ORIGINAL RESEARCH

S-Ketamine Exerts Antidepressant Effects by Regulating Rac1 GTPase Mediated Synaptic Plasticity in the Hippocampus of Stressed Rats

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Abstract

Clinical studies have found that ketamine has a rapid and lasting antidepressant effect, especially in the case of patients with major depressive disorder (MDD). The molecular mechanisms, however, remain unclear. In this study, we observe the effects of S-Ketamine on the expression of Rac1, neuronal morphology, and synaptic transmission function in the hippocampus of stressed rats. Chronic unpredictable mild stress (CUMS) was used to construct stressed rats. The rats were given a different regimen of ketamine (20 mg/kg, i.p.) and Rac1 inhibitor NSC23766 (50 μg, ICV) treatment. The depression-like behavior of rats was evaluated by sucrose preference test and open-field test. The protein expression of Rac1, GluA1, synapsin1, and PSD95 in the hippocampus was detected by Western blot. Pull-down analysis was used to examine the activity of Rac1. Golgi staining and electrophysiological study were used to observe the neuronal morphology and long-term potentiation (LTP). Our results showed that ketamine can up-regulate the expression and activity of Rac1; increase the spine density and the expression of synaptic-related proteins such as GluA1, Synapsin1, and PSD95 in the hippocampus of stressed rats; reduce the CUMS-induced LTP impairments; and consequently improve depression-like behavior. However, Rac1 inhibitor NSC23766 could have effectively reversed ketamine-mediated changes in the hippocampus of rats and counteracted its antidepressant effects. The specific mechanism of S-Ketamine's antidepressant effect may be related to the up-regulation of the expression and activity of Rac1 in the hippocampus of stressed rats, thus enhancing synaptic plasticity.

Keywords Depression · Ketamine · Rac1 GTPase · Synaptic plasticity · Long-term potentiation

Introduction

Depression is a common psychiatric illness with high morbidity and disability rates, resulting in enormous public health costs and personal suffering (Gu et al. 2013). According to the World Health Organization, depression has become the second leading cause of illness, affecting approximately 17% of the world's population. More than one million people per year are estimated to have committed

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suicide due to depression (Chapman and Perry 2008; Kessler et al. 2003). Due to the lack of conventional and effective antidepressants, the relapse rate of patients with first-onset depression is as high as 50% within the first 5 years, and the lifetime prevalence reaches 15–20% (Angst et al. 2013).

Traditional antidepressants, such as selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants, usually have slow onset for therapeutic response (takes several weeks) and a low remission rate (40–50%) (Cipriani et al. 2016; Sinyor et al. 2010). In addition, more than 30% of depression patients exhibit obvious resistance responses to currently available antidepressant medications, especially for patients with major depressive disorder (MDD) (Cipriani et al. 2016; Duman and Aghajanian 2012). In recent years, clinical studies have found that the intravenous anesthetic ketamine has a rapid and lasting antidepressant effect, especially for patients with MDD and who are treatment-resistant or show poor response to SSRIs (Wang et al. 2012; Cornwell et al. 2012). A single dose of ketamine (0.5 mg/kg, sub-anesthetic doses) can induce antidepressant effects after 40 min, and more than one-third of patients show obvious improvement one week after administration (Lenze et al. 2016). However, the underlying molecular mechanism of ketamine's antidepressant effect remains poorly understood.

A growing number of studies have shown that the pathogenesis of depression is related to changes in synaptic plasticity, including changes in synaptic structure and transmission function (Duman and Aghajanian 2012; Kang et al. 2012). Brain imaging studies found that the volume of limbic areas such as the prefrontal cortex and hippocampus (involved in emotion, mood, and cognitive functions) were reduced, accompanied by massive neuronal atrophy and a decreased number of synapses (Rajkowska et al. 1999). The autopsy analysis further confirmed that the dorsolateral prefrontal cortex (dlPFC) and hippocampus neuronal atrophy, synaptic loss, and synaptic proteins were altered in patients with MDD (Drevets 2000).

Previous studies have shown that Rac1 (a member of the small G protein Rho family, Rho-GTPase), can cause neuronal cytoskeleton remodeling and regulation of synaptic plasticity (Li et al. 2000; Luo 2002). Activation or up-regulation of Rac1 expression can lead to dramatic changes in the morphology of dendritic spines, including an extension of dendrites and an increase in dendritic spine density (Um et al. 2014). The loss of Rac1 will directly affect the formation of nerve synapses and neural circuits (Pennucci et al. 2016). Recent studies have shown that abnormal expression of Rac1 is associated with a variety of diseases, including depression, autism, and fragile X syndrome (Li et al. 2015; Chen et al. 2010; Golden et al. 2013). Chronic social frustration stress promotes down-regulation of Rac1 expression in the nucleus accumbens of mice, loss of dendritic spines, and induces depression-like behaviors such as social avoidance and anhedonia (Golden et al. 2013). However, whether Rac1 plays an important role in the antidepressant effect of ketamine is unclear.

Ketamine or (R,S)-ketamine is a racemic mixture containing equal parts of R-Ketamine and S-Ketamine. Its inhibitory constant (Ki) for N-methyl-D-aspartate receptor (NMDAR) is 0.53 μM (Wei et al. 2020). Because S-Ketamine has a higher affinity for NMDAR than R-Ketamine (0.3 μM vs. 1.4 μM), S-Ketamine has been developed as an antidepressant, and its nasal spray was approved by the U.S. Food and Drug Administration for refractory depression (Salahudeen et al. 2020). In this study, we use stressed rats with depression-like behavior to observe the effects of chronic administration of S-Ketamine on Rac1 activity, protein expression, and synaptic plasticity of the hippocampus, and elucidate the possible mechanism of ketamine's antidepressant effect.

Materials and Methods

Animals

Adult male Sprague–Dawley rats (2–3 months, 200–250 g) were obtained from the Experimental Animal Center of Hubei Institute of Selenium and Human Health. Rats were housed in standardized laboratory conditions ($23 \pm 2°$, 60% humidity, 12 h light/12 h dark, free access to water and food) for a week to allow them to adapt to the new environment. All animal experimental protocols were approved by the Ethical Committee of Hubei Institute of Selenium and Human Health (No. HB2017-003) and the animal care guidelines of the National Institute of Health were followed. The sample size is calculated using the software PASS and One-Way Analysis of Variance F-Tests, as follows: 80% test power and 5% type I error. The behavioral data (sucrose preference test) of rats (Hao et al. 2016) as the primary endpoint, an estimated 15% standard deviation of the mean between groups (σm) and 30% within-group standard deviation (σ) were detected. In other words, ketamine can increase the sucrose preference percentage in stressed rats by about 15%. The output result was the effect size of $f = 0.5$ and $N = 12$. For molecular analysis (the second end point), we estimate that 15% standard deviation of the mean between groups and 20% within-group standard deviation was detected. The output result was the effect size of $f = 0.75$ and $N = 6$. The blindness was achieved through the following methods: (1) After the rats were numbered, a researcher randomly grouped them according to the number and prepared the corresponding treatment drugs (only the number was marked); (2) Other researchers only knew the number of rats but not the grouping, and performed subsequent behavioral experiments, biochemical analysis, and statistical analysis.

Model of Stressed Rats

CUMS procedure was used to construct the model of stressed rats with depression-like behavior as previously documents (Banasr et al. 2007). Rats were kept in solitary cages and received modeling stress for 28 consecutive days, with different stressors each day. One of the nine stressors was randomly selected: horizontal shaking for 20 min; cage tilting (45°) for 24 h; continuous lighting for 24 h; clamp the tail for 1 min; damp sawdust for 24 h; food deprivation for 24 h; water deprivation for 24 h; swimming in cold water for 5 min (4 °C); social crowding (20/cage);

Experiment Treatments

Experiment 1

Twelve healthy rats (same age and batch) were included in the group C (control group), and did not receive CUMS and drug treatment. After the CUMS procedure was completed, forty-eight stressed rats were randomly assigned to four groups ($n = 12$): Group D (stressed rats), Group DK (stressed rats with S-Ketamine treatment), Group DNK (stressed rats with NSC23766 and S-Ketamine treatment), Group DN (stressed rats with NSC23766 treatment). Group D was treated with intracerebroventricular (ICV) and intraperitoneal (IP) injection saline (10 ml); group DK was treated with saline (10 µl, ICV) and S-Ketamine (20 mg/kg, IP, preparation as 2 mg/ml, H20193336, Hengrui Medicine, China); group DNK was treated with Rac1 inhibitor NSC23766 (50 µg, ICV, sc-204823A, Santa Cruz Biotechnology, USA) and S-Ketamine (20 mg/kg, IP); group DN was treated with NSC23766 (50 µg, ICV) and saline (IP). All treatments were continued for 7 days, once a day. Because our main purpose is to study the mechanism of ketamine's antidepressant effect in stressed rats. Therefore, there were no treated control groups (e.g., control/ketamine; control/NSC23766) in this study. The dosage of 50 µg NSC2376650 (ICV injection) was determined according to the results of previous studies (Zhang et al. 2009).

