Ref. No.: PNEC-D-15-00744R1

Title: Corticosterone regulates fear memory via Rac1 activity in the hippocampus

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Total number of pages: 22

Number of figures: 4

Supplemental information: 0

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ABSTRACT

Stressful events can generate enduring memories, which may induce certain psychiatric disorders such as post-traumatic stress disorder (PTSD). However, the underlying molecular mechanisms in these processes remain unclear. In this study, we examined whether the active form of the small G protein Rac1, Rac1-GTP, is involved in fear memory. Firstly, we detected the time course changes of Rac1-GTP after foot shocks (a strong stressor) and exogenous corticosterone (CORT) treatment. The data showed that stress and CORT induced the downregulation of Rac1-GTP in the hippocampus. Changes in the serum CORT level were negatively correlated with the level of Rac1-GTP. Additionally, a glucocorticoid receptor antagonist, RU38486, not only recovered the expression of Rac1-GTP but also impaired fear memory. Furthermore, systemic administration of NSC23766, an inhibitor of Rac1-GTP, improved fear memory at 1.5 and 24 h. Therefore, Rac1 activity plays a critical role in stress-related cognition and may be a potential target in stress-related disorders.

Keywords: Stress; Foot shocks; Rac1-GTP; Corticosterone; Hippocampus; Fear memory.

Abbreviations: CORT, corticosterone; FSs, foot shocks; GPCR, G-protein coupled receptors; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenocortical, MR, mineralocorticoid receptor

Introduction

The molecular mechanisms involved in fear are key points in gaining a better understanding of fear-related disorders such as posttraumatic stress disorder (PTSD) (Desmedt et al., 2015). Stressful events, which differ among individuals and daily situations, are usually remembered well; produce releasing of corticosteroids, behavior response and autonomic response, which is the interaction of physiology, psychology, and brain regions in response to the stressor. Stress has many functions. Stress events stimulate fear memory, which helps individuals to avoid dangerous situations; fear memory is believed to be the result of a salient and rapid release of stress hormones, such as corticosterone (CORT) in rats and cortisol in humans (Buchanan and Lavallo, 2001; Cornelisse et al., 2014; Marks et al., 2015; Reul and de Kloet, 1985). Contextual fear conditioning is widely used as a hippocampus-dependent model of PTSD (Desmedt et al., 2015; Eichenbaum, 2000; Grillon et al., 1996; Jiang et al., 2015)

The key intersection of stress and memory regulation is located in the hippocampus. On one hand, the hippocampus is the major region of the brain that regulates the stress response, via a negative feedback process by activating the major CORT receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). On the other hand, the hippocampus is also responsible for stress-related contextual recognition in fear memory. GRs, which are abundant in the hippocampus, are believed to be substantially activated only when CORT levels rise, thus playing a major role in stress responses (Roozendaal, 2000). GRs translocate to the nucleus after binding to CORT, where they operate as transcriptional regulators of gene expression (Herman et al., 2003; Funder, 1997; Reul and de Kloet, 1985). GRs are reported to play a key role in the consolidation of long-term fear memory (Abrari et al., 2009; Roozendaal, 2000; Keller et al., 2015; Maggio and Segal, 2012), but the underlying mechanisms remain unclear.

Ras-related C3 botulinum toxin substrate 1 (Rac1) belongs to the Ras homolog (Rho) family of small GTPases, which cycle between an active (GTP-bound) and an inactive (GDP-bound) form. When GTP loading by guanine-exchange factors (GEFs), GTP-bound active state of Rac1 engages numerous effector proteins, is involved in a series of signaling pathways, and has widespread actions, including in cognitive function, synaptic transmission, and plasticity by regulating the cytoskeleton (De Filippis et al., 2014; Fritz and Kaina, 2013; Schwechter and Tolia, 2014; Thumke et al., 2013; Um et al., 2014, Dietz et al., 2012). Decreased Rac1 signaling is reported to increase spine formation (Dietz et al., 2012), decelerate memory decay (Diana et al., 2007; Shuai et al., 2010), and therefore enhance memory (Dietz et al., 2012; Jiang et al., 2015). The activity of Rac1 may thus shift the balance between remembering and forgetting (Davis, 2010; Shuai et al., 2010).

