



## Detection of free fatty acids following a conditioned taste aversion in rats

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### Abstract

A gustatory transduction mechanism for free fatty acids (FFAs) has been described in isolated rat taste receptor cells; however, the ability of behaving rats to detect FFAs has not been characterized. Through conditioned taste aversion (CTA) methodology, this study defines the ability of rats to detect and avoid the two principal FFA components of corn oil, linoleic and oleic acid. Following taste aversion conditioning, rats avoided both linoleic and oleic acid at greater than or equal to 66  $\mu\text{M}$  and failed to avoid either 44  $\mu\text{M}$  linoleic or oleic acid. Rats demonstrated generalized avoidances between 88  $\mu\text{M}$  linoleic and oleic acid irrespective of presenting the FFAs as either unesterified acids dissolved in 5 mM ethanol or aqueous sodium salts, sodium linoleate and sodium oleate. Following a CTA to linoleic acid, rats did not show generalized avoidance of NaCl or ethanol, two potentially concomitant tastants in the oral cavity. A CTA to linoleic or oleic acid did produce a generalized avoidance to the other FFA. These results support the ability of rats to detect linoleic and oleic acid ( $>44 \mu\text{M}$ ) and suggest that the two FFAs share common orosensory properties. Furthermore, it is unlikely that the detection of the FFAs is due to an enhancement of other concomitant tastants such as saliva or the delivery solution.

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### 1. Introduction

The disease of obesity has reached epidemic proportions in industrialized countries [4,5,55]. The obesity epidemic is characterized by recent, rapid increases in waist circumference, prevalence of abdominal obesity (38% male, 60% female population) and body mass index (BMI) scores  $>30$  for more than 20% of Americans [11,35,39]. In 2000, obesity was the second leading cause of American deaths accounting for  $>20\%$  of deaths resulting from modifiable behavioral risk factors [36]. In addition to genetic traits [1,10,45,46,51], poor diet [8,6,30–33] coupled with physical inactivity [3,40,47,50] have been identified as the principal contributors to obesity [36]. A strong association exists between obesity and high daily intake of dietary fat [30,32,33] with increased palatability cited as the primary motivation for the over-consumption of dietary fat [7,30,31,52]. Surprisingly, little research has investigated the source of salient sensory perceptions underlying the high palatability of dietary fat.

Rodent research models have been used to demonstrate preferences for increased dietary fat consumption [16,44,53,54] typically employing corn and mineral oil as respective nutritive and nonnutritive stimuli with similar textural properties. The orosensory properties of corn oil produce a unanimous preference over mineral oil in two-bottle forced-choice testing using sham-fed rats to eliminate post-ingestive feedback [34]. Corn oil is preferred to control solutions of either mineral oil or xanthan gum providing evidence that texture is not the salient orosensory cue [9,16,34,42,48]. Conditioned taste aversions (CTAs) to sucrose–corn oil mixtures produce a stimulus generalization to a sucrose–linoleic acid mixture, but do not generalize to a sucrose–mineral oil mixture [44] suggesting that the salient feature is a non-textural, orosensory cue elicited by the free fatty acid (FFA) constituent of corn oil, linoleic acid. Additionally,  $\text{ZnSO}_4$  nasal treatments to block olfactory cues do not diminish the corn oil preference and anosmic rats are capable of forming conditioned place preferences using corn oil and linoleic acid as the preferred stimuli [48].

Linoleic and oleic acid constitute 83% of the FFAs found in corn oil [17] and represent likely candidates for the chemical stimuli underlying the behavioral preferences for

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dietary fat. Rodent lingual lipase can produce significant levels of FFAs from lipids within 1–5 s of exposure to the gustatory epithelium [24] and fatty acid transporter (FAT) proteins found in the gustatory epithelium [12] bind both linoleic and oleic acid [2] facilitating access to the taste receptor cells (TRCs). Evidence of a potential FFA transduction mechanism has been shown in electrophysiological recordings from isolated rat TRCs. *Cis*-polyunsaturated fatty acids (PUFA) including linoleic acid inhibit the delayed-rectifying potassium channel in TRCs from the fungiform papillae [14], while both PUFA and a subset of monounsaturated fatty acids including oleic acid inhibit the potassium channels in TRCs harvested from the circumvallate papilla [18]. Furthermore, calcium imaging in murine TRCs suggests increased intracellular  $Ca^{2+}$  within 1 min of extracellular application of linoleic or oleic acid [38]. Evidence also suggests that oral stimulation by both FFAs may produce small increases in electrophysiological recordings of the rat glossopharyngeal nerve [22,43]. In addition, there appears to be a functional significance for the orosensory recognition of FFAs, such as the initiation of pancreatic enzyme secretions [21] and elevated triacylglycerol concentrations [27,26,28].

While previous studies have suggested the behavioral detection of FFAs by rats through preference comparisons and stimulus generalizations, this study is the first direct characterization of the detection threshold for linoleic and oleic acid in rats. The ability of rats to form a conditioned taste aversion to linoleic or oleic acid and subsequently avoid future consumption of the FFAs was employed as a means to identify a threshold of detection for the FFAs. Linoleic and oleic acid can be prepared in solution either by dissolving the FFAs in a low concentration of ethanol or using the aqueous sodium salt forms, linoleate and oleate. The ability to generalize a taste aversion between linoleic acid and either ethanol or sodium chloride solutions was addressed in order to examine the role of potential concomitant tastants as cues for the behavioral detection of the FFAs. Finally, the similarity of the orosensory properties of linoleic and oleic acid were assessed through generalized conditioned taste aversion testing between the two FFAs.

## 2. Materials and methods

### 2.1. Subjects

All subjects were male Sprague Dawley rats (>90 days old; Charles River Laboratories, Wilmington, MA) individually housed in transparent plastic cages in a temperature-controlled colony room on a 12–12-h light–dark cycle with lights on at 07:00 h. All of the 1-h two-bottle access tests were conducted on the home cage of each rat during the light cycle in the animal colony room. Animals had free access to Harlan Teklad 8604 rodent chow and deionized-distilled water *ad libitum* unless otherwise noted. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the

Institutional Animal Care and Use Committee of Wofford College.

### 2.2. Chemical stimuli

Taste solutions were mixed daily from reagent grade chemicals (Sigma-Aldrich, St. Louis) dissolved in deionized-distilled water and presented at room temperature (22 °C). Prior to use, the FFAs were stored in a freezer at –20 °C. Both a minimum 99% pure, unesterified and a sodium salt form of each FFA, linoleic and oleic acid, were utilized. When the unesterified form of linoleic or oleic acid was tested, 5 mM ethanol (ETOH) was added to the solution to ensure full dissolution of the FFAs. All control solutions also contained 5 mM ETOH when being compared to or used in conjunction with the unesterified FFAs mixed in 5 mM ETOH. When the sodium salt-based forms of linoleic acid (sodium linoleate) and oleic acid (sodium oleate) were tested at 88  $\mu$ M concentrations, all control solutions contained 88  $\mu$ M sodium chloride (NaCl). The pH of the 88  $\mu$ M FFA solutions was found to be within a normal physiological range (linoleic acid in 5 mM ETOH, 6.2 pH; oleic acid in 5 mM ETOH, 6.5 pH; sodium linoleate 7.4 pH, sodium oleate 7.5 pH). Solution viscosity was measured using an Ostwald viscosimeter calibrated against water, 0.894 centipoise (cP) at 23 °C. The viscosity of the 88  $\mu$ M FFA solutions was measured as 0.908 cP for linoleic acid in 5 mM ETOH, 0.901 cP for oleic acid in 5 mM ETOH, 0.903 cP for sodium linoleate and 0.902 cP for sodium oleate. The viscosity values for the FFA solutions represent a nominal 1.5% difference from the viscosity of water. In comparison, the viscosity of dietary corn oil is between 50 and 60 cP, more than 50 times greater than the viscosity of water or the FFA solutions.

