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Regulation of stress-provoked aggressive behavior using endocannabinoids

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ABSTRACT

Reactive impulsive aggression is characterized by outbursts of rage and violence when subjects encounter threatening stressful events. Although impulsive aggression and violence create a high-cost burden on health and society, relatively little is known about treatment. Early adolescent social isolation (SI) alters brain development and functions. It induces hyper-excitatory in the ventral hippocampus (vHip) to promote acute stress-provoked outbursts of aggression, referred to as impulsive aggression, in mouse models. Cannabinoid type 1 receptors (CB1Rs) act on presynaptic sites and suppress neurotransmitter release into synapses. Given that CB1R activation inhibits neurotransmitter releases and modulates excitatory network activity, we tested the hypothesis that CB1R activation reduces impulsive aggression in SI mice through decreasing excitatory activity in the vHip. Here, we report that CB1R agonists, WIN-552122 (WIN) or arachidonylcyclopropylamide (ACPA), ameliorated acute stress-provoked attack behavior in the resident-intruder test without affecting general locomotion activity. Increasing endocannabinoids (eCBs) by inhibiting degradation enzymes in the vHip reduced impulsive aggression, and the effect was blunted by administration of AM251, a CB1R antagonist. Acute stress in SI mice induced c-Fos expression, a marker of neuronal activation, on vHip neurons projecting to the ventromedial hypothalamus (VMH), a well-known brain area that controls attack behavior. eCB augmentation inhibited c-Fos expression in VMH-projecting vHip neurons surrounded by CB1Rs. These results suggest that enhancing eCB signaling in order to activate CB1Rs suppresses impulsive aggression via suppressing vHip→VMH neural activity and point to a role of CB1R activation in ameliorating impulsive aggression in adults who have had adverse experiences during early adolescence.

1. Introduction

Impulsive aggression refers to non-goal-directed aggression that is generally triggered by threatening or frustrating events (Day and Walker, 2012). Impulsive aggression is a core symptom of intermittent explosive disorder (IED), which is characterized by outbursts of rage and violence when encountering stress, such as verbal or physical insults (Coccaro et al., 2007). Thus, acute stress-induced outbursts of aggression are defined as reactive impulsive aggression in animal models. Clinically, subjects with IED typically have a history of maltreatment in childhood (Fanning et al., 2019). Adverse juvenile experiences, including physical and emotional abuse, neglect, and household violence, are known to contribute to social, emotional, and cognitive impairment (Felitti et al., 2019; Makinodan et al., 2012). Although adverse juvenile experiences result in a cost burden on both society and public health, the best method to overcome adverse effects remains unknown. In animals, persistent social isolation after weaning is used as the model to study the adverse effects of juvenile stress on alterations in adulthood neuropsychological behavior. In male Wistar rats, socially deprived rats engaged in increased attacks on intruders, particularly to vulnerable body parts (Toth et al., 2008, 2012). In C57BL/6 mice, post-weaning socially isolated (SI) mice exhibited depression-like behavior and deficiencies in prepulse inhibition (Chang et al., 2015). SI mice and group-housing (GH) mice seldom attack intruders in low stress situations. However, acute stress has been shown to dramatically increase the attack behavior of SI mice, but not GH mice (Chang et al., 2015, 2018, 2020). The phenomenon of outbursts of acute stress-induced attacks is similar to impulsive aggression. This indicates

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that post-weaning social isolation induces maladaptive adulthood stress responses and exaggerated reactive impulsive aggression.

The hippocampus, especially in the ventral part, predominantly regulates emotion and stress responses (Levone et al., 2015; Sahay and Hen, 2007). Recent studies have reported that hippocampal excitatory outputs promote aggression in an internal state-dependent manner (Chang and Gean, 2019; Leroy et al., 2018). Furthermore, post-weaning social isolation increases excitatory activity in the ventral hippocampus (vHip) via overexpression of NMDA receptors (Chang et al., 2015). The hyper-excitatory neural circuit from the vHip to the ventromedial hypothalamus (VMH) mediates acute stress-induced aggression, referred to as impulsive aggression, in SI mice (Chang and Gean, 2019).

Endocannabinoid (eCB) is a form of retrograde feedback signaling that inhibits neurotransmitter release (Kano et al., 2009; Morena et al., 2016) and plays a critical role in mitigating anxiety, stress, and fear (Lutz et al., 2015). The main eCB system in the brain consists of cannabinoid receptor 1 (CB1R) and two endogenous ligands, N-arachidonyl ethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG). AEA is hydrolyzed by fatty acid amide hydrolase (FAAH) whereas 2-AG is degraded by monoacylglycerol lipase (MAGL) (Lutz et al., 2015; Morena et al., 2016). Previous studies have shown that 2-AG augmentation, through inhibiting its degradation by JZL184, an inhibitor of MAGL, decreases spontaneous glutamate release (Centanni et al., 2019; Folkes et al., 2020) and suppresses ventral hippocampal-amygdala glutamatergic synapses (Bluett et al., 2017). These JZL184 effects on glutamatergic synapses act in a CB1R-dependent mechanism. Additionally, CB1R activation corrects aberrant glutamatergic hyperactivity in the amygdala (Araque et al., 2017) and the hippocampus (Guggenhuber et al., 2010). These studies indicate that CB1R activation reduces excessive excitatory network activity.

Increasing evidence shows eCBs could play a role in aggression in mice. CB1R-knockout mice were found to exhibit more offensive aggression and to be susceptible to stressful conditions (Haller et al., 2004; Martin et al., 2002; Rodriguez-Arias et al., 2013). In contrast, 2-AG augmentation by JZL184 abolished aggression in male CD1 mice, a strain of mouse with aggressive characteristics (Aliczki et al., 2015). Cannabidiol, a non-psychotomimetic cannabinoid from *Cannabis sativa* plant, decreased adulthood social isolation-induced aggressive behavior via indirect CB1R activation (Hartmann et al., 2019). A low dose of Δ9-tetrahydrocannabinol (THC), the exogenous psychoactive ingredient in marijuana, decreased aggression in mice, rats, and monkeys. However, some studies have reported that THC does not affect aggression and even appears to enhance aggression in rats, which may be due to chronic daily exposure to THC (Kolla and Mishra, 2018). There was a gender-dependent effect found for adolescent THC treatment in rats previously experiencing maternal separation. In female maternally-deprived rats, THC mitigated aggression. By contrast, in male maternally-deprived rats, THC did not affect aggressiveness (Zamberletti et al., 2012). Although the deletion of CB1R induces aggression, and exogenous cannabinoids and eCBs suppress aggression, it is not known whether direct CB1R activation reduces aggression in a post-weaning social isolation model. Given that post-weaning social isolation induces maladaptive impulsive aggression and an increase in hippocampal excitatory transmission, and CB1R activation reduces excessive excitatory network activity, we studied the possibility that CB1R agonists may reduce reactive impulsive aggression in SI mice through decreased excitatory activity in the vHip.

Here, we found that CB1R agonists, WIN-552122 (WIN) and arachidonylcyclopropylamide (ACPA), decreased impulsive aggression in SI mice in the resident-intruder test. Knockdown of CB1R expression in the vHip increased impulsive aggression in the SI mice. Furthermore, increasing endogenous AEA and 2-AG, by administration of FAAH and MAGL inhibitors in the vHip, reduced impulsive aggression in a CB1R-dependent manner. Finally, we found that eCB augmentation suppressed acute stress-induced c-Fos expression, a marker of neuronal activation, in the vHip neurons projecting to the ventromedial hypothalamus (VMH), a well-known brain area in which aggression is triggered. These results suggest that enhancing eCB signaling to activate CB1Rs suppresses stress-exaggerated biting behavior and point to an essential role of CB1R activation to overcome adulthood impulsive aggression following post-weaning social isolation.

2. Materials and methods

2.1. Animals

Three-week-old male C57BL/6JNarl mice were purchased from the National Laboratory Animal Center (Taiwan) and were randomly divided into group-housing (GH, 5 mice per cage) and individual housing (one mouse per cage). Due to a critical period for social brain development (Makindodan et al., 2012), social isolation following weaning began at 3 weeks of age (postnatal days 21–28). The home cage of the socially isolated (SI) mice was changed once every two weeks. Behavioral tests were performed after five weeks of social isolation. For the resident-intruder test, the intruder mice, 3-week-old male BALB/c mice, were purchased from the National Laboratory Animal Center (Taiwan) and were group-housed in the same animal room with the C57BL/6JNarl mice. All mice were given free access to water and food in a 12 h/12 h light/dark cycle with a 22 ± 1 °C room temperature and 55–65% humidity. The experimental procedures followed the National Institutes of Health guidelines and were approved by the National Cheng Kung University Medical Center Animal Care and Use Committee.

2.2. Drugs

CB1 receptor agonist WIN 55212-2 (WIN, Tocris, 0.1 and 0.5 mg/kg body weight for the intraperitoneal injection, 1 and 2 μg/μl for micro-infusion into the vHip) (Martellotta et al., 1998; Shen et al., 2019) was dissolved in 50% DMSO (Sigma-Aldrich) and 50% normal saline solution. Arachidonylcyclopropylamide (ACPA, Abcam, 5 or 10 mg/kg body weight for the intraperitoneal injection, 4 ng/μl for micro-infusion into the vHip) (Jafari-Sabet and Karimi, 2017; Shafaroodi et al., 2004; Srisai et al., 2017) was dissolved in 100% Tocrisolve 100 (Tocris). The FAAH inhibitor URB597 (5 ng/μl, Sigma-Aldrich) (Haller et al., 2009) was dissolved in 5% DMSO and 95% normal saline solution. MAGL inhibitor JZL184 (2 μg/μl, Tocris) (Folkes et al., 2020) was dissolved in 1:1 DMSO and Tween 80 (Sigma-Aldrich) and diluted by the normal saline solution to 2% DMSO and 2% Tween 80. The CB1 receptor antagonist AM251 (2.5 ng/μl, Cayman) (Shen et al., 2019) was dissolved in 25% DMSO and 75% normal saline solution. Drug doses were based on the literature and on pilot experiments. Solutions were freshly prepared and were administrated at a volume of 0.1 ml for the intraperitoneal injection and 0.8 μl per side for the infusion into the vHip and dorsal striatum.

