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Spaced sessions of avoidance extinction reduce spontaneous recovery and promote infralimbic cortex activation

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ABSTRACT

Extinction-based therapies (EBT) are the psychological treatments of choice for certain anxiety disorders, such as post-traumatic stress disorder. However, some patients relapse and suffer spontaneous recovery (SR) of anxiety symptoms and persistence of avoidance behaviour, which underlines the need for improving EBT. In rats, recent evidence has highlighted the relevance of the temporal distribution of extinction sessions in reducing SR of auditory fear conditioning, although it has seldom been studied in procedures involving proactive avoidance responses, such as two-way active avoidance conditioning (TWAA).

We examined whether the temporal distribution of two extinction sessions separated by 24 hours or 7 days (contiguous versus spaced extinction paradigms, respectively), influences SR after 28 days of a TWAA task. c-Fos expression, as a marker of neuronal activation, was also measured by immunohistochemistry 90 min after the SR test in the amygdala and the medial prefrontal cortex.

The temporal distribution of extinction sessions did not affect the degree of extinction learning. However, only the rats that underwent the 7-day spaced extinction paradigm maintained the level of extinction in the long term, showing no SR in TWAA. This behavioural finding was consistent with a greater number of c-Fos-labelled neurons in the infralimbic cortex in the 7-day group, and in the Lateral and Central nuclei of the amygdala in the 24-hour group. These findings show that a time-spaced extinction paradigm reduces the spontaneous recovery of active avoidance behaviour, and that this behavioural advantage appears to be related to the activation of the infralimbic cortex.

1. Introduction

Extinction-based therapies (EBT), such as exposure therapy (ET), are the psychological treatment of choice for certain anxiety disorders, such as post-traumatic stress disorder (PTSD) [1][2]. In ET, patients have to confront the conditioned stimuli (CS) related to a stressful experience. Although this therapy is highly effective in reducing conditioned fear and avoidance responses, some patients relapse and suffer spontaneous recovery (SR) of these symptoms again [3].Thus, the prevention of fear and avoidance recovery has become one of the main priorities in the field of anxiety disorders and PTSD in particular.

The behavioural and neuronal features of expression and extinction of conditioned fear responses have been widely studied in animals using fear conditioning (FC) [1][4][5], a PTSD-like validated model [6]. The use of FC in rodents has revealed the relevance of time variable in extinction protocols regarding the recurrence of the fear response. Specifically, spaced intervals between extinction sessions [4] or between trials [7] [8], in both rodents and humans, have been proved to be more effective than short time intervals in reducing the long-term SR. Moreover, FC has revealed a hypothetical neural circuit for PTSD, including defective interactive connections between the amygdala and the medial prefrontal cortex (mPFC) regions [1] [2][9]. Accordingly, it has been demonstrated that microstimulation of the infralimbic cortex (IL) enhances fear extinction in rodents, whereas the opposite effect has been described after prelimbic cortex (PL) stimulation [5].

While FC reveals passive defensive responses and usually produces freezing and other conditioned reactions that obstruct avoidance, active avoidance paradigms enables the measurement of active defensive responses through avoidance responses to the CS [10].

In active avoidance conditioning, animals must learn an instrumental response to avoid a foot shock when the CS announces its imminent arrival. Interestingly, behavioural avoidance is considered a core symptom of PTSD [11] and constitutes a significant predictor of disease severity in PTSD patients [12]. Thus, avoidance as a fear response is excessive in PTSD patients and highlights the need for understanding the behavioural and neural substrates of conditioned avoidance responses to improve EBT benefits.

Pioneer studies in rats about the effects of spacing trials on resistance to the extinction in active avoidance paradigms were already performed in the 1950s [13] and [14], but there are seldom studies using the more complex two-way active avoidance (TWAA) procedure and evaluating the effect of the temporal distribution of the sessions on the resistance to the extinction and SR.

In this study, we aimed to analyse comparative effectiveness of a different temporary distribution of two extinction sessions (24 hours vs 7 days between extinction sessions) on the long-term retention of extinction (28 days after the last extinction session) of the conditioned response evaluated in a SR test, by using a TWAA paradigm. Furthermore, we attempted to elucidate the neuronal circuits underlying the effectiveness in the long term of the two distribution conditions of TWAA extinction sessions. This was done by mapping the changes in c-Fos protein expression, a well-known marker of neuron activation [15], in specific areas of the amygdala and the mPFC, two brain regions related to fear and avoidance extinction systems.

2. Methods and Materials

2.1. Subjects

A total of 32 male Wistar rats were used (90-100 days old and with a mean weight of 438.27± 41.63 g), obtained from the breeding stock of the laboratory of Psychobiology (registry number B99-00029, Autonomous University of Barcelona, UAB). All the rats were individually housed and kept on a 12hr light/dark cycle, with all TWAA sessions being conducted during the light period. All procedures were carried out in compliance with the European Community Council Directive for care and use of laboratory animals and approved by the Ethics Committee at the UAB (order number 2022).

The study was carried out in different batches of 6 rats maximum per week (up to 2 per group) to ensure that the different groups were evaluated in parallel, and only differing in terms of the independent variable "time between extinction sessions". After handling (1 daily 10-min session for 5 consecutive days), up to 2 rats per week were randomly chosen to form the Naïve group (n=8; see Figure 1), which served as a negative control of TWAA training effects on c-Fos expression, while the remaining animals underwent the procedures described below.

2.2. Two-way active avoidance (TWAA) conditioning

TWAA was conducted in two 50 x 24 x 23 cm identical automated two-way shuttle-boxes (AccuScan Instruments Inc. Columbus, Ohio, USA), enclosed in two separate sound-attenuating boxes ventilated by an extractor fan and controlled by Fusion software. Rats underwent two 5-min habituation sessions (Hab1 and Hab2) of free ambulation in the shuttle-box in order to become familiarized with the learning environment. Two days later, the rats received three 50-trial TWAA acquisition sessions, once daily (Ac1 to Ac3), consisting of the introduction of an 80 dB and 1 kHz

lasting 3s (CS) followed by a 0.6 mA and maximum 10s duration electrical foot shock (US). Both stimuli terminated simultaneously when the rat crossed, or after 13s in the case that the rat did not respond, following a delay procedure. The inter-trial interval (ITI) varied randomly from 50 to 70s. The ITI was set to be shorter than those used in other studies [10] in order to make the task more demanding, based on results of previous experiments in our laboratory. The total number of avoidance responses (considered as conditioned responses, CRs) and the mean latency (i.e. the time taken to move from one compartment to another from the CS presentation) were recorded for each session.

One day after the Ac3, rats were tested in two 50-trial TWAA extinction sessions (Ext1 and Ext2) at two different time intervals between sessions (24 h or 7 day, see Figure 1). Thus, after Ex1 rats were randomly assigned to the 24-hour (*contiguous*) or to the 7-day (*spaced*) groups. Extinction sessions were similar to the acquisition but without US presentation. Following a rest period of 28 days from the Ex2, the effects of the two extinction paradigms were tested in a single 50-trial TWAA SR test (SR-t), which replicated the characteristics of the extinction sessions. The number of CRs, here considered as the responses made during the total duration of the CS, as well as mean latency per session were also recorded.

The locomotor activity of the animals was also recorded. In the habituation sessions as the number of crossings performed during the 5-minute session duration (Hab1 and Hab2), and in the acquisition, extinction or SR sessions as the number of crossings performed in the ITI. Since individual differences in the locomotor response to a novel environment can affect fear conditioning and extinction in rats [16], the number of crossings in the habituation sessions was used as covariate in the variance analyses.