### 2.3. General conditioned taste aversion procedure

Four days prior to the conditioning day, all rats were placed on a water restriction schedule with decreasing access to two bottles containing the control solution to be used during testing (either 5 mM ETOH or 88  $\mu$ M NaCl) starting at 11:00 h such that on the first day there was 2-h access to the control solutions and on the second through fourth days the rats received 1-h access to the control solutions. At 11:00 h on the conditioning day, rats received 1-h access to two bottles each containing the conditioned stimulus. Thirty minutes following the conclusion of the 1-h two-bottle access period, the rats received intraperitoneal injections of either 150 mM LiCl or 150 mM NaCl at a dosage of 20 ml/kg body weight depending on their experimental condition. An additional 30 min after the injections, rats were observed for signs of gastric distress or malaise indicating that the LiCl injection was an effective unconditioned stimulus eliciting an unconditioned response. Beginning at 11:00 h on the following preference-testing day, rats received 1-h access to one bottle containing the conditioned stimulus and one bottle containing a control solution.

On the preference-testing day, the bottle position on the cage top (left/right) for the control solution and conditioned stimulus was randomized. Every day during water restriction and on the conditioning and preference-testing days, the intake from each bottle was calculated as the difference in bottle weight (0.1 g resolution) before and after the 1-h access period. Preference scores were calculated as a ratio of consumed conditioned stimulus/total intake of both the conditioned stimulus and the control solution in order to assess the taste aversion conditioning. Calculating preference scores in this manner produces scores of 100% for complete preference when only the conditioned stimulus and none of the control solution is consumed, 50% for indifference or equal consumption of the conditioned stimulus and control solution, and 0% for complete avoidance when only the control solution and none of the conditioned stimulus is consumed. Analyses were performed with appropriate ANOVA statistical tests and, when warranted, post hoc comparisons were made using Fisher's least significant difference test. Statistics significant at the  $p < 0.05$  level are reported. All data are graphed as the mean  $\pm$  standard error.

### 3. Experiment 1: behavioral detection of linoleic acid

#### 3.1. Subjects and procedure

A total of 42 naïve rats were assigned to 6 groups that received pairings of consumption of either 44, 66 or 88  $\mu\text{M}$  linoleic acid mixed in a 5 mM ETOH solution with intraperitoneal injections of either 150 mM LiCl or 150 mM NaCl at a dosage of 20 ml/kg (44  $\mu\text{M}$  LA and LiCl,  $n = 8$ ; 44  $\mu\text{M}$  LA and NaCl,  $n = 6$ ; 66  $\mu\text{M}$  LA and LiCl,  $n = 8$ ; 66  $\mu\text{M}$  LA and NaCl,  $n = 6$ ; 88  $\mu\text{M}$  LA and LiCl,  $n = 8$ ; and 88  $\mu\text{M}$  LA and NaCl,  $n = 6$ ). On the conditioning day, rats in the respective experimental groups received 1 h of access to two bottles both containing either 44, 66 or 88  $\mu\text{M}$  linoleic acid mixed in a 5 mM ETOH solution. At 30 min following the unconditioned stimulus injections, signs of gastric distress or malaise were observed in all rats receiving LiCl injections indicating an effective unconditioned stimulus and response. On the preference-testing day, rats received 1-h access to one bottle containing a control solution of 5 mM ETOH and one bottle containing the same concentration of linoleic acid that they had received on the prior conditioning day. ANOVA tests for differences between the LiCl and NaCl injection groups were performed for the total intake on conditioning day, bottle position-preference on conditioning day, total intake on the preference-testing day, intake of the conditioned stimulus and control solution on the preference-testing day, and preference scores.

#### 3.2. Results

There was no statistically significant difference in total intake on conditioning day between the LiCl or NaCl injection groups. There was no significant bottle position-preference on the conditioning day for the LiCl or NaCl

injection groups. The total intake on the preference-testing day did not differ from the total intake on the conditioning day for the LiCl or NaCl injection groups. As shown in Fig. 1A, there was no difference in the intake of the 44  $\mu\text{M}$  linoleic acid or control solutions for the LiCl and NaCl injection groups resulting in similar respective preference scores of 51% and 56% depicted in Fig. 1B. As shown in Fig. 1C and D, there was a significant avoidance of 66  $\mu\text{M}$  linoleic acid between the LiCl and NaCl groups ( $F_{1,13} = 13.997$ ,  $p < 0.01$ ) resulting in a significant difference in the LiCl (29%) and NaCl (50%) preference scores ( $F_{1,13} = 9.163$ ,  $p < 0.01$ ). The LiCl injection group consumed significantly less 88  $\mu\text{M}$  linoleic acid ( $F_{1,13} = 30.268$ ,  $p < 0.01$ ) and significantly more control solution ( $F_{1,13} = 32.944$ ,  $p < 0.01$ ) than the NaCl injection group (Fig. 1E). Therefore, there was a significantly different preference score ( $F_{1,13} = 45.283$ ,  $p < 0.01$ ) for 88  $\mu\text{M}$  linoleic acid between the LiCl (17%) and NaCl (72%) groups (Fig. 1F).

### 4. Experiment 2: behavioral detection of oleic acid

#### 4.1. Subjects and procedure

A total of 48 naïve rats were assigned to groups that received a single pairing of consumption of either 44, 66 or 88  $\mu\text{M}$  oleic acid mixed in a 5 mM ETOH solution with an intraperitoneal injection of either 150 LiCl or 150 mM NaCl at a dosage of 20 ml/kg (44  $\mu\text{M}$  OA and LiCl,  $n = 8$ ; 44  $\mu\text{M}$  OA and NaCl,  $n = 8$ ; 66  $\mu\text{M}$  OA and LiCl,  $n = 8$ ; 66  $\mu\text{M}$  OA and NaCl,  $n = 8$ ; 88  $\mu\text{M}$  OA and LiCl,  $n = 8$ ; and 88  $\mu\text{M}$  OA and NaCl,  $n = 8$ ). On the conditioning day, rats in the respective experimental groups received 1 h of access to two bottles both containing either 44, 66 or 88  $\mu\text{M}$  oleic acid mixed in a 5 mM ETOH solution starting at 11:00 h. Thirty minutes following the injections, signs of gastric distress or malaise were observed in all rats receiving LiCl injections indicating an effective unconditioned stimulus and response. Beginning at 11:00 h on the subsequent preference-testing day, rats received 1-h access to one bottle containing a control solution of 5 mM ETOH and one bottle containing the same concentration of oleic acid that they had received on the prior conditioning day. ANOVA tests for differences between the LiCl and NaCl injection groups were performed for the total intake on conditioning day, bottle position-preference on conditioning day, total intake on the preference-testing day, intake of the conditioned stimulus and control solution on the preference-testing day, and preference scores.

