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Phenylthiocarbamide Produces Conditioned Taste Aversions in Mice

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Abstract

Previous work has demonstrated that SWR/J (SW) mice avoid phenylthiocarbamide (PTC) to a greater degree than C3HeB/FeJ mice in 48 h, two-bottle preference tests given in ascending series. The authors hypothesized, based also on previous work, that SW mice might form a conditioned taste aversion over time due to the toxic properties of PTC. We directly tested this hypothesis by attempting to condition a taste aversion to sucrose by injections of PTC. In experiment 1, PTC was nearly as effective as a strong dose of LiCl in reducing sucrose drinking. In experiment 2, the sucrose aversions were parametrically modified by both sucrose concentration and PTC dose, a hallmark of conditioned taste aversion. We conclude that PTC can cause a conditioned taste aversion and discuss the importance of considering toxic effects of aversive tastants when analyzing behavioral strain differences.

Key words: bitter avoidance; gustatory; strain differences

Introduction

Strains of mice show considerable variation in intake amount of compounds classified by humans as tasting 'bitter' (e.g. Lush, 1981, 1982, 1984; Whitney and Harder, 1994). Such genetic-based variation led to the identification of a region on distal chromosome six (Capeless *et al.*, 1992; Lush *et al.*, 1995; Blizard *et al.*, 1999) subsequently found to harbor genes encoding putative bitter taste receptors, called T2Rs (Adler *et al.*, 2000; Chandrashekar *et al.*, 2000; Matsunami *et al.*, 2000). Only a handful of T2Rs have been functionally characterized thus far in mice or humans, but evidence for narrow or broad ligand specificity is equivocal (e.g. Chandrashekar *et al.*, 2000; Behrens *et al.*, 2004; Bufe *et al.*, 2002). In mice, polymorphisms in the *Tas2r105* gene have been linked to differential intake of the bitter stimulus cycloheximide, and the reconstituted receptor responded specifically to this substance in a heterologous expression assay (Lush and Holland, 1988; Chandrashekar *et al.*, 2000; Nelson *et al.*, 2003). However, it is a dubious proposition to assume that a direct relationship may be found between intake of a particular bitter stimulus and specific polymorphism(s) in a *Tas2r* gene. Solution intake is commonly assessed using a 24 h or 48 h two-bottle assay and such a long exposure time opens the possibility for post-ingestive effects. This may be especially true for bitter-tasting stimuli, many of which are highly toxic even at low concentrations falling within the range of detectability (Glendinning, 1994).

Previous work by Whitney and Harder (1986), following Klein and DeFries (1970), demonstrated that mice of several strains will initially not avoid 0.1 mM phenylthiocarbamide (PTC), but after a few days will develop a strong aversion to this stimulus. PTC is extremely toxic to mice (oral LD_{50} 10 mg/kg; Fisher Scientific, 2005), comparable to the rodenticide strychnine (oral LD_{50} 2 mg/kg; Environmental Protection Agency, 2005). Recent results comparing two-bottle intake tests and brief-access tests (designed to minimize possible post-ingestive factors) demonstrated that differential aversion (across inbred strains) to millimolar concentrations of PTC depended on the quantity of the stimulus that was consumed and not on immediate taste cues (Nelson *et al.*, 2003). The question remains, however, as to the specific mechanism by which aversion develops. One obvious possibility is that although mice are able to detect 0.1 mM PTC in two-bottle tests, they do not find it particularly aversive. After consuming a particular quantity of PTC, the mice become (at least mildly) ill and form a conditioned taste aversion (CTA) to the PTC, which causes subsequent avoidance.

CTA is a commonly used and well-studied paradigm to assess single-trial learning in mice and other rodent species

(e.g. Bures *et al.*, 1998; Welzl *et al.*, 2001; Riley and Freeman, 2004). An animal typically receives a pairing of a novel taste stimulus with a stimulus that produces temporary gastric distress, usually an intraperitoneal (i.p.) dose of LiCl. After pairing, the animal will avoid consumption of the conditioned taste stimulus. If PTC intake is in fact causing a CTA to develop, we reasoned that we might be able to substitute it for LiCl in a CTA design. Such a finding would provide evidence of the mechanism that causes aversion to develop in a long-term intake experiment and have important implications for the study of bitter taste.

Materials and methods

Two experiments were conducted. In experiment 1, taste aversions to sucrose were demonstrated with 5.1 mg/kg PTC as an unconditioned stimulus. This dose was chosen based upon the Nelson *et al*. (2003) preference test study: approximately this amount of PTC was consumed prior to the session in which mice clearly avoided PTC relative to water. Because the dose was an estimate and because the taste aversions in experiment 1 were not particularly robust, experiment 2 examined a dose–response curve for PTC's potency in producing taste aversions to sucrose.

