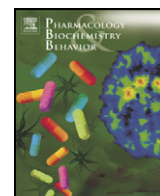Contents lists available at ScienceDirect

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Post-training corticosterone inhibits the return of fear evoked by platform stress and a subthreshold conditioning procedure in Sprague–Dawley rats

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ABSTRACT

The return of fear is an important issue in anxiety disorder research. Each time a fear memory is reactivated, it may further strengthen overactivation of the fear circuit, which may contribute to long-term maintenance of the fear memory. Recent evidence indicates that glucocorticoids may help attenuate pathological fear, but its role in the return of fear is unclear. In the present study, systemic corticosterone (CORT; 25 mg/kg) administration 1 h after fear conditioning did not impair the consolidation process but significantly suppressed the return of fear evoked by a subthreshold conditioning (SC) procedure and elevated platform (EP) stress. Compared with the SC-induced return of fear, acute stress-induced return was state-dependent. In addition, post-training CORT treatment increased the adrenocorticotropic response after EP stress, which indicates that the drug-induced suppression of the return of fear may possibly derive from its regulation effect of the hypothalamic–pituitary–adrenal axis reactivity to stress. These results suggest that post-training CORT administration may help inhibit the return of fear evoked by EP or SC stress. The possible mechanisms involved in the high-dose CORT-induced suppression of the SC- and EP-induced return of fear are discussed.

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1. Introduction

Pavlovian fear conditioning is an animal model that is used to study some of the symptoms of posttraumatic stress disorder (PTSD; *e.g.*, re-experiencing the traumatic event). Although fear extinction decreases conditioned fear responses that normally occur when a conditioned stimulus (CS) is repeatedly presented in the absence of an aversive un-conditioned stimulus (US), it does not erase the original fear memory but rather inhibits the expression of fear (Bouton and Bolles, 1979; Myers and Davis, 2002; Pavlov, 1927; Quirk and Mueller, 2008; Rescorla and Heth, 1975). Fear responses to a CS after extinction can be reinstated by presenting the US (*e.g.*, a shock) as a "reminder" (Laurent and Westbrook, 2010; Rescorla and Heth, 1975). It can also be renewed outside the extinction context (Bouton and King, 1983; Orsini et al., 2011) and recover with the passage of time (Pavlov, 1927; Rescorla, 2004a,b). Importantly, the underlying mechanisms that govern the return of fear appear to be different. For example, pre-conditioning lesions of the fornix or hippocampus abolished fear

reinstatement but had no effect on spontaneous recovery or fear renew-al (Frohardt et al., 2000; Wilson et al., 1995). Recently, two relatively novel rodent models of fear return induced by a subthreshold condi-tioning (SC) procedure and elevated platform (EP) stress were validated by Deschaux and colleagues (Deschaux et al., 2011a,b; Zheng et al., 2013).

Previous research indicated that dysfunction of the hypothalamic–pituitary–adrenal (HPA) axis contributed to the etiology of anxiety dis-orders, such as generalized anxiety disorder, panic disorder, and PTSD (de Kloet et al., 2006; Rasmusson et al., 2003; Yehuda et al., 1991). Sev-eral lines of evidence indicate that glucocorticoids may play important roles in this process (Yehuda, 1997). First, PTSD patients had lower plas-ma cortisol levels. Second, victims who exhibited a blunted glucocorti-coid response a short time after trauma were suggested to be susceptible to the later development of PTSD (Delahanty et al., 2000; McFarlane et al., 1997; Yehuda et al., 1998). Third, glucocorticoid agents have been reported to effectively alleviate the aberrant fear/anxiety symptoms that are seen in PTSD or phobia patients (Aerni et al., 2004; Schelling et al., 2004; Soravia et al., 2006). Preclinically, a blunted HPA axis response to stress has been found to be associated with the inci-dence of "extreme behavioral responses" (EBRs) after predator scent stress in rats (Cohen et al., 2006). Although recent evidence indicates that glucocorticoids may be able to attenuate pathological fear, little is

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known about the role of these agents in the return of fear, especially the two relatively novel rodent models of the return of fear induced by SC or EP stress.

In the present study, the effects of corticosterone (CORT) on the return of fear induced by SC and EP stress were tested. We selected one dose of CORT (25 mg/kg, i.p.) based on ample evidence that it effectively decreases the prevalence of EBRs after traumatic stress (Cohen et al., 2008). Our previous study also found that this dose rather than a lower one (5 mg/kg, i.p.) attenuated the renewal of fear (Wang et al., 2014). Additionally, this high dose of CORT has been shown to be associated with a decreased risk for the development of PTSD in humans (Zohar et al., 2011). Based on prior evidence that CORT may modulate fear-related behavior through various mechanisms (*e.g.*, by altering the fear memory consolidation process or acquisition and consolidation of extinction memory or directly modulating fear memory retrieval) (Abrari et al., 2008; Brinks et al., 2009; Cai et al., 2006; Cohen et al., 2008; de Quervain, 2006; Hui et al., 2004; Izquierdo et al., 2002; Roozendaal et al., 2006; Sandi and Rose, 1994; Soravia et al., 2006; Wingenfeld et al., 2012; Zorawski and Killcross, 2002), we evaluated whether CORT effectively suppresses the return of fear by directly modulating fear memory retrieval and the extinction process. We also evaluated whether CORT can regulate the HPA axis response in stressed animals and decrease shock sensitivity in the flinch–jump test.

