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School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

The possible mechanisms of analgesia produced by microinjection of morphine into the lateral habenula in the acute model of trigeminal pain in rats

Emad Khalilzadeh*, and Gholamreza Vafaei Saiah

Division of Physiology, Department of Basic Science, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, I.R. Iran.

Abstract

This study aimed to assess the effect of intra-habenular injection of morphine on acute trigeminal pain in rats. Also here, we examined the involvement of raphe nucleus opioid and $5HT_3$ receptors on the antinociceptive activity of intra habenular morphine to explore the possibility of existence of descending antinociceptive relay between the habenula and raphe nucleus. The numbers of eye wiping response elicited by applying a drop (40 µL) of NaCl (5 M) solution on the corneal surface were taken as an index of acute trigeminal nociception. Intra habenular microinjection of morphine at a dose of 2 µg was without effect, whereas at doses of 5 and 8 µg significantly produced antinociception. Microinjection of naltrexone (4 µg) and ondansetron (1 µg) into the dorsal raphe nucleus prior to intra-habenular saline did not produce any significant effect on corneal pain perception. Pretreatment of the raphe nucleus with ondansetron but not naltrexone prevented intra habenular morphine (8 µg) induced antinociception. Also, intra habenular injection of lidocaine (2%, 0.5 µL) reduced corneal pain response. Moreover, intra-habenular microinjection of L-glutamic acid (1 and 2 µg/site) did not produce any analgesic activity in this model of pain. In conclusion, the present results suggest that the activation of the habenular µ opioid receptor by microinjection of morphine or inhibition of habenular neurons by microinjection of lidocaine produced an analgesic effect in the acute trigeminal model of pain in rats. The analgesic effect of intra habenular morphine was blocked by intra-dorsal raphe injection of serotonin 5-HT_3 antagonist.

Keywords: Lateral habenula; Morphine; Ondansetrone; Dorsal raphe; Acute trigeminal pain; Rats

INTRODUCTION

The habenular complexes (include the medial and the lateral nuclei) are small bilateral epithalamic structures that modulate a wide range of physiological behaviors involving nociception, depression, stress, endocrine, reward function and addiction (1,2).

A wealth of evidence suggests a key role for the lateral habenula in central modulation of acute and chronic models of pain in the animals and human (3,4). It has been shown that the electrical stimulation of the habenula induces an antinociceptive effect in the formalin test (5).

Microinjection of morphine into the lateral habenula produced analgesia in the formalin test (6). Moreover, it is well established that µ opioid receptors are dense in the habenula but delta and kappa receptors have less density in this region (7). Also, morphine is exhibited inhibition and facilitation of excitatory synaptic transmission in the habenular nucleus neurons of rat (8).

Depending on the intensity of the noxious stimuli (but not non-noxious stimuli) lateral habenular neurons are responsive to peripheral nociceptive stimulation in excitatory or inhibitory patterns (9).

Lateral habenula received pain inputs from direct (via trigeminal nucleus and lamina I of the dorsal horn) and indirect (via structures such as the lateral hypothalamus) afferents (4,10,11).

*Corresponding author: E. Khalilzadeh
Tel: 0098 41 36378743, Fax: 0098 41 36378743
Email: e.khalilzadeh@tabrizu.ac.ir

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It has been shown that the lateral habenula (LHb) sends its projections to the dorsal and median raphe nuclei, ventral tegmental area, substantia nigra pars compacta and periaqueductal gray (12-14). Dorsal raphe nucleus is one of the important nucleus in pain modulation. This nucleus is involved in the pain perception through serotoninergic, opioidergic and GABAergic system (15). Dorsal raphe sends some afferents directly into the spinal trigeminal nucleus and spinal cord to contribute in descending serotoninergic inhibitory pain pathway (15,16).

The connection between habenula and midbrain regions such as the PAG and dorsal raphe seems to be important in the habenular modulation of nociception. Therefore the present study was aimed to investigate the effect of morphine, L-glutamic acid and lidocaine (for pharmacological blockage of the habenula) microinjection into the lateral habenula in the acute trigeminal model of pain. The possible existence of a descending pain modulatory pathway from the lateral habenula to the dorsal raphe, which is may be involved in the intra-habenular morphine induced analgesia was determined by blocking of μ opioid and $5HT_3$ serotonin receptors in the dorsal raphe by microinjection of opioid receptor antagonist naltrexone and $5HT_3$ serotonin receptor antagonist ondansetron before intra habenular injection of morphine.

