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Taste Responses to Sweet Stimuli in α-Gustducin Knockout and Wild-Type Mice

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Abstract

The importance of α-gustducin in sweet taste transduction is based on data obtained with sucrose and the artificial sweetener SC45647. Here we studied the role of α-gustducin in sweet taste. We compared the behavioral and electrophysiological responses of α-gustducin knockout (KO) and wild-type (WT) mice to 11 different sweeteners, representing carbohydrates, artificial sweeteners, and sweet amino acids. In behavioral experiments, over 48-h preference ratios were measured in two-bottle preference tests. In electrophysiological experiments, integrated responses of chorda tympani (CT) and glossopharyngeal (NG) nerves were recorded. We found that preference ratios of the KO mice were significantly lower than those of WT for acesulfame-K, dulcin, fructose, NC00174, D-phenylalanine, L-proline, D-tryptophan, saccharin, SC45647, sucrose, but not neotame. The nerve responses to all sweeteners, except neotame, were smaller in the KO mice than in the WT mice. The differences between the responses in WT and KO mice were more pronounced in the CT than in the NG. These data indicate that α-gustducin participates in the transduction of the sweet taste in general.

Key words: chorda tympani, glossopharyngeal, α-gustducin, knockout mice, sweet taste, two-bottle preference

Introduction

The transduction of sweet, bitter, and umami tastes involves binding to and activation of G protein–coupled receptors (GPCRs) by tastants (Chaudhari and Roper, 1998; Gilbertson *et al.*, 2000; Lindemann, 2001). Several sweet-, bitter- and umami-responsive GPCRs have been identified, including the sweet-responsive T1r2 + T1r3 heterodimer, the bitter-responsive T2rs, and the umami-responsive taste heterodimer T1r1 + T1r3 and mGluR4 (Chandrashekar *et al.*, 2000; Nelson *et al.*, 2001, 2002). While it has been established that these receptors couple to and activate G proteins in heterologous systems (Chandrashekar *et al.*, 2000; Nelson *et al.*, 2001, 2002), it is not completely established how many and which G proteins couple to sweet-responsive receptors *in vivo*. α-Gustducin, the alpha-subunit of a heterotrimeric G protein selectively expressed in taste receptor cells (TRCs) (McLaughlin *et al.*, 1992), has been shown to be involved *in vivo* in bitter, sweet, and umami taste responses (Wong *et al.*, 1996; Ruiz-Avila *et al.*, 2001; Margolskee, 2002, He, 2004). Thus, in long-term (48 h) two-bottle preference tests, α-gustducin knockout (KO) mice showed significantly reduced preference for sucrose and SC45647 compared to wild-type (WT) littermates (Wong *et al.*, 1996; Ruiz-Avila *et al.*, 2001; He *et al.*, 2002). In short-term (30 min) brief-access licking tests, the results with sucrose, SC45647, and maltose corroborated the results above (Glendinning *et al.*, 2005). Furthermore, the summated responses to sucrose and SC45647 in both chorda tympani (CT) and glossopharyngeal (NG) nerves were diminished in α-gustducin KO mice in comparison to WT mice (Wong *et al.*, 1996; He *et al.*, 2002).

The conclusion that α-gustducin is a key factor *in vivo* in mediating transduction of sweet taste is based on the above-mentioned experiments with the two sweeteners, sucrose and SC45647 (Wong *et al.*, 1996; Ruiz-Avila *et al.*, 2001; He *et al.*, 2002; Glendinning *et al.*, 2005). However, sweet taste can be elicited by many more compounds. In the above behavioral study, which used brief-access taste tests, the role of α-gustducin was not expanded beyond the original two

Table 1 Stimuli and concentrations used in electrophysiological and behavioral experiments

