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# Effects of β1-adrenoceptors in the Basolateral Amygdala on Spatial Memory, Passive Avoidance, Long-term Potentiation and Neuronal Arborization in the Hippocampal CA1 Region in Response to Unavoidable Stress

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**HIGHLIGHTS**

- Foot-shock stress decreased emotional and spatial memory and LTP in hippocampal CA1 synapses
- Infusion of agonist and antagonist of β1-adrenoreceptor into BLA before stress reduced memory
- Infusion of Atenolol before stress attenuated stress reduced neuronal arborization
- Extra-activation or block the β1-adrenoreceptors in the BLA could damage memory in the hippocampus

**Abstract:** Norepinephrine in the basolateral amygdala (BLA) plays a pivotal role in mediating the effects of stress on memory functions in the hippocampus, however, the functional contribution of β1-adrenergic receptors on the BLA inputs to the CA1 region of hippocampus and memory function are not well understood. In the present study the role of β1-adrenoreceptor in the BLA on memory, neuronal arborization and long-term potentiation (LTP) in the CA1 region of hippocampus was examined by infusion the β1-adrenoreceptor agonist (Dobutamine; 0.5µl/side) or antagonist (Atenolol; 0.25µL/side) bilaterally into the BLA before foot-shock stress. Passive avoidance test results showed that Step-through latency time was significantly decreased in the stress group rats one, four and seven days after the stress, which intra-BLA injection of Atenolol or Dobutamine before stress couldn't attenuate this reduction. Barnes-maze results revealed that

infusion of Dobutamine and Atenolol significantly reduced spatial memory indicators such as increased latency time, the number of errors and the distance traveling to achieve the target hole in the stress group. These learning impairments in stress rats correlated with a reduction of LTP in hippocampal CA1 synapses in-vivo, which infusion of Dobutamine and Atenolol couldn't attenuate the population spike amplitude and mean-field excitatory postsynaptic potentials (fEPSP) slope reduction induced by stress. Also, the Golgi-Cox staining demonstrated that infusion of Atenolol attenuated stress decreased CA1 region dendritic and axonal arborization. These results suggest that β1-adrenergic receptors activation or block seem to exacerbate stress-induced hippocampal memory deficits and this effect is independent of CA1 LTP modulation.

**Keywords: b**arnes maze; BLA; β1-adrenergic receptors; CA1; passive avoidance test; stress

## INTRODUCTION

Stress is usually defined as any external or internal situation that interrupts the physiological and psychological homeostasis of an organism. The study of stress and its consequences has become especially important because chronic stress is one of the most important causes of the mental health disturbances in human societies [1]. Stressful circumstances, particularly could impair cognitive function especially memory. Exposure to stressful events activates the release of noradrenaline in several neuronal populations such as the hippocampus and the amygdala, but it probably also takes place in many other brain regions [2,3]. Noradrenaline is released upon emotional arousal and its role is to regulate long-term memory storage. Thus, severe stressors disrupting this process can impair cognitive function via adrenergic mechanisms [4]. The basolateral complex of the amygdala (BLA) and hippocampus receive considerable noradrenergic innervation originating from the locus coeruleus, and levels of noradrenergic increase in the BLA in the stressful circumstances [5, 6]. Studies showed that the hippocampus and the amygdala are interconnected structures of the temporal lobe that have been involved in different aspects of learning and memory [7]. More specifically, the hippocampus is a part of a system essential for declarative or episodic memory [8], while the amygdala specialized for the processing of implicit, emotional learning and memory [9]. A large body of evidence revealed that the BLA plays an important role in the integration of hormonal and neurotransmitter effects on memory formation and critically involved in regulation of processes different parts of memory formation, such as acquisition, consolidation and retrieval in both explicit and declarative memory [10]. Also, the BLA is involved in the modulation of hippocampal plasticity and induced hippocampal long-term potentiation (LTP) and increase hippocampal output to other brain areas [11]. For instance, damage to the amygdala or the stria terminalis, a major amygdala pathway, inhibit epinephrine-induced increasing effects on memory [12]. Also, the hippocampus and BLA synchronize their activity during fear extinction and fear memory retrieval [13], while electrical stimulation of the BLA damages the induction of LTP in the hippocampal CA1 subregion [14].

It has been shown that the amygdala is involved in modulating memory consolidation via activation of norepinephrine in the BLA [15]. For instance, intra-amygdala administration of the β-receptor antagonist following inhibitory avoidance training lead to amnesia, while infusions of β-adrenergic agonists into the amygdala increased memory consolidation [16]. Moreover, post-training injection of norepinephrine or the β-adrenoreceptor agonist into the amygdala increase memory storage [15]. While, post-training intra-BLA infusions propranolol, as β-adrenoreceptor antagonists could damage [17]. A mounting evidence reveals that intra-BLA post-training administrations of norepinephrine or the β-adrenoceptor agonist enhance retention of inhibitory avoidance and water-maze training, as well as contextual fear conditioning and extinction [18]. Further, intra-BLA infusion of β-adrenoceptor antagonists selectively inhibit the memory-enhancing effects of post-training systemic injections of glucocorticoids [19]. Pre-training manipulations of norepinephrine concentration in the amygdala could also modulate the acquisition and the expression of conditioned fear memory [20].

It has been shown that NE modulates synaptic transmission, plasticity, and learning via binding to G-proteincoupled receptors (Gs), particularly β-adrenergic receptors. Then, aggregation of the second messenger cyclic adenosine monophosphate (cAMP) activates myriad downstream effectors including cAMP-dependent protein kinase (PKA) and extracellular regulated kinase (ERK) [21].

Substantial data on animal models demonstrating that stress causes dendritic atrophy and reduces spine density in principal neurons of the hippocampus and the medial prefrontal cortex [22,23]. Studies showed that acute or chronic stress impairs the LTP induction in the hippocampus [24], which the amygdala is critically involved in regulating stress effects on memory and is an important component of the neural circuitry involved

in the memory [25]. Therefore, it is believed that the pathway from BLA is important to providing information about stressful stimuli to the hippocampus in memory, neuronal arborization and allowing for proper behavioral and electrophysiological responses to stressors.

These reports, considered together with the evidence of β- adrenoreceptors subtypes within the BLA give support the view that noradrenergic effects on memory are mediated, at least in part, by an activation of β-adrenoceptors in the amygdala, but the role of β1-adrenoceptors in BLA on hippocampus LTP, emotional and spatial memory, a form of hippocampus-dependent memory, and neuronal arborization is not clear during stress situations. On the other hand, even though the interaction between BLA and hippocampus is particularly relevant with regard to their known involvement in stress responses and stress-related disorders, there is a paucity of information about how β1-adrenoreceptor of the BLA can affect the hippocampal LTP *in vivo* during stress. Thus, this study was designed to investigate the probable role of β1-adrenoreceptor of the BLA in both emotional and spatial memory and also LTP and neuronal arborization in the CA1 region of the hippocampus during unavoidable stress in greater detail by infusion of β1-adrenoreceptor agonist or antagonist into the BLA.

## MATERIAL AND METHODS

### Animals

In this study adult male Wistar rats (200–250 g) were used in all experiments (n=8 for each group). The animals were housed in groups of three per cage under a 12 h light/dark cycle with free access to food and water and the room temperature was maintained at 25 ± 2 °C. The rats were randomly assigned to different groups of the experiment. The stress exposures were performed in a separate room. The experiments were conducted in accordance with the animal care and use guidelines approved by the local ethical committee (The Baqiyatallah University of Medical Committee on the Use and Care of Animals). All efforts were made to reduce the number of animals used and their pain and suffering.

### Drugs

The following drugs were used in these experiments; Atenolol (Darou Pakhsh, Tehran, Iran) β1 receptor antagonist, Dobutamine, β1 receptor agonist, Chloral hydrate (Sigma-Aldrich, USA). Dobutamine and atenolol dissolved in sterile normal saline. Chloral hydrate was injected intraperitoneal (i.p.). The control groups received saline.

