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PERCEPTION OF SWEET TASTE IS IMPORTANT FOR VOLUNTARY ALCOHOL CONSUMPTION IN MICE

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Abstract

To directly evaluate the association between taste perception and alcohol intake, we used three different mutant mice, each lacking a gene expressed in taste buds and critical to taste transduction: α -gustducin (*Gnat3*), *Tas1r3* or *Trpm5*. Null mutant mice lacking any of these three genes showed lower preference score for alcohol and consumed less alcohol in a two-bottle choice test, as compared with wild-type littermates. These null mice also showed lower preference score for saccharin solutions than did wild-type littermates. In contrast, avoidance of quinine solutions was less in *Gnat3* or *Trpm5* knockout mice than in wild type mice, whereas *Tas1r3* null mice were not different from wild-type in their response to quinine solutions. There were no differences in null vs. wild-type mice in their consumption of sodium chloride solutions. To determine the cause for reduction of ethanol intake, we studied other ethanol-induced behaviors known to be related to alcohol consumption. There were no differences between null and wild-type mice in ethanol-induced loss of righting reflex, severity of acute ethanol withdrawal or conditioned place preference for ethanol. Weaker conditioned taste aversion to alcohol in null mice may have been caused by weaker rewarding value of the conditioned stimulus (saccharin). When saccharin was replaced by sodium chloride, no differences in conditioned taste aversion to alcohol between knockout and wild-type mice were seen. Thus, deletion of any one of three different genes involved in detection of sweet taste leads to a substantial reduction of alcohol intake without any changes in pharmacological actions of ethanol.

Keywords

knockout mice; α -gustducin; *Trpm5*; *Tas1r3*; alcohol intake

Three independent sensory systems – taste, olfaction and chemosensory somatosensation – are involved in the perception of flavor of alcohol (Bachmanov et al. 2003), which vary as a function of alcohol concentration (Mattes & DiMeglio, 2001). Likewise, rodents detect the sweet and bitter taste (see Kiefer, 1995 for rev.) and odor (Kiefer & Morrow, 1991) of alcohol. It is clear that rodents with a high preference for sweet substances consume more ethanol than rats or mice with a low preference for these substances (see Kampov-Polevoy

et al., 1999 for rev.). However, these studies provide only correlations between intake of ethanol and of sweeteners. To date, there is no direct experimental proof that genetic manipulations of taste perception lead to changes in the preference for and consumption of ethanol. The goal of our study was to directly test the role of taste in alcohol consumption using mutant mice lacking key taste signaling elements.

The detection of tastants is mediated by multiple signaling pathways present in specialized taste receptor cells of epithelial origin organized as taste buds within the lingual epithelium (see Gilbertson *et al.*, 2000; Lindemann, 2001 for rev.). The use of genome informatics to explore the molecular basis of genetic variations in taste has led to the identification of two families of G protein-coupled taste receptors (T1Rs and T2Rs), based on several *in vivo* and *in vitro* studies, T2Rs were implicated in bitter taste and the T1Rs in sweet and amino acid tastes (see Margolskee, 2002; Matsunami *et al.*, 2000; Montmayeur & Matsunami, 2002 for rev.). T1R and T2R taste receptors couple to G proteins to relay intracellular signals leading to cell depolarization, nerve transmission and, subsequently, taste perception (Kinnamon & Margolskee, 1996). One of these G proteins, α -gustducin (*Gnat3*) is expressed specifically in taste receptor cells (Boughter *et al.* 1997; McLaughlin *et al.* 1992) and a few other presumptive chemoreceptive cell types (Hofer *et al.*, 1996) and has been implicated in transducing bitter, sweet and amino acid taste. Both T2Rs and T1Rs activate the phospholipase C signaling cascade via gustducin's $\beta\gamma$ -subunits, eliciting release of Ca^{+2} from intracellular stores and activation of the monovalent-selective cation channel, TRPM5 (Liu & Liman, 2003; Ogura *et al.*, 2002; Zhang *et al.*, 2003). Thus, some taste cells could respond to sweet substances through T1R2/T1R3 and then transduce the signals by heteromeric gustducin to activation of TRPM5. Possible transduction mechanisms in vertebrate taste receptor cells were recently reviewed (Margolskee, 2002; Mombaerts, 2004; Scott, 2004; Sugita, 2006).

These molecular studies of taste signaling are supported by results obtained *in vivo* with knockout mice deficient in various components of taste pathways. Thus, *Gnat3* knockout mice show that this gene is involved in bitter, sweet and amino acid taste transduction (He *et al.*, 2004; Ming *et al.*, 1998; Ruiz-Avila *et al.*, 2001; Wong *et al.*, 1996). *Trpm5* null mice displayed diminished or absent avoidance or preference for bitter and sweet compounds, respectively, as well as greatly reduced nerve responses to bitter, sweet and umami compounds (Damak *et al.*, 2006; Zhang *et al.*, 2003). Mice lacking the sweet taste receptor component *Tas1r3* displayed no preference for artificial sweeteners and had diminished, but not abolished, behavioral and neural responses to sugars and umami compounds (Damak *et al.*, 2003; Zhao *et al.*, 2003). However, they did not show differences in responses to bitter, sour, and salty compounds. To directly study the role of taste perception in regulating alcohol intake, we used mice lacking three critical elements of taste pathways (*Gnat3*, *Tas1r3* and *Trpm5*).

