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Conditioned hedonic responses elicited by contextual cues paired with nausea or with

internal pain

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Abstract

Pairing a taste with either internal pain or nausea, despite equivalent effects on voluntary consumption, has dissociable effects on hedonic responses: only pairing with nausea results in the production of disgust reactions, while pairing with internal pain results in conditioned fear as indicated by immobility. Here, we use orofacial reactions to examine the hedonic responses elicited by contextual, nonflavor, cues paired with nausea produced by injection of LiCl or internal pain caused by injection of hypertonic saline. In Experiment 1, aversive orofacial responses were the specific context-elicited behaviors in the rats injected with LiCl, whereas immobility was seen in the animals injected with hypertonic saline. In Experiment 2, rats first received discriminative training with two contexts, where one context was paired with LiCl or hypertonic saline, and the other context with isotonic saline. After this, rats were intraorally infused with a flavor (CS+) in the paired context, and with a different flavor (CS-) in the unpaired context. Second-order conditioning was then examined in a test conducted in the unpaired context. The infusion of the CS+ flavor produced aversive orofacial responses in the rats injected with LiCl but immobility in the subjects injected with hypertonic saline. The results suggest that nonflavor cues support conditioned hedonic responses in the same way as flavor cues, which implies that the quality of aversion learning (conditioned nausea vs fear) is primarily determined by the nature of the aversive event and not the type of conditioned cue.

Keywords: context aversion, conditioned nausea, internal pain, taste reactivity, rats

In rats, a novel palatable taste paired with LiCl-induced nausea becomes not only avoided and no longer ingested, but it also elicits aversive orofacial responses (i.e., disgust reactions) when infused into the rat´s mouth via a previously-implanted oral cannula - the orofacial reactivity test[1] (Grill & Norgren, 1978), reflecting a shift in the palatability of the taste from positive to negative (Parker, 1998; Parker, Limebeer, & Rana, 2009). However, some agents, such as drugs of abuse and external pain caused by footshock, can also produce a decrease in voluntary consumption after they have been paired with a taste solution without resulting in conditioned aversive orofacial responses (e.g., Parker, 1995; Pelchat, Grill, Rozin, & Jacobs, 1983; but see Colechio & Grigson, 2014; Wheeler et al., 2011), suggesting that taste aversions caused by these events are qualitatively different than those produced by nausea-induced agents as LiCl (see Parker, 2003; 2014, for reviews; but see also Lin, Arthurs, & Reilly, 2014; 2017). According to Parker´s view, the association of a taste with nausea causes a reduction in its palatability and intake (conditioned taste aversion; CTA), whereas a taste associated with a drug of abuse or pain is avoided because it signals a potential danger or a disturbance in homeostasis regulation (taste avoidance learning; TAL).

In order to examine the distinction between CTA and TAL, we have directly compared the effects of LiCl administration with a non-emetic treatment (internal pain produced by injection of hypertonic saline). Using the orofacial reactivity method, we found that pairing a palatable taste with either nausea or internal pain leads to a subsequent reduction in consumption of that taste, as well as a reduction in the number of appetitive orofacial responses (e.g., tongue protrusions, mouth movements, and paw

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[1] This method was originally described as the Taste Reactivity Test. Although this terminology remains in common use (especially because it is most commonly applied to taste stimuli), we have chosen to emphasize the nature of the response here because we are considering its application to non-taste stimuli.

licks) elicited by the intraoral infusion of the conditioned taste. However, only pairing with LiCl resulted in an increase in aversive orofacial responses to the taste (e.g., gapes, chin rubbing, and paw treading), while pairing with hypertonic NaCl resulted in the taste eliciting immobility responses and passive dripping reflecting conditioned fear (Dwyer, Gasalla, Bura, & López, 2017). This dissociation in the impact of LiCl and hypertonic NaCl on orofacial and fear responses, despite comparable effects on voluntary consumption, demonstrates that flavors paired with internal pain or with nausea elicit divergent types of hedonic responses.

It is also known that pairing contextual cues with the aversive effects of LiCl will endow the context with at least some conditioned aversive properties, as revealed by a decrease in the amount consumed of a palatable taste in a previously LiCl-paired context (e.g., Best, Brown, & Sowell, 1984; Kwok & Boakes, 2012; Symonds & Hall, 1997). However, it is not clear whether context-nausea pairings endow the context with the same range of hedonic responses as do taste-nausea pairings. Previous studies using orofacial reactivity procedures have provided some evidence that rats not only display disgust reactions when intraorally infused with a flavored solution in a previously LiCl-paired context, but they also display aversive orofacial responses during exposure to the context in the absence of the flavored solution (Limebeer, Hall, & Parker, 2006; Limebeer, Krohn, Cross-Mellor, Litt, Ossenkopp, & Parker, 2008). Recently, we have used a blocking procedure to investigate whether nonflavor cues paired with nausea can elicit aversive orofacial reactions, and also whether nonflavor cues interfere with changes in affective responses to taste stimuli (Gasalla, Soto, Dwyer, & López, 2017). It was found that a context previously paired with LiCl-induced nausea elicited aversive orofacial responses, and also attenuated through blocking, the reduction in palatability of a saccharin solution which was paired with LiCl in that context. These results

confirm that contextual stimuli can elicit conditioned hedonic responses in the absence of any flavor component, and demonstrate that context cues can interfere with the affective aspects of taste aversion learning. However, the full nature of the affective responses conditioned to context cues has yet to be determined. In particular, it is unclear whether the dissociation in affective responses elicited by nonflavor cues paired with different classes of aversive events such as footshock or visceral pain versus nausea parallel those seen with flavor cues. Such a demonstration is particularly important for understanding the role of context in mediating qualitatively different conditioned aversive effects and its role in modulating hedonic responses. This is especially pertinent in light of the continuing interest in cue-to-consequence effects in aversion learning following classic studies apparently displaying selective flavor to nausea and nonflavor to pain learning (e.g. Garcia & Koelling, 1966). In addition, a key question in making this comparison is to determine whether taste palatability varies along two independent dimensions (appetitive and aversive hedonic responses) or should be viewed as a single continuum or dimension from positive to negative.

Based in our previous work examining the nature of the conditioned hedonic responses elicited by a flavor paired with either internal pain or with nausea (Dwyer et al., 2017), the goal of the current study was to characterize, using the analysis of orofacial reactivity, the hedonic responses elicited by contextual cues paired with nausea (LiCl administration) or internal pain produced by injection of hypertonic NaCl, and whether the hedonic changes induced by these aversive events parallel those seen with flavor cues. Specifically, in Experiment 1 we examined the conditioned hedonic responses elicited by contextual cues after repeated pairing with both USs (nausea and internal pain). In Experiment 2 we examined the reliability of this effect in a within-subject design and extended the analysis to second order conditioning with contexts as

the directly conditioned stimuli and flavors as the indirectly paired stimuli. This allows the examination of appetitive orofacial reactions which are typically only seen during fluid exposure.

Experiment 1

Because contextual, nonflavor, cues paired with nausea appear to elicit the same hedonic responses as do nausea-paired flavors, and the clearly dissociable effects of nausea and internal pain on aversive taste reactivity and fear responses (Dwyer et al., 2017; Gasalla et al., 2017), we hypothesized that context aversions induced by these aversive events might produce different types of hedonic responses, with context-nausea pairings resulting in the production of aversive orofacial responses to the context, while pairing with internal pain might result in the context producing conditioned fear as indicated by immobility.