Experiment 2

Six healthy rats were included in the control group (group C). Twenty-four stressed rats were randomly assigned to four groups ($n = 6$): group D, DK, DNK, and DN. This part was used for the electrophysiological study. The treatment schemes for rats in each group were the same as those in experiment 1 except that these rats did not undergo behavioral tests.

Intracerebroventricular Administration

50 µg Rac1-specific inhibitor (NSC23766) was dissolved in 10 µl saline. 15 min before the injection of s-ketamine, the prepared NSC23766 was injected into the left cerebral ventricle of rats. Saline was injected into the cerebral ventricle of the vehicle control rats. For intracerebroventricular administration, the rats were anesthetized using sodium pentobarbital (50 mg/kg, IP), then placed on a stereotaxic instrument (Narishige, Tokyo, Japan), and the position of the ear rods was adjusted, implanted the cannula into the left cerebral ventricle (bregm as the center, lateral 1.5 mm; anteroposterior ± 0.8 mm and depth 3.5 mm) (Zhang et al. 2009). Drug injection was administered using a microsyringe at a rate of 2 µl/min by the cannula.

Behavioral Tests

Sucrose Preference Test

Sucrose preference test was used to evaluate anhedonia (core symptom of depression) in rodents (Luo et al. 2010). The Sucrose preference test was administered after the CUMS procedures and s-ketamine treatment. In order to acculturate the rats to the sucrose solution, two bottles of 1% sucrose solution were provided during the first 24 h. After 23 h of fasting and water deprivation, the rats were allowed to drink freely for 1 h. At this time, the rat was provided with two identical bottles, one containing 1% sucrose and the other bottle was sterile water. Sucrose preference percentage = (sucrose solution consumption/sterile water consumption + sucrose solution consumption) × 100.

Open-Field Test

Depression and anxiety often coexist, and depression patients often have anxiety symptoms (Sindermann et al. 2021). The Open-Field Test was performed to evaluate the locomotor and exploratory behavior of rats in an unfamiliar environment (Hallam et al. 2004). A black wooden square platform ($100 \times 100 \times 50$ cm), placed in a quiet, dim room. The white marking line divides the floor of the apparatus into 25 square grids of equal size. Animals were placed in the center of the platform, allowed to move freely for five minutes, and count the number of crossed squares (indicating spontaneous locomotor) and the rearing frequency (rats stand completely on their hind legs, indicating exploratory activities). The test was administered after the CUMS procedure and s-ketamine treatment.

Tissue Biochemical Analysis

Tissue Preparation

After the last behavioral test was completed, 6 rats from each group were decapitated under 2% sodium pentobarbital (50 mg/kg, IP) anesthesia, and bilateral hippocampus were taken out on ice and immediately cooled in liquid nitrogen tanks, then stored in $-80\ °C$ refrigerators. This part of the hippocampus was used for real-time PCR, western blotting, and Rac1-GTP binding assay. An additional six rats were used for the Golgi staining.

Real-Time PCR Analysis

Following the manufacturer's instructions, Trizol reagent (Invitrogen Technology, Carlsbad, CA, USA) was used to extract total RNA from hippocampal tissue. Reverse transcription was performed using Goldenstar™ RT6 cDNA Synthesis Kit (Qingke Bio Inc., China). ABI PRISM 7900 sequence detection system with SYBR Green qPCR Master Mix (Applied Biosystems, CA, USA) was used for real-time PCR analysis. The reaction conditions for the holding stage were 95 ∘C for 30 s, and 40 cycling stages of 95 °C for 5 s and 55 °C and 72 °C for 30 s each. Rac1 primer sequences were 5-TCAGTTACACGACCAATGCG-3 (sense) and 5-ATGCAGGACTCACAAGGGAA-3 (antisense); Tiam1 primer sequences were 5-AGAGACCGA ACGCACCTATG-3 (sense) and 5-GGAACTCAACTT GAAACT CCACC-3 (antisense); α1-chimaerin primer sequences were 5-CCGTGAACACA CGCTTTCTG-3 (sense) and 5-CAACCACAGACAACCCAGGA-3 (antisense); Bcr primer sequences were 5-CGGTGTCAAAAT CGCTGTGG-3 (sense) and 5-CTCGC TCATCATCACGG ACA-3 (antisense).

Western Blotting Analysis

Each gram of hippocampi was homogenized with 30 µl cocktail protease inhibitor (Roche Molecular Biochemicals, Germany) and 3 ml RIPA buffer (US Biological, USA). After centrifugation at 12,000 rpm (4 °C), the supernatant was collected and stored at $-20\ °C$. BCA assay and spectrophotometry were used to assess the total protein concentration. The supernatant sample (50 µg of protein) was separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore Inc., Darmstadt, Germany). After blocking with 5% skim milk for 2 h, the membrane was incubated with primary antibody overnight: Rac1 (1:500, sc-514583, Santa Cruz Biotechnology), Tiam1 (1:500, sc-393315, Santa Cruz Biotechnology), α-chimaerin (1:500, sc-365985, Santa Cruz Biotechnology), Bcr (1:500, sc-104, Santa Cruz Biotechnology), GluA1 (1:500, sc-13152, Santa Cruz Biotechnology),

Synapsin 1(1:500, ab254349, Abcam), and PSD-95 (1:500, sc-32290, Santa Cruz Biotechnology). The specificity of these primary antibodies has been verified (Mao et al. 2017; Liu et al. 2018; Qi et al. 2004; Elzinga et al. 2013; Gu et al. 2018; Groh et al. 2014; Tsai et al. 2012). The membrane was incubated with a mixture of anti-rabbit IgG (Golden Bridge, Zhongshan, China) and horseradish peroxidase (HRP) for 1 h at room temperature. Quantity One software (Bio-Rad, USA) was used for quantitative analysis.

Rac1-GTP Binding Assay

Since the function of Rac1 was related to its activity, which was regulated by Tiam1 (GDP/GTP exchanging factor, activating Rac1), α1-Chimaerin and Bcr (GTPase-activating protein, inactivating Rac1) (Tolias et al. 2011). Therefore, we not only detected the expression levels of Tiam1, α 1-Chimaerin, and Bcr, but also analyzed the activity of Rac1. PAK1-PBD colored agarose beads (Rac1 Activation Assay Kit, 17-283MSDS, Sigma-Aldrich) was used to measure Rac1 activation. Briefly, 20 ml of PAK1-PBD agarose beads were mixed with 200 mg of sample and incubated at 4 °C for 60 min, then $MgCl_2$ was added to terminate the reaction. The treated samples were centrifuged at 12,000 rpm at 4 °C for 15 min, and the supernatants were removed. The precipitated complex was then cleaned 3 times with magnesium-containing lysis buffer, and boiled in the sample buffer. After the proteins were separated by 10% SDS-PAGE, western blot analysis was performed with anti-Rac1 antibody (1:500, sc-514583, Santa Cruz Biotechnology).

Golgi Staining

Take 6 rats from each group for Golgi staining. According to the manufacturer's instructions, solutions A, B, C, D, and E were prepared 24 h in advance, and using the PK 401/401A FD Rapid GolgiStain Kit (FD Neuro Technologies, USA) to prepare the brain. Use a cryostat microtome (Leica CM 1850, Wetzlar, Germany) to obtain 100 µm thick coronal sections at a temperature ($-20\ °C$ to $-22\ °C$). The slices were placed in a 6% sucrose solution (prepared with 0.1MDPBS) and stored at room temperature and protected from light for 72 h. Install each slice and dry overnight at room temperature. Immerse the section in the staining mixture (solutions D: solutions E: deionized water=1:1:2) for 10 min, and 50%, 75%, 95%, and 100% alcohol gradient dehydration, clarified in xylene. An appropriate amount of neutral gum was dropped and cover-slip sealed. Neurons with typical hippocampal CA1 structure were selected and the density of dendritic spines were analyzed by Image J software. Each rat randomly selects 3 slices for analysis. For each neuron, five segments of 20 mm of apical dendrites with clear traces and good isolation from neighboring dendrites were randomly selected and imaged with a 100×oil

immersion lens. The number of spines on 10 mm dendritic length was calculated as dendritic spines density.

