Modulation of cannabinoid signaling by amygdala α_2 -adrenergic system in fear conditioning

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Abstract

The noradrenergic system plays a critical role in the modulation of emotional state, primarily related to anxiety, arousal, and stress. Growing evidence suggests that the endocannabinoid system mediates stress responses and emotional homeostasis, in part, by targeting noradrenergic circuits. In addition, there is an interaction between the cannabinoid and noradrenergic system that has significant functional and behavioral implications. Considering the importance of these systems in forming memories for fearful events, we have investigated the involvement of basolateral amygdala (BLA) α_2 -adrenoceptors on ACPA (as selective cannabinoid CB1 agonist)-induced inhibition of the acquisition of contextual and auditory conditioned fear. A contextual and auditory fear conditioning apparatus for assess fear memory in adult male NMRI mice was used. Pre-training, intraperitoneal administration of ACPA decreased the percentage freezing time in contextual (at doses of 0.05 and 0.1 mg/kg) and auditory (at dose of 0.1 mg/kg) in the fear conditioning task, indicating memory acquisition deficit. The same result was observed with intra-BLA microinjection of clonidine (0.001-0.5 μ g/mouse, for both memories), as α_2 -adrenoceptor agonist and yohimbine (at doses of 0.005 and 0.05 for contextual and at dose of 0.05 μ g/mouse for auditory fear memory), as α_2 -adrenoceptor antagonist. In addition, intra-BLA microinjection of clonidine (0.0005 μ g/ mouse) did not alter ACPA response in both conditions, while the same dose of yohimbine potentiated ACPA response at the lower dose on contextual fear memory. It is concluded that BLA α_2 -adrenergic receptors may be involved in context- but not tone-dependent fear memory impairment induced by activation of CB1 receptors.

Keywords: ACPA; Clonidine; Yohimbine; Fear memory conditioning; Mice

1. Introduction

It has been suggested that stress effects on fear memory impairment or improvement require simultaneous noradrenergic and glucocorticoid activity in the basolateral amygdala (BLA) [1] and that the direction of the stress effects on fear memory depends on whether stress is experienced within or outside the context of the learning episode [2]. In addition, norepinephrine (NE) actions in modulating memory formation are dependent on the populations of adrenoceptors (ARs) in different brain locations and at different times in the sequence of memory processing [3, 4]. The BLA receives a dense noradrenergic input primarily originating from the locus coeruleus (LC), which is heavily involved in stress and stress-related pathologies [5, 6] and memory formation [7, 8]. Studies have shown that stressful stimuli such as foot shock induce NE release in the rat amygdala [9]. Also, human functional magnetic resonance imaging (fMRI) studies show that elevated NE neurotransmission enhances BLA responses to fear signals [10]. Therefore, the LC is important for providing information about aversive stimuli to the BLA and generating appropriate responses to stressors, which suggests that the LC–NE circuit in the BLA could be a potential drug target for anxiety disorders [11]. NE can interact with three families of ARs: α_1 , α_2 and $\beta_{(1-3)}$ receptors that exhibit different signal transduction. Like the β -adrenoceptor, the α_1 -adrenoceptor subtype is located post-synaptically and activated by NE. In contrast, α_2 -adrenoceptors are predominantly located pre-synaptically [12] and activation of these receptors inhibits the release of NE [13].

Growing evidence suggests an interaction between the cannabinoid and noradrenergic system that has significant functional and behavioral implications. Importantly, cannabinoids can modulate noradrenergic transmission in both noradrenergic nuclei and target regions. This modulation seems to be circuit specific and may depend on the basal status of cannabinoid and NE levels. In addition, NE seems to be important for particular cannabinoid-induced behaviors [5]. Manipulation of the endocannabinoid system results in effects on mood and cognition that

share similarities with the noradrenergic system. Briefly, increasing endocannabinoid tone has been shown to improve mood similar to increasing noradrenergic tone with antidepressants [14]. Taken together, the effects of manipulating the endocannabinoid system and modulating noradrenergic transmission suggest that the two systems may interact or share some common signaling pathways [5].