In view of the relation of Rac1 to memory decay but less known about function of Rac1 on fear memory. To better understand the role of active Rac1 in stress-related memory, rats in the present study were exposed to contextual fear conditioning consisting of 5 repeated foot shocks (FSs) as a stressor. The expression of Rac1-GTP and the serum CORT levels were measured at 0.5, 1.5, and 24 h after FSs. We found that FSs induced the downregulation of Rac1-GTP, which was negatively correlated with changes in serum CORT levels. Furthermore, the above results were mimicked by exogenous CORT treatment and blocked by a GR antagonist RU38486. Finally, fear memory was attenuated by treatment with RU38486, a GR antagonist, before FSs, while fear memory was enhanced by the Rac1-GTP inhibitor NSC23766.

1. Methods and Materials

1.1. Subjects

A total of 100 male Sprague-Dawley rats (SD rats) weighing 250-300 g were used in these experiments.

Upon arrival, rats were group-housed in standard plastic cages with a 12 h light/dark cycle (lights were on from 07:00 to 19:00), and food and water were provided ad libitum. All experimental procedures were carried out in accordance with the approved guidelines of ethics committee of Kunming Institute of Zoology, Chinese Academy of Sciences.

Fear conditioning

Contextual fear conditioning is a well-accepted animal model in fear memory research. Foot shock is often used as unconditioned stimulus (US) that is regarded as a strong stressor, to establish fear memory. FSs were carried out in a chamber ($30 \times 24 \times 21$ cm³; MED-Associates, St. Albans, VT) in which the floor consisted of 18 steel rods (4 mm in diameter) wired to a shock generator for the delivery of stimuli. Video Freeze software (Med Associates) automatically scored the freezing times; the threshold was set at 18, which excluded the background noise, including the respiratory movements of the rats. Twenty-four hours before the fear-training session, the rats freely explored the box for 5 min to habituate to the environment. In the training session, the rats were also allowed to freely explore for 2 min before receiving 5 inescapable intermittent electric FSs of 0.8 mA intensity for 2 s duration with 2 min inter-shock interval, delivered for 12 min. The rats stayed in the conditioning chamber for 2 more minutes after the fifth trial before being placed back into their home cages. At 0.5, 1.5, and 24 hours after the FSs, fear memory was tested by placing rats back in the same conditioning chamber for 5 min to measure freezing time.

1.2. Drug treatment

Corticosterone (TCI, Shanghai, China, Cat. #C0388) was dissolved in 10% dimethyl sulfoxide (DMSO) and administered intraperitoneally (i.p.) at a dose of 40 mg/kg. The GR antagonist mifepristone (RU38486, Sigma-Aldrich) was dissolved in 10% DMSO and administered at a dose of 80 mg/kg (i.p.).

Control groups received equal volumes of 10% DMSO. The Rac1-GTP inhibitor NSC23766 was dissolved in distilled water and administered (i.p.) at a dose of 1.5 mg/kg before training, and the same volume of distilled water was injected into the rats in the control group. For all the drugs, the injection volume was 0.5 ml/kg.

1.3. CORT assay

2.4.1. Serum collection

Blood was collected after the rats were rapidly decapitated under diethyl ether anesthesia 0.5, 1.5, 3, or 24 h after the FS procedure and exogenous CORT treatment. The blood was left standing for 2 h at room temperature to allow the serum to separate. After centrifugation for 10 minutes at 3,000 g, the clarified serum was carefully collected in tubes and stored at -80°C until used in an ELISA assay. As a preliminary test to check the level of serum corticosterone after stress and confirm the effective intensity of foot shock beforehand, we collected the serum of 10 samples at 5 time points: naive, 0.5, 1.5, 3, and 24 h after foot shocks. After then, total 20 samples of both the serum and entire hippocampal tissue at the same 5 time points were collected. So, the total 30 serum samples after foot shocks in 5 time points were for the CORT assay.

