Blockade of Opioid Receptors Located in the Rat Nucleus Cuneiformis Reduced the Antinociceptive Responses of Local But not Systemic Administration of Morphine in Formalin Test

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ABSTRACT

Previous studies have shown the role of opioid receptors located in the nucleus cuneiformis (CnF) in acute pain, but not in chronic pain models. In the present study, we have determined that possible effects of these receptors at the CnF on both early and late phases of formalin test following local and systemic morphine administration. Each rat was given a subcutaneous 50-µl injection of 2.5% formalin into plantar surface of hind paw. Ninety five Wistar rats bilaterally received morphine (1, 2, 4 and 8 µg/0.3 µl saline per side) into the CnF, just before the formalin test. Naloxone (1 µg/0.3 µl saline per side) was also microinjected 2 minutes before local or 28 minutes after intraperitoneal administration of morphine. The results showed that bilateral intra-CnF administration of morphine dose-dependently produced analgesia in formalin test. Naloxone administration into the CnF antagonized the analgesic response induced by morphine (4 µg/0.3 µl saline) microinjection. The results also showed that analgesic effect of systemic morphine was not significantly decreased by naloxone microinjection. We suggest that the opioid receptors located in the CnF, in part, indirectly affect the morphine-induced descending pain modulatory circuit.

1. Introduction

The midbrain periaqueductal gray (PAG), adjacent nucleus cuneiformis (CnF) and their major caudal projection target, the rostral ventromedial medulla (RVM), are important components of a descending pain modulatory system (Spinella et al., 1996; Zemlan and Behbehani, 1988). Three adjacent brain stem nuclei, i.e. the caudal CnF, PAG and deep layers of the superior colliculus, function in unison to control ventral medullary pain pathways (Zemlan and Behbehani, 1984) and the CnF plays an important role in sensory/motor integration related to pain transmission (Zemlan and Behbehani, 1988). However, the CnF modulates specifically respiration rate and motor activity (Bringmann and Klingberg, 1989). Previous studies have shown that morphine when microinjected into the CnF produces powerful analgesia in tail-flick test as a model of acute pain (Haghparast et al., 2007a, 2007b). The CnF appears to be a relay of the medullary raphe nuclei, in particular at the level of the nucleus raphe magnus (NRM) (Behbehani and Zemlan, 1986; Bernard et al., 1989). The electrolytic lesion of NRM was found to be able to reduce the analgesic response of morphine microinjected into the CnF in our recent study (Haghparast et al., 2008). Al-

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ternatively, several lines of evidence have shown that morphine and other opioid agonists, when microinjected into the PAG and NRM produce powerful analgesia (Basbaum and Fields, 1984; Dickenson et al., 1978; Manning and Franklin, 1998). Some valuable anatomical and physiological studies have demonstrated that a major source of afferents to NRM arise from a continuous band of cells located within the PAG (Beitz, 1990; Bodnar, 2000) and the CnF (Behbehani and Zemlan, 1986; Zemlan and Behbehani, 1988). Furthermore, the similarity between the CnF and PAG is already found at the ultrastructural level (Gioia and Bianchi, 1987).

Our recent behavioral study showed that administration of morphine into the CnF can induce the antinociceptive responses during both phases of formalin test (unpublished data) as a user-defined method that referring chronic pain model. A former electrophysiological study undertaken in this laboratory has also revealed that subcutaneous (s.c.) injection of formalin into the plantar surface of one hind paw can significantly increase the spontaneous activity of CnF neurons in rats (Haghparast et al., 2010). According to aforementioned evidence and the lack of investigation on possible modulatory role of the CnF in a chronic inflammatory pain model, we tried to examine the role of opioid receptors located in the CnF and analgesic responses of intra-CnF and/or systemic administration of morphine during the two phases of formalin test in the rats.

