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# A High-Throughput Method to Measure NaCl and Acid Taste Thresholds in Mice

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## Abstract

To develop a technique suitable for measuring NaCl taste thresholds in genetic studies, we conducted a series of experiments with outbred CD-1 mice using conditioned taste aversion (CTA) and two-bottle preference tests. In Experiment 1, we compared conditioning procedures involving either oral self-administration of LiCl or pairing NaCl intake with LiCl injections and found that thresholds were the lowest after LiCl self-administration. In Experiment 2, we compared different procedures (30-min and 48-h tests) for testing conditioned mice and found that the 48-h test is more sensitive. In Experiment 3, we examined the effects of varying strength of conditioned (NaCl or LiCl taste intensity) and unconditioned (LiCl toxicity) stimuli and concluded that 75–150 mM LiCl or its mixtures with NaCl are the optimal stimuli for conditioning by oral self-administration. In Experiment 4, we examined whether this technique is applicable for measuring taste thresholds for other taste stimuli. Results of these experiments show that conditioning by oral self-administration of LiCl solutions or its mixtures with other taste stimuli followed by 48-h two-bottle tests of concentration series of a conditioned stimulus is an efficient and sensitive method to measure taste thresholds. Thresholds measured with this technique were 2 mM for NaCl and 1 mM for citric acid. This approach is suitable for simultaneous testing of large numbers of animals, which is required for genetic studies. These data demonstrate that mice, like several other species, generalize CTA from LiCl to NaCl, suggesting that they perceive taste of NaCl and LiCl as qualitatively similar, and they also can generalize CTA of a binary mixture of taste stimuli to mixture components.

**Key words:** citric acid, conditioned taste aversion, LiCl, NaCl, taste threshold



## Introduction

Sodium is an important nutrient, and salt appetite is one of the main mechanisms to maintain its homeostasis. Animals are able to choose and consume sodium because they can detect it in the environment using salty taste (McCaughey and Scott 1998; Lindemann 2001). Although recent studies discovered taste receptors for bitter, sweet, umami, and possibly sour taste, a receptor protein for salty taste has not yet been unequivocally identified (reviewed in Chandrashekar et al. 2006; Bachmanov and Beauchamp 2007).

Genetic analysis of taste-evoked behavior is an efficient approach to study molecular mechanisms of taste. For example, the genetic mapping studies of sweetener consumption in mice (Phillips et al. 1994; Lush et al. 1995; Bachmanov et al. 1997; Blizard et al. 1999; Bachmanov, Li, Reed, et al. 2001; Li et al. 2001) and bitter taste responses in humans (Reed et al. 1999) and mice (Capeless et al. 1992; Lush et al. 1995; Bachmanov, Li, Li, et al. 2001) facilitated discovery of the T1R and T2R taste receptors (reviewed in Bachmanov and Beauchamp 2007). Genetic analysis of taste responses to salts also has the potential to uncover mechanisms of salty taste reception.

Previous studies using long-term (6–96 h) tests have found wide variation in voluntary NaCl consumption among inbred mouse strains (Lush 1991; Beauchamp and Fisher 1993; Bachmanov, Schlager, et al. 1998; Bachmanov, Tordoff, and Beauchamp 1998; Kotlus and Blizard 1998; Bachmanov, Beauchamp, and Tordoff 2002; Tordoff et al. 2007). However, NaCl intake in these tests can be affected not only by gustatory input but also by differences in sodium metabolism, which makes this trait less than ideal for genetic analyses of salty taste. On the contrary, NaCl taste thresholds are more likely to reflect mechanisms of peripheral taste reception directly, and thus they are a more suitable trait for genetic studies of salty taste.

In human perception studies, two types of taste thresholds are usually determined. Detection thresholds measure the lowest taste stimulus concentration that can be distinguished

from a vehicle. Recognition thresholds measure the lowest taste stimulus concentration that evokes a sensation of a certain quality (Amerine et al. 1965). Several different procedures have been developed to measure taste thresholds in nonhuman animals. Electrophysiological recordings of activity in afferent gustatory nerves have been used to determine neural response thresholds defined as the lowest concentration that evokes a signal discernible from a background activity (Pfaffmann and Bare 1950; Beidler 1953; Iwasaki and Sato 1984; Frank and Blizard 1999; Inoue et al. 2001). Behavioral responses of operant-conditioned animals have been used to assess detection thresholds defined as the minimum concentration at which a tastant can be detected against water (Carr 1952; Koh and Teitelbaum 1961; Slotnick 1982; Geran and Spector 2000; Eylam and Spector 2002, 2003; Ruiz et al. 2006).

The goal of the present study was to develop a high-throughput procedure to measure taste thresholds, which could be used in genetic experiments that require testing of large numbers of animals. To achieve this, we used the conditioned taste aversion (CTA) paradigm, which has been previously used to assess taste thresholds in rats (Tapper and Halpern 1968; du Villard et al. 1981; Scott and Giza 1987; Ramirez 1991; Yamamoto et al. 1994; Clarke et al. 2001; Scalera 2004; Curtis et al. 2005) but was less commonly used in mice (Belknap et al. 1978; Harder et al. 1989; Ninomiya et al. 1994; Blizard 2007). This approach involves conditioning animals to avoid a suprathreshold concentration of a taste solution used as a conditioned stimulus (CS). If the conditioned animals are tested with various concentrations of the same tastant, they will avoid not only the concentration used as the CS but also other concentrations within a certain range (i.e., intensity generalization range). Limits of this intensity generalization range are considered intensity generalization thresholds (Tapper and Halpern 1968; Spector and Grill 1988; Clarke et al. 2001). Although intensity generalization thresholds are not always identical to recognition thresholds, both thresholds determine the extreme tastant concentration that tastes qualitatively similar to a certain standard (e.g., CS). The intensity generalization thresholds can accurately reflect recognition thresholds under optimal conditions when the lower range of the intensity generalization includes taste stimulus concentrations near the recognition threshold. The goal of our experiments was to find such optimal CTA procedures. For convenience, we describe thresholds determined in CTA experiments of this study as avoidance thresholds, but they correspond to intensity generalization and in some cases also to recognition thresholds.

A general design of our study was the following: We first conditioned mice and then tested them in two-bottle preference tests with NaCl solutions to determine avoidance thresholds. We varied procedures for mouse conditioning and testing and compared resulting avoidance thresholds. Our goal was to find experimental conditions that would result in avoidance thresholds similar to taste thresholds reported in the literature. Such procedures would therefore be suitable for assessing taste recognition thresholds.

In our study, we used taste stimulation during oral self-administration of NaCl or LiCl as CS, and malaise-inducing postingestive effects of LiCl as an unconditioned stimulus (US). Several lines of evidence demonstrate similarity of NaCl and LiCl taste. First, humans perceive similar taste quality and intensity of these two salts (Murphy et al. 1981; van der Klaauw and Smith 1995). Second, CTA to LiCl strongly generalizes to NaCl in rats (Nachman 1963; Rolls and Rolls 1973; Lasiter and Glanzman 1985; Simbayi 1987; Loy and Hall 2002; Baird et al. 2005) and mice (Beauchamp and Fisher 1993). Third, NaCl and LiCl activate the same single fibers of the chorda tympani gustatory nerve in rats, hamsters, and macaque monkeys (Fishman 1957; Sato et al. 1975). Fourth, NaCl and LiCl evoke similar across-neuron patterns of activity in the rat nucleus of the solitary tract (Scott and Giza 1990) and in the monkey cortex (Scott et al. 1994). This similarity of NaCl and LiCl tastes allowed us to use them interchangeably as the CS. Similarly to oral self-administration of LiCl (Nachman 1963; Rolls and Rolls 1973; Lasiter and Glanzman 1985; Simbayi 1987; Beauchamp and Fisher 1993; Loy and Hall 2002; Baird et al. 2005), oral self-administration of ethanol was also used in previous studies as both CS and US to develop taste aversion to ethanol (Belknap et al. 1978).

We conducted four experiments comparing different procedures. In Experiment 1, we compared different conditioning procedures, including oral self-administration of LiCl and pairing NaCl intake with LiCl injections. In Experiment 2, we compared the sensitivity of short-term (30-min) and long-term (48-h) tests to detect aversion. Because we found that oral LiCl self-administration and testing conditioned mice in 48-h two-bottle tests is the most sensitive procedure, we used it in subsequent experiments. In Experiment 3, we examined effects of variation in CS and US intensity. This was achieved by mixing LiCl and NaCl at different concentrations so that combined concentrations of LiCl and NaCl would alter CS strength (i.e., taste intensity), and LiCl concentration would alter US strength (i.e., toxicity). In Experiment 4, we examined whether the procedure suitable for measuring NaCl taste thresholds (identified in Experiments 1–3) is also applicable for assessment of taste thresholds for other taste qualities. This was achieved by oral self-administration of a mixture of LiCl with sour-tasting citric acid. Results of these experiments allowed us to develop a simple and sensitive method to measure taste thresholds for NaCl and other taste stimuli.

