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Modulation of sweet taste sensitivities by endogenous leptin and endocannabinoids in mice

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Key points

- Potential roles of endogenous leptin and endocannabinoids in sweet taste were examined by using pharmacological antagonists and mouse models including leptin receptor deficient (\(db/db\)) and diet-induced obese (DIO) mice.
- Chorda tympani (CT) nerve responses of lean mice to sweet compounds were increased after administration of leptin antagonist (LA) but not affected by administration of cannabinoid receptor antagonist (AM251).
- \(db/db\) mice showed clear suppression of CT responses to sweet compounds after AM251, increased endocannabinoid levels in the taste organ, and enhanced expression of a biosynthesizing enzyme of endocannabinoids in taste cells.
- The effect of LA was gradually decreased and that of AM251 was increased during the course of obesity in DIO mice.
- These findings suggest that circulating leptin, but not local endocannabinoids, is a dominant modulator for sweet taste in lean mice and endocannabinoids become more effective modulators of sweet taste under conditions of deficient leptin signalling.

Abstract

Leptin is an anorexigenic mediator that reduces food intake by acting on hypothalamic receptor Ob-Rb. In contrast, endocannabinoids are orexigenic mediators that act via cannabinoid CB\(_1\) receptors in hypothalamus, limbic forebrain, and brainstem. In the peripheral taste system, leptin administration selectively inhibits behavioural, taste nerve and taste cell responses to sweet compounds. Opposing the action of leptin, endocannabinoids enhance sweet taste responses. However, potential roles of endogenous leptin and endocannabinoids in sweet taste remain unclear. Here, we used pharmacological antagonists (Ob-Rb: L39A/D40A/F41A (LA), CB\(_1\): AM251) and examined the effects of their blocking activation of endogenous leptin and endocannabinoid signalling on taste responses in lean control, leptin receptor deficient \(db/db\), and diet-induced obese (DIO) mice. Lean mice exhibited significant increases in chorda tympani (CT) nerve responses to sweet compounds after LA administration, while they showed no significant changes in CT responses after AM251. In contrast, \(db/db\) mice showed clear suppression of CT responses to sweet compounds after AM251, increased endocannabinoid (2-arachidonoyl-sn-glycerol (2-AG)) levels in the taste organ, and enhanced expression of a biosynthesizing enzyme (diacylglycerol lipase \(\alpha\) (DAGL\(\alpha\))) of 2-AG in taste cells. In DIO mice, the LA effect was gradually decreased and the AM251 effect was increased during the course of obesity. Taken together, our results suggest that circulating leptin, but not local endocannabinoids, may...
be a dominant modulator for sweet taste in lean mice; however, endocannabinoids may become more effective modulators of sweet taste under conditions of deficient leptin signalling, possibly due to increased production of endocannabinoids in taste tissue.

Excessive production of endocannabinoids in taste buds may be a dominant modulator for sweet taste in lean mice; however, endocannabinoids may become more effective modulators of sweet taste under conditions of deficient leptin signalling, possibly due to increased production of endocannabinoids in taste tissue.

Introduction

Taste is primarily devoted to the selection of foods and required nutrients, and the avoidance of potentially harmful compounds. Sweet sensing is thought to be essential for the detection of carbohydrate sources of calories. Sensory input from sweet sensing cells to the brain may serve as a signal that can provoke palatability of food to stimulate food intake, and facilitate secretion of insulin in the pancreas and other factors in the gut involved in regulating nutrient absorption and energy homeostasis. Recent studies reveal that the peripheral sweet-sensing system is modulated by the anorexigenic mediator leptin (Kawai et al. 2000) and the orexigenic endocannabinoids 2-arachidonoyl-sn-glycerol (2-AG) and anandamide (N-arachidonoylthanolamine (AEA)) (Yoshida et al. 2010).

Leptin, an adipocyte-derived hormone, primarily acts on functional leptin receptors (Ob-Rb) in the hypothalamus and reduces food intake, increases energy expenditure, and regulates body weight (Friedman & Halaas, 1998; Friedman 2004). The db/db mice have a point mutation of the db gene, lack functional leptin receptors, and are hyperphagic, massively obese and diabetic (Lee et al. 1996). In the peripheral taste system, taste cells express Ob-Rb and administration of recombinant leptin selectively suppresses taste cell, taste nerve and behavioural responses to sweet compounds without affecting responses to other basic taste stimuli (salty, sour, bitter and umami compounds) in lean control mice (Kawai et al. 2000; Shigemura et al. 2004; Yoshida et al. 2013). The db/db mice show no such leptin inhibition on sweet taste responses, but instead exhibit greater gustatory neural sensitivities (Ninomiya et al. 1995, 1998; Sako et al. 1996) and higher behavioural preferences (Ninomiya et al. 1995) for various sweet compounds compared with lean control mice.

Endocannabinoids act on cannabinoid receptor 1 (CB1 receptor) in the hypothalamus and limbic forebrain to induce appetite (Jamshidi & Taylor, 2001; Cota et al. 2003) and stimulate food intake, which opposes the action of leptin. Systemic administration of exogenous cannabinoids and endocannabinoids in rodents causes hyperphagia (Williams & Kirkham, 1999) and increases the preference for palatable substances, such as sucrose solution and sweetened food pellets (Higgs et al. 2003; Jarrett et al. 2005). Infusions of 2-AG into the pontine parabrachial nucleus, which contains third order gustatory neurons, increase intake of palatable foods, including sucrose pellets, without affecting the intake of normal chow (DiPatrizio & Simansky, 2008), suggesting that endocannabinoids may be related to hedonic aspects of sweet taste. In the peripheral taste system, comparable with their central action, systemic administration of 2-AG or AEA selectively increased taste cell, nerve and behavioural responses to sweeteners without affecting responses to salty, sour, bitter and umami compounds (Yoshida et al. 2010). Mice genetically lacking CB1 receptors showed no such enhancement of sweet taste responses by endocannabinoids and the sweet-enhancing effect was prevented by a CB1 antagonist (Yoshida et al. 2010), indicating that the effect may be mediated by CB1 receptors. Reciprocal regulation of peripheral sweet taste reception by endocannabinoids and leptin may, thus, contribute to their opposing actions on food intake and energy homeostasis. In our previous studies (Kawai et al. 2000; Yoshida et al. 2010), however, reciprocal modulation of sweet taste responses by leptin and endocannabinoids was found only in separate experiments after systemic administration of exogenous leptin and endocannabinoids to mice. Therefore, it remains unclear to what extent endogenous leptin or endocannabinoids tonically affect peripheral taste responsiveness in mice.
In the hypothalamus, leptin inhibits production of endocannabinoids (Jo et al. 2005). Conversely, hypothalamic endocannabinoids are increased in genetically obese rodents lacking functional leptin (ob/ob mice) and leptin receptors (db/db mice and Zucker rats; Di Marzo et al. 2001). These studies suggest that endocannabinoids are normally under negative control by leptin, and might be involved in the hyperphagia and obesity that accompany defects in leptin signalling. A potential site for the interaction between leptin and endocannabinoids is reported in lateral hypothalamic neurons, where leptin inhibits voltage-gated calcium channels, preventing the influx of calcium into cells and blocking endocannabinoid release and synthesis (Jo et al. 2005), a mechanism that contributes to the appetite-suppressing effect of leptin.