Electrophysiological Study

The field excitatory postsynaptic potentials (fEPSP) from the CA1 area of the hippocampus were recorded, and the method was performed as previously described (Ren et al. 2016). Briefly, six rats in each group were sacrificed under 2% sodium pentobarbital anesthesia after the completion of the behavioral test. The brain was taken out and immersed in artificial cerebrospinal fluid (ACSF) at 0–4 °C (containing: 95% O_2/5% CO_2). Hippocampal slices were cut into 400 μm thickness using a vibratome (NVSLM-1, WPI, USA) and stored for 2 h in a chamber oxygen-containing recording solution (in mM: 1.25 NaH_2PO_4, 26 $NaHCO_3$, 124 NaCl, 3 KCl, 1 $CaCl_2$, $1MgSO_4$, 2 sodium pyruvate, 10 glucose, 0.4 vitamin C, 2 sodium lactates). The slices were placed in a recording chamber with oxygen-containing recording solution. The bipolar stimulation electrode was inserted into the radiatum layer of CA3 area to activate the Schaffer collaterals, and the glass micropipette (containing recording fluid, resistance 2–3 MΩ) was inserted into the radiatum layer of CA1 area, then electrical stimulation was triggered and fEPSP was recorded. When the stimulus was sufficient to elicit a response and the slope was approximately 50% of the maximum response, the stimulus intensity was established. After the baseline fEPSP was stable for 0.5 h, the high-frequency stimulation (100 Hz with 100 pulses) was replaced to induce LTP, and recorded continuously for 1 h. The data were analyzed by Axon Instruments system (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

SPSS software (version 17.0; SPSS Inc, Chicago, III) was used for the statistical analyses. All data were expressed as mean ± SD. The Kolmogorov–Smirnov test was used to test the normality of the variables, and the Levene test was used to test the homogeneity of variance. If the variance was homogeneous ($P > 0.05$) and statistical significance was determined by one-way analysis of variance (ANOVA), followed by Bonferroni correction (post hoc tests) to multiple comparisons between the groups. Non-normal distribution or variance heterogeneity data were presented as the median and interquartile range, and the kruskal–wallis test was applied. $P < 0.05$ was considered statistically significant.

Results

S-Ketamine Increased the Sucrose Preference Percentage in Stressed Rats

Sucrose preference percentage can accurately assess the degree of anhedonia in rats (Luo et al. 2010), and we used it to validate the CUMS model and the antidepressant effect of S-ketamine. After completion of the CUMS procedure, the data of sucrose preference percentage meet normality (The P values of groups C, D, DK, DNK, and DN were 0.173, 0.275, 0.757, 0.275, 0.279) and homogeneity of variance ($F_{(4, 55)} = 0.855$, $P = 0.497$). As shown in Fig. 1A, significant intergroup differences were observed among five groups ($F_{(4, 55)} = 41.530$, $P < 0.001$). The post hoc tests showed that the sucrose preference percentage in the CUMS-treated groups (group D, DK, DNK, and DN) was significantly lower than that of the group C ($P < 0.001$, respectively), but no statistical difference was observed between the four CUMS-treated groups ($P = 1.000$, respectively). After s-ketamine and NSC23766 treatment, the data of sucrose preference percentage meet normality (The P values of groups C, D, DK, DNK, and DN were 0.157, 0.894, 0.698, 0.416, 0.667) and homogeneity of variance ($F_{(4, 55)} = 0.118$, $P = 0.976$). The intergroup statistical difference was observed among these five groups ($F_{(4, 55)} = 74.030$, $P < 0.001$). Post hoc tests showed that group DK exhibited increased levels in the sucrose preference percentage and significantly higher than that in the D group ($P < 0.001$). Compared to group DK, group DNK showed decreased levels in the sucrose preference percentage ($P < 0.001$). Figure 1D.

S-Ketamine Alleviated the Open-Field Behavioral Performance of Stressed Rats

Rearing frequency and number of crossed squares reflect the exploratory activities of rodents, and also used to evaluate the depression-like behavior in rats (Hallam et al. 2004). After completion of the CUMS procedure, the data of rearing frequency and number of crossed squares all meet normality (Rearing frequency: the P values of groups C, D, DK, DNK, and DN were 0.522, 0.385, 0.376, 0.682, 0.304; number of crossed squares: the P values of groups C, D, DK, DNK, and DN were 0.100, 0.251, 0.882, 0.292, 0.316) and homogeneity of variance (Rearing frequency: $F_{(4, 55)} = 2.116$, $P = 0.091$; number of crossed squares: $F_{(4, 55)} = 1.530$, $P = 0.206$). As shown in Fig. 1B–C, significant intergroup differences were observed among five groups in terms of rearing frequency ($F_{(4, 55)} = 53.187$, $P < 0.001$) and number of crossed
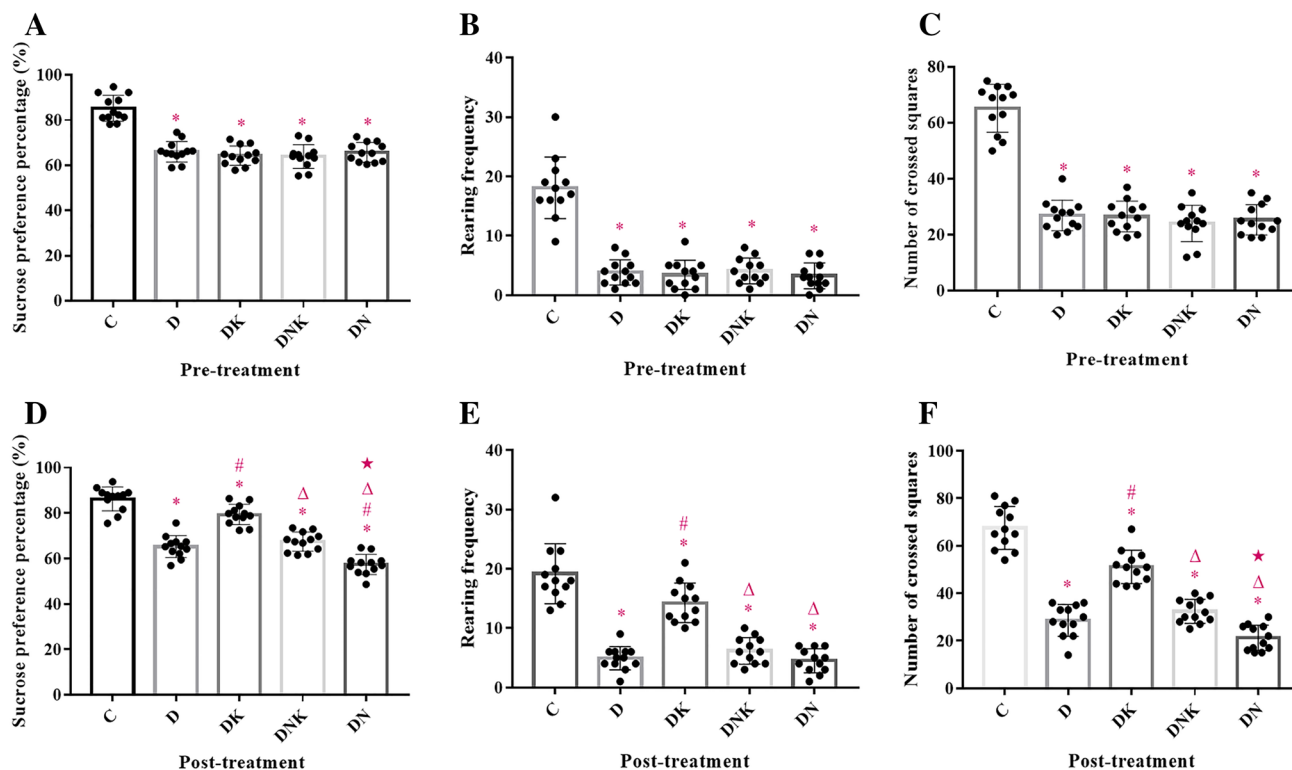
Fig. 1 S-Ketamine alleviated the depression-like behavior of stressed rats, which was effectively reversed by pre-administration of Rac1 inhibitor NSC23766. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment; Pre-treatment and post-treatment: time points before and after the rats received ket-amine and/or NSC23766 treatment. **A** and **D** The sucrose preference percentage (%) of rats; **B** and **E** the frequency of rearing (indicating exploratory activities); **C** and **F** the number of squares crossed of rats (indicating spontaneous locomotor). Data are expressed as mean ± SD ($n = 12$). *, #, Δ, ★: represents the comparison with group C, group D, group DK, and group DNK, respectively, $P < 0.05$

