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Reacquisition, Reinstatement, and Renewal of a Conditioned Taste Aversion in Preweanling Rats

ABSTRACT: *Pavlovian extinction is defined as a reduction of the conditioned response (CR) as a consequence of repeated and nonreinforced presentations of the conditioned stimulus (CS). This phenomenon has been explained through two nonexclusive associative hypotheses. One of them proposes that the CS–unconditioned stimulus (US) association is weakened during extinction, while the second one explains extinction by the formation of a new inhibitory association between the CS, and the US (CS–noUS) which competes with the excitatory one acquired at conditioning (CS–US). Research supporting this second hypothesis is based on the demonstration that the CR can be recovered after extinction. However, in preweanling rats, renewal, and reinstatement treatments have failed to recover a conditioned fear response, suggesting that extinction during this ontogenetic period may involve erasure of the CS–US association. The goal of the present study was to explore whether this conclusion can be extended to the extinction of a conditioned taste aversion by evaluating infant rats in three different procedures (reacquisition, ABA renewal, and reinstatement). The results are consistent with the idea that extinction of a taste aversive memory during infancy involves relearning about the relationship between the CS and the US, with the initial CS–US association remaining relatively intact. Extinction of a taste aversive memory and a fear memory may involve different biological mechanisms during infancy. The conclusion that the only psychological mechanism for extinction during infancy is unlearning should be confined to a particular type of memory. © 2013 Wiley Periodicals, Inc.* Dev Psychobiol 9999: 1–13, 2013.

Keywords: *extinction; infant; rat; taste aversion*

INTRODUCTION

The psychological and neurobiological basis of learning and memory are often studied within the classical conditioning framework. In a typical procedure, a relatively neutral stimulus (conditioned stimulus, CS) is presented in temporal contiguity with a naturally relevant stimulus (unconditioned stimulus, US). After this treatment, the mere presentation of the CS can induce the conditioned response (CR). It is assumed that this response reflects the formation of a hypothetical excitatory association between the CS and the US (CS–US). Pavlovian or experimental extinction is one of the classical conditioning phenomena that have received most attention from researchers, because of both its clinical and theoretical implications (Bouton, 2004; Graham & Milad, 2011; Milad & Quirk, 2012). Extinction is empirically defined as a reduction of the CR as a consequence of repeated and non-reinforced presentations of the CS (Pavlov, 1927).

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This phenomenon has been explained through two nonexclusive associative hypotheses. One of them proposes that the CS–US association is weakened during extinction. In other words, extinction induces unlearning (Rescorla & Wagner, 1972) or erases the CS–US association (Quirk et al., 2010). The alternative hypothesis, originally proposed by Pavlov, proposes that extinction produces the formation of a new inhibitory association between the CS and the US (CS–noUS) which competes with the excitatory one acquired at conditioning (CS–US) (Pavlov, 1927). Research supporting this second hypothesis is based on the demonstration that the CR can be recovered after extinction by the simple passage of time (spontaneous recovery), by the presentation of the US before testing (reinstatement) or by testing the subject in a context different from the one used during extinction (renewal; Bouton, Westbrook, Corcoran, & Maren, 2006). Another procedure that shows that the CS–US association survives extinction is reacquisition, which consists of retraining the same CS with the same US after extinction (Denniston & Miller, 2003). Reacquisition of a CR has been reported to be faster (Leung, Bailey, Laurent, & Westbrook, 2007; Napier, Macrae, & Kehoe, 1992), or slower (Denniston & Miller, 2003; Hart, Bourne, & Schachtman, 1995) than first acquisition of the same CR. In adult rats the efficacy of all these procedures (renewal, reinstatement, and reacquisition) has been proven in different paradigms, including fear (Bouton et al., 2006), and taste aversion conditioning (Berman, Hazvi, Stehberg, Bahar, & Dudai, 2003; Fujiwara et al., 2012; Hart et al., 1995; Schachtman, Brown, & Miller, 1985).

After extinction in preweanling rats, renewal, and reinstatement treatments have failed to recover the CR, and seem to be ineffective until the weaning period (J. H. Kim & Richardson, 2007; Quirk et al., 2010; Yap & Richardson, 2007). The lack of renewal and reinstatement effects in infant rats suggests that extinction during this ontogenetic period may involve a qualitatively different psychological process from during weaning, adolescence, or adulthood (J. H. Kim & Richardson, 2010). While most of the studies with adult rats have consistently shown that extinction does not completely erase the original CS–US association, in preweanling rats it has been proposed that extinction may lead to the unlearning or elimination of this association (J. H. Kim & Richardson, 2010; Quirk et al., 2010). These conclusions were drawn from studies using a fear conditioning procedure. The goal of the present study is to explore whether these conclusions can be extended to a different type of learning: an aversive taste learning. In Experiment 1, infant rats were tested for reacquisition of an extinguished taste aversion, while Experiments 2 and 3 were designed to evaluate whether renewal and reinstatement treatments recover an extinguished CR in preweanling rats.

EXPERIMENT 1A

In Experiment 1a, preweanling rats were evaluated in terms of reacquisition of an extinguished conditioned taste aversion. To our knowledge, no studies have used this procedure in early ontogeny. In adult rats, reacquisition of an extinguished conditioned taste aversion is usually retarded in comparison with a control group learning the aversion for the first time (Calton, Mitchell, & Schachtman, 1996; Hart et al., 1995). This first experiment explored whether a similar result was reproducible in infant rats. To achieve this goal, infant rats received two pairings of saccharin and LiCl on PDs 14 and 15, and then, after two extinction trials (PDs 16 and 17), they received an additional injection of LiCl following saccharin consumption (PD 18). At testing (PD 19), the magnitude of the taste aversion was compared using two control groups. The first was trained with an alternative flavor (almond), and LiCl during the first phase of the experiment (acquisition, PDs 14, and 15), and then received nonreinforced presentations (extinction) of almond during the second phase. The second group consumed water during both acquisition and extinction (see Tab. 1). The working hypothesis of this experiment is that, if extinction really does erase the CS–US association, then taste aversion will be reacquired at the same rate in all groups; on the other hand, if the CS–US association survives the extinction training, then the reacquisition of the conditioned taste aversion is expected to differ from the initial acquisition.

