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Leptin Modulates Behavioral Responses to Sweet Substances by Influencing Peripheral Taste Structures

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Leptin is a hormone that regulates body weight homeostasis mainly via the hypothalamic functional leptin receptor Ob-Rb. Recently, we proposed that the taste organ is a new peripheral target for leptin. Leptin selectively inhibits mouse taste cell responses to sweet substances and thereby may act as a sweet taste modulator. The present study further investigated leptin action on the taste system by examining expression of Ob-Rb in taste cells and behavioral responses to sweet substances in leptin-deficient *ob/ob*, and Ob-Rb-deficient *db/db* mice and their normal litter mates. RT-PCR analysis showed that Ob-Rb was expressed in taste cells in all strains tested. The *db/db* mice, however, had a RT-PCR product containing an abnormal *db* insertion that leads to an impaired shorter intracellular domain. *In situ* hybridization analysis showed that the hybridization signals for normal Ob-Rb mRNA were detected in taste cells in lean and *ob/ob* mice but not in *db/db* mice. Two different behavioral tests, one using sweet-bitter mixtures as taste stimuli and the other a conditioned taste aversion paradigm, demonstrated that responses to sucrose and saccharin were significantly decreased after ip injection of leptin in *ob/ob* and normal littermates, but not in *db/db* mice. These results suggest that leptin suppresses behavioral responses to sweet substances through its action on Ob-Rb in taste cells. Such taste modulation by leptin may be involved in regulation for food intake. (*Endocrinology* 145: 839–847, 2004)

LEPTIN, THE PRODUCT of the obese (*ob*) gene, is a hormone primarily produced in adipose cells. It regulates food intake, energy expenditure, and body weight. Leptin is thought to promote weight loss, at least in rodents, by suppressing appetite and stimulating metabolism. In *ob/ob* mice, a defect in the *ob* gene prevents leptin production. In consequence, severe obesity and diabetes develop (1). Leptin acts by binding to a specific obese receptor (Ob-R). Several isoforms exist, which are generated as splice variants of one gene (*db* gene) and differ mainly in the length of the cytoplasmic domain (2–6). The *db/db* mouse has a point mutation of the *db* gene that leads to abnormal splicing of the coding region. The resulting absence of Ob-Rb, the longer form of Ob-Rs, causes leptin insensitivity and thereby the obese, diabetic phenotype (2, 3).

Central hypothalamic targets are thought to be mainly responsible for the effects of leptin on food intake and weight loss. However, there are also direct effects on peripheral tissues, such as lymph nodes, liver, lung, uterus (7), adipose tissue, kidney, muscle (8), and pancreas (9). Recently, we proposed that the taste organ is also a peripheral target for leptin (10), because an ip injection of leptin in lean mice suppressed responses of peripheral taste nerves [chorda tympani (CT) and glossopharyngeal (GL) nerves] to sweet substances [sucrose (Suc) and saccharin (Sac)] without affecting responses to sour, salty, and bitter substances. Leptin increased outward K^+ currents in isolated taste bud cells, which leads to reduction of excitability of taste cells. The *db/db* mouse with impaired leptin receptors showed no such leptin suppression. Taste tissue containing the circumvallate papilla (innervated by the GL nerve) of lean mice expressed Ob-Rb mRNA, and some of the taste cells exhibited immunoreactivities to antibodies of the Ob-Rs. These results suggest that leptin may be a sweet-sensing modulator (suppressor) that may take part in regulation of food intake.

In our previous study (10), however, the evidence for expression of the functional leptin receptors in taste cells was limited, because tissues used for RT-PCR analysis contained not only taste buds but surrounding tissues as well, and an analysis of immunoreactivities of taste cells for Ob-Rs was not specific for Ob-Rb. Also, we had no data from the fungiform taste bud cells innervated by the CT nerve, which is reported to show much greater responses to sweet substances than the GL nerve does (11). Moreover, although we found suppression of sweetener responses of peripheral taste nerves by leptin, the magnitude of the suppression was not so pronounced (to ~70% of control) (10). This raised the question whether this effect on the peripheral taste system would be strong enough to influence behavioral responses.

In the present study, therefore, by using leptin-deficient *ob/ob* and leptin-receptor-deficient *db/db* and their normal littermates, we first examined expression of the Ob-Rb in

Abbreviations: BW, Body weight; CCK, cholecystokinin; CP, circumvallate papillae; CS, conditioned stimulus; CT, chorda tympani; CTA, conditioned taste aversion; FP, fungiform papillae; GL, glossopharyngeal; ISH, *in situ* hybridization; NTS, nucleus of the solitary tract; Ob-R, obese receptor; PLSD, projected least significant difference; Qui, quinine; Sac, saccharin; SSC, standard saline citrate; Suc, sucrose; TSA, tyramide signal amplification.

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taste cells in the fungiform and circumvallate papillae (FP and CP) located on the anterior and posterior tongue by RT-PCR and *in situ* hybridization (ISH) analyses. We next examined possible effects of leptin on behavioral responses to sweet substances by using two different short-term (10-sec) tests, one using mixtures of sweet substances (Suc or Sac) with quinine (Qui) as taste stimuli (Qui-mixture test) (12), and the other using a conditioned taste aversion (CTA) paradigm (CTA test) (13). In the former behavioral test, leptin, if effective, should decrease lick rates for Qui mixtures of Suc or Sac, whereas in the latter test leptin should increase lick rates for Suc or Sac alone in mice after conditioning to avoid these sweet taste compounds.

Materials and Methods

Experimental manipulation

All experimental procedures were approved by the committee for Laboratory Animal Care and Use at Kyushu University.

Obese, diabetic *db/db* and *ob/ob* mice [male and female, 8–20 wk of age, 50–62 g body weight (BW)] and their lean littermates (+/+, *db*/+, *ob*/+, male and female, 8–20 wk of age, 23–34 g BW) were obtained from mating pairs of the C57BL/KsJ-*db*/+ or C57BL/6J-*ob*/+ mouse strains originally supplied from The Jackson Laboratory (Bar Harbor, ME). Lean littermates with +/+, *db*/+, or *ob*/+ genotypes were used without distinction as lean controls (lean-*db* or lean-*ob*) in the behavioral Qui-mixture test, whereas in the RT-PCR analysis for expression of Ob-Rb mRNA, littermates with *db*/+ and +/+ genotypes were used separately. In the behavioral CTA test, male and female lean C57BL/KsJ mice (8–20 wk of age, 24–30 g BW) were used. In each of the two behavioral tests, mice were divided into two groups, experimental (with leptin injection) and control (with saline injection) groups. Two or three mice were housed together in plastic cages and received *ad libitum* food pellets (MF; Oriental Yeast, Osaka, Japan). Tap water was freely available except during the training and testing sessions. The mice were maintained on a 12-h light/12-h dark cycle (lights on 0800–2000 h) in a temperature- and humidity-controlled room.

