Iranian Journal of Basic Medical Sciences

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$GABA_B$ receptors within the central nucleus of amygdala may involve in the morphine-induced incentive tolerance in female rats

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Nov 11, 2016
Accepted: May 25, 2017

Keywords:
Central nucleus of amygdala
Conditioned place preference
$GABA_B$ receptors
Morphine
Tolerance

ABSTRACT

Objective(s): Central nucleus of amygdala (CeA) is the most important region for morphine-induced reward, and GABAergic system plays an important role on morphine reinforcement. The influence of CeA administration of $GABA_B$ receptor agonist and antagonist on the expression and acquisition of morphine-induced incentive tolerance using conditioned place preference (CPP) paradigm was investigated in the present study. Our purpose was to evaluate the role of CeA $GABA_B$ receptors in morphine tolerance.

Materials and Methods: Seven days after surgery and cannulation, the experiments were begun. Subcutaneous (SC) injections of morphine induced CPP. Administration of one daily dose of morphine (12.5 mg/kg) for 3 days in order to develop tolerance to the drug reduced the conditioning induced by morphine (7.5 mg/kg, SC). $GABA_B$ receptor agonist, baclofen (1.5, 6 and 12 µg/rat) or $GABA_B$ receptor antagonist, CGP35348 (1.5, 6 and 12 µg/rat) were injected into the CeA 5 min before the experiments in the test day (expression of tolerance) or 5 min before each injection of morphine (12.5 mg/kg) (acquisition of tolerance).

Results: It was shown that injections of baclofen (1.5 and 12 µg/rat) reduced acquisition, whereas the dose of 6 µg/rat of the drug exacerbated the acquisition of morphine tolerance. Baclofen at all doses significantly increased the expression of tolerance to morphine. Administration of CGP35348 (1.5, 6 and 12 µg/rat) reduced the acquisition and expression of morphine tolerance.

Conclusion: These results confirmed the importance of $GABA_B$ receptors with in the CeA in morphine tolerance in female rats.

► *Please cite this article as:*
Alavian F, Ghiasvand S. $GABA_B$ receptors within the central nucleus of amygdala may involve in the morphine-induced incentive tolerance in female rats. Iran J Basic Med Sci 2017; 20:822-828. doi: 10.22038/IJBMS.2017.9018

Introduction

The prevalence of opioid abuse is high worldwide, while the problem still remained unresolved. Several studies have shown that repeated administration of high doses of morphine may decrease its effects; which is known as morphine tolerance. Tolerance to morphine reduces its motivational effects and as a consequence, the opioid addicts need increasing drug dosage to achieve the initial effect (1, 2). Behavioral tolerance can be divided into two stages: Acquisition and expression. Acquisition is the instant neural events, and driving behavioral tolerance and expression is the long-term outcome of these initial events. It seems that the acquisition stage is generally in association with the ventral tegmental area (VTA), but the expression stage is associated with the nucleus accumbens (NA) (3, 4).

The mechanisms of tolerance to morphine have been studied extensively in part due to the negative effects of this phenomenon on long-term opiate analgesia. Despite the opioid receptor down-regulation in response to chronic morphine exposure *in vitro* and *in vivo* (1, 5), there is a strong up-regulation of the cyclic adenosine-mono-phosphate (cAMP) system (6). In addition, the N-methyl-D-Asparatate (NMDA) and metabotropic glutamate receptors have been shown to be involved in this phenomenon (7).

Various evidences prove that dopaminergic neurons activity in the VTA is modulated by GABAergic inhibitory inputs (8). Previous studies have shown that dopaminergic cell firing in the VTA can be inhibited by activation of GABA receptors. Therefore, $GABA_B$ receptors within the VTA have important role in opioid reinforcement (9, 10). On the other hand, conditioned place preference (CPP) is a learning pattern requiring communication between reward and special places, which has been widely used to study the rewarding effects of morphine (11). In this respect, administration of the $GABA_B$ receptor agonist, baclofen, into the VTA

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reduced extra-cellular dopamine in NA and blocked opioid reinforcing effects in rats (12). In addition, baclofen reduced the reinforcing effects of morphine and alcohol, as well as morphine-induced FOS expression in NA of mice (13-15). The $GABA_B$ receptor agonists also inhibit the reinforcing effects of other opioids such as heroine self-administration and heroine-induced dopamine release in NA of rats (16). By intra-VTA baclofen administration, morphine-induced CPP is reduced, and this effect can be inhibited by GABA receptor antagonist (10, 15, 17).