2. Methods

2.1. Animal Preparation and Stereotaxic Surgery

Ninety five male Wistar rats (Pasteur Institute, Tehran, Iran) weighing 230-280g were housed three per cage with freely access to chow and tap water. The vivarium was maintained on a 12:12-h light/dark cycle in a temperature controlled room (23 ± 1°C). Each animal was used once only and all experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals (the Research and Ethics committee Shahid Beheshti University, Tehran, I.R.Iran, and National Institutes of Health Publication No. 80-23, revised 1996). Tested animals were prepared with bilateral guide cannulae implantation (23 guage needle) at least 5-7 days before performing the tests. The rats were etherized with intraperitoneal (i.p.) injection of ketamine 10% (100 mg/kg) and xylazine 2% (10 mg/kg) and two cannulae were stereotaxically (Stoelting, stereotaxic apparatus, USA) implanted in the CnF. The coordinates for the CnF were determined from atlas of Paxinos and Watson (2007) from -8.4 mm caudal to bregma, ±1.9 mm lateral to midline, -6.3 mm ventral from the skull surface (guide cannulae was implanted 1 mm above the appropriate injection place). The guide cannulae were secured in place using two stainless steel screws anchored to the skull and dental acrylic cement. At the period of recovery (5-7 days) a stainless steel obdurator was inserted into the each guide cannula to prevent occlusion.

2.2. Drugs

The following drugs were used in the examination: morphine sulfate (Temad Co, Tehran, Iran) and naloxone hydrochloride (Sigma-Aldrich Inc.). Formaldehyde, 2.5%, was made from 1 part formalin (~ 36.6%; formalin, Fluka) and 13.64 parts saline. As a matter of fact a few minutes before administration, all drugs were dissolved in sterile 0.9% normal saline.

2.3. Drug Administration

All Microinjections were performed lowering a stainless steel injector cannula (30 gauge needle) with a length of 1 mm - longer than the guide cannulae - into the CnF. The injector cannula was connected to a 1-µl Hamilton syringe using a polyethylene tubing (PE-20) and 0.3 µl of drug solution or vehicle was infused over 45 sec and was remained in place for another 1 minutes following the replacement of the obdurator. The movement of an air bubble in the PE-20 tubing confirmed drug flow. Systemic treatments were performed using a 27 gauge needle at volume of 1 ml/kg.

2.4. Experimental Procedures and Groups

All experiments were carried out in a quiet room with standard temperature and light intensity while experimenters were blinded to drug administrations.

2.4.1. Formalin Test

Animals were placed individually in an open Plexiglass chamber (35 cm × 35 cm × 35 cm) with a mirror angled at 45° and positioned behind to allow an unobstructed view of the paws by the observer. The animals were habituated to the observation chamber for 30 min prior to the experimental sessions. Each rat was given a 50-µl injection of 2.5% formalin (s.c.) into the plantar surface of one hind paw using a 27-gauge needle. Observations to determine nociceptive responses began upon placing the rat into the box and continued for the next 60 min. Since all of microinjections were performed bilaterally in this study, either the right or the left paw was chosen randomly. The weighted-score or rating scale method of scoring for formalin-induced nociceptive behaviors was used to quantify nociceptive

responses in the present study (Abbott et al., 1995; Coderre et al., 1993). In this method, a nociceptive score is determined for each 5-min block during that period by measuring the amount of time spent in each of the four behavioral categories: 0, the position and posture of the injected hind paw is indistinguishable from the another hind paw; 1, the injected paw has little or no weight placed on it; 2, the injected paw is elevated and is not in contact with any surface; 3, the injected paw is licked, bitten or shaken. Then, a weighted nociceptive score, ranging from 0 to 3 was calculated by multiplying the time spent in each category by the category weight summing these products and dividing by the total time (300 sec) for each 5-min block of time (Dubuisson and Dennis, 1977).

