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An evaluation of hedonic responses in taste-potentiated odor aversion using the analysis of licking microstructure and orofacial reactivity

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ABSTRACT

Two experiments examined the hedonic responses conditioned to odor cues in the phenomenon of taste-potentiated odor aversion. Experiment 1 analyzed the microstructure of licking behavior during voluntary consumption. A tasteless odor (amyl acetate) was delivered to rats either diluted in water or mixed with saccharin before being injected with LiCl. At test, subjects which had received the odor-taste compound during conditioning showed both lower odor consumption and lick cluster size, a result indicating an increased negative evaluation of the odor. Experiment 2 examined the orofacial reactions elicited by the odor as index of its hedonic impact. During conditioning, the rats were intraorally infused with either the odor alone or the odor-saccharin compound before being injected with LiCl. At test, they were infused with the odor and their orofacial responses video recorded. More aversive orofacial responses were elicited by the odor cue in rats that had compound conditioning, again a result indicating a strengthened negative hedonic reactivity compared to animals experiencing odor aversion conditioning alone. Taken together, these results indicate that taste-mediated potentiation of odor aversion conditioning impacts on the acquisition of conditioned hedonic reactions as well as consumption.

1. Introduction

Flavor learning is a complex experience that requires the integration of multiple sensory properties of food and fluids, in particular the combination of olfactory and gustatory information (see Piqueras-Fiszman and Spence, 2016; Small and Prescott, 2005; Spence, 2015, for reviews on multisensory flavor perception). There is good evidence to suggest that associative learning has a profound contribution to the integration of olfaction and gustation in flavor learning, and particularly in flavor aversion learning (Gautam and Verhagen, 2010; Small, 2012). Many species (including humans) readily learn to avoid flavors paired with toxins that cause gastrointestinal malaise, a phenomenon termed conditioned taste aversion (CTA) (see Reilly and Schachtman, 2009, for a review). It is important to note here that although 'taste' is often used as a synonym for 'flavor' because flavors are always experienced in the mouth, flavor is more accurately defined as the result of joint stimulation of the senses of smell and taste. Despite this, there is relatively little known about odor/taste integration in flavor aversion learning and, particularly, on the contribution of the affective or hedonic properties acquired by the olfactory and gustatory cues during aversive conditioning. As suggested by Garcia et al. (1955), flavor aversion learning not only results in the subsequent reduction in consumption of the flavor, but also in changes to its palatability or hedonic qualities.

Of particular interest to understanding olfactory-gustatory interactions in flavor learning is the phenomenon termed taste-potentiated odor aversion (TPOA). In this paradigm, the reduction in intake of a weak odor (e.g., almond odor extract) produced by pairing it with a nausea-inducing event (e.g., lithium chloride) is increased when the odor is delivered in compound with a taste such as saccharin (e.g., Batsell and Blankenship, 2002; Durlach and Rescorla, 1980; Rusiniak et al., 1979). While the fact that compound conditioning results in a lower odor intake than odor-alone conditioning clearly indicates that odor-taste interactions have important effects, it should be noted that intake measures are relatively non-selective in that they are influenced by multiple factors. This is important, because prior studies of taste and flavor conditioning have revealed dissociations between measures of intake and hedonic reactions which have provided critical information regarding the learning mechanisms involved. For example, pairing

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flavors with some events, such as drugs of abuse and external pain caused by footshock, produce a reduction in voluntary consumption, without a change in affective responses as revealed by orofacial reactions (e.g., Parker, 1995; Pelchat et al., 1983), suggesting that flavor aversions caused by these events are qualitatively different than those produced by nausea-induced agents as LiCl (see Parker, 2003, 2014). According to Parker's view, the association of a taste with nausea causes a reduction in its palatability and intake (CTA), whereas a taste associated with a drug of abuse or pain is avoided because it anticipates a potential danger (taste avoidance learning [TAL]). We addressed this question by directly comparing the effects of an emetic treatment (injection of LiCl) with a nonemetic treatment (internal pain produced by injection of hypertonic NaCl) obtaining dissociable effects of LiCl and hypertonic saline on hedonic reactions and fear responses, despite equivalent effects on consumption; in addition, flavors paired with internal pain or with nausea elicited divergent types of hedonic responses (e. g., Dwyer et al. (2013, 2017); Gasalla et al. (2017). A similar result was obtained after pairing nonflavor (contextual) cues with LiCl and hypertonic saline (López et al., 2019). No previous studies of TPOA have directly assessed hedonic reactions and so it remains unknown as to whether this phenomenon impacts on such reactions. Thus, the current experiments addressed this issue by using selective hedonic measures.

In rodents, the hedonic evaluation of odors is commonly inferred from reductions in consumption using tasks such as olfactory discrimination, odor preference learning, and odor aversion conditioning (Chapuis et al., 2007; Jagetia et al., 2018; Slotnick et al., 1997; Torquet et al., 2014), but not by directly examining the affective responses elicited by the odor cue. The first truly selective method to affording a direct assessment of the hedonic impact of flavors (and odors) is the orofacial reactivity test[1] (Grill and Norgren, 1978), which is selective to disgust, in contrast to reduction in consumption that may simply reflect fluid avoidance without a change in affective responses. In this test,[2] rats are infused with a flavored solution via a cannula implanted in their oral cavity, and the orofacial reactions - stereotyped oral motor and somatic consummatory responses - elicited by the flavor are analyzed. These responses can be classified as aversive (e.g., gaping, chin rubbing, and paw treading) elicited when infused with unpleasant sour or bitter tastes, or appetitive (e.g., tongue protrusions, mouth movements, and paw licks), elicited by pleasant, sweet tastes. When infused with a palatable taste (e.g., saccharin) previously paired with LiCl-induced nausea, rats display orofacial aversive responses, reflecting a shift in the hedonic value of the fluid from positive to negative (see Parker, 2003, 2014).