Subjects

Subjects were 82 SWR/J (SW) mice of both sexes (31 in experiment 1 and 51 in experiment 2) weighing 11–30 g at the start of the experiment. Mice were either obtained from Jackson Laboratory (Bar Harbor, ME) or bred at Reed College from Jackson Laboratory parents. Mice were housed in plastic shoebox cages in a colony room where lighting (12:12), humidity and temperature were automatically controlled. Food (Harlan Teklad 7012) and water were available *ad libitum*, except where noted below under 'Procedure'. All testing occurred during the lights-on phase of the light–dark cycle.

Apparatus

Mice obtained the conditioned stimulus (sucrose) in cages that resembled the home cage but were modified to allow licks to be counted. An AC-108 contact lickometer (DiLog Instruments, Tallahassee, FL) allowed licks to be counted from eight cages simultaneously. Because mice consumed only small volumes, recording the number of licks provided a second behavioral measure that would not be affected by any fluid loss from the bottle before or after the test. In addition, the glass bottles used during the test were fitted with leakproof sipper tubes, minimizing the possibility of fluid loss from the bottles during the test. Mice were tested in the lickometer cages in the same room as their home cages.

Post-conditioning behavioral responses to sucrose (and other stimuli) were assessed in the MS-160 lickometer (DiLog Instruments). The MS-160, or 'Davis Rig', allows the presentation of up to 16 different taste stimuli within a single behavioral session, with the duration and order of stimulus presentation at the control of the experimenter (Rhinehart-Doty *et al.*, 1994; Smith, 2001; Boughter *et al.*, 2002). The test chamber consists of a plastic rectangular cage ($30 \times 14.5 \times 18$ cm) with a wire mesh floor; an oval opening centered in the front wall allows access to taste solutions contained in leak-proof sipper tubes. Fluid access can be restricted by a computer-operated shutter.

Procedure

Davis Rig training

Mice were water restricted overnight and had 20 min access to deionized water in the Davis Rig on the following 2 days. Mice that did not find the drinking spout during the first session were tested again later in the day. Other than this exception, mice were given one session per day in the Davis Rig. Following these 2 days, mice were given three sessions of 'trial training' in which the mouse was made familiar with delivery (of water) from multiple bottles. In these sessions, which were up to 25 min in duration, access to the spout remained available for up to 60 s, during which the mouse could initiate a 6 s trial by licking. The mouse could initiate up to 30 of these 6 s water trials (delivered from six separate bottles) in a randomized block design. Following the 60 s or the 6 s trial, the access door closed and the next bottle was positioned. The intertrial interval was always 7.5 s.

Taste aversion conditioning

Following the training sessions, mice began a restricted fluid access schedule in which they received fluid twice daily. In the morning, mice were transferred to lickometer cages and given access to deionized water for 15 min. Five hours later, the mice were given a second opportunity to drink water for 45 min. Intake was measured during both sessions (by weighing bottles to the nearest hundredth of a gram); number of licks was recorded during the morning session only. Mice were on this schedule for 7 days. On the seventh day, the afternoon water session was omitted so that mice would have gone for ~24 h without water prior to the following day's test in the Davis Rig. On the fifth day only, 0.3 M sucrose (the conditioned stimulus, CS) was substituted for water during the morning fluid presentation. Within 15 min of the end of this drinking test, mice were given an injection.

All injections were given in a volume of 16.5 ml/kg, except for NaCl and LiCl in experiment 1 (13.33 ml/kg). For experiment 1, concentrations of the unconditioned stimuli were: 0.15 M NaCl (n = 10), 0.15 M LiCl (n = 10) and 2 mM PTC (n = 11), resulting in doses of 2 mmol/kg for NaCl and LiCl and 0.033 mmol/kg for PTC. For experiment 2, concentrations were: 0.15 M NaCl (n = 8), 0.15 M LiCl (n = 8), 1 mM PTC (n = 9), 2 mM PTC (n = 8), 4 mM PTC (n = 9) and 8 mM PTC (n = 9), resulting in doses of 2.5 mmol/kg of NaCl and LiCl, and PTC doses of 0.017, 0.033, 0.066, and 0.132 mmol/kg respectively. In experiment

1, the PTC was mixed in saline, but in experiment 2 all PTC injectants were mixed in deionized water.

