Interaction between Morphine and Noradrenergic System of Basolateral Amygdala on Anxiety and Memory in the Elevated Plus-maze Test Based on a Test-retest Paradigm

Farhad Valizadegan PhD¹, Shahrbanoo Oryan PhD¹, Mohammad Nasehi PhD², Mohammad Reza Zarrindast PhD³,⁴,⁵,⁶

Abstract

Background: The amygdala is the key brain structure for anxiety and emotional memory storage. We examined the involvement of β-adrenoceptors in the basolateral amygdala (BLA) and their interaction with morphine in modulating these behaviors.

Methods: The elevated plus-maze has been employed for investigating anxiety and memory. Male Wistar rats were used for this test. We injected morphine (4, 5, and 6 mg/kg) intraperitoneally, while salbutamol (albuterol) (1, 2, and 4 μg/rat) and propranolol (1, 2, and 4 μg/rat) were injected into the BLA. Open-arms time percentage (%OAT), open-arms entry percentage (%OAE), and locomotor activity were determined by this behavioral test. Retention was tested 24 hours later.

Results: Intraperitoneal injection of morphine (6 mg/kg) had an anxiolytic-like effect and improvement of memory. The highest dose of salbutamol decreased the anxiety parameters in test session and improved the memory in retest session. Coadministration of salbutamol and ineffective dose of morphine presenting anxiolytic response. In this case, the memory was improved. Intra-BLA administration of propranolol (4 μg/rat) decreased %OAT in the test session, while had no effect on memory formation. Coadministration of propranolol and morphine (6 mg/kg) showed an increase in %OAT. There was not any significant change in the above-mentioned parameter in the retest session. Coadministration of morphine and propranolol with the effective dose of salbutamol showed that propranolol could reverse anxiolytic-like effect.

Conclusion: We found that opioidergic and β-adrenergic systems have the same effects on anxiety and memory in the BLA; but these effects are independent of each other.

Keywords: Anxiety, basolateral amygdala, memory, morphine

Introduction

It seems that different neurochemical mechanisms may modulate anxiety and fear.¹ Anxiety is attenuated by anxiolytic drugs, which is controlled by the amygdala and the hippocampus, while fear is resistant to anxiolytics and the periaqueductal gray may be the main site for it.² The amygdala is one of the most important structures in the brain for emotional memory acquisition and storage, a notion consistently supported by a large number of studies using different experimental paradigms and measures of conditioned fear responses.³,⁴,⁵,⁶ In addition, the amygdala also changes emotional aspects of learning in other brain regions such as the cortex and the hippocampus.⁷ From invertebrates to humans,⁷ long-term memory for an event can be increased or reduced in the minutes to hours after learning. This has led to the suggestion that memories form slowly over time, a process termed memory consolidation. The first evidence of this came when Mueller and Pilzecker⁸ reported that memory of recently learned information is disrupted by learning of other material shortly after the first learning. Later on, susceptibility of recently formed memories to postlearning manipulations was also seen with electroconvulsive shocks,⁹,¹⁰ protein synthesis inhibitors,¹¹ drug injections,¹² and electrical stimulation of discrete brain regions.¹³ Although enhancement of amygdala activity immediately after learning changes long-term retention, basolateral amygdala (BLA) lesions performed later have no effects.¹⁴,¹⁵ This implies that the amygdala is not the storage site of these memories, but the amygdala promotes memory storage processes in brain areas that are involved in declarative memory.¹⁶

Extensive evidence indicates that many neuromodulators influence memory storage through an interaction with the noradrenergic system in the amygdala.¹⁷ Beta-adrenergic antagonists infused into the amygdala block the modulating effects of peripheral stress hormones i.e., epinephrine (adrenaline) and glucocorticoids on memory for inhibitory avoidance training.¹⁸ Injection of β-adrenergic antagonists into the amygdala also block the memory-modulating effects induced by drugs affecting gamma-aminobutyric acid (GABA)ergic and opioidergic systems.¹⁹,²⁰ The present results indicate that intraperitoneal (IP) injection of the mu-opioid receptor agonist, morphine, reduced the avoidance to open arms during the test, suggesting an anxiolytic-like response.

