Original Article

Postnatal Exposure to Ethanol Increases Its Oral Acceptability to Adolescent Rats

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Abstract

The aversive flavor of ethanol limits intake by many consumers. We asked whether intermittent consumption of ethanol increases its oral acceptability, using rats as a model system. We focused on adolescent rats because they (like their human counterparts) have a higher risk for alcohol overconsumption than do adult rats following experience with the drug. We measured the impact of ethanol exposure on 1) the oral acceptability of ethanol and surrogates for its bitter (quinine) and sweet (sucrose) flavor components in brief-access lick tests and 2) responses of the glossopharyngeal (GL) taste nerve to oral stimulation with the same chemical stimuli. During the exposure period, the experimental rats had access to chow, water and 10% ethanol every other day for 16 days; the control rats had access to chow and water over the same time period. The experimental rats consumed 7–14 g/day of 10% ethanol across the exposure period. This ethanol consumption significantly increased the oral acceptability of 3%, 6% and 10% ethanol, but had no impact on the oral acceptability of quinine, sucrose or NaCl. The ethanol exposure also diminished responses of the GL nerve to oral stimulation with ethanol, but not quinine, sucrose or NaCl. Taken together, these findings indicate that ethanol consumption increases the oral acceptability of ethanol in adolescent rats and that this increased oral acceptability is mediated, at least in part, by an exposure-induced reduction in responsiveness of the peripheral taste system to ethanol per se, rather than its bitter and sweet flavor components.

Key words: adolescence, ethanol, glossopharyngeal nerve, licking responses, rats

Introduction

Ethanol elicits an aversive flavor. In humans, this flavor consists of a bittersweet taste, a burning trigeminal sensation and an aversive odor (Green 1987; Scinska et al. 2000; Mattes and DiMeglio 2001; Lanier et al. 2005). At low concentrations, the bitter taste is most salient, whereas at high concentrations, the burning sensation is most salient (Mattes and DiMeglio 2001; Allen et al. 2014; Nolden and Hayes 2015). There is evidence, however, that these aversive sensations are less intense in regular consumers of ethanol (Settle 1979; Mattes 1994; Lanier et al. 2005; Nolden and Hayes 2015; but see Mattes and DiMeglio

2001). This observation probably reflects both genetic and experiential factors. For instance, specific polymorphisms of several TAS2R bitter taste receptors (TAS2R38 and TAS2R3/4/5) predispose people to experience less bitterness when consuming ethanol (Allen et al. 2014; Nolden et al. 2016). Repeated exposure to ethanol can also make components of its flavor more acceptable. For instance, fetal alcohol exposure (FAE) increases the acceptability of ethanol's odor during adolescence (Hannigan et al. 2015), and postnatal exposure to ethanol odor increases the acceptability of the same odor in infants and toddlers (Mennella and Beauchamp 1999; Mennella 2004).

Rodents are an important model system for understanding the neural mechanisms regulating ethanol intake. When ingesting ethanol, rodents experience a quinine- and sucrose-like taste (Di Lorenzo et al. 1986; Kiefer and Lawrence 1988), a capsaicin-like burning sensation (Trevisani et al. 2002; Ellingson et al. 2009), and an aversive odor (Youngentob and Glendinning 2009). Several factors are known to make the flavor of ethanol more acceptable to rats. FAE can reduce the quinine-like bitter taste (Youngentob and Glendinning 2009), capsaicin-like burning sensation (Glendinning et al. 2012), and aversive odor (Youngentob and Glendinning 2009) of ethanol in adolescent rats. These FAE-induced behavioral changes are mediated, at least in part, by attenuated responses of 1) peripheral taste nerves (chorda tympani [CT] and glossopharyngeal [GL]) (Glendinning et al. 2017); 2) trigeminal chemosensory neurons (Glendinning et al. 2017); and 3) the peripheral olfactory epithelium (Youngentob et al. 2007). Postnatal alcohol exposure (PAE) can also improve the acceptability of ethanol's flavor in adult rats (Kiefer et al. 1994; Loney and Meyer 2018).

Here, we examined the impact of PAE in adolescent rats. We focused on adolescents because they (like their human counterparts) have a higher risk for alcohol abuse than adults (Bates and Labouvie 1997; Doremus et al. 2005). We asked 2 questions. Does PAE improve the oral acceptability of ethanol's flavor in adolescent rats? If so, are the PAE-induced changes in oral acceptability mediated, at least in part, by the peripheral taste system? In experiment 1, we measured unconditioned licking responses to ethanol, quinine, sucrose, and NaCl, both before and after PAE. In experiment 2, we measured responses of the GL nerve to oral stimulation with the ethanol, quinine, sucrose, and NaCl after PAE. We selected the GL nerve because it innervates the extensive field of circumvallate and foliate taste buds in the back of the tongue and because it responds to oral stimulation with ethanol, sucrose, quinine, and NaCl in adolescent rats (Glendinning et al. 2017).