2.3. c-Fos immunochemistry

For c-Fos immunolocalization, rats were euthanized 90 min after the SR-t (24-hour and 7-day groups) or after the last handling session (Naïve group). They were anesthetized with a lethal dose of pentobarbital (200 mg/kg body weight, i.p.) and perfused transcardially with a solution of 0.1M phosphate buffer saline (PBS), pH 7.4, followed by a solution of 4% paraformaldehyde in PBS. Brains were post-fixed in 4% paraformaldehyde in PBS solution for 2 hours and then placed in 15% sucrose in PBS for 3 days and 30% sucrose in PBS at 4 °C until they sank. Serial coronal sections of cryopreserved brains (30-µm thick) were obtained in a cryostat (Leica Biosystems CM3050 S, Barcelona, Spain) at coronal coordinates between 3.72 and 2.52 (for the PL and the IL cortices) and between -2.28 and -2.76 (for the amygdala nuclei) anteroposterior to Bregma [17], and stored at -80 °C until free-floating immunohistochemistry staining.

Frozen coronal sections were washed in TBS (0,1 M Tris-HCl pH 7.6, 0,15 M NaCl.), treated in 0.3% H_2O_2 in TBS solution for 30 minutes and incubated in 0.1% bovine serum albumin (Roche Diagnostics, SL) in TBS-T (0,03% Triton X-100 in TBS) as blocking solution for 30 minutes in a constant shaker. Sections were incubated in rabbit Anti-c-Fos antibody (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA; diluted 1:2000) overnight at 4°C in a constant shaker. After washing, sections were incubated in Biotinylated anti-Rabbit IgG antibody (Jackson Immunoresearch, Inc, USA; diluted 1:5000) one hour at room temperature in a constant shaker. Finally, samples were incubated in streptoavidin-peroxidase (Pelkin Elmer®Life Sciences, Inc., diluted 1:3600) for 2 hours at room temperature in a constant shaker, washed and incubated in DAB solution (Vector®) for 10 minutes. Lastly, sections were dehydrated, mounted and

covers slipped. No staining was observed in control slides without the primary or secondary antibodies.

2.4. Image acquisition and analysis

Microphotographs were captured with a BX-41 Olympus microscope attached to an Olympus DP-70 digital camera (Japan) from the following brain regions: the PL and the IL cortices, the lateral (LA), the basolateral (BLA) and the central (lateral and medial division, CeL and CeM, respectively) nuclei of the amygdala. The image analysis software Image-J 1.43 (http://rsb.info.nih.gov/ij/) was employed to bilaterally count the number of c-Fos immunostained nuclei using regions of interest (ROIs) (see Figures 5C and 5K), in two or three histological sections of each brain area and averaged for each animal. To remove background noise, each image was digitally smoothed and subtracted from the original. Appropriate grey threshold and particle size were set for each area and maintained for all subjects.

2.5. Statistics

Mixed 2 × 3 or 2 × 5 ANCOVA were used to analyse the effects of the temporal distribution of the extinction sessions (*GROUP* factor, 24-hour and 7-day) on TWAA acquisition (*SESSION* factor), extinction and SR (subdivided in five 10-trial blocks, *BLOCK* factor), with crossings in the habituation sessions as a covariate. A survival Kaplan-Meyer analysis was also done for the SR-t session, where the *time* variable was defined as the trial when each rat reached a previously established criterion of *extinction* (or lack of recovery of the CR) consisting of 'performing three consecutive no-responses to the CS'. The Student t-test was used to compare the locomotor activity (crossings) of the two groups in the different sessions.

For the molecular study, analyses of the number of c-Fos positive cells/mm^2 in each brain region were conducted with one-way ANOVA. Tukey post-hoc contrast was used when required. The α-level for all tests was set at .05. Statistical analysis was carried out with SPSS 15 (SPSS Inc., 2006; Chicago, IL, USA).

For the correlational study, Pearson was used with variables that followed a normal distribution (i.e. between CR and locomotor activity in the shuttle-box) and Spearman with the ones that did not fit a normal distribution (i.e. between c-Fos-labelled nuclei and latencies of response in SR-t).

3. Results

The final total number of rats was twenty-nine (24-hour, n=11; 7-day, n=10; and Naïve, n=8). As spontaneous recovery refers to the appearance of a CR after it was previously extinguished, three subjects (one in the 24-hour group and two in the 7-day group) were excluded in the analysis since they showed no signs of extinction at all (Ext1>Ac3 and Ext2 >Ac1 in number of CRs). No statistical differences between groups in weight were observed during all procedures.

Regarding locomotor behaviour, no statistical differences were observed between groups in mean crossing in habituation (Figure 2A) and mean ITI crossing in TWAA sessions (Figure 2B). It is interesting to note that the number of crossings in the habituation sessions did not correlate with the CRs performed in any of the TWAA sessions. Instead, the amount of ITI crossings performed in a given TWAA session did positively correlate with the number of CRs made in that session (Table S1 of the Supplementary Material). However, locomotor activity in habituation sessions only

shows some significant correlation with the ITI crossings of the Ex1 and SR-t sessions; this tendency to return to their basal locomotor activity in the absence of the UC can be interpreted as a normalization of exploratory behaviour by extinction.

3.1. The spaced extinction paradigm reduces spontaneous recovery in TWAA

Based on the literature that underlines the relevance of the temporal distribution of extinction sessions in reducing the reappearance of fear responses after an auditory FC paradigm [4], we hypothesized that a longer interval between extinction sessions may also reduce long-term spontaneous recovery for a TWAA task.

3.1.1 Acquisition. As shown in Figure 3A, rats' avoidance responses had a significant increase throughout the acquisition sessions (Ac1 to Ac3), following a linear function [*SESSION polynomial, first degree*: $F_{(2,36)}$= 6.132, *P*= .002]. Since neither the *GROUP* [$F_{(1,18)}$= 0.026, *P*= .873] nor its interaction × *SESSION* [$F_{(2,36)}$= 0.389, *P*= .681] were significant, it can be stated that both groups showed a similar progression regarding acquisition of TWAA and reached a similar degree of conditioning, an expected result since the experimental conditions had been identical for both groups up to that point.

3.1.2. Extinction. To study extinction with greater resolution, Ex1 and Ex2 sessions were divided in five 10-trial blocks, as depicted in Figure 3B. The number of CRs performed by the experimental groups in the Ex1 session fit similar downward linear functions [*BLOCK polynomial, first degree*: $F_{(1,18)}$= 4.820, *P*= .041; *GROUP*: $F_{(1,18)}$= 0.467, *P*= .503; *GROUP* × *BLOCK:* $F_{(1,18)}$= 0.497, *P*= .803]. In Ex2 the groups did not differ regarding the CRs performed during the session [*GROUP*: $F_{(1,18)}$= 0.049, *P*= .828; *GROUP* × *BLOCK*: $F_{(1,18)}$= 0.122, *P*= .974]. However, the number of CRs in the third

block was significantly reduced compared to the first block (*contrast*: *P*= .001), whereas it did not differ from the last block of trials (*P*= .203), showing an asymptotic progression that is not seen for Ex1. In summary, it can be assumed that both groups developed a similar degree of extinction learning, regardless of the temporal distribution of extinction sessions. Moreover, both groups showed a high degree of extinction since the number of CRs in the last Ex2 block was not statistically different from zero.

3.1.3. Spontaneous Recovery. Finally, after a rest period of 28 days from the Ex2, both groups underwent the SR-t in order to appraise the effectiveness of each extinction paradigm. SR-t was also divided in five 10-trial blocks. The detailed intra-session block study showed that the interaction factor *GROUP* × *BLOCK* was statistically significant [$F_{(1,18)}$= 5.550, *P*= .03]. Simple effects showed that the 7-day group performed a lower number of CRs than the 24-hour group in the first two blocks of the SR-t (block 1: *P*= .038; block 2: *P*= .008) (Figure 3B), revealing a decreasing effect of the more spaced extinction procedure on spontaneous recovery, which is only evident in the early trials. Survival analysis allows us to analyse the percentage of animals in each group that reached the previously established criterion of *extinction* and how many trials they needed to do so (Figure 4). According to a Kaplan-Meier analysis, significant differences between groups were shown (Breslow contrast: χ^2 = 4.941, df= 1, *P* = .026) with subjects from 7-day group requiring fewer trials to reach the *extinction* criterion than the ones in the 24-hour group. Thus, only 10% of the rats in the 24-hour group showed no relapse, whereas 40% of those in the 7-day group already had within the first 5 trials. By the end of the SR-t, 100% of the rats of both groups reached the criterion.

