Chem. Senses 34: 789–797, 2009 doi:10.1093/chemse/bjp068

Double P2X2/P2X3 Purinergic Receptor Knockout Mice Do Not Taste NaCl or the Artificial Sweetener SC45647

Meghan C. Eddy[1], Benjamin K. Eschle[1], Jennell Barrows[2], Robert M. Hallock[2], Thomas E. Finger[2] and Eugene R. Delay[1,2]

[1]Department of Biology, University of Vermont, Burlington, VT 05405, USA and [2]Rocky Mountain Taste and Smell Center, University of Colorado Denver School of Medicine, Aurora, CO 80045, USA

Correspondence to be sent to: Eugene R. Delay, Department of Biology, Marsh Life Science Room 113, University of Vermont, Burlington, VT 05405, USA. e-mail: edelay@uvm.edu

Abstract

The P2X ionotropic purinergic receptors, P2X2 and P2X3, are essential for transmission of taste information from taste buds to the gustatory nerves. Mice lacking both P2X2 and P2X3 purinergic receptors (P2X2/P2X3$^{Dbl-/-}$) exhibit no taste-evoked activity in the chorda tympani and glossopharyngeal nerves when stimulated with taste stimuli from any of the 5 classical taste quality groups (salt, sweet, sour, bitter, and umami) nor do the mice show taste preferences for sweet or umami, or avoidance of bitter substances (Finger et al. 2005. ATP signaling is crucial for communication from taste buds to gustatory nerves. *Science*. 310[5753]:1495–1499). Here, we compare the ability of P2X2/P2X3$^{Dbl-/-}$ mice and P2X2/P2X3$^{Dbl+/+}$ wild-type (WT) mice to detect NaCl in brief-access tests and conditioned aversion paradigms. Brief-access testing with NaCl revealed that whereas WT mice decrease licking at 300 mM and above, the P2X2/P2X3$^{Dbl-/-}$ mice do not show any change in lick rates. In conditioned aversion tests, P2X2/P2X3$^{Dbl-/-}$ mice did not develop a learned aversion to NaCl or the artificial sweetener SC45647, both of which are easily avoided by conditioned WT mice. The inability of P2X2/P2X3$^{Dbl-/-}$ mice to show avoidance of these taste stimuli was not due to an inability to learn the task because both WT and P2X2/P2X3$^{Dbl-/-}$ mice learned to avoid a combination of SC45647 and amyl acetate (an odor cue). These data suggest that P2X2/P2X3$^{Dbl-/-}$ mice are unable to respond to NaCl or SC45647 as taste stimuli, mirroring the lack of gustatory nerve responses to these substances.

Key words: brief access, conditioned flavor aversion, conditioned taste aversion, SC45647, transduction

Introduction

The last decade has seen exciting advancements in our understanding of how taste buds are able to initiate and transmit neural signals to the gustatory nerves. One of the more remarkable findings is the critical role of purinergic receptors in this system (Finger et al. 2005). The nerves innervating taste buds express 2 ionotropic purinergic receptor subunits, P2X2 and P2X3 (Bo et al. 1999). Genetic elimination of these receptors leaves mice with greatly diminished gustatory capabilities suggesting that ATP serves as a transmitter from taste buds to taste nerves (Finger et al. 2005). Indeed, recent studies (Huang et al. 2007; Romanov et al. 2007; Murata et al. 2008) show that Type II (receptor) cells of taste buds release ATP via a nonsynaptic mechanism.

The previous study on purinergic double knockout (KO) mice, P2X2/P2X3$^{Dbl-/-}$, showed no taste-evoked activity in the glossopharyngeal or chorda tympani nerves during stimulation of the oral cavity with bitter substances, monosodium glutamate (MSG), sucrose, artificial sweeteners, or NaCl (Finger et al. 2005). In 24-h 2-bottle preference tests, the P2X2/P2X3$^{Dbl-/-}$ mice also showed no preference for substances normally preferred by wild-type (WT) mice (e.g., MSG, sucrose, and artificial sweeteners). However, these tests also revealed limited behavioral responses by P2X2/P2X3$^{Dbl-/-}$ mice to high concentrations of bitter substances, including quinine and possibly denatonium benzoate, and near-normal responses to sour substances such as citric acid. P2X2/P2X3$^{Dbl-/-}$ mice show highly variable preferences for NaCl in these 2-bottle tests that were not statistically different from WT controls (M. Tatangelo, J. Barrows, R. Hallock, T. Finger, unpublished data) even though there is no apparent gustatory neural response to intraoral NaCl by the KO animals (Finger et al. 2005). Thus, P2X2/P2X3$^{Dbl-/-}$ mice appear to have at least some ability to detect chemical stimuli, but the exact nature of that ability is still unclear. It is possible that the P2X2/P2X3$^{Dbl-/-}$ mice respond to these substances via postingestive effects,

laryngeal or trigeminal chemoreceptors, or residual gustatory capabilities.

In order to assess the nature of possible residual responses to NaCl, animals in this study were tested with 2 complementary brief-access paradigms to assess hedonic qualities and to enhance the motivational qualities of taste stimuli while minimizing or eliminating postingestive effects. The first was a brief-access test designed to systematically assess the hedonic taste quality of NaCl over a wide range of concentrations. It is also possible that the P2X2/P2X3$^{Dbl-/-}$ mice can detect taste stimuli, but the genetic deletion alters the hedonic value of the stimulus so that it is no longer preferred or avoided. In order to test whether P2X2/P2X3$^{Dbl-/-}$ mice can detect taste stimuli in any way, we used conditioned taste aversion (CTA) methods to force the mice to assign a negative hedonic value to a taste stimulus and subsequently to avoid the substance. This way, if the mouse is capable of detecting and identifying NaCl through any form of chemosensation, it will avoid NaCl subsequent to conditioning.