MATERIALS AND METHODS

Animals

Adult male Wistar rats, weighing 250-280 g were used in this study. They were randomly housed in polyethylene cages with *ad libitum* access to food and water in a room with controlled temperature (22 ± 1 °C) and under a 12 h light–dark cycle (lights on from 07:00 h). Six rats were used in each test group. All experiments were performed between 11:00 h and 15:00 h. All research and animal care procedures were approved by the Veterinary Ethics Committee of the Faculty of Veterinary Medicine (University of Tabriz) (Registration No. D/2013.31) and were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (17).

Drugs and chemicals

Morphine sulfate was purchased from TEMAD Co. (Tehran, Iran). Naltrexone hydrochloride and ondansetron hydrochloride were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). NaCl and L-glutamic acid were purchased from Merck Chemicals Co. (Darmstadt, Germany). All drugs and chemicals were dissolved in physiological saline, and just only NaCl dissolved in distilled water.

Surgical procedure

The rats were bilaterally implanted with two guide cannulas into the habenular nucleus. In addition, all rats concurrently were implanted with a guide cannula into the dorsal raphe nucleus. Briefly, rats were anesthetized with a mixture of ketamine (80 mg/kg, Alfasan, Woerden, Holland) and xylazine (10 mg/kg, Alfasan, Woerden, Holland) injected intraperitoneally (i.p.), and then placed in a stereotaxic apparatus (Stoelting, Wood Lane, IL, USA). The scalp was incised, and the skull was leveled off around the bregma. A 23-gauge, 15 mm stainless steel guide cannula was introduced at the following coordinates: habenular complex: AP: -3.3, L: 0.7, V: 4.8 and dorsal raphe: AP: -7.8, L: 0, V: 6.4 with respect to bregma at a 0° angle. The cannulas were then fixed to the skull using three screws and dental acrylic (SR Triplex Cold, Ivoclar Vivadent AG, Liechtenstein). A 15-mm stylet was inserted on each cannula to keep them patent prior to injection. At least 10 days was allowed for recovery from the surgery (18).

Injection protocol

For intra-habenular or dorsal raphe microinjections of chemicals a 30 gauge, 15 mm injection needle was attached to a 30 cm PE10 polyethylene tube fitted to a 1 μL Hamilton syringe. After stylet removal, the rats were placed on a wooden plate for a period of 15 min for habituation, thereafter the injection needle was inserted into the guide cannula. The volume of the drug solution to be injected into the each site was 0.5 μL and the

injection was slowly made over a period of 1 min. The injection needle was left in place for a further 1 min after completion of injection to facilitate diffusion of the drug. Intra-dorsal raphe microinjection of saline, naltrexone (4 µg) and ondansetrone (1 µg) were performed 5 min before intra-habenular injection of chemicals. The possible analgesic activity of morphine (2, 5 and 8 µg/site), l-glutamic acid (1 and 2 µg/site) and lidocaine (2%) were evaluated 5, 15, 30 and 45 min after intra-habenular injection. Morphine doses were chosen from previous studies because these doses effectively prevent from nociception in the different models of pain (19,20). The glutamate, naltrexone and ondansetron doses were chosen based on the previous studies (21-23).

Cannula verification

Cannula tip placement determined at the end of the experiment. Briefly at the end of the study, rats euthanized with the high dose of ether and then methylene blue dye (0.5 µL) was injected into each nucleus. The brains were removed and placed in the 10% formalin at least for 7 days. After fixation, the brains were cut coronally, and examined under a stereo-microscope for identification of the injection site to localize in diagrams from the rat brain atlas (Fig. 1) (18). Only data obtained from animals with the correct injection site were contributed in the statistical analysis.

