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The role of 5-HT1 receptors of central amygdala upon ACPA-induced anxiolytic-like behaviors and amnesia in rat

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Abstract

Cannabinoidergic as well as serotonergic systems of the amygdala modulates anxiety like behaviors and emotional memory. The effects of cannabinergic system on the anxiety and learning process relate to the central amygdala and its interaction with serotonergic system have not studied yet. With the aim of evaluating the regulating effects of cerotonergic system on the anxiolytic effects of cannabinoids and the possibility of restoring cannabinoid caused amnesia by certonergic system, the current research was conducted using the elevated plus-maze (EPM) test-retest paradigm in the male mice. The bilateral guide-cannulae were implanted to allow intra-CeA microinjection of serotonergic agents. The formation of emotional memory was declined by intra-CeA injection of ACPA, but the anxiety reaction was not affected by the various doses of the mentioned substance. Intra-CeA injection of CP94253 Hydrocholoride, a 5-HT1 serotonin receptor agonist, diminished the formation of emotional memory and the locomotor activity, while 5-HT1 serotonin receptor antagonist, did not influence the anxiety-like behaviors and the emotional memory formation. According to the obtained findings from the current research, it seems that ACPA may trigger anxiolytic-like behaviors and prevent the emotional memory formation, mainly via the activation and deactivation of the CeA 5-HT1 serotonin receptors.

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Introduction

Without debut learning is regarded as one of the most important mental process. Learning literally occurs via experiment and exercise resulting in permanent alterations in behaviors, not reactions like, exhausting or adaptation to darkness (Heimer and Van Hoesen, 2006) In fact, the brain capability to save past experiments, events and better acclimation in the future is considered as its main characteristic. Sensory input located in the prefrontal cortex, from cortex to hypocampus and parahypocampus of the limbic system are of the importance in learning.

In human and animals, anxiety is a warning signal about the possible occurrence of danger, thereby preparing to counteract a threat (Belzung, 2001). According to the definition presented by Barlow (2002), anxiety is different from the fear; anxiety directs future behavioral reactions related to the possible close unfavorable events, whereas the fear is a warning response to exist danger felt (Davis, 2006). A cascade of biochemical and endocrine process are triggered by anxiety and stress, physiologically complicated reactions in organisms (Nutt *et al.*, 2001).

Cannabinoids with the vast pharmacological effects in human and other mammals are effective drugs in the *central nervous system* (CNS). There are two well known receptors, CB1 and CB2, for cannabinoids. CB1 receptors concentrate in the central zone and mediate many cannabinoid related effects (Hajos and Freund, 2006). CB1 receptors belonging to Gprotein-coupled family (Nasehi *et al.*, 2010) diffuse vastly in cortex, basal ganglia and amygdale, as well as its implications in the physiological and behavioral actions of cannabinoid have been well documented(Zarrindast *et al.*, 2012). In addition, recently the existence of receptors called CB3 in the nervous system has been reported(Begg *et al.*, 2005). As the activation of the endocannabinoid system occurred under the learning state modulates synaptic plasticity during the initiation process, the injection of the selective antagonist, adversely interacts with the mentioned system (De Oliveira Alvares *et al.*, 2005).

Endocannabinoids act a critical neuromodelatory role in many behavioral related reactions, including movement, anxiety, learning and memory (Lichtman *et al.*, 2002; Pacher *et al.*, 2006). The studies conducted on the mice with gene deficiencies in CB1 receptors revealed that excitements in CB1 receptors caused by cannabinoids were responsible for the memory destruction and cannabinoid system may facilitate amnesia (Varvel and Lichtman, 2002). Also, the recorded symptoms of anxiety in mice lack of CB1 receptors (Zarrindast *et al.*, 2010) and anxiety responses resulted from the application of cannabinoid ligands (Rezayof *et al.*, 2010) confirm this hypothesis that endocannabinoids are involved in the expression of anxiety behaviors(Ghiasvand *et al.*, 2009; Ghiasvand *et al.*, 2011), the destruction in short term memory, the spatial learning and anxiety-like behaviors(Zarrindast *et al.*, 2010; Degroot *et al.*, 2005; Lichtman, 2002) .

Serotonin plays vital roles in the physiological and pathological process. Transportation of serotonin possesses crucial effects in the control and regulation of mood, impulse, sleep, eating, libido and cognitive functions like learning and memory, mainly via serotonin receptors. As some of the agonists of the 5-HT1 receptor have anxiolytic effects (Nasehi *et al.*, 2010), this receptor implicates in the occurrence of anxiety disturbances. Also the function of 5HT1 of the selective ligand had the sufficient influence in the various tests related to the learning and memory (Smith *et al.*,1999; Luttgen *et al.*, 2005). At least, fifteen presynaptic and postsynaptic serotonin receptors have been so far known, classified in seven families based on their structure, activation and affinity to the different ligands as well as called $5HT_1R$ to $5HT_7R$ (Bruss *et al.*, 2000). These receptors, which are G-coupled protein, leave out for $5HT_3R$ activates adenyl cyclase, the enzyme responsible for producing of CAMP, thereby increasing the activity of CAMP-dependent protein kinase (Miquel *et al.*, 1995).

