An appetitive experience after fear memory destabilization attenuates fear retention: involvement GluN2B-NMDA receptors in the Basolateral Amygdala Complex

Roque I. Ferrer Monti,[1] Marcelo Giachero,[2] Joaquín M. Alfei,[1,3] Adrián M. Bueno,[1] Gabriel Cuadra,[2] and Victor A. Molina[2]

[1]Laboratorio de Psicología Experimental, Facultad de Psicología, Universidad Nacional de Córdoba, Enfermera Gordillo y Enrique Barros, Ciudad Universitaria, 5000 Córdoba, Argentina; [2]IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina; [3]Department of Psychology, University of Leuven, 3000 Leuven, Belgium

It is known that a consolidated memory can return to a labile state and become transiently malleable following reactivation. This instability is followed by a restabilization phase termed reconsolidation. In this work, we explored whether an unrelated appetitive experience (voluntary consumption of diluted sucrose) can affect a contextual fear memory in rats during the reactivation-induced destabilization phase. Our findings show that exposure to an appetitive experience following reactivation can diminish fear retention. This effect persisted after 1 wk. Importantly, it was achieved only under conditions that induced fear memory destabilization. This result could not be explained as a potentiated extinction, because sucrose was unable to promote extinction. Since GluN2B-containing NMDA receptors in the basolateral amygdala complex (BLA) have been implicated in triggering fear memory destabilization, we decided to block pharmacologically these receptors to explore the neurobiological bases of the observed effect. Intra-BLA infusion with ifenprodil, a GluN2B-NMDA antagonist, prevented the fear reduction caused by the appetitive experience. In sum, these results suggest that the expression of a fear memory can be dampened by an unrelated appetitive experience, as long as memory destabilization is achieved during reactivation. Possible mechanisms behind this effect and its clinical implications are discussed.

There is accumulating evidence that consolidated memories can undergo a transient destabilization process, if appropriate reactivation conditions are met (Finnie and Nader 2012). In order to persist and be available for future retrieval, reactivated traces then undergo a process of restabilization, termed reconsolidation (Nader et al. 2000). For a time-limited period, known as the "reconsolidation window," this process can be interrupted, resulting in a memory deficit (Lee 2009). Several studies have demonstrated that the expression of destabilized memories can be dampened, strengthened, or even updated through a variety of interventions (Nader and Hardt 2009). Importantly, memory destabilization and reconsolidation have been found in a wide range of species, including humans, using diverse aversive and appetitive motivated memory protocols (Agren 2014).

The possibility to manipulate this post-reactivation plasticity process offers a potential treatment for psychiatric disorders involving pathogenic or maladaptive memories (Nader et al. 2013; Schwabe et al. 2014). In fact, it has been recently proposed that effective psychotherapy techniques might work through the incorporation of contrasting emotional information into (maladaptive) destabilized mnemonic traces (Lane et al. 2015). This implies that both appetitive and aversive mnemonic traces (e.g., a drug-related memory or contextual fear memory) could be influenced by an unrelated and opposed emotional experience (such as stress or pleasure, respectively). Such experiences must occur during the labile state of a memory following its reactivation in order to affect it. Indirect support for this hypothesis comes from studies demonstrating that post-reactivation stressful experiences can dampen the retrieval of drug-related words in heroin addicts (Zhao et al. 2009) and morphine-conditioned place preference in rats (Wang et al. 2008). However, support for the inverse condition, such as unrelated appetitive experiences affecting destabilized fear memories, is currently lacking.

It is thus reasonable to explore whether an unrelated appetitive experience can affect fear memory retention. Hence, we evaluated the effects of voluntary consumption of diluted sucrose (SUC), a highly rewarding experience for rodents (Hajnal and Norgren 2001; Tõnissaar et al. 2006) after contextual fear memory (CFM) reactivation-induced destabilization.

Results

Experiment 1

Setting reactivation parameters to induce destabilization of a consolidated contextual fear memory

Memory destabilization (and therefore, reconsolidation) will not occur every time a memory is reactivated or retrieved. Rather,

this process is dependent on the interaction between learning and the reactivation conditions (Finnie and Nader 2012; Flavell et al. 2013; Alfei et al. 2015). Furthermore, the emergence of reactivation-induced instability cannot be directly observed through behavioral outputs. Hence, memory destabilization cannot be assumed unless memory expression is affected after experimental manipulations (Schwabe et al. 2014). Accordingly, our first experiment was designed to establish the reactivation conditions required to induce destabilization of the CFM. In this experimental preparation, memory destabilization has been reported to occur after reexposure to the training context without foot shock, with the reactivation time-span being critical to induce this process (Lee et al. 2008; Bustos et al. 2009).

Rats were trained in a contextual fear protocol, as previously described (Piñeyro et al. 2014) (see Materials and Methods). The fear memory was reactivated 3 d later by exposure to the conditioned context, without shock. Two reactivation lengths were used: 90 sec or 4 min. Immediately after reactivation, half of the rats in each condition were administered Midazolam (MDZ, 3 mg/kg, i.p.), a fast-acting positive modulator of the GABA-A receptor, known to disrupt memory reconsolidation of aversive and appetitive memories (Bustos et al. 2006; Robinson and Franklin 2010; Stern et al. 2012; De Oliveira Alvares et al. 2013; Piñeyro et al. 2014), while the other half received an equivalent amount of saline (SAL). To control for any unspecific effect of MDZ, a third condition was included in the experimental design. Hence, MDZ or SAL was administered without prior exposure to the conditioned context (nonreactivated control group). All groups were submitted to fear testing a day later (Fig. 1A). Since MDZ can disrupt the reconsolidation of CFM, and reconsolidation only occurs after the onset of the destabilization phase, any interfering effect of MDZ would reveal that the CFM underwent a destabilization process. In contrast, the absence of this interfering effect would reveal that the reactivation did not destabilize the memory trace.

There were no significant differences in freezing behavior among MDZ and SAL groups during reactivation ($P > 0.05$ in both reactivation conditions) (Fig. 1B). However, a factorial ANOVA (drug × reactivation condition) on the test data revealed a significant effect of drug [$F_{(1,36)} = 4.36$, $P < 0.05$, $\eta^2 p = 0.10$], reactivation condition [$F_{(2,36)} = 6.77$, $P < 0.01$, $\eta^2 p = 0.27$] and a significant drug × reactivation condition interaction [$F_{(2,36)} = 7.44$, $P < 0.01$, $\eta^2 p = 0.29$]. Post hoc analysis revealed that the group receiving a 4-min reactivation and MDZ differed from all the others ($P < 0.01$ in all cases), which did not differ between them ($P > 0.05$) (Fig. 1C).

In sum, the results from experiment 1 showed two different reactivation conditions for the CFM: one that is able to induce memory destabilization (4 min) and other one unable to initiate this process (90 sec).

Experiment 2

### Influence of the post–reactivation appetitive experience on a destabilized fear memory

Our first aim was to determine the most appetitive concentration of SUC for nondeprived rats. Accordingly, the voluntary consumption of three concentrations (10%, 20%, and 30%, w/v) and a water-only control condition were tested (see Materials and Methods). Afterward, the most appetitive concentration and the water group were compared for their effect on a destabilized fear memory.

Voluntary SUC consumption

A repeated-measures ANOVA on consumption data (group × session as factors) revealed significant effects of group [$F_{(3,28)} = 10.31$, $P < 0.001$, $\eta^2 p = 0.52$], session [$F_{(1.82,51.11)} = 8.40$, $P < 0.01$, $\eta^2 p = 0.23$] and interaction between both factors [$F_{(5.47,51.11)} = 3.49$, $P < 0.01$, $\eta^2 p = 0.27$]. Analysis of simple main effects revealed significant effects of group in the first [$F_{(3,28)} = 3.02$, $P < 0.05$, $\eta^2 p = 0.24$], second [$F_{(3,28)} = 12.56$, $P < 0.001$, $\eta^2 p = 0.57$], third [$F_{(3,28)} = 9.34$, $P < 0.001$, $\eta^2 p = 0.50$] and fourth consumption session [$F_{(3,28)} = 4.41$, $P < 0.05$, $\eta^2 p = 0.32$] (Fig. 2B). Follow up analysis revealed that the 30% concentration was the most appetitive, since it was more consumed throughout the four sessions ($P < 0.05$ in all cases). We therefore selected this concentration for further experiments.

