Title: The Modulatory Effects of CB1 Receptors on Social Behavior and Anxiety in Rats: Insights from an Empathic Pain Paradigm

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Abstract

Objectives: Cannabinoid receptor type 1 (CB1) is extensively distributed across brain regions that are crucial for emotional processing, social cognition, and anxiety, including the prefrontal cortex, amygdala, and hippocampus. Dysregulation of CB1 receptors (CB1Rs) has been associated with several disorders characterized by impaired empathy, social behavior, and anxiety. This study aimed to investigate the modulatory role of CB1Rs in social interaction and anxiety in an empathic pain model in rats.

Methods: Forty-eight adults male Wistar rats were used (n = 8 for each group). One sibling received formalin injection into the hind paw five times within a nine-day period (Demonstrator), and the other siblings reported pain (Observer) while being treated with DMSO, the cannabinoid type 1 receptor (CB1R) agonist WIN 55,212–2 (WIN; 3 mg/kg, i.p.), or the CB1R antagonist rimonabant (1 mg/kg, i.p.). Treatments were administered intraperitoneally 30 minutes prior to behavioral tests conducted on day 10, which were used to assess social behavior and anxiety.

Results: The findings indicate that empathic pain can impair social behavior and elicit anxiety-like effects. Rimonabant was effective in ameliorating deficits induced by empathic pain; conversely, WIN did not have a significant effect.

Conclusion: These results suggest that CB1Rs play a modulatory role in social contagion. This modulation may provide new therapeutic targets for conditions such as autism spectrum disorder, schizophrenia, and other psychological disorders characterized by impaired empathy and dysregulated social behavior associated with the CB1 signaling pathway.

Keywords: Cannabinoid system; Anxiety; Empathic pain; Social behavior; Rimonabant; WIN 55,212–2

1. Introduction

Empathy involves adopting another's sensory and emotional state, which is essential for social communication and interaction (1). It plays a crucial role in social behavior and interactions often linked to prosocial behavior, facilitating positive social interactions by motivating individuals to engage in actions that benefit others (2). Empathy contributes to social behavior and interaction through decoding another's feelings and emotions (3). The phenomenon of empathic pain, also referred to as social pain transfer, involves a complex interplay of neural mechanisms that include the activation of the anterior cingulate cortex (ACC) and insula, the mirror neuron system, and modulation by neurotransmitter systems such as the endocannabinoid system (1). The endocannabinoid system plays a pivotal role in social behavior, communication, and neurobehavioral functions, mainly via the cannabinoid type 1 (CB1) receptor (4, 5). These receptors are widely distributed in socioemotional regions of the brain, such as the amygdala, prefrontal cortex, nucleus accumbens, and hippocampus (6).

Social interaction is affected by various factors, including mental state, anxiety, and depression (7). Notably, the endocannabinoid system, particularly CB1 receptors, plays a pivotal role in regulating anxiety. CB1 receptors are densely concentrated in brain regions involved in anxiety and emotional behavior, such as the amygdala, hippocampus, and prefrontal cortex (8). Previous studies have revealed that anxiety, especially social anxiety, has a profound impact on social behavior, emotional responses, and autonomic regulation during social interactions (9, 10).

A growing body of literature highlights the significant role that CB1 receptors and the endocannabinoid system play in modulating empathy, social behavior, and anxiety. Numerous studies have demonstrated impaired empathy and social behavior, along with dysregulated CB1 receptors in key brain regions implicated in various psychiatric disorders, including autism

spectrum disorder (ASD), posttraumatic stress disorder (PTSD), and schizophrenia (11). Therefore, further research is necessary to clarify the mechanisms through which CB1 receptors modulate social behavior and anxiety. The present study aimed to investigate the potential modulatory role of CB1 receptors and their implications for developing targeted treatments for disorders characterized by impaired empathy, social function, and heightened anxiety.

2. Materials and methods

2.1. Animals

Male sibling rats weighing between 220 and 250 grams were utilized. The subjects were housed together beginning at birth under standard laboratory conditions (23±2 °C, 12-hour light–dark cycle; light at 07:00 a.m.) and had ad libitum access to food and water. The experimental procedures were conducted according to the Institutional Guidelines for the Care and Use of Laboratory Animals (National Institute of Health Publication, 8th edition, revised 2011). All experiments were approved by the Ethics Committee of Mazandaran University of Medical Sciences (ethics code: IR.MAZUMS.AEC.1401.028).

2.2. Drugs

The drugs used in this study included formalin (Sigma Aldrich, USA), the CB1R antagonist rimonabant (SR141716A; Cayman, USA), and the CB1R agonist WIN 55,212–2 (WIN; Sigma Aldrich, USA). The Demonstrator group received formalin (10%, 50 µl), which was dissolved in 0.9% saline. Rimonabant and WIN were dissolved in dimethyl sulfoxide (DMSO). WIN (3 mg/kg) and rimonabant (1 mg/kg) were intraperitoneally injected in a volume of 50 µl, as described in previous studies (12).