The phosphorylation of the involved enzymes by these protein kinases modulate the activity of the ion channels and consequently depolarization of

serotonin receptors occur. G_i-coupled 5-HT_1 inhibits adenyl cyclase, thereby hyperpolarizing 5-HT_1. G_q-coupled 5-HT_2 activates phospholipase C (Lam *et al.*, 2010) At least two types of serotonin receptors, including 5-HT1A, B located intensively in hypocamp, ceptom, amygdala and raphe nucleus are implicated in the expression of behaviors related to learning and anxiety (Luttgen *et al.*, 2005; Madjid *et al.*, 2006; Zarrindast *et al.*, 2010). It has been stated that vast varieties of cognitive functions, including reward, feeling, learning, memory, attention and thought correlate by amygdala (Baxter *et al.*, 2006). The receptors of the both drug types, cannabinoid and serotonine, have similarity in intracellular signal transduction mechanism, reductions in CAMP, mainly via the activation of G1 proteins (Zarrindast *et al.*, 2010).

Amygdala possesses a key role in the regulation and control of autonomical neuroendocrinal reactions and behavior related to the anxiety (Zarrindast *et al.*, 2010; Jafari *et al.*, 2007; Rezayof *et al.*, 2007). Central amygdala determines behaviors and alterations in hypocamp function related to stress and emotional memory (Schafe *et al.*, 2005). It has been proposed that the high expression rates of both cannabinoid and serotonin receptors in the amygdala, the interaction and correlation of these amygdala systems regulate anxiety and memory process (Casswell *et al.*,1973; LeDoux, 1998; Marco *et al.*, 2009). The existence of neuronal cycle related to the anxiety behaviors and memory process in amygdala has been established by the implications of the basal lateral amygdala in the control of anxiety, fear and learning reactions (Sarkisyan *et al.*, 2009). The various brain structures seem to be involved in the regulation of different types of anxiety, memory and learning. One of the most critical zone in the control of anxiety related behaviors and learning reaction is Amygdala (Pellow *et al.*, 1986; Haj-Dahmane *et al.*, 2011). The key role of amygdala in the organization and appearance of anxiety and learning has been suggested based on the experimental evidences recorded in the different *invitro* studies in various species of animals, such as rat (Moshfegh *et al.*, 2010; Zarrindast *et al.*, 2011).

With the aim of the evaluating the interaction between serotonergic receptors, located in the central amygdala, ACPA and the strong selective agonist of CB1 cannabinoid receptors, the current research was conducted. The Elevated Plus Maze test has been applied for assessing anxiety like behaviors, whereas EPM method was used to study anxiety like behaviors as well as an emotional memory in the present research. There are some studies related to the effects of ACPA on anxiety process (Chegini *et al.*, 2014; Zarrindast *et al.*, 2008), however The effects of the cannabinergic system on the anxiety, memory and learning process relate to the central amygdala nucleus and its interaction with serotonergic system have not studied yet. Therefore With the aim of evaluating the regulating effects of cerotonergic system on the anxiolytic effects of cannabinoids and the possibility of restoring cannabinoid caused amnesia by serotonergic system, the current research was conducted.

Material and methods

Male Wistar rats (250 - 300 g; Institute of pharmacology Studies, Tehran, Iran) were used in these experiments. Cages, four rats in each one, were incubated in the room in which the temperature was maintained at 23 ± 1 °C and the light: dark rhythm was controlled in a 12:12 h cycle. The animals were allowed to acclimate for seven days prior to the initiation of experiments. Eight animals were used in each experimental group. The experiments were carried out during the light phase of the cycle. Animals handling was limited to the time of home cage cleaning (each 48 h), weighing, and drugs administration only.

Drugs

The drugs used in the study were ketamine and xylazine(Alfasan Chemical Co, Woerden, Holland) for animal anesthesia.Other drugs which were supplied by Tocris, Bristol, UK were: CP94253 Hydrocholoride selective 5-HT1 agonist serotonin receptor; at 0.05, 0.5, 5 and 50 ng/rat) , GR127935 Hydrocholoride (a

selective 5-HT1 receptor antagonist; at 0.05, 0.5,5 and 50 ng/rat), and ACPA, Arachidonylcyclopropylamide (apotent and selective CB1 receptor agonist; at0.00025,0.0005, 0.0001,0.0002 µg/kg). All drugs were dissolved in sterile 0.9% saline, just before the experiments. 5-HT1 receptor agents were administered into the CeA at the volume of 0.3 l/site, while ACPAinjection was done intraperitoneally at 10 ml/kg. Control animals received saline. The infusion time and selected drug doses used in the experiments were chosen according to the pilot and published work in scientific literature (Paxinos and Watson., 2007; Motevasseli *et al.*, 2010; Nikseresht *et al.*, 2012).

Elevated plus-maze (EPM) apparatus

An EPM, made of Plexiglas and consisting of two opposite open-arms (50×10 cm) surrounded by a 1 cm high ledge, and two enclosed-arms (50×10×40 cm) was used. The maze was set up 50 cm above the floor. The junction area of the four arms (central platform) measured 10×10 cm (Zarrindast *et al.*, 2010; Eslimi *et al.*, 2011).

The Stereotactic surgery and drug infusion

Rats were intraperitoneally anesthetized using ketamine hydrochloride 10% (Alfasan, Woerden, Holland; 50 mg/kg) plus xylazine 2% (Alfasan, Woerden, Holland; 4 mg/kg) then positioned in a stereotactic frame. The upper incisor bar was set at 3.3 mm below the interaural line so that the skull aligned horizontally between bregma and lambda. Two bilateral guide-cannulae (through which an injection cannula could be inserted for drugs, saline or vehicle applications, 5-7 days later) were stereotaxically implanted over the central amygdala. Taking bregma as the reference point, the coordinates for the central amygdala were AP = -2.2 mm, ML = ±4.2 mm and DV = -6 mm, according to the atlas of Paxinos and Watson (Motevasseli *et al.*, 2010; Zarrindast *et al.*, 2010; Eslimi *et al.*,2011; Nikseresht *et al.*, 2012). The cannulae were fixed to the skull by means of acrylic resin and two stainless steel screws. By the end of the surgery, a stylet was introduced inside each guide cannula to reduce possible occlusion. After the surgery, rats were placed again in their home cages in groups of five, similar to before surgery. Five to seven days post surgery, rats received a bilateral infusion into the central amygdala using dental needles (27-gauge) introduced through guide cannulae. The injection needles were advanced until their tips reached 1 or 2 mm below the cannulae end. Then, 0.3 µl/side of solutions were injected central amygdale each one. This was done during 60 s, using a 2.5-µl glass Hamilton syringe. A polyethylene catheter was interposed between the upper end of dental needles and the microsyringes. The displacement of an air bubble inside the polyethylene catheter was used to monitor the drug flow. To allow proper infusion, needles were removed 60 s after the completion of injection.