MATERIALS AND METHODS

Animals

Null *Gnat3* (-/-) allele mice were created using homologous recombination and genotyped as previously described (Wong *et al.*, 1996). They were backcrossed three times to C57Bl/6J

and heterozygous mice from the last generation of backcross have been used to establish the experimental colony. Both *Tas1r3* knockout mice (Damak *et al.*, 2003) and *Trpm5* knockout mice (Damak *et al.*, 2006) were produced by homologous recombination in C57Bl/6J embryonic stem cells and maintained in this background. To avoid potential maternal and/or environmental effects on behavior, all behavioral analyses were performed on homozygous knockout (-/-) and wild-type (+/+) littermates generated from crosses between heterozygous animals. The original *Tas1r3* and *Trpm5* null mice were crossed with C57Bl/6J mice and heterozygotes obtained from these crosses were used for production of knockout and wild-type animals. Thus, the background of the *Gnat3* (-/-) mice and their wild-type littermates was 93.75% C57Bl/6J (6.25% 129X1/SvJ), while the *T1r3* and *Trpm5* null mice and their littermates were 100% C57Bl/6J background. Male mice were used for all studies and were at least 10-18 weeks of age at the time of analysis; within each experiment all mice were of similar age. Mice were group housed (three to five per cage) under a 12-h light/dark cycle (lights on at 07:00 h A.M.) and provided ad lib access to food and water. In experiments with voluntary intake of ethanol and different tastants and for conditioned taste aversion, mice were housed individually. After experiments with voluntary ethanol intake, the mice were allowed 2 weeks of recovery then used in experiments with voluntary intake of different tastants (saccharin, quinine and sodium chloride). In all other experiments, experimentally naive mice were used. A total of 273 wild type male mice and 289 null male mice from the three mutant colonies were used. No differences in body weight between the null strains and their wild type littermates were found. All experiments were conducted in the isolated behavioral testing rooms in the animal facility to avoid external distractions. All experiments were approved by the Institutional Animal Care and Use Committees.

Ethanol voluntary intake

Testing procedures were described earlier (Blednov *et al.*, 2001). Mice were allowed to acclimate for 1 week to individual housing. Two drinking tubes were continuously available to each mouse, and tubes were weighed daily. Food was available *ad libitum*, and mice were weighed every 4 d. After 4 d of water consumption (both tubes), mice were offered 3% (~ 0.52 M) ethanol (v/v) versus water for 4 d. Tube positions were changed every day to control for position preferences. The quantity of ethanol consumed (g/kg body weight/24 h) was calculated for each mouse and these values were averaged for every concentration of ethanol. Immediately following 3% ethanol, a choice between 6% (~ 1.04 M) ethanol and water was offered for 4 d, then 9% (~ 1.56 M) ethanol for 4 d and finally 12% (~ 2.08 M) ethanol vs. water for 4 d. Two bottles, one with water and one with the appropriate concentration of ethanol were placed into an empty cages (one cage per rack) to determine the loss of fluid by evaporation and dripping. This loss was subtracted from readings for water and ethanol obtained from experimental cages with mice.

Preference for non-ethanol tastants

Wild-type and knockout mice were also tested for saccharin, quinine and sodium chloride consumption. Mice were serially offered saccharin (0.033% and 0.066% or ~ 1.8 mM and 3.6 mM respectively), quinine hemisulfate (0.03 mM and 0.06 mM) and sodium chloride (75mM and 150 mM) and intakes were calculated. Each concentration was offered for 4 d, with bottle position changed every day. For each tastant, the low concentration was always

presented first, followed by the higher concentrations in ascending order. Between tastants, mice were presented with two bottles, each with water, for two weeks.

Conditioned Place Preference

The detailed testing procedure has been described earlier (Blednov *et al.*, 2003). Four identical acrylic boxes (30 × 15 × 15 cm) (Med Associates, St. Albans, VT) were separately enclosed in ventilated, light, and sound-attenuating chambers (Med Associates, St. Albans, VT). Each box has two compartments separated by a wall with a door. One compartment had a bar floor, the other had a floor consisting of an aluminum plate with round holes. Infrared light sources and photodetectors were mounted opposite each other at 2.5-cm intervals along the length of each box, 2.2 cm above the floor. Occlusion of the infrared light beams was used to measure general activity and location of the animal (left or right) within the box. Total activity counts and location of the animal (left or right compartment) within the box were recorded by a computer. The floors and the inside of the boxes were wiped with a damp sponge and the litter paper beneath the floors was changed between animals. The main principles of conditioned place preference procedure have been described earlier (Cunningham, 1993). Briefly, the place-conditioning study involved two habituation sessions, eight conditioning sessions, and one test session. For the habituation session, mice received an injection of saline immediately before being placed in the conditioning box for 5 min on a smooth paper floor. During habituation session both compartments were available for mice. The purpose of the habituation session was to reduce the stress associated with the novelty of experimental procedures and exposure to the apparatus. Mice were not exposed to the distinctive floor textures to avoid latent inhibition. For conditioning, mice were randomly assigned to two groups: saline and ethanol (2.0 g/kg, i.p.). Within the ethanol group, mice were randomly assigned to one of two conditioning subgroups bars (GRID+) or circles (GRID-) and exposed to a Pavlovian differential conditioning procedure. On alternating days, mice in the GRID+ group received an injection of ethanol (2 g/kg, i.p.) immediately before a 5 min session on the bars floor (CS+ sessions). On intervening days, these mice received saline immediately before exposure to the circles floor (CS- sessions). Conversely, mice in the GRID- group received ethanol paired with the circles floor and saline paired with the bars floor. Mice from the control group received a saline injection before being placed on either the bars floor or the circles floor (alternative days). During conditioning trials, all mice had access only to one of two compartments of the apparatus. The dose of ethanol (2 g/kg, i.p.) was chosen because it has previously been shown in mice to produce a strong preference for the paired tactile stimuli (Chester & Cunningham, 1998). For the 30-min test session, all mice received an injection of saline. Both compartments of each experimental box were available for exploration during the test session.