The hedonic value of flavors and odorous solutions can also be examined by analyzing the microstructure of licking behavior during voluntary consumption (Davis, 1989; Dwyer, 2012). The ingestive behavior of rodents consuming fluids consists of sustained runs of licks separated by pauses of varying length (clusters), and the mean number of licks in a cluster (lick cluster size) is directly related to the nature and concentration of the solution ingested. Lick cluster size shows a positive monotonic relationship to the concentration of palatable sweet solutions, while lick cluster size decreases monotonically with increasing concentration of unpalatable quinine solutions. In the context of flavor aversion learning, pairing an otherwise palatable taste with nausea results in a reduction of lick cluster size like that produced by exposure to unpalatable solutions. In previous work, we have used the licking behavior analysis and the orofacial reactivity test to examine the conditioned hedonic qualities acquired by flavor and non-flavor (context stimuli) cues paired with nausea and internal pain (Dwyer et al., 2017; Gasalla et al., 2017; López et al., 2019).

There is some evidence showing that rats might display conditioned changes in affective value to an odor cue paired with LiCl (Chapuis et al., 2007; Slotnick et al., 1997). In the conditioned odor aversion (COA) paradigm, rats are given exposures to an odor stimulus (usually an ingested tasteless aqueous solution of odorant) followed by a nausea-inducing agent, resulting in subsequent odor avoidance through the development of an association between olfactory information with the emotional aspects (i.e., negative hedonics) elicited by nausea. However, as noted above, in these studies the odors hedonic quality was inferred from a reduction in consumption but not by examining directly the affective responses elicited by odor. Thus, in the current study using the licking behavior analysis and the orofacial reactivity test we investigated the hedonic responses elicited by an odor diluted in water or mixed with saccharin followed by lithium-induced nausea. It is expected that, compared with rats receiving odor-alone conditioning, rats receiving odor-taste compound conditioning will display lower odor consumption and lick cluster size (Experiment 1), and an increase in the number of aversive responses elicited by the intraoral infusion of the odor (Experiment 2). We would point out that although odors can be perceived by both orthonasal (i.e., when volatiles enter the nasal cavity during inhalation) and retronasal (i.e., when volatiles released in the mouth pass into the nasal cavity during consumption) routes, we have used only retronasal presentation in these experiments because pilot studies from our laboratory comparing directly odor aversions with retronasally and orthonasally delivered odors suggested that the delivery route did not have material impacts on either lick cluster size or conditioned hedonic reactions.

2. Experiment 1

In this experiment the analysis of licking behavior was applied to examine odor hedonics in the phenomenon of taste-mediated odor potentiation. Two groups of rats had access to a tasteless odorant (0.01 % amyl acetate) diluted in water or mixed with saccharin before being injected with LiCl; a third group of rats was given the odor solution and injected with saline. On testing, all rats received the odor and the saccharin in separate sessions. An additional control group conditioned with saccharin and tested with the odor was added after the initial testing to assess generalization from the conditioned aversion to saccharin. We hypothesized that subjects receiving the odor-saccharin compound during conditioning will show both lower consumption and lick cluster size than rats being conditioned with the odor alone.

2.1. Materials and methods

2.1.1. Subjects

Twenty-four male Wistar rats, supplied by the University of Oviedo vivarium, with a mean weight of 346 g (range, 315–397 g) were used. They were individually housed in cages with food and water ad libitum in a room maintained at 21° C and illuminated under a 12 h-dark/light cycle with the light period beginning at 8:00 a.m. Throughout the experimental sessions, the rats were on a 23-h water deprivation schedule with 60-min access to water in the home cage per day. Subjects were randomly assigned (see Table 1) to one of three groups (n = 8) based in their weight: Group O-L (odor-lithium), Group OT-L (odor + taste-lithium), and Group O-S (odor-saline). A further eight rats from the same source as noted above, with a mean weight of 311 g (range, 298–343 g) were used for the additional Group O/T-L (odor/ taste-lithium). Although run separately from the other groups, this additional control was otherwise treated in the same general manner (including being run at the same time of day and in the same equipment) as the other groups.

All procedures reported here were conducted in accordance with

[1] This method was originally described as the taste reactivity test because it is most applied to taste stimuli. Here, we have chosen to emphasize the nature of the elicited responses – orofacial reactions – because we are considering its application to odor stimuli.

[2] For a detailed description of the taste reactivity method, see López et al. (2022). Bio-protocol 12(18) (2022) e4515. https://doi.org/10.21769/BioProtoc.4515.

Table 1
Design of experiments.

Experiment 1. *Licking behavior analysis*				
Group	*Familiarization*	*Conditioning*	*Test 1*	*Test 2*
O-L		Amyl → Li	Amyl	Sac
OT-L	3 x Water	Amyl+Sac → Li	Amyl	Sac
O-S		Amyl → Sal	Amyl	Sac
O/T-L		Amyl / Sac → Li	Amyl	Sac
Experiment 2. *Orofacial reactivity test*				
Group	*Familiarization*	*Conditioning*	*Test 1*	*Test 2*
O-L		Amyl → Li	Amyl	Sac
OT-L	1 x Water	Amyl+Sac → Li	Amyl	Sac
O-S		Amyl → Sal	Amyl	Sac

Keys. O: rats receiving odor alone during conditioning; OT: rats receiving the odor + taste compound during conditioning; O/T: rats receiving the odor and the taste in separate sessions; L: LiCl; S: saline. Li and Sal indicate injections of LiCl or saline; Amyl refers to the odorant amyl acetate; Sac refers to saccharin. In Experiment 1 the experimental sessions were conducted in the drinking boxes; in Experiment 2 the sessions were conducted in the taste reactivity apparatus.