2.4.2. Serum corticosterone detection

The CORT concentration of total 54 samples serum (30 samples from fear conditioning and 24 samples from exogenous CORT treatment) was determined with an ELISA assay kit (Blue Gene, Cat. #E02C0006). The CORT ELISA kit applied the competitive enzyme immunoassay technique utilizing a monoclonal anti-CORT antibody and CORT-HRP conjugate. A total of 10 µl of serum from each rat was added to the buffer and incubated together with the CORT-HRP conjugate in a pre-coated plate for 1 h. After the incubation period, the wells were decanted and washed five times, then incubated with a

substrate for the HRP enzyme. The product of the enzyme-substrate reaction formed a blue-colored complex. Finally, a stop solution was added to stop the reaction, which turned the solution yellow. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. A standard curve was plotted relating the intensity of the color (optical density, O.D.) to the concentration of standards. The CORT concentration in each sample was interpolated from this standard curve.

1.4. Rac1-GTP assay

At 0.5, 1.5, and 24 h after the 12 min FS procedure or the exogenous CORT injection, the rats were sacrificed to assay Rac1-GTP via immunoblotting. To maintain a consistent time-course, the rats in the CORT treatment groups were placed in their home cages for 12 min after the CORT injection, which was same duration as the FS procedure. Entire bilateral hippocampi were dissected on a cold plate and subsequently stored at -20°C. The tissues were used for a pull-down assay (Millipore kit, cat. #17-283). The pull-down lysates and total protein lysates were then separately used for SDS-PAGE Western blot analysis and detection.

2.5.1. Pull-down assay

The hippocampal tissue lysate was prepared by homogenization in ice-cold 1x lysis buffer (diluted from 5x Mg²⁺ Lysis Buffer, MLB, Cat. #20-168, Millipore) and placed on ice for 30 min. After 30 min, the lysate was centrifuged at 14,000 g for 15 min and the supernatant was collected. The sample concentrations were determined using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Protein p21-activated protein kinase 1 (Pak1) is an effector protein that can bind active Rac1. One mg of total protein was incubated with 10 µl PAK-1 PBD (a GST fusion protein corresponding to the p21-binding domain (PBD) of human PAK-1) agarose beads for 1 h; the beads were then washed 3 times. The agarose beads were re-suspended in 40 µl of 2x reducing sample buffer

and boiled for 5 min. The bound proteins were separated by 12% SDS-PAGE. GTP-bound Rac1 was detected by immunoblotting with an anti-Rac1 antibody (Cat. #05-389, Millipore).

2.5.2. Western blot analysis:

Western blot analysis was performed according to previous protocols (Jiang et al., 2015). The reduced samples (including bound proteins and total proteins) were separated by 12% SDS-PAGE for 30 min at 90 V followed by 75 min at 120 V and transferred to a PVDF membrane. The membrane was blocked in freshly prepared 10% BSA (Applygene, Sigma, Cat.#:P1622) in TBST for 1 h at room temperature with constant agitation and then incubated overnight at 4°C with primary antibodies diluted in freshly prepared 1% BSA in TBST (1 µg/ml of anti-Rac1, from Millipore Co, Cat.#: 05-389; 1:10000 anti-GAPDH, from Millipore Co, Cat.#: ABS16). After washing 3 times, the membrane was incubated with secondary antibodies (goat anti-mouse HRP-conjugated IgG, Kang Chen, Cat.#: kc-MM-035, 1:2000 dilution in TBST) for 1.5 h at room temperature with agitation. The PVDF membrane was washed with TBST three times (10 min for each wash). For the Western blot results, the gray values of the lanes were obtained using ImageJ software. The amount of total protein was used to control for the input immunoblot values from each individual sample, which were then normalized to the mean of the naïve control group, such that the average naïve control value equaled 100%. Total Rac1 levels were represented as the percent change from the naïve group, and the Rac1-GTP levels were represented as percent change in relation to total Rac1.

1.5. Statistical analysis

Statistical analyses were performed using SPSS for Windows software. Comparisons were conducted using Student's *t* test or one-way analyses of variance (ANOVA) followed by least significant difference comparisons. All data were reported as means $\pm$ S.E.M. Correlations were studied by means

of the Spearman's correlation coefficient. A difference was considered statistically significant if the p value was < 0.05 .

