

Reinstatement of an extinguished fear conditioned response in infant rats

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Although it is currently accepted that the extinction effect reflects new context-dependent learning, this is not so clear during infancy, because some studies did not find recovery of the extinguished conditioned response (CR) in rodents during this ontogenetic stage. However, recent studies have shown the return of an extinguished CR in infant rats. The present study analyzes the possibility of recovering an extinguished CR with a reinstatement procedure in a fear conditioning paradigm, on PD17 (Experiments 1–4) and on PD24 (Experiment 5), while exploring the role of the olfactory content of the context upon the reinstatement effect during the preweanling period. Preweanling rats expressed a previously extinguished CR after a single experience with an un signaled US. Furthermore, this result was only found when subjects were trained and tested in contexts that included an explicit odor, but not in standard experimental cages. Finally, Experiment 5 demonstrated the reinstatement effect on PD24 in a standard context. These results support the notion that extinction during infancy has the same characteristics as those described for extinction that occurs in adulthood. Instead of postulating a different mechanism for extinction during infancy, we propose that it may be more accurate to view the problem in terms of the variables that may differentially modulate the extinction effect according to the stages of ontogeny.

Extinction can be defined as a procedure consisting of repeated presentations of a conditioned stimulus (CS) after conditioning (Pavlov 1927). As a result of this training, the conditioned response (CR) usually decays progressively. It is currently accepted that the extinction effect reflects new learning, because the extinguished CR can be recovered after a retention interval (spontaneous recovery), after the presentation of the unconditioned stimulus—the US, (reinstatement), or after a context change—known as the renewal effect (Pavlov 1927; Bouton 2002; Quirk and Mueller 2008). All of these findings have been interpreted as evidence that the extinction effect is a context-dependent phenomenon (Bouton 2002, 2004).

Although extinction has been widely studied in adult organisms, only a few studies have focused on this effect during infancy, and these have yielded inconsistent results. In particular, in some of these studies the authors did not find recovery of the CR once it was extinguished (Kim and Richardson 2010), while others have reported renewal, more rapid reacquisition, and reinstatement in taste aversion learning (Revillo et al. 2014a), and spontaneous recovery (Revillo et al. 2014b) and renewal (Revillo et al. 2013, 2015) in fear conditioning. Those authors that did not find recovery of the extinguished CR suggested that in infancy, extinction is a qualitatively different phenomenon to that observed in adulthood, and that during infancy it results in erasure of the CS–US association instead of the production of new learning (Kim and Richardson 2010). However, more recent evidence of recovery after extinction in infant rats brings this argument into question, and has led us to study which variables may critically affect the persistence of extinction during infancy. This rationale is in accordance with the theoretical position raised by relevant authors in the field of developmental psychobiology (Campbell and Spear 1972; Spear 1984; Spear and Rudy 1991; Spear and Riccio 1994; Rovee-Collier and Giles 2010). These authors have pointed out

that when studying the ontogeny of learning capacities, it is critical to control procedural factors that may differentially affect the performance of subjects of different ages (Campbell and Spear 1972; Spear 1984; Spear and Rudy 1991; Spear and Riccio 1994; Rovee-Collier and Giles 2010). This issue is important because differences in learning capacities may be confounded with differences in sensitivity to experimental conditions.

One of these variables may be the sensory content of the context. It has been shown that infant rats are less competent than older subjects in performing fear conditioning tasks when the experimental contexts are primarily distinguished by visual cues (see Brassler and Spear 2004; Revillo et al. 2015). However, when the contexts are enriched with olfactory cues, infants respond much better to this type of learning paradigm and show a variety of context effects (Richardson et al. 1986; Lariviere et al. 1990; Carew and Rudy 1991; McKinzie and Spear 1995; McKinzie et al. 1996; Brassler and Spear 1998, 2004; Castello et al. 2015; Revillo et al. 2015). In agreement with these findings, it has been found that the inclusion of explicit odors in the context can critically modulate the ABA-renewal effect in infant rats (Revillo et al. 2013, 2015).

The aim of the present study was twofold. First, we intended to explore the effectiveness of a reinstatement procedure for recovering a fear conditioned response during the preweanling period. Second, we intended to assess whether the inclusion of an explicit odor in the context influences such an effect. Shedding light on this issue has theoretical implications for understanding the sources of behavioral change that are thought to occur during early ontogeny.