### Surgical procedures and cannulation in the BLA

Rats were initially anesthetized with chloral hydrate (350 mg/kg, i.p. with supplemental doses if needed) then, were placed in a stereotaxic frame (Stoelting, Wood Dale, IL). Later a small incision was made in the scalp to expose the skull. Using Bregma and lambda as landmarks, the skull was leveled in the coronal and sagittal planes. Two sterile stainless-steel guide cannulas (23-gauge) were placed bilaterally one mm above the BLA. The stereotaxic coordinates according to the Paxinos and Watson atlas (2007) [26] for the BLA were: AP = -2.8 mm, ML = ±4.8 mm, DV = 7.5. The rats were prepared with two stainless steel 23 gauge cannulas placed above the BLA (Figure 1A). At the end of the surgery, removable wire styles (30 gauge) were inserted in the cannula to maintain patency. The cannula was permanently affixed on the surface of skull by using dental acrylic cement. After surgery, animals were kept warm and returned to a clean cage (single-housed). All the rats were allowed seven days to recover after the surgery.

Dental needles head No. 30 (Alibaba; INTR), polyethylene tubes, and 2 µl Hamilton syringes were used for the microinjection of the drugs. Atenolol (1µg/rat; selective β1-adrenoceptor antagonist) or Dobutamine (0.5 µg/rat; selective β1-adrenoceptor agonist) was administered bilaterally into the BLA by 30-gauge blunts tapered needle at a rate of 0.5 ml/min, 5 min before the stress procedure. The injection needle was left in place for two min after injection to allow diffusion from the injector tip and the animals were free to move during this time.

### Stress procedure

After intra-BLA β1-adrenoceptor receptor agonist or antagonist injection, the rats were transferred to a communication box. The communication box was equipped with a grid floor composed of 0.5 cm diameter stainless steel rods placed 1.3 cm apart. The box was divided into nine smaller compartments (16 × 16 × 50 cm). Stress induction continued for four consecutive days. During the session in the foot-shock box rats received six uncontrollable and inescapable foot shocks, in which the duration and intensity of the induced

shock were controlled by a computer connected to the communication box (1 mA, 0.2 Hz, 2 s duration, every 10 s for 1 min).

## Experimental groups

Animals were randomly divided into seven groups (n=8 for each group). In the control group rats without surgery and do not receive any foot-shock stress. In the sham group rats received saline (0.5 µl/rat or 0.25 µl/side) in the both sides of the BLA and put into communication box, but do not receive any foot-shock stress. In the stress group rats received foot-shock stress without surgery. In the control of Atenolol group, β1 receptor antagonist, rats received Atenolol (0.5 µL/rat or 0.25 µL/side) in the both sides of the BLA but without the stress. In the Atenolol+stress group rats received Atenolol (0.5 µl/rat) five minutes before stress in the both sides of the BLA. In the control of Dobutamine, β1 receptor agonist, group rats received Dobutamine (1 µl/rat or 0.5 µl/side) on both sides of the BLA but without the stress. In the Dobutamine+stress group animals received Dobutamine (0.5 µl/rat) five minutes before stress on the both sides of the BLA.

## Barnes maze

Two hours after stress induction, the rats were undergoing Barnes maze. The Barnes maze was used to estimate cognitive impairment in learning and memory. Barnes maze was built as previously described [27]. Briefly, the maze was made of Plexiglas is a circular platform (90 cm in diameter), that was elevated 120 cm above the floor. Twelve holes were arranged with distance of 2cm from edges, diameter of 8 cm and a distance of 5 cm from each other.

One dark box was under the plate (escape hole or destination chamber), and the animal can hide in the box through one of the holes on the plate. The objective for the rat was to learn a spatial relationship between the target hole and visual cues (four simple papers with different color and shapes such as: circles, triangles, squares) placed around the apparatus in the experimental room. Two 150 W lamps were placed 110 cm above the maze, which was used as aversive stimuli. Before beginning each test, rats were adapted to the testing room for one hour (on the day seven after surgery). With training, animals learn to use distal visual cues to determine the spatial location of the escape hole. At the start of the experiment, rat put in the center of the maze and they moved freely in all directions until reaching the escape hole (on the day one of stress induction). The time to achieving in the escape hole (or target hole), distance traveling and the number of errors to find an escape hole were measured as criteria for detecting differences in memory between groups.

The animal was trained for 4 to 6 times in each day for four continuous days (on the day one of stress induction until four). On the fifth day after first stress (in the test phase), the animal was placed in the central of the plate and the time to achieving in target hole, distance traveling and the number of errors was measured. After testing each animal for avoiding intra-maze odor whole maze cleaning, using 70% ethanol.

## Passive avoidance test

The rats were undergoing passive avoidance (shuttle box) training, is a hippocampus-dependent learning and memory task involving the memory that previously described [27]. Briefly, the passive avoidance device had two identical compartments comprising, a white and a black room (50 × 25 × 20 cm) divided by a guillotine door. At the beginning of the experiment (on the day one before stress induction) in order to allow animals to become habituated to the device prior to testing each rat was placed in the light compartment for 10 s, after that, the door between two compartments was raised and the latency to enter the dark chamber was recorded. If the animals did not enter the dark compartment, they were eliminated from the experiment. The habituation trial was repeated after five min for the same interval. For the learning trial, after two hours, when the animal entered the dark chamber the door was closed, and the electric foot-shock (0.5 mA, 50 Hz, 2 s once) was delivered through the grid floor by an isolated stimulator. After that, the animals were transferred from the dark compartment and returned to their own cage. If the rats did not enter the dark chamber within 60 s, they were eliminated from the test and replaced with a new rat. After 5 min, the same test was conducted again, and if the rats did not enter the dark chamber by 300 s, the successful acquisition of passive avoidance response was recorded. In the retention trials, which were done 1, 4 and 7 days after the learning trial (on first and fourth day of stress induction and day 7 after first foot-shook stress), the rats were again individually placed in the light chamber with no shock. The rats were allowed to step into the dark compartment, and then the latency to re-enter the dark chamber was recorded. On the other hand, the interval between the placement in the light chamber and the entry into the dark chamber was measured as latency time (step-through latency). When a rat avoided from entering the dark compartment within 300 s, the trial was ended.

**In vivo electrophysiology procedure**

On day five after following the induction of the stress, animals were undergoing field potential recording. Animals were anesthetized with chloral hydrate. After they lost their righting reflex they were transferred to a stereotaxic apparatus (Stoelting, Wood Dale, IL). The skin was subsequently removed from the head, and using Bregma and lambda as landmarks, the skull was leveled in the coronal and sagittal planes. After that, rats were implanted with a pair of Teflon-coated stainless steel stimulating electrodes (PFA-Coated Stainless Steel, Diameter: 0.005 inch; A-M system, USA) into the Schaffer collateral pathway region, on the right sides (AP -3.1, ML 3.1; DV 3-3.5 units in mm), and with a stainless steel recording electrode in the CA1 (AP -2.8, ML 1.8; DV -2.5-3.5 units in mm) according to the atlas of Paxinos and Watson under electrophysiological control to maximize the evoked field potential. Signals were passed through an analogue to digital interface to a computer, and data were analyzed by Potentialize software (ScienceBeam). Reference screw electrode was implanted on the cortical surface of the occipital cortex. Location of the recording and stimulating electrodes was optimized by maximizing the amplitude of the evoked field potential on a digital oscilloscope (Data Acquisition ScienceBeam-B12). Extracellular evoked responses were acquired from the CA1 pyramidal cells population following stimulation of the Schaffer collateral. Extracellular field potentials were filtered (1 Hz to 3 KHz band pass) and amplified (×1000). *In vivo* recording took place five days after following the induction of the first foot-shock stress. After confirming a suitable evoked response, an input/output (I/O) curve was obtained by averaging ten population spikes induced by variation of the stimulus current (100–1200 μA). The intensity needed to evoke 40-50% of the maximum response of the population spike (PS) and the field excitatory postsynaptic potential (fEPSP) was determined from this input/output curve and then this current intensity was used for paired-pulse analysis and LTP induction. Paired-pulse ratios were determined for a range of intervals. Afterwards, a stable baseline of evoked responses to single-pulse stimuli was established for at least 20 min prior to LTP induction (100 trains).