2.3. Experimental procedures

At the beginning of the experimental procedure, empathic pain models were induced as previously described (13). Briefly, a male sibling was randomly selected from each cage and received formalin as a painful stimulus (50 µl, 10%) on the plantar surface of the hind paw on days 1, 3, 5, 7, and 9 (Demonstrator), after which he was returned to the cage. The sibling rat that observed the pain experienced by the demonstrator was designated the Observer rat. Notably, from the beginning of the work, 48 rats were divided into six groups as follows: (1) Control group: the rats that received saline in the paw according to the protocol described above; (2) Demonstrator group: the rats that received formalin; (3) Observer group: the rats that observed the Demonstrator rats; (4) Observer+DMSO group: the rats that observed the Demonstrator rats and received DMSO; (5) WIN group: the Observer rats that received WIN (3 mg/kg, i.p.); (6) Rimonabant group: the Observer rats that received rimonabant (1 mg/kg, i.p.). We adopted the dosing of rimonabant and WIN reported in a recent study (12, 14). WIN, rimonabant, or DMSO was injected 30 min before the behavioral experiments, the timing of administration was conducted in accordance with the prior studies (15, 16). As shown in Fig. 1, after drug treatment, the behavioral tests, including the social behavior test and open field test, were conducted on the 10th day at 1 h intervals. The arena was cleaned with 70% ethanol between tests. All the experiments were performed by examiners who were blinded to the treatments and groups.

2.4. Open field test (OFT)

The open field test is the basic test for anxiety assessment and is widely used to investigate the neurobiological basis of anxiety in animal studies. The open field apparatus comprises clear Plexiglas boxes (90 × 90 × 50 cm) illuminated by red light to reduce anxiety associated with bright light. Each rat was located in the center of the apparatus, and the time spent in the center and

surrounding areas was recorded for five minutes. A digital camera located on top of the box recorded the rat's activity(17).

2.5. Social behavior test (SBT)

We used social behavior as a critical component to assess social interaction. The test was conducted in a controlled environment in an open field apparatus (Plexiglas; 90 × 90 × 50 cm). Two unfamiliar rats with the same treatment and weight were randomly selected to evaluate social interaction patterns, including the total duration of climbing, crawling, following, genital investigation, and sniffing, for 10 min (18, 19).

2.6. Elevated plus maze (EPM)

Anxiety-like behavior was analyzed via an EPM apparatus made of black wood. The device consisted of two opposite open arms (50 × 10 cm) and two opposite closed arms (50 × 10 × 50 cm), separated by an open square space (10 × 10 cm). The plus shape apparatus was located inside the testing room and elevated 50 cm above the floor. Each animal was placed in the center of the EPM, and anxiety-like behavior was recorded by a video camera for 5 minutes. The frequency of entries into the open and closed arms as well as the time spent in each arm were calculated to evaluate anxiety-like behavior (20, 21).

2.6. Statistical analysis

GraphPad Prism (version 9.5) was used for the analysis of the data. The normality distribution was calculated via the Kolmogorov–Smirnov test. Normality distributed data were compared via one-way ANOVA, and Tukey's post hoc test was employed for multiple comparisons. Nonnormally distributed data were analyzed with Dunn's post hoc test. Normally distributed data are expressed

as the mean ± SEM, and nonparametric data are presented as the median and interquartile range. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Open field test

We assessed the time spent in the center and surrounding area to evaluate anxiety in the rats, with the aim of identifying the potential effects of WIN and rimonabant on the empathic pain model. Initial analyses revealed that the data had nonnormal distributions, as assessed by the Shapiro–Wilk test. Therefore, nonparametric tests were employed for further analyses. The Friedman test was conducted for time spent in the center area, followed by Dunn's post hoc test for multiple comparisons. Dunn's post hoc test revealed significant differences between the Demonstrator and Control groups ($p<0.0001$; Friedman statistic = 34.60) and between the Observer group and the Observer+DMSO group ($p= 0.0396$). However, there were no significant differences between the rimonabant treatment group ($p= 0.4876$) or the WIN treatment group and the DMSO group ($p=0.3466$). The Kruskal–Wallis test was performed for time spent in the surrounding area, followed by Dunn's multiple comparison test. Dunn's post hoc test revealed significant differences between the Demonstrator and Control groups ($p<0.0001$; Kruskal–Wallis statistic= 39.24) and between the Observer group ($p=0.0226$) and the Observer+ DMSO group. Data analysis revealed that there were no significant differences between the Rimonabant group ($p=0.1635$) or the WIN treatment group and the DMSO group ($p=0.4022$, Fig. 2A, 2B).

3.2. Social behavior tests (SBTs)

The effects of empathic pain on social behavior were measured via various parameters, including climbing, crawling, following, genital investigation, and sniffing. Analysis revealed that the time spent climbing in the Demonstrator and Observer groups was significantly lower than that in the Control group ($F_{5, 42}$=1.444 22.72, p<0.0001; Fig. 3A). Rimonabant injection, but not WIN injection, was associated with a significant increase in climbing time (p<0.01). Moreover, our results revealed that crawling time was significantly lower in the Demonstrator group ($F_{5, 42}$= 0.06819 10.12, p<0.0001; Fig. 3B) and Observer group (p<0.001) than in the Control group. Compared with the Observer+DMSO treatment, the rimonabant treatment increased the crawling time (p<0.05). Compared with that of the control group, following time in Demonstrator group was lower ($F_{5, 42}$=9.827, p<0.0001; Fig. 3C), and a similar decrease was observed in the Observer group (p<0.001). Rimonabant restored it (p<0.05). As shown in Fig. 3D, pain induction in Demonstrator rats decreased the duration of genital investigation compared with that in Control animals ($F_{5,42}$= 0.6136 3.662, p<0.05), whereas rimonabant and WIN did not significantly impact genital investigation. Further analysis indicated that the sniffing time did not differ among the groups ($F_{5, 42}$=1.739 1.516, p= 0.2056; Fig. 3E).