Modified behavioral testing

In the present study, EPM test-retest method was chosen to investigate anxiety and the aversive learning process. Recent studies have shown that using the test-retest sessions in the EPM, results in a qualitative shift in emotional state. Thus, unconditioned fear in the test session would possibly transform to a learnt avoidance during the retest (Cruz-Morales *et al.*, 2002; Gianlorenco *et al.*, 2011). In our study, animals were given a pretest intracerebral drug injection, followed by an injection free (undrugged) retest, 24 h later. Thus, drug effects on aversive learning with the subsequent long-term effects on memory, were tested 24 h later. It has been reported that prior experienced undrugged test session in the elevated plus-maze, alters behavioral responses in the retest drug-free session (Carvalho *et al.*, 2005; Stern *et al.*, 2010). It has also been suggested that EPM retest outcome depends on the test session length and the baseline anxiety state (Ghizoni *et al.*, 2006). Support for the former test-retest procedure stems from the data showing that although pretest systemic benzodiazepine administration reliably exerts anxiolytic effects, pre-retest injections fail to affect the response in the second EPM session (File *et al.*, 1993). In general, reduced open-arms exploratory behaviors of saline treated groups during retest session, indicates an

aversive learning associated with the initial exploration of this potentially dangerous environment.

All experiments were carried out in a minimally illuminated (40-lux) room, during the diurnal phase, between 9:00 AM and 15:00 PM. Five minute EPM sessions were recorded by a video camera while a monitor and a DVD-recording system were installed in the adjacent room. After each EPM session, the apparatus was cleaned and towel dried to avoid urine impregnation. The observer, who quietly sat one meter behind one of the closed arms of the maze, used chronometers to measure in the closed arms and the number of entries into the open and closed arms. Entry was considered only when all four paws were placed in the arms. The maze was cleaned with distilled water after each EPM session. The raw data were used to calculate the %OAT (Open Arm Time percentage), which is the percentage of time each mouse spent in the open arms relative to the total amount of time spent in any arm (open/total × 100). %OAE (Open Arm Entries per- centage) was recorded as a correlate for the lack of aversive memory upon retest. The sum of all closed arm entries (CAE) was used as an index for the general locomotor activity. All data were obtained by observer (but not digital-recording system) after which the measures were calculated manually. The digital-recording system was used as backup in case of lost information (Casarrubea *et al.*, 2009; Eslimi *et al.*, 2011).

Verification of cannulae placements

Upon concluding each experiment, rats were deeply anesthetized after which 1% Methylene Blue solution was injected into the central amygdala (0.5 µl/side) as described in the drug section. Each animal was then decapitated, its brain removed and placed in a 10% formalin solution. After 7-10 days, the brains were sliced and the sites of injections were verified according to the atlas of Paxinos (Paxinos and Watson., 2007). Data from rats with cannulae placements outside the intended sites were excluded from the statistical analyses.

Statistical analysis

Given the normality of distribution and the homogeneity of variance, the results were statistically evaluated using the repeated measure and two-way analysis of variance (ANOVA), in which mean ± S.E.M was employed for the comparison of outcomes between experimental groups and their corresponding controls.

Experimental design

Experiment 1

Open-arms exploratory behavior following pretest intra-central amygdala microinjections of ACPA

In order to substantiate that the intracentral amygdalamicroinjection of ACPA involves in anxiety and learling , the drug infusion took place 5 min prior to EPM testing. Naive rats were tested in the EPM, 5 min after concurrent intra- microinjection of saline (0.3 µl/rat); ACPA (0.00025, 0.0005, 0.001 and 0.002 µg/rat) .To investigate the possible carryover, intra central amygdala ACPA effects on aversive learning during test session to aversive memory in retest. Treated groups were retested in EPM 24 h later, undrugged.

Experiment 2

Open-arms exploratory behavior following pretest intra-central amygdala microinjections of CP94253 Hydrocholoride

In this experiment, the animals received concurrent intra-central amygdala microinjection of saline (0.3 µl/rat); CP94253 Hydrocholoride(0.05, 0.5,5 and 50ng/rat). Rats were tested in the EPM, 5 min after infusion. To investigate whether intra- central amygdala CP94253 Hydrocholorideaffects aversive memory acquisition, treated groups were retested in the EPM 24 h later, undrugged.

Experiment 3

the effect of subthreshold dose of CP94253 Hydrocholoride in the central amygdala upon open-arms exploratory behaviors induced byACPAin the central amygdala

Four groups of animals received concurrent intra-central amygdala microinjection of subthreshold dose

ofCP94253 Hydrocholoride(5ng/rat, intracentral amygdala) or different doses ofACPA (0.00025, 0.0005, 0.001 and 0.002µg/rat). Rats were tested in the EPM, 5 min after infusion. Treated groups were then retested in EPM, 24 h later, undrugged.