2. Materials and methods

2.1. Animals and housing

The subjects were adult male Sprague–Dawley rats (240–260 g) obtained from a commercial supplier (Vital River Animal Center, Beijing, China). The rats were individually housed in standard steel hanging cages ($28.5 \times 24 \times 21.5$ cm^3) and kept on a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) with free access to food and tap water (*ad libitum*). Experiments were performed during the lights-on phase. For the following experiments, the rats were uniformly handled to habituate to the experimenter for five days before each experiment began. This study was subjected to the review for animal use and approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Psychology, Chinese Academy of Sciences (CAS). All experiments were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Apparatus

2.2.1. Observation chamber for fear conditioning, extinction and extinction retrieval test

Four identical observation chambers ($30.5 \times 25.4 \times 30.5$ cm^3, Coulbourn Instruments, Allentown, PA) were used for fear conditioning, extinction and extinction retrieval tests. The chambers were constructed from aluminum (two side walls and ceiling) and plexiglas (rear wall and hinged front door) and were situated in sound-attenuating chests. The floor of the chamber consisted of 18 stainless-steel rods (6 mm in diameter) spaced 1.5 cm apart which were wired to a shock source and solid-state grid scrambler (Coulbourn, H13-15) for delivery of the foot shock unconditioned stimulus. A speaker was mounted on one side panel of the chamber to deliver the tone conditioned stimulus. Both shock and tone deliveries were controlled by a computerized system. A video camera was mounted on the ceiling of the chamber used for videotaping the rat behaviors.

Sensory stimuli were adjusted within the chamber to generate two different contexts (A and B). Context A was the original facility described above. A small yellow light (6 W) mounted opposite the speaker was turned on providing the illumination inside the chamber while the fluorescent room light was turned off. The chambers were scented with 75% alcohol before and after use for each rat. Context B was modified

from context A. For context B, black acrylic boards with round holes (1.5 cm in diameter) were fitted to the chamber inside walls except the ceiling and floor. The chamber light was changed to a white one and room lights were turned on. The floor was changed to a squares steel sieve-plate (1×1 cm^2). The chambers were scented with diluted perfume (1%).

2.2.2. Elevated platform

The EP stress was adapted from Xu et al. (1997). The apparatus consisted of a 12×12 cm^2 translucent plastic platform which was supported by 1.6 m-high stainless steel rod. The platform was stable during the 15-min stress period in a brightly lit room when the rats were placed on it.

2.3. Experimental protocols

2.3.1. Experiment 1: effect of post-training CORT administration on the return of fear induced by SC

Thirty-one rats were used in this experiment. The rats were subjected to five phases of manipulations: habituation, fear conditioning, extinction, subthreshold conditioning, and extinction retrieval (Fig. 1A). The following groups were formed to test whether the SC procedure induces a significant return of fear and whether drug treatment 1 h after conditioning suppresses this effect: 0.7 + veh (no SC), 0.7 + veh + SC, and 0.7 + cort25 + SC. The 0.2 + veh + SC group was used to control for the potential latent inhibition effect of exposure to the tones with low shocks on the subsequent SC procedure. Rats in this group were conditioned to the same CS–US associations but received a very mild shock (0.2 mA), which was reported to have no appreciable conditioning effect by itself (Baldi et al., 2004).

Prior to conditioning, all of the rats were habituated to context B over a 3-day period (10 min/day). A predictable tone-shock pairing procedure was used for fear conditioning (day 1 in context A). Briefly, after 3-min habituation to this environment, the rats were conditioned with either a 0.7 or 0.2 mA shock according to group assignment (Fig. 1A). Five tone–shock pairings were programmed by a computer, with the footshock as the US (0.2 or 0.7 mA, 1 s duration) and the auditory tone as the CS (2000 Hz, 75 dB, 30 s duration). The tone and shock coterminated at the last second of the tone. The average variable intertrial interval was 2 min. One minute after the last pairing, the rats were returned to their homecage. One hour after fear conditioning, the rats were administered either CORT (25 mg/kg, i.p.) or vehicle (pure sesame oil, 1.5 ml/kg, i.p.) according to group assignment (Fig. 1A). Before drug/vehicle treatment, different groups of rats (except the 0.2 + veh + SC group) were matched according to a similar level of freezing behavior during conditioning.

Forty-eight hours after conditioning (day 3), all of the rats were subjected to two sessions of extinction that were spaced 24 h apart. During each extinction session, the rats received 20 non-reinforced presentations of the CSs with an average fixed ITI of 1 min in context B. On day 5, the 0.2 + veh + SC, 0.7 + veh + SC, and 0.7 + cort25 + SC groups were subjected to the SC procedure, which was conducted in context A. In contrast, rats in the 0.7 + veh (no SC) group were left in context A for an equivalent period of time without further tone-shock pairings. For the SC procedure, similar predictable tone–shock pairings as in the first fear conditioning session were used. In the SC procedure, three CS–US pairings were given, and the shock intensity was set at 0.2 mA. Twenty-four hours after the SC procedure, all of the rats were tested for extinction retrieval in context B (day 6). Two minutes after placing the rats into the test chamber, a 2-min continuous CS was given. The behavior of the rat was video-recorded for later analysis.