Influence of voluntary SUC consumption on a destabilized fear memory

In order to test our hypothesis, the water-only and the 30% SUC groups from experiment 2 A were submitted to CFM training the day after the last consumption session. Three days later, fear memory reactivation (4-min length) was followed (after 30 min) by a fifth consumption session, since the CFM reconsolidation window is open 30 min after reactivation (Piñeyro et al. 2014). One
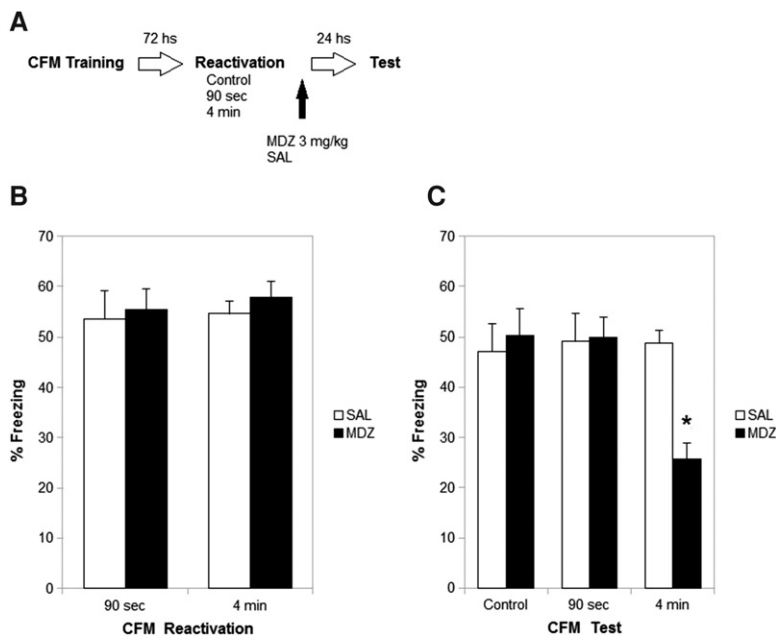
Figure 1. Experiment 1. (A) Experimental protocol. Three days after training, the CFM was reactivated for 90 sec or 4 min by reexposure to the training context. A third group served as a control without reactivation. Subjects in each group received 3 mg/kg of Midazolam (MDZ) or an equivalent amount of saline (SAL) immediately after reactivation. One day later, all groups were subjected to a 5-min test in the training context. (B) Data show the mean ± SEM of percentage time spent freezing during reactivation. (C) Data depict the mean ± SEM of percentage time spent freezing during test. (*) Denotes a significant statistical difference between the MDZ/4 min condition and the rest of the groups.
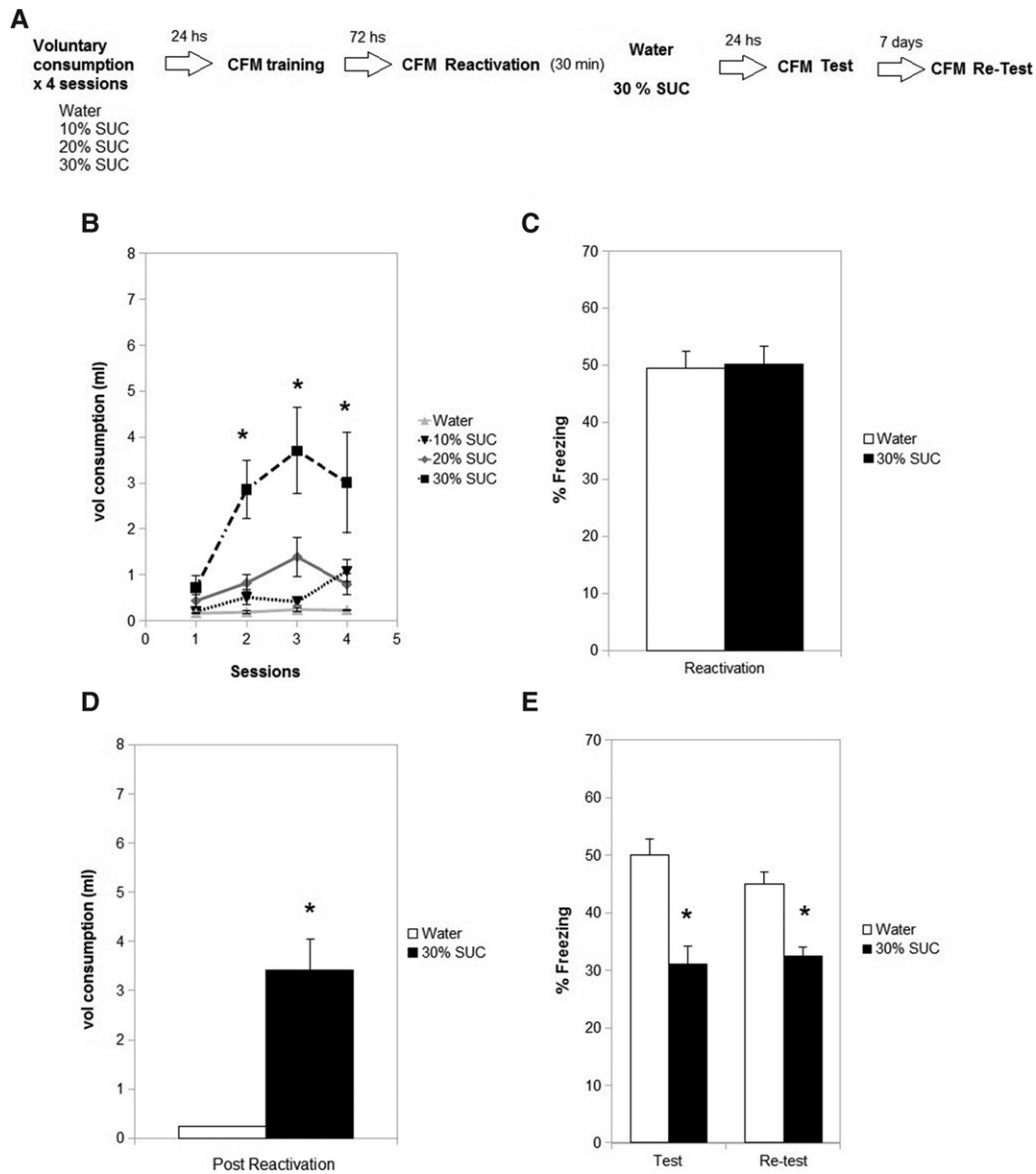
Figure 2. Experiments 2. (*A*) Experimental protocol. Rats were allowed to consume different concentrations of diluted sucrose (SUC) through four sessions (10 min each, 24 h apart). After 24 h, rats from the water and 30% SUC groups were submitted to the CFM training. Following 72 h, animals were reactivated in the training context during 4 min, without shocks. Thirty minutes later, rats were allowed to freely consume water or 30% SUC again. One day and 1 wk later, fear behavior was assessed in the conditioned context. (*B*) Data show the mean ± SEM volume (mL) of SUC consumed. (*C*) Data depict the mean ± SEM of percentage time spent freezing during reactivation. (*D*) Data represent water and 30% SUC consumption 30 min after CFM reactivation. (*E*) Data show freezing behavior for the CFM test (*left* panel) and the retest (*right* panel). (*) Denotes a significant statistical difference between the SUC 30% and water groups.

and seven days later subjects were tested for their freezing behavior in the conditioned context. There was no statistical difference between groups during memory reactivation: $t_{(14)} = 0.14$, $P > 0.05$, $d = 0.07$, suggesting that previous voluntary SUC consumption did not affect the acquisition of the CFM (Fig. 2C). There was a significant difference in voluntary consumption after CFM reactivation: $t_{(14)} = 5.05$, $P < 0.001$, $d = 2.69$, which was expected from previous consumption sessions (Fig. 2D). A repeated-measures ANOVA on fear test and retest data (group and evaluation phases as factors) revealed a significant effect of group [$F_{(1,14)} = 35.23$, $P < 0.0001$, $\eta^2 p = 0.71$], no effect of evaluation phase [$F_{(1,14)} = 1.22$, $P > 0.05$, $\eta^2 p = 0.08$] and no interaction [$F_{(1,14)} = 3.78$, $P > 0.05$, $\eta^2 p = 0.21$]. Post hoc analysis

over the group factor revealed that the water-only group expressed significantly more fear than the SUC group ($P < 0.001$) (Fig. 2E).

These data suggest that the retention of a reactivated fear memory can be affected by an unrelated appetitive experience. Furthermore, this effect remains stable for at least a week.

Influence of a nonrewarding task on fear memory reconsolidation

An alternative interpretation of the previous results could be offered. Animals in the water-control group consumed almost nothing (e.g., licking behavior was minimal or even absent) while animals in the SUC group consumed a substantial amount

(licking behavior was highly expressed). So, it could be argued that any behavior (e.g., licking) might affect the destabilized memory. To overcome this concern, we conducted an experiment that compared the effect of two different tasks on a destabilized fear memory: Open field exploration (OF) or voluntary SUC consumption. The experimental design was identical to experiment 2 B, except that the OF task was compared with SUC consumption (Fig. 3A). No fear retest was used in this experiment. Two behavioral measures were considered for the OF: quadrant crossings, to assess locomotion, and time spent in the central area of the apparatus, to assess anxiety (see Materials and Methods). If our hypothesis is correct, then OF exploration (a nonrewarding task) should not affect fear memory retention.

For the SUC group, a repeated-measures ANOVA on consumption data (session as factor) revealed a significant effect [$F_{(4,24)} = 9.19$, $P < 0.001$, $\eta^2 p = 0.60$]. Follow-up comparisons revealed that only the first session differed from all the others ($P < 0.05$ in all cases), including the one after fear memory reactivation. In other words, peak consumption levels were already present by the second session and fear memory reactivation did not seem to alter SUC consumption (Fig. 3B).

For the group in the OF task, a repeated-measures ANOVA on quadrants crossings (session as factor) revealed a significant effect

[$F_{(4,24)} = 9.46$, $P < 0.001$, $\eta^2 p = 0.61$]. Follow-up comparisons revealed that crossings remained stable throughout the four first sessions ($P > 0.05$ in all cases) and increased significantly after fear memory reactivation ($P < 0.01$) (Fig. 3C). A repeated-measures ANOVA on time spent in central area of the OF indicated a significant effect of session [$F_{(4,24)} = 8.69$, $P < 0.001$, $\eta^2 p = 0.59$] and follow-up analysis indicated that time in central area increased significantly from the first to the second session ($P < 0.05$), remained stable up to the fourth session and finally decreased significantly after fear memory reactivation ($P < 0.01$) (Fig. 3D). Therefore, these data suggest that anxiety-like behavior decreased from session one, but recovered after reexposure to the feared context. This could indicate a mild level of anxiety as a consequence of fear memory reactivation.