## General methods

### Subjects

In all experiments, naive outbred CD-1 male mice (Charles River Laboratories, Wilmington, MA) were used. At the

beginning of the experiments, they were 7–8 weeks old and had a mean body weight (BW) of 30.8 ± 0.1 g. During experiments, they were housed in individual cages in a temperature-controlled room at 23 °C on a 12:12-h light:dark cycle (7:00 AM on, 7:00 PM off) and had free access to the Teklad Rodent Diet 8604 (Harlan, Madison, WI), which includes 0.29% sodium.

### Apparatus

Construction of the drinking tubes and cage lids has been described previously (Bachmanov, Reed, et al. 2002) and is given in detail on the Monell Mouse Taste Phenotyping Project web site (Tordoff and Bachmanov 2001).

### Procedure

Mice were given deionized water in two drinking tubes at least 2 days before the start of the experiments to adapt them to the experimental setting. Fluid intakes were measured throughout the experiments. Intake measurements were made by reading fluid volume to the nearest 0.1 mL.

CTA was produced using two types of procedures. The first type (used in Experiments 1-A and 1-B) involved a 30-min presentation of a NaCl solution (CS) immediately followed by an intraperitoneal injection of LiCl (US). The conditioning was repeated three (Experiment 1-A) or six (Experiment 1-B) times. In the second type (used in Experiments 1-C, 2, 3, and 4), mice self-administered a solution containing LiCl, which was the only source of fluid for 24 h; the conditioning was repeated twice. In all experiments, CSs (NaCl or LiCl-containing solutions) were presented in both drinking tubes. Conditioning days were always separated by a recovery day when water was available in both drinking tubes. After the last conditioning session, mice had access to water in both drinking tubes for 48 h (Experiments 1C, 3, and 4) or 64 h (Experiments 1-A and 1-B) before the first 48-h test of a taste solution. This allowed mice to recover from any dehydration developed during conditioning.

During two-bottle preference tests of conditioned mice, they were presented with one tube containing a taste solution in deionized water and the other tube containing only deionized water. Each taste solution concentration was presented either for a 48-h period (on two consecutive days; Experiments 1-A, 1-B, 1-C, 3, and 4) or for two 30-min periods (on the same day; Experiment 2). When multiple concentrations of taste solutions were tested, they were presented in ascending order (Experiments 1-B, 1-C, 2, 3, and 4), starting with concentration 0 (i.e., when both tubes contained water). During the 48-h tests, mice were tested in their home cages. To control for side preferences, the positions of the tubes were switched after every 24 h in the order left–right–right–left. For the 30-min tests, mice were transferred to experimental cages from their home cages. Each solution concentration was tested twice on the same day with positions of water and solution tubes reversed between the two tests. In both 48-h and 30-min tests, there were no delays between testing different concentrations of the same compound.

### Data analysis

Preference scores were calculated as the ratio of the two-test average solution intake to the two-test average total fluid (solution + water) intake, in percent. The preference scores were analyzed by two-way repeated measures analysis of variance (ANOVA) with group as the between-subjects factor and concentration as the within-subjects factor. Newman–Keuls post hoc tests were used to evaluate differences between individual means. The avoidance threshold was estimated from the NaCl (or citric acid) preference scores using a three-parameter logistic function modified from Ritz and Streibig (2005). The preference scores for all tested concentrations except 0 mM within each treatment group were fit to a regression curve using the function: $f(x) = 50/(1 + \exp(b(\log(x) - \log(c))))$. Within the function, (x) is the stimulus concentration, (b) is the slope, and (c) is the stimulus concentration at half performance. The maximum performance was set to 50% preference as complete indifference and minimum performance was set to 0% preference as complete avoidance in the function. The 25% threshold level was chosen as a midpoint between complete indifference and avoidance, which approximates the 50% level of correct responses often used in psychophysics as a threshold value (Spector 2003; Bufe et al. 2005). In preliminary analyses, we also determined thresholds using several other approaches: 1) using a similar regression analysis to determine thresholds for each individual mouse and then analyzing group means of these individual threshold values; 2) as the lowest solution concentration for which preference score was significantly lower than preference score in an initial test with water presented in both drinking tubes; and 3) as the lowest solution concentration for which solution intake was significantly lower than intake of water presented simultaneously as the second choice. Results of all these analyses were similar, and we therefore present here only threshold values calculated using regression analysis of group data; this technique is applicable for all experiments conducted in this study, which allowed us to compare obtained threshold values across different experiments. In all experimental groups for test solution concentrations above the avoidance threshold, corresponding intakes of water and a taste solution were significantly different ($P < 0.02$, paired $t$-tests). Statistical analyses were conducted using the Statistica software package (StatSoft, Inc., Tulsa, OK) and using the statistical language and environment R (R-Development-Core-Team 2007). A $P$ value < 0.05 was used as the level of statistical significance. All data are presented as mean ± SEM (standard error of the mean).

## Experiment 1. Comparison of different conditioning procedures

In this experiment, we examined two different CTA techniques: pairing NaCl intake with LiCl injections (Experiments 1-A and 1-B) and oral self-administration of LiCl (Experiment 1-C). Pairing NaCl intake with LiCl injections was conducted either by using multiple groups (with each group conditioned by presenting the same NaCl concentration as a CS; Experiment 1-A) or by sequentially presenting mice from the same group with several different NaCl concentrations (Experiment 1-B).

### Experiment 1-A. Multiple treatment groups, each conditioned with a single NaCl concentration

In this experiment, we used different NaCl concentrations as the CS for different treatment groups. Following the CS presentation, mice from all groups were injected with LiCl as the US. After conditioning, each group was tested in 48-h two-bottle preference tests, first with the same NaCl concentration as that which had been used for conditioning and next with a series of NaCl concentrations.

#### *Method*

Seventy-four male mice were randomly divided into nine groups ($n = 8$ or 9 mice in each group). Prior to conditioning, all mice were trained for 2 days by restricting their access to water, which was presented in two tubes, to two 30-min periods per day (at 10:00 AM and 5:00 PM; Table 1). On a conditioning day, each group was given one of the following NaCl solutions (0, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 mM) as a CS in both tubes during one of the 30-min periods of access to fluid, immediately followed by an intraperitoneal injection of 150 mM LiCl. The dose of LiCl was 0.23 g/kg BW (1.09 mL of 150 mM LiCl for a mouse of 30 g BW). During the other 30-min period of access to fluid on that conditioning day, all mice received water in both tubes. Conditioning was performed three times (days 3, 5, and 7), with time of conditioning (10:00 AM or 5:00 PM) alternated. On the recovery days 4 and 6, mice were given water in both tubes for both 30-min periods. At 6:00 PM on the third conditioning day (day 7), mice were given water in both tubes overnight for recovery.

Starting on day 8, each mouse was tested in two consecutive 48-h two-bottle preference tests: first with water in both drinking tubes and then with one tube containing the concentration of NaCl used as the CS and the other tube containing water. The preference scores obtained in these two tests were analyzed by two-way repeated measures ANOVA with group as the between-subjects factor (with nine levels) and concentration as the within-subjects factor (with two levels). The avoidance thresholds were calculated using NaCl preference scores of eight groups (all treatment groups with the exception of the 0 mM group).

Starting on day 12, mice were tested with a series of NaCl solutions (0, 1, 3, 10, 30, 100, and 300 mM) presented in ascending order of concentration in 48-h two-bottle preference tests with NaCl solution in one tube and water in the other tube. The preference scores for each concentration were analyzed by two-way repeated measures ANOVA with group as the between-subjects factor (with nine levels for each CS) and concentration as the within-subjects factor (with seven levels). The NaCl avoidance threshold was calculated based on the NaCl preference scores for all tested concentrations with the exception of 0 mM.

#### *Results*

The mean NaCl preference scores obtained in the initial 48-h test with the same NaCl concentration as one used for conditioning are shown in Figure 1, with each mean score representing a separate group of mice. Two-way ANOVA of NaCl preference scores revealed the significant effects of group, $F(8, 65) = 18.04$, $P < 0.001$, and concentration, $F(1, 65) = 71.43$, $P < 0.001$, and a significant interaction

**Table 1** Schedule of Experiment 1-A

| Day | Stage | Time, solution (duration) | | | | | |
|---|---|---|---|---|---|---|---|
| 1–2 | Training | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 3 | Conditioning | 10:00 AM | NaCl (30 min) + LiCl injection | 5:00 PM | Water (30 min) | | |
| 4 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 5 | Conditioning | 10:00 AM | Water (30 min) | 5:00 PM | NaCl (30 min) + LiCl injection | | |
| 6 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 7 | Conditioning/recovery | 10:00 AM | NaCl (30 min) + LiCl injection | 5:00 PM | Water (30 min) | 6:00 PM | Water (overnight) |
| 8–9 | Preference testing | 10:00 AM | Water and water (48 h) | | | | |
| 10–11 | Preference testing | 10:00 AM | NaCl and water (48 h) | | | | |
| 12–25 | Preference testing | 10:00 AM | 0–300 mM NaCl and water (48 h) | | | | |