Conditioned taste aversion

Subjects were adapted to a water-restriction schedule (2-h water per day) over a 7-day period. At 48-h intervals over the next 10 days (days 1, 3, 5, 7, 9 and 11), all mice received 1-h access to a solution of saccharin (0.15% or ~ 8.2 mM w/v sodium saccharin in tap water). Immediately after 1-h access to saccharin, mice received injections of saline or ethanol (2.5 g/kg, i.p.) (days 1, 3, 5, 7 and 9). All mice also received 30-min access to tap water 5 h after each saccharin access period to prevent dehydration (days 1, 3, 5, 7 and 9).

On intervening days mice had 2 hrs continuous accesses to water at standard time at the morning (days 2, 4, 6, 8 and 10). Because sodium chloride (NaCl) can be also an effective flavor stimulus in mice (Risinger & Cunningham, 1995) in another series of experiments saccharin was replaced by 0.2 M NaCl solution using similar experimental design.

Acute withdrawal severity

The severity of ethanol induced acute withdrawal was assessed based on monitoring of handling induced convulsions. Mice were scored for handling induced convulsions (HIC) severity 30 min before and immediately before i.p. ethanol administration. The two predrug base-line scores were averaged (PRE). A dose of 4 g/kg of ethanol in saline was injected i.p. and the HIC score was tested every hour until the HIC level will reached base-line after withdrawal. Acute withdrawal was quantified as area under the curve but above the PRE level (Crabbe *et al.*, 1991). Briefly, each mouse is picked up gently by the tail and, if necessary, gently rotated 180°, and HIC's scored as follows: 5, tonic-clonic convulsion when lifted; 4, tonic convulsion when lifted; 3, tonic-clonic convulsion after a gentle spin; 2, no convulsion when lifted, but tonic convulsion elicited by a gentle spin; 1, facial grimace only after a gentle spin; 0, no convulsion.

Loss of righting reflex

The hypnotic effect of ethanol was assessed based on loss of righting reflex procedure. Animals were injected with ethanol (3.4 or 3.8 g/kg, i.p.) and the length of ethanol-induced loss of righting response (LORR, sleep time) was measured. These doses were selected to give LORR in all mice, but not to give a prohibitively long sleep time. Because the dependence between dose of alcohol and duration of LORR is remarkably steep, a very small increment in dosage (e.g., 3.4 and 3.8) makes a significant different in duration of LORR. Upon loss of the righting reflex, mice were placed supine in a sleep trough (~ 90° angle) and the time to regain the righting reflex measured. LORR was defined as inability of a mouse to right itself within 30 sec. Return of the righting response (RORR) was defined as the ability of a mouse to right itself twice in one minute. Sleep time was defined as the time between LORR and RORR.

Rotarod

The ataxia induced by ethanol was assessed based on motor coordination on the rotarod. Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments; speed of rod, 5 rpm), and training was complete when mice were able to remain on the rotarod for 60 s. Number of training sessions was dependent on individual mice. Usually such training took 2-3 training consecutive sessions. At the same day after completion of training mice were injected with ethanol. Every 10 min after injection of ethanol (2 g/kg, i.p.) each mouse was placed back on the rotarod and latency to fall was measured until mouse was able to stay on the rotarod for 60 sec.

Ethanol metabolism

Animals were given a single dose of ethanol (4 g/kg i.p.) and blood samples were taken from the retro-orbital sinus at 30, 60, 120, 180 and 240 min after injection. Blood alcohol

concentration (BEC) values, expressed as mg ethanol per ml blood were determined spectrophotometrically by an enzymatic assay with alcohol dehydrogenase (Lundquist, 1959).

Drugs

All ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) solutions were prepared in saline (20% v/v) and volumes for i.p. injections were adjusted to provide appropriate doses of ethanol. Control mice received a similar volume of saline.

Statistical Analysis

Data are reported as the mean $\pm$ S.E.M value. The statistics software program GraphPad Prism (Jandel Scientific, Costa Madre, CA) was used throughout. To evaluate differences between groups, analysis of variance (one-way ANOVA, two-way ANOVA or two-way ANOVA with repeated measures with post-hoc Bonferroni Test) was carried out.

RESULTS

Ethanol consumption and preference are reduced in *Gnat3*, *Tas1r3* and *Trpm5* null mice

In a two-bottle free-choice paradigm in which mice could drink either water or an ascending series of ethanol concentrations, mice lacking *Gnat3* displayed reduced preference score for ethanol (main effect of genotype – $F_{1,79} = 33.4$, $P < 0.0001$; main effect of concentration – $F_{3,79} = 12.7$, $P < 0.0001$; genotype $\times$ concentration interaction – $F_{3,79} = 2.9$, $P < 0.05$) as well as a reduction in the amount of ethanol consumed (main effect of genotype – $F_{1,79} = 24.2$, $P < 0.0001$; main effect of concentration – $F_{3,79} = 4.7$, $P < 0.01$; genotype $\times$ concentration interaction – $F_{3,79} = 2.1$, $P < 0.05$) (Fig.1 a,d). Overall, mice lacking *Gnat3* consumed more fluid than did wild-type mice (main effect of genotype – $F_{1,99} = 21.9$, $P < 0.0001$), but the differences between wild type mice and null mice become significant only during presentation of ethanol solutions with higher concentrations (9% and 12%) (Fig.1 g). *Tas1r3* null mice also showed reduced preference score for ethanol (main effect of genotype – $F_{1,79} = 19.4$, $P < 0.0001$; main effect of concentration – $F_{3,79} = 5.7$, $P < 0.01$) as well as a reduction in the amount of ethanol consumed (main effect of genotype – $F_{1,79} = 10.3$, $P < 0.01$; main effect of concentration – $F_{3,79} = 11.4$, $P < 0.001$; genotype $\times$ concentration interaction – $F_{3,79} = 2.1$, $P < 0.05$) (Fig.1 b,e). Total fluid intake in *Tas1r3* knockout mice was increased compared with wild-type littermates (main effect of genotype – $F_{1,95} = 10.2$, $P < 0.01$; main effect of concentration – $F_{4,95} = 11.2$, $P < 0.001$) (Fig.1 h). Reduction of ethanol consumption (main effect of genotype – $F_{1,79} = 35.8$, $P < 0.0001$; main effect of concentration – $F_{3,79} = 9.1$, $P < 0.001$; genotype $\times$ concentration interaction – $F_{3,79} = 25.8$, $P < 0.0001$) and preference score for ethanol (main effect of genotype – $F_{1,79} = 29.4$, $P < 0.0001$; genotype $\times$ concentration interaction – $F_{3,79} = 6.7$, $P < 0.001$) was also found with *Trpm5* null mice (Fig. 1 c,f). However, unlike the *Gnat3* and *Tas1r3* null mice, there was no difference in total fluid intake between *Trpm5* knockout mice and the wild-type controls (Fig.1 i).