MATERIALS AND METHODS

Subjects

A total of 112 Wistar pups, representative of 36 L, were utilized for the present study, including Experiments 1a ($n = 29$), 1b ($n = 30$), 2 ($n = 23$), and 3 ($n = 30$). Table 1 indicates the total number of subjects included in each independent group in each experiment. We only employed females in these experiments, and in all cases no more than one subject from a given litter was assigned to the same treatment condition, in order to avoid overrepresentation of a particular litter in any treatment (Holson & Pearce, 1992). Animals were born and reared at the vivarium of the Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC–CONICET-UNC, under conditions of constant room

Table 1. Experimental Design and Number of Subjects for Experiments 1a and 1b

Group	Conditioning PDs 14–15	Extinction PDs 16–17	Re-Conditioning PD 18	Testing PD 19	*N*
Experiment 1a					
Water	Water + vehicle	Water	Saccharin + LiCl	Saccharin	10
Almond	Almond + LiCl	Almond			9
Saccharin	Saccharin + LiCl	Saccharin			10
Experiment 1b					
Water	Water + vehicle	Water	Saccharin + LiCl	Saccharin	10
Saccharin-preexposure	Water + vehicle	Saccharin			11
Saccharin-extinction	Saccharin + LiCl	Saccharin			9

temperature (22 ± 1.0°C), on a 12-hr light–12-hr dark cycle. Births were examined daily and the day of parturition was termed postnatal Day 0 (PD0). Litters were culled to 10 pups within 48 hr after birth. Subjects were 14 days old (PD14) at the start of the experiments. All procedures were approved by the National Department of Animal Care and Health (SENASA–Argentinfe) and were in compliance with the National Institute of Health's general guidelines for the care and use of laboratory animals.

Procedures

Conditioning. Conditioning was carried out on PDs 14 and 15, one session per day. On conditioning days subjects were removed from their home cage and assigned to one of the three independent groups (Water, Almond, or Saccharin, see Tab. 1). The name of the group alludes to the solution that these groups consumed during the acquisition and extinction phases. Immediately afterwards, an intraoral cannula (PE 10 polyethylene tubing, length: 5 cm, Clay Adams, Parsippany, NJ) was implanted into the right cheek of each pup, as described previously (Arias, Molina, & Spear, 2009; Arias, Pautassi, Molina, & Spear, 2010). Briefly, a flanged end of the cannula was shaped by exposure to a heat source (external diameter: 1.2 mm). A dental needle (30-gauge Monoject, Sherwood Medical, Munchen, Germany) was attached to the nonflanged end of the cannula and positioned in the middle portion of the intraoral mucosa. The needle was inserted through the cheek, and the cannula was pulled through the tissue until the flange end rested on the mouth's mucosa. This procedure requires no more than 20 s per subject and does not induce major stress to infant rats (Spear, Specht, Kirstein, & Kuhn, 1989). Immediately afterwards, the pups' bladders were voided by gentle brushing of the anogenital area and body weights were recorded. Then subjects were placed into the conditioning context, an opaque Plexiglas chamber (15 cm × 7 cm × 2 cm) with white walls. This context was employed in all the phases of Experiments 1 (a and b), and 3. In this environment, pups received an intraoral infusion of saccharin (.15% w/v, group Saccharin), almond (Esencias del Boticario, Cordoba, Argentine; .01%, v/v, group Almond), or distillated water (group Water). The total administration volume was equivalent to 1 ml and it was delivered during 10 min at a constant rate (.1 ml/min) by means of an infusion pump (KD Scientific, Holliston, MA) connected to each pup's oral cannula by a polyethylene catheter (Clay Adams, PE 50 Parsippany, NJ). With these parameters, pups are capable of either consuming or rejecting the infused solution (Arias & Chotro, 2006; Arias et al., 2010; Revillo, Spear, & Arias, 2011). After the infusion procedure, subjects were weighed to estimate saccharin consumption scores by means of the following formula: postinfusion body weight–preinfusion body weight. Immediately after this procedure, pups were intraperitoneally injected with vehicle (.9% NaCl, group Water), or LiCl (1% of a .15 M solution, groups Almond, and Saccharin). After drug treatment, pups were reunited with their mother in their corresponding home-cage. The second conditioning trial was conducted the following day (PD15), applying the exact same procedures as those described for the first conditioning trial.

Extinction. On PDs 16 and 17 rats consumed saccharin (group Saccharin), almond (group Almond), or water (group Water) in the conditioning context, but in this case they were not injected after the intake session. The remaining procedures were identical to those described for conditioning. We opted for two extinction days, because previous studies have shown that at this age, after two extinction trials, conditioned taste aversion induced by a similar LiCl dose is completely extinguished (Arias & Chotro, 2006).

Reconditioning and testing. On PD18 all rats were injected with LiCl after consuming saccharin, and the next day (PD19) they were tested in terms of saccharin intake, in both cases in accordance with the procedures described for conditioning.

Data Analysis

Intake scores were analyzed by means of a mixed ANOVA including Group (Water, Almond, or Saccharin) as the only between-group factor, and day as the within-group variable with six levels, corresponding to two conditioning, two extinction, one reacquisition, and one testing trial. In this and subsequent experiments, significant main effects and/or interactions were further analyzed by means of follow-up ANOVAs and post-hoc analyses (Duncan). In the present study we explicitly avoided comparing scores derived from consumption of different solutions. This restriction affected only the conditioning and extinction phases, because at testing all groups consumed saccharin in all experiments. All inferential analyses employed an α level equal to .05.