Acute trigeminal test

Each rat was placed on a 50 × 50 × 1 cm wooden table and after a 15 min habituation period, one drop (40 µL) of NaCl 5 M solution was topically applied on the surface of the cornea using a pipette (Transferpette® *S* 10-100 µL Brand CO, Germany). After topical application of NaCl 5 M solution, rats always wiped with the forepaw and sometimes rapidly scratched the eye with the hind paw. The numbers of eye wipes performed with ipsilateral forepaw were counted for a period of 30 s. Also, each burst of hind paw scratches was counted as one wipe (24,25). The test was performed pre-drug and post-drug administration at the same eye of the same animal with minimum 30 min interval. The effect of drugs from the maximal possible effect (%MPE) was calculated for eye-wipes according to the following formula:

%MPE = 100× post drug wipes count - pre drug wipes count / (0 - pre drug wipes count)

Statistical analysis

Statistical differences were determined by two-way analysis of variance (ANOVA) with repeated measures, followed by Bonferroni post hoc test using GraphPad Prism® software version 5 (GraphPad Prism Software, Inc., USA). In figures, all values are expressed as Mean ± SEM. A value of $P < 0.05$ was considered statistically significant**.**

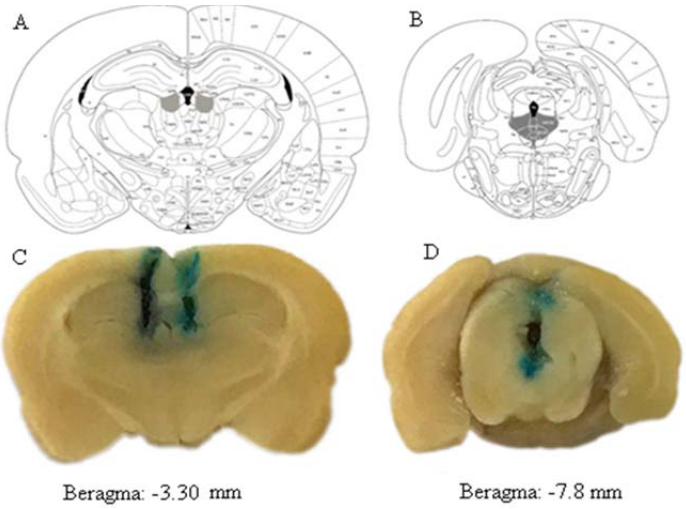

Fig. 1. Schematic illustration of coronal sections of the rat brain showing the location of (A) the lateral habenula and (B) dorsal raphe from the atlas of Paxinos and Watson (indicated by gray color). Location of the injection cannulas tip in the lateral (C) habenula and (D) dorsal raphe in this study.

RESULTS

Morphine at a dose of 2 µg was without effect, whereas at a dose of 5 µg significantly produced an antinociceptive effect 15 min (39.93 ± 7.16%, $P < 0.05$), 30 min (41.78 ± 8.90%, $P < 0.01$), and 45 min (46.35 ± 4.81%, $P < 0.01$) after intra-habenular injection. Also, morphine at a dose of 8 µg/site, significantly produced an antinociceptive effect 15 min (46.42 ± 10.74%, $P < 0.01$), 30 min (54.30 ± 7.85%, $P < 0.0001$), and 45 min (58.42 ± 9.44%, $P < 0.001$) after intra-habenular injection (treatment effect: F (3, 80) = 29.35, $P < 0.0001$; time effect: F (3, 80) = 2.80, $P = 0.0455$; treatment and time interaction effect: F (9, 80) = 0.55, $P = 0.8352$; Fig. 2). Lidocaine (2%, 0.5 µL) significantly reduced corneal pain response in 30 min (38.66 ± 13.52%, $P < 0.05$) and 45 min (42.66 ± 9.23%, $P < 0.05$) after intra-habenular injection. Moreover, intra-habenular microinjection of L-glutamic acid (1 and 2 µg/site) did not produce any analgesic activity in this model of pain (treatment effect: F (3, 76) = 15.33, $P < 0.0001$; time effect: F (3, 76) = 1.33, $P = 0.2707$; treatment and time interaction effect: F (9, 76) = 0.23, $P = 0.9887$; Fig. 3).

Microinjection of naltrexone (4 µg) and ondansetron (1 µg) into the dorsal raphe nucleus prior to intra-habenular saline did not produce any significant effect on corneal pain perception. Intra-dorsal raphe injection of ondansetron (1 µg) prior to intra-habenular morphine (8 µg) completely block morphine induced analgesia in the acute corneal pain response.