squares ($F_{(4, 55)} = 91.982$, $P < 0.001$). The post hoc tests showed that the rearing frequency and number of crossed squares in the CUMS-treated groups (group D, DK, DNK, and DN) was significantly lower than that of the group C ($P < 0.001$, respectively), but no statistical difference was observed for rearing frequency and number of crossed squares between the four CUMS-treated groups ($P = 1.000$, respectively). After S-ketamine and NSC23766 treatment, the data of rearing frequency and number of crossed squares meet normality (Rearing frequency: the P values of groups C, D, DK, DNK, and DN were 0.075, 0.488, 0.523, 0.567, 0.431; number of crossed squares: the P values of groups C, D, DK, DNK, and DN were 0.678, 0.243, 0.349, 0.459, 0.130) and homogeneity of variance (Rearing frequency: $F_{(4, 55)} = 2.502$, $P = 0.053$; number of crossed squares: $F_{(4, 55)} = 1.439$, $P = 0.233$). Significant intergroup differences were observed among five groups in terms of rearing frequency ($F_{(4, 55)} = 52.039$, $P < 0.001$) and number of crossed squares ($F_{(4, 55)} = 92.967$, $P < 0.001$). The post hoc tests showed that group DK exhibited increased levels in rearing frequency and number of crossed squares, and

significantly higher than that in the D group ($P < 0.001$, respectively). Compared to group DK, group DNK showed decreased levels in rearing frequency and number of crossed squares (both $P < 0.001$). Figure 1E–F.

S-Ketamine Up-Regulated the Level of Rac1's mRNA and Protein Expression in the Hippocampus of Stressed Rats

To determine whether Rac1 plays an important role in the antidepressant effects of S-Ketamine, we analyzed the level of Rac1's mRNA and protein expression in the hippocampus of stressed rats. The data of Rac1's mRNA and protein expression all meet normality (mRNA: the P values of groups C, D, DK, DNK, and DN were 0.588, 0.703, 0.812, 0.639, 0.687; protein expression: the P values of groups C, D, DK, DNK, and DN were 0.851, 0.692, 0.865, 0.360, 0.338) and homogeneity of variance (mRNA: $F_{(4, 25)} = 1.496$, $P = 0.233$; protein expression: $F_{(4, 25)} = 0.653$, $P = 0.630$). As shown in Fig. 2A–C, the mRNA and protein level of Rac1 among all groups showed statistically significant differences
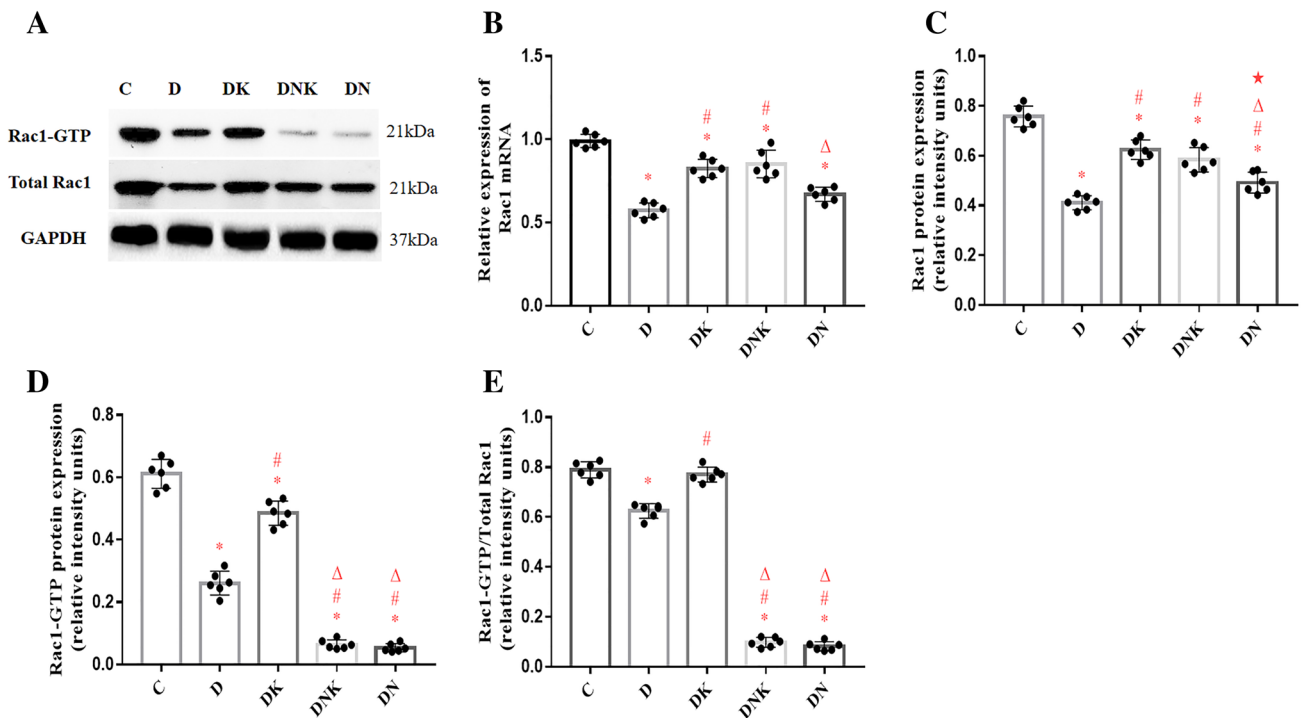
Fig. 2 S-Ketamine up-regulated the expression and activity of Rac1 in the hippocampus of stressed rats, and pre-administration of Rac1 inhibitor NSC23766 effectively reversed the up-regulation of activity induced by s-ketamine. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment. Data are expressed as mean ± SD ($n = 6$). **A** The western blotting band of Rac1 and Rac1-GTP, proteins assessed on the same loading controls and original band see (supplementary files); **B** The mRNA level of Rac1 in the hippocampus; **C** The protein expression level of Rac1 in the hippocampus; **D**, **E** The results of Rac1 activity analysis. *, #, Δ: represents the comparison with group C, group D, and group DK, respectively, $P < 0.05$

($F_{(4, 25)} = 52.234$, $P < 0.001$ and $F_{(4, 25)} = 63.423$, $P < 0.001$). Post hoc tests showed that CUMS-induced expression decrease of Rac1 in hippocampal, and the mRNA, protein level of Rac1 in the group D were significantly lower than that of the group C (both $P < 0.001$). S-Ketamine up-regulated the expression level of Rac1, and the mRNA, protein expression of Rac1 in group DK exhibited higher readings than group D (both $P < 0.001$). However, NSC23766 failed to reverse the S-Ketamine-induced expression up-regulation of Rac1, and no statistical difference was observed for the mRNA and protein expression of Rac1 between the group DK and group DNK ($P = 1.000$ and $P = 0.982$).

S-Ketamine Increased the Activity of Rac1 in the Hippocampus of Stressed Rats

Since the activated state GTP-Rac1 can reflect the function of Rac1, we further implemented the Rac1-GTP binding assay. The data of Rac1-GTP and Rac1-GTP/Total Rac1 ratio all meet normality (Rac1-GTP: the P values of groups C, D, DK, DNK, and DN were 0.762, 0.982, 0.760, 0.332, 0.369; Rac1-GTP/Total Rac1: the P values of groups C, D, DK, DNK, and DN were 0.753, 0.106, 0.797, 0.572, 0.463) and homogeneity

of variance ($F_{(4, 25)} = 2.272$, $P = 0.090$ and $F_{(4, 25)} = 0.881$, $P = 0.489$). As shown in Fig. 2D–E, the Rac1-GTP and Rac1-GTP/Total Rac1 ratio among all groups showed statistically significant differences ($F_{(4, 25)} = 340.523$, $P < 0.001$ and $F_{(4, 25)} = 1083.035$, $P < 0.001$). Post hoc tests showed that the levels of Rac1-GTP and Rac1-GTP/Total Rac1 ratio in group D were significantly lower than that of group C (both $P < 0.001$). S-Ketamine increased the activity of Rac1 in the hippocampus of stressed rats. Compared with group D, the levels of Rac1-GTP and Rac1-GTP/Total Rac1 ratio in group DK exhibited higher readings (both $P < 0.001$). However, NSC23766 reversed the s-ketamine-induced up-regulation of Rac1activity, and the Rac1-GTP and the Rac1-GTP/Total Rac1 ratio showed a very low value in group DNK, which was significantly lower than those in the DK group (both $P < 0.001$).