This evidence raises the possibility that such interaction between leptin and endocannabinoids could also be involved in modulation of sweet responsiveness of taste cells and the sweet taste modulator could be switched from leptin in lean mice to endocannabinoid in obese mice with defects in leptin signalling.

To answer these questions, we first investigated the effects of pharmacological inhibition of leptin or endocannabinoid receptors on peripheral taste responses in lean control mice with and without food deprivation, and in db/db mice. We treated mice with a leptin receptor antagonist, the leptin triple mutant L39A/D40A/F41A (LA), which is reported to increase food intake of normal chow-fed rats when infused into the lateral ventricle (Zhang et al. 2007), and the CB1 antagonist AM251, which is shown following systemic administration in mice to decrease the intake of a high-fat diet (Hildebrandt et al. 2007).
Furthermore, we investigated the molecular basis for endocannabinoid actions on the taste organ. Finally, we investigated a proposed switch of the sweet taste modulator from leptin to endocannabinoids by comparing LA and AM251 effects on sweet taste responses at different stages of development of diet-induced obese (DIO) mice.

Methods

Ethical approval

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

Animals

The study contains two major experiments: leptin experiments and endocannabinoids experiments. In leptin experiments, the subjects were adult male C57BL/6J (B6, Charles River Laboratories Japan, Yokohama, Japan) mice (8–28 weeks of age, ranging in weight from 23 to 28 g). B6 mice were housed individually in plastic cages and habituated to the animal facility for at least 1 week before the experiments. These mice were either maintained on laboratory chow (normal-fed mice) or fasted for 24 h before experiments (fasted mice, ranging in weight from 25 to 30 g before fasting). In endocannabinoids experiments, subjects were adult male and female B6 (8–20 weeks of age, ranging in weight from 20 to 27 g) and db/db mice (Jackson Laboratory, ME, USA; 8–32 weeks of age, 33–60 g, C57BL/Ksj background). For the generation of DIO mice, 4-weeks-old male B6 mice were fed the high fat diet (HFD, No.D12492, Research Diets, NB, USA) contained 60% kcal as fat for 4–12 weeks. All mice were housed under a 12:12 h light–dark cycle (lights on 08.00–20.00 h) and had ad libitum access to tap water and food pellets unless otherwise indicated.

Recording of gustatory nerve responses

Because mouse responses to sweet substances are much larger in the CT nerve innervating the anterior tongue than in the glossoopharyngeal nerve innervating the posterior tongue (Damak et al. 2003, Talavera et al. 2005) we focused on CT nerve responses. The CT nerve responses to lingual application of tastants were recorded as described previously (Kawai et al. 2000, Damak et al. 2003, Talavera et al. 2005). Mice were anaesthetized with an injection of sodium pentobarbitone (50–60 mg kg\(^{-1}\) i.p.) and maintained at a surgical level of anaesthesia with supplemental injections of sodium pentobarbitone (8–10 mg kg\(^{-1}\) i.p., if needed). The anaesthetic level was evaluated by testing the withdrawal reflex to a paw pinch. Under pentobarbitone anaesthesia, 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 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 entire nerve was placed on the Ag–AgCl electrode. An indifferent electrode was placed in nearby tissue. Neural activities were fed into an amplifier (K-1; Iyodenshikagaku, Nagoya, Japan), and

A, endogenous plasma leptin levels in normal-fed (Normal-fed) and 24 h food deprived (Fasted) mice (n = 6). Bars indicate means ± 95% CI. Symbols indicate significant differences (* P < 0.05, Student’s t test). B–D, concentration–response relationships of CT nerve responses for sucrose (B), saccharin (C) and SC45647 (D) in vehicle-treated normal-fed (Normal-fed, black squares, n = 6–8), vehicle-treated fasted (fasted, white triangles, n = 6–8) or 1 mg (kg BW)\(^{-1}\) of LA-treated fasted (Fasted LA, white circles, n = 6–7) mice. Symbols indicate significant differences (+++, ++++Normal-fed vs. Fasted; ##, ###Normal-fed vs. Fasted LA; ++, ## P < 0.01, ++++, #### P < 0.001, Tukey’s HSD test). All data are presented as the means ± SEM. Results of ANOVA tests are summarized in Table 4.
monitored on an oscilloscope and audiomoniter. Whole nerve responses were integrated with a time constant of 1.0 s and recorded on a computer using a PowerLab system (PowerLab/sp4; AD Instruments, Bella Vista, NSW, Australia). For taste stimulation, the anterior half of the tongue was enclosed in a flow chamber made of silicone rubber. Taste solutions (100 mM NH₄Cl, 100 mM NaCl, 10 mM HCl, 20 mM quinine-HCl (QHCl), 100 mM monosodium L-glutamate (MSG), 30–1000 mM sucrose (Suc), 1–20 mM saccharin (Sac), 0.1–3 mM SC45647 (SC)) were delivered to the tongue by gravity flow for 30 s. The tongue was washed with distilled water (DW) for an interval of ~1 min between successive stimulation. After recording a series of control responses, each mouse was administrated a single i.p. injection of 0.5–2 mg (kg body weight (BW))⁻¹ of LA (L39A/D40A/F41A; Protein Laboratories Rehovot, Rehovot, Israel), 0.1 mg (kg BW)⁻¹ of recombinant murine leptin (PeproTech, NJ, USA), 0.3–3 mg (kg BW)⁻¹ of CB₁ antagonist AM251 (Tocris Bioscience, MN, USA) or vehicle (physiological saline or saline–ethanol, 99:1). The recording of taste responses was continued until a particular point of time (0–120 min after injection). Only responses from stable recordings were used for data analysis. In the analysis of whole nerve responses, integrated whole-nerve response magnitudes were measured 5, 10, 15, 20, and 25 s after stimulus onset, averaged, and normalized to responses to 100 mM NH₄Cl to account for mouse to mouse variations in absolute responses. After the end of the experiment, animals were killed by the administration of an overdose of the anaesthetic. All experiments were performed at room temperature (24–25 °C).

**Measurement of plasma leptin**

After each mouse was anaesthetized with sodium pentobarbitone (50–60 mg kg⁻¹ i.p.), 100 μl of blood was collected from the tail vein and centrifuged for 20 min at 2000 g to collect plasma. Samples were immediately stored at −80°C. Plasma leptin levels were measured using a mouse leptin ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Samples were run in duplicate.

