

# Effects of Mineralocorticoid Receptors Blockade on Fear Memory Reconsolidation in Rats

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## ABSTRACT

Reconsolidation memory is defined as a process in which the retrieval of a previously consolidated memory returns to a labile state which is then subject to stabilization. Previous studies have shown that mineralocorticoid receptors (MRs) modulate distinct phases of learning and memory, which display a high concentration and distinct distribution in the hippocampus. Moreover, we found no studies that examined the role of hippocampal MRs in fear memory reconsolidation. Here, we investigated the effect of MRs blockade on fear memory reconsolidation in rats. Additionally, to test whether blockade of protein synthesis would disrupt fear memory reconsolidation in our paradigm, we tested the effect of cycloheximide, an inhibitor of protein synthesis after memory reactivation. Results indicated that systemic as well as intra-hippocampal administrations of the MR antagonist spironolactone immediately following memory reactivation did not affect on post-retrieval long-term memory. Cycloheximide given after the reactivation treatment produced a strong impairment that persisted over test sessions. These findings indicate that MRs are not required for reconsolidation of fear-based memory.

### Key Words:

Mineralocorticoid Receptor,  
Passive Avoidance Task,  
Hippocampus, Memory  
reconsolidation,  
Cycloheximide,  
Rat.

## 1. Introduction

**H**ypothalamic-pituitary-adrenal (HPA) axis is regulated by a negative feedback mechanism that occurs through a dual-receptor system of mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (Reul & de Kloet, 1985; Spencer, Young, Choo, & McEwen, 1990). This axis and glucocorticoids contained therein can significantly influence an individual's response to stress (Jurueña, Cleare, & Pariante, 2004). Glucocorticoids are released during a stressful event and bind to GRs and MRs (de Kloet, de Jong, & Oitzl, 2008). These receptors differ in their affinity for glucocorticoids, with MRs demonstrating the highest affinity for cortisol

and GRs demonstrating lower affinity for cortisol (Reul & de Kloet, 1985; Spencer et al., 1990). In addition, their distribution in rodent brain differs, with MRs predominantly in limbic areas, particularly the hippocampus, and GRs more widely distributed across all brain regions (McEwen, Weiss, & Schwartz, 1968). Thus, MR is a high affinity, low-capacity receptor, where as GR is a low-affinity, high-capacity receptor.

Memory is a process that consists of an encoding or acquisition phase, followed by an extended consolidation or stabilization phase. Memory is expressed during a retrieval phase, which can occur before or after consolidation. During memory consolidation, labile short term memories are converted into long term memory. This

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process is time dependent and requires gene expression and protein synthesis (McGaugh, 2000). Recent studies have demonstrated that when memory recalled, consolidated memory returns to a labile state which initiates another time-dependent memory similar to that seen after novel learning. This phenomenon is now referred to as reconsolidation (Nader & Einarsson, 2010). Molecular processes that underline long-term behavioral changes during consolidation and reconsolidation share some common mechanisms but also display different characteristics (Alberini, 2005).

Recent studies have shown that post-retrieval administration of glucocorticoids can influence subsequent expression of fear memory. Administration of corticosterone immediately after reactivation of a contextual fear memory disrupts subsequent recall in rats (Abrari, Rashidy-Pour, Semnani, & Fathollahi, 2008). Systemic as well as intra-amygdala injections of the GR antagonist RU38486 impaired reconsolidation of fear memory (Taubenfeld, Riceberg, New, & Alberini, 2009; Tronel & Alberini, 2007). In a recent study, we have demonstrated that systemic as well as intra-hippocampal injections of the GR antagonist RU38486 impaired fear-related memory reconsolidation in rat (Nikzad, Vafaei, Rashidy-Pour, & Haghghi, 2011). However, we have found no studies assessed the role of MRs in the hippocampus in fear memory reconsolidation. This area contains a high density of MRs (De Kloet, Vreugdenhil, Oitzl, & Joëls, 1998) and plays a crucial role in fear memory reconsolidation (Debiec, LeDoux, & Nader, 2002). Recently, we have shown that MRs play an essential role in retrieval of spatial information (Khaksari, Rashidy-Pour, & Vafael, 2007). Thus, this study was designed to examine the hypothesis that hippocampal MRs may have a role in the reconsolidation of an inhibitory avoidance, a form of contextual fear conditioning currently used as a model of a traumatic memory (Taubenfeld et al., 2009; Tronel & Alberini, 2007). The learning of this task is known to require hippocampal protein synthesis (Quevedo et al., 1999).