S-Ketamine Increased the mRNA and Protein Expression Levels of Tiam1, and Decreased the mRNA and Protein Expression Levels of α1-Chimaerin and Bcr in the Hippocampus of Stressed Rats

To study the functional status of Rac1, we further detected the expression of potential upstream activity regulatory proteins Tiam1, α1-Chimaerin, and Bcr. The data of these activity-regulating proteins all meet normality (Tiam1: the P values of groups C, D, DK, DNK, and DN were 0.457, 0.709, 0.439, 0.380, 0.689; α1-Chimaerin: the P values of groups C, D, DK, DNK, and DN were 0.653, 0.624, 0.617, 0.189, 0.427; Bcr: the P values of groups C, D, DK, DNK, and DN were 0.344, 0.501, 0.241, 0.919, 0.962) and homogeneity of variance (Tiam1: $F_{(4, 25)} = 0.472$, $P = 0.756$; α1-Chimaerin: $F_{(4, 25)} = 1.174$, $P = 0.346$; Bcr: $F_{(4, 25)} = 1.573$, $P = 0.212$). As shown in Fig. 3A–D, the protein expression levels of Tiam1, α1-Chimaerin, and Bcr among all groups showed statistically significant differences ($F_{(4, 25)} = 164.463$, $P < 0.001$; $F_{(4, 25)} = 67.307$, $P < 0.001$; and $F_{(4, 25)} = 89.617$, $P < 0.001$). Post hoc tests showed that the Tiam1 was significantly lower ($P < 0.001$), and α1-Chimaerin, Bcr were higher in group D than that of group C ($P < 0.001$ and $P < 0.001$).

Compared with group D, the protein levels of Tiam1 exhibited increased expression ($P < 0.001$), and α1-Chimaerin, Bcr exhibited decreased expression in group DK (both $P < 0.001$). Compared with the DK group, the protein levels of Tiam1 and Bcr exhibited decreased expression ($P < 0.001$ and $P = 0.002$), and α1-Chimaerinin exhibited increased expression in groups DNK ($P < 0.001$).

In order to determine whether S-Ketamine also affects these activity regulatory proteins at the transcription stage, we further detected the mRNA levels of Tiam1, α1-Chimaerin, and Bcr. These data all meet normality (Tiam1: the P values of groups C, D, DK, DNK, and DN were 0.323, 0.656, 0.190, 0.889, 0.339; α1-Chimaerin: the P values of groups C, D, DK, DNK, and DN were 0.877, 0.951, 0.738, 0.846, 0.316; Bcr: the P values of groups C, D, DK, DNK, and DN were 0.517, 0.291, 0.706, 1.000, 0.491) and homogeneity of variance (Tiam1: $F_{(4, 25)} = 2.079$, $P = 0.114$; α1-Chimaerin: $F_{(4, 25)} = 2.248$, $P = 0.093$; Bcr: $F_{(4, 25)} = 2.456$, $P = 0.072$). As shown in Fig. 3E–G, the mRNA levels of Tiam1, α1-Chimaerin, and Bcr among all groups showed statistically significant differences ($F_{(4, 25)} = 40.534$, $P < 0.001$; $F_{(4, 25)} = 67.790$, $P < 0.001$; and $F_{(4, 25)} = 28.908$, $P < 0.001$). Post hoc tests showed that the mRNA levels of Tiam1 was significantly lower ($P < 0.001$),
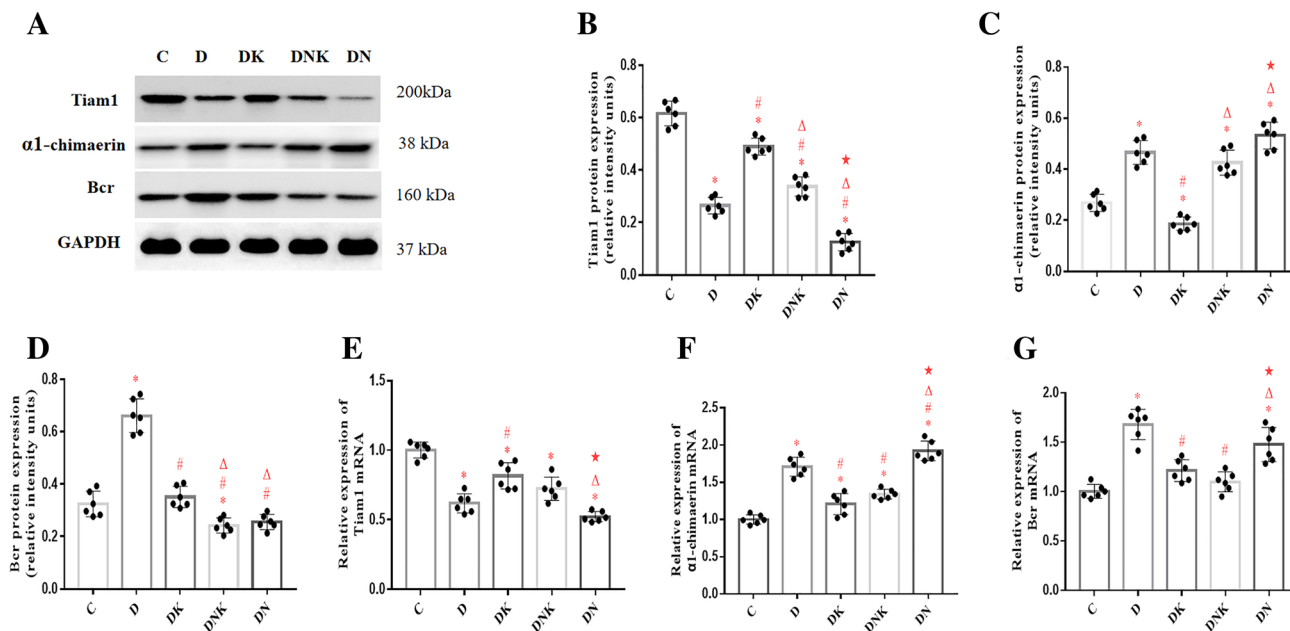

Fig. 3 S-Ketamine increased the mRNA and protein expression levels of Tiam1 (GEF), and decreased the mRNA and protein expression levels of α1-Chimaerin and Bcr (GAP) in the hippocampus of stressed rats. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment. Data are expressed as mean ± SD ($n = 6$). **A** The western blotting band of Tiam1, α1-Chimaerin, and Bcr, proteins assessed on the same pro-

teins assessed on the same loading controls and original band see (supplementary files); **B–D** The protein expression level of Tiam1, α1-Chimaerin, and Bcr in the hippocampus; **E–G** The mRNA levels of Tiam1, α1-Chimaerin, and Bcr in the hippocampus. *, #, Δ: represents the comparison with group C, group D, and group DK, respectively, $P < 0.05$. GEF: GDP/GTP exchanging factor, make Rac1 changes from an inactive to an active state; GAP: GTPase-activating protein, make Rac1 changes from an active to an inactive state

and α1-Chimaerin, Bcr were higher in group D than that of group C ($P < 0.001$ and $P < 0.001$). Compared with group D, the mRNA levels of Tiam1 exhibited increased expression ($P = 0.001$), and α1-Chimaerin, Bcr exhibited decreased expression in group DK (both $P < 0.001$). However, the mRNA levels of Tiam1, α1-Chimaerinin and Bcr were no obvious altered between the group DK and group DNK ($P = 0.331$, $P = 0.476$ and $P = 1.000$).

S-Ketamine Up-Regulated the Expression Levels of Synaptic Protein GluA1, Synapsin1, and PSD95 in the Hippocampus of Stressed Rats

Synaptic proteins GluA1, synapsin1, and PSD95 were enriched in the postsynaptic membrane, and can regulate synaptic plasticity (Colledge et al. 2003; Huang et al. 2021). So we analyzed the effect of s-ketamine on the expression levels of these proteins. The data of synaptic proteins GluA1, synapsin1, and PSD95 all meet normality (GluA1: the P values of groups C, D, DK, DNK, and DN were 0.671, 0.442, 0.902, 0.108, 0.317; synapsin1: the P values of groups C, D, DK, DNK, and DN were 0.421, 0.488, 0.384, 0.788,

0.264; PSD95: the P values of groups C, D, DK, DNK, and DN were 0.591, 0.701, 0.610, 0.871, 0.331) and homogeneity of variance (GluA1: $F_{(4, 25)} = 1.460$, $P = 0.244$; synapsin1: $F_{(4, 25)} = 0.723$, $P = 0.584$; PSD95: $F_{(4, 25)} = 0.804$, $P = 0.534$). As shown in Fig. 4A–D, the expression levels of GluA1, synapsin1, and PSD95 among all groups showed statistically significant differences ($F_{(4, 25)} = 63.077$, $P < 0.001$; $F_{(4, 25)} = 63.700$, $P < 0.001$; and $F_{(4, 25)} = 82.779$, $P < 0.001$). Post hoc tests showed that the expression levels of GluA1, synapsin1, and PSD95 were significantly lower in group D than that of group C (all $P < 0.001$). Compared with group D, their expression in group DK exhibited significantly increased levels (all $P < 0.001$). Compared with the DK group, the expression of GluA1, synapsin1, and PSD95 in groups DNK were significantly lower ($P < 0.001$, $P < 0.001$ and $P = 0.002$).

