

Research Report

Spontaneous recovery from extinction in the infant rat



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HIGHLIGHTS

- We described changes in a set of different behaviors across extinction in the infant rat.
- We described spontaneous recovery from extinction in the infant rat through the analysis of different behaviors.
- The pattern of results is compatible with the hypothesis that the original memory survives extinction training.

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ABSTRACT

Within the Pavlovian conditioning framework, extinction is a procedure in which, after conditioning, the conditioned stimulus (CS) is repeatedly presented without the unconditioned stimulus (US). During this procedure the conditioned response (CR) is gradually attenuated. It has been suggested that extinction during the early stages of ontogeny is a qualitatively different process from extinction in adulthood: during infancy, extinction may result in erasure of the memory, while during adulthood extinction involves new learning. This conclusion was supported by studies showing that renewal, reinstatement or spontaneous recovery procedures were not effective during infancy for recovering the CR once it had been extinguished. These studies used the freezing response as the only behavioral index, although some recent evidence indicates that the absence of freezing after conditioning or after extinction does not necessarily imply a deficit in memory, and that other behavioral indexes may be more sensitive to detecting conditioning effects. The goal of the present study was to analyze extinction in preweanling rats by examining the possibility of the spontaneous recovery of a conditioned fear response, measured through a different set of mutually-exclusive behaviors that constitute an exhaustive ethogram, and including control groups (Experiment 1: US-Only and CS-Only; Experiment 2: US-Only, CS-Only and Unpaired) in order to examine whether non-associative learning may explain quantitative or qualitative changes in the frequency of specific responses during extinction or recovery. Extinction produced changes in the expression of freezing, grooming and exploration, and the clearest evidence of spontaneous recovery came from the analysis of freezing behavior. The pattern of behavior observed during extinction is compatible with theoretical approaches which consider different dynamic behavioral systems, and it also fit in well with a molar approach to the analysis of behavior, which considers that extinction involves a transition from one allocation of time among behaviors to another allocation, rather than a loss of strength in any particular discrete response. These results have implications for the study of extinction during infancy, since they are compatible with the hypothesis that the original memory survives extinction, and highlight the importance of control conditions for detecting this effect during this ontogenetic period.

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1. Introduction

Within the Pavlovian conditioning framework, extinction can be defined as a procedure in which, after conditioning, the conditioned stimulus (CS) is repeatedly presented without the unconditioned

stimulus (US). During this procedure the conditioned response (CR) is gradually attenuated, an effect that has also been referred to as “behavioral extinction” [1]. Within the associative framework, the CR is assumed to reflect a hypothetical association between mental representations of the CS and US [2]. Several psychological theories of learning have proposed associative hypotheses aimed at explaining the extinction effect. These theories can be grouped into two major categories. Some propose that extinction involves “unlearning” [3], or that the association between the CS and the

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US is weakened or even erased during extinction training [4]. However, an alternative, but not exclusive, associative explanation suggests that extinction implies new learning that competes with the expression of the previously acquired conditioning [1,4]. The latter hypothesis has found empirical support in results showing expression of the CR after extinction, by means of procedures of renewal, reinstatement, spontaneous recovery or rapid reacquisition [1,2,5]. The fact that the magnitude of the CR is usually lower after than before extinction has been interpreted as evidence that the association between the CS and the US is also weakened during extinction training [6]. Over recent decades several neurobiological studies have been carried out with the aim of identifying the neuronal correlates of this hypothetical new learning acquired during extinction training [7,8], although some recent evidence also seems to provide partial support for the “unlearning” hypothesis [9].

Behavioral extinction has also been described in young laboratory rats [10–12]. However, in some cases, renewal, reinstatement or spontaneous recovery procedures were found not to be effective during this ontogenetic period for recovering the CR once it had been extinguished [12–14]. These results have prompted some authors to postulate that extinction during the early stages of ontogeny is a qualitatively different process from extinction in adulthood [4]: during infancy, extinction results in unlearning or erasure of the memory, while during adulthood, the extinction effect mainly consists of an expression of new learning. However, this conclusion is not supported by recent findings that show renewal, reinstatement or rapid reacquisition during infancy using a conditioned taste aversion procedure [15]. Since those studies that failed to find evidence of CR recovery all used a foot-shock as the US, it may be that the ontogenetic difference in extinction was dependent on the type of learning analyzed. However, in a more recent study, evidence of recovery from extinction during infancy was extended to a fear conditioning procedure, and a procedural manipulation was identified that critically modulates performance [10]. Specifically, an ABA-renewal design enabled the recovery of an extinguished CR, using a foot-shock as the US, only when contexts A and B differed in their odor content, as well as in their visual appearance, but not when they differed exclusively in visual cues [10]. In light of a previous study with adult rats which found comparable results (with an operant conditioning procedure) [16], it seems that this finding does not reflect a particular characteristic of the infantile period. The authors demonstrated that the effectiveness of an ABA-renewal procedure depends also on the features shared by contexts A and B, as well as on the sensory content of the context [16]. These results prompted us to explore additional forms of relapse from extinction, such as the spontaneous recovery effect.