RT-PCR

Each mouse was anesthetized with ip injections of pentobarbital sodium (40–50 mg/kg) and then killed by cervical dislocation and their tongues rapidly removed. After washing of the tongues with a normal extracellular solution [NES; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM sodium pyruvate, and 10 mM HEPES-NaOH (pH 7.4)], 0.2 ml of NES containing 1.0 mg/ml elastase (Roche, Indianapolis, IN) was injected between the epithelium and muscle layers of the tongue. After incubation in NES continuously bubbled with 95% O_2 and 5% CO_2 for 15–20 min at 26 C, the epithelial sheet containing FP and CP was peeled off from the underlying muscle. The epithelium was washed with NES, pinned serosal side up in a silicone dish, and incubated in divalent cation-free extracellular solution containing 2 mM EDTA for 20 min at room temperature. Taste buds were individually removed from FP and CP, respectively, by sucking with a 100-μm transfer pipette. One hundred taste buds from the taste papillae or 1 × 1-mm block of the epithelial tissue without taste buds were transferred to an Eppendorf tube containing 100 μl (1 vol) of lysis buffer [4 M guanidine thiocyanate, 25 mM trisodium citrate (pH 7.0), 0.5% sodium *N*-lauroyl-sarcosine, and 0.1 M 2-mercaptoethanol] and homogenized. Yeast tRNA (1.0 μg) was used as a carrier. Sequentially, 0.1 vol of 2 M sodium acetate (pH 4.0), 1 vol of acidic phenol, and 0.2 vol of chloroform-isoamyl alcohol (49:1) were added, the mixture being vortexed after each addition. After incubation at 4 C for 15 min and centrifugation at high speed, the aqueous phase was recovered and RNA was precipitated with 1 vol of isopropanol. After centrifugation, the pellet was resuspended in 1 vol of lysis buffer and RNA was reprecipitated with isopropanol. After washing twice with 75% ethanol, the pellet was dried and dissolved in water. A cDNA was generated by RT [oligo $(dT)_{12–18}$ primer] with the superscript preamplification system (Gibco BRL, Gaithersburg, MD). PCRs were carried out with an equivalent of 10 taste buds per reaction. Genomic DNA did not contribute to the signal as suggested by two protocols. In the first, RNA was treated in parallel in the presence and absence of reverse transcriptase, and the material was then used for PCR. In the absence of reverse transcriptase, there were no amplification fragments having the expected size. Primers were chosen to span one or more introns to exclude confusion with amplified fragments from genomic DNA. PCR led in this case to the amplification of two bands, either specific for the genomic DNA (characterized by a longer size owing to the presence of at least one intron) or for the reverse transcribed cDNA. In our preliminary experiments, we found no detectable PCR products for Ob-Rb produced in the case of amplifying with 30 PCR cycles, although primers for β-actin and α-gustducin (taste-cell-specific G protein) (14) produced strong bands with 25 or 30 cycles, respectively. To obtain greater amplifications in the present experiments, therefore, we used a nested PCR protocol for Ob-Rb. The primers used for DNA amplification were as follows: Ob-Rb outer primers, 5′-TTCCTGGACACTGTCACCTGATGAT-3′ and 5′-CTTGTGACTGTGCGTGGAACAGGTT-3′ (GenBank accession number U58861; the expected sizes of PCR products from mRNA, 1192 bp); Ob-Rb inner primers, 5′-AATTGTTCCTGGGCACAAGGACTGA-3′ and 5′-TTACTGGAGATGCAGTTGCTGACAG-3′ (U58861; 413 bp); α-gustducin, 5′-AGATGGGAAGTGGAATTAGTTCAGA-3′ and 5′-GCTCAGAAGAGCCCACAGTCTTTGA-3′ (X65747; 1069 bp); β-actin, 5′-GGTTCCGATGCCCTGAGGCTC-3′ and 5′-ACTTGCGGTGCACGATGGAGG-3′ (X03765; 360 bp). PCR was performed on PE9700 with the following conditions: 95 C for 2 min (1 cycle); 94 C for 15 sec, 58 C for 30 sec, and 68 C for 40–80 sec (25–40 cycles); and 72 C for 5 min (1 cycle). The PCR solution contained 10 mM Tris/HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 200 μM deoxyribonucleoside 5′-triphosphate (dNTP), and 0.05 U/μl of KOD-Plus-*Taq* polymerase (TOYOBO, Osaka, Japan). Authenticity of each gene amplification was confirmed by direct sequencing. The resulting amplification products were visualized in a 2% agarose gel with 0.5 μg/ml ethidium bromide.

ISH

Because results from our preliminary experiment suggest that Ob-Rb mRNA in taste cells could not be detected by conventional ISH protocol, in the present study we used the ISH protocol combined with tyramide signal amplification (TSA) method, which has been adopted as a means of enhancing signal strength for immunoblotting and immunocytochemistry (15, 16).

Mouse Ob-Rb (obtained by inner PCR primers) and α-gustducin DNA fragments were purified and cloned into the pGEM T-Easy vector (Promega, Madison, WI), which was digested with appropriate restriction enzymes. Biotin-labeled antisense RNA probes were generated by *in vitro* transcription using biotin-RNA labeling mix (Roche) and SP6 or T7 RNA polymerase. This Ob-Rb riboprobe corresponds to base positions 2714–3126 in the intracellular domain of Ob-Rb, which contains the region inserted in the additional sequence (106 bp) in *db/db* mice, as previously shown in other tissues (2, 3). Frozen blocks of the dissected tongue embedded in the OCT compound (Sakura Finetechnical, Tokyo, Japan) were sectioned into 10-μm-thick slices, which were mounted on silane-coated glass slides. The cryosections were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, treated twice with 0.1% diethyl pyrocarbonate in PBS for 15 min, washed with 5× standard saline citrate (SSC) for 15 min at room temperature, and then prehybridized in 5× SSC/50% formamide for 2 h at 58 C. Hybridization was carried out in a hybridization buffer containing 50% formamide, 5× SSC, 5× Denhardt's solution, 500 μg/ml denatured salmon testis DNA, 250 μg/ml denatured baker's yeast tRNA, 1 mM dithiothreitol, and 20–200 ng/ml antisense riboprobe for 18 h at 58 C. After hybridization, sections were washed twice in 5× SSC/50% formamide for 5 min each and twice in 0.2× SSC/50% formamide for 30 min each at the same temperature as that used for hybridization. Subsequently, the sections were immersed in Tris-buffered saline consisting of 50 mM Tris/HCl (pH 7.5) and 150 mM NaCl for 5 min at room temperature, put in the blocking solution containing 0.5% blocking reagent (Roche) in Tris-buffered saline for 30 min, and incubated with streptavidin conjugated with horseradish peroxidase (1:200 dilution) in the blocking solution for 30 min at room temperature. The sections were washed three times in TNT buffer consisting of 50 mM Tris/HCl (pH7.5), 150 mM NaCl, and 0.05% Tween 20 for 5 min each. Biotinyl-tyramide was diluted (1:50 in the diluent

