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Glucagon-like peptide-1 is specifically involved in sweet taste transmission

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ABSTRACT **Five fundamental taste qualities (sweet, bitter, salty, sour, umami) are sensed by dedicated taste cells (TCs) that relay quality information to gustatory nerve fibers. In peripheral taste signaling pathways, ATP has been identified as a functional neurotransmitter, but it remains to be determined how specificity of different taste qualities is maintained across synapses. Recent studies demonstrated that some gut peptides are released from taste buds by prolonged application of particular taste stimuli, suggesting their potential involvement in taste information coding. In this study, we focused on the function of glucagon-like peptide-1 (GLP-1) in initial responses to taste stimulation. GLP-1 receptor (GLP-1R) null mice had reduced neural and behavioral responses specifically to sweet compounds compared to wild-type (WT) mice. Some sweet responsive TCs expressed GLP-1 and its receptors were expressed in gustatory neurons. GLP-1 was released immediately from taste bud cells in response to sweet compounds but not to other taste stimuli. Intravenous administration of GLP-1 elicited transient responses in a subset of sweet-sensitive gustatory nerve fibers but did not affect other types of fibers, and this response was suppressed by pre-administration of the GLP-1R antagonist Exendin-4(3-39). Thus GLP-1 may be involved in normal sweet taste signal transmission in mice.—Takai, S., Yasumatsu, K., Inoue, M., Iwata, S., Yoshida, R., Shigemura, N., Yanagawa, Y., Drucker, D. J., Margolskee, R. F., and Ninomiya, Y. Glucagon-like peptide-1 is specifically involved in sweet taste transmission. *FASEB J.* 29, 000–000 (2015). www.fasebj.org**

Key Words: gustation · transmitter · incretin

THE SENSE OF TASTE IS VITALLY important for life; it provides information on which prospective foods are nutritious, and warns as to those that are noxious. Sweet, bitter, salty, sour, and umami (amino acid) tastes are recognized to represent the five basic taste qualities experienced by humans, rodents, and other mammals. Sweet and umami tastes allow the detection of nutritious substances, bitter and sour tastes prevent the ingestion of toxic chemicals, and salty taste provides for the detection of certain electrolytes (1). Each of the five taste qualities is thought to be detected initially by dedicated TCs in taste buds, and these differentiated TCs can be classified into at least 3 cell types: types I, II, and III according to their cytologic, ultrastructural, and functional features (2, 3). Among the taste qualities, sweet, bitter, and umami tastes are initiated by type II cells, which express TAS1R and TAS2R G-protein-coupled taste receptors (1, 4, 5). Activation of these taste receptors leads to cell-depolarization, generation of action potentials, and release of ATP (6–9). It is well established that ATP and its receptors, ionotropic purinergic receptor 2/3 (P2X2/3), play a crucial role in taste signaling pathways; for example, P2X2/3 double knockout mice exhibit a near total loss of neural and behavioral responses to all taste qualities including sour and salty taste (10). Hence, taste signal transmission may depend principally on extracellular ATP.

Previous studies suggested that the 5 basic tastes may be discriminated at the receptor cell level by the expressions of different taste receptors, or electrophysiological response profiles to taste stimuli (1, 5), and that taste information is transferred to the nerve fibers without major modification (3). However, it is not understood how taste signal specificity can be maintained, because sweet, bitter, and umami-sensitive type II TCs lack conventional synaptic

Abbreviations: CCK, cholecystokinin; CT, chorda tympani; CV, circumvallate; DW, distilled water; DPP4, dipeptidyl peptidase-4; FP, fungiform papillae; GAD67, glutamate decarboxylase 67; GFP, green fluorescent protein; GG, geniculate ganglion; GL, glossopharyngeal; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor;

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connections (11). In addition, type III TCs, which are thought to be activated by sour stimulus, have not been shown to liberate ATP (7, 8). Thus other mechanisms may be involved in specifying taste quality during signal transmission from TCs to nerve fibers.

Recent studies have demonstrated that subsets of TCs express several gut peptides, including GLP-1 (12), neuropeptide Y (NPY) (13), glucagon (14), ghrelin (15), and vasoactive intestinal peptide (VIP) (16). Furthermore, some of these peptides are secreted in response to various taste stimuli, and the secretion patterns of peptides, such as GLP-1, glucagon, and NPY, are correlated with specific taste qualities, implying that these gut peptides may contribute to taste quality coding (17, 18). Among them, GLP-1 was originally identified as an insulinotropic incretin hormone, which is expressed in enteroendocrine L cells in gut. Enteroendocrine L cells express multiple taste transduction molecules, including the sweet taste receptor taste receptor type 1 member 2 (T1R2)/taste receptor type 1 member 3 (T1R3) and the taste G-protein gustducin, and release GLP-1 in response to stimulation with sweet compounds (19–21). GLP-1 is reported to activate the vagus nerve, and this signal may play an important role in glucose homeostasis (22, 23). In taste buds, it was reported that incubation for 2 h with apically applied sweet or umami compounds elicited GLP-1 release from isolated lingual epithelia containing circumvallate (CV) papillae taste buds (17), although from this *ex vivo* study it is unclear how GLP-1 contributes to the neurotransmission of taste quality information in linking individual TCs to nerves. From studies in humans, it is known that the perception and discrimination of taste qualities can occur within a couple of seconds (24), thus we focused on the role of GLP-1 in acute responses for signal transmission between TCs and gustatory nerve fibers.

In the present study we report that GLP-1 is expressed in a subset of T1R3-immunopositive TCs (as had been previously reported) (25), and its receptors are expressed in gustatory neurons. We detected transient release of GLP-1 from sweet-sensitive TCs immediately after stimulation with sweet compounds, indicating that GLP-1 might activate sweet-sensitive gustatory nerve fibers. GLP-1-elicited activation of sweet-sensitive nerve fibers was inhibited by pretreatment with a GLP-1R antagonist. Altogether, these results indicate that in mice GLP-1 is specifically involved in transmission of sweet taste signals.

MATERIALS AND METHODS

All experimental protocols and procedures were approved by the committee for Laboratory Animal Care and Use at Kyushu University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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MPG, monopotassium glutamate; MSG, monosodium glutamate; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; P2X2/3, purinergic receptor 2/3; T1R2, taste receptor type 1 member 2; T1R3, taste receptor type 1 member; TCs, taste cells; TNT, Tris-NaCl-Tween; VIP, vasoactive intestinal peptide; WT, wild-type

Experimental animals

T1R3-green fluorescent protein (GFP) mice, gustducin-GFP mice, glutamate decarboxylase 67 (GAD67)-GFP mice, and GLP-1R$^{-/-}$ mice were described previously (26–29). The genetic background for T1R3-GFP, gustducin-GFP, and GAD67-GFP mice is C57BL/6. GLP-1R$^{-/-}$ mice were originally produced from CD-1 morulae (29), and we back-crossed them to C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) for 5 generations to breed heterozygous mice (30). These mice were interbred to generate GLP-1R$^{-/-}$ mice in the C57BL/6 background and used in this study. WT controls were C57BL/6J mice. All mice were maintained on a 12/12 hour light-dark cycle and fed standard rodent chow.

Gustatory nerve recordings

Gustatory nerve responses to lingual application of tastants were recorded from the chorda tympani (CT) or the glossopharyngeal (GL) nerve as described (30, 31). Under pentobarbital anesthesia (50–60 mg/kg body weight), the trachea of each mouse was cannulated and the mouse was then fixed in the supine position with a head holder to allow dissection of the CT or the GL nerve. The right CT nerve was dissected free from surrounding tissues after removal of the pterygoid muscle and cut at the point of its entry to the bulla. The right GL nerve was exposed by removal of the digastricus muscle and posterior horn of the hyoid bone. The GL nerve was then dissected free from underlying tissues and cut near its entrance to the posterior lacerated foramen. The entire nerve was placed on an Ag/AgCl electrode. For single-fiber recordings, a single fiber or a few fibers of the CT nerve were teased apart with a pair of needles and placed on the electrode. An indifferent electrode was placed in nearby tissue. Neural activities were fed into an amplifier (K-1; Iyodenshikagaku, Nagoya, Japan), and monitored on an oscilloscope and audio monitor. Whole-nerve responses were integrated with a time constant of 1.0 s and recorded on a computer using a PowerLab system (PowerLab/sp4; ADInstrument, Bella Vista, NSW, Australia). For taste stimulation of fungiform papillae (FP), the anterior half of the tongue was enclosed in a flow chamber made of silicone rubber. For taste stimulation of the CV and the foliate papillae, an incision was made on each side of the animal's face from the corner of the mouth to just above the angle of the jaw, then the papillae exposed and their trenches opened by slight tension applied through a small suture sewn in the tip of the tongue. Taste solutions [100 mM NH_4Cl, 10–1000 mM sucrose, 10–100 mM NaCl, 0.1–10 mM HCl, 0.1–20 mM quinine-HCl, 10–1000 mM monosodium glutamate (MSG) + 100 μM amiloride, 500 mM glucose, 500 mM maltose, 500 mM fructose, 20 mM saccharin, 1 mM SC45647, 10 mM citric acid, 20 mM denatonium, 100 mM monopotassium glutamate (MPG)] were delivered to each part of the tongue by gravity flow and flowed over the tongue for 30 s (CT) or 60 s (GL). The tongue was washed with distilled water (DW) for an interval of ~1 min between successive stimulation. Only responses from stable recordings were used in data analysis. For recordings of responses to GLP-1, 20 μg/kg body weight of GLP-1 were administrated from the femoral vein. In some cases the GLP-1R antagonist Exendin-4(3–39) (40 μg/kg body weight; Phoenix Pharmaceuticals, Burlingame, CA, USA) (32) was administrated intraperitoneally 20 minutes before recording responses of CT nerve fibers. For measurement of plasma GLP-1 concentration after intravenous injection of GLP-1, 20 μl plasma samples were collected from carotid artery at some time points. These samples were diluted to 100 μl with the ELISA kit buffer (Shibayagi, Shirokawa, Japan) containing 50 μM dipeptidyl peptidase-4 (DPP4) inhibitor (Millipore, Billerica, MA, USA) to prevent the degradation of GLP-1 and stored at −80°C for GLP-1 quantification.