#### 4.2. Results

As can be seen in Fig. 2, there were no significant differences between LiCl and NaCl injection groups for the total intakes on either the conditioning or preference-testing day for 44, 66 or 88  $\mu\text{M}$  oleic acid. On the preference-testing day, there was no significant difference in the intake of 44  $\mu\text{M}$  oleic acid or the control solution producing preference scores of 64% for the LiCl injection group compared to 58% for the

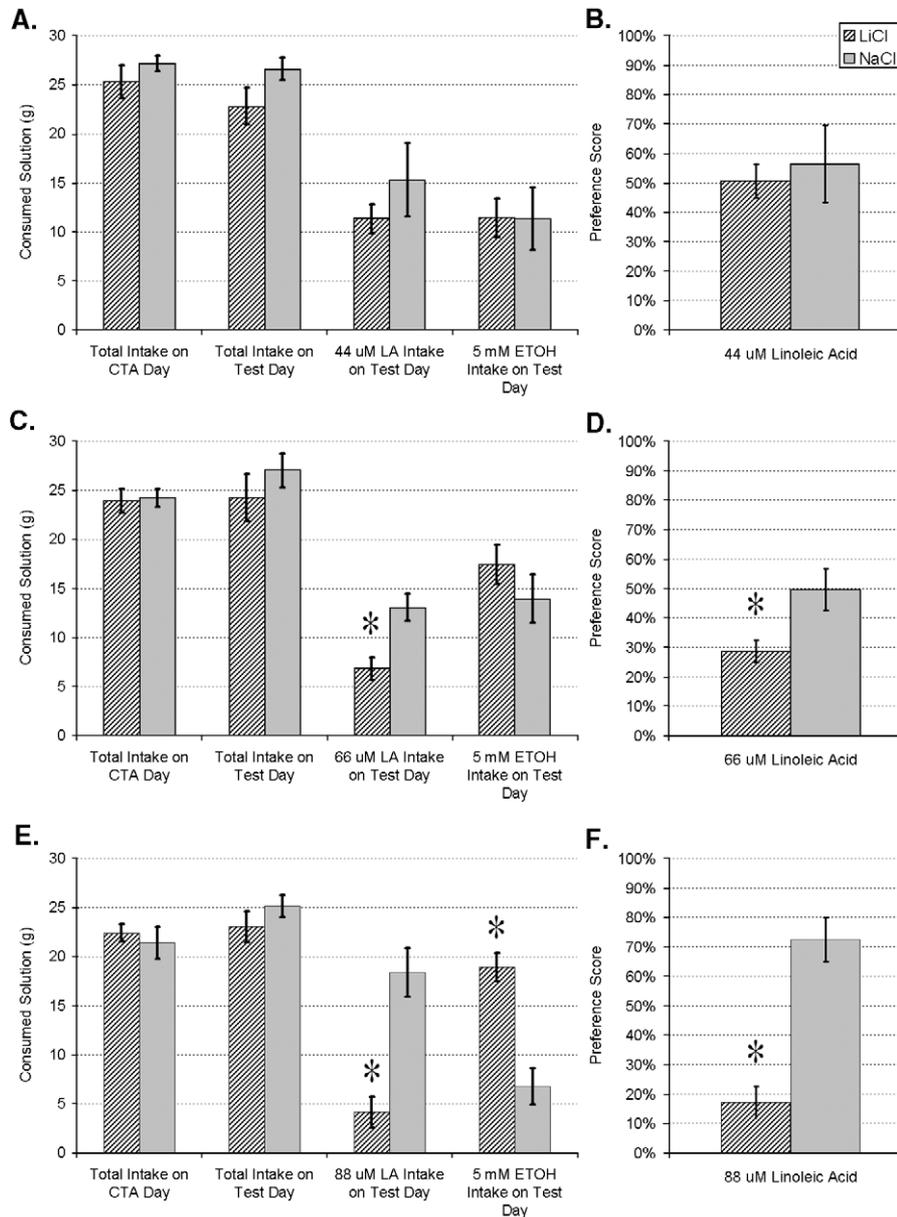


Fig. 1. Mean ( $\pm$ S.E.) intake and preference score ratios for 1-h two-bottle testing of linoleic acid (LA) CTA formation. There were no significant differences of intake (A) or preference score (B) between the LiCl (hatched bars) and NaCl (filled bars) injection groups for 44  $\mu$ M. A CTA was evidenced by significantly less consumption of 66 (C) and 88 (E)  $\mu$ M linoleic acid by rats receiving LiCl injections resulting in a significant reduction in the preference scores for 66 (D) and 88 (F)  $\mu$ M linoleic acid as compared to control groups. Stars indicate  $p < 0.01$  significant differences between injection groups.

NaCl injection group (Fig. 2B). The LiCl injection group consumed significantly less 66  $\mu$ M oleic acid ( $F_{1,15}=4.691$ ,  $p < 0.05$ ) and significantly more control solution ( $F_{1,15}=8.727$ ,  $p < 0.01$ ) than the NaCl injection group. As shown in Fig. 2D, these intake differences produced a significantly lower preference score ( $F_{1,15}=8.869$ ,  $p < 0.01$ ) for the LiCl injection group (35%) compared to the NaCl injection group (65%). As can be seen in Fig. 2E, the LiCl injection group consumed significantly less 88  $\mu$ M oleic acid ( $F_{1,15}=10.917$ ,  $p < 0.01$ ) and significantly more control solution ( $F_{1,15}=4.378$ ,  $p < 0.05$ ) than the NaCl injection group. This resulted in a significantly lower preference score ( $F_{1,15}=9.272$ ,  $p < 0.01$ ) for the LiCl injection group (37%) compared to the NaCl injection group (70%) as shown in Fig. 2F.

## 5. Experiment 3: stimulus generalization between linoleic acid and ethanol

### 5.1. Subjects and procedure

Linoleic and oleic acid when in their unesterified form must be dissolved in a low concentration of ethanol. Although an identical concentration of ethanol was used as the control solution in the linoleic and oleic acid detection experiments, it may be possible for the FFAs to increase the perceived intensity of the ethanol. If rats are avoiding the FFA solutions on the basis of a perceived stronger ethanol solution compared to the control solution, then there should be a stimulus generalization between