Method

Subjects. Thirty naive male Wistar rats from the University of Oviedo vivarium (Spain) were used. They were approximately 90 days old and with a mean free-feeding weight of 331 g (range = 289-428 g) at the start of the experiment. Upon arrival, they were individually housed in opaque plastic cages, under constant temperature (21° C), and a 12hr/12hr light/dark cycle (light on at 08:00 h). All experimental manipulations took place during the light phase of the cycle. Throughout the experiment, water and food were always available in the home cages. All procedures reported here were conducted in accordance with Spanish (RD 53/2013) and European (2010/63/UE) legislation for animal experimentation.

Fluids and apparatus.   The fluids used as unconditioned stimulus (US) were solutions of lithium chloride (0.15 M LiCl), hypertonic sodium chloride (1.5 M NaCl), and isotonic sodium chloride (0.15 M NaCl). The US solutions were administered intraperitoneally (i.p.) at a volume of 10 ml/kg of body weight.

The behavioral procedures took place in a conditioning chamber located in a room without natural light. The chamber was made of clear Plexiglas sides (26 cm long x 24 cm wide x 14 cm high) with a plastic lid, and was placed on a table with a clear Plexiglas top. Two 50-W white lights on each side of the table provided a bright lighting. A mirror beneath the chamber on a 45° angle allowed the recording of the rats during the experimental sessions. While the rats were placed in the conditioning chamber, their orofacial responses and the time spent immobile were videotaped using a video camera (Sony Optical 20 X, Sony corporation, Tokyo, Japan) connected to a computer that enabled recordings to be directly stored. The videotapes were scored manually by two raters blind to the experimental groups using the Observer XT 9.0 (Noldus Information Technology, Sterling, VA) event recording program.

Procedure.   The experimental design is summarized in Table 1. The rats were randomly assigned to one of three groups (n=10) based on their weight: Group Lithium, Group Hypertonic, and Group Isotonic. The training phase consisted of four contextual conditioning trials (one per day). On each of the conditioning trials, the rats were placed in the conditioning chamber for 5 min before being injected with either lithium (Group Lithium), hypertonic saline (Group Hypertonic), or isotonic saline (Group Isotonic). After the injections, the animals were kept for 30 min in the conditioning chamber before being returned to the home cages. The observable signs of illness induced by lithium and discomfort/pain produced by hypertonic saline lasted approximately 20 min. A recovery day followed each two conditioning trials. Two days after the final

conditioning session, the orofacial reactivity test occurred. During this session, each rat was placed in the conditioning chamber for 5 min where their orofacial responses were recorded.

Based on the method followed by Parker (1984), and as previously used in our laboratory (e.g. Dwyer et al., 2017; Gasalla et al., 2017; López et al., 2010), the aversive orofacial responses scored included the frequency of the responses 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). Forelimb flails (rapid horizontal movements of the forelimbs for remove fluid from the fur) and head shakes (rapid side-to-side head movements with the mouth open in order to remove the fluid out of the mouth) were also scored as aversive responses. These scores were summed to provide a total aversive response score. In addition, the total time spent immobile by the rats in the contexts (scored as suppression of all the movements in the rat with the exception of those required for respiration) was assessed to measure fear. Appetitive responses (e.g., mouth movements, tongue protrusions, and paw licks) related to consummatory behavior were not scored in this experiment because the rats did not receive exposure to flavors. The interrater reliability ($rs > 0.83$) for each behavior scored was highly significant.

Data analysis. The aversive responses evoked by the context during training and test sessions were analyzed with 3 (group) x 5 (session) mixed ANOVAs. A similar ANOVA was used to analyze the time spent immobile by the animals during training sessions and the test. All analysis was performed on the responses recorded in the 5 min prior to US delivery in training (and the 5 min test period). When a significant

interaction between factors was revealed, pairwise comparisons were performed. All test reported here used a criterion for significance of $p = .05$.

Results

Figure 1 shows the data over context training and test sessions for groups Lithium, Hypertonic, and Isotonic (Figure 1A, aversive responses; Figure 1B, immobility data). Group Lithium displayed more aversive responses during the 5 min prior to the US injection to the context than groups Hypertonic and Isotonic, which did not differ from each other. The ANOVA conducted with these scores revealed main effects of session, $F(4,108) = 9.87$, $p < .001$; group, $F(2,27) = 13.58$, $p < .001$; and a significant session by group interaction, $F(8,108) = 9.91$, $p < .001$. An exploration of the interaction with pairwise comparisons revealed no differences between groups on sessions 1-2 (largest $t(27) = 1.72$, $p = .097$ between Group Lithium and Isotonic). More importantly, Group Lithium displayed higher aversive responses on the remaining sessions than either Group Hypertonic (lowest $t(27) = 3.0$, $p = .006$ on session 3) or Group Isotonic (lowest $t(27) = 3.11$, $p < .004$ on session 3). Critically, Groups Hypertonic and Isotonic showed equivalent aversive reactions over the training and test sessions (largest $t(27) = 0.89$, $p = .394$ on session 2).

Figure 1B shows the mean time spent immobile (indicative of fear) by the groups during training and test sessions. Groups did not differ on the first training session, and Group Hypertonic showed increased fear responses compared to Groups Lithium and Isotonic over the remaining sessions. ANOVA revealed main effects of session, $F(4,108) = 29.12$, $p < .001$; group, $F(2,27) = 15.39$, $p < .001$; and a significant session by group interaction, $F(8,108) = 5.82$, $p < .001$. Pairwise comparisons revealed no differences between groups on the first session (largest $t(27) = 0.47$, $p = .639$ for the

difference between Groups Hypertonic and Isotonic). Group Hypertonic displayed higher immobility responses on the remaining sessions than either of Groups Isotonic (lowest $t(27) = 3.19$, $p = .004$ on session 3) and Lithium (lowest $t(27) = 3.34$, $p = .002$ on session 2). Groups Lithium and Isotonic did not themselves differ on immobility responses over training and test (largest $t(27) = 1.04$, $p = .307$ on test session).

In summary, this experiment serves to extend previous studies demonstrating that not only flavors paired with nausea, but also nausea-paired contexts, can elicit conditioned disgust reactions in rats (Gasalla et al., 2017; Limebeer et al., 2006; Limebeer et al., 2008). In addition, the present results show that pairing the same context with internal pain produced by an injection of hypertonic NaCl resulted in conditioned fear as indicated by immobility responses. Thus, this study demonstrates that contextual cues paired with nausea or with internal pain elicit divergent types of hedonic reactions. These dissociable effects of LiCl and hypertonic NaCl on aversive and fear responses elicited by nonflavor cues are similar to those previously observed after pairing flavor cues with nausea or with internal pain (Dwyer et al., 2017).