To induce LTP tetanic stimulation pattern or high-frequency train stimulation (HFS) (10 bursts of 20 pulses; duration 200 μs; burst frequency 400 Hz; intraburst pulse frequency every 2 Sec) was delivered to the Schaffer collateral pathway. During each recording session in CA1 region, baseline fEPSPs at 1.5×threshold intensity were recorded, at 20 minutes before and one hour after tetanus. The mean of 72 responses within 12 min (1-12, 13-24, 25-36, 37-48, 49-60 min) was again recorded at 60 min after tetanization (360 trains at 60 min). For each rat, the average fEPSP slope and PS amplitude before the tetanus were normalized to 100% (baseline average), and slope measures at each times were normalized by the baseline. The group average is the average of all normalized slope measures for all rats in the group.

**The Golgi-Cox staining**

The Golgi-Cox method is one of the best neurohistologic test that based on metallic impregnation of neurons and used to determine the cytoarchitecture of the brain and accurate morphology of neurons with high clarity and visualization of cell soma, axons, dendrites, and spines [28]. Twenty-four hours after the final stress episode, stressed and control animals were decapitated after with chloral hydrate anesthesia, and brains were removed for modified Golgi-Cox staining. In this study, we standardized Golgi-Cox protocol in our laboratory to study morphologic changes of CA1 hippocampus neurons and their arborization.

To perform the staining following solutions used:

**Golgi-Cox solution**: 40ml of 5% potassium chromate + 50ml of 5% potassium dichromate + 50ml of 5% mercuric chloride + 100ml of dd-$H_2O$. After mixing the solution in a glass bottle it kept in dark at least 48hr before used to precipitate the formations.

**Developing solutions**: **a.** Ascending ethanol series (50, 70, 90 and 100), **b.** Xylene, **c.** 3:1 ammonia: dd-$H_2O$ (prepared with 200ml ammonia with 100ml dd-$H_2O$), **d.** 5% sodium thiosulfate. All solutions were kept at room temperature and dark.

**Staining protocol**: For each brain samples in impregnation step, gently take 10 ml of Golgi-Cox solution from the upper clear part of the solution and dispensed into a small bottle. The brains cut into two halves and each one put in the individual bottle and kept in dark for 7-10 day. After that samples were sliced in 100-200 μm and loaded on albumin coated slides and kept in dark for 2 days. In developing step slides put in dd-$H_2O$ twice for 5min each and continue as follow; 5 min in 50% ethanol, 8 min in 3:1 ammonia, dd-$H_2O$ twice for 5min each, 10 min in 5% sodium thiosulfate, dd-$H_2O$ twice for 1 min each, 70, 95 and 100% ethanol for 6 min each and xylene for 6 min. Finally slides were mounted with glass cover slips and histological glue.

### Histological verification

At the end of the experiments to the verified the location of stimulating (Shaffer collateral) and recording electrodes (CA1), the animals were deeply anesthetized and transcardial perfusion was performed with 10% formaldehyde. The animals then were decapitated and brains were removed and fixed with 10% formaldehyde. After that, brains were dehydrated and paraffin embedded blocks prepared. Whole-brain paraffin embedded were dissected coronally on a microtome at a thickness of 5 µm and mounted onto glass slides. Then, the sections on glass slides were deparaffinized and stained using hematoxylin and eosin dyes to the verified Shaffer collateral and CA1 area (Figure 1B). Only animals with correct electrode locations were included in the final results.

### Statistical analysis

Data are shown as the mean±S.E.M. for 8 animals. One-way analysis of variance (ANOVA) was performed to assess specific group comparisons followed by a Tukey post hoc test. Also, Two-way ANOVA with repeated measure on the factor, time was used in the Barnes maze. Differences with $P<0.05$ were considered statistically significant.

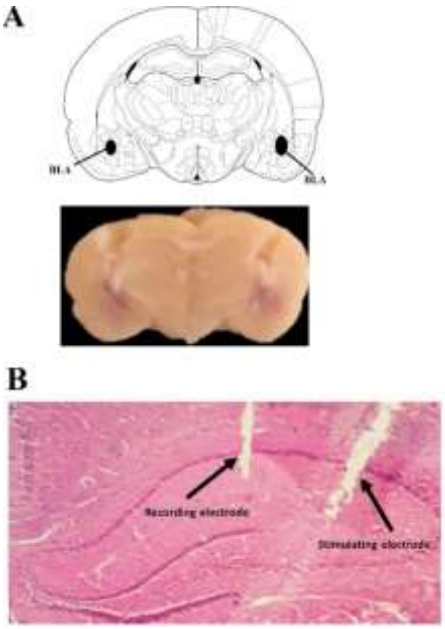


**Figure 1. A)** Photomicrograph showing the infusion cannula tip placements bilateral into the BLA. **B)** A representative photomicrograph of a hematoxylin and eosin stained showing the location of stimulating electrode in Shaffer collateral and recording electrode in the CA1 area of the hippocampus.

## RESULTS

### Behavioral tests

Behavioral tests (Barnes maze and passive avoidance test) were done in order to assess the effect of β1-adrenoreceptor on the BLA in both spatial and emotional memory during unavoidable stress.

### Effects of BLA injection of β1-adrenoreceptor drugs on passive avoidance test performance

Rats received saline sham (n = 8), Atenolol (n = 8) or Dobutamine (n = 8) bilaterally infusion into the BLA five minutes prior to stress induction. Also, Atenolol (n = 8) or Dobutamine (n = 8) bilaterally infusion into the BLA without stress induction. The animals that received the foot-shock stress one day after the passive avoidance training trial (i.e., the first day of foot-shock stress) showed significantly shorter (n=7) Step-through latency time than the control (n=8; $P<0.01$) and sham groups (n=8; $P<0.01$) (Figure 2 A). When the experiment was done on day four of the foot-shock stress, the Step-through latency time was significantly decreased in the stress animals compared to the control and sham groups (Figure 2 B). In addition, when the experiment was performed seven days after the shock experience (7 days after first foot-shock stress), the Step-through latency time was significantly decreased in the stress animals relative to the control and sham-treated rats (Figure 2 C). Overall, Step-through latency time on days 1, 4, and 7 post foot-shock stress animals were profoundly decreased than the control and sham animals. The animals in the control group that

were treated with Atenolol (n=8) in the both sides of BLA revealed a significant memory impairment compared to the control and sham groups in days one, four (P<0.001) and seven (P<0.001) after first foot-shock stress. Administration of Atenolol in the both sides of BLA five minutes before induction of stress (Atenolol+stress group; n=8) more significantly reduced Step-through latency time than the control, sham and stress groups on day one, four and seven. Administration of Dobutamine in the both sides of BLA in the control group also, caused a more significant decreased in the Step-through latency time to cross into the dark compartment compared to the control, sham and stress groups. However, injection of Dobutamine five minutes before induction of foot-shock stress, increased Step-through latency time than Dobutamine+control group, but still with significant decreased compared to the control, sham and stress groups. Overall, the administration of agonist and antagonist of the β1 receptors into BLA reduced emotional memory in both control and during foot-shock stress condition.