Results

Figure 1a represents intake from the different groups (Water, Almond, and Saccharin) as a function of the conditioning, extinction, reconditioning, and testing trials. The mixed ANOVA revealed a significant Group × Day interaction [$F(10, 130) = 3.56$, $p < .05$]. To further analyze this interaction, one-way between-factor ANOVAs were conducted with the intake scores from each day. These analyses indicated a significant main effect of Group on PD 19 [$F(2, 26) = 4.49$, $p < .05$], coinciding with the testing day. Post-hoc analyses revealed that on this day, the Saccharin group consumed less saccharin than the other groups (Water or Almond). This result demonstrates that relearning in the Saccharin group was faster than in the Water or Almond conditions. It is important to note that all groups consumed the same amount of saccharin during the trial conducted before testing (PD18).

The one-way between-factor analysis did not demonstrate that the Almond and Saccharin groups acquired aversion after the conditioning trials (C1 and C2). With similar parameters, we have found strong evidence of aversion in previous studies (e.g., Arias et al., 2010; Revillo, Arias, & Spear, 2012), but in

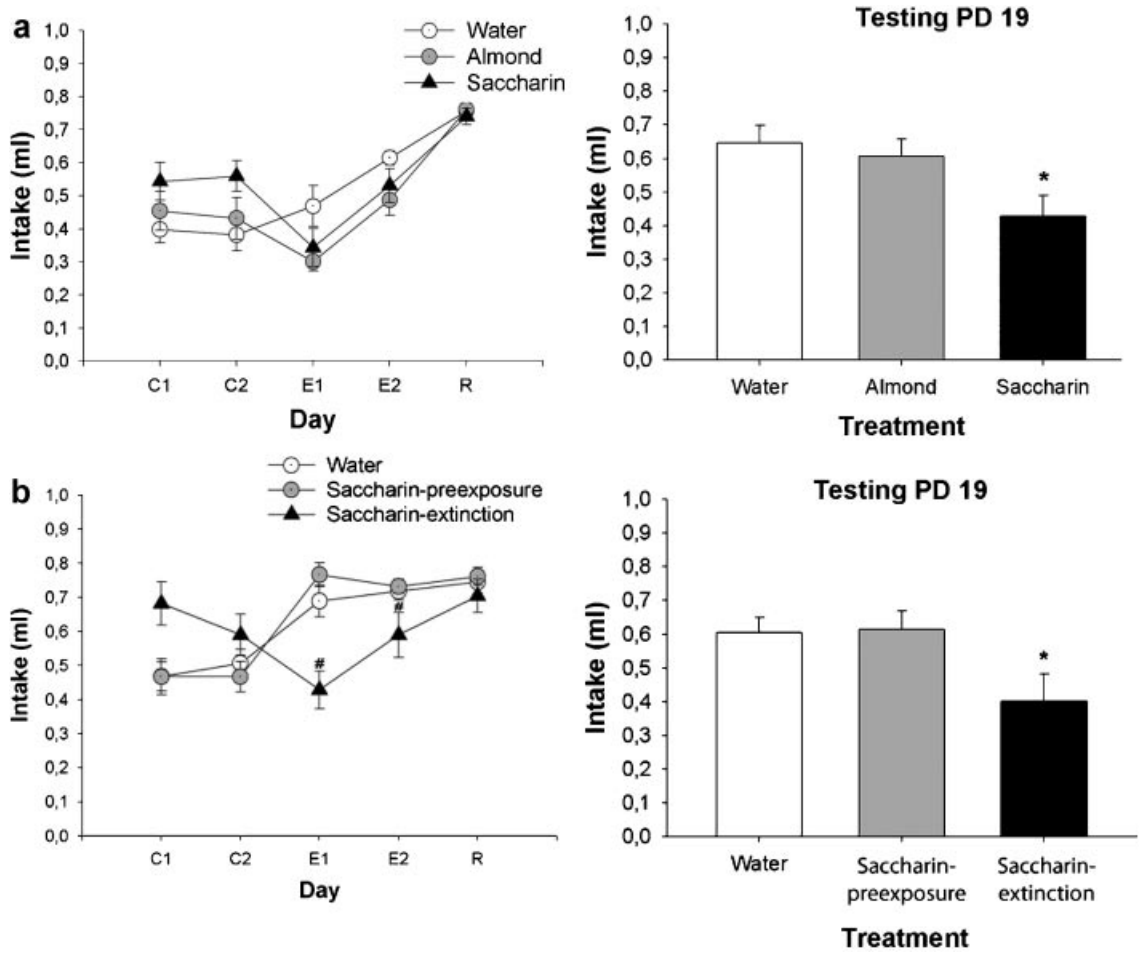

FIGURE 1 **a**: Intake data from Experiment 1a. Scores represent the mean saccharin consumption as a function of Group (Water, Almond, or Saccharin), and Day [Conditioning Days 1 and 2 (C1, C2), Extinction days 1 and 2 (E1, E2), and Reacquisition (R) and Testing days]. The left side of the figure includes intake during conditioning, extinction, and reacquisition, while the right side, intake at testing. Vertical bars represent the standard error of the means (SEM). $^{*}p < .05$ versus the remaining groups at testing. **b**: Intake data from Experiment 1b. Scores represent the mean saccharin consumption as a function of Group (Water, Saccharin-preexposure, or Saccharin-extinction) and Day [Conditioning days 1 and 2 (C1, C2), Extinction days 1 and 2 (E1, E2), and Reacquisition (R) and Testing days]. The left side of the figure includes intake during conditioning, extinction, and reacquisition, while the right side, intake at testing. Vertical bars represent the standard error of the means (SEM). $^{\#}p < .05$ versus the remaining groups during extinction; $^{*}p < .05$ versus the remaining groups at testing.

these cases we included different and more appropriate control conditions (unpaired or CS-only) in the design. However, acquisition of the aversion can be detected in the present study through repeated measure ANOVAs, comparing intake scores within each group across trials. These analyses indicate a significant main effect of day in both conditioned groups (Almond and Saccharin) [$F(5, 40) = 10.44$, $p < .05$; $F(5, 45) = 7.44$, $p < .05$, respectively]. Post-hoc tests were used specifically to explore the acquisition of the aversion after conditioning. These analyses indicate that consumption during the first extinction trial was significantly lower than that registered the first conditioning day on both groups, Almond and Saccharin.

