Original Article

Cannabinoid System of the Lateral Septum in the Modulation of Anxiety-like Behaviors in Rats

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Abstract

Backgrounds: A large body of evidence suggests that the cannabinoid CB_1 receptor plays a key role in the regulation of emotional behaviors. The present study was designed to evaluate the effects of CB_1 agonist and antagonist on anxiety-like behaviors in the lateral septum (LS) region of the rat brain using elevated plus maze test.

Method: Rats were anesthetized with ketamine and xylazine and special cannulas were inserted stereotaxically into the LS region. After 1 week of recovery, the effects of intra-LS administration of the CB_1 receptor agonist, WIN 55,212-2 and CB_1 receptor antagonist, AM251, on %OAT and %OAE were measured. Moreover, the effects of pretreatment with AM251 on the response induced by intra-LS administration of WIN 55,212-2 were also assessed.

Results: Intra-LS administration of WIN 55,212-2 (0.001, 0.005, and 0.5μg/rat) decreased the %OAT and %OAE but not locomotor activity, showing an anxiogenic-like response. Intra-LS injection of different doses of AM251 (0.001, 0.01, and 0.1 μg/rat) did not significantly alter the anxiety-like parameters on the plus-maze test. However, intra-LS injections of AM251 (0.01 μg/rat) significantly reversed WIN 55,212-2-induced anxiogenic-like effects.

Conclusions: The results suggest that the cannabinoid system of the lateral septum modulates anxiety-like behavior through CB_1 receptor.

Key words: Anxiety, cannabinoid, lateral septum, plus-maze test, rat

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Introduction

Cannabinoids are a class of psychoactive compounds that exert a wide range of effects in a large number of species. Two different cannabinoid receptors have been identified to date: the CB_1 receptor, mainly located in the central nervous system (CNS), and CB_2 receptors which are mostly present in peripheral tissues, mainly in the immune system.[1,2] These cannabinoid receptors are metabotropic receptors coupled to Gi/o proteins, whose activation is followed by an inhibition of adenylyl cyclase activity.[3] CB_1 receptors are particularly present in the cortex, hippocampus, lateral septum, nucleus accumbens, amygdala and periaqueductal gray (dPAG).[4–7]

The cannabinoid system in the CNS is implicated in anxiety, motor activity, nociception, memory and learning, cognitive processes, neuroendocrine regulation, neurodegenerative processes, appetite, body temperature, emesis and brain reward.[8] Moreover, cannabis-based medications have reportedly been helpful in patients with pain, spasticity, nausea, loss of appetite and vomiting.[9]

Cannabinoids modulate the release of several transmitters implicated in the control of anxiety-related behaviors. There is abundant anatomical, neurochemical and functional evidence suggesting that CB_1 receptor activation restrains neuronal activity by inhibiting the release of neurotransmitters associated with anxiety, including glutamate and GABA.[10]

The lateral septal area, considered to interconnect with a number of limbic, diencephalic and midbrain regions, plays a critical role in the modulation of processes related to mood and motivation.[11] The role of the lateral septum in anxiety is reported with controversies. A variety of reports, including electrical stimulation studies, suggest anxiogenic and anxiolytic functions of the lateral septum.[12–14] In this context, microinjection of midazolam or the $GABA_A$ receptor agonist muscimol into the lateral septum produced anxiolytic-like effects through facilitation of GABAergic transmission.[15,16] CB_1 receptor is mainly located presynaptically where it can inhibit the release of other neurotransmitters.[17] Neurons expressing CB_1 receptors are of diverse nature. Studies were able to detect them in terminals that release GABA and glutamate as the main inhibitory and excitatory neurotransmitters, respectively.[18,19]

Previous studies showed that cannabinoid agonists induce complex and often contradictory effects on anxiety in both humans and experimental animals. The mechanisms mediating the effects of cannabinoids on anxiety-related responses appear to be mediated by both CB_1 and non-CB_1 cannabinoid receptors.[20] Considering different sites of the brain which are involved in the cannabinoid-induced anxiety-like behaviors, we hypothesized that the lateral septum could represent another brain site for the cannabinoid system in the modulation of anxiety. Since no study has evaluated the role of lateral septal cannabinoid system in the modulation of anxiety-like behavior, the purpose of the present study is to elucidate the effects of CB_1 receptor agonist and antagonist microinjected into the lateral septum on anxiety-related behaviors.