Experiment 2

The results of Experiment 1 demonstrate that contextual, nonflavor, cues support conditioned hedonic responses in the same way as flavor cues, suggesting that the quality of aversion learning (conditioned nausea vs conditioned fear) is primarily determined by the nature of the event (US) inducing the aversion and not the type of conditioned cue (flavor vs nonflavor cues). However, as noted in the introduction, in our previous work examining affective responses to flavors paired with internal pain or with nausea (Dwyer et al., 2017) there was an equivalent effect of both USs on appetitive orofacial responses to the CS flavor: LiCl and hypertonic NaCl produced a

reduction in appetitive responses compared with control rats injected with isotonic saline. Since appetitive reactions cannot be assessed in the absence of consummatory behavior (i.e., with nonflavor cues alone), in the present experiment we examined the ability of a flavor to acquire secondary conditioning when paired with a context that has previously been associated with internal pain or with nausea. There is some evidence by using the second-order conditioning paradigm that rats will avoid drinking a flavor associated with a previously illness-paired context (e.g., Best, Best, & Mickley, 1973; see also Bond & Harland, 1975). In this paradigm a CS (CS2) acquires the ability to elicit a conditioned response (CR) by being paired with other CS (CS1), rather than being directly paired with a US; thus second-order conditioning is evident if CS2 elicits the same CR elicited by CS1. In this paradigm, CS2 is thought to be associated mainly with the emotional aspects (i.e., positive and/or negative hedonics) of the US (e.g., Gewirtz & Davis, 2000; Holland & Rescorla, 1975).

The design of this experiment is summarized in Table 1. During context training phase, two groups of rats were injected with either LiCl (Group Lithium) or with hypertonic NaCl (Group Hypertonic) in one context (US-paired context), and with isotonic saline in a different context (unpaired context). Having learned this discrimination, the rats were now intraorally infused in successive days with a flavor (CS+) in the paired context, and with a different flavor (CS-) in the unpaired context. Finally, the rats were tested for second-order conditioning in an orofacial reactivity test conducted in the unpaired context. When the flavors are added in the second-order conditioning phase, they elicit unconditioned appetitive responses that could only be modified by the prior conditioning to the context – thus potentially revealing effects of the conditioned contexts on appetitive reactions. We hypothesized that a flavor previously experienced in a context previously paired with LiCl will elicit conditioned

nausea as revealed by aversive reactions in the orofacial reactivity test, but will result in conditioned fear (as revealed by immobility) after pairing with a context previously associated with the internal pain produced by hypertonic saline.

Method

Subjects, fluids, and apparatus.   Twenty four naive male Wistar rats, obtained from the University of Oviedo vivarium (Spain) at approximately 90 days old and with a mean free feeding weight of 404 g (range = 387-467) at the start of the experiment, served as subjects. They were maintained as in Experiment 1. The fluids used as CSs were a 0.1% (w/w) sodium saccharin solution and a 1% (w/w) sodium chloride solution. The solutions used as USs were the same as those in Experiment 1: LiCl (0.15 M); hypertonic NaCl (1.5 M), and isotonic saline (0.15 M). These solution were i.p. administered at a volume of 10 ml/kg of body weight.

In this experiment, two contexts were selected to be distinctly different along various dimensions, including floor texture, shape and size of the conditioning chamber, background noise, and illumination. One of the contexts was the same as that in Experiment 1, a square compartment made of transparent plastic with a plastic lid placed on a clear glass surface, and with bright illumination. The other context consisted of a cylindrical cage made of black plastic (25 cm diameter x 20 cm high) placed on a table with a clear Plexiglas top. The floor of the cage was made of wire mesh, and the roof was covered with a dark lid. The chamber was dimly illuminated by a 25-W red bulb positioned in a corner of the room close to the cage and contained a speaker delivering a click with an intensity of 75 dB and a frequency of 300 Hz. For both contexts, a mirror beneath the chamber on a 45º angle facilitated viewing of the ventral surface of the rats during the experimental sessions.

In this study, in addition to the aversive and immobility responses evoked by the contexts during discrimination training, the appetitive and aversive orofacial reactivity responses displayed by the rats during the intraoral infusion of the flavors through the implanted cannula (described below) were recorded. The appetitive responses scored were 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). The total number of seconds that the rats displayed the responses was used as the appetitive response score. Appetitive and aversive responses were scored on different scales (duration vs frequency) because they display very different properties: appetitive responses are typically displayed over extended periods of time, while aversive responses occur as isolated behaviors (Berridge, 2000). The time spent immobile by the animals over the infusion period and the frequency of passive-dripping (each occasion on which a drop of fluid was allowed to leak out of the mouth to the floor without other orofacial actions) were also scored. Passive dripping and immobility were scored independently such that time spent dripping was not recorded as immobile. The interrater reliability for each behavior scored was highly significant ($rs > 0.91$).

Cannulation surgery and intraoral infusion.   The rats were surgically implanted with an intraoral cannula under anesthesia using the procedure described by Dwyer et al. (2017). The surgical anesthesia preparation included administration of an intraperitoneal (i.p.) injection of ketamine HCl (50 mg/kg) combined with the analgesic drug medetomidine HCl (0.15 mg/kg). Following surgery, the rats were administered ketofren (1.5 mg/kg, s.c.), an anti-inflammatory drug, and the antibiotic enrofloxacin (0.3 mg/kg, s.c.). 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 PE-20 Intramedic polyethylene tubing (Clay Adams) with an inner diameter of 0.38 mm and an outer diameter of 1.09 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, during which they received 0.3 mg/kg of enrofloxacin to prevent any infection. The cannula flushed daily with a solution of chlorhexidine in distilled water. For the purpose of intraoral infusion, the 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.

Procedure.   The subjects were randomly assigned to one of two groups (n=12) based on their weight: Group Lithium, and Group Hypertonic (see Table 1). The context training phase consisted of four 2-days cycles (one session per day). A recovery day followed each two cycles. In one of the sessions in each cycle, the rats were placed in the US-paired context for 5 min before being injected with either lithium (Group Lithium) or hypertonic NaCl (Group Hypertonic). After the injection, the animals were kept for 30 min in the conditioning chamber before being returned to the home cages. In the other session of each cycle, the animals were placed in the unpaired context for 5 min before being injected with isotonic saline, and immediately after the injection placed in the context for 30 min. Contexts were counterbalanced: thus, for half of the subjects in each group, the conditioning chamber with the square transparent cage served as the paired context and the chamber with the cylindrical black cage as the unpaired context; for the remaining animals, the assignment was reversed. Also, for half

of the animals in each subgroup the sequence of training trials started with an US

injection (ABBAABBA), with the remaining animals starting with a saline injection

(BAABBAAB). During context training sessions, the rats´ orofacial responses and

immobility were recorded.

The day following the final context training session, the rats were implanted

with intraoral cannulas following the procedure above described. Four days after the

surgery, the rats were habituated to the intraoral infusion method receiving a 2 ml water

infusion (1 ml/min) in the home cages. The next day, the second-order conditioning

phase (2 daily sessions) started. In one of the sessions, the animals were placed in the

paired context for 2 min before being infused with the CS+ flavor (counterbalanced

between 0.1% saccharin and 1% saline) for 5 min (infusion rate: 1ml/min). After the

infusion, the animals remained for 10 min in the trained context before being returned to

the home cages. In the other session of this phase, the rats were placed in the unpaired

context for 2 min before receiving an intraoral infusion of the CS- flavor

(counterbalanced between 1% saline and 0.1% saccharin) for 5 min. The rats remained

in the context for 10 min after the infusion. The sequence of second-order conditioning

trials was counterbalanced, with half of the rats receiving first the CS+ flavor in the

paired context and later the CS- flavor in the unpaired context; for the remaining

animals, the assignment was reversed. During the course of these sessions, the

appetitive and aversive orofacial responses elicited by the flavor infusion, passive

dripping events, and immobility responses were recorded.

Finally, all subjects were tested for second-order conditioning in orofacial

reactivity tests performed in the unpaired context. In each of two sessions, the rats were

intraoral infused with one of the flavors (CS+ or CS-: in a counterbalanced order) for 2

min. During the infusions, appetitive and aversive taste reactivity responses, passive dripping, and immobility were recorded.