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Table 1. Displays the number of subjects per group in each of the experiments from the present study, with the main variables that define procedures used in each case

Exp	Context	Odor	Group	Number of conditioning trials	<i>n</i>	Age
Exp 1a	AA	Yes	Paired	2	7	PD17
			Unpaired	2	7	
	Paired		2	8		
	Unpaired		2	8		
Exp 1b	AA	Yes	Paired	6	7	PD17
			Unpaired	6	7	
	Paired		6	9		
	Unpaired		6	9		
Exp 2	AAA	Yes	Paired-Reminder	6	11	PD17
			Paired-No reminder	6	11	
			Unpaired-Reminder	6	10	
			Unpaired-No reminder	6	10	
	ABB		Paired-Reminder	6	8	
			Paired-No reminder	6	8	
			Unpaired-Reminder	6	8	
			Unpaired-No reminder	6	8	
Exp 3	AAA	No	Paired-Reminder	6	8	PD17
			Paired-No reminder	6	8	
			Unpaired-Reminder	6	9	
			Unpaired-No reminder	6	9	
	ABB		Paired-Reminder	6	10	
			Paired-No reminder	6	10	
			Unpaired-Reminder	6	11	
			Unpaired-No reminder	6	11	
Exp 4a	ABB	Yes	Paired-Reminder	2	12	PD17
			Paired-No reminder	2	12	
			Unpaired-Reminder	2	12	
			Unpaired-No reminder	2	12	
Exp 4b	ABB	No	Paired-Reminder	2	10	PD17
			Paired-No reminder	2	10	
			Unpaired-Reminder	2	9	
			Unpaired-No reminder	2	9	
Exp 5	ABB	No	Paired-Reminder	2	11	PD24
			Paired-No reminder	2	11	
			Unpaired-Reminder	2	11	
			Unpaired-No reminder	2	11	

Results

Experiment I

The goal of the first experiment was to analyze the effectiveness of two different conditioning protocols (two or six conditioning trials) for demonstrating a particular conditioned response (freezing) in the presence of a tone CS in preweanling rats. In a previous study, we have shown that a context change between conditioning and testing facilitated the detection of Pavlovian conditioning in same-age subjects (Revillo et al. 2014b). In order to replicate this finding, after conditioning, half of the subjects were given an extinction session in the same context as the one used during conditioning, while the remaining subjects were given the extinction treatment in a different context (see Apparatus

section). The results of this study will allow us to establish the conditioning and extinction parameters to be used in subsequent experiments. Table 1 displays the number of subjects per group in each of the experiments from the present study, with the main variables that define procedures used in each case.

Experiment 1a

Figure 1A represents freezing scores during the extinction session from subjects trained with two conditioning trials, as a function of the Group and Context treatment (Experiment 1a). The ANOVA revealed a significant Group by Context treatment by Block interaction, $F_{(2,44)} = 3.47$, $P < 0.05$. This interaction reflects the fact that in the AB condition, scores from the Paired group were

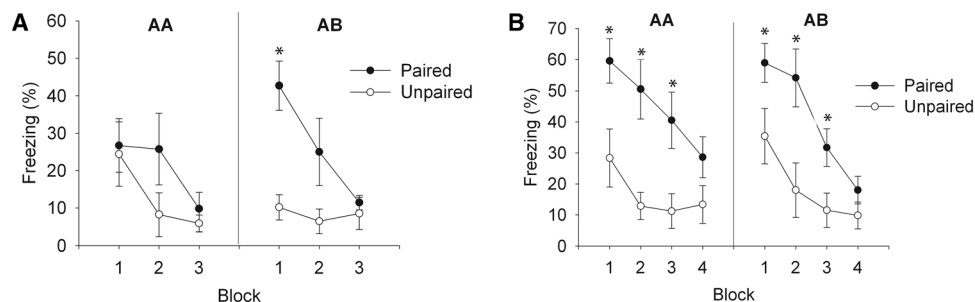


Figure 1. (A) Depicts extinction data from Experiment 1a, after two conditioning trials. Scores represent the percentage of freezing as a function of Group and Context treatment. The *left* side of the figure shows freezing scores from animals trained and tested in Context A, while the *right* side shows freezing scores of animals trained in Context A and tested in Context B. Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing. (B) Includes extinction data from Experiment 1b, after six conditioning trials. Scores represent the percentage of freezing as a function of Group and Context treatment. The *left* side of the figure shows freezing scores from animals trained and tested in Context A, while the *right* side shows freezing scores of animals trained in Context A and tested in Context B. Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing.

significantly higher than those from the Unpaired one, but only on the first conditioning trial [$F_{(1,14)} = 19.49, P < 0.05$]. This CR was rapidly extinguished. In the AA condition no between group differences were detected in any of the blocks.

Experiment 1b

Figure 1B represents extinction scores from Experiment 1b in which subjects were given six conditioning trials during training. In this case, Pavlovian conditioning was observed in both context conditions, AA and AB. The ANOVA revealed a significant Group by Block interaction [$F_{(3,72)} = 2.82, P < 0.05$]. In both context conditions, freezing scores from the Paired group were significantly higher than those from the Unpaired group on the first three blocks [AA condition: all $F_{S(1,12)} > 7.03$, all $P_s < 0.05$. AB condition: all $F_{S(1,16)} > 4.64$, all $P_s < 0.05$], but not in the last one.

These results show Pavlovian conditioning after two conditioning trials when extinction and conditioning were carried out in distinctly different contexts, and an effect with six trials independent of a context change between conditioning and testing. In addition, these results show that the freezing CR is extinguished within a single session.

