GABA\textsubscript{B} receptors within the central nucleus of amygdala may involve in the morphine-induced incentive tolerance in female rats

Firoozeh Alavian 1*, Saeedeh Ghasvand 2

1Department of Basic Sciences, Farhangian University, Tehran, Iran
2Departments of Biology, Faculty of Science, Malayer University, Malayer, Iran

**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\textsubscript{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\textsubscript{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 conditioned induced by morphine (7.5 mg/kg, SC). GABA\textsubscript{B} receptor agonist, baclofen (1.5, 6 and 12 µg/rat) or GABA\textsubscript{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\textsubscript{B} receptors within the CeA in morphine tolerance in female rats.

---

**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-monophosphate (cAMP) system (6). In addition, the N-methyl-D-aspartate (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\textsubscript{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\textsubscript{B} receptor agonist, baclofen, into the VTA
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-tolerance induced 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 (TEMAx, 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), ±1.4 mm middle-lateral (ML) and -2.12 mm anterior-posterior (AP). Cannulas were secured to anchor jeweler’s screws with dental acrylic. All animals were allowed one week to recover from surgery and gain 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 (4th 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 the entire 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).

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

Animals were received different doses of baclofen (the GABAB receptor agonist), (1.5, 6 and 12 µg/rat) or CGP35348 (the GABAB 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.

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 evidence 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 emphasize that female rats are 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\(_B\)R1a, GABA\(_B\)R1b and GABA\(_B\)R2 subtypes. Radio ligand binding and in situ hybridization studies suggested that GABA\(_B\)R1a subtypes are located primarily pre-synaptic, GABA\(_B\)R1b subtypes are located primarily post-synaptic, and GABA\(_B\)R2 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 BAGABA 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-monophosphate (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 these 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 interactions...
results. However, the final judgment in this case requires further investigations.

Conclusion

The present study suggested that GABA 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.