Testing

Mice were tested for avoidance of the CS in two sessions in the Davis Rig identical to the trial training sessions except that taste solutions (rather than just water) were available in some of the six bottles. In experiment 1, mice were tested with two concentrations of the CS (0.1 and 0.3 M sucrose) as well as PTC (0.2 and 2 mM), NaCl (0.15 M) and deionized water. The primary intent was to verify that any aversion conditioned to sucrose was specific to this taste compound. In experiment 2, mice were tested with three concentrations of sucrose (0.1, 0.3 and 1 M); the other three bottles contained deionized water.

Data analysis

The primary measure was the number of licks during the 6 s trials during the test session. To standardize for individual differences in lick rate, the lick rate to the test solutions was standardized to the lick rate to water by dividing the mean licks to a tastant over the mean licks to water on an individual animal basis ('lick ratio'). These lick ratios range from 0 (complete suppression) to ~1 (equal licks to tastant and water; water lick rates were presumed to be roughly maximal in these water restricted mice). Taste aversions to a given substance (e.g. sucrose) would be indicated if the lick ratio for a given stimulus was reliably less than that of saline-injected controls. Lick ratio data were analyzed by analysis of variance (ANOVA); when significant differences were found, *post hoc* tests were used to indicate significant differences from saline-injected controls (taken as evidence of taste aversion).

Results

Experiment 1

The LiCl-injected mice moderately avoided the CS, whereas saline-injected controls licked the CS at the same rate as water. Mice injected with PTC demonstrated intermediate avoidance (Figure 1). Interestingly, NaCl was also a moderately aversive taste stimulus, but was avoided equally by all mice regardless of injection condition. Statistically, a Group × Stimulus ANOVA indicated a main effect of Group [$F(2,28) = 3.48$, $P = 0.045$] and Stimulus [$F(4,112) = 15.09$, $P = 0.00001$], as well as a Group × Stimulus interaction [$F(8,112) = 2.58$, $P = 0.013$]. Separate one-way ANOVAs for each stimulus indicated group differences only for the 0.3 M sucrose CS [$F(2,28) = 5.75$, $P = 0.008$] and the lower concentration of sucrose [$F(2,28) = 5.08$, $P = 0.013$]. Post-hoc *t*-tests confirmed that the LiCl-injected group differed from the saline-injected controls (0.3 M: $P = 0.0021$; 0.1 M: $P = 0.0034$). There was only a trend towards the PTC-injected mice differing from the saline-injected group (0.3 M: $P = 0.071$; 0.1 M: $P = 0.079$). Importantly, these *t*-tests did not provide enough evidence to discriminate the LiCl-injected mice from the PTC-injected mice (*P*-values > 0.12), suggesting that CS avoidance in these mice fell in between that for the LiCl-injected and saline-injected mice. These results prompted a parametric analysis of PTC dose in experiment 2.

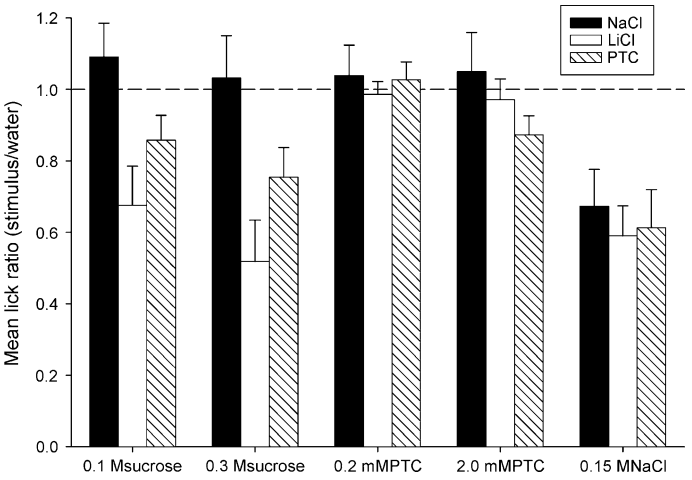

Figure 1 Mean (+ SE) lick ratios (licks to stimulus over licks to water) in 6 s trials for LiCl-injected (open bars), saline-injected (filled bars) and PTC-injected (hatched bars) mice. The conditioned stimulus for all groups was 0.3 M sucrose.