provided), and sections were incubated in the dark for no longer than 10 min at room temperature in this substrate. After three washes of 5 min each in TNT buffer, sections were incubated for 30 min at room temperature with streptavidin conjugated with AP buffer consisting of 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ (1:200 dilution) in the blocking solution. After three washes of 5 min each in TNT buffer, sections were immersed in AP buffer for 5 min. The signals were developed using nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate as chromogenic substrates. Then, the reaction was stopped by rinsing slides in Tris-EDTA buffer and mounted. The signal specificities of the Ob-Rb and α-gustducin mRNA in the taste tissues were tested by using a sense probe as a negative control.

Behavioral experiments

We used two different short-term (10-sec) tests for studying the leptin effects on behavioral responses, one using Suc- or Sac-Qui mixtures as taste stimuli without aversion conditioning (Qui-mixture test) and the other using the CTA paradigm (CTA test).

Qui-mixture test. Details of procedures for this test were described in our previous paper (12). Briefly, each animal with 23-h water deprivation was placed in a test box on d 1 of training and given free access to distilled water during a 1-h session. The licks were detected by a lick meter with a laser beam lick sensor (Yutaka Electronics Co., Gifu, Japan) and recorded on a strip chart recorder. Days 2–5 were the training session. During this period, the animal was trained to drink distilled water on an interval schedule, consisting of 10-sec periods of presentation of the distilled water alternated with 20-sec intertrial intervals. On the d 6, 7, and 8, the number of licks for each test stimuli and distilled water given by each animal was counted during the first 10 sec after the animal's first lick. On each test day, the first test stimulus given to the animal was distilled water. Then mixtures of 3 mM Qui with Suc of six different concentrations (0.01–1.0 M) and Sac of eight different concentrations (0.01–30 mM) were tested in a descending order. After this measurement, the remaining test stimuli (300 mM NaCl, 10 mM HCl, and 3 mM Qui) were tested in a randomized order. As the concentration of Qui for Qui-Suc or -Sac mixture, we chose 3 mM, because no clear strain difference in lick rates for 3 mM Qui alone was observed and good concentration-response curves for Qui-sweet stimuli mixtures were obtained (12). Measurements of the number of licks were made in experimental groups before and 10–40 min after ip injection with 100 ng/g BW of murine leptin (Petro Tech, Inc., Rocky Hill, NJ) dissolved in 10 mM PBS (pH 7.4, 0.0125 ml/g). This time period was chosen because our previous electrophysiological experiments (10) demonstrated that the leptin suppression of taste nerve responses to sweet substances started approximately 7 min after the leptin injection and continued more than an hour. Within this time period, testing continued until the mice no longer licked the distilled water within 7 sec after the animal's first lick on a certain trial. Control groups were injected with the same volume of PBS as that injected in experimental groups. The mean number of licks across 3 d was obtained for each of the test stimuli in each animal. Data are shown as means ± SE. Comparisons between groups were made using *post hoc* Fisher's projected least significant difference (PLSD) test preceded by two-way ANOVA. To obtain threshold for Suc and Sac, comparison in lick rates between 3 mM Qui alone and mixtures of 3 mM Qui and Suc or Sac at various concentration was made by use of Student's *t* test. The level of significance was taken as $P < 0.05$. Statistical comparisons were made using Statview software (Calabasa, CA).

CTA test. Details of procedures for this test were described in our previous paper (13). Procedures for the training session from d 1–5 were the same as those used for the Qui-mixture test. On d 6, each animal with 23-h water deprivation was given access to 0.1 M Suc [conditioned stimulus (CS)] during the interval schedule for more than 20 trials and then given an ip injection of 0.15 M LiCl (0.23 mg/g, 0.036 ml/g; unconditioned stimulus) to induce gastrointestinal malaise. Day 7 was a recovery period, but the training of drinking distilled water for 30 min was still carried out on this day. On d 8–10, the number of licks for each of 13 test stimuli including the CS and distilled water (0.01–1 M Suc, 1–20 mM Sac, 300 mM NaCl, 10 mM HCl, and 0.3 mM Qui) was counted before and 10–40 min after ip injection of leptin (100 ng/g BW) for the experimental group and PBS for control group. On the first trial of the test session the stimulus was always distilled water, followed by the CS on the second trial. This procedure ensured that the animal would drink water but avoid the CS. Animals that did avoid the CS were repeatedly presented with distilled water and each of the test stimuli in a randomized order until the mice no longer licked the distilled water within 7 sec after the animal's first lick on a certain trial. Then, the response of each animal to each stimulus was measured by the mean number of licks during the repeated 10-sec presentations. Statistical analysis used in CTA test was the same as that used for the Qui-mixture test.

Results

Expression of Ob-Rb in taste tissue in nondiabetic and diabetic mice

Expression of Ob-Rb in taste cells in *ob/ob* and *db/db* mice and their lean littermates (+/+ and *db/+*) was first examined by using the nested PCR protocol. As shown in Fig. 1, a band of correct size (413 bp) for functional Ob-Rb was evident in taste buds of *ob/ob* and lean mice. The mRNAs of Ob-Rb were expressed in FP and CP, but not in the epithelial tissue without taste buds, in +/+ mice. Similarly, the RT-PCR products were also expressed in FP (data not shown) and CP in *ob/ob* mice. The *db/db* mice have the longer RT-PCR product (519 bp) in FP (data not shown) and CP than lean mice do (Fig. 1). Sequence analysis of the longer product indicates that it contains an abnormal insertion (106 bp) with a stop codon within the coding region for the intracellular domain of Ob-Rb, as previously shown in other tissues (2, 3). In heterozygous *db/+* mice, RT-PCR products of both the normal and the longer size were detected. All control experiments in which the reverse transcriptase enzyme was omitted (RT–) yielded the negative results as expected.

