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mGluR5-Mediated eCB Signaling in the Nucleus Accumbens Controls Vulnerability to Depressive-Like Behaviors and Pain After Chronic Social Defeat Stress

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Abstract

Stress contributes to major depressive disorder (MDD) and chronic pain, which affect a significant portion of the global population, but researchers have not clearly determined how these conditions are initiated or amplified by stress. The chronic social defeat stress (CSDS) model is a mouse model of psychosocial stress that exhibits depressive-like behavior and chronic pain. We hypothesized that metabotropic glutamate receptor 5 (mGluR5) expressed in the nucleus accumbens (NAc) normalizes the depressive-like behaviors and pain following CSDS. Here, we show that CSDS induced both pain and social avoidance and that the level of mGluR5 decreased in susceptible mice. Overexpression of mGluR5 in the NAc shell and core prevented the development of depressive-like behaviors and pain in susceptible mice, respectively. Conversely, depression-like behaviors and pain were exacerbated in mice with mGluR5 knockdown in the NAc shell and core, respectively, compared to control mice subjected to 3 days of social defeat stress. Furthermore, (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), an mGluR5 agonist, reversed the reduction in the level of the endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) in the NAc of susceptible mice, an effect that was blocked by 3-((2-methyl-1, 3-thiazol-4-yl) ethynyl) pyridine hydrochloride (MTEP), an mGluR5 antagonist. In addition, the injection of CHPG into the NAc shell and core normalized depressive-like behaviors and pain, respectively, and these effects were inhibited by AM251, a cannabinoid type 1 receptor (CB1R) antagonist. Based on these results, mGluR5-mediated eCB production in the NAc relieves stress-induced depressive-like behaviors and pain.

Xiaotao Xu, Kaixuan Wu, Xiaqing Ma and Wenying Wang contributed equally to this work.

Highlights

- Chronic social defeat stress-induced depressive-like behaviors and pain by decreasing mGluR5 levels in the nucleus accumbens.
- Overexpression or activation of mGluR5 in the nucleus accumbens prevented the development of depressive-like behaviors and pain following stress.
- The enhancement of endocannabinoid signaling in the NAc by targeted pharmacological activation of mGluR5 in the NAc alleviates depressive-like behaviors and relieves pain in mice exposed to stress.

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Introduction

Major depressive disorder (MDD) and related pain disorders affect a significant portion of the global population. These conditions lead to reduced productivity, personal suffering and significant healthcare costs [1]. Chronic pain is usually accompanied by increased anxiety and depression that influence quality of life [2]. Conversely, patients with depressive disorder are vulnerable to pain [3]. Studies exploring the relationships between MDD and chronic pain are helpful for developing effective clinical interventions for these syndromes.

The chronic social defeat stress (CSDS) procedure, which is used to establish an animal model of psychosocial stress, consists of two independent phases: the induction phase and testing phase [4]. This stress-based model of depressive-like behaviors has been widely used to simulate the symptomatology of human depression [5, 6]. In this model, stress evokes behavioral and physiological changes, such as social avoidance, anhedonia, and altered corticosterone levels [7]. Interestingly, some mice subjected to the CSDS model are resilient and do not exhibit the depressive-like behavior of social avoidance [5]. Similarly, not every individual exposed to chronic stress develops psychopathological conditions [8]. In addition, the CSDS model was recently reported to induce hyperalgesia, which is not associated with the depressive-like behavior of social avoidance [9]. Based on these results, stress is an important factor triggering both depressive-like behaviors and related chronic pain.

The clinical manifestations of comorbid depression and pain indicate that common or interacting neural circuits, neuroanatomical structures, and neurotransmitter systems underlie the persistence of negative mood and physical pain [10, 11]. Previous studies involving human subjects and animal models have revealed the critical role of the nucleus accumbens (NAc) in concurrent pain and depression [12, 13]. Metabotropic glutamate receptor (mGluR)-mediated neuronal changes are of particular interest. mGluR5, a G protein-coupled receptor, is important for modulating plastic changes in neural circuits [14, 15]. mGluR5 is also well known to be involved in various neurological disorders, including chronic pain and mood disorders such as depression and drug addiction [16–18]. mGluR5 expressed in the NAc is critical for promoting resilience to chronic stress [19]. According to more recent reports, however, mGluR5 is expressed in the medial prefrontal cortex (mPFC), and its expression levels in the mPFC are changed in response to chronic pain and depressive disorders [20]. For instance, prominent upregulation of mGluR5 was detected in the mPFC of animals with chronic neuropathic pain [21]. Therefore, the elucidation of the contribution of mGluR5 to MDD and related pain will help us understand the underlying mechanisms.

Endogenous cannabinoids (eCBs) are lipid mediators with essential modulatory functions in the brain [22] that play important roles in a wide variety of physiological processes, including affective and nociceptive responses [23]. The two major eCBs, anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), are produced in the postsynapse and signal in a retrograde direction to modulate synaptic strength via the activation of presynaptic cannabinoid type 1 receptor (CB1R) [24]. Activation of CB1R leads to acute depression of synaptic transmission that induces endocannabinoid-mediated long-term depression (LTD), which was originally discovered in the NAc [25], in combination with enhanced eCB signaling. The restoration of eCB signaling in the NAc protects against CSDS-induced anxiety-like behaviors by enhancing 2-AG signaling. The production of eCB is associated with the activity of mGluR5 [26–28]. The phosphorylation of mGluR5 at tyrosine residues is critical for maximal signaling [29], illustrating that a reduction in phosphorylation might subsequently alter eCB production and signaling.

Based on these findings, we designed a series of experiments to ask whether mGluR5 in the NAc plays an important role in stress-induced pain threshold alterations and depressive-like behaviors through eCB signaling. The elucidation of the mechanism that mediates depression and nociceptive symptoms in the context of psychological stress may lead to the development of novel therapeutic strategies for the management of chronic pain states and depressive-like behaviors in patients.

Materials and Methods

Animals

All experiments and procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Eighth Edition) published by the National Research Council (USA) and were approved by the Institutional Animal Care and Use Committee of Sixth People's Hospital Affiliated with Shanghai Jiao Tong University. Specific pathogen-free (SPF) male C57BL/6J mice (12–20 weeks old; Fudan University Medical Animal Center, Shanghai, China) were used. The mice were housed in a temperature- and humidity-controlled environment (lights on 07:00–19:00) and provided a standard rodent diet and water *ad libitum*.

Social Defeat Stress and Social Interaction

The social defeat stress procedure was conducted as described previously [30]. Briefly, each C57BL/6J male mouse was subjected to an unfamiliar aggressive resident CD1 male mouse for 5 min for physical defeat. After defeat, both the intruder mouse and the resident CD1 mouse were housed together, but separated by a perforated plastic divider that allows visual, olfactory, and auditory contact, but not physical interaction, for the remainder of the 24-h period. The same test mouse was subjected to social defeat from a different aggressive CD1 resident mouse each day for 3 consecutive days or 10 consecutive days (10-day CSDS procedure). Nondefeated control mice were housed opposite another C57BL/6 mouse instead of a CD1 mouse. Twenty-four hours after the last social defeat session, the social interaction test (defeated mice and nondefeated control mice) was conducted in an open-field arena (43.5 cm × 43.5 cm) equipped with a wire mesh cage (10 × 6.5 cm). Social avoidance behavior was recorded with a video tracking system and measured according to a two-stage interaction test. In the first "no target" stage, an empty wire mesh cage was placed in the interaction zone, and the behavior of the C57BL/6J mice was recorded for 10 min. For the second "target" stage, a novel CD1 aggressor was placed in the wire mesh cage. Both the test mouse and the target CD1 mouse maintained visual, olfactory, and auditory contacts but made no direct physical contact. The time spent by the mouse in the "interaction zone" (26.0 × 14.5 cm) and "corner zone" (43.5 × 8.0 cm) of the arena was recorded with video-tracking software (SMART 2.5, Panlab). The social interaction test was performed starting 24 h after the final day of the CSDS procedure. The social interaction ratio was defined as [time spent in the interaction zone / (time spent in the interaction zone + time in the corner zone) × 100 (%)] [31]. According to the social interaction ratio, defeated mice were divided into susceptible and resilient subpopulations. If the social interaction ratio was greater than the lower endpoint of the 95% confidence interval of that of control mice, a defeated mouse was considered resilient.