For similar reasons, CTA methods were also chosen to evaluate potential residual taste for sweet stimuli. The artificial sweetener, SC45647, was chosen for these CTA experiments to avoid potential confounding by side tastes sometimes associated with other artificial sweeteners and/or postingestive effects that might occur with carbohydrates such as sucrose. Mice with a genetic deletion of the T1R3 receptor (important for detecting sweet substances) initially show reduced or no preference for sucrose unless the concentration is high (Damak et al. 2003; Zhao et al. 2003; Dotson and Spector 2007; Zukerman et al. 2009). However, Zukerman et al. (2009) found that if these KO mice are tested a second time with an ascending series of concentrations of sucrose, during the second series they will show preferences for sucrose that are similar to WT mice. This emergent preference for sucrose may be a result of the KO mice associating residual oral sensation elicited by sucrose with the postingestive effects of its ingestion, and, subsequently, the mice may be able to identify sucrose by these oral sensations. Consequently, SC45647 was used for conditioning in this study to further analyze the gustatory capacity of P2X2/P2X3$^{Dbl-/-}$ mice. SC45647 is a nonnutritional artificial sweetener with minimum postingestive effects but is preferred by WT mice (Nofre et al. 1990; Finger et al. 2005). Finally, to test whether the P2X2/P2X3$^{Dbl-/-}$ deletion generally impairs the KO mice's ability to learn a CTA, we conditioned mice with a stimulus combination of SC45647 and amyl acetate (an odorant).

Methods and materials

Subjects

The KO mice in these experiments were B6;129-P2rx2^{tm1Ckn}/P2rx3^{tm1Ckn} (identified here as P2X2/P2X3$^{Dbl-/-}$ mice) developed by Debra Cockayne (Roche Pharmaceuticals; Cockayne et al. 2000, 2005). The WT control mice (B6;129, also identified as P2X2/P2X3$^{Dbl+/+}$ mice) were on a mixed 129Ola and C57BL/6 background. Thus, there is genetic variation in the KO and the WT mouse populations. The P2X2/P2X3$^{Dbl-/-}$ mice weighed between 28 and 35 g, and the WT mice weighed between 26 and 33 g at the start of the experiments. For the brief-access experiment, 5 P2X2/P2X3$^{Dbl-/-}$ and 5 WT adult male mice were maintained in a vivarium at the University of Colorado Denver School of Medicine. These mice were water deprived for 16–20 h when tested. These mice had previous experience with NaCl in the lickometer 2 days prior to the NaCl testing reported here. In the original test, the mice were deprived of water for more than 20 h and consequently failed to reject any stimulus, including high concentrations of NaCl. The water deprivation was then decreased, and the data reported below were collected.

For the conditioned aversion experiments, 31 adult P2X2/P2X3$^{Dbl-/-}$ mice and 17 adult male WT mice were kept on a 22-h water deprivation schedule at the University of Vermont. Because the supply of WT and KO mice was limited, most of the animals were tested in 2 conditioned aversion experiments with a minimum of 3 weeks separating the 2 experiments. Two of the KO mice were tested in the saline injection condition in all 3 experiments. All mice were allowed 1 h of water access in the home cage during a 24-h period beginning 30 min after the end of each 30-min session. All animals in both colonies were housed individually, were maintained on a 12-h light:dark schedule, and had food available ad libitum throughout the course of all experiments. The P2X2/P2X3$^{Dbl-/-}$ animals are maintained as double KOs, and occasionally individuals are genotyped by polymerase chain reaction to ensure quality control of the colony. All experiments reported herein were approved by the institutional animal care and use committees for the University of Colorado Denver School of Medicine and the University of Vermont.

Apparatus

Brief-access and conditioned aversion procedures were conducted in lickometers (Davis MS160; DiLog Instruments). Each MS160 lickometer consists of a Plexiglas chamber with a shutter covering an opening on one end, behind which a mobile tray of up to 16 bottles with stainless steel sipper tubes can be mounted. For each trial, a computer positions the assigned bottle, opens the shutter to give the mouse access to the bottle's sipper tube with a 2.5-mm diameter opening, and counts each contact with the tube.

Brief-access experiment

Procedure

Brief-access testing procedures were similar to those described in Glendinning et al. (2002). Briefly, water-deprived mice were acclimated to the apparatus and trained to lick water from the lickometer for 2 days. Following initial training, mice were tested with 7 concentrations of NaCl (0, 50,

100, 175, 300, 600, and 1000 mM; Sigma-Aldrich) mixed in deionized water (Millipore). Test sessions lasted 30 min, during which time the mouse could initiate as many trials as possible. Each trial was 5 s in duration beginning when the mouse licked the sipper tube the first time. Intertrial intervals were 7.5 s. Concentrations were presented in randomized blocks in which each stimulus was presented once before beginning a new block. Because all animals completed either 6 or 7 blocks of trials in a single session; the data from the first 6 blocks (42 trials) were used for analysis.

A nearly identical experiment was completed before this experiment with 5 different WT and 5 different P2X2/P2X3$^{Dbl-/-}$ mice tested with 0, 3, 10, 30, 100, 270, 450, and 1000 mM NaCl. Because there were more low concentrations of NaCl tested in the earlier experiment, those mice managed to sample each concentration only 4–5 times during the session. Consequently, the present experiment used one less concentration and fewer low concentrations of NaCl to increase the number of trials of each test stimulus that the mice sampled.