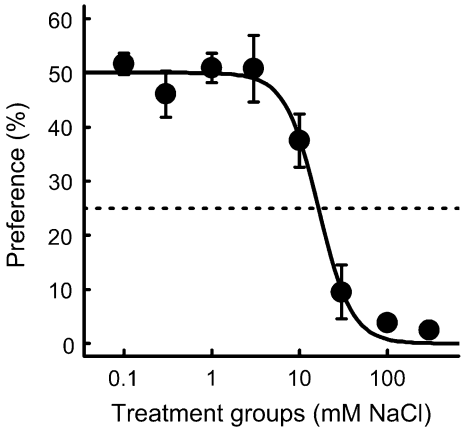


**Figure 1** Experiment 1-A: NaCl preference scores (Mean ± SEM) in 48-h two-bottle tests of mice from eight groups conditioned to avoid a corresponding NaCl solution. During conditioning, each mouse was exposed to a single NaCl concentration followed by LiCl injections and then was tested with the same NaCl solution. Each mean represents a preference score of a separate group. A dotted horizontal line shows a 25% preference score corresponding to the avoidance threshold. The curve was fit to the CS preference scores of all treatment groups using the function described in the text (see Methods section). The avoidance threshold (i.e., the stimulus concentration at the intersection of the regression curve with the 25% preference level) was 16 mM.

between group and concentration, $F(8, 65) = 19.26$, $P < 0.001$. When water was presented to conditioned mice in both tubes in the first 48-h test, the preference scores were close to 50% (data not shown), and there were no significant differences between the groups (Newman–Keuls post hoc tests). In contrast, the preference scores for the CS obtained in the second 48-h test were different among the groups: Mice conditioned with 30–300 mM NaCl had significantly lower preference scores for the CS than mice conditioned to avoid 0–10 mM NaCl (Figure 1). The avoidance threshold was 16 mM.

The mean NaCl preference scores obtained in the following 48-h tests with series of NaCl concentrations are shown in Figure 2. Each group of mice was conditioned with a different NaCl concentration but tested with the same concentration series. ANOVA of NaCl preference scores revealed significant effects of group, $F(8, 66) = 26.2$, $P < 0.001$, and NaCl concentration, $F(6, 396) = 57.6$, $P < 0.001$, and a significant interaction between group and concentration, $F(48, 396) = 4.97$, $P < 0.001$. The avoidance thresholds for all nine groups are shown in Table 2. Mice conditioned with 0–10 mM NaCl had avoidance thresholds ~300 mM or higher, similar to avoidance thresholds in mice without prior conditioning (Lush 1991; Bachmanov, Tordoff, and Beauchamp 1998; Bachmanov, Beauchamp, and Tordoff 2002). Mice conditioned with 30–300 mM NaCl had lower avoidance thresholds (ranging from 6 to 27 mM), which is consistent with suppression of NaCl consumption in these groups in the initial test (Figure 1).

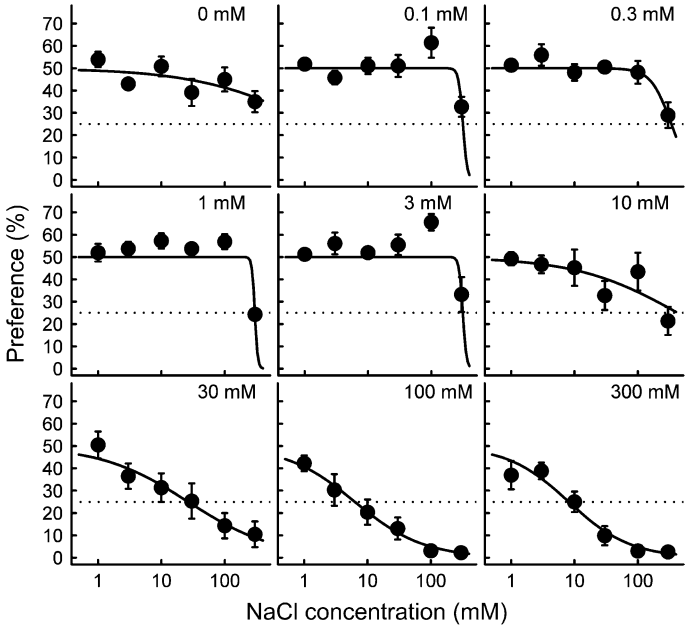


**Figure 2** Experiment 1-A: NaCl preference scores (Mean ± SEM) in 48-h two-bottle tests of mice from nine groups conditioned to avoid a NaCl solution (0, 0.1, 0.3, 1, 3, 10, 30, 100, or 300 mM). Mice were tested with series of NaCl solutions after initial tests of single concentrations shown in Figure 1. The curve was fit to NaCl preference scores using the function described in the text (see Methods section). Avoidance thresholds for each group are shown in Table 2. Other descriptions are the same as in Figure 1.

**Table 2** NaCl avoidance thresholds of mice from nine groups conditioned to avoid NaCl (Experiment 1-A)

| Groups[a] | Threshold (mM) |
|---|---|
| 0 mM | 2559 |
| 0.1 mM | 315 |
| 0.3 mM | 337 |
| 1 mM | 299 |
| 3 mM | 314 |
| 10 mM | 425 |
| 30 mM | 27 |
| 100 mM | 6 |
| 300 mM | 8 |

[a]Values in this column show NaCl concentration used as a CS.

### Experiment 1-B. A single treatment group conditioned with multiple NaCl concentrations

In this experiment, we exposed each mouse to multiple concentrations of NaCl used as the CS. We hypothesized that if mice develop CTA to several different NaCl concentrations rather than to a single concentration, this would extend their intensity generalization range. To increase the probability

that intensity generalization range includes a recognition threshold, we have chosen to use as CS those NaCl concentrations, which were near or slightly above reported NaCl taste thresholds (Table 3; see also Experiments 1-A, 1-C, and 3). Following the CS presentation, mice were injected with LiCl as the US. After conditioning, all mice were tested with a series of NaCl concentrations using 48-h two-bottle preference tests.

### *Method*

Sixteen male mice were randomly divided into two groups, conditioned ($n = 8$) and control ($n = 8$). Prior to conditioning, all mice were trained to a restricted access to water as described in Experiment 1-A. Mice from both groups were given NaCl to drink during six 30-min conditioning sessions similar to the conditioning sessions described in Experiment 1-A, with the exception that NaCl concentrations varied during each session (3, 10, or 30 mM; presented in the order described in Table 4). Each mouse was presented with each NaCl concentration twice (Table 4). Immediately after the 30-min access periods to NaCl, mice from the conditioned group were injected with LiCl as described in Experiment 1-A, and mice from the control group were injected with equivalent volume of 150-mM NaCl. On the days when mice received NaCl to drink during one of the two 30-min sessions, they received water in both tubes during the other 30-min access period to fluid. During the recovery days 4, 6, 8, 10, and 12 separating the conditioning days, mice were given water to drink in both tubes for both 30-min sessions. After completion of the last conditioning on day 13, mice were given water in both tubes overnight starting from 6:00 PM for recovery. Starting on day 14, mice were tested with a series of NaCl solutions (0, 0.3, 1, 3, 10, and 30 mM) presented in ascending order of concentration in 48-h two-bottle preference tests with NaCl solution in one tube and water in the other tube. The preference scores and NaCl avoidance thresholds were analyzed as in Experiment 1-A.

### *Results*

The mean NaCl preference scores of the conditioned (LiCl-injected) and control (NaCl-injected) groups are shown in Figure 3. ANOVA of NaCl preference scores revealed a significant group effect, $F(1, 14) = 37.09$, $P < 0.001$, and a significant interaction between group and concentration,

**Table 3** Summary of reported NaCl taste thresholds

| Species | Method (type of threshold) | Strain | Reported threshold (mM) | Reference |
|---|---|---|---|---|
| Mouse | Chorda tympani nerve—electrophysiology (neural response threshold) | ddy | 1 | Iwasaki and Sato (1984) |
| | | C57BL/6J | 3–10 | Frank and Blizard (1999) |
| | | DBA/2J | 3–10 | Frank and Blizard (1999) |
| | Operant conditioning—discrimination test (detection threshold) | C57BL/6J | 3 | Ruiz et al. (2006) |
| | | C57BL/6J | 62 | Eylam and Spector (2002) |
| | | C57BL/6J | 47 | Eylam and Spector (2003) |
| | | DBA/2J | 49 | Eylam and Spector (2003) |
| | CTA—preference test (intensity generalization/ recognition threshold) | CD-1 | 2–4 | This study |
| Rat | Chorda tympani nerve—electrophysiology (neural response threshold) | Wistar | 1–2 | Pfaffmann and Bare (1950) |
| | | Lashley | 2 | Beidler (1953) |
| | Operant conditioning— discrimination test (detection threshold) | NA | 1.5 | Carr (1952) |
| | | Albino | 0.7–0.8 | Koh and Teitelbaum (1961) |
| | | NA | 0.7 | Slotnick (1982) |
| | | Sprague-Dawley | 4 | Geran and Spector (2000) |
| | CTA—licking suppression test (intensity generalization threshold) | Albino | 60[a] | Scott and Giza (1987) |
| | | Wistar | 30 | Yamamoto et al. (1994) |
| | CTA—preference test (intensity generalization threshold) | Fischer 344 | 1–2 | Clarke et al. (2001) |
| | | Wistar | 1–2 | Clarke et al. (2001) |

[a]The threshold is estimated from data shown in Figure 2 using the critical value given in the paper. The paper also reports a 29 mM threshold for discrimination between different NaCl concentrations. NA: No information about the strain used in the study was available.