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Materials and Methods

Animals

Male Wistar rats (Pasteur institute; Amol, Iran) weighing 220 ± 20 g at the time of surgery were used. The animals were housed four/cage, in a colony room with a 12/12–hr light/dark cycle (7:00–19:00) at 22 ± 2%. They had free access to food and tap water except during the time of experiments. Rats were handled for 5 min each day prior to behavioral testing. All experiments were performed between 9:00 h and 13:00 h and each animal was used only once. Seven animals were used in each group of experiments. A total number of 84 animals were used in the experiments. The study protocol was approved by the Local Ethical Committee and was performed in accordance with the Declaration of Helsinki.

Drugs

The drugs used in the present study were WIN 55,212-2 mesylate, AM251 (*N*-(piperidin-1-yl)-5-(4iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide;Tocris, Bristol, UK). WIN 55,212-2 and AM251 were dissolved in dimethyl sulfoxide, sterile 0.9% saline (up to 10% v/v) and two drops of Tween 80.

Stereotaxic surgery

Rats were anesthetized intraperitoneally with ketamine hydrochloride (50 mg/kg) and xylazine (4 mg/kg) and placed in a Stoelting stereotaxic instrument (Stoelting Co, Illinois, USA). According to Paxinos and Watson,[21] stereotaxic coordinates for injection into the lateral septum were: +0.8 anterior to bregma, 0.8 mm lateral to the midline, and 4.8 mm ventral of the dorsal surface of the skull

Microinjections

A stainless steel guide cannula (22-gauge) was unilaterally implanted in the lateral septum, 1 mm above the site of injection. It was then fixed to the skull with acrylic dental cement. To prevent clogging, the stainless steel stylet (27-gauge) was placed in the guide cannula until the animal was given the injection. The animals were allowed seven days to recover before the test. For drug infusion, the stylet was withdrawn and replaced by the injection cannula (27-gauge stainless steel tubing), terminating 1 mm below the tip of the guide. Each injection cannula was connected by polyethylene tubing to a 2-μL Hamilton syringe (Hamilton, Reno, NV, USA). Animals received an injection of 1 μL of each solution over a 60 s period (1 μL/rat). To allow diffusion of the solution, the injection cannula was left in place for an additional 60s. Seven days after implantation, the effects of intra-lateral septum (LS) injection of drugs were tested in the elevated plus-maze test.

Behavioral test (Plus-maze test)

The elevated plus-maze test (EPM), one of the many tests for identification of anxiolytic or anxiogenic-like effects of drugs, exploits the aversion of rodents to novel, elevated and open spaces. The method is basically the same as described by Pellow, et al.[22] The apparatus consists of two open (50 × 10 cm) and two closed (50 × 10 cm) arms in in the shape of a plus sign. The center of the maze is a square platform of 10 × 10 cm. The maze was elevated to a height of 50 cm. At least 1 hour before testing, animals were placed in the room used for experiments. For testing, the rats were placed in the center of the maze facing an open arm and allowed 5 min of free exploration. The following parameters were measured: the number of entries into the open/closed arms and the total time spent in the open/closed arms. After each test, the floor was cleaned with water. Entry was defined as all four paws in the arms. The percentage of open arm entries (%OAE) and open arm time (%OAT) as the standard anxiety indices were calculated as follow: %OAT (the ratio of times spent in the open arms to total times spent in any arms × 100); %OAE (the ratio of entries into the open arms to total entries in any arms × 100). Smaller %OAE and %OAT ratio scores indicate that rats are more anxious. Closed arm entries were considered as animal locomotor activity.[22]

Experiments

Experiment 1: effects of AM251 on anxiety-like behaviors

The animals received intra-LS injection of vehicle or one of the three doses of AM251 (0.001, 0.01 and 0.1 μg/rat). The test session took place5 min after intra-LS injection. %OAT, %OAE and locomotor activity were measured as described in the method section (Figure 1).