Additionally, latency results for Ex2-SR-t sessions corroborated the effects observed in CRs (Figure S1).

3.2. The spaced extinction paradigm increases the number of c-Fos-labelled nuclei in the IL cortex after the SR-t

We sought to find out if the observed differences in spontaneous recovery after two different temporal distributed extinction sessions could be associated with changes in the activation of particular brain regions related specifically to the extinction of conditioned fear, such as the IL [5]. Figure 5 shows representative photomicrographs of c-Fos immunohistochemistry and bar charts of the number of c-Fos positive nuclei/mm^2, in 24-hour and 7-day groups compared to the Naïve group, in mPFC (Figures 5A and 5B) and in amygdala regions (Figures 5G to 5J).

3.2.1. mPFC. One-way ANOVA revealed that the extinction paradigm had a significant effect on the number of c-Fos-labelled nuclei within the IL [$F_{(2,26)}$ = 4.277, P= .026]. Post-hoc tests confirmed that the 7-day group showed a higher number of IL c-Fos-labelled nuclei compared with both the 24-hour (P= .025) and the Naïve groups (P= .049; Figures 5A and 5D to 5F). However, no effect was observed in the PL [$F_{(2,26)}$ = 0.640, P= .536; Figure 5B). Significant positive correlations were consistently observed between IL c-Fos expression and SR performance (mean latency in the first block of SR-t) (Spearman's rho = .482; P = .036) (Table 1).

3.2.2. Amygdala. The extinction paradigm had a significant effect on the number of c-Fos-labelled nuclei within the LA [$F_{(2,16)}$ = 3.757, P= .049] (Figure 5I) and the CeM [$F_{(2,16)}$ = 4.63, P = .029] (Figure 5H). However, the extinction paradigm had no significant effect either within the BLA (P= .123; Figure 5J) or within the CeL (P= .536; Figure 5G). Post-hoc tests confirmed that the 24-hour group, unlike the spaced one, showed a statistically greater number of c-Fos-labelled nuclei compared with the Naïve group both in the LA (P= .048), the CeM (P= .018) (see Figures 5L and 5N), and

a trend towards significance in BLA (P= .077). Finally, a tendency (P= .08) towards significance between the 7-day and the 24-hour groups was also observed in LA, the 24-hour group being the one that shows a greater expression of c-Fos positive cells.

Significant negative correlations were detected between the mean latency in some blocks of the SR-t and the c-Fos expression in BLA and CeL nuclei of the amygdala (Table 1). This would also favour a positive relationship between activation of the amygdala and a greater conditioned fear.

4. Discussion

This study provides data about the effects of the temporal distribution of extinction sessions on the 28-day spontaneous recovery test in a TWAA task. Our results showed that 1) two spaced sessions of extinction are more effective than two contiguous extinction sessions to prevent long term spontaneous recovery of the conditioned response in a TWAA task; and that 2) the spaced extinction paradigm induced a significant increase of c-Fos expression in the IL cortex after the spontaneous recovery test.

4.1. Role of timing between extinction sessions on long-term spontaneous recovery for TWAA conditioning.

To the best of our knowledge, this is the first study analysing the effect of the temporal distribution of extinction sessions on long-term spontaneous recovery in a TWAA conditioning. Our results support a higher effectiveness of a 7-day than a 24-hour interval extinction paradigm on the maintenance of long-term extinction and suggest that the rest period between extinction sessions could be crucial to avoid the long-term

relapse. However, the reduction of spontaneous recovery after the spaced extinction paradigm was only significant in the early trials. At the end of the SR-t session, the CR was extinguished equally in the two groups. Therefore, the group subjected to the spaced paradigm maintained a better extinction level and needed less extinction trials to reduce CRs than the 24-hours group. Nevertheless, present results did not allow us to discard that the conditioned response weakening showed by the 7-day group in the SR-t could be alternatively attributed to the longer period between the Ex1 and SR-t sessions. This was because our design prioritized equalizing the extinction recovery assessment interval (28 days) for the two extinction procedures instead of the time elapsed between the Ex1 and SR-t sessions (29 days for the 24-hour group and 36 days for the 7-day group). However, an additional experiment including rats subjected to the same extinction procedures, but varying the number of days between Ex2 and SR-t to 36 for the 24-hour group (24-hour_36, n=4) and 29 days for the 7-day group (7-day_29, n=3), has shown that the 7-day_29 group continued to perform a significant lower number of CRs than the 24-hour_36 group in the first block of trials of the SR-t (P= .021; see Figure S2). No differences were found between 7-day and 7-day_29 groups or between 24-hour and 24-hour_29 in any session. These results support our hypothesis that reduction of SR observed in the 7-day group is caused by the spaced paradigm, and that variations of one week in the length of the time period between the Ex1 and the SR-t do not significantly affect the long-term recovery of extinction in present conditions. Thus, present results are consistent with previous data on contextual fear extinction in mice, in which extinction sessions at spaced intervals are more effective than consecutive extinction sessions also for fear memory [4].

Since both the 24-hour and the 7-day groups showed no differences in TWAA conditioning and reached a similar high level at the end of the acquisition phase, we can

rule out that the observed differences in spontaneous recovery could be related to the level of acquisition of the task achieved. The differential level of relapse in SR-t does not appear to be related to locomotor behaviour either, since no differences in ITI or in habituation were observed between both groups. Moreover, both spaced and contiguous procedures would have caused similar levels of anxiety or stress in the subjects, since both groups showed an expected warm up effect throughout the different phases of the experiment. This effect only disappears in rats highly vulnerable to anxiety [19].

In contrast, an unexpected result was the lack of differences between the 24-hour and the 7-day groups in the extinction level at the end of Ex2. This finding could be attributable to a floor effect in both groups in the short term, which disappeared in the extinction recall test. It seems, therefore, that the effect of the temporal procedure was only manifested in the long term (i.e. SR-t). This idea agrees with the fact that extinction memories are slow to form in comparison to aversive memories [20]. It also reinforces the notion that spacing extinction sessions may be a more suitable method for enabling the underlying molecular processes related to the maintenance of extinction, at least for this kind of task. In fact, extinction in TWAA, being considered as a new learning [21] and a striatum-related task [22], might benefit from the spacing of training trials and/or sessions [23]. Spaced training schedule has also been demonstrated to be more effective than a massed one in other implicit memory tasks, such as taste aversion conditioning [24] and an associative body-turn response task in a plus-maze [23], all of which are dorsal striatum dependent tasks.

The total number of extinction sessions should also be considered when assessing the effect of temporal distribution of extinction sessions on the extinction paradigm. It has been reported that five extinction sessions at short intervals are also effective in preventing spontaneous recovery of fear memory [4][25]. Therefore, a spaced extinction

paradigm might be beneficial when there is fewer extinction sessions, as in our case, but the same effect could also be achieved by increasing the number of sessions in a contiguous extinction paradigm.

4.2. c-Fos expression in the mPFC and the amygdala during extinction recall

In this study, we have also demonstrated that the 7-day group showed a higher c-Fos expression in the IL cortex than the 24-hour group, meaning that rats subjected to an extinction procedure that causes a higher long-term extinction recall also showed c-Fos overexpression in the IL. Together with the positive correlation in IL between c-Fos and latency response in the first block of trials in the SR-t, present results support the idea that IL activation seems to be related to lower spontaneous recovery of avoidance conditioning levels.

