A comparison of behavioral and pharmacological interventions to attenuate reactivated fear memories.

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ABSTRACT

Two experiments using rats in a contextual fear memory preparation compared two approaches to reduce conditioned fear: 1) pharmacological reconsolidation blockade and 2) reactivation-plus-extinction training. In experiment 1 we explored different combinations of reactivation-plus-extinction parameters to reduce conditioned fear and attenuate reacquisition. In experiment 2, memory reactivation was followed by extinction training, or administration of Midazolam (vs vehicle) to reduce conditioned fear and attenuate spontaneous recovery. We found both treatments to be equally effective in both experiments. This study suggests that parameters leading to memory destabilization during reactivation are critical to observe long-lasting effects of MDZ or reactivation-plus-extinction.

Anxiety disorders (i.e., general anxiety disorder, phobias and post-traumatic stress disorder) are most effectively treated with exposure therapy, which consists of the controlled presentation of feared stimuli or contexts, eventually leading to fear reduction (Craske et al. 2008). Experimental extinction is a laboratory model for exposure therapy, based on the assumption that one of the multiple components of anxiety disorders are associative memories driving the expression of conditioned responses (CR), acquired through pairing of a conditioned stimulus (CS) with an aversive unconditioned stimulus (US). Following this rationale, one of the multiple effects of exposure therapy is the reduction of CR expression as a consequence of repeated presentation of the CS without the US (extinction). Clinical and laboratory evidence supports these assumptions (Scheveneels et al. 2016). Indeed, the benefits and pitfalls of exposure therapy are observed in extinction learning. For example, a major challenge for exposure therapy is relapse, or the return of fear after treatment in a variety of circumstances that represent a change from the (internal or external) context of exposure training to that of testing (Urcelay 2012). This is captured by the notion that extinction training promotes new (context dependent) inhibitory learning, rather than the modification of the original fear memory (Bouton 2002). As a result, after extinction training, the original fear memory co-exists with an extinction memory, with the retrieval context (or other critical cues present at the time of retrieval) determining which memory controls behavior at a given time (Bouton 2004). Great research efforts have been devoted to enhancing extinction learning and maximizing the benefits of exposure therapy (Urcelay, Lipatova & Miller, 2009; Urcelay, Wheeler & Miller, 2009), but the

endurance of the original fear memory always makes the return of fear a possibility (Craske et al. 2008, 2014).

Prospects for a more robust prevention of return-of-fear come from studies that target fear memory more directly (Beckers and Kindt, in press). Retrograde amnesia can be induced for discrete consolidated memories (Misanin et al. 1968), if properly reactivated (Finnie and Nader 2012). The reconsolidation hypothesis states that reactivation of a consolidated memory trace can induce its destabilization and subsequent reconsolidation during a time-limited period: the "reconsolidation window" (Nader et al. 2000). It is currently unknown whether the retrograde amnesia for reactivated and destabilized memories reflects interference with memory storage or retrieval mechanisms. However, there is strong experimental evidence supporting retrieval-failure explanations (Briggs and Olson, 2013; Guisquet-Verrier et al. 2015; see Riccio, Millin and Bogart 2006 for a review). Regardless, clinical applications of reconsolidation interference are currently the focus of several research programs (Beckers and Kindt, in press).

Two main approaches have been reported to disrupt or permanently alter reactivated pavlovian CS-US memories: pharmacological reconsolidation blockade (Nader et al. 2000) and the reactivation-plus-extinction procedure (R+E; Monfils et al. 2009). The former consists in pharmacologically disrupting memory reconsolidation, while the later entails conducting extinction training during the reconsolidation window. Previous work in our lab has been aimed at better understanding the psychological variables that determine the effectiveness of these manipulations. Using contextual fear conditioning (CFC) in rats, we previously reported that permanent reduction of fear through the R+E procedure requires that

memories are destabilized during reactivation (Piñeyro et al. 2014). We also reported that memory destabilization, and therefore the possibility of pharmacologically blocking the reconsolidation process, requires the occurrence of an US temporal prediction error during memory reactivation (Alfei et al. 2015), a result reported by other groups in other experimental preparations (Díaz-Mataix et al. 2013; Pedreira et al. 2004).