Intra-dorsal raphe injection of naltrexone (4 µg) prior to intra-habenular morphine (8 µg) did not alter morphine induced analgesia 15 min (54.34 ± 9.35%, $P < 0.001$), 30 min (56.70 ± 10.07%, $P < 0.0001$), and 45 min (62.30 ± 8.34%, $P < 0.001$) after intra-habenular injection of morphine (treatment effect: F (4, 100) = 17.43, $P < 0.0001$; time effect: F (3, 100) = 1.06, $P = 0.3711$; treatment and time interaction effect: F (12, 100) = 0.84, $P = 0.6109$; Fig. 4).

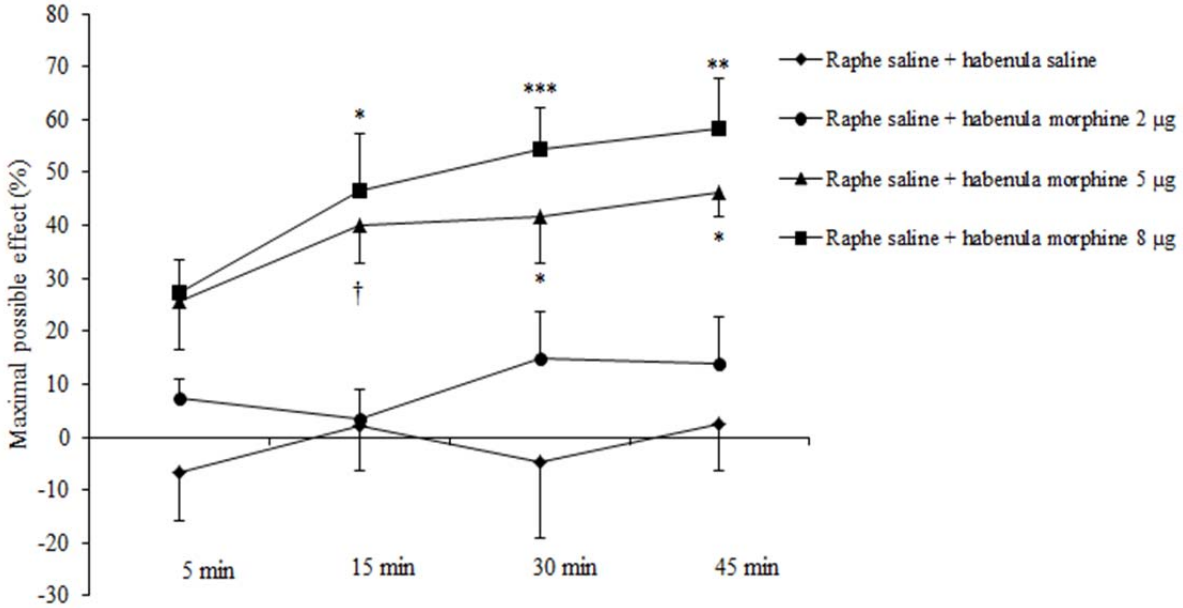

Fig. 2. Effect of intra-habenular microinjection of morphine after intra-dorsal raphe microinjection of saline (0.5 µL/site) on corneal pain response induced by NaCl 5 M solution applied to the corneal surface in rats. Maximal possible effect (MPE%) is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). The values are expressed as mean ± SEM (n = 6 per group). The data are compared with two-way analysis of variance (ANOVA) followed by bonferroni post hoc test; ***$P < 0.0001$, **$P < 0.001$, *$P < 0.01$ and $^{\dagger}P < 0.05$ as compared to normal saline treated group. Raphe, dorsal raphe; habenula, lateral habenula.

