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Short Communication

SARS-CoV-2 recombinant proteins-induced degeneration of taste buds in rat circumvallate papillae

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Abstract Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections cause loss or alteration of taste and smell as early symptoms and sequelae, but the detailed mechanism remains unclear. This study investigated whether coronavirus disease 2019-induced taste disorders are caused by direct effects on taste bud cells. SARS-CoV-2 recombinant spike and nucleocapsid proteins were applied to circumvallate papillae of male Sprague–Dawley rats. Immunohistochemistry and image analysis were used to compare the number of taste buds, and taste bud cells and area, together with confirmation of angiotensin-converting enzyme 2 (ACE2) expression. Immunohistochemical analysis revealed ACE2 expression in the taste buds of rat circumvallate papillae. Decreases in the number of taste buds, taste bud cells, and their area were observed at 12 days after application of SARS-CoV-2 recombinant spike and nucleocapsid proteins. These data suggest that SARS-CoV-2 proteins induce degeneration of taste buds.
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Introduction

Clinical manifestations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) range from cold-like symptoms typically associated with respiratory tract infections, such as cough and fever, to severe pneumonia with respiratory failure. Patients also frequently experience smell and taste disorders. These mainly consist of a decrease or loss of smell (hyposmia and anosmia) and taste (hypogeusia and ageusia).[1,2] Recently, the angiotensin-converting enzyme 2 (ACE2) receptor was identified as the pathway for viral entry into host cells during SARS-CoV-2 infection. ACE2 is expressed in the taste buds of humans and rats.[3]

However, the direct effect of SARS-CoV-2 on taste cells and the inference mechanism remain unclear. In this study, we investigated whether SARS-CoV-2 has a direct effect on taste cells and its potential contribution to coronavirus disease 2019 (COVID-19)-induced taste impairment.

Material and methods

All experimental procedures were reviewed and approved by the Animal Research Committee of Niigata University (approval number: SA00970) and were performed in accordance with the guidance of the National Institutes of Health on animal care and use.

Animals

Male Sprague—Dawley rats (5 weeks old, 150—200 g; The Jackson Laboratory, Yokohama, Japan) were housed as pairs and maintained in a 12/12-h light/dark cycle at about 23 °C with free access to food and water for 1 week before experiments.

Immunohistochemistry

The animals were deeply anesthetized with three mixed anesthetics (2.5 mg/kg butorphanol, 0.375 mg/kg medetomidine, and 2 mg/kg midazolam, *i.p.*) followed by chloral hydrate (600 mg/kg, *i.p.*) and transcardially perfused with 0.02 M phosphate-buffered saline (PBS; pH 7.2), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.2). Circumvallate papillae were carefully harvested and fixed with 4% paraformaldehyde in 0.1 M PB for at least 48 h. After soaking in 20% sucrose in PBS overnight for cryoprotection, the tissues were embedded in Tissue Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in −80 °C. Frozen sections were cut transversely at 15 μm thicknesses using a cryostat (Leica Biosystems, Nussloch, Germany), followed by immunofluorescence staining. Images were obtained under a fluorescence microscope (BZ-X800; Keyence, Itasca, IL, USA).

Immunostaining of ACE2 in rat taste buds

Sections were blocked with 1% BSA/PBS in the humidified box for 30 min. Then, the sections were rinsed with 0.01 M PBS and permeabilized with a 0.3% Triton X-100 solution before reacting with the primary antibody. A primary antibody against ACE2 (1:400, 21115-1-AP; Proteintech, Rosemont, IL, USA) was applied to the specimen at 4 °C overnight. A fluorescent secondary antibody (1:500, Alexa Fluor 488, The Jackson Laboratory) was applied to the specimen for 1 h. Then, the sections were washed three times with PBS for 5 min each.