Tail Suspension Test (TST)

Mice were individually suspended by the tail from a metal hanger with adhesive tape in the tail suspension box approximately 20 cm above the ground. The immobility time during the last 4 min of the 6-min TST session was recorded for each mouse. Immobility was defined as only minor limb movements or no movement at all.

von Frey Filament Test

The nociceptive mechanical threshold of the right hind paw was measured with an electronic von Frey device (Bioseb, France). Briefly, each mouse was individually placed in a cage with a mesh floor and adapted to the environment before testing. After a 30-min habituation period, a series of von Frey filaments with increasing pressure (0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, and 2.0 g) was applied to the plantar surface of the right hind paw until the mouse withdrew its paw. The filaments, starting with the 0.04-g filament, were perpendicularly applied from underneath the mesh floor to stimulate the plantar surface of the paw for 5–6 s in each trial. We recorded the force applied at the time of withdrawal, and responses are reported in grams. The cutoff threshold was set to 2 g. Stimulation was conducted via the up-down method, and the 50% withdrawal threshold was determined from the results. The threshold value was determined by averaging three consecutive trials at 5-min intervals.

Mass Spectrometry Detection of Anandamide (AEA) and 2-Arachidonoylglycerol (2-AG)

The NAc region was dissected from the brain. Tissue samples were weighed and placed in borosilicate glass culture tubes containing 2 ml of acetonitrile with 5 pmol of [d8] AEA and 5 nmol of [d8] 2-AG for extraction. The tissues were homogenized with a glass rod and sonicated for 30 min in an ice-cold water bath. The supernatants were transferred to new glass tubes and evaporated to dryness under N_2 gas. The samples were resuspended in 300 μl of acetonitrile to recapture any lipids that had adhered to the glass tube and dried again under N_2 gas. Finally, lipid extracts were suspended in 20 μl of acetonitrile and stored at – 80 °C until analysis. Tissue AEA and 2-AG levels were determined using liquid chromatography-mass spectrometry (LC-MS/MS) as described previously [32].

Virus Preparation

A chemically synthesized mGrm5 transcript, NM_001081414, was amplified by PCR using the following primer sets: mGrm5-F: 5'-AGGTCGACTCTAGAGGATCCCGCCACCATGGTCCTTCTGTTGATTCTGTCAGTC-3' and mGrm5-R: 5'-TCCTTGTAGTCCATACCCAACGATGAAGAACTCTGCGTGTAATC-3'. The amplified fragment was inserted into the BamH I and Age I restriction enzyme sites of the GV314 plasmid (CMV-MCS-3FLAG-SV40-EGFP) (Genechem, Shanghai).

A chemically synthesized miR30-based shRNA designed to knock down mGluR5 expression was amplified by PCR using the following primer sets: miR-30 (mGlu5)-F: 5'-ACGAGCTGTACAAGGCTAGCTAAGCCTTGTTAAGTGCTCGCTTCG-3' and miR-30 (mGlu5)-R: 5'-GTTGATTATCGATAACCGGTCGCGTCGCCGCGTGTTTAAACGC-3'. The amplified fragment was inserted into the Nhe I and Age I restriction enzyme sites of the

GV412 plasmid (AAV9-CMV-EGFP-MCS) (Genechem, Shanghai). All viral vectors were stored in aliquots at – 80 °C.

Stereotaxic Injection and Cannula Implantation

Mice were anesthetized with pentobarbital sodium (45 mg·kg^{-1}, i.p.) and then fixed in a stereotaxic frame [33]. Two hundred nanoliters of the virus were injected bilaterally into the NAc shell (from the bregma: + 1.6 mm anteroposterior; ± 0.5 mm lateral; − 4.1 mm dorsoventral) or into the NAc core (from the bregma: + 1.3 mm anteroposterior; ± 1.0 mm lateral; − 4.1 mm dorsoventral) at a slow rate (20 nl·min^{-1}) using a syringe pump. The needle was withdrawn 10 min after the end of infusion. The location of the injection sites was confirmed in each mouse by observing coronal sections (50 μm) containing the NAc, and mice in which the virus was injected into the incorrect site were excluded from the experiments.

For microinjection of the drugs into the NAc shell and core, a guide cannula (26 gauge, 5-mm long; Plastics One) was chronically implanted + 1.6 mm anteroposterior, ± 0.5 mm lateral, and − 3.1 mm dorsoventral from the bregma and + 1.3 mm anteroposterior, ± 1.0 mm lateral, and – 3.1 mm dorsoventral from the bregma and secured with dental acrylic cement. Obturators were placed in the guide cannulas after implantation.

Intracranial Microinjection of Drugs

An internal cannula (33 gauge; Plastics One) connected to a 1-μl syringe (Hamilton) via polyethylene (PE)-20 tubing was inserted into the guide.

3-((2-Methyl-1,3-thiazol-4-yl)ethynyl) pyridine hydrochloride (MTEP hydrochloride, Tocris Bioscience) (10 μg per side) was unilaterally microinjected into the NAc of susceptible mice immediately before CHPG (10 μg per side) treatment. AM251, vehicle control or (RS)-2-chloro-5-hydroxyphenylglycine (CHPG, Tocris Bioscience) was unilaterally microinjected into the NAc core or shell of susceptible mice in a volume of 0.5 μl per side over 30 s. After 1 min, the internal cannula was withdrawn and the obturator was replaced. The mice received a microinjection of AM251 (MedChem Express, 0.8 μg per side), vehicle control or CHPG (10 μg per side) once a day for 3 consecutive days. The dose and treatment time of drug administration, alone or in combination, were chosen based on previous studies [19, 34].

Immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde (wt/vol) in 0.1 M PBS (pH 7.4). The brains of the mice were removed, fixed overnight with the paraformaldehyde solution, and then stored in 30% sucrose in PBS. All brains were then frozen in O.C.T. (Sakura Finetek, Inc.) and cut into 30-μm coronal sections using a cryostat (Leica Biosystems). For mGluR5 immunofluorescence staining, the sections were blocked with PBS containing 3% normal donkey serum and 0.3% Triton X-100 for 1 h at room temperature. Thereafter, the sections were incubated with an anti-mGluR5 antibody (1:200; 2237-1, Epitomics) in PBS containing 0.5% Triton X-100 (vol/vol) at 18–20 °C overnight. Sections were then washed with PBS three times and incubated with a secondary antibody conjugated to Alexa Fluor in 0.5% Triton X-100 in PBS for 2 h at room temperature. Images were captured using a Leica TCS SP5II confocal microscope with LAS AF Lite software (Leica Microsystems).

Immunoblotting

The brains were placed in a coronal brain matrix (1-mm slice interval; ASI-instrument). Single-edged blades were inserted into the appropriate slits. Coronal slices with a 1–2-mm thickness were placed in a dish containing ice-cold PBS for NAc dissection. The NAc was dissected rapidly and immediately stored at – 80 °C. Individual tissues were thawed on ice as needed and placed in RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a protease inhibitor (Calbiochem, Schwalbach, Germany). The tissue lysates were centrifuged at 12000×*g* for 15 min at 4 °C, and the supernatant was collected to measure the protein concentration using a BCA kit (Beyotime Institute of Biotechnology, Haimen, China). The samples were then resolved on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% nonfat dry milk plus 0.05% Tween 20 (vol/vol) in PBS for 1 h at room temperature and then incubated with the appropriate primary antibodies against mGluR5 (1:1000; ab76316, Abcam) and β-actin (1:2000; catalog no. 4970, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. The membranes were washed three times with 1× TBST and then incubated with an HRP-conjugated secondary antibody (1:2000; catalog no. 7074, Cell Signaling Technology, Danvers, MA, USA) for 1 h. The bands were detected using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, USA) with an enhanced chemiluminescence (ECL) detection kit (Millipore, USA), and the band densities were quantified using ImageJ software (version 4.0.0, USA).