2.3. Lentivirus for CB1R knockdown

Lentiviral particles with CB1 shRNA (sc-39911-V) or copGFP control (sc-108084) were purchased from Santa Cruz. 1 μl volume of 1×10^6 infectious units of lentivirus was delivered into the vHip according to the manufacturer's instructions. We used western blotting to evaluate CB1R protein expression two weeks later.

2.4. Surgery

The mice were anesthetized with Zoletil (50 mg/kg; VIRBAC, France) and xylazine hydrochloride (Rompun, 5 mg/kg; Bayer, Korea) and were placed on a stereotaxic apparatus (Kopf). The mice were implanted with cannulas (26 gauge stainless steel) into the vHip (anterioposterior, −3.2 mm; mediolateral, ±3.2 mm; dorsoventral, −4.0 mm) or dorsal striatum (anterioposterior, 0 mm; mediolateral, ±1.8 mm; dorsoventral, −2.0 mm). After 10 days of resting, these mice underwent behavioral

experimental procedures. To track the vHip projection to the VMH, red retrobead tracers (0.5 μl/side with 1:4 dilutions, Lumafluor Inc.) were infused into the VMH (anterioposterior, −1.5 mm; mediolateral, ±0.4 mm; dorsoventral, −5.5 mm). For micro-infusion, lentivirus, drugs, or a vehicle were infused bilaterally into the vHip or dorsal striatum at a rate of 0.1 μl/min through the implanted cannulas. After the behavioral tests, mice were sacrificed to identify the location of the cannula implantation. The mice were excluded from all data analysis if the cannula location was not in the targeted regions.

2.5. Resident-intruder test

To assess attack behavior, the resident-intruder (RI) test was given according to previous studies (Chang et al., 2020). To maintain the territory of the resident mice, the home cage bedding of the resident mice was not changed on the first day before the RI test or during the RI test. To trigger stress-induced outbursts of aggression (referred to as impulsive aggression), the resident mice were placed on a foot-shock grid (San Diego Instrument, USA) and received five 0.1 mA, 1 s, foot-shocks at random intervals, 30 min before the RI test. In the RI test, the intruder (a male BABL/c mouse) was placed in the home cage of the resident mice for 15 min. Behavioral videos were recorded with a SONY digital camera and were analyzed by a blinded observer. The observed behaviors referred to the descriptions provided in previous studies (Fish et al., 1999; Koolhaas et al., 2013). Biting behavior included incidences of the resident mice biting the vulnerable parts (belly and paws) and the non-vulnerable parts (back) of the intruder mice. Offensive behaviors comprised mounting, pursuit, boxing, and tail rattling. Non-aggressive behaviors included social sniffing, social grooming, self-grooming, and inactivity.

2.6. Open field test

To assess general locomotion activity, mice were placed into the corner of a 40 cm × 40 cm × 40 cm square box with black walls and a white floor. The Noldus video tracking system with EthoVision X8.1 software recorded and analyzed the total distance that the mice traveled in the box for 10 min. Anxiety-like behavior was determined by the duration that the mice stayed in the central zone of the test box, the distance that the mice traveled in the central zone (as central distance), and the frequency that the mice entered into the central zone. The central zone was defined as the central part of a 4 by 4 array of box. 75% ethanol was used to clean the box to prevent olfactory cues after every trial.

2.7. Immunofluorescence

The mice were anesthetized with Zoletil (50 mg/kg) and xylazine hydrochloride (Rompun, 5 mg/kg) and were perfused with 4% paraformaldehyde. After a 30% sucrose incubation, coronal brain sections (40 μm thickness) were obtained with a cryostat (CM3050S, Leica). Floating sections were subjected to antigen retrieval in a saline sodium citrate (SSC) solution at 85 °C for 15 min and then were cleaned with 1% Triton X-100 diluted in PBS for 1h at room temperature. The sections were blocked in 0.1% Fab fragment goat anti-mouse IgG dissolved in CAS-block reagent (Invitrogen) for 2 h. Primary antibodies, mouse anti-cFos (1:200, Genetex), and rabbit anti-CB1R (1:200, Abcam), were dissolved in CAS-block reagent and incubated with the brain sections overnight at 4 °C. Next, the sections were incubated in the CAS-block reagent with DyLight-405 Fab fragment goat anti-mouse (1:500, Jackson ImmunoResearch #115-477-185) and Alexa-488 goat anti-rabbit (1:500, Jackson ImmunoResearch #111-545-003) for 90 min. For the lentiviral injections, the brain sections were incubated in 0.1% DAPI (Sigma-Aldrich) dissolved in PBS for 15 min. Images were acquired with a Leica DM2500 Microscope with Zyla sCMOS (ANDOR, OXFORD instruments) and MetaMorph software (Molecular Devices). For the confocal images, the stellaris confocal system was used (Leica).

2.8. Western blotting

The vHip tissues were dissected out of 400 μm thick coronal slices prepared using a DTK-1000 microslicer (D.S.K.) and then homogenized by sonication (UP50H ultrasonic processor, Hielscher) in a 0.32 M sucrose buffer [10 mM Tris-HCl, pH 7.4, 0.32M sucrose, protease and phosphatase inhibitor cocktail (Roche)] and were centrifuged at 9200×*g* for 20 min. 30 μg total proteins were denatured in the loading buffer (40 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 100 mM Dithiothreitol, 197 mM Bromphenol Blue) and were run in 10% SDS-PAGE. Rabbit anti-CB1R (1:1000, Genetex) and mouse anti-tubulin (1:2000, Millipore) were used for the primary antibodies. Image J software was used to analyze the images and the respective internal control-normalized target proteins for each sample run in at least triplicate.

2.9. Statistics

Using GraphPad Prism 6 software (GraphPad Software, San Diego), the data were analyzed with a Student's *t*-test or an ANOVA. For between-group comparisons, a Student's *t*-test was used. For comparison of multiple groups in one factor, the ordinary one-way ANOVA was used. For comparison of the multiple groups in two factors shown in Fig. 1B, Fig. 4D, and Fig. 4E, a mixed two-way ANOVA was used. Bonferroni's multiple comparisons test was used for *post hoc* comparisons of the ANOVA. All data were expressed as mean ± SEM. The level of significance was set at $p < 0.05$.

3. Results

3.1. SI mice exhibit maladaptive impulsive aggression

Previous studies were repeated to confirm the effects of acute stress on aggression in post-weaning socially isolated mice. Mice at postnatal day (PND) 21 were randomly assigned to group-housing (GH) or socially isolated (SI) groups. After five weeks, the resident-intruder (RI) test without acute stress was tested. On day 2, the mice received five foot-shocks (as acute stress) 30 min before the second RI test (n = 8 per group) (Fig. 1A). A mixed two-way ANOVA showed a significant interaction effect of social isolation × acute stress ($F_{(1,14)} = 9.825$, $p = 0.0073$) (Fig. 1B). The *post hoc* test revealed that the SI mice exhibited an increased number of attack bites toward the intruder after acute stress ($p < 0.01$) In contrast, acute stress did not affect the amount of biting in the GH mice ($p > 0.05$). The percentage of SI mice that engaged in more than the maximum level of biting of the non-stress-treated GH mice rose from 12.5% up to 62.5% after acute stress (Fig. 1C). This finding suggested that the SI mice exhibited maladaptive impulsive aggression. These results replicated our previous reports (Chang et al., 2015, 2018), showing that the stress-induced exacerbation of attack biting behavior was restricted to the SI mice. In the open field test, the SI mice traveled less time in the central zone of the open field box ($t = 3.26$, $p < 0.01$) (Fig. 1 D). There were no different levels of total traveling distance found in the SI and GH mice ($t = 1.79$, $p > 0.05$) (Fig. 1 E). These results suggested that the SI mice exhibited anxiety-like behavior.

