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Behavioral Evidence for a Role of α-Gustducin in Glutamate Taste

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Abstract

The taste perception of monosodium glutamate (MSG) is termed 'umami'. Two putative taste receptors for glutamate have been identified, a truncated form of mGluR4 (taste-mGluR4) and the presumed heterodimer T1R1 + T1R3. Both receptors respond to glutamate when expressed in heterologous cells, but the G protein involved is not known. Gα-Gustducin mediates the transduction of several bitter and sweet compounds; however, its role in umami has not been determined. We used standard two-bottle preference tests on α-gustducin knockout (KO) and wildtype (WT) mice to compare preferences for ascending concentrations of MSG and MSG + 5′-inosine monophosphate (IMP). A Latin Square was used to assign the order of tastants presented to each mouse. Statistical comparisons between KO and WT mice revealed that whereas WT mice preferred solutions of MSG and MSG + IMP over water, KO mice showed little preference for these stimuli. Denatonium and sucrose served as control stimuli and, as shown previously, WT mice prefered sucrose and avoided denatonium significantly more than did KO mice. Naïve mice were also tested, and while prior exposure to taste stimuli influenced the magnitude of the preferences, experience did not change the overall pattern of intake. These data suggest that α-gustducin plays a role in glutamate taste.

Key words: α-gustducin, MSG, taste transduction, two-bottle preference, umami

Introduction

The taste perception of monosodium glutamate (MSG) has been termed 'umami', meaning delicious or savory, and was first described by Ikeda (Ikeda, 1908; Lindemann *et al.*, 2002). MSG is found in a wide variety of foods and is a key molecule determining the flavor of those foods. A characteristic feature of umami taste is its potentiation by 5′-ribonucleotides. Although controversial, in humans umami taste is thought to be a unique taste quality distinct from salty, sour, bitter and sweet stimuli (Yamaguchi, 1967). However, there appear to be species differences in the qualitative perception of umami stimuli (Ninomiya and Funakoshi, 1989; Stapleton *et al.*, 2002) and quantitative differences in behavioral measures reported for several different mouse strains (Ninomiya *et al.*, 1992; Bachmanov *et al.*, 2000).

The mechanisms involved in glutamate taste transduction are not well understood. Two taste cell-expressed G protein coupled receptors have been proposed to mediate umami taste; the combination of TIR1+TIR3 (Nelson *et al.*, 2001; Li *et al.*, 2002), and a truncated variant of mGluR4 (taste-mGluR4) (Chaudhari *et al.*, 1996; Chaudhari *et al.*, 2000). Both of these receptors respond to L-glutamate when expressed in heterologous cells. Although the role of TIR1+TIR3 in glutamate transduction *in vivo* has not been determined, several lines of evidence suggest that taste-mGluR4 may be involved: (i) the taste of L(+)-2-amino-4-phosphonobutyric acid (L-AP4), a specific agonist for mGluR4, generalizes to the taste of MSG in rats (Chaudhari *et al.*, 1996) and in humans elicits umami taste (Kurihara and Kashiwayanagi, 2000); (ii) L-AP4 elicits responses in taste receptor cells (Hayashi *et al.*, 1996; Bigiani *et al.*, 1997; Lin and Kinnamon, 1999); (iii) in heterologous systems, the cloned receptor responds to glutamate at concentrations that elicit umami taste (Chaudhari *et al.*, 2000); and (iv) glutamate elicits decreases in cAMP in taste cells, which is consistent with the role of mGluR4 in brain tissue (Zhou and Chaudhari, 1997). The signaling events downstream of

receptor binding have not been identified. For example, it is not known which G protein is linked to the receptor, what the effector enzymes are or which target channels are involved.

Several G proteins are present in taste cells, including three found to decrease cAMP levels in taste cells: gustducin, transducin and Gαi (McLaughlin *et al.*, 1992, 1993; Kusakabe *et al.*, 2000). Activation of α-gustducin or α-transducin stimulates phosphodiesterase, which degrades intracellular cAMP, while activation of Gαi inhibits adenylyl cyclase, also resulting in a decrease of cAMP levels. α-Gustducin has been shown to be important in the detection of sweet and bitter compounds (Wong *et al.*, 1996), but its role in glutamate taste has not been investigated. The objective of the current experiments was to determine if α-gustducin is involved in the transduction of glutamate as a taste stimulus. To do this, we utilized a knockout (KO) mouse in which the coding sequence for α-gustducin was deleted (Wong *et al.*, 1999). Standard two-bottle preference tests were used to compare taste preferences for MSG and 5′-ribonucleotides, in α-gustducin KO mice and wildtype (WT) littermates.