Experiment 3: effects of WIN 55,212-2 alone or with AM251 on anxiety-like behaviors

The animals received intra-LS injection of vehicle or one of the three doses of WIN 55,212-2 (0.001, 0.005 and 0.5 μg/rat). Five minutes after the injections, animals were submitted to the EPM test. Four other groups of rats received vehicle or one of three doses of WIN 55,212-2 (0.001, 0.005, and 0.5 μg/rat) 5 min after intra-LS injection of AM251 (0.01 μg/rat). The test session took place 5 min after final intra-LS injections. %OAT, %OAE and locomotor activity were measured (Figure 2).

Verification of cannula placements

After completion of the experimental sessions, each animal was sacrificed with chloroform. Subsequently, 1.0 μL of methylene blue was injected into the lateral septum by injection cannula to aid in histological verification. The brains were removed and fixed in 10% formalin solution 10 days before sectioning. Sections were examined to determine the location of the cannula aimed for the lateral septum. The cannula placements were verified using the atlas of Paxinos and Watson.[21] Data from rats with cannula placement outside the lateral septum were excluded from the analysis (Figure 3).

Statistical analysis

One-way ANOVA was used to compare different experimental groups. Two-way ANOVA was used to evaluate interactions between drugs. Post hoc analysis (Tukey test) was performed to assess specific group comparisons. Statistical significance was set at $P < 0.05$.

Results

Effects of selective CB1 receptor antagonist, AM251 on anxiety-like behaviors

In the first experiment (Figure 1), injection of AM251 changed neither %OAT [$F(3, 24) = 2.64$; $P > 0.05$], nor %OAE [$F(3, 24) = 0.98$; $P > 0.05$] or locomotor activity [$F(3, 24) = 1.71$; $P > 0.05$] significantly. In other words, in these doses, AM251 does not affect animal behavior significantly in the EPM.

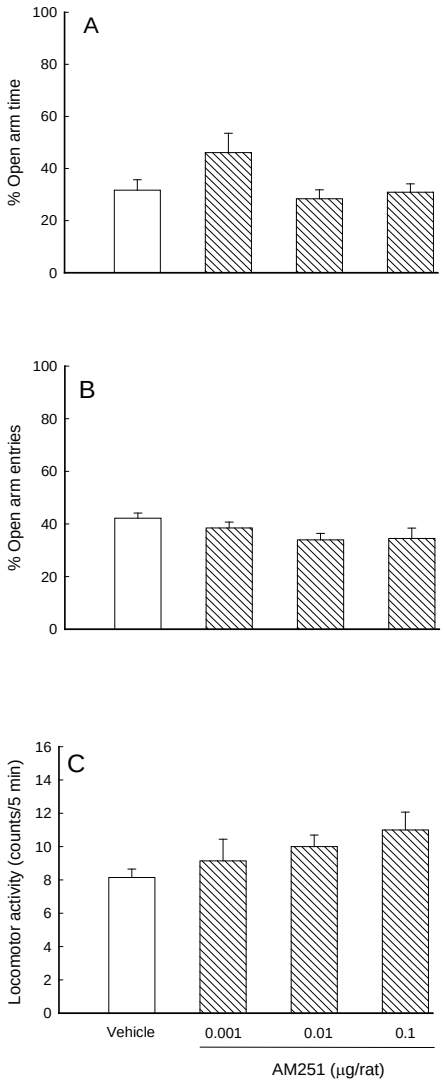

Figure 1. The effects of intra-lateral septum injection of AM251 on anxiety-like behaviors. Rats were injected vehicle (1 μL/rat) or AM251 (0.001, 0.01, and 0.1 μg/rat). The tests were performed 5 min after intra-LS injections. Each bar represents mean ± S.E.M. %OAT **(A)**, %OAE **(B)** or locomotor activity **(C)**. n = 7.