Previous studies have shown that inhibition of protein synthesis impair memory reconsolidation in a variety of tasks and species (Tronson & Taylor, 2007). Specifically, blockade of protein synthesis by systemic as well as intra-cerebral injections of protein synthesis inhibitors can impair substantially fear related memories (Debiec et al., 2002), indicating that such memories require protein synthesis to reconsolidate. However, not all reports using protein synthesis inhibitors have shown disruption of reconsolidation. For example, reconsolidation of an inhibitory avoidance memories were not disrupted by

hippocampal injections of protein synthesis inhibitors (Power, Berlau, McGaugh, & Steward, 2006; Taubenfeld, Milekic, Monti, & Alberini, 2001). Presently, the reasons of these null results are not known. However, systemic injections of protein synthesis inhibitor anisomycin can block reconsolidation of an inhibitory avoidance memory (Tronel & Alberini, 2007). These findings together indicate that an inhibitory avoidance memory can undergo reconsolidation that does not de novo protein synthesis in the hippocampus. Another aim of the present study was thus to probe further the role of systemic protein synthesis inhibition in reconsolidation of an inhibitory avoidance memory.

## 2. Materials and Methods

### 2.1. Animals

Adult male Wistar rats (230–260 g) were housed four per cage (60×40×20 cm) in a room with natural light cycle and constant temperature (24±2 °C). Food and water were available ad libitum. Behavioral procedures were performed during the light phase of the cycles between 10:00 and 13:00. Also all procedures were conducted in agreement with the National Institutes of Health Guide for care and use of laboratory animals and were approved by the Ethics Committee of Semnan University of Medical Sciences, Iran.

### 2.2. Surgery Procedure

Approximately one week prior to the initiation of the behavioral experiments, the rats were anesthetized with ketamine hydrochloride (70 mg/kg, i.p.) plus xylazine (14 mg/kg, i.p.). The rats were fixed in a stereotaxic apparatus, and a midline incision of the skin in the cranial region was made. The skull was dried and cleaned of fascias. Two permanent stainless steel guide cannulae (22 gauge, 10 mm) were aimed at 1 mm above the dorsal hippocampus at the following coordinates relative to the bregma: AP -3.6 mm; L ±2.48 mm (midline); DV -3.4 from skull; with nose bar -3.30 mm below the inter-aural lines, implanted bilaterally (Paxinos & Watson, 2005). The cannulae were affixed to the skull with dental acrylic; stylets were inserted into the cannulae to keep them patent.

### 2.3. Drugs and Injection Procedures

The MR antagonist spironolactone (SPR, purchased from Sigma) was dissolved in propylene glycol and subsequently diluted in 0.9% saline to a final concentration of 0.3, 3, 30 or 100 ng/μl for intra-hippocampal

and 5, 25, 50 and 100 mg/kg for systemic injections. The final concentration of propylene glycol was 5%. These doses of SPR were sufficient to interfere with behavioral responses (Adamec, Muir, Grimes, & Pearcey, 2007; Herman & Spencer, 1998; Kumar et al., 2007; Ninomiya et al., 2010). The protein synthesis inhibitor cycloheximide (CHX) was dissolved in saline and administered (IP) at dose of 2.8 mg/kg (Milekic, Brown, Castellini, & Alberini, 2006).

Intra-hippocampal infusions of SPR (0.3, 3, 30 or 100 ng in 1  $\mu$ l vehicle/hemisphere) or vehicle (1  $\mu$ l/hemisphere) were performed (1  $\mu$ l/min) through an injection needle (30 gauge, 11 mm) attached to a 10- $\mu$ l Hamilton syringe via polyethylene tubing. The needle was equipped with a stopper limiting the depth of insertion to 1 mm beyond the tip of the cannula. The injection needle remained in the cannula for 1 min following the infusion in order to maximize diffusion away from the needle tip and to minimize dorsal diffusion. The volume and duration of intra-hippocampal injections were chosen based on previously published reports. Systemic injections were intraperitoneal and given in injection volumes of 2 ml/kg. Animals received SPR (5, 25, 50 and 100 mg in 2 ml/kg) or vehicle (2ml/kg). All injections applied immediately after reactivation (Test 1).