In sum, these results indicate that prior conditioning and extinction of a given CS facilitates later acquisition of the CR. The fact that the Saccharin group expressed stronger aversion at testing than the Almond condition suggests that prior exposure to the US, or prior aversive learning with an alternative CS, is not sufficient to facilitate learning in preweanling rats, a result that rules out sensitization as explanation of the faster reacquisition effect in the Saccharin group.

EXPERIMENT 1B

Using a taste aversion preparation, it has been shown that reacquisition in adult rats is slower in comparison with similar control conditions to those employed in Experiment 1a (Hart et al., 1995). This retardation effect was interpreted in terms of inhibitory strength gained by the CS during extinction (Denniston & Miller, 2003). However, further studies questioned this interpretation. Specifically, if reacquisition of the extinguished aversion was compared with a group that received the same amount of nonreinforced presentations of the CS, it was shown that the retardation effect was susceptible to being interpreted in terms of mere exposure to the CS (at least in taste aversion conditioning; Aguado, de Brugada, & Hall, 2001). In some cases, preexposure to a CS has been found to facilitate conditioning in infant rats (Hoffmann & Spear, 1989). Hence, it is plausible that the mere exposure to saccharin could facilitate conditioning. With the aim of testing this possibility we repeated Experiment 1a with a different control group in the design. This group consumed water during the first phase of the experiment (PDs 14 and 15), but then during extinction was exposed to saccharin. The experimental design is summarized in Table 1.

Procedures

All the procedures employed in this experiment were identical to those used in Experiment 1a, the only exception being that the Almond group was replaced by the Saccharin-preexposure group, which received water at conditioning (PDs 14 and 15), and saccharin during the remaining phases of the experiment (extinction, reconditioning, and testing). The statistical processing of the data was also identical to that described for Experiment 1a.

Data Analysis

Intake scores were analyzed by means of a mixed ANOVA including Group (Water, Saccharin-preexposure, and Saccharin-extinction) as the only between-group factor and day as the within-group variable with six levels, corresponding to two conditioning, two extinction, one reacquisition, and one testing trial.

Results

Data from Experiment 1b are represented in Figure 1b. The Group $\times$ Day ANOVA revealed a significant interaction between these factors [$F(10, 135) = 8.02$, $p < .05$]. To explore this interaction, follow-up one-way ANOVAs were performed, considering Group as the only between-group factor. These analyses revealed a significant effect of Group on extinction Day 1 [$F(2, 27) = 14.82$, $p < .05$], extinction Day 2 [$F(2, 27) = 3.72$, $p < .05$], and testing [$F(2, 27) = 3.72$, $p < .05$]. The acquisition of the aversion by the Saccharin-extinction group was evidenced by the post-hoc analyses with intake scores from extinction Day 1. According to these analyses, saccharin acceptance was significantly lower in the Saccharin-extinction group than in the Saccharin-preexposure group. Post-hoc analyses also revealed significant differences between these groups on extinction Day 2, indicating that extinction was not fully completed until the reacquisition trial, when no statistical differences were detected between groups. Interestingly, according to the post-hoc analyses, at testing, rats from the Saccharin-extinction group consumed significantly less than those from the other conditions (Water or Saccharin-preexposure).

These results replicated those obtained in Experiment 1a, showing that reacquisition of the conditioned taste aversion is facilitated after extinction. Additionally, according to the present experiment, this effect cannot be explained by mere nonreinforced exposure to the CS during extinction. In Experiment 1b, mere exposure to the CS (Saccharin-preexposure group) did not affect the acquisition rate of conditioning, a result that it is consistent with previous studies that failed to observe latent inhibition or facilitation in preweanling rats in taste aversion learning (Nicolle, Barry, Varonesi & Stanton, 1989; but see Chotro & Alonso, 1999 for the opposite result). On a whole, the evidence from

Experiments 1a and 1b do not support the hypothesis that extinction, in preweanling rats, erases the CS–US association. In fact, these results indicate that the conditioned taste aversive memory survives extinction training in infancy. Since this conclusion is derived from experiments in which it was employed a procedure that was not previously used with preweanling rats, in the following experiments we decided to test whether evidences of the CS–US association can be detected after extinction by means of a renewal or a reinstatement procedure.

EXPERIMENT 2

In Experiment 2 we employed an ABA renewal design. After acquiring an aversion to saccharin in context A, groups of rats received extinction trials in context B. At testing, subjects were assessed in terms of saccharin consumption in context A (group ABA saccharin), or in context B (ABB saccharin). An additional control group received water and LiCl (ABA water) in context A, while during extinction consumed saccharin in context B. These subjects were also tested in context A. This group was included to test whether the contextual conditioning that may occur in context A could explain a possible difference in consumption at testing between groups ABA and ABB. The renewal effect has not been observed in infant rats, but this procedure has only been assessed in fear conditioning preparations (Yap & Richardson, 2007).

Apparatus

Context A was an opaque Plexiglas chamber (15 cm × 7 cm × 12 cm) with white walls and a small bag on the top of the cage containing coffee grains (6 g). The floor of this context was a piece of clean, white absorbent paper towel. Context B differed from context A in size, color, texture, and odor. Context B consisted of a square, polystyrene box (10 × 10 × 10). The internal walls were covered with smooth black fabric, the floor of the context was made of thin wire mesh and the cage was completely covered with a piece of red cellophane. Finally, a piece of cotton wool was placed on the top of the cage containing .2 ml of an almond scent (Esencias del Boticario, Cordoba, Argentine).

Procedures

Conditioning. Conditioning was carried out on PDs 14 and 15, one session per day. Procedures during conditioning were identical to those described for Experiment 1a for the ABA saccharin and ABB saccharin groups. The procedures applied to the ABA water group were also similar, with the only exception being that this group received intraoral water instead of saccharin.

Extinction. On PDs 16 and 17 all rats consumed saccharin in context B.

Testing. On PD18 rats from the ABA (water or saccharin) groups were tested in terms of saccharin intake in context A, while rats from the ABB group were evaluated in context B. The experimental design of this experiment is summarized in Table 2. Infusion parameters used during extinction and testing were similar to those described for conditioning in Experiment 1a.