S-Ketamine Increased the Spines Density in the Hippocampal CA1 Region of Stressed Rats

To determine whether the antidepressant effect of S-Ketamine is related to changes in synaptic structure, we analyzed
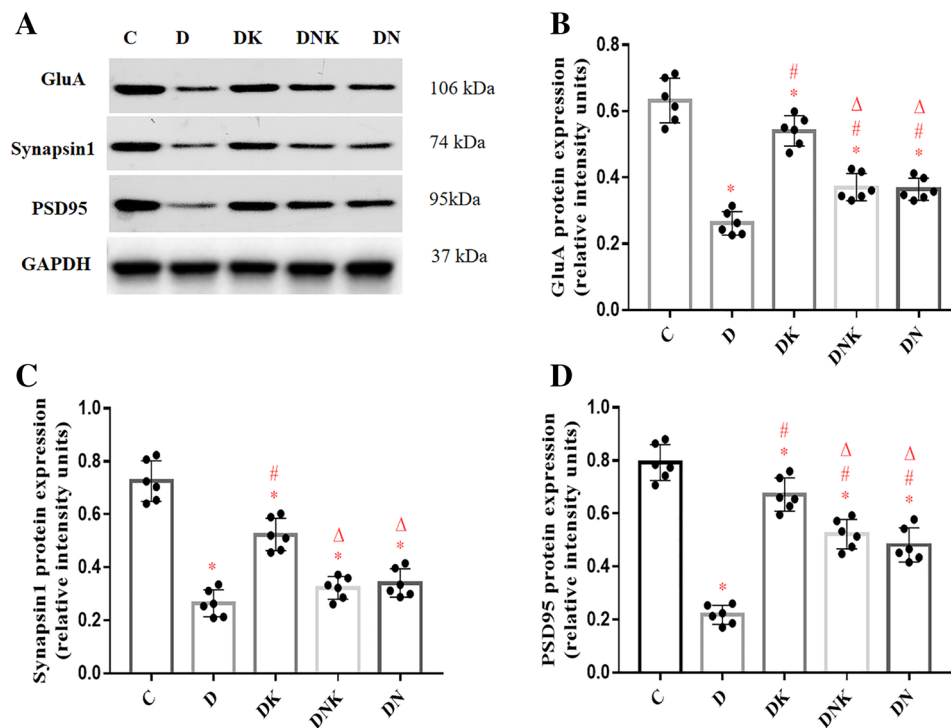

Fig. 4 S-Ketamine up-regulated the expression of synaptic protein GluA1, synapsin1, and PSD95 in the hippocampus of stressed rats, which was effectively reversed by pre-administration of Rac1 inhibitor NSC23766. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment. Data are expressed as mean ± SD ($n = 6$). **A** The western blotting band of GluA1, synapsin1, and PSD95, proteins assessed on the same proteins assessed on the same loading controls and original band see (supplementary files); **B** The expression level of GluA1 in the hippocampus; **C** The expression level of synapsin1 in the hippocampus; **D** The expression level of PSD95 in the hippocampus. *, #, Δ: represents the comparison with group C, group D, and group DK, respectively, $P < 0.05$

Fig. 5 S-Ketamine increased the spines density in the hippocampal CA1 region of stressed rats, which was effectively reversed by pre-administration of Rac1 inhibitor NSC23766. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment. **A** Representative image of dendrite spines in the hippocampal CA1 region. Scale bars = 10 μm; **B** The quantitative analysis of dendritic spines density in the hippocampal CA1 region. **C** The molecular mechanism schematic for the antidepressant effect of S-Ketamine. Data are expressed as mean ± SD ($n = 6$). *, #, Δ: represents the comparison with group C, group D, and group DK, respectively, $P < 0.05$
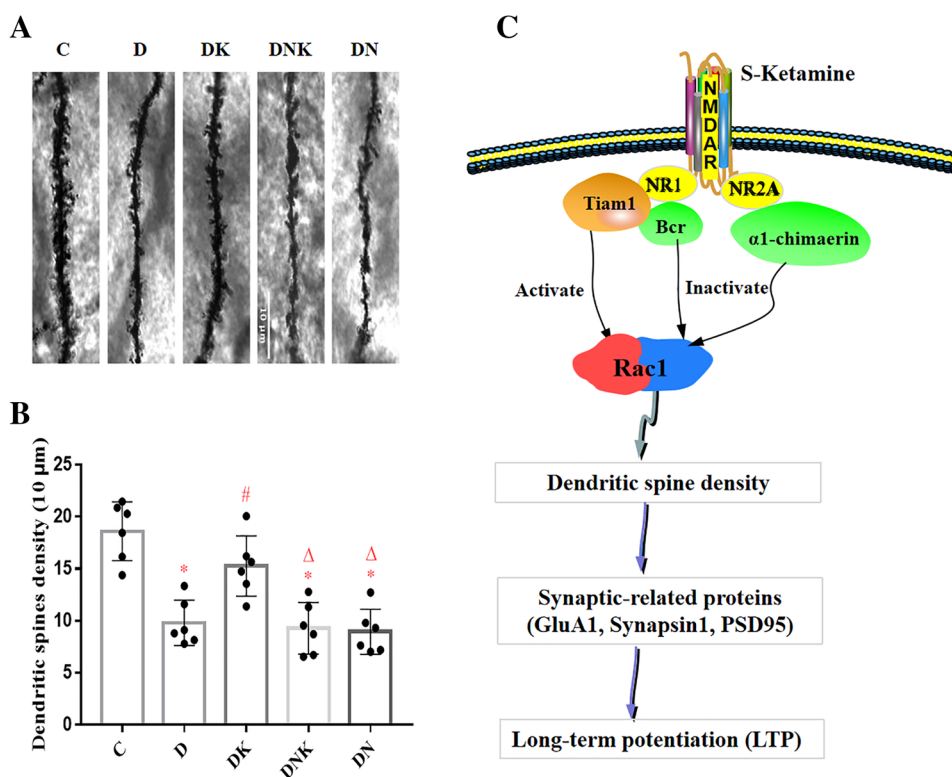
the dendritic spines density of hippocampal CA1 region in stressed rats. The data of spines density meet normality (The P values of groups C, D, DK, DNK, and DN were 0.446, 0.221, 0.888, 0.627, 0.245) and homogeneity of variance ($F_{(4, 25)} = 0.177$, $P = 0.948$). As shown in Fig. 5A–B, the spine density among all groups showed statistically significant differences ($F_{(4, 25)} = 17.544$, $P < 0.001$). Post hoc tests showed that the spine density was significantly lower in group D than that of group C ($P < 0.001$). Compared with group D, group DK exhibited increased levels of spines density ($P = 0.010$). Compared with the DK group, the spines density in groups DNK were lower ($P = 0.004$).

S-Ketamine Alleviated the LTP Impairments of Schaffer-CA1 in the Hippocampus of Stressed Rats

Since LTP can intuitively reflect the process of synaptic plasticity, we implemented electrophysiological experiments through brain slices to study the internal mechanism of S-Ketamine on the antidepressant effects in stressed rats. Figure 6A shows the original trace of fEPSP and data of fEPSP slope, which was calculated as % of baseline over 1 h of recording. We statistically analyzed the average of fEPSP slope in the last 30 min after the high-frequency stimulation. As shown in Fig. 6B, the average of fEPSP slope meet normality (The P values of groups C, D, DK, DNK, and DN were 0.971, 0.693, 0.890, 0.693, 0.857) and homogeneity

of variance ($P = 0.985$). The average of fEPSP slope among all groups showed statistically significant differences ($F_{(4, 25)} = 45.444$, $P < 0.001$). Post hoc tests showed that CUMS-induced LTP impairment in group D as compared to group C (fEPSP slope: $145.9 \pm 5.2\%$ vs. $177.7 \pm 6.6\%$) ($P < 0.001$). S-Ketamine effectively alleviated the LTP impairment caused by CUMS, as shown by the fEPSP slope being higher in the DK group ($164.47 \pm 5.6\%$) than in the D group ($P < 0.001$). However, Rac1 inhibitor NSC23766 effectively reversed the protective effect of S-Ketamine. Compared with the DK group, the fEPSP slope in groups DNK ($148.9 \pm 5.2\%$) was lower ($P = 0.001$). Figure 6A, B.