**Table 4** Schedule of Experiment 1-B

| Day | Stage | Time, solution (duration) | | | | | |
|---|---|---|---|---|---|---|---|
| 1–2 | Training | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 3 | Conditioning | 10:00 AM | 30 mM NaCl (30 min) + LiCl (or NaCl) injection | 5:00 PM | Water (30 min) | | |
| 4 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 5 | Conditioning | 10:00 AM | Water (30 min) | 5:00 PM | 3 mM NaCl (30 min) + LiCl (or NaCl) injection | | |
| 6 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 7 | Conditioning | 10:00 AM | Water (30 min) | 5:00 PM | 10 mM NaCl (30 min) + LiCl (or NaCl) injection | | |
| 8 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 9 | Conditioning | 10:00 AM | 3 mM NaCl (30 min) + LiCl (or NaCl) injection | 5:00 PM | Water (30 min) | | |
| 10 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 11 | Conditioning | 10:00 AM | 10 mM NaCl (30 min) + LiCl (or NaCl) injection | 5:00 PM | Water (30 min) | | |
| 12 | Recovery | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 13 | Conditioning/recovery | 10:00 AM | Water (30 min) | 5:00 PM | 30 mM NaCl (30 min) + LiCl (or NaCl) injection | 6:00 PM | Water (overnight) |
| 14–27 | Preference testing | 10:00 AM | 0–30 mM NaCl and water (48 h) | | | | |

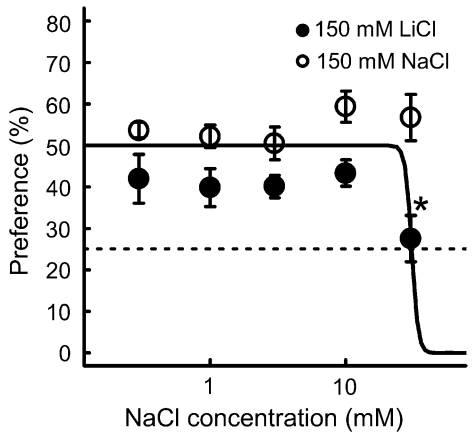


**Figure 3** Experiment 1-B: NaCl preference scores (Mean ± SEM) in 48-h two-bottle tests of mice exposed during conditioning to multiple (3, 10, and 30 mM) NaCl concentrations followed by LiCl injections (conditioned group; filled circles) or NaCl injections (control group; open circles). The curve was fit to NaCl preference scores using the function described in the text (see Methods section). The avoidance threshold in the conditioned group was 30 mM. Asterisks show concentrations at which control and conditioned groups significantly differ ($P < 0.05$, Newman–Keuls post hoc tests). Other descriptions are the same as in Figure 2.

$F(5, 70) = 2.44$, $P = 0.04$, but the effect of concentration was not significant, $F(5, 70) = 2.23$, $P = 0.06$. The conditioned mice had lower preference scores than did control mice for 30 mM but not other NaCl concentrations ($P < 0.01$, Newman–Keuls post hoc tests). The NaCl avoidance threshold for mice from the conditioned group was 30 mM. None of the tested NaCl solutions was significantly avoided by control mice, and therefore, avoidance threshold could not be estimated in this group.

### Experiment 1-C. A single treatment group conditioned with oral self-administration of 150 mM LiCl

In this experiment, mice were presented with LiCl solution to drink for conditioning. The taste stimulation elicited by LiCl was a CS, and postingestive toxicity of LiCl was a US. After conditioning, all mice were tested with a series of NaCl concentrations using 48-h two-bottle preference tests.

#### *Method*

Seventeen male mice were randomly divided into two groups, conditioned ($n = 9$) and control ($n = 8$). Mice were given two 24-h periods of access to 150 mM LiCl (for the conditioned group) or 150 mM NaCl (for the control group) presented in both drinking tubes, separated by one 24-h period of access to water given in both tubes (Table 5). The LiCl and NaCl intakes during conditioning periods were expressed as solution volume per 30 g BW (the approximate weight of an adult mouse) and as LiCl weight per 1 kg BW (Table 6). Beginning from day 4, mice were tested with a series of NaCl solutions (0–300 mM) presented in ascending order of concentration using 48-h two-bottle preference tests with a NaCl solution given in one tube and water given in the other tube. Because during the first 48-h test mice were

presented with water in both tubes (0 mM), they were hydrated by the time they were exposed to NaCl solutions. The preference scores and NaCl avoidance thresholds were analyzed as in Experiments 1-A and 1-B.

### *Results*

The intakes of the CS solutions and the doses of LiCl self-administered during conditioning are shown in Table 6. The mean NaCl preference scores in each group of mice are shown in Figure 4. Although the testing procedure took 18 days, the CTA did not extinguish by the end of testing. All mice in the conditioned group strongly avoided 150 mM NaCl (the individual preference scores ranged from 0.8% to 4.6%), which is expected to be perceptually similar to the CS, 150 mM LiCl. ANOVA of NaCl preference scores revealed significant effects of group, $F(1, 15) = 26.56$, $P < 0.001$, and concentration, $F(8, 120) = 9.08$, $P < 0.001$, and a significant interaction between group and concentration, $F(8, 120) = 8.55$, $P < 0.001$. The conditioned mice had lower preference scores than did control mice for 30 and 150 mM but not for other NaCl concentrations ($P < 0.05$, Newman–Keuls post hoc tests). The NaCl avoidance thresholds were 4 mM in the conditioned group and 338 mM in the control group (Table 7).

Mice from the control group drank significantly less 300 mM NaCl than water available as the second choice ($P < 0.001$, paired $t$-test). This is consistent with results of previous studies, which have shown that mice typically avoid 300 mM NaCl without prior conditioning (Lush 1991;

**Table 5** Schedule of Experiment 1-C

| Day | Stage | Solution (duration) |
|---|---|---|
| 1 | Conditioning | 150 mM LiCl (or NaCl) (24 h) |
| 2 | Recovery | Water (24 h) |
| 3 | Conditioning | 150 mM LiCl (or NaCl) (24 h) |
| 4–21 | Preference testing | 0–300 mM NaCl and water (48 h) |

The procedures in Experiments 2 and 4 were similar to those described in this table, with the exceptions of the solutions used for conditioning and preference testing.

**Table 6** Average daily CS solution intake and LiCl dose orally self-administered during conditioning in Experiments 1-C, 2, 3, and 4 involving LiCl ingestion

| Experiment | CS solution | CS intake (ml/30g BW) | | LiCl dose (g/kg BW) | |
|---|---|---|---|---|---|
| | | First exposure | Second exposure | First exposure | Second exposure |
| 1-C | 150 mM LiCl | 1.7 ± 0.2$^{c}$ | 0.3 ± 0.1$^{c,*}$ | 0.37 ± 0.05 | 0.07 ± 0.02* |
| | 150 mM NaCl | 13.3 ± 0.8$^{a}$ | 12.9 ± 0.8$^{a}$ | | |
| 2 | 150 mM LiCl | 1.9 ± 0.2$^{c}$ | 0.5 ± 0.1$^{c,*}$ | 0.40 ± 0.04 | 0.10 ± 0.01* |
| | 150 mM NaCl | 11.1 ± 1.1$^{a,b}$ | 10.9 ± 0.8$^{a,b}$ | | |
| 3 | 75 mM LiCl | 3.1 ± 0.3$^{c}$ | 1.6 ± 0.2$^{c,*}$ | 0.32 ± 0.03 | 0.17 ± 0.03* |
| | 75 mM LiCl + 75 mM NaCl | 3.3 ± 0.6$^{c}$ | 1.6 ± 0.4$^{c,*}$ | 0.35 ± 0.06 | 0.17 ± 0.04* |
| | 150 mM LiCl + 150 mM NaCl | 1.4 ± 0.1$^{c}$ | 0.7 ± 0.1$^{c}$ | 0.30 ± 0.03 | 0.14 ± 0.02* |
| | 300 mM LiCl | 0.9 ± 0.1$^{c}$ | 0.5 ± 0.1$^{c}$ | 0.37 ± 0.04 | 0.21 ± 0.05* |
| | 300 mM NaCl | 10.5 ± 0.7$^{a,b}$ | 8.6 ± 1.0$^{b,*}$ | | |
| | Water | 9.1 ± 0.6$^{b}$ | 9.4 ± 0.7$^{b}$ | | |
| 4 | 150 mM LiCl + 10 mM citric acid | 1.3 ± 0.1$^{c}$ | 0.9 ± 0.05$^{c}$ | 0.28 ± 0.03 | 0.20 ± 0.01 |
| | 150 mM NaCl + 10 mM citric acid | 10.6 ± 0.8$^{a,b}$ | 8.0 ± 0.4$^{b,*}$ | | |

$^{a,b,c}$Group means within a column that do not share any common superscribed letters significantly differ ($P < 0.05$, Newman–Keuls tests), whereas those labeled with at least one letter in common do not.