Experiment 1

The purpose of the first experiment was to compare taste preferences of α-gustducin KO and WT mice using solutions of MSG, MSG + IMP, sucrose and denatonium. A Latin Square model was used to randomly assign the order of tastants presented to each mouse. The advantage of a Latin Square model is that fewer animals are required for testing and it is presumed to produce less experimental error.

Materials and methods

Subjects

Twenty KO mice (10 females and 10 males) and 20 WT mice (7 females and 13 males) were used for this experiment. All were adult mice. The mice were housed in individual clear plastic cages with food (Teklad Rodent Diet 8640, Harlan Sprague Dawley) and water available *ad libitum* throughout the experiment. The colony was maintained on a 12 h light/dark cycle with the onset of lights at 6 a.m. The temperature of the room was kept at 20°C. Each mouse was tested at approximately the same time of day.

Genotype analysis

For the current experiments the gustducin KO mice were generated by replacing one gustducin allele with the neomycin (Neo) resistant gene producing heterozygous mice that were then bred to produce WT (+/+) and KO (–/–) mice (Wong *et al.*, 1996). Pups were weaned at 21–30 days of age and reared in same-sexed groups of 1–5. The genotype of each mouse was identified by polymerase chain reaction (PCR) analysis of genomic DNA. PCR products for either α-gustducin (800 bp) or neo (500 bp) were separated by agarose gel electrophoresis. Knockout mice were identified by the presence of neo and absence of α-gustducin, indicating that neo had replaced both α-gustducin alleles in the genome. Wildtype mice were positive for α-gustducin and lacked a neo gene. Once the mice were identified based on their genetic profile, they were housed individually for experiments.

Behavioral procedures

Daily fluid intake was measured using two-bottle preference test paradigms. Construction of drinking tubes was accomplished by using a sipper tube placed through a hole cut in a plunger of a 20 ml plastic syringe attached to a 25 ml glass graduated cylinder (hereafter, 'bottle'). The drinking tubes were positioned to the left of the feeder with their tips 3 cm apart and extending ~1 cm into the cage. Each stainless steel sipper tube had a 2–3 mm diameter hole from which the mice could lick fluids. We tested four taste stimuli: MSG, MSG + IMP, sucrose and denatonium. Each was presented at five concentrations in ascending order. Individual mice were picked to receive tastants in a sequence selected randomly from a Latin Square model. The concentrations were as follows: (i) MSG 1, 3, 10, 30 and 100 mM; (ii) MSG + IMP, same concentrations of MSG with 100 μM IMP added to each; (iii) sucrose, 0.5, 5, 50, 150 and 500 mM; (iv) denatonium, 0.01, 0.1, 1, 10 and 50 mM (Delay *et al.*, 2000).

Testing consisted of presenting each mouse with a test solution in one bottle and water in the other bottle. Each test lasted 48 h and the locations of the bottles were switched after 24 h to control for side preferences. When the bottles were changed, the quantity consumed was measured, and new solution was added. There were no rest periods between the different taste stimuli.

Data analyses

The data were analyzed to determine if a particular taste stimulus was preferred, neutral or avoided compared to water. A preference or aversion was defined as a response that deviated two standard deviations from 50% (i.e. from equal consumption of test solution and water).

To analyze the results statistically, data were converted into preference ratios:

$$\text{preference ratio} = (\text{total stimulus ingested in 48 h} / \text{total fluid ingested in 48 h}) \times 100$$

Repeated-measures analyses of variance (ANOVA) design was performed on the preference ratios. Simple effects tests for genetic type were performed at each concentration. The degrees of freedom were approximated using Satterthwaite's formula (Littell *et al.*, 1996).

