane integrity in taste bud cells from db/db mice. **F.** Each taste bud is separated from the underlying connective tissue by the basement membrane (arrowheads). Beneath the basement membrane, several unmyelinated axons (black arrows) can be seen containing neurofibrils and mitochondria. A basal lamina covering the outer aspect and mesaxon is indicated (white arrows). Scale bars: A, B, 5 µm; C-F, 2 µm.

located separately in type I and type II taste bud cells, respectively, and therefore were considered to be characteristic of these different cells. Furthermore, in *db*/*db* mouse taste bud cells, organelles such as the mitochondria (m), Golgi apparatus (G), and rough endoplasmic reticulum (rER) were readily visible in the cytoplasm (Fig. 1E). There was no change in the morphology of these organelles. The taste bud was located on a basement membrane (Fig. 1F, arrowheads), above the connective tissue of the papillae. The basal region of the taste bud was surrounded by glossopharyngeal nerve fibers which terminated upon the taste cells (Fig. 1F). Most of the nerve fibers close to the basal region of the taste bud were unmyelinated (Fig. 1F, black arrows). Mitochondria and neurofibrils were also clearly present in the cytoplasm of the nerve axons. Thus, according to our observations, there was no significant difference between the ultrastructural features of the taste bud cells in Wt and *db*/*db* mice.

Previous studies indicated that dense granules were an important prerequisite for taste reception in the taste pit of adult mammals (Murray, 1973). Putative production sites were identified as type I cells that contain similar electron-dense granules, displaying the same lectin binding pattern as the pore mucus (Ohmura et al., 1989). In the gustatory organ of vertebrates, lectins have been used to demonstrate the important function of carbohydrate residues on the surface of the olfactory epithelium and taste bud. In the current study, the number of dense granules in the apical cytoplasm of type I taste cells was quantified and the results indicated that the mean number of dense granules per unit area of cytoplasm was lower in *db*/*db* than Wt mice (Wt 4.12±1.2, *db*/*db* 3.40±1.14, n=10, Table 1), but not significantly different.