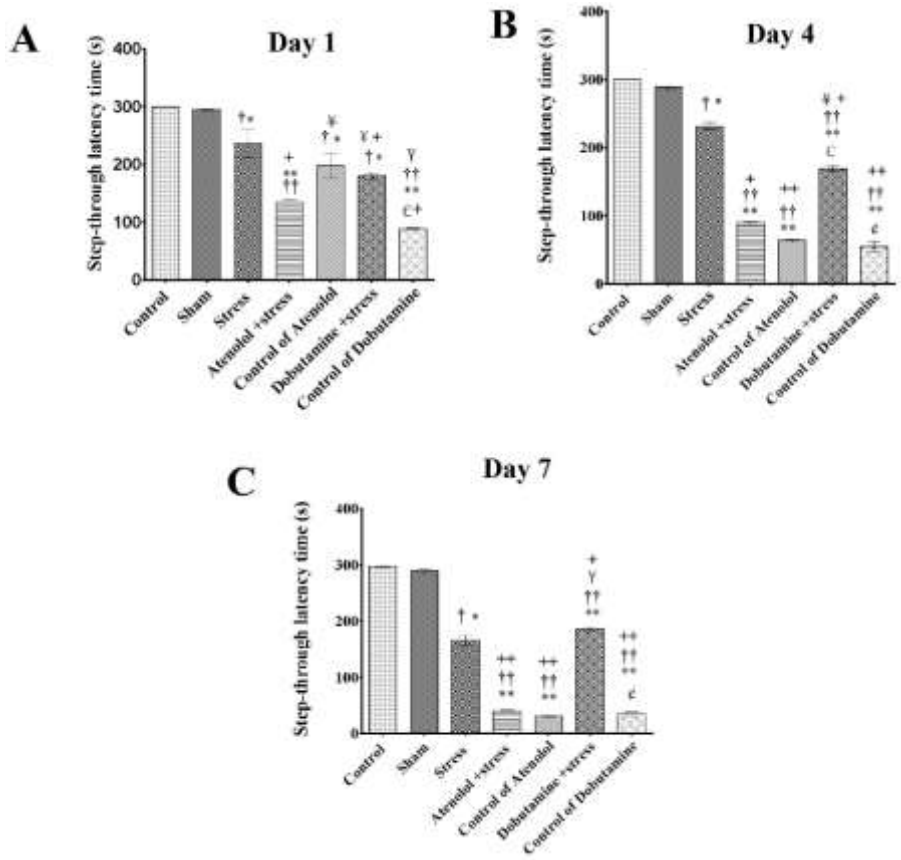


**Figure 2.** Foot-shock stress decreased rate of learning acquisition relative to the control and sham-treated rats in passive avoidance memory. **A)** Bilateral injection of β1-adrenergic receptor agonist and antagonist into BLA 1 day after the passive avoidance training trial in both control and during foot-shock stress condition showed significantly shorter Step-through latency time to enter the dark chamber than the control and the sham group. **B)** After 4 days foot-shock stress, the Step-through latency time to enter the dark chamber was significantly decreased when β1-adrenergic receptors agonist and antagonist injected into BLA compared to the control and sham groups. **C)** Moreover, 7 days after the first retention trial (on day seven after first foot-shock stress) the Step-through latency time was significantly reduced when β1-adrenergic receptor agonist and antagonist injected into BLA compared to the control and sham-treated animals. * p<0.05 as compared to the control group, ** p<0.01 as compared to the control group, † p < 0.05 as compared to the sham group, †† p < 0.01 as compared to the sham group, + p <0.05 as compared to the stress group. ++ p <0.01 as compared to the stress group. € as compared to the Atenolol+control. ¥ as compared to the Atenolol+stress. ¢ as compared to the Dobutamine+stress. Analysis used a one-way ANOVA with Turkey's post-hoc test.

## Effects of BLA injection of β1-adrenoreceptor drugs on the spatial cognitive functions

On day 1 of stress induction, the animals were underwent Barnes maze to evaluate spatial cognitive functions in all groups. The results revealed that the latency time to achieving the target hole during the five-day (probe day) training sessions of the Barnes maze test was decreased with enhanced training sessions in the control and sham groups. On the other hand, this time on day five was meaningfully reduced than that on day one for control and sham groups, but as shown in Figure 3A, the rats that received four consecutive days foot-shock stress (stress group, n=8), the animals that were treated with Atenolol in the both sides of

BLA (Atenolol+control group, n=8) and when Atenolol administrated in the both sides of the BLA five minutes before four consecutive days induction of stress (Atenolol+stress group, n=7) exhibited significantly longer the latency time to achieving the target hole on day five compared to the control and sham-treated group (n=7). Also, the latency time to achieving the target hole on day five (probe day) was increased when Dobutamine injected in the both sides of BLA (Dobutamine+control group, n=7) or when Dobutamine injected in the both sides of BLA five minutes before induction of stress (Dobutamine +stress group, n=8) compared to the control and sham-treated groups (Figure 3B).

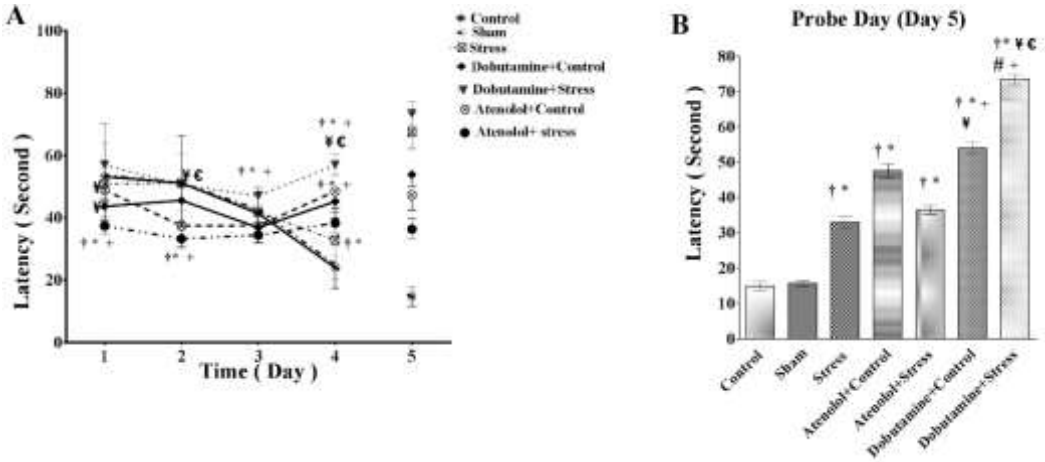


**Figure 3.** Effect of intra-BLA administration of Atenolol and Dobutamine in stress induced spatial memory impairment in the Barnes maze task. **A)** Mean latency time to achieving the target hole (Sec). **B)** Mean latency time to achieving the target hole in the probe day (day 5). Values represent the mean±SEM. * p<0.05 as compared to the control group, † p < 0.05 as compared to the sham group, + p <0.05 as compared to the stress group. € as compared to the Atenolol+control. ¥ as compared to the Atenolol+stress. # as compared to the as compared to the Dobutamine+control

In addition, the number of errors to find escapable hole was measured in all groups. The results showed that the number of errors to find the target hole during the five days (probe day) of training sessions on the Barnes maze test was reduced with enhanced training sessions in the all groups, except for the stress group. On the other hand, as shown in Figure 4., the rats that received the Dobutamine or Atenolol in the both sides of the BLA five minutes before induction of stress showed significantly (P<0.05) decreased the number of errors to find the target hole on day five compared to the stress group.

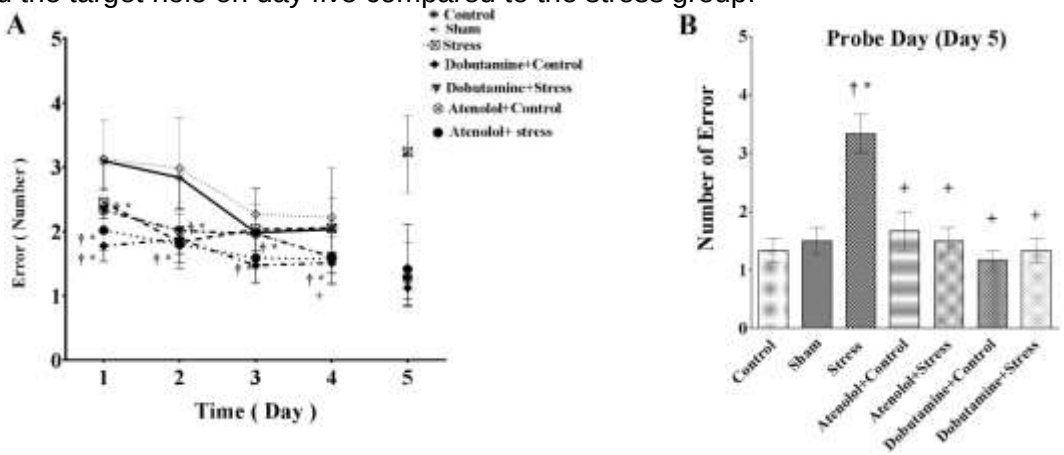


**Figure 4.** Effect of intra-BLA administration of Atenolol and Dobutamine on the mean number of errors in the Barnes maze task. **A)** Mean the number of errors during five days of training sessions in different groups. **B)** Mean the number of errors during the probe day (day 5). Values represent the mean±SEM. * p<0.05 as compared to the control group, † p < 0.05 as compared to the sham group, + p <0.05 as compared to the stress group.