Behavioral tests

Taste behavior was assayed by a short term (10 seconds) lick test with sweet or umami-bitter mixtures as test stimuli to detect concentration-dependent changes in lick rates more clearly (33). On the first day of training, each animal was water deprived for 23 h, and then placed in the test cage and given free access to DW during a 1 h session. Days 2–5 were training sessions. During this period, the animal was trained to drink DW on an interval schedule, consisting of 10-second periods of presentation of DW alternating with 20 seconds intertrial intervals. On days 6–8, the number of licks for each test stimulus and DW were counted during the first 10 seconds after the animal's first lick using a lick meter (Yutaka Electronics Co., Gifu, Japan). On each test day the first test stimulus given to the animal was DW. Then, test stimuli (30–1000 mM sucrose + 1 mM quinine, 10–1000 mM NaCl, 1–30 mM HCl, 0.1–10 mM quinine, 10–300 mM MSG + 1 mM quinine + 30μM amiloride, 0.1–20 mM saccharin + 1 mM quinine) were tested in a randomized order. Even after water deprivation mice cannot drink adequate amounts of solutions to include all stimuli at all concentrations to be tested in one session, so multiple sessions were required. In a given session, testing continued until the mice no longer licked the DW within 7 seconds after the animal's first lick of a given trial. The mean number of licks across 3 day was obtained for each of the test stimuli at each concentration in each animal.

TC recording

The recording procedures were the same as used previously (5, 8). Animals were anesthetized with ether and killed by cervical dislocation. The anterior part of the tongue was removed and injected with 100 μl of Tyrode solution (in mM: NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 5, glucose 10, sodium pyruvate 10, HEPES 10; pH 7.4 adjusted with NaOH) containing 0.5–1 mg/ml elastase (Elastin Products, Owensville, MO, USA). After incubation for 10–20 minutes at room temperature, the lingual epithelium was peeled and pinned out in a Sylgard-coated culture dish (Dow Corning, Midland, MI, USA). Individual fungiform taste buds with a piece of surrounding epithelium were excised from this sheet, and the mucosal side was drawn into the orifice of the stimulating pipette. Gentle suction on the stimulating pipette was maintained by a peristaltic pump to perfuse taste solutions and to hold the taste bud in place. Tyrode solution was perfused inside the stimulating pipette except during the period of recording. Taste bud cells containing GFP were identified under confocal laser scanning microscopy (excitation 488 nm, emission 500–600 nm, FV-1000, Olympus, Tokyo, Japan) and were approached by a recording electrode (inner diameter: 3–5 μm, inner solution: 20 μl Tyrode solution with 50 μM DPP4 inhibitor to prevent the degradation of GLP-1 by DPP4) from the basolateral side to record action potentials of TCs extracellularly. Electrical signals from TCs were recorded by a high-impedance patch-clamp amplifier (Axopatch 200B; Axon Instruments, Sunnyvale, CA, USA) interfaced to a computer by an analog-to-digital board (Digidata 1320A; Axon Instruments) at room temperature (25°C). The apical side of the taste bud was preadapted for at least 30 seconds with DW, and then the taste stimulus applied for 60 seconds. After recording, the solution in the recording electrode was collected immediately and stored at −80°C for GLP-1 quantification. For the collection of GLP-1 released from single taste buds in response to taste stimulation, a collecting pipette covering the whole single taste bud (inner diameter: about 30 μm, inner solution: 20 μl Tyrode solution with 50 μM DPP4 inhibitor) was used. The inner solution was collected immediately after taste stimulation (60 seconds) and stored at −80°C for GLP-1 quantification. In this session, we monitored spontaneous activity to exclude those cells that gave extraordinary responses to the touch of the pipette.

Quantitative determination of GLP-1

GLP-1 concentrations were measured by using an ELISA kit for active GLP-1 (Shibayagi), according to the manufacturer's instructions. This ELISA kit is able to measure from 0.47 pmol/l to 15.16 pmol/l of GLP-1(7-36) amide and has no cross reactivity (<0.1%) to mouse/rat GLP-1(7–37), GLP-1(9–36)amide, GLP-2, or glucagon. Samples were diluted to 50 μl with the buffer included in the kit before GLP-1 measurement. The enzyme activity for GLP-1 was measured spectrophotometrically by increased absorbance at 450 nm, corrected from absorbance at 630 nm with a Multiscan JX microplate reader (Thermo Fisher Scientific, MA, USA).

Immunohistochemistry

Immunohistochemistry was performed as described previ ously (30, 33). The dissected tongues of GAD67-GFP mice ($n = 7$, 8–20 wk of age) were fixed in 4% paraformaldehyde in PBS for 30–60 min at 4°C. The geniculate ganglion (GG), which contains neuronal cell bodies for gustatory nerve fibers coming up from the anterior tongue through the CT nerve and from the palate through the greater petrosal nerve, was obtained from WT and GLP-1R$^{-/-}$ mice and fixed in 4% paraformaldehyde in PBS for 10–15 minutes at 4°C. After dehydration with sucrose solution (10% for 1 hour, 20% for 1 hour, 30% for 3 hours at 4°C), the frozen block of fixed tongue or the GG was embedded in Optimal Cutting Temperature compound (Sakura Finetek, Tokyo, Japan), sectioned into 8 μm thick slices, which were mounted on silane-coated glass slides. Frozen sections were washed with Tris-NaCl-Tween (TNT) buffer, refixed in 4% paraformaldehyde in PBS for 10 minutes, and washed with TNT buffer. Then sections of the tongue were incubated in Histo VT One (Nakalai Tesque, Kyoto, Japan) for 30 minutes at 80°C, washed with TNT buffer. Sections of GG were incubated in 1% H_2O_2 for 15 minutes, washed with TNT buffer, incubated in avidin/biotin blocking (Dako, Glostrup, Denmark) for 10 minutes each, and then washed again with TNT buffer. Next all sections were incubated overnight in Blocking One solution (Nakalai Tesque) at 4°C. After blocking, sections were incubated overnight at 4°C with primary antibodies for GFP (1:60, rabbit anti-GFP, Cat# sc-8334; Santa Cruz Biotechnology, Santa Cruz, CA, USA), T1R3 (1:60, goat anti-T1R3, Cat# sc-22458; Santa Cruz Biotechnology), GLP-1 (1:100, mouse anti-human GLP-1, Cat# G2040-35-C; US Biologic, Salem, MA, USA), P2X2 (1:300, rabbit anti-P2X2; Alomone Labs, Jerusalem, Israel) or GLP-1R (1:740 biotinylated mouse monoclonal GLP-1R antibody 7F38, which was kindly provided by Dr. Charles Pyke, Novo Nordisk A/S, Maaloev, Denmark) in Blocking One solution. After washing with TNT buffer, tissues were incubated for 2 h at room temperature with secondary antibodies for GFP (1:300, Alexa Fluor 488 donkey anti-rabbit IgG, Cat# A21206; Invitrogen, Carlsbad, CA, USA), T1R3 (1:300, Alex Fluor 633 donkey anti-goat IgG, Cat# A21082, Invitrogen), GLP-1 (1:300, Alexa Fluor 568 donkey anti-mouse IgG, Cat# A10037, Invitrogen), P2X2 (1:300, Alex Fluor 555 donkey anti-rabbit IgG, Cat# A-31572; Invitrogen) or GLP-1R (1:300; streptavidin CF dye 488A conjugated, Cat# 29034; Biotium, Hayward, CA, USA) in Blocking One solution, and washed with TNT buffer. Immunofluorescence of labeled TCs was observed using a laser scanning microscope (FV-1000; Olympus); images were obtained using Fluoview software (Olympus). To determine the number of cells expressing GAD67-GFP, GLP-1, and T1R3, we counted positive cells in each taste bud in horizontal sections of FP and CV (the number of taste buds; FP: 121 CV: 211). Image-ProPlus (version 4.0; Mediacybernetics, Warrendale, PA, USA) was used to exclude artifactual signals: cells showing a signal density greater than the mean plus two standard deviations of the density in TCs in the negative control (primary antibodies omitted) were considered positive. The same cells found on the contiguous sections were counted only once.