The statistical analyses for CS intakes and LiCl doses were conducted with all data shown in this table combined, using two-way repeated measures ANOVA followed by Newman–Keuls post hoc tests. CS intakes were significantly affected by CS solution, $F(11, 87) = 92.06$, $P < 0.001$, exposure, $F(1, 87) = 79.57$, $P < 0.001$, and an interaction between CS solution and exposure, $F(11, 87) = 4.68$, $P < 0.001$. Intakes of LiCl-containing solutions were lower than intakes of water or NaCl solutions. In some, but not all, groups, CS intakes were lower during the second exposure than during the first exposure. This decrease tended to be larger for intakes of LiCl-containing solutions. LiCl doses were significantly affected by exposure, $F(1, 52) = 148.17$, $P < 0.001$, and an interaction between CS solution and exposure, $F(6, 52) = 3.91$, $P = 0.003$, but not by CS solution, $F(6, 52) = 0.75$, $P = 0.62$. Correspondingly, self-administered LiCl doses were significantly lower during the second exposure than during the first exposure (in all groups with the exception of the 150 mM LiCl + 10 mM citric acid group), but they did not differ among the groups. The average doses of orally self-administered LiCl in Experiments 1-C, 2, 3, and 4 (0.22–0.29 g/kg) were similar to the dose of injected LiCl in Experiments 1-A and 1-B (0.23 g/kg). Lower intakes of LiCl-containing solutions compared with water or NaCl solution intakes and decrease in LiCl consumption from the first to the second exposure demonstrate a negative postingestive effect of self-administered LiCl and successful aversive conditioning after the first exposure.

*Significant difference between the first and second exposures, $P < 0.05$, Newman–Keuls tests.

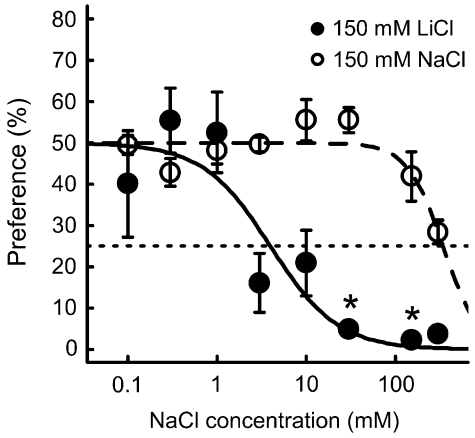


**Figure 4** Experiment 1-C: NaCl preference scores (Mean ± SEM) in 48-h two-bottle tests of mice conditioned by self-administration of 150 mM LiCl (conditioned group; filled circles) or 150 mM NaCl (control group; open circles). The avoidance threshold was 4 mM in the conditioned group and 338 mM in the control group. Other descriptions are the same as in Figure 2.

**Table 7** Relative stimulus toxicity and taste intensity, and NaCl avoidance thresholds of mice conditioned by oral LiCl self-administration and control mice (Experiments 1-C and 2)

| Groups[a] | Experiment | Toxicity[b] | Taste intensity[c] | Threshold (mM) |
|---|---|---|---|---|
| Experimental groups | | | | |
| 75 mM LiCl | 3 | 1/2 | 1/2 | 3 |
| 75 mM LiCl + 75mM NaCl | 3 | 1/2 | 1 | 2 |
| 150 mM LiCl | 1-C | 1 | 1 | 4 |
| 150 mM LiCl + 150 mM NaCl | 3 | 1 | 2 | 4 |
| 300 mM LiCl | 3 | 2 | 2 | 19 |
| Control groups | | | | |
| Water | 3 | 0 | 0 | 327 |
| 150 mM NaCl | 1-C | 0 | 1 | 338 |
| 300 mM NaCl | 3 | 0 | 2 | 21 |

[a]This column shows self-administered solutions.
[b]The toxicity was expressed relative to 150 mM LiCl.
[c]The taste intensity was expressed as total salt concentration relative to 150 mM.

Bachmanov, Tordoff, and Beauchamp 1998; Bachmanov, Beauchamp, and Tordoff 2002), probably because of its aversive sensory and/or postingestive properties.

In summary, Experiment 1 compared three different procedures for conditioning an aversion to NaCl. The lowest avoidance threshold (4 mM) was observed in Experiment 1-C that involved conditioning using oral self-administration of LiCl. The avoidance thresholds observed in Experiments 1-A and 1-B (involving NaCl consumption paired with LiCl injection) were higher (6 mM and up) than the avoidance threshold observed in Experiment 1-C. Thus, the oral LiCl self-administration conditioning procedure was the most sensitive method to assess NaCl taste thresholds.

## Experiment 2. NaCl avoidance thresholds measured in 30-min two-bottle tests

In Experiment 1, NaCl avoidance thresholds were measured in long-term 48-h two-bottle tests. Short-term tests have several potential advantages compared with long-term tests. First, testing multiple concentrations of taste solutions could be done faster when using short-term tests compared with long-term tests. For example, the testing procedure in this experiment (9 days) was half as long as those in Experiment 1 (18 days). Second, a shorter exposure to a nonreinforced CS may delay extinction of the conditioned aversion. Third, the short-term tests may be advantageous for certain types of experiments, for example, when short-lasting pharmacological agents are administered before the tests or when taste-modifying compounds with postingestive effects are used. Therefore, in this experiment, we conditioned mice by oral self-administration of LiCl exactly as in Experiment 1-C and then tested mice with a similar series of NaCl concentrations but in 30-min two-bottle tests instead of the 48-h tests. Comparison of NaCl avoidance thresholds observed in this experiment and in Experiment 1-C allowed us to assess which testing procedure is more sensitive.

### Method

Seventeen mice were randomly divided into the conditioned group ($n = 9$) exposed to 150 mM LiCl and the control group ($n = 8$) exposed to 150 mM NaCl. Prior to conditioning, all mice were trained to a restricted access to water for 2 days as described in Table 8. During the period between the last training session and the first exposure to LiCl or NaCl, they were provided with water in both tubes overnight. After that, mice were conditioned using the same procedure as in Experiment 1-C. After the second exposure to LiCl or NaCl, mice received 6-h access to water in both drinking tubes, and then they were water deprived overnight and given an additional day of training to the restricted access to water. Starting on day 8, the mice were tested with a series of NaCl solutions (0–300 mM) presented in ascending order of concentration using 30-min two-bottle preference tests with NaCl solution in one tube and water in the other tube. Mice were tested twice per day with a 6.5-h interval during daytime and 16.5-h interval during nighttime. The preference scores and NaCl avoidance thresholds were analyzed as in Experiments 1-A, 1-B, and 1-C.

### Results

The intakes of the CS solutions and the doses of LiCl self-administered during conditioning are shown in Table 6. The

**Table 8** Schedule of Experiment 2

| Day | Stage | Time, solution (duration) | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | Training | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 2 | Training | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | 6:00 PM | Water (overnight) |
| 3 | Conditioning | 10:00 AM | 150 mM LiCl (or NaCl) (24 h) | | | | |
| 4 | Recovery | 10:00 AM | Water (24 h) | | | | |
| 5 | Conditioning | 10:00 AM | 150 mM LiCl (or NaCl) (24 h) | | | | |
| 6 | Recovery | 10:00 AM | Water (6 h) | | | | |
| 7 | Training | 10:00 AM | Water (30 min) | 5:00 PM | Water (30 min) | | |
| 8–16 | Preference testing | 10:00 AM | 0–300 mM NaCl and water (30 min) | 5:00 PM | 0–300 mM NaCl and water (30 min) | | |

mean NaCl preference scores for each group are shown in Figure 5. ANOVA of NaCl preference scores revealed significant effects of group, $F(1, 15) = 8.66$, $P = 0.01$, and concentration, $F(8, 120) = 2.72$, $P = 0.01$, and a significant interaction between group and concentration, $F(8, 120) = 3.96$, $P < 0.001$. However, differences between the preference scores of conditioned and control mice did not reach the threshold of statistical significance at any of the concentrations tested ($P > 0.05$, Newman–Keuls post hoc tests; the difference was marginally significant for 300 mM NaCl preference sores, $P = 0.09$). None of the tested NaCl solutions was significantly avoided by control mice, and therefore avoidance threshold could not be estimated in this group. In the conditioned group, the NaCl avoidance threshold was 71 mM. This is higher than the threshold of similarly conditioned mice tested in 48-h two-bottle tests (4 mM; Experiment 1-C). This demonstrates that the 30-min tests are less sensitive for detecting conditioned aversion than are the 48-h tests.