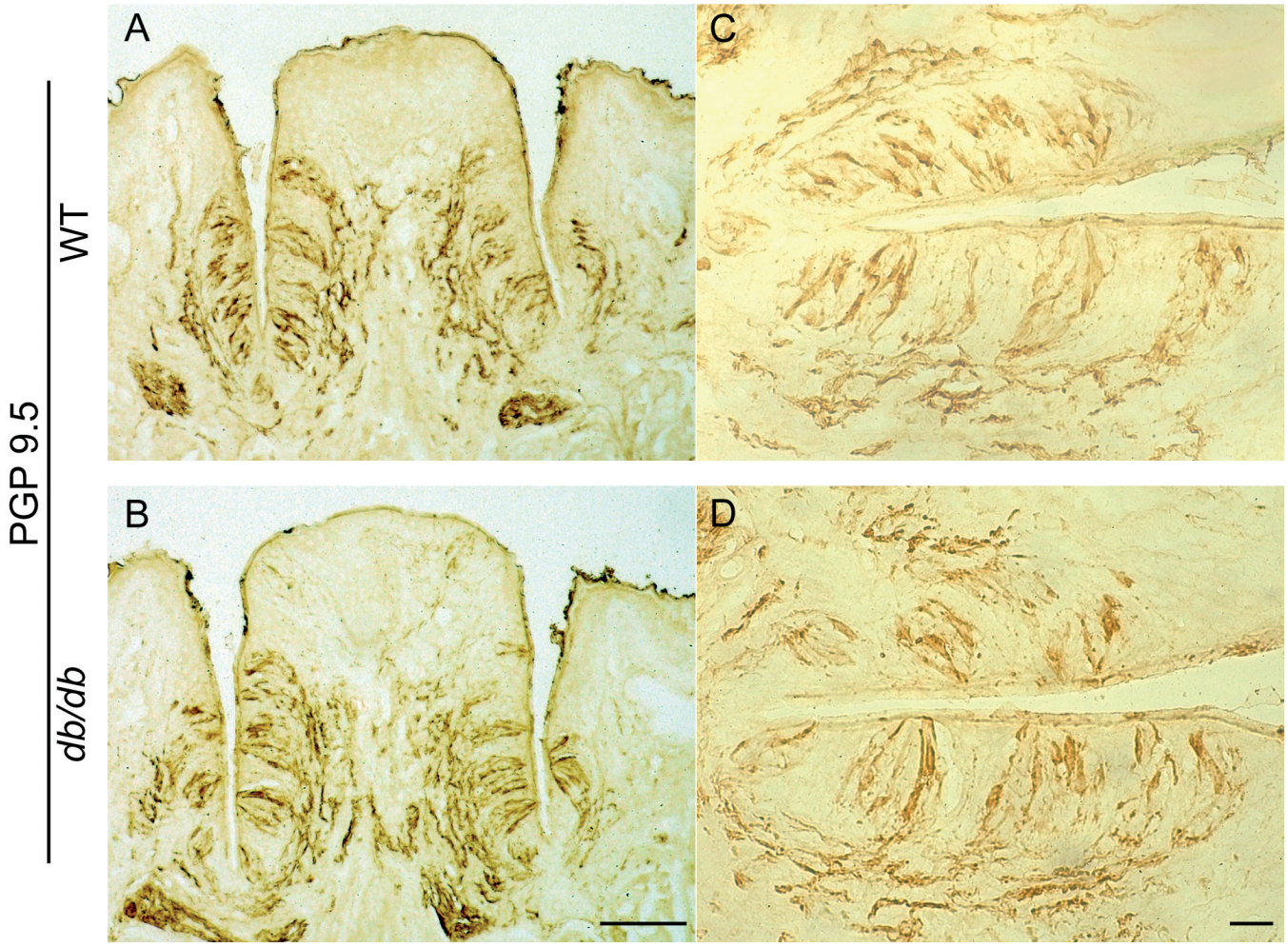


Fig. 2. PGP 9.5 immunoreactivity in circumvallate papillae from Wt **(A)** and db/db **(B)** mice. High magnification photomicrograph of the lateral side of the circumvallate papilla illustrating nerve staining by PGP 9.5 in Wt **(C)** and db/db **(D)** mice. Small bands of PGP 9.5 immunoreactive nerve fibers are present at the base of the taste bud and penetrate into the taste bud. Scale bars: A, B, 5 µm; C, D, 1 µm.

Taste bud innervation in db/db mice

The mechanosensory CVP of the posterior tongue, the epithelial cells of CVP, as well as the taste buds per se are innervated by somatosensory fibers emanating from the trigeminal ganglia, and trigeminal fibers support taste buds in cross-innervation studies. In order to test whether the somatosensory fiber innervation of taste buds in *db/db* mice was altered, we used PGP9.5, a general marker of somatosensory and gustatory nerve fibers (Kanazawa and Yoshie, 1996), to examine the intragemmal (within buds) and perigemmal (surrounding buds) innervation of taste buds (Fig. 2, Wt: A, C and *db/db*: B, D). Analysis revealed that there was no