Data analysis

Integrated whole-nerve response magnitudes were measured 5, 10, 15, 20, and 25 s (for the CT) and 5, 10, 20, 30, and 40 seconds (for the GL) after stimulus onset, averaged, and normalized to responses to 100 mM NH_4Cl to account for mouse-to-mouse variations in absolute responses. This relative response was used for statistical analysis (2-way ANOVA and the *post hoc t* test). In the analysis of single-fiber responses, single fibers were identified by their uniform spike height, singular wave form and intervals between contiguous spikes (31). In the analysis of TC responses, action potential waveforms were analyzed in respect to the following parameters: time of peak–peak, peak amplitude/antipeak amplitude ratio, antipeak amplitude, and peak amplitude (34). In both single-fiber recordings and TC recordings, the number of spikes per unit time was counted throughout the recording. The mean spontaneous impulse discharge for each unit was calculated by averaging the number of spikes over the 10-second period in which DW flowed over the taste pore or the tongue prior to each stimulus. The magnitude of response to taste stimuli was obtained by counting the total number of impulses for the first 10 s after the onset of stimulus application and subtracting the spontaneous impulse discharge. We used data from single fibers and single TCs that were defined by the following criteria: 1) the number of spikes evoked by taste stimulation was larger than the mean plus 2 standard deviations of the spontaneous discharge and 2) at least +15 spikes (for 60 seconds) were evoked by taste stimulation. Two-way ANOVA and *post hoc t* test were used to statistically evaluate the difference between WT and GLP-1R$^{-/-}$ mice. One-way ANOVA and *post hoc* Tukey honest significant difference test were used to statistically evaluate the differences in responses to intravenous injection of GLP-1 among each type of fiber. Student's *t* test was used to statistically evaluate the GLP-1 release from TCs, the effect of intravenous injection of GLP-1 and the difference between WT and GLP-1R$^{-/-}$ mice. Statistical analyses were performed using the statistical software packages IBM SPSS Statistics (IBM, White Plains, NY, USA).

RESULTS

GLP-1R$^{-/-}$ mice have reduced responses to sweeteners

We first recorded gustatory whole-nerve responses to taste stimuli representing different taste qualities from the CT

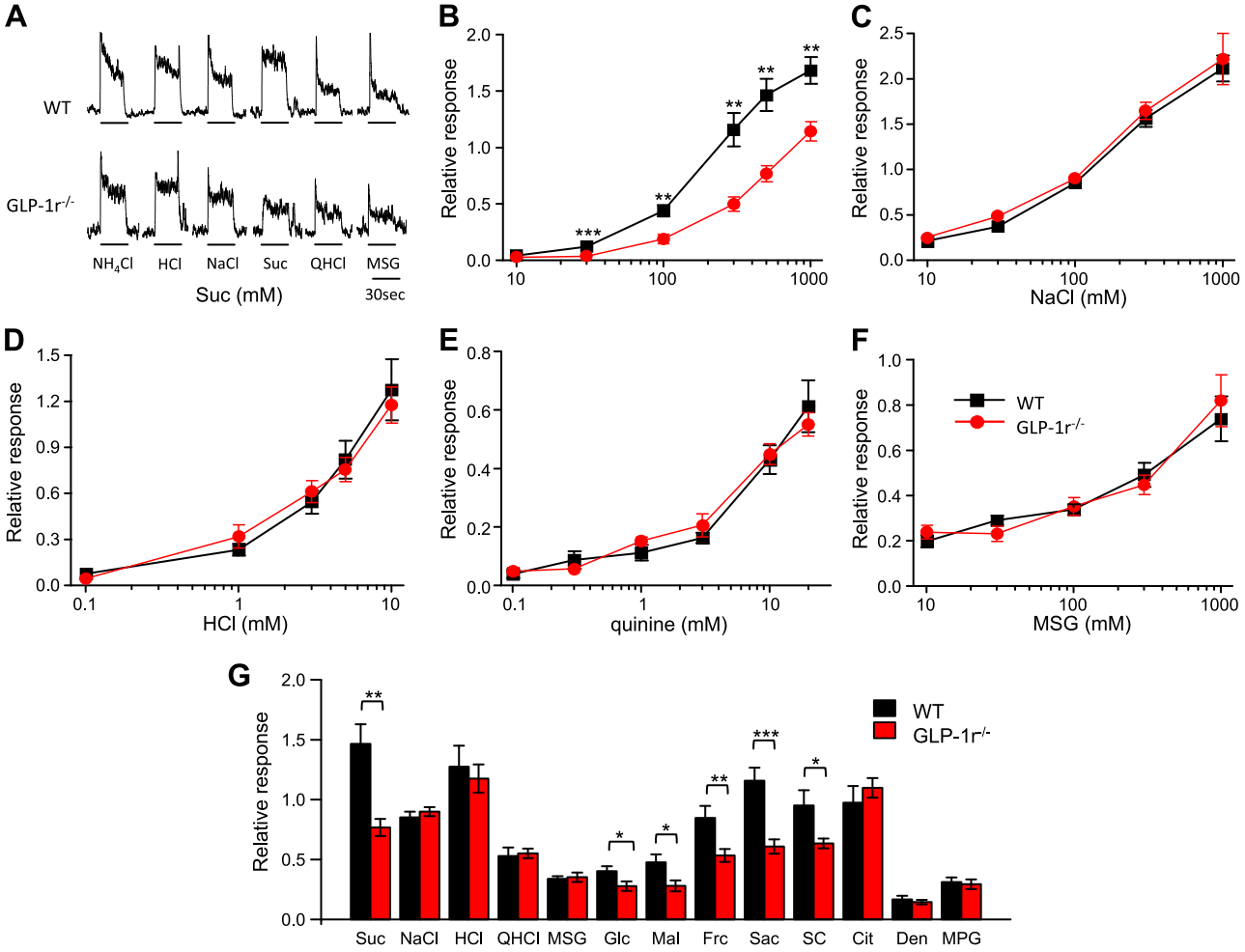

Figure 1. Reduction of CT nerve responses to sweet stimuli in GLP-1R$^{-/-}$ mice. *A*) Typical examples of CT nerve responses of WT and GLP-1R$^{-/-}$ mice to various tastants. *B–F*) Concentration-dependent CT nerve responses to sucrose (*B*), NaCl (*C*), HCl (*D*), quinine (*E*), and MSG (*F*) in WT (black squares, $n = 5–8$) and GLP-1R$^{-/-}$ (red circles, $n = 5–6$) mice. *G*) CT nerve responses of WT (black) and GLP-1R$^{-/-}$ (red) mice stimulated by sweet (Suc: 500 mM sucrose, Glc: 500 mM glucose, Mal: 500 mM maltose, Frc: 500 mM fructose, Sac: 20 mM saccharin, SC: 1 mM SC45647), salty (NaCl: 100 mM NaCl), sour (HCl: 10 mM HCl, Cit: 10 mM citric acid), bitter (QHCl: 20 mM quinine-HCl, Den: 20 mM denatonium), and umami (MSG: 100 mM MSG + 100 μM amiloride, MPG: 100 mM MPG) compounds ($n = 5–7$). All data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test.

and GL nerves of WT and GLP-1R$^{-/-}$ mice, finding that nerve responses to sweeteners were smaller in GLP-1R$^{-/-}$ mice than in WT mice (**Figs. 1 and 2**). The CT nerve responses to 30–1000 mM sucrose were significantly smaller in GLP-1R$^{-/-}$ mice than in WT mice (Fig. 1*B*, $F_{1,72} = 43.4$, $P < 0.001$, ANOVA). In contrast, the CT nerve responses to 10–1000 mM NaCl ($F_{1,50} = 1.2$, $P > 0.1$), 0.1–10 mM HCl ($F_{1,46} = 0.02$, $P > 0.1$), 0.1–20 mM quinine-HCl (QHCl, $F_{1,65} = 0.02$, $P > 0.1$), and 10–1000 mM MSG + 100 μM amiloride ($F_{1,49} = 0.03$, $P > 0.1$) did not differ significantly in GLP-1R$^{-/-}$ mice from those in WT mice (Fig. 1*C–F*); similar results were obtained in the GL nerve responses (Fig. 2). CT nerve responses to other sweeteners tested (glucose, maltose, fructose, saccharin, and SC45647) were also smaller in GLP-1R$^{-/-}$ mice than in WT mice (Fig. 1*G*).