Augmented behavioral responses to aversive stimuli, including freezing, has been reported in a number of different learning and memory paradigms in animals subjected to prior stressful experiences [15, 16]. The memory of learned fear can be assessed quantitatively using a Pavlovian fear-conditioning paradigm [17]. There is evidence suggests that both acute and chronic stress enhance fear conditioning, but the degree to which they might affect learning and memory processes requires more extensive analysis [18].

Given this background knowledge, the present study was designed to evaluate the role of α_2 -adrenoceptors of the BLA in mediating arachidonylcyclopropylamide (ACPA; selective CB1 cannabinoid receptor agonist) fear learning. Therefore, we studied whether ACPA-induced fear learning deficit could be affected by intra-BLA microinjections of α_2 -adrenoceptor agonist/antagonist.

2. Material and methods

2.1. *Animals*

All animal care and experimental studies were in compliance with the international laws on animal experimentation and approved by the committee of Ethics of Tehran University of Medical Sciences, Tehran, Iran. All procedures were carried out in accordance with the guidelines for animal care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 2010). Male NMRI mice were used in all experiments (Institute

for Cognitive Science Studies; Tehran, Iran). At the beginning of the experiments, mice were weighed 28–33 g. The animals were maintained under controlled temperature ($22 \pm 2^{\circ}\text{C}$) and light (light–dark cycle from 8:00 to 20:00 h), with free access to food and water. The experiments were carried out between 9:00 am and 14:00 pm. The same conditions were maintained for all the behavioral tests.

2.2. Cannulae implantation

Prior to surgery mice were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) plus xylazine (5 mg/kg) and fixed in a stereotaxic frame. A stainless steel guide cannula (22 gauge) was implanted bilaterally in the amygdaloid complex (Basolateral amygdala nuclei). Stereotaxic coordinates for the guide cannulae placements were anterior–posterior (AP) = -1.5 mm from bregma, medial–lateral (ML) = ± 2.8 mm, and dorsal–ventral (DV) = -4.5 mm [19]. Guide cannulae were anchored to the skull with screws and dental acrylic. At the end of the surgery each cannulae was temporarily sealed with a stainless steel wire to protect it from obstruction. Animals were given 5-7 days of post-operative recovery prior to the start of behavioral training. Cannulae placements were verified by dye (a 0.3 μl /side; 1% aquatic methylene blue solution) injections followed by coronal sectioning on the vibroslice. Animals in which the cannulae showed an incorrect placement were removed from the analyses.

2.2. Treatments

Drugs used were: cannabinoid CB1 receptor agonist, ACPA [Arachidonylcyclopropylamide] and α_2 -adrenoceptor antagonist, yohimbine from Tocris, UK; and α_2 -adrenoceptor agonist; clonidine hydrochloride from Sigma Chemical Co., St Louis, CA, USA. Clonidine and yohimbine were dissolved in sterile 0.9% saline, while ACPA was stored at -20°C as a prepared solution dissolved in anhydrous ethanol at a concentration of 5

mg/ml and then was diluted to the required volume using sterile 0.9% saline before related experiments. The highest concentration of ethanol and DMSO contained in a dose was used as the control vehicles 1 and 2, respectively. ACPA was administered intraperitoneally at 10 ml/kg, while other drugs were microinjected into the BLA at the volume of 0.3 μ l/ each side. The wait time for test sessions after drug injection was 5 min. In combination studies, each animal received intraperitoneal administration of ACPA, and after 5 min intra-BLA injection of α_2 -adrenoceptor agents. Each mouse was tested once.

2.3. *Intra-BLA microinjections*

The animals were gently held in hand. A thin dental needle (27 gauge) was introduced through the guide cannula until its lower end was 1 mm below its tip. The injection needle was connected to a 2.5 μ l syringe Hamilton by a polyethylene tube. A volume of 0.3 μ l of the drug was injected over 60 s. The displacement of an air bubble inside the polyethylene tubing was used to monitor the microinjection.