Spanish (RD 53/2013) and European (2019/63/UE) legislation for animal experimentation.

2.1.2. Fluids and apparatus

The fluids used as conditioned stimulus (CS) were a 0.01 % (w/v) amyl acetate solution and a 0.05 % saccharin solution retronasally delivered (i.e., dissolved in water in drinking boxes) to the rats. These two solutions were mixed to produce the compound CS. The odor concentration was chosen because amyl acetate is known to be tasteless up to 0.1 % to male Wistar rats (Slotnick et al., 1997). The unconditioned stimulus (US) was an intraperitoneal injection of 0.15 M lithium chloride (LiCl) administered at a volume of 2 ml/kg of body weight. Control rats were injected with isotonic saline (0.9 % at 2 ml/kg).

The behavioral procedures took place in an artificially illuminated room containing 16 custom-made drinking boxes (42 × 25 × 20 cm) with acrylic walls and flooring. 50 ml drinking bottles with metal spouts could be inserted at one end of each box. A contact sensitive lickometer registered the licks made by rats to the nearest 0.01 s, and MED-PC software (Med Associates, Inc.) controlled the equipment and recorded the behavioral data.

2.1.3. Procedure

The experimental design is shown in Table 1. Initially, the rats were supplied with water in the drinking boxes on three consecutive days to adapt them to the apparatus and the 10-min sessions. During the conditioning trial, the rats were given the CS solution for 10 min before being injected with either LiCl (groups OT-L and O-L) or saline (group O-S). After this, the rats received a recovery day in which they were given water for 23 h in their home cages. On the next two test sessions (one per day) all rats had access to a bottle containing the odor solution (Test 1) or the saccharin solution (Test 2) for 10 min. Rats in subsequently added Group O/T-L received an additional pre-training session in which they were given the odor alone for 10 min in the drinking boxes, and they received (similar to groups OT-L and O-L) a single conditioning trial with the saccharin CS followed by injection of the LiCl US.

2.1.4. Data analysis

The amount of fluid consumed during the experimental sessions was calculated by weighing bottles before and after consumption and converting the difference to ml. For the analysis of lick cluster size, a cluster was defined as a series of licks separated by pauses no more than 0.5 s interval, a criterion used in our previous studies examining taste aversion learning by licking analysis (e.g., Dwyer et al., 2012, 2013, 2017). Data from familiarization sessions with water were analyzed by a 4 (group) × 3 (sessions) mixed ANOVA. Consumption and lick cluster size data recorded during conditioning and testing were analyzed by separate one-way ANOVAs with group as between-group factor. Post hoc independent t-tests were conducted to determine significant differences between pairs of groups. All tests reported here used a significance value of $p = .05$.

2.1.5. Results

The mixed ANOVA conducted with the water consumption data (descriptive data not shown) during the familiarization sessions revealed a significant effect of session, $F(2,56) = 160.01$, $p < .001$, $\eta_p^2 = .85$, 95 % CI [.69,.91], but there was neither an effect of group, $F(3,28) = 1.19$, $p = .330$, $\eta_p^2 = .11$, 95 % CI [.01,.38], nor a significant session x group interaction, $F(6,56) = 1.75$, $p = .134$, $\eta_p^2 = .24$, 95 % CI [.01,.22]. A similar analysis with the lick cluster size data showed that there were no effects of session, $F(2,56) = 1.25$, $p = .293$, $\eta_p^2 = .04$, 95 % CI [.01,.27], or group ($F<1$), nor an interaction between these two factors ($F<1$). The one-way ANOVA performed with the data from the conditioning session revealed that there were no significant differences between groups in the amount of CS fluid consumed, $F(3,28) = 1.31$, $p = .289$, $\eta_p^2 = 1.23$, 95 % CI [0,.29], or lick cluster size ($F< 1$), indicating a comparable hedonic evaluation of the odor and the odor-taste compound prior to conditioning. A post hoc comparison between pair of groups showed that there were no significant differences between groups in either consumption (largest $t(14) = 0.84$; $p = .412$, $d = .42$, 95 % CI [.05, 1.4], for the difference between groups OT-L and O-S) or cluster size (largest $t(14) = 1.43$; $p = .173$, $d = .71$, 95 % CI [1.72,.30], for the difference between groups O-L and O-S). The mean (± SEM) consumption (ml) for the different groups were: group O-L: 9.1 (± 0.41); group OT-L: 9.37 (± 0.62); group O-S: 8.33 (± 1.05); group O/T-L: 7.6 (± 0.51). The mean lick cluster size for each group was: group O-L: 20.14 (± 2.36); group OT-L: 21.17 (± 3.67); group O-S: 25.83 (± 3.18); group O/T-L: 21.23 (± 2.42).