Experiment 2

Avoidance of sucrose at the CS concentration (0.3 M) and higher concentrations (1 M) appears to be parametrically related to the dose of PTC (Figure 2). The parametric nature of the data and the similarities of experiments 1 and 2 support this conclusion, although statistically, this effect can best be described as 'mild', perhaps due to an unexpected amount of variability in the saline-injected mice. A Group × Sucrose Concentration ANOVA indicated a main effect of Group [$F(5,45) = 3.64$, $P = 0.0075$] and Concentration [$F(2,90) = 19.42$, $P = 0.00001$] but no interaction. Accordingly, simple effects were tested by *t*-tests comparing the average response to sucrose. These tests indicated that the mice in the LiCl ($P = 0.042$), 0.066 mmol/kg PTC ($P = 0.031$) and 0.132 mmol/kg mM PTC ($P = 0.00029$) groups differed in their responses to sucrose relative to saline-injected controls. As in the first experiment, the 0.033 mmol/kg PTC group did not differ from controls ($P = 0.171$), but likewise there was no evidence that this group differed from the LiCl-injected mice ($P = 0.486$). The orderly data suggest that 0.033 mmol/kg mM PTC and LiCl both cause moderate conditioned taste aversions under the conditions of these experiments, but that PTC at high doses clearly produces aversions even to a naturally preferred sucrose solution.

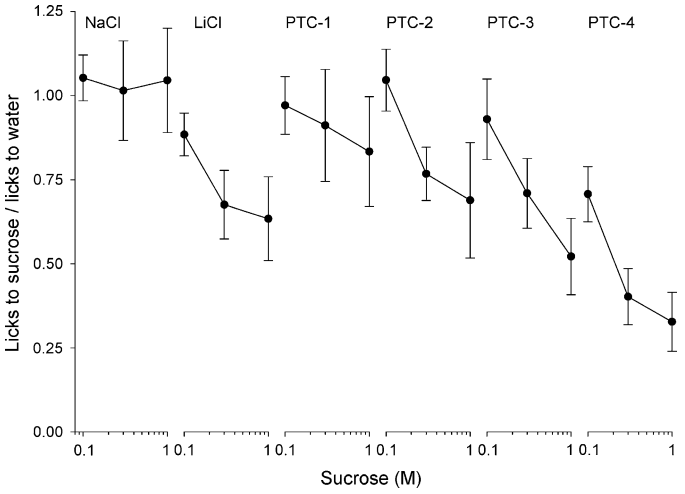

Figure 2 Mean (± SE) lick ratios (licks to sucrose over licks to water) for saline-injected (NaCl), LiCl-injected (LiCl) and PTC-injected mice. PTC-injected mice in groups 1, 2, 3 and 4 were given doses of PTC of 0.017, 0.033, 0.066 and 0.132 mmol/kg respectively. The conditioned stimulus for all groups was 0.3 M sucrose.

Discussion

This study was prompted most directly by the results of Nelson *et al.* (2003), who showed that SW mice were more sensitive to PTC than C3HeB/FeJ mice in 48 h, two-bottle preference tests, but were actually *less sensitive* in a brief-access licking paradigm using the same apparatus (the Davis Rig) used in the current study. Brief-access paradigms are thought to more faithfully reflect orosensory contributions to behavior than long-term tests (Grill *et al.*, 1987; Boughter *et al.*, 2002; Glendinning *et al.*, 2002), which are prone to post-ingestive feedback and learning phenomena. The authors suggested that the preference test results could therefore represent strain differences not in gustatory sensitivity but in other domains. Because PTC is toxic, and because higher concentrations were offered every 2 days, the mice might show enhanced avoidance of PTC through the development of a conditioned taste aversion. Strain differences in PTC preference could potentially be explained as differences in the speed of learning, sensitivity to PTC or simply volume of PTC consumed. The possibility of nongustatory influences in the preference tests was reinforced both by the fact that SW mice consumed more PTC than C3HeB/FeJ mice by the day that PTC avoidance was noted, and by previous work that had indicated that PTC avoidance develops after repeated presentations of an initially unavoided concentration (Whitney and Harder, 1986).

In order to provide direct evidence that PTC can be an unconditioned stimulus in a taste aversion paradigm, we attempted to condition an avoidance to sucrose following PTC injections. Our procedure has the merits of experimentally disentangling the conditioned stimulus and the unconditioned stimulus so that strength of the gustatory cue is not confounded by strength of the unconditioned effects (gastrointestinal malaise). The task also allows assessment of the specificity of any aversion (experiment 1) as well as a dose–response analysis (experiment 2) in a design that eliminates many of the uncontrolled variables present in overnight intake tests and guarantees uniformity in the amount of PTC delivered. That said, it must be recognized that injecting PTC into the peritoneal cavity is substantially different than offering PTC for free ingestion. Certainly, the rate at which PTC acts must differ. In experiment 1, we attempted to match the dose of PTC to the amount mice drank prior to showing PTC avoidance.