As mentioned above, most ontogenetic studies of extinction have used a procedure in which the US was a foot-shock and the CS a tone or an odor [4,10,12–14,17–19]. In these studies the behavioral index used to estimate the state of the “fear memory” or the “CS–US association” was the freezing response. Freezing is a species-specific defensive behavior that belongs to a wider category of activities that are displayed by the organism in response to threats [20–22]. It has been proposed that the defensive system of an organism can be divided into three stages [22] or modes [23], depending on the proximity of the threat. The pre-encounter level refers to the defensive responses that take place in an area where a threat was encountered previously, but is not present at the moment. The next level is the post-encounter one, in which the defensive system is activated when the predator is identified at a certain distance. And finally, the circa-strike defensive system refers to the behaviors displayed in the presence of the predator [22]. In the rat, the freezing response belongs to the hypothetical post-encounter level, and this behavior is evoked in the laboratory in response to CSs or contexts previously paired with a foot-shock

[22]. However freezing is not the only behavior which undergoes changes in frequency in response to fear conditioning treatments. After conditioning, CSs can induce changes in heart and breathing rate, blood pressure and pupillary dilation [24]. They can also affect the expression of behaviors other than freezing, such as exploration (horizontal and vertical) of the environment in which the threat was encountered, and can inhibit non-defensive behaviors, such as grooming or exploration [20,24–26]. Moreover, the extinction of freezing does not necessarily imply the extinction of other conditioned behaviors, and it has been shown, for example, that evidence of conditioning can still be detected in adult rats by analyzing different overt behaviors, such as inhibition of grooming [27]. Interestingly, a recent study with preweanling and weanling rats demonstrated that, at this ontogenetic stage, freezing is not necessarily the most sensitive index of memory in a contextual conditioning preparation using a low intensity foot-shock [28]. Similarly to the results found by Whor et al. [27], inhibition of grooming or wall climbing was found to be even more effective than freezing in the case of a low-intensity foot-shock, while freezing was as sensitive as these alternative measures when a high-intensity foot-shock was used [28]. Obviously, increases in freezing necessarily result in a decrease of other behaviors, but even when a conditioning treatment does not induce freezing, other exploratory behaviors may still be attenuated [28]. These results indicate that the absence of freezing after conditioning or after extinction does not necessarily imply a deficit in memory [24], and we should be cautious when drawing conclusions about the preservation or erasure of a memory based only on the observation of this behavior [29].

The goal of the present study was to analyze extinction in preweanling rats by examining the possibility of spontaneous recovery. Only one previous study has explored the possibility of spontaneous recovery during infancy in mice [14]. The authors showed that when conditioning and extinction occurred during the preweanling period subjects showed no evidence of CR recovery, a result that was interpreted as additional evidence in support of the theory that extinction during infancy erases the CS–US association [14]. However, in this study the authors only examined the freezing response. In the present study we decided to measure a different set of mutually-exclusive behaviors that constitute an exhaustive ethogram that has proven effective in revealing context learning during infancy [28]. Additionally, our experimental design also included control groups (Experiment 1: US-Only and CS-Only; Experiment 2: US-Only, CS-Only and Unpaired) in order to examine whether non-associative learning may explain quantitative or qualitative changes in the frequency of specific responses during extinction or recovery [30].

2. Experiment 1

2.1. Materials and methods

2.1.1. Subjects

For the present experiment we used a total of 27 male and female Wistar rats, representative of 7 litters. In both experiments carried out in present study no more than one subject of each sex from a given litter was assigned to the same treatment condition, 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 (P0). Subjects were 17 days old at the start of the experiment. 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.

2.1.2. Apparatus

Conditioning, extinction and the spontaneous recovery test were carried out in the same context, which consisted of a Plexiglas chamber (29 cm × 17 cm × 20 cm) with white opaque walls and vertical black lines (2 cm wide), spaced 5 cm apart, all along the walls except for the front one that was transparent to enable the testing sessions to be videotaped. The grid floor (30 cm × 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. – FCEFyN, UNC, Cordoba, Argentina) that delivered 0.6 mA foot-shocks (duration: 1 s). The room was dimly lit with a constant low noise (80 dB) generated by an air extractor.

2.1.3. Procedures

Conditioning: Conditioning was carried out on PD 17. On this day, subjects from each litter were randomly assigned to the experimental groups (CS-only, US-only and Paired), removed from their home-cage and individually placed in the experimental chamber for conditioning. After 1 min of contextual adaptation, the CS (a 90 dB tone) was presented for 10 s and immediately after, the US foot-shock (0.6 mA, 1 s) was delivered. Rats received a total of eight conditioning trials with an intertrial interval (ITI) of 50 s. Thirty seconds after the last trial, subjects were returned to their home-cage.

Extinction: Four extinction sessions (one each day) were conducted between PDs 18–21. Pups were placed in the experimental chamber and after 1 min were exposed twice to the tone-CS (2 min tone) in the absence of the US, with a 1 min interval between each tone-CS presentation. Immediately after the second tone, rats were returned to their home-cage. Hence, extinction training involved eight CS presentations (CS1–CS8).

Spontaneous recovery test: Ten days after the last extinction session (PD 31), behavior was analyzed in an additional extinction session (CS9).

The parameters used for conditioning, extinction and testing were extracted and adapted from previous studies with infant rats [10]. The extinction and spontaneous recovery testing sessions were videotaped and analyzed by two trained researchers blind to the experimental conditions of the animals. The inter-rater reliability in accordance with Pearson's p was always higher than 0.98. Based on a previous study [28], four dependent variables were registered: (1) Horizontal exploration: time (s) spent exploring the chamber with all four paws on the grid floor. (2) Vertical exploration (including wall climbing and rearing): subjects were considered to be wall climbing when standing on their rear limbs with their forepaws against the wall of the testing chamber. (3) Grooming: time (s) spent paw licking or grooming, which was defined as a sequence of movements beginning with rapid elliptical strokes by the paws over the nose. Occasionally, the amplitude of such movements progressively increases and is followed by bilateral strokes. This sequence of movements ends with a postural turn followed by a period of body licking directed at the flank. (4) Freezing: time (s) with no movement except respiration. These categories are mutually-exclusive, or in other words, any behavior displayed by the rat was included in one of these categories, and no behavior was included in more than one category.

2.1.4. Statistical analysis

For the present study we operationalized spontaneous recovery as a change in behavior in CS9 relative to CS8 (within-group), but also in terms of between-group differences (difference between

the Paired and control groups) in the spontaneous recovery test (CS9). Although within-group analyses have been more frequently used in spontaneous recovery studies than between-group ones, we believe that the latter approach may be informative in ontogenetic studies in which subjects' responses may undergo important variations throughout the duration of the experiment. In Experiment 1, extinction sessions were carried out on PDs 18–21, and the spontaneous recovery test on PD31. During this ontogenetic period important variations occur, for example, in locomotor activity [31], and the sensitivity of some of the behavioral indexes measured, such as vertical and horizontal explorations, may be affected by these ontogenetic changes.