Fig. 1 (panel A) shows the mean consumption of the odor and the saccharin solutions during testing. The one-way ANOVA conducted with these data revealed that they significantly differed in both odor consumption, $F(3,28) = 56.46$, $p < .001$, $\eta_p^2 = .85$, 95 % CI [.71,.89], and saccharin intake, $F(3,28) = 36.88$, $p < .001$, $\eta_p^2 = .79$, 95 % CI [.6,.85]. Post hoc t-tests showed that the group O-S had greater odor intake than both the OT-L and O-L groups (lowest $t(14) = 7.64$, $p < 0.001$, $d = 3.82$, 95 % CI [5.51, 2.0], for the difference between the groups O-L and O-S), and, importantly, that group OT-L consumed less of the odor solution than group O-L, $t(14) = 3.22$, $p = .006$, $d = 1.61$, 95 % CI [.44, 2.73]. The group OT-L had a lower odor consumption than group O/T-L, $t(14) = 10.73$, $p < .001$, $d = 5.36$, 95 % CI [7.54, 3.15], which in turn did not differ from group O-S ($t(14) = 1.60$, $p = .130$), $d = .8$, 95 % CI [2.33, 1.81], a result suggesting that there was no simple generalization to the odor cue of the aversive properties acquired by the saccharin. As for saccharin intake, the t-tests revealed that the group OT-L had a lower consumption than the groups O-L and O-S (lowest $t(14) = 9.30$, $p < 0.001$, $d = 4.65$, 95 % CI [2.66, 6.59], for the difference between groups OT-L and O-L), which did not differ from each other, $t(14) = 0.92$, $p = .373$, $d = .46$, 95 % CI [1.44,.54]. In addition, group O/T-L had a lower consumption than groups O-L and O-S (lowest $t(14) = 6.20$, $p < 0.001$, $d = 3.1$, 95 % CI [1.57, 4.58], for the difference between groups O/T-L and O-S). There was no significant difference between the saccharin consumptions of groups OT-L and O/T-L, ($t(14) = 1.27$, $p = .231$), $d = .63$, 95 % CI [1.63,.38].

The cluster size data during testing are shown in Fig. 1 (panel B). With respect to the odor solution, the one-way ANOVA revealed a significant effect of group, $F(3,28) = 41.15$, $p < .001$, $\eta_p^2 = .81$, 95 % CI [.63, 0.86], and post hoc comparisons showed that lick cluster size was higher for group O-S than for both OT-L and O-L groups (lowest $t(14) = 7.72$, $p < .001$, $d = 3.88$, 95 % CI [5.59, 2.13], for the difference between the groups O-L and O-S). Importantly, group OT-L had a lower cluster size than group O-L, $t(14) = 2.74$, $p = .016$, $d = 1.37$, 95 % CI [.24, 2.45]. In addition, group O/T-L had a higher cluster size than groups OT-L and O-L (lowest $t(14) = 4.81$, $p < 0.001$, $d = 2.4$, 95 % CI [3.7, 1.06], for the comparison between groups O-L and O/T-L). There was no significant

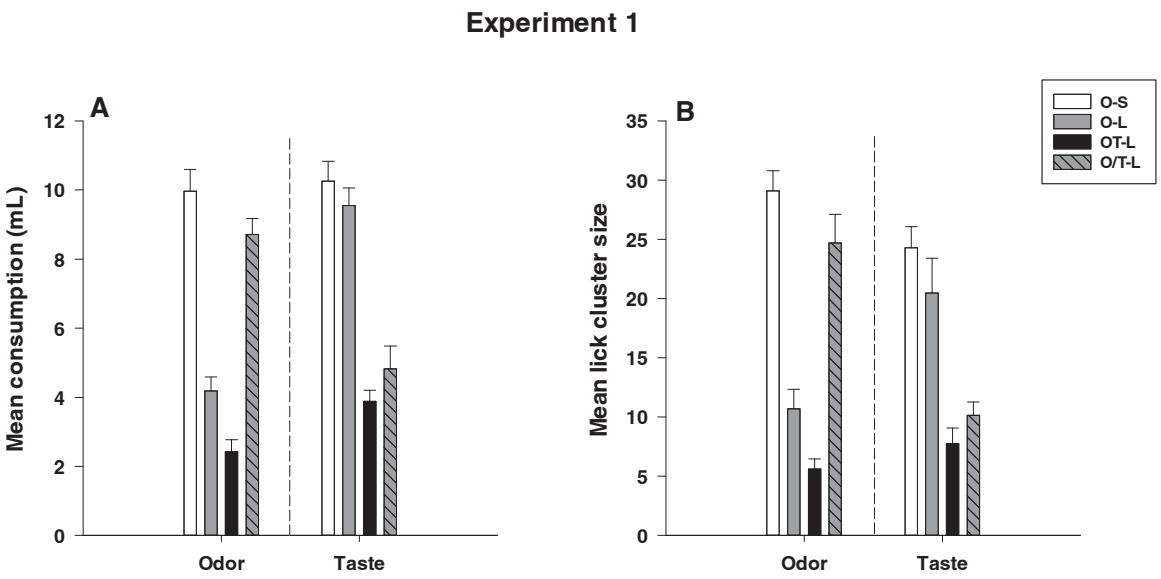

Fig. 1. Experiment 1. (A) Mean odor and saccharin intakes for the different groups during testing. (B) Mean lick cluster size for the odor and saccharin solutions during testing. Error bars represent *SEM*. (Note the different upper limits of frequency scale on the two panels).

difference in cluster size between groups O-S and O/T-L ($t(14) = 1.49$, $p = .158$, $d = .74$, 95 % CI [.28, 1.75]). Regarding the saccharin solution, the ANOVA showed that there were significant differences between groups, $F(3,28) = 17.13$, $p < .001$, $\eta_p^2 = .64$, 95 % CI [.35,.74]. The post hoc t-tests revealed that group OT-L had a lower cluster size than both O-L and O-S groups (lowest $t(14) = 3.93$, $p = .002$, $d = 1.96$, 95 % CI [.72, 3.16], for the difference between groups O-L and OT-L), which did not differ between themselves, $t(14) = 1.11$, $p = .284$, $d = .55$, 95 % CI [1.54,.45].

These results indicate that the presence of saccharin during conditioning increased the magnitude of the conditioned properties, including the aversive hedonic responses, acquired by the odor. In addition, the results argue against the possibility that the odor cue acquires negative hedonic qualities by simple generalization from the conditioned aversion to saccharin.