Data Analysis

Intake scores were analyzed by means of a mixed ANOVA including Group (ABA water, ABB saccharin, or ABA saccharin) as a between-group factor and day as a within-group variable with 5 levels, corresponding to two conditioning, two extinction, and one testing trial.

Results

The results obtained in this experiment are represented in Figure 2. The Group × Day ANOVA revealed a significant interaction [$F(8, 80) = 5.20$, $p < .05$]. Following the statistical strategy from Experiment 1, one-way between-group ANOVAs were further conducted to explore this interaction. In these analyses, Group was considered as the only factor, and the dependent variable was the intake data from each experimental day. These analyses revealed a significant main effect of Group on the first extinction trial [$F(2, 20) = 4.46$, $p < .05$], a result indicative of an acquired aversion to saccharin in the ABA saccharin and ABB saccharin groups. Post-hoc analyses corroborated this interpretation, showing that rats treated with saccharin and LiCl (ABA saccharin and ABB saccharin groups) consumed less saccharin than the ABA water group. By the second

Table 2. Experimental Design and Number of Subjects for Experiment 2

Group	Conditioning PDs 14–15	Extinction PDs 16–17	Test PD18	*N*
Experiment 2				
ABA water	Water LiCl	Saccharin	Saccharin (A)	8
ABB saccharin	Saccharin LiCl		Saccharin (B)	7
ABA saccharin	Saccharin LiCl		Saccharin (A)	8

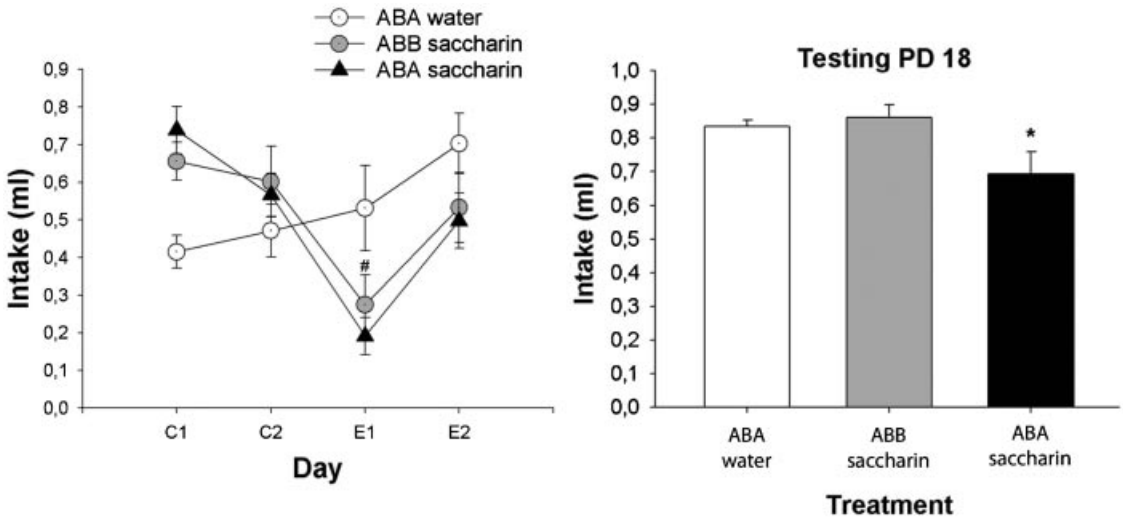

FIGURE 2 Represents data from Experiment 2. Scores represent the mean saccharin consumption as a function of Group (ABA water, ABB saccharin, or ABA saccharin), and Day [Conditioning days 1 and 2 (C1, C2), Extinction days 1 and 2 (E1, E2), and Testing]. The left side of the figure includes intake during conditioning and extinction, while the right side, intake at testing. Vertical bars represent the standard error of the means (SEM). $^{\#}p < .05$ ABB saccharin and ABA saccharin versus Water during extinction; $^{*}p < .05$ versus the remaining groups at testing.

extinction trial, however, the effect of Group no longer reached statistical significance. Finally, the corresponding ANOVA with intake data collected at testing revealed a significant effect of Group [$F(2, 20) = 3.92$, $p < .05$]. According to post-hoc tests, subjects from the ABA saccharin condition drank less than those from the other groups (ABB saccharin or ABA water).

These results demonstrate renewal in preweanling rats using a taste aversion preparation. Differences observed at testing between the ABA saccharin and ABB saccharin groups cannot be interpreted in terms of contextual conditioning induced by LiCl, since the ABA saccharin group also consumed less saccharin than the ABA water group.

EXPERIMENT 3

The goal of Experiment 3 was to test, in preweanling rats, the effectiveness of a reinstatement procedure to recover the CR after extinction of a conditioned taste aversion. Two conditioning trials were conducted (PDs 14 and 15) in which infant rats received a LiCl (US) injection after saccharin (CS) consumption. On PDs 16 and 17, rats received nonreinforced presentations of the CS. Finally, upon testing (PD17), subjects were tested in terms of saccharin intake after receiving half of the LiCl dose. Appropriate control groups were included in the design to control possible unspecific effects of the LiCl treatment at conditioning, and possible unconditioned effects of LiCl at testing. In preweanling rats, no evidence of reinstatement has been observed in fear conditioning (Kim & Richardson, 2007), but this procedure has not been assessed in conditioned taste aversion.

Procedures

The experimental design for the present experiment (summarized in Tab. 3) includes four independent groups: Saccharin +/−, Vinegar +/+, Saccharin −/+, and Saccharin +/+. The names of the groups allude to the solution received at conditioning (saccharin or vinegar), and to the conditioning (vehicle or LiCl), and reinstatement (vehicle or LiCl) treatments. The working hypothesis is that, if reinstatement is indeed effective, the Saccharin +/+ group will consume less saccharin at testing than the other conditions and the Saccharin +/− group will, at testing, show the same consumption level reached after acquisition and extinction of the saccharin aversion. The Saccharin −/+ group controls for the possible unconditioned effects of LiCl at the moment of testing, and the vinegar +/+ group controls for possible effects of LiCl at conditioning on the reinstatement treatment (possible sensitization) and possible unspecific effects of a prior aversive learning.