Authors’ affiliations: ¹Faculty of Biological Sciences, Tarbiat Moallem (Kharazmi) University, Tehran, Iran, Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Garmser Branch, Semnan, Iran, Department of Pharmacology and Iranian National Center for Addiction Studies, Tehran University of Medical Sciences, Tehran, Iran, ²School of Cognitive Science, Institute for Studies in Theoretical Physics and Mathematics, Tehran, Iran, ³Institute for Cognitive Science Studies, Tehran, Iran, ⁴Department of Neuroscience, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran.

‡&RUUHVSRQGLQJ DXWKRU DQG UHSULQWV Medical Sciences, Tehran, Iran.

§XWKRUV¶ DI¿OLDWLRQV Neurosciences, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran, ⁶Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Garmsar Branch, Semnan, Iran, ⁵Department of Cognitive Science, Institute for Studies in Theoretical Physics and Mathematics, Tehran, Iran, ⁶School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran. P.O. Box: 13145-784.

Tel: +9821-66402569, Fax: +9821-66402569, E-mail: zarinmr@ams.ac.ir.

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in the elevated plus-maze (EPM). Moreover, the anxiolytic effects of morphine emerged in the retest session. The above effect suggests that the aversive memory acquisition is improved in these groups. It seems that morphine’s route of administration and the doses used are critical elements defining the morphine effect on learning and memory. Moreover, posttraining intramygdala infusions of norepinephrine (noradrenaline) (NE) and β-adrenergic agonists and antagonists enhance and impair, respectively, retention of inhibitory avoidance and spatial water-maze training. These findings suggest that the release of NE within the amygdala induced by training and drugs is involved in integrating neuro-modulatory influences on memory storage.

BLA was chosen for our investigation because of its role in anxiety and memory. Since no studies have been conducted examining the modulation of BLA by noradrenergic and morphinergic systems in anxiety-like behavior, the purpose of the present study was to elucidate, first, the effects of the NE β-receptor agonist and antagonist microinjected into the BLA and their possible roles in anxiety-related behavior and memory formation using the EPM test; second, the effects of morphine in this site; and finally, possible interactive roles of these two systems on mentioned behaviors.

Materials and Methods

Animals
Male Wistar rats (Pasteur Institute; Tehran, Iran) weighing 220 ± 20 g at the time of surgery were used. The animals were housed four per standard rat cage, in a colony room with a 12/12 hours light/dark cycle (7:00 – 19:00 lights on) at 22 ± 1 °C. Commercial rodent pellets and tap water were available ad libitum. They were allowed to adapt to the laboratory conditions for at least one week before surgery. Rats were handled about five minutes each day prior to behavioral testing. Six animals were used in each group of experiments. A total number of 200 animals were used in the experiments. All procedures were carried out in accordance with institutional guidelines for animal care and use.

Stereotactic surgery and drug infusion
Rats were anesthetized using ketamine hydrochloride 10% (Alfasan, Woerden, Holland; 50 mg/kg) plus xylazine 2% (Alfasan, Woerden, Holland; 4 mg/kg) IP, and then positioned in a stereotactic frame. The upper incisor bar was set at 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. Two bilateral guide-cannulae (through which injection cannulae could be inserted for drugs or saline applications five to seven days later) were stereotaxically implanted into the BLA. Taking bregma as a reference point and according to the atlas of Paxinos and Watson, the coordinates for the BLA were: AP = -2.8mm, ML = ± 5mm, and DV = -6.5mm. 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 prevent possible occlusion. Postsurgery, rats were placed again in their home cages in groups of four, in a same condition as before surgery. Five to seven days later, rats received a bilateral intra-BLA infusion via dental needles (27-gauge) introduced through guide cannulae until their tips reached 1mm below the cannulae end. One Fl/side of solutions was injected into BLA over 60 s, using a 2.5-Fl glass Hamilton syringe. A polyethylene catheter was interposed between the upper end of dental needles and the microsyringe. The displacement of an air bubble inside the polyethylene tube was an indicator to monitor the drug flow. Needles were removed 60 s after the infusions were completed.