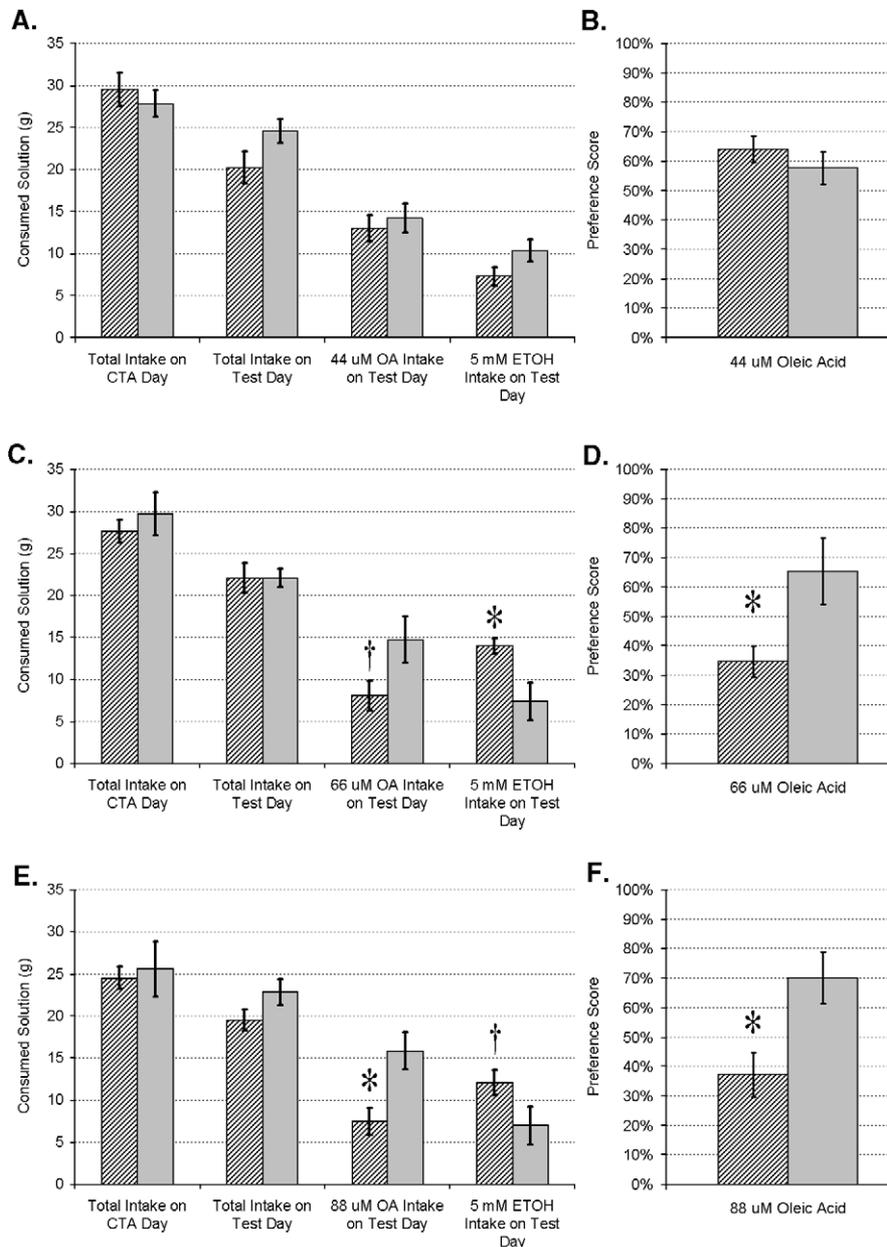


Fig. 2. Mean ( $\pm$ S.E.) intake and preference score ratios for 1-h two-bottle testing of oleic acid CTA formation. There were no significant differences of intake (A) or preference score (B) between the LiCl (hatched bars) and NaCl (filled bars) injection groups for 44  $\mu$ M. A CTA was evidenced by significantly less consumption of 66 (C) and 88 (E)  $\mu$ M oleic acid by rats receiving LiCl injections resulting in a significant reduction in the preference scores for 66 (D) and 88 (F)  $\mu$ M oleic acid as compared to control groups. Crosses indicate  $p < 0.05$  and stars indicate  $p < 0.01$  significant differences between injection groups.

linoleic acid dissolved in ethanol and ethanol presented alone. To assess this potential confound in the detection of linoleic and oleic acid, 31 naïve rats were divided into 2 groups that received pairings of consumption of 88  $\mu$ M linoleic acid dissolved in a 5 mM ETOH solution with intraperitoneal injections of either 150 mM LiCl ( $n=16$ ) or 150 mM NaCl ( $n=15$ ) at a dosage of 20 ml/kg. Approximately 30 min after the injections, signs of gastric distress or malaise were observed in all rats receiving LiCl injections indicating an effective unconditioned stimulus and response. Beginning at 11:00 h on the first preference-testing day, all rats received 1-h access to one bottle

containing a control solution of 5 mM ETOH and one bottle containing a test solution of 15 mM ETOH. Beginning at 11:00 h on the second preference-testing day, all rats received 1-h access to one bottle containing a control solution of 5 mM ETOH and one bottle containing a test solution of 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH. The bottle position (left/right) for the control and test solutions was randomized on each preference-testing day. ANOVA tests for differences between the LiCl and NaCl injection groups were performed for the total intake on conditioning day, bottle position-preference on conditioning day, total intake on each preference-testing day,

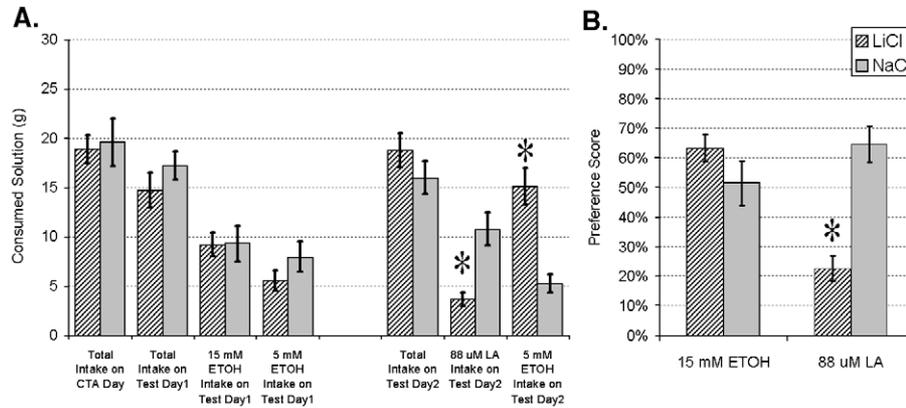


Fig. 3. Mean ( $\pm$ S.E.) intake and preference score ratios for 1-h two-bottle testing of 88  $\mu$ M linoleic acid CTA formation and stimulus generalization to 15 mM ETOH. Significant differences in intake (A) and preference score (B) indicates CTAs were formed to 88  $\mu$ M linoleic acid by the LiCl injection group (hatched bars) as compared to the NaCl injection group (filled bars). No differences in intake measurements and preference score provide evidence that the CTA to linoleic acid did not generalize to ethanol. Stars indicate  $p < 0.01$  significant differences between injection groups.

intake of the tested and control solutions on each preference-testing day and the preference scores for each two-bottle test.

## 5.2. Results

As can be seen in Fig. 3A, there were no significant differences between the total intakes on the conditioning day, preference-testing day 1 or preference-testing day 2 for the LiCl and NaCl injection groups. Furthermore, there was no significant difference in the consumption of 15 mM ETOH or the control solution for the LiCl and NaCl injection groups and thus no difference in the preference score for each group (LiCl 63%, NaCl 51%). Although there was no demonstrated aversion to ethanol, the two-bottle preference test on the second day revealed a conditioned aversion to 88  $\mu$ M linoleic acid with the LiCl injection group showing a significant decrease in the consumption of linoleic acid ( $F_{1,30}=17.116$ ,  $p < 0.01$ ) and a significant increase in the consumption of the control solution ( $F_{1,30}=23.243$ ,  $p < 0.01$ ) compared to the NaCl injection group. As shown in Fig. 3B, the preference score for the LiCl injection group (23%) was significantly lower ( $F_{1,30}=34.580$ ,  $p < 0.01$ ) than the NaCl injection group (65%) during assessment of a CTA to linoleic acid.

## 6. Experiment 4: stimulus generalization between linoleic acid and NaCl

### 6.1. Subjects and procedure

The sodium content of saliva represents another potential concomitant tastant that may interact with the FFAs to provide a cue permitting avoidance of linoleic and oleic acid during the previous preference testing. To assess the ability to generalize an aversion between linoleic acid and NaCl, 16 naïve rats received pairings of consumption of 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH with intraperitoneal injections of either 150 mM LiCl ( $n=8$ ) or 150 mM NaCl

( $n=8$ ) at a dosage of 20 ml/kg. Following the injections, signs of gastric distress or malaise were observed in all rats receiving LiCl injections indicating an effective unconditioned stimulus and response. Beginning at 11:00 h on the first preference-testing day, all rats received 1-h access to one bottle containing a control solution of 5 mM ETOH and one bottle containing a test solution of 150 mM NaCl dissolved in 5 mM ETOH. Beginning at 11:00 h on the second preference-testing day, all rats received 1-h access to one bottle containing a control solution of 5 mM ETOH and one bottle containing a test solution of 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH. The bottle position (left/right) for the control and test solutions was randomized on each preference-testing day. ANOVA tests for differences between the LiCl and NaCl injection groups were performed for the total intake on conditioning day, bottle position-preference on conditioning day, total intake on each preference-testing day, intake of the tested and control solutions on each preference-testing day and the preference scores for each two-bottle test.