Data analysis. The aversive and immobility responses evoked in the 5 min prior to US delivery by the contexts during discrimination training were analyzed with 2 (group: Lithium vs Hypertonic) x 2 (context: paired vs unpaired) x 4 (session) mixed ANOVAs. The orofacial reactivity responses (appetitive and aversive) elicited by the infusion of the flavors during the second-order conditioning phase (5 min) and during testing (2 min), as well as immobility and passive dripping events, were analyzed by analyses of variance (ANOVAs) with group (Lithium vs Hypertonic) as between-subject factor and a within-subject factor of flavor (CS+ vs CS-). All test reported here used a significance value of $p = .05$.

Results

Figure 2 shows the data (aversive responses and immobility) over the cycles of context training for Groups Lithium and Hypertonic. Figure 2A displays the mean number of aversive orofacial responses evoked by the US-paired context and the unpaired context (P and U, respectively in the figure). It may be seen that for Group Lithium the number of aversive responses increased over training cycles in the paired context, whereas it remained low across the cycles in the unpaired context. For Group Hypertonic, however, the number of aversive responses remained low over training sessions in both contexts. A comparison of the aversive responses in the paired and unpaired contexts across the 4 cycles, with one between-subjects factor (group) and two within-subjects factors (cycle and context), revealed significant main effects of cycle, $F(3,66) = 19.53$, $p < .001$; context, $F(1,22) = 29.79$, $p < .001$; and group, $F(1,22) = 71.92$, $p < .001$. The interactions involving these factors were all significant: cycle x

group, $F(3,66) = 11.33$, $p = .001$; context x group, $F(1,22) = 28.52$, $p < .001$; cycle x

context, $F(3,66) = 16,8$, $p < .001$; and cycle x context x group, $F(3,66) = 12.95$, $p <$

.001. A simple effect analysis of the triple interaction revealed that Group Lithium

displayed significantly more aversive responses in the paired than the unpaired context

on cycles 3, $t(11) = 5.09$, $p < 0.001$, and 4, $t(11) = 6.68$; $p < .001$, and that there was no

significant difference between the number of aversive responses shown in these

contexts for subjects in Group Hypertonic (highest $t(11) = 1.02$, $p = .314$ for the

difference on cycle 1).

Figure 2B shows the mean time spent immobile (reflecting fear) for Groups

Lithium and Hypertonic in the paired and unpaired contexts across training. As shown

in the figure, Groups did not differ on the cycles 1-2, and Group Hypertonic showed

increased immobility responses compared to Groups Lithium over the remaining

sessions. The mixed ANOVA conducted with the data shown in Figure 2B revealed

main effects of cycle, $F(3,66) = 44.41$, $p < .001$; context, $F(1,22) = 101.05$, $p < .001$;

and group, $F(1,22) = 211.94$, $p < .001$. The interactions involving these factors were all

significant: cycle x group, $F(3,66) = 39.97$, $p = .001$; context x group, $F(1,22) = 84.88$,

$p < .001$; cycle x context, $F(3,66) = 30.97$, $p < .001$; and cycle x context x group,

$F(3,66) = 31.23$, $p < .001$. The simple effects analysis showed that Group Hypertonic

spent significantly more time immobile in the paired context than in the unpaired

context on cycles 2-4 (lowest $t(11) = 14.96$, $p = .025$ for the difference on cycle 2), and

that there was no significant difference between the time spent immobile in each of the

contexts for subjects in Group Lithium (largest $t(11) = 1.64$, $p = .113$ for the difference

on cycle 1).

The data (aversive and appetitive responses, immobility, and passive-dripping

events) during the second-order conditioning phase are displayed in Figure 3. Figure 3A

shows the number of aversive orofacial responses displayed during the infusion of the

CS+ and the CS- flavors for each group. Because this was the first exposure to these

flavors, responses seen during this session reflect the effects of prior context

conditioning. It is clear from this figure that rats in Group Lithium displayed more

aversive responses when being infused in the paired context (i.e., with the flavor that

will be the second order CS+), than when infused in the unpaired context (i.e., with the

flavor that will be the second order CS-). In contrast, Group Hypertonic appeared to

show a similar number of aversive responses when infused with both flavors. The

ANOVA conducted on these data, with group and flavor as the factors, revealed a

significant effect of group, $F(1,22) = 23.19$, $p < .001$; flavor, $F(1,22) = 19.62$, $p < .001$;

and a significant interaction between these two factors $F(1,22) = 14.19$, $p < .001$. An

exploration of the interaction with pairwise comparisons showed that Group Lithium

displayed significantly more aversive responses to the CS+ flavor than to the CS-

flavor, $t(11) = 5.79$, $p < .001$, and that there was no significant difference between the

numbers of aversive responses to the flavors for subjects in Group Hypertonic, $t(11) =$

.46, $p = .644$.

The mean duration of the appetitive responses displayed by the groups during

the infusion of the flavors CS+ and CS- is shown in Figure 3B. Both Group Lithium and

Group Hypertonic displayed fewer appetitive responses to the CS+ flavor than to the

CS- flavor. Again, this reflects the effects of context conditioning, because the flavors

were novel at this point. The ANOVA conducted with these data revealed a significant

effect of flavor, $F(1,22) = 21.12$, $p < .001$, but no effect of group, $F(1,22) = 2.26$, $p =$

.147, or a significant interaction between these factors ($F < 1$). Pairwise comparisons

revealed that both groups displayed significantly less appetitive responses when infused

with the CS+ flavor than when infused with the CS- flavor, $t(11) = 3.27$, $p =.003$, and $t(11) = 3.22$, $p = .004$, for Groups Lithium and Hypertonic, respectively.

Figure 3C shows the mean time spent immobile for each group when infused with the flavors during the second-order conditioning phase. The ANOVA conducted with these data revealed significant main effects of group, $F(1,22) = 78.56$, $p < .001$; and flavor, $F(1,22) = 93.72$, $p < .001$; and a significant interaction between these factors, $F(1,22) = 88.79$, $p < .001$. Pairwise comparisons revealed that Group Hypertonic spent more time immobile when infused with the CS+ flavor than when infused with the CS- flavor, $t(11) = 13.50$, $p <.001$, and that there was no significant difference between the numbers of immobility responses in Group Lithium when infused with both flavors, $t(11) = 182.67$, $p = .857$. Finally, as shown in Figure 3D, the analysis of the passive dripping events revealed that the infusion of the CS+ flavor elicited more passive dripping in Group Hypertonic than in Group Lithium. The ANOVA conducted on these data revealed significant main effects of group, $F(1,22) = 48.51$, $p < .001$; and flavor, $F(1,22) = 48.21$, $p < .001$; and a significant interaction between these two factors $F(1,22) = 39.01$, $p < .001$. Subsequent pairwise comparisons showed that rats in Group Hypertonic displayed significantly more passive dripping when infused with the CS+ flavor than when infused with the CS- flavor, $t(11) = 9.32$, $p <.001$, and that there was no difference between the numbers of passive-dripping events to the flavors for subjects in Group Lithium, $t(11) = 0.49$, $p = .627$.