Preliminary ANOVAs revealed that behavioral scores were statistically equivalent in males and females, and that Sex did not interact with Group. Hence, behavioral scores were analyzed and represented in the figures considering Group as the only between-group variable. In order to avoid Type I errors the statistical analyses were driven by the operational definition of spontaneous recovery. We first ran a mixed ANOVA to explore behavioral changes as a consequence of the extinction training, including data from the first (CS1) and last (CS8) CS presentations during extinction. This analysis included Group as the only between-group factor, and CS-presentation as the within-group variable. The second mixed ANOVA was performed to explore the possible spontaneous recovery effect, including Group as the between-group variable and CS-presentation as a within-group factor. In this case the latter variable had two levels, corresponding to the last CS presentation during extinction (CS8) and the CS-presentation during the spontaneous recovery test (CS9). Interactions were followed by individual ANOVAs with data from each CS-presentation, and significant effects were analyzed by means of post-hoc tests (Newman–Keuls), with the p level set at 0.05.

3. Results

Fig. 1(a–d) represents variations in the different behaviors contemplated in the ethogram (including freezing, horizontal and vertical exploration and grooming) in the CS-Only, US-Only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9). The first remarkable characteristic is that while in the first CSs the percentage of time dedicated to (horizontal) exploration or grooming was low in subjects from the Paired group, more and more time was progressively dedicated to these behaviors as extinction progressed. On the other hand, freezing behavior in this group followed the opposite pattern, decreasing progressively across the extinction training. Interestingly, the US-Only group displayed a similar profile during extinction, but not in the spontaneous recovery test. In order to minimize the possibility of Type I errors caused by a high number of analyses, we conducted specific ANOVAs to statistically corroborate that a behavioral change did indeed occur during the extinction phase (between CS1 and CS8), and to explore the possibility of spontaneous recovery (comparing CS8 with CS9). The first mixed ANOVA was conducted with scores from CS1 and CS8, and the second was conducted with scores from CS8 and CS9, this latter session corresponding to the spontaneous recovery test conducted 10 days after the last extinction session. In these ANOVAs Group was the only between-group factor. We have organized the description of the analyses into separate subsections for each behavior.

Freezing: The ANOVA conducted with the freezing scores from the extinction sessions (CS1 and CS8) revealed a significant Group by CS-presentation interaction [$F(2,24) = 11.08, p < 0.05$]. To further explore this interaction we conducted one-way between-group ANOVAs with freezing scores from CS1 and CS8. The ANOVA with scores from CS1 revealed a significant main effect of Group