**Measurement of 2-AG**

After each mouse was anaesthetized with sodium pentobarbitone (50–60 mg kg⁻¹ i.p.), the epithelial tissue (containing papillae and lingual aponeurosis) of the whole tongue was peeled off the lingual muscle with fine forceps. The peeled epithelial tissue was separated into three parts:

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**Figure 3. Leptin suppresses gustatory nerve responses to sweeteners in fasted mice**

A–C, concentration–response relationships of CT nerve responses for sucrose (A), saccharin (B) and SC45647 (C) in vehicle-treated (white triangles, n = 6–8) or leptin-treated (100 ng (g BW)⁻¹, black circles, n = 6–8) fasted mice. D, summary of the effect of LA and leptin on CT nerve responses to sweeteners (vehicle-treated normal-fed mice (Normal-fed, white bars, n = 6–8), LA-treated normal-fed mice (Normal-fed LA, light grey bars, n = 6), vehicle-treated fasted mice (Fasted, shaded bars, n = 6–7), LA-treated fasted mice (Fasted LA, dark grey bars, n = 6–8), leptin-treated fasted mice (Fasted Lep, black bars, n = 6–7)). Symbols indicate significant differences (+P < 0.05; ++P < 0.01; +++P < 0.001; Tukey’s HSD test). All data are presented as the mean ± SEM. Results of ANOVA tests are summarized in Tables 5 and 6.
the anterior part containing the majority of fungiform papillae (FP), the middle part without taste papillae, and the posterior part containing a circumvallate papilla (VP) and foliate papillae. Each section of tissue was immediately snap frozen in liquid nitrogen. Frozen tissues were weighed and homogenized in 1 ml of methanol containing the internal standards, $[^3]$H$_2$]-2-arachidonoyl-sn-glycerol (Cayman Chemical, San Diego, CA, USA). Lipids were extracted with chloroform (2 vol) and washed with water (1 vol). Organic phases were collected and fractionated

Figure 4. AM251 suppresses gustatory nerve responses to sweeteners in db/db mice

A, typical examples of CT nerve responses of db/db mice 30 min after i.p. injection of vehicle (upper traces) or 3 mg (kg BW)$^{-1}$ of AM251 (lower traces). B, CT nerve responses of db/db mice to 300 and 500 mM sucrose (Suc), 20 mM saccharin (Sac), 1 mM SC45647 (SC), 20 mM quinine-HCl (QHCl), 100 mM NaCl (NaCl), 10 mM HCl (HCl) and 100 mM MSG (MSG) 10–30 min after administration of vehicle (white bars, n = 5–14) or 3 mg (kg BW)$^{-1}$ of AM251 (black bars, n = 6). Asterisk indicates significant differences, (*P < 0.05, Student’s t test). C, concentration–response relationships of CT responses for sucrose in vehicle-treated (white squares, n = 6) or AM251-treated (3 mg (kg BW)$^{-1}$, black circles, n = 6) db/db mice and vehicle-treated B6 mice (white triangles, n = 6). Symbols indicate significant differences (**P < 0.001, for db/db vehicle vs. db/db AM251, +++P < 0.001 for db/db vehicle vs. B6 vehicle, Tukey’s HSD test). D, CT nerve responses to 300 and 500 mM sucrose (Suc), 20 mM saccharin (Sac), 1 mM SC45647 (SC), 20 mM quinine-HCl (QHCl), 100 mM NaCl (NaCl), 10 mM HCl (HCl), and 100 mM MSG (MSG) in AM251-treated db/db mice (3 mg (kg BW)$^{-1}$, black bars, n = 6) or vehicle-treated B6 mice (white bars, n = 6). E, time-dependent changes in CT nerve responses to 500 mM Suc (black circles), 20 mM Sac (white circles), 100 mM NaCl (black triangles), 100 mM MSG (white triangles), 10 mM HCl (black squares) and 20 mM QHCl (white squares) before and 5–120 min after administration of 3 mg (kg BW)$^{-1}$ of AM251 in db/db mice (n = 6–13). *P < 0.05 for Suc, **P < 0.01 for Sac, Tukey’s HSD test). F, dose-dependent effect of AM251 (white bars, 0.3 mg kg$^{-1}$; grey bars, 1.0 mg kg$^{-1}$; black bars, 3 mg kg$^{-1}$) on CT nerve responses to 300 mM and 500 mM Suc, 20 mM Sac and 1 mM SC in db/db mice (n = 6–9). Responses to sweeteners after administration of AM251 were normalized to those after administration of vehicle. Symbols indicate significant differences (+P < 0.05; ++P < 0.01; Tukey’s HSD test). All data are presented as the means ± SEM. Results of ANOVA and t test are summarized in Tables 1–3 and 5.

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by open-bed silica gel column chromatography, as previously described (Astarita & Piomelli 2009; DiPatrizio et al. 2011). Eluted fractions were dried under N₂ and reconstituted in 100 μL of methanol for lipid analyses. We used a 1100-liquid chromatography system coupled to a 1946D-mass spectrometer detector (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an electrospray ionization interface for lipid analyses. Lipids were separated on a XDB Eclipse C18 column (50 × 4.6 mm i.d., 1.8 μm, Zorbax), eluted by a gradient of methanol in water (from 85% to 90% methanol in 2.5 min) at a flow rate of 1.5 ml min⁻¹. Column temperature was kept at 40°C. MS detection was in the positive ionization mode, capillary voltage was set at 3 kV, and fragmentor voltage at 120 V. Lipids were quantified with an isotope-dilution method (Giuffrida et al. 2000) monitoring sodium adducts of the molecular ions [M + Na]⁺ in the selected ion-monitoring mode.