### 6.2. Results

The results of the NaCl generalized avoidance test are illustrated in Fig. 4. There was no significant difference between the LiCl and NaCl injection groups for the total intake on the conditioning day or preference-testing day 1. In addition, there was no significant difference between the injection groups for the consumption of 150 mM NaCl or the control solution resulting in similar preference scores (LiCl 52%, NaCl 51%) for the NaCl generalized avoidance-testing day. On the second preference-testing day, there was a significant decrease in the total intake of the LiCl injection group compared to the NaCl injection group ( $F_{1,15}=16.754$ ,  $p < 0.01$ ) due to a significant decrease in the consumption of 88  $\mu$ M linoleic acid by the LiCl injection group ( $F_{1,15}=13.737$ ,  $p < 0.01$ ) and no significant difference in the consumption of the control solution between the two injection groups. As shown in Fig. 4B, the significant decrease ( $F_{1,15}=13.462$ ,

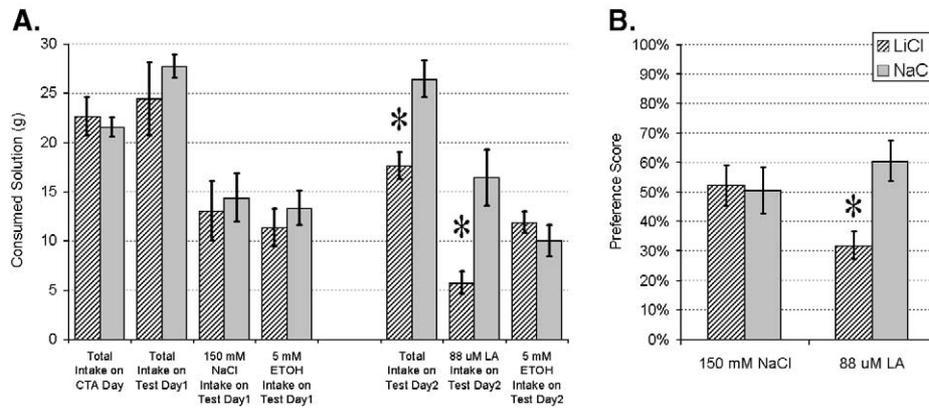


Fig. 4. Mean ( $\pm$ S.E.) intake and preference score ratios for 1-h two-bottle testing of 88  $\mu$ M linoleic acid CTA formation and stimulus generalization to 150 mM NaCl. A significant reduction in linoleic acid intake (A) and preference score (B) provides evidence that a CTA formed to 88  $\mu$ M linoleic acid by the LiCl injection group (hatched bars) as compared to the NaCl injection group (filled bars). No differences in the intake measurements and preference score provide evidence that the CTA to linoleic acid did not generalize to ethanol. Stars indicate  $p < 0.01$  significant differences between injection groups.

$p < 0.01$ ) in preference score for 88  $\mu$ M linoleic acid for the LiCl injection group (32%) compared to the NaCl injection group (60%) demonstrates that an aversion to linoleic acid was conditioned.

## 7. Experiment 5: stimulus generalization between linoleic acid and oleic acid

### 7.1. Subjects and procedure

Based on the previous experiments, it appears that rats can detect linoleic and oleic acid and that concomitant tastants, such as ETOH and NaCl, are not being used as a cue to detect the FFAs. It is possible to dissolve the sodium salt form of linoleic and oleic acid in water without adding ethanol to the solution. In this experiment, stimulus generalizations between the unesterified FFAs and the sodium salt FFAs were examined. Furthermore, the ability to generalize an aversion between linoleic and oleic acid was also assessed. There were two phases of this experiment. In the first phase, 32 naïve rats were assigned to four experimental groups receiving pairings of consumption (1 h) of either 88  $\mu$ M linoleic acid mixed in a 5 mM ETOH solution (LA-ETOH) or 88  $\mu$ M sodium linoleate (LA-SALT) with intraperitoneal injections of either 150 mM LiCl or 150 mM NaCl at a dosage of 20 ml/kg (88  $\mu$ M LA-ETOH and LiCl,  $n = 8$ ; 88  $\mu$ M LA-ETOH and NaCl,  $n = 8$ ; 88  $\mu$ M LA-SALT and LiCl,  $n = 8$ ; and 88  $\mu$ M LA-SALT and NaCl,  $n = 8$ ). Thirty minutes following the injections, signs of gastric distress or malaise were observed in all rats receiving LiCl injections indicating an effective unconditioned stimulus and response. Using two-bottle 1-h preference tests on preference-testing days 1 and 2, a CTA to the conditioned stimulus of either 88  $\mu$ M linoleic acid mixed in a 5 mM ETOH solution or 88  $\mu$ M sodium linoleate was confirmed and a stimulus generalization to the alternative FFA form (salt-based or unesterified acid in ETOH) was measured using a counterbalanced design such that one half of the rats in each experimental condition received one bottle containing a control

solution of 5 mM ETOH and one bottle containing the test solution of 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH while the other half of the rats received one bottle containing a control solution of 88  $\mu$ M NaCl and one bottle containing a test solution of 88  $\mu$ M sodium linoleate and vice versa on the second preference-testing day. On the third preference-testing day, a stimulus generalization to oleic acid was measured with a two-bottle 1-h preference test such that rats having received 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH as the conditioned stimulus were given access to one bottle containing a control solution of 5 mM ETOH and one bottle containing the test solution of 88  $\mu$ M oleic acid dissolved in 5 mM ETOH. Likewise, rats having received 88  $\mu$ M sodium linoleate as the conditioned stimulus were given access to one bottle containing a control solution of 88  $\mu$ M NaCl and one bottle containing the test solution of 88  $\mu$ M sodium oleate.

In phase 2, 28 naïve rats were placed on a 23-h water restriction schedule as previously described. Following the 4-day water restriction schedule, the conditioning and testing procedure described above in phase 1 was repeated using a 15-min access period to either 88  $\mu$ M oleic acid dissolved in 5 mM ETOH (OA-ETOH) or 88  $\mu$ M sodium oleate (OA-SALT) as the conditioned stimuli paired with either NaCl or LiCl injections as the unconditioned stimuli (88  $\mu$ M OA-ETOH and LiCl,  $n = 7$ ; 88  $\mu$ M OA-ETOH and NaCl,  $n = 7$ ; 88  $\mu$ M OA-SALT and LiCl,  $n = 7$ ; and 88  $\mu$ M OA-SALT and NaCl,  $n = 7$ ). Using a two-bottle 15-min preference test on preference-testing days 1 and 2, confirmation of the CTA and stimulus generalization to the alternative form of FFA was assessed using the counterbalanced procedure described in phase 1. Likewise, on the third preference-testing day of phase 2, a stimulus generalization from oleic to linoleic acid was measured using a two-bottle 15-min preference test in the same manner as described in phase 1. On all preference-testing days, the bottle position (left/right) for the control and test solutions was randomized. A mixed-design ANOVA tested the effect of the repeated measure, days and injection condition in the comparison of total intake on conditioning days, bottle position-preference on conditioning days, total intake on the preference-

testing days, intake of the test and control solutions on the preference-testing days, and the preference scores for each two-bottle test.

7.2. Results

The consumption measurements for phase 1 using either 88  $\mu$ M linoleic acid dissolved in 5 mM ETOH (LA-ETOH) or 88  $\mu$ M sodium linoleate (LA-SALT) as the conditioned stimuli are shown in Fig. 5. There was no effect of days on the counterbalanced preference testing procedure for either conditioned stimulus, LA-ETOH or LA-SALT. The data for assessment of the conditioned taste aversion to the conditioned stimulus and the generalization of the aversion to the

complementary FFA form (unesterified acid or sodium salt) collected respectively on preference-testing days 1 and 2 ( $n=4$  each group) and vice versa for the other half of the subjects ( $n=4$  each group) were combined to form two representative groups ( $n=8$ ). There were no significant differences between the LiCl and NaCl injection groups for the total intake on conditioning day or any of the preference-testing days. The total intake on conditioning day is redrawn on each component graph in Fig. 5 to allow comparisons with the total intake for each preference-testing day.