3.3. Anxiety-like behavior in the EPM test

One-way ANOVA demonstrated that the time spent exploring the open arms was significantly lower in the pain group than in the control group ($F_{5, 42}$=7.616, p<0.001). Moreover, the findings indicated that the time spent in the open arms was lower in the Observer group than in the Control group (p<0.01). In addition, compared with the Observer+DMSO group, the Rimonabant group but not the WIN group spent more time in the open arms (p< 0.05; Fig. 4A). The frequency of entries into the open arms was lower in the pain and observer groups than in the control group ($F_{5,}$

$_{42}$=19.42; p<0.0001), whereas compared with the Observer+DMSO group, the Rimonabant group, but not the WIN group, presented a marked increase (p<0.01; Fig. 4B). The present data revealed that the time spent in the closed arms was greater in both the Demonstrator and Observer groups than in the Control group ($F_{5, 42}$=20.18, p<0.0001; Fig. 4C). Rimonabant ameliorated this effect (p<0.05). Moreover, the results revealed that the number of entries into the closed arms significantly increased in the Demonstrator ($F_{5, 42}$=10.47, p<0.0001) and Observer (p<0.001; Fig. 4 D) groups. Compared with Observer+DMSO, rimonabant decreased the frequency of entry into the closed arms (p<0.05). Collectively, these results suggest that antagonism of the CB1R may lead to anxiolytic effects against anxiety induced by empathic pain.

4. Discussion

The primary aim of the present study was to investigate the modulatory role of CB1Rs in social behavior and anxiety in an empathic pain model. The present findings indicate that empathic pain can impair social behavior and elicit anxiety-like behavior. Notably, rimonabant was effective in ameliorating deficits induced by empathic pain, whereas WIN did not have a significant effect.

Our results revealed that formalin injection significantly decreased the time spent in the center and increased the time spent surrounding the open field arena, indicating pain-induced anxiety-like behavior. Specifically, the pain group spent less time in the open arms of the EPM test. Consistent with these observations, hind paw Freund's adjuvant (CFA) injection in rats results in significant anxiety-like behavior in the EPM and dark/light tests in mice (22, 23). Pain has been shown to alter the functioning of different brain regions involved in anxiety and emotional regulation, particularly the amygdala, prefrontal cortex, and hippocampus (24). Evidence suggests that

dysregulation of cannabinoid receptor signaling, particularly CB1Rs, is associated with increased anxiety in pain models. (25). Furthermore, altered pain perception, such as hyperalgesia, can contribute to anxiety-like behavior (24, 26).

In alignment with our findings, Nazeri et al. (27) reported that exposure to home-cage pain induced anxiety in observer rats. Recent optogenetic studies have confirmed the activation of specific neural populations in the anterior cingulate cortex during both direct pain experiences and when observing pain in others (1). The social transfer of pain-induced anxiety among rats involves both visual and chemical cues (28). These cues significantly influence social transfer and the expression of empathic pain (29, 30). The modulatory role of CB1Rs in anxiety and social behavior suggests their potential involvement in the social transfer of anxiety (31).

Moreover, we assessed the effects of injecting a CB1R agonist or antagonist on empathy-induced anxiety. Our data indicated that the CB1R agonist WIN did not significantly affect anxiety-like behavior in observers; however, the CB1R antagonist rimonabant had an anxiolytic effect on the Observer group during the EPM test but not in the open field test. The differential effects of rimonabant on anxiety across these tests may be attributed to their varying sensitivities to different aspects of anxiety-like behavior. The EPM is particularly sensitive to anxiolytic effects and evaluates anxiety related to open space and height (32). Conversely, the open field test assesses general exploratory behavior within a novel environment (33). The greater sensitivity of the EPM test to anxiolytic compounds may explain why Rimonabant exhibited more pronounced effects in this test than in the OFT. Importantly, WIN seems to exhibit anxiolytic or anxiogenic effects in a dose-dependent manner; lower doses may induce anxiolytic effects, whereas higher doses may have no impact on anxiety responses (34, 35). In line with our findings, Zador et al. (35) reported that rimonabant at a low dose (1 mg/kg) could decrease anxiety-like behavior in the EPM and

dark/light tasks in mice. The specific contribution of CB1 receptors to the social transfer of anxiety may involve their regulation of several neurotransmitter systems.

Pain can significantly reduce social behaviors such as climbing, following, crawling, and genital investigation through various physiological and psychological mechanisms. Our findings corroborate those of Santos et al. (36), who demonstrated that formalin injection decreased climbing behavior in mice. Studies have indicated that complete Freund's adjuvant (CFA)-induced pain leads to reduced activity levels (37). Persistent inflammatory pain can substantially alter climbing ability through several factors, such as mechanical hypersensitivity, reduced locomotor activity, and altered movement patterns (37). Chronic pain influences social dynamics, leading to changes in the social interactions of rats with their environment and conspecifics. For example, chronic pain increases anxiety-like behavior, which may lead to heightened caution or withdrawal from social interactions, including sniffing or exploring new scents (38). In addition, the selective decrease in active social behavior observed in the current study suggests that pain not only leads to a general decrease in social behavior but also disrupts the motivational neural circuits that drive social interaction (39).

Previous studies have shown that the social transfer of pain occurs through a combination of behavioral and neurobiological mechanisms. These mechanisms include the interplay among the social environment, olfactory communication, and neurobiological factors, highlighting the complex relationship between social dynamics and pain perception (40-42).