3.2. Administration of CB1R agonists reduces impulsive aggression in SI mice

Next, to examine whether CB1R activation affected the level of impulsive aggression, the SI mice received intraperitoneal injections of WIN 55212-2 (WIN), a CB1R agonist, at 0, 0.1, or 0.5 mg/kg 30 min before acute stress (n = 5 per group, Fig. 1F). A one-way ANOVA showed that the WIN treatment decreased acute stress-induced biting behavior in the SI mice compared to the vehicle ($F_{(2,12)} = 36.49$, $p < 0.01$) (Fig. 1G). Furthermore, the SI mice with 0.5 mg/kg WIN exhibited less

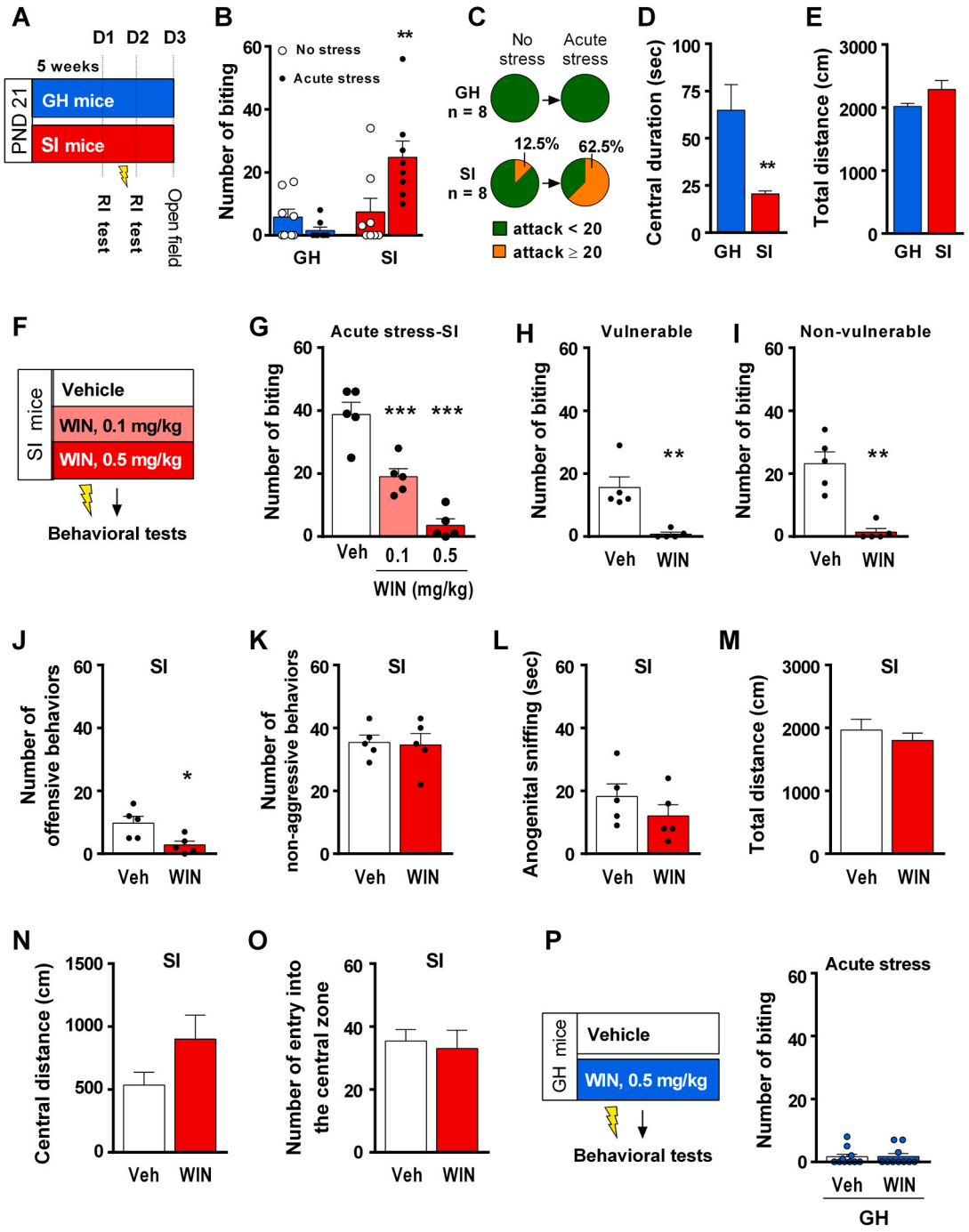

Fig. 1. Administration of WIN 55212-2 inhibits impulsive aggression in post-weaning socially isolated mice, A. The experimental procedure. Mice at postnatal day 21 were randomly divided into group-housing (GH) or socially isolated (SI) mice (n = 8 per group). 5 weeks later, these mice underwent behavioral tests., B. In the no stress condition, there were no differences in the biting behavior between the SI and GH mice. Acute stress increased biting behavior in the SI mice but not in the GH mice. $p < 0.01$, acute stress vs. no stress in SI mice., C. The percentage of SI mice that engaged in more than the maximum level of biting of the non-stress-treated GH mice rose from 12.5% up to 62.5% after acute stress, D. The SI mice stayed less time in the central zone of the open field box compared to the GH mice ($p < 0.01$), suggesting that the SI mice were exhibiting anxiety-like behavior., E. There were no differences in the total distance traveled in the open field box between the SI and GH mice, suggesting no difference in general locomotor activity., F. The experimental procedure for the intraperitoneal injection of WIN 55212-2 (WIN), a CB1R agonist. Another three groups of SI mice received the vehicle, 0.1 and 0.5 mg/kg WIN, respectively (n = 5 per group). 30 min later, these mice received foot-shocks to induced impulsive aggression in the RI test., G. The administration of 0.1 and 0.5 mg/kg WIN decreased biting behavior compared to the vehicle dose. $p < 0.001$., H. 0.5 mg/kg WIN administration in SI mice decreased the level of biting toward vulnerable parts of intruders. $p < 0.01$. n = 5 per group., I. WIN treatment decreased the level of biting toward non-vulnerable parts of intruders. $p < 0.01$., J. WIN treatment decreased offensive behavior in the SI mice. $p < 0.05$., K. WIN treatment did not affect non-aggressive behavior in the SI mice. $p > 0.05$., L. There was no difference in the duration of ano-genital sniffing between the vehicle and 0.5 mg/kg WIN treatments. $p > 0.05$., M. 0.5 mg/kg WIN administration had no effect on the total distance the SI mice traveled in the open field box compared to the vehicle dose. $p > 0.05$. n = 5 per group., N and O. 0.5 mg/kg WIN treatment did not affect anxiety-like behavior. There were no differences in the distance traveled in the central zone (**N**, $p > 0.05$) or the frequency of entry into the central zone (**O**, $p > 0.05$)., P. Two groups of GH mice received the vehicle and 0.5 mg/kg WIN, respectively, and then received acute stress 30 min later (n = 10 per group). The WIN treatment had no effect on biting behavior compared to the vehicle. $p > 0.05$.Data represent as mean ± SEM.