Statistical Analysis

All data are presented as the means ± SEM. The statistical analysis was performed with GraphPad Prism 7.00 software (GraphPad Software Inc., San Diego, CA, USA). Differences between the means were analyzed by one-way analysis of variance (ANOVA). One-way ANOVA followed by Bonferroni's post hoc test was performed to compare multiple

independent groups. Unpaired Student's t tests were used to compare data from two groups. $P < 0.05$ was considered statistically significant.

Results

Stress Induces Depressive-Like Behaviors and Pain

Male mouse were exposed to an aggressive retired breeder CD1 mouse as a social defeat stressor for 10 days to explore the effect of stress on depression and pain. Twenty-four hours after the last defeat stress exposure, the social interaction behaviors of mice were tested in an open-field arena with an interaction zone (Fig. 1A). After the last defeat episode, the mice were separated into two groups based on social interaction ratios: the susceptible group and resilient group (Fig. 1B). The time spent in the interaction zone in the absence of a target mouse was similar among the different groups (Fig. 1C). The time spent in the interaction zone in the presence of a target mouse was shorter for the susceptible group than for the control and resilient groups (Fig. 1D). Based on this result, susceptible mice exhibited a strong social avoidance behavior. We also detected the depressive-like phenotypes of mice using the TST. The immobility time in the TST was longer for susceptible mice than for control and resilient mice (Fig. 1E). The electronic von Frey test was used to evaluate mechanical nociception and the effect of stress on pain. The mechanical thresholds were decreased in susceptible and resilient mice compared with control mice. However, the mechanical thresholds were not different between susceptible and resilient mice (Fig. 1F). Thus, mice that underwent CSDS (resilient and susceptible) exhibit a higher sensitivity to pain in the mechanical nociceptive test.

Stress Induces Changes in mGluR5 Expression in the NAc

A Western blot assay was performed to measure the level of mGluR5 in the NAc of mice and to determine whether the mGluR5 level in the NAc changed after 10 days of CSDS. Lower expression of the mGluR5 protein was observed in the NAc of susceptible mice than in the NAc of control and resilient mice (Fig. 2A and B).

Overexpression of mGluR5 in the NAc Shell or Core Alleviates Stress-Induced Depression-Like Behaviors and Pain, Respectively

We showed that mGluR5 expression was decreased in the NAc of susceptible mice after CSDS, consistent with a previous study [19]. Moreover, this study revealed that virus-induced mGluR5 expression in the NAc shell of mGluR5$^{-/-}$ mice plays an important role in protecting against stress-induced depression-like behaviors [19]. We therefore tested whether viral rescue of mGluR5 expression in the NAc shell is critical for preventing stress-induced depression-like and pain behaviors in susceptible mice (Fig. 3A). An in vitro experiment showed an increased level of mGluR5 in cells transfected with mGluR5-AV compared with cells transfected with control-AV (Fig. 3B). After the CSDS procedure, an adenovirus expressing mGluR5 was stereotaxically injected into the NAc shell of susceptible mice, and these adenoviruses were expressed effectively in vivo (Fig. 3C). Western blot results showed an increased expression level of mGluR5 in the NAc shell of susceptible mice 5 days after the injection (Fig. 3D). Thus, all subsequent behavioral procedures were performed 5 days after the injection. The time spent in the interaction zone in the absence of a target mouse was not different between the two groups (Fig. 3E). Moreover, compared with control-AV-injected susceptible mice, upregulation of mGluR5 expression in the NAc shell of susceptible mice significantly increased the interaction time in the presence of a target mouse (Fig. 3F). Compared with control-AAV-injected susceptible mice, rescue of mGluR5 expression in the NAc shell of susceptible mice decreased the immobility time in the TST (Fig. 3G). However, compared with control-AV-injected susceptible mice, rescue of mGluR5 in the NAc shell of susceptible mice did not change the mechanical thresholds (Fig. 3H). These results show that mGluR5 expression in the NAc shell in susceptible mice contributes to resilience to depression-like behaviors and does not influence pain sensitivity.

Furthermore, we determined whether viral rescue of mGluR5 expression in the NAc core plays a critical role in preventing stress-induced depression-like and pain behaviors in susceptible mice (Fig. 4A). After the CSDS procedure, an adenovirus expressing mGluR5 was stereotaxically injected the NAc core of susceptible mice, and the adenoviruses were expressed effectively (Fig. 4B). All behavioral procedures were performed 5 days after the injection. Compared with control-AV-injected susceptible mice, upregulation of mGluR5 expression in the NAc core of susceptible mice did not change the interaction time (Fig. 4 C and D). Similarly, compared with control-AV-injected susceptible mice, rescue of mGluR5 expression in the NAc core of susceptible mice did not alter the immobility time in the TST (Fig. 4E). However, compared with control-AV-injected susceptible mice, rescue of mGluR5 expression in the NAc core of susceptible mice increased the mechanical thresholds (Fig. 4F). Based on these data, mGluR5 expression in the NAc core of susceptible mice reduces pain sensitivity but does not change depression-like behaviors.

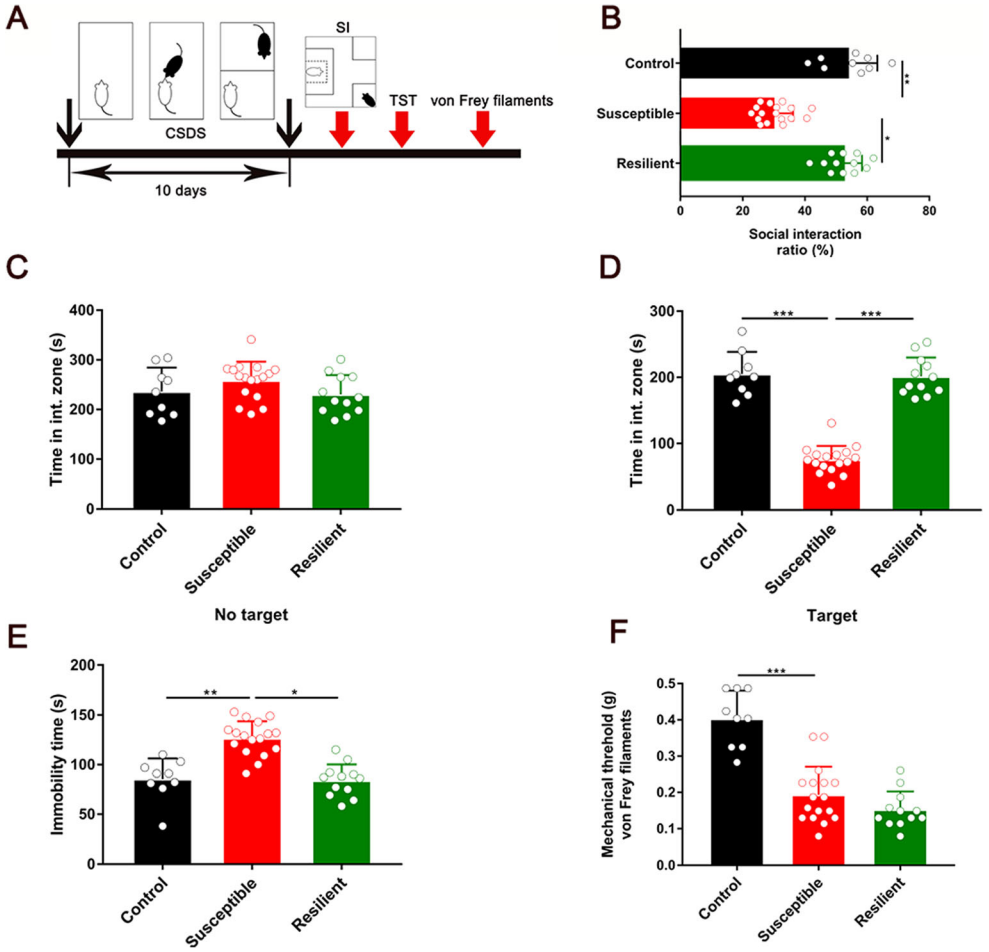

Fig. 1 Stress induces depressive-like behaviors and hyperalgesia. **A** Timeline of the experimental procedure. **B** Segregation of defeated mice into susceptible and resilient groups based on their social interaction ratios (n = 9, 17, and 12 mice in the control, susceptible, and resilient groups, respectively; means ± SEMs; one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons; $*P < 0.05$ and $**P < 0.01$). **C**–**D** Social interaction test. Time spent in the interaction zone after 10 days of chronic defeat stress in the presence of no target (**C**) and a target (**D**) (n = 9, 17, and 12 mice in the control, susceptible, and resilient groups, respectively; means ± SEMs; one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons; $***P < 0.001$). **E** TST. Immobility time was recorded during the last 4 min of the 6-min suspension period. Susceptible mice displayed an increase in immobility time (n = 9, 17, and 12 mice in the control, susceptible, and resilient groups, respectively; means ± SEMs; one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons; $*P < 0.05$ and $**P < 0.01$). **F** The von Frey filaments test was used to determine the effect of stress on nociception (n = 9, 17, and 12 mice in the control, susceptible, and resilient groups, respectively; means ± SEMs; one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons; $*P < 0.05$ and $***P < 0.001$)