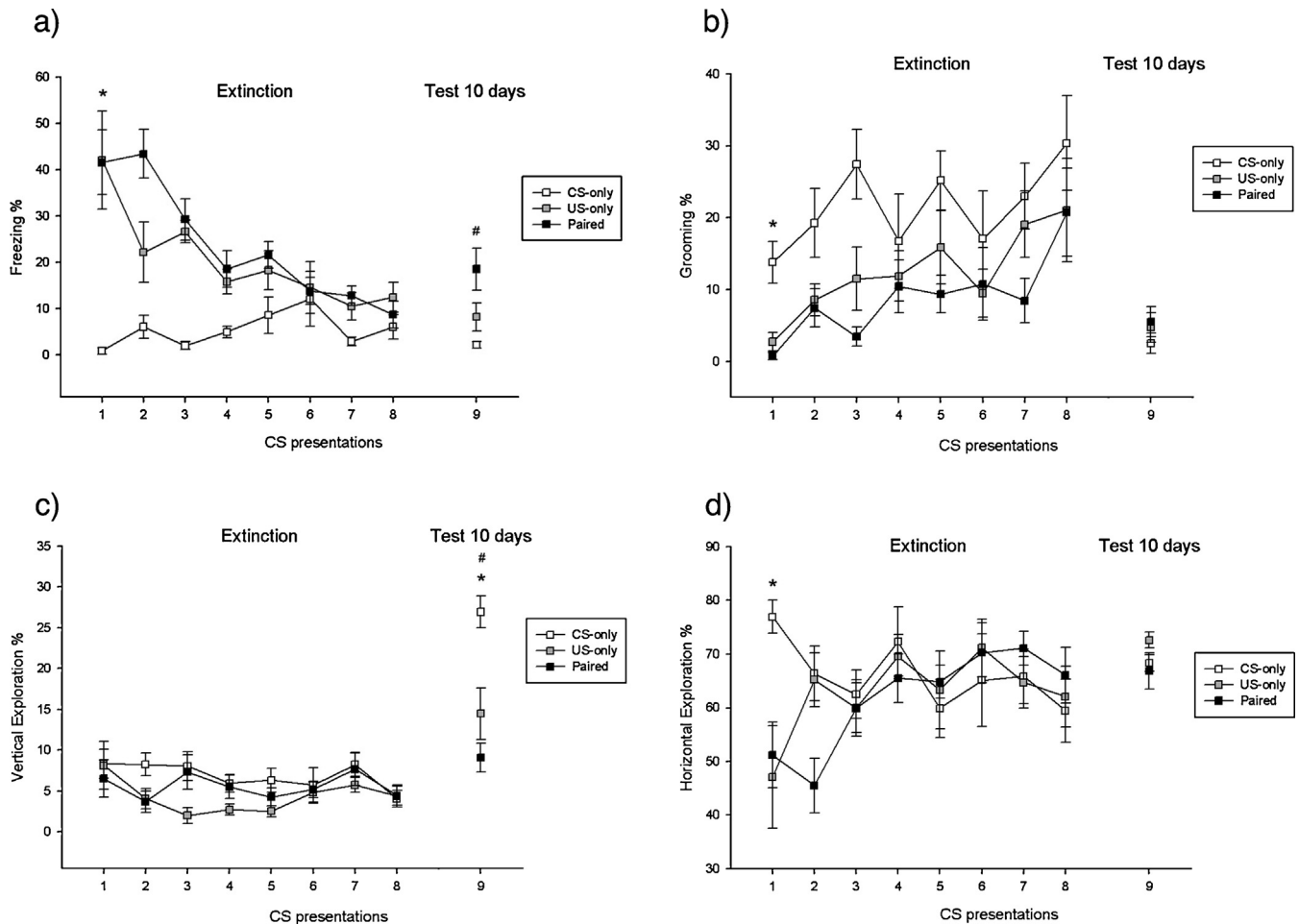


Fig. 1. (a) It represents variations in freezing (percentage of time) in the CS-only, US-only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9), 10 days after the last extinction session. Vertical bars represent the standard error of the means (SEM); * $p < 0.05$ CS-Only versus the other groups; # $p < 0.05$ Paired versus the other groups. (b) Grooming scores (percentage of time) in the CS-only, US-only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9). Vertical bars represent the standard error of the means (SEM); * $p < 0.05$ CS-Only versus the other groups. (c) Vertical exploration (percentage of time) in the CS-only, US-only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9). Vertical bars represent the standard error of the means (SEM); * $p < 0.05$ CS versus the other groups; # $p < 0.05$ Paired versus the other groups. (d) Horizontal exploration (percentage of time) in the CS-only, US-only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9). Vertical bars represent the standard error of the means (SEM); * $p < 0.05$ versus the other groups.

[$F(2,24) = 11.30$, $p < 0.05$], indicating, according to the post-hoc tests, that exposure to the CS led to a significant increase in freezing in the Paired and US-Only groups in comparison with the CS only control group. These differences were completely abolished in CS8. These results demonstrate that repeated exposure to the CS produced a decrease in freezing scores, although the responses by the US-Only and Paired groups during the extinction training were statistically indistinguishable as regards magnitude.

The ANOVA conducted to explore the possibility of spontaneous recovery (including CS8 and CS9) also revealed a significant Group by CS-presentation interaction [$F(2,24) = 11.08$, $p < 0.05$], due to significant between-group differences in CS9, [$F(2,24) = 6.49$, $p < 0.05$], but not in CS8. Specifically, the Paired group spent significantly more time freezing than the CS-Only and US-Only groups. The within-group ANOVAs only revealed a significant increase in the Paired group in freezing scores in the spontaneous recovery test (CS9) relative to scores from CS8 [$F(1,9) = 5.83$, $p < 0.05$], but not in the other conditions. In sum, during the spontaneous recovery test (CS9), ten days after extinction, freezing scores were higher than those from the other control conditions, and the only group that showed an increase in this behavior in the spontaneous recovery test, in comparison with the last extinction trial (CS8), was

the Paired group. These results are compatible with the spontaneous recovery effect from both a between- and a within-group perspective.

Grooming: Although grooming scores from the Paired and US-Only groups were lower on CS1 [$F(2,24) = 15.14$, $p < 0.05$] and during most of the extinction trials than those from the CS-Only control group, the ANOVA comparing scores from CS1 and CS8 revealed no significant effect or interaction. The ANOVA conducted to explore changes in grooming between CS8 and CS9 found no significant effects or interactions.

Vertical exploration: No significant between-group differences were detected by the ANOVA during extinction (CS1 and CS8), and this analysis only revealed a significant decrease in the time spent engaged in vertical exploration in CS8 relative to CS1 [$F(1,24) = 5.89$, $p < 0.05$], regardless of the experimental condition. However, during the spontaneous recovery test, this behavior clearly increased in the CS-Only and US-Only groups, in comparison with the extinction trials. The ANOVA conducted with CS8 and CS9 (spontaneous recovery test) revealed a significant Group by CS-presentation interaction [$F(2,24) = 12.11$, $p < 0.05$]. The statistical analysis confirmed that vertical exploration differed between groups in the spontaneous recovery test [$F(2,24) = 16.88$, $p < 0.05$], with the Paired

group scoring lower in vertical exploration than the CS-Only and US-Only groups (scores from these groups also were statistically different). Within-group analyses also revealed a significant increase in vertical exploration in CS9 relative to CS8 in groups CS-Only [$F(1,8) = 86.60, p < 0.05$] and US-Only [$F(1,7) = 10.05, p < 0.05$], but not in the Paired condition.

Horizontal exploration: Finally, the ANOVA conducted with horizontal exploration scores during extinction (CS1 and CS8) revealed a significant Group by CS-presentation interaction [$F(2,24) = 4.81, p < 0.05$]. Between-group differences only reached statistical significance in CS1, [$F(2,24) = 14.88, p < 0.05$], with the Paired and US-Only groups scoring lower than the CS-Only group for this behavior. No significant effects or interactions were detected by the ANOVA conducted with CS8 and CS9.