Considerable evidences suggest that the CeA is also involved in morphine rewarding effect (18). It has been shown that morphine tolerance might alter gating receptor modulated K+ channels on amygdala neurons in rats (19).

However, the role of $GABA_B$ receptors within the CeA in morphine-induced tolerance in female rats was not established earlier. Considering the difference between male and female animals in response to morphine (20-22), the aim of this study is to provide further clarification for the role of $GABA_B$ receptors in morphine tolerance in female rats. For this purpose, we use the CPP paradigm as a model for investigation of morphine reinforcing properties.

Materials and Methods

Animals

Female Wistar rats (225±25 g, Pasture Institute, Tehran, IRAN) were used throughout the study (6-9 rats for each experiment). Animals were housed in groups of 4 per cage in a 12/12 hr light/dark cycle (lights on at 07.00 AM), with *ad libitum* food and water available. The animals were randomly allocated to different groups of the experiment. All experiments were conducted in accordance with standard Ethical Guidelines and approved by the Local Ethical Committee (The Baqiyatallah (A.S.) University of Medical Committee on the use and care of Animals).

Drugs

The following drugs were used in these experiments: morphine sulfate (TEMAD, Iran), baclofen and CGP35348 (Novartis Basel, Switzerland), ketamine hydrochloride and Xylazine (Alfasan Worden, Holland). The drugs were dissolved in sterile saline. Morphine was injected subcutaneously in a volume of 1 ml/kg; baclofen and CGP35348 were given intra-CeA in doses of 1.5, 6 and 12 µg/rat and were prepared before use (10, 23). The control groups received saline.

Surgical procedures

Rats were anesthetized with intra-peritoneal (IP) injection of ketamine hydrochloride (70 mg/kg) + Xylazine (10 mg/kg), and two stainless steel cannulas (23-gauge) were placed stereotaxically (Stoelting instruments, USA) into the CeA. Stereotaxic coordinates according to the Paxinos and Watson (1986) were: incisor bar (-3.3 mm), -7.8 mm dorsal-ventral (DV), ±4.1 mm middle-lateral (ML) and -2.12 mm anterior-posterior (AP). Cannulas were secured to anchor jewelers' screws with dental acrylic. All animals were allowed one week to recover from surgery and clear anesthetic.

Injection into the central nucleus of amygdala

In order to intra-CeA injections, each animal received the drugs via a 30-gauge (0.3 mm outer diameter) blunt tapered needle (0.25 µl/side), at a rate of 0.5 µl/min. After the completion of each injection, needle remained in the guide cannula for 1 additional min and then was removed from guide cannula, and after 2 min animal was placed in the apparatus.

Apparatus

A two compartment CPP apparatus (30X60X30 cm) was used in these experiments. Place conditioning was conducted using an unbiased procedure, with minor changes to the design previously described (24). The apparatus was made of wood. Both compartments were identical in size (the apparatus was divided into two equal-sized compartments by means of a removable white wall) and shading (both were white), but distinguishable by texture and olfactory cue. To provide the tactile difference between the compartments, one of the compartments had a smooth floor, while the other compartment had a nylon white mesh floor. A drop of menthol was placed at the right center of the compartment with a textured (nylon mesh) floor, to provide the olfactory difference between the compartments. Two compartments were differently striped black on their sides. In this apparatus, rats showed no consistent preference for either compartment.

Induction of morphine tolerance

Tolerance to morphine was achieved based on the method described in our previous work (25). Animals were randomly treated subcutaneously with morphine (12.5, 25 and 50 mg/kg, SC) once daily (9:00 AM), for a period of 3 days. On the fourth day, the CPP paradigm was induced by morphine (7.5 mg/kg, SC). However, the doses of 25 and 50 mg/kg killed more than 60% of the animals.