Regarding freezing behavior, there were no significant differences between groups at the fear reactivation phase: $t_{(12)} = 0.53$, $P > 0.05$, $d = 0.30$ (Fig. 3E). However, groups differed 24 h later, during fear test: $t_{(12)} = 2.87$, $P < 0.05$, $d = 1.65$. Hence, fear memory retention seems to be vulnerable to appetitive experiences or consummatory behaviors (licking SUC) but not to a neutral experience or even a mildly aversive one (OF exploration avoiding central area) (Fig. 3F). Boccia et al. (2005) reported a similar result with different experimental tasks.
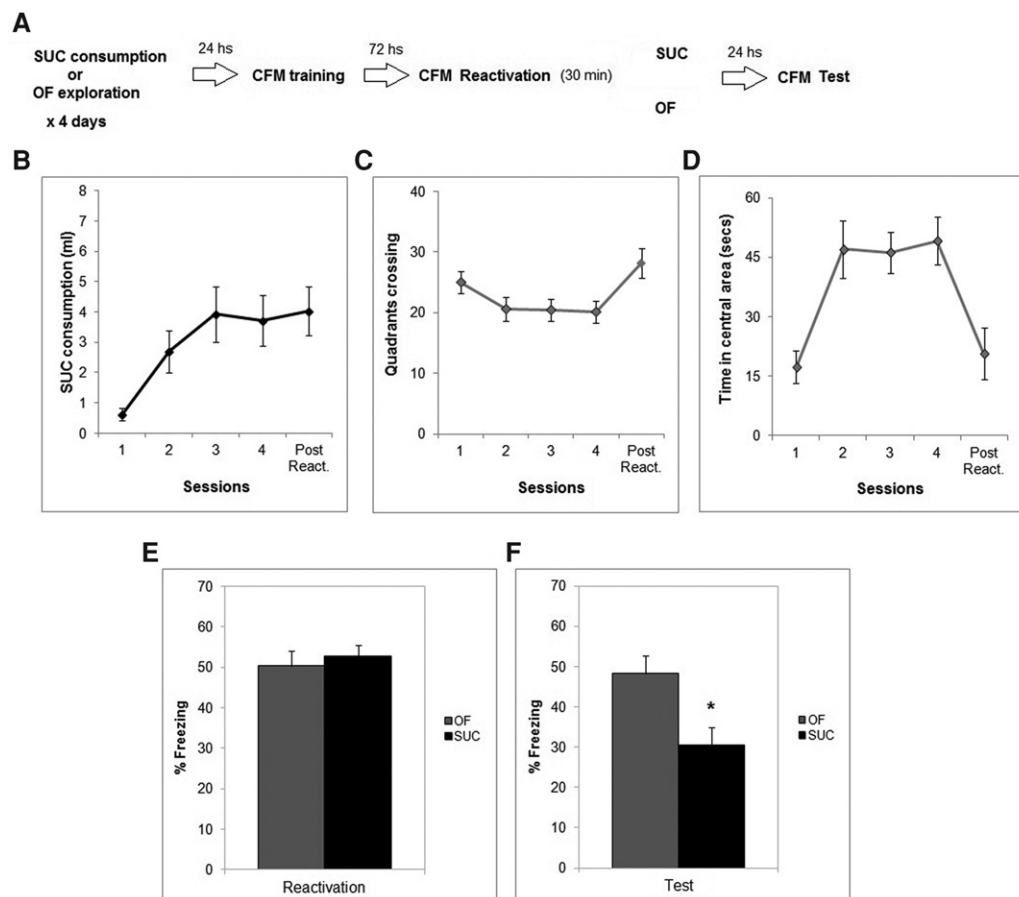
Figure 3. Experiment 2B. (*A*) Experimental protocol. Rats were allowed to consume SUC or were exposed to an open field (OF) apparatus during four sessions, like the previous experiment. After 24 h, rats from both groups were submitted to the CFM training. Following 72 h, animals were reactivated in the training context during 4 min, without shocks. Thirty minutes later, rats were reexposed to the OF apparatus or allowed again to consume SUC. One day later, fear behavior was assessed in the conditioned context. (*B*) Data show the mean ± SEM volume (mL) of SUC consumed. (*C*) Data show the mean ± SEM of quadrants crossed in the OF apparatus. (*D*) Data illustrate the mean ± SEM of time spent in the central area of the OF apparatus. (*E*) Data depict the mean ± SEM of percentage time spent freezing during reactivation. (*F*) Data show freezing behavior for the CFM test. (*) Denotes a significant statistical difference between groups.

Experiment 3

Role of the destabilization process in the influence of the appetitive experience on fear memory

According to the previous results, an appetitive experience can alter the expression of a fear memory after reactivation. However, it is not clear from the above experiments if this memory trace needs to be critically destabilized by its reactivation, as the reconsolidation hypothesis suggests (Flavell et al. 2013). It could be argued that the appetitive experience affects fear memory expression through other mechanisms, thus making memory destabilization not necessary. To test this hypothesis, two groups of rats were allowed to freely consume SUC, as previously described, and submitted to the CFM training 24 h later. Three days after training, animals were subjected to the reactivation session. One group was reactivated for 90 sec (this reactivation does not induce destabilization, according to experiment 1), while the other was reactivated for 4 min (reactivation induces destabilization). After 30 min, SUC was freely available for both groups. The next day, fear testing was conducted (Fig. 4A).

A repeated-measures ANOVA on consumption data revealed a significant effect of session [$F_{(2.94, 44.14)} = 8.37$, $P < 0.001$, $\eta^2 p = 0.36$], no effect of group [$F_{(1,15)} = 0.02$, $P > 0.05$, $\eta^2 p = 0.00$], and no significant interaction [$F_{(2.94, 44.14)} = 0.49$, $P > 0.05$, $\eta^2 p = 0.03$] (Fig. 4B). Follow-up analysis on the effect of session revealed that SUC consumption increased from the first to the second session ($P < 0.01$) and remained stable throughout the rest of the experiment, replicating previous results. Freezing behavior during reactivation was not compared statistically since both groups had different reactivation lengths. However a visual inspection of the reactivation data shows similar levels of freezing (Fig. 4C). Both groups expressed statistically different levels of freezing at test: $t_{(15)} = 3.79$, $P < 0.01$, $d = 1.95$ (Fig. 4D). These results can then be explained through the destabilization process (induced by a 4 min, but not by a 90-sec memory reactivation session), since there were no differences between groups in SUC consumption either before or after reexposure to the conditioned context.
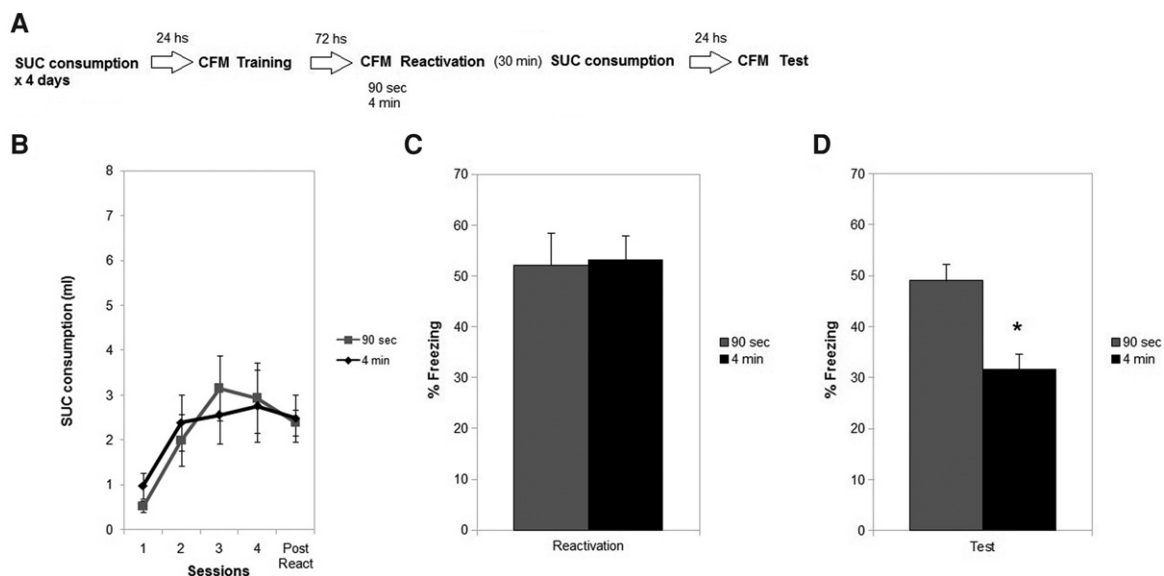
Experiment 4

*Isolated effects of the appetitive experience or the reactivation session*

A standard procedure in reconsolidation studies is to control the effects of the interfering manipulation and those of the reactivation session (Nader and Hardt 2009). Accordingly, four groups of rats were allowed to consume sucrose and later trained in the CFM. After 3 d, one group received a 4-min reactivation session followed 30 min later by free access to sucrose (R + SUC group). Another group received only the memory reactivation session (R group), a third group was solely allowed to consume sucrose (SUC group), and the control group remained untreated. All four groups were tested for their fear behavior 24 h later (Fig. 5A).