Experiment 2

The goal of the second experiment was to explore whether the delivery of a mild footshock 24 h after extinction can reinstate the extinguished CR. In this experiment, we used six conditioning trials. For consistency, we kept the two context conditions from the previous experiment, since with six conditioning trials Pavlovian conditioning was not affected by the context change. Therefore, for half of the subjects conditioning, extinction, and testing occurred in the same context (AAA), and for the remaining half extinction and testing a different context than that used during conditioning (ABB). In Experiment 2, as in the previous one, contexts A and B included the explicit odor.

The results obtained during extinction are represented in Figure 2 (left panel), which shows freezing scores during extinction as a function of Group, Context treatment, and Block. The ANOVA revealed a significant Group by Block interaction, $F_{(6,132)} = 3.95, P < 0.05$. Follow-up ANOVAs were run to analyze between-group differences in each block. These ANOVAs revealed a significant main effect of Group on the first two blocks for the AAA condition [all $F_{S(2,29)} > 4.64$, all $P_s < 0.05$] and on the first three blocks for the ABB condition [all $F_{S(2,21)} > 7.66$, all $P_s < 0.05$]. According to the post hoc tests, in all of these blocks subjects from the Paired groups (Paired-Reminder and Paired-No reminder) spent more time engaged in the freezing response than those from the Unpaired-Reminder group. No between-group dif-

ferences were observed in the last extinction block in any of the context conditions, indicating that the CR was extinguished by the end of the extinction session.

At testing, the behavioral profile was again similar across the two context conditions (see Fig. 2, right panel). The ANOVA revealed a significant main effect of Group [$F_{(2,44)} = 9.83, P < 0.05$], and the interaction Group by Context condition and Group by Block were far from significant. According to the post hoc tests, freezing scores from the Paired-Reminder group were significantly higher than those from the remaining groups (Paired-No reminder and Unpaired-Reminder groups). In sum, the results from Experiment 2 replicate those from Experiment 1b, showing clear evidence of Pavlovian conditioning and extinction in the AAA and ABB conditions. Interestingly, administration of a mild footshock before testing reinstated the extinguished CR, an effect that was also observed in both context conditions.

Experiment 3

The goal of Experiment 3 was to test whether the reinstatement effect observed in the previous experiment could be dependent on the context including an explicit odor. We have previously observed that the odor content of the context is critical for the recovery of an extinguished CR in preweanling rats by means of a renewal procedure, and other authors have reported the importance of the odor content of the context for context effects and context learning during the preweanling period (Revillo et al. 2015).

Extinction scores are represented in Figure 3 (left panel). The ANOVA revealed a significant interaction Group by Block [$F_{(6,132)} = 2.39, P < 0.05$]. Although the interaction between these factors and Context treatment did not reach statistical significance, we explored between group differences separately in each context condition. These analyses revealed a significant effect of Group in each block in the ABB condition [all $F_{S(2,28)} > 4.99$, all $P_s < 0.05$], and no between-group differences in any block of the AAA condition, apart from a trend on the first block [$F_{(2,22)} = 3.18, P = 0.06$]. Freezing scores recorded during the test phase are depicted in Figure 3 (right panel). In this experiment, the reinstatement treatment was not effective in recovering the CR. The ANOVA revealed a significant effect of Block [$F_{(3,132)} = 17.67, P < 0.05$], indicating a progressive decay of the freezing responses across the testing session. This effect was independent of the Group and Context treatments.

Experiment 4

In order to give more consistency to the conclusions derived from Experiments 2 and 3, we conducted this experiment to explore

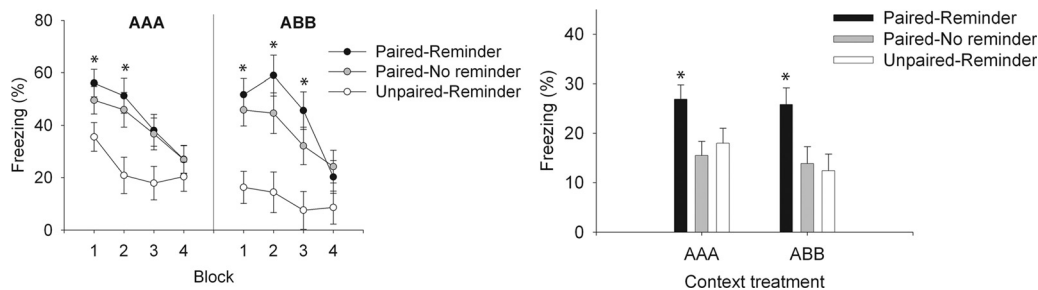


Figure 2. Represents extinction and testing data from Experiment 2, after six conditioning trials and using contexts enriched with salient odors. The left side of the figure shows extinction data as a function of Group (Unpaired-Reminder, Paired-No reminder, and Paired-Reminder), and Context treatment (AAA and ABB). Freezing scores at testing are presented on the right side of the figure as a function of Group (Unpaired-Reminder, Paired-No reminder, and Paired-Reminder), and Context treatment (AAA and ABB). Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing.