Although administering amnestic drugs and conducting extinction training upon memory reactivation have both been shown to induce robust reduction of conditioned fear and prevent fear recovery, it is currently unknown which one is more effective. To our knowledge, only one study has addressed this problem, using a human differential fear conditioning protocol (Soeter and Kindt 2011). In that report both the beta-blocker Propranolol (PROP) or extinction training were applied after CS reactivation, with R+PROP preventing several forms of fear reappearance (spontaneous recovery, reinstatement, reacquisition and fear generalization). The R+E procedure prevented only spontaneous recovery, failing to attenuate reinstatement, reacquisition or generalization. One possibility is that extinction trials felt short to fully alter the reactivated memory, as suggested by the own authors. Another possible explanation for those results is that different interference procedures might require different reactivation conditions to alter the target memory. However, in that study R+PROP and R+E were compared across two experiments, rather than in the same experiment. Hence, a direct comparison in a single experiment of both strategies has not been yet conducted, and the question of the relative efficacy of each approach remains elusive. The experiments presented here were designed to compare directly both strategies

(Exp 2), after determining the critical role of reactivation conditions to induce different plasticity process over the target memory (Exp 1).

We used CFC in rats and Midazolam (MDZ), a fast-acting positive modulator of the GABA-A receptor known to interfere with the consolidation and reconsolidation of aversive memories (Bustos et al. 2006; Kroon and Carobrez 2009), and compared it with experimental extinction following reactivation. Because memory reactivation can lead to different mnemonic outcomes (mere retrieval, destabilization, or the formation of a novel memory trace), and we have previously shown that memory destabilization during reactivation is critical for the success of the R+E procedure (Piñeyro et al. 2014), our first step was to investigate different reactivation conditions combined with extinction. This was done to identify the optimal reactivation conditions for the R+E procedure before comparing it against R+MDZ. We evaluated reactivation conditions that our previous work suggested would induce mere retrieval (no CR reduction and insensitivity to MDZ when tested 24 hs after reactivation), destabilization (no CR reduction in control conditions but vulnerability to MDZ), insensitivity to MDZ (neither CR reduction nor MDZ vulnerability) and extinction (CR reduction that can be blocked by MDZ).

Subjects were naïve, adult male Wistar rats (60–65d old, weighing 270–320 g at experimental onset), bred in our own colony in the Laboratorio dePsicología Experimental, Facultad de Psicología, Universidad Nacional de Córdoba, Argentina. Animals were housed in standard laboratory Plexiglas cages (60 cm long $\times$ 40 cm wide $\times$ 20 cm high) in groups of 3–4 per cage. Food and water

were available ad libitum. Animals were maintained on a 12-h light–dark cycle (lights on at 8 am), at room temperature of 21°C–23°C (following standards of the NIH Guide for the Care and Use of Laboratory Animals). The number of animals and their suffering was kept to the minimum possible to achieve the goals of this research. The experimental apparatus employed to conduct the CFC was a 24 long × 22 wide × 22 cm high Plexiglas chamber with opaque gray walls and a removable transparent ceiling, the floor consisting of 20 parallel stainless steel grid bars each measuring 3 mm in diameter, spaced 1 cm apart and connected to a device to provide adjustable footshocks (the Automatic Reflex Conditioner 7501, Ugo Basile, Milan, Italy). All experiments were video-taped from above for later off-line analyses. Freezing behavior was used as an index of fear, defined as the total absence of body and head movements except for that associated with breathing (Blanchard and Blanchard 1969). Freezing was scored minute-by-minute with a stop-watch by an observer blind to the experimental condition of each animal, and expressed as % of time (in seconds). Inter-observed reliability was previously established with a different set of data (Pearson's r =0.95). Data was analyzed with "t" tests, one-way, repeated measures or mixed ANOVAs, followed by the Tukey HSD test as post-hoc analyses for significant effects reported by the ANOVAs.