By using the ISH protocol combined with the TSA method (15, 16), signals of mRNA for Ob-Rb were detected in some spindle-shaped cells of fungiform and circumvallate taste

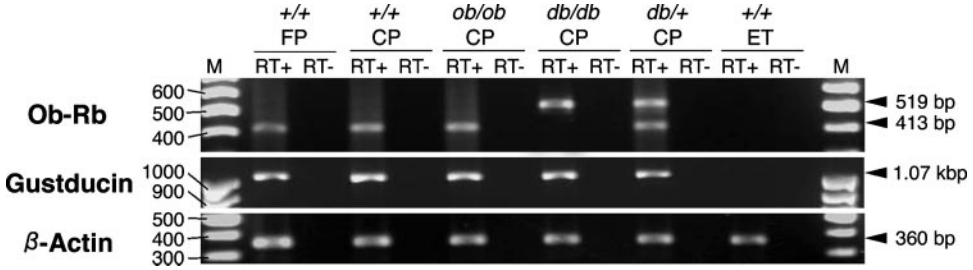

FIG. 1. RT-PCR analysis of leptin receptor, Ob-Rb, α-gustducin, and β-actin in diabetic (*ob/ob* and *db/db*) and nondiabetic (+/+ and *db/+*) mouse FP, CP, and tongue epithelium (ET). The RT-PCR products of normal size (413 bp) were expressed in *ob/ob* and lean control (+/+ and *db/+*), and the longer products (519 bp) were detected in *db/db* and *db/+*. M, 100-bp size markers; RT+, with reverse transcriptase; RT–, without reverse transcriptase.

buds in *ob/ob* and lean (+/+) mice, although the signal detected was much less intense compared with that for gustducin (Fig. 2). In *db/db* mice, the hybridization signals for Ob-Rb mRNA were not detected (Fig. 2). The staining in controls using sense probe was negative. These results, together with the data from RT-PCR analysis, strongly suggest that functional Ob-Rb mRNA is expressed in taste receptor cells of taste buds located on both the anterior and posterior tongue in *ob/ob* and lean mice.

Leptin effects on behavioral responses to sweet substances

Behavioral preferences for sweet substances mixed with Qui before and after ip injection of PBS are shown in Fig. 3. PBS injections produced no significant changes in the mean number of licks to all test stimuli in all strains tested [ANOVA, $F_{1,8-140} = 0.001–0.01$ ($P > 0.05$)]. Lick rates for mixtures of 3 mM Qui and 0.01 M Suc or 0.01 mM Sac were significantly greater than those for 3 mM Qui alone in all mouse strains tested (*t* test, $P < 0.05$). This indicates that thresholds for Suc and Sac are 0.01 M and 0.01 mM or less, respectively, in all mouse strains. Also, lick rates for 3 mM Qui alone were approximately 10 licks/10 sec among all mouse strains, suggesting no strain difference in sensitivities to Qui.

Behavioral preferences for the sweet-Qui mixtures before and after ip injection of leptin (100 ng/g BW; 500 ng/g BW for *db/db* mice) are shown in Fig. 4. In lean-*ob* mice, the mean number of licks to the Qui-mixed Suc (0.01–1.0 M) and Sac (0.01–30 mM) after leptin injection was significantly lower than that before leptin [ANOVA, $F_{1,40} = 6.02$ ($P < 0.05$) for Suc, and $F_{1,56} = 10.78$ ($P < 0.05$) for Sac]. The Fisher's PLSD *post hoc* test indicates significant differences in lick rates for Suc at lower concentrations, such as 0.01 and 0.03 M ($P < 0.05$) and Sac at higher concentrations, such as 5–30 mM ($P < 0.01–0.05$). In lean-*db* mice, leptin injection also decreased lick rates for sweet-Qui-mixtures [$F_{1,100} = 6.26$ ($P < 0.05$) for Suc, and $F_{1,140} = 12.0$ ($P < 0.01$) for Sac]. The *post hoc* test indicated that decreases in lick rates were significant for Suc at concentrations from 0.01 to 0.3 M ($P < 0.05$), and for Sac at all concentrations tested ($P < 0.01–0.05$). In *ob/ob* mice, again, similar leptin inhibition on lick rates for sweet-Qui mixtures was observed [$F_{1,80} = 5.21$ ($P < 0.05$) for Suc and $F_{1,112} = 6.06$ ($P < 0.05$) for Sac]. The *post hoc* test indicated significant reduction in lick rates for Suc at 0.03 and 0.3 M ($P < 0.05$) and for Sac at 0.01, 0.03, 10, and 30 mM ($P < 0.01–0.05$). In contrast, no such effect of leptin was observed in *db/db* mice even if a much higher dose of leptin (500 ng/g BW) was injected [$F_{1,40} = 0.004$ ($P > 0.05$) for Suc, and $F_{1,56} = 0.15$ ($P > 0.05$) for Sac]. Injections with leptin produced no significant changes in lick rates for 300 mM NaCl [$F_{1,8-20} = 0.01–0.72$ ($P > 0.05$)], 10 mM HCl [$F_{1,8-20} = 0.001–2.18$ ($P > 0.05$)], or 3 mM Qui [$F_{1,8-20} = 0.074–3.19$ ($P > 0.05$)] in all strains tested (Fig. 5).

Fig. 6 shows data obtained from the CTA test. In this test, ip injection with leptin (100 ng/g BW) significantly increased lick rates for Suc and Sac [$F_{1,90} = 22.20$ ($P < 0.01$) for 0.01–1.0 M Suc, and $F_{1,36} = 7.46$ ($P < 0.05$) for 1–20 mM Sac]. The *post hoc* test indicated a significant increment in lick rates for 0.03 and 0.05 M Suc ($P < 0.01$) and 1 mM Sac ($P < 0.05$). Injections with leptin produced no significant changes in lick rates for 300 mM NaCl [$F_{1,18} = 0.212$ ($P > 0.05$)], 10 mM HCl [$F_{1,18} = 0.022$ ($P > 0.05$)], or 0.3 mM Qui [$F_{1,18} = 0.19$ ($P > 0.05$)]. PBS injections also did not affect lick rates for all test stimuli, including sweet substances [$F_{1,18-90} = 0.001–0.006$ ($P > 0.05$)].