Experiment 4

Open-arms exploratory behavior following pretest intra-central amygdala microinjections of GR127935 (5-HT1 serotonin receptor antagonist)

In this experiment, the animals received concurrent intra-central amygdala microinjection of saline (0.3 µl/rat); GR127935(0.05, 0.5,5 and 50ng/rat). Rats were tested in the EPM, 5 min after infusion. To investigate whether intra- central amygdala GR127935 affects aversive memory acquisition, treated groups were retested in the EPM 24 h later, undrugged.

Experiment 5

The effect of subthreshold dose of GR127935in the central amygdala upon open-arms exploratory behaviors induced byACPAin the central amygdala.

Four groups of animals received concurrent intra-central amygdala microinjection of subthreshold dose ofGR127935(5µg/rat, intracentral amygdala) or different doses ofACPA (0.00025, 0.0005, 0.001 and 0.002µg/rat). Rats were tested in the EPM, 5 min after infusion. Treated groups were then retested in EPM, 24 h later, undrugged.

Results

Results from the experiment 1

Effects of the pretest intra-CeA microinjection of ACPA on the open-arms exploratory behaviors

Repeated measure and posthoc analysis between test and retest days showed that intra-BLA injection of ACPA at applied doses did not alter the transfer latency [Intra-groups: $F(1, 35) = 8.95$, $P < 0.01$, Inter-groups: $F(4, 35) = 1.63$, $P > 0.05$, Inter-Intra groups inter-action: $F(4, 35) = 3.66$, $P > 0.05$; Fig. 1, panels 1A and 2A], %OAT[Intra-groups: $F(1, 35) = 15.09$, $P < 0.001$, Inter-groups: $F(4, 35) = 2.32$, $P > 0.05$, Inter-Intra groups interaction: $F(4, 35) = 4.31$, $P < 0.01$;Fig. 1, panels 1B and 2B], %OAE [Intra-groups: $F(1, 35) = 32.40$, $P < 0.001$, Inter-groups: $F(4, 35) = 2.77$, $P > 0.05$, Inter-Intra groups interaction: $F(4, 35) = 8.45$, $P < 0.001$; Fig. 1, panels 1C and 2C] and locomotor activity [Intra-groups: $F(1, 35) = 0.24$, $P > 0.05$, Inter-groups: $F(4, 35)=3.712$, $P > 0.05$, Inter-Intra groups interaction: $F(4,35) = 497.4$, , $P < 0.001$; Fig. 1, panels 1D and 2D] on test or retest days as compared to the own control groups, suggesting that ACPA applied doses does not appear to alter anxiety-like behaviors and aversive memory acquisition.

Results of the experiment 2

Effects of the pretest intra-CeA microinjection of CP94253 Hydrocholoride on the open-arms exploratory behaviors

Repeated measure and posthoc analysis between test and retest days showed that intra-CeA injection of CP94253 HCL at applied doses did not alter the transfer latency [Intra-groups: $F(1, 35) = 32.68$, $P < 0.001$, Inter-groups: $F(4, 35) = 2.52$, $P > 0.05$, Inter-Intra groups inter-action: $F(4, 35) = 1.405$, $P > 0.05$; Fig. 2, panels 1A and 2A], %OAT[Intra-groups: $F(1, 35) = 44.48$, $P < 0.001$, Inter-groups: $F(4, 35) = 9.57$, $P < 0.001$, Inter-Intra groups interaction: $F(4, 35) = 1.050$, $P > 0.05$;Fig. 2, panels 1B and 2B], %OAE [Intra-groups: $F(1, 35) = 15.58$, $P < 0.001$, Inter-groups: $F(4, 35) = 0.906$, $P > 0.05$, Inter-Intra groupsinteraction: $F(4, 35) = 1.45$,$P > 0.05$; Fig. 2, panels 1C and 2C] andlocomotor activity [Intra-groups: $F(1, 35) = 6.78$, $P < 0.01$, Inter-groups: $F(4, 35)= 2.63$, $P > 0.05$, Inter-Intra groups interaction: $F(4,35) = 1.68$, , $P > 0.05$; Fig. 2, panels 1D and 2D] on test or retest daysas compared to the own control groups, suggesting that reduced the emotional memory formation and locomotor activity but ineffective treatment on the anxiety -like behaviors.

Results of the experiment 3

The effects of the pretest intra-CeA microinjection of CP94253 HCL on the ACPA-induced open-arms exploratory behaviors

Two-way ANOVA and posthoc analysis showed that intra-CeA injection of the sub threshold dose CP94253 HCL decreased %OAT [Intra-groups: F (1,