2.3.2. Experiment 2: effect of post-training CORT administration on the return of fear induced by EP stress

Thirty-five rats were used in this experiment. The behavioral manipulations were the same as in Experiment 1, with the exception that EP
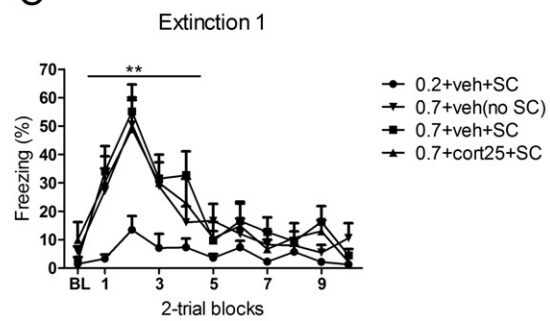
A Timeline (experiment 1)

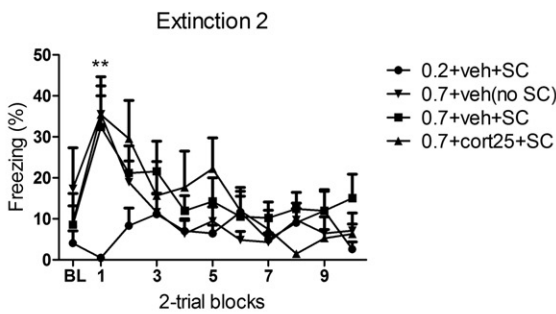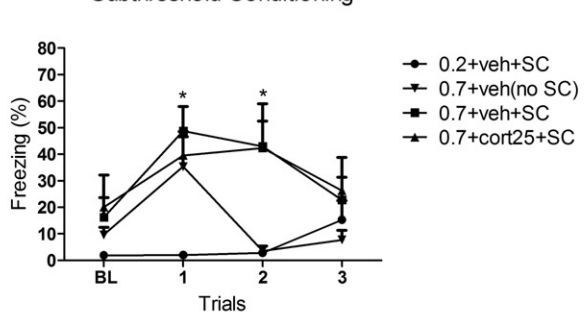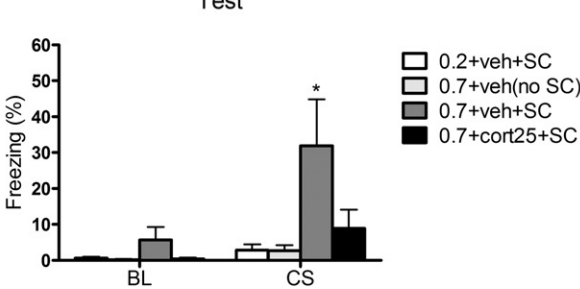| Groups	Day 1 FC (A)	Day 3 Ext1 (B)	Day 4 Ext2 (B)	Day 5 SC (A)	Day 6 Test (B)
0.2+veh+SC	5 × CS-0.2 mA/veh	20 trials	20 trials	3 × CS-0.2mA	2 min CS
0.7+veh(no SC)	5 × CS-0.7 mA/veh	20 trials	20 trials	A -exposure	2 min CS
0.7+veh+SC	5 × CS-0.7 mA/veh	20 trials	20 trials	3 × CS-0.2mA	2 min CS
0.7+cort25+SC	5 × CS-0.7 mA/cort25	20 trials	20 trials	3 × CS-0.2mA	2 min CS
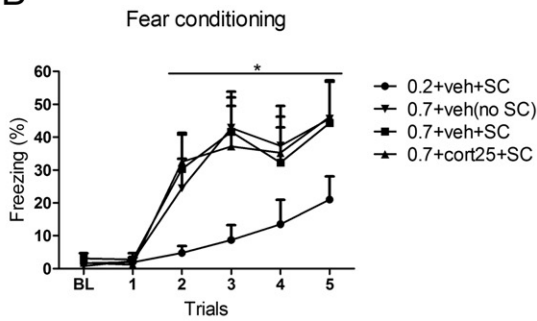
Fig. 1. Effects of CORT (25 mg/kg, i.p.) administration 1 h after fear conditioning on the return of fear induced by SC. (A) Experimental timeline and group assignment. (B) Freezing behavior for the baseline (BL) context and conditioned tone during fear conditioning (the 0.2 + veh + SC group *vs.* each of the other groups during the last four trials: *$p < 0.05$). (C, D) Freezing behavior for the BL context and tone during the first and second extinction sessions (20 tone-alone extinction sessions were binned into 10 blocks of two-trial averages). The rats shocked with 0.7 mA showed higher freezing during the first four blocks of extinction 1 and the first block of extinction 2 (the 0.2 + veh + SC group *vs.* each of the other groups: **$p < 0.01$). (E) Freezing behavior for the BL context and tone during the SC procedure. For the 0.7 + veh (no SC) group, freezing levels were fear response to context in the same time intervals which three trials of tone were present during SC procedure (the 0.2 + veh + SC group *vs.* each of the other groups during the first trial: *$p < 0.05$; the 0.2 + veh + SC or 0.7 + veh (no SC) groups *vs.* the 0.7 + veh + SC or 0.7 + cort25 + SC groups during the second trial: *$p < 0.05$). (F) Freezing behavior for the BL context and tone during the retrieval test (the 0.7 + veh + SC group *vs.* the 0.2 + veh + SC or 0.7 + veh (no SC) groups: *$p < 0.05$). The data are expressed as mean (%) ± SEM. $n = 7$–8 per group. FC, fear conditioning; Ext, extinction; veh, vehicle.