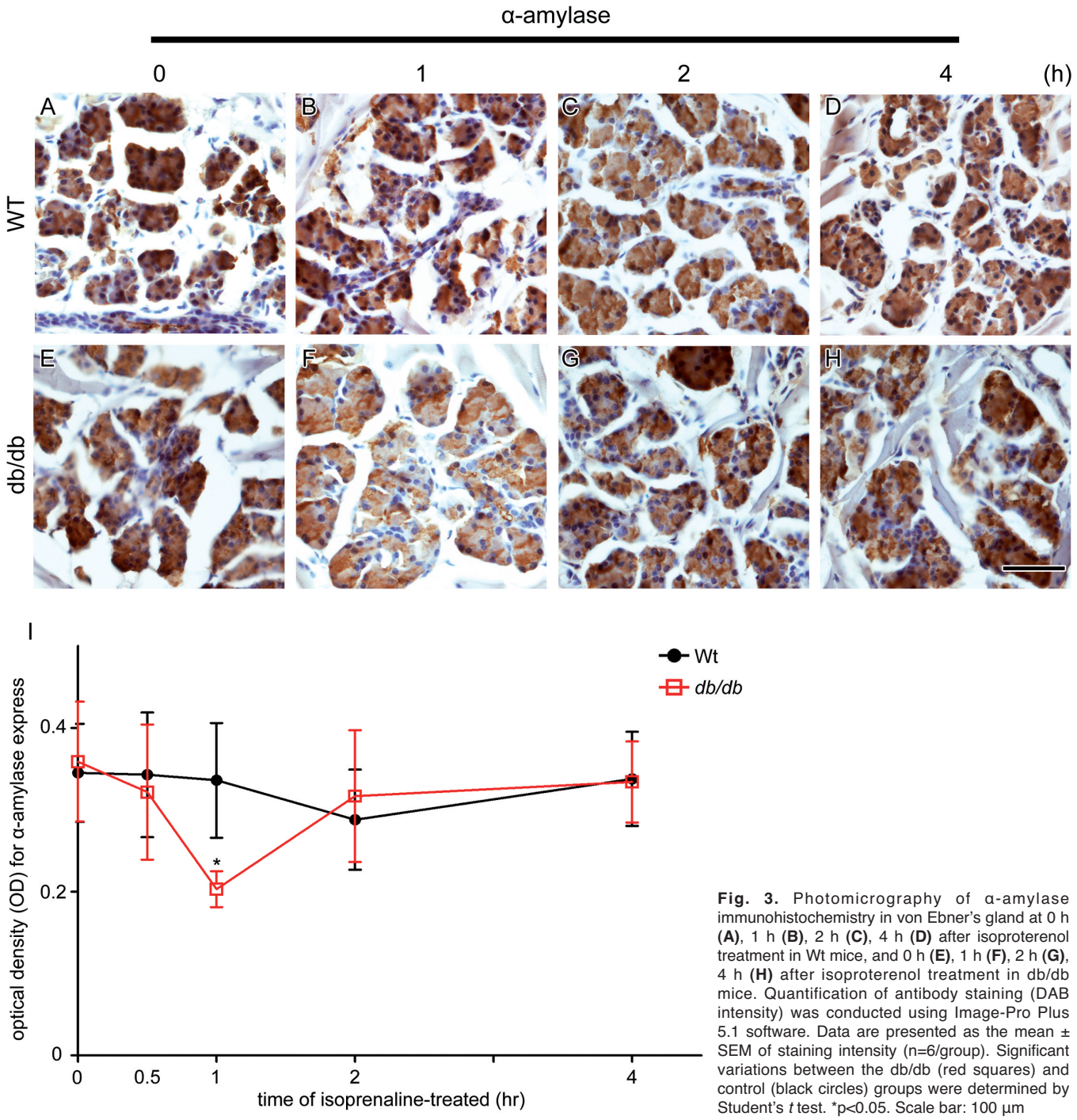


Fig. 3. Photomicrography of α-amylase immunohistochemistry in von Ebner's gland at 0 h **(A)**, 1 h **(B)**, 2 h **(C)**, 4 h **(D)** after isoproterenol treatment in Wt mice, and 0 h **(E)**, 1 h **(F)**, 2 h **(G)**, 4 h **(H)** after isoproterenol treatment in db/db mice. Quantification of antibody staining (DAB intensity) was conducted using Image-Pro Plus 5.1 software. Data are presented as the mean ± SEM of staining intensity (n=6/group). Significant variations between the db/db (red squares) and control (black circles) groups were determined by Student's *t* test. *p<0.05. Scale bar: 100 μm

significant difference in the density of PGP 9.5 positive fibers in Wt and *db*/*db* mice (Table 1, Wt 50±2.84%, db/db 48±8.79%).

Immunohistochemistry and ultrastructure of the isoproterenol-induced secretory cycle in von Ebner's gland

As a major VEG protein, α-amylase can be detected in mice and humans by immunohistochemistry. Studies indicate that the main function of α-amylase is to degrade glycogen and starch by hydrolyzing α-glycosidic bonds into smaller polysaccharides or individual glucose molecules, and the activity of α-amylase in saliva reflects the functional status of VEGs. α-Amylase immunoreactivity was present in VEGs (Fig. 3, Wt: A-D, and *db*/*db*: E-H) and quantitative measurements of the immunoreactivity optical densities (ODs) were made as a reflection of α-amylase abundance (Fig. 3I, Table 2). In these studies, in the absence of isoproterenol treatment, there was no significant difference in α-amylase ODs in *db*/*db* (Fig. 3E) and Wt mice (Fig. 3A) (*db*/*db* 0.36±0.07, Wt 0.35±0.06). In regard to the cycle of VEG α-amylase secretion in Wt mice following isoproterenol treatment, a small decrease in α-amylase OD was observed within 1 h (Fig. 3B). At 2 h, the levels of α-amylase were reduced to 83.4% of control (Fig. 3C) (α-amylase OD 0.29±0.06), and had returned to levels equivalent to initial control values after 4 h (Fig. 3D). However, in *db*/*db* mice, the levels of VEG α-amylase were rapidly reduced to a minimum value of 56.6% of control values by 1 h after isoproterenol treatment (Fig. 3F) (α-amylase OD 0.20±0.02). Thereafter, α-amylase immunoreactivity was restored to initial values by 2 h (Fig. 3G) (*db*/*db* α-amylase OD 0.32±0.08). Analysis revealed a significant main effect of time. These results demonstrate a very fast and large increase in VEG secretion induced by isoproterenol in *db*/*db* mice.