Stimuli	CT recordings	NG recordings	Two-bottle preference tests
	Concentration (WT[a], KO)	Concentration (WT[a], KO)	Range of concentrations (WT, KO)
Dulcin	5 (3, 4); 10 (5, 3)	5 (3, 3); 10 (4, 4)	0.1–10 (10, 9)
Fructose	300 (17, 9); 600 (13, 7)	300 (17, 7); 600 (15, 6)	
NC00174	0.07 (10, 8); 0.14 (15, 6)	0.07 (10, 7); 0.14 (15, 6)	0.0014–0.058 (10, 10)
Neotame	5 (5, 3); 10 (6, 5); 15 (5, 3); 20 (5, 3)	5 (3, 5); 10 (8, 7); 15 (3, 5); 20 (4, 5)	0.1–20 (10, 9)
D-phenylalanine	100 (17, 9); 150 (11, 9)	100 (19, 7); 150 (11, 8)	1.0–60 (9, 8)
L-proline	100 (9, 5), 1000 (9, 5)	100 (12, 8), 1000 (15, 10)	1–1500 (9, 8)
Saccharin	10 (4, 0); 50 (9, 5); 100 (9, 5)	10 (12, 2); 50 (14, 7); 100 (17, 8)	0.05–30 (10, 10)
SC45647	8 (7, 5)	1 (4, 2); 8 (5, 6); 10 (4, 2)	0.01–1 (13, 6)
Sucrose	500 (10, 3)	100 (4, 2); 250 (4, 2); 500 (13, 4)	10–230 (13, 6)
D-tryptophan	10 (7, 5); 50 (7, 5)	10 (7, 6); 25 (7, 4); 50 (11, 9)	0.1–20 (10, 10)
Citric acid	20 (18, 20)	20 (41, 20)	
NaCl	100 (23, 14)	100 (45, 24)	
NH_4Cl	100 (27, 14)	100 (51, 29)	0.3–300 (11, 9)
QHCl	10 (21, 11)	10 (43, 20)	

WT, wild type; KO, α-gustducin KO mice. Figures outside parentheses are concentrations in mM, and figures inside parentheses are the number of WT and KO recordings, respectively.
[a]WT group comprising GUS/GUS and GUS/gus mice.

sweeteners and maltose (Glendinning *et al.*, 2005). Therefore, we here address the question how general is the role of α-gustducin in transduction of sweet taste by comparing the behavioral and electrophysiological responses of α-gustducin KO and WT mice to 11 different sweeteners, representing carbohydrates, artificial sweeteners, and sweet amino acids.

Materials and methods

Electrophysiological experiments

Animals and surgery

Recordings were obtained from a total of 86 male mice: 30 α-gustducin KO (gus/gus) mice, which had been backcrossed into the C57BL6/J background (mice were 97% C57BL6/J and 3% 129SvEmsJ), their 24 heterozygous (GUS/gus) littermates and 32 WT homozygotes (GUS/GUS) in the pure C57BL6/J background. The animals weighed 20–39 g and were 9–18 months at the time of recording.

Anesthesia was initiated with an intramuscular injection of a mixture of 1.75 mg/ml ketamine and 1.75 mg/ml xylazine in saline (5 μl/g body weight). The mouse was then intubated and the anesthesia maintained with 0.8–0.9% halothane. Body temperature and heart rate were continuously monitored.

The CT nerve was dissected free at its junction with the lingual nerve rostral to the tympanic bulla. The NG was accessed through the same incision as the CT and cut near its exit from the posterior lacerated foramen (Danilova *et al.*, 2002).

In 42 mice, we recorded first from the CT and then from the NG. In five mice, we recorded only from the CT. In 39 mice, we recorded only from the NG. To insure that the foliate and vallate taste buds were stimulated, we had to stretch the tongue.

Stimuli and stimulation

Table 1 lists stimuli and concentrations used in both electrophysiological and behavioral experiments. The first column lists compounds tested. In the second and third column, the figures in front of each parenthesis show concentration used, while inside the parentheses, the first figure shows the number of recordings in WT mice and the second figure the number of recordings from KO mice. The fourth column lists ranges of concentrations used in two-bottle preference (TBP) experiments. The figures in parentheses show numbers of WT and KO mice tested. All compounds except further specified were obtained from Sigma Chemical Co, St Louis, MO. Neotame, SC45647, NC00174, and dulcin were gifts from NutraSweet Company. The information and structural formula for SC45647 and NC00174 can be found in Nagarajan *et al.* (1996) and for neotame in Neotame Scientific Overview Brochure (2002).

The stimuli were delivered to the tongue with an open-flow system controlled by a computer under conditions of constant flow and temperature (33°C) (Hellekant and Roberts, 1995). Each stimulation lasted for 20 s with 50 s rinsing time between stimulations.

All compounds were dissolved in artificial saliva. Between stimulations the tongue was rinsed with the same artificial saliva as in our previous mouse study (He *et al.*, 2002). We use artificial saliva to maintain the taste buds in good condition and because it produced stable and reproducible nerve recordings for many hours.

