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ORIGINAL ARTICLE

Acute and chronic stress exert opposite effects on formation and contextual-related fear conditioning in rats

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Abstract

OBJECTIVE: Stress and fear conditioning are both involved in the development of affective disorders, but their interconnected relationship remains unclear. Here in this study we employed acute and chronic stress model to investigate their respective effect on fear conditioning and the CRFR1 signaling change in the limbic areas including mPFC, hippocampus and BLA.

METHODS: Male rats were subjected to acute restraint stress or chronic unpredictable mild stress before open field test and fear condition test. *In situ* hybridization was used to investigate CRFR1 mRNA expression in limbic region including mPFC, hippocampus and BLA.

RESULTS: Our results demonstrated that acute and chronic stress have opposite effects on the acquisition of fear conditioning, which is correlated to CRFR1 mRNA expression in hippocampus; however, they have similar effects on fear extinction and both facilitated contextual-related fear conditioning.

CONCLUSION: Our findings revealed acute and chronic stress led to distinct behavioral responses in fear conditioning and indicated CRFR1 is involved in the interaction of stress and fear conditioning, which help understand the connection between stress and fear memory.

Abbreviations:

mPFC	- medial prefrontal cortex
CRF	- corticotropin-releasing factor
BLA	- the basolateral amygdala
AS	- acute restraint stress
CS	- chronic stress
CUMS	- chronic unpredictable mild stress
OFT	- open field test

INTRODUCTION

Recently, increasing efforts regarding the translation of basic research achievements to the therapy of clinical disorders have led to a growing interest in investigating the neural mechanisms of fear conditioning(Delgado *et al.* 2008). Fear conditioning is widely used to investigate neurobiological mechanisms and identify potential treatments for affective disorders (Ochsner *et al.* 2005). In addition, it has been determined that stress has a significant role in anxiety (Chiba *et*

al. 2012) and depressive disorders (Swaab *et al.* 2005), and these disorders involve functional disruptions in mPFC and amygdala. The amygdala complex contains CRF cell bodies, nerve terminals and CRF receptors, and it is critical in the control of emotional and autonomic responses to stress (Zohar *et al.* 2011). Specifically, the BLA is closely related to fear and anxiety-like affective disorders (Hubbard *et al.* 2007, Kolber *et al.* 2008), which were associated with a high density of CRFR1 mRNA (Van Pett *et al.* 2000). Substantial evidence points to a key role of the CRFR1 in the mediation of CRF-elicited effects in anxiety, depressive disorders and other stress-associated disorders (Muller *et al.* 2004). Furthermore, CRFR1 in the BLA have been demonstrated to play a role in mediating the effect of CRF on the fear memory consolidation process (Roozendaal *et al.* 2002). Collectively, evidence indicates the complicated relationship between stress and fear conditioning.

In contrast to these findings, little is known about the overlap between the neural circuitry regulation of emotion and conditioned fear. Previous work has demonstrated that exposure to both acute and chronic stress has substantial effects on learning and memory. Equally important but not fully understood is the precise role of the CRFR1 in cognitive function, such as emotional learning and memory. Thus, several questions arise, such as how would the CRFR1 react following auditory cued fear conditioning, and if different types of stress, including acute and chronic stress, were induced in rat models prior to fear conditioning, what would comprise the behavioral and psychological outcomes?

Pavlovian fear conditioning is a widely used model for investigating the neurobiology of fear(Maren 2001), and the fear conditioning paradigm of this study was mainly adapted from two reports (Zhang *et al.* 2013, Kwapis *et al.* 2014).We have developed an experimental paradigm that combines the pre-process of stress conditions and post-stress fear conditioning. Our research aims to investigate the different effects of acute and chronic stress on fear conditioning in adult male rats and to investigate CRFR1 signaling change in the limbic areas including mPFC, hippocampus and BLA, which may provide valuable information to help understand how differentially stressed brains become engaged during the acquisition, extinction and retrieval of fear memory.

MATERIALS AND METHODS

Animals

Adult male SD rats (10 weeks, n=30) were purchased from Beijing Vital Rival Laboratory Animal Technology Co., Ltd. All rats were housed in the same room, with 5 animals per cage, in cages lined with wood shavings and free access to water and food. The housing room was maintained on a 12:12 h dark-light cycle (lights on between 06:00 AM and 06:00 PM), with an ambient temperature of 23±1°C. To minimize the pain and discomfort of the animals, the rats were handled for one week prior to the initiation of the experimental procedures. All protocols and experimental procedures involving animals were approved by the Committee on the Ethics of Animal Care and Use of University of Science and Technology of China.