stress was used as the inducing condition after extinction. A total of five groups were used (Fig. 2A). The EP alone (1 h) group was used to demonstrate that 15-min EP exposure itself 1 h prior to the test did not induce significant fear in non-shocked animals. The 0.7 + veh (no EP), 0.7 + veh + EP (1 h), 0.7 + veh + EP (2 h), and 0.7 + cort25 + EP (1 h) groups were used to test whether EP stress 1 or 2 h prior to the

A Timeline (experiment 2)

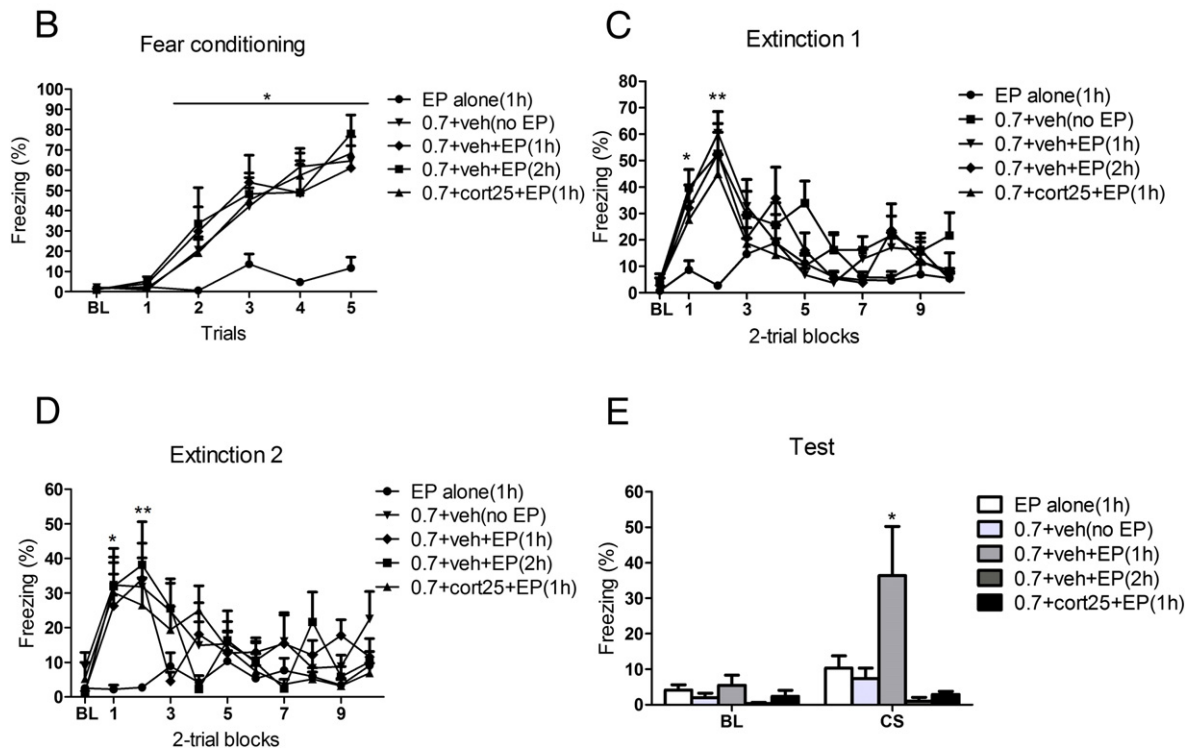| Groups	Day 1 FC (A)	Day 3 Ext1 (B)	Day 4 Ext2 (B)	Day 6(a) EP stress	Day 6(b) Test (B)
EP alone (1h)	5 × CS	20 trials	20 trials	EP	2-min CS
0.7+veh (no EP)	5 × CS - 0.7mA/veh	20 trials	20 trials		2-min CS
0.7+veh+EP (1h)	5 × CS - 0.7mA/veh	20 trials	20 trials	EP	2-min CS
0.7+veh+EP (2h)	5 × CS - 0.7mA/veh	20 trials	20 trials	EP	2-min CS
0.7+cort25+EP (1h)	5 × CS - 0.7mA/cort25	20 trials	20 trials	EP	2-min CS

Fig. 2. Effects of CORT (25 mg/kg, i.p.) administration 1 h after fear conditioning on the return of fear induced by EP stress. (A) Experimental timeline and group assignment. (B) Freezing behavior for the baseline (BL) context and conditioned tone during fear conditioning (the EP alone (1 h) group vs. each of the other groups during the last four trials: *p < 0.05). (C, D) Freezing behavior for the BL context and tone during the first and second extinction sessions (20 tone-alone extinction sessions were binned into 10 blocks of two-trial averages). The rats shocked with 0.7 mA showed higher freezing during the first two blocks of extinction 1 and 2 (the EP alone (1 h) group vs. each of the other groups: *p < 0.05; **p < 0.01). (E) Freezing behavior for the BL context and tone during the extinction retrieval test (the 0.7 + veh + EP (1 h) group vs. each of the other groups: *p < 0.05). The data are expressed as mean (%) ± SEM. n = 6–7 per group. Abbreviations as in Fig. 1.

test induces significant fear return and whether CORT can suppress it. Some previous research has found time-limited effects of acute EP stress. For example, EP stress could facilitate the induction of stable homosynaptic long-term depression (LTD) and block long-term potentiation (LTP) in the hippocampus, however these effects could be lost within 20 min after the animals were removed from the EP (Xu et al., 1997), which was accompanied with the decrease of plasma corticosterone to the baseline 1 h after the removal of EP stress (Degroot et al., 2004). Therefore, in this experimental design, two intervals 1 h or 2 h between the EP stress and the final retrieval test were set to assess whether the return of fear evoked by acute stress was time-limited.