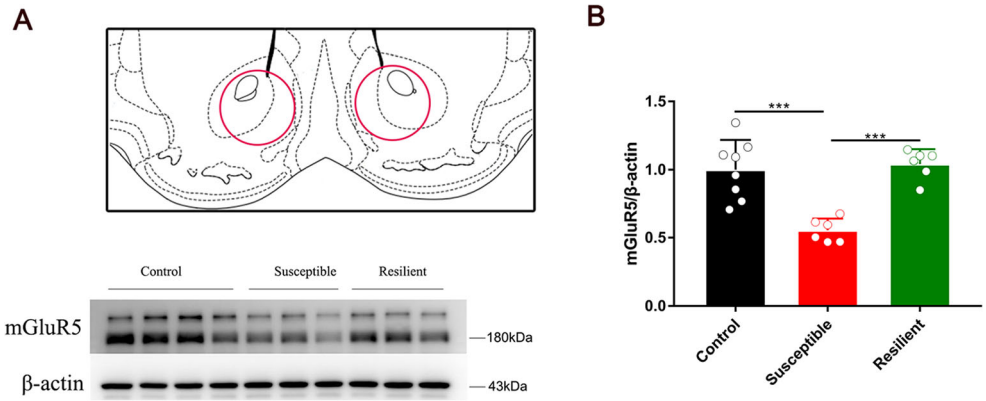

Fig. 2 The level of mGluR5 in the NAc is reduced in susceptible mice. **A** The level of mGluR5 in the NAc of mice in each group following CSDS was detected using Western blotting. **B** Quantification of mGluR5 expression in the NAc of each group of mice following CSDS (n = 8, 6, and 6 mice in the control, susceptible, and resilient groups, respectively; means ± SEMs; one-way ANOVA followed by Bonferroni's test; $***P < 0.001$)

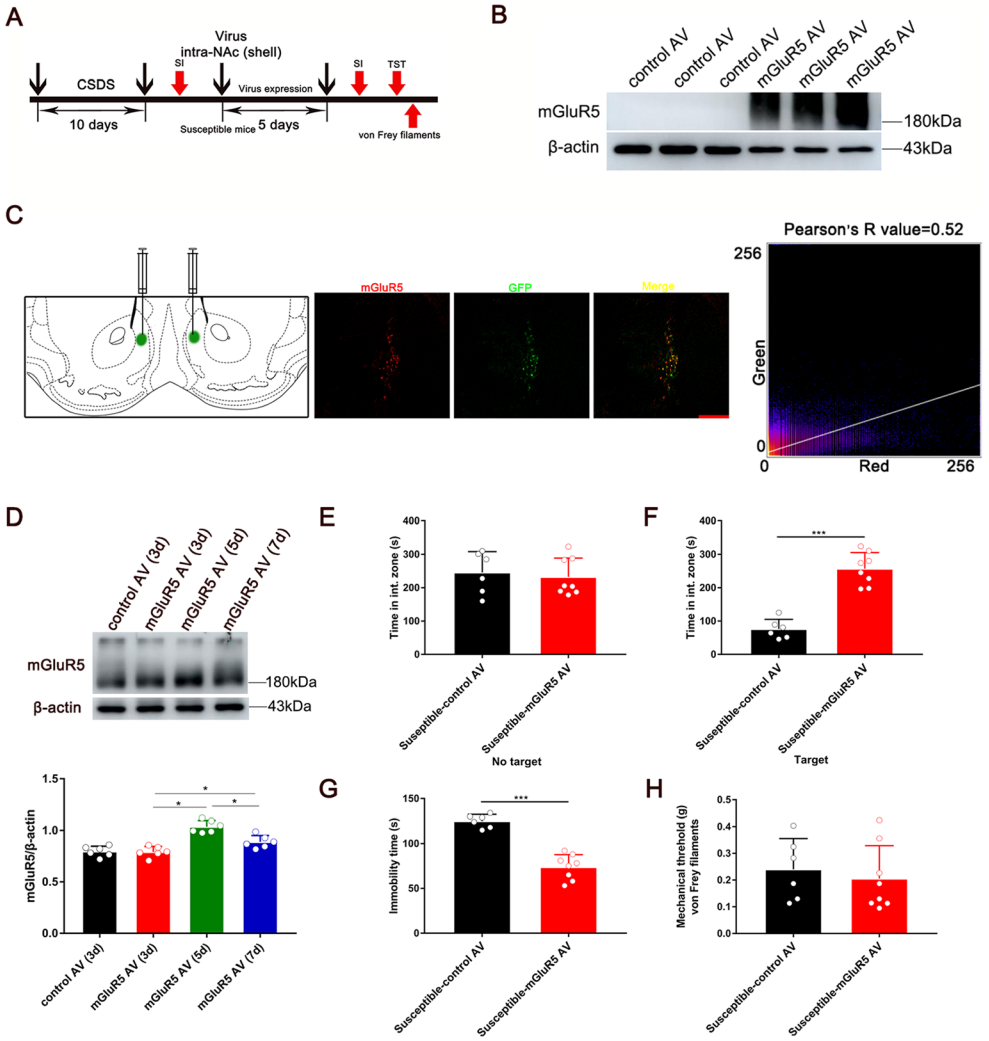

Fig. 3 Overexpression of mGluR5 in the NAc shell rescues social defeat stress-induced social avoidance but does not change pain sensitivity. **A** Timeline of the experimental procedure. **B** Validation of the effect of the mGluR5-targeting adenovirus. Western blot analysis of mGluR5 expression in HEK293 cells 24 h after transduction of the control-AV (adenovirus) or mGluR5-AV at low, medium, and high concentrations. **C** Schematic representation of the injection sites of mGluR5-AV or control-AV into the NAc shell. For each mouse used, the correct location of the virus injection was confirmed by visualizing the coexpression of GFP with mGluR5 induced by the AV by immunofluorescence staining. The scale bars represent 200 μm. The Pearson's R value of co-localization of each was quantified by Image J. **D** Western blot analysis of mGluR5 expression in the NAc shell 3, 5, and 7 days after injection of the control-AV (adenovirus) or mGluR5-AV. (**E**–**F**) Social interaction test. Time spent in the interaction zone by AV-injected susceptible mice in the presence of no target (**E**) and a target (**F**) (n = 6 mice in the control-AV group and 8 in the mGluR5-AV group; means ± SEMs; Student's *t* test; $*P < 0.05$ and $**P < 0.01$). **G** Summary of the TST data for the mGluR5-AV and control-AV-injected mice. The graphs show a decrease in the immobility for the mGluR5-AV-injected susceptible mice (n = 6 mice in the control-AV group and 8 in the mGluR5-AV group; means±SEMs; Student's *t* test; $*P < 0.05$ and $**P < 0.01$). **H** Control-AV- and mGluR5-AV-injected susceptible mice displayed no change in the pain phenotype in von Frey filament tests (n = 6 mice in the control-AV group and 8 in the mGluR5-AV group; means ± SEMs; Student's *t* test)