Results

There was a significant increase in MSG preference with increasing concentration [$F(4,80) = 6.63$, $P = 0.0001$]. Importantly, simple effects tests revealed that WT mice preferred MSG significantly more than did the KO mice at 100 mM

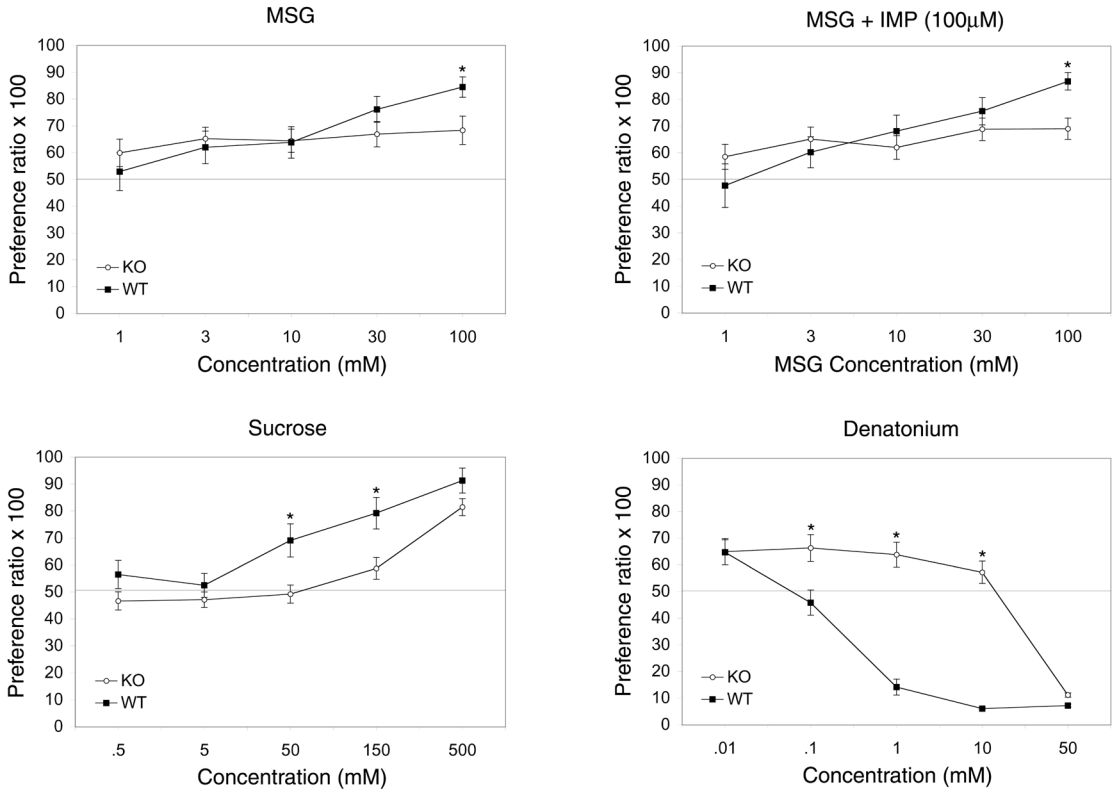

Figure 1 Data represents experiment 1, Latin Square paradigm. Preference ratios for KO and WT mice for MSG, MSG + IMP, sucrose and denatonium in experiment 1. Mean preference ratios are reported with standard error bars in this and all subsequent figures. The * represents a significant difference ($P < 0.05$) between KO (open circles) and WT (closed squares) in this and all subsequent figures. The 50% level indicates no preference for the taste stimulus versus water. The concentrations of the taste stimuli were presented in ascending order. Since each mouse saw all four taste stimuli, n = 20 for each mouse type for each taste stimulus.

MSG (P = 0.0339) (Figure 1). Similarly, there was an increased preference for MSG + IMP with increasing concentration [$F(4,138)$ = 10.33, $P \leq 0.0001$]. WT mice preferred 100 mM MSG + 0.10 mM IMP more than did KO mice (P = 0.0201). Surprisingly, there was no synergistic effect of IMP on the preferences for MSG in either WT or KO mice.

Consistent with previous studies (Wong *et al.*, 1996), our data showed that KO mice were compromised in their responses to sucrose and denatonium. Data for sucrose showed a significant difference between WT and KO mice due to concentration [$F(4,81) = 23.84$, $P = <.0001$]. WT mice preferred 50 mM (P = 0.0018) and 150 mM (P = 0.0013) sucrose significantly more than did KO mice. For denatonium there was a significant difference due to concentration [$F(4,141) = 66.65$, $P \leq 0.0001$]. WT mice avoided 0.1 mM (P = 0.0002), 1 mM ($P \leq 0.0001$) and 10 mM ($P \leq 0.0001$) denatonium significantly more than did KO mice (Figure 1). The data on sucrose and denatonium confirm previous findings (Wong *et al.*, 1996).