In Experiment 1, rats were submitted to a CFC protocol that yields a fear memory with a precise temporal expectation of US arrival (Alfei et al. 2015). Animals were handled and habituated to the experimenter during three consecutive days. A day later, animals were introduced in the experimental apparatus for 1 min (pre-shock period), after which two foot-shocks (1 mA, 3-s duration, intershock

interval of 30 s) were delivered. Immediately after, animals were returned to the home-cage. Seventy-two hours later, memory reactivation was achieved by re-exposing animals to the conditioned context, without foot-shocks. Four non-reinforced reactivation lengths were used in order to trigger different mnemonic processes: 30 s (mere retrieval), 2 min (destabilization), 6 min (insensitive state) or 15 min (extinction). Following a 10-min interval in the home cage after reactivation, half of the animals in the 30 s, 2 min and 6 min conditions were submitted to a second context exposure of 14.5, 13, and 9 min respectively, to achieve a total of 15 min of exposure in all animals (hence, accomplishing conditions prone to induce fear extinction according to Alfei et al. 2015). The other half had the second exposure omitted, to serve as controls. The group submitted to a straight 15 min exposure did not receive a second exposure trial, as conditions to induce extinction had already been met. After 24 h, subjects were submitted to a reacquisition procedure, consisting in the administration of a single 0.5 mA shock for 3 seconds after a 93 s pre-shock period, to recover the extinguished CR (Piñeyro et al. 2014; Ferrer Monti et al. 2016). An additional control group, without any previous training, was included to quantify the amount of CR expression produced by the US employed during the reacquisition procedure. The final test, a 5 min context re-exposure without shock, was conducted a day later. Groups were labeled as follows: R 30 s / Ext 14.5 min (n=7); control: R 30 s (n=6); R 2 min / Ext 13 min (n=7); control: R 2 min(n=6); R 6 min / Ext 9 min (n=6); control: R 6 min (n=6); Ext 15 min (n=6); Reacq Only (n=7) (see Figure 1A).

Results: unpaired, two-tailed t-tests revealed no significant differences at reactivation when comparing each of the groups that received both R+E to its

respective control ($P > 0.05$ in all cases, Figure 1B). The Ext 15 min group lacked an identical control during reactivation and was not compared at this phase. A one-way ANOVA (group as factor) on the pre-shock period of the reacquisition phase yielded a significant effect of group [$F(7, 43) = 12.41$, $P = .00000$, $\eta^2p = .66$]. Post-hoc analyses revealed that the groups submitted to R+E and the 15 min extinction group expressed significantly less fear than the control groups (R 30 s, R 2 min and R 6 min) and similar levels of fear as the untrained Reacq Only group. In other words, combining reactivation and extinction as well as straight extinction reduced CRs relative to reactivation controls (Figure 1C). However, 24 h after reacquisition, the only group to still show similar low levels of freezing as the Reacq Only group was the R 2 min / Ext 13 min group, with both groups expressing significantly lower levels of CR than the rest. This was confirmed by a one-way ANOVA [$F(7, 43) = 36.98$, $P = .00000$, $\eta^2p = .85$] and by the post hoc analysis. Hence, the R+E procedure prevented rapid CR reacquisition only when reactivation was timed such as to be optimal for inducing memory destabilization (Figure 1D). This confirms, with different parameters, our previous findings regarding the critical role of memory destabilization prior to extinction in order to observe superior effects of R+E over regular extinction training (Piñeyro et al. 2014). Our results are in line with the idea that memory destabilization is achieved by the induction of a temporal prediction error; the discrepancy between information presented during acquisition (i.e., US onset at the 1st minute) and memory reactivation (i.e., US absence at the expected time), which renders the memory unstable and vulnerable to pharmacological (Alfei et al. 2015) or behavioral interventions (present results). It is important to note that every group submitted to both reactivation and extinction had