#### 2.4. Inhibitory Avoidance Training and Testing

The experimental apparatus was a shuttle box (Ugo Basile, Spain) divided into dark and light compartments. Both compartments had a grid floor (3 mm stainless steel rods spaced at 9 mm) connected to a shock generator. An automated apparatus registered the latency of passage from the light to the dark side of the box. The apparatus was located in the sound attenuated room.

For the study of an inhibitory avoidance memory reconsolidation, we used the same protocol of our recent study (Nikzad et al., 2011). All experimental rats were first habituated to the apparatus. The rat was placed in the illuminated compartment and the guillotine door was raised 7 s later. Upon entering the dark compartment, the door was closed and the rat was taken from the dark compartment into the home cage. The acquisition trial was done 30 min later during which the door was closed and a 50 Hz, 1 mA constant current shock was applied for 3 s immediately after the rat had entered the dark compartment. The rat was removed from the dark compartment about 10 s after receiving the shock and returned to his home cage.

Forty-eight hours after training, memory reactivation was occurred (Test 1). The rat was again placed in the illuminated compartment and the guillotine door was opened. Rats that entered the dark compartment and returned to their home cages immediately after entering. For rats that did not enter the dark side, the test was terminated at 540 sec. Foot shock was not delivered during the retention test.

Two (Test 2), and four days (Test 3) after memory reactivation (Test1), animals were retested for fear memory retention. To determine whether memory could re-emerge, immediately after Test 3, rats were exposed to a reminder shock (0.5 mA, 1.5 s) in a different box and retested 7 days and 9 days later (Test 4 and 5). All retention tests (Tests 1–5) were done as described for Test 1.

### 2.5. Experimental Protocol

#### 2.5.1. Experiment 1. Effects of MRs blockade on fear memory reconsolidation.

To determine the role of MRs in fear memory reconsolidation, rats were randomly divided into 10 groups ( $n=9$  in each group) and given different treatment immediately after Test 1. Five groups of rats were systemically injected with vehicle or SPR (5, 25, 50 and 100 mg/kg). Five groups received bilateral intra-hippocampal injections of vehicle or SPR (0.3, 3, 30 or 100 ng/ $\mu$ l). Control groups for systemic or intra-hippocampal injections of SPR received the same volume of vehicle.

#### 2.5.2. Experiment 2. Effects of protein synthesis blockade by CHX on fear memory reconsolidation

It is well established that protein synthesis plays a crucial role in memory reconsolidation as injections of protein synthesis inhibitors after memory reactivation impair memory reconsolidation in a variety of tasks (Taubenfeld et al., 2001). This experiment was designed to see whether blockade of protein synthesis would impair memory reconsolidation in our paradigm. Another aim of this experiment was to test reliability of our paradigm for study of fear memory reconsolidation. Rats were randomly divided into 2 groups ( $n=8$  in each group) and trained according to procedures described in Section 2. Immediately following memory reactivation, the animals received saline or CHX (2.8 mg/kg). Rats were tested at the same intervals mentioned in Expt1. Dose of CHX was chosen on basis of previous studies (Milekic et al., 2006). This dose of CHX reduces brain protein synthesis by 70% within 60 min of administra-

tion (Milekic et al., 2006). To see whether the effect of CHX was dependent to reactivation of memory, one additional group was received CHX two days after training in the absence of memory reactivation and was tested two days later.

## 2.6. Statistical Analysis

Data were expressed as a mean  $\pm$  SEM. Data were analyzed by analysis of variance (ANOVA). Tukey's posthoc test was performed to determine the source of detected significant differences. Student's t-test was used to compare two independent groups. Values of  $P < 0.05$  were considered significant.

## 2.7. Histology

After completion of the behavioral tests, the rats were anesthetized with an overdose of Ketamine (100 mg/kg, i.p). The brains were removed and placed in a 10% formalin solution for approximately 1 week, then sectioned into 40  $\mu$ m slices with a freezing microtome and stained with cresyl violet. Cannula location (Fig.1) was determined using a light microscope and atlas plates (Paxinos and Watson, 2005) by an observer blind to the behavioral results. Only rats with both needle tips terminating within the dorsal hippocampus were included in the behavioral analysis.