70) = 0.18, P > 0.05, Inter-groups: F(4, 70) = 3.63,P < 0.001, Inter-Intra group interaction: F (4, 70) = 1.009, P > 0.05;Fig. 1, panels 1B and 3B] and %OAE [Intra-groups: F (1, 70) = 0.869,P > 0.05, Inter-groups: F(4, 70) = 0.625, P > 0.05, Inter-Intra group interaction: F (4, 70) = 2.14, P < 0.01; Fig. 1, panels 1C and 3C] while did not alter the transfer latency [Intra-groups: F(1, 70) = 9.52, P < 0. 01,Inter-groups: F(4, 70) = 0.76, P > 0.05, Inter-Intra group interaction: F(4, 70) = 0.98, P > 0.05; Fig. 1, panels 1A and 3A] and locomo-tor activity [Intra-groups: F(1, 70) = 23.82, P < 0.001, Inter-groups: F(4, 70) = 3.78, P < 0.01, Inter-Intra group interaction: F(4, 70) = 0.532,P > 0.05; Fig. 1, panels 1D and 3D] already induced by ACPA on test day as compared to the respective control group, indicating thatCP94253 HCL blocks the ACPA-induced anxiolytic-like behaviors. Moreover, two-way ANOVA indicated that the subthresholddoseCP94253 HCL decreased the transfer latency [Intra-groups: F(1,70) = 7.05, P < 0. 01, Inter-groups: F(4, 70) = 2.58, P < 0.05, Inter-Intragroups interaction: F(4, 70) = 2.051, P > 0.05; Fig. 1, panels 2A and4A] and %OAT [Intra-groups: F(1, 70) = 6.37, P < 0.05, Inter-groups: F(4, 70) = 4.17, P < 0. 01, Inter-Intra group interaction: F(4, 706) = 17.92 ,P < 0.001; Fig. 1, panels 2B and 4B] while did not alter %OAE [Intra-groups: F(1, 70) = 2.164, P > 0.05, Inter-groups: F(4, 70) = 1.84, P > 0.05,Inter-Intra group interaction: F(4, 70) = 12.77, P < 0. 001; Fig. 1, panels2C and 4C] and locomotor activity [Intra-groups: F(1, 70) = 0.019,P > 0.05, Inter-groups: F(4, 70) = 2.813, P < 0.05, Inter-Intra group interaction: F(4, 70) = 2.925, P < 0.05; Fig. 1, panels 2D and 4D] already induced by ACPA on retest day as compared to the respective control group. In conclusion, the data revealed that the intra-CeAinjection of CP94253 HCL restored the ACPA-induced aversive memory deficits.

Results of the experiment 4

Effect of the pretest intra-CeA microinjection of GR127935 on the open-arms exploratory behaviors

The repeated measure and posthoc analysis showed that the intra-BLA injection of GR127935 increased transfer latency in retest day; Intra-groups: F(1, 35) = 35.36, P < 0.001,Inter-groups: F (4, 35) = 1.98, P > 0.05, Inter-Intra group interaction: F (4, 35) = 6.29, P < 0.001; Fig. 2, panels 1A and 2A], %OAT in retest day; Intra-groups: F (1,35) = 39.33,P < 0.001, Inter-groups: F (4, 35) = 6.288, P < 0.001, Inter-Intra group interaction: F (4, 35) = 0.47, P > 0.05; Fig. 2, panels 1B and 2B],%OAE in retest day; Intra-groups: F (1, 35) = 33.90, P < 0.001, Inter-groups: F (4, 35) = 4.24, P < 0.01, Inter-Intra group interaction: F(4, 35) = 2.14, P < 0.05; Fig. 2, panels 1Cand 2CA], while alter locomotor activity [Intra-groups: F (1,35) = 6.56, P < 0.05, Inter-groups: F(4, 35) = 2.64, P < 0.05, Inter-Intragroups interaction: F (4, 35) = 2.87, P < 0.05; Fig. 2, panels 1D and 2D].

Results of the experiment 5

The effects of the pretest intra-CeA microinjection of GR127935 on the ACPA-induced open-arms exploratory behaviors

Two-way ANOVA and post hoc analysis showed that the intra-CeA injection of the sub threshold dose GR127935 did not alter the exploratory behaviors already induced by the sub threshold and effective doses of ACPA both on test and retest days when compared to the respective controls. The measured indices for compared to test days were transfer latency [Intra-groups: F(1, 70) = 6.53,P < 0.05, Inter-groups: F(4, 70) = 2.14, P > 0.05, Inter-Intra groups interaction: F(4, 70) = 3.069, P < 0.05; Fig. 1, panels 1A and 5A], %OAT[Intra-groups: F(1, 70) = 0.009, P >0.05, Inter-groups: F(4, 70) = 9.565,P < 0.001, Inter-Intra group interaction: F(3, 56) = 3.1, P > 0.05; Fig. 3,panels 1B and 5B], %OAE [Intra-groups: F(1, 70) = 5.32, P < 0.05,Inter-groups: F(4, 70) = 2.38, P > 0.05, Inter-Intra group interaction: F(4, 47) = 2.47, P > 0.05; Fig. 1, panels 1C and 5C] and locomotor activity [Intra-groups: F(1, 70) = 14.98, P < 0.001, Inter-groups: F(4,70) = 3.21, P < 0.01, Inter-Intra groups interaction: F(4, 70) = 3.13, P > 0.05; Fig. 1, panels 1D and 5D].The measured indices for compared of retest days were transfer latency [Intra-groups: F(1, 70) = 1.47, P > 0.05, Inter-groups: F(4,70) = 0.65, P > 0.05, Inter-Intra groups interaction: F(4, 70) = 4.18, P < 0.01; Fig. 1, panels 2A and 6A], %OAT [Intra-groups: F(1,70) = 0.22, P >

0.05, Inter-groups: F(4, 70) = 0.324, P > 0.05, Inter-Intra groups interaction: F(4, 70) = 4.461, P < 0.001; Fig. 1, panels2B and 6B], %OAE [Intra-groups: F(1, 70) = 0.068, P > 0.05, Inter-groups: F(4, 70) = 0.072, P > 0.05, Inter-Intra groups interaction: F(4,70) = 11.23, P < 0.001; Fig. 1, panels 2C and 6C] and locomotor activity[Intra-groups: F(1, 70) = 0.59, P > 0.05, Inter-groups: F(4, 70) = 0.406,P > 0.05, Inter-Intra groups interaction: F(4, 70) = 3.64, P < 0.01; Fig. 1,panels 2D and 6D].In conclusion, our data suggested that the intra-CeA injectionof GR127935 does not alter the ACPA-induced exploratory behaviors upon EPM test and retest.