The different pattern of secretion within granular acinar cells of *db*/*db* and Wt mice was confirmed by transmission electron microscopy. The ultrastructure of the secretory granules during the isoproterenol-induced secretory cycle was very similar to that observed in the immunohistochemical study (Fig. 4, Wt: A-B, and *db*/*db*: C-D), and a quantitative assessment of the secretory granules in both groups was conducted (Fig. 4E, Table 2). Prior to isoproterenol injection, the acinar cells contained granules of various sizes in similar quantities in both *db*/*db* and Wt mice. In Wt mice, the granules were slightly depleted from acinar cells at 2 h after isoproterenol treatment (Fig. 4A), while small granules were observed to display a persistent discharge at 4 h (Fig. 4B). Furthermore, the lumens were gradually enlarged and numerous small-sized granules were located near the lumen. In contrast, in *db*/*db* mice, the acinar cells were far more sensitive to isoproterenol treatment. The majority of granules were released by 2 h after isoproterenol injection (Fig. 4C), and at 4 h after isoproterenol, secretory granule levels had recovered to those observed initially (Fig. 4D). Therefore, isoproterenol induced a different VEG secretory cycle in *db*/*db* and Wt mice, suggesting that a deficit in leptin receptor signaling affects the secretory cycle of VEGs and may cause taste disorder.

Table 2. Summary of quantitative analysis of the isoproterenol-induced secretory cycle in von Ebner's gland.

Time after isoproterenol treatment	Optical densities of α-amylase		Number of secretory granules	
	Wt	*db/db*	Wt	*db/db*
0 h	0.35±0.06	0.36±0.07	767±41	851±11
0.5 h	0.34±0.08	0.32±0.08	764±17	651±45*
1 h	0.34±0.07	0.20±0.02*	759±19	598±27*
2 h	0.29±0.06	0.32±0.08	698±21	248±21*
4 h	0.34±0.05	0.33±0.05	666±25	729±43

*means significant difference between Wt and *db/db* mice.