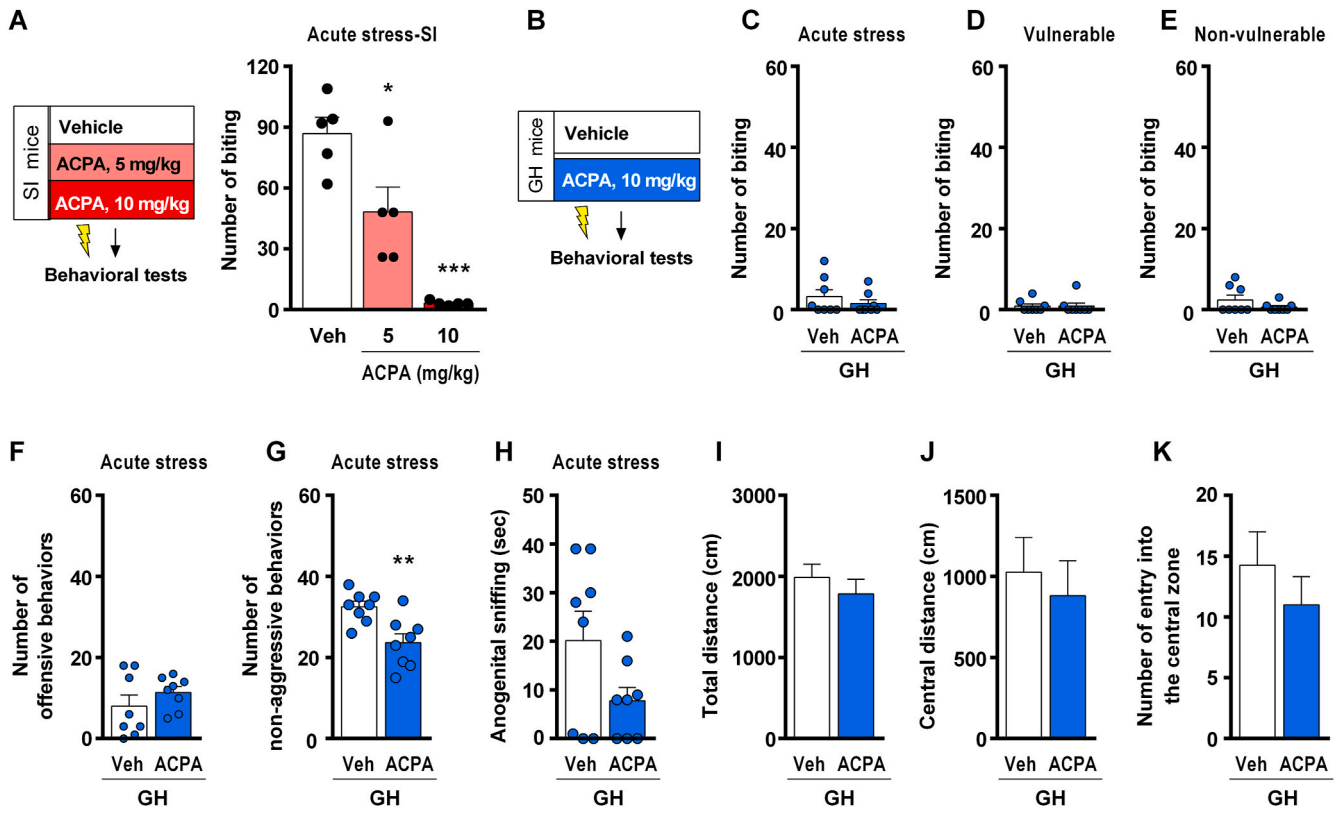

Fig. 2. ACPA administration inhibits impulsive aggression in SI mice but not in GH mice, A. Another three groups of SI mice received the vehicle, 5 and 10 mg/kg ACPA, another CB1R agonist, respectively (n = 5 per group). 30 min later, these mice received foot-shocks to induced impulsive aggression in the RI test. ACPA administration in the SI mice decreased impulsive aggression compared to the vehicle dose ($p < 0.05$ in 5 mg/kg ACPA; $p < 0.001$ in 10 mg/kg ACPA)., B. Another two groups of GH mice received the vehicle and 10 mg/kg ACPA, respectively (n = 8 per group)., C. The biting levels were 3.25 ± 1.63 and 1.50 ± 0.93 in the vehicle and ACPA, respectively. An unpaired Student *t*-test showed no differences between the vehicle and ACPA ($p > 0.05$). Data represented as mean ± SEM., D and E. Detailed analysis of biting behavior. The vehicle and 10 mg/kg ACPA in the GH mice did not induced biting toward vulnerable parts of intruders (**D**, mean ± SEM as 0.875 ± 0.52 and 0.875 ± 0.74, respectively, $p > 0.05$) and non-vulnerable parts of intruders (**E**, mean ± SEM as 2.37 ± 1.19 and 0.63 ± 0.38, respectively, $p > 0.05$)., F. 10 mg/kg ACPA in GH mice did not induced offensive behavior compared to the vehicle. $p > 0.05$., G. The level of non-aggressive behavior in ACPA was lower than in the vehicle. $p < 0.01$., H. There were no differences in the duration of ano-genital sniffing between the vehicle and ACPA. $p > 0.05$., I. ACPA in the GH mice did not affect the total distance traveled compared to the vehicle. $p > 0.05$., J and K. ACPA in the GH mice did not affect anxiety-like behavior in the open field test. The indicators of anxiety-like behavior in the distance traveled in the central zone (**J**, $p > 0.05$) and in the frequency of entry into the central zone (**K**, $p > 0.05$). Data represented as mean ± SEM.

biting toward both the vulnerable and non-vulnerable parts of intruders ($t = 4.31$ and 5.54, $p < 0.01$, Fig. 1H and I, respectively). A 0.5 mg/kg WIN injection also reduced offensive behavior ($t = 2.84$, $p < 0.05$) (Fig. 1J) but did not affect non-aggressive behavior ($t = 0.19$, $p > 0.05$) (Fig. 1K) and ano-genital sniffing ($t = 1.15$, $p > 0.05$) (Fig 1L) compared to the mice receiving the vehicle injection. These results indicated that 0.5 mg/kg WIN injection reduced aggressive behavior but did not affect social interaction in the SI mice. In addition, there were no differences in total traveling distance ($t = 1.72$, $p > 0.05$) (Fig. 1M), the distance traveling in the central zone ($t = 1.69$, $p > 0.05$) (Fig. 1N), and the frequency of entry into the central zone ($t = 0.35$, $p > 0.05$) (Fig. 1O) in the open field test between the vehicle- and WIN-injected SI mice, indicating that 0.5 mg/kg WIN did not affect general locomotion activity and anxiety-like behavior in the SI mice. Furthermore, 0.5 mg/kg WIN did not affect biting behavior in the GH mice ($t = 0.078$, $p > 0.05$; n = 10 per group) (Fig. 1P), indicating that CB1R activation did not induce impulsive aggression in the GH mice.

Next, another CB1R agonist, arachidonylcyclopropylamide (ACPA), was used to examine its anti-aggressive effects in SI mice (Fig. 2A). ACPA also dose-dependently decreased biting behavior in the SI mice compared to those with the vehicle injection ($F_{(2,12)} = 24.54$, $p < 0.001$; n = 5 per group) (Fig. 2A), indicating that CB1R activation reduces impulsive aggression in SI mice. Due to the higher biting levels in the vehicle control for ACPA than was the case in the other vehicle controls, ACPA was intraperitoneally injected in GH mice to examine whether the vehicle (100% Tocrisolve 100) by itself affected impulsive aggression (n = 8 per group, Fig. 2B). The vehicle and ACPA did not induce impulsive aggression in the GH mice (mean ± SEM = 3.25 ± 1.63 and 1.50 ± 0.93 in biting levels, $t = 0.93$, $p > 0.05$) (Fig. 2C). Furthermore, 10 mg/kg ACPA did not induce biting toward vulnerable ($t = 0$, $p > 0.05$) (Fig. 2D) and non-vulnerable parts of intruders ($t = 1.40$, $p > 0.05$) (Fig. 2E) or offensive behavior ($t = 1.10$, $p > 0.05$) (Fig. 2F). 10 mg/kg ACPA decreased non-aggressive behavior ($t = 3.46$, $p < 0.01$) (Fig. 2G) but did not affect ano-genital sniffing ($t = 1.86$, $p > 0.05$) (Fig. 2H). These results indicated that ACPA did not affect impulsive aggression, offensive behavior, or social interaction in the GH mice. Additionally, there were no differences in the total traveling distance ($t = 0.85$, $p > 0.05$) (Fig. 2I), the distance traveled in the central zone ($t = 0.48$, $p > 0.05$) (Fig. 2J), and the frequency of entry into the central zone ($t = 0.91$, $p > 0.05$) (Fig. 2K) between the ACPA and vehicle groups, indicating that 10 mg/kg ACPA did not affect general locomotion and anxiety-like behavior in the GH mice. Taken together, CB1R activation by intraperitoneal injection of WIN and ACPA reduced impulsive aggression in the SI mice.

3.3. *The infusion of CB1R agonists into the hippocampus reduces impulsive aggression*

Next, we tested whether ventral hippocampal (vHip) CB1R

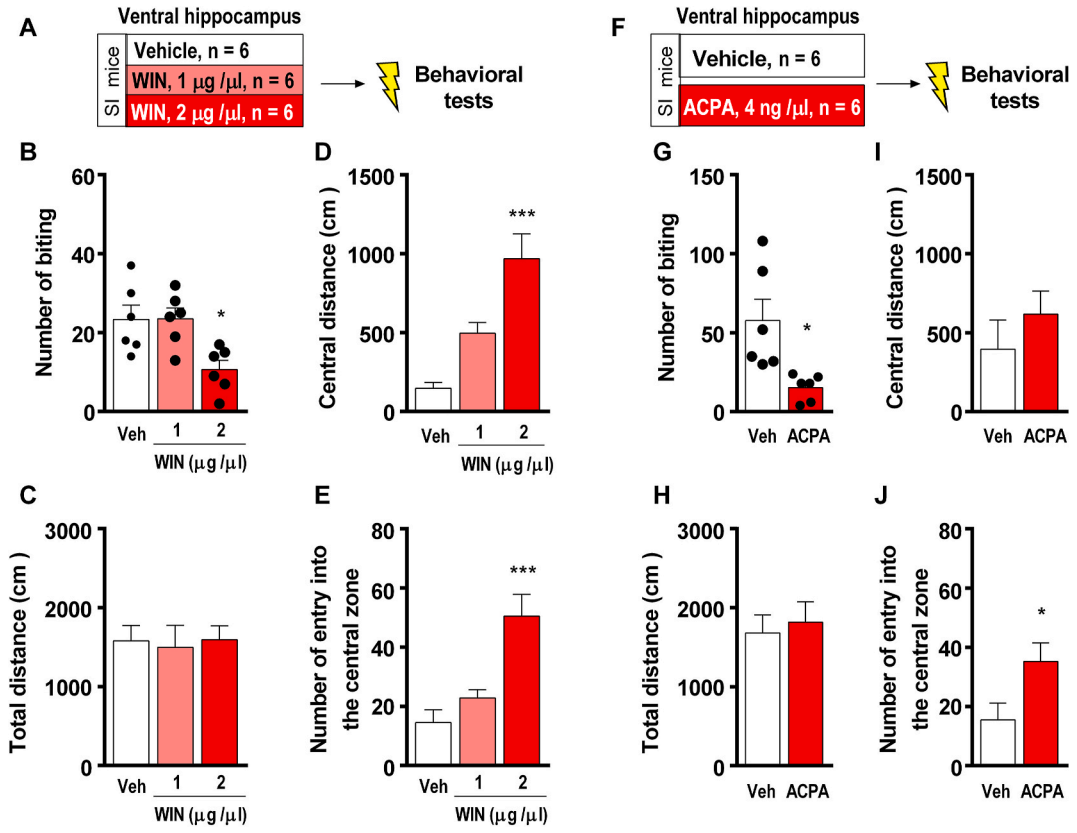

Fig. 3. Intra-hippocampal infusion of CB1R agonists in SI mice inhibits impulsive aggression., A. The experimental procedure for WIN infusion into the vHip. The vehicle, 1, and 2 μg/μl WIN were infused into the vHip of three groups of SI mice, respectively. 30 min later, these mice received foot-shocks to induce impulsive aggression. n = 6 per group., B. 2 μg/μl WIN decreased biting levels compared to the vehicle. *Post hoc* test, $p < 0.05$ in 2 μg/μl WIN vs. the vehicle. There were no differences between 1 μg/μl WIN and the vehicle. *Post hoc* test, $p > 0.05$ in 1 μg/μl WIN vs. the vehicle., C. There were no differences in the total distance traveled among the dose-groups in the open field test. The one-way ANOVA, $p > 0.05$., D and E. The 2 μg/μl WIN infusion into the vHip decreased anxiety-like behavior in the open field test. 2 μg/μl WIN increased the distance traveled in the central zone (**D**) and the frequency of entry into the central zone (**E**). *Post hoc* test, $p < 0.001$ in 2 μg/μl WIN vs. the vehicle. The 1 μg/μl WIN had no effects on anxiety-like behavior compared to the vehicle. *Post hoc* test, $p > 0.05$., F. Another two groups of SI mice received the vehicle and 4 ng/μl ACPA infusion into the vHip, respectively. n = 6 per group., G. ACPA infusion in SI mice decreased impulsive aggression compared to the vehicle. $p < 0.05$., H. The ACPA infusion into the vHip had no effects on general locomotor activity in the open field test. $p > 0.05$., I. ACPA infusion in SI mice had no effects on the distance traveled in the central zone. $p > 0.05$., J. The ACPA infusion into the vHip increased the frequency of entry into the central zone. $p < 0.05$.Data represented as mean ± SEM.