Measurement of CPP

The experimental period of CPP started immediately on the day after tolerance inducement. CPP consisted of three phases: pre-conditioning, conditioning and post-conditioning (26):

Pre-conditioning: On the first day of conditioning paradigm section (4^{th} day of the experiments), each morphine-tolerated rat was placed separately into the apparatus for 20 min, with free access to all

compartments.

Conditioning: This phase consisted of a 3-day schedule of conditioning sessions (days 5-7 of the experiments). In this phase, animals received three trials in which they experienced the effects of morphine (7.5 mg/kg, SC) while confined in one compartment for 40 min, and three trials in which they experienced the effects of saline while confined in the other compartment. Access to the compartments was blocked on these days.

Post-conditioning phase: On the 5th day of conditioning paradigm section (8th day of experiments), the partition was removed, and the rats could access entire the apparatus. The mean time for each rat spent in each compartment during a 10 min period, was determined as the preference criteria. No injection was given in the acquisition tests.

Induction of expression and acquisition

In this stage, the animals received 1.5, 6 and 12 µg/rat of the mentioned drug ($GABA_B$ receptor agonist or antagonist) via intra-cannula injection (i-CeA) 5 min before the experiments on the test day (8th day of the experiments) for expression, or 5 min before each morphine (12.5 mg/kg, SC) injection for acquisition. In order to prevent drug relapse, injection cannula was kept still.

Histology

After the completion of testing, all animals were anesthetized and received a transcardial perfusion with 0.9% normal saline followed by 10% buffered formalin. The brains were removed, blocked and cut coronally in 40 µm sections through cannula placement. The tissue stained with crystal violet was examined by light microscopy by an observer unfamiliar with the behavioral data. Only animals with confirmed cannula placements included in the data analysis. Cannula location accuracy was compared with the figure from the Paxinos and Watson Atlas (Figure 1).

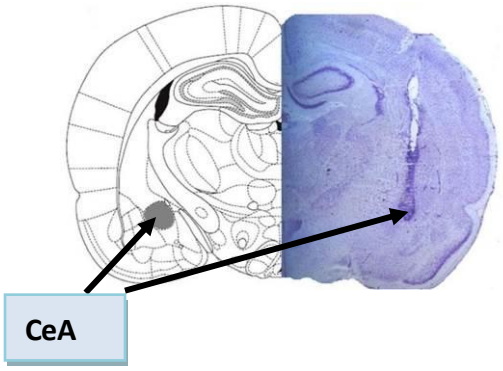

Figure 1. Schematic design of the histological instauration showing the localization of the guide cannula (27); where cannula tips were correctly placed in the central nucleus of amygdala (CeA) in accordance with Paxinos and Watson Atlas (28): incisor bar (-3.3mm), -7.8 mm dorsal-ventral (DV), ±4.1 mm middle-lateral (ML) and -2.12 mm anterior-posterior (AP)

Statistical analysis

The conditioning scores represent the time spent in the drug-paired place minus the time spent in the saline-paired place, given as the mean±SEM for 6-9 animals (24). In order to test the hypothesis, one way analysis of variance (ANOVA) followed by Tukey's test and t-test were performed to assess specific group comparisons. Differences with $P<0.05$ were considered statistically significant.

Results

Morphine dose-response on CPP paradigm

The effects of morphine have been shown in Figure 2. Injection of different doses of morphine sulfate (0.5, 1, 5, 7.5, 10 mg/kg, SC) to rats caused a significant increase in time spent in the drug-paired compartment compared to that spent in the saline-paired compartment. According to the data obtained, 7.5 mg/kg subcutaneous dose was used in further stages as an effective dose, which could induce CPP. Subcutaneous injection of saline to the animals (saline control group) in the conditioning compartments did not produce any preference or aversion for either place.

Effect of 12.5 mg/kg morphine on CPP inhibition in tolerated animals

Compared with the control group, 12.5 mg/kg dose of morphine could induce tolerance in the animals and thus inhibit conditioned place preference (CPP) (Figure 3).

Effects of intra-CeA injections of $GABA_B$ agonist and antagonist on the expression of morphine tolerance

Animals were tolerated to morphine (12.5 mg/kg, SC) and conditioned with morphine (7.5 mg/kg, SC) as described in the method section. Different doses of baclofen (1.5, 6 and 12 µg/rat) or CGP35348 (1.5, 6 and 12 µg/rat) were administered to the animals 5 min before the experiments on the test day (8th day of the experiments). The results are shown in Figure 4A and 4B.