Discussion

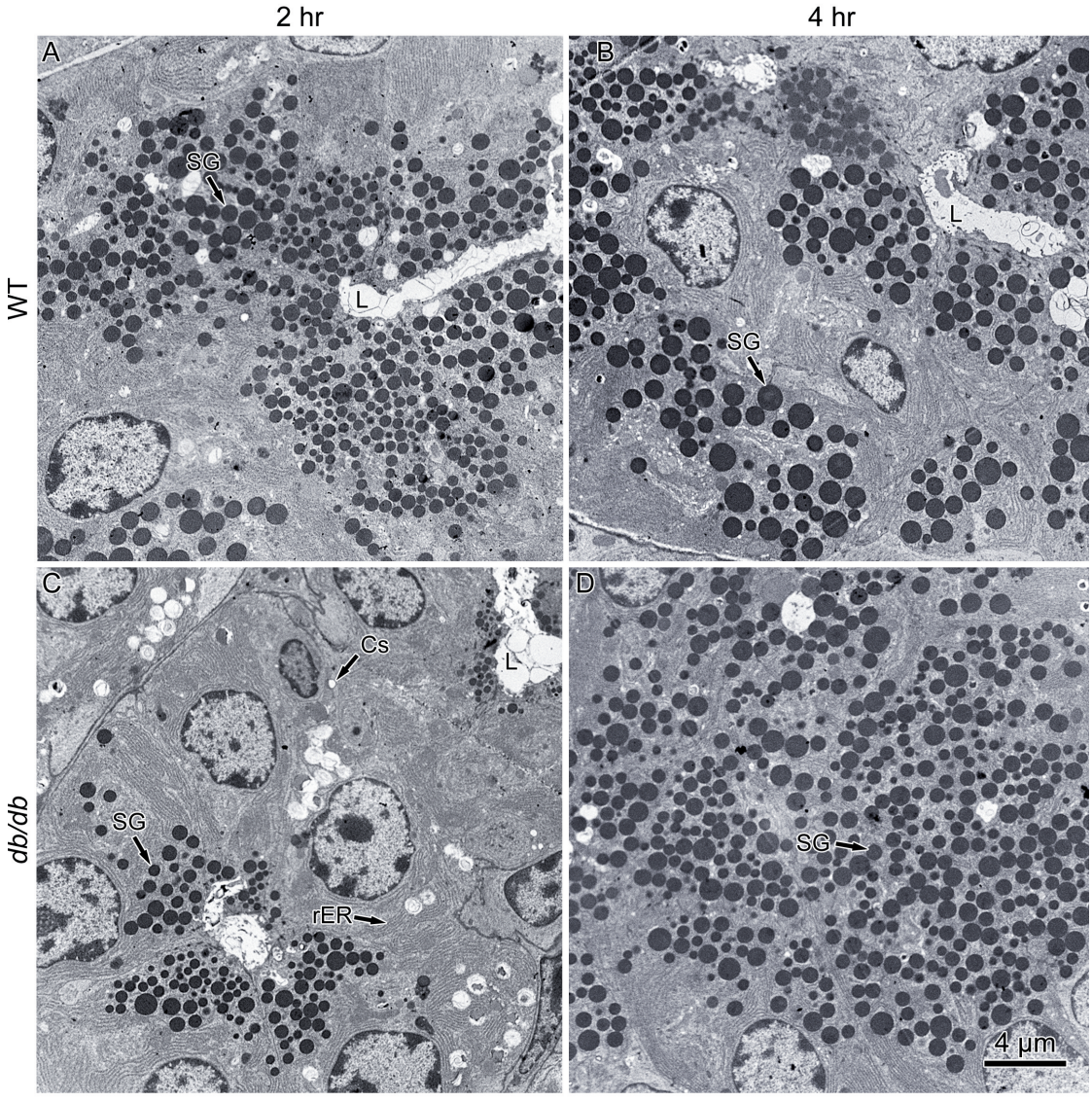
The present study employing light and electron microscopy did not reveal any significant differences in the nerve distribution or structure of taste buds in *db*/*db* and Wt mice. It also demonstrated that in addition to taste bud fine structure, morphology (papilla thickness and taste bud number) was not affected in *db*/*db* mice. However, the isoproterenol-induced α-amylase release and VEG secretory cycle was different in *db*/*db* and Wt mice. Thus, our results may provide evidence to explain the reported difference in taste performance of *db*/*db* mice (Ninomiya et al., 1995, 1998; Sako et al., 1996).

Antibodies against PGP9.5 are the most commonly used for quantifying nerve fiber density (Wilkinson et al., 1989; Kanazawa and Yoshie, 1996). The number of PGP-9.5-immunoreactive cells per taste bud profile decreased in streptozotocin (STZ) induced diabetic mice (Pai et al., 2007), but until now, there has not been a report on PGP9.5 staining in taste buds of *db*/*db* mice.

An earlier study used immunodetection of PGP9.5 in cryosectioned skin samples to quantify the intra-epidermal nerve fiber (IENF) density in *db*/*db* mice, and reported that the IENF density was negatively correlated with diabetes. However, *db*/*db* mice displayed a significant decrease in IENF density from 18 (but not 10) weeks of age, compared to their Wt littermates, and these differences worsened in 26- and 32-week-old *db*/*db* mice, indicating progressive impairments that correlate with disease evolution.