Table 3. Experimental Design and Number of Subjects for Experiment 3

Group	Conditioning PDs 14–15	Extinction PDs 16–17	Test PD18	*N*
Experiment 3				
Saccharin +/−	Saccharin LiCl	Saccharin	Vehicle saccharin	7
Vinegar +/+	Vinegar LiCl		LiCl saccharin	7
Saccharin −/+	Saccharin vehicle		LiCl saccharin	8
Saccharin +/+	Saccharin LiCl		LiCl saccharin	8

Conditioning. All the conditioning procedures were identical to those described for the previous experiments. Following these procedures, pups from the Saccharin +/− and Saccharin +/+ groups received LiCl after saccharin consumption, while pups from the Vinegar +/+ group were given LiCl after vinegar (1 ml of a .15%,v/v solution) intake. Finally, rats from the Saccharin −/+ condition received a vehicle injection after saccharin consumption.

Extinction. On PDs 16 and 17, all rats consumed saccharin following the procedures employed in the previous experiments.

Testing. On PD18, all rats were tested for saccharin intake. Procedures were identical to those described for the previous phases with the only exception being that, 10 min before testing, rats were injected with vehicle (Saccharin +/−), or with half the LiCl dose (.5% of the .15 M solution; Vinegar +/+, Saccharin −/+, and Saccharin +/+ groups). This LiCl dose was selected in a pilot study because it was shown not to affect saccharin consumption in naïve subjects of the same age.

Data Analysis

Intake scores were analyzed by means of a mixed ANOVA including Group (Saccharin +/−, Vinegar +/+, Saccharin −/+, and Saccharin +/+) as a between-group factor and day as a within-group variable with five levels, corresponding to the two conditioning, two extinction, and one testing trial.

Results

Figure 3 represents intake scores from the different groups as a function of the experimental day. The

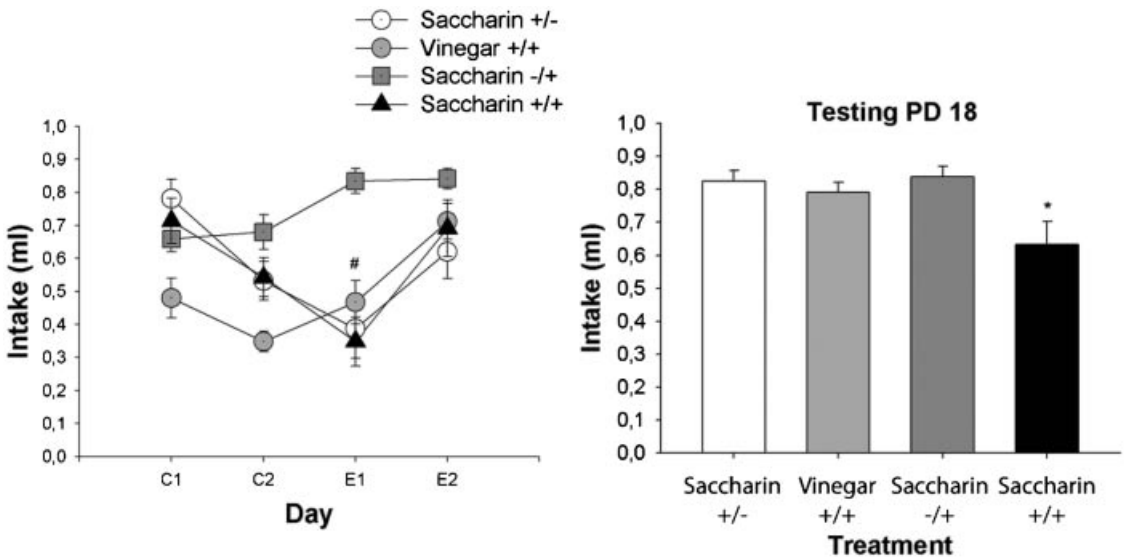

FIGURE 3 Represents data from Experiment 3. Scores represent the mean saccharin consumption as a function of Group (Saccharin +/−, Vinegar +/+, Saccharin −/+, or Saccharin +/+), and Day [Conditioning days 1 and 2 (C1, C2), Extinction days 1 and 2 (E1, E2), and Testing]. The left side of the figure includes intake during conditioning and extinction, while the right side, intake at testing. Vertical bars represent the standard error of the means (SEM). $^{\#}p < .05$; Saccharin +/−, Vinegar +/+, and Saccharin +/+ versus Saccharin −/+ during extinction; $^{*}p < .05$ versus the remaining groups at testing.

ANOVA revealed a significant Group × Day interaction [$F(12, 104) = 6.01$, $p < .05$], that was further explored by one-way between-group ANOVAs. These analyses indicated a significant effect of Group on extinction Day 1 [$F(3, 26) = 11.13$, $p < .05$], and at testing [$F(3, 26) = 4.50$, $p < .05$]. The effect of Group was not significant during the second extinction trial. Post-hoc analyses revealed that, on the first extinction day, rats from the Saccharin −/+ group consumed more saccharin than those from the other conditions. This result indicates that rats from the Saccharin +/+ and Saccharin +/− groups acquired an aversion to saccharin. Moreover, the reduced saccharin intake in the Vinegar +/+ group may be indicative of generalization between the vinegar and saccharin solutions. The most important result for the aim of the present study is the one observed at testing. On this day the Saccharin +/+ group consumed less saccharin than the other conditions.

These results show the effectiveness of a reinstatement procedure in preweanling rats, and demonstrate that a taste aversive memory can survive extinction training. The reinstatement effect cannot be explained by an effect of the prior LiCl experience, or previous acquisition of a conditioned taste aversion, since the group trained at conditioning with vinegar also consumed more saccharin at testing than the Saccharin +/+ group.