The rats were gently placed on the EP for 15 min in a brightly lit room (500 lx) that was adjacent to the main experimental room where conditioning and extinction/retrieval occurred. Occasionally, some of the rats fell from the platform, particularly during the initial few minutes. When an animal accidentally fell from the platform, it was gently put back on it. Approximately one in 10 rats fell from the platform, but this incidence was not different between groups. If an animal fell from the platform three or more times, then its data were excluded from the subsequent analysis. During stress, the animals exhibited behavioral freezing and sometimes urination and defecation. The rats were returned to their homecage immediately after the end of EP exposure.

2.3.3. Experiment 3: effect of post-training CORT administration on CORT and ACTH reactivity evoked by EP stress

Thirty-two rats were used in this experiment. Four groups were formed using a 2 × 2 factorial design according to whether the rats received CORT (25 mg/kg, i.p.) after conditioning (day 1; the procedure was the same as in Experiment 2) and whether they were subjected to 15-min EP stress (the procedure was the same as in Experiment 2) 6 days after conditioning. On day 6, half of the rats were decapitated

10 min after EP stress. Trunk blood was collected for CORT and ACTH analysis. The remaining animals were placed in a third room for 10 min but remained in their homecage. Trunk blood was collected from these animals following decapitation. Efforts were made to avoid any influence of nonspecific stress beyond the purported EP stress.

2.3.4. Experiment 4: effect of CORT administration on shock sensitivity

Twenty rats were used in this experiment. Half of the rats randomly received CORT (25 mg/kg, i.p.) or vehicle (pure sesame oil, 1.5 ml/kg, i.p.). After 5 days, all of the rats individually underwent auditory fear conditioning to test shock sensitivity. After a 3-min period of habituation to the test chamber, the shock intensities were increased in a stepwise manner (0.05 mA steps, 0.05–0.8 mA range), depending on the responsiveness of the rat. When a jump reaction was observed, no further footshocks were given. The flinch threshold was defined as the lowest shock intensity that elicited any detectable response. The vocalization threshold was defined as the lowest shock intensity that elicited vocalization. The jump threshold was defined as the lowest shock intensity that elicited the simultaneous removal of at least three paws (including both hindpaws) from the grid floor. The flinch, vocalization, and jump thresholds (in milliamperes) were defined for each rat. The interval between shocks was 30 s, and each animal was tested only once (Takahashi et al., 2006).

2.4. Drugs

Preparation of the CORT solution was described by Hellsten et al. (2002). Corticosterone (Sigma) was first suspended in 100% sesame oil to reach the appropriate concentration and then sonicated for 1 h to ensure an even suspension of the drug. Prior to each drug injection (25 mg/1.5 ml/kg, i.p.), the solution was vigorously shaken.

2.5. Behavioral scoring and statistical analysis

Freezing behavior, defined as the absence of any movement except respiration (Fanselow, 1994), was automatically quantified using Freeze-Frame software (ACT-100, Coulbourn Instruments). Freezing was defined as continuous inactivity that lasted at least 1 s and further confirmed by an experimenter who was blind to group assignment. The level of freezing is expressed as the percentage of the time spent freezing during the 30 s tone presentation. The conditioning and extinction data were analyzed using two-way repeated-measures analysis of variance (ANOVA), which followed by the Least Significant Difference (LSD) post hoc test or simple-effect analysis when appropriate. The fear return data were analyzed using a one-way ANOVA followed by the LSD post hoc test. Corticosterone and ACTH levels were analyzed using two-way ANOVA followed by simple-effect analysis. Statistical significance was set to $p < 0.05$.

2.6. Corticosterone and ACTH enzyme-linked immunosorbent assay

To minimize the effects of circadian rhythms on CORT concentrations, blood was collected between 9:00 AM and 12:00 PM ($n = 7$–8 per group). Plasma concentrations of CORT and ACTH were measured using an enzyme-linked immunosorbent assay (ELISA) as previously reported (Guo et al., 2011). Briefly, trunk blood was collected, and samples were centrifuged at 3000 ×g for 15 min at 4 °C. Plasma was stored at − 20 °C until the assay. Corticosterone and ACTH were extracted from the plasma, added to ethanol, and measured by ELISA. The ELISA kits' cross reactivity with other steroids was < 0.01%. The sensitivity of the CORT assay was 0.5 μg/dl. The intra- and inter-assay coefficients of variation were less than 5% and 8%, respectively. The ACTH assay sensitivity was 5 pg/ml, and the intra- and inter-assay coefficients of variation were less than 5% and 8%, respectively.