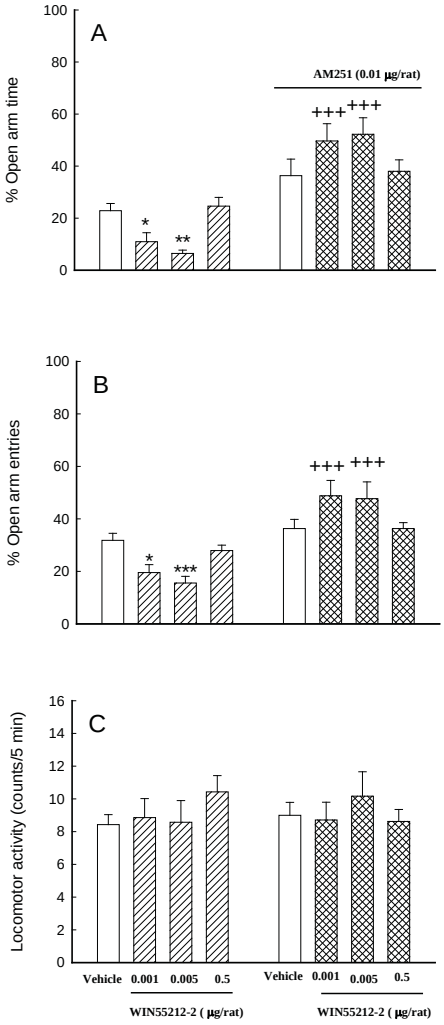

Figure 2. The effects of intra-LS injection of WIN 55,212-2 alone or with AM251 on anxiety-like behaviors. Rats were injected saline (1 μL/rat) or WIN 55,212-2 (0.001, 0.005, and 0.5 μg/rat) alone or 5 min after injection of AM251 (0.01 μg/rat). The tests were performed 5 min after final intra-LS injections. Each bar represents mean ± S.E.M. %OAT **(A)**, %OAE **(B)** or locomotor activity **(C)**. *n* = 7. *: $P < 0.05$, **: $P < 0.01$ compared to the vehicle treated rats. +++: $P < 0$. compared to the WIN 55,212-2 treated rats.

Effects of the cannabinoid receptor agonist WIN 55,21255,212-2 alone or with AM251, on anxiety-like behaviors

In the second experiment (Figure 2), the effects of WIN 55,212-2 alone or with AM251 on anxiety-like parameters were evaluated. A two-way ANOVA between the response of WIN 55,212-2 (0.001, 0.005, and 0.5μg/rat) and WIN 55,212-2 plus AM251 (0.01 μg/rat), using post hoc analysis revealed that WIN 55,212-2 decreased %OAT [$F(3, 48) = 9.87$, $P < 0.001$] and %OAE [$F(3, 48) = 8.3$, $P < 0.01$] indicating an anxiogenic-like response by WIN 55,212-2. No significant change in the locomotor activity was observed following administration of WIN 55,212-2 [$F(3, 48) = 0.26$, $P > 0.05$]. Administration of AM251 at a dose of 0.01 μg/rat 5 min before injection of WIN 55,212-2 was able to reverse the decreased %OAT [$F(3, 48) = 7.27$; $P < 0.001$] and %OAE [$F(3, 48) = 7.41$; $P < 0.001$] induced by WIN 55,212-2 at the dose of 0.001 and 0.005 WIN 55,212-2, but did not change locomotor activity [$F(3,48) = 0.76$; $P > 0.05$]. Post hoc analysis indicated that AM251did not change anxiety-related parameters by itself, but reversed WIN 55,212-2-induced anxiogenic-like response.