Apparatus
The EPM was a wooden cross-shaped maze, consisting of four arms arranged in the shape of a plus sign. Two of the arms have no side or end walls (open arms; 50 × 10 cm). The other two arms have side walls and end walls, but are open on the top (closed arms; 50 × 10 × 40 cm). Where the four arms intersect, there is a square platform of 10 × 10 cm. The maze was elevated 50 cm above the floor level. The animals were randomly allocated to treatment conditions and tested in counterbalanced order. The rats were individually placed in the center of the maze facing a closed arm and allowed five minutes of free exploration. The number of entries into open arms, the number of entries into closed arms, and the total time spent in the open arms and total time spent in the closed arms were measured. Entry was defined as all four paws in the arms.

General conditions and data collection
We used the EPM to examine memory and anxiety-like behaviors in test-retest fashion as previously outlined. Rats were left undisturbed at the testing room one hour prior to the test so that to adapt to the experiment environment. They were then individually placed on the initial part of the open arms of the maze facing the center and allowed for five minutes of free exploration. Experiments were under a low-illuminance (60-lux) condition, over the diurnal phase, between 13:00 and 17:00 hours. The five-minute EPM sessions were recorded by a video camera while a monitor and a digital recording system were installed in the next room. The observer, who quietly sat one meter behind one of the closed arms of the maze using chronometers, measured the time spent in open arms, the time spent in the closed arms, and the number of entries into open and closed arms. Entry was considered only when all four paws were in the arms. The maze was cleaned with distilled water after each EPM session. The recorded raw data were used to calculate the %OAT (open-arms time percentage) which is percentage of time each rat spent in the open arms relative to the total amount of time spent in any arm (open/total × 100). Open-arms entries percentage (%OAE) was recorded as a correlate for lack of aversive memory during retest. The sum of all open- and closed-arms entries was used as an index for general locomotor activity. Findings during EPM behavioral assessments also relate to emotion, as the behaviors exhibited on test arise from a conflict between the motivation to explore the maze and the natural tendency to avoid open spaces. The test-retest procedure in the EPM is an appropriate method to test the features related to anxiety and memory. In general, animals acquire information with regard to safe and dangerous areas in the maze on test. Repeated testing in the EPM (usually in 24 hours) induces experience-dependent behavioral changes and represents an index of memory acquisition, consolidation, and retention. When rodents are exposed to a retest session, their open-arms exploration time reduces and this pertains to the presence of their aversive learning and memory.

Drugs
The drugs used in this study were ketamine and xylazine (Alfasan Chemical Co, Woerden, Holland) for animal anesthesia, morphine sulfate (as a mu-opioid receptor agonist, for IP injec-
tion, Temad, Tehran, Iran), salbutamol (as a β2-adrenoceptor agonist, IranDaru, Tehran, Iran), and propranolol (as a β-adrenoceptor antagonist, Iran Daru, Tehran, Iran) for intra-BLA injection. Morphine, salbutamol, and propranolol were dissolved in sterile 0.9% saline just before being injected. Control animals received saline.

Verification of cannula placements
After completion of the experimental sessions, each animal was killed with an overdose of chloroform. Subsequently, 1 μL of ink (1% aquatic methylene blue solution) was injected into the BLA by a 27-gauge injection cannula, which projected a further 1 mm ventral to the tip of the guide to aid in histologic verification. The brains were removed and fixed in a 10% formalin solution 10 days before sectioning. Sections were examined to determine the location of the cannula aimed for the BLA. The cannula placement was verified using the atlas of Paxinos and Watson.22 Data from rats with cannula placement outside the BLA were excluded from the analyses.

Statistical analysis
Given the normality of distribution and homogeneity of variance, the results were statistically evaluated by one-way analysis of variance (ANOVA). Mean ± SEM was used to compare outcomes between experimental groups with their controls. Where F-value was significant, post-hoc analysis (Tukey test) was performed. Differences with P < 0.05 between groups were considered statistically significant.