Turning to the expression of second order conditioning, Figure 4 shows the results of testing the CS+ and CS- flavors conducted in the unpaired context. As shown in Figure 4A, Group Lithium displayed more aversive responses than Group Hypertonic when infused with the CS+ flavor (i.e., the flavor previously infused to the rats in the paired context). In addition, for Group Lithium the CS+ flavor elicited more aversive

responses than the CS- flavor (the flavor infused in the unpaired context). The ANOVA conducted on these data revealed a significant effect of group, $F(1,22) = 24.40$, $p <$ .001; flavor, $F(1,22) = 16.18$, $p = .001$; and a significant interaction group by flavor, $F(1,22) = 11.06$, $p = .003$. Subsequent pairwise comparisons revealed that rats in Group Lithium displayed significantly more aversive responses to the CS+ flavor than to the CS- flavor, $t(11) = 5.19$, $p < 001$, and that there was no difference between the numbers of aversive responses to the CS+ and CS- flavors for subjects in Group Hypertonic, $t(11) = .49$, $p = .627$.

Figure 4B shows the mean duration of the appetitive responses for each group when infused with the CS+ flavor and the CS- flavor during the taste reactivity test. Groups Lithium and Hypertonic showed an equivalent number of appetitive responses to the infusion of the CS+ flavor and, importantly, fewer appetitive responses when infused with the CS+ flavor than whereas infused with the CS- flavor. The ANOVA performed with these scores revealed main effects of flavor, $F(1,22) = 21.47$, $p < .001$; and group, $F(1,22) = 6.51$, $p = .018$, but no significant interaction between them ($F < 1$). Pairwise comparisons revealed that both groups Lithium and Hypertonic displayed significantly fewer appetitive responses to the CS+ flavor than to the CS- flavor, $t(11) = 3.26$, $p = .004$; and $t(11) = 3.29$, $p = .003$, for groups Lithium and Hypertonic, respectively.

Figure 4C shows the immobility data for each group when infused with the CS+ flavor and the CS- flavor during the second-order test. Group Hypertonic spent more time immobile than Group Lithium when infused with the CS+ flavor, but not when infused with the CS- flavor. The ANOVA conducted with the immobility data revealed significant main effects of group, $F(1,22) = 151.82$, $p < .001$; and flavor, $F(1,22) = 147.58$, $p < .001$; and a significant interaction between these factors, $F(1,22) = 155.33$, p

< .001. The pairwise comparisons revealed that rats in Group Hypertonic showed significantly more immobility when infused with the CS+ flavor than when infused with the CS- flavor, $t(11) = 17.40$, $p <.001$; and that there was no significant difference in the time spent immobile by rats in Group Lithium when infused with any of the flavors, $t(11) = .522$, $p = .826$.

Finally, as shown in Figure 4D, the analysis of passive-dripping events revealed that the infusion of the CS+ flavor elicited more passive dripping in Group Hypertonic than in Group Lithium. The ANOVA conducted on these scores revealed significant main effects of group, $F(1,22) = 76.23$, $p < .001$; and flavor, $F(1,22) = 51.94$, $p < .001$; and a significant group by flavor interaction $F(1,22) = 50.13$, $p < .001$. The pairwise comparisons revealed that Group Hypertonic displayed significantly more passive dripping when infused with the CS+ flavor than when infused with the CS- flavor, $t(11) = 10.10$, $p <.001$, and that there was no significant difference between the numbers of passive-dripping events to each flavor in Group Lithium, $t(11) = .08$, $p = .929$.

In summary, this experiment yielded three major findings. First, analysis of the context discrimination training and responses during flavor infusions during the second order conditioning phase confirmed that contexts paired with lithium and hypertonic saline elicit different types of reactions (aversive orofacial reactions and immobility respectively) but had similar effects on appetitive responses (a reduction in both cases). Second, it confirmed the central result of a previous work by Sticht, Leach, Wilson, and Parker (2015) demonstrating through a second-order conditioning procedure that rats display orofacial aversive responses to a novel flavor presented in a previously lithium-paired context. Third, it demonstrated that a flavor presented in a previously LiCl-paired context results in the production of orofacial aversive responses (reflecting conditioned nausea) whereas the same flavor experienced in a context previously paired with

hypertonic NaCl results in the flavor eliciting immobility responses and passive dripping (reflecting conditioned fear). This difference was observed at the same time as it was found that pairing the flavor CSs with contexts previously followed by either LiCl or hypertonic NaCl injections had a common effect on appetitive responses with both producing fewer appetitive responses to the CS+ flavor than to the CS- flavor, even though the first-order context cues did not elicit appetitive responses themselves. It should be noted that in the present experiment the final orofacial reactivity test was conducted in the context never paired with the US injection indicating that the hedonic responses elicited by flavor cues were the result of second-order conditioning and not simply generalization from the paired context.

Discussion

The experiments reported here examined the nature of the conditioned hedonic responses elicited by contextual cues after their pairing with nausea (produced by LiCl administration) or with internal pain (produced by injection of hypertonic saline). Experiment 1 replicated previous findings (Limebeer et al., 2006; 2008) showing that rats display orofacial aversive responses to a nausea-paired context - confirming that contextual cues can elicit orofacial reactivity responses in the absence of any flavor component. In addition, the experiment demonstrated selective conditioning effects between internal pain and lithium-induced nausea: Pairing a context with LiCl resulted in the production of orofacial aversive responses to the context, but pairing the same context with internal pain resulted in the production of conditioned fear as indicated by immobility responses. Experiment 2, using a second-order conditioning paradigm, found that the intraoral infusion of a novel flavor that had been experienced in a context previously paired with LiCl elicited aversive orofacial reactions indicative of

conditioned nausea, whereas the infusion of the same flavor in a context that had previously been paired with hypertonic NaCl produced immobility responses reflecting conditioned fear. Thus, these findings show that contextual cues paired with internal pain or with nausea elicit divergent types of hedonic responses. It is important to note, however, that both lithium-induced nausea and internal pain caused reductions in appetitive taste reactivity responses when the animals were infused with the flavor previously experienced in the US-paired context.

The clearest implication of the results obtained here is that that contextual cues support conditioned hedonic responses in qualitatively the same way that flavor cues. As mentioned before, a previous study from our laboratory (Dwyer et al., 2017) used orofacial reactivity analysis to examine the hedonic responses elicited by flavor cues paired with internal pain produced by hypertonic NaCl or LiCl-induced nausea. This showed that only pairing with nausea results in the flavor eliciting aversive responses whereas pairing with internal pain results in the production of immobility responses. Also, as in the current study, it was found that either internal pain or nausea resulted in the reduction of appetitive orofacial responses. That is, the pattern of responses elicited by flavors and context (i.e.nonflavor) cues after pairing with LiCl-induced nausea or hypertonic saline-induced pain was the same. In addition, we have reported (Gasalla et al., 2017) that contextual cues paired with lithium-induced nausea interfere, through blocking, with changes in hedonic responses to flavor cues: not only did a context previously paired with LiCl elicited aversive orofacial responses, it also attenuated the reduction in palatability of a saccharin solution which was paired with LiCl in that context, demonstrating that contextual stimuli can interfere with the affective aspects of taste aversion learning. This complements the current observation of second order conditioning between context and flavor cues – reinforcing the idea that not only do

flavor and nonflavor cues engage in aversion conditioning in similar ways when trained separately, but that these cue types interact in learning. In short, it appears that the quality of aversion learning (conditioned nausea vs conditioned fear) is primarily determined by the nature of the US (LiCl vs hypertonic NaCl). This is not to say that the nature of the CS is entirely without influence on the form of the conditioned responses in the current experiments: in particular, the contextual cues did not elicit appetitive orofacial reactions or support their conditioned reduction, unlike flavor cues. However, as with Holland (1977) and the demonstration of different conditioned responses to light or tone stimuli paired with food, the evidence from second order conditioning (Experiment 2) and blocking (Gasalla, et al., 2017) suggests that the content of the CS-US association is similar across stimulus modality. Moreover, the rate at which aversive responses emerged after contexts were paired with nausea and immobility emerged after contexts were paired with pain was similar in the current experiments and the same pattern was seen in prior studies using flavor CSs and the same USs (Dwyer et al., 2017). These results seem incompatible with the strong assumption of highly selective cue-to-consequence learning stemming from classic taste aversion studies (e.g. Garcia & Koelling, 1966).