Stress treatments

After one week of handling, 30 rats were randomly allocated to the acute restraint stress (AS) group, the chronic stress (CS) group, and the control (CON) group. Next CS group rats received 4 weeks chronic unpredictable mild stress (CUMS) treatment and chronic stress was primarily adapted and modified according to a previous study (Isgor *et al.* 2004) and was different from the CUMS procedure (Ge *et al.* 2013). The chronic stress regimens included 4 randomly assigned stressors: 5 min of restraint stress, 24 h of a wet cage, 24 h of food and water deprivation, and 5 min of a cold swim at 8-10°C. The different stressors were randomly distributed at an interval of 4 days, and all stressors were administered seven times within 28 days. On the 28th day of chronic stress treatment AS group rats were subjected to a 15-min forced swim test by placing each rat in a transparent cylinder (60×30 cm), which contained water at 22±2°C with a depth of 35 cm. The control rats were placed in the next room from the location of swimming to avoid being affected. On the next day all rats had undergone the open-field test. Then all rats were exposed to the formation test of fear conditioning. 24h later, the contextual related fear conditioning was examined. On the next day, the extinction training of fear conditioning was conducted, the condition of extinction retention was examined 36 h later. Immediately following all fear conditioning tests, the rats were anesthetized and the brains were removed to produce frozen sections, followed by in situ hybridization experiments of CRFR1.

Open-Field Test

The open field test (OFT) apparatus was applied to analyze the spontaneous and exploratory activities of animals to a novel environment (Prut *et al.* 2003). The testing room was temperature-, humidity- and illumination-controlled. The test box was composed of black wood and consisted of a floor (96 × 96 cm) with 50 cm walls; it was equipped with photocell emitters and receptors, which created an x-y grid of invisible infrared beams (Tatem *et al.* 2014). The box floor was painted with white lines (6 mm) to form 16 equal squares, and the central four squares were defined as the center area. The test was performed under bright ambient room light. Each rat was placed in the center of the open field and left to freely explore the unfamiliar arena for 5 min. The total locomotion distance, velocity, rearing times and grooming times were recorded (Cai *et al.* 2010).

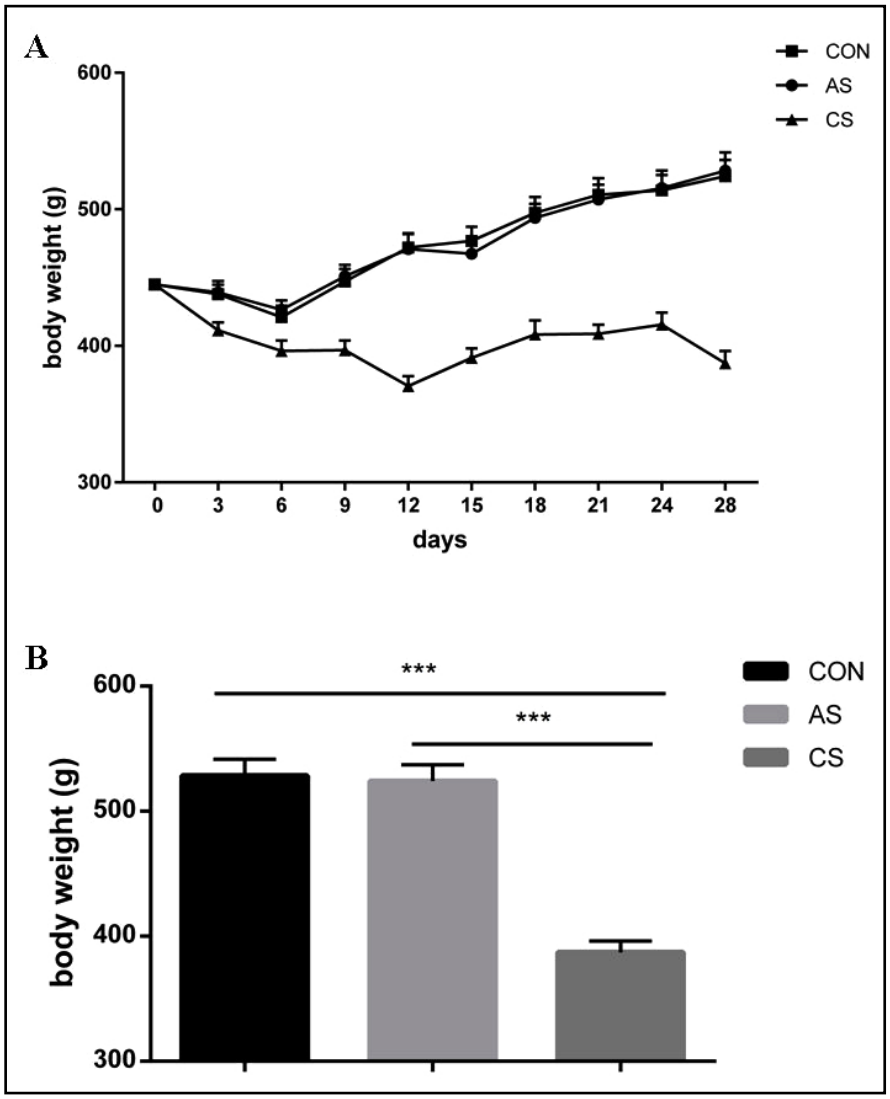

Fig. 1. Body Weight curves. A, The 4-weeks body weight curves ranged from the nest day after handling to the last day of chronic stress, which was recorded every three days for all three groups. B, body weight before fear conditioning. Each weight was collected at 10 o'clock in the morning. The data are shown as mean ±S.E.M; *** $P<0.001$ using ANOVAs with a student Newman-Kuels post hoc test, n=10. CON, control; AS, acute stress; CS, chronic stress.