Results
Effects of morphine on anxiety-like behavior and memory retrieval
Figure 1 shows the effects of IP injections of morphine on anxiety-related parameters and memory formation in the EPM. Rest test session was performed 24 hours later. A one-way ANOVA revealed that morphine dose-dependently increased %OAT [F (3, 40) = 4.298, P < 0.05] indicating an anxiolytic response and
improvement of memory by morphine. No significant change in the %OAE [F (3, 40) = 5.761, P < 0.01], and locomotor activity [F (3, 40), P > 0.05] was observed following administration of morphine.

Effects of salbutamol alone or with morphine on anxiety-like behavior and memory retrieval

The effects of salbutamol alone or with morphine on anxiety and memory storage have been shown in Figure 2. One-way ANOVA indicated that salbutamol (1, 2, and 4 μg/rat, intra-BLA) has different effects dose-dependently on anxiety-like behavior. Low dose(s) of salbutamol decreased %OAT [F (3, 40) = 15.718, P < 0.001] and %OAE [F (3, 40) = 6.311, P < 0.01] indicating an anxiogenic effect, but the highest dose decreased the anxiety parameters in test session and improved the memory in retest session.

**Figure 3.** The effect of the intra-BLA microinjection of propranolol on exploratory-like behaviors in rats subjected to EPM in presence and absence of morphine. Rats were tested in the EPM, five minutes after microinjection of saline (1μL/rat) or propranolol (1, 2, and 4 μg/rat) in those which were treated by saline (1mL/kg) or effective dose of morphine (6 mg/kg) IP previously. Twenty four hours later, all groups were retested in the EPM, undrugged. A) % open-arms time, B) open- arm entries, C) locomotor activity. Values are expressed as mean ± SEM (n = 6 in each group). *P < 0.05 different from saline/saline or saline/morphine control group on test day. +P < 0.05, +++P < 0.001 different from test group in the same session. ###P < 0.001 different from test group in left panel.

**Figure 4.** The effect of intra-BLA propranolol microinjection on exploratory-like behaviors induced by ‘salbutamol’ and ‘salbutamol plus morphine’. Rats were tested in EPM, five minutes after microinjection of saline (1μL/rat) or salbutamol (1, 2, and 4 μg/rat) in those which were treated by saline (1mL/kg) or subthreshold dose of propranolol (1 μg/rat) in presence or absence of subthreshold dose of morphine (4 mg/kg). Twenty four hours later, all groups were retested in the EPM, undrugged. A) % open arm time, B) open arm entries, C) locomotor activity. Values are expressed as mean ± SEM (n = 6 in each group). *P < 0.05, **P < 0.01, ***P < 0.001 different from saline/saline control group on test day. +P<0.05, +++P < 0.001 different from test group in the same session.
ing and improving effect on anxiety [F (3, 40) = 9.493, P < 0.001] and memory parameters (% OAT), respectively. But had no effects on %OAE [F (3, 40) = 0.993, P < 0.05]. Further analysis also showed that the anxiolytic effect of 4 µg/rat of salbutamol was significantly increased by morphine (4 mg/kg), given five minutes before salbutamol synergistically. It should be noted that no significant change in the locomotor activity was observed following administration of salbutamol [F (3, 40) = 0.337, P > 0.05] and salbutamol plus morphine [F (3, 40) = 0.821, P > 0.05]. There was not significantly between any test group in right panel and their group in left panel in % OAT [F (6, 40) = 19.249, P < 0.001], % OAE [F (6, 40) = 3.886, P < 0.01], and locomotor activity [F (6, 40) = 0.391, P > 0.05].

Effects of propranolol alone or with morphine on anxiety-like behavior and memory retrieval