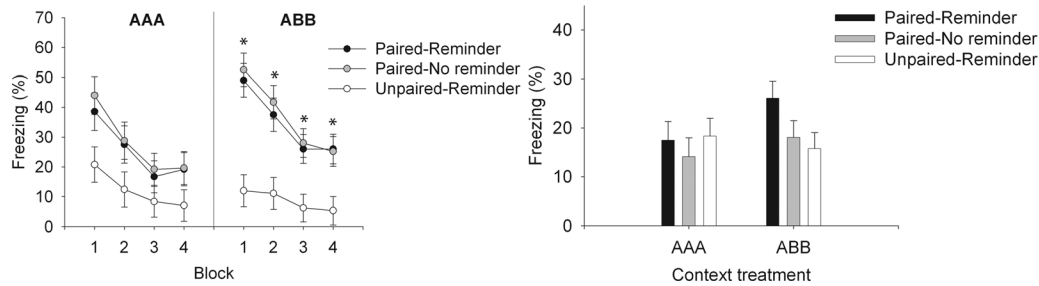


Figure 3. Depicts extinction and testing scores from Experiment 3, after six conditioning trials and using standard contexts (without odors). The *left* side of the figure shows extinction data as a function of Group (Unpaired-Reminder, Paired-No Reminder, and Paired-Reminder), and Context treatment (AAA and ABB). Freezing scores at testing are presented on the *right* side of the figure as a function of Group (Unpaired-Reminder, Paired-No Reminder, and Paired-Reminder), and Context treatment (AAA and ABB). Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing.

whether the reinstatement effect observed in Experiment 2a could also be observed with two conditioning trials, and whether in this case the odor is also necessary for this effect. Given that in Experiment 1a, after two conditioning trials, Pavlovian conditioning was only observed in the ABB condition, in Experiment 4 we used this context condition. In Experiment 4a, contexts A and B included the odor, while in Experiment 4b they did not.

Experiment 4a

The results from extinction corresponding to Experiment 4a are depicted in Figure 4a (left panel). These results parallel those obtained in Experiment 1b, in which the CR was evident on the first extinction trial, but rapidly decayed thereafter. The ANOVA revealed a significant Group by Block interaction [$F_{(4,60)} = 2.91$, $P < 0.05$], which reflects between-group differences only in the first block [$F_{(2,33)} = 5.38$, $P < 0.05$], in which, according to

post hoc tests, the Paired groups scored higher than the Unpaired one.

An ANOVA conducted on the test scores (Fig. 4a, right panel) revealed a significant effect of Group [$F_{(2,30)} = 4.73$, $P < 0.05$], indicating that subjects in the Paired-Reminder group spent more time engaged in the freezing response than those in the remaining two conditions.

Experiment 4b

Freezing scores from extinction and testing corresponding to Experiment 4b are depicted in Figure 4b. The ANOVAs conducted on this data did not find any significant main effect or interaction during extinction or reinstatement, indicating that in this experiment, no evidence of Pavlovian conditioning or reinstatement was observed. Again, reinstatement was only observed in the odor condition, a result that supports the conclusions derived from Experiments 2 and 3.

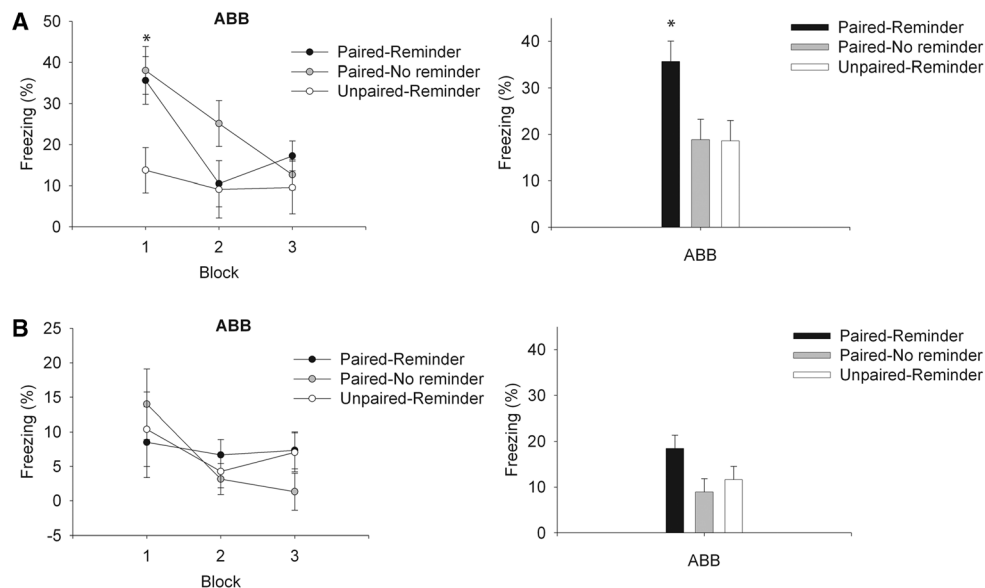


Figure 4. (A) Includes extinction and testing data from Experiment 4a, after two conditioning trials and using contexts with salient odors. The *left* side of the figure shows extinction data, while freezing scores at testing are presented on the *right* side of the figure. Data from extinction and testing are shown as a function of Group (Unpaired-Reminder, Paired-No Reminder, and Paired-Reminder). Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing. (B) Depicts extinction and testing data from Experiment 4b, after two conditioning trials and using standard contexts. The *left* side of the figure shows extinction data, while testing are presented on the *right* side. Data from extinction and testing are shown as a function of Group (Unpaired-Reminder, Paired-No Reminder, and Paired-Reminder). Animals were 17 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM).