activation affected impulsive aggression in SI mice (Fig. 3A). The intra-vHip infusion of 2 μg/μl WIN significantly reduced biting behavior compared to vehicle and 1 μg/μl WIN groups in the SI mice (one-way ANOVA, $F_{(2,15)} = 6.3$, $p < 0.05$; *post hoc* test, $p < 0.05$ in 2 μg/μl WIN vs vehicle; n = 6 per group) (Fig. 3B). The intra-vHip WIN infusion had no effect on the total distance traveled among groups in the open field test (one-way ANOVA, $F_{(2,15)} = 0.05$, $p > 0.05$) (Fig. 3C). Interesting, the intra-vHip WIN infusion increased the distance traveled in the central zone (one-way ANOVA, $F_{(2,15)} = 16.4$, $p < 0.01$; *post hoc* test, $p < 0.001$ in 2 μg/μl WIN vs vehicle) (Fig. 3D) and the frequency of entry into the central zone (one-way ANOVA, $F_{(2,15)} = 13.3$, $p < 0.01$; *post hoc* test, $p < 0.001$ in 2 μg/μl WIN vs vehicle) (Fig. 3E) in a dose-dependent manner. The results indicated that WIN-mediated CB1R activation in the vHip reduced impulsive aggression and anxiety-like behavior in the SI mice.

Similarly, the ACPA infusion (4 ng/μl) into the vHip of SI mice significantly reduced biting behavior compared to the vehicle ($t = 3.04$, $p < 0.05$; n = 6 per group) (Fig. 3F and G) but had no effect on the total general locomotion activity ($t = 0.04$, $p > 0.05$) (Fig. 3H). The intra-vHip ACPA infusion did not affect the distance traveled in the central zone ($t = 0.94$, $p > 0.05$) (Fig. 3I) but significantly increased the frequency of entry into the central zone ($t = 2.32$, $p < 0.05$) (Fig. 3J). Taken together, pharmacological CB1R activation in the vHip of SI mice reduced impulsive aggression and anxiety-like behavior.

To rule out general effects on the brain, 2 μg/μl WIN (n = 5 per group, Supplemental Fig. 1A) or 4 ng/μl ACPA (n = 6 per group, Supplemental Fig. 1F) was infused into the dorsal striatum of SI mice with acute stress. The dorsal striatum belongs to a part of the basal ganglia and regulates reinforcement-based habit formation and motor control. The dorsal striatum has been not reported as one of the brain areas in the neural circuit of aggression (Nelson and Trainor, 2007). Thus, the dorsal striatum was used for the controls. The data showed that the WIN infusion did not affect biting behavior ($t = 1.75$, $p > 0.05$) (Supplemental Fig. 1B) or general locomotion ($t = 0.568$, $p > 0.05$) (Supplemental Fig. 1C). In the analysis of anxiety-like behavior, the WIN infusion into the dorsal striatum was not found to affect the distance traveled in the central zone ($t = 1.10$, $p > 0.05$) (Supplemental Fig. 1D) or the frequency of entry into the central zone ($t = 0.33$, $p > 0.05$) (Supplemental Fig. 1F). In addition, the administration of ACPA into the dorsal striatum had no effects on the number of attacks ($t = 0.173$, $p > 0.05$) (Supplemental Fig. 1G), the total distance traveled ($t = 0.036$, $p > 0.05$) (Supplemental Fig. 1H), the distance traveled in the central zone ($t = 0.27$, $p > 0.05$) (Supplemental Fig. 1I), or the frequency of entry into the central zone ($t = 0.55$, $p > 0.05$) (Supplemental Fig. 1J). These results indicated CB1R activation in the dorsal striatum by 2 μg/μl WIN and 4 ng/μl ACPA did not affect impulsive aggression and anxiety-like behavior in the SI mice.

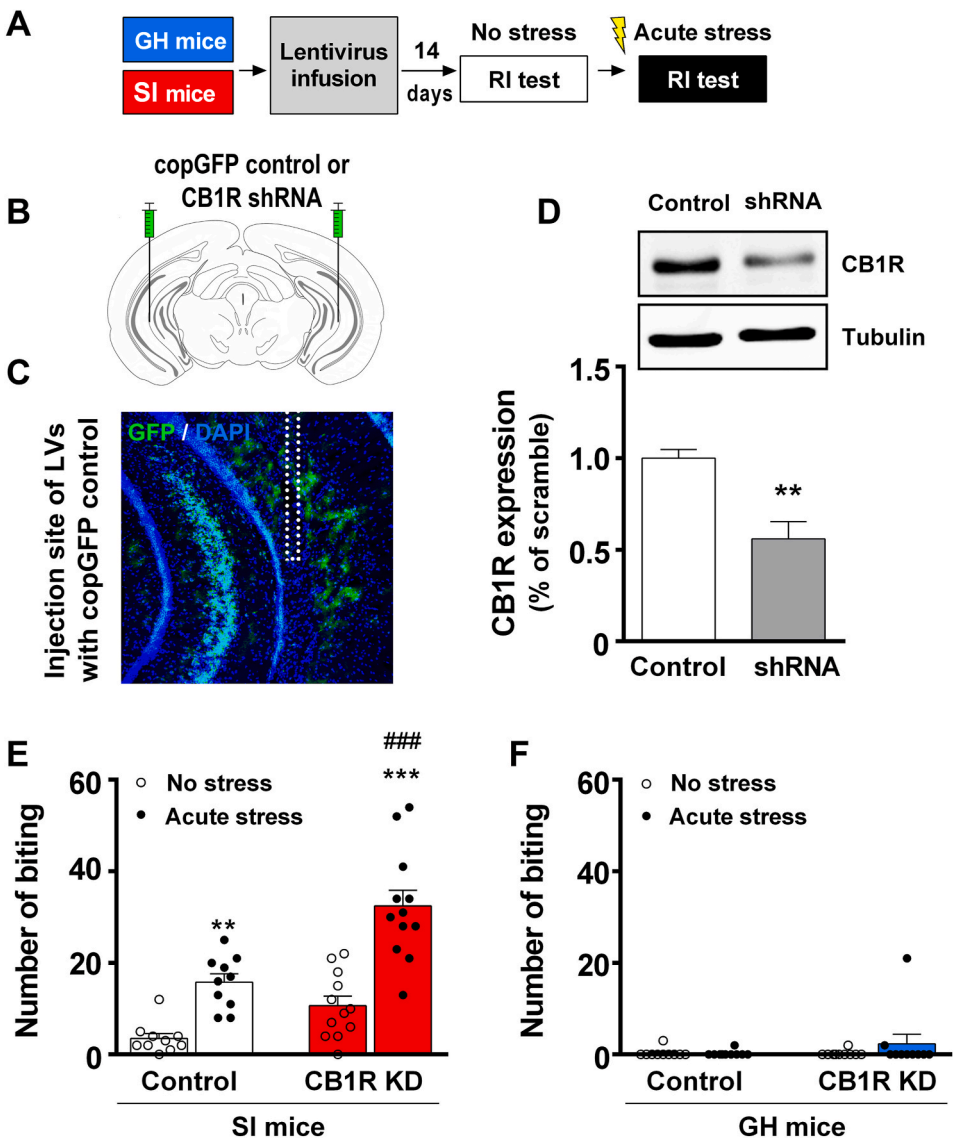

Fig. 4. Decreasing CB1R expression in the vHip exaggerates impulsive aggression in SI mice but not in GH mice., A and B. The experimental schema for CB1R knockdown (KD) (**A**). Lentiviruses carrying CB1R shRNA or the copGFP control were infused into the vHip (**B**). After 2 weeks of recovery, these mice received the RI test under no stress and then in a state of acute stress one day later (**A**)., C. Representative image showing the injection location of the lentivirus carrying the copGFP control. Green, GFP; blue, DAPI. D. Western blotting confirmed that the transduction of CB1R shRNA significantly decreased CB1R protein expression in the vHip. n = 7., E. CB1R knockdown in the SI mice further exaggerated impulsive aggression. There was a significant interaction effect of CB1R-KD and stress ($p < 0.05$). Acute stress increased biting behavior both in the control- and CB1R-KD-treated mice (no stress vs. acute stress, $^{**}p < 0.01$ and $^{***}p < 0.001$ in the control and CB1R-KD mice, respectively). Importantly, CB1R-KD mice under acute stress exhibited a higher level of biting behavior compared to control mice under acute stress. *Post hoc* test, $^{###}p < 0.001$. n = 10 and 12 in copGFP control and CB1R shRNA, respectively., F. CB1R knockdown in the GH mice had no effects on impulsive aggression. The interaction effects of CB1R-KD and stress were not significant in the biting behavior of the GH mice. $p > 0.05$. n = 10 both in the control and shRNA.Data represented as mean ± SEM. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. CB1R knockdown in the hippocampus exaggerates impulsive aggression in SI mice

Since pharmacological activation of hippocampal CB1Rs reduced adulthood impulsive aggression in mice that had experienced early adolescent social isolation, we next examined the effects of decreased CB1R expression on aggression. Using lentiviruses carrying either a specific CB1R shRNA or copGFP control, we transduced these lentiviruses into the hippocampus two weeks before the resident-intruder test (Fig. 4A and B). Fig. 4C shows the injected location of the lentivirus carrying the copGFP control. Transduction with CB1R shRNA significantly decreased CB1R expression in the hippocampus ($t = 4.12$, $p < 0.01$, n = 7) (Fig. 4D). In the SI mice (n = 10 and 12 in control and CB1R–KO respectively), the mixed two-way ANOVA showed a significant interaction effect between CB1R knockdown (KD) and acute stress on biting behavior ($F_{(1,20)} = 4.85$, $p < 0.05$) (Fig. 4E). The *post hoc* test revealed that acute stress provoked biting behavior in the control and CB1R-KD SI mice (no stress vs. acute stress, $p < 0.01$ and 0.001 in the control and CB1R-KD mice, respectively). Furthermore, during acute stress, the CB1R-KD mice exhibited higher levels of biting behavior compared to the control groups ($^{###}p < 0.001$). This indicated that decreases in hippocampal CB1R expression further exaggerated impulsive aggression in the SI mice.