Materials and methods

Animals and maintenance

We purchased adolescent Long Evans Hooded rats from Envigo (<http://www.envigo.com>). They arrived on postnatal day 26 (i.e., P26), weighing 71–95 g. There were an equal number of males and females. The rats were housed individually in standard polycarbonate cages in a temperature- and humidity-controlled vivarium on a 12-h light/dark cycle. Water was provided through sipper tubes, and laboratory chow (5001, PMI Nutrition International) was available through the cage lid. Rats were weighed weekly. Food and water were provided ad libitum, unless specified otherwise. All experimental procedures were approved by the Institutional Animal Care and Use Committees at Columbia University. The protocols were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Impact of PAE on oral acceptability of ethanol and its flavor components (experiment 1)

Using a within-subject design, we measured the oral acceptability of 4 chemical stimuli (ethanol, quinine, sucrose, and NaCl) in a gustometer, using the brief-access lick test (Glendinning et al. 2002). We measured licking responses of the rats both before and after they were subjected to an intermittent ethanol exposure regime (Simms et al. 2008; Loney and Meyer 2018). During this exposure regime, the rats were each provided ad libitum access to chow plus 2 sipper tubes, every other day for 16 days. For the experimental rats ($n = 6$

males, 6 females), one sipper tube dispensed 10% ethanol and the other water; for the control rats ($n = 6$ males, 6 females), both sipper tubes dispensed water. We measured daily intake of each solution gravimetrically. During the “off” days, the rats had ad libitum access to chow plus one bottle of water.

We conducted gustometer training and the first phase of lick testing from P29 to P38, ethanol exposure from P39 to P55, and the second phase of lick testing from P56 to P61. In the following sections, we provide the methodological details.

Lick testing paradigm

We tested a range of concentrations of each chemical stimulus, in the following order: ethanol (1%, 3%, 6%, 10%, 20% v/v), quinine dihydrochloride (0.006, 0.01, 0.03, 0.1, 0.3, 1.0 mM), NaCl (0.03, 0.1, 0.3, 0.6, 1 M), and sucrose (0.03, 0.1, 0.2, 0.3, 0.6, 1.0 M). All chemicals were obtained from Sigma–Aldrich. We included NaCl as a positive control stimulus. Prior studies demonstrated that the indicated concentrations of each chemical stimulus span the dynamic range of licking responses in adolescent rats (Youngentob and Glendinning 2009). We dissolved each of the chemical stimuli in distilled water and tested each solution at room temperature.

In a test session, a rat was offered the range of concentrations of one chemical stimulus. We measured the number of licks to each concentration during successive 10-s trials, over a 30-min test session. The intertrial interval was 7.5 s. A computer-controlled gustometer (Davis MS160; DiLog Instruments) recorded licks and presented the different concentrations of each chemical stimulus according to a randomized block design. The rats could initiate up to 102 trials during each test session.

Each rat underwent 3 days of training in the gustometer. To motivate licking, it was water deprived for 23 h prior to each training session. On day 1, the rat was allowed to lick freely for water from a single stationary sipper tube for 30 min. On days 2 and 3, it was allowed to lick from 5 different sipper tubes, each during different trials. At the end of each trial, the shutter was closed and then reopened after a different sipper tube was positioned in the center of the slot.

We tested each chemical stimulus on separate days. To motivate licking for the aversive solutions (ethanol, QHCl, and NaCl), the rats were water-deprived for 23 h before testing. To motivate licking for the sucrose solutions, the rats were food-deprived for 23 h before testing.

We converted licking responses to the aversive solutions to a tastant/water lick ratio. This involved dividing the mean number of licks per trial for a given taste stimulus concentration by the mean number of licks per trial for water alone. A tastant/water lick ratio of 1.0 reveals that the rats licked for the stimulus solution at the same rate as they did for water, whereas a ratio approaching 0.0 indicates that the rats licked much less frequently for the stimulus solution than they did for water.

We converted licking responses to sucrose to a standardized lick ratio (SLR). This involved several steps. First, we determined the mean interlick interval (ILI), based on the population of ILIs < 200 ms during the first training session; then, we took the reciprocal of this mean to determine lick rate (i.e., licks per second). Next, we multiplied the lick rate by a scaling factor of 10 to estimate the maximal number of licks that a rat could generate if it licked continuously across the 10-s trial. Finally, we calculated the SLR by dividing the average number of licks per trial for a given chemical stimulus by the maximal potential licks per trial. An SLR approaching 0.0 indicates that the taste stimulus elicited sporadic licking, whereas one approaching 1.0 indicates that it elicited nearly continuous licking across each 10-s trial.