Overall, the results from this experiment are compatible with the spontaneous recovery effect. The clearest evidence of this effect came from the analysis of freezing behavior, since only subjects from the Paired condition increased the time engaged in this behavior on the spontaneous recovery day (CS9) relative to the last CS-presentation during the extinction phase (CS8). Moreover, freezing scores in the Paired group were higher than those in the other groups in the spontaneous recovery test, while in the last extinction trial they were statistically equivalent across groups. This result may represent additional evidence supporting the idea that extinction during the preweaning period does not erase the fear memory. However, there are some important differences between our extinction protocol and the one used in previous extinction studies with preweaning rats, and it is difficult to compare results across studies. Firstly, our extinction protocol involved two CS-presentations each day across four days, while previous studies used usually one and at the most two extinction sessions with several CS-presentations [12–14,17,19,32]. For this reason, our extinction training lasted until PD21, ending closer to the pre-adolescent period than in previous studies, which conducted extinction on PD18 or PD19. These procedural differences may have affected our pattern of results, including the spontaneous recovery effect. Furthermore, the US-Only control condition revealed a similar behavioral profile to the Paired group during most of the extinction training (although not during the spontaneous recovery test), a finding which may be interpreted as an obstacle to considering that our data provide clear evidence of conditioning, extinction, and hence spontaneous recovery from extinction. The behavioral pattern displayed by the US-Only group may reflect sensitization to the tone-CS after exposure to the shock [30]. This effect may be influenced by contextual fear conditioning, since we employed an AAA design, in which extinction and spontaneous recovery sessions were carried out in the same context (see the following citations for evidences of long-term contextual fear conditioning in P18 or younger rats [28,33–37]). For all these reasons we conducted an additional experiment with significant variations in the protocol, with the aim of exploring possible recovery from extinction when the extinction protocol is carried out during the preweaning period, using procedures more similar to those employed in previous studies. Firstly, conditioning was conducted on PD17 and extinction on PD18 (only 1 session). We also used an ABB design in order to reduce the response of the US-Only group, and included a better control for Pavlovian conditioning (Unpaired group). In order to ensure that extinction of fear conditioning was completed within a single session, and to reduce the response of the US-Only group, we also used a weaker conditioning protocol, with fewer conditioning trials (6 instead of 8) and a slightly weaker foot-shock (0.5 mA instead of 0.6 mA). Finally, during both extinction and testing, the CS-presentation lasted 30 s, similarly to the only previous study that explored spontaneous recovery in preweaning rodents [14]. Once extinction was completed, subjects were evaluated in response to the CS, one (PD19) and eleven days (PD29) later.

4. Experiment 2

4.1. Materials and methods

4.1.1. Subjects

For the present experiment we used a total of 36 (20 males and 16 females) Wistar rats, representative of 5 litters, reared under the same conditions as those described for Experiment 1.

4.1.2. Apparatus

As mentioned above, for Experiment 2 we used an ABB design to reduce the response of the US-Only group during extinction. Conditioning was carried out in context A, and the other phases (extinction and testing) in context B. Context A consisted of a Plexiglas chamber (29 cm × 17 cm × 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 cm × 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. – FCEfYn, UNC, Cordoba, Argentina) that delivered, 0.5 mA footshocks (duration: 1 s). 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 Boticario, Cordoba, Argentina). Context B consisted of a similar Plexiglas chamber (29 cm × 17 cm × 20 cm) with black opaque walls and scented with orange odor (0.5 ml of pure orange scent, Esencias Banglaesh, Buenos Aires, Argentina). 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.

4.1.3. Procedures

Conditioning: Conditioning was carried out on PD 17 following a procedure very similar to that used in Experiment 1. On this day, subjects from each litter were randomly assigned to the experimental groups (CS-Only, US-Only, Unpaired and Paired), removed from their home-cage and individually placed in the experimental chamber (Context A) for conditioning. After 1 m of contextual adaptation, the Paired group was exposed to the first CS (a 90 dB tone) for 20 s, with a US foot-shock being delivered on second 19 (0.5 mA, 1 s). Rats received a total of six conditioning trials with a mean intertrial interval (ITI) of 70 s (between 25 s and 130 s). Thirty seconds after the last trial, subjects were returned to their home-cage. Subjects from the US-Only, Paired and CS-Only control groups received the same amount of context exposure. During this time subjects from the US-Only and CS-Only conditions received the same amount of exposures to the US and the CS, respectively. These stimuli were delivered at the same time-points as the Paired condition. Subjects from the Unpaired group received the same amount of CSs and USs as the Paired group, but these stimuli were separated by an interval of 45 s, starting with a foot-shock 2 min after initial exposure to the context.

Extinction: The extinction session was conducted in context B 24 h later, on PD18. Pups were placed in the experimental chamber and after 1 m were exposed to the eight tone-CSs (30-s tone) in the absence of the US (CS1–CS8), with a 30 s interval between each tone-CS presentation. Immediately after the last tone, the pups were returned to their home-cage.

Spontaneous recovery tests: One (PD19) and eleven (PD 29) days after the last extinction session, behavior was analyzed in response to the CS (30-s) after being placed in context B for 2 min.

Similarly to in Experiment 1, the extinction and testing sessions were videotaped, and the same behaviors were measured: horizontal and vertical exploration, freezing and grooming.