Conditional Deletion of mGluR5 in the NAc Shell or Core Induces Depressive-Like Behaviors and Increased Pain Sensitivity, Respectively

We next evaluated the level of mGluR5 in brain regions that may be responsible for the depressive-like and pain phenotypes of mice exposed to social defeat stress. We designed a series of experiments (Fig. 5A) and stereotaxically injected an AAV containing miR30-mGluR5-shRNA or control-shRNA into the bilateral NAc shell of naïve mice. These adenoviruses were expressed effectively in vivo (Fig. 5B). Western blot results showed decreased expression levels of mGluR5 in the NAc shell of mice 2 weeks after the injection (Fig. 5C). Thus, all subsequent procedures were performed 2 weeks after the injection. We subjected animals to 3 d of social defeat stress 2 weeks after the injection. The time spent in the interaction zone in the absence of a target mouse was not different between the mGluR5-shRNA and control-shRNA groups

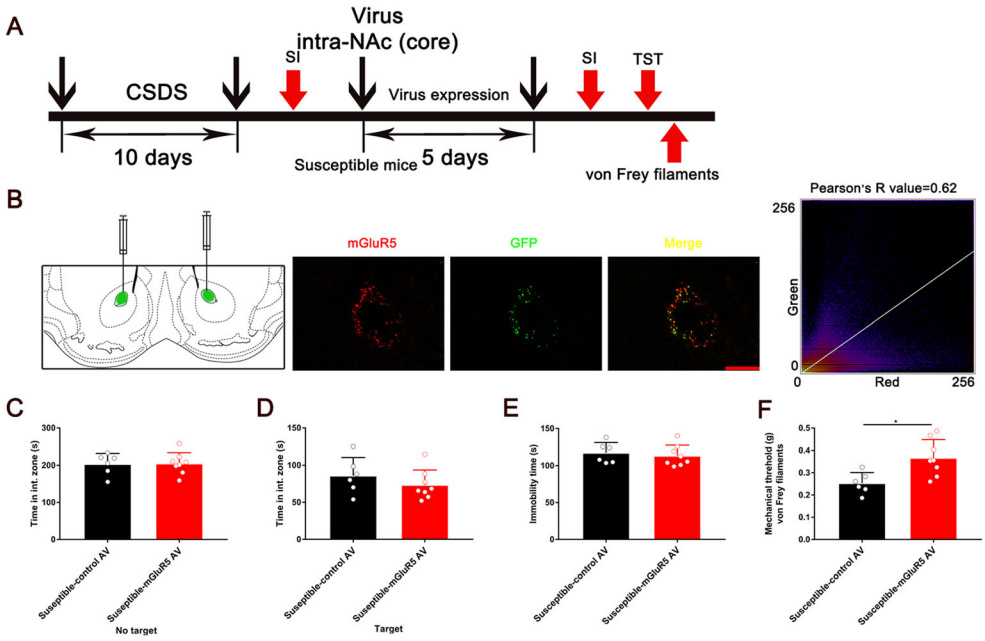

Fig. 4 Overexpression of mGluR5 in the NAc core alleviates social defeat stress-induced hyperalgesia but does not affect social avoidance. **A** Timeline of the experimental procedure. **B** Schematic representation of the injection sites of mGluR5-AV and control-AV into the NAc core. For each mouse used, the correct location of the virus injection was confirmed by visualizing the coexpression of GFP with mGluR5 induced by the AV by immunofluorescence staining. The scale bars represent 200 μm. The Pearson's R value of co-localization of each was quantified by Image J. **C**–**D** Social interaction test. The amount of time spent in the interaction zone by susceptible mice microinjected with mGluR5-AV into the NAc core was similar to that spent by control-AV-injected susceptible mice in the presence of no target (**C**) and a target (**D**) (n = 6 mice in the control-AV group and 8 in the mGluR5-AV group; means ± SEMs; Student's *t* test). **E** Summary of the TST data for the control-AV and mGluR5-AV-injected susceptible mice. The graphs show no difference in immobility time between the control-AV- and mGluR5-AV-injected susceptible mice (n = 6 mice in the control-AAV group and 8 in the mGluR5-AAV group; means±SEMs; Student's *t* test). **F** mGluR5-AV-injected susceptible mice displayed a decrease in the pain phenotype in the von Frey filaments tests compared to their respective control-AV-injected susceptible mice (n = 6 mice in the control-AV group and 8 in the mGluR5-AV group; means ± SEMs; Student's *t* test; $*P < 0.05$ and $**P < 0.01$)

(Fig. 5D). Notably, mGluR5-shRNA mice spent less time in the interaction zone in the presence of a target mouse and exhibited a longer immobility time in the TST than control-shRNA mice (Fig. 5E–F). However, the mechanical thresholds were similar between the mGluR5-shRNA-treated mice and control-shRNA-treated mice (Fig. 5G). Therefore, conditional knockdown of mGluR5 in the NAc shell of mice increases both stress-induced social avoidance and depression-like behaviors but does not change pain sensitivity.

Furthermore, we wanted to determine whether conditional knockdown of mGluR5 expression in the NAc core contributes to stress-induced depressive-like behaviors and pain (Fig. 6A). We stereotaxically injected an AAV containing miR30-mGluR5-shRNA or control-shRNA into the bilateral NAc core of naïve mice (Fig. 6B). The animals were subjected to 3 days of social defeat stress 2 weeks after the injection. The time spent in the interaction zone in the absence of a target mouse was not different between the mGluR5-shRNA and control-shRNA groups (Fig. 6C). The time spent in the interaction zone in the presence of a target mouse and immobility time in the TST were similar between the mGluR5-shRNA-treated mice and control-shRNA-treated mice (Fig. 6D–E). However, the mechanical thresholds increased in mGluR5-shRNA-treated mice compared with control-shRNA-treated mice (Fig. 6F). These results indicated that conditional knockdown of mGluR5 in the NAc core of mice increases stress-induced pain sensitivity but does not change either stress-induced social avoidance.

mGluR5-Mediated Production of eCB Within the NAc Is Impaired in Susceptible Mice

The eCB system is composed of CB1R and cannabinoid receptor type 2 (CB2R) [35–37]. Two major endogenous ligands, AEA and 2-AG, bind to CB1R [35, 38]. According to previous reports, sustained disruption of the levels of the eCB 2-AG within the amygdala following CSDS results in pathological states of anxiety, while an increase in 2-AG levels in the NAc increases behavioral resiliency to chronic stress in mice [32, 34]. Together, these studies indicate that eCB signaling is an important regulator of depression and anxiety. Thus, we postulated that the susceptible mice might