Data analysis

We analyzed licking responses to each chemical stimulus separately, using a mixed-model analysis of variance (ANOVA). The between factors were ethanol exposure (i.e., control vs. ethanol exposure regime) and sex, and the within factors were stimulus concentration and time (i.e., licking responses before vs. after exposure). We compared licking responses across exposure regimens at a given concentration, using Sidak's multiple comparison test, to evaluate significant interaction terms. In this and all subsequent tests, we set the alpha level at 0.05 and controlled for sphericity by adjusting the degrees of freedom (df) according to the Greenhouse–Geisser correction. We conducted the statistical analyses with IBM SPSS Statistics 25 (<https://www.ibm.com/us-en/marketplace/analytics>).

Impact of PAE on responsiveness of the GL nerve to ethanol and its flavor components (experiment 2)

Here, we asked whether the intermittent ethanol exposure regime (as described in experiment 1) alters responsiveness of the GL nerve to oral stimulation with ethanol or its bitter and sweet flavor components. We exposed the experimental ($n = 3$ males, 4 females) and control ($n = 4$ males, 3 females) rats to their respective ethanol or control fluid exposure regimen from P39 to P55 and conducted the GL nerve recordings from P56 to P60.

Test solutions

We tested 4 concentrations of each of 4 chemical stimuli in the following order: NaCl (0.03, 0.1, 0.3, 1 M), sucrose (0.03, 0.1, 0.3, 1 M), quinine (1, 3, 10, 20 mM), and ethanol (1%, 3%, 6%, 10%). We used 0.5 M NH_4Cl as the reference stimulus and as a control for any potential time-dependent changes in responsiveness. We recorded responses to 0.5 M NH_4Cl both before and after each concentration series for a taste stimulus. All chemicals were dissolved in an artificial saliva solution (Ogawa et al. 1972), which consisted of 17.52 g of NaCl, 32.8 g of KCl, 6.7 g of CaCl_2 , and 1.16 g of MgCl_2 dissolved in 20 L of deionized water.

Anesthesia and surgery

The rats were anesthetized with isoflurane (Baxter). Afterwards, we secured each rat in a nontraumatic head holder and maintained it on a circulating-water heating pad (HTP-1500, Adroit Medical Systems) set at 37 °C.

We accessed the GL nerve with a ventral approach, similar to that described by Martin and Sollars (2015). In brief, we removed the posterior digastric muscle, posterior horn of the hyoid bone, and overlying tissue. We used the hypoglossal nerve as a guide to locate the GL nerve where it exits the skull. A 10-cm polyethylene tube (with a flange on its back end) was inserted through the mouth and into the esophagus; it exited through a small incision approximately halfway to the stomach. We sutured the esophagus anterior to the incision point. The flange on the back end of the polyethylene tube 1) prevented the back end of the tube from entering the esophagus; 2) ensured that the back end of the polyethylene tube rested at the entrance to the pharynx, permitting taste solutions to access the circumvallate and foliate papillae; and 3) prevented taste stimuli from entering the pharynx.

Stimulus application

For each stimulation trial, we infused 5 mL of chemical stimulus into the back of the mouth (at 35 °C). The taste stimulus was left in the mouth for 20 s, after which it was flushed out with artificial saliva solution until

nerve activity returned to baseline. One of the challenges of stimulating taste buds in the posterior tongue is that they are recessed within the circumvallate and foliate trenches. To open the entrance of the trenches, we applied traction to the tongue via a suture in the median eminence. To help “push” the chemical solutions into the trenches, we rapidly infused the 5 mL of chemical stimulus into the back of the mouth (i.e., within 3–5 s). Although the oral infusion procedure produced a transient mechanical response, it was excluded from the analysis.

Electrophysiological recordings

The GL nerve was desheathed prior to placing it on the platinum wire electrode. We attached the ground electrode to a nearby salivary gland. Electrophysiological responses were amplified via a bio-amplifier (ISO-80, World Precision Instruments), passed through a band-pass filter (300–3000 Hz), and then digitized (sampling rate: 1000 samples/s), transformed (root mean square), and integrated (time constant: 1 s) (Biopac Software).