actually a total of 15 minutes of context re-exposure between both instances. Hence, any difference among groups cannot be attributed to differences in total time spent in the context without US delivery, but to the particular effect of memory reactivation. In fact, we have previously observed that separating the first and second context re-exposures for a longer delay (6 h) prevents the effect of extinction (or other interference procedures as MDZ or contrasting emotional information) over the destabilized memory, presumably because the reconsolidation window is closed by that time (Piñeyro et al. 2014; Alfei et al. 2015; Ferrer et al. 2016).

Experiment 2 was designed to directly compare the effects of MDZ administration and extinction training after reactivation (R+MDZ versus R+E). Rats were trained as in experiment 1 and memory was reactivated 72 h later. Three reactivation lengths were employed: 2 min (destabilization), 6 min (insensitive state) and 15 min (extinction). Animals in each reactivation condition were randomly assigned to one of three treatments: MDZ 3 mg/kg (i.p, Alfei et al. 2015), saline (SAL) or a second context re-exposure (after a 10 min break in the home-cage) to complete the 15 min necessary to achieve extinction (EXT, which lasted 13 or 9 min for the groups that received 2 or 6 min of context exposure during initial reactivation, respectively). Animals submitted to a straight 15 min reactivation had the second re-exposure omitted, because this length of exposure leads to extinction (Alfei et al., 2015; Piñeyro et al. 2014). Subjects were then tested and retested for 5 min in the conditioned context (without US) 1d and 7d following memory reactivation, respectively. For all groups, n=6 (see Figure 2A).

Results: although all experimental conditions were run simultaneously, reactivation lengths were different for the 2 min, 6 min and 15 min conditions. Accordingly, reactivation data were analyzed separately (treatment as factor), with no significant differences between treatments for any of the three reactivation conditions (P>.05 in all cases). Furthermore, a one-way mixed ANOVA on the minute-by-minute freezing data for the 3 groups that completed 15 min of context re-exposure (group and time as factors) revealed no effect of group [F (2, 14) =.00, P=.99348], a significant effect of time [F (14, 196) = 3.25, P=.00011, η^2p=.18], but no interaction [F (28, 196) =.58, P=.95078]. Post hoc analyses revealed that freezing peaked by the 2nd min, as previously reported for animals trained under these conditions (Alfei et al. 2015; see Figure 3). Regarding the effects of the different treatments, a mixed 3 (reactivation: 2, 6 or 15 min) x 3 (treatment: EXT, MDZ or SAL) x 2 (session: test and retest) ANOVA revealed significant effects of reactivation [F (2, 45) =12.18, P=.00006, η^2p=.35], treatment [F (2, 45) =13.54, P=.00002, η^2p=.37], session [F (1, 45) =27.49, P=.00000, η^2p=.37], reactivation x treatment [F (4, 45) =9.71, P=.00001, η^2p=.46], reactivation x session [F (2, 45) =9.52, P=.00036, η^2p=.29], treatment x session [F (2, 45) =8.51, P=.00073, η^2p=.27] and critically, a reactivation x treatment x session interaction [F (4, 45) =4.48, P=.0039, η^2p=.28]. To break down the three-way interactions, mixed ANOVAS were performed for each reactivation condition separately (treatment and session as factors). For the 2 min condition, the ANOVA revealed a significant effect of treatment [F (2, 16) =26.08, P=.00001, η^2p=.76], no effect of session [F (1, 16) =.00, P=.924], and no interaction [F (2, 16) =.33, P=.71781]. Post-hoc analyses revealed that MDZ and EXT reduced CR compared to SAL at test, and a week