Assessment of the taste bud number, area, and cells after application of SARS-CoV-2 recombinant proteins to rat circumvallate papillae

Animals were anesthetized by three mixed anesthetics (2.5 mg/kg butorphanol, 0.375 mg/kg medetomidine, and 2 mg/kg midazolam, *i.p.*) and randomly divided into four groups. In the saline group, saline was applied to the tongue as a negative control. In the nerve transection group, both sides of glossopharyngeal nerves were transected as a positive control. In the SARS-CoV-2 group, SARS-CoV-2 recombinant spike and nucleocapsid proteins (diluted in saline at 50 μg/50 μl each, AG30689 and AG30676; Proteintech) were applied. In the denatured SARS-CoV-2 group, SARS-CoV-2 recombinant spike and nucleocapsid proteins denatured by heat (95 °C, 60 min) were applied. Tissues from all groups were collected at 12 days after treatments.

To label taste buds, they were subjected to lectin histochemistry for *Ulex europaeus* agglutinin-I (UEA-I).[4] Briefly, sections were incubated with Texas Red-conjugated UEA-I (1 μg/ml; E-Y Laboratories, San Mateo, CA, USA) for 2 h at room temperature while protected from light. After brief incubation with 4′,6-diamino-2-phenylindole (DAPI) for 10 min and washing in PBS, the sections were mounted on glass slides and coverslipped.

A typical taste bud contains elongated cells that form a rosebud-shaped structure. An individual taste bud from an intact tongue was generally found every 30—60 μm in preliminary experiments. To avoid double counting, one out of four consecutive sections (every 15 $\mu m \times 4 = 60 \mu m$) from a tongue specimen was used for measurements. Thus, taste buds were non-repetitively counted across images of all serial sections. In each section, the taste bud was outlined, taste buds and DAPI-positive taste bud cells were counted, and the taste bud area was measured with ImageJ Fiji (NIH, Bethesda, MD, USA). The average value of all sections for the number and area of taste buds and the summed value of DAPI-positive taste bud cells were determined as a representative value of each rat.

Statistical analysis

Data are expressed as the mean ± SEM. One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test was used to compare differences in the number of taste buds, taste bud cells, and area of taste buds. Statistical analyses were performed using GraphPad Prism 8 (GraphPad, San Diego, CA, USA). A significant difference was determined at $P < 0.05$.

Results

Rat taste cells express ACE2 receptor

Immunohistochemical analyses showed that ACE2 was expressed at cell membranes in the taste buds of circumvallate papillae from intact rats (Fig. 1).

Application of SARS-CoV-2 recombinant proteins to rat circumvallate papillae decreases the number of taste buds, taste bud area, and DAPI-positive taste cell count

In the saline group, the specimens showed a normal tissue structure and taste buds were orderly (Fig. 2A). In the nerve transection group, significant decreases were observed in the taste bud number and area ($P < 0.01$) compared with the saline group (Fig. 2B—H). The SARS-CoV-2 group, but not denatured SARS-CoV-2 group, showed significant decreases in the taste bud number ($P < 0.01$, $P = 0.99$), taste bud area ($P < 0.05$, $P = 0.52$), and DAPI-positive taste cell count ($P < 0.05$, $P = 0.64$) compared with the saline group (Fig. 2C—H).

Discussion

Evidence suggests that SARS-CoV-2 enters cells via the ACE2 receptor. In particular, ACE2 is abundantly expressed in the oral cavity, including oral mucosal epithelial cells and taste buds, in humans and rats.[3] In this study, we confirmed that ACE2 receptor was expressed in taste buds of rat circumvallate papillae.

In this study, the saline group was used as a negative control, which showed histologically normal taste bud structures, and the nerve transection group was used a as a positive control, which showed atrophy of taste buds after innervating nerve transection,[5] to compare with the SARS-CoV-2 group. The tissue was examined at 12 days SARS-CoV-2 recombinant proteins application, which corresponds to the normal physiological turnover cycle (12—14 days). This is because we assumed if the taste cells were affected, the next turnover might not occur successfully. The SARS-CoV-2 group, but not denatured-SARS-CoV-2 protein group, showed significant decreases in the taste bud number, taste bud area, and DAPI-positive taste cell count compared with the saline group. Thus, it appears that SARS-CoV-2 proteins may be responsible for the effect on taste bud tissue seen in this study.