We also examined these data to determine whether the preference scores of KO or WT mice were influenced by their prior exposure to any of the four tastants. We tested preference scores for denatonium depending on whether it was tested first, second, third or fourth, irrespective of the solutions that were tested previously. Separate one-way repeated-measure ANOVAs were conducted on the data for each concentration of each substance with both types of mice to examine the effects of order of testing. All analyses revealed significant effects related to order of testing. For denatonium at the lowest concentration, the KO [$F(3,15)$ = 5.43, P = 0.0099] and WT mice [$F(3,15) = 3.75$, P = 0.0343] showed a significant effect depending on the order of testing. There was also a significant effect on the order of testing for sucrose at the lowest concentration for the WT mice [$F(3,16)$ = 7.74, P = 0.0002], for MSG at the higher concentrations for the KO mice [$F(3,15)$ = 3.77 or greater, P = 0.0337 or smaller] and WT mice [$F(3,15) = 4.3$, P = 0.0223]. Finally, significant differences of order were observed for MSG plus IMP at the higher concentrations for the KO mice [$F(3,15)$ = 3.67, P = 0.0366] and for the WT mice [$F(3,15)$ = 4.07 or greater, $P < 0.03$ or smaller]. Closer examination of the data revealed that the mice consumed more of each substance with more prior exposure to the other taste substances.

Thus, even though a Latin Square design was used to minimize the effects of order of stimulus presentation, these

mice showed significant shifts in preference scores with experience. While Smith and Foster (1980) did not find any carry-over effects on two-bottle preference testing of rats related to order of presenting different glucose and saccharin mixtures, a variety of conditioning paradigms have shown that exposure to one taste substance can influence subsequent flavor-based avoidance or preferences (cf. Sclafani *et al.*, 1999; Dwyer and Mackintosh, 2002; Blair and Hall, 2003; Forestell and LoLordo, 2003). Even though no overt conditioning procedure was used, the two-bottle intake data of the mice in this experiment appeared to be influenced by prior exposure to taste stimuli and any conclusions drawn from these data might be compromised. Consequently, a second experiment was conducted with naïve mice to test our conclusions further.

Experiment 2

Taste preferences of *naïve* KO and WT mice presented with solutions of MSG, MSG + IMP, IMP, NaCl and denatonium were calculated and compared.

Materials and methods

Subjects

Ten KO mice (4 females and 6 males) and 9 WT mice (5 females and 4 males) were used in the experiment with MSG as the taste stimulus. Nine KO mice (4 females and 5 males) and 10 WT mice (4 females and 6 males) were used in the experiment with MSG + IMP as the taste stimulus. Ten KO mice (5 females and 5 males) and 10 WT mice (4 females and 6 males) were used in the experiment with IMP as the taste stimulus. Ten KO mice (6 females and 4 males) and 10 WT mice (3 females and 7 males) were used in the experiment with NaCl as the taste stimulus. Adult mice that had never been exposed to any taste stimulus testing prior to this experiment ('naïve mice') were used. The mice were housed in the same manner as the mice in experiment 1 and genetic analyses were also conducted as in experiment 1.

Behavioral procedures

Experimental procedures were the same as in experiment 1. We tested four different taste stimuli with mice that had not been tested previously (naïve mice): MSG, MSG + IMP, IMP, NaCl, and denatonium as a control. There were seven concentrations of MSG, MSG plus IMP, and NaCl; and five concentrations of IMP and denatonium, presented in ascending order. The concentrations were as follows: (i) MSG, 1, 3, 10, 30, 100, 200 , and 300 mM; (ii) MSG + 1 mM IMP, same concentrations of MSG with 1 mM IMP added to each; (iii) IMP, 0.01, 0.1, 1, 10 and 100 mM; (iv) NaCl, 1, 3, 10, 30, 100, 200 and 300 mM. Denatonium (0.01, 0.1, 1, 10 and 50 mM) was presented to all mice following each experiment as a control to verify that the mice were behaving as expected for the KO phenotype (Wong *et al.*, 1996).