A repeated-measures ANOVA on consumption data revealed a significant effect of session [$F_{(2.3, 50.59)} = 16.63$, $P < 0.001$, $\eta^2 p = 0.43$], no effect of group [$F_{(3,22)} = 0.26$, $P > 0.05$, $\eta^2 p = 0.03$], or significant interaction [$F_{(6.89, 50.59)} = 1.12$, $P > 0.05$, $\eta^2 p = 0.13$] (Fig. 4B). Follow-up analysis on the effect of session revealed that sucrose consumption increased from the first to the second session ($P < 0.01$) and remained stable throughout the rest of the experiment. There were no differences between R + SAC and R groups during fear reactivation: $t_{(12)} = 0.40$, $P > 0.05$, $d = 0.23$ (Fig. 5C). At the same time, no significant difference in sucrose consumption was observed between the SUC and the R + SUC groups: $t_{(11)} = 1.31$, $P > 0.05$, $d = 0.71$ (Fig. 5D). Finally, regarding fear test data, a factorial ANOVA (fear reactivation and consumption as factors) revealed a main effect of reactivation [$F_{(1,22)} = 7.37$, $P < 0.05$, $\eta^2 p = 0.25$], consumption [$F_{(1,22)} = 5.37$, $P < 0.05$, $\eta^2 p = 0.19$] and a significant reactivation × consumption interaction [$F_{(1,22)} = 5.89$, $P < 0.05$, $\eta^2 p = 0.21$]. Post hoc analysis revealed that the R + SUC group expressed significantly less fear than the other groups ($P < 0.01$ in all cases), which did not differ among them ($P > 0.05$) (Fig. 5E).

In sum, these data suggest that destabilization or sucrose consumption on their own do not affect fear behavior at testing, at least under the present experimental conditions. Both memory

Figure 4. Experiment 3. (*A*) Experimental protocol. Rats were allowed to freely consume 30% SUC through four consecutive sessions spaced by 24 h. One day later, rats were trained in the CFM protocol. Three days later, half of the animals were reactivated with a 4-min session and the other half with a 90-sec session. After 30 min, rats were allowed to freely consume 30% SUC. One day later, fear was assessed at testing. (*B*) Data show the mean ± SEM volume (mL) of SUC consumed. (*C*) Data depict the mean ± SEM of percentage time spent freezing during reactivation. (*D*) Data show freezing behavior in the CFM test. (*) Denotes a significant statistical difference between groups.
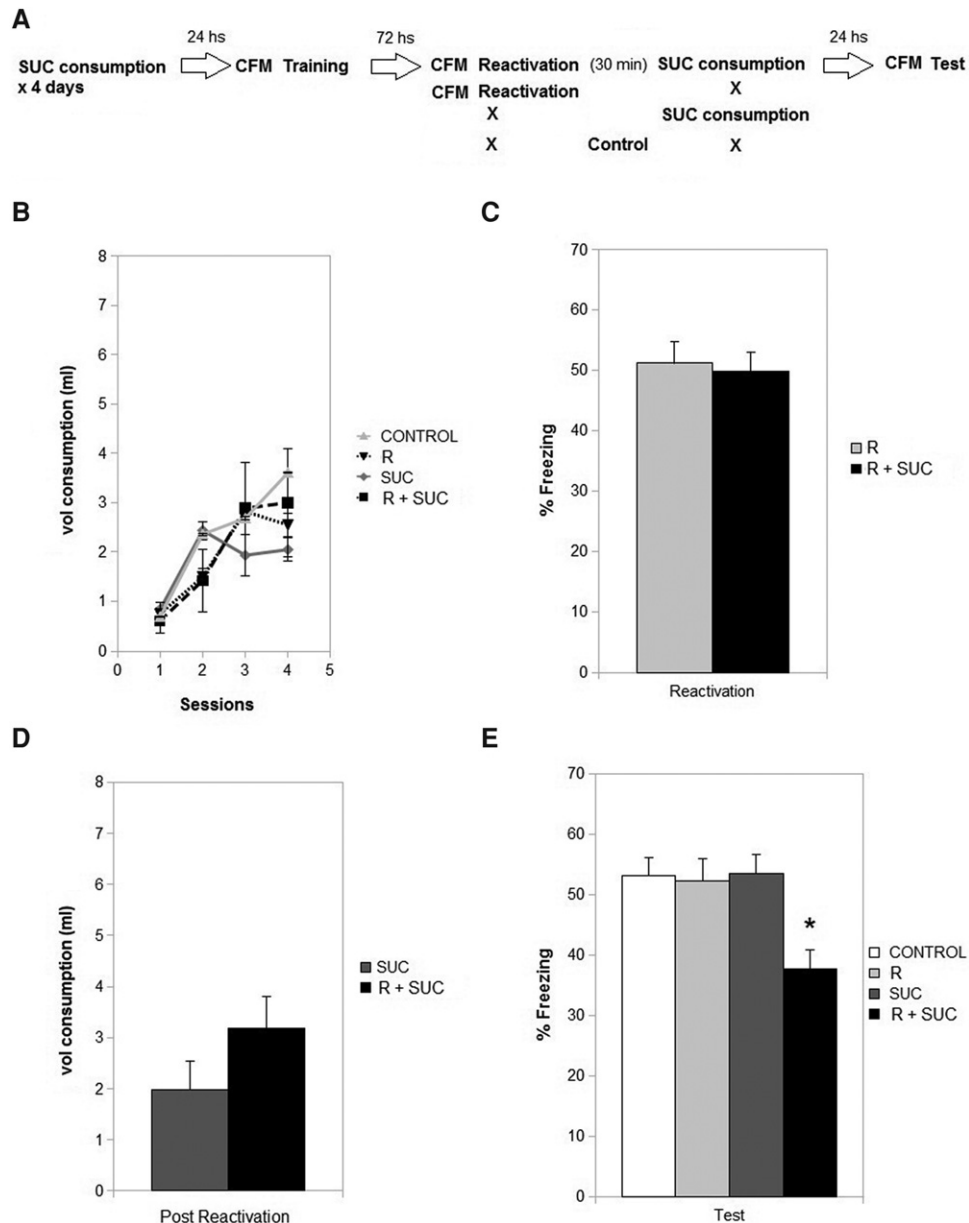
Figure 5. Experiment 4. (*A*) Experimental protocol. Rats were allowed to freely consume sucrose through four sessions and 1 d later they were trained in the CFM. After 3 d, animals from one group were submitted to a 4-min CFM reactivation followed 30 min later by sucrose consumption (R + SUC). A second group underwent only CFM reactivation (R), while a third one received solely sucrose consumption (SUC). Finally, a fourth control group remained untreated (control). One day later, fear behavior of all groups was assessed in the test session. (*B*) Data show the mean ± SEM volume (mL) of consumed sucrose. (*C*) Data depict the mean ± SEM of percentage time spent freezing during reactivation. (*D*) Data represent sucrose consumption after CFM reactivation. (*E*) Data show freezing behavior in the CFM test. (*) Denotes a significant statistical difference between the R + SUC condition and the rest of the groups.

destabilization and the appetitive experience must co-occur in order to affect fear memory retention.

Experiment 5

Effects of the appetitive experience during or after the fear reconsolidation window

It is widely accepted that the reconsolidation process is time-dependent. Once the memory becomes labile after its reactivation, it will take a limited amount of time to restabilize (Nader

and Hardt 2009). The "reconsolidation window" refers to the fact that the amnesic gradient decreases with the interval between reactivation and the experimental procedure (Przyby-slawski and Sara 1997). For contextual fear memories, the reconsolidation window has been reported to be closed after at least 2 h (Bustos et al. 2006; Piñeyro et al. 2014; Alfei et al. 2015).

If the effect reported in the above experiments is mediated through destabilization process, then it would be reasonable to expect that increasing the interval between fear memory reactivation and sucrose consumption would result in a reduced effect. This experiment was designed to test this hypothesis.

Accordingly, three groups of rats were allowed to consume sucrose and received contextual fear conditioning, as described in previous experiments. Three days after fear training, the reactivation took place followed by voluntary sucrose consumption either after 30 min, 1 h, or 6 h. One day later, all groups were subjected to the fear test (Fig. 6A).

A repeated-measures ANOVA on consumption data revealed a significant effect of session [$F_{(2.43,51.17)} = 18.85$, $P < 0.001$, $\eta^2 p = 0.47$], no effect of group [$F_{(2,21)} = 0.06$, $P > 0.05$, $\eta^2 p = 0.00$], or significant interaction [$F_{(4.87,51.17)} = 0.63$, $P > 0.05$, $\eta^2 p = 0.05$] (Fig. 4B). Follow-up analysis on the effect of session revealed that sucrose consumption increased from the first to the second session ($P < 0.01$) and remained stable throughout the rest of the experiment. A one-way ANOVA over fear reactivation data revealed no effect of group [$F_{(2,21)} = 0.05$, $P > 0.05$, $\eta^2 p = 0.00$] (Fig. 6C). However, there was a significant effect of group during the fear test [$F_{(2,21)} = 7.65$, $P > 0.01$, $\eta^2 p = 0.42$]. The post hoc analysis showed that the 30 min and 1 h groups were not statistically different from each other, but both differed from the 6 h group ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 6D).

In sum, this result indicates that extending the time interval between CFM reactivation and sucrose consumption reduces the effects of the appetitive experience on fear memory retention, adding further support to our hypothesis.

Experiment 6

### Effects of the appetitive experience after memory destabilization or extinction

An alternative explanation of the above results could be offered, since we consistently observed a decrease in the conditioned response at testing. This decrease took place after nonreinforced reactivations, which is the standard procedure to induce extinction learning (Pavlov 1927). It is generally accepted that extinction represents a form of new learning (CS–no US), which interferes with the expression of the original association (CS–US), but does not erase the original memory (Bouton 2002, 2004). It could be argued that the reduced fear observed at testing can be explained as a weak extinction learning (induced by a nonreinforced 4-min reactivation of the fear memory) enhanced through sucrose consumption, given the enhancing effects of glucose in memory (Rodriguez et al. 1999). Alternatively, the sucrose consumption could also act as an unspecific interference procedure, affecting any on-going and learning-related plasticity process (e.g., reconsolidation of a fear memory or consolidation of extinction).