The results from two different tests clearly indicate that leptin selectively suppresses behavioral responses to sweet substances, but not to salty, sour, and bitter stimuli.

Discussion

In the present study, by using RT-PCR analysis, we revealed that the functional leptin receptor Ob-Rb mRNA (as

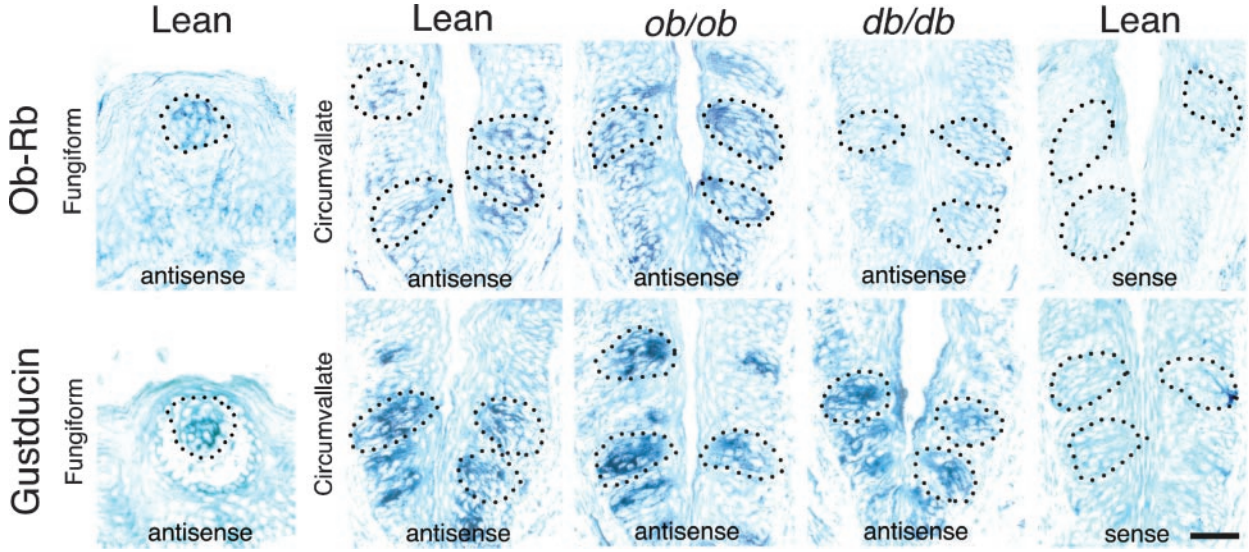

FIG. 2. ISH analysis of Ob-Rb and α-gustducin mRNA in nondiabetic lean +/+ and diabetic *ob/ob* and *db/db* mouse FP and CP. Biotin-labeled RNA antisense or sense probe for Ob-Rb or gustducin mRNA were hybridized to sections of FP and CP. The biotin epitope was amplified by the TSA method and visualized by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The *dotted lines* indicate the outlines of sample taste buds. *Bar*, 50 μm.