Our study collected tongue sections from *db*/*db* mice of 8-10 weeks of age and used PGP9.5 to detect nerve fibers. These studies revealed that the innervation of taste bud cells was similar in *db*/*db* and Wt mice. In addition, nerves from *db*/*db* mice displayed very few or no degenerated profiles upon ultrastructural examination. These nerves play an important role in the

maintenance and formation of the number and size of the taste buds (Hall et al., 1999; Suzuki, 2008). Many experiments have indicated a dependence of taste buds on their sensory innervation, i.e., taste buds disappear after denervation via apoptosis and reappear following reinnervation (Iwayama, 1970; Takeda et al., 1996;



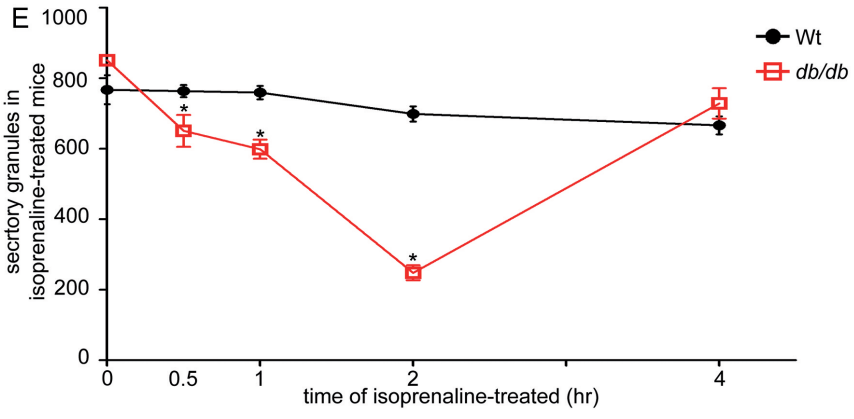


Fig. 4. Transmission electron microscopy of glandular acini from von Ebner's gland, 2 and 4 hours after an isoproterenol injection. In Wt mice, at 2 h after an isoproterenol injection, there were various-sized secretory granules (SG, arrow) in the cells and the luminal zone (L) was grossly enlarged **(A)**. At 4 h after isoproterenol, the cells were considerably depleted of smaller granules and the larger sized granules were in surplus in the acini cell **(B)**. In *db/db* mice, at 2 h after an isoproterenol injection, the cells were largely depleted of their granules. The luminal (L) and canalicular spaces (Cs) were grossly enlarged and often invaginated into the cellular volume. At this stage, the rough-surfaced endoplasmic reticulum (rER) occupied much of the cell volume **(C)**. At 4 h after the isoproterenol injection, reaccumulation of secretory granules had begun in most cells **(D)**. **E.** Time course of VEG secretion in *db/db* (red squares) and Wt (black circles) mice, following isoproterenol treatment. *p<0.05, indicates a significant difference between the time course of secretion, as measured by Student's *t* test (mean ± SEM, n=6).

Huang and Lu, 2001; Sollars, 2005). This relationship is consistent with the finding that the number of taste buds remained constant and no significant change was observed between *db*/*db* and Wt mice. Electron microscopy revealed all types of taste bud cells with distinctive nuclear morphology, various cytoplasmic electron densities, and characteristic subcellular organelles in the circumvallate taste bud from Wt and *db*/*db* mice. Ultrastructural analysis revealed no significant differences between the groups.

The *db*/*db* mouse is a leptin receptor-deficient rodent model and the mutation leads to the functional replacement of the long form of the leptin receptor (Ob-Rb) by short forms of the leptin receptor (Ob-Ra). The defective leptin receptor leads to over production of extracellular leptin, but a lack of intracellular leptin, which is generated via Ob-Rb (Chen et al., 1996; Lee et al., 1996). Expression of leptin receptors (LepRbs) on taste bud cells has been confirmed by immunohistochemistry (Kawai et al., 2000), polymerase chain reaction and *in situ* hybridization (Shigemura et al., 2003). Roper reported that leptin decreased sweet stimulus-evoked release of ATP from taste bud cells (Roper, 2013) and Ninomiya and others reported that peripheral responses to sweet stimuli were larger in obese *db*/*db* mice which lack functional LepRbs (Ninomiya et al., 1998). However, to our knowledge, there is a lack of direct morphological evidence that a deficit in relevant LepRbs alters the structure of taste buds. Therefore, our data provide the first observations that taste buds of *db*/*db* mice are morphologically similar to those in Wt mice.