Next, we used a short-term lick test to measure behavioral responses to sweet stimuli of GLP-1R$^{-/-}$ and WT mice. To more clearly detect concentration-dependent changes in lick rates for preferred solutions, we used as test solutions mixtures of 1 mM quinine with 30–1000 mM sucrose, 0.1–20 mM saccharin, or 10–300 mM MSG (following a sweet-bitter mixture paradigm procedure) (33). In both WT and GLP-1R$^{-/-}$ mice, clear concentration dependent changes in lick rates were observed for NaCl, HCl, quinine (decreasing); and for MSG, sucrose and saccharin (increasing) (**Fig. 3**). Mean lick rates for sucrose-quinine mixtures and saccharin-quinine mixtures were significantly smaller in GLP-1R$^{-/-}$ mice than in WT mice ($F_{1,174} = 74.4$, $P < 0.001$ for sucrose, $F_{1,174} = 70.7$, $P < 0.001$ for saccharin, Fig. 3*A*, *B*). Mean lick rates for NaCl ($F_{1,174} = 0.58$, $P > 0.1$), HCl ($F_{1,145} = 0.79$, $P > 0.1$), quinine ($F_{1,145} = 0.07$, $P > 0.1$), and MSG ($F_{1,145} = 0.24$, $P > 0.1$) were not significantly different between WT and GLP-1R$^{-/-}$ mice (Fig. 3*C–F*). Together, the nerve and behavior data demonstrate a specific reduction of sweet responses in GLP-1R$^{-/-}$ mice, indicating that GLP-1 plays a role in peripheral gustatory responses to sweet compounds but not to nonsweet compounds.

Sweet tastants evoke GLP-1 secretion from sweet-sensitive TCs

To determine if GLP-1 might be released from TCs, we first used immunohistochemistry to examine its pattern of expression in TCs, similarly to a previous study (25). We used T1R3, a component of sweet and umami receptors, as a marker for sweet TCs, and GAD67 as a marker for type III TCs. In WT mice ~50–53% TCs expressing GLP-1 co-expressed T1R3; ~25–28% of TCs expressing T1R3 also expressed GLP-1; ~13–16% of TCs expressing GLP-1 co-expressed GAD67; ~10–14% of TCs expressing GAD67 expressed GLP-1 (**Fig. 4*A*, *B***, and **Table 1**). We also examined the distribution of GLP-1R in taste tissues and gustatory neurons. In the GG of WT mice, a subset of neurons expressed GLP-1R but such GLP-1R expression was not observed in neurons in the GG of GLP-1$^{-/-}$ mice (Fig. 4*C*). If primary antibodies were omitted, we did not detect any immunohistochemical signal in

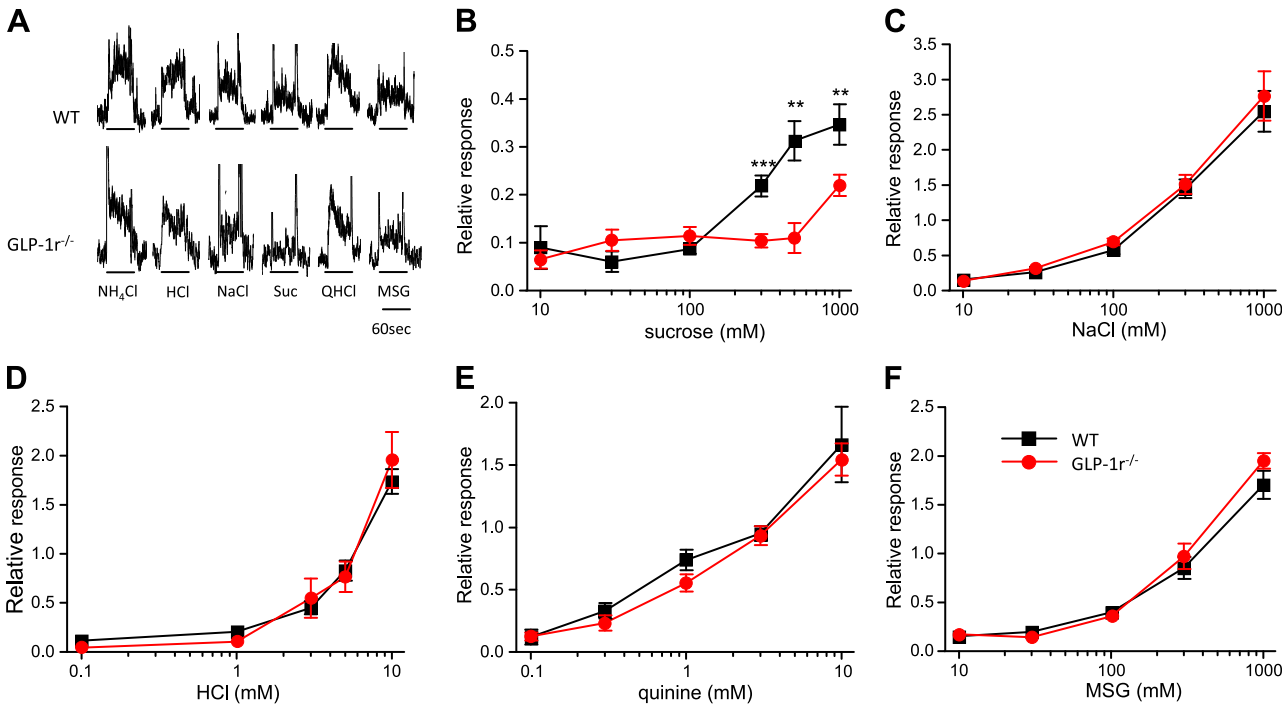

Figure 2. Reduction of GL nerve responses to sweet stimuli in GLP-1R$^{-/-}$ mice. *A*) Typical examples of GL nerve responses of WT and GLP-1R$^{-/-}$ mice to various tastants. *B–F*) Concentration-dependent GL nerve responses to sucrose (*B*), NaCl (*C*), HCl (*D*), quinine (*E*), and MSG (*F*) in WT (black squares, $n = 5–8$) and GLP-1R$^{-/-}$ (red circles, $n = 5–7$) mice. Responses to sucrose were significantly smaller in GLP-1R$^{-/-}$ mice than in WT mice ($F_{1,58} = 18.2$, $P < 0.001$ for sucrose, $F_{1,45} = 0.99$, $P > 0.1$ for NaCl, $F_{1,49} = 0.04$, $P > 0.1$ for HCl, $F_{1,49} = 1.18$, $P > 0.1$ for quinine, $F_{1,48} = 1.52$, $P > 0.1$ for MSG, ANOVA). All data are presented as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test.

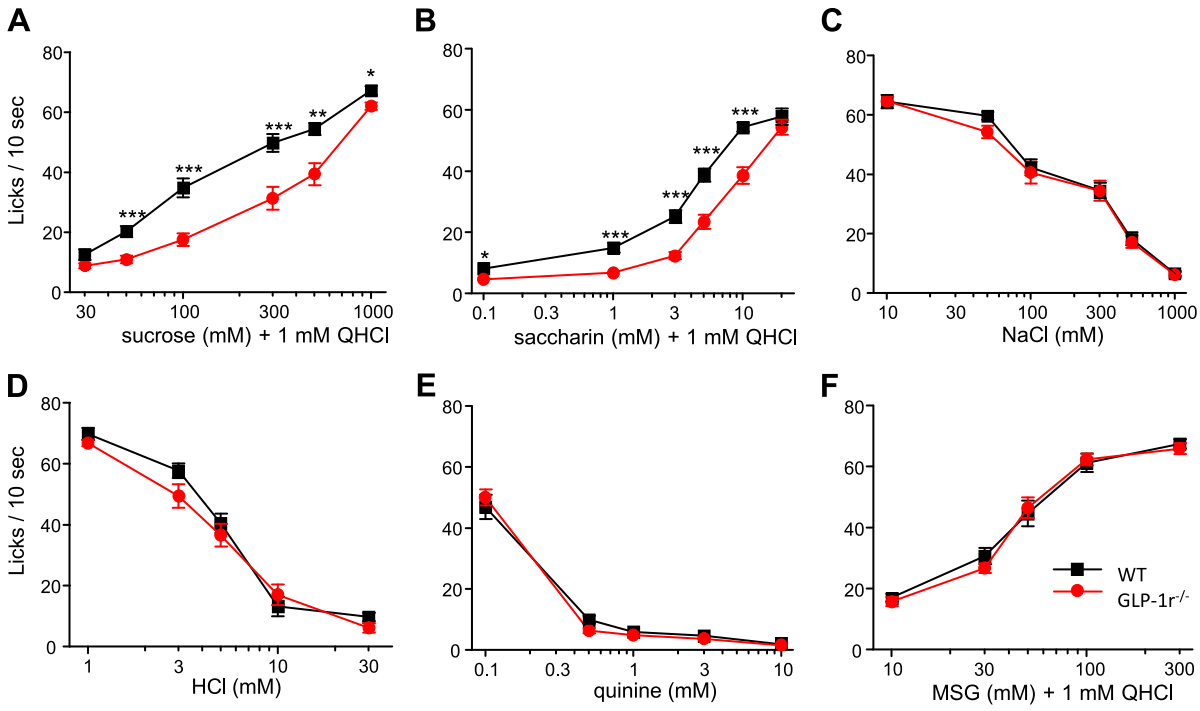

Figure 3. Reduction of lick responses to sweet stimuli in GLP-1R$^{-/-}$ mice. *A–F*) Concentration-dependent lick responses to sucrose + 1 mM quinine (*A*), saccharin + 1 mM quinine (*B*), NaCl (*C*), HCl (*D*), quinine (*E*), MSG + 100 μM amiloride + 1 mM quinine (*F*) in WT (black squares, $n = 15$) and GLP-1R$^{-/-}$ (red circles, $n = 16$) mice. All data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Student's *t* test.

either taste tissues or GG (Supplemental Fig. 1). These results indicate that a subset of TCs and gustatory nerve fibers express GLP-1 and GLP-1R, respectively.