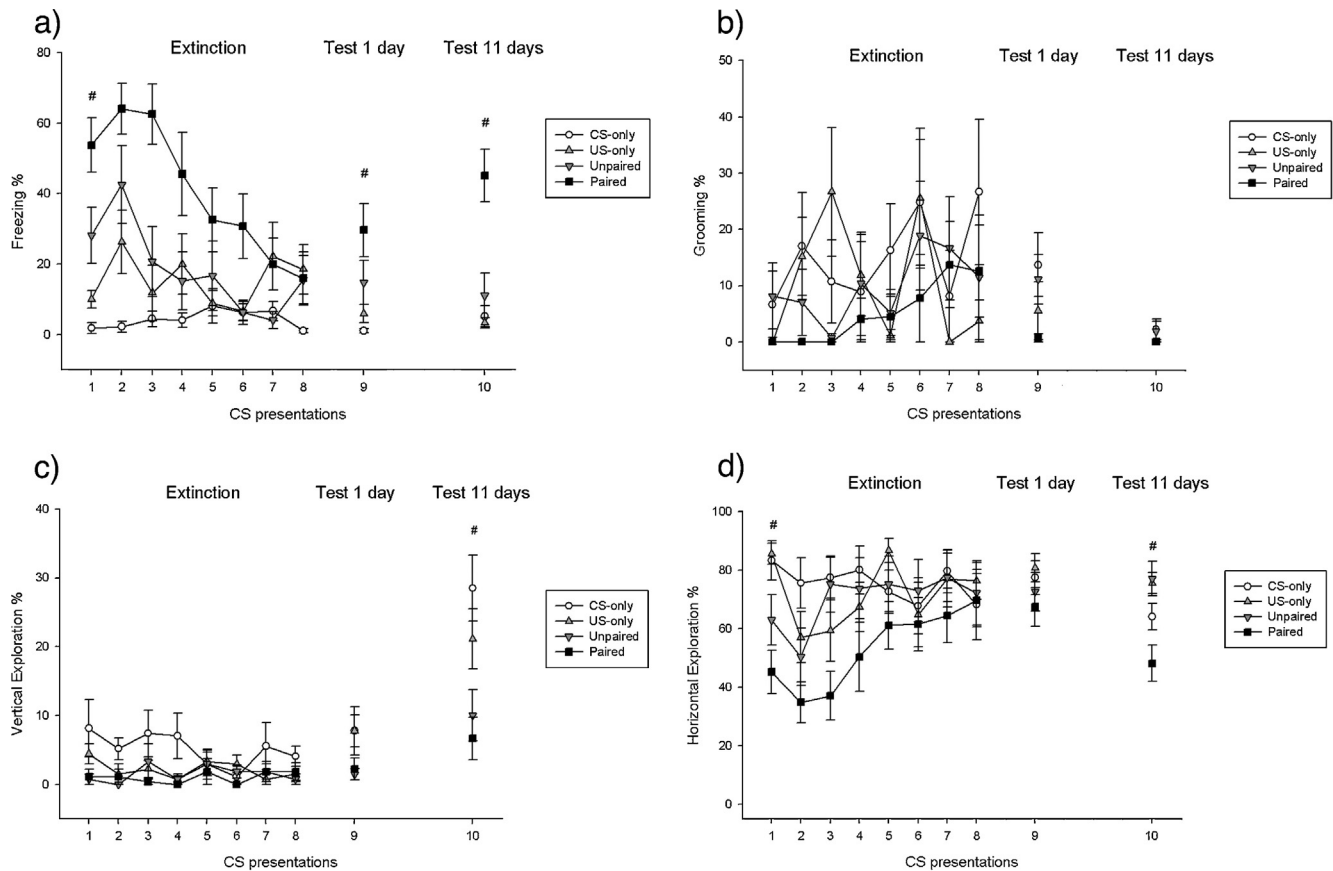


Fig. 2. (a) It represents variations in freezing (percentage of time) in the CS-only, US-only, Unpaired and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery tests, 24 h (CS9) or 11 days (CS10) after extinction. Vertical bars represent the standard error of the means (SEM); # $p < 0.05$ Paired versus the other groups. (b) Grooming scores (percentage of time) in the CS-only, US-only, Unpaired and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery tests (CS 9 and CS10). Vertical bars represent the standard error of the means (SEM). (c) Vertical exploration (percentage of time) in the CS-only, US-only, Unpaired and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery tests (CS9 and CS10). Vertical bars represent the standard error of the means (SEM); # $p < 0.05$ Paired versus CS-Only and US-Only groups. (d) Horizontal exploration (percentage of time) in the CS-only, US-only and Paired groups across the extinction training (CSs 1–8) and during the spontaneous recovery test (CS9). Vertical bars represent the standard error of the means (SEM); # $p < 0.05$ Paired versus CS-Only and US-Only groups on CS1; $p < 0.05$ Paired versus the other groups on CS10.

4.1.4. Statistical analysis

Following the same strategy as in Experiment 1, the statistical analyses were driven by the operational definition of spontaneous recovery. The ANOVAs were conducted firstly to corroborate conditioning and extinction, and secondly to explore the possible spontaneous recovery effect 1 and 11 days after extinction. Similarly to in Experiment 1, we defined spontaneous recovery as a change in behavior at testing, one (CS9) or eleven (CS10) days after the extinction session relative to the last CS-presentation during the extinction session (CS8) (within-group definition), but also in terms of between-group differences (Paired versus Controls) in the spontaneous recovery tests (CS9 and CS10). The first mixed ANOVA was conducted to explore possible between-group differences indicative of conditioning during extinction, as well as to corroborate that extinction was completed in a single extinction session. This analysis included data from the first (CS1) and last (CS8) CS presentation from the extinction session, and Group was the only between-group factor. To explore spontaneous recovery 1 day after extinction, we ran a mixed ANOVA including data from CS8 and CS9. A similar analysis was conducted to explore spontaneous recovery 11 days after extinction, including CS8 and CS10. Interactions were followed by individual ANOVAs with data from each CS-presentation, and 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, behavioral scores

were analyzed and represented in the figures considering Group as the only between-group variable.

5. Results

Freezing: The ANOVA conducted with extinction scores revealed a significant Group by CS-presentation interaction [$F(3,32) = 11.08$, $p < 0.05$] (Fig. 2a), due to the between-group differences observed in CS1 [$F(3,32) = 16.05$, $p < 0.05$], but not in CS8. According to the post-hoc analysis, in CS1, freezing scores from the Paired group were significantly higher than those from the other control conditions. This pattern of results indicates that our training protocol was sufficient to induce conditioning and extinction. The Group by CS-presentation interaction, considering CS8 and CS9, failed to reach statistical significance, but guided by our working hypothesis, we explored scores collected at testing, which revealed a significant main effect of group [$F(3,32) = 6.09$, $p < 0.05$], indicating that subjects from the Paired group spent more time engaged in freezing than their counterparts in the other groups. In this case, the within-group analysis revealed no statistical differences in any group for CS9 in comparison with CS8. The ANOVA to explore spontaneous recovery 11 days after extinction revealed a significant Group by CS-presentation interaction [$F(3,32) = 7.05$, $p < 0.05$], due to the notable between-group differences in CS10 [$F(3,32) = 14.47$, $p < 0.05$], indicating that freezing scores from the

Paired group were significantly higher than those from all three control conditions (CS-Only, US-Only and Unpaired). Moreover, in this case, only the Paired group showed a significant increase in this behavior in CS10 relative to the last extinction trial [$F(1,8)=24.82$, $p<0.05$]. These results are compatible with the spontaneous recovery effect, from both a within-group and a between-group perspective.