2.4. *Behavioral assessment*

Fear conditioning was conducted using a protocol developed by Shoji and colleagues [20] for assessing conditioned fear in the mice. The fear conditioning apparatus consisted of a square chamber (55 cm $\times$ 53 cm $\times$ 67 cm) with acoustic walls and a light source, two sound generators, and video camera attached to the ceiling above the apparatus. During training, mice were placed in a square Plexiglas chamber (25 cm $\times$ 25 cm $\times$ 25 cm) with an electrifiable grid floor, which constantly illuminated (24 w) the fear conditioning chamber and they are typically allowed to freely explore the chamber for 120 sec. Thereafter, a tone (conditioned stimulus, CS, 4 kHz, 35 dB) is presented for 30 sec, and a footshock (unconditioned stimulus, US, 1 mA, 50 Hz) is

given to the mice during the last 2 sec of the sound. Mice were removed from the fear conditioning box 30 s after shock termination in order to avoid aversive association with the handling procedure. Before entrance of each mouse into the task, box was thoroughly cleaned via 70% ethanol solution.

In order to test context dependent retention, animals were placed in the same conditioning box 24 after first day for a period of 300s without CS and US presentations.

Tone dependent memory test was conducted one hour after the context dependent memory assessment. In this test, mice are placed into another testing chamber with very different properties in texture, bedding and shape, providing a novel context that is unrelated to the conditioning chamber. The procedure was repeated with CS presentation for 3 min. A video was used to record the animal behaviors. The observed behaviors were manually recorded as raw data. Freezing, defined as the lack of movement besides respiration and heartbeat, was recorded using a stopwatch. Latency to the freezing, grooming and rearing were scored as percentages of the time during which these behaviors were respectively expressed. Experiments were carried out by someone blinded to the experimental groups.

2.5. Experimental design

2.5.1. Experiment 1: effect of pre-training microinjection of clonidine or yohimbine into the BLA on contextual and auditory conditioned fear learning

In this experiment, 9 groups of animals received intra-BLA microinjection of saline (0.6 μ l/mouse), clonidine (0.0005, 0.001, 0.005 and 0.05 μ g/mouse) or yohimbine (0.0005, 0.001, 0.005 and 0.05 μ g/mouse) 5 min before training. Contextual and auditory conditioned fear memory retention tests were performed 24 h after training; context followed by tone after a 1 hour interval.

2.5.2. Experiment 3: Effect of pre-training intra-BLA microinjection of saline, a subthreshold dose of clonidine or yohimbine on ACPA-induced inhibition of the acquisition of contextual and auditory conditioned fear

In this experiment, 12 groups of animals were used. The animals were divided into three sets of four groups. The mice received vehicle (10 ml/kg) or different doses of ACPA (0.005, 0.05 and 0.1 mg/kg) 15 min before training, intraperitoneally. These treated groups also received saline (0.6 µl/mouse; four groups), clonidine (0.0005 µg/mouse; four groups) or yohimbine (0.0005 µg/mouse; four groups) into the BLA, 5 min before starting of training session. Contextual and auditory fear memory retention tests were performed 24 h after training. The assessment of auditory-memory retention was done 1 h after context condition.

2.6. Statistical analysis

Data were expressed as mean $\pm$ SEM. Analysis of data was performed using one-way and two-way analyses of variance (ANOVA). In combination studies, two-way ANOVA was performed with four levels (0, 0.005, 0.05 and 0.1 mg/kg) of ACPA as intra-group factor, and two levels: clonidine vs. saline and yohimbine vs. saline as inter-group factors. Following a significant F-value, post-hoc analysis (Tukey-test) was performed for assessing specific inter-group comparisons. In all cases, a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of pre-training microinjection of clonidine or yohimbine into the BLA on contextual conditioned fear learning

Fig. 1A-D (left panel) shows the effect of pre-training intra-BLA microinjection of clonidine on contextual conditioned fear learning. One-way ANOVA and Tukey's test post-hoc

comparisons indicated a marked decrease in %freezing [$F(4, 35) = 9.75, P < 0.001$; at doses of 0.001, 0.005 and 0.05 $\mu\text{g}/\text{mouse}$], while increased latency [$F(4, 35) = 10.78, P < 0.001$; at doses of 0.005 and 0.05 $\mu\text{g}/\text{mouse}$]. Similar analysis showed that this intervention did not alter % rearing [$F(4, 35) = 3.52, P < 0.05$] and % grooming [$F(4, 35) = 0.69, P > 0.05$]. It should be noted that, however there is a significant value for F ANOVA result of %rearing, post hoc analysis did not show any significant level for treated groups vs. control in this parameter.