Data analysis

To calculate the relative response of the GL nerve to each taste solution, we determined the mean integrated response (in millivolt per second) over 20 s prior to stimulation (baseline response) and during the 20 s of steady stimulation by a chemical stimulus (taste response). Note that, we considered the 20-s response to have begun after the transient mechanical response ended. We calculated the absolute response (taste response – baseline response). We determined the relative response by dividing the absolute response to a taste stimulus (e.g., 0.3 M NaCl) by the mean absolute responses to 0.5 M NH_4Cl (determined both before and after each concentration series).

We analyzed the relative nerve responses separately for each chemical stimulus, using a mixed-model ANOVA. We treated stimulus concentration as a within factor and adolescent ethanol exposure (i.e., control vs. experimental) and sex as between factors. In the event of a significant interaction between adolescent ethanol exposure regimen and concentration of a given stimulus, we compared nerve responses across exposure regimens at each concentration, using Sidak's multiple comparison test. We also compared responses at a given concentration to zero, using a 1-sample *t*-test with Bonferroni correction.

Results

Impact of PAE on oral acceptability of ethanol and its flavor components (experiment 1)

Supplemental Figure 1A shows total daily fluid intake over the course of the exposure period by control and experimental rats. There was a significant main effect of time on total intake, reflecting slightly higher daily fluid intake during the middle of the exposure period (Supplemental Table 1). Supplemental Figure 1B illustrates ethanol intake (g/kg rat) by experimental rats. There was no significant effect of sex or time on mass-specific ethanol intake (mean intake/day was 4.5 g/kg rat; Supplemental Table 1). Supplemental Figure 1C shows intake from the 2 water bottles by control rats (left panel) and from the water and ethanol bottles by experimental (right panel). For control rats, there was no significant difference in intake from each of the water bottles (Supplemental Table 1). In contrast, the experimental rats consumed significantly less 10% ethanol than water. The absence of a significant main effect of time, or interaction of intake $\times$ time shows that the experimental rats consistently drank less of the 10% ethanol (overall mean = 9.9 g/day) than water (overall mean = 26.2 g/day) over the exposure period. The significant

main effect of sex reflects the fact that experimental males consumed more total fluid (i.e., water plus 10% ethanol) than did experimental females, independent of fluid treatment.

Figure 1 illustrates how short-term licking responses to ethanol differed between control and experimental rats. In the top row of panels, we show mean lick ratios; in the bottom row, we show lick ratios from individual rats. There was a significant main effect of ethanol concentration, revealing that the lick ratio decreased with increasing ethanol concentration in all rats (Table 1). Furthermore, there was a significant main effect of time (i.e., pre- vs. postexposure) and interaction of fluid treatment $\times$ time. These results, together with visual inspection of Figure 1, reveal that whereas the concentration–response (C–R) curve for control rats did not change over the exposure period, that for experimental rats shifted significantly to the right. Indeed, a multiple comparison test revealed that the lick ratios for 3%, 6%, and 10% ethanol were significantly higher postexposure in experimental rats. This establishes that ethanol exposure increased oral acceptability of the intermediate concentrations of ethanol.

Figure 2 shows how ethanol exposure altered licking for quinine, NaCl, and sucrose in the experimental rats. For quinine and NaCl, there was a significant main effect of stimulus concentration (Table 2), revealing that the oral acceptability of both chemical stimuli decreased with increasing concentration. For sucrose, there was a significant main effect of concentration, showing that its oral

acceptability increased with concentration. There was also a significant interaction of concentration $\times$ time. A post hoc test demonstrated that the lick ratios for 0.1 M sucrose decreased significantly over the exposure period in both control and experimental rats; whereas the lick ratios for 1 M sucrose increased significantly over the exposure period in both control and experimental rats.

Impact of PAE on responsiveness of the GL nerve to ethanol and its flavor components (experiment 2)

Supplemental Figure 2A shows total daily fluid intake over the exposure period by control and experimental rats. There was a significant main effect of time, revealing an overall increase in fluid intake across the 8 exposure days, independent of fluid treatment (Supplemental Table 2). Supplemental Figure 2B reveals mass-specific ethanol intake (g/kg rat) across the exposure period by experimental rats. The effect of time was significant, reflecting the fact that mass-specific ethanol intake decreased over the exposure period (Supplemental Table 2). Although this time-dependent decrease in mass-specific ethanol intake was not observed in experiment 1, the mean mass-specific ethanol intake per day was indistinguishable across both experiments (4.4 vs. 4.5 g/kg rat in experiments 1 and 2, respectively). Supplemental Figure 2C shows intake from each of the bottles by control and experimental rats. For control rats, there was no significant main effect of fluid treatment or sex, but there was a significant main effect of time. The latter result reflects an overall

Figure 1. Ethanol exposure increased the oral acceptability of 3%, 6%, and 10% ethanol. The top row of panels shows mean ($\pm$ standard error) tastant/water lick ratios, whereas the bottom row illustrates each individual rat's tastant/water lick ratios as a line. We show licking responses of rats both before (in red) and after (in blue) exposure to the control or ethanol regime. A tastant/water lick ratio approaching 1.0 indicates that the rat licked for the chemical stimulus at the same rate as it did for water, whereas a ratio approaching 0 indicates the chemical stimulus strongly inhibited licking. The lick ratios are analyzed in Table 1. An asterisk indicates the concentrations at which the postexposure lick ratios are significantly greater than the pre-exposure lick ratios ($P < 0.05$, Sidak's multiple comparison test).