Histology

Fig. 1 shows locations of the injection cannulae tips in the central amygdala. illustrate the representative sections taken from the rat's brain atlas of Paxinos and Watson [44]. Shaded and dark areas represent the approximate points in which the cannulae were positioned for each animal. Data from the animals with injection sites located outside the central amygdala were not used in the analysis.

Discussion

The effects of ACPA on anxiety like behaviors and learning

The obtained results of the present study indicated that the pretest injections of various concentrations of ACPA, the selective agonist of the CB1 receptor, into the central amygdala led to the amnesia, while were ineffective on anxiety related behaviors. By receiving signals from basolateral amygdale, central amygdale nucleus which is the critical region implicated in learning and anxiety process, sent messages to targets, including hypothalamus and brain stem, the area involved in the most authonomical and electrophysiological behaviors caused by fear, anxiety and emotional learning (Walker *et al*., 2003). Based on the behavioral studies, the anxiety like behaviors have been mentioned as one of the main effects of cannabinoids in animal and human models(Lichtman *et al*., 2002; Zarrindast *et al*., 2010).

The anxiolytic impacts of agonists of CB1 receptors have been supported by the induced changes by the applications of Win 55,229(Haller *et al*., 2007) and CP55,940 [55, 56], in contrast to antagonists of CB1, such as AM251(Haller *et al*., 2002; Rodrigues *et al*., 2004). Δ9-THC, a non selective cannabinoid agonist, and annandamid, an endogenic cannabinoid ligand, elevated the presence of mice in the close arm, indicator of anxiogenic effects, which are in consistent with our finding. Morover, there are some reports reflecting the cannabinoid agonists led to the anxiety response (Zarrindast *et al*., 2008; Zarrindast *et al*., 2010). In addition, the consumption of cannabinoid drugs in human caused the anxiety reaction (Hall *et al*., 1998). It has been stated that the animal responses to cannabinoids (McGregor IS *et al*., 1996) and the diffusions of the cannabinoid receptors in the brain structure are variable dependent to the species (Arnold *et al*., 2001).

*Canabinoid antagonist reverses the various anxiety related behaviors i*nduced by Cannabis sativa (Hall *et al*., 1998).Overall, it seems that the dose of cannabinoid agonist and experimental animal species play critical roles in anxiety related responses caused by cannabinoids.

The recorded findings from the current study clearly reflected that ACPA damaged memory. Based on the various behavioral studies, the destructions in short term memory and spatial learning have been proposed as the main effects of cannabinoids (Moshfegh *et al*., 2010; Nasehi *et al*., 2010). The firing of amygdale neurons during formation of short term memory were inhibited by Cannabinoids (Wilson *et al*., 2002; Wilson *et al*., 2002; Moore *et al*., 2010). The systemic pretest usage of Delta-9-Tetrahydrocannabinol ruined working memory while restored (Nava *et al*., 2000; Lopez-Moreno *et al*., 2008) and improved memory process by antagonist of CB1 receptor (Da and Takahashi., 2000).

Also, there are evidences indicating agonist of CB1 receptor may affect achievement and/or fixation of memory (Da and Takahashi, 2000; Robinson *et al*., 2007) The high rate of CB1 receptors in the area, like

amygdale and the implication of cannabinoid system of amygdala in memory process have been confirmed by different researches (Viveros *et al.*, 2005; Collin *et al.*, 2007; Marco *et al.*, 2009). The modulating role of the cannabinoid system in the memory consolidation process has been proposed (BRUSCO 2008) and seems that the mentioned system may act as a dual neuromodulator (Moreira *et al.*, 2008). The reductions in release of glutamate and acetyl colin in amygdala and some other related regions, including hypocamp could be responsible for destroying the memory.