However, Fig. 1A-D (right panel) shows the effect of pre-training intra-BLA microinjection of yohimbine on contextual conditioned fear learning. One-way ANOVA and post hoc analysis indicated that yohimbine decreased %freezing [$F(4, 35) = 12.22, P < 0.001$; at doses of 0.005 and 0.05 $\mu\text{g}/\text{mouse}$], while increased latency [$F(4, 35) = 12.32, P < 0.001$; at dose of 0.05 $\mu\text{g}/\text{mouse}$]. Similar analysis showed that this intervention did not alter % rearing [$F(4, 35) = 6.32, P < 0.01$] and % grooming [$F(4, 35) = 1.96, P > 0.05$].

[Fig. 1 somewhere near here]

3.2. Effect of pre-training microinjection of clonidine or yohimbine into the BLA on auditory conditioned fear learning

Fig. 2A-D (left panel) shows the effect of pre-training intra-BLA microinjection of clonidine on auditory conditioned fear learning. One-way ANOVA and Tukey's test post-hoc comparisons indicated a marked decrease in %freezing [$F(4, 35) = 18.69, P < 0.001$; at doses of 0.001, 0.005 and 0.05 $\mu\text{g}/\text{mouse}$] and % rearing [$F(4, 35) = 4.18, P < 0.01$; at doses of 0.001 and 0.005 $\mu\text{g}/\text{mouse}$], while increased % grooming [$F(4, 35) = 6.07, P < 0.01$; t doses of 0.005 and 0.05 $\mu\text{g}/\text{mouse}$] but did not alter latency [$F(4, 35) = 1.19, P > 0.05$].

However, Fig. 2A-D (right panel) shows the effect of pre-training intra-BLA microinjection of yohimbine on auditory conditioned fear learning. One-way ANOVA and Tukey's test post-hoc comparisons indicated a decrease in %freezing [$F(4, 35) = 3.27, P < 0.05$;

at dose of 0.05 µg/mouse] and % rearing [$F(4, 35) = 13.31, P < 0.001$; at doses of 0.001 and 0.005 µg/mouse], while increased % grooming [$F(4, 35) = 2.82, P < 0.05$; at dose of 0.05 µg/mouse] and latency [$F(4, 35) = 7.58, P < 0.001$; at dose of 0.05 µg/mouse].

[Fig. 2 somewhere near here]

3.3. Effect of ACPA in the absence or presence of either clonidine or yohimbine on the acquisition of contextual conditioned fear

Fig. 3A-D (left panel) shows the effect of pre-training intraperitoneal administration of ACPA on the acquisition of contextual conditioned fear. One-way ANOVA and Tukey's test post-hoc comparisons indicated ACPA decreased %freezing [$F(3, 28) = 15.10, P < 0.001$; at doses of 0.05 and 0.1 mg/kg], while increased % grooming [$F(3, 28) = 7.43, P < 0.01$] and latency [$F(3, 28) = 2.81, P > 0.05$; at dose of 0.1 mg/kg] but not % rearing [$F(3, 28) = 1.30, P > 0.05$].

Two-way ANOVA and post hoc analysis indicated that clonidine did not alter ACPA response on %freezing [intra-groups: $F(3, 56) = 18.80, P < 0.001$; inter-groups: $F(1, 56) = 4.36, P < 0.05$; inter-intra groups interaction: $F(3, 56) = 1.57, P > 0.05$; Fig. 3A, middle panel], % rearing [intra-groups: $F(3, 56) = 0.56, P > 0.05$; inter-groups: $F(1, 56) = 6.37, P < 0.05$; inter-intra groups interaction: $F(3, 56) = 0.72, P > 0.05$; Fig. 3C, middle panel] and latency [intra-groups: $F(3, 56) = 4.69, P < 0.01$; inter-groups: $F(1, 56) = 0.33, P > 0.05$; inter-intra groups interaction: $F(3, 56) = 0.81, P > 0.05$; Fig. 3D, middle panel], while decreased ACPA response on % grooming [intra-groups: $F(3, 56) = 5.25, P < 0.01$; inter-groups: $F(1, 56) = 11.28, P < 0.01$; inter-intra groups interaction: $F(3, 56) = 8.46, P < 0.001$; Fig. 3B, middle panel].