In summary, results of Experiments 1 and 2 have shown that oral self-administration of LiCl solutions and testing conditioned mice in 48-h two-bottle tests result in the lowest NaCl avoidance thresholds. We therefore used these procedures for conditioning and testing mice in subsequent experiments.

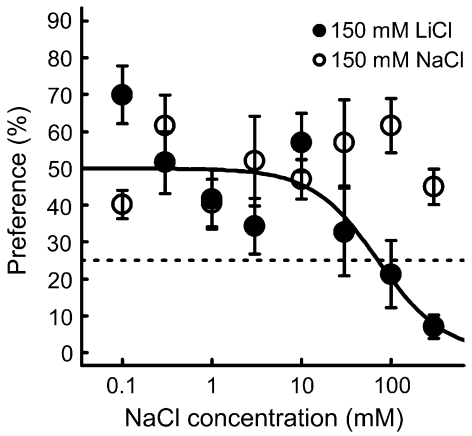


**Figure 5** Experiment 2: NaCl preference scores (Mean ± SEM) in 30-min short-term tests of mice conditioned by self-administration of 150 mM LiCl (conditioned group; filled circles) or 150 mM NaCl (control group; open circles). The avoidance threshold in the conditioned group was 71 mM. Other descriptions are the same as in Figure 2.

## Experiment 3. Effects of varying CS and US intensities on oral self-administration conditioning

This experiment was conducted to optimize the oral LiCl self-administration conditioning procedure. It is known that intensity of CS and US may influence CTA (Nachman and Ashe 1973; Nowlis 1974). Therefore, our goal was to vary intensities of the CS and US independently. To achieve this, we manipulated the concentrations of NaCl, LiCl, and their mixtures. Because there is strong evidence that mice perceive similar taste quality and intensity of LiCl and NaCl, we attempted to vary the strength of the CS and US independently by substituting LiCl with NaCl. We assumed that combined concentrations of LiCl and NaCl determine CS strength (i.e., taste intensity), and LiCl concentration determines US strength (i.e., toxicity). Although these assumptions were not always true (see details in Discussion), this experiment is nevertheless important for practical purposes of optimizing the taste threshold measurement technique. We used multiple treatment groups, each exposed to a different CS. After conditioning, all mice were tested with a series of NaCl concentrations using 48-h two-bottle preference tests.

### Method

Forty-eight male mice were randomly divided into six groups ($n = 8$ per group). Mice were conditioned and tested using a procedure identical to that described in Experiment 1-C (Table 5), with two exceptions. The first exception was that different solutions were used for conditioning than in Experiment 1-C. The four groups were presented with one of the following solutions containing LiCl: 75 mM LiCl, a mixture

of 75 mM LiCl and 75 mM NaCl, a mixture of 150 mM LiCl and 150 mM NaCl, or 300 mM LiCl. The other two groups were controls given solutions without LiCl: 300 mM NaCl or water. The relative toxicity and taste intensity of these solutions are described in Table 7. The second exception was that in Experiment 3, the NaCl concentration series tested in the conditioned mice included 100 mM NaCl instead of 150 mM NaCl used in Experiment 1-C. The preference scores and NaCl avoidance thresholds were analyzed as in Experiments 1-A, 1-B, 1-C, and 2.

## Results

The intakes of the CS solutions and the doses of LiCl self-administered during conditioning are shown in Table 6. The mean NaCl preference scores for each of the six groups are shown in Figure 6. The CTA did not extinguish during the preference tests: all mice in the LiCl-exposed groups strongly avoided NaCl solutions at concentrations similar to the corresponding CS (e.g., individual preference scores for 100 mM NaCl ranged from 2.6% to 7.4%). ANOVA of NaCl preference scores in these groups revealed significant effects of group, $F(5, 42) = 15.23$, $P < 0.001$, and concentration, $F(8, 336) = 46.77$, $P < 0.001$, and a significant interaction between group and concentration, $F(40, 336) = 3.57$, $P < 0.001$.

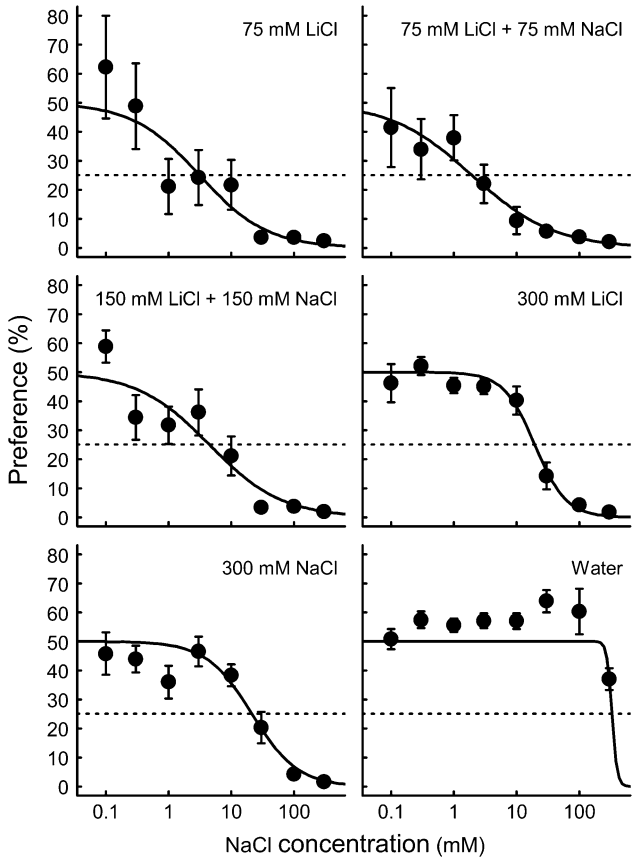


**Figure 6** Experiment 3: NaCl preference scores (Mean ± SEM) in 48-h two-bottle tests of mice conditioned by self-administration of different solutions. The self-administered solutions and corresponding avoidance thresholds (in parentheses) are: 75 mM LiCl (3 mM), 75 mM LiCl + 75 mM NaCl (2 mM), 150 mM LiCl + 150 mM NaCl (4 mM), 300 mM LiCl (19 mM), 300 mM NaCl (21 mM), water (327 mM). Other descriptions are the same as in Figure 2.

The avoidance thresholds for all eight groups used in Experiments 1-C and 3 are summarized in Table 7. Among the five LiCl-conditioned groups used in Experiments 1-C and 3, in four groups thresholds were similarly low, ranging from 2 to 4 mM (the groups exposed to 75 mM LiCl + 75 mM NaCl [2 mM], 75 mM LiCl [3 mM], 150 mM LiCl [4 mM], and 150 mM LiCl + 150 mM NaCl [4 mM]), whereas the fifth group (exposed to 300 mM LiCl) had a substantially higher threshold (19 mM). For stimuli of similar toxicity, altering taste intensity did not result in consistent changes in avoidance thresholds (Table 7). For example, for stimuli with relative toxicity value of 1/2 (75 mM LiCl and 75 mM LiCl + 75 mM NaCl), stronger taste intensity was associated with a marginal decrease in avoidance threshold (from 3 to 2 mM). For stimuli with relative toxicity value of 1 (150 mM LiCl and 150 mM LiCl + 150 mM NaCl), thresholds were similar (4 mM) regardless of differences in taste intensity. However, for stimuli of similar taste intensity, increased toxicity tended to be associated with higher threshold values. For instance, for stimuli with relative taste intensity value of 1 (75 mM LiCl + 75 mM NaCl and 150 mM LiCl), doubling toxicity resulted in approximately 2-fold increase in avoidance threshold (from 2 to 4 mM), and for stimuli with relative taste intensity value of 2 (150 mM LiCl + 150 mM NaCl and 300 mM LiCl), doubling toxicity resulted in approximately 5-fold increase in avoidance threshold (from 4 to 19 mM). Overall, avoidance thresholds were similarly low (2–4 mM) in mice self-administering solutions with toxicity ranging from 75 to 150 mM LiCl and taste intensity ranging from 75 to 300 mM LiCl and/or NaCl. A higher threshold was found in mice exposed to 300 mM LiCl (with the highest toxicity).

In a water-exposed control group, avoidance threshold was 327 mM, which is similar to the threshold in control mice exposed to 150 mM NaCl in Experiment 1-C (338 mM). Interestingly, avoidance threshold in another control group that was exposed to 300 mM NaCl was 21 mM, which is lower than thresholds of the other two control groups and is close to the threshold of mice conditioned with 300 mM LiCl (19 mM). This suggests that the mice forced to ingest 300 mM NaCl developed a conditioned aversion to NaCl. This is consistent with the reduction of the CS (300 mM NaCl) intake from the first to the second conditioning exposure (Table 6). Consumption of osmotically hypertonic 300 mM NaCl available as the only fluid for 24 h must have made animals thirsty without them being able to satisfy their thirst during the 24-h period. This likely provided a negative reinforcement that conditioned mice to avoid NaCl taste. This effect is similar to suppression of NaCl consumption when NaCl concentrations are tested in the descending order (Bachmanov, Tordoff, and Beauchamp 1998).