Care was exercised to make sure that the tongue was optimally stimulated. During the CT experiments, the flow was directed over the fungiform papillae and was easily visible. During the NG recordings, we checked with a surgical microscope that the flow ran over the vallate and foliate papillae. Furthermore, at the beginning of a recording sequence, we tested whether the summated response had a sharp onset to acids. The sharp onset of these responses revealed if the taste buds on the back of the tongue were adequately stimulated and diminished the possibility that a lack of a phasic component of the responses was not artifactual but related to the property of the stimulus.

Recording and analysis

Nerve impulses were recorded with a custom-made amplifier, monitored over a loudspeaker and an oscilloscope, and fed into a recorder (Gould ES 1000) and into an IBM computer via a DAS–Keithley interface. For the whole nerve recordings, the nerve impulses were processed by a smoothed absolute value circuit integrator (Hellekant and Roberts, 1995) and changed to a direct current potential whose amplitude was related to the nerve impulse frequency, here called the summated response. This signal and a code related to the tastant on the tongue were fed to the computer. Its program sampled the summated response before, during, and after stimulation and displayed it on a monitor.

The integrated response during stimulation was calculated by subtracting the area of spontaneous nerve activity (preceding stimulation) from that during stimulation. Thus, it reflects the level of activity during 20 s of stimulation time. The responses to all compounds were expressed relative to the response to 0.1 M NH_4Cl.

The GUS/GUS and GUS/gus groups were pooled into one group. To justify this, we compared the CT as well as NG responses to the stimuli tested in both groups (data not shown). Because we found no statistically significant differences between the average responses of the two groups, we used GUS/GUS and/or GUS/gus as representative of the WT group.

To assess differences between the WT and KO groups, separate analyses were carried out for comparisons of responses in either the CT or NG. Responses were first evaluated with two-way analysis of variance (ANOVA) with mice assigned at random to groups and varying concentrations. If significant effect of group was found, then *post hoc* comparisons using protected Fisher's least squares differences test were performed to check differences between WT and KO groups at specific concentrations. For stimuli presented at single concentrations, the differences were assessed with two-tailed *t*-tests. *P* values for all statistical tests are shown in Table 2. The first column lists compounds. The second and third columns show concentrations and *P* values, within parentheses, for each concentration in the CT and NG, respectively. *P* value less than 0.05 was considered significant.

Table 2 *P* values for statistical tests between WT and gustducin KO mice

	Results in the CT	Results in the NG
Acesulfame-K	25 (0.004); 50 (0.013)	10 (0.1); 25 (0.006); 50 (<0.001)
Dulcin	5 (0.31); 10 (0.009)	5 (0.25); 10 (0.64)
Fructose	300 (0.011); 600 (<0.001)	300 (0.07); 600 (0.016)
NC00174	0.07 (0.001); 0.14 (<0.001)	0.07 (0.29); 0.14 (0.006)
D-phenylalanine	100 (0.02); 150 (<0.001)	100 (0.051); 150 (0.007)
L-proline	100 (0.07), 1000 (0.86)	100 (0.65), 1000 (<0.001)
Saccharin	50 (0.057); 100 (0.08)	10 (0.11); 50 (<0.001); 100 (<0.001)
SC45647	8 (0.029)	8 (0.006): 10 (0.18)
Sucrose	500 (<0.001)	100 (0.6); 250 (0.042); 500 (0.037)
D-tryptophan	10 (0.77); 50 (0.01)	10 (0.19); 50 (0.01)
Citric acid	20 (0.7)	20 (0.18)
NaCl	300 (0.28)	300 (0.44)
QHCl	10 (0.6)	10 (<0.01)
Neotame	NS	NS

Figures outside parentheses are concentrations (mM), and figures inside parentheses are *P* values for each concentration tested.

Behavioral experiments

Two-bottle preference tests were carried out with 16 KO gus/gus and 23 WT GUS/gus mice housed individually. Table 1 indicates the range of concentrations of the stimuli and the number of animals used. We could not use as high concentration of NC00174 and D-tryptophan in TBP tests as in the nerve recordings because they precipitated during the 48-h test period. Some of the animals used in behavioral experiments were later used in electrophysiological experiments.