In addition, two-way ANOVA and post hoc analysis indicated that yohimbine increased ACPA response on % freezing [intra-groups: $F(3, 56) = 37.90, P < 0.001$; inter-groups: $F(1, 56) = 31.06, P < 0.001$; inter-intra groups interaction: $F(3, 56) = 2.41, P > 0.05$; Fig. 3A, right

panel] and latency [intra-groups: $F(3, 56) = 7.84$, $P < 0.001$; inter-groups: $F(1, 56) = 18.00$, $P < 0.001$; inter-intra groups interaction: $F(3, 56) = 1.68$, $P > 0.05$; Fig. 3D, right panel], while decreased ACPA response on % grooming [intra-groups: $F(3, 56) = 5.01$, $P < 0.01$; inter-groups: $F(1, 56) = 0.65$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 7.10$, $P < 0.001$; Fig. 3B, right panel] but did not alter ACPA response on % rearing [intra-groups: $F(3, 56) = 2.40$, $P > 0.05$; inter-groups: $F(1, 56) = 2.32$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 0.20$, $P > 0.05$; Fig. 3C, right panel].

[Fig. 3 somewhere near here]

3.4. Effect of ACPA in the absence or presence either clonidine or yohimbine on the acquisition of auditory conditioned fear

Fig. 4A-D (left panel) shows the effect of pre-training intraperitoneal administration of ACPA on the acquisition of auditory conditioned fear. One-way ANOVA and Tukey's test post-hoc comparisons indicated that ACPA decreased %freezing [$F(3, 28) = 7.06$, $P < 0.01$; at dose of 0.1 mg/kg] but not % rearing [$F(3, 28) = 3.00$, $P < 0.05$], % grooming [$F(3, 28) = 0.08$, $P > 0.05$] and latency [$F(3, 28) = 1.31$, $P > 0.05$].

Two-way ANOVA and post hoc analysis indicated that clonidine did not alter ACPA response on % freezing [intra-groups: $F(3, 56) = 15.11$, $P < 0.001$; inter-groups: $F(1, 56) = 0.30$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 1.66$, $P > 0.05$; Fig. 4A, middle panel], % grooming [intra-groups: $F(3, 56) = 1.03$, $P > 0.05$; inter-groups: $F(1, 56) = 0.99$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 0.53$, $P > 0.05$; Fig. 4B, middle panel], % rearing [intra-groups: $F(3, 56) = 2.38$, $P > 0.05$; inter-groups: $F(1, 56) = 0.01$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 1.94$, $P > 0.05$; Fig. 4C, middle panel] and latency [intra-groups: $F(3, 56) = 4.80$, $P < 0.01$; inter-groups: $F(1, 56) = 0.12$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 0.93$, $P > 0.05$; Fig. 4D, middle panel].

In addition, two-way ANOVA and post hoc analysis indicated that yohimbine increased ACPA response on %grooming [intra-groups: $F(3, 56) = 1.83$, $P > 0.05$; inter-groups: $F(1, 56) = 8.74$, $P < 0.01$; inter-intra groups interaction: $F(3, 56) = 2.85$, $P < 0.05$; Fig. 4B, right panel] but not % freezing [intra-groups: $F(3, 56) = 13.84$, $P < 0.001$; inter-groups: $F(1, 56) = 11.59$, $P < 0.01$; inter-intra groups interaction: $F(3, 56) = 1.33$, $P > 0.05$; Fig. 4A, right panel], % rearing [intra-groups: $F(3, 56) = 7.25$, $P < 0.001$; inter-groups: $F(1, 56) = 3.75$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 1.03$, $P > 0.05$; Fig. 4C, right panel] and latency [intra-groups: $F(3, 56) = 3.35$, $P < 0.05$; inter-groups: $F(1, 56) = 2.96$, $P > 0.05$; inter-intra groups interaction: $F(3, 56) = 0.31$, $P > 0.05$; Fig. 4D, right panel].