Data analysis

To normalize lick rates, a lick ratio was calculated by dividing the mean number of licks for each taste solution by the mean number of licks for water. Analysis of variance (ANOVA) and *t*-tests with Bonferroni corrections were applied to assess differences between WT and P2X2/P2X3$^{Dbl-/-}$ mice in lick ratio across the concentration range. The α' is reported when the correction is applied.

Conditioned aversion experiments

General conditioned aversion method

All conditioned aversion training and testing took place in a Davis MS160 lickometer, as described above. CTA procedures were similar to those described previously (Stapleton et al. 1999; Heyer et al. 2003; Eschle et al. 2008). For each experiment, water-deprived mice were trained to lick water for 4–5 days to ensure consistent licking in the test apparatus, regardless of their experience with the CTA procedure. When the mice were licking consistently in the test apparatus, the mice were given their first conditioning session in the lickometer in which they were given a minimum of 15 presentations of the conditioned stimulus (CS). Immediately following CS exposure, the mice were given an intraperitoneal injection of either 225 mM LiCl (0.1 mL/10 g body weight; 0.954 mg/10 g body weight) to induce gastric distress or saline (control). Preliminary studies showed that this dose was most effective for conditioning a CTA in both WT and KO mice. The next day was a recovery day in which the mice were presented only water in the apparatus. To further improve the effectiveness of conditioning, 2 days after the first conditioning session all mice were similarly exposed to the CS a second time, and conditioning was repeated. During the next 2 days, the mice were again presented with water to extinguish contextual conditioning and to ensure the animals' motivational states were stable. During the next session, each mouse was tested with an array of concentrations of the CS. Each concentration was tested twice, once in each of 2 blocks of trials. Licks during each of the 5-s trials were counted by the computer. The order of stimulus concentrations within a block was randomized for each mouse using a modified Latin square procedure. Each stimulus was separated by 1–3 water rinse trials. Intertrial intervals were 10 s. The mice were given one more recovery day and then tested again. All training and conditioning procedures were identical for each experiment. After conditioned flavor aversion (CFA) conditioning, the mice were tested with a stimulus mixture (taste plus smell) and with the individual components of the mixture. All solutions were mixed fresh in deionized water (Millipore) on conditioning and test days. Mice tested in more than one experiment were randomly assigned to either the saline or LiCl injection condition for their first experiment and were assigned to the same condition in the subsequent experiment in which they participated.

Sodium chloride CTA

In this experiment, mice were conditioned with 300 mM NaCl as the CS. This concentration was selected because it was clearly salient and only slightly aversive to the WT mice in the brief-access experiments. There were 17 P2X2/P2X3$^{Dbl-/-}$ mice with 11 randomly assigned to the LiCl injection condition and 6 to the saline injection condition. Five of the LiCl-injected mice and 3 of the saline-injected KO mice were naive to CTA procedures prior to this conditioning, whereas the rest of the mice had received previous conditioning with either SC45647 or the stimulus mixture of SC45647 + amyl acetate. Of the 17 WT mice in this experiment, 11 (5 naive) received LiCl injections and 6 (4 naive) received saline injections. Group sizes were weighted more heavily toward the LiCl condition in this and the other experiments to ensure that the experimental condition (LiCl injection) had sufficient animals to optimize the estimate of the effects of LiCl conditioning for statistical comparisons. On test days, the lick rates of these animals were measured when presented with 0, 50, 100, 300, and 600 mM NaCl (Sigma–Aldrich).

SC45647 CTA

The results of the NaCl experiment indicated that the P2X2/P2X3$^{Dbl-/-}$ mice did not respond to any of the stimulus concentrations. Consequently, we tested mice for their ability to form a CTA to an artificial sweetener, 0.05 mM SC45647 (a nonnutritional substance normally preferred by mice and perceived as "sweet" by humans; Nofre et al. 1990). In previous 24-h 2-bottle testing, WT mice showed a strong preference for SC45647 at this concentration (Finger et al. 2005). In this experiment, 8 P2X2/P2X3$^{Dbl-/-}$ mice (7 naive)

received LiCl injections and 8 (8 naive) received saline injections. Of the WT mice, 5 WT (3 naive) mice received LiCl injections and 3 (1 naive) received saline injections. During the test sessions, all of these mice were tested with 0.0, 0.005, 0.01, 0.025, 0.05, and 0.1 mM SC45647.

SC45647 + amyl acetate CFA

The apparent inability of the LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice to form an aversion to either NaCl or SC45647 raised additional questions about the ability of the P2X2/P2X3$^{Dbl-/-}$ mice to learn a conditioned aversion. To examine this issue, mice were tested in a CFA experiment to determine whether the P2X2/P2X3$^{Dbl-/-}$ mice can form a conditioned aversion that included nontaste cues by conditioning with a mixture of 0.1 mM SC45647 + 0.001% amyl acetate (an odor). In this experiment, the concentration of SC45647 was increased to see if the KO mice could respond to a higher concentration of SC45647. The concentrations of amyl acetate selected for conditioning and testing are well above absolute thresholds in WT mice (Van Houten et al. 2008) and have little effect on taste detection (Slotnick et al. 1997). Eleven P2X2/P2X3$^{Dbl-/-}$ mice were randomly assigned to the LiCl injection condition, and 6 P2X2/P2X3$^{Dbl-/-}$ mice were assigned to the saline injection condition. Four KO mice in each injection condition were naive to CTA conditioning. Ten WT (3 naive) mice were assigned to the LiCl injection condition, and 3 WT (1 naive) mice were assigned to the saline injection condition. During each test session, the lick rates of these animals were measured when presented with 1) water (0 mM), 2) SC45647 (0.05 and 0.1 mM), 3) amyl acetate (0.0005% and 0.001%), and 4) the stimulus mixture (0.1 mM SC45647 + 0.001% amyl acetate).