To rule out the gain-of-function effect of CB1R knockdown on aggression, CB1R knockdown was performed on the GH mice (n = 10 both in the control and CB1R–KO mice) (Fig. 4F). There was no interaction effect of CB1R-KD and acute stress on biting behavior ($F_{(1,18)} = 1.04$, $p > 0.05$). CB1R knockdown did not induce aggression in either the no stress or acute stress situations in the GH mice. This suggested that CB1R knockdown did not affect trait aggressive characteristics and impulsive aggression in the GH mice.

3.5. eCB augmentation used to activate CB1Rs reduces impulsive aggression

Since pharmacological CB1R activation reduces impulsive aggression and CB1R-KD exaggerated impulsive aggression in SI mice, we examined whether increasing endogenous eCBs in order to activate CB1Rs affects impulsive aggression. To augment endogenous AEA and 2-AG, we administrated URB597 (5 ng/μl) and JZL184 (2 μg/μl), the inhibitors of FAAH and MAGL, respectively, into the vHip of SI mice before the RI test (Fig. 5A). Using pharmacological eCB augmentation, the combination of URB597 and JZL184 (n = 6) significantly decreased acute stress-provoked biting behavior compared to the vehicle treatment (n = 7) (one-way ANOVA, $F_{(3,23)} = 21.8$, $p < 0.001$; *post hoc* test, $p < 0.01$) (Fig. 5B). The effect of enhancing eCBs was blocked by the CB1R

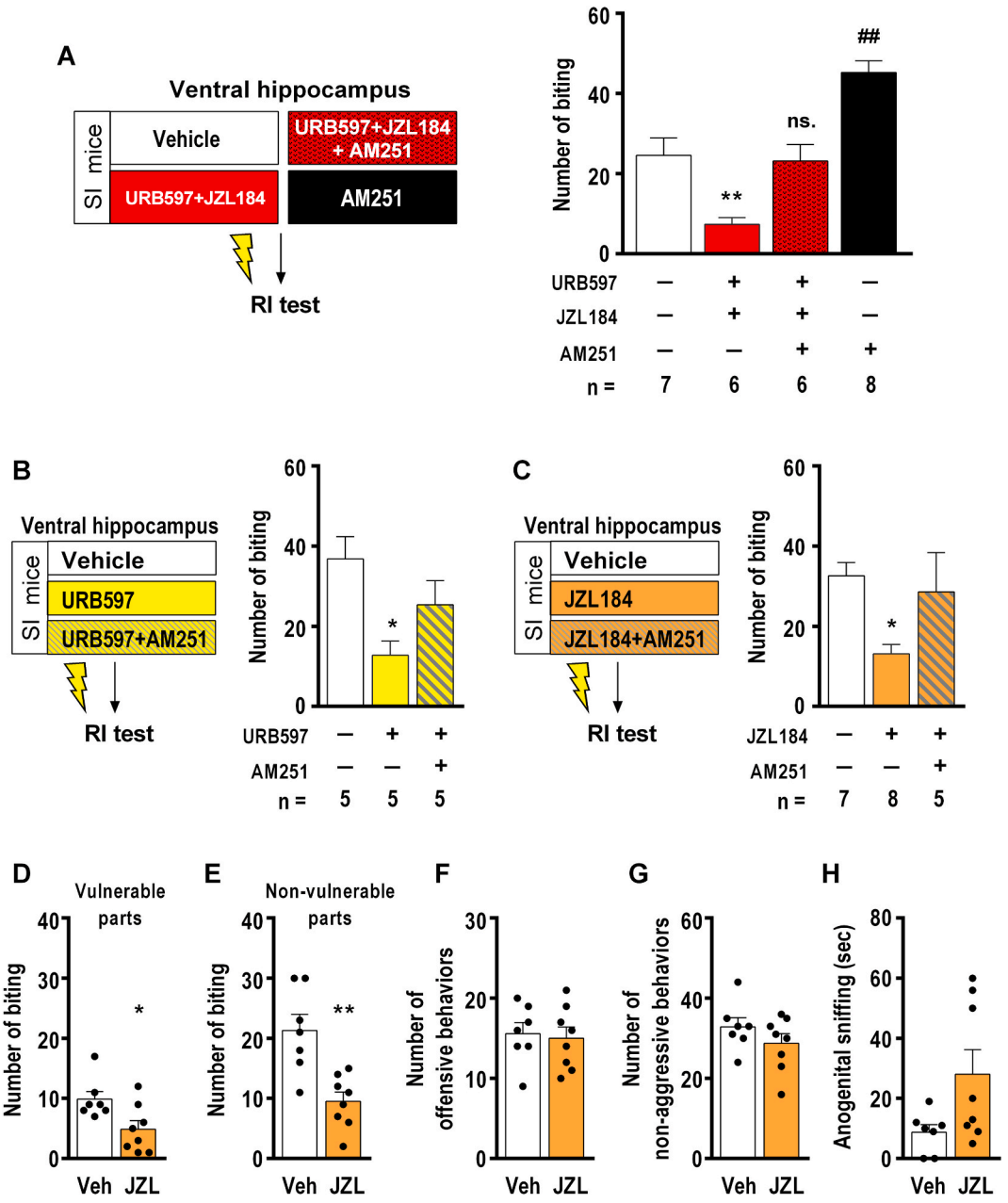

Fig. 5. Increasing endocannabinoids by inhibiting degradation enzymes reduces impulsive aggression in a CB1R-dependent manner., A. The experimental schema for eCB augmentation in the vHip (left). Four groups of SI mice received the intra-vHip infusion of the vehicle (n = 7), UBR597+JZL184 (n = 6), URB597+JZL184+AM251 (n = 6), and AM251 only (n = 8), respectively. 5 ng/μl URB597 and 2 μg/μl JZL184 inhibited FAAH and MAGL degradation enzymes to increase AEA and 2-AG, respectively. AM251 (2.5 ng/μl) blocked CB1R activation. These mice encountered acute stress 30 min after drugs were infused into the vHip. The right panel shows that pharmacological eCB augmentation via the combination of URB597 and JZL184 treatment decreased impulsive aggression compared to the vehicle treatment. *Post hoc* test, $p < 0.01$. AM251 infusion blocked the anti-aggressive effects of eCB augmentation. *Post hoc* test, $p > 0.05$ in vehicle vs URB597+JZL184+AM251. Inhibiting CB1Rs via AM251 treatment increased impulsive aggression compared to the vehicle. *Post hoc* test, $p < 0.01$ in vehicle vs AM251., B. Another three groups of SI mice received the intra-vHip infusion of the vehicle, URB597, and URB597+AM251, respectively. n = 5 per group. AEA augmentation reduced impulsive aggression compared to the vehicle, the effects of which were blocked by AM251 infusion. *Post hoc* test, $p < 0.05$ in URB597 vs. the vehicle; $p > 0.05$ in URB597+AM251 vs. the vehicle., C. Another three groups of SI mice received the intra-vHip infusion of the vehicle (n = 7), JZL184 (n = 8), and JZL184+AM251 (n = 5), respectively. 2-AG augmentation reduced impulsive aggression compared to the vehicle, the effect of which was blocked by AM251 infusion. *Post hoc* test, $p < 0.05$ in JZL184 vs. the vehicle; $p > 0.05$ in JZL184+AM251 vs. the vehicle., D and E. 2-AG augmentation in the vHip reduced the level of biting toward the vulnerable parts (**D**, $p < 0.05$) and non-vulnerable parts of intruders (**E**, $p < 0.01$). n = 7 and 8 in vehicle and JZL184, respectively. F ~ H. 2-AG augmentation via JZL184 did not affect offensive behavior (**F**, $p > 0.05$), non-aggressive behavior (**G**, $p > 0.05$), or the duration of ano-genital sniffing (**H**, $p > 0.05$). n = 7 and 8 in the vehicle and JZL184, respectively.Data represented as mean ± SEM.

antagonist, AM251 (2.5 ng/μl) (n = 6, *post hoc* test, $p > 0.05$) (Fig. 5B), suggesting that the eCB-mediated decrease in impulsive aggression was acting as a CB1R-dependent mechanism. Furthermore, AM251 infusion into the vHip (n = 8) increased acute stress-provoked biting behavior compared to the vehicle treatment (*post hoc* test, $p < 0.001$) (Fig. 5B). This result was consistent with the results of the CB1R knockdown experiment.