Furthermore, the distance traveling to achieve the target hole was measured in the all groups. The results showed that the distance traveling to achieve the escapable hole during the probe day of training sessions was reduced with enhanced training sessions in the all groups. But, as shown in Figure 5A, the animals that received the Dobutamine or Atenolol in the both sides of the BLA five minutes before induction of stress showed significantly increased the distance traveling to achieve the target hole on day five compared to the

control, sham and stress groups. Also, administration of Atenolol (Atenolol+control group) and Dobutamine (Dobutamine+control group) into the both sides of the BLA increased the distance traveling to achieve the target hole on day five compared to the control and sham groups (Figure 5B). Thus, it seems that injection of both agonist and antagonist of the β1 adrenergic receptors into BLA could reduce the spatial memory function in the Barnes maze in the stress condition.

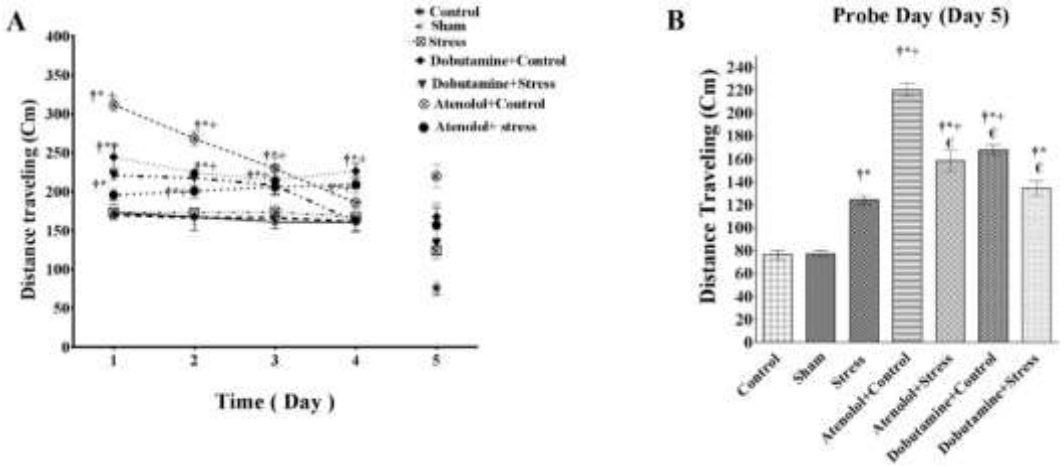


**Figure 5.** Effect of intra-BLA administration of β1-adrenergic receptors agonists and antagonists in the distance traveling to achieving the target hole in the Barnes maze task. **A)**. Distance traveling to achieving the target hole (cm). **B)** Distance traveling to achieving the target hole in the probe day (day 5). Values represent the mean±SEM. * $p<0.05$ as compared to the control group, † $p < 0.05$ as compared to the sham group, + $p <0.05$ as compared to the stress group. € as compared to the Atenolol+control.

### In vivo electrophysiology

EPSPs were evoked by stimulation pulses applied through an electrode implanted in the Shaffer collateral pathway and recorded for 60 min by electrode through CA1 cells following the LTP protocol from rats (Figure 6). On the other hand, long-term plasticity was investigated for 60 minutes (0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min) after a theta burst pattern of tetanic stimulation in the CA1 neurons. During the 60 min after HFS, PS amplitude was significantly larger in the control and sham groups (n=6, $P<0.01$), also amplitude increased in the stress group (n=7, $P<0.05$) when compared with baseline. However, this increase was lower in the stress group than in the control and sham groups. On the other side, the results showed an overall significant elevation in PS amplitude in the control and sham group compared with baseline during sixty min after HFS (0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min after HFS), with lower changes in the stress group compared with baseline.

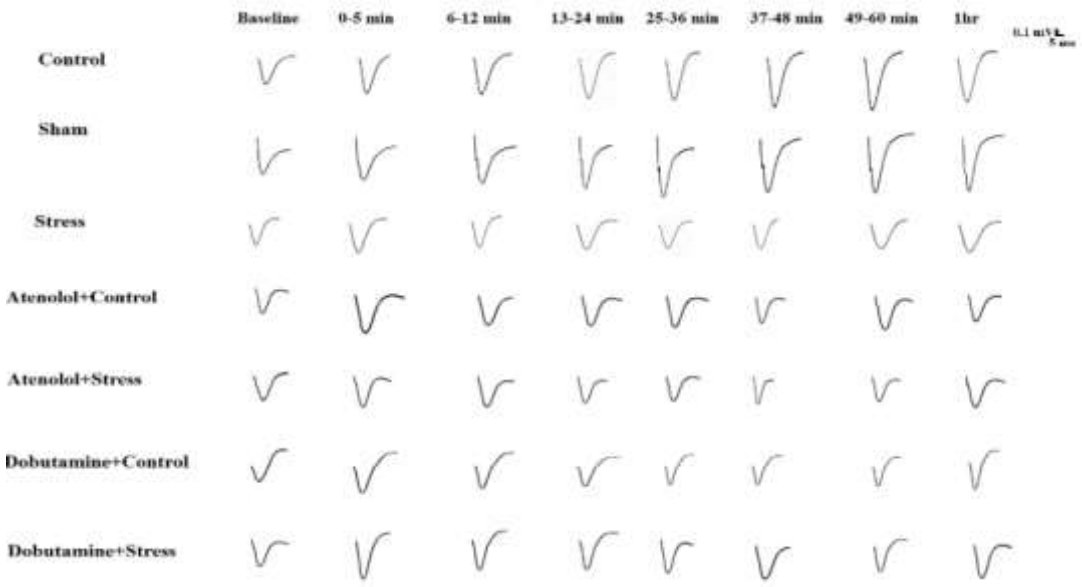


**Figure 6.** Effect of intra-BLA infusion of β1-adrenergic receptor agonist and antagonist on foot-shock stress induced impairment on the induction of CA1 LTP from *in vivo* rats. Evoked potentials recorded from the CA1 cell layer in the control, sham, stress, Atenolol+control, Atenolol+stress, Dobutamine+control, Dobutamine+stress. Long-term plasticity was investigated for 60 minutes (0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min) after a theta burst pattern of tetanic stimulation in the CA1 neurons. Traces show field potentials immediately before (pre) and 60 min after (post) tetanic stimulation.

We then examined whether the intra-BLA administration of β1-adrenergic receptors agonist or antagonist could affect hippocampal CA1 neuron LTP, which reduced by stress. Figure 7A shows that injection of Atenolol and Dobutamine five minutes before induction of stress couldn't significantly increase PS amplitude during the 60 min after HFS compared with stress group (146.06±9.45 %, n=7; 148.29±10.55 %, n=6, in the Atenolol+stress and Dobutamine+stress group, respectively). Moreover, administration of Atenolol and Dobutamine alone significantly reduced PS amplitude compared with the control and sham groups (146.47±13.01 %, n=6; 158.04±12.36 %, n=6, in the Atenolol+control and Dobutamine+control groups, respectively).

Furthermore, the induction of LTP was determined by the increasing fEPSP slope and recordings were continued for 60 min as LTP maintenance. The results indicated the mean fEPSP slope immediately after applying HFS as E-LTP induction, significantly ($P<0.05$) increased in the all groups in comparison with baseline during 60 min. However, injection of the Atenolol in the both sides of BLA before stress induction more significantly increased the fEPSP slope during 60 min compared with baseline (168.75±10.41;163.82±9.09; 143.51±15.60; 156.08±14.69; 222.60±22.10; 168.73±16.89; 164.15±15.50 in the control, sham, stress, Atenolol+control, Atenolol+stress, Dobutamine+control, Dobutamine+stress groups, respectively) (Figure 7B). Also, the same results were observed in mean fEPSP slope during 0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min immediately after applying HFS. This significant potentiating remained stable during the recording period, which LTP induction was obvious at all timepoints of tested.