later (attenuated spontaneous recovery; Figure 2B). For the 6 min condition, the ANOVA revealed a significant effect of treatment [F (2, 15) =4.88, *P*=.02325, η^2p=.39] and session [F (1, 15) =13.36, *P*=.00234, η^2p=.47] and a significant interaction [F (2, 15) =10.68, *P*=.00131, η^2p=.58]. Post-hoc analyses revealed that EXT reduced CR expression compared to MDZ and SAL at test, but this difference disappeared a week later (spontaneous recovery; Figure 2C). For the 15 min condition, the ANOVA revealed a significant effect of treatment [F (2, 14) = 4.23, *P*=.003634, η^2p=.37] and session [F (1, 14) =21.36, *P*=.0004, η^2p=.60] and a significant interaction [F (2, 14) =5.92, *P*=.01368, η^2p=.45]. Post-hoc analyses showed that MDZ blocked the CR reduction observed for the EXT and SAL groups at test, but this difference disappeared again after a week (spontaneous recovery; Figure 2D).

In summary, this experiment: a) revealed that R+MDZ and R+E are equally effective at reducing fear CRs, provided that memory destabilization is achieved during reactivation; b) confirms that, unlike standard extinction, R+E prevents spontaneous recovery if destabilization is triggered before extinction (Piñeyro et al. 2014); c) suggests that the beneficial effects of R+E are not due to a differential pattern of CR expression during re-exposure to feared cues; and d) replicates previous findings from different groups, including ours, that hint to the existence of a particular mnemonic state between destabilization and extinction memory formation which is insensitive to pharmacological and behavioral interference (Alfei et al. 2015; Briggs and Olson 2013; Flavell and Lee 2013; Merlo et al. 2014; Sevenster et al. 2014).

These findings suggest similar effectiveness of pharmacological and behavioral interventions to modify reactivated aversive memories. This is partially at odds with the report of Soeter and Kindt (2011), who found that the R+E procedure prevented spontaneous recovery but failed to attenuate reinstatement and reacquisition. Here we find that the R+E procedure attenuates both spontaneous recovery (Exp 2) and reacquisition (Exp 1). These somewhat contrasting results might reflect species (human vs rats), task (cued vs contextual fear) or other procedural differences, such as the temporal lapse to observe spontaneous recovery after extinction (1 vs 7 days). One important feature of the present results is the suggestion that memory reactivation in itself does not necessarily lead to memory destabilization; prediction error at the time of reactivation is a critical moderator of the occurrence of post-retrieval malleability (Exton-McGuinness et al. 2015; Fernandez et al. 2016). In line with this notion, we used reactivation conditions previously shown in pharmacological work to induce destabilization through a temporal prediction error (Alfei et al. 2015). Although the importance of prediction error has been acknowledged in the reactivation-extinction literature (Auber et al. 2013; Fricchione et al. 2016; Golkar et al. 2012; Hutton-Bedbrook and McNally 2013; Ishii et al. 2015; Oyarzún et al. 2012), only a few studies have actually used reactivation conditions that were optimally suited for inducing memory destabilization (as deduced from pharmacological blockade of memory reconsolidation in independent experiments). In those studies, CR recovery was fully or partially prevented (Rao-Ruiz et al. 2011; Soeter and Kindt 2011; Flavell et al. 2011; Piñeyro et al. 2014). Lack of prediction error at the time of reactivation might help explain why some groups have failed to replicate the results

originally reported by Monfils et al. (2009; see Auber et al. 2013; Exton-Mcguinness et al. 2015 for discussion). Furthermore, it is worth mentioning that two recent reports in hazardous drinkers also suggest a critical role of memory destabilization, through the induction of prediction errors (via guided expectancy violations about alcoholic-related cues), for observing endurable effects of reappraisal and counterconditioning (Das et al. 2015; Hon et al 2016). Similar results have recently been reported in animals as well (Haubrich et al. 2015; Ferrer Monti et al. 2016).