**Immunohistochemistry**

The procedures for immunohistochemistry were similar to those used previously (Yoshida et al. 2010, Shigemura et al. 2013). Mice used for immunostaining for serotonin were injected with 5-hydroxytryptophan (80 mg kg⁻¹ i.p.) 1 h before dissection. Dissected tongues of B6 or db/db mice were fixed in 4% paraformaldehyde in phosphate buffer saline for 30–45 min at 4°C. After dehydration with sucrose solution (10% for 1 h, 20% for 1 h, and 30% for 3 h at 4°C), the frozen block of fixed tongue was embedded in optimum cutting temperature compound (Sakura Finetechanical, Tokyo, Japan) and sectioned into 8-μm-thick slices, which were mounted on Silane-coated glass slides. Frozen sections were washed with TNT buffer, treated with 1% blocking reagent (Roche, Mannheim, Germany) for 1 h at room temperature, and incubated overnight with primary antibodies for diacylglycerol lipase α (DAGLα, 1:50, rabbit anti-DAGLα N13, Santa Cruz Biotechnology, CA, USA), monoacylglycerol lipase (MAGL, 1:200, rabbit anti-monoacylglycerol lipase antibody, Abcam, Cambridge, UK), T1R3 (1:50, goat anti-T1R3 N20 polyclonal, Santa Cruz Biotechnology), serotonin (1:400, goat anti-serotonin polyclonal, Immunostar, WI, USA), or glutamate and aspartate transporter (GLAST, 1:200, guinea-pig anti-GLAST polyclonal, Millipore, MA, USA) in 1% blocking reagent. After washing with TNT buffer, tissues were incubated for 2 h at room temperature with secondary antibodies for DAGLα or MAGL (1:500, peroxidase-conjugated AffiniPure donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories) and for T1R3, serotonin (1:200, Alexa Fluor 546 donkey anti-goat IgG, Molecular Probes) or GLAST (1:500, Alkaline phosphatase-conjugated AffiniPure F(ab')² fragment donkey anti-guinea-pig IgG, Jackson ImmunoResearch Laboratories) in 1% blocking reagent and washed with TNT. When peroxidase-conjugated second antibodies were used, tissues were incubated for 30 min at room temperature with tyramide-Alexa 488 substrate (TSA kit no. 22, Invitrogen, CA, USA). When alkaline phosphatase conjugated second antibodies were used, tissues were incubated for 30 min at room temperature with HNPP/FastRed AP (HNPP fluorescent detection set, Roche Applied Science, Basel, Switzerland). The immunofluorescence of labelled taste cells was observed using a laser scanning microscope (FV-1000, Olympus, Tokyo, Japan) and images were obtained using Fluoview software (Olympus). Image-Pro Plus (Media Cybernetics, MD, USA) was used to exclude artifactual signals: cells showing a density signal greater than the mean plus two SDs of the density in taste cells in the negative control (primary antibodies omitted) were considered positive. To examine the number of cells expressing DAGLα, MAGL, T1R3, serotonin and GLAST we counted positive cells in each taste bud in horizontal sections of FP and VP. The same cells found on the contiguous sections were counted only once.

**Data analysis**

The effect of LA (Fig. 1B), AM251 (Figs 4B and D and 5B and E) and difference in plasma leptin level between normal-fed and fasted mice (Fig. 2A) were evaluated by Student’s t test. Time-dependent effects of LA (Fig. 1C) and AM251 (Figs 4E and 5D), dose-dependent effects of LA (Fig. 1D) and AM251 (Fig. 4F) and the effect of LA and leptin on CT nerve responses to sweeteners (Fig. 3D) were evaluated by one-way factorial analysis of variance (ANOVA) followed by post hoc Tukey’s HSD test. Significant differences among groups or concentrations (Figs 2B–D, 3A–C, 4C and 5C), among groups and tongue regions (Fig. 6) and among pharmacological treatments and HFD feeding periods (Fig. 9) were evaluated by two-way ANOVA followed by post hoc Tukey’s HSD test. Calculations were performed using the statistical software packages IBM SPSS Statistics (IBM, Armonk, NY, USA), and differences were considered significant at values of P < 0.05. Data are presented as means ± SEM or means ± 95% CI.

**Results**

**Effect of LA on gustatory nerve responses in normal-fed mice**

We first examined potential effects of the leptin antagonist LA on gustatory nerve responses to various taste stimuli in normal-fed B6 mice. We recorded CT taste responses
after I.P. administration of vehicle (saline) or LA. CT nerve responses of normal-fed B6 mice to sweeteners (sucrose, saccharin and SC45647) but not salty (NaCl), bitter (quinine), sour (HCl) or umami (MSG) compounds, increased significantly following treatment with LA, when compared to vehicle control (Student’s $t$ test, Fig. 1A and B, Table 1), suggesting that the effect of LA is sweet-specific. Increased responses to sweet compounds by LA (120~140% of control for 300 mM sucrose) were observed at 10–40 min post-injection, and then reverted to the control level at 60–90 min post-injection (one-way ANOVA, Fig. 1C, Table 2). In contrast, LA had no such effect on CT nerve responses to NaCl, quinine, HCl or MSG (one-way ANOVA, Fig. 1C, Table 2). The effect of LA on sweet responses was dose dependent (one-way ANOVA, Fig. 1D, Table 3).

Figure 5. AM251 does not affect gustatory nerve responses in both normal-fed and fasted mice

A, typical examples of CT nerve responses of normal-fed mice to various tastants 30 min after I.P. injection of vehicle (upper traces) or 3 mg (kg BW)$^{-1}$ of AM251 (lower traces). B, CT nerve responses of normal-fed mice to 300 and 500 mM sucrose (Suc), 20 mM saccharin (Sac), 1 mM SC45647 (SC), 20 mM quinine-HCl (QHCl), 100 mM NaCl (NaCl), 10 mM HCl (HCl) and 100 mM MSG (MSG) 10–40 min after administration of vehicle (white bars, $n = 6–8$) or 3 mg (kg BW)$^{-1}$ of AM251 (black bars, $n = 6–8$). C, concentration–response relationships of CT nerve responses for sucrose in vehicle-treated (white triangles, $n = 6–8$) or AM251-treated (3 mg (kg BW)$^{-1}$, black circles, $n = 6–8$) normal-fed mice. D, time-dependent changes in CT nerve responses to 500 mM Suc (black circles), 20 mM Sac (white circles), 100 mM NaCl (black triangles), 100 mM MSG (white triangles), 10 mM HCl (black squares) and 20 mM QHCl (white squares) before and 5–120 min after administration of 3 mg (kg BW)$^{-1}$ of AM251 in normal-fed mice ($n = 6–10$). E, CT nerve responses of 24 h fasted mice to 300 and 500 mM Suc, 20 mM Sac, 1 mM SC, 20 mM QHCl, 100 mM NaCl, 10 mM HCl and 100 mM MSG 10–40 min after administration of vehicle (white bars, $n = 5–6$) or 3 mg (kg BW)$^{-1}$ of AM251 (black bars, $n = 5–6$). All data are presented as the means ± SEM. Results of ANOVA and $t$ tests are summarized in Tables 1–3 and 5.
Table 1. Results of Student’s t test for the effect of LA or AM251 on CT nerve responses (Figs 1B, 4B and 4D, 5B and E)