It has been reported that microstimulation of the IL enhances extinction in FC [5][26], whereas the inactivation of the IL cortex impairs extinction both in FC [9] and in an avoidance task [27]. It has also been pointed out that FC decreases the excitability of the IL neurons and that fear extinction reverses the depressed excitability [28]. Considering SR-t not only as a recall test but as an additional extinction session in which animals continue to reduce the number of CRs, differences in c-Fos levels in IL would also be in agreement with results by Cruz et al. [29], which emphasizes the importance of the IL activation during the consolidation of extinction. Additionally, present results regarding the 24-hour group also are consistent with the reduction in IL neurons excitability recorded in animals that showed spontaneous recovery of fear 17 days after extinction [29]. Thus, according to the proposed role of IL in fear extinction, our results suggest that spacing extinction sessions could be a useful way to promote IL activation and reduce relapse after extinction. This was reinforced by the fact that there were no c-Fos

expression differences in the IL cortex between the 24-hour group, which showed spontaneous recovery, and the Naïve animals, which had no experience in TWAA.

Our results using TWAA, similar to the classical FC, expand the role of IL cortex in inhibition of maladaptive affective responses to more complex, decision-based, naturalistic paradigms [30]. Considering its inhibitory influence on proactive avoidance behaviour, such as that in TWAA conditioning, ventral medial PFC could also be involved in coping and resilience, as proposed elsewhere [31][32].

Similar results to those obtained for the IL cortex were expected in the CeL amygdalar nuclei. Microstimulation of the IL excites GABAergic neurons in the CeL that, in turn, would inhibit the CeM and the expression of fear [5]. However, the absence of differences between groups in the CeL could indicate that the actions of the IL cortex on the CeM in TWAA extinction must involve other regions or other mechanisms. For instance, the GABAergic intercalled cells of the amygdala, situated between the BLA and the central nucleus of the amygdala, are important for inhibitory control over the amygdala [5].

PL cortex has also been related to fear expression [33]. Therefore, we were expecting to observe higher c-Fos activation in the PL and the BLA regions after the 24-hour extinction paradigm. However, no differences among groups were detected. Even though it has been claimed that the inhibition of the PL cortex enhances fear extinction [9] and PL and BLA activation increases fear expression and impairs extinction in FC [5], these regions have not been implicated in the extinction of an avoidance task [27]. Moreover, a recent study has reported that BDNF expression in the IL, but not in the PL, is necessary and sufficient for fear extinction [34]. Thus, opposed roles of the IL

and the PL cortices in avoidance responses may not be crucial to reducing or enhancing spontaneous recovery.

Interestingly, consistent with the higher level of spontaneous recovery observed in the 24-hour group, we found a higher number of c-Fos-labelled nuclei in LA and the CeM regions compared with the Naïve group. The LA is where the US and the CS are associated [35], whereas the CeM is the area from which the outputs of fear are sent [5]. Moreover, it seems that the direct pathway between the LA and the CeM is sufficient to mediate aversive conditioning [35], without the need for a relay in the BLA. Further studies are required to study possible connections, apart from the regions mentioned above, between the IL and the LA-CeM in extinction of TWAA.

4.3. Conclusion

Taken together, our results highlight the relevance of the temporal distribution of extinction sessions in long-term spontaneous recovery of active avoidance responses. The behavioural advantage provided by a more spaced extinction paradigm in active avoidance seems to be mediated by appropriate activation of the IL cortex, a specific neural substrate involved in extinction. Thus, a significant differential long term response of IL depending on the temporal extinction paradigm applied was demonstrated after the recovery test. Extinction studies with laboratory animals would be useful to explore potential parameters to optimize extinction-based therapies for anxiety-related disorders. Future experiments analysing c-Fos activation at different time points could aid to better understand the contribution of the IL and other extinction related areas throughout the acquisition, consolidation and recall of the extinction.

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Figure captions

Figure 1: Scheme, experimental design of the study, in which the main procedural characteristics of each experimental group are specified (Ac: *acquisition sessions*; Ex: *extinction sessions*; SR-t: *spontaneous recovery test*). Sacrifice for c-Fos immunodetection was performed at 90 min.

Figure 2: Locomotor behaviour in the Shuttle-box. Crossings in the shuttle-box showed by the 24-hour and the 7-day groups in: **A**. the two 5-min habituation sessions (Hab1 and Hab2), and **B.** the inter-trial intervals of the TWAA sessions (Ac1 to Ac3: *acquisition sessions*; Ex1 and Ex2: *extinction sessions*; SR-t: *spontaneous recovery test*). No differences were observed between groups. The locomotor activity in the habituation sessions was used as a covariate in the study of the effects of temporal distribution of extinction sessions on TWAA performance.

Figure 3: Effects of the temporal distribution of extinction sessions on extinction and spontaneous recovery levels in TWAA. Conditioned responses (CRs) along the different phases of TWAA showed by the 24-hour the 7-day groups are represented for: **A**) the three acquisition sessions and **B**) the blocks of 10 trials in which the two Extinction sessions and the Spontaneous Recovery test were divided for detailed study. Data presented as mean ± SEM. **P*< .05.

Figure 4: Survival curve of the Spontaneous Recovery test. It is showed the accumulated proportion of subjects that in each trial are reaching the criterion of *extinction*. Spaced paradigm (7day) resulted in a higher and faster percentage of subjects showing no relapse (Breslow, *P* = .026).

Figure 5: Effects of the temporal distribution of extinction sessions on c-Fos expression in the mPFC (upper part) and the amygdala (bottom). In the bar charts is represented the mean number of c-Fos immunopositive cells for the different regions studied: A) infralimbic cortex (IL), B) prelimbic cortex (PL), G) the lateral division of the central nucleus (CeL), H) the medial division of the central nucleus of the amygdala (CeM), I) the lateral amygdala (LA), and J) the basolateral amygdala (BLA). Data presented as mean of c-Fos-labelled nuclei/mm^2 ± SEM. *P< .05.

Regions of interest (ROIs) for the quantification of c-Fos immunopositive nuclei in the mPFC (C) and in the amygdala (K) are shown superimposed on coronal sections adapted from Paxinos and Watson's atlas (2007). To the right of C and K, respectively, are shown representative photomicrographs of c-Fos immunopositive-nuclei from one subject in each experimental group, 24-hour, 7-day and Naïve, from mPFC (D, E and F) and from the amygdala nuclei (I, M and N). Analysis was performed bilaterally. (azp, *azygous pericallosal artery*; BLA, *basolateral amygdala*; CeL, *lateral division of the central nucleus of the amygdala*; CeM, *medial division of the central nucleus of the amygdala*; ec, *external capsule*; fmi, *forceps minor of the corpus callosum*; IL, *infralimbic cortex*; LA, *lateral amygdala*; PL, *prelimbic cortex*).

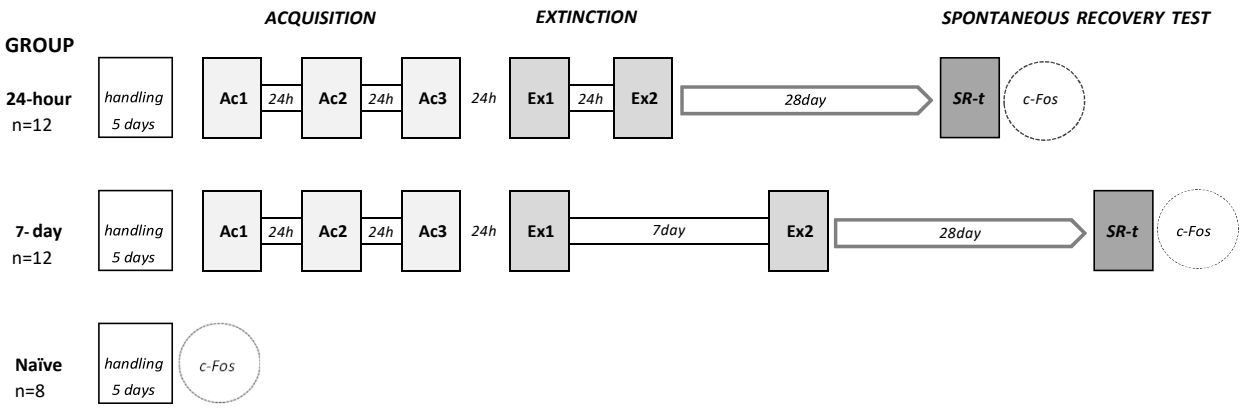
Table 1: Correlations between the performance in the Spontaneous Recovery test (mean latency of response) and c-Fos expression. Spearman's rho value and bilateral significance (below in italics) are expressed in each box. Significant correlations are highlighted in bold. * P < .05