[Fig. 4 somewhere near here]

4. Discussion

Experiment 1 and 2 demonstrate that pre-training intra-BLA microinjection of clonidine or yohimbine, as α_2 -adrenoceptor agonist or antagonist, decreased freezing time percentage in the both contextual and auditory fear conditionings, suggesting an inhibition of the acquisition of contextual and auditory conditioned fear memories. The higher dose of above mentioned drugs increased latency to the freezing. One interpretation of this effect is that learning is the basis of memory. If there is no learning, there can be no memory later [21]. Thus, a long-lasting delay was observed in performance of freezing behavior. However, two higher doses of clonidine or the highest dose of yohimbine increased % grooming, while two middle doses of clonidine or yohimbine increased % rearing in the auditory fear conditioning. It has been reported that stressors such as electric shock, loud noise, tail-pinch, and immobilization as well as stress hormones activate noradrenergic input to the basolateral amygdala (BLA) from locus coeruleus (LC) [22-27]. In addition, some papers have confirmed the role of the noradrenergic projection to the BLA in memory processing [28]. This modulatory role is complex and extensive behavioral pharmacological evidence suggests that it occurs by interaction with

opioids, corticosteroids, acetylcholine, dopamine and other neurotransmitters within the BLA. There is evidence shows that norepinephrine (NE) facilitates GABA release through action at both α_1 and β receptors; as a result, noradrenergic input to the BLA exerts a tonic inhibitory influence on this structure [9]. On the other hand, α_{2A} ARs are expressed in the amygdala where they are located on both the postsynaptic amygdala neurons or presynaptically on noradrenergic terminals [29]. Thus, NE release from the ascending noradrenergic tracts is also under presynaptic control of both α_{2C} and α_{2A} ARs [30], where they act as autoreceptors on noradrenergic terminals. It has been reported that α_2 agonists such as dexmedetomidine may reduce anxiety and the responses to the memories of a stressful situation [31], whereas α_2 antagonists such as yohimbine increase anxiety in posttraumatic stress disorder (PTSD) patients [32] and to enhance fear memory when infused into rat amygdala [13]. Yohimbine is specific in its antagonism of the α_2 “inhibitory” subtype, and is used clinically as a probe to identify abnormal physiological and affective responses to increased noradrenergic signaling. Although yohimbine has been suggested or reported to interact with receptors for other monoamines [33], the α_2 receptor agonist clonidine reverses yohimbine-induced anxiety, and the ability of yohimbine to increase cFos expression and establishment of long-term memory formation in the cortex, amygdala, and other brain regions in rats is attenuated or blocked by pretreatment with clonidine or propranolol, a post-synaptic β -adrenergic receptor antagonist [34-36]. Yohimbine increases neurotransmitter release from noradrenergic axon terminals by blocking presynaptic feedback inhibition of release, and simultaneously reduces the ability of NE to exert inhibitory post-synaptic effects while leaving the excitatory effects of NE at postsynaptic α_1 and β receptors unopposed [37]. It seems that clonidine via the activation of presynaptic α_2 -adrenoceptors inhibits NE release induced by electric foot shock. In this way, clonidine impairs fear memories. In addition, it may be suggest that yohimbine via blocking of presynaptic α_2 -adrenoceptors increases NE release. It is likely that electric shock stress also reinforce yohimbine effect on NE release. Thus, NE release at high concentration may activate

postsynaptic α_2 -ARs in addition to α_1 - and β -ARs. Activation of inhibitory signal transduction pathways due to activation of postsynaptic α_2 -ARs may inhibit the effect of NE postsynaptic α_1 - and β -ARs. These events may produce a fear memory deficit.