Grooming: The ANOVA to explore grooming during extinction and spontaneous recovery tests revealed no significant effects or interactions.

Vertical exploration: The ANOVAs with vertical exploration scores revealed no significant effects or interactions during either extinction or when the scores from CS8 and Testing 1 were compared. However, the ANOVA conducted to compare scores from CS8 and CS10 revealed a significant interaction, explained by between-group differences in CS10 [$F(3,32)=6.18$, $p<0.05$], although not in CS8. According to the post-hoc tests, scores from the Paired group were lower than those from the CS-Only and US-Only groups. This result replicates the observations from Experiment 1, since no differences in this behavior were observed during extinction, although they were found in the spontaneous recovery test conducted with adolescent rats. Interestingly, as in Experiment 1, the CS-Only and US-Only groups showed a significant increase in this behavior in CS10 relative to CS8 [CS-Only: $F(3,32)=31.22$, $p<0.05$; US-Only: $F(3,32)=16.12$, $p<0.05$]. This difference almost reached statistical significance in the Unpaired condition, $F(3,32)=5.26$, $p=0.05$, and was far from significant in the Paired group, as in Experiment 1.

Horizontal exploration: The Group by CS-presentation interaction was not statistically significant in any of the mixed ANOVAs conducted either to explore horizontal exploration scores during extinction (CS1 and CS8), or to compare scores from the last extinction trial (CS8) with scores at testing (CS9 or CS10). However, between-group differences were evident in CS1 [$F(3,32)=7.76$, $p<0.05$] and CS10 (11 days after extinction) [$F(3,32)=6.72$, $p<0.05$]. On these days, the Paired group scored significantly lower in horizontal exploration than either the US-Only or CS-Only controls, and on CS10 scored also lower than the Unpaired group, while no difference was observed between the Unpaired group and these control conditions.

6. Discussion

Both experiments show spontaneous recovery after extinction in subjects that received conditioning and extinction training during the preweaning period. These results have implications for the study of extinction during infancy, because they are compatible with the hypothesis that the original memory survives extinction training, an idea which contradicts conclusions derived from previous studies that failed to find renewal, reinstatement or spontaneous recovery during infancy [4].

The only antecedent that explicitly explored the possibility of spontaneous recovery after extinction during infancy is the study by Gogolla et al. [14]. In addition to the fact that these authors used mice and we used rats, there are also several more methodological differences between Gogolla's study and the present experiments. Probably the most important difference is the experimental design. Firstly, our conclusions are based on within-group comparisons, i.e. comparisons of the scores from the last extinction trial and the spontaneous recovery tests; and secondly, we also observed the spontaneous recovery effect from a between-group perspective, because by the end of the extinction training (CS8) in both experiments, the groups scored identically in all the behaviors analyzed. However, when subjects were tested again after extinction, between-group differences emerged, with the

Paired group scoring differently from the control conditions, particularly in terms of freezing. In Gogolla et al.'s study, none of these analyses were conducted, and the conclusions drawn by the authors are based on comparisons of the freezing scores of subjects that received conditioning and extinction sessions during the preweaning or weaning period, without controls for classical conditioning. Hence, it is possible that the responses observed in that study and in ours may be the result of different learning processes, since we observed, for example, that mere exposure to the foot-shock can induce a strong freezing response in the presence of a novel tone (see the US-Only group in Experiment 1).

In addition to the long-term (after 10 or 11 days) spontaneous recovery effect, our study also detected recovery from extinction 24 h after extinction (on PD 19, Experiment 2), although in this case this effect was only detected by the between-group analysis. Although this effect is common in the literature concerning adult rodents, it is new in the infant rat and its importance lies in that it is not compatible with the hypothesis which holds that extinction erases the fear memory during infancy.

In the second experiment we used an ABB design, while in Experiment 1 we used an AAA one. Since the expression of extinction critically depends on the presence of the extinction context [5,38,39], a stronger spontaneous recovery effect is to be expected in the first than in the second experiment. However, in Experiment 2, freezing scores for CS10 were over 40%, and almost reached the magnitude of the CR at the beginning of the extinction training, while in Experiment 1 freezing scores for CS9 (around 20%) were equivalent to half of the magnitude observed in CS1. This is even more surprising considering that in Experiment 2 we used fewer conditioning trials than in Experiment 1. This difference across experiments may be related to procedural issues, such as, for example, the length of the CS: 2 min in Experiment 1, and 30 s in Experiment 2. We observed that the freezing response is induced rapidly by the presence of the CS and decreases progressively throughout the time for which the CS lasts. It is therefore likely that shorter CSs result in higher scores, when scores are translated into percentage of time. Moreover, in Experiment 1, extinction was carried out over the course of four days, while in Experiment 2 it was completed within a single extinction session, and it is well-known that spacing extinction trials can prevent recovery from extinction [40].

The clearest evidence of spontaneous recovery came from the analysis of freezing. In both experiments freezing was the only behavior in which subjects from the Paired group consistently differed from the control groups during the spontaneous recovery tests (CS9 in Experiment 1, and CS9 and CS10 in Experiment 2). In Experiment 1, during extinction training, subjects from the US-Only group scored similarly in this behavior to the Paired group, but during the spontaneous recovery test these groups differed in their expression of this behavior. In Experiment 2 subjects from the Paired group scored higher in freezing not only during the spontaneous recovery tests, but also during the extinction training. The analysis of the other behaviors (grooming and vertical and horizontal exploration) added no critical information to this conclusion. The general pattern of behavior expressed by subjects from the Paired group involved a reduction in grooming and horizontal exploration at the beginning of the extinction training, and a reduction in vertical exploration (Experiments 1 and 2) or horizontal exploration (Experiment 2) in the spontaneous recovery test. These results are compatible with prior studies involving both infant [28] and adult rats [27], showing that fear conditioning can result in the inhibition of these behaviors, although in our experiment a similar pattern of results was observed also in a lesser extent in control groups exposed to the US at conditioning (Unpaired and US-Only).