Fear conditioning test

This procedure was adapted from previous reports (Cordero *et al.* 2003, Baran *et al.* 2009, Zhang *et al.* 2013). Each chamber was enclosed by an audio speaker, a house light, an infrared LED light and a ceiling mounted digital camera that was sensitive to light in the IR range; the set-up was connected to a personal computer that ran video-tracking software, which detected and recorded behavior. On the first day, immediately following the 2 minutes of habituation, there were 25 pairings of the tone (10 seconds, 1500 HZ, 75 dB) with a foot shock (1 mA, 2 seconds, which was the last two seconds before the end of the tone) that co-terminated with the tone. After the fear conditioning test, the rats were transported to the colony room for normal feeding. On the second day, the context induced fear conditioning was measured in the same chamber. The testing process consisted of 2 minutes of free activities without a tone or foot shock. On the fourth day, the extinction of fear conditioning was measured: 2 minutes of habituation followed by 25 trials of a tone without foot shock. This step was repeated after 24 hr. The intertribal interval (ITI) was 60 seconds, and each rat was removed from the conditioning chamber 30 seconds after the final shock. Freezing was defined as the absence of all movements with the exception of movements associated with respiration. The freezing time during each trial (10 second tone) was calculated; it was subsequently used as an index of conditioned fear and converted to a percentage (Σ ([time of freezing/10 seconds] $\times$ 100)).

Brain preparation and in situ hybridization (ISH)

All brains were prepared in accordance with a previously described procedure (Poulin *et al.* 2008). In

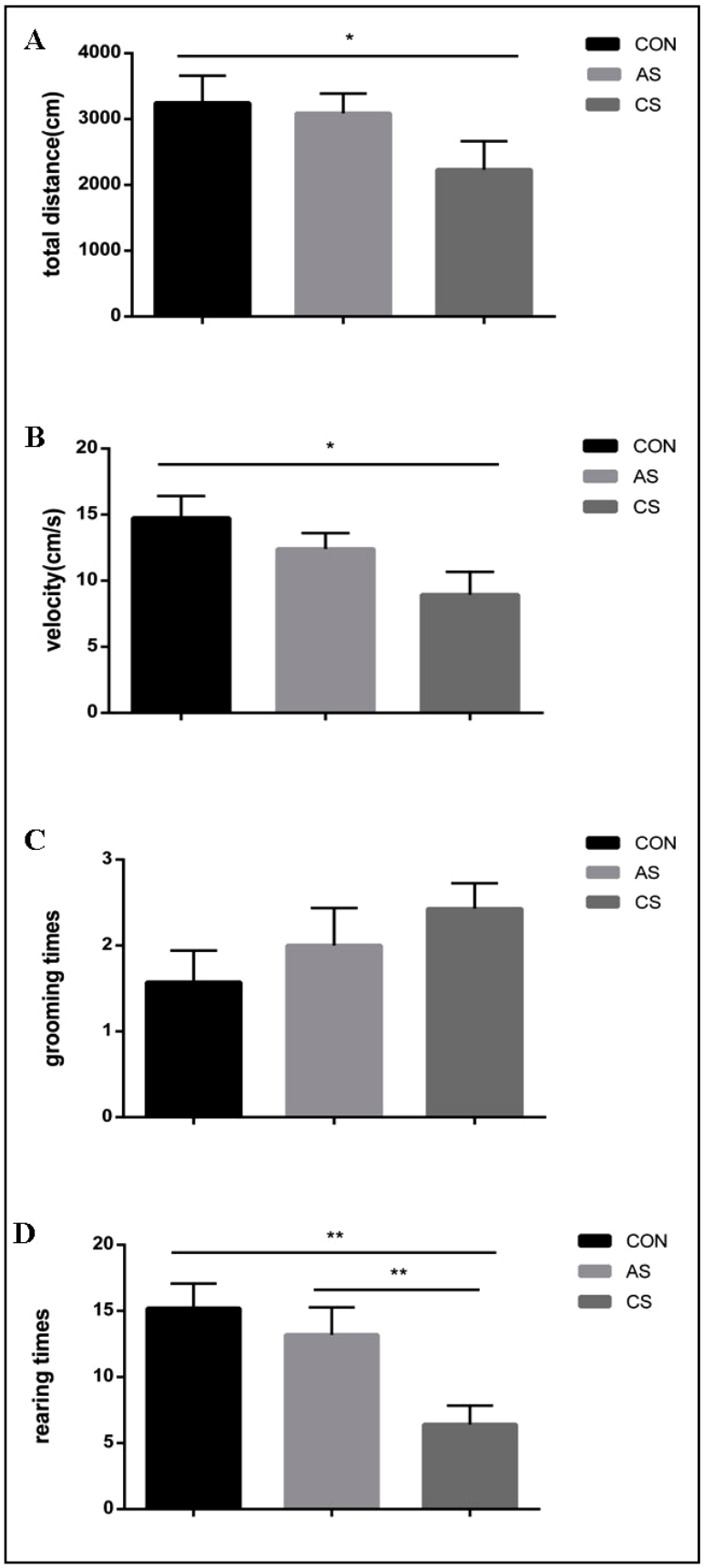

Fig. 2. Open field test data for rats subjected to acute or chronic stress. A, total distance; B, velocity; C, grooming times; D, rearing times. The presented data are showed as mean ±S.E.M with n=10 for each group, **P*<0.05, ***P*<0.01 using ANOVAs with a student Newman-Kuels post hoc test. CON, control; AS, acute stress; CS, chronic stress.