## 3. Results

### 3.1. Experiment 1

When systemic administration of SPR (5, 25, 50 and 100 mg/kg) was done following memory reactivation (Test 1), memory did not impair significantly at subsequent tests (Fig. 2B). A Two-way ANOVA on latencies data indicated no significant effects of groups ( $F_{4,96} = 0.54$ ,  $P = 0.7$ ), a significant effect of tests ( $F_{2,96} = 5.5$ ,  $P = 0.01$ ) and no significant interaction between both factors ( $F_{8,96} = 0.8$ ,  $P = 0.8$ ).

As shown in Figure 2C, injection of SPR in the hippocampus after memory reactivation did not impair subsequent memory retentions. A two-way ANOVA that compared the vehicle-injected and the SPR-injected groups at Tests 1 to 3 revealed no significant effects of groups ( $F_{4,96} = 1.25$ ,  $P = 0.26$ ), of tests ( $F_{2,96} = 3.25$ ;  $P = 0.056$ ), and no significant interaction between both factors ( $F_{8,96} = 0.5$ ,  $P = 0.84$ ). Also, application of a weak reminder shock reemerged strength memory retention in all groups (Fig. 2C).



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**Figure 1.** Diagrammatic representation of a histology section, redrawn from Paxinos and Watson (2005). The dark areas define the range of cannula tips location within the hippocampus.

Also, the retention levels of the rats injected with the vehicle or SPR 48 hours after training in the absence of memory reactivation (Test 1), did not differ during retention test which was done two days later (Fig. 3B) ( $P = 0.4$  and  $P = 0.1$  for systemic and intra-hippocampal comparisons, respectively).

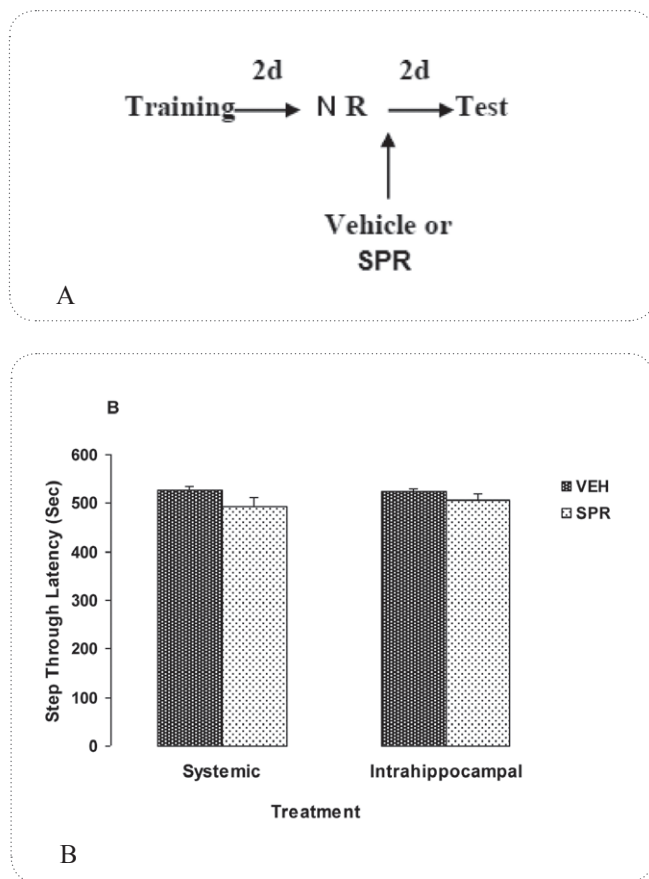
### 3.2. Experiment 2

As expected, CHX administration following memory reactivation impaired subsequent expression of memory (Fig. 4B). Two-way ANOVA on latencies data indicated a significant effect of groups ( $F_{1,42} = 7.83$ ,  $P = 0.01$ ), a significant effect of tests ( $F_{2,42} = 26.19$ ,  $P < 0.0001$ ) and a significant interaction between both factors ( $F_{2,42} = 5.34$ ,  $P = 0.01$ ). Post-hoc comparison indicated that there is a significant difference between the saline group and CHX groups ( $P < 0.01$ ) in tests 2 and 3, indicating a permanent disrupting effects of CHX on memory retention. Also, application of a weak reminder shock after test 3 strengthened the memory in saline-injected, but not the CHX-injected group. Finally, retention latency ( $510 \pm 25.6$ ) of rats that received CHX in the absence of memory reactivation was not different from that of control group ( $527 \pm 11.9$ ) in Expt.2. ( $t_{16} = 1.54$ ;  $P = 0.87$ ).