2. Results

2.1. Fear conditioning induces an increase in the level of serum CORT and the down-regulation of Rac1-GTP expression

Serum and entire bilateral hippocampi were collected from the same rats 0.5, 1.5, or 24 h after FS (**Figure 1A**). We found that the level of serum CORT in the rats was increased after exposure to FSs and reached a peak at 1.5 h; the level recovered to the baseline by 24 h. The level of serum CORT in 1.5 h group was significantly different compared with the naïve and 24 h groups ($F_{4, 25} = 4.250$, $p < 0.05$) (**Figure 1B**). At the same time, the expression of Rac1-GTP was decreased at 0.5 h but recovered at 1.5 h after the FSs ($F_{3, 12} = 5.536$, $p < 0.05$; (**Figure 1C₁₋₂**). However, the expression of total Rac1 remained unchanged after the FSs ($F_{3, 12} = 0.589$, $p > 0.05$; **Figure 1C₃**), which suggested that the fear stimuli stressor inhibited Rac1 activation.

2.2. Exogenous CORT mimics the effect of stress on Rac1-GTP expression

To further demonstrate whether the stress hormone CORT was the key factor in the downregulation of hippocampal Rac1-GTP, we treated rats with an exogenous CORT injection instead of the FS stressor. We found decreased Rac1-GTP expression at 0.5 h (**Figure 2C_{1-C₂}**) was accompanied by increased serum CORT level ($F_{3, 20} = 4.214$, $p < 0.05$; **Figure 2B**). Twenty-four hours after systemic CORT injection, the Rac1-GTP level had recovered ($F_{3, 12} = 5.973$, $p < 0.05$; **Figure 2C_{1-C₂}**). However, the total Rac1 expression remained unchanged after FS ($F_{3, 12} = 0.525$, $p > 0.05$; **Figure 2C₃**)

2.3. The expression of Rac1-GTP is negatively correlated with the level of serum CORT

A correlation coefficient was computed to assess the relationship between hippocampal Rac1-GTP and

serum CORT levels. Western blot analysis was used to assess the extracted proteins from twelve rats, for which the serum CORT levels were determined by ELISA analysis (see Methods 2.4, 2.5). Rac1-GTP (y-axis) was ranked from greatest to least, while CORT (x-axis) was ranked from least to greatest. We found a significant negative correlation between the expression of Rac1-GTP and the level of CORT (Spearman's coefficient: -0.08292, $*p < 0.05$; **Figure 3**).

2.4. A GR antagonist blocks Rac1-GTP downregulation as well as fear memory retrieval impairment induced by stress

To further investigate the role of GRs on the expression of Rac1-GTP, rats were randomly divided into 2 groups that received either RU (80 mg/kg, i.p., n=10) or vehicle 30 minutes before the treatment with CORT (40 mg/kg, i.p., n=10). The rats were put back into their home cages for 12 min and a further 1.5 h before sample collection. The expression of Rac1-GTP was then determined by a pull-down assay and SDS-PAGE detection. The data showed that RU38486 prevented the downregulation of Rac1-GTP after exogenous CORT treatment compared with the CORT group ($F_{2,9} = 14.612$, $p < 0.05$; **Figure 4B₁₋₂**). At the same time, total Rac1 was not altered ($F_{2,9} = 1.489$, $p > 0.05$; **Figure 4B₃**). Furthermore, we also tested fear memory at 1.5 and 24 h after 5 FSs of 0.4 mA for 2 s each, a medium stimuli which could be induced to a greater or lesser effect by RU38486; the results showed no difference in the learning curve between the vehicle and RU38486 treatment groups ($F_{1,24} = 2.210$, $p = 0.150$; **Figure 4C**). The 24-hour fear memory was significantly decreased in the RU treatment group compared with the vehicle group ($F_{1,18} = 9.225$, $p < 0.05$; **Figure 4C**).

2.5. A Rac1-GTP inhibitor improves fear memory

To confirm that fear memory is regulated by changes in Rac1-GTP mediated by glucocorticoid receptors, fear memory was tested 1.5 and 24 h after systemic NSC23766 administration (1.5 mg/kg,

i.p.) 30 min before 5 trials of FS at 0.2 mA for 2 s each. This was a weak stimulus, a floor effect, which might be improved by the Rac1-GTP inhibitor treatment. The data showed that NSC23766 significantly improved the fear memory 1.5 and 24 h after foot shocks ($F_{1,26} = 5.413$, $p < 0.05$; **Figure 4F**).