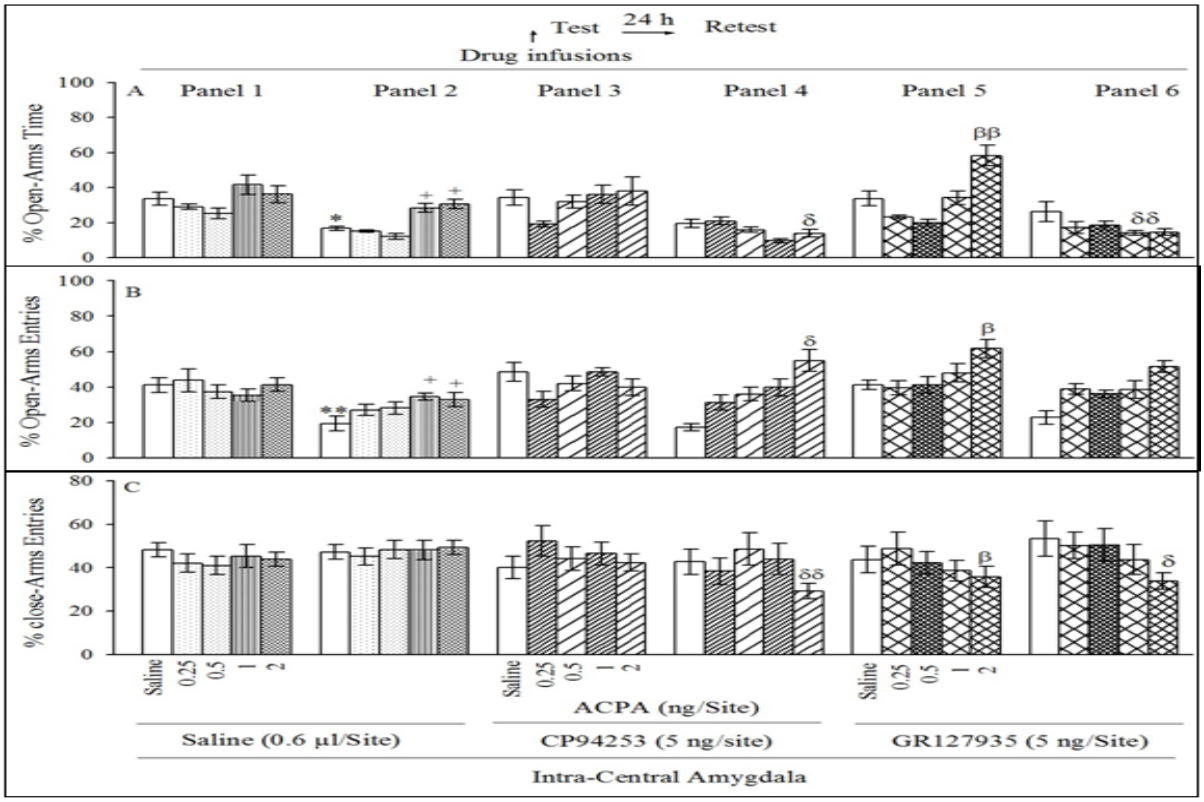

Fig. 1. (panels 1 and 2) shows open-arms exploratory behaviors following N the pretest intraperitoneal injection of ACPA. After 24 h, all groups were retested in the EPM,undrugged. %Open-Arms Time (A); %Open-Arms Entries (B) and number of Closed Arm Entries (C). Values are expressed as mean ± S.E.M (n = 8 in each group).*P < 0.05 and**P < 0.01 different from saline group in panel 1. P < 0.01 and P < 0.001 different from the respective saline group in panel 1.+<0.05 and++<0.01different from the saline group in panel 2. In addition, Fig. 1 (panels 3 and 4 for CP94253 HCL, while panels 5 and 6 for GR127935) shows the effect of the intra-CeA pre-test injectionof the subthreshold dose CP94253 HCL and GR127935on open-arms exploratory-like behaviors induced by both the subthreshold and effective doses of ACPA. After 24 h, all groups were retested in the EPM, undrugged. Values are expressed as mean ± S.E.M (n = 8 in each group). For panels 3 and 4,δ<0.05 as compared to the respective group in panel 1(comparison of the two drugs on test day), while ˛β< 0.05 is as compared to the respective group in panel 2 (comparison of the two drugs on retest day).

Effect of intra-CeA microinjection of agonist and antagonist of 5HT1 receptor on anxiety behaviors and memory

Intra-CeA microinjection of CP Hcl and GR (respectively agonist and antagonist of 5HT1B serotonin receptor) at high concentrations, immediately pretest, were ineffective on the anxiety however, led to amnesia. The highest levels of $5HT1_{A, B}$ receptors have been recorded in hypocamp, septom, cortex and amygdala, locations of post synaptic receptors (Davis and Claridge, 1998).

Conditional fear studies revealed that 5HT inhibited the inducing signals to the amygdala nucleus

reflecting the critical involvement of amygdala in Conditional fear reaction (LeDoux, 1998; Armony *et al.*, 1998). Analysis of the found data of this study showed that anxiety like behaviors were not influenced by agonist or antagonist of 5HT1B. These results are contradictory to the findings of Dimmler 2004 and Ramboz 1998 shown that the various types of 5HT1 agonists mediated the anxiogenic effects in animal and human models. Also, it has been stated that 5HT1A agonist acts as an anxiolytic compound in animals and human model systems (Ramboz *et al.*, 1998; El-Khodor *et al.*, 2004).