Table 1. Results of a 4-way mixed-model ANOVA performed on the lick ratios in control and experimental rats, separately for each chemical stimulus (data in [Figures 1](#) and [2](#))

Chemical stimulus	Source of variation	df	F ratio	P value	Significant?
Ethanol	EE	1, 20	0.23	0.64	
	Time	1, 20	24.4	<0.001	Yes
	SC	2.8, 56.0	307.7	<0.001	Yes
	Sex	1, 20	<0.1	0.88	
	EE × SC	2.8, 56.0	1.5	0.23	
	EE × Time	1, 20	7.9	0.01	Yes
	EE × Sex	1, 20	<0.1	0.76	
	Time × Sex	1, 20	<0.1	0.81	
	Time × SC	2.7, 54.3	7.0	0.001	Yes
	SC × Sex	2.8, 56.0	0.6	0.61	
	EE × Time × SC	2.7, 54.3	1.1	0.35	
	EE × Time × Sex	1, 20	<0.1	0.78	
	EE × SC × Sex	2.8, 56.0	1.0	0.38	
	Time × SC × Sex	2.7, 54.3	1.4	0.26	
	EE × Time × SC × Sex	2.7, 54.3	0.8	0.54	
Quinine	EE	1, 20	<0.1	0.96	
	Time	1, 20	0.3	0.60	
	SC	2.7, 53.8	267.1	<0.001	Yes
	Sex	1, 20	0.9	0.37	
	EE × SC	2.9, 57.2	1.1	0.37	
	EE × Time	1, 20	0.8	0.38	
	EE × Sex	1, 20	0.1	0.73	
	Time × Sex	1, 20	0.1	0.75	
	Time × SC	2.9, 57.2	1.0	0.38	
	SC × Sex	2.7, 53.8	1.0	0.40	
	EE × Time × SC	2.9, 57.2	1.0	0.41	
	EE × Time × Sex	1, 20	1.0	0.38	
	EE × SC × Sex	2.7, 53.8	0.4	0.56	
	Time × SC × Sex	2.9, 57.2	1.3	0.29	
	EE × Time × SC × Sex	2.9, 57.2	1.1	0.37	
Sucrose	EE	1, 20	0.25	0.62	
	Time	1, 20	0.2	0.70	
	SC	1.4, 28.9	48.4	<0.001	Yes
	Sex	1, 20	0.6	0.43	
	EE × SC	1.4, 28.9	2.4	0.13	
	EE × Time	1, 20	0.4	0.54	
	EE × Sex	1, 20	0.5	0.50	
	Time × Sex	1, 20	0.5	0.49	
	Time × SC	3.0, 60.9	11.0	<0.001	Yes
	SC × Sex	1.4, 28.9	0.2	0.79	
	EE × Time × SC	3.0, 60.9	0.5	0.70	
	EE × Time × Sex	1, 20	4.0	0.06	
	EE × SC × Sex	1.4, 28.9	0.3	0.64	
	Time × SC × Sex	3.0, 60.9	0.7	0.58	
	EE × Time × SC × Sex	3.0, 60.9	0.8	0.48	
NaCl	EE	1, 20	3.6	0.07	
	Time	1, 20	0.2	0.69	
	SC	2.4, 47.6	293.5	<0.001	Yes
	Sex	1, 20	0.2	0.69	
	EE × SC	2.4, 47.6	0.4	0.56	
	EE × Time	1, 20	2.0	0.15	
	EE × Sex	1, 20	<1.0	0.81	
	Time × Sex	1, 20	0.7	0.41	
	Time × SC	2.1, 42.3	2.1	0.14	
	SC × Sex	2.4, 47.6	0.1	0.90	
	EE × Time × SC	2.1, 42.3	0.3	0.76	
	EE × Time × Sex	1, 20	3.1	0.09	
	EE × SC × Sex	2.4, 47.6	0.29	0.34	
	Time × SC × Sex	2.1, 42.3	1.6	0.22	
	EE × Time × SC × Sex	2.1, 42.3	1.1	0.34	

The between factors were ethanol exposure (i.e., control vs. ethanol exposure regime) and sex, and the within factors were stimulus concentration and time (i.e., before vs. after exposure to the diet). We controlled for sphericity by adjusting the df with the Greenhouse–Geiser correction. EE, ethanol exposure; SC, stimulus concentration.