Experiment 5

The aim of the final experiment was to test whether reinstatement in weaning rats can be observed in a context lacking the explicit odors. In this condition, preweaning rats did not show reinstatement after extinction (Experiments 3 and 4b). We therefore conducted a single experiment with 2 conditioning trials, using the ABB condition described in Experiment 4b.

During extinction, scores from the different groups varied differentially across blocks [$F_{(6,81)} = 2.55$, $P < 0.05$]. Between-group differences were observed in blocks 1, 2 and 3 [all $F_{(2,30)} > 4.37$, all P s < 0.05], in which, according to post hoc tests, the Paired groups scored higher than the Unpaired-Reminder condition (see Fig. 5, left panel). At testing, a clear reinstatement effect was observed (see Fig. 5, right panel). The ANOVA revealed a significant main effect of Group [$F_{(2,26)} = 7.71$, $P < 0.05$], and post hoc tests indicated higher freezing scores in the Paired-Reminder than in the remaining groups.

Discussion

The present results show that a fear CR can be reinstated after extinction by the presentation of the US in preweaning rats. This effect was only found when subjects were trained and tested in a salient context with an explicit olfactory component (Experiments 2 and 4a), which is consistent with prior research showing the relevance of olfactory contexts in regulating behavior and learning in preweaning rats (Brasser and Spear 2004; Revillo et al. 2015). Consistent with the present results, we have previously reported reinstatement, renewal, and more rapid reacquisition in preweaning rats using a conditioned taste aversion paradigm (Revillo et al. 2014a), and renewal and spontaneous recovery using fear conditioning procedures (Revillo et al. 2013, 2014b). Taken together, all of these findings suggest the possibility of recovering an extinguished CR during infancy by means of a variety of procedures.

The procedural variables experimentally manipulated in our experiments add valuable information for the study of Pavlovian conditioning phenomena during infancy. First, a context change allowed for the detection of conditioning after two conditioning trials when the contexts included an explicit odor (Experiment 1a), and after six conditioning trials when the contexts were not enriched with the odor (Experiment 4). Second, in some cases the presence of the odor enhanced the strength of the CR elicited by the tone CS. For instance, detection of Pavlovian conditioning after two conditioning trials required, in addition to a context change, the presence of the odor in the conditioning context (Experiment 1a, 4a, and 4b). Similarly, with six conditioning trials and without a context change, the olfactory component present

during training also seems to facilitate conditioning (when comparing results from Experiment 1b, 2, and 3). To explain how the presence of the odor favors conditioning in preweaning rats is beyond the scope of the present study. However, Spear and his colleagues have proposed a hypothesis that may help to understand this effect (McKinzie and Spear 1995; Brasser and Spear 1998, 2004). These authors observed that during conditioning, preweaning rats showed an increase in the orientation response to the CS (Kraebel et al. 1998) and enhanced responses to the US (Brasser and Spear 1998) when trained in a sensory-enriched context and when the CS and US were contiguous. According to these authors, these changes may favor (particularly during infancy) processing of the events that constitute the conditioning episode, thereby facilitating learning (Brasser and Spear 1998; Kraebel et al. 1998). Given that Spear and his collaborators limited their studies to the acquisition phase of conditioning, it would be interesting to extend these observations to extinction training, in order to explore whether the salience of the context also affects processing of the CS during extinction in preweaning rats. In spite of its plausibility, however, this explanation is not sufficient to account for the entire pattern of results that emerged from our study. For example, with six conditioning trials, following a context change (ABB), clear evidence of Pavlovian conditioning was observed regardless of the presence of the odor in the context (Experiment 3). More research is therefore required to improve our understanding of the contribution of procedural factors to Pavlovian conditioning during infancy.

A further novel aspect of our study was the inclusion of an unpaired control group, which was not included in most of the previous studies aimed at analyzing fear extinction learning in preweaning rats. It is critical to include this group in studies of this sort, since it permits us to rule out less interesting alternative explanations to those based on conditioning-induced behaviors, such as sensitization (Rescorla and Heth 1975), and together with the context-change treatment that we implemented, it also helps to rule out any possible role of direct odor–shock associative learning upon the reinstatement effect. Without this unpaired group, it would be difficult to know which of the experimental conditions (with or without a context change; with or without the odor) produced results compatible with Pavlovian conditioning and extinction. Finally, as mentioned in the Procedures section, we chose for the reminder a footshock intensity that was sufficiently low enough to not significantly increase the freezing behavior in unpaired controls, ensuring that the effect observed in the Paired-Reminder group in Experiments 2, 4a, and 5 is not the result of an unspecific effect of the footshock on the freezing response.