3. Experiment 2

This experiment used the orofacial reactivity procedure to examine the hedonic properties acquired by the odor in the phenomenon of taste-mediated odor potentiation. The experiment replicated the design of the three original groups in Experiment 1 (see Table 1) with the only difference that rats received intraoral infusion of the CS fluids during conditioning and testing. During conditioning, the rats were intraorally infused with either the odor diluted in water or mixed with saccharin before being injected with LiCl or saline. At test, the orofacial responses elicited by the odor and the saccharin were examined in separate sessions. Here, we assessed whether compound conditioning increases the magnitude of the conditioned aversive hedonic responses elicited by the odor cue as revealed by the taste reactivity test.

3.1. Materials and methods

3.1.1. Subjects, fluids and apparatus

Twenty-four male Wistar rats (University of Oviedo vivarium) weighing from 291 to 383 g (mean 328 g) were used. The housing and deprivation conditions were the same as in Experiment 1. Each subject was implanted with an oral cannula using the procedure described below. Subjects were randomly assigned to three groups (8 rats per group): Group O-L (odor-LiCl), Group OT-L (odor + taste-LiCl), and Group O-S (odor-saline). Two rats lost their cannula during the experiment, so that the number of subjects in each group was: Group O-L (n = 7); Group OT-L (n = 8); and Group O-S (n = 7). The fluids used as CSs and US, as well as the dose levels, were the same as in Experiment 1.

The behavioral procedures took place in a conditioning chamber located in a dark room. The chamber was made of clear Plexiglas sides (26 cm × 23 cm × 14 cm) with a dark lid and was placed on a table with a clear Plexiglas top. Two 50-Watt white lights on each side of the table provided illumination. A mirror beneath the chamber on a 45° angle facilitated viewing of the ventral surface of the rat during the intraoral infusion. Fluids were administered to the animals through an infusion pump (KD Scientific) connected to the implanted cannula. While the rats were infused with the fluids, their orofacial responses were recorded using a video camera (Sony Optical 20 X) connected to a computer. The videos were manually scored using the Observer XT 9.0 (Noldus Information Technology, Sterling, VA) event recording program. The videos were scored by two raters blind to the experimental groups.

3.1.2. Orofacial response scoring

Based on the procedure followed by Parker (1995), and as previously used in our studies on taste aversion learning (Dwyer et al., 2017; Gasalla et al., 2017), the aversive behaviors scored included the frequency of the disgust reactions of gaping (rapid, large-amplitude opening of the mandible with retraction of the corners of the mouth), chin rubbing (mouth or chin in direct contact with the floor or wall of the chamber and body projected forward), and paw treading (forward and backward movement of the forepaws in synchronous alternation). These scores were summed to provide a total disgust reaction score. In addition, appetitive behaviors were scored as follows: the number of seconds that the rats displayed ingestion reactions of tongue protrusions (extension of the tongue out the mouth), mouth movements (movement of the lower mandible without opening the mouth), and paw licks (midline extension of the tongue directed to the forepaws) was also summed to create a total ingestion reaction score. Appetitive and aversive responses were scored on different scales (duration vs. frequency) because they play very different properties: Appetitive responses are typically displayed over extended periods of time whereas aversive responses occur as isolated behavior (see Berridge, 2000).

3.1.3. Cannulation surgery

The rats were surgically implanted with an intraoral cannula using the method described in López et al. (2022). The surgical anesthesia

preparation included administration of an i.p. injection of ketamine (50 mg/kg) combined with medetomidine hydrochloride (0.15 mg/kg), a drug with analgesic properties. Following surgery, the rats were administered ketoprofen (1.5 mg/kg, s.c.), an anti-inflammatory drug, and the antibiotic enrofloxacin (0.3 mg/kg, s.c.). In order to implant the cannula a thin-walled 15-gauge stainless steel needle was inserted at the back of the neck, directly subcutaneously around the ear and brought out behind the first molar inside mouth. A length of intramedic polyethylene tubing with an inner diameter of 0.86 mm and an outer diameter of 1.27 mm was then run through the needle after which the needle was removed. Two square elastic discs were placed over the tubing and drawn to the exposed skin at the back of the neck for the purpose of stabilizing the cannula. The tubing was held secure in the oral cavity by an O-ring, which was sealed behind the tubing prior to cannulation surgery. Following surgery, rats were monitored for four days and had their cannula flushed daily with chlorhexidine to prevent infection. For the purpose of fluid infusion, the cannula was connected to the infusion pump by slipping the tubing of the cannula inside a second polyethylene tubing (inner diameter 1.19 mm; outer diameter 1.70 mm) attached to the infusion pump.

3.1.4. Procedure

The experimental procedure is shown in Table 1. All rats had recovered from the oral cannulation surgery within four days and were then placed on a water-deprivation schedule, comprising 1-h access to water each day, given approximately 2 h after the experimental sessions. The rats were then given a 1-min session with water infusion in the conditioning chamber to familiarize them with the apparatus and the intraoral infusion method (infusion rate 1 ml/min). During the conditioning session, rats were placed in the conditioning chamber, and they were infused with the CS solution for 2 min (1 ml/min) while their orofacial responses were video-recorded. Rats in groups O-L and O-S were infused with the odor (0.01 % amyl acetate) before being injected with 2 ml/kg of 0.15 M LiCl (group O-L) or saline (groups O-S); rats in group OT-L were intraoral infused with the odor-saccharin compound and injected with LiCl. The rats were returned to the home cages after the injection and given a water recovery day. On the next two days the test sessions were conducted. During these sessions, each rat was placed in the taste reactivity apparatus with their cannula attached to the infusion pump. On Test 1, the animals were intraorally infused with the odor solution (amyl acetate) for 2 min at a rate of 1 ml/min. On Test 2, the rats were infused with the saccharin for another 2 min (1 ml/min). During the fluid infusions the rats´orofacial responses were video recorded for subsequent quantification.