Overall, the findings presented in this report suggest that behavioral and pharmacological interventions can be equally effective to reduce conditioned fear. Extended to clinical settings, this implies that patients reluctant to take psychiatric drugs might choose exposure therapy. Similarly, patients not willing to re-experience fear stimuli extensively might benefit from a pharmacologic alternative. Thus, both therapeutic interventions might capitalize on memory destabilization and reconsolidation processes as primary allies in the treatment of psychiatric conditions mediated by aberrant memories. In particular, the R+E procedure seems a promising strategy to boost CR reduction in a behavioral and non-invasive way. Future studies should, however, establish reliable on-line indices of fear memory destabilization, otherwise it will be difficult to translate this insight into clinical applications, because behavioral or pharmacological interventions might be applied while memory is unsusceptible to environmental influence (i.e., while merely retrieved or in an insensitive state). For example, a critical aspect of memory reactivation to achieve destabilization in this and other reconsolidation studies seems to be prediction error (Díaz-Mataix et al. 2014; Pedreira et al. 2014; Sevenster et al. 2013). But prediction error also leads to new learning, so extended

exposure results in a new memory trace (i.e., extinction learning) being established and this can lead to no effect of the intervention, or extinction learning can be the target of the intervention, as we have observed in this and previous reports. . Although advances on this topic have been made by measuring on-line US expectancies during fear memory reactivation (Sevenster et al. 2013), further research in this direction is clearly needed to translate basic reconsolidation research to clinical settings.