The spike onset latency 60 min after applying HFS didn't the significant change in the all groups (Figure 7C). Also, the same results were observed in mean fEPSP slope during 0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min immediately after applying HFS.

Thus, it seems that injection of both agonist and antagonist of the β1 adrenergic receptors into BLA could reduce the LTP induction in the neurons in the hippocampal CA1 region in the stress condition.

### Administration of β1-adrenoreceptor antagonist into the BLA attenuated stress decreased CA1 region dendritic spine density

The loss of soma, axon and dendritic spines in the hippocampus may be a major cause of learning and memory impairment. Therefore, twenty-four hours after the end of the four days of foot-shock stress episode the soma, axon and dendritic spines density of pyramidal neurons from the CA1 region of the hippocampus in the stressed and control animals were analyzed (Figure 8A). Four days of foot-shock stress significantly

(P<0.05) reduced number of branches along the dendrites in the pyramidal neurons from the CA1 region (n=12) as compared with the control (n=11) and sham (n=12) animals. Intra-BLA infusion of Dobutamine without stress condition (Dobutamine+control group) significantly (P<0.05) increased the dendritic spine density in the CA1 area (n=14) relative to the sham and stress groups, however, injection of Dobutamine before stress (Dobutamine+stress group) did not significantly (n=12, P<0.05) affect the when compared to the sham group, but it was higher than in the stress group. No differences, in the number of dendritic spines, were observed in the dendrites of the pyramidal neurons of the Atenolol+control group (n=13) versus sham rats, but there was significantly greater dendritic arborization in the Atenolol+control group compared to the stress group (P < 0.05). Interestingly, Intra-BLA administration of Atenolol before stress (Atenolol+stress group, n=14) significantly (P<0.05) increased dendritic arborization as compared with the control, sham, stress and Atenolol+control groups (Figure 8B).

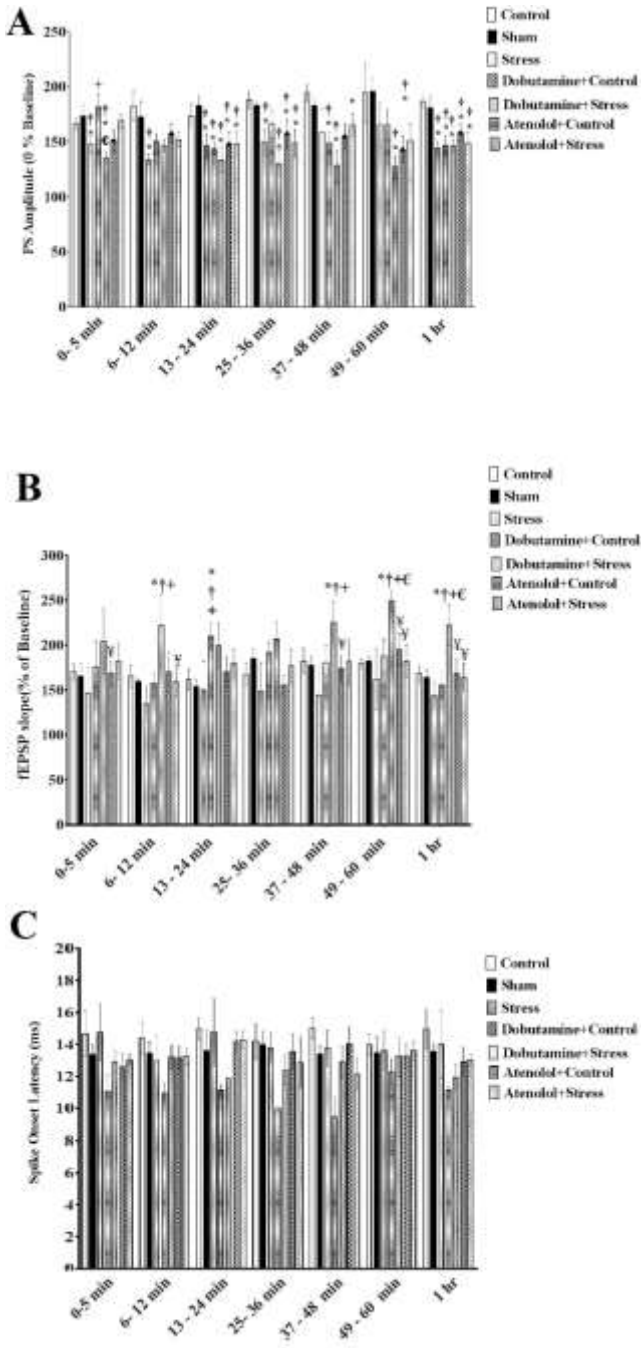


**Figure 7. A)** Effect of intra-BLA injection of Atenolol and Dobutamine on foot-shock stress induced impairment on the induction of CA1 LTP from *in vivo* rats. **A)** The population spike (PS) amplitude was defined as the mean of the amplitude from the first positive peak to the succeeding negative peak and the amplitude from the negative peak to the second positive peak. Results showed that during the 60 min after HFS (0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min), PS amplitude was significantly larger in the control and sham groups, also amplitude increased in the stress group when compared with baseline, but with lower changes in the stress group compared with baseline. Atenolol and Dobutamine

administration five minutes before induction of foot-shock stress couldn't significantly increase PS amplitude during the 60 min after HFS compared with stress. **B)** The induction of LTP was investigated by the increasing fEPSP slope and recordings were continued for 60 min (0-5, 6-12, 13-24, 25-36, 37-48 and 49-60 min) as LTP maintenance. The fEPSP slope was measured as the slope between the baseline and the peak of the first positive wave. **C)** Spike onset latency was taken from the initial positive peak of the spike. The spike onset latency 60 min after applying HFS didn't the significant change in the all groups when compared to baseline. Each point on the graph represents the mean±S.E.M. * $p<0.05$ as compared to the control group, † $p < 0.05$ as compared to the sham group, + $p <0.05$ as compared to the stress group. € as compared to the Atenolol+control. ¥ as compared to the Atenolol+stress.

The results showed that compared to the sham group, foot-shock stress significantly decreased the number of axonal arbors ($P<0.05$), but intra-BLA infusion of both β1-adrenoreceptor agonist or antagonist prevented this arborization loss in the stress group and enhanced the number of arborization in the pyramidal neurons from the CA1 region of hippocampus compared to the stress group (Figure 8C). As shown in Figure 8D., repeated foot-shock stress did not affect significantly soma arborization of the CA1 pyramidal neurons ($P<0.05$) when compared to the control and sham groups. Also, intra-BLA infusion of Dobutamine with and without stress condition did not affect soma arborization. However, results showed a significant decrease in soma arborization when Atenolol injected into BLA in normal condition (Atenolol+control group) as compared to the sham rats, while Atenolol infusion into BLA before stress (Atenolol+stress group) significantly increased soma arborization ($P<0.05$) relative to stress, Dobutamine+stress, and Atenolol+control groups. Overall, foot-shock stress reduced the neuronal arborization in the CA1 region of hippocampus, however, the administration of agonist and antagonist of the β1 receptors into BLA attenuated neuronal arborization reduction in the CA1 region of hippocampus induced by foot-shock stress.

## DISCUSSION

Despite several studies have investigated the interaction between BLA and hippocampus, there is a lack of information about the role of β1-adrenoceptors in BLA on hippocampus LTP, emotional and spatial memory during stress situations. Therefore, in the present experiments, we investigated whether activation or inactivation of β1-adrenergic receptors in the BLA could change emotional and spatial memory and hippocampus LTP in the CA1 pyramidal neurons in response to unavoidable stress. On the other hand, this study tested the hypothesis that β1-adrenergic receptors on the BLA contributes to emotional and spatial memory and hippocampus LTP in the CA1 pyramidal neurons in response to stress.