brief, the rats were rapidly anesthetized via pentobarbital sodium (30 mg/kg) and subsequently perfused with 200 ml of ice-cold saline, followed by 500 ml of a 4% paraformaldehyde (PFA) solution. All brains were carefully maintained in a 4% PFA solution at 4°C overnight and then transferred to 15% and 30% sucrose solutions successively until they settled to the bottom of the bottles prior to being cut in the cryostat microtome (Leica CM1950, Germany). Thirty micrometer thick sections were obtained and stored at -20°C until use.

DNA probe based *in situ* hybridization was performed (Sylwestrak *et al.* 2016) to localize the CRFR1 mRNA in the frozen brain sections. The ISH kit (Item No.MK1615) was purchased from Wuhan Boster Biological Engineering Co., LTD, and the protocol used was mainly adapted from the manufacturer's instructions.

In brief, 2 drops of concentrated pepsin were added to 1 ml of 3% citric acid, followed by mixing and digesting for 5-120 seconds at room temperature. The brain sections were washed in PBS three times for 5 min each time. The sections were subsequently immobilized in stationary liquid for 10 min at room temperature and rinsed in distilled water three times. Pre-hybridization: 20 μL of a preliminary hybridization solution were added to every section under a constant temperature (38-42°C) treatment for 2-4 h. After hybridization, the processing condition was the same as the previous step with the exception that the action time was 24 h. On the following day, the sections were washed with 2× SSC twice for 5 minutes/time, followed by 0.5× SSC for 15 minutes and then washed again with 0.2× SSC for 15 minutes. The temperature of all solutions was maintained at 37°C. Blocking buffer was added to the sections. The sections were incubated for 30 minutes at 37°C, and biotinylated anti-rat digoxin was subsequently added and incubated for 60-120 minutes at 37°C. The sections were washed in PBS four times for 5 minutes each time. SABC was added to the sections and incubated for thirty minutes at room temperature, followed by washing in PBS three times for 5 minutes each time. The next step included the addition of biotinylated peroxidase to the sections for 30 minutes at room temperature. Finally, DAB staining was performed for at least 20 minutes, followed by washing with distilled water, dehydration, transparent and mounting.

RESULTS

Body weight and behavior change induced by chronic stress

Body weight changes were assessed for 28 consecutive days. The line of the AS group was consistent with the control; however, the rats that were chronically subjected to stress did not exhibit weight gain over time (Figure 1A). At the end of the chronic stress, there was a significant change in body weight among three groups ($F_{2,27}$=43.99, P<0.001), particularly only body weights of chronic stressed rats were significantly decreased compared with the controls (P<0.001) or AS group (P<0.001) (Figure 1B), which was consistent with one of the typical physiological alterations identified in rats in a depression model, as well as patients with depressive disorders.

In open field test, it showed that there was a significant difference among three groups in terms of total locomotion distance ($F_{2,27}$=4.13, P=0.04, Figure 2A) and velocity ($F_{2,27}$=3.97, P=0.04, Figure 2B). Particularly the total distance and velocity were significantly decreased in CS group compared with the control group (P<0.05), whereas there was no significant difference between the AS and control group. Regarding the grooming times, there was no significant difference among the three groups ($F_{2,27}$=0.03, P=0.74, Figure 2C). The rearing times of the CS group were significantly decreased compared with the control or AS groups ($F_{2,27}$=7.72, P<0.01, Figure 2D).

Different effects of chronic stress and acute stress on fear conditioning

With respect to the formation of fear conditioning results, there was a significant difference in freezing ration among three groups ($F_{2,27}$=12.72, P<0.001, Figure3A), particularly the rats subjected to acute stress exhibited a significantly lower freezing ratio compared with the control group (P<0.01) and CS group (P<0.001), whereas the rats subjected to chronic stress exhibited a little higher freezing ratio than control rats (P<0.05), which suggest that acute and chronic stress exerted opposite effects on the formation process of fear conditioning. For the contextual-related fear conditioning test (Figure 3B), we put animals into another test box with the same context 24 h after the initial test. There was a significant difference among the three groups ($F_{2,27}$=3.81, P=0.03). Specifically, the rats in