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Figures and Legends

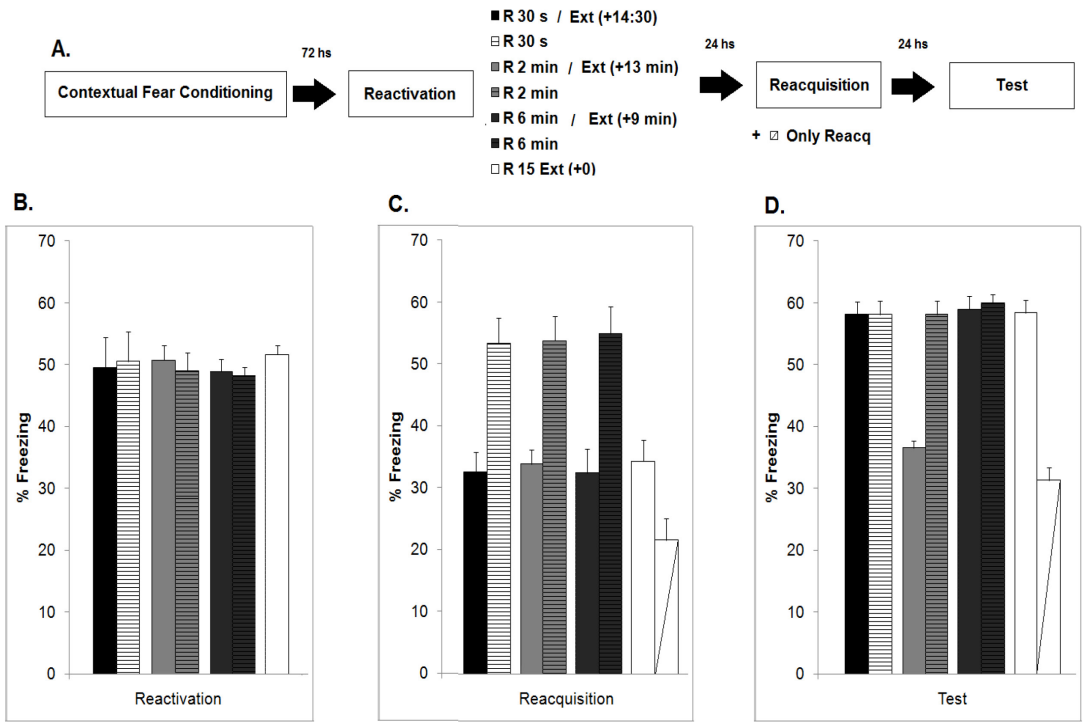

Figure 1. Experiment 1 (panel A). Three days after training, contextual fear memory was reactivated through 30 s, 2 min, 6 min or 15 min of re-exposure to the training context (panel B, showing total freezing percent during each reactivation length). Half of the subjects in each group were submitted to a second context exposure (after a 10-min interval in the home-cage) to complete the 15 min needed to achieve extinction, except for those submitted to a straight 15 min of reactivation (data not shown, but see Figure 3 showing similar data for Experiment 2). After 24 h, all groups were submitted to a reacquisition protocol (panel C: pre-shock freezing). At this stage, a group of untrained (naive) animals was added, which only received reacquisition. All groups were tested a day later (panel D). None of the groups completing 15 min of context re-exposure at reactivation differed from an untrained group in pre-shock freezing to the context 24 h later (during reacquisition). However, 1 day after reacquisition, the group submitted to reactivation conditions apt to trigger memory destabilization was the only one comparable to the previously untrained controls. Data shows the mean ± SEM of percentage time spent freezing during reactivation, pre-shock period of reacquisition and test.