Experiment 3 and 4 illustrate that pre-training intraperitoneal administration of ACPA, as a selective cannabinoid CB1 receptor agonist, inhibited the acquisition of the both contextual and auditory memories. The higher dose of the drug increased % grooming and latency to the freezing in the contextual fear conditioning. Moreover, pre-training intra-BLA microinjection of a subthreshold dose of clonidine did not alter ACPA response in both conditions, while a subthreshold dose of yohimbine potentiated ACPA response at the lower dose in contextual fear conditioning. Subthreshold dose of clonidine or yohimbine decreased ACPA response at the higher dose on % grooming in the contextual fear conditioning, while yohimbine increased ACPA (0.05 μ g/mouse) response on % grooming in the auditory fear conditioning. In addition, yohimbine increased ACPA response at two lower doses on latency to the freezing. The effects of (endo) cannabinoid signalling on behavior during the initial acquisition phase of conditioned fear have not been studied extensively. In wild type mice, one study reported increased freezing during the conditioning session after injection of the CB1 receptor antagonist AM251 [38]. This effect seemed to be specific for paired conditioned stimulus (CS)–unconditioned stimulus (US) presentations, as it was not seen when the mice received presentations of the CS only (tone) or the US only (1 s, 0.8 mA footshocks) [38], which appears to exclude an effect on pain processing. The possible involvement of the endocannabinoids in early phases of fear memory processing was also suggested by the finding of a 20% increase in CB1 receptor mRNA expression in the infralimbic cortex 48 hours after contextual fear conditioning [39]. A proper interpretation of the participation of the endocannabinoids in the phase of fear memory acquisition can be compromised by the fact that the endocannabinoids regulates pain processing [40]. CB1 receptors are coupled to Gi/o, as are α_2 receptors, so these two receptor systems may converge on the same signal transduction pathways in cells where they are co-localized, making

cross-regulation possible [41]. Regarding the proposed mechanism for yohimbine effect, it seems that yohimbine may potentiate the inhibitory effect of ACPA on GABAergic neurons. Thus, combination of subthreshold doses of yohimbine and ACPA produce a fear memory impairment.

Acknowledgement

Authors wish to thank the Iran National Science Foundation (INSF) for providing financial support to this project.

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Legends

Fig. 1: Effect of pre-training microinjection of clonidine or yohimbine into the BLA on contextual conditioned fear learning. Nine groups of animals received intra-BLA microinjection of saline (0.6 μ l/mouse), clonidine (0.0005, 0.001, 0.005 and 0.05 μ g/mouse) or yohimbine (0.0005, 0.001, 0.005 and 0.05 μ l/mouse) before training. Contextual fear memory retention test was performed 24 h after training. % freezing (A), % grooming (B), % rearing (C) and latency to the freezing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). **P < 0.01 and ***P < 0.001 different from saline control group.

Fig. 2: Effect of pre-training microinjection of clonidine or yohimbine into the BLA on auditory conditioned fear learning. Nine groups of animals received intra-BLA microinjection of saline (0.6 μ l/mouse), clonidine (0.0005, 0.001, 0.005 and 0.05 μ g/mouse) or yohimbine (0.0005, 0.001, 0.005 and 0.05 μ l/mouse) before training. Auditory fear memory retention test was performed 24 h after training. % freezing (A), % grooming (B), % rearing (C) and latency to the freezing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). **P < 0.01 and ***P < 0.001 different from saline control group.

Fig. 3: Effect of pre-training intra-BLA microinjection of a subthreshold dose of clonidine or yohimbine on ACPA response in the contextual fear conditioning. 12 groups of animals received ACPA (0, 0.005, 0.05 and 0.1 mg/kg) plus saline (0.6 μ l/mouse), clonidine (0.0005 μ g/mouse) or yohimbine (0.0005 μ g/mouse) before training. Contextual fear memory retention test was performed 24 h after training. % freezing (A), % grooming (B), % rearing (C) and latency to the freezing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). *P < 0.05 and ***P < 0.001 different from saline control group; +++P < 0.001 different from saline/ACPA respective group, \$P < 0.05, \$\$P < 0.01 and \$\$\$P < 0.001 different from saline/ACPA respective group.

Fig 4: Effect of pre-training intra-BLA microinjection of a subthreshold dose of clonidine or yohimbine on ACPA response in the auditory fear conditioning. 12 groups of animals received ACPA (0, 0.005, 0.05 and 0.1 mg/kg) plus saline (0.6 μ l/mouse), clonidine (0.0005 μ g/mouse) or yohimbine (0.0005 μ g/mouse) before training. Auditory fear memory retention test was performed 24 h after training. % freezing (A), % grooming (B), % rearing (C) and latency to the freezing (D). Values are expressed as mean $\pm$ S.E.M (n = 8 in each group). ***P < 0.001 different from saline control group; \$P < 0.05 different from saline/ACPA respective group.

Fig. 1

Fig. 2

Fig. 3

Fig. 4