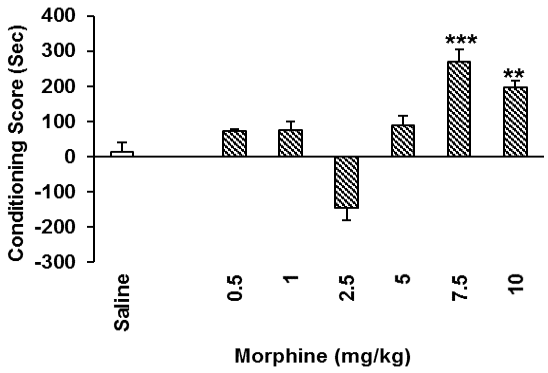

Figure 2. Conditioned place preference induced by morphine. Animals received different doses of morphine (N ≥8, data as mean ± SEM and $^{**}P<0.01$, $^{***}P<0.001$)

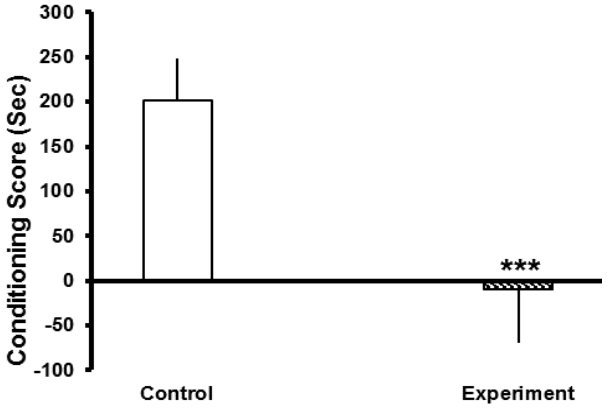

Figure 3. Effect of morphine (subcutaneous 12.5 mg/kg) in inducing conditioned place preference in the group with induced tolerance. (N ≥8 and ***P<0.001)

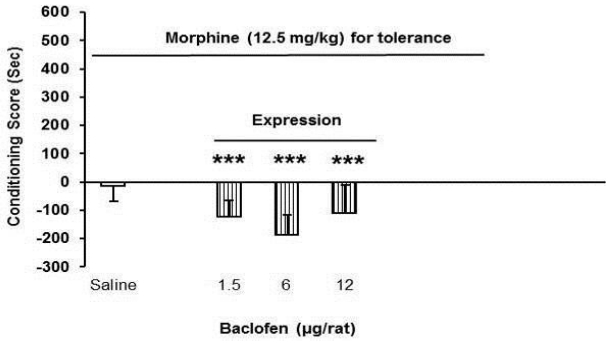

Figure 4A. Effects of the intra-central nucleus of amygdala administration of baclofen on the expression of conditioned place preference in morphine-induced tolerance. Animals received baclofen (1.5, 6 and 12 µg/rat) 5 min before the test (N ≥8, data as mean± SEM and ***P<0.001)

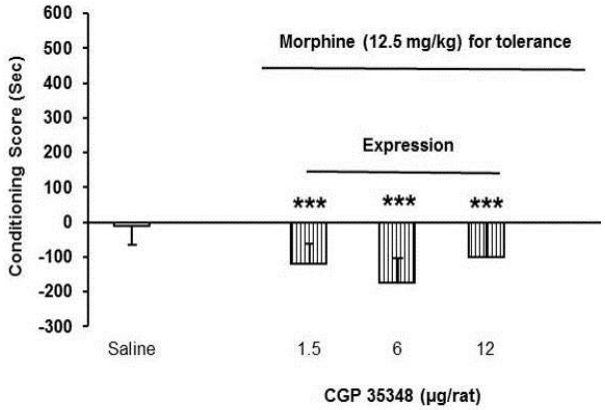

Figure 4B. Effects of the intra-central nucleus of amygdala administration of CGP35348 on the expression of conditioned place preference in morphine-induced tolerance. Animals received CGP35348 (1.5, 6 and 12 µg/rat) 5 min before the test. (N ≥8 data, data as mean±SEM and ***P<0.001)