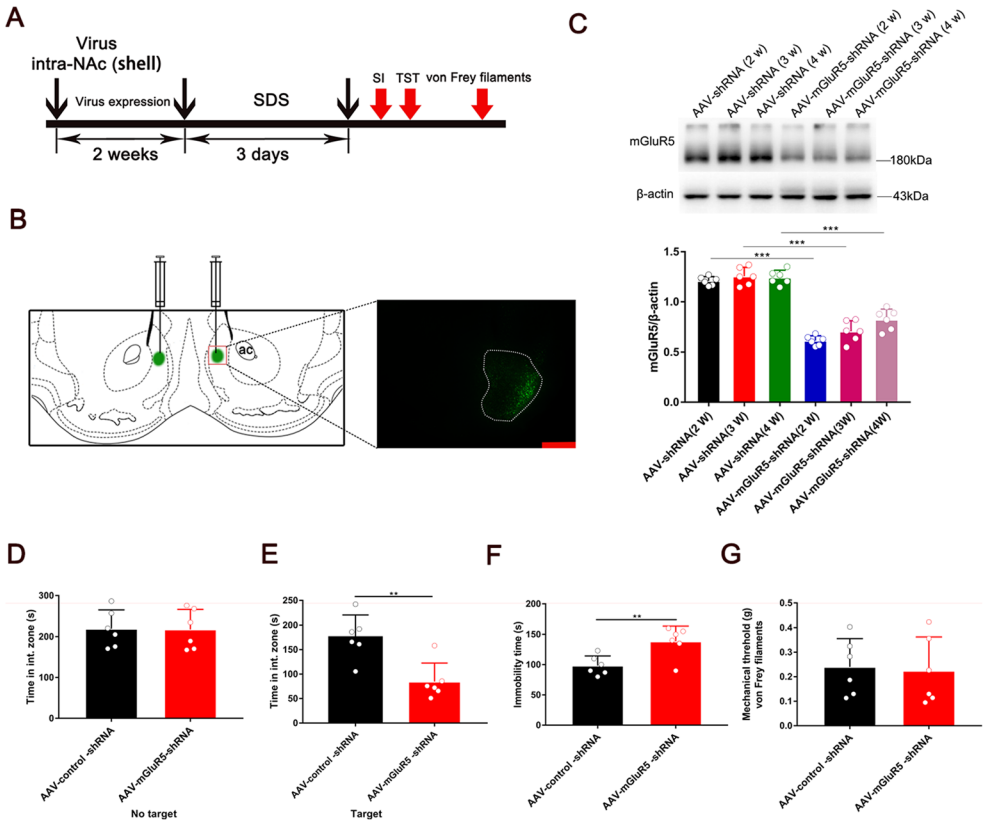

Fig. 5 Conditional deletion of mGluR5 in the NAc shell induces social avoidance but does not change pain sensitivity. **A** Timeline of the experimental procedure. **B** Schematic representation of the injection sites of the AAV expressing mGluR5-shRNA (AAV-mGluR5-shRNA) and the AAV expressing control-shRNA (AAV-control-shRNA) in the NAc shell. The precise location of the virus injection was confirmed by visualizing GFP in each mouse by immunofluorescence staining. The scale bar represents 50 μm. **C** Western blot analysis of mGluR5 expression in the NAc shell at 2, 3 and 4 w after the injection of AAV-control-shRNA or AAV-mGluR5-shRNA. **D**–**E** Social interaction test. Time spent in the interaction zone by AAV-injected mice in the presence of no target (**D**) and a target (**E**) after 3 days of defeat stress (n = 6 mice per group; means ± SEMs; Student's *t* test; $*P < 0.05$ and $**P < 0.01$). **F** Summary of the TST data for AAV-control-shRNA- and AAV-mGluR5-shRNA-injected mice. The graphs show an increase in the immobility time of AAV-mGluR5-shRNA-injected mice (n = 6 mice per group; means ± SEMs; Student's *t* test; $*P < 0.05$ and $**P < 0.01$). **G** The mechanical thresholds in the von Frey filament tests after 3 d of social defeat stress were not different between AAV-mGluR5-shRNA-injected mice and their respective AAV-control-shRNA mice (n = 6 mice per group; means ± SEMs; Student's *t* test)

exhibit impaired eCB induction. We measured eCB levels in the NAc of susceptible mice to test this hypothesis. The level of 2-AG in the NAc was decreased in susceptible mice compared with control mice (Fig. 7A). Furthermore, activation of mGluR5 is known to increase the induction of eCB signaling [26]. We therefore determined whether pharmacological activation of mGluR5 reversed the induction of eCB signaling within the NAc of susceptible mice. We injected either vehicle or CHPG in the presence or absence of MPEP into the NAc of susceptible mice once per day for 3 days and quantified eCB levels 24 h after the final injection. As expected, CHPG restored 2-AG induction in susceptible mice, but the effect was blocked by MPEP, confirming that 2-AG induction is mediated by mGluR5 in the NAc after CSDS (Fig. 7A). However, the level of AEA in susceptible mice was similar among different groups (Fig. 7B).

Enhancement of eCB Signaling in the NAc Shell or Core by Targeted Pharmacological Activation of mGluR5 in the NAc Shell or Core Alleviates Depressive-Like Behaviors and Relieves Pain in Mice Exposed to CSDS, Respectively

We next asked whether pharmacological modulation of mGluR5 within the NAc shell counteracted stress-induced depressive-like behaviors and pain though mGluR5-mediated eCB signaling (Fig. 8A). We injected either the vehicle or CHPG in the presence or absence of AM251 into the NAc shell of susceptible mice once per day for 3 days (Fig. 8B). Mice in the different groups spent a similar amount of time spent in the interaction zone in the absence of a target mouse (Fig. 8C). Susceptible mice that received an injection of CHPG into the NAc shell spent more time in the interaction zone in the presence of a target mouse and exhibited

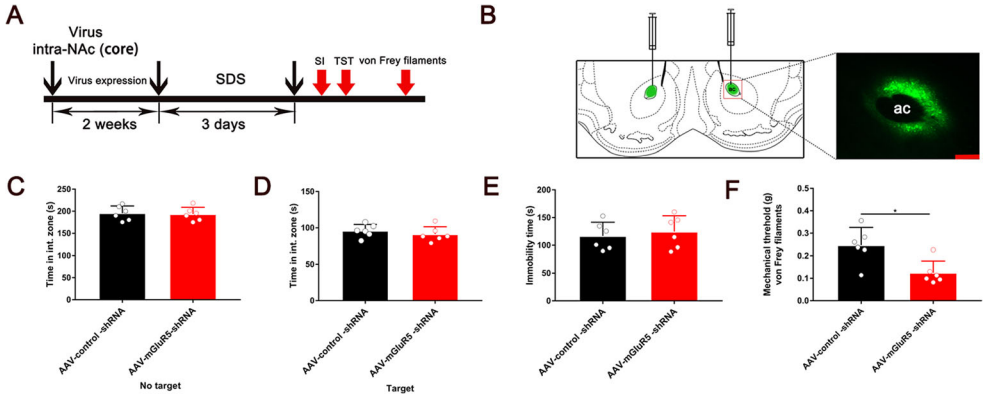

Fig. 6 Conditional deletion of mGluR5 in the NAc core results in pain hypersensitivity but does not alter depressive-like behaviors. **A** Timeline of the experimental procedure. **B** Schematic representation of the injection sites of AAV-mGluR5-shRNA or AAV-control-shRNA in the NAc core. The precise location of the virus injection was confirmed by visualizing GFP in each mouse by immunofluorescence staining. The scale bar represents 50 μm. **C**–**D** Social interaction test. Time spent in the interaction zone by AAV-injected mice after 3 days of defeat stress in the presence of no target (**C**) and a target (**D**) (n = 6 mice per group; means ± SEMs; Student's *t* test). **E** Summary of the TST data for AAV-control-shRNA- and AAV-mGluR5-shRNA-injected mice. The graphs show that the immobility time was not different between AAV-control-shRNA- and AAV-mGluR5-shRNA-injected mice (n = 6 mice per group; means ± SEMs; Student's *t* test). **F** AAV-mGluR5-shRNA-injected mice displayed an increase in the pain phenotype in the von Frey filament test after 3 days of social defeat stress compared to their respective AAV-control-shRNA-injected mice (n = 6 mice per group; mean ± SEMs; Student's *t* test; $^{*}P < 0.05$ and $^{**}P < 0.01$)

a decreased immobility time in the TST compared with susceptible mice in the vehicle group. Moreover, the effect of CHPG on stress-induced depressive-like behaviors in susceptible mice was blocked by an injection of AM251 into the NAc shell (Fig. 8D-E). However, the mechanical thresholds were similar between susceptible mice from different groups (Fig. 8F).