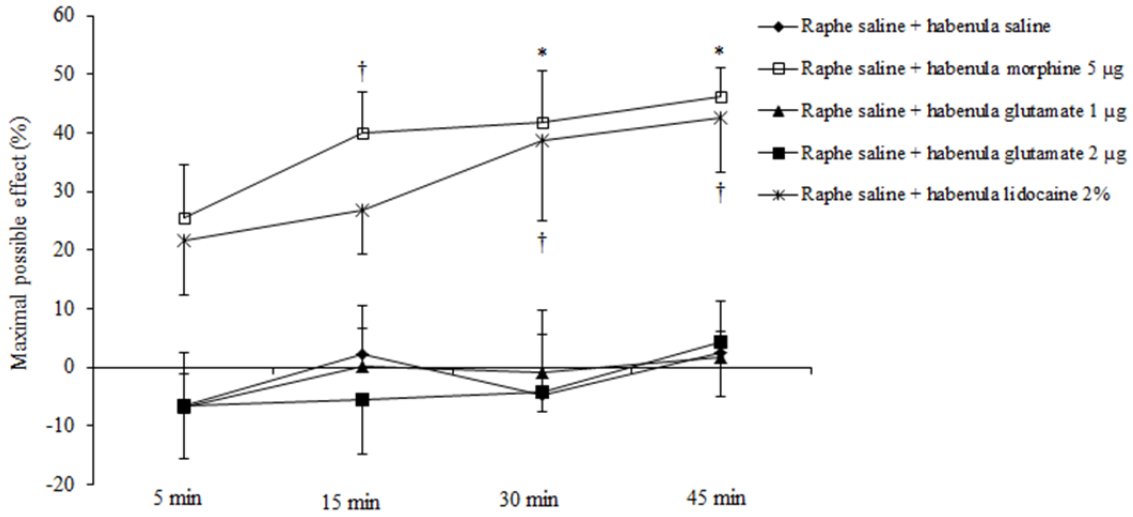

Fig. 3. Effect of intra-habenular microinjection of l-glutamic acid and lidocaine after intra-dorsal raphe microinjection of saline (0.5 µL/site) on corneal pain response induced by NaCl 5 M solution applied to the corneal surface in rats. Maximal possible effect (MPE%) is considered as an index for comparison between the results of two tests (for calculation of % MPE see the text). The values are expressed as mean ± SEM (n = 6 per group). The data are compared with two-way analysis of variance (ANOVA) followed by bonferroni post hoc test; $^{*}P < 0.01$ and $^{\dagger}P < 0.05$ as compared to normal saline treated group. Raphe, dorsal raphe; habenula, lateral habenula; glutamate, L-glutamic acid.

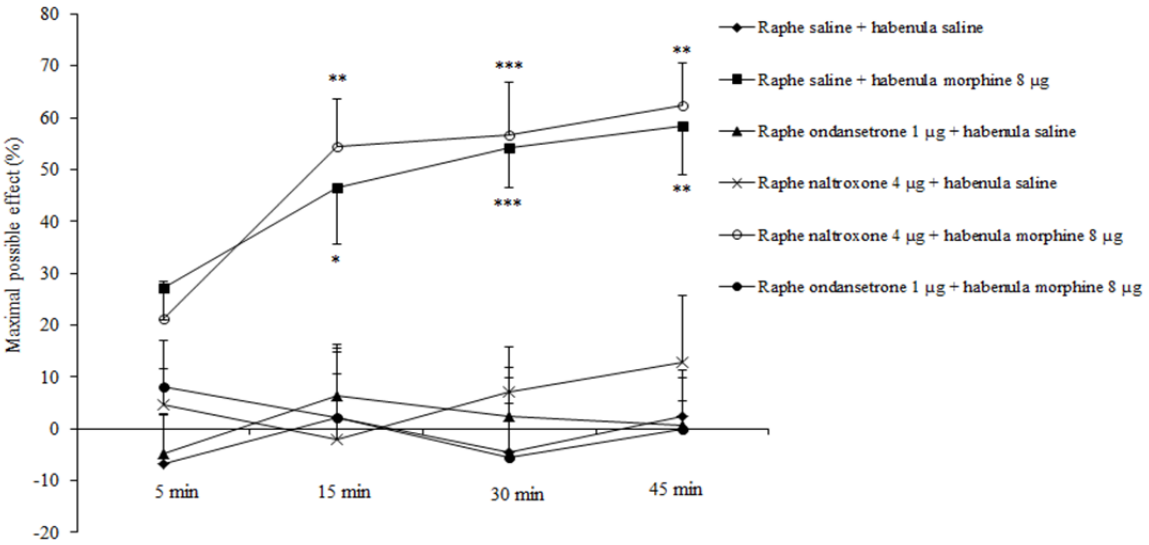

Fig. 4. Effect of intra-dorsal raphe microinjection of naltrexone and ondansetron before intra-habenular injection of saline (0.5 µL/site) and morphine on corneal pain response induced by NaCl 5 M solution applied to the corneal surface in rats. The values are expressed as mean ± SEM (n = 6 per group). The data are compared with two-way analysis of variance (ANOVA) followed by bonferroni post hoc test; $^{***}P < 0.0001$, $^{**}P < 0.001$ and $^{*}P < 0.01$ as compared to normal saline treated group. Raphe, dorsal raphe; habenula, lateral habenula.