Previously, a reinstatement procedure with infant rats was not effective in terms of recovering the extinguished CR (Kim

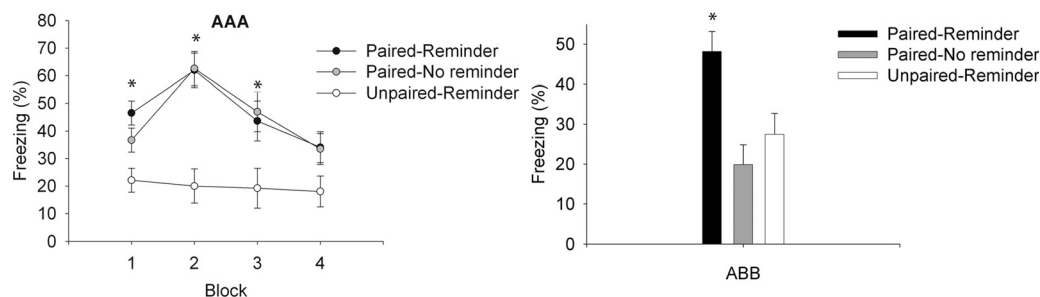


Figure 5. Represents extinction and testing data from Experiment 5, after two conditioning trials and using standard contexts. The left side of the figure shows extinction data, while freezing scores at testing are presented on the right side. Data from extinction and testing are shown as a function of Group (Unpaired-Reminder, Paired-No Reminder, and Paired-Reminder). Animals were 24 d old at the beginning of the experiment. Vertical bars represent the standard error of the means (SEM). (*) $P < 0.05$ versus the remaining groups at testing.

and Richardson 2007a). This result, together with the lack of observation of renewal or spontaneous recovery, led the authors to suggest that extinction in infancy is mediated by a rather different set of processes to those that govern extinction in adulthood (Kim and Richardson 2010), a hypothesis that was strengthened by the lack of activation of prefrontal structures (infra and prelimbic) during acquisition and expression of extinction (Kim and Richardson 2010). Therefore, while in adults extinction involves new learning (mediated by a hippocampal–prefrontal lobe circuit), during infancy extinction was proposed as an inflexible phenomenon producing erasure of the CR, probably because the hippocampal–prefrontal lobe circuit was not engaged during extinction training (Kim and Richardson 2010). This possibility may imply, according to the authors, that exposure therapies (theoretically linked to the extinction phenomenon) may be more effective during infancy than later in ontogeny, since infants may be less susceptible to relapse (Kim and Richardson 2010). If we carefully consider the procedures used in these studies, our results are not necessarily incompatible with their findings. In the studies that reported an absence of renewal (Kim and Richardson 2007b; Yap and Richardson 2007), reinstatement (Kim and Richardson 2007a), or spontaneous recovery (Gogolla et al. 2009) with infant rats, the contexts were composed mainly of visual cues, without explicit odors. The olfactory component of the context was critical for observing reinstatement in our study—this effect was not found in any of the experiments in which we used contexts without odors. The inclusion of the explicit odor in the context was also necessary for renewal during infancy (Revillo et al. 2013, 2015). In brief, those studies that did not find these context effects during infancy were conducted under experimental conditions that, at least in our laboratory, clearly hinder the detection of Pavlovian conditioning, reinstatement, and renewal. It is likely that, under conditions in which infants respond in a similar way to adults when given experimental treatments aimed at producing recovery from extinction, the hippocampal–prefrontal lobe circuit is also engaged during extinction training. This is compatible with the idea that the behavioral expression in a theoretically hippocampus-dependent task may depend on the interaction between the developmental status of the hippocampus itself and the different behavioral and perceptual systems involved in the task (Stanton 2000).

The hypothesis that extinction during infancy has the same characteristics as those described for extinction that occurs in adulthood is compatible with results from both our laboratory and those of other studies. For instance, in adult rats a context change after extinction does not always result in recovery of an extinguished response, and the renewal effect also appears to be dependent on the type of context used (Thomas et al. 2003), similar to what we observed with infant rats. Interestingly, recovery from extinction has been demonstrated in both preverbal (Rovee-Collier and Cuevas 2009) and verbal human infants (Byrne et al. 2015). In the light of these findings, rather than postulating a qualitatively different mechanism for extinction during infancy, it may be more appropriate to view the problem in terms of which variables could differentially modulate the extinction effect across the different stages of ontogeny. In answering this question it seems important to consider that infants can respond to experimental conditions in a way that is different—not only quantitatively but also qualitatively—to older organisms (Campbell and Spear 1972; Spear 1984; Spear and Riccio 1994; Rovee-Collier and Giles 2010). For example, Spear and collaborators reported procedures that favored contextual or trace conditioning in infants, but not in older animals—such procedures sometimes retarding learning in the latter case (McKinzie and Spear 1995; Brassler and Spear 1998, 2004). In our study we found, for instance, that with two conditioning trials, the CR displayed by