The intake of water and tastant was recorded during two consecutive 24-h periods with the graduated cylinders switched after 24 h. For each mouse, the preference ratio was calculated for a 24-h period as the amount of a tastant consumed divided by the total amount of liquid consumed from both cylinders. Data for 2 days were averaged, and the mean preference ratio was calculated for the WT and

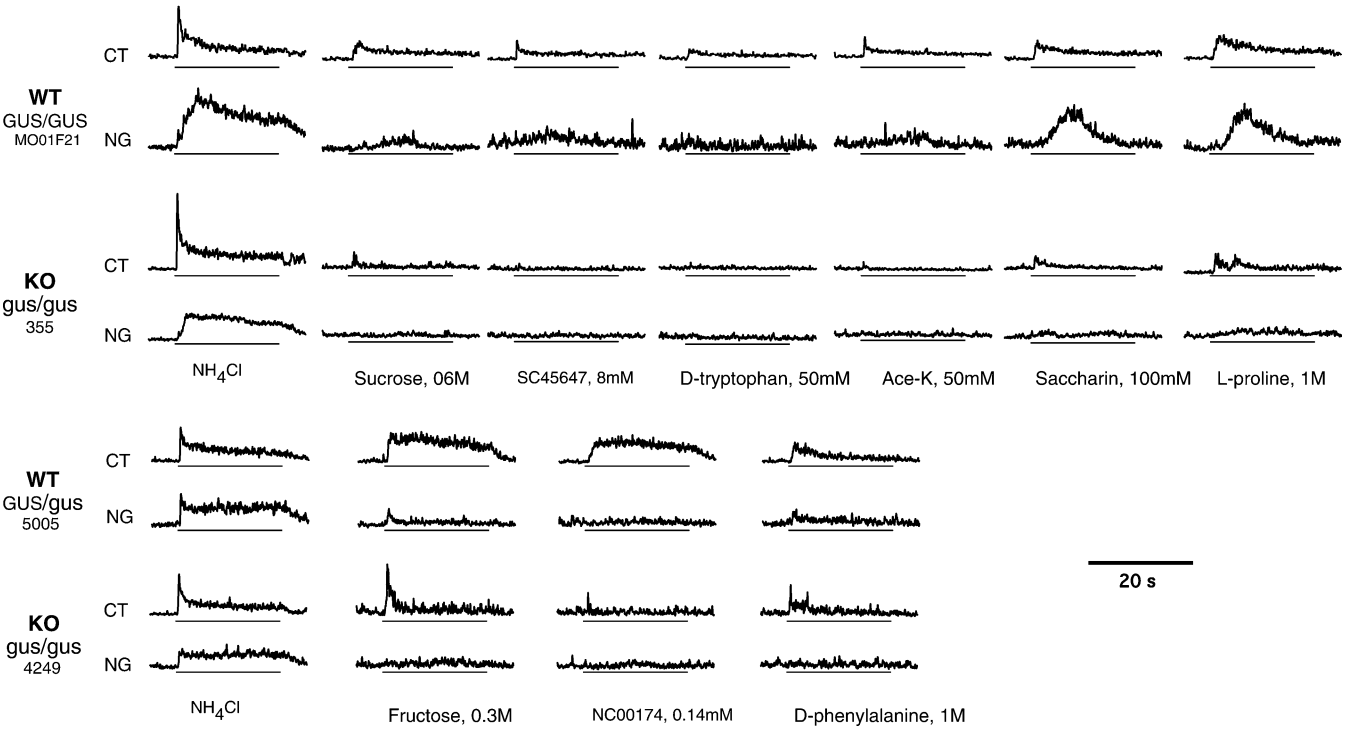

Figure 1 Summated responses from the CT and NG nerves during stimulation of the tongue in WT mouse MO01F21 (GUS/GUS) and 5005 (GUS/gus) and α-gustducin KO mouse 355 and 4249 (gus/gus). In all four animals, the responses were obtained from both the CT and NG nerves. The bar at the bottom of each recording indicates stimulation (20 s).

KO groups. Equal consumption of both liquids (indifference) yields a preference ratio of 0.5, and complete preference yields a preference ratio of 1.

Responses of the two groups were first compared by ANOVA, treating groups as between-subject variable and concentrations as within-subject variable. If significant interactions were found, then *post hoc* comparisons using protected Fisher's least squares differences test were performed to check differences between WT and KO groups at specific concentrations. For all statistical tests, $P < 0.05$ was considered significant.