Next we determined if GLP-1 could be released from an individual taste bud after apical application of tastants. The basolateral side of an entire single FP taste bud was covered by a collecting pipette (inner φ 30 μm) to collect secreted GLP-1, then taste stimuli were applied to the apical side for 60 s and the GLP-1 concentration of the solution in the collecting pipette was analyzed by ELISA (**Fig. 5*A*** inset). GLP-1 concentration in the collecting pipette solution was significantly increased when taste buds were stimulated with sweet compounds such as saccharin, sucrose, and glucose (Fig 5*A*). The effect of sweet taste stimuli was concentration dependent; higher concentrations of sweet taste stimuli produced higher GLP-1 concentrations in the collecting pipette. In contrast, the GLP-1 concentration in the collecting pipette after stimulation with salty (NaCl), sour (HCl), bitter (quinine-HCl), or umami (MSG) compounds was not significantly different from that after stimulation with DW (Fig. 5*A*).

We then set out to measure tastant-stimulated GLP-1 release from an individual TC. In this case, a thin recording electrode (inner φ 3–5 μm) was used to record taste responses and simultaneously collect secreted GLP-1 from a single TC with a similar procedure to that described previously (8). To identify sweet-sensitive and bitter-sensitive TCs, T1R3-GFP mice and gustducin-GFP mice were used, respectively (5, 30). We collected the recoding electrode solution from T1R3-GFP TCs, which responded to the application of sweet-tasting solutions, and from gustducin-GFP TCs, which showed responses to stimulation with bitter-tasting solutions (Fig. 5*B*). The mean response magnitude was not significantly different between sweet-responsive T1R3-GFP TCs (52.2 ± 5.67 impulses/60 s) and bitter-responsive gustducin-GFP TCs (53.7 ± 5.03 impulses/60 s, *t* test, $P > 0.1$), whereas the mean amount of released GLP-1 was significantly higher in sweet-sensitive TCs than in bitter-sensitive TCs (Fig. 5*C*). Thus, apical stimulation with sweet compounds elicits GLP-1 release from sweet-sensitive TCs.

GLP-1 activates sweet-sensitive CT nerve fibers

To determine whether GLP-1 secreted from sweet-sensitive TCs would activate particular types of gustatory nerve fibers, we recorded single CT fiber responses to various taste stimuli and to intravenous injection of GLP-1. Among 40 CT fibers tested, 17, 5, 5, and 13 fibers were classified as S-type (sweet-best), N-type (NaCl-specific), M-type (MPG-best), and E-type (electrolyte-responsive), respectively. Among them, a subset of S-type fibers showed large transient responses (more than 30 impulses/10 s) to intravenous injection of GLP-1 (**Fig. 6*A–C***). Responses of S-type fibers to intravenous injection of GLP-1 were significantly greater than those of other type of fibers (Fig. 6*D*, *E*). S-type fibers could be further classified into GLP-1-sensitive and -insensitive fibers (**Fig. 7*A***). In GLP-1-sensitive S-type fibers, increases in impulse discharges started 41.7 ± 8.0 s after intravenous injection of GLP-1 (Fig. 6*A*, $n = 7$). This time course was similar to changes in plasma GLP-1 concentration after intravenous injection of GLP-1 (Fig. 7*B*). We

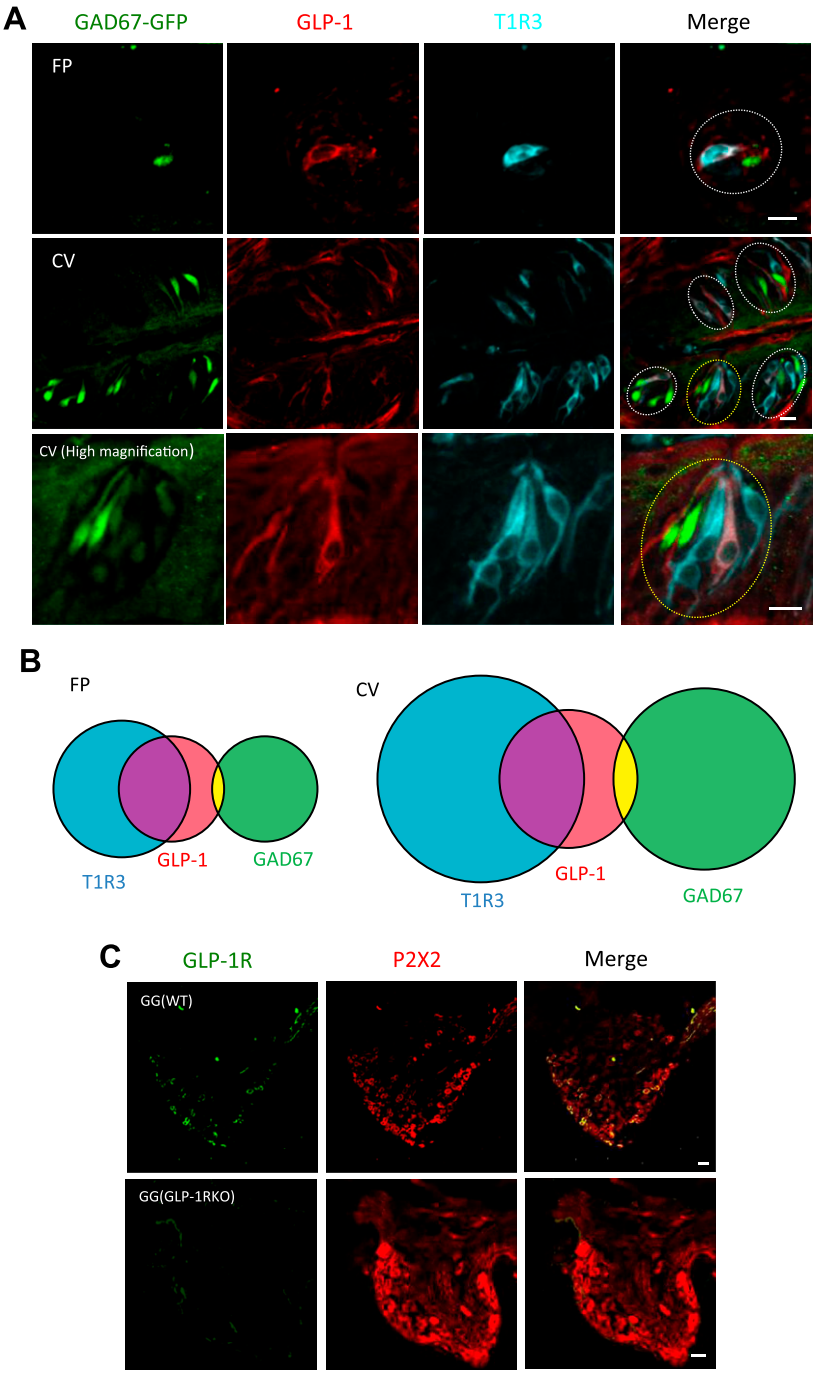

Figure 4. Presence of GLP-1 in TCs and GLP-1R in gustatory neurons. *A*) Immunofluorescence of GAD67-GFP (green), GLP-1 (red), T1R3 (cyan), and merged images in mouse FP (upper) and CV (lower) papillae taste buds. *B*) Quantitative diagrams of the overlapping distribution patterns of the expression of T1R3, GAD67, and GLP-1 in FP (left) and CV (right). Each circle is quantitatively proportional to the number of cells expressing each peptide. *C*) Immunofluorescence of GLP-1R (green) and P2X2 (red) in the geniculate ganglion (GG) of WT (upper) and GLP-1R$^{-/-}$ (lower) mice. No GLP-1R signal was observed in the GG of GLP-1R$^{-/-}$ mice. Scale bars, 10 μm.

tested whether responses of S-type fibers to intravenous injection of GLP-1 were suppressed by pre-administration of Exendin-4(3-39), which is an analog of GLP-1 that acts as a potent GLP-1R antagonist (32). Indeed, GLP-1 responses of S-type fibers were blocked by pre-administration of Exendin-4(3-39) (**Fig. 8**). These results indicate that GLP-1 activates a subset of sweet-sensitive gustatory nerve fibers via GLP-1R.