weaning rats was much stronger than the one displayed by preweanlings with the same conditioning protocol (Experiment 5). Furthermore, for detecting Pavlovian conditioning after two conditioning trials, the presence of the odor was necessary for preweanling (Experiment 4) but not for weaning rats (Experiment 5). Finally, after extinction, weaning rats showed a clear reinstatement effect, an important result considering that the contexts were not enriched with the explicit odor (Experiment 5). Overall, these results support the theoretical approach followed by researchers such as Norman Spear and Carolyn Rovee-Collier, sometimes referred to as the ecological hypothesis when studying the ontogeny of learning phenomena (see Rovee-Collier and Giles 2010). According to this view, infantile learning and retention capacities are not necessarily poorer or weaker than those of the adult organism; rather, these capacities allow the infant organisms to respond adaptively to their environment in each ontogenetic stage. Therefore, when infant organisms are evaluated in memory tasks whose testing demands are adapted to their perceptual and motor capacities, they are capable of acquiring complex forms of learning and can also respond accurately in tasks considered to be hippocampus dependent (Spear 1984; Rovee-Collier and Cuevas 2009; Rovee-Collier and Giles 2010).

Materials and Methods

Subjects

We used a total of 62 Wistar rats (32 males and 30 females), taken from 9 litters for Experiment 1. Thirty subjects were used for Experiment 1a (2 conditioning trials) and 32 for Experiment 1b (6 conditioning trials); for Experiment 2 we used a total of 56 Wistar rats (29 males and 27 females), taken from 7 litters; for Experiment 3 a total of 56 Wistar rats (28 males and 28 females), taken from 7 litters; for Experiment 4a a total of 18 males and 18 females Wistar rats, representative of 6 litters, and for 4b 14 male and 15 female Wistar rats, representative of 5 litters, and finally, for Experiment 5 we used a total of 17 male and 16 female Wistar rats, representative of 6 litters (see Table 1). In all of the experiments conducted in the present study no more than one subject of each sex from a given litter was assigned to the same treatment condition, in order to avoid overrepresentation of a particular litter in any treatment. 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 temperature ($22 \pm 1.0^\circ\text{C}$), on a 12 h light–12 h dark cycle with food and water provided ad libitum. Births were examined daily and the day of parturition was termed postnatal day 0 (PD0). Subjects were 17 d old at the start of Experiments 1–4, and 24 d old on Experiment 5. All procedures were approved by the National Department of Animal Care and Health (SENASA—Argentina) and were in compliance with the National Institute of Health's general guidelines for the Care and Use of Laboratory Animals.

Apparatus

In the present study, we used two different contexts. Conditioning was always carried out in context A, while the remaining phases (extinction and testing) in context A or B. Context A consisted of a Plexiglas chamber ($29 \times 17 \times 20$ cm) with white opaque walls, except for the front one that was transparent, to enable the testing sessions to be videotaped. The grid floor (30×18 cm) consisted of stainless steel rods (0.2 cm in diameter) separated by a distance of 1 cm. The grid was wired to a scrambling electrical stimulation generator (L.I.A.D.E.—FCFyN, UNC) that delivered footshocks (the intensity varied across experiments). Context B consisted of a similar Plexiglas chamber ($29 \times 17 \times 20$ cm) with black opaque walls. Both contexts were placed in the same room during conditioning, extinction, and testing. This room was illuminated with dim light and had a constant low noise (80 dB) generated by an air extractor. In some of the experiments (1, 2, and 4a)

a small piece of cotton located at the top of the chamber was used to scent the context with almond odor (0.5 mL of pure almond scent, Esencias del Botanicario) for context A, and with orange odor (0.5 mL of pure orange scent, Esencias Bangladesh) for context B. In Experiments 3, 4b, and 5, the same contexts were used but without the odor. According to the results of previous experiments conducted in our laboratory, 17 d old rats are able to discriminate between these contexts—at least when they contain the explicit odors (Revillo et al. 2015).

Procedures

Experiment 1

Conditioning: In Experiments 1a and 1b conditioning was carried out on PD 17 in Context A (including the almond odor). On this day, subjects from each litter were randomly assigned to the experimental groups (Paired or Unpaired), removed from their home cage and individually placed in the experimental chamber for conditioning. After 1 min of contextual adaptation, the subjects in the Paired group were exposed to the first CS (a 90 dB tone) for 20 sec, with a footshock US being delivered on second 19 (0.5 mA, 1 sec). Rats received a total of 2 (Experiment 1a) or 6 (Experiment 1b) conditioning trials with a mean inter-trial interval (ITI) of 70 sec (between 25 and 130 sec). Thirty seconds after the last trial, subjects were returned to their home cage. Subjects from the Unpaired group received the same number of CSs and USs as the Paired group, but these stimuli were separated by an interval of 45 sec, starting with a footshock delivered 1 min after initial exposure to the context.

Extinction: The extinction session was conducted 24 h later (on PD18) in context A for one half of the animals and in B for the remaining half. These contexts contained the explicit odors described in the Apparatus section. Pups were placed in the experimental chamber and after 2 min they were exposed to 6 (Experiment 1a) or 8 (Experiment 1b) tone CSs (30-sec tone) in the absence of the US, with a 30-sec interval between each tone CS presentation. According to previous results from our laboratory, this amount of tone CSs should be sufficient to completely extinguish the CR. Immediately after the last tone, the pups were returned to their home cage.