<table>
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<tr>
<th></th>
<th>Fig. 1B</th>
<th>Fig. 4B</th>
<th>Fig. 4D</th>
<th>Fig. 5B</th>
<th>Fig. 5E</th>
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<td>$T_{(20)} = 2.58$</td>
<td>$T_{(10)} = 0.063$</td>
<td>$T_{(11)} = -0.669$</td>
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<td>Sac</td>
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<td>$T_{(14)} = 2.85$</td>
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<td>$T_{(12)} = 0.143$</td>
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<td>$T_{(12)} = -0.227$</td>
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<td>$T_{(11)} = -0.144$</td>
<td>$T_{(10)} = 0.139$</td>
<td>$T_{(10)} = 0.589$</td>
<td>$T_{(12)} = 0.119$</td>
<td>$T_{(9)} = 0.588$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.888$</td>
<td>$P = 0.892$</td>
<td>$P = 0.569$</td>
<td>$P = 0.907$</td>
<td>$P = 0.571$</td>
</tr>
<tr>
<td>HCl</td>
<td>$T_{(11)} = 0.826$</td>
<td>$T_{(13)} = 0.053$</td>
<td>$T_{(13)} = -1.357$</td>
<td>$T_{(10)} = 0.248$</td>
<td>$T_{(8)} = -0.219$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.427$</td>
<td>$P = 0.959$</td>
<td>$P = 0.198$</td>
<td>$P = 0.809$</td>
<td>$P = 0.832$</td>
</tr>
<tr>
<td>MSG</td>
<td>$T_{(14)} = 0.594$</td>
<td>$T_{(10)} = -0.427$</td>
<td>$T_{(10)} = -0.295$</td>
<td>$T_{(12)} = -0.129$</td>
<td>$T_{(8)} = -0.204$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.562$</td>
<td>$P = 0.678$</td>
<td>$P = 0.774$</td>
<td>$P = 0.9$</td>
<td>$P = 0.843$</td>
</tr>
</tbody>
</table>

Table 2. Results of one-way ANOVA test for time dependent changes in CT nerve responses after LA or AM251 treatment

<table>
<thead>
<tr>
<th></th>
<th>Effect of LA in B6 mice (1C)</th>
<th>Effect of AM251 in db/db mice (4E)</th>
<th>Effect of AM251 in B6 mice (5D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc</td>
<td>$F_{(6,50)} = 2.41$</td>
<td>$F_{(6,45)} = 3.374$</td>
<td>$F_{(6,39)} = 0.073$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.04$</td>
<td>$P = 0.008$</td>
<td>$P = 0.998$</td>
</tr>
<tr>
<td>Sac</td>
<td>—</td>
<td>$F_{(6,37)} = 2.51$</td>
<td>$F_{(6,40)} = 0.122$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.866$</td>
<td>$P = 0.039$</td>
<td>$P = 0.993$</td>
</tr>
<tr>
<td>NaCl</td>
<td>$F_{(5,32)} = 0.128$</td>
<td>$F_{(6,34)} = 0.133$</td>
<td>$F_{(6,43)} = 0.315$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.985$</td>
<td>$P = 0.991$</td>
<td>$P = 0.925$</td>
</tr>
<tr>
<td>MSG</td>
<td>$F_{(4,30)} = 0.282$</td>
<td>$F_{(5,30)} = 0.255$</td>
<td>$F_{(5,53)} = 0.338$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.887$</td>
<td>$P = 0.934$</td>
<td>$P = 0.888$</td>
</tr>
<tr>
<td>HCl</td>
<td>$F_{(5,37)} = 0.88$</td>
<td>$F_{(5,30)} = 0.04$</td>
<td>$F_{(5,30)} = 0.404$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.994$</td>
<td>$P = 0.999$</td>
<td>$P = 0.842$</td>
</tr>
<tr>
<td>QHCl</td>
<td>$F_{(4,28)} = 0.259$</td>
<td>$F_{(6,33)} = 0.182$</td>
<td>$F_{(6,54)} = 0.121$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.902$</td>
<td>$P = 0.98$</td>
<td>$P = 0.993$</td>
</tr>
</tbody>
</table>

Table 3. Results of one way ANOVA test for dose dependent effect of LA or AM251 (Figs 1D and 4F)

<table>
<thead>
<tr>
<th></th>
<th>Effect of LA in B6 mice (1D)</th>
<th>Effect of AM251 in db/db mice (4F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300 mM Suc</td>
<td>$F_{(2,17)} = 9.036$</td>
<td>$F_{(2,14)} = 5.485$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.002$</td>
<td>$P = 0.017$</td>
</tr>
<tr>
<td>500 mM Suc</td>
<td>$F_{(2,16)} = 4.58$</td>
<td>$F_{(2,14)} = 0.004$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.027$</td>
<td>$P = 0.005$</td>
</tr>
<tr>
<td>20 mM Sac</td>
<td>$F_{(2,15)} = 4.632$</td>
<td>$F_{(2,12)} = 4.253$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.027$</td>
<td>$P = 0.04$</td>
</tr>
<tr>
<td>1 mM SC</td>
<td>$F_{(2,16)} = 3.936$</td>
<td>$F_{(2,16)} = 6.547$</td>
</tr>
<tr>
<td></td>
<td>$P = 0.041$</td>
<td>$P = 0.008$</td>
</tr>
</tbody>
</table>

Effect of leptin and LA on gustatory nerve responses in fasted mice

Plasma leptin levels were significantly lower in 24 h food-deprived mice (fasted mice, $2.46 \pm 0.16$ ng ml$^{-1}$, $n = 6$) than in normal-fed mice ($5.22 \pm 0.89$ ng ml$^{-1}$, $n = 6$, $T_{(10)} = 3.03$, $P = 0.013$, Student’s $t$ test, Fig. 2A). We next asked if lower leptin levels would alter the effect of LA and leptin on gustatory nerve responses in mice. We
Table 4. Results of two-way ANOVA test for the effect of LA on CT nerve responses of fasted B6 mice (Fig. 2B–D)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc</td>
<td>$F(2,87) = 37.781$ P &lt; 0.001</td>
<td>$F(4,87) = 493.1$ P &lt; 0.001</td>
</tr>
<tr>
<td>Sac</td>
<td>$F(2,65) = 11.373$ P &lt; 0.001</td>
<td>$F(3,65) = 193.9$ P &lt; 0.001</td>
</tr>
<tr>
<td>SC45647</td>
<td>$F(2,65) = 8.358$ P &lt; 0.001</td>
<td>$F(3,65) = 275.8$ P &lt; 0.001</td>
</tr>
</tbody>
</table>

Table 5. Results of two-way ANOVA test for the effect of leptin on CT nerve responses of fasted B6 mice (Fig. 3A–C)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc</td>
<td>$F(1,63) = 124.9$ P &lt; 0.001</td>
<td>$F(4,63) = 452.2$ P &lt; 0.001</td>
</tr>
<tr>
<td>Sac</td>
<td>$F(1,46) = 19.9$ P &lt; 0.001</td>
<td>$F(3,46) = 107.2$ P &lt; 0.001</td>
</tr>
<tr>
<td>SC45647</td>
<td>$F(1,46) = 43.0$ P &lt; 0.001</td>
<td>$F(3,46) = 308.5$ P &lt; 0.001</td>
</tr>
</tbody>
</table>

Table 6. Results of one-way ANOVA test for the effect of leptin and LA on CT nerve responses (Fig. 3D)