Results

Nerve responses to sweet compounds

Figure 1 shows representative traces from CT and NG nerve recordings from two WT mice, MO01F21 (GUS/GUS) and 5005 (GUS/gus), and two α-gustducin KO (gus/gus) mice, 355 and 4249. MO01F21 and 355 belonged to one series in which the animals were tested with sucrose, SC45647, D-tryptophan, acesulfame-K, saccharin, and L-proline. Mouse 5005 and 4249 belonged to another series in which the animals were tested with fructose, NC00174, and D-phenylalanine. NH_4Cl used as a standard was tested in all series in all animals.

Figure 2 compares the integrated nerve responses to the sweet stimuli in the WT and KO mice. In general, the nerve responses to most sweeteners tested were smaller in the KO mice than in the WT mice. In comparison to the WT, the responses of KO mice to acesulfame-K, fructose, D-phenylalanine, SC45647, sucrose, and D-tryptophan were significantly smaller in both nerves, whereas responses to dulcin and NC00174 were significantly smaller only in the CT and to L-proline and saccharin only in the NG. The CT and NG responses to neotame did not differ between the two groups.

Responses to nonsweet stimuli

To determine if α-gustducin is also involved in transduction of other taste qualities, stimuli representing sour (citric acid), salty (NaCl), and bitter [quinine hydrochloride (QHCl)] taste qualities were also tested in the WT and KO mice. Figure 3 shows the results. In the CT, we found no significant differences between the WT and KO responses to NaCl, citric acid, and QHCl. In the NG, the responses to NaCl and citric acid again did not differ between the WT and KO mice, whereas the responses to QHCl were significantly smaller in the KO mice than in the WT (Table 2).

Two-bottle preference experiments

Figure 4 compares the 48-h preference ratios of WT and KO mice for acesulfame-K, dulcin, NC00174, neotame, D-phenylalanine, L-proline, D-tryptophan, saccharin, SC45647,