DISCUSSION

The results from the present series of experiments are not consistent with the hypothesis that extinction in preweanling rats erases the CS–US association. Evidence against this hypothesis was gathered by means of three different procedures. After extinction, reacquisition of the conditioned response was facilitated by prior conditioning of the CS (Experiment 1, Fig. 1a and b). In Experiment 2, with an ABA design, recovery of the CR was observed when subjects were evaluated in a different context (the training context) from the one employed during extinction (Fig. 2). Finally, in Experiment 3, after extinction, the CR was also recovered by exposure to the US before testing (reinstatement, Fig. 3).

Previous studies failed to observe either reinstatement or renewal effects during the infantile period of the rat (J. H. Kim & Richardson, 2010). One of the most critical differences that may explain the discrepancy between the results of these studies and those obtained during the present experiments is the type of memory analyzed. Experiments in which renewal or reinstatement effects were not detected used a fear conditioning preparation, while here we used a conditioned taste aversion paradigm. Also, regardless of the type of memory under analysis, the procedures employed in the present study may be more sensitive to detecting renewal and reinstatement effects in preweanling rats than those employed in the studies using the fear conditioning paradigm. However, if this were true, it would be difficult to explain why those procedures that failed to detect renewal or reinstatement of a fear conditioned response in infant rats were highly sensitive a few days later, after weaning (J. H. Kim & Richardson, 2007, 2010; Yap & Richardson, 2007). Another possibility is that the mechanisms underlying extinction of a fear memory are qualitatively different from those which underlie the extinction of a taste aversive memory, and that those mechanisms mature at a different rate in infant rats.

Our results are consistent with the most widely accepted theoretical view of extinction, that is, that this process involves a relearning of the relationship between the CS and the US, with the initial CS–US association remaining relatively intact (Bouton, 2004; Bouton et al., 2006). In this sense it is accepted that during extinction training an inhibitory learning (CS–noUS) is acquired which is strongly modulated by context, and which competes with the CS–US excitatory association. This observation has stimulated neurobiological research into the mechanisms that may support the formation of the inhibitory association. This research has focused on different levels of analysis, including molecular and cell biology, neuropharmachology, and the neuroanatomy of the circuits involved in learning and memory (Milad & Quirk, 2012; Sotres-Bayon, Cain, & LeDoux, 2006). At these levels of analysis there are important differences and similarities between the mechanisms underlying taste aversive and fear memories. For example, from a functional neuroanatomy perspective, the dorsal hippocampus seems to be an important structure for contextual learning induced by a footshock (Anagnostaras, Gale, & Fanselow, 2001; Fanselow, 2000), or LiCl (Aguado, Hall, Harrington, & Symonds, 1998). Hence, it is not surprising that the dorsal hippocampus participates in renewal in taste aversion (Fujiwara et al., 2012), or fear conditioning (Ji & Maren, 2005; Maren & Hobin, 2007) in adult rats. Similarly, the infralimbic prefrontal cortex and the amygdala also participate in the extinction of both types of memory in adult animals (J.J. Kim & Jung, 2006; Mickley, Kenmuir, Yocom, Wellman, & Biada, 2005). The specific role of the infralimbic prefrontal cortex has been linked to its function in behavioral inhibition (Quirk, Garcia, & Gonzalez-Lima, 2006). This brain area projects to the basolateral amygdala and to the intercalated neurons,

forming a pathway for the potential inhibition of the central nucleus of the amygdala. Through this pathway, it is believed that information about where extinction occurs (contextual information) modulates the expression of extinction.

In preweanling rats it has been demonstrated that extinction of a fear conditioned response does not require the participation of the infralimbic cortex (J. H. Kim & Richardson, 2010; Li, Kim, & Richardson, 2012). During infancy, but not during adulthood, the extinction of this kind of response seems to depend exclusively on a single structure (the amygdala; Li et al., 2012), and this explains why extinction at this age is inflexible (J. H. Kim & Richardson, 2010), or may produce erasure of the CS–US association (Quirk et al., 2010). The fact that at this age, a taste aversive memory can survive extinction training may be explained by the involvement of different structures (in addition to the amygdala) in extinction learning. Some brain structures are preferentially involved in taste processing, such as, the nucleus of the tractus solitarius, the parabrachial nucleus, or the insular cortex (more specifically, the gustatory cortex) (Nunez-Jaramillo, Ramirez-Lugo, Herrera-Morales, & Miranda, 2010). In these brain areas, both taste and visceral information converge. The parabrachial nucleus participates in the acquisition but not the retention of the conditioned taste aversive memory, while the nucleus of the tractus solitarius seems to be related to the expression of the conditioned taste aversion (Nunez-Jaramillo et al., 2010). Hence, it is unlikely that these structures (parabrachial nucleus and the nucleus of the tractus solitarius) can maintain the CS–US association after extinction. Retrieval of the conditioned taste aversion requires the functionality not only of the amygdala, but also of the insular cortex, a brain area critically involved in taste memory (Mickley et al., 2005; Nunez-Jaramillo et al., 2010). Moreover, the insular cortex is connected with the amygdala and the prefrontal cortex, structures critically involved in extinction. During taste aversion learning, the insular cortex also processes information about the US, and it has been proposed as an important candidate for the long-term storage of the association between the CS and the US in taste aversion learning (Rosenblum, Meiri, & Dudai, 1993). Hence, the insular cortex seems to be an accurate candidate for preserving, in infant rats, a conditioned taste aversive memory after extinction. Interestingly, some studies have found evidence of the participation of the insular cortex in the extinction of a conditioned taste aversion (Akirav et al., 2006; Fresquet, Angst, Schleef, Gobaille, & Sandner, 2007). Future research is required to explore the plausibility of this hypothesis.