DISCUSSION

In the present study intra-habenular injection of l-glutamic acid did not alter eye wiping responses, but microinjection of morphine and lidocaine into the habenular complex produced significant analgesia in acute trigeminal model of pain in rats. More importantly, analgesia produced by intra habenular injection of morphine, blocked by intra dorsal raphe injection of ondansetron but µ-opioid receptor antagonist naltrexone did not prevent the analgesic effect of intra-habenular morphine. These results probably indicate that

activation of 5-HT_3 serotonin receptor in the dorsal raphe nucleus following morphine microinjection into the habenula, could cause analgesia in the acute trigeminal model of pain in rats. Habenula receives direct pain signals from trigeminal nucleus and lamina I of the dorsal horn and indirectly receive signals from other brain structures that contribute in pain processing like lateral hypothalamus, limbic forebrain structures and nucleus accumbens (4,10,11). Projections from habenula are terminated on the some well-known structures that involved in pain processing like periaqueductal gray and dorsal raphe nuclei (4,12). These anatomical evidences are supported by some other electrophysiological (9), behavioral (6) and immunohistological (26) evidences that confirming pain processing role of the habenular complex in the brain. Moreover, habenula is one of the few regions in the brain that play an important role in the regulation of both dopamine and serotonin systems (2).

It has been reported that stimulation of lateral habenula can modulate dorsal raphe neuronal activity (27). Lidocaine injection into the habenular complex or its lesion could improve depressive signs via increasing of serotonin level in dorsal raphe nucleus of depressed rats (28). Also, the increase in serotonin release is reported in the striatum after electrical stimulation of lateral habenula and this effect blocked by microinjection of the non-selective N-methyl-D-aspartate receptor coupled glycine B receptor NMDA/GLYB receptor antagonist kynurenic acid into the dorsal raphe nucleus (29,30). Furthermore, extracellular level of serotonin in the dorsal raphe significantly decreased following intra-habenular microinjection of l-glutamic acid in normal rats (28). On the other hand, in the behavioral study, it was observed that activation of the lateral habenula by microinjection of l-glutamic acid decreased tail flick latency in rats (31). However, in this study, we found that microinjection of L-glutamic acid into the habenular complex did not produce any effect on hypertonic saline induced eye wiping response.

Analgesia produced by microinjection of morphine into the habenular complex has been reported previously in the formalin and the tail flick test (6,32). Habenula is one of the brain sites with high levels of μ opioid receptor expression (7). Microinjection of morphine into the lateral habenula induced both inhibition and facilitation of glutamatergic synaptic transmission (8). There are some reports indicate that habenulo-raphe projection is excitatory and synapses on GABA interneurons, which in turn inhibit serotonergic projections and reduced serotonin level, particularly in the dorsal raphe (33-35). Our results suggest that inhibition, but not activation of habenular complex by morphine (like lidocaine effect) may lead to decrease of glutamate release from habenulo-raphe excitatory projection thus decrease GABAergic interneurons activity and subsequently increase serotonin release from dorsal raphe serotonin-containing neurons.

It is well established that serotonin is one of the key neurotransmitters in the descending pain inhibitory pathways (36) that also involved in morphine-induced antinociception (37). Serotonin 5-HT_3 receptors have both pro- and antinociceptive role depending on their expression site (peripheral or central). The existence of 5-HT_3 receptors in dorsal raphe has been demonstrated by Laporte, *et al*. (38). Serotonin 5-HT_3 receptors have an undeniable role in the antinociceptive activity of serotonin in the central nervous system (39-41). The analgesic effect of intrathecal injection of serotonin and 1-(m-chlorophenyl)-bi-guanide (5-HT_3 receptor agonist) inhibited by intrathecal pretreatment of rats with tropisetron and granisetron (5-HT_3 receptor antagonists) (41).

CONCLUSION

In conclusion, the present results suggest that the activation of the habenular μ opioid receptor by microinjection of morphine or inhibition of habenular neurons by microinjection of lidocaine produced an analgesic effect in the acute trigeminal model of pain in rats. The analgesic effect of intra habenular morphine was blocked by intra-dorsal raphe injection of serotonin 5-HT_3 antagonist.

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