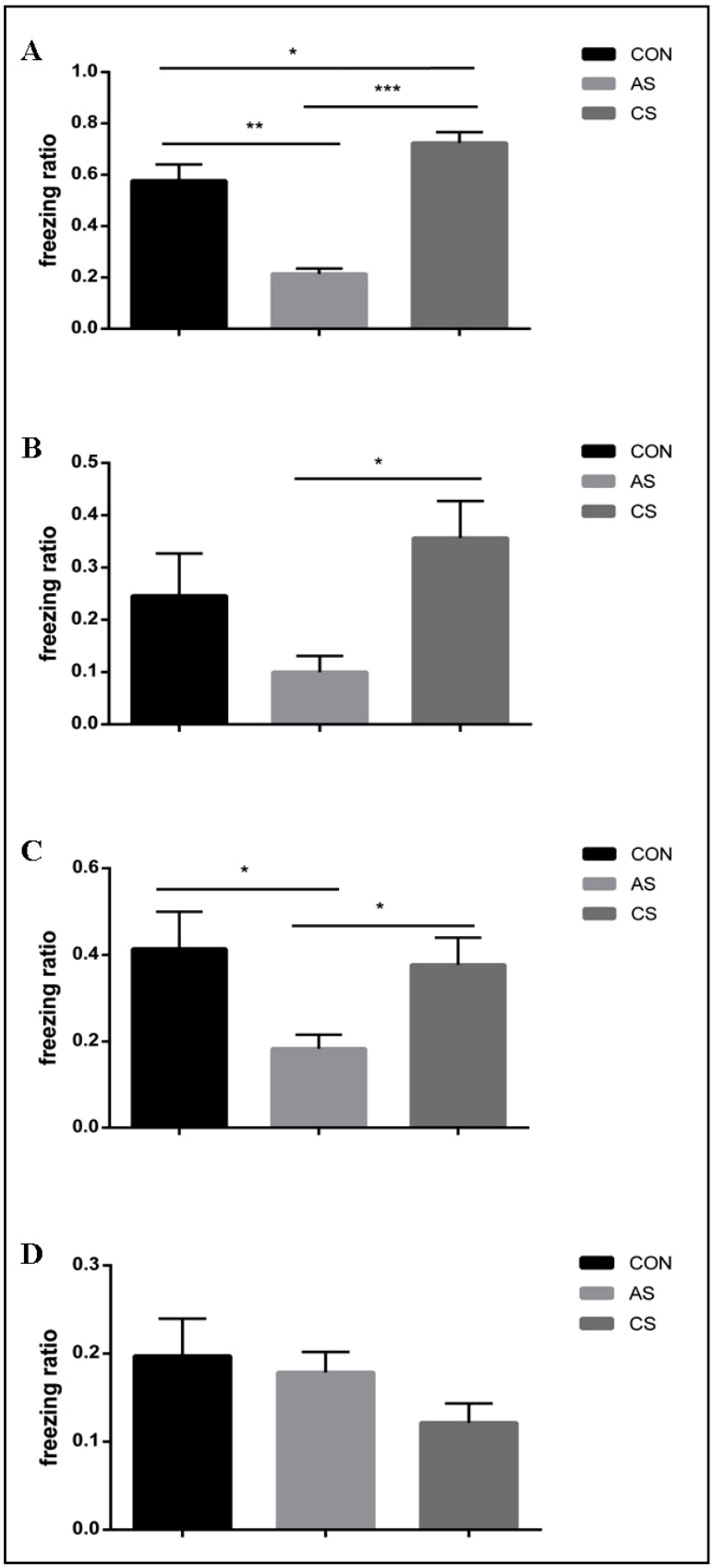

Fig. 3. Freezing ratio for rats subjected to acute or chronic stress at different stages in fear conditioning test. A, Formation of fear conditioning; B, contextual related fear conditioning test; C, fear extinction stage1; D, fear extinction stage 2. The presented data are showed as mean ±S.E.M with n=10 for each group, *P<0.05, **P<0.01, *** P<0.001 using ANOVAs with a student Newman-Kuels post hoc test. CON, control; AS, acute stress; CS, chronic stress.