<table>
<thead>
<tr>
<th>Effect of treatment</th>
<th>300 mM Suc</th>
<th>500 mM Suc</th>
<th>20 mM Sac</th>
<th>1 mM SC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F(4,29) = 14.05$ P &lt; 0.001</td>
<td>$F(4,28) = 10.99$ P &lt; 0.001</td>
<td>$F(4,25) = 5.958$ P = 0.002</td>
<td>$F(4,27) = 12.316$ P &lt; 0.001</td>
</tr>
</tbody>
</table>

recorded CT nerve responses to different concentrations of sucrose, saccharin and SC45647 in fasted mice 10–40 min after administration of vehicle (Fasted), 1 mg (kg BW)$^{-1}$ of LA (Fasted LA) or 100 ng (g BW)$^{-1}$ of leptin (Fasted Lep) and in normal-fed mice 10–40 min after injection of vehicle (normal-fed). Concomitant with the reduction of circulating leptin, fasted mice showed significantly larger CT nerve responses to sweeteners compared to normal-fed mice (two-way ANOVA, Fig. 2B–D, Table 4; one-way ANOVA, Fig. 3D, Table 6). In addition, LA did not affect CT nerve responses to sweeteners in fasted mice (two-way ANOVA, Fig. 2B–D, Table 4; one-way ANOVA, Fig. 3D, Table 6). In contrast, leptin significantly reduced CT nerve responses to sweeteners in fasted mice (two-way ANOVA, Fig. 3A–C, Table 5; one-way ANOVA, Fig. 3D, Table 6), indicating that the sweet suppressive effect of leptin is intact in fasted mice. Thus, the results suggest that endogenous leptin may chronically affect gustatory nerve responses to sweeteners in normal-fed mice (about 5 ng ml$^{-1}$ of plasma leptin level) but not in fasted mice (about 2.5 ng ml$^{-1}$ of plasma leptin level).

Effect of AM251 on gustatory nerve responses in normal-fed, fasted and db/db mice

Previous studies demonstrated that db/db mice showed greater CT nerve responses to sweet compounds than lean control mice (Ninomiya et al. 1995, 1998; Sako et al. 1996). Endocannabinoids may contribute to such responses to sweeteners in db/db mice because they enhance sweet taste responses (Yoshida et al. 2010). To
leptin receptor deficient db/db mice but not in B6 mice, we quantified the levels of the abundant endocannabinoid 2-AG in the taste organs of db/db and B6 mice. We collected the epithelial tissue of the tongue from each of db/db and B6 mice and divided it into three parts: the anterior part containing the majority of fungiform papillae, the middle part without taste papillae, and the posterior part containing a circumvallate papilla and foliate papillae. We then measured 2-AG levels by HPLC/MS in each part of epithelial tissues (Fig. 6). 2-AG levels were significantly higher in db/db mice than in B6 mice (F(1,30) = 11.564, P = 0.002 for genotype; F(2,30) = 2.721, P = 0.082 for tissue region; F(2,30) = 1.312, P = 0.284 for interaction; two-way ANOVA, Fig. 6). These results suggest that higher 2-AG levels in taste tissues of db/db mice lead to greater sweet responses in db/db mice.

Expression of DAGLα and MAGL in taste buds

2-AG is synthesized by DAGL and degraded by MAGL (DiPatrizio & Piomelli, 2012). Genetic deletion of DAGLα leads to significant decreases in brain 2-AG levels (Tanimura et al. 2010). Conversely genetic deletion of MAGL leads to large increases in brain 2-AG levels (Taschner et al. 2011). Therefore we speculate that enhanced 2-AG levels in taste tissues of db/db mice are possibly due to enhanced expression of DAGL and/or reduced expression of MAGL in taste tissues. We analysed the expression of DAGL and MAGL in taste buds of db/db and B6 mice by immunohistochemistry. Taste bud cells are morphologically and functionally classified into three subtypes (types I, II and III cells) whose apical membrane is exposed to the taste pore and could be stimulated by taste substances (Murray, 1971). We used three cell type markers: GLAST for type I cells (Lawton et al. 2000), T1R3 for sweet and umami receptor component expressed in type II cells (Max et al. 2001) and serotonin for type III cells (Yee et al. 2001) to investigate coexpression patterns of DAGL or MAGL and these cell type markers. Some taste cells in FP and VP expressed DAGLα or MAGL in both db/db (Fig. 7) and B6 mice (Fig. 8). In taste buds of both db/db and B6 mice, about 40–65% of DAGLα positive cells expressed T1R3 (Table 8). On the other hand, about 10–20% of MAGL positive cells expressed T1R3 (Table 8). MAGL was well coexpressed with the type I cell marker GLAST (about 50–65%, Table 8) and about 25–40% of DAGLα positive cells expressed GLAST.
Serotonin-expressing type III cells did not express either DAGLα or MAGL (0–6%, Table 8). When comparing the expression of DAGLα and MAGL in the various cell types in B6 and db/db mice, the number of DAGLα–T1R3 double positive cells was significantly larger in db/db mice for both FP and VP taste buds (Table 9). Expression of DAGLα in GLAST positive cells of VP taste buds was also likely to be higher in db/db mice than in B6 mice (Table 9). Furthermore, the number of MAGL–GLAST double positive cells in VP taste buds was larger in db/db mice than in B6 mice (Table 9). Taken together, we speculate that increases in DAGLα (2-AG production) in T1R3 positive cells may lead to enhanced 2-AG levels in taste tissues of db/db mice, especially in FP taste buds.

Effect of LA and AM251 on sweet responses in DIO mice

Finally, we asked if the effects of endogenous leptin and endocannabinoids on sweet responses would change during the development of obesity using DIO model mice. Four-week-old male B6 mice were fed HFD for 4, 6, 8 or 12 weeks and used to record CT nerve responses to 500 mM sucrose before and after i.p. administration of LA (1 mg (kg BW)^{-1}) or AM251 (3 mg (kg BW)^{-1}). The enhancement of sweet responses by administration of LA began to decrease at 4–6 weeks of HFD feeding and had almost disappeared at 8–12 weeks of HFD feeding, and the suppression of sweet responses by administration of AM251 began to increase at 6–8 weeks of HFD feeding and reached maximal

Figure 7. Coexpression of DAGLα or MA gl with taste cell markers in db/db mice

Immunofluorescence of DAGLα (green) and GLAST (A, red), T1R3 (C, red) or serotonin (E, red) and merged images in fungiform (FP, upper) and circumvallate (VP, lower) taste buds of db/db mice. Immunofluorescence of MAGL (green) and GLAST (B, red), T1R3 (D, red) or serotonin (F, red) and merged images in FP and VP taste buds of db/db mice. G, immunostaining without primary antibodies in db/db mice (negative control). Dotted lines indicate the outline of taste buds. Scale bars, 10 μm. Quantitative data are shown in Table 8. Statistical data are shown in Table 9.
at 8–12 weeks of HFD feeding ($F_{(4,50)} = 6.961, P < 0.001$ for HFD feeding period; $F_{(1,50)} = 52.457, P < 0.001$ for pharmacological treatment; $F_{(4,50)} = 0.318, P = 0.864$ for interaction, two-way ANOVA, Fig. 9). These results indicate that chronic modulators for sweet taste responses shift from endogenous leptin to endocannabinoids during the development of obesity.