The trench surrounding the CVP is regarded as the distal portion of the VEG ductal system. Generally, the microenvironment of most lingual taste buds is provided by VEGs. Serous secretion of VEGs removes tastants so that taste buds can receive and respond to new gustatory stimuli, as demonstrated by electrophysiological recordings of taste responses from CVP taste buds (Sbarbati et al., 1999). Several studies have demonstrated interactions between taste and salivary gland function. For example, the neurophysiological taste response to tastants was reduced after the salivary volume of VEGs was decreased by the parasympathetic nervous system antagonist, atropine (Gurkan and Bradley, 1988). Other investigators have also examined the chronic effect of reduced salivary secretion on taste performance (Kawamura and Yamamoto, 1978). In addition, Leinonen et al. (2001) proposed that VEGs abundantly express carbonic anhydrase VI (CA VI) and deliver it directly into the trenches of the CVP in the immediate proximity of the taste buds. CA VI is an enzymatically active member of the α-CA gene family that is only present in the salivary gland and, accordingly, the enzyme's presence in VEG saliva might be directly linked to taste bud function (Leinonen et al., 2001). All these studies suggest that a decrease in secretion of saliva might reduce taste sensitivity. Our research demonstrates that the expression of VEGs is affected, and we speculate this could be a reason for the reduced taste sensitivity of *db*/*db* mice.

Previous studies have reported that salivary α-amylase levels in diabetics were significantly higher than in a control group (Hu et al., 1992; Aydin, 2007; Malathi et al., 2013). A behavioral test also demonstrated that *db*/*db* mice displayed enhanced neural responses to, and increased behavioral preference for, sweet stimuli (Kawai et al., 2000). Our study revealed that α-amylase expression in db/db mice was a little higher than in Wt (*db*/*db* 0.36±0.07, Wt 0.35±0.06). Furthermore, salivary α-amylase levels of diabetic VEGs were rapidly reduced within 1 h of isoproterenol treatment (Fig. 3). Chemosensory information might be generated by a local accumulation of sugar during carbohydrate hydrolysis; when directly exposed to a high sugar concentration, amylase-expressing cells and/or neighboring cells might operate as sensors for sweetness. As sweet taste sensitivity and amylase concentration seem to be associated (Marquezin et al., 2016; Rodrigues et al., 2017), our study provides morphological evidence that differences in amylase abundance between *db*/*db* and Wt mice might impair sweet taste perception.

Our results showed that in *db*/*db* mice, α-amylase levels significantly decreased after 1 h of treatment with isoproterenol and restored to initial levels by 2 h. Besides, the ultrastructural analysis of secretory granules in *db*/*db* mice was correspondingly decreased from 0.5 h to 2 h after treatment with isoproterenol and came back to initial levels by 4 h. Previous studies showed that injection of isoproterenol caused total granule depletion in the parotid gland of mammals, and significant reduction in the amylase content of mammalian parotid glands (Lillie and Han, 1973; Emmelin and Gjörstrup, 1976). VEGs of mice also seemed to follow this general rule (Gurkan and Bradley, 1987), which was also the case for Wt mice in this study. However, when functionality is affected in *db*/*db* mice, this rule may be disrupted. This may be one of the reasons that the levels of amylase and degranulation in our results were out of synchronization in *db*/*db* mice. Furthermore, proteins are synthesized by the rER and transported to Golgi apparatus for the modification and sorting before the formation of secretory granules from the trans-Golgi network (Tooze and Stinchcombe, 1992). A previous study indicated that metabolic pathways of diabetes were accompanied with a variety of posttranslational protein modifications that manifest diabetic glucotoxicity (Zheng et al., 2016), suggesting that many protein modifications in Golgi apparatus may be affected in DM. Besides, the α-amylase is synthesized by rER in the acinar cells of the saliva glands, which is followed by the packaging of the synthesized proteins by the Golgi apparatus and then stored in secretory granules before released (Cwikel et al., 1976; Takano et al., 1991). Therefore, we speculated that α-amylase could be normally synthesized in rER and its protein level reached initial levels by 2 h in *db*/*db* mice. However, the

formation of secretory granules may be affected by Golgi apparatus dysfunction in *db*/*db* mice, which led to delayed restoration of secretory granules until 4 h after treatment with isoproterenol. Further study could investigate the function of Golgi apparatus in *db*/*db* mice, which was not evaluated in this study.

Together, our results suggest that the diabetic metabolic disturbance in *db*/*db* mice does not interfere with the structure and innervation of taste buds in the CVP (Table 1). However, the VEG secretory pattern was affected in *db*/*db* mice (Table 2) and might disturb taste sensation. This hypofunctional symptom could have negative effects on social functioning and quality of life.

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