Figure 2. Ethanol exposure did not alter the oral acceptability of a range of concentrations of quinine, NaCl and sucrose. We show licking responses of rats to each stimulus both before (open circles) and after (closed circles) the control or ethanol exposure. Licking responses (mean $\pm$ standard error) to quinine and NaCl are presented as a tastant/water lick ratio. A tastant/water lick ratio of approaching 1.0 (dashed line) indicates that the rat licked for the chemical stimulus at the same rate as it did for water, whereas a ratio approaching 0 indicates rat licked less rapidly for the chemical stimulus (relative to water). Licking responses to sucrose are presented as an SLR. An SLR near 0 indicates that the rats licked sporadically for sucrose, whereas an SLR approaching 1.0 indicates that the rats licked throughout the 10-s trial for sucrose. The analysis of the lick ratios is presented in Table 1, separately for each chemical stimulus. When there was a significant main effect fluid treatment for a given chemical stimulus, we compared SLRs from before and after exposure to the control or experimental diet, using Sidak's multiple comparison test (* $P < 0.05$).

increase in daily water intake over the exposure period. For experimental rats, there was a significant main effect of fluid treatment and time. This establishes that the experimental rats consistently drank less 10% ethanol (overall mean = 10.4 g/day) than water (overall mean = 22 g/day; range = 17–20) over the exposure period, but that daily intake of both solutions increased over the exposure period (Supplemental Table 2).

Figure 3 shows typical integrated whole-nerve responses of the GL nerve to oral stimulation with ethanol, quinine, sucrose, and NaCl. Figure 4 shows relative responses to the same chemicals. There was a significant main effect of concentration on GL nerve responses to

ethanol, quinine, sucrose, and NaCl (Table 2), revealing concentration-dependent increases in responsiveness to all chemical stimuli. In addition, there was a significant main effect of fluid treatment on GL nerve responses to ethanol, but not to sucrose, quinine, or NaCl. A Sidak's multiple comparison test revealed that the relative response to 6% ethanol was significantly lower in experimental than control rats. Furthermore, a 1-sample t -test showed that for control rats, the relative responses to 3%, 6%, and 10% ethanol were all significantly greater than 0 (in each comparison, t value > 4.2 , $df = 6$, $P < 0.006$), whereas for experimental rats, the relative responses to 10% ethanol alone was significantly greater than 0 (t value = 3.2, $df = 6$, $P < 0.012$).

Table 2. Results of 3-way mixed-model ANOVAs performed on GL nerve responses in adolescent rats, separately for each chemical stimulus (data in Figure 4)

Chemical stimulus	Source of variation	df	F ratio	P value	Significant?
Ethanol	EE	1, 10	9.0	0.013	Yes
	SC	2.6, 25.6	6.2	0.004	Yes
	Sex	1, 10	2.3	0.16	
	EE × SC	2.6, 25.6	0.3	0.79	
	EE × Sex	1, 10	2.7	0.13	
	SC × Sex	2.6, 25.6	0.7	0.52	
	EE × SC × Sex	2.6, 25.6	0.3	0.79	
Quinine	EE	1, 10	0.5	0.48	
	SC	2.1, 21.4	8.0	0.002	Yes
	Sex	1, 10	<0.1	0.97	
	EE × SC	2.1, 21.4	<0.1	0.94	
	EE × Sex	1, 10	0.7	0.42	
	SC × Sex	2.1, 21.4	1.1	0.37	
	EE × SC × Sex	2.1, 21.4	1.4	0.27	
Sucrose	EE	1, 10	<0.1	0.84	
	SC	1.5, 15.1	9.0	0.005	Yes
	Sex	1, 10	1.2	0.30	
	EE × SC	1.5, 15.1	0.4	0.63	
	EE × Sex	1, 10	0.3	0.59	
	SC × Sex	1.5, 15.1	0.9	0.42	
	EE × SC × Sex	1.5, 15.1	0.4	0.61	
NaCl	EE	1, 11	1.0	0.34	
	SC	1.1, 12.6	33.6	<0.001	Yes
	Sex	1, 11	0.9	0.37	
	EE × SC	1.1, 12.6	0.9	0.38	
	EE × Sex	1, 11	1.9	0.20	
	SC × Sex	1.1, 12.6	0.4	0.58	
	EE × SC × Sex	1.1, 12.6	0.9	0.37	

The between factors were ethanol exposure and sex, and the within factor was stimulus concentration. We controlled for sphericity by adjusting the degrees of freedom with the Greenhouse–Geiser correction. EE, ethanol exposure; SC, stimulus concentration.