Cellular damage due to prolonged viral shedding, a chronic immune inflammatory response, and procoagulant state induced by SARS-CoV-2 infection are suggested mechanisms that contribute to sequelae.[6] The present model used only recombinant proteins and not actual viruses containing replicable nucleic acids. Replicated viruses infect neighboring taste bud cells and subsequent newborn taste buds, resulting in residual taste dysfunction. The so-called clinical sequelae in actual viral infections, prolonged dysgeusia, and viral shedding suggest anatomical reservoirs for SARS-CoV-2, which act as sources for active or latent taste dysfunction in the long-term for COVID-19 patients.[6] SARS-CoV-2 spike protein induces apoptosis and necrosis in macrophages,[7] peripheral blood mononuclear cells[7] and adipocytes,[8] which support our results. A recent report using several cell lines also revealed that SARS-CoV-2 spike protein promotes local inflammation and apoptosis in infected cells through apoptosis-related intracellular signaling.[9,10] Thus, direct infection of taste bud cells via ACE2 along with spike protein-induced intracellular signaling may lead to apoptosis and degeneration of taste buds, although nucleocapsid protein may also contribute. Therefore, further investigations of taste sensory transmission systems are required in the future.

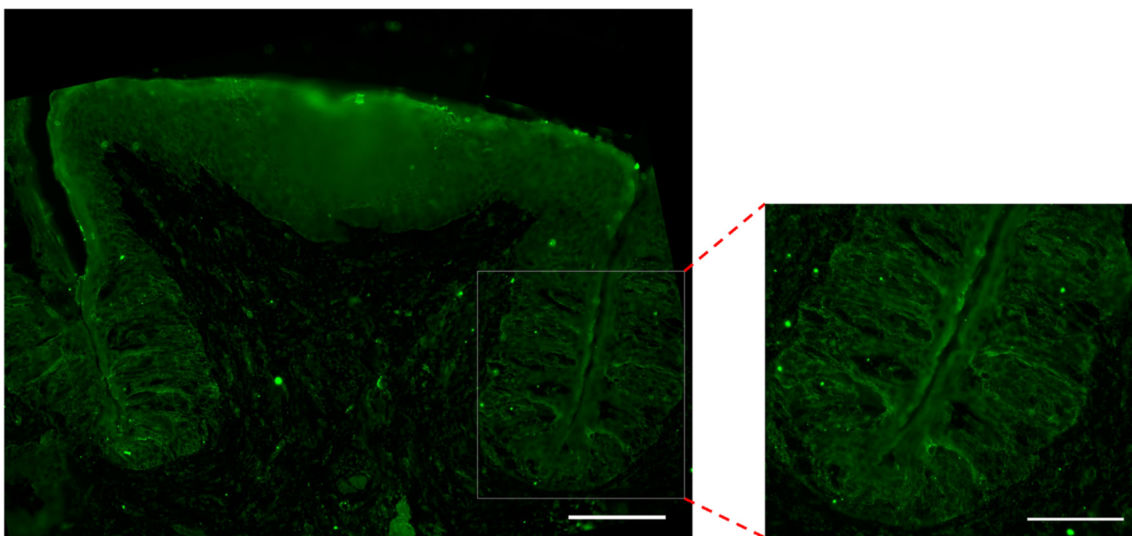

Figure 1 Expression of ACE2 receptor in rat taste buds of circumvallate papillae. Primary: anti-ACE2 (21115-1-AP; Proteintech), 1:400. Secondary: Alexa Fluor 488 (The Jackson Laboratory), 1:500. Scale bar = 100 μm (50 μm in the enlarged window).