Reduced preference scores for sweet solutions in *Gnat3*, *Tas1r3* and *Trpm5* null mice

Mice lacking *Gnat3*, *Tas1r3* or *Trpm5* displayed significantly reduced preference score for saccharin solutions (main effect of genotype – $F_{1,39} = 32.7$, $P < 0.0001$; main effect of

concentration - $F_{1,30} = 19.1$, $P < 0.001$; for *Gnat3* colony; $F_{1,31} = 48.1$, $P < 0.0001$; main effect of concentration - $F_{1,31} = 5.1$, $P < 0.05$; for *Tas1r3* colony and main effect of genotype - $F_{1,39} = 118$, $P < 0.0001$; genotype $\times$ concentration interaction - $F_{1,39} = 8.1$, $P < 0.01$; for *Trpm5* colony) (Fig.2 a,b,c). Only *Gnat3* knockout mice showed slightly reduced avoidance of bitter quinine solutions (main effect of genotype - $F_{1,23} = 5.1$, $P < 0.05$; genotype $\times$ concentration interaction - $F_{1,23} = 7.4$, $P < 0.05$) whereas *Trpm5* null mice demonstrated only interaction between genotype and concentration ($F_{1,39} = 12.8$, $P < 0.01$) (Fig.1 d,f). Comparison of avoidance of quinine for *Tas1r3* null mice and corresponding wild-type mice revealed only dependence on concentration ($F_{1,31} = 18$, $P < 0.001$) (Fig.2 e). There was no difference in preference score for sodium chloride solutions between wild-type and any of the null mice (Fig.2 g,h,i). A dependence on concentration for the sodium chloride solutions was found for *Tas1r3* null mice ($F_{1,31} = 7.8$, $P < 0.05$) and *Trpm5* colony ($F_{1,39} = 6.2$, $P < 0.05$).

The response to conditioned stimulus is critical for ethanol-induced conditioned taste aversion in *Gnat3*, *Tas1r3* and *Trpm5* null mice

Consumption of saccharin solution on trial 0 (before conditioning) was greatest for most of null mice and least for wild-type mice (*Gnat3* colony: 63.6 ± 1.9 g/kg for wild type mice and 54.5 ± 2.2 g/kg for null mice; *Tas1r3* colony: 55.7 ± 2.9 g/kg for wild type mice and 46.8 ± 3.9 g/kg for null mice; *Trpm5* colony: 67.9 ± 4.0 g/kg for wild type mice and 66.7 ± 4.0 g/kg for null mice). To attempt to correct for these initial differences in saccharin intake and facilitate presentation of the data, intake was calculated as a percentage of the trial 0 consumption for each subject by dividing the amount of saccharin solution consumed on subsequent conditioning trials by the amount of saccharin solution consumed on trial 0 (before conditioning). Ethanol-saccharin pairings produced reductions in saccharin intake across trials when compared with saline-saccharin paired group, indicating the development of conditioned taste aversion (CTA) in all three wild type mouse strains (main effect of treatment: $F_{1,80} = 98.9$, $P < 0.0001$ for *Gnat3* colony; $F_{1,50} = 208.5$, $P < 0.0001$ for *Tas1r3* colony; $F_{1,60} = 89.4$, $P < 0.0001$ for *Trpm5* colony and main effect of trial: $F_{4,80} = 3.1$, $P < 0.05$ for *Gnat3* colony; $F_{4,50} = 5.0$, $P < 0.01$ for *Tas1r3* colony; $F_{4,60} = 4.8$, $P < 0.01$ for *Trpm5* colony) as well as for null mice (main effect of treatment: $F_{1,85} = 24.0$, $P < 0.0001$ for *Gnat3* colony; $F_{1,47} = 6.4$, $P < 0.05$ for *Tas1r3* colony; $F_{1,60} = 4.5$, $P < 0.05$ for *Trpm5* colony and main effect of trial: $F_{4,85} = 1.5$, $P > 0.05$ for *Gnat3* colony; $F_{4,47} = 1.1$, $P > 0.05$ for *Tas1r3* colony; $F_{4,60} = 0.9$, $P > 0.05$ for *Trpm5* colony). There were no differences between saline-treated groups for wild-types and any of the three knockout strains (Fig. 3 a,b,c). On the contrary, in ethanol-treated groups of all three strains of null mice consumed more saccharin than ethanol-treated groups of corresponding wild-type mice (main effect of genotype: $F_{1,90} = 72.7$, $P < 0.0001$ for *Gnat3* colony; $F_{1,57} = 64.2$, $P < 0.0001$ for *Tas1r3* colony; $F_{1,85} = 51.9$, $P < 0.0001$ for *Trpm5* colony and main effect of trial: $F_{4,90} = 3.5$, $P < 0.05$ for *Gnat3* colony; $F_{4,57} = 6.8$, $P < 0.05$ for *Tas1r3* colony; $F_{4,85} = 5.2$, $P < 0.05$ for *Trpm5* colony) (Fig. 3 a,b,c).