Our data demonstrated that social behaviors, such as climbing, crawling, and following, decreased in both demonstrator and observer rats. This transfer of impaired social behavior appears to be mediated primarily through emotional contagion, a process in which the emotional state of a rat influences the behavior of its cagemates (43). Additionally, neurobiological factors, particularly

alterations in neurotransmitter signaling pathways (e.g., oxytocin and endocannabinoids), play a significant role in modulating social behaviors (6, 44). Interestingly, pain not only increases neural sensitivity but also alters neuromodulatory systems, particularly the endocannabinoid system, leading to decreased motivation for social interactions (45, 46). Our study revealed a critical role of the endocannabinoid system in modulating empathy–induced social behavior deficits in rats.

The administration of rimonabant to observers improved social behaviors such as following, climbing, and crawling. This result aligns with previous reports suggesting that CB1 receptor blockade can improve social impairment in various neuropsychiatric conditions (47). Trezza et al. (48) demonstrated that rimonabant could improve social play behavior deficits in mature rats exposed to early-life stress. Rimonabant has multiple functions that may help restore impairments in social behavior. By blocking CB1 receptors, rimonabant can increase dopamine and serotonin levels in brain regions critical for social behavior, such as the nucleus accumbens (49). Additionally, the anxiolytic effects of rimonabant may indirectly improve social interactions by reducing social anxiety (16).

Interestingly, our data indicated that WIN did not significantly affect social behavior tests in the empathic pain model. This finding stands in contrast to those of several prior studies suggesting that CB1 agonists can modulate social behavior (48). The lack of a significant impact of WIN on social behavior may be attributed to several potential mechanisms. **First,** chronic pain conditions may lead to desensitization and downregulation of CB1Rs due to prolonged activation, potentially diminishing social behavior(50). Second, chronic inflammatory pain may alter the properties of CB1Rs in regions responsible for pain processing, thereby reducing WIN effectiveness in modulating social behavior (51). **Third, evidence has revealed that** chronic pain may disrupt multiple neurotransmitter systems involved in social behavior, such as (e.g., oxytocin, dopamine),

which could explain WIN's lack of effect due to complex interactions with these disrupted systems (44).

Finally, there is evidence that WIN exerts its effect in a dose-dependent manner; thus, the dosage used in this empathic pain model may not have been optimal for enhancing social behavior (48). In the present study, no significant difference in climbing or crawling activity was detected between the WIN-treated group and the control group. This finding suggests that WIN may not disrupt basic exploratory behaviors such as climbing or crawling; however, it could influence more complex social interactions (6).

Taken together, our results demonstrate that while empathic pain can lead to notable deficits in social interactions and induce anxiety-like behaviors, the administration of the CB1R antagonist rimonabant effectively ameliorates these impairments. These findings suggest that targeting CB1Rs may provide a therapeutic avenue for addressing social dysfunction associated with pain. Future studies that consider other aspects of social behavior such as novel object memory and social memory should further explore these mechanisms and consider broader implications for treatment strategies for patients with neuropsychiatric conditions.

Declaration of competing interests

The authors declare that they have no conflicts of interest.

Author contributions

H.GH. and M.SH. conceived, designed and planned the experiments. F.R.SH. carried out the experiments. R.R., S.M.S.T. and M.KH. contributed to the interpretation of the results. F.R.SH.

and H. GH contributed to manuscript writing. All the authors reviewed and approved the manuscript.

Data availability

Data will be made available on request. The requests should be addressed to H.GH.

Competing interests

The authors declare that they have no competing interests.

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Figures and figure legends:

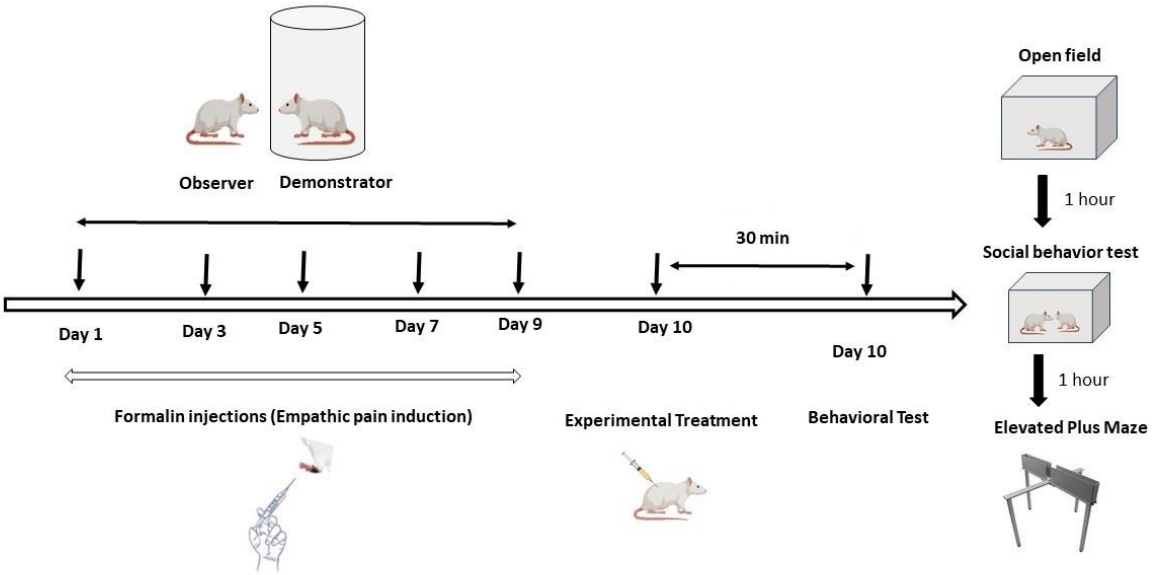


Fig. 1. Timeline of the experimental procedures used in the present work.