Therefore, two possibilities can be predicted: (a) If sucrose consumption is enhancing a weak extinction process, then normal extinction learning might also be enhanced by sucrose or become more resistant to recovery; or (b) if sucrose consumption acts interfering with any on-going learning process, then it would impair both reconsolidation and consolidation of extinction. The present experiment was conceived to address these possibilities. We used a reinstatement protocol, which consists in presenting the US in the acquisition context after extinction, which typically leads to the reemergence of the conditioned response (Urcelay, 2012). All relevant groups were included in this experiment to avoid a cross-experimental comparison.

Rats were allowed to freely consume sucrose and then were fear conditioned, just as in the above experiments. After 3 d, one group was reactivated for 4 min in the training context without further treatment (R). Another group was reactivated and allowed to consume sucrose after 30 min (R + SUC). A third group received a prolonged reactivation session of 15 min to induce extinction learning (E). The fourth group also received extinction learning, followed 30 min later by the possibility of consuming sucrose (E + SUC). A fifth group remained untreated (control). All groups were subjected 1 d later to the conditioned context for 3 min, at the end of which a weak shock (0.5 mA, 3 sec) was administered, since this procedure has been previously shown in our laboratory to recover extinguished conditioned responses of fear memories (Piñeyro et al. 2014). The day after, all groups were submitted to the final fear test (Fig. 7A).

A repeated-measures ANOVA on consumption data revealed a significant effect of session [$F_{(3,105)} = 25.98$, $P < 0.001$, $\eta^2 p = 0.42$], no effect of group [$F_{(4,35)} = 0.24$, $P > 0.05$, $\eta^2 p = 0.02$] or significant interaction [$F_{(12,105)} = 0.66$, $P > 0.05$, $\eta^2 p = 0.07$]
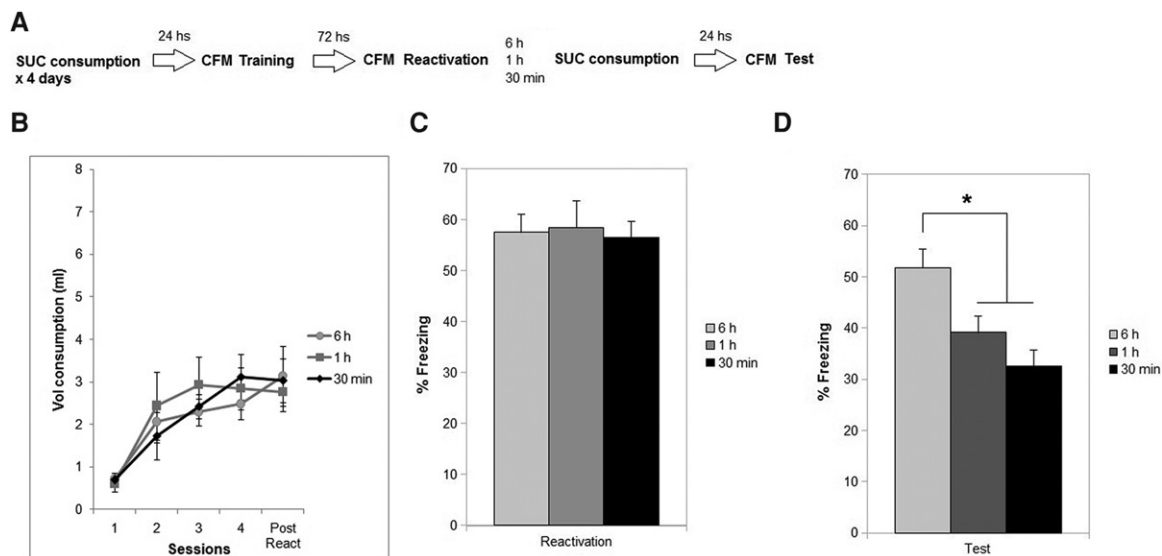
Figure 6. Experiment 5. (*A*) Experimental protocol. Rats were allowed to freely consume 30% SUC through four sessions and 1 d later they were trained in the CFM. Three days later, animals were exposed to the 4-min reactivation session followed by SUC consumption either after 30 min, 1 h, or 6 h. One day later, fear behavior was assessed in the test session. (*B*) Data show the mean ± SEM volume (mL) of consumed SUC. (*C*) Data depict the mean ± SEM of percentage time spent freezing during CFM reactivation. (*D*) Data show freezing behavior in the CFM test. (*) Denotes a significant statistical difference between the 6 h condition and the other two groups.
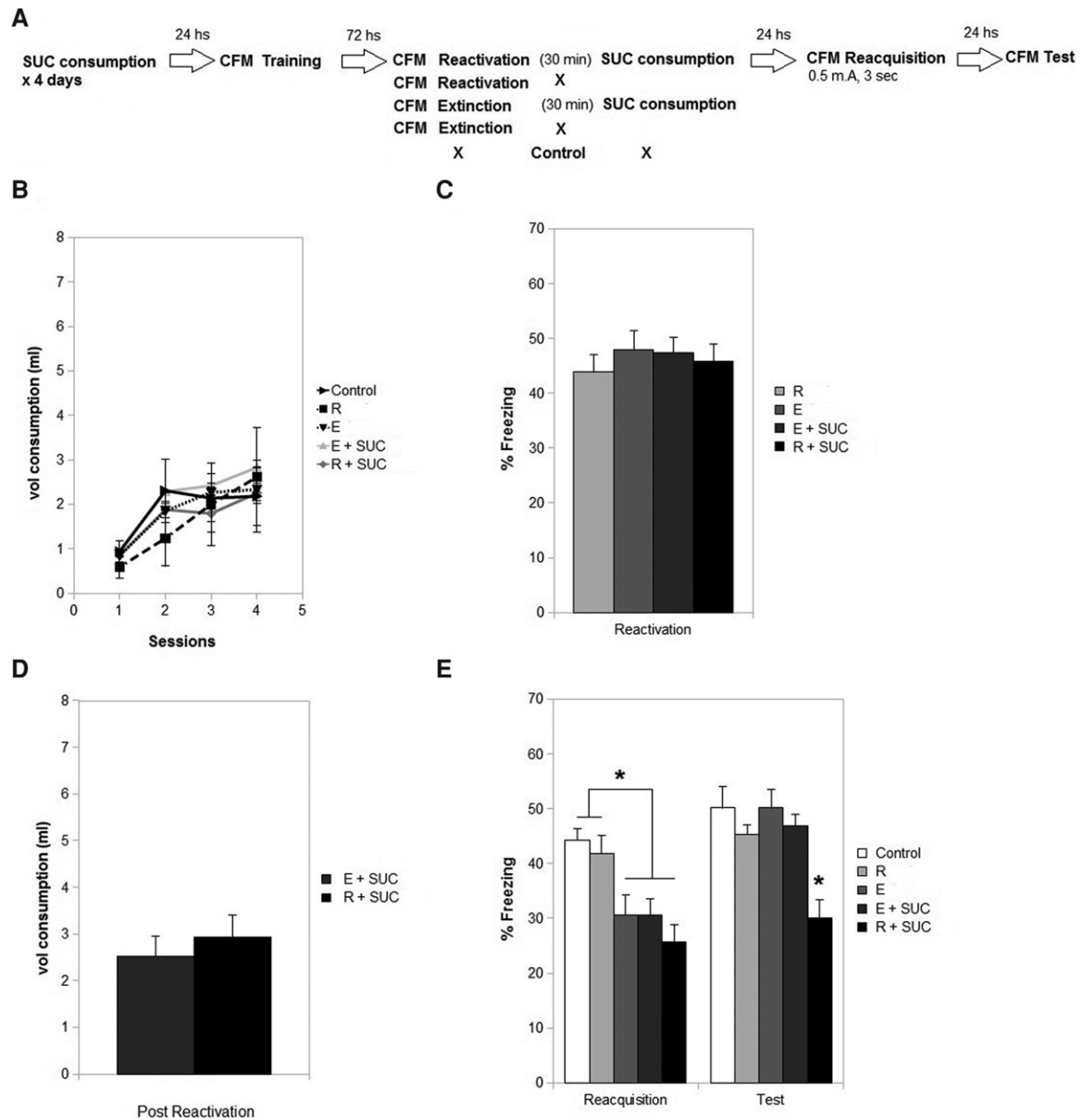
Figure 7. Experiment 6. (*A*) Experimental protocol. Rats were allowed to freely consume 30% SUC through four sessions and 1 d later they were trained in the CFM. Three days later, animals were either submitted to CFM reactivation with 4 or 15 min of context exposure or remained untreated (control). Half of the animals in each reactivated condition were allowed to consume SUC and the other half not. The day after, fear behavior was assessed in the conditioned context during 3 min for all groups and immediately after a mild shock was administered. One day later, fear behavior was again evaluated in the conditioned context (test). (*B*) Data show the mean ± SEM volume (mL) of consumed SUC. (*C*) Data depict the mean ± SEM of percentage time spent freezing during CFM reactivation. (*D*) SUC consumption after CFM reactivation. (*E*) Data shows freezing behavior in conditioned context during the pre-shock phase of reinstatement (*left* panel) and during the final CFM test (*right* panel). (*) Denotes a significant statistical difference between groups.

(Fig. 4B). Follow-up analysis on the effect of session revealed that sucrose consumption increased from the first to the second session ($P < 0.01$) and remained stable throughout the rest of the experiment. (Fig. 7B). There were no differences between groups submitted to fear reactivation: $t_{(14)} = 0.51$, $P > 0.05$, $d = 0.27$. There were no significant differences either between groups receiving fear extinction: $t_{(14)} = 0.25$, $P > 0.05$, $d = 0.13$ (Fig. 7C). Finally, there were no significant differences in sucrose consumption after either a 4 min or a 15-min reactivation, since both R + SUC and E + SUC groups consumed an equivalent amount: $t_{(14)} = 0.41$, $P > 0.05$, $d = 0.21$ (Fig. 7D).