There was no difference between wild-type and mutant mice of each strain in the preconditioning consumption of sodium chloride solution (*Gnat3* colony: 96.6 ± 3.9 g/kg for wild type and 103.8 ± 3.9 g/kg for null mice; *Tas1r3* colony: 74.0 ± 3.1 g/kg for wild type and 71.1 ± 2.9 g/kg for null mice; *Trpm5* colony: 81.2 ± 3.6 g/kg for wild type and $80.1 \pm$

4.6 g/kg for null mice). Nevertheless, to offset minor initial differences in sodium chloride intake and make presentation of the data similar with presentation of saccharin data, intake was calculated as for the experiment with saccharin. Figure 3 (d,e,f) depicts mean ($\pm$ SEM) sodium chloride intakes for each genotype over the course of the five sodium chloride access periods. Ethanol produced dose-dependent reductions in sodium chloride intake over trials in wild type mice (main effect of treatment: $F_{1,65} = 14.5$, $P < 0.001$ for *Gnat3* colony; $F_{1,65} = 555$, $P < 0.0001$ for *Tas1r3* colony; $F_{1,60} = 236$, $P < 0.0001$ for *Trpm5* colony) as well as for knockout mice (main effect of treatment: $F_{1,65} = 42.5$, $P < 0.001$ for *Gnat3* colony; $F_{1,65} = 793$, $P < 0.0001$ for *Tas1r3* colony and main effect of treatment - $F_{1,65} = 301$, $P < 0.0001$, main effect of trial - $F_{4,65} = 5.9$, $P < 0.001$ and genotype $\times$ trial interaction - $F_{4,65} = 5.5$, $P < 0.001$ for *Trpm5* colony) indicating the development of conditioned taste aversion. However, comparison of ethanol-treated groups of all three types of knockout mice with ethanol-treated littermate wild-type mice demonstrated similar levels of conditioned aversion, showing only dependence on trial ($F_{4,90} = 2.5$, $P = 0.05$ for *Gnat3* colony; $F_{4,80} = 16.3$, $P < 0.0001$ for *Tas1r3* colony; $F_{4,75} = 13.4$, $P < 0.0001$ for *Trpm5* colony) but not on genotype (no main effect of genotype).

Conditioned place preference for ethanol is not altered in *Gnat3*, *Tas1r3* and *Trpm5* null mice

Neither null mice nor wild-type mice injected with saline showed original preference to any floor texture (data not shown). Analysis of the test data obtained from all three mutant strains indicated the development of significant conditioned place preference (CPP) (main effect of conditioning group - $F_{1,16} = 30.9$, $P < 0.0001$ for *Gnat3* strain; $F_{1,29} = 20.2$, $P < 0.0001$ for *Tas1r3* strain; $F_{1,16} = 23.8$, $P < 0.0001$ for *Trpm5* strain) without difference between genotypes (no main effect of genotype) (Fig.4 a,c,e).

Activity during each 5-min ethanol (CS+) and saline (CS-) conditioning trial are depicted in Fig. 4 b,d,f. No differences in activity between any of knockout strains and correspondent wild type mice were found after the first saline injection (Fig 4 b,d,f). The first injection of ethanol (Trial 1) produced a significant increase in activity relatively to saline injections in all mouse strains (main effect of treatment: $F_{1,50} = 32.7$, $P < 0.0001$ for *Gnat3* colony; $F_{1,62} = 15.7$, $P < 0.001$ for *Tas1r3* colony and $F_{1,70} = 13.8$, $P < 0.001$ for *Trpm5* colony) (Fig 4 b,d,f). However, the ethanol-induced motor stimulation was similar in null mutant mice and wild type mice (no main effect of genotype). Injections of ethanol in the following trials (2, 3 and 4) did not induce further motor activation in any null mouse strains or in wild type mice (no effect of treatment, one-way ANOVA). On the contrary, activity on saline trials decreased across trials in all null mouse strains (one-way ANOVA, effect of treatment: $F_{3,52} = 3.2$, $P < 0.05$ for *Gnat3* colony; $F_{3,60} = 3.8$, $P < 0.05$ for *Tas1r3* colony and $F_{3,76} = 4.6$, $P < 0.01$ for *Trpm5* colony) and in wild type mice (one-way ANOVA, effect of treatment: $F_{3,48} = 11.9$, $P < 0.001$ for *Gnat3* colony; $F_{3,64} = 4.4$, $P < 0.01$ for *Tas1r3* colony and $F_{3,64} = 3.3$, $P < 0.05$ for *Trpm5* colony). Activity levels during the test day were similar for wild-type and corresponding mutants (data not shown).

Hypnotic effect of ethanol, severity of ethanol-induced acute withdrawal and metabolism of ethanol are not altered in *Gnat3*, *Tas1r3* and *Trpm5* null mice

The severity of ethanol induced acute withdrawal was assessed based on monitoring of handling induced convulsions (HIC). A single 4 g/kg ethanol dose suppressed basal HIC in all three knockout strains as well as in the wild-type controls for about 5-6 h, followed by increased HIC (Fig. 5 a,c,e). No differences between levels of basal HIC were seen between any of the knockout strains and their wild-type controls. All knockout strains and wild-type mice showed similar HIC scores over the entire period of observation (Fig. 5 a,c,e). There were no differences in area under the curves for HIC during withdrawal between null mutants and wild-type mice (Fig. 5 b,d,f).