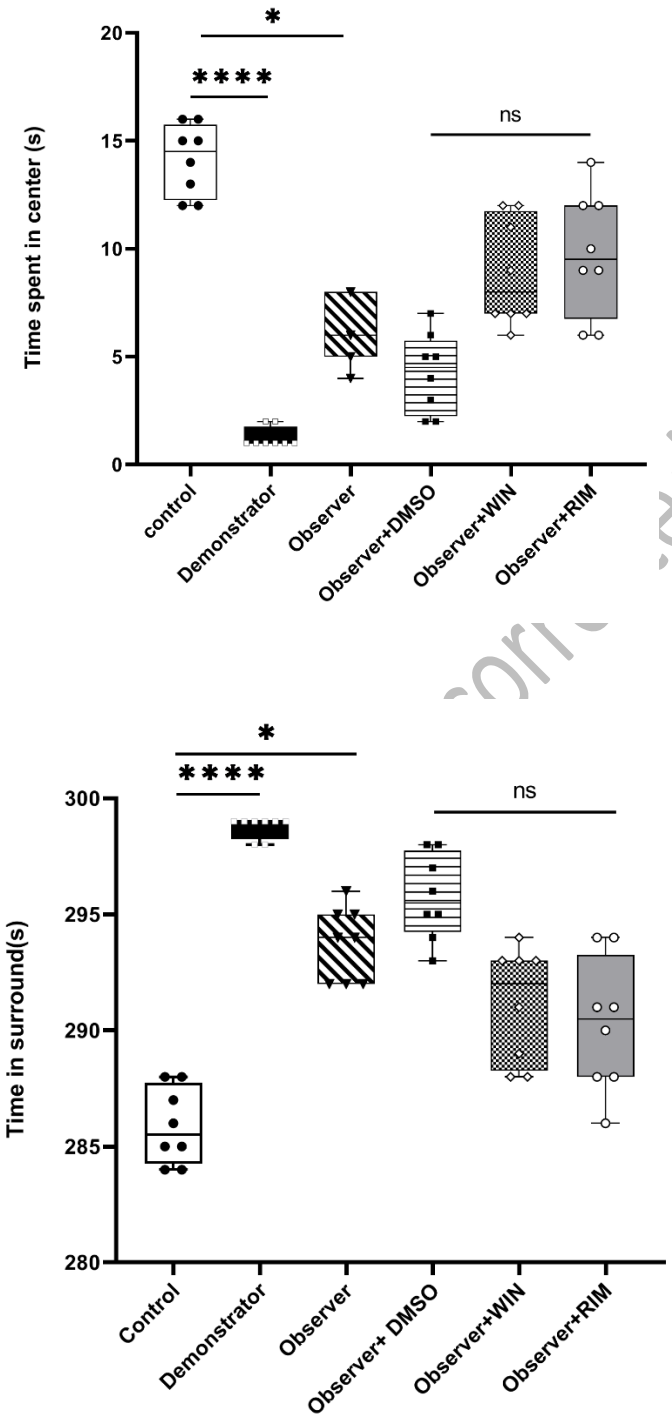
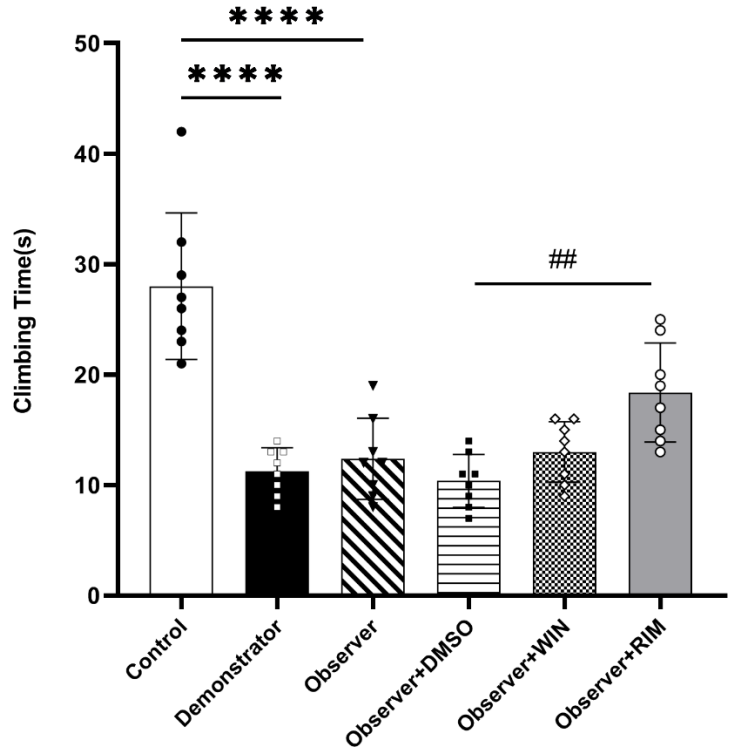
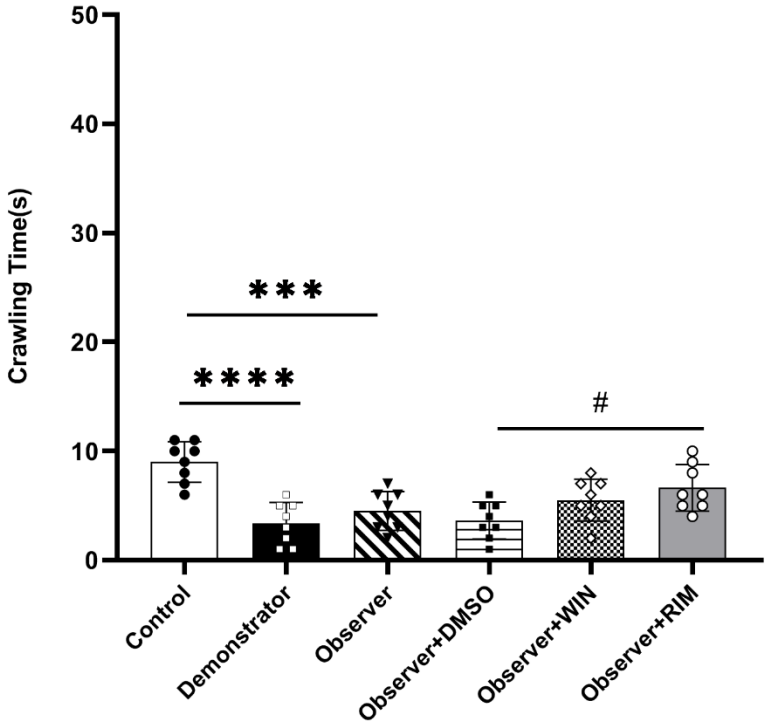