### Discussion

In the present study, we demonstrated that pharmacological blockade of leptin receptor activation with the leptin receptor inhibitor LA leads to selective enhancement of taste nerve responses to sweet compounds. The ability for LA to enhance neural responses to sweet compounds is suggested to be caused by an inhibition of the suppressive effects of leptin on sweet responsive cells. Food deprivation for 24 h reduced circulating leptin levels of lean mice from $\approx 5.0 \text{ ng ml}^{-1}$ to $\approx 2.5 \text{ ng ml}^{-1}$ (Fig. 2A), leading to increased basal sweet taste responses before LA administration, and no further enhancement of the responses after LA administration (Fig. 2). This result suggests that the threshold of leptin’s effect on sweet taste responses may be $\approx 2.5 \text{ ng ml}^{-1}$ in the taste system. The fasting-induced enhancement of responses to sweet compounds was abolished by exogenous leptin treatment (Fig. 3), indicating that the enhancement may be primarily due to reductions in leptin levels by fasting in lean mice. These findings strongly suggest an involvement in lean mice of endogenous leptin in normal sweet taste responses at levels between $\approx 2.5$ and 10 ng ml$^{-1}$, which is similar to the effective concentration of leptin in sweet sensitive taste cells (R. Yoshida et al., unpublished data) and CT nerve responses (Kawai et al. 2000). A link between sweet taste sensitivities and circulating leptin levels has also been shown in non-obese humans (Nakamura et al. 2008). For example, taste recognition thresholds of non-obese humans for sweet compounds, such as sucrose, glucose and saccharin, have a diurnal variation that parallels variation in leptin levels, being lowest in the morning and highest in the night under normal three-meal conditions. When leptin levels were phase shifted following imposition of one or two meals per day, the diurnal variation of thresholds for sweet taste shifted in parallel. This diurnal variation is sweet taste specific: recognition thresholds for NaCl, citric acid, quinine and monosodium glutamate did not exhibit such diurnal variation. Also, parallel longitudinal decreases in sweet taste thresholds and circulating leptin levels during weight loss in obese females has been reported (Umabiki et al. 2010). Collectively, comparable linkage between circulating leptin levels and sweet taste sensitivities, both in mice and humans, suggests that endogenous leptin is involved in modulating basal sensitivities of sweet responsive cells via its chronic action on Ob-Rb.

The present and previous studies demonstrate that leptin specifically inhibits sweet taste responses in mice (Kawai et al. 2000; Shigemura et al. 2004). This effect may account for enhanced preference for sweet taste in vaso-active intestinal peptide (VIP)–null mice, which exhibited reduced expression of Ob-Rb in taste tissue (Martin et al. 2010). Other groups, however, have shown that leptin increases temperature-dependent gustatory nerve responses to sucrose in mice (Lu et al. 2012). These discrepancies may be due to methodological differences between these studies, including temperature ($24–25 \text{ °C}$ vs. $35 \text{ °C}$), the solvent and rinse solution for stimuli.
Table 9. Results of Fisher’s exact test for comparison of the expression of DAGLα and MAGL in the various cell types in B6 and db/db mice (Figs 7 and 8, Table 8)

<table>
<thead>
<tr>
<th></th>
<th>FP</th>
<th>VP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+DAGL</td>
<td>−DAGL</td>
</tr>
<tr>
<td>GLAST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>66</td>
<td>196</td>
</tr>
<tr>
<td>db/db</td>
<td>22</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>251</td>
</tr>
<tr>
<td>T1R3</td>
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<td></td>
</tr>
<tr>
<td>B6</td>
<td>76</td>
<td>112</td>
</tr>
<tr>
<td>db/db</td>
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<tr>
<td>Total</td>
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<td>167</td>
</tr>
<tr>
<td>5-HT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
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</tr>
<tr>
<td>db/db</td>
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</tr>
<tr>
<td>Total</td>
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</tr>
<tr>
<td>GLAST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+MAGL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>db/db</td>
<td>26</td>
<td>42</td>
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<tr>
<td>Total</td>
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<tr>
<td>T1R3</td>
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<td></td>
</tr>
<tr>
<td>B6</td>
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<td>140</td>
</tr>
<tr>
<td>db/db</td>
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<tr>
<td>Total</td>
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<tr>
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<td>1</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>100</td>
</tr>
</tbody>
</table>

Each column indicates number of cells expressing each marker (GLAST, T1R3 or 5-HT) with (+) or without (−) expression of DAGLα (upper) or MAGL (lower). N.D., no significant difference.

transcription 3 (STAT3) induced by leptin (0.1 mg l$^{-1}$) in Chinese hamster ovary (CHO) cells expressing Ob-Rb, showed that 2.5 mg l$^{-1}$ LA inhibited STAT3 phosphorylation by ~90% of control, close to the maximum inhibition level (Shpilman et al. 2011). In our previous study, a 0.1 mg kg$^{-1}$ leptin injection increased plasma leptin levels from 3.5 to 10 ng ml$^{-1}$ (6.5 ng ml$^{-1}$ change) and generated maximum suppression of sweet taste responses (~65% of control), while further elevations in plasma leptin did not increase the suppression level above this maximum (Kawai et al. 2000). LA, at 2.0 mg kg$^{-1}$ used in this study, opposed the action of leptin and elicited the maximum rise in nerve responses (~140%). These results suggest that the magnitude of enhancement in response to sweet compounds may well accord with the potency of the leptin antagonist measured in the in vitro study using CHO cells.

The time course for LA (1 mg (kg BW)$^{-1}$) on sucrose responses (peak at 20–40 min and recovery to the control levels at 60–90 min after administration, Fig. 1C) is comparable with that of leptin’s (0.1 mg (kg BW)$^{-1}$) effects on sucrose responses in our previous study (Kawai et al. 2011).
The maximum enhancement of sucrose responses by LA administration (2.0 mg (kg BW)⁻¹) is ~140% of control (Fig. 1D), which is also comparable to the maximum suppression of the response (~65% of control) by exogenous leptin (Kawai et al. 2000). LA has a 16 kDa molecular weight (Salomon et al. 2006), which is the same as that for endogenous leptin. It is assumed that exogenously administrated endogenous leptin and LA would be degraded primarily via the kidney, and have a half-life of 8–30 min based on the molecular weight (Haffner et al. 1994). Therefore, the similar effective time periods of exogenous wild-type leptin and LA may be associated with their similar degradation periods in the periphery.