A dependence on dose ($F_{1,16} = 28.8$, $P < 0.0001$ for *Gnat3* colony; $F_{1,27} = 39.5$, $P < 0.0001$ for *Tas1r3* colony and $F_{1,31} = 64.8$, $P < 0.0001$ for *Trpm5* colony) but not on genotype (no main effect of genotype) was found in hypnotic effect of ethanol, which was assessed based on loss of righting reflex (Fig. 6 a,b,c).

There were no differences in metabolism of ethanol between wild-type and knockout mice in each colony. Thus, ethanol clearance expressed in mg of ethanol/dl of blood $\times$ 1 hour was: 53 ± 2 and 50 ± 5 for wild type and *Gnat3* knockout mice respectively; 56 ± 5 and 51 ± 4 for wild type and *Tas1r3* knockout mice respectively and 49 ± 7 and 54 ± 4 for wild type and *Trpm5* knockout mice respectively.

Ataxia induced by ethanol is not altered in *Gnat3*, *Tas1r3* and *Trpm5* null mice

The ataxia induced by ethanol was assessed based on motor coordination on the rotarod. Acute administration of ethanol (2 g/kg) produces motor incoordination followed by time-dependent recovery in all three mouse strains (main effect of time: $F_{6,83} = 60.6$, $P < 0.0001$, for *Gnat3* colony; $F_{7,95} = 62.5$, $P < 0.0001$, for *Tas1r3* colony; $F_{6,83} = 106.3$, $P < 0.0001$, for *Trpm5* colony) (Fig. 7 a,b,c). However, no differences were found between wild type and correspondent null mice in ethanol-induced acute ataxia (no main effect of genotype).

DISCUSSION

The data presented in this study provide the first direct demonstration that genetic manipulation of taste results in substantial changes in alcohol preference and consumption. It is clear that deletion of any of these three signaling proteins – α -gustducin, T1R3 or TRPM5- leads to substantial reduction of alcohol consumption and ethanol preference in mutant mice. The most likely explanation for this reduction is the loss of sweet taste found in all three null mutant models because only preference for alcohol and saccharin were consistently reduced in all three null strains. However, there are several other possible mechanisms for decreased ethanol intake in the two-bottle choice paradigm (Blednov & Harris, 2006). For example, mice can reduce consumption of ethanol because of increased aversive postingestive properties of alcohol or because of increased (or decreased) ethanol reward (see Chester & Cunningham, 2002 for rev.). However, our studies of CTA and CPP in these null mice indicate that changes in these properties of ethanol do not account for the decreased alcohol consumption. On the contrary, significant differences between wild-type

and all three null mouse strains in CTA test suggest that these gene deletions make ethanol less aversive. However, it is possible that the decreased CTA response to ethanol in these three strains of knockout mice with saccharin as conditioned stimulus may reflect the decrease of rewarding properties of saccharin rather than decrease of aversive properties of ethanol. Thus, according to Grigson (2000), CTA behavior can be based on a process termed “reward comparison” related to the concept of anticipatory contrast, in which consumption of a preferred conditioned factor (e.g. saccharin) is reduced in situations when it predicts availability of a more rewarding factor (drug administration). In accordance with this hypothesis, in the CTA paradigm, reduction in intake of a preferred flavor represents greater preference for the drug. Indeed, in two-bottle choice paradigm all three null strains showed significant reduction of preference for sweet solutions of saccharin in comparison with the very high preference for saccharin in wild-type mice. The replacement of saccharin for sodium chloride showed no difference in ethanol-induced CTA, likely because all three knockout strains and wild-type mice showed similar preference for consumption of salt solutions. Reduced sweetener preference or indifference would be expected as a result of decreased sweet taste sensitivity, but in our study all three null strains show avoidance of saccharin solutions. Because saccharin possesses an intrinsic lingering bitter aftertaste (Horne *et al.*, 2002), the avoidance of saccharin suggests that in knockout mice responsiveness to saccharin sweetness is diminished, but responsiveness to an aversive component of saccharin taste is not changed or increased. These data are also consistent with the idea that effect of gene disruption on CTA of saccharin can be due to differential effective salience (perceptual effectiveness) of this conditioned stimulus for knockout and wild type mice (Artigas *et al.*, 2006; Smith *et al.*, 2004).

Many studies have shown a negative correlation between the hypnotic (loss of righting reflex) effect of ethanol and voluntary ethanol consumption (Hodge *et al.*, 1999; Spanagel *et al.*, 2002; Thiele *et al.*, 1998, 2000). However, deletion of the three taste genes did not alter the hypnotic effect of ethanol. Alcohol withdrawal severity is also inversely correlated with alcohol consumption in mice (Metten *et al.*, 1998) raising the possibility that increased alcohol withdrawal could account for the differences in alcohol consumption. However, all three tested knockout strains did not differ from the corresponding wild-type mice in the severity of acute ethanol-induced withdrawal. Together, these data show that deletion of *Gnat3*, *Tas1r3* or *Trpm5* do not change the pharmacological effects of alcohol linked to ethanol consumption.

A positive relationship between ethanol and sweet intake had been known for more than 40 years (Forgie *et al.*, 1988; Ramirez & Sprott, 1978; Rodgers *et al.*, 1963; Rodgers & McClearn, 1964). These findings have been confirmed in many studies in inbred strains of mice (Bachmanov *et al.*, 1996; Belknap *et al.*, 1993), outbred rats (Gosnell & Krahn, 1992), genetically selected alcohol preferring rats (Kampov-Polevoy *et al.*, 1995; Sinclair *et al.*, 1992; Stewart *et al.*, 1994) and monkeys (Higley & Bennett, 1999). Furthermore, rats selected for high or low saccharin consumption consumed more or less ethanol, respectively (Dess *et al.*, 1998). However, these studies provide only a correlation between intake of ethanol and different sweeteners.