3. Results

3.1. Experiment 1: effect of post-training CORT administration on the return of fear induced by SC

Fig. 1A shows the group assignments and timeline of the experiment. Data in Fig. 1B–D show that the rats receiving strong 0.7 mA shock exhibited higher levels of freezing than the rats that received 0.2 mA shock during the processes of fear acquisition and the first several blocks of each extinction session. Two-way repeated-measures ANOVA performed on the data of fear conditioning (Fig. 1B; four groups × the last four trials from 2 to 5) revealed main effects of group ($F_{3,26} = 4.104$, $p < 0.05$) and trial ($F_{3,24} = 4.403$, $p < 0.05$). However, there was no Group × Trial interaction. Post hoc LSD tests indicated that the 0.2 + veh + SC group differed from each of the other three groups receiving the 0.7 mA shock (0.7 + veh (no SC), 0.7 + veh + SC and 0.7 + cort25 + SC groups; $p < 0.05$). Two-way repeated-measures ANOVA was performed on the first four blocks of two extinction sessions (Fig. 1C–D; four groups × four blocks). For the first extinction session (Fig. 1C), there were significant main effects of group ($F_{3,56} = 4.116$, $p = 0.01$) and block ($F_{3,54} = 10.817$, $p < 0.01$), but no significant Group × Block interaction effect. The post hoc LSD tests further revealed that the 0.2 + veh + SC group differed from each of the other groups (0.7 + veh (no SC), 0.7 + veh + SC and 0.7 + cort25 + SC groups; $p < 0.05$). However, three groups exposed to strong shock did not differ from each other ($p > 0.05$). For the second extinction session (Fig. 1D), there was significant Group × Block interaction effect ($F_{9,168} = 2.390$, $p < 0.05$), but no main effects of group and block. The following simple effect analysis confirmed that three groups exposed to the 0.7 mA shock still presented higher levels of freezing than rats conditioned with the 0.2 mA shock in the first block ($p < 0.01$). These results indicate that rats shocked with 0.7 mA, but not 0.2 mA, were conditioned. And CORT did not significantly influence the consolidation of fear memory or the extinction process compared with rats that received vehicle treatment.

During the SC procedure in traumatic context A (Fig. 1E), freezing levels of the 0.7 + veh (no SC) group were fear response to context A in the same time intervals which three trials of conditioned tone were present for the other three groups. Two-way repeated-measures ANOVA performed on these data (four groups × three trials) revealed main effects of group ($F_{3,27} = 6.345$, $p < 0.01$) and trial ($F_{2,26} = 3.121$, $p = 0.061$), and significant Group × Trial interaction effect ($F_{6,54} = 2.411$, $p < 0.05$). The following simple-effect analysis confirmed that the conditioned rats (0.7 + veh (no SC), 0.7 + veh + SC and 0.7 + cort25 + SC groups) exhibited higher levels of freezing than the rats receiving sub-threshold shock (0.2 + veh + SC group; $p < 0.05$) in the first trial. However, three groups exposed to the 0.7 mA shock did not differ from each other ($p > 0.05$). It indicates that both traumatic context and conditioned tone in traumatic context induced significant fear response for the conditioned rats. For the second trial, the SC procedure maintained the higher freezing levels for the conditioned rats, compared with the 0.2 + veh + SC or 0.7 + veh (no SC) groups ($p < 0.05$). However, there were no differences between four groups during the third trial ($p > 0.05$). No differences were observed for the conditioned rats with vehicle and CORT treatments during the SC procedure, which means that CORT did not influence the fear response to CS in traumatic context.

Fig. 1F shows the mean levels of freezing for the baseline (BL) context prior to the CS and the entire 2-min CS during extinction retrieval. The one-way ANOVA performed on the data of conditioned tone indicated different levels of freezing behavior among groups ($F_{3,24} = 3.411$, $p < 0.05$). The conditioned rats with vehicle treatment and SC procedure (0.7 + veh + SC group) exhibited higher levels of freezing, compared with each of the remaining three groups (0.2 + veh + SC group, $p < 0.05$; 0.7 + veh (no SC) group; $p < 0.05$; 0.7 + cort25 + SC group; $p = 0.05$). However, no differences were observed between

the remaining three groups ($p > 0.05$). These results indicate that the SC procedure induced the return of fear for the conditioned rats, which could be suppressed by post-training CORT administration significantly.

3.2. Experiment 2: effect of post-training CORT on the return of fear induced by EP stress

Fig. 2A shows the group assignments and timeline of Experiment 2. As shown in Fig. 2B, two-way repeated-measures ANOVA performed on the data of fear conditioning (five groups × the last four trials) revealed main effects of group ($F_{4,28} = 3.491$, $p < 0.05$) and trial ($F_{3,26} = 13.199$, $p < 0.01$), but no Group × Trial interaction effect. The following post hoc LSD tests indicated that the rats receiving 0.7 mA shock (0.7 + veh (no EP), 0.7 + veh + EP (1 h), 0.7 + veh + EP (2 h) and 0.7 + cort25 + EP (1 h) groups) exhibited higher levels of freezing than the rats with no shock (EP alone (1 h) group; $p < 0.05$). Two-way repeated-measures ANOVA were performed on the data of two extinction sessions (five groups × the first four blocks). For the first extinction session (Fig. 2C), there were significant main effects of group ($F_{4,71} = 4.550$, $p < 0.01$) and block ($F_{3,69} = 10.357$, $p < 0.01$), and Group × Block interaction effect ($F_{12,213} = 2.145$, $p < 0.05$). For the second extinction session (Fig. 2D), significant main effects of group ($F_{4,71} = 4.550$, $p < 0.01$) and block ($F_{3,69} = 10.357$, $p < 0.01$), and the Group × Block interaction effect ($F_{12,213} = 2.145$, $p < 0.05$) were observed. The following simple-effect analysis found that the EP alone (1 h) group were lower freezing levels, compared with four groups receiving the 0.7 mA shock ($p < 0.05$) in the first and second blocks of two extinction sessions. However, four groups exposed to the 0.7 mA shock did not differ from each other during fear conditioning and two extinction sessions ($p > 0.05$). Consistent with the findings of Experiment 1, these results indicated that CORT did not significantly modulate the expression and extinction of fear memory compared with rats that received vehicle treatment. In the extinction retrieval test (Fig. 2E), the groups exhibited different levels of freezing to conditioned tone. The one-way ANOVA revealed a significant main effect of group ($F_{4,25} = 3.392$, $p < 0.05$). Rats in the 0.7 + veh + EP (1 h) group exhibited higher levels of freezing compared with the other four groups ($p < 0.05$). The latter four groups did not differ from each other.