Nociceptive score = (t0 × 0) + (t1 × 1) + (t2 × 2) + (t3 × 3)/t0 + t1 + t2 + t3

By utilizing this method, an ordinal scale of nociceptive scores was generated with a range of 0-3 (Coderre et al., 1993).

2.4.2. Dose-Response for Bilateral Morphine Microinjection into the Nucleus Cuneiformis

Saline (0.3 µl/side) either as vehicle or morphine (1, 2, 4 and 8 µg/0.3 µl saline per side), for determining possible effects of morphine microinjection into the CnF on both early and late phases of formalin test - to calculate morphine ED50% - was slowly injected into the CnF. In this experiment, forty rats were used for microinjection of morphine (n= 32) or 0.9% saline (n = 8) into the CnF, and just 2 min later, animals received formalin (50 µl; s.c.) into the plantar surface of a hind paw.

2.4.3. Effect of Naloxone Microinjection into the Nucleus Cuneiformis on Morphine-Induced Antinociception

In order to assess the inhibitory effect of naloxone, an opiate receptor antagonist, on morphine induced analgesia after both local and systemic morphine administration, naloxone hydrochloride (1 µg/0.3 µl saline per side) was microinjected into the CnF. In the first set of experiments, animals bilaterally received saline (0.3 µl/side) or morphine (ED50%; 4 µg/0.3 µl saline per side) into the CnF two min after intra-CnF administration of naloxone and immediately formalin test was carried out. In the second set of experiments, to determine if microinjection of naloxone into the CnF could preclude the inhibition produced by systemic morphine, naloxone (1 µg/0.3 µl saline per side) was slowly injected into the CnF, 28 min after saline (1 ml/kg; i.p.) or morphine (ED50%; 6 mg/kg; i.p.) administration. In these experiments, two separate groups of animals received saline (0.3 µl) instead of naloxone into the CnF in order to confirm that the volume of injection was not affecting the pain scores in formalin test. These groups were used as control-saline groups. Aforesaid doses of systemic morphine administration and naloxone microinjection was chosen based on previous reports. A pilot study was performed in order to determine the effectiveness of systemic morphine treatments.

2.5. Statistical Analysis

The results obtained are expressed as mean ± SEM (standard error of mean). An average of the scores obtained in the first 5 min was considered as phase 1, and the area under curve (AUC) of pain scores obtained using the trapezoidal rule during 15-60 min after formalin injection was considered as phase 2. Data were analyzed by GraphPad Prism® (Version 5.0) software. The calculated and normalized AUC values in all groups were subjected to one-way and/or two-way ANOVA followed by protected Tukey's or Bonferroni's test for multiple comparisons respectively, as needed. P-values less than 0.05 were considered to be statistically significant.

2.6. Histological Verification

Upon the completion of behavioral testing, the animal was deeply anaesthetized with an overdose of ketamine and xylazine solution and transcardially perfused with 60 ml of heparinized saline followed by 150 ml of buffered 10% Formalin. Then, the brain was removed and stored in buffered 10% formalin prior to sectioning using a vibratome. The drug injection sites subsequently examined in coronal sections (50 µm) stained with Cresyl violet by an observer unfamiliar with the behavioral data. The most ventral point of the microinjector tips were mapped onto schematics of the appropriate plates using a rat brain atlas (Paxinos and Watson, 2007). The data reported here are only from animals in whom the placement of cannulae was histologically verified. In case of misplacement, related data were excluded from analysis.

3. Results

In the present study, the saline control group considered as a group without any antinociceptive responses and the area under the curves of weighted pain scores in early and late phase in all experimental groups were normalized by AUC values in respective saline control groups. Therefore, the baseline values are equal to zero according to normalization of AUC values in experimental groups. On the other hand, the percentage

of decrease in AUC was considered as a drug-induced antinociception during two phases of formalin test.