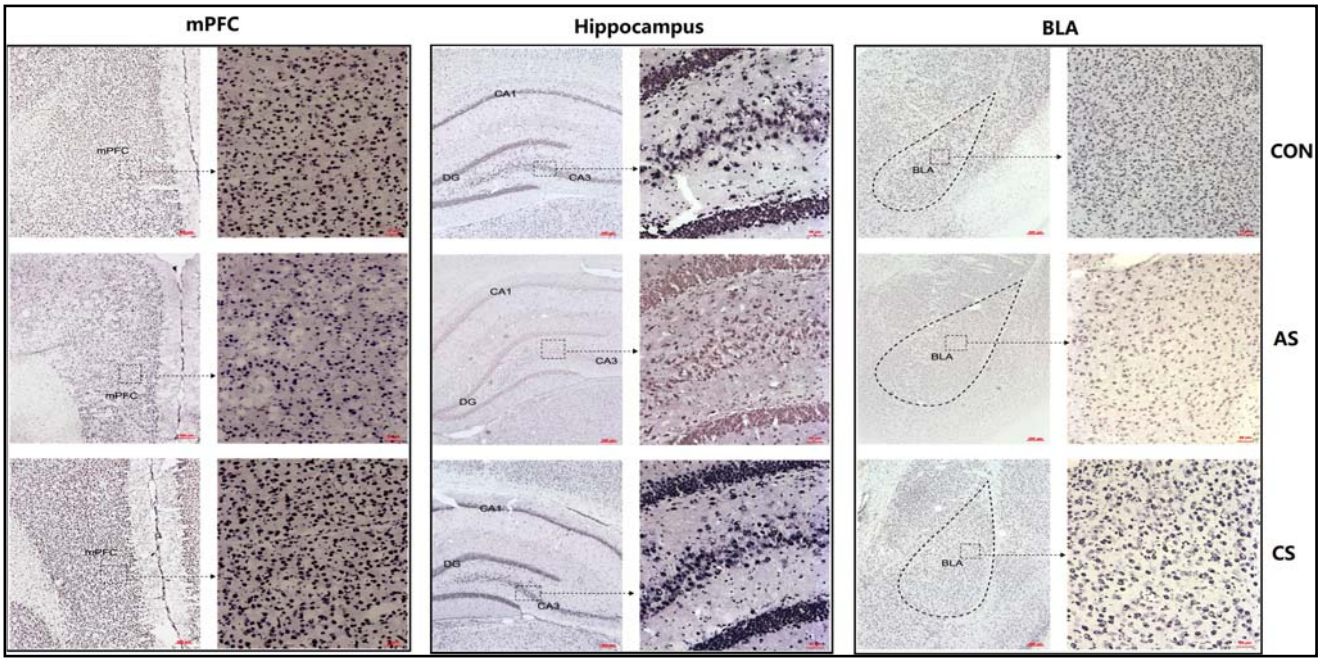

Fig. 4. CRFR1 mRNA expression in mPFC (left panel) Hippocampus (middle panel) and BLA (right panel). The CRFR1 mRNA was detected by *in situ* hybridization. Left: magnification of ×5, Bar =200 μm; Right: Magnification of ×20, Bar =50μm (CON, control; AS, acute stress; CS, chronic stress).

the CS group exhibited a significantly increased freezing ratio compared with the AS group ($P<0.05$), which indicated that acute and chronic stress exerted distinct and significant effects on the response to the same surroundings.

During fear extinction test, as shown in Figure 3C (stage I) and 3D (stage II), the difference of freezing ration among three groups was significant at stage I ($F_{2,27}=3.34$, $P=0.02$) and no significant difference was identified at stage II 36 h later ($F_{2,27}=2.81$, $P=0.08$). In particular, at stage I there was a significant difference of freezing ratio between the AS and control group or CS group ($P<0.05$). However, When the CS group was compared with the controls, the former was less than the control group, and the difference was not significant ($P>0.05$). At stage II of fear extinction test, there was no significant difference between any two groups in terms of freezing ratio.

Hippocampal CRFR1 mRNA expression related to chronic stress and acute stress

Limbic system areas such like mPFC, amygdala and hippocampus are mainly involved in stress response and fear conditioning. Since CRF signaling is a key mediator in stress response, we investigated CRFR1 mRNA expression by *in situ* hybridization in the above areas (Figure 4: Left panel, mPFC; middle panel, Hippocampus; right panel, BLA). Compared with control group, a significant increased CRFR1 mRNA expression was only in CS group ($P<0.01$, Figure 5A) in mPFC while a significant decreased mRNA level of CRFR1 in BLA were found either in AS or CS group ($P<0.01$, Figure 5A). In the hippocampus, there was a significant difference ($F_{2,27}=24.51$, $P<0.01$) among the three groups in the CRFR1-positive cell numbers (Figure 5A). Compared with control rats, the number of CRFR1 positive cells was significantly increased in chronically stressed rats ($P<0.05$), whereas decreased in the acutely stressed rats ($P<0.01$). More interestingly, there was a significant correlation between freezing ratio at fear acquisition phase and CRFR1 mRNA expression in hippocampus ($R^2=0.56$, $P<0.01$, Figure 5B), which suggest hippocampus play a vital role in mediating stress response and fear conditioning.

DISCUSSION

This study was designed to investigate whether there was a difference between acutely and chronically stressed adult male SD rats in terms of the behavioral responses in a fear conditioning test. The novel findings indicated that acutely stressed rats exhibited a decreased freezing ratio in the formation and contextual related fear conditioning, whereas chronic stress exerted the opposite role. Both acute and chronic stress decreased the freezing ratio in fear extinction. 36 hours later, the chronically stressed rats continued to exhibit a lower freezing ratio compared with the controls; in contrast, the acutely stressed rats had returned to approximately the same degree as the controls. It was clear that the response to chronic stress was completely different from the response to acute stress. The current findings indicate that there may be overlaps between the mechanisms of stress regulation and fear conditioning, which may provide useful information regarding how to overcome and diminish fear.