3.3. Experiment 3: effect of post-training CORT administration on CORT and ACTH evoked by EP stress

As shown in Fig. 3A, drug treatment and EP stress induced no significant changes in CORT levels. Two-way ANOVA performed on CORT levels (drug treatment × EP stress) revealed neither main effects of drug and stress, nor their interaction effect. For ACTH levels (Fig. 3B), a significant drug × stress interaction ($F_{1,27} = 11.647$, $p < 0.01$) was

Fig. 4. Effects of CORT on shock sensitivity. The shock thresholds for flinch, vocalization, and jump reactions were determined in rats treated with sesame oil (VEH group) and rats treated with CORT (25 mg/kg; CORT group). No difference was observed between the CORT and VEH groups. n = 10 per group.

found, but main effects of drug and stress was not significant. The following simple-effect analysis revealed that EP stress induced marginally blunted ACTH reactivity in animals that did not receive drug treatment ($p = 0.094$). However, in rats that received drug treatment, EP stress significantly increased ACTH levels ($p < 0.01$). Furthermore, the drug treatment significantly increased ACTH reactivity to EP stress ($p < 0.01$). These results indicate that CORT administration 1 h after conditioning reversed the blunted HPA reactivity in conditioned rats.

3.4. Experiment 4: effect of CORT administration on shock sensitivity

Fig. 4 shows that high-dose CORT (25 mg/kg, i.p.) had no effect on shock sensitivity. Independent t-tests did not reveal significant differences between groups in the rat flinch reaction ($t_{18} = 0.287$, $p > 0.05$), vocalization reaction ($t_{18} = 0.531$, $p > 0.05$), or jump reaction ($t_{18} = 0.421$, $p > 0.05$).

4. Discussion

The main finding of the present study was that both the SC procedure and EP stress reactivated the extinguished fear. These two stressful stimuli did not induce significant fear in response to the CS (tone) when presented alone. Therefore, the reemergence of fear after extinction reflected the return of previous fear memory. High-dose CORT administration 1 h after fear conditioning suppressed the return of fear induced by the SC procedure and acute EP stress, which was consistent with previous studies that showed that CORT plays an important role in preventing the development of anxiety (Cohen et al., 2008; Rao et al.,
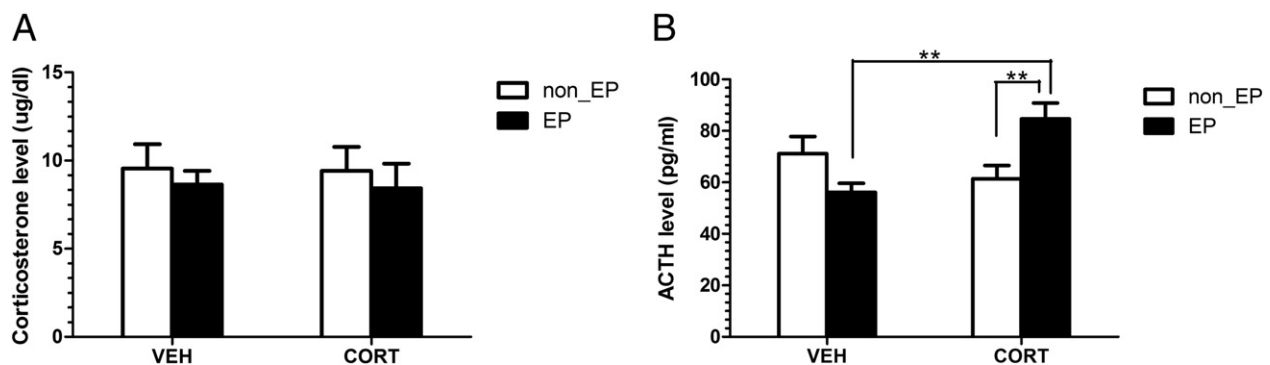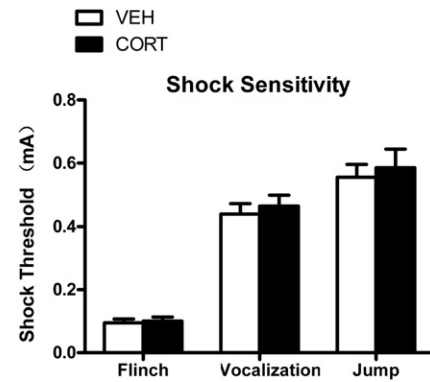

Fig. 3. Effect of CORT administration after conditioning on CORT and ACTH evoked by EP stress. (A) Corticosterone treatment and EP stress did not interact to modulate the CORT response. (B) Corticosterone treatment significantly interacted with EP stress to modulate the ACTH response (the group with drug treatment and EP stress vs. the group with vehicle treatment and EP stress or the group with drug treatment but no EP stress: **$p < 0.01$). The data are expressed as the mean (μg/dl) ± SEM corticosterone level and mean (pg/ml) ± SEM ACTH level. $n = 7$–8 per group.