3.1. Dose- Response Effect of Morphine Microinjection into the Nucleus Cuneiformis on Time-Course of Formalin-Induced Pain Behaviors

In this set of experiment, dose-response effects of different doses of morphine microinjected into the CnF were examined. Two-way ANOVA for repeated measures over time followed by Bonferroni's test for obtained pain score values [treatment main effect: $F(4,417)=115.8$, $P<0.0001$; time main effect $F(4,417)=9.769$, $P<0.0001$; treatment×time interaction effect: $F(44,417)=1.548$, $P=0.0168$] shown in Fig.1A revealed significant differences in time-course of formalin-induced pain behaviors between doses of morphine at 4 and 8 µg/side as compared to saline control group. On the other hand, one-way ANOVA followed by the Tukey's post-hoc test for normalized AUC values in Fig. 1B showed that bilateral morphine microinjected into the CnF, dose-dependently increases the antinociceptive responses during both early [$F(4,39)=10.19$, $P<0.0001$] and late phases [$F(4,39)=39.51$, $P<0.0001$]. Nevertheless, Fig. 1B shows that normalized decrease in AUC values as an analgesic index is not significantly different in saline and two low doses of morphine (1 and 2 µg) during neither early nor late phase of formalin test. Furthermore, there were no significant differences in the AUC values at the doses of 4 and 8 µg morphine microinjected into the CnF. However, in order to examine the role of opioid receptors located in the CnF in morphine induced antinociception, the dose of 4 µg of morphine near to ED50% was microinjected into the CnF because of its significant, but not maximal analgesic effect.

3.2. Effects of Naloxone Microinjection into the Nucleus Cuneiformis on Analgesic Response of Local Morphine Administration

In this experiment, animals received saline (0.3 µl/side) or naloxone (1 µg/0.3 µl saline per side) into the CnF, 2 min before morphine (4 µg/side) or saline (0.3 µl/side) in the same site. Two-way ANOVA for repeated measures over time followed by Bonferroni's test for obtained pain score values shown in Fig.2A revealed a significant difference in time-course of formalin-induced pain behaviors between Naloxone+Morphine and Saline+Morphine groups [treatment main effect: $F(3,240)=65.49$, $P<0.0001$; time main effect $F(3,240)=3.727$, $P<0.0001$; treatment×time interaction effect: $F(33,240)=1.012$, $P=0.4554$]. On the other hand, one-way ANOVA followed by the Tukey's post-hoc test for normalized AUC values in Fig. 2B shows that pre-administration of naloxone into the CnF significantly decreases the morphine-induced antinociception in both early ($P<0.05$) and late ($P<0.01$) phases. Nevertheless, Fig. 2B shows that normalized decrease percentages of AUCs as an analgesic index in Naloxone+Saline and