Data analyses

Data were analyzed as in the previous experiment.

Results

As found in experiment 1, there was a significant difference between KO and WT preferences over the concentration ranges tested [$F(6,61) = 11.31$, $P < 0.0001$]. Simple effects tests revealed that naïve WT mice preferred 100 mM MSG significantly more than did naive KO mice ($P = 0.0113$). The analyses of the preference scores for MSG + IMP (1 mM) revealed that there was a significant difference between KO and WT mice over concentrations [$F(6,60) = 10.72$, $P < 0.0001$]. The WT mice preferred 30, 100 and 200 mM MSG + IMP significantly more than did KO mice; 30 mM ($P = 0.0496$), 100 mM ($P = 0.0006$), and 200 mM ($P = 0.0417$).

We compared MSG with MSG + IMP and found no significant potentiation ('synergy'). Thus, we tested naïve KO and WT mice with a series of IMP concentrations to determine if these mice preferred IMP over water. No differences were observed between KO and WT mice in response to IMP alone (Figure 2).

NaCl was also tested to determine if the KO and WT mice were influenced by the sodium component of MSG. No differences were observed between naïve KO and WT mice in response to NaCl. However, NaCl was aversive to both KO and WT mice at the higher concentrations, particularly at the concentrations MSG was most preferred by WT mice (Figure 2).

Effect of prior experience on preferences for tastants

We further investigated the question of whether prior experience influenced preference scores by comparing preference ratios for the first five concentrations of MSG between mice in experiment 1 (Latin Square) and mice in experiment 2 (naïve) of the same genotype (Figure 3). KO mice from experiment 1 had significantly different preference scores from the KO mice in experiment 2 [$F(1,27) = 6.68$, $P = 0.0155$]. With the exception of the lowest concentration (1 mM), simple effects tests revealed that MSG was preferred by the KO mice in experiment 1 significantly more than the KO mice in experiment 2 at all concentrations (all Ps < 0.05).

The WT mice from experiment 1 also preferred MSG significantly more than did the WT mice from experiment 2 [$F(1,30) = 5.35$, $P = 0.0277$], but in contrast to the KO groups, the WT mice in experiment 1 and the WT mice in experiment 2 only showed significant preference differences at the two highest concentrations of MSG (30 mM, $P = 0.0277$); (100 mM MSG, $P = 0.0267$). Thus, experience affects both WT and KO mice, but differently.

Effect of prior experience on responses to denatonium

All of the 'naïve' mice were tested with denatonium following each taste stimulus to ensure that the mice were expressing the appropriate phenotype. We compared the