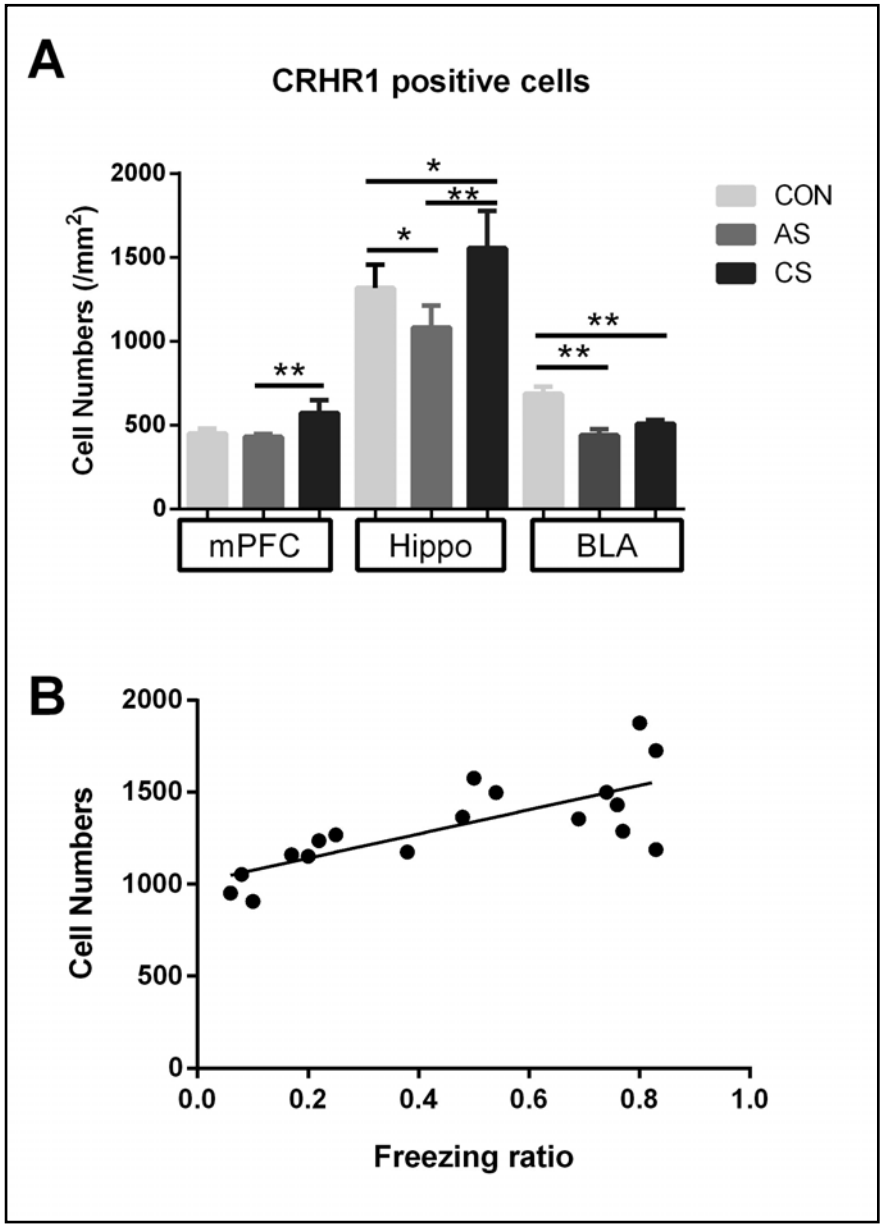

Fig. 5. Statistics for CRFR1 positive cells. A, The number of CRFR1 positive cells in mPFC, hippocampus and BLA. B, The linear correlation between CRFR1 positive cells in hippocampus (6 plots, each group) and freezing ration at the acquisition stage. The data are shown as mean ±S.E.M; **P*<0.05, ***P*<0.01, *** *P*<0.001, using ANOVAs with a student Newman-Kuels post hoc test. CON, control; AS, acute stress; CS, chronic stress.

The data indicated that chronic stress considerably decreased the body weight gain in rats, which was consistent with the previous findings (Lenglos *et al.* 2013) (Retana-Marquez *et al.* 2014). The locomotive and exploratory behaviors of animals following chronic stress exposure have been comprehensively measured in the open field test (Prut *et al.* 2003, Tatem *et al.* 2014), and the CS group exhibited significantly decreased total distance traveled and rearing times compared with the controls, which suggested that chronic stress affect the exploratory behavior in adult male rats.

In the formation of fear conditioning, which is also referred to as acquisition, chronic stress increased the freezing time, but acute stress reduced it. Similar results have indicated that the exposure of rats to chronic stress for 21 days enhanced subsequent cued fear conditioning, and the stress involved both moderate (Cordero *et al.* 2003) and highly stressful levels at training(Sandi *et al.* 2001). But a recent study indicated that repeated restraint stress did not significantly impact the acquisition of fear conditioning compared with the non-restraint controls (Zhang *et al.* 2013). This discrepant result was intriguing: the primary dissimilarity was the form and duration of the chronic stress. Repeated restraint stress has age-dependent effects on both the amygdala physiology and amygdala-dependent affec-

tive disorder. Regarding the use of the acute stress model, a previous experiment demonstrated that acute exposure to inescapable swim stress persistently facilitated the acquisition and performance of the classically conditioned eye blink response (Shors 2001). Thus, the intrinsic nature of acute stress may play a key role in the acquisition of fear conditioning. In general, the current findings revealed more regarding what was not critical rather than what was critical in terms of facilitated fear conditioning in response to different types of stress. The findings suggested that facilitated fear conditioning may be mediated by the activated psychological response and the intrinsic nature of the stress.