Discussion

Our results showed that CUMS down-regulated the expression and activity of Rac1, decreased the dendritic spine density and the expressions of synaptic-related proteins such as GluA1, Synapsin1, and PSD95, impaired the LTP in the hippocampus, and induced depression-like behavior in rats. Ketamine can up-regulate the expression and activity of Rac1 in the hippocampus of stressed rats, increase the dendritic spine density and the expression of GluA1, Synapsin1, and PSD95, effectively reduce the CUMS-induced LTP impairments, and thus improve the depression-like behavior of rats. However, Rac1-specific inhibitor NSC23766 effectively reversed these ketamine-mediated changes in

the hippocampus of rats and counteracted its antidepressant effects.

The animal model by CUMS has been proved to be very similar to humans in simulating the pathogenesis of depression, so it has good apparent, predictive, and structural validity (Nestler and Hyman 2010). The core symptom of depression is anhedonia, which can be assessed by the sucrose preference in rats. Thus it can make an accurate assessment of the degree of depression in them (Zhu et al. 2015). In this study, compared with the control group, the sucrose preference percentage, number of crossed squares, and rearing frequency of rats in the CUMS treatment group were significantly lower, indicating that the establishment of the rat depression model in this study was successful.

Synaptic plasticity is a phenomenon wherein the transmission function of the synapse is enhanced or weakened with neural activity (Lynch 2004). Changes in dendritic morphology or dendritic spine density affect the transmission function of synapses (Stein et al. 2021; Muellerleile et al. 2020). Numerous studies have shown that the pathogenesis of depression is related to changes in synaptic plasticity (Yoshino et al. 2021; Lorenzetti et al. 2020). The classic SSRIs fluoxetine and vortioxetine can not only induce synaptogenesis and enhance synaptic transmission function but also have a good antidepressant effect (Bath et al. 2012; Waller et al. 2017). Several studies have shown that ketamine can increase synaptogenesis, up-regulate the expression of synaptic proteins, such as α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor and PSD95 in the hippocampus of animals, and induce LTP- like changes (Li et al. 2011, 2019; Aleksandrova et al. 2020). Our results also found that CUMS can not only induce depression-like behavior in rats but also cause dendritic spine loss and LTP impairments in the hippocampal CA1 region. Ketamine treatment can increase dendritic spine density and reduce LTP impairments in the hippocampus, effectively improving the depression-like behavior of stressed rats. However, pre-administration of Rac1 inhibitor reversed the ketamine-mediated synaptic plasticity changes in the hippocampus and effectively counterbalanced its antidepressant effect on stressed rats. Our results further confirm that changes in synaptic plasticity are associated with the pathogenesis of depression, and Rac1 plays a key role in the antidepressant effect of ketamine.

In this study, we found that ketamine can up-regulate the expression and activity of Rac1 in stressed rats, while pretreatment with Rac1 inhibitor reduced the activity of Rac1, and the antidepressant effect of ketamine was significantly reversed. This indicates that in addition to the expression level of Rac1, the activity or functional status of Rac1 also play an important role in the antidepressant effect of ketamine. The activity of Rac1 is coregulated by its upstream guanine nucleotide exchange factors (GEFs), which catalyzes GDP/GTP exchange to activate Rac1 and GTPase-activating proteins (GAPs), which enhance its intrinsic GTPase activities to inactivate Rac1 (Tolias et al. 2011). It has been found that Tiam1 as GEF and α1-chimaerin, Bcr as GAP, can specifically act on Rac1 and regulate its activity and functions (Tolias et al. 2011; Oh et al. 2010). Further research revealed that α1-chimaerin binds the NMDA receptor subunit NR2A (Van de Ven et al. 2005), and Tiam1, Bcr would form a complex coupled with NMDA receptors subunit NR1 (Tolias et al. 2005; Smith et al. 2017). Therefore, the functional status of NMDA receptors will directly affect the expression and function of Tiam1, Bcr, and α1-chimaerin. In this study, we found that ketamine increased the expression of Tiam1, and decreased the expression of α1-chimaerin and Bcr, and up-regulated the activity of Rac1. This may be attributed to the antagonistic effect of ketamine on NMDA receptors. It is somewhat puzzling that our results also show that NSC23766 (Rac1 inhibitor) can affect the expression of Tiam1, Bcr, and α1-chimaerin. This may be attributed to the compensatory feedback of these upstream regulatory factors on the changes in Rac1 expression and activity, and the influence of NSC23766 itself on their expression. Studies have shown that over-expression of a constitutively active Rac1 mutant was sufficient to cause an accumulation of α1-chimaerin through a phospholipase C-dependent mechanism (Marland et al. 2011). NSC23766, a small chemical compound, can down-regulate the expression of Tiam1 (Elias et al. 2010). In addition, NMDA-receptor antagonists have an inhibitory effect on the expression and function of Bcr and NSC23766 has an antagonistic effect on NMDA receptors (Hou et al. 2014; Simma et al. 2014).