A one-way ANOVA (group as factor) on the data from the pre-shock period of the reinstatement phase revealed a significant effect of group [$F_{(4,35)} = 10.25$, $P < 0.0001$, $\eta^2 p = 0.53$]. Post hoc analysis revealed that the R and Control groups did not differ ($P > 0.05$), which indicates that the sole reactivation of the fear memory does not affect its behavioral expression, replicating findings from experiment 4. At the same time, both groups expressed significantly more fear than the remaining three (R + SUC, E and E + SUC) ($P < 0.01$ in all cases), which did not differ from each other. This implies that both extinction and destabilization followed by sucrose consumption are capable of diminishing

conditioned fear. Also, these results indicate that sucrose consumption does not enhance extinction learning, since groups E and E + SUC did not differ from each other ($P > 0.05$) (Fig. 7D, left panel). Although fear expression levels did not decline during the 15-min context reexposure (groups E and E + SUC), this does not mean that extinction learning was not achieved. In fact, fear expression was reduced for both groups 24 h later when compared with the nonreactivated control or the group receiving a shorter reactivation (4 min, group R). Hence, although extinction was not expressed within the reactivation session, it actually took place. This in line with reports both in rodents (Plendl and Wotjak 2010) and humans (Prenoveau et al. 2013), showing that reduction of fear responding during reexposure to conditioned stimuli is not necessary for or indicative of the strength of extinction learning when evaluated after 24 h.

It is still possible that sucrose-enhanced extinction is manifested not as a more profound fear reduction, but as a resistance to reinstatement after reassociating the context with a mild shock. However, data from the test phase (24 h after reinstatement) does not support this prediction. A one-way ANOVA on the test data revealed a significant effect of group [$F_{(4,35)} = 8.44$, $P < 0.0001$, $\eta^2 p = 0.41$]. The post hoc analysis revealed that the R + SUC group expressed significantly less fear than any of the other groups ($P < 0.001$ in all cases), which did not differ among themselves ($P > 0.05$) (Fig. 7D, right panel).

In sum, this set of data suggests that sucrose consumption did not exert its effects enhancing extinction learning, whether this is expressed as more reduced conditioned responding or as a resistance to reinstatement. At the same time, these results indicate that sucrose consumption did not exert its effects as a general interfering procedure, able to disrupt any ongoing learning-related plasticity process. Furthermore, the only condition under which fear responding did not reemerge after reinstatement was when fear memory destabilization was followed by sucrose consumption.

Experiment 7

Involvement of the GluN2B–NMDA receptors within the basolateral amygdala on the effect of the appetitive experience on fear memory expression

Several studies support the view that glutamatergic signaling mechanisms at the basolateral complex of the amygdala (BLA) are critically involved in both fear memory destabilization and reconsolidation. Particularly, there is evidence suggesting that the GluN2B-NMDA receptor subtype in this particular brain area is essential for fear memory destabilization (Wang et al. 2009). In fact, various findings show that intra-BLA infusion of Ifenprodil (IFN, a GluN2B-NMDA antagonist) prevented fear memory destabilization (Ben Mamou et al. 2006; Milton et al. 2013). Considering that the experiments reported in this work point toward memory destabilization as the mechanism behind the effect under study, we investigated the involvement of GluN2B-NMDA sites in the BLA in the influence of the appetitive experience on the conditioned fear memory.

In this experiment, four groups of rats were bilaterally cannulated at the BLA as previously reported (Giachero et al. 2013). After recovery, all animals underwent voluntary sucrose consumption and fear training, as in previous experiments. After 72 h, subjects were administered intra-BLA with either IFN or vehicle (VEH). Immediately after, half of the animals in each condition were submitted to fear memory reactivation and 30 min later allowed to freely consume sucrose (IFN and VEH groups, respectively). The remaining two groups were subjected to the same procedure, except that fear reactivation was omitted and animals were allowed to consume sucrose freely (IFN-NR and VEH-NR groups). Total

time between infusion and sucrose consumption was held constant between groups. One day after, all groups were submitted to the test session to assess fear behavior (Fig. 8A).

A repeated-measures ANOVA on consumption data revealed a significant effect of session [$F_{(2.74,68.58)} = 39.61$, $P < 0.001$, $\eta^2 p = 0.61$], no effect of group [$F_{(3,25)} = 0.35$, $P > 0.05$, $\eta^2 p = 0.04$] or significant interaction [$F_{(8.23,68.58)} = 0.42$, $P > 0.05$, $\eta^2 p = 0.04$] (Fig. 8B). Follow-up analysis revealed that sucrose consumption increased significantly from session to session, including the one after fear memory reactivation ($P < 0.05$ in all cases). There were no significant differences between IFN and VEH groups during fear reactivation: $t_{(13)} = 0.33$, $P > 0.05$, $d = 0.19$ (Fig. 8C). Confirming previous reports (Milton et al. 2013), IFN intra-BLA infusion does not affect the behavioral expression of the reactivated fear memory when compared with the VEH condition. Also, the current data suggest that neither intra-BLA infusion of IFN or fear reactivation affected voluntary SUC consumption.

A factorial ANOVA (drug and fear reactivation as factors) of the fear test data revealed a significant effect of drug [$F_{(1,25)} = 13.38$, $P < 0.01$, $\eta^2 p = 0.34$], reactivation [$F_{(1,25)} = 16.17$, $P < 0.001$, $\eta^2 p = 0.39$] and a significant interaction between both factors [$F_{(1,25)} = 9.07$, $P < 0.01$, $\eta^2 p = 0.26$]. Post hoc analysis revealed that the VEH group was the only one expressing less fear at testing compared with the remaining three ($P < 0.001$ in all cases) (Fig. 8D). Moreover, IFN-treated animals showed similar fear values to nonreactivated animals, suggesting that the appetitive experience only affects fear memory after reactivation-induced instability mediated by a GluN2B-NMDA-dependent process within the BLA complex.

General discussion

The main finding reported in this study is that exposure to an unrelated appetitive experience following fear memory reactivation reduces fear retention. This effect was noticeable even after 1 wk. Importantly, such reduction was not observed with a nonrewarding task. These results cannot be explained as enhanced extinction learning or a general interfering effect of sucrose on learning processes. Our results also show that this effect takes place as long as the appetitive experience occurs after the destabilization process initiated by fear memory reactivation, mediated through GluN2B-NMDA receptors within the BLA complex.

An unexpected finding in this study was that fear memory reactivation apparently induced anxiety, as measured in the OF apparatus. It is worth noting that animals in the OF group of experiment 2-c showed a habituation pattern along the four sessions. Exploration decreased (although not significantly) from session 1, as well as anxiety (as measured by time spent in the central area of the apparatus). However, fear memory reactivation seems to alter such behavioral patterns back to session 1 levels: animals augmented exploration away from the central area. Although there was no control group for this unexpected effect (like a nonreactivated group submitted only to the OF for a fifth session), this is consistent with recent unpublished results from our group. Furthermore, they suggest that fear memory retrieval might function as an unpleasant experience, functionally equivalent to an unconditioned stimulus (Giachero et al. 2013).