Chronically stressed rats exhibited an increased freezing ratio in contextual-related fear conditioning; however, acute stress had the opposite result. We identified a decreased freezing ratio by acute stress, which was inconsistent with a previous study in which rats that experienced the stressful situation of a single exposure to restraint stress 2 days before training exhibited enhanced contextual fear conditioning (Cordero *et al.* 2003). However, the big difference in this study was that there was exposure to stress 2 days before training, whereas in our study, contextual fear conditioning was evaluated immediately after acute stress. Interestingly, this effect was identified when the animals were trained as early as 30 minutes after exposure to the stressor was completed (Shors 2001) and remained present if the training occurred 48 hours post-stress. One potential hypothesis for this effect is that animals become sensitized to shock during or after exposure to the stressor (Ryoke *et al.* 2014), and the effect transfers to the training condition in which the foot shock is also a shock. Similarly, one study reported that acute stress (1 hr. of neonatal isolation) impairs context-induced fear conditioning in adult male rats (Kosten *et al.* 2006). In conclusion, these findings support the idea that chronic stress experiences that precede exposure to new types of stressors facilitate the development of contextual fear conditioning in adult male rats; however, a single exposure to aversive stimulation was sufficient to decrease context-dependent fear conditioning. Moreover, previous evidence indicates that increased glucocorticoid release at training (Cordero *et al.* 2003) may be implicated in the regulation of contextual related fear conditioning following the experience of different stresses.

The extinction of fear conditioning was trained or tested for 24 hours following the test of contextual fear conditioning. Our data demonstrated a significantly decreased freezing time in the acute stress rats compared with the controls, whereas there was no remarkable difference between the controls and chronically stressed rats. In addition, a history of chronic stress reduced the freezing time to a tone at 36 hours after the first extinction session, nevertheless acute stress only had a minimal impact on the extinction retention. A recent study indicated that 7 days of repeated restraint stress disrupted the acquisition of the extinction of fear conditioning in adolescent rats; however, it had no effect in adult rats. Several previous studies did not identify an effect of chronic stress on fear extinction in adult rats (Miracle *et al.* 2006). One potential explanation for the inefficacy of chronic stress on fear extinction was that these studies only measured the initial trials of extinction, whereas there were 25 trials recorded in our experiment. Furthermore, our chronic stress regimen had four types of stress; however, they only applied to one type of repeated stress. Thus, different stressful events applied to different maturities of adult rats may exert different damages to the structure and function of the limbic areas and may have therefore caused adverse behavioral consequences on fear extinction.

The hippocampus is not only important in spatial memory formation and contextual-related fear conditioning (Fabri *et al.* 2014) but is also highly sensitive to stress and is strongly affected by the HPA axis (Muller *et al.* 2004). CRFR1 signaling has been indicated as an important contributor to fear conditioning in rats. Evidence indicates the importance of the limbic CRF and CRFR1 neural pathways as a promising therapeutic target for affective disorders, such as depression, or the consequences of stress (Muller *et al.* 2003). Based on our results, after the fear conditioning tests, the acutely stressed rats exhibited a significantly decreased CRFR1 mRNA expression in the hippocampal CA3 compared with the controls; in contrast, chronic stress increased the CRFR1 mRNA expression. More interestingly, there was a significant correlation between hippocampal CRFR1 positive cells and freezing ration at the acquisition stage among three groups, which indicate hippocampus play a role in crosslinking stress response to fear. Furthermore, in the hippocampus, there was a significant difference in the CRFR1-positive cell numbers among the three important subdivisions, including the DG, CA2 and CA3, and the DG had the largest number of CRFR1 positive cells, which suggest a key role of DG involved in stress and fear interconnected neural pathway. Our data suggest that acute stress and chronic stress differentially affect the acquisition of fear and it was correlated with CRFR1 expression in hippocampus.

In conclusion, our data demonstrated acute and chronic stress led to distinct behavioral responses in fear conditioning and indicated CRFR1 is involved in the interaction of stress and fear conditioning, which help understand the connection between stress and fear memory.

STATISTICAL ANALYSIS

Statistical analysis was performed with IBM SPSS Statistics 19.0 for Windows (SPSS Inc., Chicago, IL, USA). The data are presented as the mean ± SEM (n=10 per group, and a P-value <0.05 was considered statistically significant). The comparisons among these three groups were performed with one-way analyses of vari-

ance (ANOVA), followed by a student Newman-Kuels post hoc test, and all diagrams were created by GraphPad Prism 6.0.

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