Effects of intra-CeA of amygdala injections of $GABA_B$ receptor agonist and antagonist on the acquisition of morphine tolerance

Animals were received different doses of baclofen (the $GABA_B$ receptor agonist), (1.5, 6 and 12 µg /rat) or CGP35348 (the $GABA_B$ receptor antagonist), (1.5, 6 and 12 µg/rat) 5 min before each morphine (12.5 mg/kg, SC) injection, during induction of morphine tolerance; and conditioned with morphine (7.5 mg/kg, SC) during conditioning section. These animals were tested in the test day (8th day of the experiments) in drug free state. The results are shown in Figure 5A and 5B.

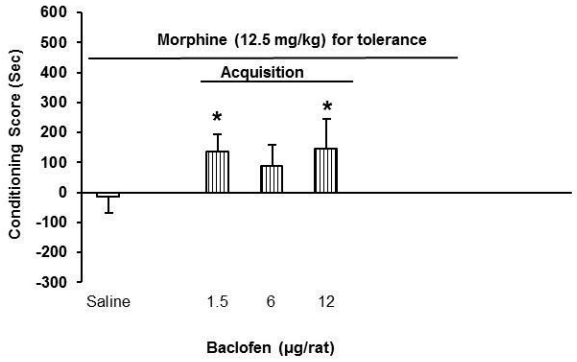

Figure 5A. Effects of intra- central nucleus of amygdala injections of baclofen on the acquisition of morphine-induced tolerance. Animals received baclofen (1.5, 6 and 12 µg/rat) 5 min before morphine injection during the induction of tolerance. (N ≥8, data as mean±SEM and *P<0.05)

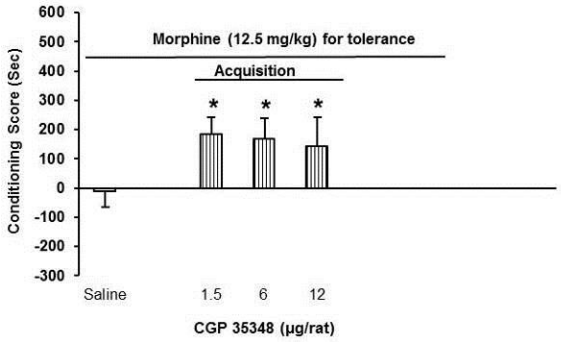

Figure 5B. Effects of intra- central nucleus of amygdala injections of CGP35348 on the acquisition of morphine-induced tolerance. Animals received CGP35348 (1.5, 6 and 12 µg/rat) 5 min before morphine injection during the induction of tolerance (N ≥8, data as mean ± SEM and *P<0.05)

Discussion

In accordance with previous studies in male rats (24), as well as female mice (29), the present study showed that morphine administration increased the time spent in the drug-paired side in female rats, and induced CPP while subcutaneous injections of saline did not induce any response.

Several lines of evidences have demonstrated that morphine exerts its positive reinforcing effect via activation of μ-opioid receptors located in the VTA (30, 31). In agreement with these data, some investigators suggested that μ-opioid receptor knockout mice did not show motivation to the morphine (32, 33). However, investigators emphasis that female rat is more sensitive to the morphine in CPP paradigm than male rats (22, 34). In addition, injection of high dose of morphine produced tolerance in rats. These animals showed less sensitivity for morphine than morphine-non-tolerated rats; indicating tolerance to morphine. This result is in agreement with previous studies (25), which showed that morphine-tolerated mice have less propensities for morphine.

Despite several investigations regarding the role of $GABA_B$ receptors on morphine tolerance and positive reinforcing properties (26, 35, 36), there is limited information on the role of $GABA_B$ receptors within the CeA on morphine-induced CPP in morphine-tolerated rats.

In the present study, intra-CeA injections of $GABA_B$ receptor agonist, baclofen, and also $GABA_B$ receptor antagonist, CGP35348, produced an interesting response on the expression of morphine-induced conditioned place preference in morphine-tolerated rats. These findings may suggest that there may be $GABA_B$ receptor subtypes in the CeA, which play a role in the expression of morphine CPP in morphine-tolerated rats. Excitation and/or inhibition of these receptors reduced animal response to the morphine. Many studies confirmed that morphine tolerance is considered as the major problem in the treatment with morphine (37). Some data have shown that pre-synaptic $GABA_B$ receptor inhibition leads to more GABA release from the GABAergic neurons and increases the GABA-mediated inhibitory responses (38).