Some human studies have also found an association between a hedonic (pleasurable) response to sweet solutions and dependence on alcohol (Kampov-Polevoy et al., 1997, 2001), as alcohol-dependent patients gave the highest pleasantness rating to the most concentrated sucrose solution (0.83 M). Conversely, most control subjects gave the highest palatability ratings to one of the lower sucrose concentrations (0.05, 0.10, 0.21, or 0.42 M). More recent studies suggested that sweet liking is associated with a genetic risk of alcoholism, as measured by a paternal history of alcoholism, rather than by the alcoholic status of an individual (Kampov-Polevoy et al., 2003a,b). However, at least two studies (Kranzler et al., 2001; Scinska et al., 2001) failed to replicate some of these findings.

In rodents, common chromosomal loci with pleiotropic effects on alcohol and sweetener consumption are known (Bachmanov *et al.*, 2002; Foroud *et al.*, 2002). The *Ap3q* (alcohol preference 3 QTL on distal mouse chromosome 4) locus overlaps with the *Sac* (saccharin preference in subtelomeric region of mouse chromosome 4) locus controlling sensitivity to some sweeteners (Lush *et al.*, 1995) and corresponds to the *Tas1r3* gene, encoding the sweet taste receptor T1R3 (Max *et al.*, 2001). Reed *et al.* (2004) showed that polymorphisms in the *Tas1r3* region significantly associated with saccharin preference in 30 strains of inbred mice. This region has conserved synteny with a subtelomeric region of a short arm of human chromosome 1 (1p36). A locus influencing vulnerability to human alcoholism has been mapped to human chromosome 1 (1p13-35) (Nurnberger et al. 2001).

The natural origin of ethanol gives another link between sweet taste and alcohol intake. In nature, sugars within fruits represent the predominant substrate for fermentation and formation of alcohol. The occurrence of ethanol in ripe and decaying fruit and the substantial heritability of alcoholism in humans may suggest an evolutionary association between primate frugivory and alcohol consumption (Dudley, 2000; 2002).

In cells of fungiform papillae T1R3 and T1R2 receptors are co-expressed with α -gustducin and TRPM5 (Kim *et al.*, 2003; Zhang *et al.*, 2003). These results raise the possibility that some taste cells could respond to sweet substances through T1R2/T1R3, and then transduce the signals by gustducin and TRPM5. This speculation is in agreement with our and previously published data showing substantial reduction of response to some sweet substances in *Gnat3* null mice (He *et al.*, 2002; Wong *et al.*, 1996) as well as in *Tas1r3* knockout mice (Damak *et al.*, 2003; Zhao et al., 2003). Recently Lemon *et al.* (2004) showed that orally applied ethanol produces a concentration-dependent activation of central sucrose-responsive neurons of the nucleus of the solitary tract (NST), suggesting that ethanol stimulation of the anterior tongue and palate evokes a pattern of activity in gustatory circuits in the NST resembling that produced by sweeteners.

In agreement with earlier data (Damak *et al.*, 2006; Wong *et al.*, 1996; Zhang *et al.*, 2003), we found that mice lacking *Gnat3* and *Trpm5* showed reduced avoidance of bitter solutions of quinine, whereas mice lacking *Tas1r3* did not show altered avoidance of quinine. However, all three mutant strains showed strong reduction of ethanol preference and consumption. Although it depends on experimental conditions, during continuous access to alcohol the reduction of avoidance of bitter solutions should lead to an increase, not a decrease in ethanol consumption. For example, the alcohol consumption in randomly bred

albino rats during the first week of access to alcohol was positively correlated with the prior intake of quinine, suggesting that individual gustatory differences influence the initial alcohol acceptance (Kampov-Polevoy *et al.*, 1990). Similar relationships between ethanol and quinine intake during continuous access drinking were found in Lewis rats (Goodwin *et al.*, 2000). Therefore, in our study the effects of the gene deletions on consumption of bitter solutions are unlikely to be important for the reduced alcohol consumption.

The ability of different mutations to reduce preference for ethanol varied in the order: *Trpm5* > *Gnat3* > *Tas1r3*. Thus, deletion of the most distant downstream target – *Trpm5* (which is involved in more taste functions than simply sweet taste detection) produced the greatest reduction in ethanol preference, whereas the primary target – *Tas1r3* (probably the most specific for sweet taste recognition) induced the weakest effect on ethanol preference. Therefore, the effect of mutations on preference for alcohol can be more complex than simply the effect of reduction of sweet taste recognition. This conclusion is supported by data obtained by the Collaborative Study on the Genetics of Alcoholism (COGA) showing the association between risk for alcoholism and TAS2R16 gene encoding a taste receptor for β -glucopyranosides (Edenberg & Foroud, 2006).

Nevertheless, the strong reduction of voluntary ethanol intake revealed in mice lacking one of three genes underlying taste transduction (*Gnat3*, *Tas1r3* and *Trpm5*) in this study can be explained mainly by differences in perception of sweet taste rather than by differences in pharmacological effects of ethanol such as reward, aversion, hypnosis or severity of acute withdrawal. These data support the idea that ethanol and sweet consumption share some common molecular mechanism(s).