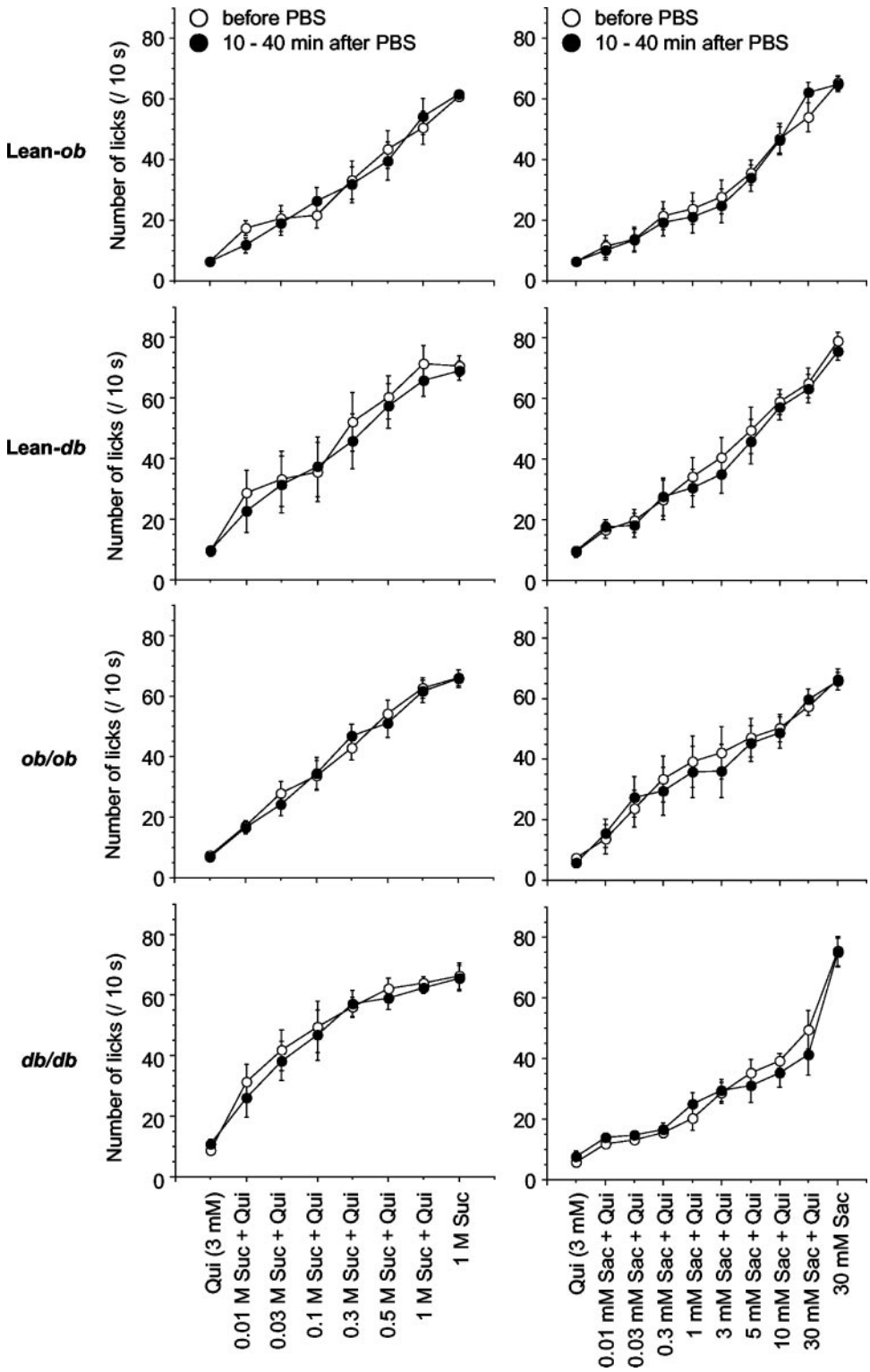

FIG. 3. Concentration-response (mean number of licks/10 sec) relationships to Suc (0.01–1.0 M) or Sac (0.01–30 mM) with Qui (3 mM) mixtures before (*open circles*) and 10–40 min after (*closed circles*) ip injection of PBS in lean-*ob* (n = 9), lean-*db* (n = 7), *ob/ob* (n = 6), and *db/db* (n = 4) mice. There is no significant difference in the mean number of licks between two groups before and after ip injection of PBS (ANOVA, $P > 0.05$).

RT-PCR products of 413 bp) is expressed in the fungiform and circumvallate taste buds in *ob/ob* mice and normal lean littermates of *ob/ob* and *db/db* mice. In *db/db* mice, the RT-PCR product was longer (519 bp) than the normal size. The heterozygous *db/+* mice possessed both products. Sequence analysis indicates that the longer product contains an abnormal insertion with a stop codon within the coding region for the intracellular domain of Ob-Rb. This insertion was shown to lead to the shorter intracellular domain of Ob-Rb and impairments of the rest of the transduction process after ligand-receptor binding in *db/db* mice (2, 3).

ISH analysis revealed that Ob-Rb mRNAs were expressed in some of the fungiform and circumvallate taste bud cells of *ob/ob* and lean control mice, although no such expression

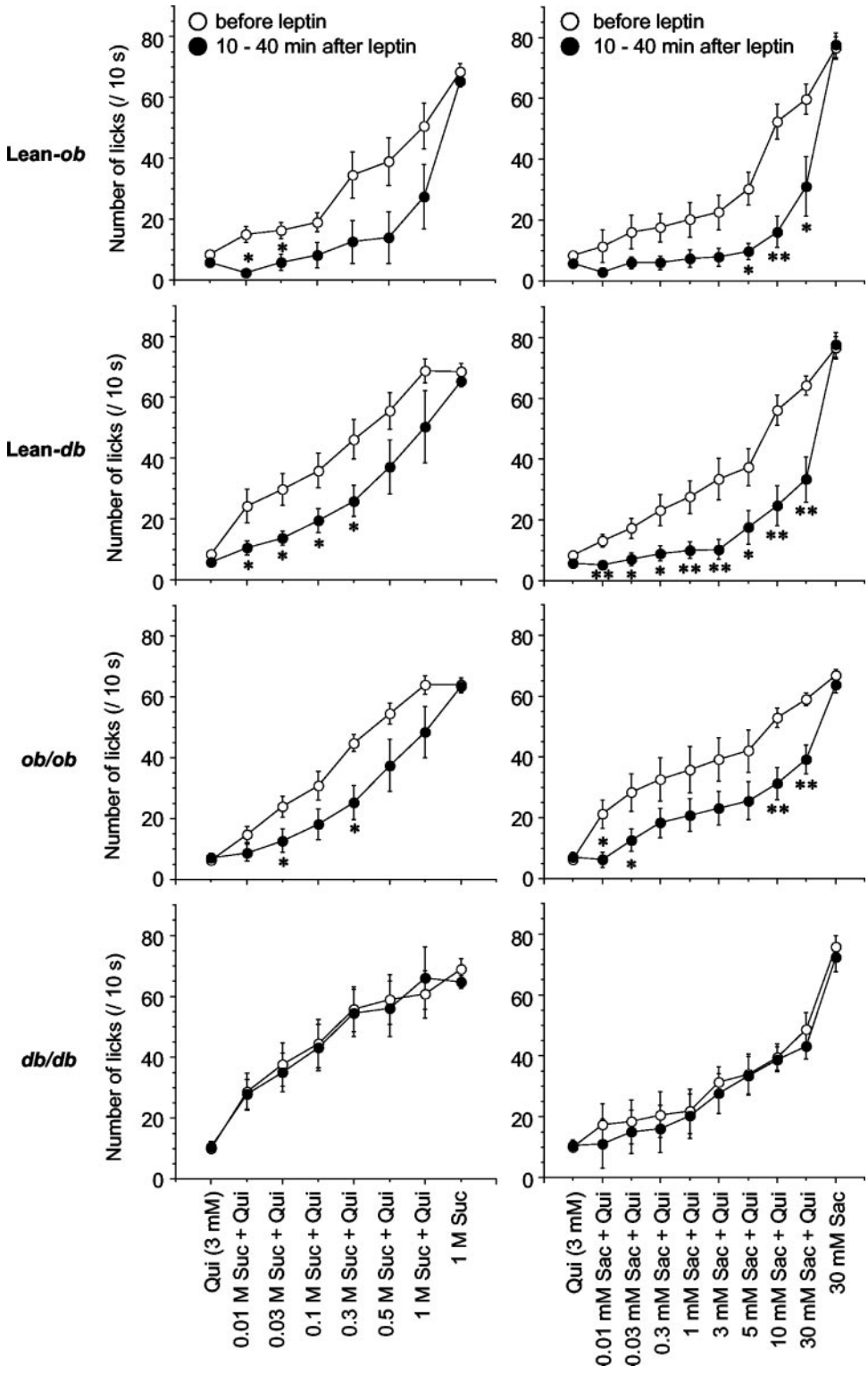

FIG. 4. Concentration-response (mean number of licks/10 sec) relationships to Suc (0.01–1.0 M) or Sac (0.01–30 mM) with Qui (3 mM) mixtures before (*open circles*) and 10–40 min after (*closed circles*) ip injection of murine leptin (100 ng/g BW; 500 ng/g for *db/db*) in lean-*ob* (n = 5), lean-*db* (n = 11), *ob/ob* (n = 9), and *db/db* (n = 5) mice. The mean numbers of licks between before and after ip injection of leptin were statistically analyzed by a Fisher's PLSD *post hoc* test preceded by ANOVA. *, $P < 0.05$; **, $P < 0.01$ *vs.* the group before ip injection of leptin.