As we all know, Rac1, PSD95, synapsin1, and AMPA receptors are enriched in the postsynaptic membrane. These synaptic proteins interact and regulate synaptic plasticity (Colledge et al. 2003; Huang et al. 2021). Previous studies have shown that the antidepressant effect of ketamine may be related to the up-regulation of brain-derived neurotrophic factor (BDNF) and enhancement of AMPA receptor function (Li et al. 2019, 2010). Activating Rac1 can up-regulate the expression of tropomyosin-related kinase B (TrkB), the receptor of BDNF in the hippocampus, and induce more AMPA receptors to aggregate at the synapse and enhance the excitatory postsynaptic potential (Pandya et al. 2017; Martinez and Tejada-Simon 2011; Benoist et al. 2013). In this study, we found that ketamine (a non-competitive antagonist of NMDA receptor) can up-regulate the expression of PSD95, synapsin1, and AMPA receptors subunit 1 (GluA1) in the hippocampus of stressed rats, while Rac1 inhibitor can reverse the up-regulation effect of ketamine on these proteins. Our results suggest that these synaptic-related proteins are involved in the regulation of ketamine on the synaptic plasticity in the hippocampus of stressed rats, and Rac1 plays a key role in this regulation.
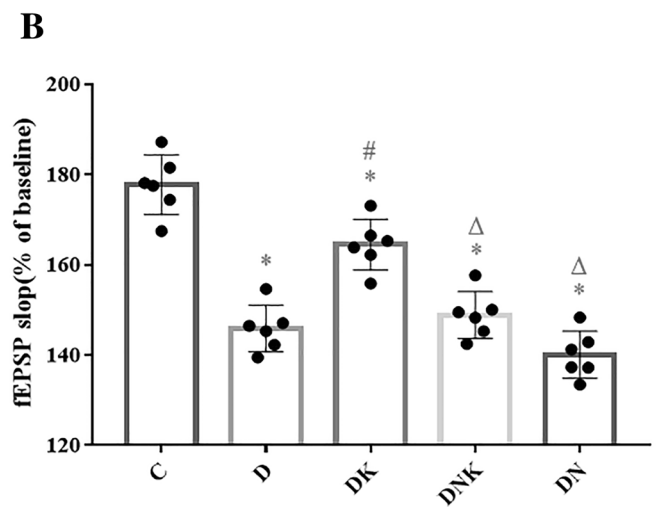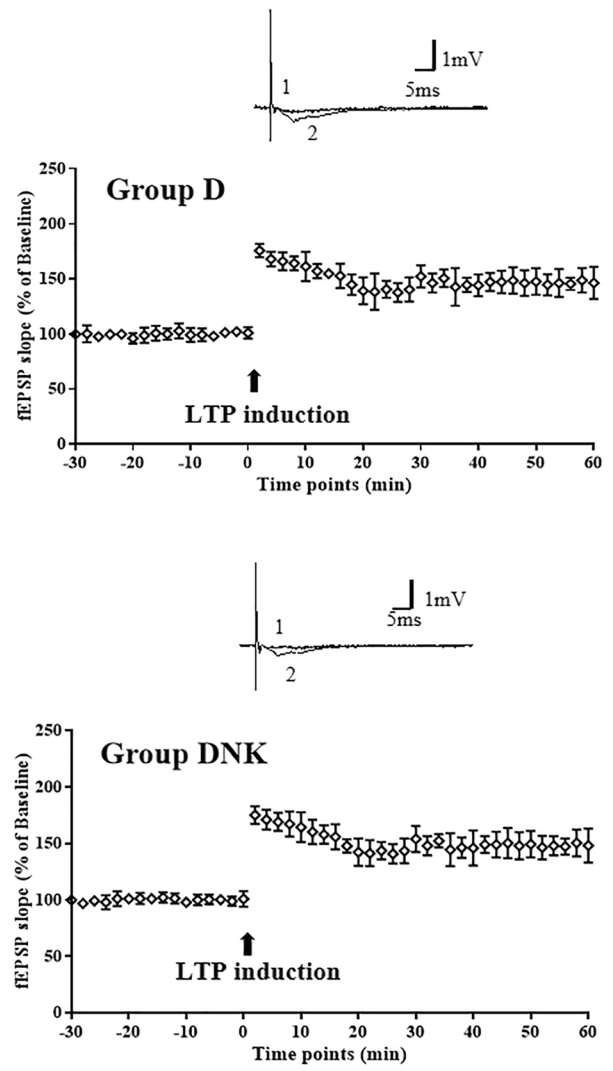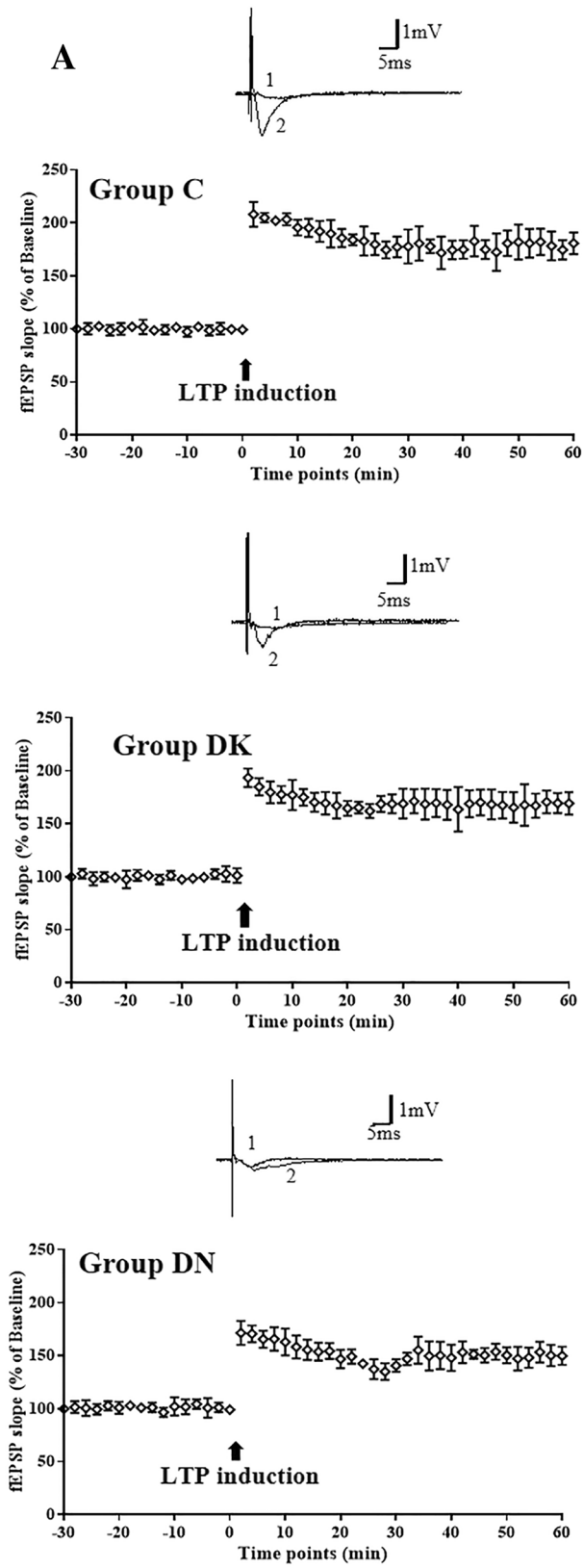
◄**Fig. 6** S-Ketamine alleviated the CUMS-induced LTP impairments of Schaffer-CA1 in the hippocampus of stressed rats, which was effectively reversed by pre-administration of Rac1 inhibitor NSC23766. Group C: healthy control rats; Group D: stressed rats; Group DK: stressed rats with ketamine treatment; Group DNK: stressed rats with NSC23766 and ketamine treatment; Group DN: stressed rats with NSC23766 treatment. **A** Shows the data of fEPSP slope, which was calculated as % of baseline over 1 h of recording. The insets above the fEPSP are the original trace of fEPSP (trace 1 represents the baseline, and trace 2 represents the post-stimulation). The scales of the horizontal and vertical lines are 5 ms and 1 mv, respectively. **B** Shows the statistical analysis results of average of fEPSP slope in the last 30 min after the high-frequency stimulation between groups. Data are expressed as mean ± SD ($n = 6$). *, #, Δ: represents the comparison with group C, group D, and group DK, respectively, $P < 0.05$. LTP: long-term potentiation; fEPSP: field excitatory postsynaptic potential

Several NMDAR antagonists/modulators, such as MK-801 and phencyclidine-induced rapid antidepressant effects in rodents, but clinical data have shown that the antidepressant effect of ketamine in MDD patients was more potent and longer-lasting than non-ketamine NMDAR antagonists (Newport et al. 2015; Kishimoto et al. 2016; Hillhouse et al. 2014). These evidences indicated that non-NMDAR inhibition may also play a role in the antidepressant effects of ketamine. In addition, (S)-Norketamine is the main metabolite of S-Ketamine, similar to S-Ketamine, (S)-Norketamine elicited rapid-acting and sustained antidepressant-like effects in an animal model of depression, but no obvious mental side effects been found (Yang et al. 2018). Ketamine-induced side effects are associated with its NMDAR inhibition (Hashimoto 2020). (Hashimoto 2019) reported that the inhibitory constants (Ki) for NMDAR of ketamine and its metabolites were (R,S)-Ketamine (Ki = 0.53), S-Ketamine (Ki = 0.3), R-Ketamine (Ki = 1.4), and (S)-Norketamine (Ki = 1.7). Therefore, R-Ketamine and (S)-Norketamine could be a safer antidepressant than (R,S)-Ketamine and S-Ketamine. A study showed that no abnormal mental behavior was observed in rodents after intraperitoneal injection of ketamine below 25 mg/kg (Hunt et al. 2006). Our previous study also found that 20 mg/kg of ketamine had a good antidepressant effect, but it declined with the increase in the dose (Zhu et al. 2017). Therefore, we chose 20 mg/kg of S-Ketamine as the treatment dose for this study. Previous animal experiments showed that 50 μg NSC23766 ICV injection would be the optimal dose according to the dose–response curve, which could effectively inhibit the activation of Rac1 GTPase in the hippocampus without showing any significant behavioral side effects (Zhang et al. 2009). In this study, we found that 50 μg of NSC23766 not only significantly down-regulated the activity of Rac1 in the hippocampus of rats, but also effectively reversed the antidepressant effect of ketamine.

The mechanism of the rapid antidepressant effect of ketamine is still unclear. Our results extend the existing work and knowledge on depression by examining whether Rac1 plays an important role in the antidepressant effect of ketamine. However, the pathogenesis of depression is complex and involves multiple brain areas such as the hippocampus, prefrontal cortex, and amygdala (Qiu et al. 2021; Rezaei et al. 2021). In this study, we only observed the effect of ketamine on the hippocampus of rats. In addition, in this study, we also found that NSC23766 completely inhibited the activity of Rac1, but the downstream events of Rac1 signaling, such as spines density and LTP, were not completely blocked after NSC23766 administration. Previous studies have confirmed that the small G protein Rho family, including Rac1, Cdc42, and RhoA, were involved in the regulation of structural and functional of dendritic spines (Duman et al. 2021). Rac1 and Cdc42 promote dendritic growth and synaptic strength, while RhoA has the opposite effect (Govek et al. 2005; Duman, et al. 2015). Therefore, whether Cdc42 and RhoA also play a role in the antidepressant effect of ketamine is currently unclear, and further research is needed. Our results also found that ketamine affected the expression of many Rac1 activity regulators such as Tiam1, α1-chimaerin, and other synaptic-related proteins, but the upstream and downstream signaling pathways were not further clarified. It would be valuable to further study the effects of ketamine on other depression-related brain regions and Rac1 upstream signaling pathways.

In conclusion, S-Ketamine has a good antidepressant effect. The specific mechanism of its antidepressant effect may be related to the enhancement of synaptic plasticity by up-regulating the expression and activity of Rac1 protein in the hippocampus of stressed rats, and then affecting synaptic morphology, related protein expression, and transmission function.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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