DISCUSSION

In the present study, first we demonstrated that GLP-1R$^{-/-}$ mice exhibited reduced CT and GL nerve responses to sweet stimuli. GLP-1R$^{-/-}$ mice exhibited ~60–70% of the nerve responses to sweet compounds found in WT (Fig. 1*G*, 2). Consistently, in immunohistochemical assays, around a quarter of T1R3 immunopositive TCs in both FP and CV also express GLP-1 (Fig. 4*A*, Table 1), implying that GLP-1 might be related to only a portion of sweet taste signaling. A previous study reported that GLP-1R$^{-/-}$ mice display reduced sensitivity to sweeteners in brief-access tests, and our study corroborated their results (35). We observed a decrease specifically of sweet taste sensitivity in short-term lick tests (Fig. 3), which corresponded well with the results of nerve recordings (Figs. 1, 2). Furthermore, the only significant increases in GLP-1 release from taste buds were in response to sweet-

TABLE 1. *Summary of immunohistochemical data on detection of GLP-1, T1R3, and GAD67-GFP in FP and CV taste buds*

	FP	%	CV	%
GLP-1/GAD67-GFP	15/109	13.8	39/397	9.8
GLP-1/T1R3	56/202	27.7	129/524	24.6
GAD67-GFP/GLP-1	15/113	13.2	39/244	16
T1R3/GLP-1	56/113	49.6	129/244	52.9

The value on the right of the slash (/) is the number of immunopositive or GFP-positive cells for the given marker. The value on the left of the slash is the number of cells expressing both markers.

tasting compounds (Fig. 5). Thus GLP-1 may be involved specifically in sweet taste sensitivity. In CV and FP taste buds, GLP-1 was expressed most often in type II cells, but also in some type III cells (13.8% and 9.8% of GAD-GFP cells in FP and CV, respectively: Table 1). It has been reported that all type III cells express the polycystic kidney disease 2-like 1 protein (36), which is a candidate sour receptor (37, 38), although in both behavioral and neural assays, we found no significant differences of sour responses between WT and GLP-1R$^{-/-}$ mice. The function of GLP-1 in type III cells is presently unclear; perhaps it relates to transmission of sweet coding information between type II and type III cells, but it does not impact on sour taste sensitivity of mice.

In behavioral assays, mice can perceive and discriminate the quality and intensity of taste stimuli within a couple of seconds (a few licks); therefore, we focused on the role of GLP-1 in immediate responses to taste stimuli. In a previous study, glucose stimulation evoked biphasic GLP-1 release from STC-1 cells, with the first phase starting immediately after stimulation and some GLP-1 being released within 1 minute from exocytotic granules (39). In the present study, sweet-sensitive TCs showed GLP-1 release in response to sweet taste stimuli within 60 s (Fig. 5). Next, to examine the direct effect of GLP-1 on taste nerve fibers, we applied GLP-1 from the femoral vein. It has already been shown that i.v. injection of a transmitter candidate, such as norepinephrine, can affect the activity of taste nerves directly (40). We found that the injected GLP-1 elicited a transient increase of nerve activities in a subset of sweet-best (S-type) gustatory nerve fibers (Fig. 6) through the activation of GLP-1Rs, which are expressed on a subset of gustatory neurons (Fig. 4*C*). In most tissues, GLP-1 is quickly degraded in a time-dependent manner by the degradative enzyme DPP4 widely expressed in blood vessels (41). However, taste buds do not appear to express DPP4 (25). In our preparation, as the blood concentration of GLP-1 rapidly decreased (Fig. 7*B*), the impulse discharge of single nerve fibers likewise rapidly disappeared (Fig. 6*A*). Thus, in the microenvironment around a taste bud, blood circulation might provide enough local DPP4 to inactivate GLP-1 from TCs. Altogether, GLP-1 would be released from TCs just after sweet taste stimulation and could activate its receptors on adjacent sweet-sensitive nerve fibers, finally being quickly inactivated by DPP4 to limit signal duration. These observations are consistent with GLP-1 functioning as

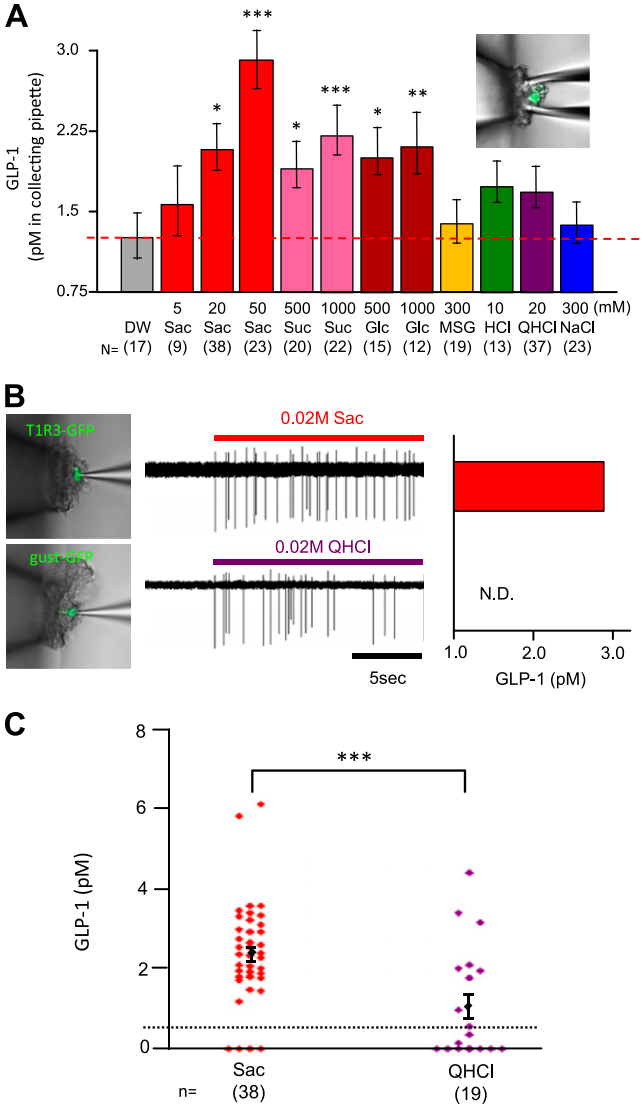

Figure 5. GLP-1 secretion from sweet-sensitive TCs. *A*) GLP-1 secretion from single taste buds. Apical side of an individual taste bud was stimulated for 60 seconds with DW, saccharin (Sac; 5, 20, or 50 mM Sac), sucrose (Suc; 500 or 1000 mM Suc), glucose (GLC; 500 or 1000 mM Glc), MSG (300 mM MSG), HCl (10 mM HCl), quinine-HCl (20 mM QHCl), or NaCl (300 mM NaCl) then the GLP-1 concentration in a collecting pipette was analyzed by ELISA. Inset shows the set up for GLP-1 collection (green: T1R3-GFP fluorescence). Differences from DW: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's t test. *B*) Representative GLP-1 release elicited by sweet or bitter stimulus from individual GFP-positive cells of T1R3-GFP (upper) or gustducin-GFP (lower) mice. The GLP-1 collection setup (left), taste response measurements (middle), and GLP-1 concentration measured in the recording electrode solution (right) are shown. N.D., not detectable (detection limit, 0.47 pM). *C*) Summary of GLP-1 release from single sweet- (T1R3-GFP) or bitter-sensitive (gustducin-GFP) TCs. The dotted line indicates 0.47 pM. All data are presented as the mean ± SEM. $***P < 0.001$, Student's t test.

a specific neurotransmitter for sweet taste in peripheral taste signaling.