According to some authors, preweanling rats have important limitations as regards acquiring and expressing contextual conditioning (Rudy & Morledge, 1994), a result that has been hypothetically linked to a lack of functional maturity of the dorsal hippocampus (Raineki et al., 2010; Schiffino, Murawski, Rosen, & Stanton, 2011). However, this hypothesis is questioned by studies showing that under conditions in which contextual cues are highly salient, preweanling rats can acquire strong contextual conditioning, similarly to weaning rats (Brasser & Spear, 2004; Pugh & Rudy, 1996). This empirical background raises the question of whether the contextual conditioning deficits observed in some studies in preweanling rats are due to memory or perceptual ontogenetic limitations. In this regard, a recent study confirmed that infant rats can acquire and express long-term contextual conditioning even when a standard conditioning cage (without explicit odors) is employed (Pisano, Ferreras, Krapacher, Paglini, & Arias, 2012), but detection of this learning required the inclusion in the experimental design of appropriate age-matched control groups, as well as a statistical analysis of multiple dependent variables (not only freezing, which is the one normally used), with the time-course of the expression of these variables across the testing session being taken into account also. It is important to notice that in this study contextual conditioning in the infant rat was observed using a paradigm that is highly dependent on the hippocampus, at least in adult and weaning rats (the context preexposure facilitation effect; Schiffino et al., 2011). In the light of these antecedents, it is difficult to assume that the lack of renewal in fear conditioning in infant rats is related to a deficit in contextual learning. As mentioned above, renewal in adult rats (in taste aversion learning or in fear conditioning) is also hippocampus-dependent (Fujiwara et al., 2012; Ji & Maren, 2005), and we did observe renewal after extinction of a conditioned taste aversion. These data also suggest the need for a more detailed analysis of the possible role of the hippocampus in the modulation of extinction and other interference paradigms, such as the unconditioned stimulus preexposure effect (Castello, Bobbio, Orellana, & Arias, 2011; Revillo et al., 2012), or latent inhibition (Yap & Richardson, 2005), which seem also to be context-independent in preweanling rats. If the hippocampus is definitively not completely functional in this period, then further research is required to determine which other structure may be assuming its role in modulating extinction expression in infant rats (in taste aversion learning).

Rapid reacquisition after extinction has been interpreted by some authors as another example of the

renewal, or context change, effect (Bouton, 2004). In these terms, the presentation of the US after the CS at conditioning provides a particular context which is different from that formed by the presentation of the CS alone during extinction. However, in taste aversion learning, reacquisition after extinction in adult rats is usually retarded when compared with the acquisition of a control group learning the aversion for the first time (Calton et al., 1996; Hart et al., 1995). This retardation effect has been explained in terms of the effect of the mere exposure to the CS during the extinction phase (latent inhibition; Aguado et al., 2001). These results question the possibility that a given CS can gain inhibitory strength during extinction training, at least in taste aversion learning (but see Denniston & Miller, 2003). The present results cannot be interpreted in this way, since reacquisition was faster than acquisition of the aversion for the first time. It is interesting to note that during infancy, short preexposures to a CS can in fact facilitate conditioning, an effect that has been rarely observed in adult rats (Hoffmann & Spear, 1989). However, our results cannot be explained by the mere exposure to the CS during extinction, since in this case, no facilitation was observed in the group that was exposed to the CS during extinction (Group Saccharin exposure, Experiment 1b). Alternatively, facilitated reacquisition could result from the prior conditioning experience, which may improve nonspecific acquisition of a subsequent conditioning. The learning experience may have made subjects "better learners". Nevertheless, following through with this argument, it is hard to explain why the same effect was absent in the almond group (that previously learned aversion to almond). The most plausible explanation seems to be that during the reacquisition trial, exposure to the same conditioning episode facilitates retrieval of the original CS–US association.

In adult rats, reinstatement of a conditioned taste aversion response has not always been observed (Bouton, 1982). Schachtman et al. (1985) observed the recovery of a taste aversive memory after extinction when LiCl was given before testing. Sensitivity for detecting this effect seems to depend on the duration of the extinction training. Specifically, Schachtman et al. observed the reinstatement effect after a short (three trials) but not long (six trials) extinction training. Our results are consistent with this result, since the recovery induced by the reinstatement treatment was evident after a short extinction treatment (two trials). Several associative explanations have been proposed for the reinstatement effect. The first one alludes to the strengthening of the context-US association during the presentation of the US before testing. Hypothetically, this association would contribute to an increased response to the CS at testing. However, this explanation cannot easily account for the results obtained by Schachtman et al., since the US was presented in a different context from the one employed at testing (Schachtman et al., 1985). It is also difficult to explain the results obtained in the present study in terms of the US-context association. In this case, the US (LiCl) was given immediately before testing and animals were placed in the testing context at the same time as they started to consume the CS (saccharin). Hence, to explain this result in terms of this associative explanation, it is necessary to assume that the US-context association contributes to the expression of the CR at the same time as the association is being reinforced. In addition, the LiCl dose that we employed at testing was not sufficient by itself to generate a change in the intake response. An alternative explanation may be to consider the US as a reminder that facilitates the recovery of the CS–US association (Schachtman et al., 1985). In this sense, the LiCl effects that were not strong enough to affect consumption in control groups were enough to act as a remainder for the experimental condition (Saccharin +/+), thus allowing the animals to recover their initial taste aversive memory.

The discrepancy between the present findings and those from previous studies on extinction in early development may be interpreted in terms of the hypothesis which postulates that the developmental onset of "higher-order" learning processes occurs earlier when they involve behavioral systems that mature earlier (Stanton, 2000; Rudy, 1992). The fact that the extinction process seems to work in an adult-like way when the CS cue is a taste (the present data) rather than a tone (e.g., J. H. Kim & Richardson, 2007) is coherent with the fact that the taste system is functional in a earlier stage of development than the auditory system. However, this interpretation is hard to reconcile with the results of a previous study in which no recovery of the fear memory was observed and in which the CS was an olfactory cue (Yap & Richardson, 2007).

In sum, the present results show that a conditioned taste aversive memory can survive extinction in preweanling rats, suggesting that extinction of a fear and extinction of a taste aversive memory may involve different biological mechanisms during infancy. The conclusion that the only psychological mechanism for extinction is unlearning should be confined to a particular type of memory (fear memory). In taste aversion learning at least, extinction seems to work similarly in infant and adult rats, creating a new inhibitory memory that competes with the one acquired at conditioning.

NOTES

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