To rule out the intrinsic effects of the AM251 dose, 2.5 ng/μl AM251

was infused into the vHip of GH mice (n = 9 per group, Supplemental Fig. 2A). Compared to the control, AM251 infusion did not induce biting behavior ($t = 1.17$, $p > 0.05$) (Supplemental Fig. 2B) and did not affect offensive behavior ($t = 2.01$, $p > 0.05$) (Supplemental Fig. 2C). Although the AM251 infusion decreased non-aggressive behavior ($t = 2.33$, $p < 0.05$) (Supplemental Fig. 2D), there was no difference in ano-genital sniffing ($t = 0.12$, $p > 0.05$) (Supplemental Fig. 2E). Additionally, the AM251 infusion did not affect general locomotion activity ($t = 1.08$, $p > 0.05$) (Supplemental Fig. 2F) or anxiety-like behavior ($t = 2.03$ and 2.05, $p > 0.05$, in Supplemental Figs. 2G and 2H, respectively). The results indicated that a 2.5 ng/μl AM251 infusion into the vHip had no effects on impulsive aggression in the GH mice.

Overall, pharmacological eCB augmentation via administration of UBR597 and JZL184 decreased impulsive aggression in the SI mice. Next, we examined which of UBR597, JZL184, or both were responsible for the anti-aggressive effects. The one-way ANOVA revealed that the intra-vHip infusion of URB597 (5 ng/μl) decreased biting behavior compared to the vehicle group ($F_{(2,12)} = 5.51$, $p < 0.05$; *post hoc* test, $p < 0.05$) (Fig. 5B). AM251 treatment blocked the effect of URB597 pretreatment on biting behavior (*post hoc* test, AM251+URB597 vs vehicle, $p > 0.05$) (Fig. 5B). Similarly, 2-AG augmentation via JZL184 infusion (2 μg/μl) reduced biting behavior compared to the vehicle group (one-way ANOVA, $F_{(2,17)} = 4.94$, $p < 0.05$; *post hoc* test, $p < 0.05$) (Fig. 5C). Blocking CB1R activation via AM251 treatment reduced the effects of JZL184 pretreatment on biting behavior (*post hoc* test, $p > 0.05$) (Fig. 5C). These results indicated that endogenous AEA and 2-AG augmentation reduced adulthood impulsive aggression in the mice that had experienced early adolescent social isolation.

We further analyzed other behaviors of the JZL-treated mice. Compared to the control (n = 7), JZL184 infusion (2 μg/μl, n = 8) reduced biting toward the vulnerable parts ($t = 2.59$, $p < 0.05$) (Fig. 5D) and non-vulnerable parts of intruders ($t = 3.94$, $p < 0.01$) (Fig. 5E). JZL184 infusion did not affect offensive behavior ($t = 0.29$, $p > 0.05$) (Fig. 5F), non-aggressive behavior ($t = 1.23$, $p > 0.05$) (Fig. 5G), or ago-genital sniffing ($t = 2.12$, $p > 0.05$) (Fig. 5H). Thus, 2-AG augmentation via JZL184 reduced impulsive aggression but not offensive and social behavior.

Furthermore, we analyzed the effects of URB597, JZL184, and AM251 infusion into the vHip in the open field test (Supplemental Fig. 3A). There was no difference in the general locomotion activity among the vehicle, URB597, JZL184, and AM251 infusion into the vHip (one-way ANOVA, $F_{(3,25)} = 0.54$, $p > 0.05$) (Supplemental Fig. 3B). URB597 treatment increased the distance traveled in the central zone (one-way ANOVA, $F_{(3,25)} = 4.47$, $p < 0.05$; *post hoc* test, $p < 0.01$ in the vehicle vs. URB597) (Supplemental Fig. 3C). Both URB597 and JZL184 infusion increased the frequency of entry into the central zone compared to the control (one-way ANOVA, $F_{(3,25)} = 4.55$, $p < 0.05$; *post hoc* test, $p < 0.05$) (Supplemental Fig. 3D). The results suggested that eCB augmentation reduces anxiety-like behavior in SI mice. Also, the dose of AM251 infusion had no effects on the SI mice in the open field test. Taken together, pharmacological AEA and 2-AG augmentation via UBR597 and JZL184 treatment decreased impulsive aggression in the SI mice in a CB1R-dependent manner.

3.6. The eCB augmentation blunts the activation of vHip neurons projecting to the VMH

The neural circuit of the vHip neurons projecting to the ventromedial hypothalamus (VMH) has been reported to mediate acute stress-provoked aggression in SI mice (Chang and Gean, 2019). Thus, we examined whether eCB augmentation was associated with the activity of the vHip neurons projecting to the VMH. Red retrobeads, a retrograde tracer, were infused into the VMH, and the tips of the drug cannula were fixed into the vHip. After 2 weeks of tracer retrograde, URB597 (5 ng/μl) or JZL184 (2 μg/μl) was infused into the vHip of SI mice. One hour after drug pretreatment, these SI mice received foot-shocks and then were sacrificed 90 min later. (Fig. 6A). Fig. 6B showed the injection site of the red retrobeads in the VMH. Since c-Fos expression (blue) is the marker of neuronal activation, in the ventral CA1 (vCA1), the signals of red retrobeads were co-localized with c-Fos-positive (blue) cells (Fig. 6D), indicating that acute stress activated the ventral CA1 neurons projecting to the VMH. Importantly, presynaptic CB1Rs (green) surrounded the c-Fos-positive (blue) cells (Fig. 6D). In the vehicle control, c-Fos-positive cells surrounded by CB1R signals (green) were co-localized with the most retrobead (red)-positive cells (the top in Fig. 6C), suggesting that acute stress activated the VMH-projecting vCA1 neurons surrounded by presynaptic CB1R-positive synapses. However, the URB597 or JZL184 treatment significantly reduced c-Fos expression compared to the vehicle control (the middle and bottom of Fig. 6C, respectively). A quantitative analysis revealed that the percentage of c-Fos-positive cells in the retrobead-positive cells in the URB597 and JZL184 treatment was significantly lower than in the control (one-way ANOVA, $F_{(2,33)} = 50.17$, $p < 0.001$; *post hoc* test, $p < 0.001$; n = 12 brain slices from 3 mice per group) (Fig. 6E). These results suggested that pharmacological AEA and 2-AG augmentation via UBR597 and JZL184 decreased the activation of VMH-projecting vCA1 neurons surrounded by presynaptic CB1R-positive synapses.

4. Discussion

This study found that pharmacological activation of CB1Rs via WIN or ACPA administration reduced acute stress-provoked attack behavior in SI mice without affecting general locomotion activity. Through increasing endogenous AEA and 2-AG by URB597 and JZL184, respectively, eCB augmentations reduced impulsive aggression and suppressed the activation of vHip neurons projecting to the VMH. Furthermore, the anti-aggressive effect of eCB augmentation was blunted by AM251, a CB1R antagonist. This indicated that eCB augmentation acted as a CB1R-dependent mechanism. In contrast, the knockdown of hippocampal CB1R expression or the use of AM251 treatment to decrease or block CB1Rs exaggerated impulsive aggression in the SI mice. Thus, CB1R activation by eCBs plays a significant role in reducing impulsive aggression in mice that have experienced adverse early adolescence.

4.1. Presynaptic CB1R activation ameliorated impulsive aggression in SI mice by alleviating excessive excitatory activity

Long-term adverse early adolescent stress, such as social neglect, dampens brain development and induces maladaptive responses in neuropsychological behaviors. Post-weaning social isolation induces aberrant hyper-excitatory in vHip neurons and makes mice susceptible to acute stress (Chang et al., 2015, 2019). Since it is important to maintain an optimal balance between excitatory and inhibitory activity in neural networks, the function of presynaptic CB1Rs to quell neurotransmitter release is likely to be beneficial to alleviate excessive excitatory network activity. Our results support the view that CB1R activation reduces vHip hyperactivity and in turn decreases impulsive aggression in the SI mice. The intra-vHip infusion of CB1R agonists and eCB degradation inhibitors mediated presynaptic CB1Rs in the vHip and decreased impulsive aggression in the SI mice. Indeed, CB1R activation decreases the amplitude of excitatory postsynaptic currents (EPSCs) and enhances paired-pulse facilitation (PPF) without affecting the amplitude of spontaneous miniature EPSCs (mEPSCs) in the glutamatergic cells of the hippocampus (Misner and Sullivan, 1999). Overexpression of CB1Rs in hippocampal pyramidal neurons alleviates seizure-induced excessive excitatory network activity (Guggenhuber et al., 2010). CB1R activation due to pharmacological 2-AG augmentation also reduces glutamate release and EPSC frequencies (Araque et al., 2017; Folkes et al., 2020). In this study, we found that eCB augmentation reduced c-Fos expression, a marker of neuronal activation, in VMH-projecting vHip neurons. Direct CB1R activation via WIN or ACPA and indirect CB1R activation by eCB augmentation both reduced acute stress-provoked biting