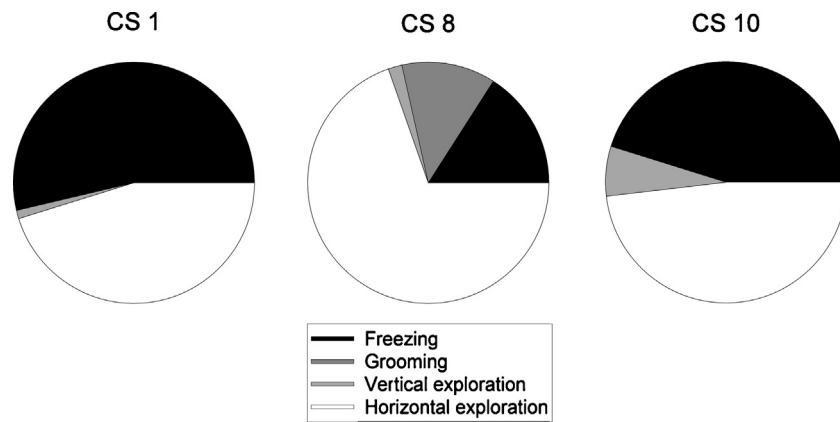


Fig. 3. Transition from one distribution of time among behaviors in CS1 (first extinction CS) to another allocation in CS8 (last extinction CS) and in CS10 (last spontaneous recovery test) in the Paired group from Experiment 2.

The present results are compatible with theoretical approaches which consider different dynamic behavioral systems [22,23,41]. Within this framework, behavior is considered to be organized around important functions for survival in different response subsystems, such as feeding, reproduction or defense [23,41]. Within each subsystem, the sequential and temporal organization of behavior varies along a continuum ranging from general activity patterns to specific responses to predictive stimuli, and is modified by experience (learning) at different levels. As described in the introduction, it has been proposed that the defensive system of an organism can be divided into three categories, depending on the proximity of the threat (pre-encounter, post-encounter and circa strike) [22]. In rodents, freezing is the predominant behavior in the post-encounter stage (i.e. in response to threat detection) [22], although the expression of other behaviors (grooming or exploration) may also vary [25]. It has also been suggested that better predictive stimuli may control more focused behaviors, whereas less predictive stimuli may regulate those related to general search patterns [41]. Given that for rodents, freezing may be the most effective response in terms of probability of survival [22], it is not surprising that animals from the Paired group engaged in this behavior when they detected the CS after conditioning, when it strongly predicted the possible occurrence of the US. However, between an absolute lack of movement (i.e. freezing) and the type of exploration engaged in when the presence of a threat is not expected (for example, in the control subjects), there are intermediate types of exploration, such as, for example, when subjects explore the environment with slow movements, sometimes with reduced rearing or vertical behavior [26]. The inhibition of exploration and grooming was observed not only in the Paired group, but also in the controls that received foot-shock during conditioning (the US-Only and Unpaired groups). This is compatible with the hypothesis that inhibition of grooming or exploration is elicited by stimuli that are not good predictors of the presence of the threat. The fact that behavioral differences between the Paired and US-only or Unpaired groups were only quantitative, not qualitative, in terms of the time spent engaged in specific behaviors, underscores the importance of including these control conditions in behavioral and neurobiological studies during this ontogenetic stage.

As mentioned in the introduction, associative models usually assume that the association between mental representations of the CS and US results in the elicitation of a CR, and use the magnitude of the CR to infer the strength of this association [42]. Other authors have frequently used the magnitude of the CR to infer the intensity of the emotion that hypothetically underlies or mediates behavioral expression (“fear” in the case of freezing) [43,44], and a significant

attenuation of the CR has been used to infer memory loss or amnesia [45,46]. In general, it is common for conclusions in memory studies to be based on the analysis of a single behavioral index or conditioned response, such as freezing, even though it has been acknowledged that conditioning can inhibit the expression of other measures. One theoretical approach which offers an alternative to molecular theories (which are based on the analysis of a single response) is the molar analysis of behavior. This paradigm takes the behavior (activities) of the whole organism as its unit of analysis, rather than just a single response [47,48]. In Baum’s terms [48], behavior requires time, and so increases in one behavior result in a reduction in the time available for other behaviors. This approach also assumes that phylogenetically important events (a category in which USs are included) that reduce fitness (such as predators), induce defensive activities (such as hiding or freezing) which alleviate the danger. Therefore, increases in the frequency of freezing mean that less time is available for alternative behaviors that may be induced by the experimental setting. In the control condition, the context plus the tone-CS induced exploration and grooming, but after conditioning, in the Paired group, the tone-CS plus the context induced defensive reactions, which are not compatible with exploration of the environment or grooming behavior. For Baum [48,49], extinction involves a transition from one allocation of time among activities to another allocation, rather than a loss of strength in any particular discrete response. In our experiments, for example, the freezing induced by the tone-CS progressively decreased during extinction and the level of exploration and grooming increased. Allocation of these behaviors changed again during the spontaneous recovery test (see, for example, Fig. 3, corresponding to data from the Paired group from Experiment 2).

In sum, together with recent findings, the present results challenge the hypothesis that extinction is a qualitatively different phenomenon during infancy than during adulthood. This hypothesis is based on the lack of memory recovery after extinction in preweanling rats, which systematically failed to show effects such as spontaneous recovery, renewal or reinstatement [4]. However, evidence of these phenomena has recently been found, although in addition to certain procedural changes, their detection has always required also that scores from preweanling rats be analyzed separately from those obtained by older age-groups. For example, reinstatement, renewal and rapid re-acquisition after extinction were observed during infancy using a conditioned taste aversion preparation [15]; by enriching the salience of the context with explicit odors we were able to detect renewal [10]. Thus, procedural issues seem to be important in order to detect these effects early in ontogeny.

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