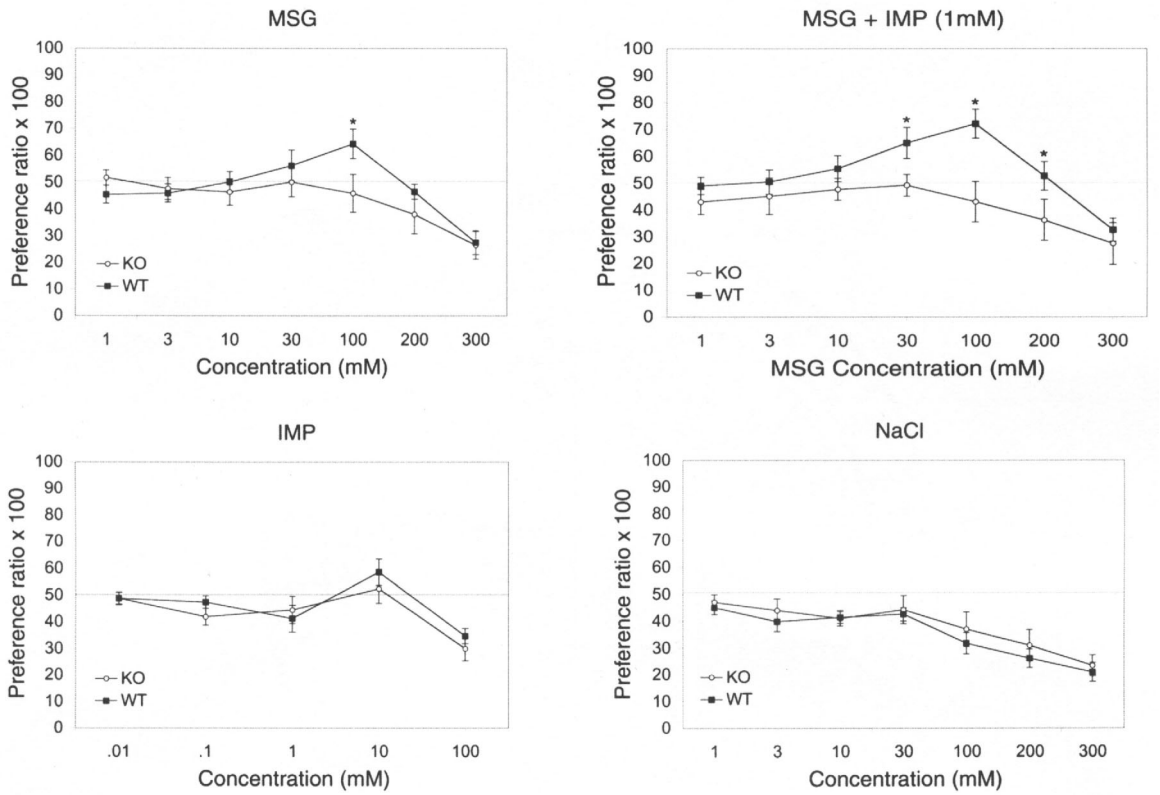

Figure 2 Preference ratios for naïve KO and WT mice for MSG, MSG + IMP, IMP, and the NaCl. The 50% level indicates no preference for the taste stimulus vs water. The concentrations of the taste stimuli were presented in ascending order. Sample sizes were as follows: MSG, n = 10 (KO) and n = 9 (WT); MSG + IMP, n = 9 (KO) and n = 10 (WT); IMP and NaCl, n = 10 for each mouse type.

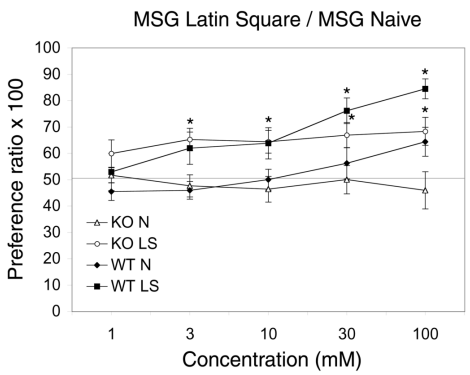

Figure 3 Comparison of preference ratios for KO and WT mice for MSG in experiment 1 and experiment 2. The 50% level indicates no preference for the taste stimulus versus water. The concentrations of the taste stimuli were presented in ascending order. There was significance for the KO mice at concentrations 3, 10, 30 and 100 mM. There was significance for the WT mice only at concentrations 30 and 100 mM.

preference scores for the lowest concentration (and the first to be presented) of denatonium for all of these mice to see if prior exposure to another substance affected their drinking. Due to prior exposure to a positive (i.e., MSG + IMP) or a negative (i.e. NaCl) stimulus, their preference for the lowest concentration of denatonium was significantly affected [$F(3,70) = 3.57$, $P = 0.0184$] (data not shown). There was a downward shift in preference for both KO and WT mice after prior exposure to NaCl. Prior exposure also influenced the extent of difference between KO and WT mice: larger differences were observed after exposure to a positive stimulus than to a negative stimulus.

Discussion

The principal finding from this study is that α-gustducin knockout mice are compromised in their preference for, and presumably detection of, glutamate, as well as for sweet and bitter compounds. WT mice preferred MSG and MSG + IMP, but α-gustducin KO mice showed little or no preference for any concentration of MSG or MSG + IMP. Further, differences between KO and WT mice were not due to the sodium component of MSG, since both WT and KO mice had similar preferences for NaCl. These data indicate that α-gustducin is involved in the transduction of glutamate, just as α-gustducin is involved in the transduction of sweet and bitter compounds. Our data are consistent with the data of Wong *et al.* (1996), in showing that α-gustducin KO mice are compromised in their preference for bitter and sweet compounds and that the taste for bitter is affected more than that for sweet. One possible confound with two-bottle preference testing is that preference ratios can reflect

post-ingestive effects, since the mice consume the taste stimuli over a two-day period. Thus, we cannot rule out post-ingestive effects contributing to the results.