Results of behavioral investigation using the passive avoidance test on day 1 and 4 of stress induction and 7 days after first stress showed passive avoidance memory impairment. Also, assessment of spatial learning and memory by a Barnes maze during the stress, showed impairment of spatial learning and memory. It shows that stress could induce spatial and emotional memory deficit [29-31]. Consistent with these studies, the results of the present study also observed reduced spatial memory in the Barnes maze task and emotional memory in the passive avoidance test after unavoidable stress, as evaluated by increased latency time to achieving the target hole, increased the number of errors to find the escapable hole and the increased the distance traveling to achieve the target hole in the Barnes maze task and decreased Step-through latency time in the passive avoidance test.

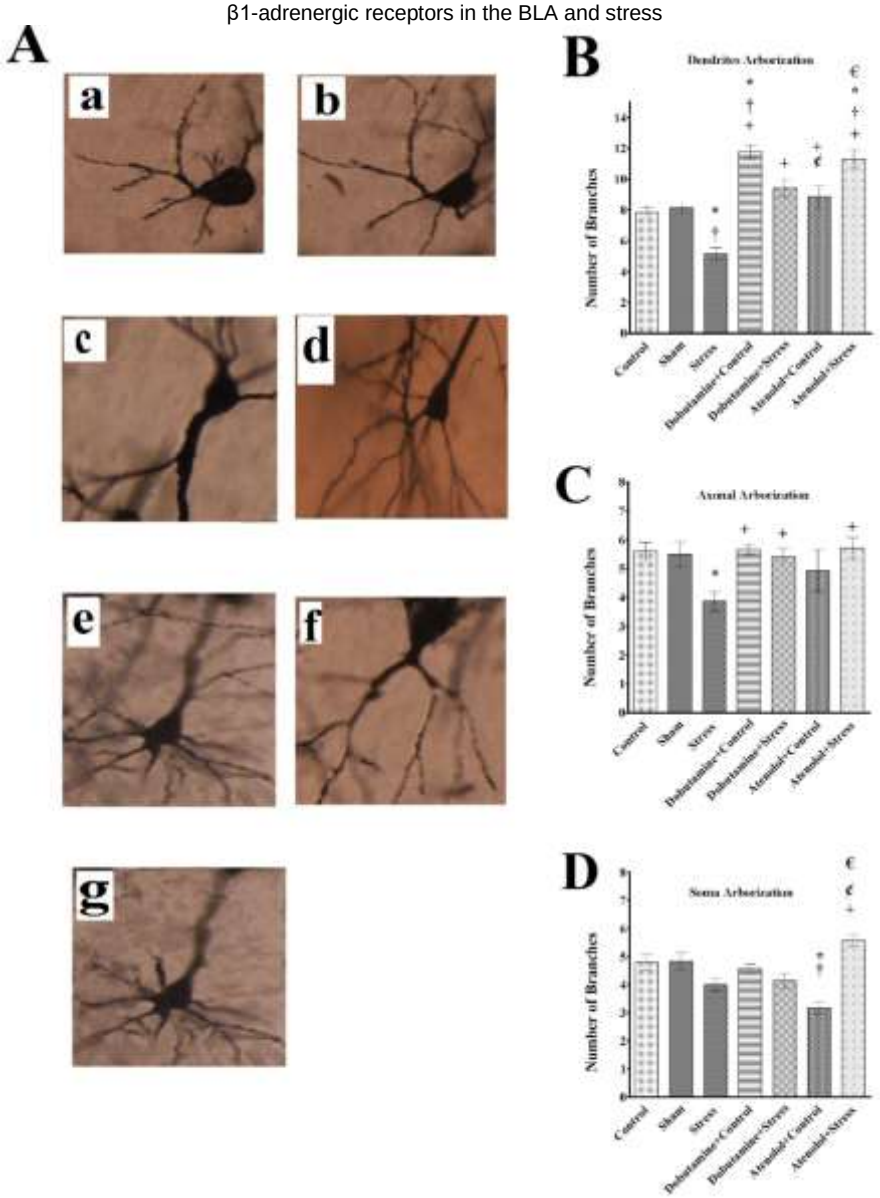


**Figure 8.** Effect of intra-BLA infusion of β1-adrenergic receptor agonist and antagonist on spine density by Golgi-Cox staining in the CA1 neurons. **A)** Representative examples of dendritic, axon and soma arborization of CA1 hippocampal pyramidal neurons; a) Control, b) Sham, c) Stress, d) Dobutamine+control, e) Dobutamine+stress, f) +control, g) Atenolol+stress groups. Scale bar represents 100 μm. Bar graphs display the dendritic **(B)**, axon **(C)**, and soma **(D)** arborization of hippocampal CA1 pyramidal cells one day after four consecutive days foot-shock stress. Each point on the graph represents the mean±S.E.M. * $p<0.05$ as compared to the control group, † $p < 0.05$ as compared to the sham group, + $p <0.05$ as compared to the stress group. € as compared to the Atenolol+control. ¢ as compared to the Dobutamine+stress

In the present study, bilateral administration of selective agonist (Dobutamine) and antagonist (Atenolol) of β1-adrenergic receptors into the BLA before stress induction, couldn't improve the spatial memory function in the Barnes maze and emotional memory in passive avoidance test during foot-shock stress condition. It has been shown that the amygdala is a pivotal part of the systems mediating many aspects of the stress response, spatial and emotional learning and memory for emotionally evocative events [9,25]. The results of the present study demonstrate that the suppressive actions of norepinephrine in the BLA in learning and memory were mimicked by injection of β1-agonist Dobutamine and β1-antagonist Atenolol into BLA, suggesting that norepinephrine has its inhibitory effects on learning and memory at least in part via β1-receptors.

To consist with the results of the present study, Hatfield and McGaugh have shown that post-training injections of norepinephrine into the BLA increased memory for spatial learning in a water maze whereas intra-BLA infusions of the β-adrenoceptor antagonist propranolol impaired memory on the same task [32]. Also, Ferry and McGaugh found that the administrations of the β-adrenoceptor agonist clenbuterol into the BLA increased inhibitory avoidance retention [33]. Studies showed that epinephrine affects memory

consolidation by involving noradrenergic activation in the amygdala. For instance, McGaugh (2002) revealed that norepinephrine in the BLA increases learning and consolidation of tasks involving aversive stimuli [34]. Also, intra-amygdala administration of the β-adrenergic antagonist propranolol or depletion of norepinephrine in the amygdala by the neurotoxin N-2-chloroethyl-N-ethyl-bromobenzylamine (DSP-4) inhibit the memory-increasing effect of the epinephrine [19]. Moreover, Goode and coauthors showed that injection of the propranolol, directly into the BLA blocks the fear-enhancement of habit memory, and they suggest that the mnemonic effects of conditioned emotional stimuli might similarly depend on both the noradrenergic system and a modulatory role of the BLA. On the other hand, they showed that noradrenergic activity specifically within the BLA is needed for emotional arousal to influence dorsolateral striatum (DLS)-dependent habit memory [35]. In addition, when β-adrenergic activation is inhibited by systemic injection of propranolol, the emotional enhancement effect of arousing material as well as the subsequent memory effect in the amygdala is reduced [36].

One may possibly interpret the predominantly suppressive effect of norepinephrine via β1-adrenergic receptors in the BLA observed here as being contradictory to the facilitative effect exerted by norepinephrine on behavior. The data of the present study showed that the actions of norepinephrine in the BLA through β1 receptors are, in fact, inhibitory with stress or without stress condition. It has been shown that norepinephrine having suppressive effects in the BLA via different functions. One possible mechanism is that norepinephrine having an overall suppressive effect on the majority of neurons of the BLA could serve to increase the signal-to-noise ratio of those select units that are excited. This would enhance sending of those excited units onto downstream BLA targets to subserve behavioral responding [37]. Another possible mechanism is that, an inhibition of the BLA could cause a disinhibition of the central nucleus of the amygdala (CeA), as the BLA has a mainly suppressive effect over the CeA [38]. Disinhibition of the CeA can cause activation of downstream brain regions involved in the behavioral response. The CeA sends excitatory fiber in many brain areas that heavily involved in the production of the stress response and stress-related behaviors, such as corticotropin-releasing hormone (CRH)-containing projection to the locus ceruleus [39] and paraventricular nucleus of the hypothalamus (PVN) [40].