The importance of ATP has been well established in signal transmission in the peripheral taste system; gustatory nerve fibers express ionotropic purinergic receptors,

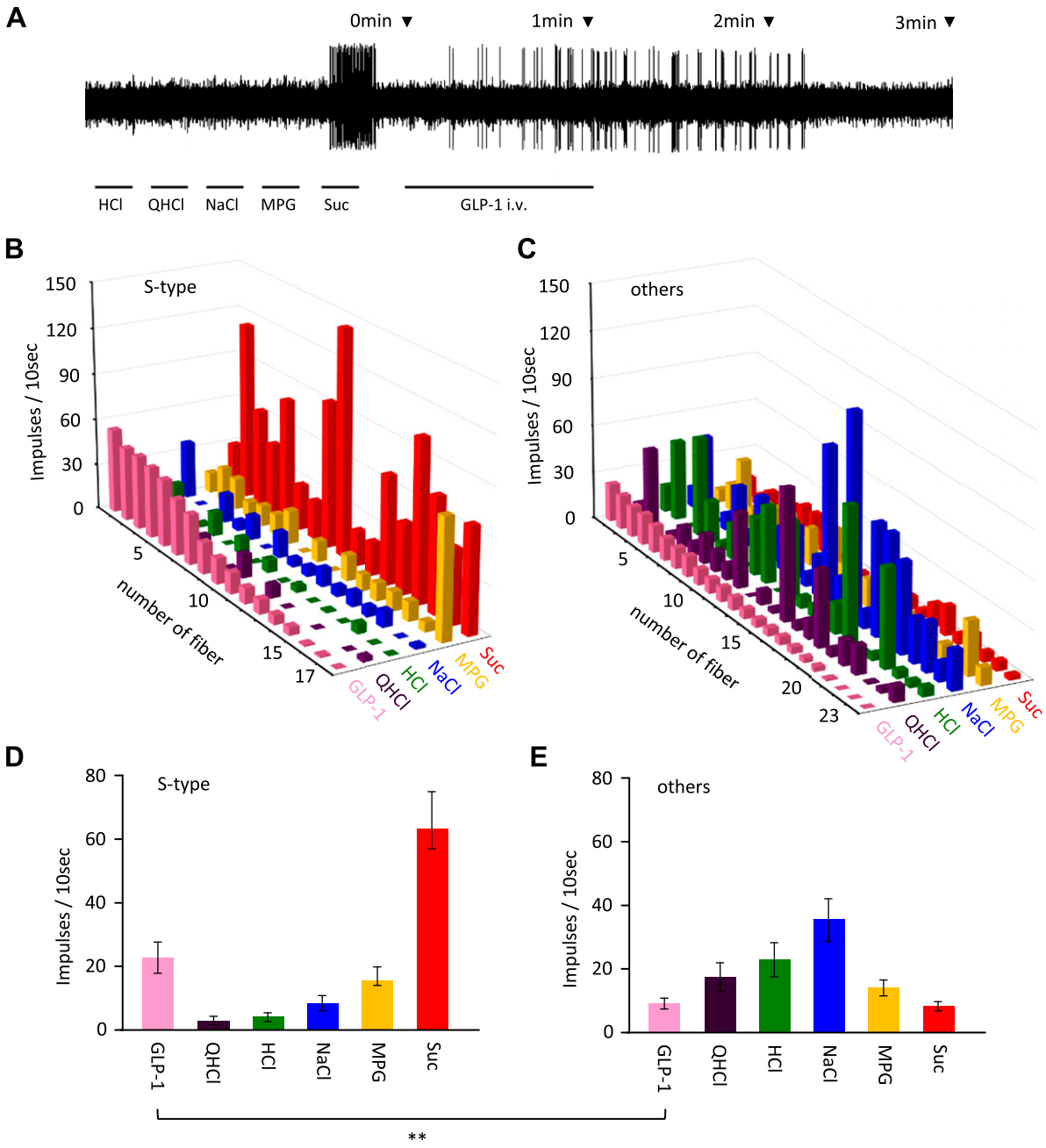

Figure 6. GLP-1 activates sweet-best S-type gustatory nerve fibers. *A*) A representative recording from a sweet-best CT fiber responding to tastants and intravenous injection of GLP-1. Taste stimuli were 10 mM HCl, 20 mM QHCl, 100 mM NaCl, 100 mM MPG, and 500 mM sucrose (Suc). GLP-1 (20 μg/kg body weight) was injected from the femoral vein. *B, C*) Response profiles of S-type (*B*) and single fibers other than S-type ("others") (*C*) in the CT nerve of WT mice. CT fibers are arranged according to the magnitude of response to GLP-1 injection. *D, E*) Summary of responses to tastants (10 mM HCl, 20 mM QHCl, 100 mM NaCl, 100 mM MPG, 500 mM Suc) and intravenous injection of GLP-1 in S-type (*D*, $n = 17$) and other types (*E*, $n = 23$) of single CT fibers. All data are presented as the mean ± SEM. $^{**}P < 0.01$, Student's *t* test.

P2X2/X3 (42), and mice genetically lacking these ATP receptors show markedly diminished nerve responses to all taste qualities (10). ATP is released from type II cells in response to sweet, bitter, or umami taste stimuli, although it is not clear whether salty- and sour-sensitive TCs release ATP in response to taste stimulation (7, 8). In addition, NTPDase2 knockout mice show an accumulation of ATP in taste tissue and a concomitant decrease of neural taste responses (43). All of these data indicate that ATP plays a crucial role in taste signal transmission. According to present and previous studies, subsets of sweet responsive type II TCs might release both GLP-1 and ATP simultaneously, and both are capable of independently producing a transient increase of nerve activities. Additionally, GLP-1Rs are expressed in gustatory nerves, but not on TCs. All GG neurons in rodents express P2X3, and approximately 60% of neurons express P2X2 (44), whereas only a subset of GG neurons express both GLP-1R and P2X2 (Fig. 4*C*). Taken together, GLP-1 is likely to play a role as an ancillary but functionally important neurotransmitter in

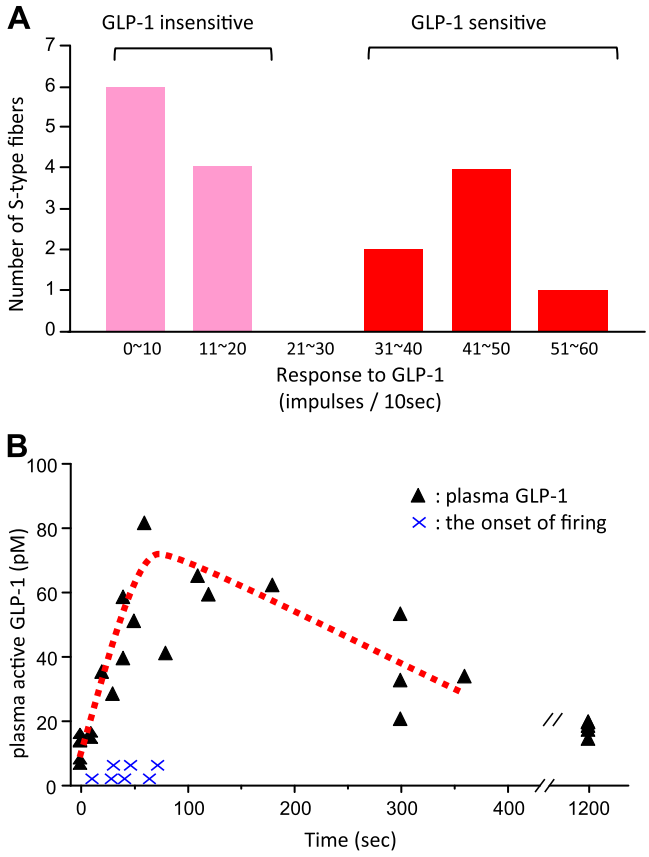

Figure 7. The effect of intravenous injection of GLP-1. *A*) GLP-1-sensitive and -insensitive S-type fibers. S-type fibers that elicited more than 30 impulses/10 s in response to i.v. injection of GLP-1 were classified as GLP-1-sensitive fibers. *B*) Time-dependent changes in plasma GLP-1 level after intravenous injection of GLP-1 (triangles). Mice were administrated with 20 μg/kg GLP-1 from the femoral vein. Data were obtained from 5 WT mice. The onsets of firing activities after i.v. injection of GLP-1 in GLP-1-sensitive S-type fibers were indicated by X marks (n = 7).

cooperation with ATP, and this signaling might be required for maximal activation of sweet nerve fibers.

Aside from GLP-1, taste bud cells also express several bioactive peptides. For example, glucagon is frequently co-expressed with T1R3 and the glucagon receptor (14) and sweet taste stimuli inhibit basal glucagon secretion from taste epithelia (17), suggesting that glucagon may function as a feedback signal in sweet-sensitive TCs. NPY, VIP, and cholecystokinin (CCK) are often co-expressed in taste buds and the majority of TCs expressing these peptides co-expressed gustducin but not T1R2 (45), suggesting that these may be bitter-sensitive TCs, although NPY is released from taste buds in response to sour and salty taste stimuli (17). Exogenous application of CCK inhibits K^+ currents in isolated TCs (46) and CCK-sensitive cells show Ca^{2+} responses to bitter taste stimuli (47), indicating that CCK may regulate bitter sensitivity of TCs in an autocrine fashion. Exogenous application of NPY activates K^+ currents in isolated TCs (13). The VIP receptor is expressed in type II cells (18). Thus, NPY, VIP, and CCK may also function as autocrine or paracrine signals in taste buds. In addition, it is possible that these peptides may be involved in signal transmission of particular taste qualities from TCs to gustatory nerve fibers.