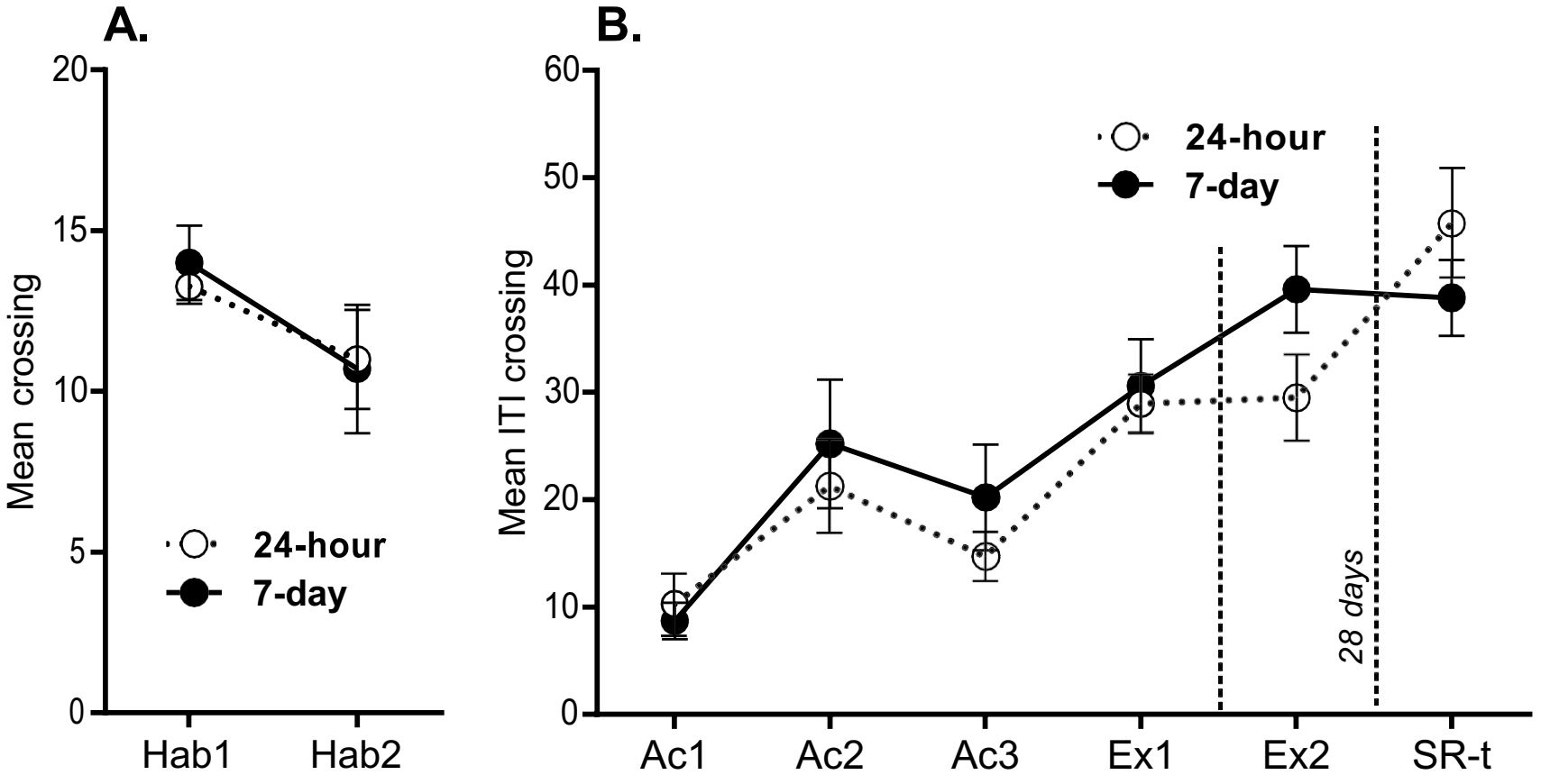
<u>Supplementary Material</u>: Figure and Table captions

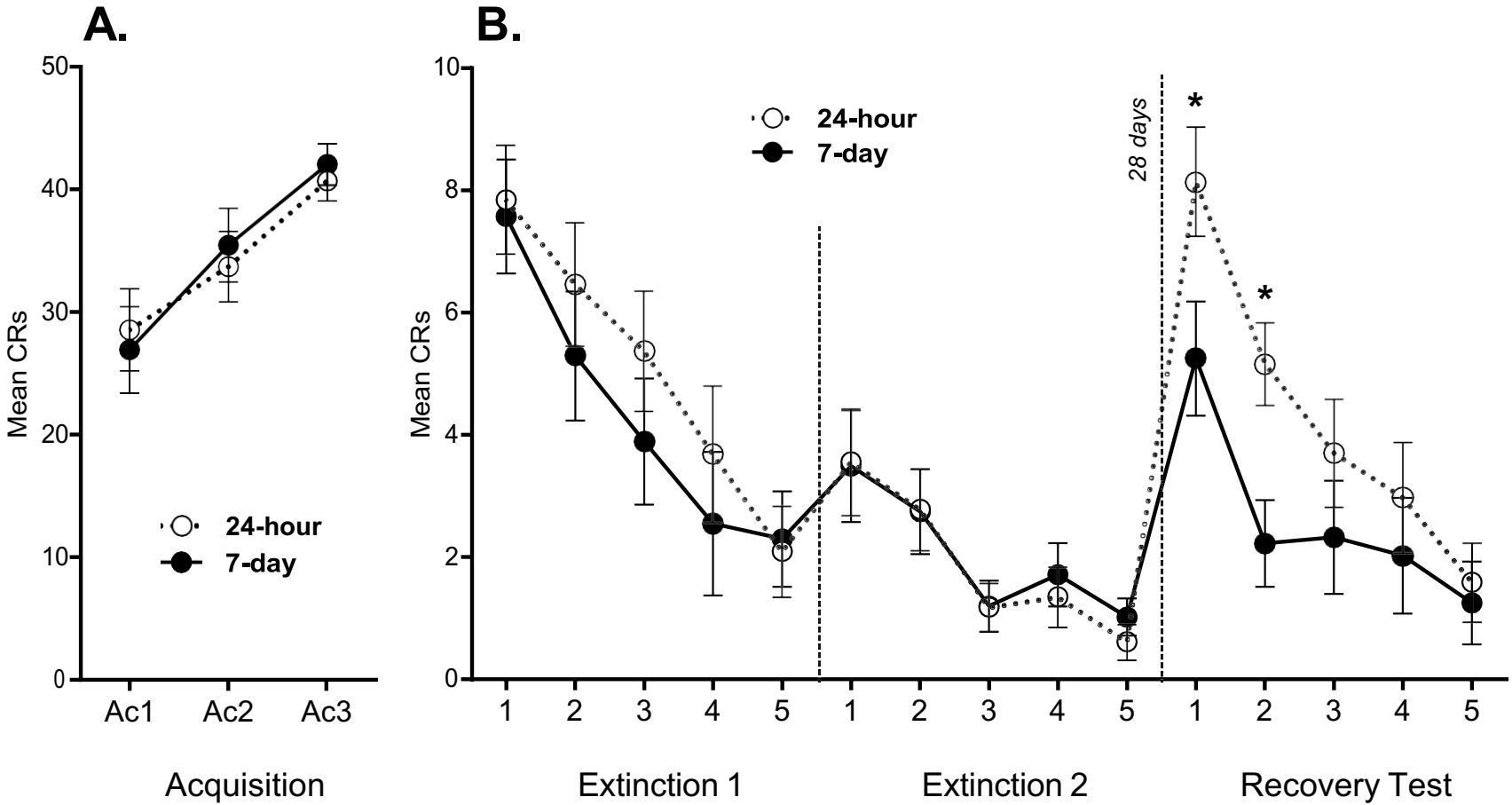
Figure S1: **Effects of the temporal distribution of extinction sessions on extinction and spontaneous recovery levels in TWAA. Latencies of response**. Black arrow line points out the different interval between extinction sessions. Considering Ex2 and SR-t sessions, groups evolved differently between Ex2 and SR-t [*GROUP* × *SESSION: P*= .001]. Simple effects within each group showed a significant decrease of latencies in the 24-hour group (*P*< .001) but not in the 7-day group (*P*= .563). Considering SR-t as a whole, the 7-day group showed a significantly higher mean latency than 24-hour group (*P*= .034), which suggests a clear lowering effect on the spontaneous recovery of the spaced procedure. The analysis considering the mean latency of response corroborated the effects observed in CRs (see Figure 3). Data presented as mean ± SEM. * *P*<0.05.