2012; Wang et al., 2014; Zohar et al., 2011). A translational study found that high-dose hydrocortisone (100–140 mg) given in the first few hours after a traumatic experience decreased the risk of subsequently developing PTSD (Zohar et al., 2011). We extended this finding and found that post-training CORT administration suppressed the return of fear evoked by the SC procedure and EP stress after extinction.

In both Experiments 1 and 2, drug treatment within the period of memory consolidation did not decrease the level of freezing in response to the tone in the first extinction session in rats that received strong shocks (Figs. 1C, 2C). This is consistent with our previous study (Wang et al., 2014), in which high-dose CORT administration did not impair the consolidation or retrieval of fear memory. Although some investigations found that extrinsic stress/corticosteroid administration within the period of memory consolidation may strengthen (Hui et al., 2004, 2006; Roozendaal et al., 2006; Zorawski and Killcross, 2002) or impair (Cohen et al., 2008; Sandi and Rose, 1994) fear memory, the effects of CORT on memory depended on the interaction between the aversiveness of the task and dose of the stress hormone (Conrad, 2005; Sandi and Pinelo-Nava, 2007). In contrast to previous studies, no facilitatory effect of the drug on the extinction process was observed (Figs. 1D, 2D). An important reason might be the different behavioral paradigms or drug doses used, and the effect of CORT on facilitating extinction has been shown to be strain-dependent.

The return of fear after extinction can be considered as a result of competition between the initial fear memory and the subsequent extinction memory. If extinction memory in the final retrieval test was retrieved well, the return of fear would not be observed. Importantly, a recent series of work by van Ast and colleagues demonstrated that cortisol did not alter emotional memory *per se*, but its slow effects enhanced the memory contextualization process during retrieval (van Ast et al., 2012, 2013). In other words, the contextual control over memory retrieval (*i.e.* context-specific retrieval) was enhanced by cortisol. CORT might have significantly strengthened and facilitated the context-specific retrieval of extinction memory in extinction context, thereby suppressing the return of fear after the SC procedure and EP stress (Figs. 1F, 2E). In the present study, the major differences between extinction retrieval on day 4 (Ext2, considered the retrieval test for Ext 1) and day 6 (the final retrieval test) were the prior fear-induction procedures. Compared the vehicle-treated rats, extinction memory in the final retrieval test was retrieved better in the safe extinction context for the drug-treated rats, even when stressful stimuli (the SC procedure or EP stress) were prior to the test. However, the enhanced retrieval of extinction memory could not generalize to the traumatic context (Fig. 1E).

Although CORT suppressed the return of fear induced by the SC procedure or acute EP stress, this does not mean that these two models share the same behavioral or neural mechanisms. The return of fear was observed 24 h after the SC procedure, whereas the time delay was 48 h in other studies (Deschaux et al., 2011a,b, 2013). Compared with the return of fear induced by the SC procedure, the acute stress-induced return of fear might be state-dependent. In Experiment 2, the EP stress 1 h rather than 2 h prior to the retrieval test induced the obvious return of fear, which was consistent with previous investigations on the transient effects of EP stress (Degroot et al., 2004; Xu et al., 1997). This time-limited effect might be related with the post-stress circulating corticosterone levels, which displayed an inverted U-shape curve over time. In addition to the study of Degroot et al. (2004) mentioned above, peak corticosteroid levels in the brain were probably reached 1 h after 15-min forced swimming stress, and normalization took place only after 1–2 h (Droste et al., 2008). Furthermore, acute stress or glucocorticoids disrupted memory retrieval in a time-dependent manner (de Quervain et al., 1998; Roozendaal et al., 2004; Wong et al., 2007). The retention performance in a water-maze spatial task for rodents was impaired 30 min but not 2 min or 4 h after footshock (de Quervain et al., 1998). Therefore, the post-stress corticosterone levels might regulate the return of fear by affecting the retrieval of extinction memory: the high levels 30–60 min after acute stress impaired or suppressed the retrieval of extinction memory, whereas the baseline level of corticosterone 2 h after stress had no effect on the retrieval of extinction memory and could not induce the return of fear.

It is observed that corticosterone interacted with EP stress to influence the ACTH response (Fig. 3B). In detail, the rats with post-training CORT treatment showed an increased ACTH response to EP stress, compared with the ones that received vehicle treatment. And the EP stress induced marginally blunted ACTH reactivity in vehicle-treated rats ($p = 0.094$). These results indicate that the acute stress-induced return of fear may be causally related to altered HPA reactivity during the fear reactivation process. And the suppression of CORT on the return of fear induced by acute stress appears to mainly derive from its interference with such fear reactivation process. In contrast, CORT did not influence the effects of the SC procedure in the traumatic context (Fig. 1E) or decrease shock sensitivity (Fig. 4) but suppressed the return of fear in the extinction context after the SC procedure. The enhancement of extinction memory contextualization induced by drug treatment might be the main reason for the suppression of the return of fear after the SC procedure.

5. Conclusions

In summary, the present study found that systemic CORT administration suppressed the return of fear in extinguished rats evoked by a SC procedure or acute EP stress. The drug-induced suppression of the SC procedure-induced return of fear appears to derive from its ability to facilitate the context-specific retrieval of extinction memory. The drug-induced suppression of the acute stress-induced return of fear appears to mainly derive from its interference with the fear reactivation process, which was temporally paralleled by a blunted ACTH response after EP stress. Further studies are needed to elucidate the mechanisms of the preventive effects of CORT on the return of fear.

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