Discussion

Elevated plus-maze is one of the most widely used experimental models for investigating anxiety in rodents. In this test, we addressed the aversion of rodents to novel, open and high spaces.[23] Different physiological mechanisms appear to be involved in regulation of anxiety-like behaviors. GABA, serotonin and norepinephrine are neurotransmitters that are mostly implicated in the modulation of anxiety. However, many other neurotransmitters and modulators, including endocannabinoids, endovanilloids,

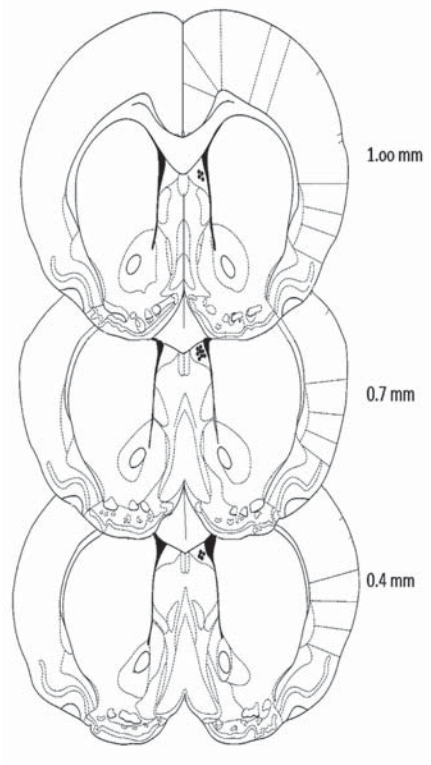

Figure 3. The approximate sites of injected drugs in the lateral septum are presented by circles. Representative sections of the lateral septum (1, 0.7, and 0.4 mm from bregma, respectively) are adopted from Paxinos and Watson.[21]

adenosine, histamine and acetylcholine, have also been implicated.[24,25]

The data of the present study showed that microinjection of WIN 55,212-2, as CB_1 and CB_2 receptor agonist, into the lateral septum decreased the percentage of time spent in the open arms (%OAT) and caused a decrease in the percentage of open arm entries (%OAE) at the doses of 0.001 and 0.005 μg/rat, whereas no significant effect was observed for locomotor activity. However, at the dose of 0.5 μg/rat, the animal's behavior did not change significantly in the EPM. This indicates that WIN 55,212-2 exerts anxiogenic-like effects in the lateral septum at low and medium doses but not at high dose. It is noteworthy that lesions of the lateral septum cause anxiolytic-like effects.[12] Consistent with the present data, it has been shown that intra-CA1 administration of WIN 55,212-2 induces anxiogenic-like effects.[26] Moreover, administration of anandamide, which is an endogenous ligand for cannabinoid receptors, also induced anxiogenic-like responses in three different strains of mice.[27] Anxiogenic responses have also been reported following administration of Delta(9)-tetrahydrocannabinol, as major psychoactive constituent of C*annabis sativa,* in social interaction test.[28] In line with our findings, Arnold *et al.* (2001) reported that the cannabinoid receptor agonist, CP 55,940, increased anxiety-related behaviors in Wistar but not Lewis rats in the conditioned ultrasonic vocalization task representing a genetic basis in cannabinoid-induced anxiety.[29] The same results were reported by Genn, et al. (2004) using CP55,940.[30] In contrast, some previous studies have reported anxiolytic, rather than anxiogenic-like effects for cannabinoid agonists. Microinjection of WIN 55,212-2 into the basolateral amygdala reduced stress-induced rise in corticosterone levels.[31] Furthermore, intra-dPAG administration of 2-Arachidonoylglycerol promoted anxiolytic-like effects in the elevated plus-maze test.[32] In separate studies, intraperitoneal administration of WIN 55,212-2, ACEA and CP55,940 as CB_1 receptor agonists, promoted anxiolytic-like effects, which were suppressed by pretreatment with AM251.[33–35] Interestingly, similar anxiolytic-like effects have been documented for WIN 55,212-2-treated marmoset monkeys in an open-field test.[36] As mentioned before, despite its anxiogenic-like effects at lower doses, intra-LS administration of WIN 55,212-2 at higher dose (0.5 μg/rat) did not affect animal's behavior significantly in the EPM. A similar shift in cannabinoid response at higher dose has been reported elsewhere.[37] These controversies have been attributed to the effects of cannabinoids and endocannabinoids on TRPV1 receptors.[37] Previous studies show that WIN 55,212-2, similar to anandamide, inhibits TRPV1 receptors.[38] Since inhibition of TRPV1 receptors has been linked to anxiolytic-like responses,[25] it may be suggested that at least some of the controversies in those effects of endocannabinoids and cannabinoids are mediated by their interaction with TRPV1 receptors. Therefore, it is possible that inhibition of TRPV1 receptors by WIN 55,212-2 at higher dose hindered its anxiogenic-like effects. This bimodal action or discussed controversies may also be dependent on different brain regions, expression of receptors on different cell-types (GABA vs. glutamate neurons), environmental conditions and species differences (mice vs. rats).[36]