We classified S-type fibers into 2 groups according to their responsiveness to GLP-1 (Fig. 7). Previous reports had demonstrated that S-type nerve fibers can be classified into at least 2 distinct groups according to their sensitivity to gurmarin (48), which is a sweet taste inhibitor in rodents (49), suggesting that the variation in responsiveness to GLP-1 by S-type fibers might correlate with their sensitivity to gurmarin. However, further studies are required to validate this possibility.

GLP-1 is secreted from intestinal endocrine L-cells, which also express taste signaling elements such as T1R2, T1R3, gustducin, PLCβ2, and transient receptor potential cation channel subfamily M member 5. Gustducin knockout mice are deficient in intestinal secretion of GLP-1 after ingestion of glucose, and GLP-1 release from the human L-cell line NCI-H716 cells was promoted by sugars and the noncaloric sweeteners and blocked by the sweet receptor antagonist lactisole (21), suggesting that sweet taste-signaling components contribute to GLP-1 release in enteroendocrine L-cells. However, in *in vivo* experiments, ingestion of artificial sweeteners alone did not affect secretion of GLP-1 (50, 51), indicating that *in vivo* GLP-1 may not be secreted in response to activation of T1R2/T1R3-dependent transduction pathways alone. To induce GLP-1 release from enteroendocrine L-cells *in vivo*, another mechanism such as glucose sensing by glucose transporters and K_{ATP} channels may be required in addition to T1R signaling pathways. In contrast, sweet-sensitive TCs released GLP-1 in response to both sugars (glucose and sucrose) and a noncaloric sweetener (saccharin) (Fig. 5). Reduction of gustatory nerve responses to various sweeteners in GLP-1R$^{-/-}$ mice (Fig. 1) suggests that multiple sweet compounds including noncaloric sweeteners induce GLP-1 release from sweet-sensitive TCs.

GLP-1 stimulates insulin secretion from pancreatic islet β cells and suppresses glucagon secretion from α cells via GLP-1Rs on these cells, thereby functioning to reduce the level of plasma glucose. However, it is not clear if GLP-1 released from TCs affects secretion of these hormones and/or blood glucose levels. In rats and healthy humans, oral infusion of artificial sweeteners does not increase plasma GLP-1 levels (50, 51). The amount of GLP-1 released from TCs may be small or limited locally, thus systemic effects of GLP-1 released from TCs may be below detectable levels. GLP-1 released from enteroendocrine cells is also able to activate the vagus nerve (22, 23), which may in turn activate neurons in the nucleus of the solitary tract (NTS). Such signals may be reflexively transmitted to pancreas, stomach, and intestine to regulate the secretion of hormones (23). Neural signals elicited by GLP-1 released from TCs may also contribute to this reflex pathway because these signals are also transmitted to NTS via the CT and the GL nerves. During a meal, GLP-1-induced neural signals would occur in turn from the tongue, intestine, and then portal vein. Such timing-dependent neural signals and/or cumulative neural signals to the NTS may be crucial for the regulation of hormonal releases and glucose homeostasis.

How might sweet-sensitive TCs secrete GLP-1? Sweet-sensitive TCs release ATP in response to sweet stimuli via nonvesicular mechanisms, such as pannexin or connexin

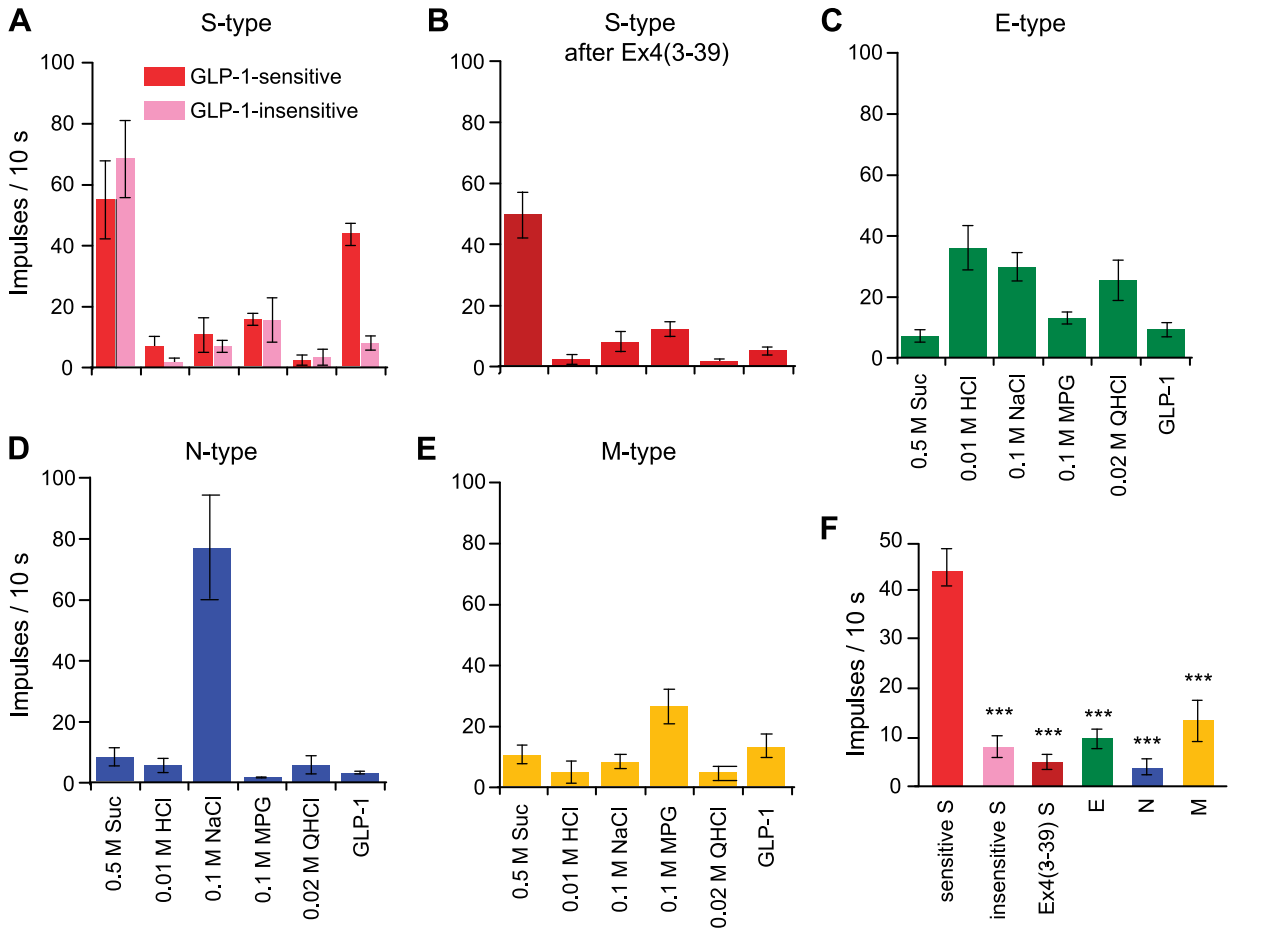

Figure 8. Summary of single-fiber responses. *A–E*) Response profiles of S-type (GLP-1-sensitive: $n = 7$, GLP-1-insensitive: $n = 10$), S-type after Exendin 4(3-39) treatment ($n = 9$), electrolytes-responsive type (E-type, $n = 13$), NaCl-specific type (N-type, $n = 5$), and MPG-best type (M-type, $n = 5$) single fibers in the CT nerve of WT mice. *F*) Mean response to intravenous injection of 20 μg/kg body weight of GLP-1 in S- [GLP-1-sensitive, -insensitive and with pretreatment of Exendin 4(3-39)], E-, N-, and M-type single CT fibers. Suc, sucrose. All data are presented as the mean ± SEM. ***$P < 0.001$, post hoc Tukey honest significant difference test, ANOVA: $F_{5,43} = 38.5$, $P < 0.001$).

hemichannels, and CALHM1 (6–9). However, these channels would not allow peptide hormones like GLP-1 to pass through their pore. In STC-1 cells, initial secretion of GLP-1 is released from two types of granules, previously docked granules and newcomers (39). Such vesicular mechanisms are likely to be involved in GLP-1 secretion from TCs. However, type II cells do not show voltage- and Ca^{2+}-dependent increases in capacitance (52). At present there is no histochemical evidence for vesicles containing GLP-1 in TCs. Further studies are required to reveal the mechanisms underlying GLP-1 release from sweet-sensitive TCs.

Our results suggest that GLP-1, an incretin hormone that regulates pancreatic hormone release and thereby glucose homeostasis, also plays a role in the peripheral sweet taste system, which is important for the detection of carbohydrates in caloric foods and drinks. Thus, GLP-1 may function as an important glucostatic hormone to link brain, gut, and taste systems. FJ

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