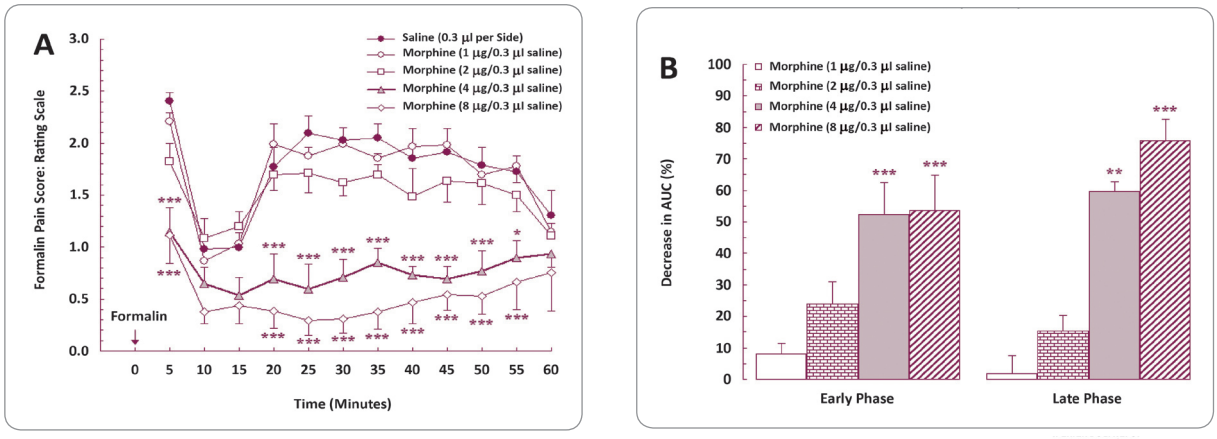

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Fig. 1. (A) Time-course of formalin-induced pain behaviors by bilateral intra-CnF microinjection of saline (3 µl/side) or different doses (1, 2, 4 and 8 µg/0.3 µl saline per side) of morphine. The 50% effective dose of morphine was close to 4 µg/0.3 µl saline per side (bold line). Each point is the mean ± SEM for 8 rats. (B) The percentage of decrease (analgesic effect) in area under the curves (AUC) of weighted pain scores using the time-response curves shown in A for rats receiving different doses (1, 2, 4 and 8 µg/0.3 µl saline per side) of morphine into the nucleus cuneiformis (CnF) during the early (0-5 min) and late (15-60 min) phases of formalin test. Normalized data are represented as mean ± SEM.
* $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to saline control group

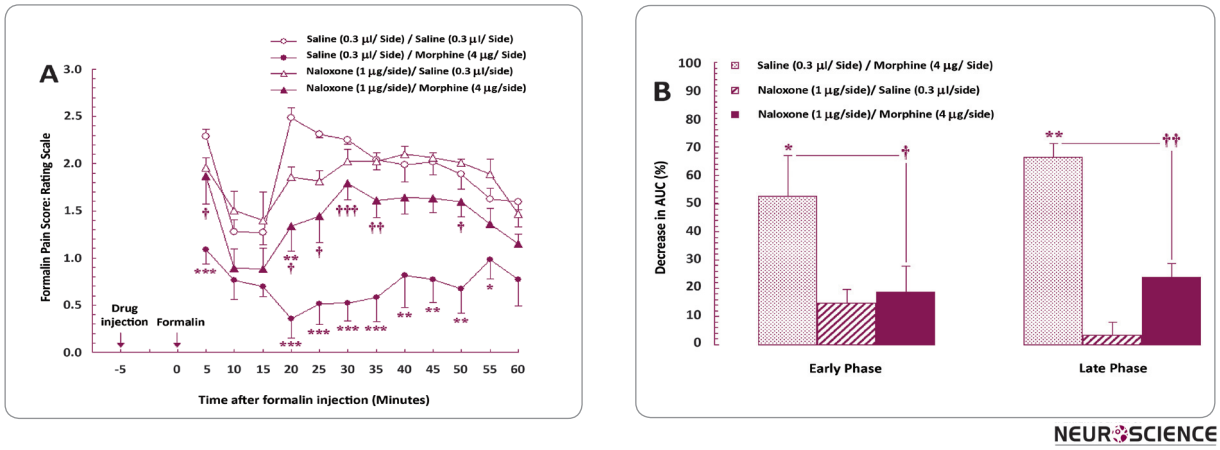

Fig. 2. (A) Effects of naloxone microinjection into the nucleus cuneiformis (CnF) on analgesia induced by local administration of morphine in the same site during formalin test. Animals received bilaterally naloxone (1 µg/0.3µl saline per side), 5 min before morphine microinjection (4 µg/0.3 µl saline per side). Each point is the mean ± SEM for 5-8 rats. (B) The percentage of decrease (analgesic effect) in area under the curves (AUC) of weighted pain scores using the time-response curves shown in A during the early (0-5 min) and late (15-60 min) phases of formalin test. Normalized data are represented as mean ± SEM.
* P<0.05; ** P<0.01; *** P<0.001 compared to saline control group
† P<0.05; †† P<0.01; ††† P<0.001 compared to morphine control group