Another part of our results indicate that intra-LS administration of AM251, a selective CB_1 receptor antagonist, altered neither the anxiety-related parameters nor the locomotor activity significantly. These results are consistent with previous studies showing that local injections (intra-dorsal hippocampus, intra-ventral hip-

pocampus, intra-dPAG) of AM251 did not induce any behavioral change in rats exposed to the EPM.[25,26,39] Furthermore, Rutkowska, et al. (2006) reported that intraperitoneal injection of AM281, as a selective CB_1 receptor antagonist, did not influence anxiety-related behaviors of the mice in the light/dark box test when administered alone.[40] Our findings possibly imply that endocannabinoids in the lateral septum are not tonically involved in modulation of anxiety-like behaviors. Similar to the cannabinoid agonists, there is controversy regarding the effects of cannabinoid receptor antagonists on anxiety. There are reports showing anxiolytic-like effects following administration of AM251.[26,36] Moreover, injection of rimonabant, a selective CB_1 receptor antagonist, into the central amygdala of the rats precipitated anxiety-like behaviors.[41] Conversely, Litvin, et al. (2013) showed that AM251 induced anxiety-like effects in both CB_1 knock-out and wild type animals toward a novel male conspecific.[42] Furthermore, this antagonist promoted an anxiogenic-like effect in the elevated T maze at higher dose.[35] These controversies may occur due to the same reasons described previously for cannabinoid agonists. Moreover, both AM251 and rimonanant are antagonist/inverse agonists and part of their effects may be due to their bimodal action on CB_1 receptors. Recent studies show that AM251 is also an agonist of a novel receptor, namely GPR55 (a potential CB_3 receptor).[43] Although the role of this receptor in the modulation of anxiety-like behaviors is unclear. Activation of GPR55 receptors by AM251 is a possible alternative mechanism in those diverse effects of AM251 on anxiety-like behaviors. Another possible mechanism involved in anxiogenic or anxiolytic actions of cannabinoids is the sympathetic nervous system. A recent study showed that rimonabant-induced freezing following both systemic and intracerebroventricular administration requires increased peripheral sympathetic nervous system activation and blockade of peripheral β-adrenergic receptors prevented the anxiogenic effect of both systemic and intracerebroventricular rimonabant injections.[44] This study shows that CB_1 receptor blockade acts centrally to increase peripheral norepinephrine release, and this may in turn lead to anxiety-like behaviors.[44]

In another part of our experiments, we assessed the effects of pretreatment with AM251 (0.01 µg/rat) on the response induced by intra-LS administration of WIN 55,212-2. These data showed that administration of AM251 prevented anxiogenic-like effect of WIN 55,212-2 at low and medium doses. This finding implies that part of the effects of WIN 55,212-2 on anxiety is CB_1 receptor mediated. Moreover, the inability of AM251 to affect higher dose of WIN 55,212-2 is possibly explained by our previous hypothesis regarding the involvement of TRPV1 receptors in the actions of WIN 55,212 at high dose. Therefore, based on our present results, the cannabinoid system in the lateral septum region of the rat brain may play an important role in modulation of anxiety-like behaviors.

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