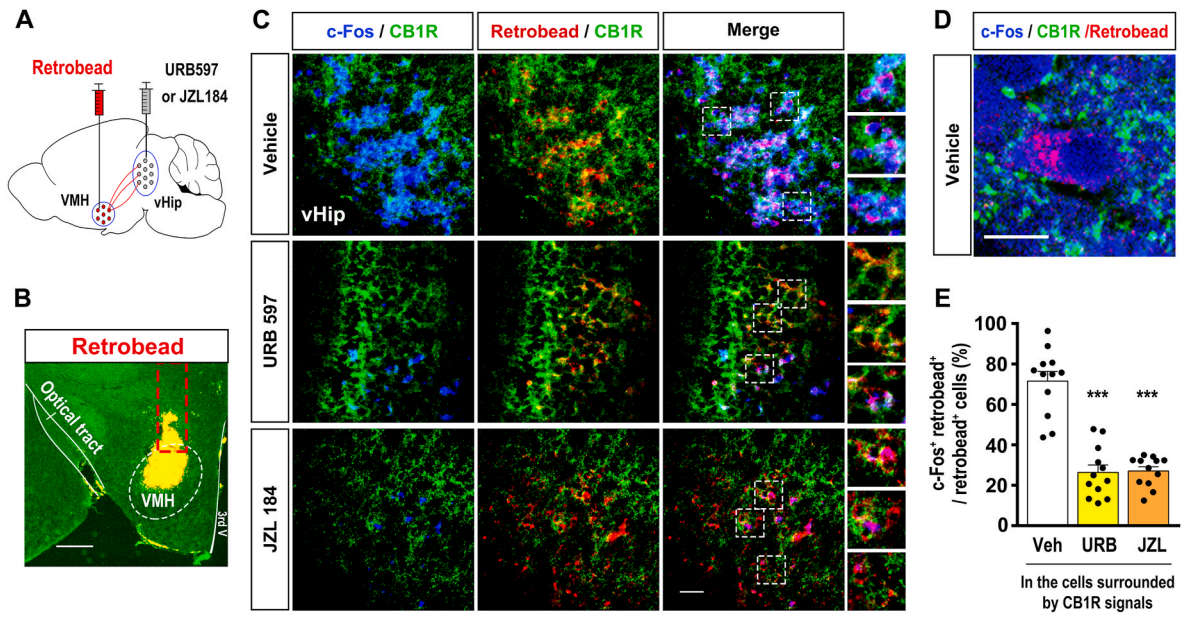

Fig. 6. eCB augmentation inhibits the activation of vHip neurons projecting to the VMH. A. Schema of the experimental design. Red retrobeads were infused into the VMH, and tips of drug cannulas were implanted in the vHip of SI mice. After two weeks of recovery, the three retrobead-treated groups of mice received an intra-vHip infusion of the vehicle, 5 ng/μl URB597, and 2 μg/μl JZL184, respectively. One hour after the drug infusion, these SI mice received foot-shocks to induce c-Fos expression and then were sacrificed 90 min later. n = 3 per group., B. Representative image showing the injected location of retrobead infusion in the VMH. Scale bar: 550 μm, C. Representative images showing signals of red retrobeads (red), CB1Rs (green), and c-Fos (blue) in the ventral CA1 of the vehicle (top), UBR597 (middle), and JZL184 (bottom) -infused mice. Acute stress induced c-Fos expression (blue) surrounded by CB1Rs (green). c-Fos signals were co-localized with red retrobeads. URB597 infusion and JZL184 infusion significantly decreased c-Fos expression, suggesting that AEA and 2-AG augmentation suppressed acute stress-provoked vHip activation. Scale bar: 40 μm. The right panels show the magnified insets as white dotted squares., D. Representative confocal image showing c-Fos-positive cells co-localized with red retrobeads. The CB1R signals surrounded the c-Fos-positive cells. Scale bar: 10 μm., E. Quantitative analysis of Fig. 6C. In the vHip, URB597 infusion and JZL184 infusion decreased c-Fos expression surrounded by CB1R signals in retrobead-positive cells compared to the vehicle. *Post hoc* test, $p < 0.001$; n = 12 brain slices from 3 mice per group. Data represented as mean ± SEM. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

behavior in the SI mice. CB1R may mediate this action at the presynaptic terminals of local axons of vHip neurons or at those of axons projecting from distant brain areas. Furthermore, AM251 administration to block the CB1R function exaggerated impulsive aggression in the SI mice with hyperactive vHip but not in the GH mice. Thus, activating CB1Rs plays an important buffering role in protection against acute stress susceptibility in SI mice.

On the other hand, CB1R knockdown in the vHip further increased aggression levels in the SI mice, implying that disturbing modulation within the hippocampal microcircuit by decreasing CB1R expression exaggerates impulsive aggression. The commercial lentiviruses with CB1R shRNA used in this study knocked down gene expression of CB1R in multiple types of hippocampal cells. Recently, astroglial CB1R was demonstrated to mediate glucose metabolism and in turn to affect social behavior (Jimenez-Blasco et al., 2020). Thus, it is complex which postsynaptic cell types that regulate impulsive aggression are affected by the decrease of CB1R expression in the vHip microcircuit. In the present study, acute stress activated the vHip neurons projecting to the VMH, and the activated neurons were surrounded by presynaptic CB1Rs, indicating that CB1Rs work on these vHip neurons to mediate impulsive aggression. Further studies should electrophysiologically analyze the cell-type-specific neuronal population that presynaptic CB1Rs act on.

4.2. CB1R knockdown in adult SI mice exaggerate impulsive aggression

Previous studies have shown that knockout of the CB1R gene induces aggression (Haller et al., 2004; Martin et al., 2002; Rodriguez-Arias et al., 2013). This indicates that deleting the CB1R gene in the embryonic stage alters brain development and induces trait aggression. Given that CB1R activation provides feedback control of neurotransmission release, decreasing CB1R expression in adulthood eliminates the buffering role in neurotransmission control. This may exaggerate vHip hyper-excitatory activities and promote sensitivity to acute stress in SI mice. Indeed, in this study, knockdown of CB1R expression in the vHip exaggerated impulsive aggression in the SI mice but not in the GH mice. Similarly, AM251, a CB1R antagonist, also exaggerated impulsive aggression in the SI mice, supporting the view that decreasing the role of CB1R in the feedback control of neurotransmitter release exaggerates abnormal phenotypes in SI mice. Further studies should examine the effects of CB1R knockdown on the EPSC and IPSC characteristics of vHip neurons in SI mice.

In the present study, CB1R knockdown did not induce impulsive aggression in the GH mice under either no stress or acute stress, and blocking hippocampal CB1Rs by AM251 did not induce aggression in the GH mice. This suggests that decreasing CB1R function is likely to be not necessary to induce impulsive aggression. However, it is possible that decreasing CB1R function could lead to a loss of the protection against acute stress due to the development of pathological states induced by an adverse juvenile environment, such as those observed in the SI mice. Indeed, CB1R knockdown and blocking CB1Rs via AM251 further exaggerated impulsive aggression in the SI mice. On the other hand, CB1R activation and enhancing eCBs in the vHip were sufficient to protect against acute stress and decrease anxiety-like behavior and impulsive aggression in the SI mice. Our data suggest that pharmacological eCB augmentation to activate CB1Rs could serve as a therapeutic approach to preventing impulsive aggression.

4.3. The relationship between impulsive aggression and anxious status

Impulsive aggression is induced by acute stress and thus is accompanied by anxious states. The eCB-CB1R activation has been reported to promote stress resilience (Bluett et al., 2017; Shen et al., 2019) and

produce anxiolytic effects (Aliczki et al., 2015; Bluett et al., 2017; Busquets-Garcia et al., 2011; Sciolino et al., 2011). Our data supported this view. In this study, CB1R activation and eCB augmentation in the vHip reduced anxiety-like behavior in the SI mice. This raised the question as to whether CB1R activation selectively inhibits impulsive aggression or decreases anxiety to sequentially quell impulsive aggression. Because the vHip is the hub to integrate multiple information from the amygdala, the prefrontal cortex, and other limbic brain areas (Fanselow and Dong, 2010; Strange et al., 2014), CB1R activation in the vHip may have multiple effects on emotional behavior. Further studies could use real-time calcium images and electrophysiology in free-moving mice to detect brain activity in the context of anxiety and impulsive aggression.

4.4. Sex differences in CB1R density

Previous studies reported that males have higher CB1R binding sites than females in human and rodents (Laurikainen et al., 2019; Rubino and Parolaro, 2011). Furthermore, maternal deprivation and chronic unpredictable stress reduced the hippocampal CB1R protein levels in male rats while increased CB1R levels in female rats (Llorente-Berzal et al., 2013; Reich et al., 2009; Suárez et al., 2009). Δ9-THC mitigated aggression in female maternally-deprived rats rather than in male maternally-deprived rats (Zamberletti et al., 2012). These raise the possibility that adverse juvenile experiences might modulate CB1R expression in a manner of sex differences and that pharmacological eCB augmentation is likely to display sex different effects in the post-weaning social isolation. Further researches could examine the effect of post-weaning social isolation in female rodents in CB1R expression and treatment.

5. Conclusion

In summary, the present study demonstrates that pharmacological CB1R activation via WIN and ACPA prevented acute stress-induced outbursts of aggression, referred to as impulsive aggression, in SI mice. WIN treatment reduced biting behavior and offensive behavior but did not affect non-aggressive behavior. This indicated that CB1R activation suppressed impulsive aggression rather than other social behaviors. Furthermore, hippocampal AEA and 2-AG augmentation via URB597 and JZL184 reduced impulsive aggression in a CB1R-dependent manner. AEA and 2-AG augmentation suppressed the activation of VMH-projecting vHip neurons surrounded by presynaptic CB1R-positive synapses. In conclusion, the pharmacological CB1R activation reduced the acute stress-triggered vHip activation and prevented impulsive aggression in the SI mice. It suggests that therapeutics targeting the eCB system may represent an alternative strategy in the control of emotional outbursts.

CRediT authorship contribution statement

Chih-Hua Chang: Investigation, Methodology, Validation, Formal analysis, Writing – review & editing, Visualization. **Yu-Chen Liu:** Investigation, Formal analysis, Writing – original draft. **Chih-Yang Sun:** Validation, Formal analysis. **Chun-Lin Su:** Conceptualization, Supervision, Project administration. **Po-Wu Gean:** Conceptualization, Visualization, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2021.100337.

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