We now turn to the broader issues of how different classes of orofacial reactivity reactions should be interpreted, and the implications this has for the conceptual analysis of aversion learning. Soon after the taste reactivity test was established (Grill & Norgren, 1978) a two-dimensional account of palatability was proposed (Berridge & Grill, 1983, 1984) partially on the basis that taste compounds can be evaluated simultaneously as positive and as negative, with the compound eliciting appetitive and aversive responses in alternation. Supporting this view, it has been reported that the number of aversive responses may be increased by taste manipulations that do not affect

the number of appetitive responses. For example, the frequency of aversive responses to a compound of sucrose and quinine, that originally elicits a mixture of appetitive and aversive responses, may be increased by increasing the concentration of quinine without affecting the number of appetitive responses (e.g., Berridge & Grill, 1983).

This idea that there are two separate dimensions to the taste reactivity test, with aversive reactions reflecting the degree of disgust and appetitive reactions reflecting the degree of positive palatability was reinforced by the observation of selective conditioning effects whereby some aversive stimuli paired with flavors (in particular LiCl and other emetics) resulted in a reduction in consumption as well as an increase in aversive reactivity responses, while others (in particular pain from footshock and some drugs of abuse) resulted in reductions in consumption without increases in aversive reactivity responses (for reviews, see Parker, 2003, 2014). Parker's interpretation of these results was that nausea-inducing events support a "true" conditioned taste aversion (CTA) based on conditioned disgust indicated by orofacial aversive responses, whereas non-nausea negative events support a fear-based process of taste avoidance learning (TAL) indicated by the suppression of consumption without aversive orofacial responses. It might also be the case that the dissociation of hedonic responses observed in the current study reflect two distinct aspect of the conditioned response. As proposed by Konorski (1967; see also Wagner & Brandon, 1989), the US can be represented by multiple nodes in the memory system, including the representation of the emotional and the sensory characteristics of the US. According to such an analysis, reduction in appetitive responses may primarily reflect the overall emotional value of the US, but immobility and aversive orofacial response may primarily is reflecting the sensory-specific features of the US. Regardless of the mechanism, it is clear that the form of the conditioned response differs between USs.

However, these views have not been unchallenged. An alternative interpretation is that hedonic reactivity to taste stimuli can be considered as varying along a single continuum or dimension of palatability from highly positive (many appetitive reactions), through neutrality (few appetitive or aversive reactions), to highly negative (many aversive reactions: e.g., Breslin, Grill, & Spector, 1992; Young, 1977). This one-dimension interpretation of orofacial informed a critique of Parker's ideas by Reilly and colleagues (see Lin et al., 2014, 2017) which emphasized the fact that all negative USs which result in a reduction in consumption when paired with flavors also result in the reduction of appetitive orofacial reactions. Moreover, using an alternative method for assessing taste palatability in rodents, the analysis of the microstructure of licking behavior during voluntary consumption (Davis, 1989; Dwyer, 2012), Reilly´s group examined the effect of pairing a novel taste with the administration of gallamine hydrochloride (a drug inducing paralysis and pain in muscle tissue), hypertonic NaCl (inducing visceral pain), or amphetamine (Arthurs, Lin, Amodeo, & Reilly, 2012; Lin, Arthurs, Amodeo, & Reilly, 2012; Lin, Arthurs, & Reilly, 2013). They found that these substances not only produced a reduction in flavor consumption, but also decreased lick cluster size (mean number of licks per cluster), reflecting a reduction in taste palatability. Thus, contrary to our view, these authors suggest that the differences in taste aversions elicited by nausea, internal pain, and drugs of abuse might be quantitative rather than qualitative; that is, internal pain or drugs of abuse might produce only a mild conditioned aversion (reflected in a reduction in appetitive taste reactivity responses and decreased lick cluster size, without an increase in aversive responses), while nausea might produce a strong taste aversion reflected in an increase in aversive responses as well as a decrease in appetitive taste reactivity responses.

While Reilly's critique is important in highlighting the fact that many treatments can suppress appetitive responses and consumption, the present results appear to be more consistent with a two-dimensional account of hedonic reactions as well as with a division between nausea-based conditioned disgust and pain-based conditioned fear. This was shown most clearly in Experiment 2 where flavors experienced in context previously paired with LiCl or in a context previously paired with hypertonic saline subsequently resulted in equal reductions in appetitive orofacial responses, but only the flavor experienced in the LiCl-paired context elicited aversive orofacial reactions, while the flavor experienced in the hypertonic saline-paired context elicited immobility. A one dimensional account of palatability would suggest that treatments which produce equivalent effects on appetitive responses should also have equivalent effects on aversive responses – which is not the case here. Moreover, in a previous study (Dwyer et al., 2017), we demonstrated that pairing a flavor with either nausea or internal pain to the point that both appetitive responses and consumption were completely suppressed (and lick cluster size greatly reduced), only nausea-paired flavors showed increases in aversive reactions, while the pain-paired flavor elicited greater levels of immobility. In summary, many negative USs share the ability to reduce positive aspects of hedonic reactions, but there are reliable differences between them (in particular between emetic and non-emetic treatments) in the ability to induce either conditioned disgust or conditioned fear that cannot be explained simply in terms of differing levels of aversion (for an extended discussion of this issue, see Dwyer et al., 2017).

In conclusion, the present study provides evidence confirming that pairing a context with internal pain or nausea has dissociable effects on the conditioning of hedonic responses: only pairing with nausea results in the production of aversive responses, while pairing with internal pain results in conditioned fear as indicated by

immobility. This reproduces the response pattern observed after pairing flavor cues with internal pain or nausea confirming the proposed interpretation: that the quality of conditioned aversions (nausea vs fear) is primary determined by the nature of the aversive event and not the type of conditioned cue (flavor vs context). Future research will elucidate more precisely the learning mechanisms responsible for the conditioning of hedonic reactions with different aversive events, including drugs of abuse.

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Conflict of interest

The authors declare that they have no conflict of interest, financial or otherwise, related to this work.

TABLE 1. Experimental designs

_____

Experiment 1

Group	*Training (4×)*	*Testing*
Lithium	Context (5min) → LiCl → Context (30min)	Context (5min)
Hypertonic	Context (5min) → Hypertonic → Context (30min)	Context (5min)
Isotonic	Context (5min) → Isotonic → Context (30min)	Context (5min)

Experiment 2

Group	*Context training (4×)*	*Second-order conditioning*	*Testing*
Lithium	P → LiCl & U → Isotonic	P: CS+ flavor (IO) & U: CS- flavor (IO)	U: CS+ flavor (IO) & U: CS- flavor (IO)
Hypertonic	P → Hypertonic & U → Isotonic	P: CS+ flavor (IO) & U: CS- flavor (IO)	U: CS+ flavor (IO) & U: CS- flavor (IO)

_____

Note. In Experiment 2, P and U designate paired and unpaired contexts, respectively (the identity of the contexts were counterbalanced). During context training the rats were placed in the corresponding context for 5 min before being injected, and were kept 30 min in the context before being returned to the home cages. In the second-order conditioning phase, the rats were placed in the corresponding context before being intraorally infused for 5 min, and were kept 10 min in the context after the infusion. In the testing sessions, the rats were infused each of the flavors for 2 min in separate sessions.