$GABA_B$ receptors are further subdivided into $GABA_BR1a$, $GABA_BR1b$ and $GABA_BR2$ subtypes. Radio ligand binding and in situ hybridization studies suggested that $GABA_BR1a$ subtypes are located primarily pre-synaptic, $GABA_BR1b$ subtypes are located primarily post-synaptic, and $GABA_BR2$ subtypes are located at both pre and post-synaptic sites (39, 40).

In our study, both baclofen and CGP35348 showed a similar response in the expression of morphine-induced CPP in morphine-tolerated rats; excitation and inhibition of $GABA_B$ receptors within CeA causing a sharp strengthening of morphine tolerance. The results may indicate that both pre- and post-synaptic $BAGA_B$ receptors within the CeA are involved in the expression of morphine CPP in morphine-tolerated rats. Probably, excitation and inhibition of specific subgroups of $GABA_B$ receptors led to the weakening of the animal's response to morphine.

Injection of baclofen and CGP35348 into the CeA reduced the acquisition of morphine CPP in morphine-tolerated rats. The response was dose-dependent for baclofen. Considering the responses obtained in the present study from baclofen, it can be concluded that activation of $GABA_B$ receptors during tolerance development may activate mechanism(s), which inhibits morphine tolerance as shown by CPP paradigm. The result is very interesting since it indicated that baclofen might be a useful medication in treatment of morphine tolerance. Previous studies have shown that baclofen inhibits morphine tolerance in rats (35), which is in agreement with the results obtained in our research.

The inability of CGP35348 for reduction of morphine CPP in morphine-tolerated rats indicated that physiologic GABA within the CeA might have not a significant role in this phenomenon. Our data, may be in agreement with previous studies, showed that the $GABA_B$ receptors were involved in morphine-induced behavioral sensitization in mice (41, 42) and also inhibited the morphine-induced CPP in rats (43), mice (15) and associated induction of Fos in cortical and limbic regions in mice (41, 42).

The role of $GABA_B$ receptors in reduction of heroin (16) and cocaine (44) self-administration in rats is also well documented. So, it is not surprising that baclofen could inhibit the acquisition of morphine CPP in morphine-tolerated rats.

Cellular and molecular mechanism(s) underlying the baclofen or CGP35348 effects are not well understood. It is known that baclofen reduces cyclic adenosine-mono-phosphate (cAMP) in target cells. However, it is clear that the main role of $GABA_B$ receptors is to inhibit the release of several neurotransmitters in the central nervous system (39, 40). Since, it is well known that during morphine tolerance, concentration of cAMP is increased in morphine-targeted cells, and the release of some neurotransmitters is decreased (45), baclofen or CGP35348 may involve in theses mechanisms and morphine tolerance may be reduced as a result.

Differential effects of both drugs on expression and acquisition of morphine represents the difference effectiveness of the drug in different times, which may be related to hormonal fluctuations in the sexual cycle of female rats. On the other hand, CeA area is reciprocally connected to more rostral forebrain structures(46). Thus, the CeA receives direct excitatory, inhibitory, or peptidergic input from different areas. The neurotransmitters that are released from these inputs act on excitatory or inhibitory receptors at pre- and post-synaptic levels (46, 47) .Also inside the CeA, there are multiple synapses between GABAergic neurons whose final output may be inhibitory or excitatory (46) .These complex connections and complex interactions between cells may be the reason of these complex

results. However, the final judgment in this case requires further investigations.

Conclusion

The present study suggested that $GABA_B$ receptors within the CeA may play an important role on morphine CPP in morphine-tolerated rats and they may be good targets for treatment of opioid tolerance.

Acknowledgment

The authors would like to thanks Morteza Golzar for his technical assistance. This work was supported by the grant from the Behavioral Sciences Research Center (BSRC), Baqiyatallah (A.S.) University of Medical Sciences, Tehran, Iran.

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