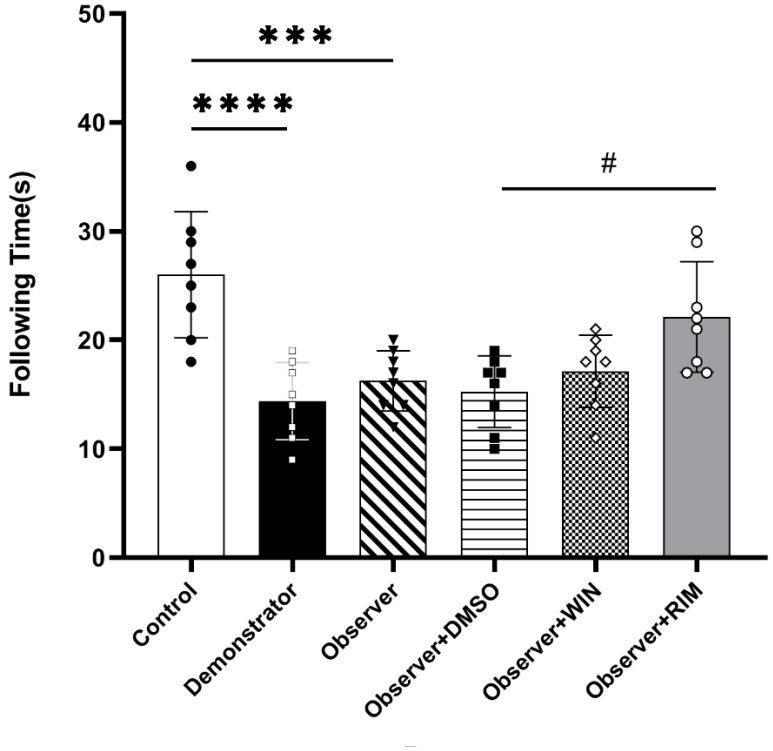
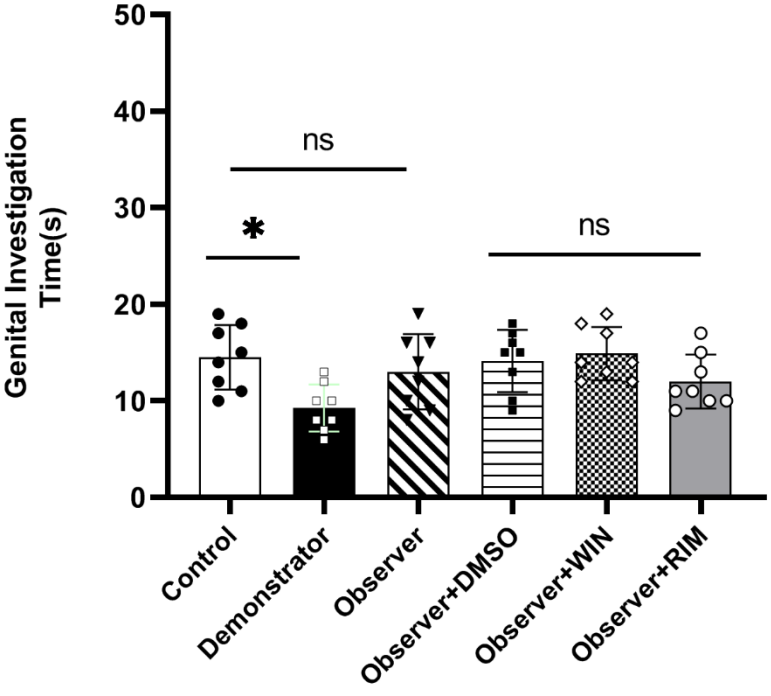