Furthermore, we explored whether pharmacological modulation of mGluR5 within the NAc core would counteract stress-induced depressive-like behaviors and pain though mGluR5-mediated eCB signaling (Fig. 9A). We injected either vehicle or CHPG in the presence or absence of AM251 into the NAc core of susceptible mice once per day for 3 days (Fig. 9B). Mice in the different groups spent similar amounts of time in the interaction zone in the absence of a target mouse (Fig. 9C). The time spent in the interaction zone in the presence of a target mouse and immobility time in the TST were not different between the CHPG group and vehicle group after CHPG was injected into the NAc core of susceptible mice (Fig. 9D–E). However, the mechanical thresholds increased in susceptible mice treated with CHPG compared to susceptible mice treated with the vehicle (Fig. 9F). Moreover, the inhibitory effect of the CHPG injection into the NAc core of susceptible mice on stress-induced pain behaviors was blocked by the injection of AM251 (Fig. 9F). These results suggest that social avoidance behavior and pain sensitivity are altered after exposure to CSDS. Collectively, these data indicate that mGluR5 signaling in the NAc regulates stress-

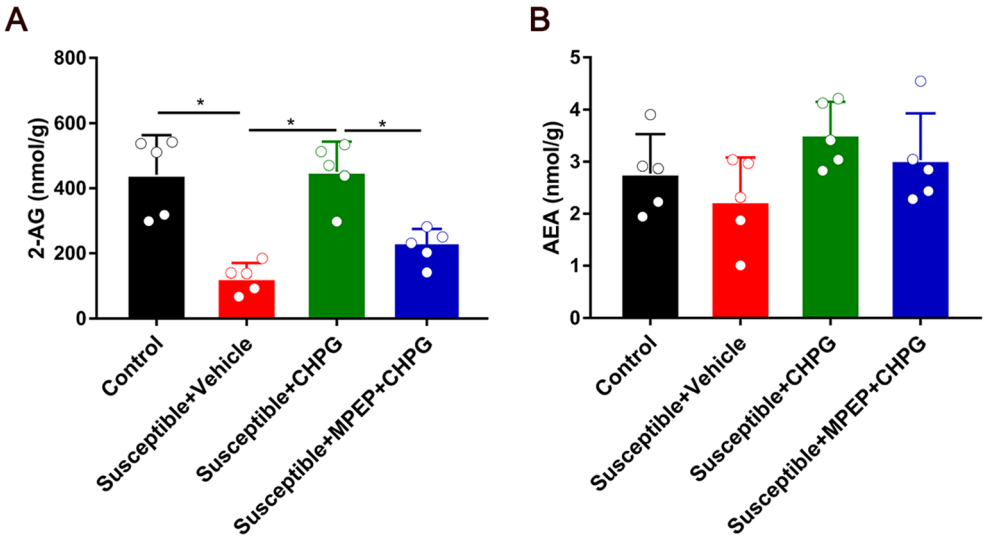

Fig. 7 Activation of mGluR5 restores eCB signaling within the NAc in susceptible mice. Either vehicle or CHPG was injected in the presence or absence of MPEP into the NAc of susceptible mice once per day for 3 days, and 2-AG and AEA levels in the NAc were quantified 24 h after the final injection (n = 5, 5, 5, and 5 mice in the control, susceptible plus vehicle, and susceptible plus CHPG, susceptible plus MPEP and CHPG groups; means ± SEMs; one-way ANOVA followed by Bonferroni's test; $^{*}P < 0.05$)

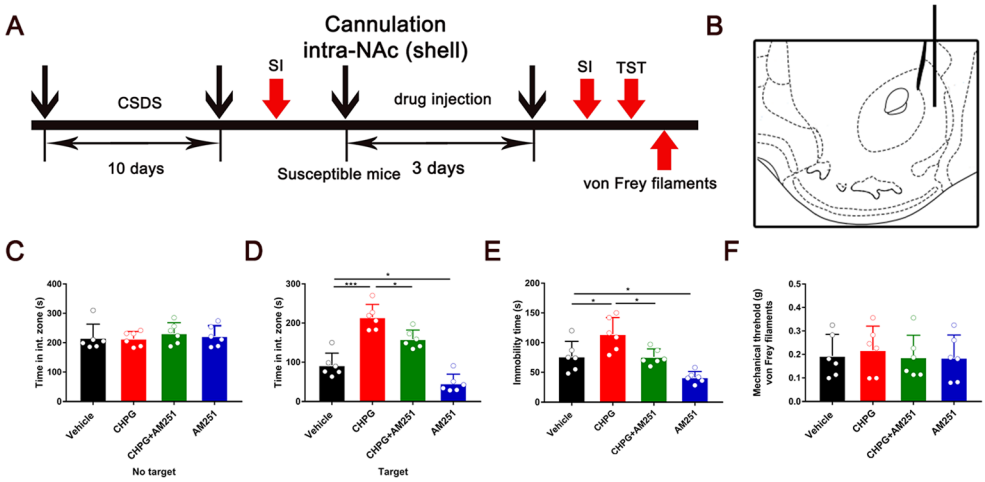

Fig. 8 The enhancement of eCB signaling in the NAc shell by targeted pharmacological activation of mGluR5 reverses anxiety-like behaviors but does not relieve pain in mice exposed to CSDS. **A** Timeline of the experimental procedure. **B** Schematic representation of the injection sites of vehicle and CHPG in the presence or absence of AM251 in the NAc shell of susceptible mice. **C**–**D** Social interaction test. The amount of time spent in the interaction zone by susceptible mice that received microinjections of different drugs into the NAc shell in the presence of no target (**C**) and a target (**D**) was recorded (n = 6 mice per group; means ± SEMs; one-way ANOVA followed by Bonferroni's test; $^*P < 0.05$). **E** The immobility time of susceptible mice treated with different drugs in the TST was recorded (n = 6 mice per group; means ± SEMs; one-way ANOVA followed by Bonferroni's test; $^*P < 0.05$). **F** The mechanical thresholds of susceptible mice treated with different drugs in the von Frey filament test were determined (n = 6 mice per group; means ± SEMs; one-way ANOVA followed by Bonferroni's test)

induced depressive-like and pain behaviors through an eCB-dependent mechanism.

Discussion

CSDS induced a decrease in mGluR5 protein levels in the NAc in susceptible mice, which exhibited depressive-like and pain behaviors that were reversed by overexpression of mGluR5 in the shell and core of the NAc, respectively. Mice with a conditional deletion of mGluR5 in the shell and core of the NAc that were exposed to 3 days of social defeat stress were susceptible to depressive-like and pain behaviors, respectively. Furthermore, CSDS induced a decrease in eCB levels in the NAc of susceptible mice. This decrease was reversed by an injection of CHPG, and the effect of CHPG was blocked by MTEP. In addition, an injection of CHPG into the shell and core of the NAc ameliorated stress-induced depressive-like behaviors and pain, respectively. The effect of CHPG on CSDS-induced depressive-like behaviors and pain

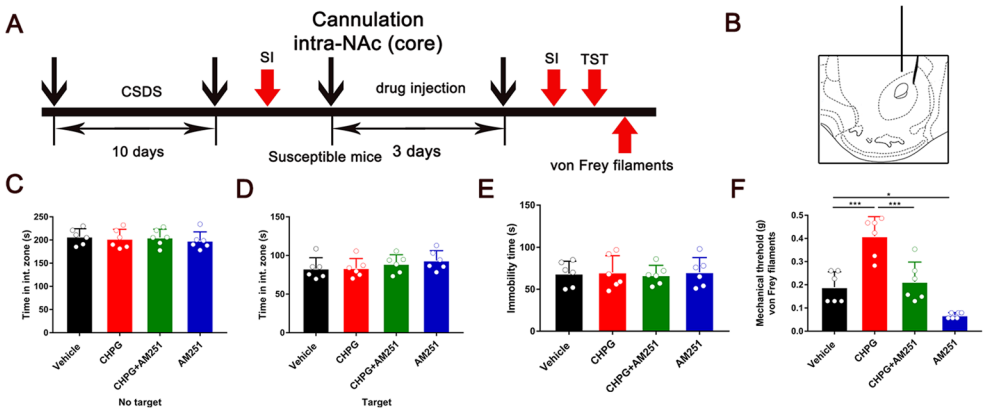

Fig. 9 The enhancement of eCB signaling in the NAc core by targeted pharmacological activation of mGluR5 in the NAc core relieves pain and does not modify anxiety-like behaviors in mice exposed to CSDS. **A** Timeline of the experimental procedure. **B** Schematic representation of the injection sites of vehicle and CHPG in the presence or absence of AM251 in the NAc core of susceptible mice. **C**–**D** Social interaction test. The amount of time spent in the interaction zone by susceptible mice that received microinjections of different drugs into the NAc shell in the presence of no target (**C**) and a target (**D**) was recorded (n = 6 mice per group; means ± SEM; one-way ANOVA followed by Bonferroni's test). **E** The immobility time of susceptible mice treated with different drugs in the TST was recorded (n = 6 mice per group; means ± SEMs; one-way ANOVA followed by Bonferroni's test). **F** The mechanical thresholds of susceptible mice treated with different drugs in the von Frey filament tests were determined (n = 6 mice per group; means ± SEMs; one-way ANOVA followed by Bonferroni's test; $^*P < 0.05$)

was blocked by AM251. These data support the hypothesis that mGluR5 mitigates stress-induced depressive-like behaviors and pain through eCB signaling in the NAc.