References

1. Morris BJ, Herz A. Control of opiate receptor number in vivo: Simultaneous μ-receptor down-regulation and δ-receptor up-regulation following chronic agonist/antagonist treatment. Neurosci 1989; 29:43-44.
2. Farvardin S, Moghimi M, Eskami P, Masoudi A. α-Terpinene attenuates morphine-induced physical dependence and tolerance in mice; role of nitric oxide. Iran J Basic Med Sci 2016; 19:201-208.
3. Khatibi A, Haghiparast A, Shams J, Dianati E, Komakli A, Kamalinejad M. Effects of the fruit essential oil of Cuminum cyminum L. on the acquisition and expression of morphine-induced conditioned place preference in mice. Neurosci lett 2008; 448:94.
4. Sahraei H, Falahi M, Zarrindast MR, Sabetkasaei M, Arout CA, Caldwell M, Rossi G, Kest B. Spinal and supraspinal N-methyl-d-aspartate and melanocortin-1 receptors contribute to a qualitative sex difference in morphine-induced hyperalgesia. Physiol Behav 2015; 147:364-372.
5. Chao J, Nestler EJ. Molecular neurobiology of drug addiction. Annu Rev Med 2004; 55:113-132.
6. Trujillo KA, Akil H. Inhibition of morphine tolerance and dependence by the NMDA receptor antagonist MK-801. Science 1991; 251:85.
7. Chalabi Yani D, Sahraei H, Meftahi GH, Hosseini SB, Sadeghi-Gharahedaghi S, Beig HA, et al. Effect of transient inactivation of ventral tegmental area on the expression and acquisition of nicotine-induced conditioned place preference in rats. Iran Biomed J 2015; 19:214.
8. Bijani S, Sadeghi-Gharahedaghi S, Zardoos H, Ghoshooni H, Eid A, Shams J, et al. Influence of nitric oxide in the central amygdala on the acquisition and expression of morphine-induced place preference in morphine sensitized rats. Basic Clin Neurosci 2011; 2:36-46.
9. Chen X, Marrero HG, Murphy R, Lin Y-J, Freedman JE. Altered gating of opiate receptor-modulated K+ channels on amygdala neurons of morphine-dependent rats. Proc Nat Acad Sci 2000; 97:14692-14696.
10. Arout CA, Caldwell M, Rossi G, Kest B. Spinal and supraspinal N-methyl-d-aspartate and melanocortin-1 receptors contribute to a qualitative sex difference in morphine-induced hyperalgesia. Physiol Behav 2015; 147:364-372.
11. Carroll ME, Lynch WJ, Roth ME, Morgan AD, Cosgrove KP. Sex and estrogen influence drug abuse. Trends Pharmacol Sci 2004; 25:273-279.
12. Cicero TJ, Ennis T, Ogden J, Meyer ER. Gender differences in the reinforcing properties of morphine. Pharmacol Biochem Behav 2000; 65:91-96.
13. Karami M, Zarrindast MR, Sepehri H, Sahraei H. Role of nitric oxide in the rath hippocampal CA1 area on morphine-induced conditioned place preference. Eur J Pharmacol 2002; 449:113-119.
14. Zarrindast MR, Faraji N, Rostami P, Sahraei H, Ghoshooni H. Cross-tolerance between morphine and nicotine-induced conditioned place preference in mice. Pharmacol Biochem Behav 2003; 74:363-369.
15. Zarrindast MR, Rezayof A. Morphine-induced place preference: interactions with neurotransmitter systems. Iran J Pharm Res 2010; 3:15.
16. Poletos-Burgess IA, Pentkowski NS, Der-Ghazarian T, Neisewander JL. Effects of the S-HT2C receptor agonist CP809101 in the amygdala on reinstatement of cocaine-seeking behavior and anxiety-like behavior. Int J Neuropsychopharmacol 2014; 17:1751-1762.
17. Poxinos G, Watson C. The rat brain in stereotaxic atlas. London: Academic; 1986.
29. Sahraei H, Ghazzaghi H, Zarrindast M‐R, Ghoshooni H, Sepehri H, Haeri‐Rohan A. The role of alpha‐adrenoceptor mechanism(s) in morphine‐induced conditioned place preference in female mice. Pharmacol Biochem Behav 2004;78:135‐141.
30. Koob GF, Bloom FE. Cellular and molecular mechanisms of drug dependence. Science 1988;242:715‐723.
31. Koob GF. Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol Sci 1992;13:177‐184.
32. Loh HH, Liu H‐C, Cavalli A, Yang W, Chen YF, Wei LN. μ Opioid receptor knockout in mice: effects on ligand‐induced analgesia and morphine lethality. Mol Brain Res 1998;54:321‐326.
33. Spanagel R, Weiss F. The dopamine hypothesis of reward: past and current status. Trends Neurosci 1999;22:521‐527.
34. Karami M, Zarrindast MR. Place aversion by morphine in offspring born of female morphine administered wistar rats. Iran J Pharm Res 2011;10:577.
35. Bexis S, Ong J, White J. Attenuation of morphine withdrawal signs by the GABA B receptor agonist baclofen. Life Sci 2001;70:395‐401.
36. Macey DJ, Froestl W, Koob GF, Markou A. Both GABA B receptor agonist and antagonists decreased brain stimulation reward in the rat. Neuropharmacology 2001;40:676‐685.
37. Hyman SE, Malenka RC. Addiction and the brain: the neurobiology of compulsion and its persistence. Nat Rev Neurosci 2001;2:695‐703.
38. Bernard C, Cossart R, Hirsch JC, Esclapez M, Ben‐Ari Y. What is GABAergic inhibition? How is it modified in epilepsy? Epilepsia 2000;41:S90‐S95.
39. Bettler B, Kaufmann K, Mosbacher J, Gassmann M. Molecular structure and physiological functions of GABAB receptors. Physiol Rev 2004;84:835‐867.
40. Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, et al. International union of pharmacology. XXXIII. Mammalian γ‐aminobutyric acidB receptors: structure and function. Pharmacol Rev 2002;54:247‐264.
41. Leite‐Morris KA, Fukudome EY, Kaplan GB. Opiate‐induced motor stimulation is regulated by P‐aminobutyric acid type B receptors found in the ventral tegmental area in mice. Neurosci Lett 2002;317:119‐122.
42. Leite‐Morris KA, Fukudome EY, Shoeb MH, Kaplan GB. GABAB receptor activation in the ventral tegmental area inhibits the acquisition and expression of opiate‐induced motor sensitization. J Pharmacol Exp Ther 2003;308:667‐678.
43. Tsuji M, Nakagawa Y, Ishibashi Y, Yoshii T, Taka shima T, Shimada M, et al. Activation of ventral tegmental GABA B receptors inhibits morphine‐induced place preference in rats. Eur J Pharmacol 1996;313:169‐173.
44. Shoaib M, Swanner LS, Beyer CE, Goldberg SR, Schindler CW. The GABAB agonist baclofen modifies cocaine self‐administration in rats. Behav Pharmacol 1998;9:195‐206.
45. Williams JT, Christie MJ, Manzoni O. Cellular and synaptic adaptations mediating opioid dependence. Physiol Rev 2001;81:299‐343.
46. Veinante P, Yakim J, Barrot M. The amygdala between sensation and affect: a role in pain. J Mol Psychiatry 2013;1:9.
47. Nuss P. Anxiety disorders and GABA neurotransmission: a disturbance of modulation. Neuropsychiatr Dis Treat 2015;11:165.