Although our results demonstrate the critical importance of memory destabilization for the appetitive experience to alter conditioned fear, we do not know what precise mechanism is responsible for the fear reduction, limiting the conclusions that can be offered from this data set. A candidate mechanism is "counterconditioning," which consists in pairing an emotionally valenced CS with a (new) US of opposite valence (Pavlov 1927). This procedure can lead to either proactive interference or retroactive interference on the acquisition or expression of
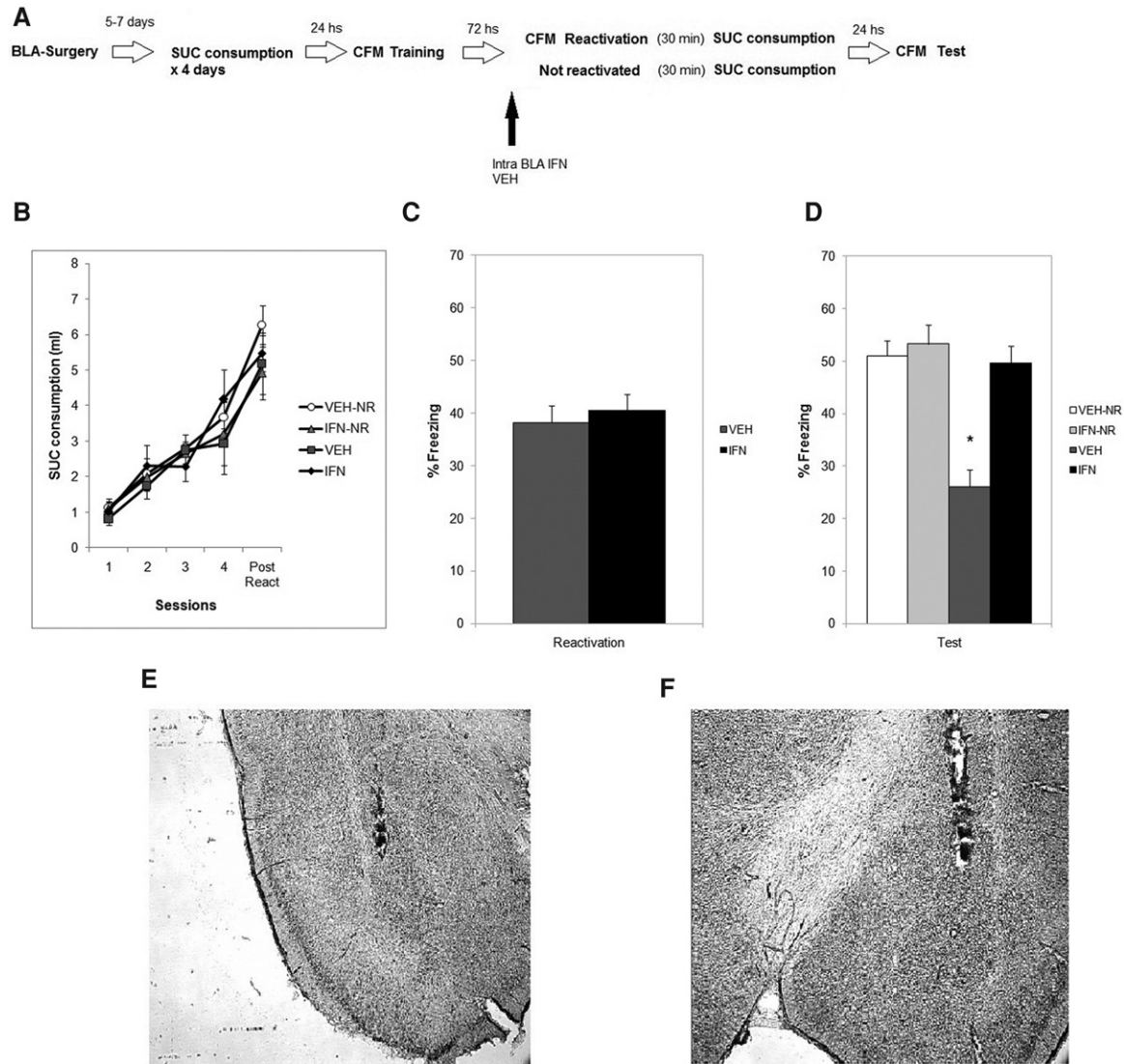
Figure 8. Experiment 7. (*A*) Experimental protocol. After cannulation aimed at the BLA (from bregma: anterior, −3.0 mm; lateral, ±5.0 mm; ventral, −6.2 mm) and following a 5–7 d recovery period, rats were allowed to freely consume 30% SUC through 4 sessions and 1 d later trained in the CFM protocol. Three days later, animals were administered Ifenprodil (IFN, 2 μg/μL) or vehicle (VEH) intra-BLA. A total of 1 μL was administered in either condition. Immediately after, half of the rats in each condition were reactivated for 4 min and 30 min later allowed to consume SUC. The other half was not reactivated (NR) but was allowed to consume SUC. All groups were tested for freezing behavior 24 h later. (*B*) Data show the mean ± SEM volume (mL) of SUC consumed. (*C*) Data depict the mean ± SEM of percentage time spent freezing during CFM reactivation. (*D*) Data show freezing behavior during the CFM test. (*E,F*) Photomicrographs of coronal brain sections showing infusion site locations in the BLA. (*) Denotes a significant statistical difference between the group receiving VEH and the rest of the groups.

the second or first association (Bouton 1993). Richardson et al. (1982, 1987), using a similar approach to that reported here but with different experimental tasks, arrived to similar results. The authors argued that counterconditioning procedures can change the hedonic value of the information contained in the reactivated memory.

An alternative interpretation is "memory updating." Several authors have speculated that an essential function of memory destabilization is to allow the updating of memories with novel environmental information (Pedreira et al. 2004; Hupbach et al. 2007; Lee 2010; Forcato et al. 2010; Finnie and Nader 2012; Sevenster et al. 2013; Díaz-Mataix et al. 2013). In addition, reactivation-induced destabilization is a necessary requirement for updating to take place (Hupbach et al. 2008; Lee 2010; De Oliveira Alvares et al. 2013). This implies that new information

is actually incorporated to the reactivated trace, then leading to an altered response or even to new responses. Olshavsky et al. (2013) found that pairing a previously appetitive CS with foot shocks retarded later reacquisition of the appetitive response. Haubrich et al. (2015) recently demonstrated that when a fear-conditioned context was reassociated with the presence of appetitive food, the conditioned fear response was substantially reduced. Finally, in an elegant and ground-breaking paper using optogenetic techniques to directly activate neurons involved in memory acquisition, Redondo et al. (2014) bidirectionally changed the hedonic value of both appetitive and aversive memories. In that study, authors reported that appetitive responses actually appeared in an aversive context, if the memory of such context was previously reactivated (with optogenetic stimulation) while subjects where submitted to appetitive information.

Another possibility is that both the fear memory and the memory of the appetitive experience might become linked, forming a new combined mnemonic trace responsible for the reduction we observed in fear behavior during testing. In fact, a recent report using optogenetic technology showed that this is conceivable. Ohkawa et al. (2015) demonstrated that independent contextual and aversive information can become integrated into a single NMDA and protein synthesis-dependent mnemonic trace. This integrated new memory seems to be formed through coactivation of both traces, in a hebbian-like mechanism. This possibility was advanced by Lewis (1979), who suggested that independent memories in active states might become associated with each other.

Based on the current evidence, we cannot conclude which is the precise mechanism responsible for the reduction of fear memory retention reported in this study. In fact, none of the experiments described in this work were designed to answer such question and new experiments are necessary to unveil what type of neurocognitive processes might be responsible for the observed effect.

Our results demonstrate that the onset of memory destabilization is a prerequisite for the effect of the appetitive experience on the reactivated fear memory. This pattern is coherent with previous results from our laboratory (Piñeyro et al. 2014) and with others that indicate the critical importance of memory destabilization in order to be able to affect memory traces, both in rodents (Lee et al. 2008) and humans (Sevenster et al. 2012, 2013). Haubrich et al. (2015) and the present findings revealed that those reactivation sessions that were unable to destabilize the fear memory preclude the influence of the appetitive experience. Moreover, there is agreement regarding the pivotal role of the GluN2B subunit of the NMDA receptor in the BLA in fear memory destabilization (Ben Mamou et al. 2006; Wang et al. 2009; Milton et al. 2013). The intra-BLA administration of a selective antagonist of this NMDA receptor subtype prevents the instability induced by fear memory reactivation (Ben Mamou et al. 2006; Milton et al. 2013). In support of this view, the present results showed that preventing memory destabilization with IFN infusion into the BLA, a primary site for fear memory formation and retrieval (LeDoux 2000; Pape and Pare 2010), prevented the influence of the appetitive experience on conditioned fear.

In summary, the evidence presented here along with other reports suggests that understanding and manipulating memory destabilization is a promising path for an effective and noninvasive treatment of pathological memories: neural representations of past experiences that lead to exaggerated or maladaptive emotional responses, whether the subject is aware or not of the elicitation of those responses or the circumstances that lead to their acquisition (Lane et al. 2015). If memory destabilization is achieved, then extinction learning (Monfils et al. 2009; Clem and Huganir 2010; Piñeyro et al. 2014) or contrasting emotional experiences (Wang et al. 2008; Zhao et al. 2009; Olshavsky et al. 2013; Haubrich et al. 2015) may profoundly affect the fate of the memory trace.

Materials and Methods

Subjects

Subjects were experimentally naïve, adult male Wistar rats (60–65 d old, weighing 270–320 g at the beginning of the experiments). Animals were bred in our colony in the Laboratorio de Psicología Experimental, Facultad de Psicología, Universidad Nacional de Córdoba, Argentina. All animals were housed in standard laboratory Plexiglas cages (60 cm long × 40 cm wide × 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 a.m.), at a room temperature of 21°C–23°C. The standards of the NIH Guide for the Care and Use of Laboratory Animals were respected. The number of animals and their suffering was kept to the minimum possible to achieve the goals of this research.

Drugs

Midazolam (MDZ, Gobbi Novag SA, Buenos Aires) was diluted in sterile isotonic saline (SAL, 0.9% w/v) to a concentration of 3 mg/mL, and administered intraperitoneally (i.p.). The total volume of drug or equivalent amount of SAL was 1.0 mL/kg in all cases (Piñeyro et al. 2014). Ifenprodil (IFN, Sigma) was dissolved in sterile isotonic saline as vehicle (VEH) to a concentration of 2 µg/µL and administered into the Basolateral Amygdala (Ben Mamou et al. 2006; Milton et al. 2013). The total amount of IFN or VEH administered was 1 µL.

Apparatus

Contextual fear conditioning was conducted in a 24 cm long × 22 cm 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 foot shocks (Automatic Reflex Conditioner 7501, Ugo Basile). The entire chamber was cleaned with water and dried with paper towels before and after all subjects. Recording of behavior (for off-line analysis) was made with a DCR-SR21 Sony Handycam digital video camera placed 50 cm above the conditioning chamber. Background noise was supplied with ventilation fans. All CFM procedures were performed in a sound-insulated experimental room separated from the colony room.

Voluntary sucrose consumption (SUC) was carried out in individual 40 cm long × 30 cm wide × 20 cm high plastic chambers, with a removable bar ceiling that allowed bottle placement for liquid consumption. The SUC chambers were cleaned with water and dried with paper towels before and after all subjects. SUC chambers were always placed in groups of 4 on a work bench inside the colony room. SUC and fear chambers were located in clearly distinct environments to avoid contextual overlapping between tasks.

Open field exploration (OF) was carried out in a circular, 100 cm diameter × 50 cm high, black painted plastic tank. The entire apparatus was cleaned with water and dried with paper towels before and after all subjects. The OF was always placed inside the colony room, like SUC chambers.