Figure 3. Typical whole-nerve integrated responses of the GL nerve (from a control and experimental rat) to oral stimulation with 0.5 M NH_4Cl , 1 M NaCl, 20 mM quinine, 1 M sucrose, and 9% ethanol. We identify several features of the integrated neural response directly on the traces for 0.5 M NH_4Cl : 1) transient responses to infusion of the chemical stimulus or the artificial saliva rinse into the oral cavity; 2) the taste response to the chemical stimulus (the dashed line indicates the time period over which the taste response was quantified); and 3) a time-scale bar.

Finally, our use of “relative response” as the dependent measure assumed that fluid treatment did not alter GL nerve responses to 0.5 M NH_4Cl (the normalizing stimulus). To test this assumption, we compared the absolute response of the GL nerve to 0.5 M

NH_4Cl across the experimental and control rats. That the absolute responses did not differ between control and experimental rats (unpaired t value = 2.2, df = 12, P = 0.35) justifies our use of 0.5 M NH_4Cl as the normalizing stimulus.

Figure 4. Ethanol exposure reduced responses of the GL nerve to intraoral infusions of ethanol. We show relative responses (mean $\pm$ standard error) of the GL nerve in control and experimental rats to a range of concentrations of (A) sucrose, (B) ethanol, (C) quinine, and (D) NaCl. The relative response was calculated by dividing the integrated response to a given taste stimulus by that to 0.5 M NH_4Cl . The analyses of the relative responses are presented in Table 2. If the main effect of ethanol exposure on the response to a particular stimulus was significant, then we compared relative response with each concentration across control and experimental rats (separately for each age class), using Sidak's multiple comparison test ($*P < 0.05$). We asked whether responses to select ethanol concentrations were significantly greater than 0, using a 1-sample *t*-test. For control rats, we did so for 3%, 6%, and 10% ethanol ($*P < 0.006$), and for experimental rats, we did so for 10% ethanol ($*P < 0.012$).

Discussion

We discovered that intermittent access to 10% ethanol increased the oral acceptability of 3%, 6%, and 10% ethanol, but had no impact on the oral acceptability of quinine, sucrose, or NaCl in adolescent rats. Similar observations have been made previously in adult rats. For example, intermittent exposure to 20% ethanol enhanced licking for 10% and 20% ethanol, but not quinine (Loney and Meyer 2018). Likewise, continuous exposure to 10% ethanol increased the frequency of appetitive orofacial responses (and decreased the frequency of aversive orofacial responses) to intraoral infusions of ethanol (Kiefer et al. 1994). Taken together, these results indicate that PAE can improve the flavor of ethanol in both adolescent and adult rats, but that the effect does not include changes in the sweet and bitter taste components of ethanol's flavor.

The control rats in the present study did not experience a shift in oral acceptability of ethanol across the exposure period. In contrast, the control rats in the study by Loney and Meyer (2018) did—they were more accepting of ethanol after the exposure period. We can propose 2 non-mutually exclusive explanations for these discrepant results. First, Loney and Meyer tested adult rats; we tested adolescent rats. The ethanol exposure that occurred during the initial brief-access lick test (i.e., before the exposure period) may have been sufficient to shift the oral acceptability of ethanol in adult, but not adolescent rats. Second, we used a lower range of ethanol concentrations (1%–20%) than did Loney and Meyer (1.25%–40%). Exposure to the higher (i.e., 40%) concentration of ethanol during the initial brief-access lick test could have made the taste of ethanol more acceptable during the second brief-access lick test.

We were surprised that PAE did not alter behavioral or GL nerve responses to sucrose and quinine. This is because previous research indicated that ethanol, quinine, and sucrose all activate overlapping taste pathways. For instance, sucrose and ethanol stimulated the same primary afferent fibers in the CT nerve of macaque (Hellekant et al. 1997) and taste-responsive neurons in the nucleus of the solitary tract (NST) of rat (Lemon et al. 2004). Likewise, quinine and ethanol activated the same primary afferent fibers in the CT nerve of

macaque (Hellekant et al. 1997) and taste-responsive neurons in the NST of rat (Lemon et al. 2004). Furthermore, the sweet taste receptor, $\text{T1r2} + \text{T1r3}$, was implicated in behavioral and neurophysiological processing of sucrose and ethanol taste in mouse (Brasser et al. 2010). Finally, FAE reduced behavioral and peripheral taste responses of adolescent rats to ethanol, sucrose, and quinine (Youngentob and Glendinning 2009; Glendinning et al. 2017). Our results thus indicate that PAE attenuated an ethanol-specific sensory pathway.