## Experiment 4. Conditioning using oral self-administration of a mixture of LiCl and citric acid

The goal of this experiment was to examine whether a CTA procedure suitable for measuring NaCl taste thresholds can also be used to measure mouse taste thresholds for compounds with taste qualities other than salty (NaCl-like). It is known that CTA to binary mixtures of taste substances generalizes to mixture components in rats and hamsters (Nowlis and Frank 1981; Frank et al. 2003). Consistent with this, CTA to orally self-administered mixtures of LiCl and carbohydrates was used to measure carbohydrate taste thresholds in rats (Ramirez 1991). Following this example, we combined 150 mM LiCl with 10 mM citric acid, a sour taste stimulus, to test if our technique could measure a citric acid avoidance threshold that would be consistent with taste thresholds identified using other techniques. The 10 mM citric acid evokes robust neural and behavioral taste responses in mice (Bachmanov et al. 2000; Danilova and Hellekant 2003; McCaughey 2007), suggesting that it is a salient taste stimulus for conditioning.

### Method

Seventeen mice were randomly divided into the conditioned group ($n$ = 9) and control group ($n$ = 8). Mice were conditioned and tested using a procedure similar to that described in Experiment 1-C (Table 5) but with different solutions for conditioning and testing. For conditioning, mice were given two 24-h periods of access to a mixture of 150 mM LiCl and 10 mM citric acid (conditioned group) or to a mixture of 150 mM NaCl and 10 mM citric acid (control group) presented in both drinking tubes. After conditioning, mice were tested with a series of citric acid solutions (0–30 mM) presented in the ascending order of concentrations using 48-h two-bottle preference tests with a citric acid solution in one tube and water in the other tube. The preference scores and avoidance thresholds were analyzed as in Experiments 1-A, 1-B, 1-C, 2, and 3.

### Results

The intakes of the CS solutions and the doses of LiCl self-administered during conditioning are shown in Table 6. The mean citric acid preference scores for each group are shown in Figure 7. ANOVA of citric acid preference scores revealed significant effects of group, $F(1, 15) = 12.58$, $P = 0.003$, and concentration, $F(8, 120) = 49.25$, $P < 0.001$, and a significant interaction between group and concentration, $F(8, 120) = 5.24$, $P < 0.001$. The conditioned mice had lower preference scores than did control mice for 1 and 3 mM but not other citric acid concentrations ($P < 0.05$, Newman–Keuls post hoc tests). The citric acid avoidance thresholds were 1 mM in the conditioned group and 7 mM in the control group. The threshold of the control group is consistent with results of a previous study with nonconditioned mice (Bachmanov et al. 2000).

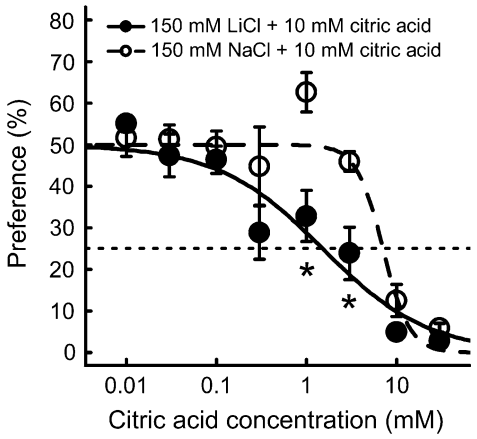


**Figure 7** Experiment 4: Citric acid preference scores (Mean ± SEM) in 48-h two-bottle tests of mice conditioned by self-administration of 150 mM LiCl + 10 mM citric acid (conditioned group; filled circles) or 150 mM NaCl + 10 mM citric acid (control group; open circles). The avoidance threshold was 1 mM for the conditioned group and 7 mM for the control group. Other descriptions are the same as in Figure 2.

## Discussion

In the present study, we examined whether CTA can be used to assess taste thresholds in mice and how different conditioning and testing procedures influence the sensitivity of the method. We varied several parameters, including the routes of US administration, strength of CS and US, and procedures for testing of conditioned mice. We found that a simple technique involving conditioning by oral self-administration of 75–150 mM LiCl or its mixtures with NaCl and testing conditioned mice with a series of ascending NaCl concentrations in 48-h two-bottle preference tests is a sensitive method to estimate NaCl taste thresholds. The NaCl taste thresholds obtained using this technique (in Experiments 1-C and 3) were 2–4 mM. These thresholds are similar to NaCl taste thresholds of mice and rats reported in several other studies using different methods (Table 3). This suggests that the simple CTA-based method developed in this study is suitable for measuring taste recognition thresholds.

### Suitability for genetic studies

Some genetic experiments, for example, linkage analyses of segregating crosses or screening mutagenized mice for phenotypical deviations, require (i) testing large numbers of animals and (ii) obtaining reliable individual phenotypical characteristics. Both requirements could be satisfied using the method involving conditioning by oral self-administration of LiCl and subsequent testing with NaCl in 48-h preference tests.

Although duration of conditioning and testing periods used in this study is relatively long (21 days for experiments 1-C, 3, and 4), intake measurements take very little time so that large numbers of mice could be tested simultaneously using this technique. For example, one full-time technician could test simultaneously up to ~200 mice. Genetic crosses used for linkage analyses often use ~200 hybrid mice. Thus, collection of phenotypical data for such experiment can be completed in 3 weeks. If needed to increase throughput, the procedure could be shortened by excluding test solution concentrations that are less informative (e.g., those below and above taste thresholds in both progenitor strains for a cross). Conditioned mice could even be tested with a single test solution concentration, which is below threshold for one progenitor strain and above threshold for another progenitor strain. This would reduce the duration of the experiment to ~1 week.

Taste thresholds can also be measured using electrophysiological and operant conditioning techniques. These techniques potentially could be used for genetic analyses that require high-throughput phenotyping. However, throughput of these techniques is lower than throughput of the CTA-based method. In electrophysiological experiments, an experienced researcher usually obtains successful gustatory nerve recordings from one or two mice per day. Thus, characterizing ~200 mice would take ~100 to 200 working days. Although we used the electrophysiological approach in our genetic studies of hybrid mice (Inoue et al. 2004; Shigemura et al. 2008), we found that it is much more laborious than the preference tests. Furthermore, the published studies on mouse NaCl taste thresholds obtained using operant conditioning techniques have lower throughput than the CTA-based technique. For example, training and testing took 87–110 days in experiments of Eylam and Spector (2002, 2003), and it appears that it took a month or more in experiments of Ruiz et al. (2006). Thus, even if ~200 mice could be tested in a single day using these techniques, these tests would last longer than the CTA-based tests.

In addition to the throughput issues, operant conditioning techniques have several other features that make them less suitable for genetic studies compared with the CTA-based technique. First, they typically involve fluid restriction, which may affect sodium metabolism (Weisinger et al. 1985) and taste perception (Scalera 2004). On the contrary, the CTA-based technique involves tests of nondeprived mice. Second, operant conditioning procedures typically require some level of individualization of training. For example, Ruiz et al. (2006) individually adjusted intensity of electric shock. Eylam and Spector (2002, 2003) individually adjusted duration of some training phases until a mouse had reached a certain criterion of performance. Such variation in the operant conditioning procedures increases a possibility that genetic effects on obtained taste thresholds may be due to nontaste factors, such as variation in pain sensitivity or learning ability. On the contrary, CTA-based conditioning and testing procedures are uniform for all mice.

In this paper, we present taste thresholds based on regression analyses using group data. However, for experiments where each mouse was tested with multiple test solution concentrations, we also calculated threshold values for individual mice and conducted statistical analyses using these individual threshold values (results were similar for both approaches; see details in Methods). Such individual threshold values could also be determined in genetic experiments involving linkage analyses or mutation screening. If conditioned mice are tested only with a single test solution concentration, individual preference scores could be used as a substitute for thresholds (they positively correlate, i.e., mice with lower thresholds tend to have stronger avoidance of peri-threshold concentrations).