was evident in *db/db* mice. The positive hybridization signal became evident by using the ISH protocol combining the TSA method. This method is known to enzymatically catalyze the deposition of more biotin molecules at the site of the biotinylated riboprobe-mRNA hybrids. The signal is then further amplified by binding streptavidin-alkaline phosphatase to the biotin. Therefore, TSA offers greater resolution and enhances sensitivity *vs.* radiometric techniques (15). By using the ISH protocol combining the enzyme-labeled fluorescent and TSA methods, an intense signal of Ob-Rb mRNA was detected in the hypothalamic neurons where Ob-Rb mRNA is present in relatively low abundance (16). Usually,

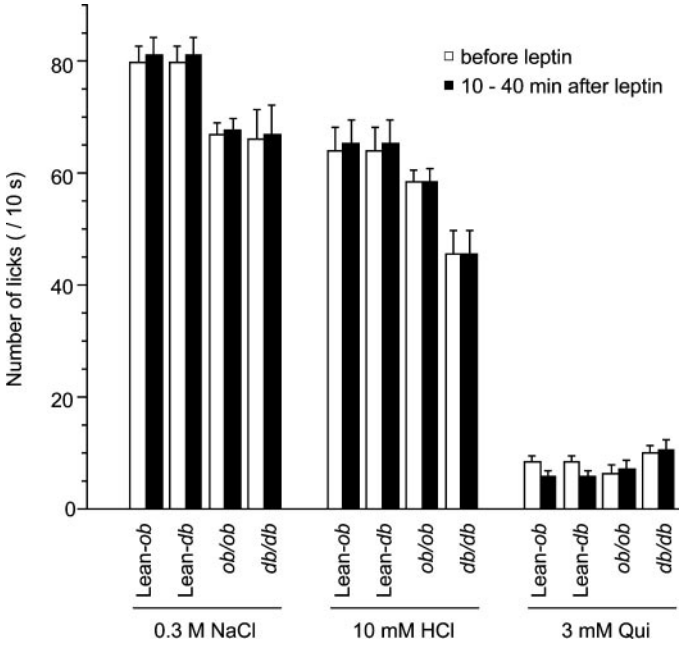

FIG. 5. Responses (mean number of licks/10 sec) to NaCl (0.3 M), HCl (10 mM), and Qui (3 mM) before (*open bars*) and 10–40 min after (*closed bars*) ip injection of 100 ng/g BW (500 ng/g for *db/db*) murine leptin in lean-*ob* (n = 5), lean-*db* (n = 11), *ob/ob* (n = 9), and *db/db* (n = 5) mice. There is no significant difference in the mean number of licks between two groups before and after ip injection of leptin.

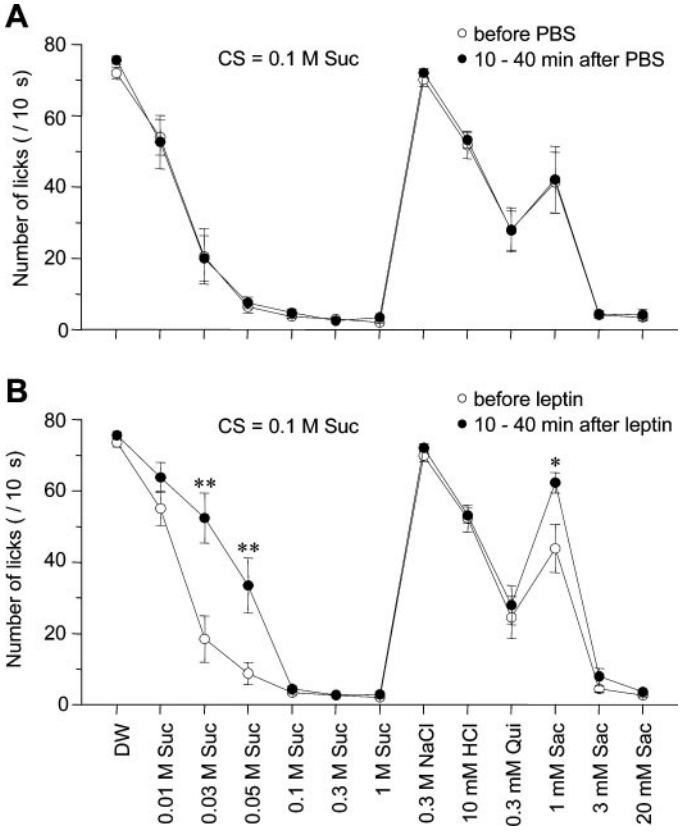

FIG. 6. Responses (mean number of licks/10 sec) of mice (C57BL/KsJ strain, n = 6–10 for each group) to the various taste stimuli before (*open circles*) and 10–40 min after (*closed circles*) ip injection with PBS (A) or 100 ng/g murine leptin (B) by using the CTA paradigm. The mean numbers of licks between before and 10–40 min after ip injection with PBS or leptin were statistically analyzed by Fisher's PLSD *post hoc* test preceded by ANOVA. *, $P < 0.05$; **, $P < 0.01$.

such signal amplification is accompanied by increasing nonspecific background. In the present study, however, we prepared negative controls with sense probe or without probe that exhibited neither general background nor nonspecific staining of cells as shown in Fig. 2.

Because the riboprobe used for ISH was complementary to the coding region for intracellular domain of Ob-Rb, the signal for mRNA was not detected in *db/db* mice, which have mutant Ob-Rb mRNA. Previous studies showed that *db/db* mice have a G→T point mutation in the genomic Ob-R sequence. This mutation generates a donor splice site that converts the 106-bp region to a novel exon retained in the Ob-Rb transcript (2, 3). This suggests that the riboprobe used in this study may be unable to bind the mutant form in *db/db* mice. In addition, the riboprobe may not recognize mRNA for Ob-Ra, which is the Ob-R short form and is expressed abundantly and ubiquitously in various tissues including taste buds (Refs. 7–9; and Shigemura, N., H. Miura, Y. Kusakabe, A. Hino, and Y. Ninomiya, unpublished observation).