Because we made whole-nerve recordings, we could not determine the relative contribution of the taste versus nociceptive fibers in the GL nerve (Sweazey and Bradley 1989; Hayakawa et al. 2010) to the integrated responses. The available evidence indicates, however, that the nociceptive fibers made minimal contributions. First, one study found that ethanol concentrations $\geq 20\%$ are required to elicit consistent responses from nociceptor fibers in the lingual nerve of rats (Sostman and Simon 1991). We obtained consistent GL nerve responses to ethanol concentrations as low as 3%. Second, ethanol is thought to stimulate nociceptive fibers by diffusing through the oral epithelium (Mistretta 1971) and then activating TrpV1 (Trevisani et al. 2002; Ellingson et al. 2009) and G protein-coupled inwardly rectifying potassium channels (Bodhinathan and Slesinger 2014; Chung et al. 2014). This stimulation process appears to take 40–60 s to onset, based on whole-nerve recordings from the lingual nerve of rat (Simon and Sostman 1991). We obtained robust GL nerve responses in <5 s.

There are several lines of support for the existence of ethanol-selective taste fibers. For example, electrophysiological studies revealed that some of the primary afferent fibers in the GL and CT nerves of macaque (Hellekant et al. 1997; Danilova and Hellekant 2000) and CT nerve of rat (Sako and Yamamoto 1999) respond more strongly to oral stimulation with ethanol than sucrose or quinine. These ethanol-selective taste responses could have been mediated by a large-conductance calcium-sensitive potassium (BK) channel in taste cells (Akabas et al. 1990). BK channels are not only ethanol responsive, but their expression levels are modulated by ethanol consumption (Bettinger and Davies 2014).

It is likely that the increased acceptability of ethanol's flavor was mediated at least in part by the attenuating effect of PAE on the GL

nerve response. However, PAE may have caused additional physiological changes, which further increased the acceptability of ethanol's flavor. For example, PAE could have 1) attenuated the aversive olfactory (Youngentob et al. 2007) and oral trigeminal (Glendinning et al. 2017) attributes of ethanol or 2) conditioned a preference for ethanol's flavor based on its postoral nutritive actions (Ackroff and Sclafani 2001, 2002). At this point, we cannot determine the relative contribution of these different physiological changes to the observed increase in ethanol's acceptability.

Response to sucrose

In brief-access lick tests, we found that the slope of the C–R curve for sucrose became steeper over the course of the exposure period (Figure 3). Because the change in slope occurred in both control and experimental rats, it probably reflects a normal developmental process rather than an effect of PAE. We attribute the steeper C–R curves to an increased attractiveness of 0.6–1.0 M sucrose. This elevation in attractiveness would have made the low sucrose concentrations proportionally less attractive, owing to a negative contrast effect (Flaherty et al. 1995). Given that CT nerve responses to sucrose decline over the course of adolescence (i.e., P21–P56) in rats (Harada and Maeda 2004), it is likely that the increase in acceptability of the taste of 0.6–1.0 M sucrose reflects a developmental change in central evaluation of sucrose taste input.

Sex differences

A prior study reported that adolescent male and female rats exhibit similar preferences for 6% and 10% ethanol during 24-h preference tests (Vetter-O'Hagen et al. 2009). We, too, did not observe any sex-related differences in GL nerve responses to (or lick rates for) ethanol and its flavor components. Taken together, these results indicate that adolescent male and female rats are similar with respect to their evaluation of ethanol's flavor.

Future directions

It is notable that that even though the intermittent exposure regime increased the acceptability of ethanol's flavor, it did not reliably increase daily intake of ethanol (i.e., it did so in experiment 2, but not in experiment 1). This latter observation implies that the increased acceptability of ethanol's flavor was not sufficient to overcome the aversive postingestive actions of ethanol. In future studies, we will attempt to mask the aversive flavor components of ethanol with sweeteners and then determine whether this increases ethanol intake across the intermittent exposure regime. This approach was inspired by prior reports of rats increasing daily intake of 10% ethanol when it was presented in 1) a sweetened, chocolate-flavored solution (Hosová and Spear 2017) or 2) a 3% glucose + 0.125% saccharin solution (Ji et al. 2008). Likewise, adolescent humans consume sweetened alcoholic beverages more avidly than unsweetened ones, in large part because of the flavor-masking effects of sweeteners (Hughes et al. 1997; Copeland et al. 2007; Siegel et al. 2011).

Supplementary material

Supplementary data are available at *Chemical Senses* online.

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Conflict of interest

None declared.

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