In the other part of the experiments unavoidable foot-shock stress impairs the LTP induction in the hippocampus CA1 pyramidal neurons in vivo. On the other hand, the present findings show that stress-induced deficit in the CA1 hippocampal LTP and hippocampal-dependent spatial memory in rats. Intra-BLA infusion of β1-adrenoceptor agonist and antagonist, also showed impaired in CA1 pyramidal neurons LTP in vivo, during normal conditions or under stress. These results are in line with many studies which investigated the effects of beta-adrenoceptor drugs on the BLA neural activity. For instance, Buffalari and Grace showed that systemic administration of propranolol, caused a decrease in spontaneous activity of BLA neurons [41]. In vitro studies revealed that noradrenaline increases excitatory neurotransmission in the BLA through β-adrenergic receptors [42]. These data suggest that the BLA, via its projection to the hippocampus might be involved in mediating stress effects on hippocampal functioning and enhances hippocampal output to other brain areas [43].

Anatomically, BLA sends indirect fiber through the entorhinal cortex projections to the hippocampus, and direct fiber via the magnocellular and parvicellular divisions to the CA1 and subiculum, which it can effect on hippocampal functions [44]. It has been shown that stimulation of the amygdala elevated synaptic transmission, plasticity, and LTP in the dentate gyrus of the hippocampus [45]. Moreover, the evidence showed synchronization of the amygdala and hippocampal theta rhythm during retrieval of conditioned fear [46]. There is also evidence for norepinephrine-induced increase of cellular excitability, plasticity and synaptic transmission in rat dentate gyrus [47]. It has been demonstrated that the BLA can regulate consolidation of memories in other regions of the brain [48]. Activation of β-adrenergic receptors could lead to facilitation of synaptic transmission via the mechanism involving elevates in the intracellular cAMP concentration and new protein synthesis, therefore contributing to the memory acquisition and maintenance [16]. On the other hand, β-adrenergic receptors in neuronal membrane are positively coupled to adenylate cyclase (AC) via Gs protein. When activated, AC triggers the formation of cAMP, which activates PKA and leads to phosphorylate many synaptic and intracellular protein substrates, such as response element binding protein (CREB).

It is possible that β-adrenergic receptors in neuronal membrane share this common signaling pathway to regulate synaptic plasticity and memory formation [49]. Indeed, it has been showed that β-adrenergic receptor stimulation increases NMDA receptor-mediated synaptic transmission and plasticity in the BLA, and facilitates the formation of conditioned fear [34]. Also, activation of norepinephrine in the BLA promotes the induction of LTP and the expression of activity-regulated cytoskeletal protein (Arc), implicated in mechanisms of synaptic plasticity and memory formation, in the hippocampus [50] and increased immediate-early gene,

which involved in hippocampal synaptic plasticity and memory consolidation processes [51]. In addition, inactivation of the BLA with administration of lidocaine impairment memory consolidation and reduced Arc protein levels in the hippocampus [50]. Bass and Manns revealed that electrical stimulation of the BLA after rodents have seen novel objects cause increased memory for those objects, as well as enhanced synchrony in the gamma frequency range in the hippocampus [52].

Results showed a reduction in the dendritic and axonal arborization in the CA1 pyramidal neurons after four days of foot-shock stress. To consist with these results, previous studies in rodents have shown that chronic stress can induce atrophy of the apical dendritic tree of CA1 pyramidal neurons, including a retraction of their dendritic terminal segments [53]. Also, it has been shown that that stress caused remodeling in the CA3 pyramidal neurons by significantly reducing the number of dendritic branch points and the total length of the apical dendritic tree [54]. Moreover, similar results have been obtained in the CA1 region of animal's subjected to chronic stress, where apical dendritic atrophy and spine density reduction have been observed [55,56]. It is possible that the reduction and atrophy of the dendritic and axonal arborization in the CA1 pyramidal neurons in the foot-shock stress may induce reductions in the number of synaptic contacts and their function and this has an impact on spatial and emotional and LTP memory impairment.

In line with these results several studies showed that in rodents stress effects in the hippocampus and impair LTP and memory that these functional deficits often are accompanied by diminished dendritic arborization [57-59]. The results of present study showed that intra-BLA infusion of β1-adrenoreceptor antagonist could reduce stress decreased the CA1 pyramidal neurons dendritic and axonal spine density, however, injection of Dobutamine into BLA before stress did not significantly reduce the dendritic arborization when compared to control and sham group, but it was higher relative to the stress group. The adrenergic system that activates β-adrenoreceptor is a key regulator of synaptic plasticity in the hippocampus. The β-adrenoreceptor potentiate LTP in the dentate gyrus and the CA3 regions and stimulate the mitogen-activated protein kinases/extracellular signal-regulated kinases (ERK) signaling cascade via the cAMP-dependent pathway to promote protein synthesis, a pivotal step in the LTP formation [60].

The findings of the present study provide evidence that activation of β1-adrenoceptor in the BLA in the foot-shock stress is critical for dendritic and axonal spine density impairment in the CA1 region of the hippocampus. However, there is little support at present for a role of β1-adrenoreceptor in the BLA in structural plasticity in the CA1 area and the mechanisms by which Atenolol in the BLA blocks stress-induced dendritic and axonal arborization in the of CA1 pyramidal neurons loss need to be elucidated.

As mention above, results showed that bilateral intra-BLA infusion of selective agonist (Dobutamine) of β1-adrenergic receptors before stress induction, also couldn't prevent the spatial and emotional memory deficit during foot-shock stress condition. Consist with these results Roozendaal and coauthors revealed that stimulation of β1-adrenergic receptors with systemic administration of the selective agonist xamoterol induces memory retrieval damages relative to that seen after corticosterone infusion which, they suggest that glucocorticoid effects on memory retrieval destruction involve activation of noradrenergic mechanisms [61]. BLA β-adrenergic receptor activity has been frequently shown to affect memory consolidation, according to an inverted U-shape function, such that extremely low or extremely high β-adrenergic receptor activation is harmful to memory consolidation than moderate activation, which is useful [4]. The data of the present study showed that the relationship between BLA β1-adrenergic receptor activity and spatial and emotional memory to repeat stress is also could describe by an inverted U-shape. Repeated foot-shock stress produced hippocampus memory impairment that were enhanced by pharmacological stimulation of β1-adrenergic receptors (Dobutamine), suggesting that further enhancing β1-adrenergic receptor activation in the BLA could decrease the hippocampal memory formation. One explanation of the current results is that β1-adrenergic receptor activity in the BLA may cause the increases of memory deficit in the hippocampus. That is, either increasing of β1-adrenergic receptor activity in the BLA (by using β1-adrenergic receptor agonist) or decreasing (by using β1-adrenergic receptor antagonist) could promote memory impairment. On the other hand, moderate activation of β1-adrenergic receptors in the BLA is beneficial for the hippocampal memory formation. However, the use of more selective and additional doses of pharmacological agents in future experiments will be important in reinforcing the findings of the present study.

## CONCLUSIONS

The present findings support that modulation of β1-adrenergic receptors in the BLA can influence on spatial and place avoidance memory, a hippocampus-dependent task. The results of the present study showed that the spatial memory as assessed with the Barnes maze task and place avoidance memory, can be impaired by treatments the additional activation or by blocking the β1-adrenergic receptors in the BLA and supports further evidence for the view that the β-adrenergic receptors in the BLA plays an important role in

memory storage. Also, the β1-adrenoceptors in the BLA seems to modulate the magnitude of LTP and dendritic and axonal spine density in the CA1 region of the hippocampus during normal conditions or under stress. Thus, these results extended those findings that the β-adrenergic receptors in the BLA are a pivotal part of a memory-modulator system, which doing this, in part, through the β1-adrenoceptors and the BLA exerts its modulatory effect via its interactions with hippocampus that is involved in the formation of different types of memory.

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**Conflict of interest:** The authors declare that they have no conflict of interest.

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