Data analysis

Prior to analysis of the CTA or CFA data, the lick rates for each subject were normalized. This was accomplished by dividing the mean lick rates for each taste stimulus, including 2 water trials (each preceded by at least one water rinse trial) treated as taste stimuli, by the mean lick rate for the water rinse trials of that subject. An initial set of ANOVA procedures were completed for each CS condition to determine if mice without prior CTA experience performed differently than mice that had already been through the CTA experiment with the CS. No significant differences between any of these groups were detected, so the normalized data for naive and nonnaïve mice were combined for all subsequent analyses. The normalized data were then subjected to a 3-factor ANOVA procedure for mixed designs treating the injection condition (LiCl or saline) and mouse type (WT or P2X2/P2X3$^{Dbl-/-}$) as between-subject variables and test solution/concentration as a within-subject variable. For significant interactions, simple effects tests and *t*-tests with Bonferroni corrections were then used as needed to partition the data to determine where differences existed between WT and KO mice (Howell 2007). An unconditioned aversion is indicated if the lick rates for a taste stimulus by control mice are significantly lower than their lick rates for water. A conditioned aversion is revealed when there is a suppression of drinking by the LiCl-injected group compared with the saline-injected group, showing that this aversion has been learned and was not due to a naturally occurring (unconditioned) aversive quality.

Results

Brief-access test

Whereas the WT mice avoided licking for the higher concentrations of NaCl, the P2X2/P2X3$^{Dbl-/-}$ mice did not. A 2-way ANOVA was conducted to compare the normalized preference ratios of mouse type (2 levels) for each concentration of NaCl (6 levels) in the brief-access tests. This analysis revealed a significant genotype, $F_{1,8} = 320.19$, $P < 0.001$; concentration, $F_{5,40} = 60.92$, $P < 0.001$; and genotype-by-concentration interaction, $F_{5,40} = 65.89$, $P < 0.001$ (Figure 1). Independent samples *t*-tests with a Bonferroni correction to account for multiple comparisons were then used to compare the preference ratios of WT and P2X2/P2X3$^{Dbl-/-}$ mice at each concentration. WT mice licked significantly less than the P2X2/P2X3$^{Dbl-/-}$ mice at the 3 highest concentrations of NaCl (all *P*'s <0.001). Additionally, WT mice licked significantly less to each of the 3 highest concentrations of NaCl than water (all *P*'s <0.002). Conversely, P2X2/P2X3$^{Dbl-/-}$ mice did not lick differently to any concentration of NaCl (all *P*'s >0.1). Thus, even the highest concentration of NaCl tested (1000 mM) was not avoided by the P2X2/P2X3$^{Dbl-/-}$ mice, unlike their WT counterparts. The results of this experiment are similar to the findings of the preliminary NaCl brief-access test with a different group of mice. The results

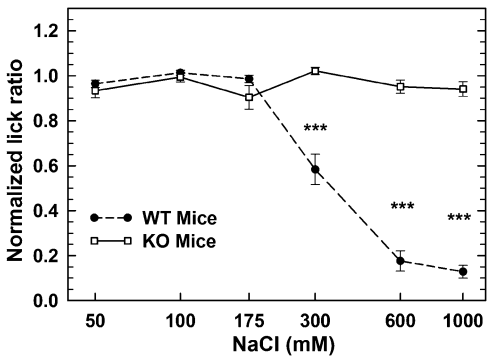

Figure 1 Comparison of the mean (±SEM) normalized lick ratios for various concentrations of NaCl in WT (dash line) and P2X2/P2X3$^{Dbl-/-}$ mice (solid line) during brief-access testing. Lick ratios were normalized by dividing the mean number of licks for each taste solution by the mean number of licks for water. The normalized lick ratios (ordinate) are plotted against the corresponding concentration of NaCl (abscissa). WT mice avoid concentrations of 300 mM and higher whereas the P2X2/P2X3$^{Dbl-/-}$ mice do not. ***$P < 0.001$.

of the preliminary experiment are shown in the Supplementary Figure 1 for comparison.

Conditioned aversion experiments

Sodium chloride CTA

We performed a CTA test to determine whether P2X2/P2X3$^{Dbl-/-}$ mice could learn to avoid NaCl when it was associated with postingestive illness. LiCl-injected WT mice learned to avoid NaCl at 50 mM, well below concentrations that normally drive aversion in water-deprived mice (>300 mM), but LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice did not. A 3-way ANOVA for mixed designs comparing mouse type (2 levels) and injection (2 levels) as between-subject variables and concentration (5 levels) as a repeated measures variable indicated that all main effects and interactions were significant, including the 3-way interaction between mouse type, injection condition, and concentration, $F_{4,116} = 4.72$, $P < 0.002$ (Figure 2). Simple effects tests of the WT data showed that WT saline-injected mice significantly decreased their licking of 600 mM NaCl compared with their licking of 0, 50, 100, or 300 mM ($P < 0.01$), indicating that NaCl is aversive (unconditioned) to the WT control mice at the highest concentration tested. The LiCl-injected WT mice had significantly lower lick ratios than saline-injected WT mice at all concentrations except 0 mM NaCl (all P's <0.01). P2X2/P2X3$^{Dbl-/-}$ mice, however, showed no significant avoidance at any concentration regardless of conditioning (all F's <1.0). The lick ratios of the WT control mice and the P2X2/P2X3$^{Dbl-/-}$ mice were compared at each concentration using a 1-way ANOVA followed by t-test comparisons. These ANOVAs found group differences in lick rates at 50 mM NaCl and higher, $F_{3,30} \geq 8.64$, P's <0.001. t-Tests with Bonferroni corrections indicated that the lick ratios of the LiCl-injected WT were significantly lower than all other groups at all concentrations above 0.0 mM (P's <0.006), including the saline-injected WT ($P < 0.05$). In addition, the lick ratios of the saline-injected WT mice were significantly lower than either KO group at 600 mM (P's <0.001). In summary, even though WT mice learned to avoid NaCl at concentrations of 50 mM and higher or showed an unconditioned aversion for 600 mM, the P2X2/P2X3$^{Dbl-/-}$ animals did not show any evidence of avoidance.