## 4. Discussion

The main purpose of the present study was to investigate the effects of blockade of MRs on memory reconsolidation. Our results indicate that systemic as well as intra-hippocampal administrations of the MR antagonist spironolactone after memory reactivation did not effect on subsequent expression of memory.

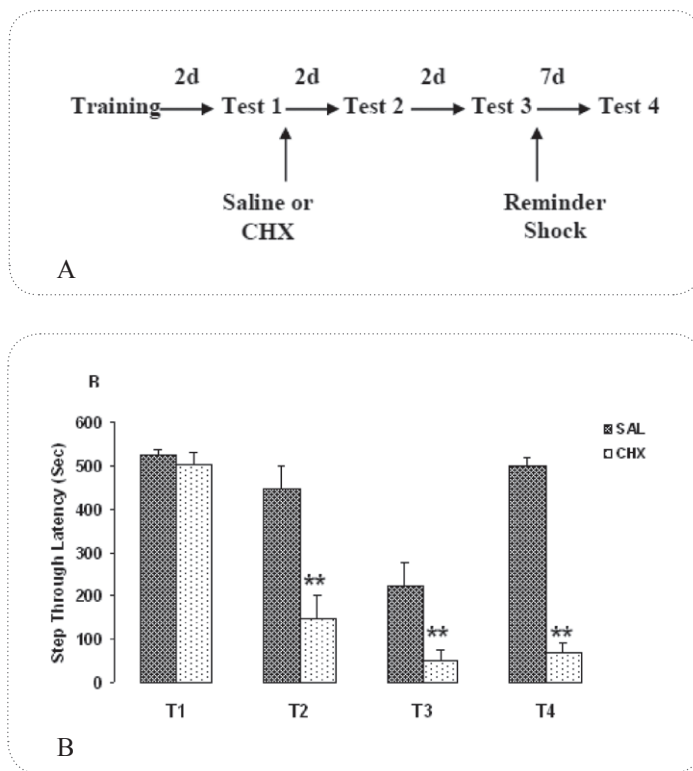




**Figure 3.** Effects of SPR administration in the absence of memory reactivation (NR) on fear memory reconsolidation. A: Passive avoidance training/testing and drug administration schedule. B: Mean latencies ± SEM of groups of rats systemically (50 mg/kg) or intra-hippocampally (3ng in 1μ per side) injected with SPR or vehicle (VEH; n = 8) in the absence of memory reactivation and tested two days. VEH: vehicle, NR: No memory reactivation.

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**Figure 4.** Effects of cycloheximide administration following memory reactivation on fear memory reconsolidation. A: passive avoidance training/testing and drug administration schedule. B: Mean latencies ± SEM of groups of rats systemically injected with 2.8 mg/Kg of cycloheximide (CHX; n = 8) or saline (SAL; n = 8) immediately after test 1 and retested two days (test 2), 7 days (test 3) and after a remainder shock (test 4). \*\*P < 0.01 as compared with SAL group. SAL: saline; CHX: cycloheximide