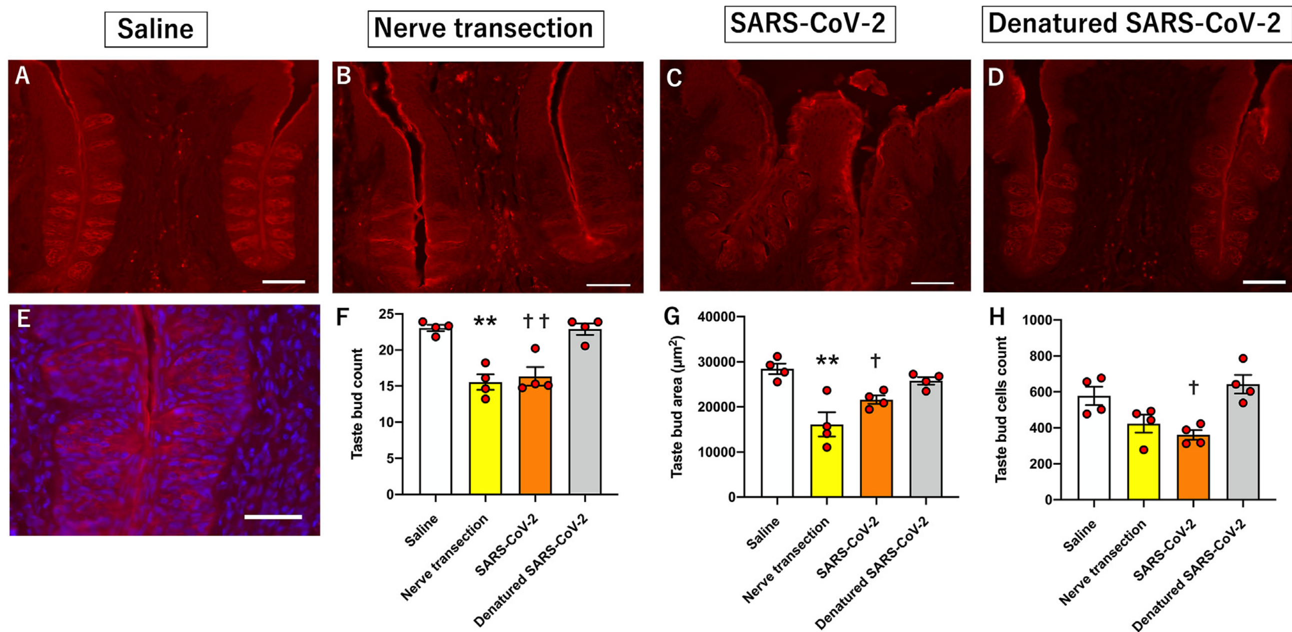

Figure 2 Histological changes in rat taste buds after application of SARS-CoV-2 proteins. Application of SARS-CoV-2 recombinant spike and nucleocapsid proteins, but not SARS-CoV-2 proteins denatured by heat, decreased the taste bud number, taste bud area, and taste bud cell number. (A) Saline group; (B) Nerve transection group (C); SARS-CoV-2 protein group; (D) Denatured SARS-CoV-2 protein group. (E) Double staining of taste bud cells with UEA-I and DAPI. (F) Taste bud count; (G) Taste bud area; (H) Taste bud cells count 12 days after treatment. Scale bar = 100 μm $^{*,\dagger}P < 0.05$, $^{**,\dagger\dagger}P < 0.01$, vs Saline group, one-way ANOVA, followed by Dunnett's multiple comparison test, n = 4.

This study demonstrated that SARS-CoV-2 proteins degenerated the taste buds of rat circumvallate papillae, suggesting that the influence of SARS-CoV-2 proteins on taste buds is involved in taste dysfunction after infection. Further investigations should extend our understanding of the intracellular mechanisms responsible for taste disorders caused by COVID-19 infection.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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