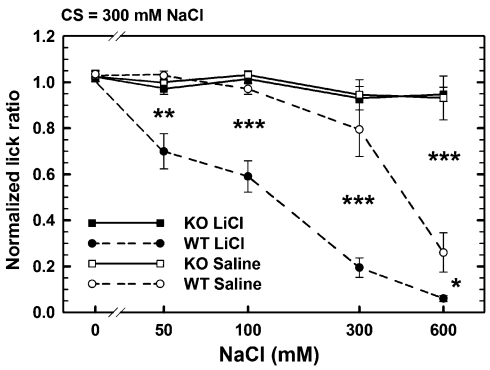

Figure 2 Comparison of water-deprived WT and P2X2/P2X3$^{Dbl-/-}$ mice on a CTA test for NaCl. Lick rates for each taste solution were normalized as a ratio of water intake by dividing the mean lick rate for each taste solution by the mean lick rate for water. The mean (±SEM) normalized lick ratios (ordinate) are plotted against the corresponding concentration of NaCl (abscissa). Saline-injected (control) WT mice found 600 mM NaCl innately (unconditioned) aversive. The 0 mM scores were derived from 2 water trials selected from those preceded by at least one other rinse trial. The LiCl-injected WT mice show a learned avoidance (lower lick ratios) for 50–600 mM when conditioned to avoid 300 mM NaCl. The P2X2/P2X3$^{Dbl-/-}$ mice show no unconditioned or learned avoidance to NaCl at any concentration. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

SC45647 CTA

Although WT mice learned a CTA for NaCl, the KO mice did not. To test whether the apparent inability of P2X2/P2X3$^{Dbl-/-}$ mice to learn to avoid NaCl was specific to that quality, we tested whether these mice could form a CTA to an artificial sweetener, SC45647. A 3-way ANOVA for mixed designs comparing mouse type (2 levels) and injection (2 levels) as between-subject variables and concentration (6 levels) as a repeated-measures variable. This analysis indicated that the main effects for mouse type, $F_{1,20} = 6.15$, $P < 0.025$; injection, $F_{1,20} = 12.82$, $P < 0.005$; and concentration, $F_{5,100} = 16.15$, $P < 0.001$ were significant. In addition, the 2-way interactions between mouse type and injection, $F_{1,20} = 7.49$, $P < 0.015$; concentration and mouse type, $F_{5,100} = 6.54$, $P < 0.001$; concentration and injection condition, $F_{5,100} = 6.47$, $P < 0.001$; and the 3-way interaction, $F_{5,100} = 6.04$, $P < 0.001$; reached significance (Figure 3). The data were then partitioned to compare groups at each concentration. Significant group differences were found at 0.025, 0.05, and

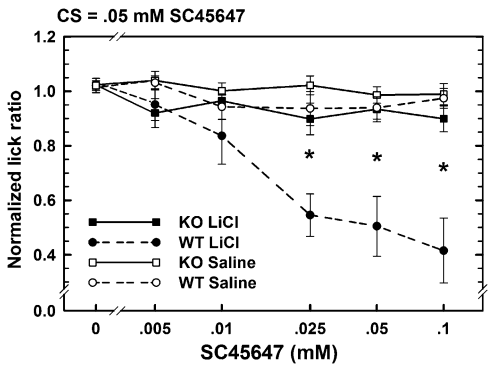

Figure 3 Comparison of water-deprived WT and P2X2/P2X3$^{Dbl-/-}$ mice on a CTA test with the artificial sweetener SC45647. Lick rates for each taste solution were normalized as a ratio by dividing the mean lick rate for each taste solution by the mean lick rate for water. The mean (±SEM) normalized lick ratios (ordinate) are plotted against the corresponding concentration of SC45647 (abscissa). The 0 mM scores were derived from 2 water trials selected from those preceded by at least one other rinse trial. LiCl-injected WT mice learned to avoid concentrations of 0.025, 0.05, and 0.1 mM when conditioned with a CS of 0.05 mM SC45647. P2X2/P2X3$^{Dbl-/-}$ mice did not. *$P < 0.025$.

0.1 mM, $F_{3,20} \geq 7.27$, P's <0.005. t-Tests revealed significantly lower lick ratios for LiCl-injected WT mice compared with saline-injected WT mice at 0.025 ($P = 0.011$), 0.05, and 0.1 mM (both P's <0.005). The lick ratios of LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice were not significantly different from the saline-injected group or from saline-injected WT mice at any concentration.