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Previous studies have shown that that systemic or intra-amygdala infusions of spironolactone did not impair memory consolidation of an inhibitory avoidance task in rats (Quirarte, Roozendaal, & McGaugh, 1997). Findings of other studies also indicated that spironolactone, when administered prior to a test session, reduced freezing time in contextually fear-conditioned animals, indicating that spironolactone, and perhaps other MR antagonists, increase the extinction of aversive memories. But this effect was not observed when the drug was administered after the test session, suggesting that this drug does not act by enhancing the consolidation of new learning (Ninomiya et al., 2010). Other studies also have demonstrated that MRs mediate the interpretation of novel information (Oitzl & de Kloet, 1992) and memory retrieval (Conrad, Lupien, Thanasoulis, & McEwen, 1997). Administration of the MR agonist (aldosterone) was shown to enhance spatial memory, as assessed with the Y maze (Conrad et al., 1997) and conversely, central administration of spironolactone impaired performance in the water-maze (Yau, Noble, & Seckl, 1999). These results suggest the critical importance of MRs occupancy for the processing of some aspects of hippocampal-dependent memory. In the present study, using a wide range of the MR antagonist spironolactone, we did not find any effects of spironolactone on fear memory reconsolidation following systemic or intra-hippocampal injections. Our findings suggest that MRs is not required for reconsolidation of an inhibitory avoidance memory. Lack of MRs blockade on fear memory reconsolidation may not due to lesser occupancy of MRs by spironolactone, since doses of SPR used in the present work were sufficient to interfere with behavioral responses in other studies (Adamec et al., 2007; Herman & Spencer, 1998; Kumar et al., 2007; Ninomiya et al., 2010).

In a recent study, we have found that that systemic as well as intra-hippocampal injections of the GR antagonist RU38486 impaired reconsolidation of inhibitory avoidance task (Nikzad et al., 2011), indicating that GRs are required for reconsolidation of fear-based memory. Previous studies have shown that GRs and MRs appear to play different roles in memory and behavior. GR appears to be involved in the consolidation of recently acquired information, whereas MR appears to be involved in the interpretation of environmental stimuli and the selection of an appropriate response (Khaksari et al., 2007; Oitzl & de Kloet, 1992). Studies in rats and chickens suggest that activation of the MRs is essential during sensory storage (i.e. encoding), whereas normal levels of activation of the GRs (in addition to the already activated MRs) is essential during memory consolidation and retrieval (Tytherleigh, Vedhara, & Lightman, 2004).

Findings of the present and previous studies show that GRs and MRs may play differential role in fear-related memory reconsolidation. As mentioned before, these differential effects of GRs and MRs blockade also were found on other aspects of memory processing.

Previous studies indicated that post-retrieval systemic administration of protein synthesis inhibitors after memory reactivation produces a long-lasting impairment of subsequent recall of different kinds of memory in a variety of tasks (Taubenfeld et al., 2001), suggesting a critical role of protein synthesis in memory reconsolidation. Our results indicate that administration of a protein synthesis inhibitor cycloheximide after fear memory reactivation induced a long term retrograde amnesia. In fact, fear memory does not recover over time nor reinstate with further with a remainder shock. Previously, it has been shown that the same dose of cycloheximide (2.8 mg/kg) impairs consolidation of inhibitory avoidance memory (Gold & Sternberg, 1980). These findings together suggest that consolidation and reconsolidation of an inhibitory avoidance memory share some common mechanisms. On the basis of these experiments, the anatomical sites of cycloheximide actions are not clear. Given the role of basolateral amygdala in fear memory reconsolidation (Duvarci & Nader, 2004; Duvarci, Nader, & LeDoux, 2005), it is likely that the systemic manipulations in the present experiments have affected memory reconsolidation, at least in part, through actions in this structure.

Our findings are in agreement with the pioneer work of Tronel et al. (2005) reporting that systemic anisomycin can impair reconsolidation of an inhibitory avoidance memory, but are in disagreement with other studies have failed to show the disruptive effects of intra-hippocampal anisomycin on reconsolidation of such memory (Power et al., 2006; Taubenfeld et al., 2009). Obviously, due to differences in experimental paradigms (e.g. systemic versus intra-hippocampal injections of protein synthesis inhibitors), a direct comparison of these apparently contrast results are not possible. However, it should be noted that some technical issues such as the drug dose, site of injection and training paradigm might influence the results. Finally, it should be stressed that failure to disrupt reconsolidation of an inhibitory avoidance memory by intra-hippocampal anisomycin does not rule out the role of the hippocampus in such process because the various protein synthesis independent mechanisms might be required for memory reconsolidation.

In conclusion, the finding of the present study indicated that blockade of MRs did not effect on memory reconsolidation, suggesting that these receptors are not required for fear memory reconsolidation. Together with recent studies (Nikzad et al., 2011), these results strongly suggest that the effects of glucocorticoids on fear memory reconsolidation are mediated by GRs.

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