3.1.5. Data analysis

The orofacial reactivity scores during conditioning were analyzed by one-way ANOVAs with group as the between-group factor. The appetitive and aversive orofacial responses elicited by the odor and the saccharin during testing were also analyzed by separate one-way ANOVAs with group as between-group factor. Where group differences were observed follow-up pairwise comparisons were performed as t-tests. The inter-rater reliability (r´s > 0.89) for each behavior scored was highly significant.

3.1.6. Results

The ANOVA conducted with the data from the conditioning session revealed that there were no significant differences between groups in the number of aversive ($F<1$) or appetitive responses, $F(2,19) = 1.61$; $p = .226$, $\eta_p^2 = .14$, 95 % CI [0,.37], elicited by the infusion of the CS fluids, indicating a comparable hedonic valuation of the odor and the odor-taste compound. The mean (± SEM) number of aversive responses for the different groups were: group O-L: 1.42 (± 1.11); group OT-L: 0.87 (± 0.35); group O-S: 2.14 (± 1.18). The mean (± SEM) duration (seconds) of the appetitive responses for each group was: group O-L: 68.28 (± 2.99); group OT-L: 73.03 (± 2.68); group O-S: 67.22 (± 1.37).

Fig. 2 (panel A) shows the mean number of aversive responses elicited by the infusion of the odor and the saccharin during testing (the mean number of aversive responses by categories, gaping, chin rubbing, and paw treading, is shown in Table 2). As shown in the Fig. 2A, the rats in group OT-L, which had received compound conditioning, displayed more aversive responses to odor than the subjects from the other two groups. Importantly, group OT-L showed more aversive responses than group O-L. The one-way ANOVA conducted with these scores revealed a significant effect of group, $F(2,19) = 14.88$; $p < .001$, $\eta_p^2 = .61$, 95 % CI [.24,.74]. Post hoc t-tests confirmed that group OT-L significantly differed from group O-S, $t(13) = 5.40$; $p < .001$, $d = 2.79$, 95 % CI [1.3, 4.24], and, importantly that group OT-L displayed significantly more aversive responses than group O-L, $t(13) = 2.91$; $p = .012$, $d = 1.08$, 95 % CI [2.03,.02]. The groups O-L and O-S differed significantly from each other in the number of aversive responses, $t(12) = 2.43$; $p = .031$, $d = 1.08$, 95 % CI [.65, 1.63].[3]

The statistical analysis performed with the aversive reactions elicited by saccharin during testing revealed a significant effect of group, $F(2,18) = 5.21$; $p = .016$, $\eta_p^2 = .36$, 95 % CI [.01,.57]. The post hoc comparisons showed that group OT-L displayed more aversive reactions to saccharin than groups O-L and O-S (lowest $t(12) = 2.49$; $p = .028$, $d = 1.34$, 95 % CI [.13, 2.5], for the difference between the groups OT-L and O-S). Groups O-L and O-S did not differ from each other, $t(13) = 1.87$; $p = .087$, $d = 1.04$, 95 % CI [.14, 2.19].

Fig. 2 (panel B) shows the mean duration (in seconds) of appetitive responses elicited by the odor and the saccharin during testing (see Table 2 for the mean duration of the appetitive responses by separate categories, tongue protrusions, mouth movements, and paw licks). Relative to the odor test, the one-way ANOVA revealed significant differences among the three groups, $F(2,19) = 13.83$; $p < .001$, $\eta_p^2 = .59$, 95 % CI [.22,.73]. The post hoc comparisons showed that rats in group O-S exhibited more appetitive responses than groups OT-L and O-L (lowest, $t(12) = 3.73$; $p = .002$, $d = 1.93$, 95 % CI [3.16,.65], for the difference between groups OT-L and O-S), and that groups OT-L and O-L did not differ, $t(13) = 1.04$; $p = .316$, $d = .53$, 95 % CI [1.56,.50]. The absence of differences between groups OT-L and O-L - reflecting no evidence of potentiation in the measure of appetitive reactions, could be attributed to a floor effect. As for the appetitive responses elicited by the saccharin infusion, the ANOVA conducted with these scores revealed a significant effect of group, $F(2,18) = 7.17$; $p = .005$, $\eta_p^2 = .44$ 95 % CI [.06,.62]. The post hoc pairwise comparisons showed that group OT-L exhibited less appetitive responses than groups O-L and O-S (lowest, $t(13) = 2.40$; $p = .032$, $d = 1.24$, 95 % CI [.10, 2.34], for the difference between groups OT-L and O-L), which did not differ from each other, $t(11) = 0.82$; $p = .429$, $d = .45$, 95 % CI [1.55,.65].

The results of this experiment confirm those found in Experiment 1 examining odor hedonics in taste-potentiated odor aversion using licking behavior analysis and show that the presence of the taste during conditioning increase the negative hedonic responses to the odor; that is, odor + taste compound conditioning strengthened the conditioned properties, including affective responses, acquired by the odor cue.

4. Discussion

This study explored the hedonic responses conditioned to an odor cue in the taste-potentiated odor aversion paradigm. Analyzing the

[3] It should be noted that there was an increase in aversive responses to the odor in group O-S between conditioning and test, and also a reduction in the duration of appetitive responses from conditioning to test in this group. Despite these (presumably unconditioned) changes in a group receiving only saline injections, this does not question the analysis in terms of conditioned effects based on LiCl injections because groups O-L and O-S differed significantly from each other in the number of aversive responses, and in the duration of appetitive responses.