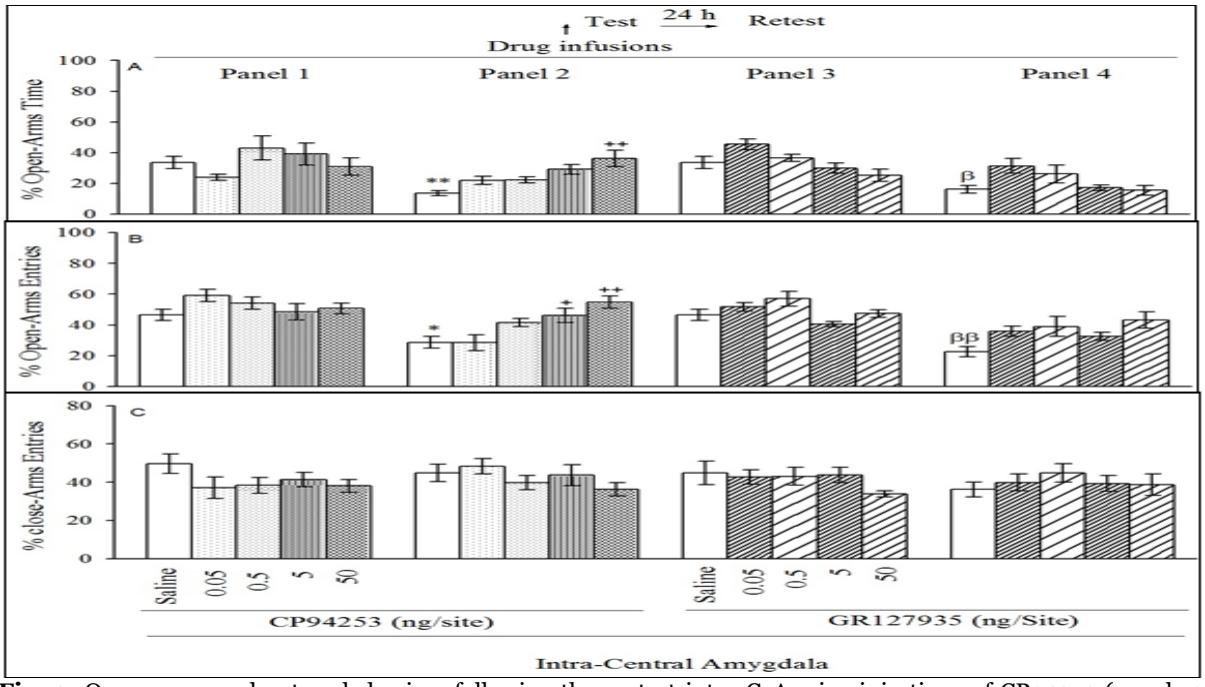

Fig. 2. Open-arms exploratory behaviors following the pretest intra-CeA microinjections of CP94253 (panels 1 and 2) and GR127935 (panels 3 and 4). After 24 h, all groups were retested in the EPM, undrugged. %Open-ArmsTime (A); %Open-Arms Entries (B) and number of Closed Arm Entries (C). Values are expressed as mean ±S.E.M (n = 8 in each group).*P < 0.05 and**P < 0.01 different from the respective saline group in panel 1.+P < 0.05 and ++P < 0.051 different from the control saline group in panel 3.βP < 0.05 and ββP < 0.01 different from the respective saline group in panel 4.

The complete depletion of 5HT1 receptors led to the anxiety like behaviors (Gross *et al.*, 2002). The anxiolytic impacts of serotonin have been well documented and there are more concerns about 5HT1 receptors for improving the anxiety related disorders (Laaris *et al.*, 1997; Belzung *et al.*, 2001; Menard *et al.*, 2007). There is a positive correlation between 5HT concentrations in brain and anxiety rate (Nasehi *et al.*, 2010). 5HT plays a vital role in the development and continuity of anxiety disorders (Zarrindast *et al.*, 2008). The concentrations of 5HT in brain of different mouse and rat species were variable and there were close relation between 5HT contents in central neurone system (CNS) and anxiety related behaviors (Zarrindast *et al.*, 2008). The increases in 5HT levels in various regions of CNS have been recorded in animals showing the anxiety behaviors (Gomez-Merino *et al.*, 2001; Kusserow *et al.*, 2004). According to the obtained results from the present study it could be stated that the high fear enhances the concentrations of 5HT in serotonergic area and there is a correlation between 5HT levels and anxiety in animals.

In this study, the recorded destruction of memory caused by injection of agonist of 5HT1 receptor is in agreement with findings of Luttgen (Luttgen *et al.*, 2005). The disturbance in the learning process induced by the different doses of 8-OHPAT, agonist of 5HT1A receptor, in PA and water Maze tests have

been reported (Carli *et al.*, 1999; Luttgen *et al.*, 2005). In the current research, the antagonist of 5HT1B did not influence memory process which inconsistence with the results of Madjid (Madjid *et al.*, 2006) and Luttgen (Luttgen *et al.*, 2005) reflecting the improving impacts of 5HT1A antagonist on the learning process under the various kinds of tests, including EPM, PA and MAZE tests. It has been shown by the different studies that 5HT1 antagonist affects spatial memory via the hypocamp and amygdala as well as the activity of 5HT1 receptor may indirectly involve in the mechanisms of strategical and spatial learning (Horisawa *et al.*, 2011).

The interaction of serotonin and cannabinoid receptors on anxiety behaviors and memory

Based on the obtained results from the present study, the simultaneous injections of different doses of ACPA and ineffective dose of serotonin agonist were ineffective treatments on anxiety like behaviors, in contrast to ineffective dose of serotonin antagonist which had the anxiolytic effects. During retest the destruction of memory achievement caused by ACPA restored by the injection of the ineffective dose of this drug. Based the evaluations done on the serotonin and the cannabinoid system in the amygdale, the both CB1 and 5HT1A are the ligand dependent channels and the activation of CB1 receptor may influence the stimulation of raphe nuclei by the control of stimulating and inhibitory inputs or by direct inhibiting of serotonergic neurons (Hermann *et al.*, 2002; Haj-Dahmane and Shen., 2011). The both cannabinoid and serotonin receptors are present in gabargic neurons of amygdala and hypocamp structures (Akimova *et al.*, 2009). There is an interaction between cannabinoid and serotonergic systems in neuronal transmissions. The both CB1 receptors and serotonin transporter protein are located in rat amygdale (Kleijn *et al.*, 2011). CB1 receptors presented on the serotonergic fibers of raphe nucleus forms synaps in amygdala; therefore it has been concluded that there is an interaction between cannabinoid and serotonergic systems (Brailov *et al.*, 2000).

The activation of the CB1 receptor decreased Ca^{+2}-dependent release of serotonin from the frontal cortical terminals of the mouse (Darmani and Pandya, 2000; Yang *et al.*, 2010). Cannabinoid presynapsis CB1 receptors prevent the secretion of the many neurotransmitters, including γ-aminobutyric acid (GABA), glutamate, dopamine, noradernaline, acetylcolin and 5- hydroxytryptamine (5-HT). The reduction in 5HT secretion by the activation of cannabinoid CB1 have been confirmed by the functional electrophysiological studies in neocortex (Nakazi *et al* ., 2000) and it seems that this decrease possesses the considerable inhibitory effects. In the current research serotonin agonist partially alleviated anxiolytic impacts of ACPA, but it was not significant. Also the destroying impacts of ACPA on the memory process were restored by the agonist and antagonist of serotonin. It could be explained by the substitution of ACPA-prevented neurotransmitters by serotonin agonist. The damaging effects of ACPA on the learning and memory could be attributed to the presynapsis inhibition of serotonin release by the cannabinoid receptors.

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