Figure S2: Effects of the time elapsed between the acquisition of extinction and the long-term memory test (29 vs. 36 days) on the spontaneous recovery of the conditioned response. Conditioned responses (CRs) along the different phases of TWAA showed by the groups from the additional experiment (24-hour_36 and 7-day_29) are shown superimposed on those of the original study groups (the 24-hour and 7-day) for: **A**) the three acquisition sessions and **B**) the blocks of 10 trials in which the two Extinction sessions and the Spontaneous Recovery test were divided. An 2 × 2 ANOVA for the SR-t session pointed out significant effects of Extinction procedure [$F_{(4,96)}$= 3.112, *P*= .019] but not of the time elapsed between Ex1 and SR-t [$F_{(4,96)}$= 0.097, *P*= .983]. Specifically, the spaced paradigm caused lower spontaneous recovery in blocks 1 and 2 (*P*= .05, *P*= .022, respectively), regardless of the time elapsed between Ex1 and SR-t sessions [interaction factor: $F_{(4,96)}$= 0.410, *P*= .801]. Data presented as mean ± SEM. **P*< .05.

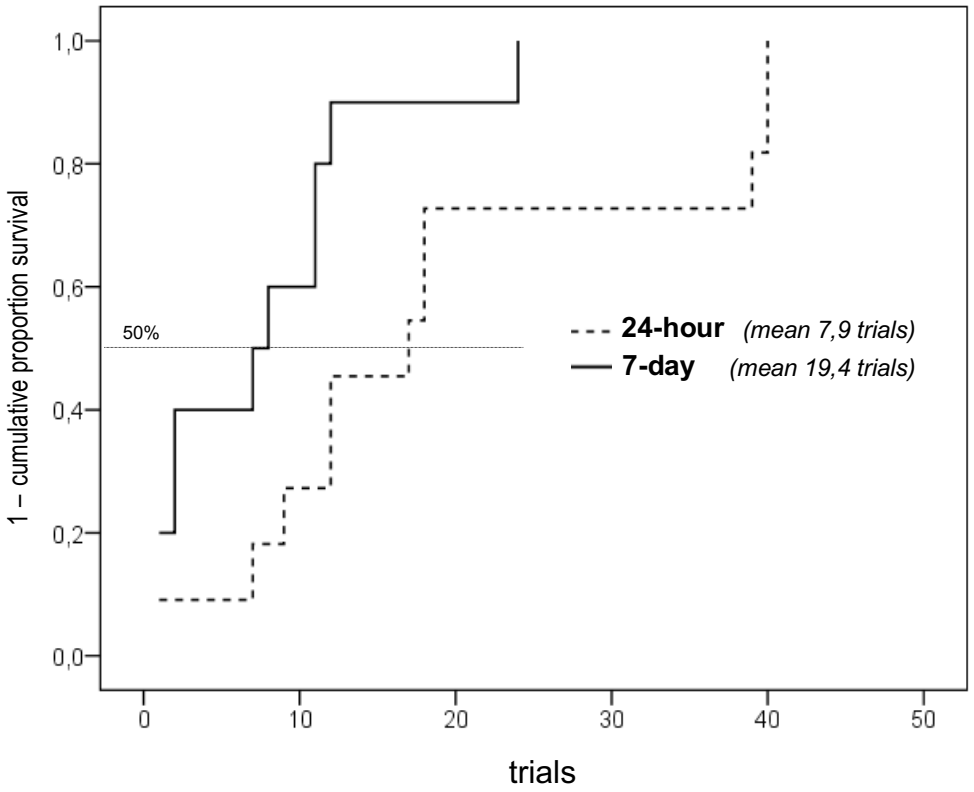
Table S1: Correlations among Conditioned Responses (CRs) and locomotor behaviour in the Shuttle-box. To highlight: 1) the lack of correlation between the crossings performed in the habituation sessions and the execution in the TWAA; 2) the significant positive correlation between ITI crossings and CRs within the same session, except for Ex1 (yellow); 3) locomotor activity in SR-t also correlates with CRs in extinction sessions (green); 4) number of CRs in a given TWAA session positively correlates with execution in the following TWAA session (blue), and 5) the learning level shown in the first acquisition session (Ac1) positively correlates with the level of CRs shown in the SR-t (violet).


ACQUISITION
EXTINCTION
SPONTANEOUS RECOVERY TEST
GROUP
24-hour
n=12
handling
5 days
Ac1
24h
Ac2
24h
Ac3
24h
Ex1
24h
Ex2
28day
SR-t
c-Fos
7- day
n=12
handling
5 days
Ac1
24h
Ac2
24h
Ac3
24h
Ex1
7day
Ex2
28day
SR-t
c-Fos
Naïve
n=8
handling
5 days
c-Fos