3. Discussion

Stressful experiences are usually well remembered, but the underlying mechanisms are not well known. Previous research has revealed that GRs activated by stress in the hippocampus can modify structural plasticity (Finsterwald and Alberini, 2014) and other molecular activity, thereby enhancing the memory of stressful events (Fritz and Kaina, 2013; Schwechter and Tolias, 2013; Thumkeo et al., 2013). In this study, we found that Rac1-GTP is a key molecule in this process. First, the expression of Rac1-GTP was sensitive to stress and could be downregulated by either a foot-shock stressor or exogenous CORT. Second, the changes in Rac1-GTP expression occurred after fear memory formation. Third, enhanced fear memory could be achieved by treatment with the Rac1-GTP inhibitor NSC23766. Finally, and most important, fear memory could be attenuated by the GR antagonist RU38486, which also blocked the downregulation of Rac1-GTP.

Response to stress starts with the release of hormones and comprises the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis, GTPase-coordinated trafficking of G-protein coupled receptors (GPCRs) from the endoplasmic reticulum to the plasma membrane. At the plasma membrane they are available for binding hormones and thus influence the magnitude of signal transduction, and physiological responses mediated by these receptors, including cell plasticity via the actin cytoskeleton (Wang and Wu, 2012). Rac1 is a binary molecular switch GTPase. Rac1 also adjusts rapid nuclear activation by stress kinases, such as SAPK/JNK and p38 kinase, as well as transcription factors (AP-1, NFκB) (Fritz and Kaina, 2013). When cells are exposed to stress, the GEFs for Rac1 become activated

and Rac1 binds with GTP. Rac1-GTP then couples with different effector proteins, leading to a series of cascade effects: upregulated PAK, polymerized actin cytoskeleton, and nuclear binding to topoisomerase II. As a consequence, Rac and its downstream effectors, including p21-activated kinase (PAK) and phosphatidylinositol-3 kinase (PI3K)/Akt, promote neuronal survival through activation of mitogen-activated protein kinase (MAPK) pathways and inhibition of Bcl-2 family proteins (Stankiewicz and Linseman, 2014).

Rac1 activity may regulate the memory process by modulating the morphology and connectivity of the dendritic tree through neurotransmission, synaptic plasticity (Cahill et al., 2009; Waltereit et al., 2012), or polymerized actin activity (Edwards et al., 1999). Dendritic spines are actin-rich protrusions. In the primary molecular phase of learning and memory, NMDA-type glutamate receptors are activated and calcium flows into excitatory synapses, initiating a program of cytoskeletal rearrangement that results in larger spines with stronger synapses. Recent research showed that the GEFs link calcium flux to both spine enlargement and synaptic strengthening through Rac1 activity. Additionally, acute Rac1 activation is sufficient to enhance synaptic transmission. Rac1 is a major regulator of cytoskeleton synaptic strengthening in molecular phase of learning and memory; it regulates the activity of actin-binding proteins such as cofilin, profilin, and the Arp2/3 complex to initiate the cytoskeletal effects (Schwechter and Tolias., 2013). Inhibition of Rac1 activation inhibits the long-term potentiation that causes AMPAR trafficking to the synapse (Martinez and Tolias, 2011; Schwechter and Tolias, 2013). Rac1 also promotes stress fiber formation, which is necessary for the formation of lamellipodia.

Rac1 also functions at different stages of memory. It is reported that inhibition of the activity of Rac1 leads to a slower decay of early memory, which suggests that downregulation of Rac1-GTP may enhance memory length (Davis, 2010). In our study, Rac1-GTP was downregulated during the cellular

consolidation period (Jiang et al., 2015), which lasts several hours after memory formation, suggesting that Rac1-GTP may enhance memory by regulating the pathway involved in memory consolidation. Rac1 inhibition strengthened the fear memory before memory acquisition, indicating the important role of RAC1 in memory processes. Furthermore, we also found that the fear memory was more long-lasting (24 h after stress) than the downregulation of Rac1-GTP (0.5 h after stress), which indicates that the regulation of fear memory via Rac1-GTP is a strictly time-limited process similar to a molecular switch with an active (GTP-bound) and inactive (GDP-bound) state. Shan found that PAK1, downstream of Rac1, also modulated memory consolidation (Shan et al., 2015). PAK1 activity was sensitive to environmental conditions; light may act as a switch of PAK1 activity. Rac1 involvement in downstream effectors constitutes a complex molecular regulation system for memory. Interestingly, a previous study reported that long-lasting Rac1-GTP activity also enhances fear memory (Diana et al., 2007). This indicates that the regulation of Rac1 and downstream effectors is a complex cascade pathway.