In contrast to the effect of LA, inhibiting CB₁ receptors with AM251 did not show any significant effect on taste responses in lean mice with or without 24 h food deprivation (Fig. 5). This is consistent with the previous finding that mice genetically lacking CB₁ did not show any significant changes in taste responses compared to lean control mice (Yoshida et al. 2010). This also strongly supports the hypothesis that the enhancement of sweet responses by fasting may solely be due to a reduction in the suppressive effect of leptin and not an increase in endocannabinoid activity in lean mice. In this sense, the endocannabinoid system in the peripheral taste organ may be somewhat different from that in the lateral hypothalamus where 24 h food deprivation increased endocannabinoid

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**Figure 8. Coexpression of DAGLα or MAGL with taste cell markers in B6 mice**

Immunofluorescence of DAGLα (green) and GLAST (A, red), T1R3 (C, red) or serotonin (E, red) and merged images in fungiform (FP, upper) and circumvallate (VP, lower) taste buds of B6 mice. Immunofluorescence of MAGL (green) and GLAST (B, red), T1R3 (D, red) or serotonin (F, red) and merged images in FP and VP taste buds of B6 mice. G, immunostaining without primary antibodies in B6 mice (negative control). Dotted lines indicate the outline of taste buds. Scale bars, 10 μm. Quantitative data are shown in Table 8. Statistical data are shown in Table 9.
levels, which has been proposed to enhance food intake in rats (Kirkham et al. 2002).

In db/db mice, unlike lean mice, inhibiting CB1 receptors with AM251 clearly decreased responses to sweet compounds without affecting responses to salty, sour, bitter and umami taste stimuli (Fig. 4), suggesting the effect is sweet-specific, similar to that of exogenous administration of 2-AG or AEA in lean mice (Yoshida et al. 2010). The time course of effect for AM251 on sucrose responses (peak at around 30 min, recovery at around 60 min, maximum decreases ~70% of control) is also comparable to that of exogenous 2-AG in lean control mice, although the effect is opposite (maximum increases ~140% of control by 2-AG, Yoshida et al. 2010). In addition, blocking CB1 activation with AM251 abolished the enhanced component of sweet taste responses in db/db mice, which led to their overall taste responsiveness being indistinguishable from that of lean control mice (Fig. 4). Collectively, these results suggest that the enhancement of sweet taste responses in db/db mice may be exclusively due to tonic activation of CB1 receptors in taste cells by endocannabinoids. Consistent with previous reports showing enhanced endocannabinoid levels in hypothalamus of Zucker rats, db/db and ob/ob mice (Di Marzo et al. 2001), we demonstrated enhancement of 2-AG levels in taste tissue of db/db mice (Fig. 6). Such enhancement may be involved in tonic activation of CB1 receptors in taste cells in db/db mice.

The major pathway for the biosynthesis of 2-AG in the brain comprises sequential hydrolysis of arachidonic acid-containing inositol phospholipids by phospholipase C (PLC) and DAGL (Jung et al. 2005, 2007). DAGL is an enzyme hydrolysing diacylglycerol at the sn-1 position to yield monoacylglycerol and free fatty acid (Wang and Ueda, 2009). The enzyme was cloned as two isozymes, DAGLα and β. DAGLα appeared to be more abundant in the adult brain and DAGLβ more abundant in the developing brain (Bisogno et al. 2003). We have for the first time identified expression of DAGLα and MAGL – the primary degradative enzyme for 2-AG – in the taste organ of wild-type and db/db mice. Furthermore DAGLα expressing taste cells co-expressed T1R3. Our previous study showed that about 60% of taste cells expressing T1R3 co-expressed CB1 (Yoshida et al. 2010). Collectively, our data suggest that 2-AG might be produced by taste stimuli (especially sweet taste) and mediate an autocrine feedback mechanism in taste cells with T1R3 in both wild-type and db/db mice. Further studies, however, are needed to clarify this possibility.

In DIO mice, the effect of LA administration on sweet responses began to decrease at 4–6 weeks of HFD feeding and almost disappeared at 8–12 weeks of HFD feeding (Fig. 9), indicating that sweet sensitive taste cells become leptin resistant during the course of obesity development by HFD feeding. Along with the development of leptin resistance in taste cells, CT nerve responses to sweet stimuli began to be suppressed by AM251 administration in these animals (Fig. 9). These results indicate that the dominant basal modulator for sweet taste would shift from leptin to endocannabinoids during the development of obesity. Because db/db mice also showed suppression of sweet taste responses by AM251, we speculate that deficiency of the leptin effect, such as leptin resistance and leptin receptor mutation, may promote endocannabinoid activity in the peripheral taste system. As shown in db/db mice, 2-AG levels in taste tissues were higher than in lean mice (Fig. 6).

In addition, it has been reported that HFD consumption over 14 days elicited a long-lasting increase in hypothalamic 2-AG levels (Higuchi et al. 2012). Therefore, we speculate that increased 2-AG levels in taste tissues may occur along with the development of leptin resistance. One possible mechanism for enhanced 2-AG level may be up-regulation of DAGL in taste cells as shown in db/db mice. However, further studies are required to elucidate why the endocannabinoid system would be up-regulated by development of leptin resistance in peripheral taste tissues.

It has been reported that a number of proteins contributing to Ob-Rb trafficking and/or signalling such as Bardet-Biedl syndrome protein (Rahmouni et al. 2008), suppressor of cytokine signalling-3 (SOCS3), protein tyrosine phosphatase 1B (PTP1B) and SH2B adaptor protein 1 (SH2B1; Morris & Rui, 2009, White et al. 2008, de

![Figure 9. Changes in the effect of LA and AM251 on sweet responses during 0–12 weeks of feeding of high fat diet in B6 mice](image-url)
Lartigue et al. (2011) may be involved in the development of leptin resistance. Endoplasmic reticulum (ER) stress, which also inhibits leptin receptor signalling, is another possible mechanism for induction of leptin resistance (Ozcan et al. 2009). To date, however, the mechanisms for induction of leptin resistance in taste tissue are entirely unknown. These mechanisms should be clarified by future studies.

In conclusion, the results of the present study suggest that circulating leptin, but not endocannabinoids, may act as a modulator that tonically affects basal sweet sensitivity of lean control mice. Endocannabinoids may become more effective when there are defects in leptin receptors (i.e. in db/db mice) or a reduced inhibitory activity of leptin on sweet taste.

References


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Additional information

Competing interests
All authors declare no competing financial interests.

Author contributions
Y.N. conceived and designed the experiments. M.N., M.J., R.Y., K.Y. and N.V.D. performed the experiments. M.N., M.J., R.Y., N.V.D., D.P., N.S. and Y.N. analysed the data. M.N., R.Y. and Y.N. wrote the paper. All authors approved the final version of the manuscript.

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