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FIG.1. Mice lacking *Gnat3*, *Tas1r3* or *Trpm5* show markedly reduced preference for and consumption of alcohol

A. *Gnat3* knockout mice. Pure ethanol (EtOH) consumption (g/kg/day); n=7 for wild-type and n=10 for knockout mice. B. *Tas1r3* knockout mice. Pure ethanol consumption (g/kg/day); n=8 for each genotype. C. *Trpm5* knockout mice. Pure ethanol consumption (g/kg/day); n=7 for wild-type, n=10 for knockout mice. D. *Gnat3* knockout mice. Preference score for ethanol. E. *Tas1r3* knockout mice. Preference score for ethanol. F. *Trpm5* knockout mice. Preference score for ethanol. G. *Gnat3* knockout mice. Total fluid intake (g/kg/day). H. *Tas1r3* knockout mice. Total fluid intake. I. *Trpm5* knockout mice. Total fluid intake. * - p<0.05; ** - p<0.01; *** - p<0.001 – significant differences relative to wild-type mice for the same concentration of ethanol (post-hoc Bonferroni Test).

FIG.2. *Gnat3*, *Tas1r3* and *Trpm5* null mice show markedly reduced preference for saccharin
A. *Gnat3* knockout mice. Preference score for saccharin. n=7 for wild-type. n=10 for knockout mice. B. *Tas1r3* knockout mice. Preference score for saccharin; n=8 for each genotype. C. *Trpm5* knockout mice. Preference score for saccharin. n=7 for wild-type. n=10 for knockout mice. D. *Gnat3* knockout mice. Preference score for quinine. E. *Tas1r3* knockout mice. Preference score for quinine. F. *Trpm5* knockout mice. Preference score for quinine. G. *Gnat3* knockout mice. Preference score for sodium chloride (NaCl). H. *Tas1r3* knockout mice. Preference score for NaCl. I. *Trpm5* knockout mice. Preference score for NaCl. * - p<0.05; *** - p<0.001 – significant differences relative to wild-type mice for the same concentration of tastants (post-hoc Bonferroni Test).

FIG.3. *Gnat3*, *Tas1r3* and *Trpm5* null mice displayed markedly reduced conditioned taste aversion (CTA) to ethanol when saccharin was used as the conditioned stimulus
 A. *Gnat3* mouse colony. Saccharin as the conditioned stimulus. n=5 - for saline-treated groups. n=10 - for ethanol-treated groups. B. *Tas1r3* mouse colony. Saccharin as the conditioned stimulus. n=5 - for saline-treated groups. n=7 - for ethanol-treated groups. C. *Trpm5* mouse colony. Saccharin as the conditioned stimulus. n=5 - for saline-treated groups. n=9 - for ethanol-treated groups. D. *Gnat3* mouse colony. NaCl as the conditioned stimulus. n=10 - for ethanol-treated groups. E. *Tas1r3* mouse colony. NaCl as the conditioned stimulus. n=6 - for saline-treated groups. n=9 - for ethanol-treated groups. F. *Trpm5* mouse colony. NaCl as the conditioned stimulus. n=6 - for saline-treated groups. n=8-9 - for ethanol-treated groups.

FIG.4. No difference in ethanol-induced conditioned place preference (CPP) was observed between wild-type and the null mice

A. *Gnat3* mouse colony. Mean time (% of total) spent on the grid floor during a 30-min test by mice from GRID+ and GRID- groups. n=10 for all groups. B. *Gnat3* mouse colony. Motor activity during each 5-min ethanol conditioned trials (CS+) and saline conditioned trials (CS-). C. *Tas1r3* mouse colony. Mean time (% of total) spent on the grid floor during a 30-min test by mice from GRID+ and GRID- groups. n=10 for all groups. D. *Tas1r3* mouse colony. Motor activity during each 5-min ethanol conditioned trials (CS+) and saline conditioned trials (CS-). E. *Trpm5* mouse colony. Mean time (% of total) spent on the grid floor during a 30-min test by mice from GRID+ and GRID- groups. n=10 for all groups. F. *Trpm5* mouse colony. Motor activity during each 5-min ethanol conditioned trials (CS+) and saline conditioned trials (CS-). * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$, between [GRID+] and [GRID-] groups of the same genotype (post-hoc Bonferroni test).

FIG.5. There were no differences in severity of ethanol-induced withdrawal between *Gnat3*, *Tas1r3* and *Trpm5* knockout and wild-type mice

The severity of ethanol induced acute withdrawal was assessed based on monitoring of handling induced convulsions (HIC). A. HIC scores after acute ethanol administration for *Gnat3* mouse colony. n= 7-8 per genotype. B. Area below HIC curve and above the basal level for *Gnat3* mouse colony. C. HIC scores for *Tas1r3* mouse colony. n= 6-7 per genotype. D. Area below HIC curve and above the basal level for *Tas1r3* mouse colony. E. HIC scores for *Trpm5* mouse colony. n= 7 per genotype. F. Area below HIC curve and above the basal level for *Trpm5* mouse colony.

FIG.6. There were no differences in hypnotic effect of ethanol between *Gnat3*, *Tas1r3* and *Trpm5* knockout and wild-type mice

The hypnotic effect of ethanol was assessed based on loss of righting reflex (LORR) procedure. A. Duration of LORR for *Gnat3* mouse colony. n= 5 per dose and genotype. B. Duration of LORR for *Tas1r3* mouse colony. n= 7-8 per dose and genotype. C. Duration of LORR for *Trpm5* mouse colony. n= 8-10 per dose and genotype.

FIG.7. There were no differences in ataxic effect of ethanol between *Gnat3*, *Tas1r3* and *Trpm5* knockout and wild-type mice

The ataxia induced by ethanol was assessed based on motor coordination on the rotarod. A. *Gnat3* mouse colony. Time on the rotarod. n= 6 per genotype. B. *Tas1r3* mouse colony. Time on the rotarod. n= 6 per genotype. C. *Trpm5* mouse colony. Time on the rotarod. n= 6 per genotype.