### Effects of conditioning procedures

In Experiment 1, we compared two different types of conditioning procedures, one involving NaCl consumption by water-deprived mice followed by LiCl injection (Experiments 1-A and 1-B) and the other one involving LiCl consumption by nondeprived mice (Experiment 1-C). After aversion conditioning, mice were tested with NaCl solutions in 48-h two-bottle tests. The procedure involving oral LiCl self-administration resulted in lower avoidance thresholds than the procedure involving pairing NaCl intake with LiCl injection (cf. Figures 1–3 with Figures 4 and 6, and Table 2 with Table 7). Under conditions when CS concentration and testing procedures were equivalent, thresholds of mice that self-administered LiCl (2–4 mM in mice conditioned with 75–150 mM LiCl or its mixtures with NaCl in Experiments 1-C and 3) were on average twice lower than thresholds of LiCl-injected mice (6 mM in mice conditioned with 100 mM NaCl as CS in Experiment 1-A). Injected and orally self-administered LiCl doses were similar (Table 6), suggesting that the difference between the two types of conditioning is due not to dose but to some other factors, such as (i) the route of LiCl administration, (ii) the numbers of conditioning episodes, or (iii) duration of CS–US intervals. First, a possible role of route of administration is indicated by data showing that intraperitoneal and oral LiCl administrations involve different mechanisms of CTA learning (Simbayi 1987). Second, the numbers of conditioning episodes could be involved because when NaCl intake was paired with LiCl injection, LiCl was administered only once per conditioning session, but when mice consumed LiCl, they may have experienced multiple pairings between the CS (oral stimulation with LiCl) and US (LiCl-induced malaise) during the 24-h conditioning exposure period. Therefore, it is likely that a greater number and/or frequency of CS–US pairings resulted in a more efficient conditioning when LiCl was self-administered. Finally, when LiCl was injected, intervals between the end of the exposure to NaCl (CS) and LiCl administration (US) could have been up to 30 min, but during oral LiCl self-administration intervals between the CS and US were shown to be shorter (9 min or less;

Nachman 1963; Baird et al. 2005). However, no differences in CTA strength were found between experiments with CS–US intervals 15 and 30 min (Schafe et al. 1995) or 5 and 10 min (Yamamoto et al. 1994). Therefore, shorter CS–US intervals are unlikely to be responsible for more efficient conditioning when LiCl was self-administered. Thus, the route of LiCl administration and number and/or frequency of CS–US pairings are the most likely factors responsible for the difference between the two types of conditioning. However, additional studies are needed to examine their role.

The oral LiCl self-administration procedure has advantages compared with injecting LiCl when there are genetic differences in sensitivity to toxic effects of LiCl (e.g., Smith 1978; El-Kassem and Singh 1983; Risinger and Cunningham 2000). When animals have access to a LiCl solution, they drink LiCl until they experience symptoms of intoxication strong enough to act as the US. Thus, depending on individual sensitivity to toxic effects of LiCl, each animal controls the US strength by self-administering a LiCl dose sufficient to condition taste aversion. It is more difficult to determine LiCl doses that produce equal toxic effect in such animals if LiCl injections are used.

### Effects of testing procedures

When mice were conditioned by oral LiCl self-administration, NaCl avoidance thresholds measured in the 48-h two-bottle tests (Experiment 1-C) were lower than the thresholds measured in the 30-min two-bottle test (Experiment 2; cf. Figures 4 and 5). This difference in sensitivity of the two testing procedures may be explained by several factors. First, compared with the 30-min tests, during the 48-h tests mice performed a much greater number of drinking bouts, which may have allowed them to discriminate better between available choices. Second, during the 30-min tests, mice were motivated to drink by prior water restriction, which was shown in rats to suppress aversive taste responses (Scalera 2004). Third, dehydration was shown to induce negative sodium balance and to evoke sodium appetite (Weisinger et al. 1985), which may have counteracted the conditioned aversion to NaCl.

### Effects of CS and US intensity

Results of Experiments 1 and 2 showed that the most sensitive procedure to determine avoidance thresholds involves conditioning by oral self-administration of LiCl and subsequent testing of conditioned mice in 48-h two-bottle tests. To further optimize the oral LiCl self-administration conditioning procedure, we examined how changes in concentration and composition of self-administered solutions affect obtained avoidance thresholds (Experiment 3). In this experiment, we exposed mice to different concentrations of NaCl, LiCl, and their mixtures. We have found that 75–150 mM LiCl or its mixture with NaCl are optimal solutions for oral self-administration conditioning to measure avoidance thresholds.

Results of Experiment 3 also provide some insight into the role of CS and US intensity in CTA. In regard to the CS intensity, we found that variation in taste intensity had little effect on avoidance thresholds. This contradicts results of Nowlis (1974), who paired presentation of NaCl solutions of different concentrations with injections of cyclophosphamide in rats and found that stronger CS intensity (higher NaCl concentration) is associated with stronger aversion. This discrepancy can be attributed to differences between these two studies in animal species, conditioning and testing procedures, US used and exact CS (NaCl) concentrations used. In addition, although we attempted to vary intensities of the CS and US independently, we probably were not always able to achieve this. For example, we found that avoidance threshold in a presumably control group exposed to 300 mM NaCl was lower than thresholds of the other two control groups (exposed to water and 150 mM NaCl) and was similar to the threshold of mice conditioned with 300 mM LiCl. Thus, mice forced to ingest 300 mM NaCl have developed a conditioned aversion to NaCl, which indicates that our assumption that NaCl concentration affects only CS (taste) intensity is not accurate in this case. It is likely that when a combined LiCl + NaCl solution concentration was osmotically hypertonic, NaCl could also have contributed to the US strength.

In regard to the US intensity, we found that higher LiCl concentrations tended to be associated with higher avoidance thresholds. This relationship could be explained based on analyses of CS solution intakes during conditioning (Table 6). Mice from all conditioned groups self-administered similar doses of LiCl regardless of solution concentration. However, they differed in CS intakes. When mice were exposed during conditioning to solutions of similar taste intensity but different toxicity (LiCl concentration), higher toxicity tended to be associated with lower solution intakes, which may have resulted in smaller numbers of CS–US pairings and subsequently weaker conditioning and higher thresholds.

### Acid taste thresholds

In Experiment 4, we examined whether the oral LiCl self-administration conditioning technique could be used to measure taste thresholds for other, nonsalty, taste qualities. We modified this technique to measure the taste threshold for citric acid. Using this modified technique, which involves conditioning by oral self-administration of a mixture of 150 mM LiCl and 10 mM citric acid, we estimated the citric acid avoidance threshold as 1 mM (Figure 7). This value is comparable to citric acid taste thresholds obtained in studies with rats using different behavioral techniques (0.085 mM; Thaw and Smith 1992; 0.09–0.2 mM; Scalera 2004). This suggests that the intensity generalization threshold for citric acid

determined using our method reflects the citric acid recognition threshold, as is the case with NaCl thresholds. Moreover, we suggest that it is possible to use this procedure to estimate taste thresholds for taste stimuli with other taste qualities, such as sweet, bitter, and umami, with a caveat that mixture component interaction could alter the perceived taste intensity (e.g., Breslin and Beauchamp 1995; Frank et al. 2003).

One useful application of this technique for genetic studies is that it can serve as a control for taste specificity of differences in NaCl taste thresholds. For example, if strains differ in NaCl taste thresholds determined using the oral LiCl self-administration technique, it is important to demonstrate that the difference is due to NaCl taste sensitivity and not to different performance in this experimental paradigm. There is little genetic variation in acid taste responsiveness (Bachmanov et al. 2000). Thus, if mouse strains differ in NaCl taste thresholds but not in acid taste thresholds determined using a similar self-administration procedure, this will demonstrate that the strain differences involve NaCl taste sensitivity rather than performance under these experimental conditions (e.g., see Ishiwatari and Bachmanov 2007).

### Taste perception in mice

In addition to method development, results of this study illustrate several mechanistic aspects of taste perception and CTA in mice. We have shown that like humans and rats (Nachman 1963; Murphy et al. 1981; van der Klaauw and Smith 1995; Loy and Hall 2002; Baird et al. 2005;), mice generalized CTA from LiCl to NaCl, which suggests that they perceive taste of NaCl and LiCl as qualitatively similar. Although both cation and anion can contribute to the taste of salts (Beidler 1953; Murphy et al. 1981; Ye et al. 1991; Rehnberg et al. 1993), results of several experiments suggest that similarity of NaCl and LiCl tastes is mostly determined by the cations. In rats, CTA to 500 mM NaCl generalized to sodium salts regardless of the anion but not to $Cl^-$ containing compounds (KCl, $NH_4Cl$, or HCl) (Hill et al. 1990). Consistent with this, rats that self-administered 120 mM LiCl had the strongest CTA generalization and the most difficult discrimination with NaCl compared with other chlorides (KCl and $NH_4Cl$) (Nachman 1963). Analysis of the taste quality profiles in humans has shown that the taste of LiCl is similar not only to NaCl but also shows strong similarity to non-chloride sodium salts (van der Klaauw and Smith 1995). Finally, across-neuron patterns of activity in the monkey cortex were similarly close for both chlorides and bromides of Na and Li (Scott et al. 1994). $Cl^-$ itself can contribute to the taste of NaCl and LiCl, but its taste seems to be detectable only in presence of amiloride (Formaker and Hill 1988; Hill et al. 1990).

We have also shown that like several other species (Nowlis and Frank 1981; Frank et al. 2003), mice can generalize CTA of a binary mixture of taste stimuli to mixture components. Our data illustrate relationships among different types of taste thresholds (intensity generalization, recognition, and detection) in nonhuman animals. We have shown that CTA develops most efficiently under conditions similar to natural, when the same stimulus acts as both CS and US.

## Concluding remarks

In summary, we established a simple and sensitive behavioral method to assess taste thresholds for NaCl and other taste stimuli. This method is suitable for high-throughput genetic studies that require testing large numbers of mice. In our ongoing studies, we are using this technique to compare NaCl taste sensitivity of inbred, hybrid and genetically engineered mice (Ishiwatari and Bachmanov 2007; Nelson et al. 2008). We believe that this method is a useful tool to study the mechanisms of salty taste perception in mammals.

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