Behavioral procedures

In all experiments, rats were identified, weighed and handled for 5 min on three separate days to habituate them to experimental manipulation. In the experiments involving voluntary SUC consumption (experiments 2–7), animals were habituated to the contextual stimuli where the consumption sessions would take place by exposing the home-cages 1 h/day for 2 d to the work bench in the colony room before identification and handling. Rats participating in experiments involving i.p. administration were injected with 1ml/kg SAL after handling was complete to habituate them to this procedure.

Contextual fear conditioning: to form a contextual fear memory (CFM), rats were taken out individually from their home-cage, transported into the experimental room and exposed to the conditioning chamber for 3 min (preshock period), after which two foot shocks (1.0 mA, 3-sec duration, with an intershock interval of 30 sec) were delivered, serving as unconditioned stimulus (US). Immediately after the second shock ended, rats were removed from the chamber, transported back to the colony room and replaced in their home cages.

CFM reactivation session: reactivations were always carried out 72 h after conditioning. Rats were reexposed to the conditioning chamber, without foot shock, for different periods of time (90 sec, 4 or 15 min, depending on the experiment) and transported back to the home cages afterward.

CFM reinstatement: reinstatement was used only in experiment 6 and was carried out 24 h after CFM reactivation. It consisted of a 3-min preshock period followed by only one 3-sec shock of 0.5 mA (half the number [2] and intensity [1.0] of US compared with initial conditioning), after which rats were immediately removed from the chamber and taken back to their home cages in the colony room.

CFM test: consisted of a 5-min exposure session to the conditioning chamber, without shock, 24 h after the last experimental treatment.

CFM retest: identical to test, but 7 d later (only used in experiment 2).

CFM behavioral scoring: all experiments were video-taped for later off-line analysis. Freezing behavior, defined as the total absence of body and head movements except for that associated with breathing, 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).

Voluntary sucrose (SUC) consumption: SUC consumption sessions lasted 10 min each and consisted of placing the animal in the consumption chamber with only one bottle. This procedure was carried out four times, each 24-h apart, to reduce neo-phobia. Animals were not deprived at any time to avoid stress, which can affect the destabilization of CFM (Bustos et al. 2010). Hence, consumption was entirely voluntary. Sessions were conducted in individual chambers, using four chambers at the same time, on a workbench inside the colony room. All the animals in the same home-cage were run at the same time. After completion of this phase, animals were submitted to the contextual fear conditioning. A fifth SUC consumption session was carried out in most experiments and for most groups. In every case, this last SUC consumption occurred individually, preceded by a CFM reactivation (experiments 2–7), an intra-BLA drug infusion (experiment 7) or in isolation (experiment 4, group only SUC). SUC concentrations (10%, 20%, or 30%, w/v) were made with commercially available sugar. Consumption levels were determined by weighing bottles before and after each session.

Open field (OF) exploration: animals that underwent this protocol received four OF sessions, 10 min each, 24 h apart. A fifth session was used 30 min after CFM reactivation. Exploration was assessed by two means: quadrants crossings and total time spent in the central area of the apparatus. For quadrants crossings, the apparatus was divided into four virtual quadrants and every time the four paws of the animal crossed the dividing lines a crossing was counted. The central area was determined by dividing the apparatus in two virtual concentric rings. Diameter of the inner ring (central area) was of 50 cm and the remaining 50 cm represented the outer ring. A stopwatch was initiated every time the animal entered with his four paws into the central area and stopped when the animal abandoned such zone. Behavior was videotaped from above and analyzed later off-line.

Surgery, intracranial infusion and histological procedures

Intra-basolateral amygdala (BLA) cannula implantation procedures were described in detail in Giachero et al. (2013). The coordinates used were (from bregma): anterior, -3.0 mm; lateral, ± 5.0 mm; ventral, -6.2 mm (Paxinos and Watson 2009), with only those animals with adequate injection sites being considered for statistical analysis. Stereotaxic surgery was performed in a stereotaxic (Stoelting) device. Behavioral protocols started after a 5–7 d recovery period. Intra-BLA infusion procedures were also previously described in Giachero et al. (2013). Briefly, animals were bilaterally infused with IFN or VEH at a 0.25 μL/min rate with 30 gauge infusion cannulas that protruded 2.0 mm beyond the guide cannulas. These infusion cannulas were connected to 10 μL microsyringes (Hamilton) through polyethylene tubing (PE 10, Becton Dickinson). Microsyringes were mounted on a microinfusion pump (Cole-Parmer 74900—Series). Infusion cannulas were left in place for an additional min in order to permit diffusion of the drug. After behavioral tests, rats were sacrificed, their brains immediately removed and immersed in a 4% formalin fixative solution. Frontal sections were cut in a cryostat (Leica). An observer,

blind to the experimental condition, verified cannula placement in the BLA under light microscope. Animals with inaccurate cannula placement or extensive damage were excluded from data analysis.

Experiment 1

Rats were subjected to contextual fear conditioning. Three days later, they were randomly assigned to one of three reactivation conditions: 90 sec, 4 min, or a control group (no reactivation at all or 0 min). Immediately after reactivation, half of the rats in each condition received a 3 mg/kg MDZ injection (i.p.) and the other half received an equivalent amount of SAL. Groups were labeled by drug (MDZ or SAL) and reactivation condition (90 sec, 4 min or control). A day after reactivation, all groups were subjected to a 5-min test. Group sizes were as follows: 90 sec MDZ ($n = 8$) and SAL ($n = 7$), 4 min MDZ ($n = 7$) and SAL ($n = 7$), control MDZ ($n = 6$) and SAL ($n = 7$).

Experiment 2

Subjects were allowed to freely consume one SUC concentration (10%, 20%, or 30%, w/v) during four 10 min sessions, 24 h apart. A fourth group was added to the experimental design to serve as control, with access to water instead of diluted SUC. For all groups, $n = 8$.

Subjects from 30% SUC and water groups were submitted to CFM training one day after the last consumption session. Three days later both groups received CFM reactivation by a 4-min context exposure. After a 30 min delay, both groups were submitted to a final consumption session. Freezing behavior was evaluated in the conditioned context 1 d (test) and 1 wk (retest) later.

Two groups of rats participated in this experiment. One group was exposed to the OF apparatus four times, 10 min each, every 24 h. The other group was allowed to consume SUC freely in four sessions, like in the previous experiment. Both groups were fear-conditioned the day after the last SUC or OF session. After 72 h, both groups received CFM reactivation, followed 30 min later by an OF or SUC final session. The day after both groups was tested for freezing behavior in the conditioned context. For both groups, $n = 8$.

Experiment 3

Subjects were allowed to consume a 30% SUC solution freely through four sessions, as in the previous experiment. The day after the last session, fear conditioning was conducted. After 3 d, CFM reactivation took place. Animals were reactivated for 90 sec ($n = 8$) or 4 min ($n = 9$). Thirty minutes after reactivation, both groups were allowed to consume 30% SUC once again. CFM testing took place a day later.

Experiment 4

Four groups of rats were submitted to both voluntary SUC consumption and fear conditioning, like in the previous experiment. However, after 72 h, one group received a 4 min CFM reactivation followed 30 min later by a consumption session (R + SUC, $n = 7$). A second group received only CFM reactivation (R, $n = 7$) and a third only SUC consumption (SUC, $n = 6$). Finally, a fourth group remained untreated (control, $n = 6$). All groups were submitted to CFM testing a day later.

Experiment 5

Three groups of rats were submitted to the same protocol as group CFM + SUC from Experiment 4, except that the interval between tasks was either of 30 min, 1 h, or 6 h. CFM testing took place a day after. For all groups, $n = 8$.

Experiment 6

Animals were submitted to the SUC consumption sessions and fear conditioning protocols described in earlier experiments. After 72 h, animals received CFM reactivation with a 4- or

15-min context exposure. Half the animals in each condition were allowed, after 30 min, to freely consume 30% SUC, while the other half omitted SUC consumption. A fifth group served as control, remaining untreated. The day after, all groups were submitted to a previously reported reinstatement protocol (Piñeyro et al. 2014). Briefly, animals were reexposed to the conditioning context for 3 min, after which a single 3 sec, 0.5-mA shock was delivered (this represents half the number and intensity of US compared with the training phase). The day after, all groups underwent CFM testing. For all groups, $n = 8$.

Experiment 7

After stereotaxic surgery to implant guide cannulas aimed at the BLA complex, animals were left undisturbed for a 5–7 d period to allow recovery. Afterward, they were submitted to the 4 SUC consumption sessions, followed a day later by fear conditioning. After 72 h, half the animals were intra-BLA infused with IFN, while the other half received VEH instead. Immediately after, half the animals in each drug group were submitted to a CFM reactivation session of 4 min. The other half omitted CFM reactivation. Finally, all groups were allowed to consume 30% SUC. Total time between the end of the infusion procedure and SUC consumption was held constant among groups. Group sizes: IFN ($n = 7$), VEH ($n = 8$), IFN-NR ($n = 7$) and VEH-NR ($n = 7$).

Statistical analysis

Results were expressed as mean ± the standard error of the mean (SEM) of (a) volume of consumed SUC, (b) percentage time the animal spent freezing, (c) quadrants crossed in the OF apparatus, or (d) total time spent in the central area of the OF. Data were analyzed through ANOVAS or unpaired two-tailed "t" tests. Since CFM reactivations had different time lengths than CFM tests they were analyzed apart. For those cases where repeated-measures ANOVAS were appropriate, data was first checked for sphericity and the Greenhouse–Geisser correction was adopted when sphericity was violated. For significant factorial or one-way ANOVAS, the Tukey post hoc test was used, while exploration of simple main effects was conducted for repeated-measures ANOVAS. Effect size estimates were analyzed by Cohen's d (for "t" tests) or $\eta^2 p$ (ANOVAS). In all cases, $P < 0.05$ was the statistical threshold.

Acknowledgments

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