There was a sharp rise of CORT in the circulation after stressors, which could provide rapid effects on behavior by rapidly altering neuronal activity and excitability in a number of brain areas (de Kloet et al., 2008; Groeneweg et al., 2011). The HPA axis is the primary circuit mediating the physiological response to stressors, regulates the level of circulating glucocorticoid hormones, and homeostatically maintains resting and stress-related physiologic reactions. Hormones may be released at several to even a hundred times the baseline, decided by stressors or individual reactions (Gong et al., 2015; Wynne-Edwards et al., 2013). Furthermore, high levels of hormones are usually a marker of pathological situations (Shen et al., 2016). Our experiments showed that treatment with exogenous high doses of CORT (40mg/kg, i.p.) also induced a very rapid increase in serum CORT, a similar change in scope compared with the baseline. High doses of exogenous CORT produced decreased Rac1 activity, similar

to the effect of a strong stressor (foot shocks). The level of CORT then recovered to the baseline in the stage of recovery and adaptation to the stress. In a stressful situation, the hippocampus controls the interaction of stress and memory. Glucocorticoids(GCs) are produced and they modulate hippocampus-dependent memory and site-preferential effects on hippocampal neurons (Roozendaal, 2000; Liao et al., 2013; Rohleder et al., 2010). In our study, rapid changes of Rac1-GTP accompanied the changes in CORT levels induced by fear conditioning. Furthermore, downregulation of Rac1-GTP in the hippocampus was prevented by RU38496, a GR antagonist, and downregulation of Rac1-GTP promoted fear memory, indicating that the hippocampus is an anatomical site for the interaction of Rac1-GTP and stress. Previous research also reported that systemic injection of GR inhibitors prior to contextual fear conditioning decreased fear memory when tested 24 h later (Cordero et al., 1998; Pugh et al., 1997). These results showed that Rac1 is an important molecular for the hippocampus-dependent regulation of memory.

The ‘Yerkes-Dodson Law’ states that “just the right amount of stress” is beneficial to memory (Yerkes and Dodson, 1908). Adverse or excessive responses to stressful experiences are two of the main causes of psychiatric diseases. Fear learning is beneficial for survival in stressful situations by guiding subsequent behavior, but fear learning is also a critical initiating factor for stress-related disorders, such as PTSD. In our experiments, Rac1 was a key modulator in memory retrieval, and as mentioned above, Rac1 regulates different stages of memory. Based on these important roles in memory, further study of Rac1 and Rac1-GTP may be beneficial for memory-deficit-related diseases, such as Alzheimer’s disease.

4. Conclusions

Our data suggest that Rac1-GTP in the hippocampus is one of the key proteins involved in fear memory.

A decreased level of Rac1-GTP via GRs after acute stress is one molecular pathway to improve fear memory.

Acknowledgements

We especially thank Mrs. Pei-Yu Zhang for technical assistance in the behavioral experiments. This work was supported by the 973 program from the Ministry of Science and Technology of China (2013CB835103 to L.X.), an NSFC-CIHR joint grant (81161120536 to L.X.), and grants from The National Science Foundation of China (31100775 and 31371141 to Q-X.Z., 81171294 to Y-Y. X.), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB02020002), and Science and Technology Program of Yunnan Province (2013GA003).