Fig. 2. Effects of the CB_1R agonist (WIN) and antagonist (Rimonabant) on anxiety; time spent in the center area in the open field test (A), time spent in the surrounding area (B). Data are shown as the median and interquartile range, and n=8 rats in each group. **** $P<0.0001$ and $P<0.05$ compared with the Demonstrator and Observer groups, respectively.









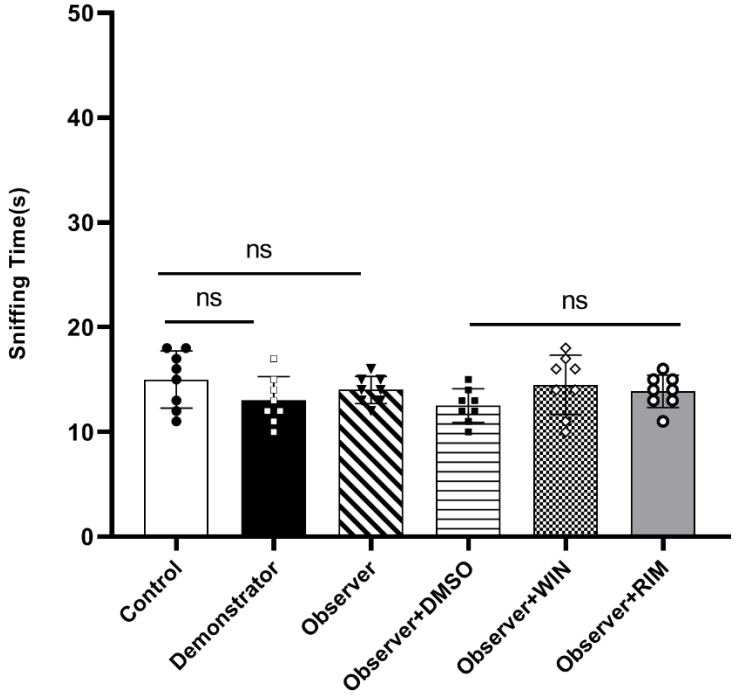
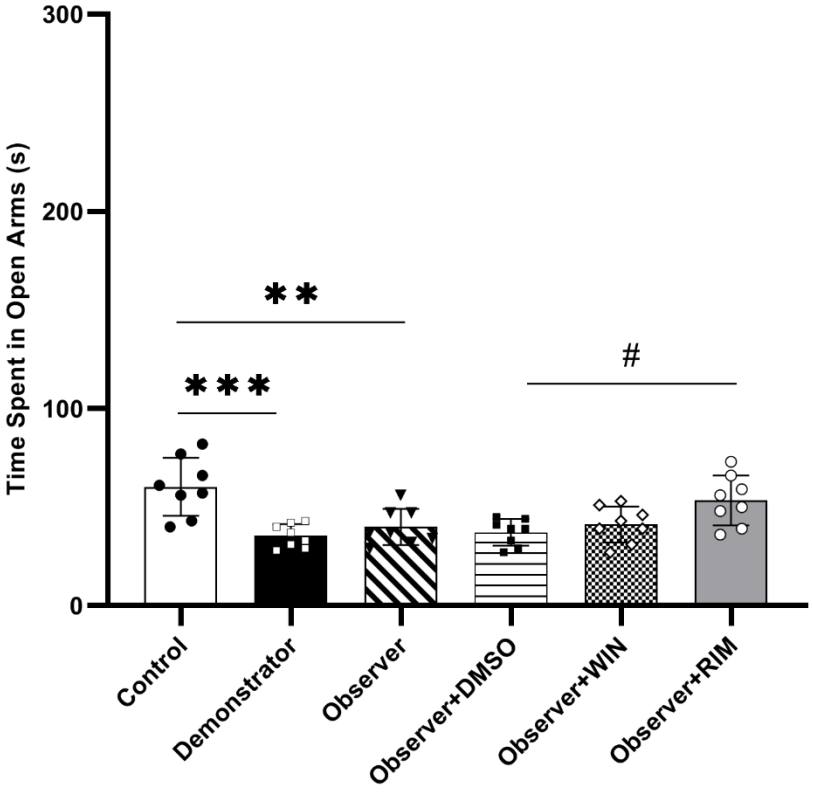
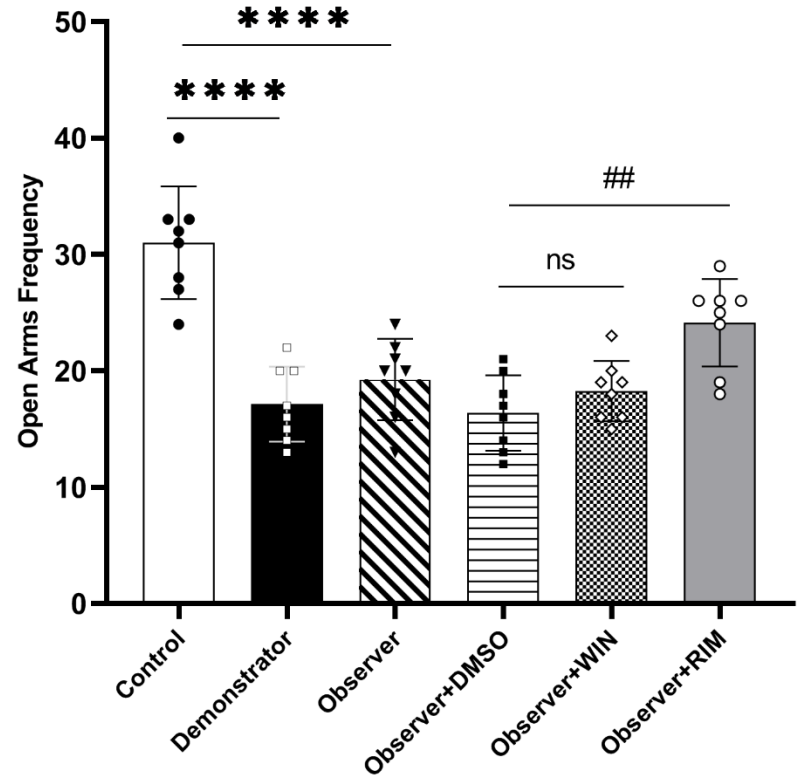
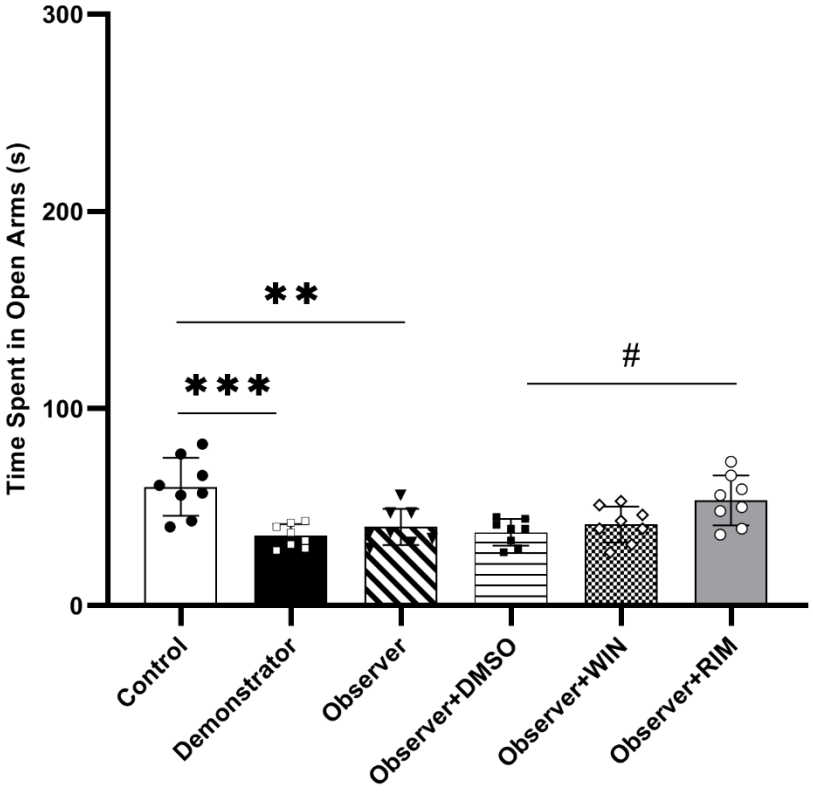