There was a significant main effect of injection group for the consumption of the test solutions ( $F_{1,42}=37.369, p<0.01$ ) and the control solutions ( $F_{1,42}=8.966, p<0.01$ ) by the groups receiving LA-ETOH as the conditioned stimulus as well as the

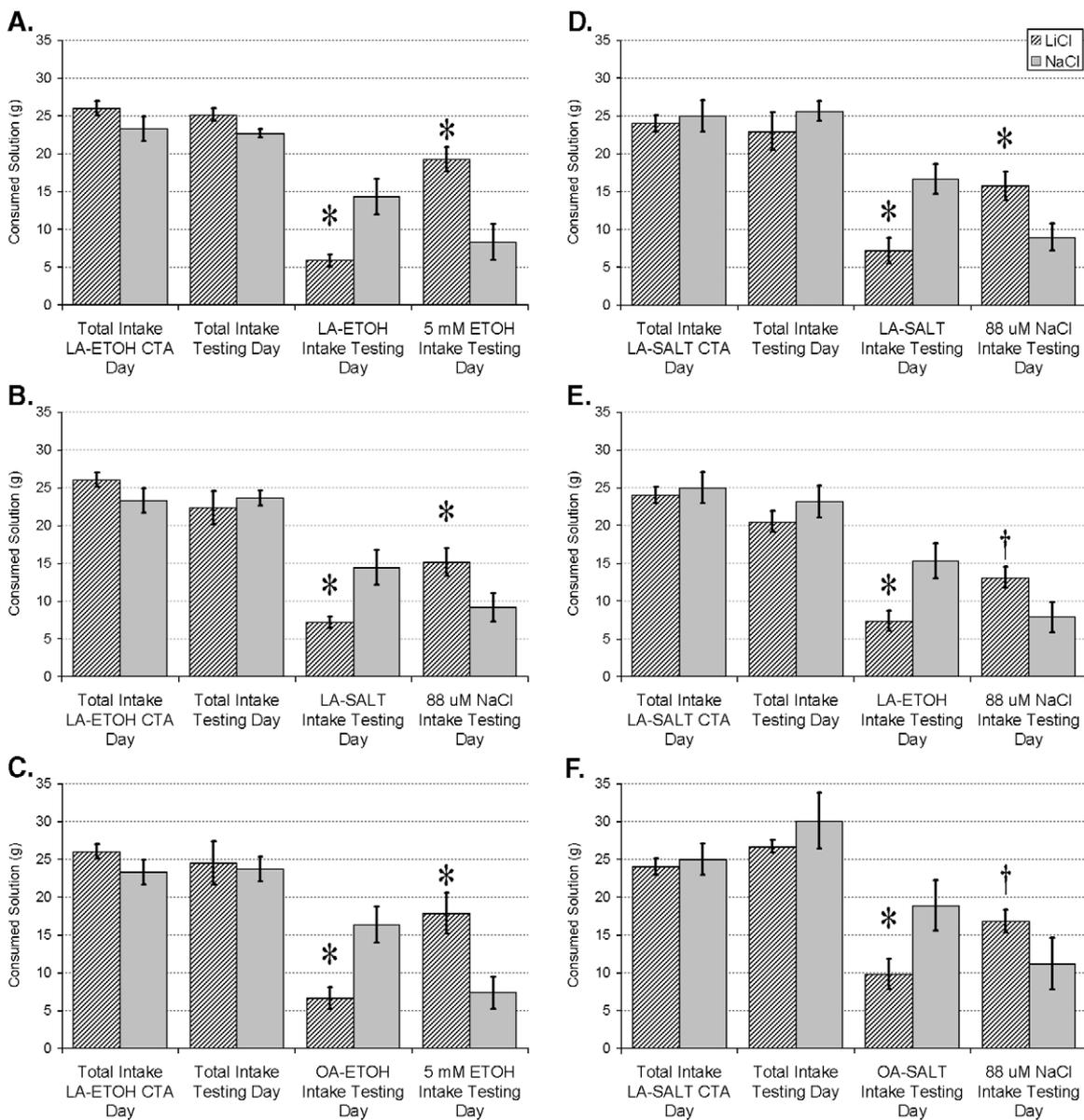


Fig. 5. Reductions in 88  $\mu$ M linoleic acid intake for the LiCl injection group (hatched bars) compared to the control group (filled bars) demonstrates CTA formation to unesterified linoleic acid in 5 mM ETOH (A) and sodium linoleate (D). Reductions in FFA intake for the LiCl injection group compared to the control group indicates a stimulus generalization associated between unesterified linoleic acid and sodium linoleate (B, E) and between linoleic acid and oleic acid (C, F). The total intake on the CTA day is redrawn on each graph for comparison to total intake on test days. Crosses indicate  $p < 0.05$  and stars indicate  $p < 0.01$  significant differences between injection groups.

groups receiving LA-SALT as the conditioned stimulus (test solutions  $F_{1,42}=27.563$ ,  $p<0.01$ ; control solutions  $F_{1,42}=13.471$ ,  $p<0.01$ ). Fig. 5A and B show the results of the conditioned taste aversion to the conditioned stimulus of either LA-ETOH or LA-SALT. Post hoc tests revealed that the LiCl injection groups demonstrated formation of a taste aversion to the conditioned stimulus by consuming significantly less LA-ETOH ( $p<0.01$ ) or LA-SALT ( $p<0.01$ ) and significantly more of the respective control solutions, 5 mM ETOH ( $p<0.01$ ) or 88  $\mu$ M NaCl ( $p<0.01$ ), than the NaCl injection groups. Fig. 5B and E show a clear stimulus generalization between LA-ETOH and LA-SALT with the LiCl injection groups consuming significantly less LA-SALT ( $p<0.01$ ) or

LA-ETOH ( $p<0.01$ ) and significantly more of the respective control solutions, 88  $\mu$ M NaCl ( $p<0.01$ ) or 5 mM ETOH ( $p<0.05$ ), than the NaCl injection groups. Finally, on preference-testing day 3, a stimulus generalization was demonstrated between linoleic and oleic acid as shown in Fig. 5C and F. The LiCl injection groups consumed significantly less OA-ETOH ( $p<0.01$ ) or OA-SALT ( $p<0.01$ ) and significantly more of the matched control solution, 5 mM ETOH ( $p<0.01$ ) or 88  $\mu$ M NaCl ( $p<0.05$ ), than the NaCl injection groups.

For phase 2, there was no effect of days on the counter-balanced preference testing procedure so the groups were combined as previously mentioned above in phase 1. There