The effects of propranolol alone or with morphine on anxiety and memory storage have been shown in Figure 3. One-way ANOVA indicated that propranolol (4 µg/rat, intra-BLA) decreased %OAT [F (3, 40) = 12.523, P < 0.001], but had no effect on %OAE [F (3, 40) = 6.295, P > 0.01] and locomotor activity [F (3, 40) = 0.498, P > 0.05]. Administration of different doses of propranolol had no effect on memory in retest sessions. One-way ANOVA indicated that coadministration of propranolol (4 µg/rat, intra-BLA) with effective dose of morphine (6 mg/kg, IP) had a decreasing effect on % OAT [F (3, 40) = 1.398, P > 0.05], but had no effect on memory retrieval. In this experiment, propranolol (4 µg/rat) reversed the decreasing and improving effects of morphine (6 mg/kg) on anxiety and memory, respectively. Mentioned coadministration did not show any effects on %OAE [F (3, 40) = 0.335, P > 0.05] and locomotor activity [F (3, 40) = 0.523, P > 0.05] in test or retest sessions. In all test groups in right panel, there was an increase in % OAT compared with their retest groups in left panel [F (6, 40) = 4.676, P < 0.001], but not in % OAE [F (6, 40) = 1.953, P > 0.05] and locomotor activity [F (6, 40) = 0.251, P > 0.05].

Effects of salbutamol and propranolol or salbutamol and propranolol with morphine on anxiety-like behavior and memory retrieval

Figure 4 shows the effects of intra-BLA propranolol microinjection on exploratory-like behaviors induced by salbutamol and salbutamol plus morphine. Rats were tested in EPM, five minutes after microinjection of saline (1mL/rat) or salbutamol (4µg/rat) in those which were treated by saline (1µL/rat) or subthreshold dose of propranolol (1 µg/rat) in presence or absence of subthreshold dose of morphine (4 mg/kg).Twenty four hours later, all groups were retested in the EPM, undrugged. Injection of subthreshold dose of propranolol reduced the %OAT [F (6, 50) = 19.045, P < 0.001] and %OAE [F (6, 50) = 8.961, P < 0.001] induced by salbutamol (4µg/rat) either in test or retest sessions. This reduction was less in presence of morphine.

Discussion

The basolateral structures of amygdala (BLA) are cortex-like. This complex consists of a majority of glutamatergic neurons and a minority of local GABAergic interneurons. The medial structures (CEA) are striatum-like, with the vast majority of neurons being GABAergic and exhibiting medium spiny-type morphology.25 Projections between nuclei of amygdala generally follow from dorsal to ventral and lateral to medial parts (e.g., from LA to BA and from BLA to CEA).25 One of the main flows of information within the amygdala follows a serial path in the direction of the main internuclear projections, while other parallel inputs and outputs exist.26,27 Lateral amygdala (LA) receives multimodal and early sensory information from the thalamus and cortex.28,29 Systemic or local treatments that increase GABAergic transmission produce anxiolytic effects30 and can interfere with the acquisition or expression of memory responses.31,32 In contrast, pharmacologic manipulations that decrease GABAergic transmission induce anxiogenic–like effects33,34 and can improve learning or retrieval of memories.35,36 Anxiety behavior and acquired anxiety responses are subject to modification by neuromodulators and neuropeptides. In the amygdala, inhibitory neurons are major targets of neuromodulatory systems.7,37–41 This may allow inhibition-dependent functions of amygdala networks to be adjusted according to the environmental conditions and the behavioral state of the animal. Plasticity at sensory inputs to the BLA is the most important neural mechanisms of acquisition and expression of memory. Many studies support the notion that the LA is an essential site where early, NMDA receptor-dependent changes in neuronal activity are required for the acquisition of memory.42–48 Neurmodulators that are released in the amygdala upon stress can gate induction of plasticity by transiently suppressing pre- or postsynaptic inhibition. LA projection neurons receive substantial GABAergic feedforward inhibition, which tightly controls their activity.49 This inhibition can be suppressed or enhanced by neuromodulators. While dopamine, noradrenaline, or opioids block feedforward inhibition, and thereby promote LTP induction postsynthetically,50 other modulators, such as gastrin-related peptide and serotonin, enhance inhibition, thereby possibly constraining LTP induction.51

Recent evidence shows that there is an inverse relationship between GABAergic inhibitory tone in the BLA and behavioral anxiety.52 Results of previous studies indicate that morphine via mu-opioid receptors exert anxiolytic effects, probably by interacting with the GABAergic system.53

The present results indicate that rats treated with morphine showed a selective increase in the percentage of time spent in the open arms of the EPM with no significant effects on the locomotor activity in the maze.