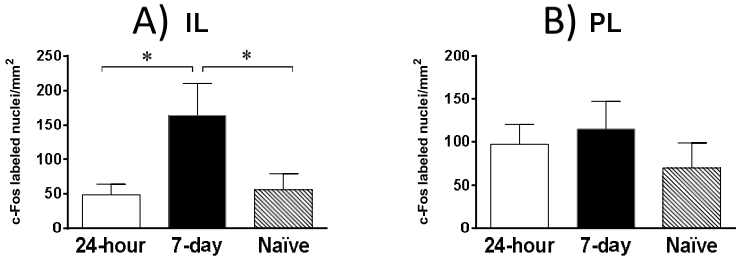

A.
B.
Mean crossing
Mean ITI crossing
24-hour
7-day
28 days
Hab1
Hab2
Ac1
Ac2
Ac3
Ex1
Ex2
SR-t
0
5
10
15
20
30
40
50
60

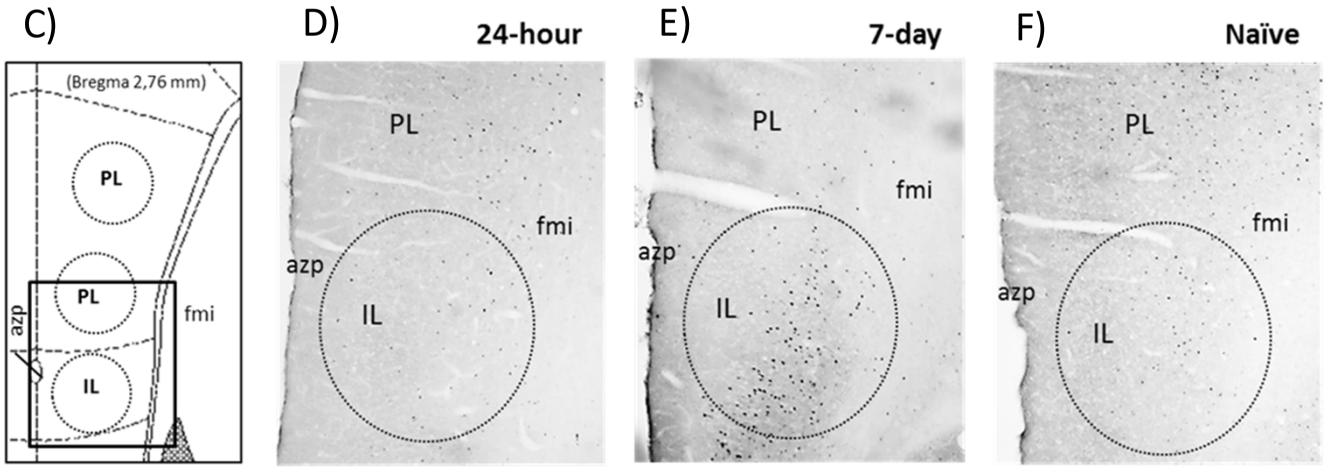

A.
B.
Mean CRs
24-hour
7-day
Ac1
Ac2
Ac3
Acquisition
28 days
*
*
Extinction 1
Extinction 2
Recovery Test

Spontaneous Recovery test

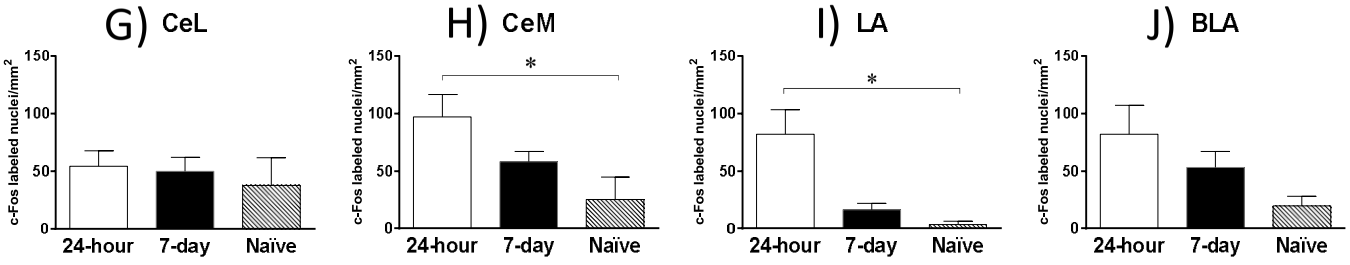


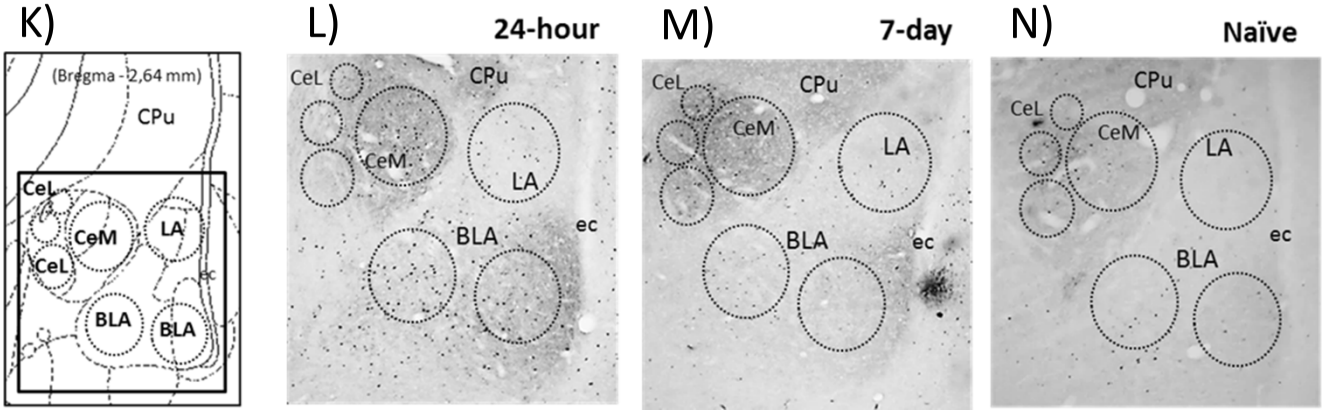
Medial PreFrontal Cortex

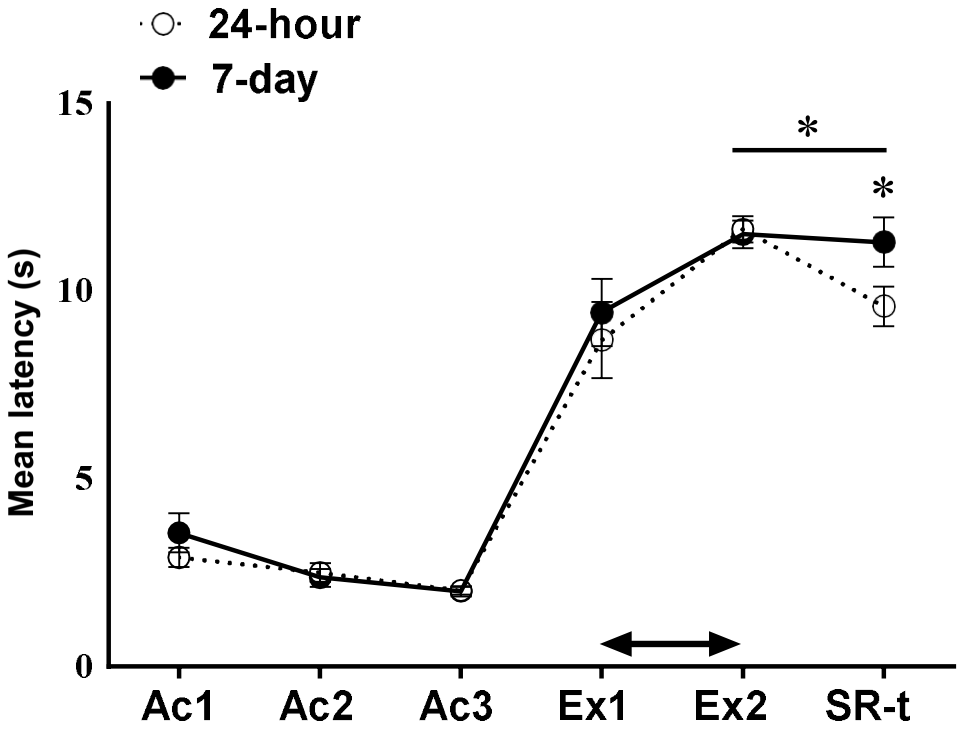


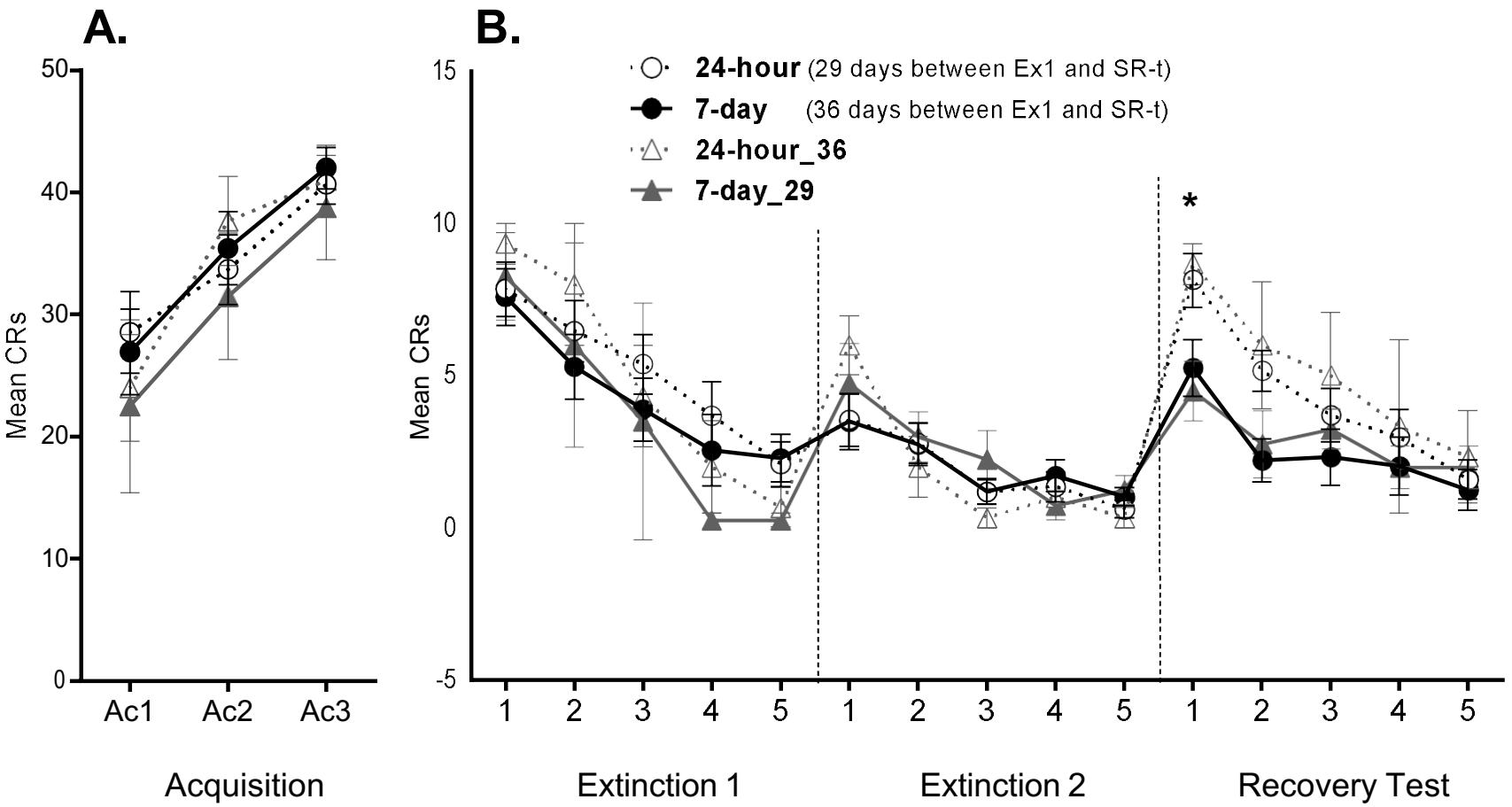


Amygdala






24-hour
7-day
Mean latency (s)
15
10
5
0
*
*
Ac1
Ac2
Ac3
Ex1
Ex2
SR-t


A.
B.
24-hour (29 days between Ex1 and SR-t)
7-day (36 days between Ex1 and SR-t)
24-hour_36
7-day_29
Mean CRs
50
40
30
20
10
0
Ac1
Ac2
Ac3
Acquisition
15
10
5
0
-5
1
2
3
4
5
*
Extinction 1
Extinction 2
Recovery Test

		c-Fos expression					
		mPFC		Amygdala			
		IL	PL	LA	BLA	CeM	CeL
Mean latency of response	block 1	**0,482** ***0,036 ****	0,538 *0,058*	-0,006 *0,986*	**-0,613** ***0,026 ****	-0,014 *0,964*	0,061 *0,843*
	block 2	0,202 *0,408*	0,117 *0,634*	-0,083 *0,787*	**-0,594** ***0,032 ****	-0,446 *0,127*	-0,084 *0,785*
	block 3	0,351 *0,141*	0,247 *0,309*	0,155 *0,613*	-0,547 *0,053*	-0,335 *0,263*	-0,398 *0,178*
	block 4	0,097 *0,694*	-0,102 *0,678*	-0,119 *0,698*	**-0,58** ***0,038 ****	-0,496 *0,085*	-0,389 *0,189*
	block 5	0,285 *0,237*	0,168 *0,492*	-0,145 *0,637*	-0,42 *0,153*	-0,457 *0,117*	**-0,619** ***0,024 ****
	SR-t total	0,402 *0,088*	-0,19 *0,937*	-0,465 *0,109*	-0,487 *0,091*	-0,197 *0,52*	-0,128 *0,678*

		Ac1	Ac2	Ac3	Ex1	Ex2	SR-t	cross_ Hab1	cross_ Hab2	cross_ ITI_Ac1	cross_ ITI_Ac2	cross_IT_ Ac3	cross_ ITI_Ex1	cross_ ITI_Ex2	cross_ ITI_SR-t
Ac1	Pearson	1	,390	,634**	,562**	,287	,461*	,035	-,108	,380	,084	,330	,150	,331	,317
	Sig. (bilateral)		,080	,002	,008	,207	,035	,882	,642	,089	,719	,144	,517	,143	,161
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
Ac2	Pearson	,390	1	,553**	,238	,230	,178	-,086	,090	,029	,559**	,444*	,218	,350	,174
	Sig. (bilateral)	,080		,009	,300	,316	,440	,710	,699	,901	,008	,044	,342	,120	,450
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
Ac3	Pearson	,634**	,553**	1	,543*	,230	,192	-,256	-,165	,285	,316	,515*	,253	,374	,124
	Sig. (bilateral)	,002	,009		,011	,316	,404	,262	,474	,211	,163	,017	,269	,095	,591
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
Ex1	Pearson	,562**	,238	,543*	1	,562**	,673**	,222	,092	,331	,112	,293	,236	,493*	,437*