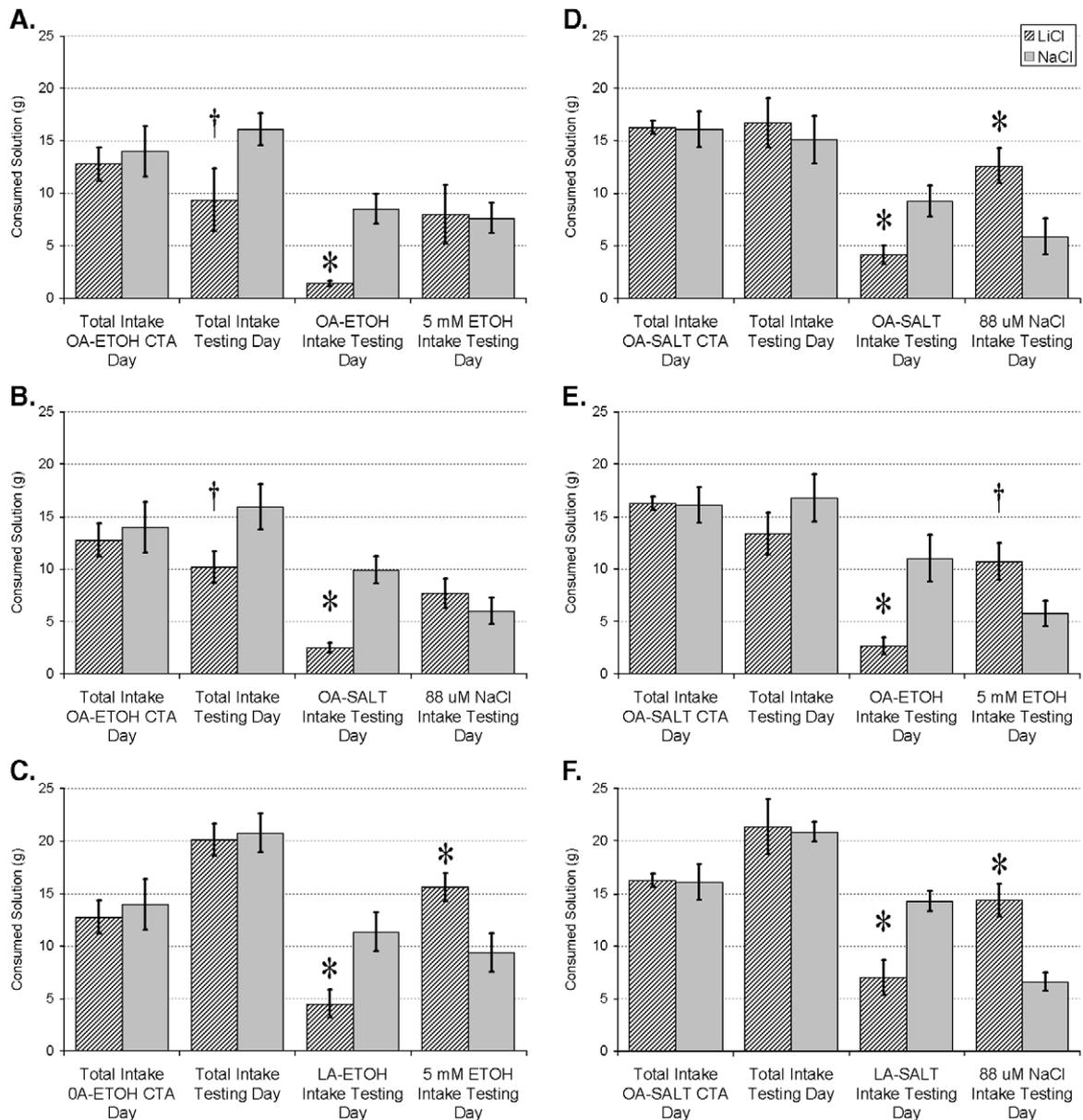


Fig. 6. Reductions in 88  $\mu$ M oleic acid intake for the LiCl injection group (hatched bars) compared to the control group (filled bars) demonstrates CTA formation to unesterified oleic acid in 5 mM ETOH (A) and sodium oleate (D). Reductions in FFA intake for the LiCl injection group compared to the control group indicates a stimulus generalization associated between unesterified oleic acid and sodium oleate (B, E) and between oleic acid and linoleic acid (C, F). The total intake on the CTA day is redrawn on each graph for comparison to total intake on test days. Crosses indicate  $p < 0.05$  and stars indicate  $p < 0.01$  significant differences between injection groups.

was no difference between the LiCl and NaCl injection groups for the total intake on the conditioning day for either conditioned stimulus. The total intake on the conditioning day is redrawn on each component graph of Fig. 6 to allow comparisons with the total intakes on each preference-testing day.

During the preference testing for the OA-ETOH conditioned stimulus group, there was a significant main effect of days on the total solution intake ( $F_{2,36}=7.941$ ,  $p<0.01$ ) and on the intake of the control solution ( $F_{2,36}=6.347$ ,  $p<0.01$ ). Post hoc tests identified the source of the significant differences as an increase in control solution intake ( $p<0.01$ ) and subsequently the total solution intake ( $p<0.01$ ) on the LA-ETOH preference-testing day with no significant differences in control solution or total solution intakes between the counterbalanced OA-SALT and OA-ETOH preference-testing days 1 and 2. In addition for the OA-ETOH group, there was a significant main effect of injection on the total solution intake ( $F_{1,36}=8.963$ ,  $p<0.01$ ), intake of test solution ( $F_{1,36}=85.034$ ,  $p<0.01$ ) and intake of the control solution ( $F_{1,36}=3.958$ ,  $p=0.05$ ). There were no significant interactions between days and injection variables for the OA-ETOH conditioned stimulus group. As shown in Fig. 6A and B, post hoc tests revealed a significant decrease ( $p<0.05$ ) in total solution intake for the LiCl injection group on the preference-testing days for OA-ETOH and OA-SALT with no significant difference in total intake on the LA-ETOH preference-testing day (Fig. 6C). Post hoc tests also confirmed significant decreases ( $p<0.01$ ) in the consumption of the test solution for the LiCl injection group across all three preference-testing days, indicating a conditioned avoidance to OA-ETOH (Fig. 6A) and a generalized avoidance of OA-SALT (Fig. 6B) and LA-ETOH (Fig. 6C). Post hoc tests indicated a significant increase ( $p<0.01$ ) in control solution intake on the LA-ETOH preference-testing day for the LiCl injection group.

When the conditioned stimulus was OA-SALT, there was no significant main effect of days, but there was a significant main effect of injection on the intake of the test solution ( $F_{1,36}=38.175$ ,  $p<0.01$ ) and the intake of the control solution ( $F_{1,36}=29.716$ ,  $p<0.01$ ). There were no significant interactions between the days and injection variables for the OA-SALT conditioned stimulus group. Post hoc tests indicated significant decreases for the intake of the test solution on every preference-testing day for the LiCl group suggesting formation of conditioned and generalized avoidances to all of the FFAs. During the two-bottle preference testing, the animals conditioned to avoid OA-SALT consumed significantly less OA-SALT ( $p<0.05$ ), OA-ETOH ( $p<0.01$ ) and LA-SALT ( $p<0.01$ ) than the NaCl injection group as shown respectively in Fig. 6D, E and F. As there were no significant differences in the total solution intake between the two injection groups, the reduction in consumption of the test solution by the LiCl injection group was compensated with an increase in the consumption of control solutions compared to the NaCl injection group.

Fig. 7 displays the preference score results for the stimulus generalizations between the unesterified and sodium salt forms of linoleic and oleic acid. There was a main effect of injection

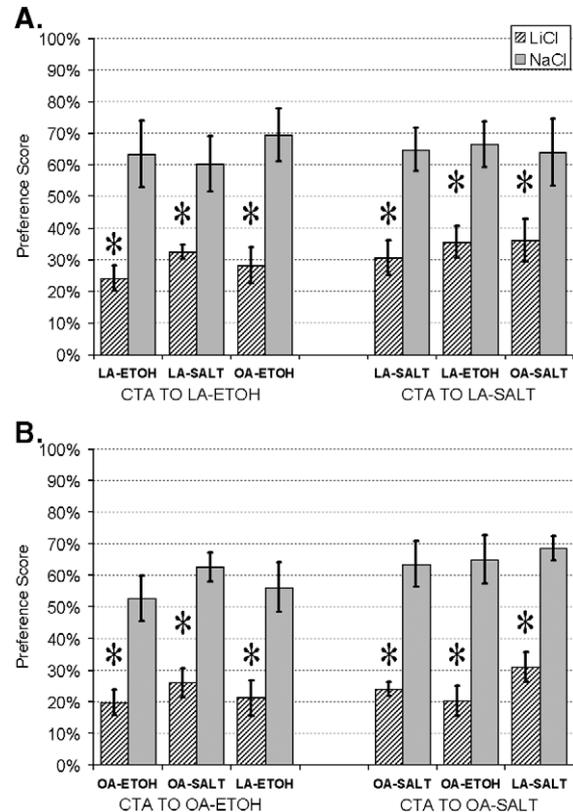


Fig. 7. Significant reductions in preference scores for the LiCl injection groups (hatched bars) compared to the control groups (filled bars) provide evidence of CTA formation and stimulus generalizations within and between the unesterified and salt based forms of the FFAs when the conditioned stimulus is linoleic acid (A) or oleic acid (B). Stars indicate  $p<0.01$  significant differences between injection groups.