Two G protein-coupled receptors that may mediate glutamate taste have been identified: taste-mGluR4 (Chaudhari *et al.*, 1996, 2001), and the combination of T1R3 + T1R1 (Nelson *et al.*, 2002; Li *et al.*, 2002). Both of these receptors are expressed in subsets of taste cells, and in principle, either could couple to α-gustducin. α-Gustducin is very similar to α-transducin and appears to activate taste cell phosphodiesterase (PDE) resulting in a decrease in intracellular cAMP levels (Yan *et al.*, 2001). This mechanism is thought to mediate bitter transduction. Namely, T2R bitter receptors are thought to activate α-gustducin to decrease cAMP, while its βγ partners stimulate phospholipase C to produce IP_3 and DAG (Margolskee, 2002). Similarly, glutamate elicits decreases in cAMP in taste buds of vallate papillae (Zhou and Chaudhari, 1997) as well as in heterologous cells expressing taste-mGluR4 (Chaudhari *et al.*, 2000). Further, glutamate produces increases in IP_3 in fungiform taste buds (Ninomiya *et al.*, 2000) and causes release of Ca^{2+} from intracellular stores (Lin *et al.*, 2003). Thus, the phospholipase C pathway likely plays a role in glutamate as well as bitter taste transduction. Consistent with these findings, a recent study reported that mice deficient in PLCβ2 have deficits in the transduction of both bitter stimuli and amino acids, which suggests that these stimuli share similar downstream signaling mechanisms (Zhang *et al.*, 2003).

While taste-mGluR4 is a strong candidate for activating α-gustducin, the T1R1 + T1R3 combination may also activate α-gustducin. When T1R3 is co-expressed with T1R2 in heterologous cells, the receptor responds to sweet compounds (Nelson *et al.*, 2001). However, when T1R3 is co-expressed with T1R1, the receptor responds to L-amino acids, including glutamate (Li *et al.*, 2002; Nelson *et al.*, 2002). It is not known whether activation of T1R1 + T1R3 by glutamate decreases cAMP. However, α-gustducin KO mice are compromised in their preference for sweet stimuli (Wong *et al.*, 1996), suggesting that T1R2 + T1R3 is coupled to α-gustducin. If so, then T1R1 + T1R3 may also be coupled to α-gustducin. Further studies will be required to determine whether both of the candidate glutamate taste receptors, taste-mGluR4 and T1R1 + T1R3, couple to α-gustducin *in vivo*.

Surprisingly, the preference for MSG was not enhanced by the presence of IMP, either at 100 μM or at 1 mM in either KO or WT mice. Previous studies with *rats* (Delay *et al.*, 2000) have shown a strong potentiation of behavioral responses to MSG by IMP, and the response of T1R1 + T1R3 in heterologous cells is potentiated by 5′-ribonucleotides (Li *et al.*, 2002; Nelson *et al.*, 2002). One possible cause for the lack of potentiation in the present study is that IMP by itself was not preferred at any concentration tested. Thus, the lower concentrations used in the MSG + IMP study may not have been detected or recognized by the mice. The sensitivity to glutamate has been found to vary widely, depending on the background strain of mice (Bachmanov *et al.*, 2000). The mice used in the present study were predominately of the 129/J strain. Mice with a 129/J background have a lower preference for MSG than C57BL/6J mice. Our data for WT mice are consistent with this, since our preference ratios for MSG or MSG + IMP never exceeded 75%, which is less than the 95% preference ratios observed for C57BL/6J mice (Bachmanov *et al.*, 2000). In addition, the Na^+ component of MSG may have affected the overall preference for MSG + IMP, since NaCl was aversive at the concentrations that should have shown potentiation.

Finally, our data suggest that prior experience may affect the response of mice to subsequent compounds, even in a Latin Square model which is designed to minimize these effects. The main finding is that the KO mice in experiment 1 showed a greater preference to all concentrations of MSG and MSG + IMP than did the naïve KO mice (see Figure 3). Similar effects of prior experience were observed in experiment 2 when the naïve mice were tested with denatonium following exposure to MSG + IMP, NaCl, or IMP. These data, taken together, suggest that prior experience during 2-bottle preference testing may influence subsequent responses to taste stimuli, and these can influence differences observed between control and experimental groups.

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