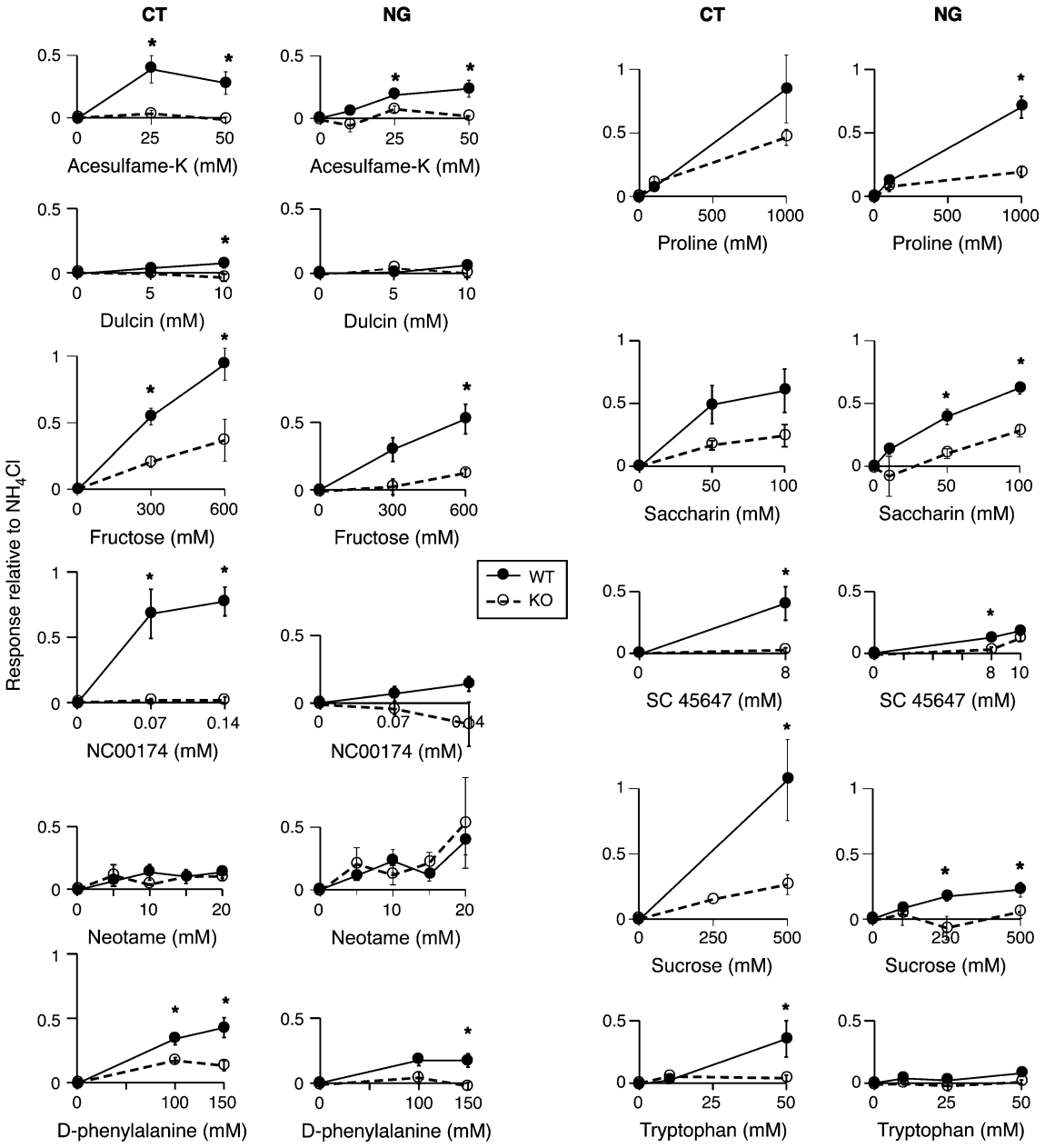

Figure 2 Nerve responses to sweeteners in WT (black circles, solid line) and KO mice (open circles, dashed line). Error bars are SEs. Statistically significant differences between the responses of two groups at particular concentrations are indicated by asterisks ($P < 0.05$).

sucrose, and NH_4Cl. Five features should be noted. First, the WT mice preferred all the sweeteners except neotame. For most sweeteners the preference ratio for WT mice increased with concentration until it reached a plateau. However, acesulfame-K, which was preferred at lower concentrations, became less attractive at higher concentrations. Neotame was never preferred and became aversive at higher concentrations. Second, preference ratios of the KO mice were significantly lower than those of WT for all sweeteners except neotame. For neotame, the aversion was weaker in the KO mice than in WT mice. Third, the KO mice showed a preference only at high concentrations of some sweeteners, for example, acesulfame-K, saccharin, SC45647, and sucrose. Fourth, the KO mice showed no preference for any concentration of dulcin, NC00174, or D-tryptophan. Fifth, there were no significant differences between the WT and KO mice in the preference ratios for NH_4Cl, which was increasingly avoided as concentrations increased.

Discussion

To determine if α-gustducin is generally involved in sweet taste, we examined the behavioral and nerve responses of

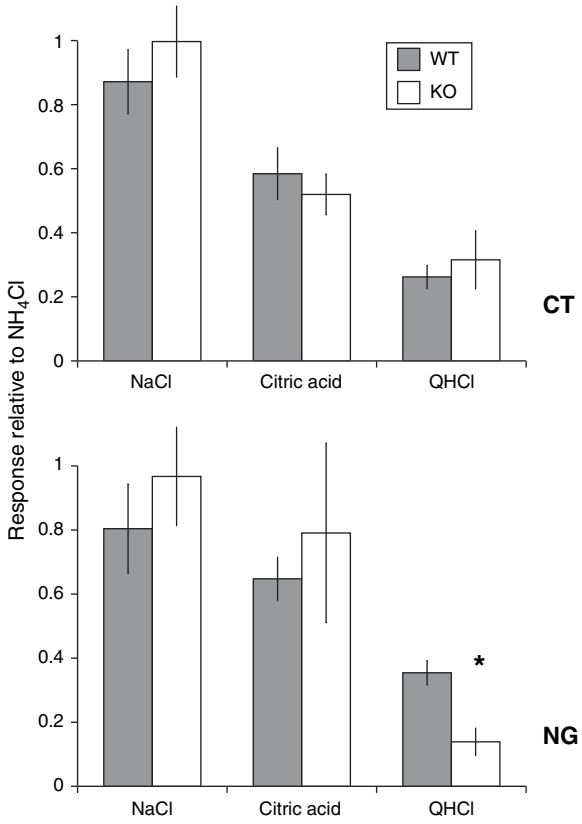

Figure 3 Comparison of the nerve responses to nonsweet stimuli of WT (black bars) and KO mice (white bars). Chorda tympani, CT; glossopharyngeal nerve, NG. Error bars are SEs. Statistically significant differences between the responses of two groups at particular concentrations are indicated by asterisks ($P < 0.05$).