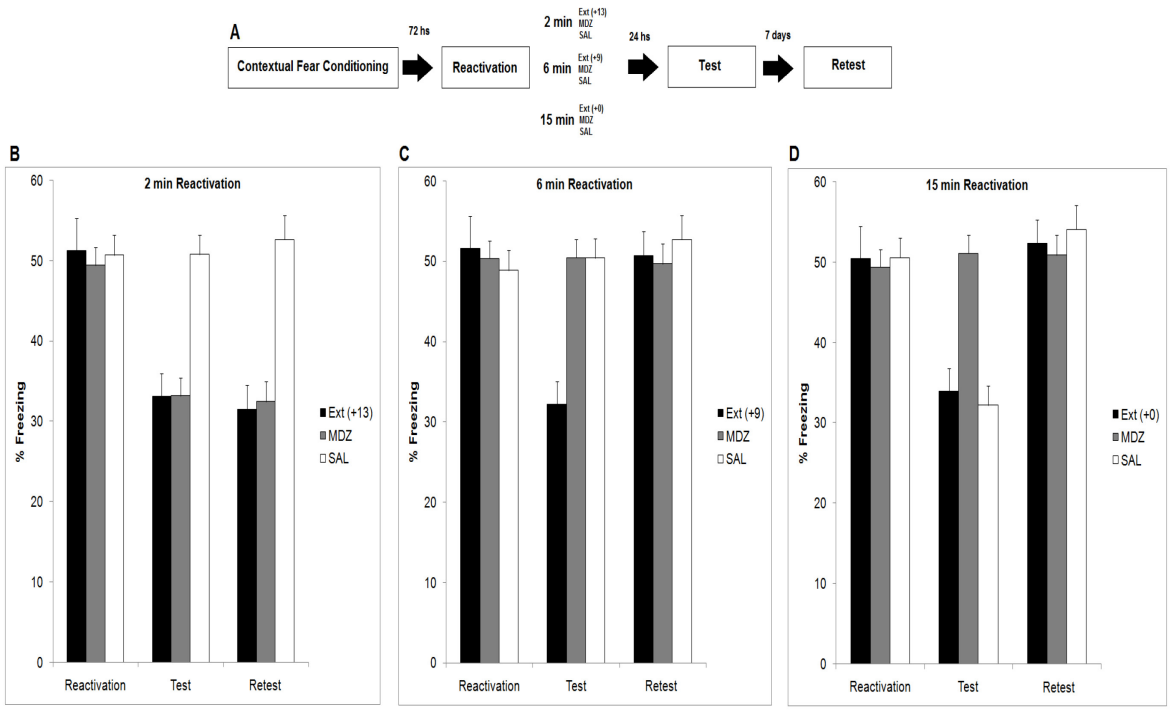

Figure 2. Experiment 2 (panel A). Three days after training, contextual fear memory was reactivated during 2 min (panel B), 6 min (panel C) or 15 min (panel D) by re-exposure to the training context. Subjects in each condition received either 3mg/kg of Midazolam (MDZ), saline (SAL) or a second re-exposure (after a 10-min brake in the home-cage) to achieve conditions for inducing extinction (EXT). One day and one week later, all groups were subjected to a 5-min test and re-test in the training context. MDZ and EXT were equally effective at reducing CR and attenuating spontaneous recovery when reactivation was optimal for inducing destabilization (2 min reactivation). In contrast, MDZ was unable to reduce CR and EXT failed to prevent spontaneous recovery when reactivation was not attuned to inducing destabilization (6 min reactivation). Finally, straight extinction (15 min reactivation) reduced CR at test but failed to prevent spontaneous recovery. Extinction was blocked by MDZ in this condition. Data shows the mean ± SEM of percentage time spent freezing during reactivation, test and re-test.

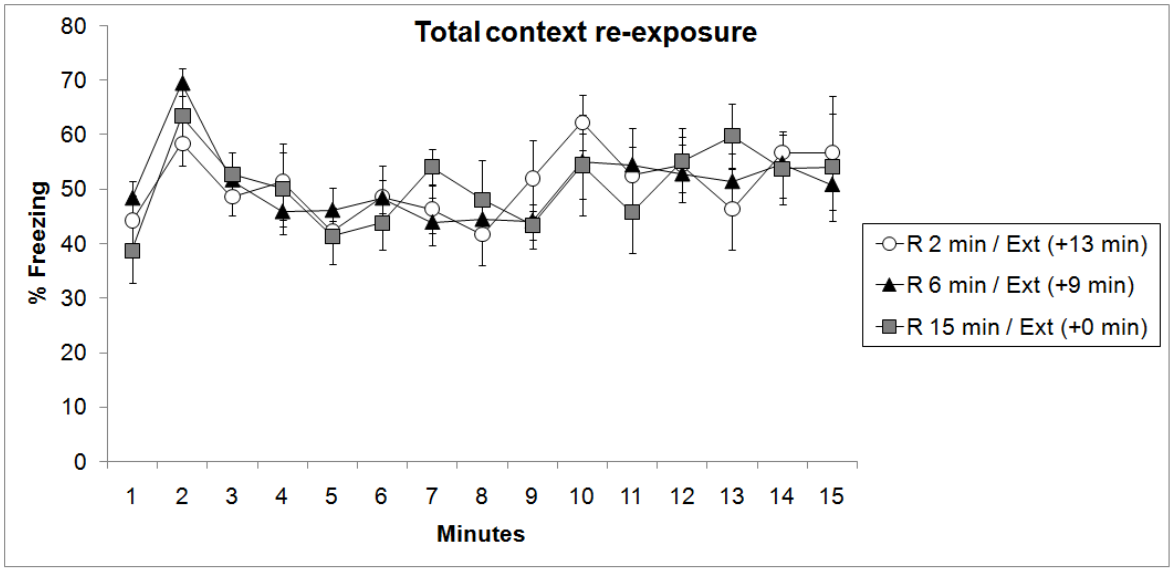

Figure 3. Experiment 2. Data shows the mean ± SEM of percentage time spent freezing during the entire 15 minutes of context re-exposure for groups R 2 min / Ext 13 min, R 6 min / Ext 9 min and R 15 min (straight extinction). For all groups, the peak of CR expression was at min 2. There was no difference between groups at any time during context re-exposure. Hence, attenuated spontaneous recovery for the R 2 min / Ext 13 min group cannot be attributed to enhanced CR reduction during context re-exposure.