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Figure legends

Figure 1. The downregulation of hippocampal Rac1-GTP after fear conditioning was accompanied by increased levels of serum CORT in rats. **(A)** Experimental protocol: The rats underwent 5 foot shocks (0.8 mA and 2 s each with 2-min intervals between every shock) in 12 min. The sera and hippocampi were collected at different time points for CORT (at 0.5, 1.5, 3, or 24 h, n=6) and Rac1-GTP detection (at 0.5, 1.5, or 24 h, n = 4) in separate groups of rats. **(B)** Time course for the serum CORT level after fear conditioning. The value of serum CORT is normalized to the naïve serum CORT level and is expressed as the mean $\pm$ S.E.M. %, n = 6 in each group. * $p < 0.05$, compared with the naïve and 24 h groups. **(C1-3)** Time course for Rac1-GTP expression in the hippocampus after fear conditioning. Rac1-GTP levels are normalized to the total Rac1 (T-Rac1) levels and to the naïve group. **(C2)** Data are normalized to GAPDH levels. **(C3)** All the data are expressed as the mean $\pm$ S.E.M. %, n = 4 in each group. * $p < 0.05$, compared with naïve, 1.5, and 24 h groups.

Figure 2. Time course of stress-induced hippocampal Rac1-GTP downregulation is mimicked by exogenous CORT treatment. **(A)** Experimental protocol: The rats were administered exogenous CORT (40 mg/kg, i.p.) and put back into the home cages for 12 min, in accordance with the fear conditioning training duration. **(B)** Serum CORT detection by ELISA assay at 0.5, 1.5, or 3 h. The value of serum CORT is normalized to the naïve serum CORT level and is expressed as the mean $\pm$ S.E.M. %, n = 6 in each group. ** $p < 0.01$, compared with naïve, 0.5 h, and 3 h groups. **(C1-3)** Expression of Rac1-GTP at 0.5, 1.5, or 24 h, respectively. The gray value is normalized to the Rac1-GTP expression in the naïve group and is expressed as the mean $\pm$ S.E.M. %, n = 4 in each group. * $p < 0.05$, compared with the naïve and 24 h groups.

Figure 3. Negative correlation between Rac1-GTP and CORT. (Spearman's correlation coefficient: -0.8292 ; $p < 0.05$). The value represents the percentage of the naïve group. The point 1 represents fear conditioning and point 2 shows CORT treatment. Moreover, the white represents the naïve group, the pink represents the 24 h group, the blue represents the 0.5 h group, the light-blue represents the 3 h groups and the green represents the 1.5 h groups.

Figure 4. The GR antagonist RU38486 not only recovered the expression of Rac1-GTP after exogenous CORT treatment but also impaired long term fear memory at 24 h. **(A)** Experimental protocol: The rats received RU38486 treatment (80 mg/kg, i.p.) 30 min before CORT administration (40 mg/kg, i.p.). Hippocampi were collected at 1.5 h for Rac1-GTP detection by Western blot analysis. **(B₁₋₃)** RU38486 treatment prevented downregulation of Rac1-GTP induced by CORT. The value represents the percentage of the naïve group and is expressed as the mean $\pm$ S.E.M., $n = 10$, ANOVA with Newman-Keuls *post-hoc* test, $*p < 0.05$ compared with the RU treatment group. **(C)** RU38486 behavioral experimental protocol: RU38486 treatment (80 mg/kg, i.p.) 30 before the session of fear conditioning (total 5 FSs in 12 min at 0.4 mA for 2 s in each trial with 2-min intervals between every trial), and then the fear memory was tested at 1.5 h and 24 h after training. **(D)** The 24 h long-term fear memory was significantly weakened by RU38486, *t*-test, $*p < 0.05$ compared with vehicle control. **(E)** NSC23766 behavioral experimental protocol: NSC23766 treatment (1.5 mg/kg, i.p.) 30 min before fear conditioning (total 5 FSs in 12 min at 0.2 mA for 2 s in each trial with 2-min intervals between every trial), and then the fear memory was tested at 1.5 and 24 h. **(F)** NSC, the inhibitor of Rac1-GTP, enhanced the long-term fear memory at 24 h. The freezing level was the percentage of freezing time compared to the inter-shock interval and is expressed as

the mean $\pm$ S.E.M. %, n = 10 in each group, ANOVA with Newman-Keuls *post-hoc* test. * $p < 0.05$ compared with vehicle control.

Gan et al Figure 1

Gan et al Figure 2

Gan et al Figure 3

Gan et al Figure 4