Figure legends

Fig.1. Experiment 1 data over context training and test sessions for Groups Lithium, Hypertonic, and Isotonic: (A) Mean number of aversive orofacial responses, and (B) mean time spent immobile (sec). Data is shown for the 5 min period prior to US delivery in training (and the same 5 min period at test) and error bars represent the standard errors of the mean (*SEM*).

Fig. 2. Experiment 2 data over context training for Groups Lithium and Hypertonic: (A) mean number of aversive orofacial responses, (B) mean time spent immobile (sec). Over the cycles, the US was i.p. administered in the paired context (P), but not in the unpaired context (U). Data is shown for the 5 min period prior to US delivery and error bars represent the standard errors of the mean (*SEM*).

Fig. 3. Experiment 2 data for Groups Lithium and Hypertonic from the second-order conditioning phase: (A) mean number of aversive responses elicited during infusion of CS+ flavor in the paired context and the CS- flavor in the unpaired context, (B) mean duration of appetitive responses, (C) mean time spent immobile, and (D) mean number of passive-dripping events. Error bars represent the standard errors of the mean (*SEM*).

Fig. 4. Experiment 2 data for Groups Lithium and Hypertonic from the taste reactivity test: (A) mean number of aversive responses elicited by the CS+ and the CS- flavors, (B) mean duration of appetitive responses, (C) mean time spent immobile, and (D) mean number of passive-dripping events. The flavors were intraorally infused in the unpaired context. Error bars represent *SEM*.
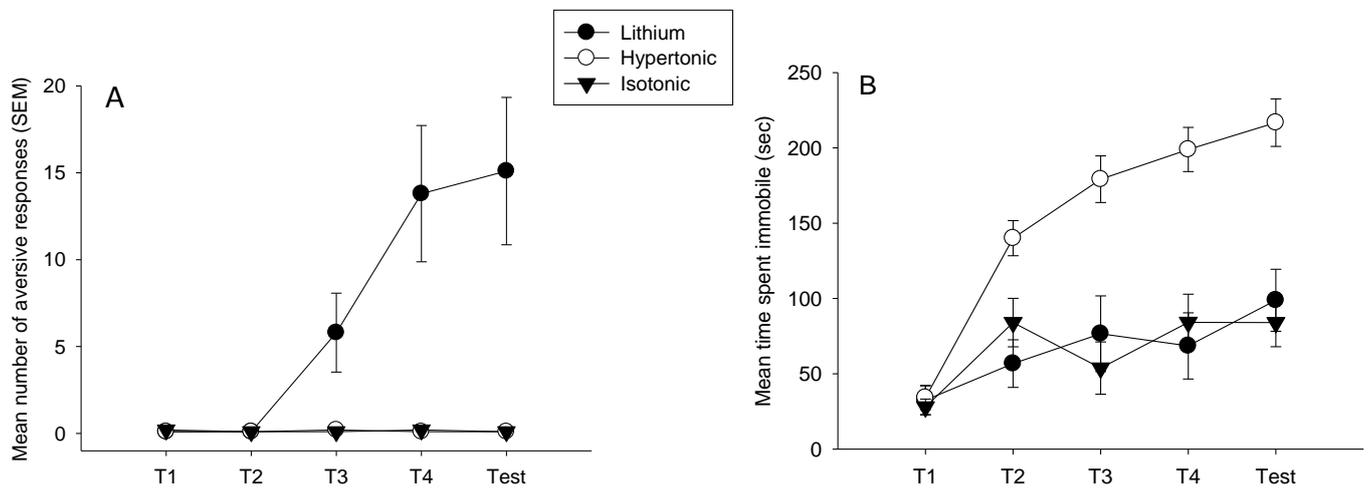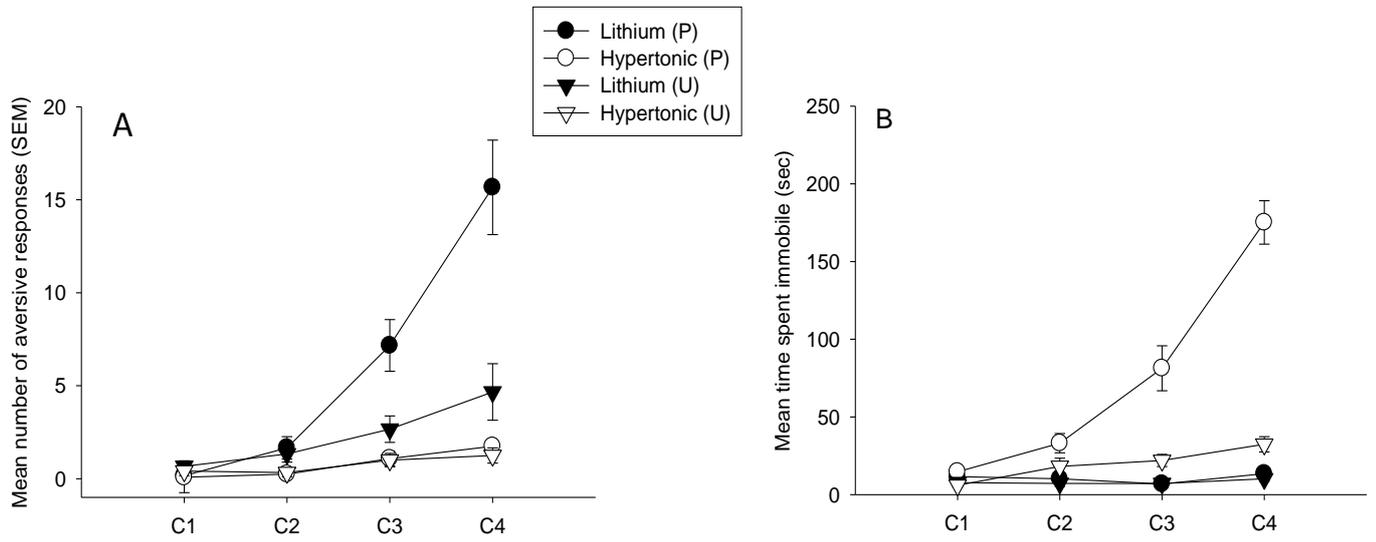
Figure 1

Figure 2

Figure 3

Aversive responses

CS- Flavor
CS+ Flavor

A

Mean number of aversive responses

Lithium   Hypertonic

Appetitive responses

B

Mean duration of appetitive responses (s)

Lithium   Hypertonic

Immobility

C

Mean time spent immobile (sec)