Chronic pain is well known to induce depressive-like behaviors. Depression also affects the prognosis and treatment of pain [39]. According to a previous study, a longer duration of depression can lead to greater severity of pain at baseline, and a long-term course of pain significantly increases the risk of developing depression after 2 years [40]. This finding implies the existence of shared underlying supraspinal brain mechanisms that modulate chronic pain and depression or anxiety. Several recent studies have elucidated that stressed mice (resilient and susceptible) exhibit a higher sensitivity to pain than unstressed mice in mechanical and chemical tests [9]. However, mice subjected to 30- and 60-min restraint stress show analgesia in both the hot plate and plantar tests of thermal pain [41]. This difference may be attributed to the intensity, duration, or quality of the stressor. In the present study, although CSDS was shown to lead to pain and depression, these two conditions may present independently, since resilient mice that did not manifest depressive-like behaviors displayed higher sensitivity to pain in the mechanical pain assay after exposure to CSDS.

One possible explanation for this behavioral phenotype is the alteration of mGluR5 expression in the brain. mGluR5 plays an important role in modulating neuronal excitability and is involved in the pathophysiology of various psychiatric and neurological disorders. Changes in mGluR5 expression and the functional impacts of these changes in different brain regions have been documented in studies of negative mood disorders and chronic pain [42–47]. However, the antidepressant phenotype in mice with mGluR5 loss-of-function is controversial among studies.

The expression of mGluR5 in the NAc was reported to be significantly downregulated in susceptible mice. The role of mGluR5 in the NAc is critical for the development of stress resilience via the modulation of cellular resilience mechanisms such as ΔFosB expression in response to stress [19]. Conversely, susceptible mice show a significant increase in mGluR5 protein levels in the hippocampus [7], confirming the regional specificity of CSDS-induced changes in mGluR5 expression. Consistent with this study [19], we observed an antidepressant phenotype in mice with mGluR5 gain-in-function in the NAc shell in our study, illustrating that mGluR5 expressed in different brain regions has a distinct role in modulating depressive-like behaviors. The PFC provides top-down control of affective and sensory processes. Animal and human imaging studies have shown that activation of the mPFC inhibits pain behaviors. A key projection target for the PFC is the NAc, which was also reported to be involved in pain regulation [48]. mGluR5 expressed in other brain regions has become the focus of studies exploring the mechanisms by which depression and pain are encoded. For instance, in neuropathic pain models, a PET analysis showed the downregulation of mGluR5 expression in the NAc and insular cortex [49]. The present study also focused on the NAc. In our study, the level of mGluR5 was decreased in the NAc of susceptible mice. Our behavioral data suggest that rescue or activation of mGluR5 expression in the shell of the NAc ameliorates stress-induced depressive-like behaviors and that rescue or activation of mGluR5 expression in the core of the NAc mitigates stress-induced pain. This study revealed the distinct roles of mGluR5 expressed in the core and shell of the NAc in stress-induced depressive-like behaviors and pain. Furthermore, we found that resilient mice exhibit pain, which may be attributed to the recovery of mGluR5 levels in the shell, but not in the core of the NAc.

However, little is known about the mechanism by which mGluR5 mediates antinociceptive and antidepressant effects after CSDS. eCB signaling is an important modulator of synaptic transmission and plasticity in the brain and interacts with mGluR5 through the phospholipase C-diacylglycerol lipase α (DAGLα) pathway, which leads to the formation of 2-AG and CB1-mediated homo- or heterosynaptic inhibition of transmitter release [50–52]. Chronic unpredictable stress (CUS) induces depression- and anxiety-like behaviors in rodents [53], and the CUS-induced increase in pain sensitivity is due to an impairment in eCB signaling in the brain [54]. Importantly, URB597, an inhibitor of the AEA-degrading enzyme fatty acid amide hydrolase (FAAH), and JZL184, an inhibitor of the 2-AG-degrading enzyme monoacylglycerol lipase (MAGL), increase eCB levels in the brain and periphery and are both effective at alleviating CUS-induced depressive-like behaviors and thermal hyperalgesia [55]. Based on these results, eCB signaling plays an important role in stress-induced depressive-like behaviors and thermal hyperalgesia. Serum 2-AG levels are decreased in rodents exposed to chronic stress and individuals exposed to traumatic stress [56]. An increase in 2-AG levels in the NAc reverses anxiety-like behaviors and synaptic plasticity following CSDS [34]. In the present study, CHPG reversed the decrease in the level of 2-AG in the NAc of susceptible mice, an effect that was blocked by MPEP. This result confirmed that 2-AG production was mediated by mGluR5. We also demonstrated that an injection of CHPG into the NAc shell of susceptible mice inhibited stress-induced depressive-like behaviors. However, the effect of CHPG on stress-induced depressive-like behaviors was blocked by a reverse agonist of eCBs, AM251. In addition, an injection of CHPG into the NAc core of susceptible mice decreased stress-induced pain sensitivity, and this effect was also blocked by AM251. These results suggest that mGluR5 in the NAc alleviates CSDS-induced depressive-like behaviors and pain through eCB signaling.

The current study has some limitations in terms of understanding social stress induced depressive-like behaviors and pain. First, animal models do not actually mimic patients with

chronic pain and depression in clinical settings. Second, the present study explored the relationship between depression and chronic pain using a social stress context. We only investigated whether depression would predispose an animal to pain chronification, but did not assess whether chronic pain would predispose mice to depressive-like behaviors. One important limitation of the study is that these studies are restricted to male animals due to the requirement for aggressive resident-intruder interactions. Chronic social stress is mechanistically different from chronic stresses without social components. Future studies should investigate changes in mGluR5 levels in the NAc in response to additional chronic stress procedures, such as chronic variable stress or newly established repeated social stress models that have enabled inclusion of female mice.

Conclusions

Overall, these data demonstrate that impairment in mGluR5 signaling in the NAc is a synaptic signature for behavioral adaptability following social stress. Restoration of mGluR5 signaling protects against CSDS-induced depressive-like behaviors and pain. However, knockdown of mGluR5 exacerbates CSDS-induced depressive-like behaviors and pain. Finally, activation of mGluR5 in the NAc alleviates CSDS-induced depressive-like behaviors and pain through eCB signaling. Thus, the relationship between mGluR5 and eCB signaling may prove to be relevant to our understanding of the relationship between stress, pain, and emotional behavior.

5**Abbreviations** *MDD,* Major depressive disorder; *CSDS,* Chronic social defeat stress; *NAc,* Nucleus accumbens; *eCB,* Endocannabinoid; *2-AG,* 2-Arachidonoylglycerol; *CHPG,* (RS)-2-chloro-5-hydroxyphenylglycine; *MTEP,* 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl) pyridine hydrochloride; *CB1R ,* Cannabinoid type 1 receptor; *mGluR5,* Metabotropic glutamate receptor 5; *mPFC,* Medial prefrontal cortex; *AEA ,* Anandamide; *LTD,* Long-term depression; *SPT,* Sucrose preference test; *TST,* Tail suspension test

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Author Contribution X.X., K.W., X.M., W.W., data curation, investigation, and methodology; H.W., M.H., methodology and software; H.S. and T.Y., formal analysis; S.C., visualization and funding acquisition; J.H., A.W. and T.X., writing and supervision.

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Data Availability All the data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author upon reasonable request.

Declarations

Ethics Approval All experiments and procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Eighth Edition) published by the National Research Council (USA) and were approved by the Institutional Animal Care and Use Committee of Sixth People's Hospital Affiliated with Shanghai Jiao Tong University.

Consent to Participate Not applicable

Consent for Publication Not applicable

Conflict of Interest The authors declare no competing interests.

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