α-gustducin KO and WT mice to 11 compounds that are sweet to humans. We included carbohydrates, artificial sweeteners, and sweet amino acids. In addition, because the involvement of α-gustducin with sucrose and SC45647 has only been examined in the CT nerve (Wong *et al.*, 1996), we recorded from both the CT and NG nerves. In general, the electrophysiological and behavioral tests gave concordant results, with the KO mice significantly less responsive than WT mice to several sweeteners.

KO mice showed no nerve or behavioral responses to dulcin or NC00174, indicating that α-gustducin is required for the detection of NC00174 or dulcin over the range of concentrations used here. However, the KO mice showed "residual" nerve and behavioral responses to sucrose, fructose, D-phenylalanine, L-proline, and saccharin. In regard to residual preference of high concentrations of SC45647 in the KO mice, the behavioral response is determined by the input from all taste nerves. One of the taste nerves, greater superficial petrosal, has been shown in rats to respond best to "sweet" compounds (Travers and Norgren, 1991). Because we did not record from that nerve, we cannot exclude that the input from the palate taste area was responsible for the behavioral results with SC45647.

The residual nerve responses of the KO mice to sucrose and some other sweeteners suggest that either other G protein α-subunits can partially substitute for α-gustducin in coupling to the sweet receptor or that pathways independent of the sweet receptor and α-gustducin are involved. Other G protein α-subunits expressed in taste tissue that might couple to T1r2 + T1r3 to transduce this residual response include $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, $G\alpha_s$, $G\alpha_{14}$, $G\alpha_{15}$, α-transducin, and Gαq (Ruiz-Avila *et al.*, 1995). He *et al.* (2002) demonstrated that transgenic expression of α-transducin, another G protein α-subunit expressed in TRCs, can partly restore the ability of α-gustducin KO mice to taste sweet compounds. However, knocking out α-transducin alone did not alter the nerve or behavioral responses to sucrose and SC45647, indicating that α-transducin plays a lesser role in the transduction of these sweet compounds (He *et al.*, 2004). Furthermore, double KO mice lacking both α-transducin and α-gustducin were no more diminished in their responses to sucrose and SC45647 than were the single α-gustducin KO mice.

Both neural and behavioral responses to the new sweetener neotame were recorded here for the first time in mice. We found that both the WT and KO groups avoided neotame, but the rejection was stronger in WT mice (Figure 4). Earlier we had observed that C57BL/6J mice also avoided neotame in TBP tests (our unpublished data). Our data in mice corroborates behavioral and molecular data in rats. Thus, in toxicological studies with neotame, rats preferred a basal diet to diet with neotame (Mayhew *et al.*, 2003), and *in vitro*, neotame did not activate the rat sweet receptor (Xu *et al.*, 2004). The rejection of a compound sweet to humans by an animal species is not uncommon (Jakinovich and Sugarman, 1989; Danilova *et al.*, 1998).

It is interesting that at very high concentration neotame has a strong unpleasant bitter component in humans (our unpublished data). The interpretation can be that in humans both sweet and one or many bitter receptors interact with neotame, although the affinity for the bitter receptors might be low. The rejection of neotame by mice in behavioral experiments indicates that in this species neotame interacts only with bitter-responsive taste receptors.

There is seemingly a contradiction between the strong preference for dulcin and the virtually no nerve response. Although for each stimulus there is a correlation between the size of nerve response and the sensation evoked, this relationship may vary between compounds. A small response in peripheral nerves elicited by one compound may have the same behavioral effect as a large peripheral response to another compound. The measure of response here was average activity over 20 s of stimulation. Consequently, a small phasic response would be hidden in the average. Furthermore, the behavioral response (preference ratio) has a ceiling at 100%, while the nerve response can continue to increase.

In one type of short-access test, Glendinning *et al.* (2005) reported that the lick responses of α-gustducin KO mice did