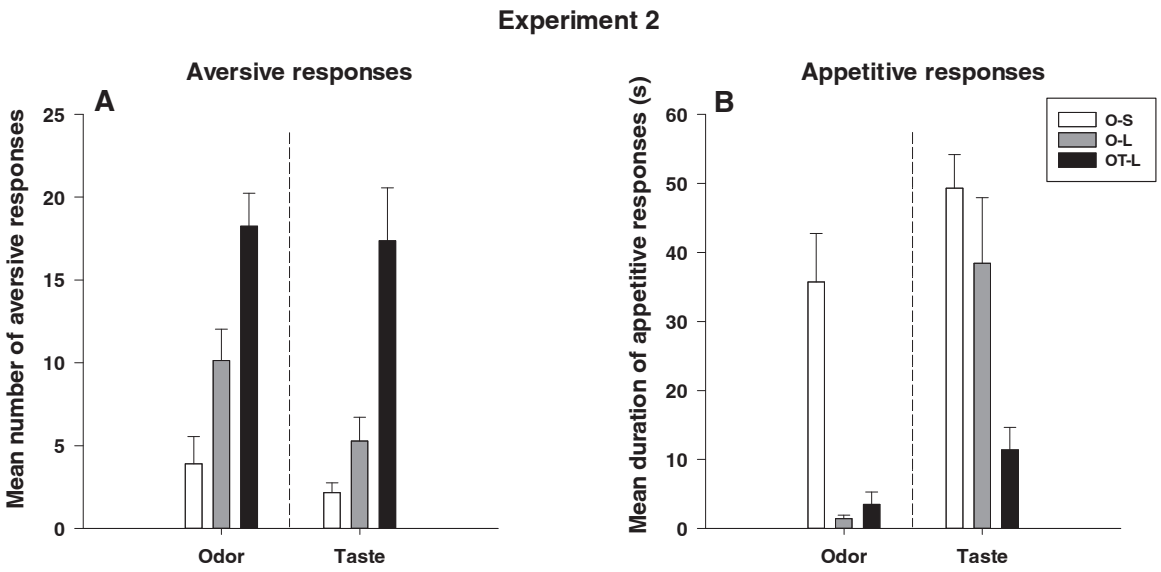

Fig. 2. Experiment 2. Data from the intraoral test sessions for the different groups: (A) Mean number of aversive orofacial responses. (B) Mean duration (s) of appetitive orofacial responses. Error bars represent *SEM*. (Note the different upper limits of frequency scale on the two panels).

Table 2
Experiment 2. Mean number of aversive responses and mean duration of appetitive responses by separate categories elicited by the odor and the taste during testing. Standard error of mean (± *SEM*) is shown in brackets.

Odor test						
Aversives				Appetitives		
Group	*Gaping*	*Chin rubbing*	*Paw treading*	*Mouth movements*	*Tongue protrusions*	*Paw licks*
O-L	5.57 (1.25)	3,42 (.57)	1.14 (.45)	1.02 (.45)	0.40 (.11)	0.0 (0.0)
OT-L	9.37 (1.35)	7.25 (.77)	1,62 (.46)	2.73 (1.17)	0.76 (.76)	0.0 (0.0)
O-S	1.28 (.83)	2.57 (.92)	0.14 (.14)	8.99 (3.53)	20.74 (7.16)	5.98 (4.34)
Taste test						
Aversives				Appetitives		
Group	*Gaping*	*Chin rubbing*	*Paw treading*	*Mouth movements*	*Tongue protrusions*	*Paw licks*
O-L	2.85 (1.03)	1.85 (.40)	57 (.42)	1.99 (.9)	19.15 (9.47)	17.26 (6.45)
OT-L	7.12 (1.59)	7.0 (3.82)	3.25 (1.03)	3.48 (1.54)	5.99 (2.66)	2.0 (.76)
O-S	2.16 (.60)	0.0 (0.0)	0.0 (0.0)	2.4 (.80)	29.33 (4.39)	20.92 (4.52)

microstructure of licking behavior in Experiment 1 showed that rats receiving compound conditioning (odor + taste associated with nausea) displayed both lower odor consumption and lick cluster size than rats receiving odor-alone conditioning, a result reflecting an increased negative evaluation of the odor. It was also found that there was no generalization to the odor cue of the aversive hedonic responses acquired by the taste during aversive conditioning with the taste alone. Experiment 2 examined the orofacial reactions displayed by rats during the intraoral infusion of the odor solution. There were increased aversive orofacial responses in rats that had compound conditioning, again a result indicating a strengthened negative hedonic reactivity in taste-potentiated odor aversion. While taste-potentiated odor aversion learning has been reported previously, the current experiments are the first to demonstrate that this effect extends to producing enhanced hedonic reactions in addition to consumption changes.

It is worth noting that the fact that the potentiation effect was observed here (Experiment 2) with a relatively short infusion time (2 min) does not necessarily imply that different results would be obtained with longer infusion times. Certainly, Westbrook et al. (1983) found that the duration of the exposure to the odor-taste compound could be a factor determining between overshadowing or potentiation, with longer durations producing potentiation in their experiments. However, other studies (e.g., Coburn et al., 1984) have demonstrated taste-mediated odor potentiation with exposure as short as 2 min, as in the present study. That said, the results reported by these authors are based on voluntary consumption and our data on oral infusion, so the two procedures that cannot be directly compared. In addition, we would like to note that in our previous works using the orofacial reactivity method, we usually administered the fluids to the rats at an infusion rate of 1 ml/min over a period of 2 min (e.g., Dwyer et al., 2017; López et al., 2023). It is known that longer infusion rates tend to result in an increase in passive dripping and unconditioned aversive orofacial reactions to sweet and bitter taste solutions in the rat (e.g., Cagniard and Murphy, 2009).