SC45647 + amyl acetate CFA

Because the P2X2/P2X3$^{Dbl-/-}$ mice did not appear to learn either CTA, we conditioned and tested these mice on a CFA to determine if they were capable of forming a conditioned aversion to any chemosensory stimulus. Specifically, we tested the mice with a stimulus mixture of the sweetener SC45647 and the odorant amyl acetate. The P2X2/P2X3$^{Dbl-/-}$ mice exhibited normal acquisition of the CFA task. However, their avoidance behavior was related to the presence of the odor cue but not the taste cue. A 3-way ANOVA was calculated to examine the effects of mouse type (2 levels), injection (2 levels), and test stimulus (6 levels) on lick ratios. The main effects for injection condition, $F_{5,28} = 17.82$, $P < 0.001$; and test stimulus, $F_{5,140} = 12.55$, $P < 0.001$; plus the interactions between test stimulus and mouse type, $F_{5,140} = 2.33$, $P < 0.05$; and between test stimulus and injection condition, $F_{5,140} = 7.62$, $P < 0.001$; were significant (Figure 4), but the 3-way interaction was not. Data were then partitioned to examine how the responses of WT and P2X2/P2X3$^{Dbl-/-}$ mice were affected by the CFA conditioning for each specific stimulus and to compare the responses of the WT mice with the KO mice.

The ANOVA examining the lick rates of the WT mice found significant main effects due to injection, $F_{1,11} = 9.12$, $P < 0.02$; test stimulus, $F_{5,65} = 4.08$, $P < 0.003$; and a significant interaction between the 2 variables, $F_{5,65} = 2.96$, $P < 0.025$ (Figure 4). Independent samples t-tests with Bonferroni corrections were used to compare saline and LiCl injection WT groups. The LiCl-injected WT mice licked significantly less of all the stimuli (except water) than the saline-injected WT mice (all P's <0.01). Paired t-tests also showed that the lick ratios for LiCl-injected WT mice for each concentration of SC45647 and amyl acetate were significantly less than for water (all P's <0.01), but these ratios did not differ from each other. In addition, the lick ratios of LiCl-injected WT mice were significantly lower for the stimulus mixture than for 0.1 mM SC45647 ($P < 0.001$) or 0.001% amyl acetate ($P < 0.02$).

The ANOVA of the lick ratios of the P2X2/P2X3$^{Dbl-/-}$ mice indicated significant effects due to injection, $F_{1,17} = 6.25$, $P < 0.025$, test stimulus, $F_{5,85} = 14.54$, $P < 0.001$, and the interaction between injection and stimuli, $F_{5,85} = 7.27$, $P < 0.001$ (Figure 4). LiCl-injected KO mice licked significantly less of the stimulus mixture ($P < 0.01$), 0.0005% ($P < 0.05$), and 0.001% amyl acetate ($P < 0.01$) than the saline-injected KO mice. LiCl conditioning (vs. saline conditioning) did not affect lick ratios of the KO mice for either concentration of SC45647. Paired samples t-tests indicated that the lick ratios of the LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice for 0.0005% amyl acetate were significantly

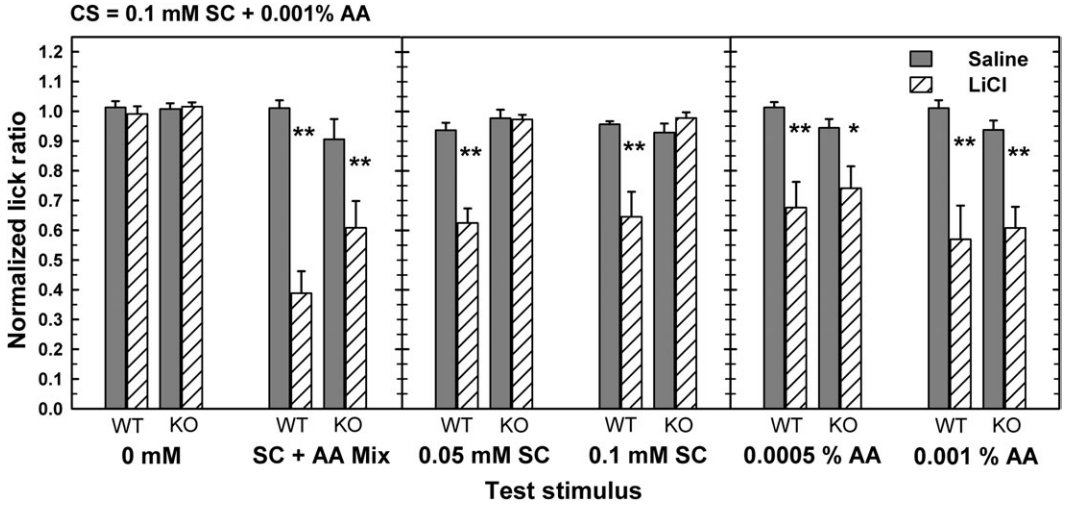

Figure 4 Comparison of water-deprived WT and P2X2/P2X3$^{Dbl-/-}$ mice on a CTA test after conditioning with a stimulus mixture (CS compound) of 0.1 mM SC45647 + 0.001% amyl acetate. Lick rates (ordinate) were normalized as a ratio in the same manner as described for Figures 2 and 3. Mean (±SEM) normalized ratios (ordinate) are plotted for each test solution identified on the abscissa. The 0 mM scores were derived from water trials preceded by at least one other rinse trial. Saline-injected (Control = Solid bars) WT and KO mice did not avoid any stimulus. The LiCl-injected (LiCl = striped bars) WT mice reduced their lick ratios for the individual SC45647 and amyl acetate stimulus solutions compared with the water stimulus (P's <0.05). They also had significantly lower lick ratios (greater avoidance) for the mixture compared with the individual substances (P's ≤0.015). The LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice avoided the stimulus mixture ($P < 0.01$) and 0.001% amyl acetate ($P < 0.01$) to the same degree, relative to their lick ratios for the water stimulus. However, their lick ratios for both concentrations of SC45647 were not affected by LiCl conditioning. This shows that the P2X2/P2X3$^{Dbl-/-}$ mice are capable of learning the association between an odor cue and gastric distress but are not capable of identifying the taste components of the stimulus mixture.

lower than their lick ratios for water ($P < 0.006$). Furthermore, these KO mice licked 0.001% amyl acetate and the stimulus mixture less than they licked 0.0005% amyl acetate (both P's < .05). However, no differences between their lick ratios for 0.001% amyl acetate and the stimulus mixture were detected ($P = 0.641$).