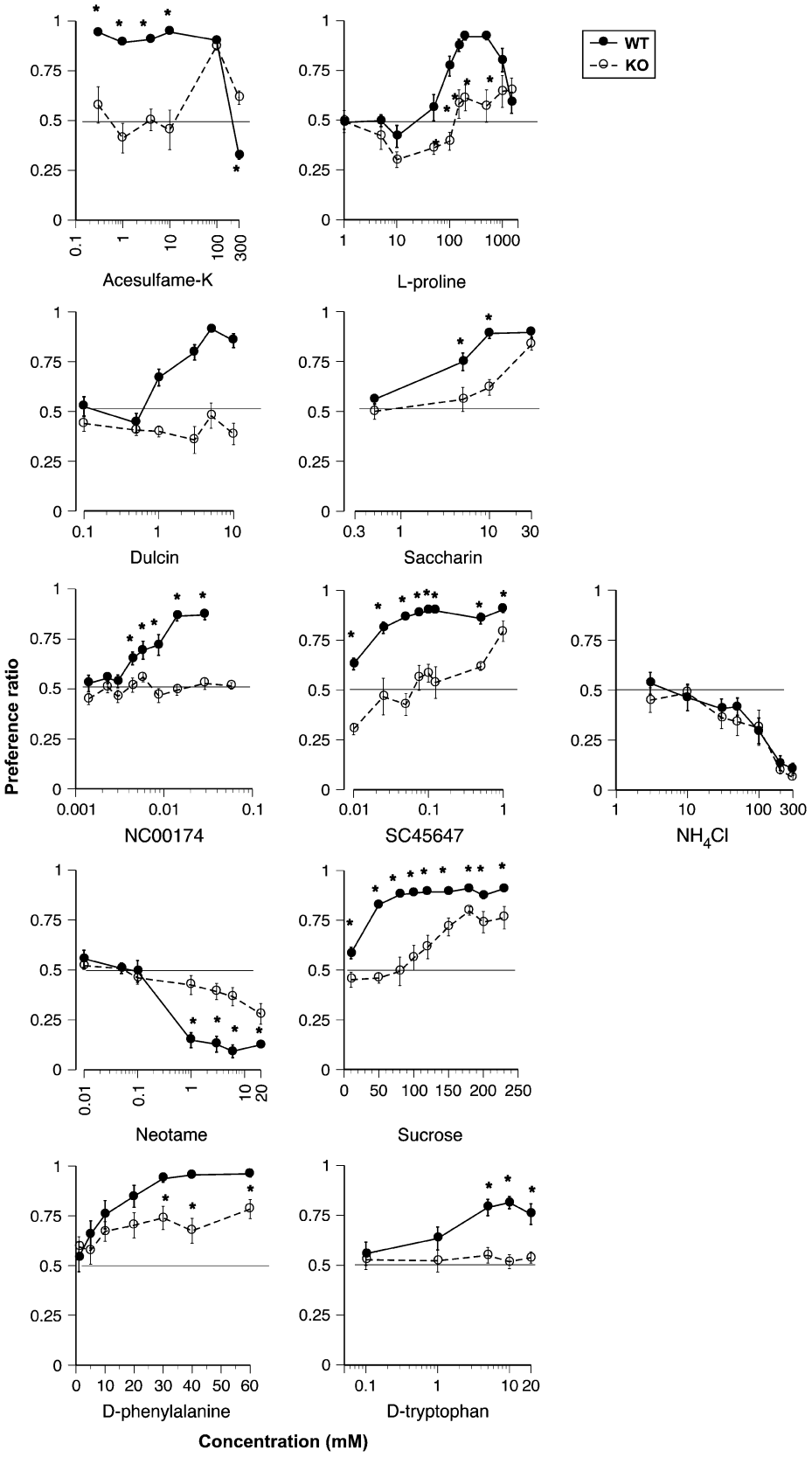

Figure 4 Results of two-bottle preference tests in WT (black circles, solid line) and KO mice (open circles, dashed line). Error bars are SEs. Statistically significant differences between the responses of two groups at particular concentrations are indicated by asterisks ($P < 0.05$).

not differ from those of WT mice to several sweet compounds (SC45647, maltose, fructose, polycose, and maltooligosaccharide). However, these authors found that the results depend on "specific testing conditions," and in another short-access two-bottle preference test, they reported significant differences between KO and WT mice in their preferences for sucrose, SC45647, and maltose. Our results with 48-h two-bottle preference tests and nerve recording (CT and NG) agree with their second short-access two-bottle preference test and previous data that show a clear reduction of the response of α-gustducin KO mice to sucrose and SC45647. Although postingestive effects may have complicated the interpretation of 48-h two-bottle preference tests, the consistent reduction in both behavioral and nerve responses shown here indicates that the primary effects we are observing are for the most part taste related.

In conclusion, we have extended the analysis of the effect of α-gustducin KO in mice to acesulfame-K, dulcin, fructose, NC00174, D-phenylalanine, L-proline, D-tryptophan, saccharin, and neotame, which are all sweet to humans. Except for neotame, which is not attractive to mice, our results show that α-gustducin is involved in transduction of sweet taste.

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