The present study provided evidence for expression of Ob-Rb not only in the circumvallate but also fungiform taste bud cells. This is comparable with data from our previous electrophysiological experiments (10) showing that leptin injection suppresses neural responses of both CT and GL nerves to sweet substances. The fungiform taste bud cells innervated by the CT nerve are known to show much greater sensitivities to sweet substances than the circumvallate taste bud cells innervated by the GL nerve (11). Our previous study using mice with bilateral denervation of the CT or the GL nerve indicated that taste information conveyed by the CT played more important roles in behavioral discrimination between sweet and other taste compounds than did the GL nerve (Ninomiya, Y., unpublished observation).

The difference in the presence or absence of functional Ob-Rb in taste cells between *db/db* and *ob/ob* mice was consistent with the difference in effects of leptin on behavioral responses to sweet substances in these mouse groups. In the present behavioral study, we examined taste responses within the limited time period from 10–40 min after the leptin injection. During this time period, the numbers of licks for distilled water in water-deprived animals did not reach the level for terminating the experiment (when the mice no longer licked the distilled water for 7 sec after the animal's first lick). Concentration-response curves for Qui-Suc or -Sac mixtures were also clearly evident. Thus, during this time period, a decrease in thirst due to hydration might not largely influence the results of this test, although lick rates for distilled water tended to decrease. Under such conditions, lick rates for mixtures of Qui with Suc or Sac at particular concentration ranges were found to significantly decrease after the ip injection of leptin in *ob/ob* and lean littermates (*ob/+* and *db/+*), whereas no such effect of leptin was observed in *db/db* mice. Because the saline-injected control group in each strain showed no changes in lick rates for the mixtures, the observed decrease in mice other than *db/db* mice is suggested to be caused by the leptin injection.

It has been shown that in both humans and rodents (17–19), responses to a Suc-Qui mixture are lower than the sum of responses to each component of mixture presented alone. This so-called mixture suppression between Suc and Qui is proposed to involve both central and peripheral mechanisms (17–19). Electrophysiological studies in the hamster CT nerve demonstrated that suppressive effects of Qui on Suc responses occur in Suc-best fibers, suggesting that mixture suppression may occur at the intracellular level of Suc-responsive taste cells (19). In the present study, the concentration-response curve for Suc-Qui mixtures in the Qui-mixture test corresponds to that for Suc alone in the CT nerve responses in C57BL mice (20). The behavioral threshold for Suc (0.01 M or less) is approximately the same as that (0.01 M) for Suc responses of the CT nerve (21) or that from a long-term (48-h) two-bottle preference test in the same C57BL mice without water deprivation (20). These results suggest that peripheral mixture suppression may be negligible in the present mouse behavioral test. Thus, the Qui-mixture short-term (10-sec) test may provide a tool for repeated measurements of taste-guided behavioral responses to sweet substances within a limited time period in mice, and thereby the observed leptin suppression on behavioral responses to sweet substances in the present study is suggested to be reliable.

For behavioral response measurements, we also used a CTA paradigm. In this method, leptin suppression of sweet perception, if any, is evidenced by an increase in the number of licks. The results of the CTA experiment clearly showed that lean mice conditioned to avoid Suc increase their licks for low (0.03–0.05 M) but not high (0.1–1.0 M) concentrations of Suc after leptin injection. In the control group, the number of licks of 0.03–1.0 M Suc was not significantly changed after saline injection. The increase of consumption was also observed in response to 1.0 mM but not 3–20 mM Sac. Because the saline-injected control group showed no changes in numbers of licks for any stimulus, the observed increase in lick rates for Suc and Sac is, again, likely caused by the leptin injection.

One of the main effects of leptin via central hypothalamic targets is thought to reduce appetite and ingestive behavior. Kinetic studies, however, indicate that upon a single iv or ip injection, leptin decreases food intake only after several hours in mice (22). Thus, changes in behavioral responses to sweet substances observed within 40 min after leptin injection in the present study might not be involved in such long-term regulation of food intake through central hypothalamic pathways. Reduction of short-term food intake (first 3 h after injection) has, however, been reported to occur when leptin was coinjected with cholecystokinin (CCK) or CCK-inducible gastric nutrient preload (23). CCK is normally secreted from small intestinal cells in response to food ingestion and produces a postprandial satiety signal by interacting with vagal afferent fibers. Recent studies in rats and mice (24–26) suggest the importance of the nucleus of the solitary tract (NTS) as a site for the synergistic interaction between leptin and CCK, because the NTS receives primary vagal afferent input (25) and expresses the Ob-Rb (26). Responses to gastric loads in some NTS neurons have been reported to be enhanced by third-ventricular administration of leptin in rats (25). These meal-related ascending signals through the NTS may modify short-term food intake, although it remains unclear whether such behavioral modification occur through hypothalamic signaling pathways. In the present study, we found a decrease in lick responses of lean and *ob/ob* mice to Suc and Sac after leptin in the Qui-mixture test. This decrease may, therefore, be partially due to negative feedback control on the intake by the synergistic interaction between leptin and CCK that might occur after leptin with CCK-inducible nutrient preloads during repeated trials for control lick measurements in the test. Likewise, increases in consumption of low concentrations of aversive Suc or Sac solutions after leptin observed in CTA experiments may also be due to some modulation of ascending aversive taste signals by leptin at the brainstem. There is, however, no direct evidence that leptin may modulate taste signals, at least arising from the oral cavity in the central nervous system. Therefore, increases in consumption of aversive Suc and Sac solutions may be more reasonably explained by leptin suppression of responses to sweet substances at the taste receptor cell level (10), if the strength of the behavioral aversion for these sweet substances is paralleled by the strength of the perceived taste intensity. Collectively, the results obtained from two different tests suggest that suppression by leptin of behavioral responses to sweet substances occurs at least partially through its functional receptor, Ob-Rb, in mouse taste cells.

Our previous study (10) revealed that receptor cells of the taste sensory system were affected by leptin. The *db/db* mouse, which has defects in the functional receptor Ob-Rb, displays enhanced neural responses and elevated behavioral preference to sweet stimuli (20, 21) because of defects in leptin suppression of sweet responses in the taste system that healthy mice normally possess (10, 27). Here, we showed expression of the functional Ob-Rb receptor in taste cells of lean and *ob/ob* mice but not in the *db/db* mice. By binding the receptor in taste cells, the hormone partially suppresses taste cell responses and inhibits afferent signals indicating sweet taste. The blunting of sweet taste leads to a decrease in consumption of sweet solutions in mice demonstrated by the present study. Thus, leptin may influence food intake not only through the central nervous system but also at a peripheral level acting as a sweet-sensing modulator.

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