To further characterize and compare the responses of WT and P2X2/P2X3$^{Dbl-/-}$ mice, the number of trials initiated by each mouse type in the 32-trial sessions was also examined using ANOVA procedures to compare mouse type (2 levels) and injection (2 levels) conditions for each CS. No significant differences (all F's <2.00) for either variable or their interaction were detected for any CS. For all 3 conditioned aversion experiments, the mean trials initiated were 25.94 (±1.56 standard error of the mean [SEM]) by WT mice and 27.81 (±1.51 SEM) by the P2X2/P2X3$^{Dbl-/-}$ mice. The lick ratios during water trials were also compared using the same type of ANOVA analysis, and again no significant differences were revealed by the analysis. For the 3 experiments, the mean number of licks per trial was 45.71 (±0.58 SEM) by WT mice and 43.86 (±0.45 SEM) by the P2X2/P2X3$^{Dbl-/-}$ mice. These results suggest that the WT mice and the P2X2/P2X3$^{Dbl-/-}$ mice exhibited similar motivational states and sampled comparable numbers of taste solutions during testing.

The results of the CFA experiment indicate that the LiCl-injected WT mice exhibited a learned aversion to both the taste and odor components of the conditioning stimulus, but as anticipated these mice showed a greater aversion for the mixture of the 2. Second, this experiment revealed that the P2X2/P2X3$^{Dbl-/-}$ mice were capable of learning a conditioned aversion task but responded only to the odor. That is, the taste component of the mixture did not elicit any avoidance behavior by the P2X2/P2X3$^{Dbl-/-}$ mice.

Discussion

Our results indicate that, unlike WT mice, the P2X2/P2X3$^{Dbl-/-}$ mice do not exhibit a preference for or avoidance of NaCl at a wide range of concentrations. In addition, P2X2/P2X3$^{Dbl-/-}$ mice cannot identify NaCl or SC45647 as a taste stimulus in a CTA paradigm. These findings are consonant with the previous findings of a lack of gustatory neural response to NaCl or SC45647 in the P2X2/P2X3$^{Dbl-/-}$ mice (Finger et al. 2005).

Previous experiments with P2X2/P2X3$^{Dbl-/-}$ mice have suggested that these mice are apparently non or minimally responsive to most taste substances, particularly sweet, umami, and bitter, that is, those qualities involving a G-protein-coupled receptor transduction pathway associated with Type II receptor cells (Finger et al. 2005). In those previous studies, behavioral effects of the KO were measured via 24-h 2-bottle preference testing in which the hedonic value of a substance can motivate ingestive behavior. However, it is possible that P2X2/P2X3$^{Dbl-/-}$ mice are still able to detect some sort of taste signal, but this signal lacks the potency for the P2X2/P2X3$^{Dbl-/-}$ mice to assign a particular hedonic value that will motivate behavioral changes. To further test the extent to which this KO decreases or even eliminates the capability to taste, we utilized a combination of behavioral methods to study both inherent (unconditioned) and conditioned hedonic characteristics of taste stimuli.

P2X2/P2X3$^{Dbl-/-}$ mice do not avoid high concentrations of NaCl as do WT mice in the brief-access tests. Brief-access testing is able to assess the inherent hedonic value of a taste stimulus, without the postingestive effects seen with 24-h 2-bottle preference tests (Spector 2003). WT mice exhibited a clear decrease in the lick rates at NaCl concentrations of 300 mM and above, especially for the 1000 mM solution that was avoided almost completely. These data indicate that the WT mice found the higher concentrations increasingly aversive. If the P2X2/P2X3$^{Dbl-/-}$ mice were able to detect and perceive NaCl in the same manner as WT mice, then they should alter their lick rates at high concentrations. However, the KO mice continued to drink even the highest concentrations of NaCl (up to 1000 mM) as if they were drinking water.

Brief-access tests can help determine the inherent hedonic value of a taste stimulus to a mouse, but they often require a mild state of deprivation to motivate the mouse to drink in the test apparatus (Glendinning et al. 2002). This deprivation also increases drinking for water, and because preferences for a substance are assessed against water consumption, a mild preference or aversion for a substance may go undetected in this paradigm, as might be the case for the WT mice at the lower concentrations of NaCl in the brief-access experiment. On the other hand, CTA methods used in this study exposed mice to a novel taste substance and then immediately afterward the animal was injected with LiCl to induce internal malaise. This causes a lasting association of the taste with the malaise leading to avoidance of solutions associated with the taste (Spector 2003). CTA can be used behaviorally to assess whether or not an animal is capable of detecting and identifying a taste, even if the taste signal is weak, because it forces the animal to assign an associated negative hedonic value to the taste. In each of the CTA experiments, the animals were exposed to the paired CS injection conditions twice to ensure conditioning. The CTA experiment with 300 mM NaCl used a CS that, for WT mice, is highly salient and can be readily associated with the effects of the injection. The WT mice injected with LiCl avoided NaCl at concentrations as low as 50 mM. P2X2/P2X3$^{Dbl-/-}$ mice, on the other hand, responded as if they either could not detect the stimulus or could not make an association between the CS and the effects of the injection.