With these caveats in mind, there is strong evidence that PTC can cause a conditioned taste aversion in mice. In experiment 1, sucrose was paired with an intraperitoneal injection of PTC as well as an effective dose of LiCl; both groups avoided sucrose to a similar degree. In contrast, PTC-injected mice did not avoid NaCl more than saline-injected mice, confirming that the aversion was specific to the conditioned stimulus (one defining feature of taste aversion learning). Furthermore, these mice did not avoid PTC when offered at the drinking spout. The PTC, in this context, was novel—although it was the compound that made the mice sick, they were naïve to its taste. Even at 2 mM, a concentration that C3HeB/FeJ mice will avoid in brief-access tests similar to this (Nelson *et al.*, 2003), the PTC-injected SW mice in this experiment showed no avoidance. In two-bottle preference tests, however, SW mice show a pronounced avoidance of 0.3 mM (Nelson *et al.*, 2003)—at least in an ascending series where PTC is not novel. That is, given the constellation of findings, it appears that SW mice avoid PTC not because it is unconditionally aversive, but rather because they develop a taste aversion over repeated exposures (cf. Whitney and Harder, 1986).

The PTC-induced aversions to sucrose in experiment 1 were not large (the LiCl-induced aversions were likewise only moderate). Given differences in the route of administration of PTC, the duration of illness, and behavioral context in the Nelson *et al.* (2003) study it would be unwise to try evaluating whether the modest aversion seen in experiment 1 could fully explain the preference test data. In order to ascertain whether the weakness of the aversion seen in experiment 1 was a function of the identity of the unconditioned stimulus or its dose, we paired sucrose with four doses of PTC in a naïve set of mice and examined behavioral responses to higher and lower concentrations of the conditioned stimulus. It is noteworthy that responses to the conditioned stimulus (0.3 M sucrose) were virtually identical in experiments 1 and 2 at the dose used commonly in both experiments. More important, licking responses were parametrically modified by both the concentration of sucrose and the dose of PTC. This finding establishes further that the sucrose avoidance was due to a classical conditioned taste aversion, as parametric effects of both conditioned stimulus concentration and

unconditioned stimulus dose are definitive features of conditioned taste aversion. Second, it establishes that PTC-induced taste aversions can be quite strong: even in highly motivated mice, lick rate to the conditioned stimulus averaged just 40% that of water. In the context of a two-bottle, 48-h preference test, the effects of these higher doses might be relevant as the test proceeds because more PTC is consumed and higher concentrations are introduced.

The work of Nelson *et al*. (2003; see also Whitney and Harder, 1986) thus represents a dramatic example of the power of using multiple behavioral tasks in taste research. The use of the brief access test would suggest that SW mice are largely insensitive to the taste of PTC, whereas the use of the two-bottle preference test would suggest that SW mice are more sensitive to PTC than other mouse strains. Considering both tasks along with the current results presents a more subtle picture: SW mice apparently do not find PTC *hedonically aversive*, but do possess the ability to sense PTC at low concentrations. That is, if mice are capable of forming a conditioned taste aversion to PTC presented orally (i.e. our interpretation of the results of Nelson *et al.*, 2003; Whitney and Harder, 1986), then they must be able to discriminate PTC from water. Despite the label 'conditioned *taste* aversion', PTC could be recognized by an olfactory cue (PTC has a notable odor to humans) or even by sensory receptors of the gastrointestinal tract (Tracy *et al.*, 2004). Alternatively, PTC may indeed have a taste to SW mice that was not revealed by brief-access testing of thirsty mice, which relies on the taste being hedonically aversive. Further behavioral studies can provide us still more information. For example, can mice detect PTC in an operant signal detection task? Would these detection thresholds be altered by gustatory nerve transection or $ZnSO_4$ treatment of the nasal epithelium? Would performance be maintained even if PTC were directly intubated into the stomach?

Regardless of the specifics of the current study, its importance may be more as a reminder that, when assessing strain differences, the conclusions are always limited (and *informed*) by the behavioral task employed. Many compounds that are aversive on the basis of taste are also toxic and can cause behavioral avoidance through a variety of mechanisms, not just taste. The discovery of the *Tas2r* gene cluster suggests the possibility that a strain difference in taste sensitivity could be useful in assigning roles to the various T2Rs. However, as noted by Nelson *et al*. (2003), the assignment of SW and C3HeB/FeJ mice as 'tasters' or 'nontasters' of PTC would be opposite depending on the behavioral task employed—if one assumed that the results of a two-bottle intake test reflected gustatory processing. To be sure, the two-bottle intake test offers a convenient way to assess taste preference and gives results that are more often than not consonant with the results of other procedures. The exceptions, such as PTC, remind us that the limitations of each procedure must always be held in mind.

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