for the preference scores of each conditioned stimulus group (LA-ETOH  $F_{1,42}=43.022$ ,  $p<0.01$ ; OA-ETOH  $F_{1,36}=76.560$ ,  $p<0.01$ ; LA-SALT  $F_{1,42}=31.666$ ,  $p<0.01$ ; OA-SALT  $F_{1,36}=92.650$ ,  $p<0.01$ ). The ability of rats to form a conditioned taste aversion to 88  $\mu\text{M}$  linoleic acid (Fig. 7A) and 88  $\mu\text{M}$  oleic acid (Fig. 7B) and subsequently avoid future consumption of the FFA in a two-bottle preference test was confirmed through post hoc tests (all comparisons at a level of  $p<0.01$ ). Furthermore, rats avoided linoleic and oleic acid regardless of whether the tested stimulus was the unesterified FFA (LA-ETOH  $p<0.01$ , OA-ETOH  $p<0.01$ ) or sodium salt FFA (LA-SALT  $p<0.01$ , OA-SALT  $p<0.01$ ) when the conditioned stimulus was the alternative form of the FFA. Finally, there were stimulus generalizations between both linoleic and oleic acid. Linoleic acid formed stimulus generalizations with oleic acid in the unesterified form ( $p<0.01$ ) and in the sodium salt form ( $p<0.01$ ). Oleic acid formed stimulus generalizations with linoleic acid in both the unesterified form ( $p<0.01$ ) and in the sodium salt form ( $p<0.01$ ).

## 8. Discussion

This study is the first to characterize the detection threshold of linoleic and oleic acid in rats. In a conditioned taste aversion paradigm, rats can detect and avoid concentrations of linoleic

and oleic acid that are greater than or equal to 66  $\mu\text{M}$  but fail to avoid 44  $\mu\text{M}$  concentrations of either FFA. The detection and avoidance of linoleic and oleic acid is independent of whether an unesterified form of the FFA is dissolved in 5 mM ethanol or the aqueous sodium salt form of the FFA is used as the conditioned or tested stimulus. The conditioning of a taste aversion to linoleic acid resulted in a generalized stimulus avoidance of oleic acid and likewise the conditioning of a taste aversion to oleic acid resulted in a generalized avoidance of linoleic acid. These results suggest that linoleic and oleic acid share similar orosensory properties. Furthermore, the avoidance of linoleic acid following a CTA pairing did not generalize to either ethanol or NaCl suggesting that linoleic acid is the salient stimulus as opposed to another stimulus concomitantly present, such as the delivery solution or saliva.

Previous research has demonstrated a preference for dietary fat when textural cues [9,16,34,42,48] and post-ingestive cues [16,34] have been controlled or olfactory cues eliminated [48]. Furthermore, a specific preference for FFAs has been demonstrated in studies controlling for textural cues [13,24,48,49] or blocking olfactory cues [13,48]. This preference of FFAs was confirmed in the present study with the control injection groups consistently drinking two to three times the amount of 88  $\mu\text{M}$  linoleic or oleic acid than the control solutions resulting in preference scores ranging from 60% to 75%. An innate taste preference for FFAs is beneficial in identifying and promoting the consumption of essential fatty acids, such as linoleic acid, that are necessary for good health and can not be produced by animals.

Since an 88  $\mu\text{M}$  solution of linoleic or oleic acid has a negligible difference in viscosity from water and a very low nutritional content (0.014 cal/60 ml), it is highly unlikely that rats in the current study are detecting the FFAs on the basis of textural or post-ingestive cues. It also seems unlikely that detection of the FFAs was based on an olfactory cue as the low concentration of ethanol added to the linoleic acid solution as the conditioned stimulus solution for experiment 3 can clearly be detected through olfaction and yet rats did not form a generalized aversion to ethanol in the absence of linoleic acid. The selective avoidance demonstrated in experiment 3 suggests that linoleic acid was the salient stimulus rather than an olfactory cue from ethanol.

Our results indicate that the detection threshold for linoleic and oleic acid is in the micromolar concentration range that is only slightly higher than the concentrations of FFAs that elicit responses in patch clamp recordings of isolated taste receptor cells [14,15,18]. An orosensory signal is the most likely cue used by the rats to detect and avoid linoleic and oleic acid. This hypothesis is supported by data from ongoing chorda tympani transection experiments conducted by our laboratory. Preliminary results suggest that the ability of rats to detect linoleic acid is eliminated when the afferent neural pathways of the gustatory system are compromised [19,29,41]. We are continuing this investigation using short duration stimulus presentations (30 s) and additional gustatory nerve transections (chorda tympani and glossopharyngeal nerves).

Recent work in humans suggests that the ability to detect FFAs through orosensory stimulation is not unique to the rodent animal model [23,37]. Human subjects identified as being able to taste 6-*n*-propyl thiouracyl (PROP) were able to successfully discriminate the presence of 10  $\mu\text{M}$  linoleic acid [23]. Similar to our findings in rats, these subjects were able to detect linoleic acid in the absence of other taste stimuli. Human tasters of PROP have also been able to correctly identify the addition of linoleic acid to vanilla ice cream [37] suggesting that linoleic acid is either detectable when other salient taste stimuli are concomitantly present or the presence of linoleic acid may produce detectable changes in the sensation of other concomitant taste stimuli. The proposed gustatory transduction mechanism of FFAs, inhibition of a delayed-rectifying potassium channel [14], could theoretically produce independent taste sensations as well as enhance the perceived stimulus intensity of concomitant tastants. The results of the current study suggest that, in rats, linoleic and oleic acid generate a detectable orosensory sensation independent of other concomitant taste stimuli. However, dietary fat has been identified as being able to increase the palatability of food [7,30,31,52] and evidence from another research project underway in our laboratory suggests that the addition of linoleic or oleic acid to taste solutions can in fact alter rats' consumption of the tastants in a manner reflective of an increase in perceived stimulus intensity [20,25]. Therefore, there is behavioral evidence in rats and humans of both predicted outcomes of the proposed FFA gustatory transduction mechanism that FFAs may produce independent sensations as well as enhance the perceived intensity of other taste stimuli.

As evidence supporting a role of the gustatory system in the detection of FFAs accumulates, several major questions remain to be answered. Currently, we are conducting experiments examining the ability of rats to detect FFAs during brief stimulus trials following gustatory nerve transections in order to identify a signal pathway from the oral cavity to the brain. In addition, we are examining the ability of rats to form stimulus generalizations between FFAs and stimuli representing the four prototypical taste categories, sweet, sour, salt and bitter. Finally, the applicability of the characteristics of the rodent ability to detect and prefer free fatty acids to a human application remains to be sufficiently investigated. The results of this study reporting an identification of a behavioral detection threshold for linoleic and oleic acid in rats represents a significant contribution toward understanding the rodent chemoreception of FFAs, which, in turn, adds to the collective knowledge of the influence of FFAs on the gustatory system. In time, future exploration in this new chemoreceptive ability may hold the promise of being able to manipulate the pre-ingestive influences of FFAs in order to aid in the control of obesity due to high dietary fat consumption.

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