In order to assess whether the lack of responsiveness to NaCl was quality specific, we tested whether P2X2/P2X3$^{Dbl-/-}$ mice could develop a CTA to a nonnutritional sweetener, SC45647. Previously published work with 24-h 2-bottle preference testing with this substance has shown that WT mice readily prefer the artificial sweetener

SC45647 over water, whereas the P2X2/P2X3$^{Dbl-/-}$ mice exhibit little or no preference for the sweetener (Finger et al. 2005). In the current study, WT mice in the CTA experiment readily learned the aversion to SC45647, whereas the KO mice did not. Thus, the results of both studies indicate that P2X2/P2X3$^{Dbl-/-}$ mice are incapable of detecting the taste qualities of SC45647.

P2X2 and P2X3 receptors have been reported in other sensory systems, most notably peripheral nociceptors signaling visceral function and gastric mechanoreceptors responsive to stomach distension (Cockayne et al. 2005; McIlwrath et al. 2009; Mo et al. 2009). If these same receptors contribute to the signal associated with gastric distress produced by LiCl injections, it is conceivable that genetic deletion of the $P2X_2$ and $P2X_3$ subunits might have negatively affected the ability of the P2X2/P2X3$^{Dbl-/-}$ mice to learn any conditioned aversion. That is, the strength of the unconditioned stimulus (LiCl) supporting CTA learning and subsequent effects on behavior would be weakened (Bouton 2007), and the KO mice might not be able form a conditioned aversion using LiCl. We tested this in the CFA experiment in which mice were exposed to a CS consisting of both a taste (SC45657) and odor (amyl acetate) component. In this experiment the P2X2/P2X3$^{Dbl-/-}$ mice exhibited the same amount of aversion for the stimulus mixture as the WT mice, thus showing that the genetic deletion did not have an adverse effect on the ability of these mice to make the association between a chemosensory stimulus and gastric distress. This suggests that the KO mice were not only capable of sensing the effects LiCl, but that LiCl at the concentration used in these experiments was as effective at forming the aversion in KO mice as in WT mice.

A closer examination of the CFA experiment is enlightening. When a stimulus mixture or rather, to correctly use learning terminology, a stimulus compound (Bouton 2007) combines an odor stimulus with a taste stimulus to serve as the CS in the CFA, 1 of 3 outcomes are expected. In general, if both stimuli are salient to the mouse, the strength of the aversion to the compound is the combined strength of the aversion to each of the 2 elements of the compound (Bouton 2007). Presented separately, each cue can elicit avoidance behavior, but the amount of avoidance is less than that elicited by the compound. If one stimulus is more salient than the other stimulus of the compound, then overshadowing or compound potentiation may occur. In overshadowing, the more salient of the 2 stimuli forming the compound is more strongly associated with the aversion. To detect overshadowing, the 2 stimuli are paired during the learning phase. Then during the test session the stimuli are presented separately and as a unit. When tested separately, the more salient of the 2 stimuli elicits more avoidance than the less salient stimulus. On the other hand, compound potentiation can occur when a strongly salient odor stimulus is paired with a weak taste as the CS during conditioning (Slotnick et al. 1997), such as might occur with the KO mice in this experiment. This possibility can be detected when one compares the learned behavior of a mouse conditioned with just the taste stimulus to the behavior of a mouse conditioned with the odor and taste stimuli paired during conditioning. If a weakly salient taste stimulus by itself is conditioned with LiCl in a naive mouse, the taste stimulus might elicit little or no learned avoidance during testing. However, if this same weak (but detectable) taste stimulus is combined with a stronger odor stimulus during conditioning in a naive mouse, the odor stimulus may potentiate or enhance the avoidance elicited by the taste stimulus compared with the level of avoidance elicited when the taste stimulus is conditioned by itself. In the present CFA experiment, WT mice showed similar reductions in licking for the odor and for the taste stimulus when each was presented alone, and greater response suppression when the 2 stimuli were presented together as a mixture. These results would be anticipated if the 2 stimuli were similarly salient to the mouse and are sharing the associative value of the LiCl-induced aversion. In comparison, the LiCl-injected P2X2/P2X3$^{Dbl-/-}$ mice showed a similar degree of response suppression to the stimulus mixture as the WT mice. However, they exhibited response suppression only to the odorant and no evidence of avoiding the taste component of the stimulus mixture, ruling out both overshadowing and compound potentiation. This finding is consistent with the interpretation that the P2X2/P2X3$^{Dbl-/-}$ mice are incapable of detecting and identifying many classical taste stimuli.

Taken together, our results support the conclusion, initially based on gustatory nerve recordings and 2-bottle preference tests (Finger et al. 2005), that P2X2/P2X3$^{Dbl-/-}$ mice are largely incapable of detecting and identifying NaCl and the sweetener SC45647. Our use of brief-access tests minimized the possibility that the mice could utilize postingestive cues and therefore provide clear behavioral results entirely consistent with the lack of gustatory nerve responses to these substances in the previous study. The residual avoidance behaviors elicited by sour and some bitter substances seen in 24-h 2-bottle preference tests reported by Finger et al. (2005) are likely attributable to nongustatory cues. In addition, the results of the conditioned aversion tests generally rule out weak, ineffective hedonic properties of taste stimuli or the lack of ability to associate a stimulus with an associated response consequence for the KO mice. Instead, the results of this study are consistent with hypothesis that the P2X2/P2X3$^{Dbl-/-}$ mice lack the ability to identify the taste of either NaCl or SC45647.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

Funding

National Institute on Deafness and Other Communication Disorders at the National Institutes of Health [R01

DC06070, R01 DC00766, and P30 DC04657 to T.E.F. and Diego Restrepo (University of Colorado Denver School of Medicine)]; and National Institute on Deafness and Other Communication Disorders at the National Institutes of Health [R15 DC007617 to E.R.D.].

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Accepted September 7, 2009