	Sig. (bilateral)	,008	,300	,011		,008	,001	,334	,691	,143	,628	,197	,302	,023	,048
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
Ex2	Pearson	,287	,230	,230	,562**	1	,766**	,171	,025	,547*	,414	,533*	-,038	,505*	,477*
	Sig. (bilateral)	,207	,316	,316	,008		,000	,458	,915	,010	,062	,013	,871	,020	,029
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
SR-t	Pearson	,461*	,178	,192	,673**	,766**	1	,303	-,019	,590**	,321	,218	-,078	,300	,603**
	Sig. (bilateral)	,035	,440	,404	,001	,000		,181	,936	,005	,156	,342	,736	,187	,004
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ Hab1	Pearson	,035	-,086	-,256	,222	,171	,303	1	,451*	,018	-,052	,092	,156	,119	,562**
	Sig. (bilateral)	,882	,710	,262	,334	,458	,181		,040	,939	,824	,692	,498	,606	,008
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ Hab2	Pearson	-,108	,090	-,165	,092	,025	-,019	,451*	1	,135	,097	-,079	,468*	,356	,233
	Sig. (bilateral)	,642	,699	,474	,691	,915	,936	,040		,559	,674	,733	,032	,114	,310
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_Ac1	Pearson	,380	,029	,285	,331	,547*	,590**	,018	,135	1	,478*	,161	-,084	,339	,345
	Sig. (bilateral)	,089	,901	,211	,143	,010	,005	,939	,559		,028	,486	,716	,133	,126
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_Ac2	Pearson	,084	,559**	,316	,112	,414	,321	-,052	,097	,478*	1	,486*	,070	,331	,161
	Sig. (bilateral)	,719	,008	,163	,628	,062	,156	,824	,674	,028		,026	,764	,143	,485
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_Ac3	Pearson	,330	,444*	,515*	,293	,533*	,218	,092	-,079	,161	,486*	1	,215	,349	,201
	Sig. (bilateral)	,144	,044	,017	,197	,013	,342	,692	,733	,486	,026		,349	,121	,381
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_Ex1	Pearson	,150	,218	,253	,236	-,038	-,078	,156	,468*	-,084	,070	,215	1	,561**	,360
	Sig. (bilateral)	,517	,342	,269	,302	,871	,736	,498	,032	,716	,764	,349		,008	,108
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_Ex2	Pearson	,331	,350	,374	,493*	,505*	,300	,119	,356	,339	,331	,349	,561**	1	,656**
	Sig. (bilateral)	,143	,120	,095	,023	,020	,187	,606	,114	,133	,143	,121	,008		,001
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21
cross_ ITI_SR-t	Pearson	,317	,174	,124	,437*	,477*	,603**	,562**	,233	,345	,161	,201	,360	,656**	1
	Sig. (bilateral)	,161	,450	,591	,048	,029	,004	,008	,310	,126	,485	,381	,108	,001	
	N	21	21	21	21	21	21	21	21	21	21	21	21	21	21

**. 0,01 (bilateral).

*. 0,05 (bilateral).