Lithium   Hypertonic

Passive dripping

D

Mean number of passive dripping events
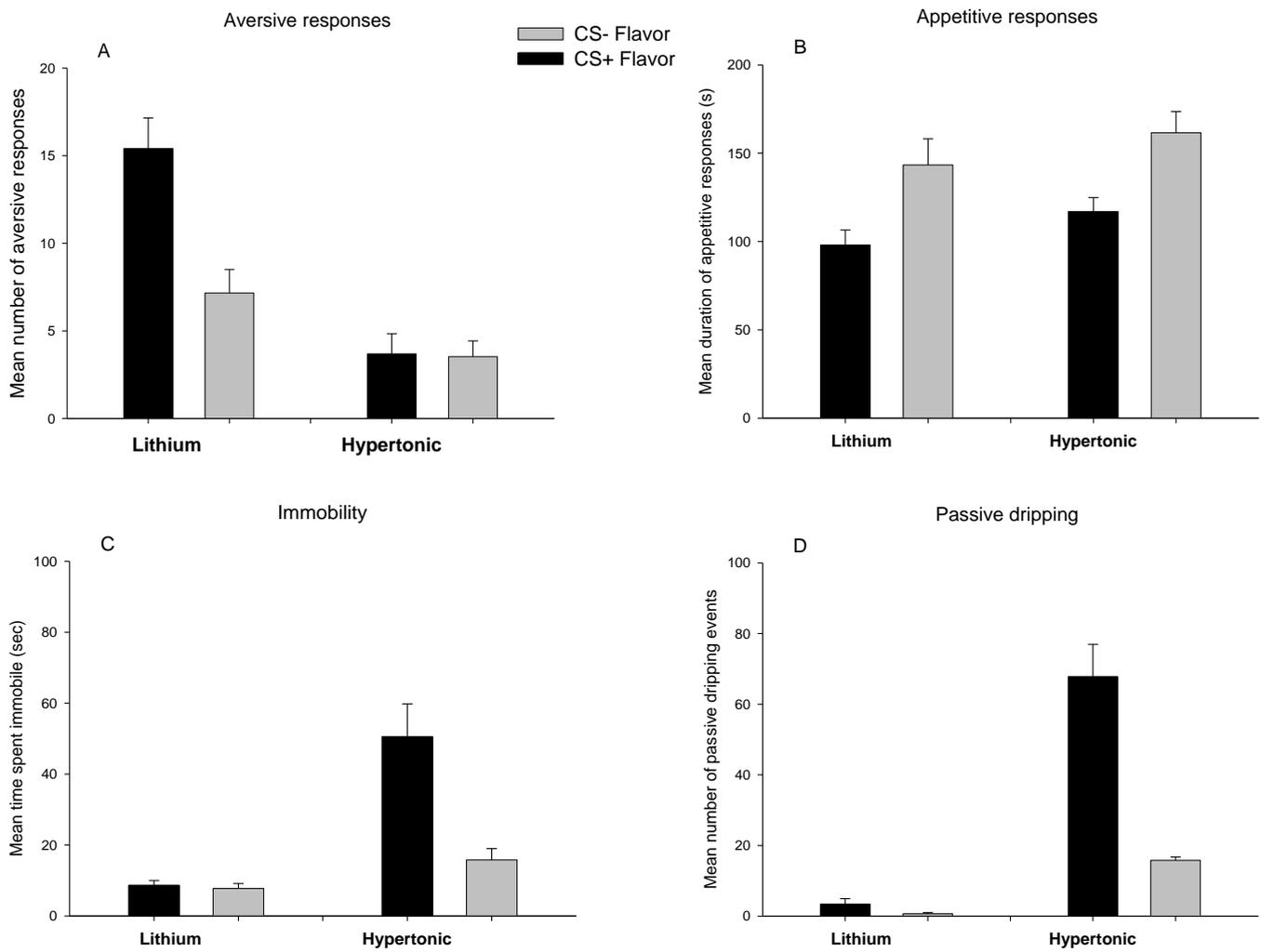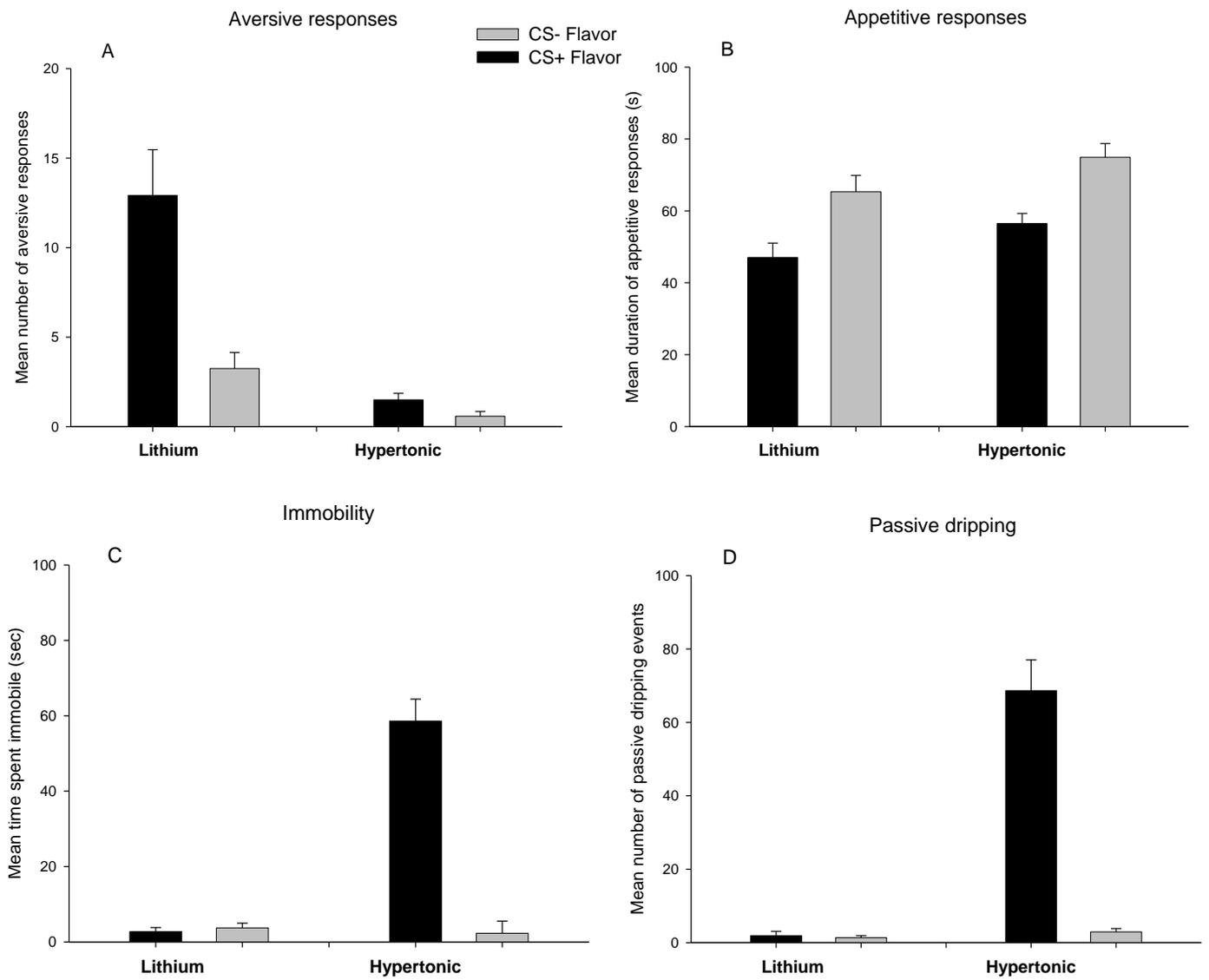
Lithium   Hypertonic

Figure 4

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