It should be noted that the current experiments also extend previous work from our laboratory examining the nature of the conditioned hedonic responses elicited by flavor and non-flavor cues after their pairing with nausea. As noted in the introduction, we have demonstrated, using orofacial reactivity assessment, that contextual stimuli paired with nausea produced by LiCl injections can elicit aversive orofacial responses as LiCl-paired flavors (López et al., 2019), and that context cues interfere, through blocking, with the reduction in palatability of a taste paired with LiCl in that context (Gasalla et al., 2017). We have also demonstrated that flavors paired with nausea or with internal pain produced by hypertonic saline elicit divergent types of hedonic responses: Only pairing with nausea results in the production of aversive orofacial responses to the flavor whereas pairing with internal pain results in the flavor eliciting immobility (reflecting fear), despite equivalent reductions in consumption (Dwyer et al., 2017). Taken together, these results are consistent with the idea that aversion learning, including affective responses, is governed by general associative mechanisms, and that the quality of the conditioned hedonic responses is primarily determined by the nature of the aversive event (nausea, pain) and not the type of conditioned cue (taste, odor, context).[4]

[4] There is abundant evidence that the nature a CS can impact on the nature of the CR in some circumstances (e.g., Trost and Batsell, 2004; for a recent discussion see Honey and Dwyer, 2022), thus this summary is specific to our previous aversion learning experiments and the way they speak against the idea of selective taste-illness learning mechanisms.

Returning to the issue of TPOA, although the phenomenon itself is very well established, there is little consensus as to the mechanism(s) underpinning the effect. Early theoretical analysis centered on the comparison of two ideas: the "sensory gate" hypothesis (Garcia et al., 1985), referring to the idea that aversion learning is a specific gut defense mechanism that would not normally process odors as a cue for illness, but presentation of a taste alongside the odor would allow access for odors (or other non-taste cues) to the gut defense mechanism; and "within-compound associations" (e.g., Durlach and Rescorla, 1980), referring to the idea that presenting the odor and taste together would form associations between them allowing the odor to retrieve the taste, which in turn could retrieve the representation of illness. More recently, the possibility that combined presentation of taste and odor would produce a configural odor/taste representation that would generalize to either odor or taste alone has been considered (e.g., Batson et al., 2008; Trost and Batsell, 2004; see also Urcelay and Miller, 2009). While the general observation that TPOA can be observed for hedonic reactions as well as intake measures is not strictly incompatible with any of these possible mechanisms, the details of the current results are perhaps most consistent with a configural account. For example, the sensory gate hypothesis would suggest that odor conditioning alone should not engage the gut defense mechanism on which true aversion learning is purportedly based, yet the odor alone controls in the current experiments did display some (albeit moderate) levels of conditioned hedonic responses. There is also evidence that aversions based on within-compound associations between highly distinct "basic" taste stimuli such as sweet, salty, sour, (as opposed to those conditioned directly) predominantly affect intake measures compared to hedonic assays (e.g., Dwyer et al., 2012), while the current TPOA effects were observed clearly in both intake and hedonic measures.

The idea that the present results are most consistent with a configural understanding of the integration of olfactory and gustatory information complements recent work of our laboratory (López et al., 2023) examining odor hedonics using the taste-mediated odor aversion paradigm, i.e., examining responses to an odor that had previously been paired with a taste after conditioning an aversion to that taste alone (Holland, 1981; Holland and Wheeler, 2009). Using microstructural analysis of ingestive behavior, we found that saccharin devaluation with LiCl after odor-saccharin pairings resulted in both reduced intake of the odor solution and lick cluster size, a result indicating a reduced hedonic evaluation of the odor cue. Also, by examining the orofacial responses elicited by the infusion of the odor after saccharin devaluation we observed an increase in the number of aversive responses in rats that had prior odor-saccharin experience as compared with subjects receiving previously the odorant alone, a result again showing a change in the hedonic value of the odor from positive to negative. As discussed in López et al. (2023) these results, and their contrast to the effects observed with highly distinct basic tastes as examined by Dwyer et al. (2012), are in line with the suggestion by Gautam and Verhagen (2010), that the odor could acquire a specific taste quality (i.e., the odor was perceived as sweet) after odor-taste pairings.

Regardless of the precise mechanism(s) underpinning TPOA, it should be noted that the current observation of concurrent hedonic and consumption effects in TPOA was only observed with retronasal presentation of odors. Although our pilot studies suggested both retronasal and orthonasal presentation of odors allowed for hedonic changes after direct odor-aversion conditioning, there is at least some suggestion that TPOA effects may be impacted by the route of odor presentation (e.g., Bouton et al., 1986: albeit that this impact seems to stem from the delivery route affecting the conditionability of the odors rather than directly affecting the presence or absence of potentiation). Thus, future research should examine hedonic reactions in TPOA across different olfaction modalities. However, while the behavioral and neural mechanisms involved in the functional integration of olfactory and gustatory information in flavor aversion learning may require further investigation, the current experiments clearly establish that the TPOA effect is evident in hedonic reactions as well as intake measures, and this observation constrains the potential mechanisms underpinning the effect.

CRediT authorship contribution statement

Conceptualization, Methodology, Formal analysis, Manuscript preparation: M.L. and D.M.D.; Data collection, Data analysis: M.L., C.J., A.B., and E.A.; Funding acquisition: A.B., E.A., and D.M.D.

Declaration of Competing Interest

The authors declare no competing financial and non-financial interests.

Data availability

Data will be made available on request.

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