NE-containing neurons can be found throughout the nervous system and point therefore to a prominent role for this neurotransmitter in the central nervous system. Adrenaline and its agonists increase memory performance. Beta-adrenergic antagonists, unlike, lead to decreased memory performance, e.g., longer retention times.7 Beta-blockade also decreased the performance that was raised artificially by agonistic-working agents that were injected concurrently.54 Infusions of noradrenaline into the BLA of rats increased the consolidation of memory, whereas infusion of β-adrenoceptor antagonists impaired this consolidation.55 Several evidences indicate that noradrenergic activity in the BLA has pivotal role in mediating the modulatory effects of other hormones and neurotransmitters on memory consolidation. Many investigations have reported that intra-BLA injections of the GABAergic receptor antagonist bicuculline enhance memory consolidation and that GABAergic receptor agonists impair memory.56 Antagonists for β-adrenoceptors injected into the amygdala block the memory-enhancing effects of bicuculline infused concurrently.57 It is shown that activation of the CB1 endocannabinoid receptor in the BLA increases memory consolidation whereas blockade of
CB1 receptors induces memory impairment. Endocannabinoids might enhance memory consolidation by inhibiting GABAergic activity in the BLA, thereby presynaptically increasing noradrenergic transmission. Glucocorticoids also require noradrenergic activity in the BLA to enhance the consolidation of memory. On the other hand, the activation of glucocorticoid receptors (GRs) in the BLA facilitates memory consolidation through a rapid potentiation of the noradrenaline signaling cascade; conversely, intra-BLA infusions of a GR antagonist attenuated the dose–response effects of a β-adrenoceptor agonist on retention enhancement. These findings suggest that glucocorticoids facilitate the effects of noradrenergic stimulation in the BLA on memory consolidation through an interaction with β-adrenoceptor-AMP cascade.

Beta-adrenoceptor antagonist propranolol, administered systemically or directly into the BLA, blocked the corticosterone-induced memory enhancement. It is shown that BLA mediates the modulatory effects of stress hormone sat the behavioral level. Activation of α2-adrenoceptors had inhibitory effects, but noradrenaline also had smaller, excitatory effects that were mediated by activation of β-adrenoceptors. These results show that noradrenaline enhanced excitatory neurotransmission through β-adrenoceptors and produced inhibitory effects through α2-adrenoceptors. Thus, β-adrenoceptors mediate both the facilitative effects of noradrenaline on memory consolidation and the excitatory effects in the BLA.

In this study, injection of salbutamol (intra-BLA) has different effects dose-dependently on anxiety-like behavior. Low dose (s) of salbutamol, decreased %OAT and %OAE indicating an anxiogenic effect, but the highest dose decreased the anxiety parameters and improved the memory in retest session. Co-administration of saline and different doses of salbutamol with subthreshold dose of morphine had a decreasing and improving effect on anxiety and memory parameters, respectively. Anxiolytic effect of salbutamol was significantly increased by morphine synergistically.

Administration of propranolol (into the BLA) decreased %OAT, but no effect on %OAE and locomotor activity. Administration of different doses of propranolol had no effect on memory in retest sessions. Co-administration of propranolol with effective dose of morphine (IP) had a decreasing effect on %OAT, but had no effect on memory retrieval. In this experiment, propranolol (4 μg/rat) reversed the decreasing and improving effects of morphine (6 mg/kg) on anxiety and memory, respectively. Mentioned coadministration did not show any effects on %OAE and locomotor activity in test or retest sessions.

Summarizing, some neuromodulatory peptides such as noradrenaline and morphine effects on motivational arousal induced memory in the BLA, via changing in GABAergic tone in this site. On the other hand, several agents like endocannabinoids and glucocorticoids might change inhibiting GABAergic activity thereby increasing noradrenergic transmission in the BLA. Our investigation showed that 1) activation of mu-opioid receptors in BLA induced anxiolytic-like behaviors and improved emotional memory, 2) activation and deactivation of β2-adrenoceptors induced anxiolytic and anxiogenic-like behaviors, respectively in this site. These interventions in BLA however, altered emotional memory and 3) effects of these two systems on anxiety and memory may be independent to each other in BLA.

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