Fig. 3. Effects of the CB_1R agonist (WIN) and antagonist (Rimonabant) on social behavior, including climbing (A), crawling (B), following (C), genital investigation (D), and sniffing (E). The data are shown as the means±SEMs, and n=8 rats in each group. * $P<0.05$, *** $P<0.001$, and **** $P<0.0001$ compared with the control group; # $P<0.05$ and ## $P<0.01$ compared with the Observer +DMSO group.







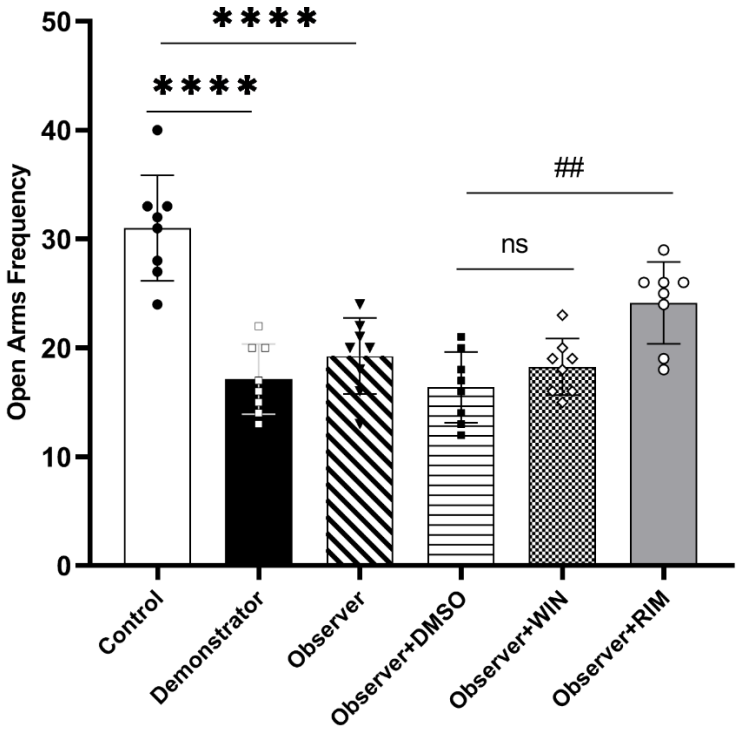


Fig. 4. Effects of the CB_1R agonist (WIN) and antagonist (Rimonabant) on anxiety-like behavior in Observer rats, including time spent in the open arms (A), entry frequency into the open arms (B), time spent in the closed arms (C), and entry frequency into the closed arms (D). The data are shown as the means±SEMs, and n=8 rats in each group. ** $P<0.01$, *** $P<0.001$, and **** $P<0.0001$ compared with the control group; # $P<0.05$ and ## $P<0.01$ compared with the Observer+DMSO group.