In these and in the following experiments the experimental session was videotaped and analyzed by two trained researchers blind to the experimental conditions of the animals. The interrater reliability in accordance with Pearson's P was always higher than 0.98. In the present study, the only behavior measured was the freezing response, defined as the time (s) with no movement except respiration.

Experiment 2

Conditioning: Conditioning was carried out on PD17. On this day, subjects from each litter were randomly assigned to the experimental groups (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder). The first term of the name refers to the conditioning treatment, while the second term refers to the reinstatement treatment. Conditioning parameters were exactly the same as those described in Experiment 1. The Paired-Reminder and Paired-No reminder received the treatment described for the Paired group, while the Unpaired-Reminder group was treated identically to the Unpaired condition from Experiment 1.

Extinction: The extinction session was identical for all the subjects and identical to the test session in Experiment 1b, in which all subjects received eight exposures to the tone CS. Approximately half of the subjects were assigned to the AAA condition, while the remaining half were assigned to the ABB condition.

Reinstatement: Twenty-four hours after extinction, subjects were returned to the extinction context and 1 min later subjects from the Paired-Reminder and Unpaired-Reminder groups were given a 0.3 mA (1s) footshock, while subjects from the Paired-No reminder condition did not. After the second min, all subjects were exposed to eight 30-sec tone CSs, with a 30-sec inter-

val between each tone. In this and in the following experiments the CS was presented the same amount of times during the reinstatement test as during extinction training.

The footshock intensity chosen for the reminder is weaker than the one used at testing. In a preliminary study, we were able to confirm that this footshock does not significantly increase freezing in unpaired controls. With this aim in mind, we compared the percentage of freezing displayed at testing by Unpaired-Reminder and Unpaired-No reminder subjects, after the conditioning and extinction phases described in this section. The ANOVA did not reveal a significant effect of the footshock, $F_{(1,22)} = 2,37$, $P = 0.14$ (Group Unpaired-Reminder: mean = 18.5, SD = 13.7, $n = 12$; Group Unpaired-No reminder: mean = 11.4, SD = 8.3, $n = 12$). Based on this result, we did not include the Unpaired-No reminder group in the experimental design.

Experiment 3

Conditioning, Extinction, and Reinstatement procedures were identical to those described for Experiment 2, with the only exception that in the present experiment contexts A and B did not include the explicit odor.

Experiment 4

Conditioning: Following the procedures of Experiment 1a, conditioning was carried out on PD17 with 2 conditioning trials. Groups were the same as in Experiment 2 (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder). The odors were included in contexts A and B in Experiment 4a, but not in Experiment 4b.

Extinction: The extinction session was identical for all the subjects and identical to the extinction session from the previous experiment, except that subjects received six non-reinforced exposures to the tone CS, instead of eight.

Reinstatement: This phase was identical to the one described for the previous experiment, with only six 30-sec tone CSs, with a 30-sec interval between each tone CS presentation.

Experiment 5

Conditioning: Following the procedures of Experiment 4b, conditioning was carried out on PD24, with two conditioning trials. The groups were the same (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder), and the context did not include the explicit odor.

Extinction: The extinction session was identical for all the subjects and identical to the extinction session from the previous experiment, except that subjects received eight nonreinforced exposures to the tone CS, instead of 6, because the magnitude of the CR was greater to the one induced by the CS in the preweaning rats with the same amount of conditioning trials.

Reinstatement: This phase was identical to the one described for the previous experiment, with eight 30-sec tone CSs, with a 30-sec interval between each tone CS presentation.

Experimental design and statistical analysis

The experimental design of Experiments 1a and 1b is mixed, with three between-group variables: Group (Paired vs Unpaired), Context treatment (AA or AB) and Sex (Male or female). For subjects from the AA condition extinction was carried out in the conditioning context, while for subjects from the AB condition extinction took place in the alternative context. In this and in the following experiments the dependent variable analyzed was the percentage of time engaged in the freezing response in the presence of the tone CSs (% freezing). In all the experiments freezing data were collapsed into blocks of two consecutive CSs. A mixed ANOVA was conducted to explore possible between-group differences in this behavior, and to confirm that the CR was extinguished during the extinction session. Experiments 2 and 3 used a mixed design, with three between-group variables: Group (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder),

Context treatment (AAA and ABB) and Sex (Male vs female). Mixed ANOVAs were conducted to explore possible between-group differences in this behavior during extinction and reinstatement, with Block as the within-group factor. Experiments 4a and 4b used a mixed design with two between-group variables: Group (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder) and Sex (Male versus female). Mixed ANOVAs were conducted to explore possible between-group differences in this behavior during extinction and reinstatement. Finally, Experiment 5 used a mixed design with two between-group variables: Group (Paired-Reminder, Paired-No reminder, or Unpaired-Reminder) and Sex (Male vs Female). Mixed ANOVAs were conducted to explore possible between-group differences in this behavior during extinction and reinstatement. In all of the experiments significant effects were analyzed by means of post hoc tests (Newman-Keuls), with the *P* level set at 0.05. Since Sex did not interact with Group in any measure in any experiment, behavioral scores were represented in the figures with Group and Context treatment as the between-group factors.

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