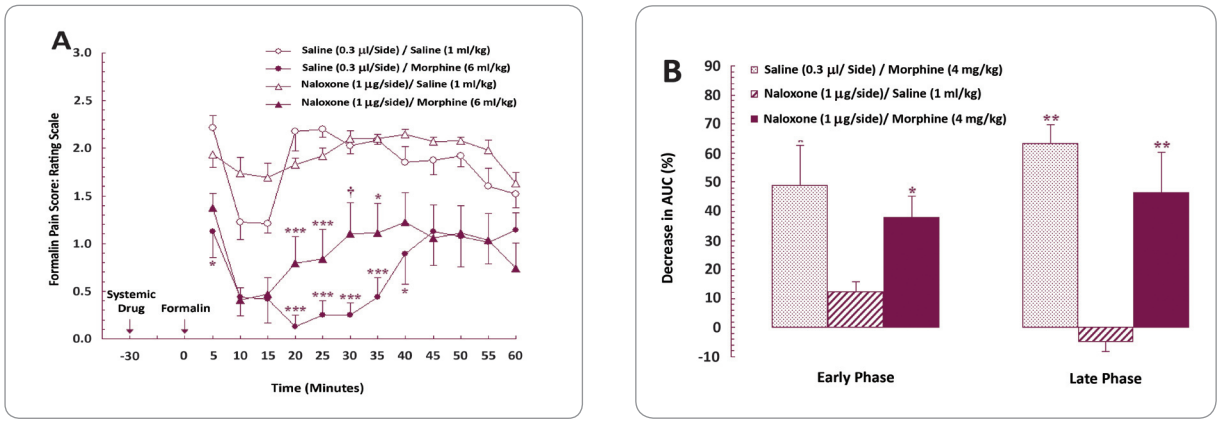

Fig. 3. (A) Effects of naloxone microinjection into the nucleus cuneiformis (CnF) on analgesia induced by systemic administration of morphine during formalin test. Animals received bilaterally saline (0.3 µl/side) or naloxone (1 µg/0.3µl saline per side), 28 min after intraperitoneal injection of saline or morphine (6 mg/kg). Each point is the mean ± SEM for 7-8 rats. (B) The percentage of decrease (analgesic effect) in area under the curves (AUC) of weighted pain scores using the time-response curves shown in A during the early (0-5 min) and late (15-60 min) phases of formalin test. Normalized data are represented as mean ± SEM.
* P<0.05; ** P<0.01; *** P<0.001 compared to saline control group
† P<0.05 compared to morphine control group

Naloxone+Morphine are not significantly different from the baseline in both early and late phases of formalin test.

3.3. Effects of Naloxone Microinjection into the Nucleus Cuneiformis on Analgesic Response of Systemic Morphine Administration

In this set of experiment, animals received saline (0.3 µl/side) or naloxone (1 µg/0.3 µl saline per side) into the CnF, 28 min after intraperitoneal administration of morphine (6 mg/kg) or saline (1 ml/kg) and immediately formalin test was carried out. Two-way ANOVA for repeated measures over time followed by Bonferroni's test for obtained pain score values [treatment main effect: $F(3,324)=89.63$, $P<0.0001$; time main effect:

F(11,324)=3.836, P<0.0001; treatment×time interaction F(33,324)=1.286, P=0.1411] shown in Fig.3A revealed that time-course of formalin-induced pain behaviors in Naloxone+Morphine group have been increased as compared to morphine control (Saline+Morphine) group in the time period between 20-40 min after formalin injection. However, there were not any significant differences in pain scores between two aforementioned groups at any time points except 30-min time after formalin injection (Fig. 3A). On the other hand, one-way ANOVA followed by Tukey's multiple comparison test for normalized AUC values showed that pre-administration of naloxone into the CnF did not affect the systemic morphine-induced antinociception by decrease in normalized AUC values in both early and late phases (Fig. 3B). It was mentioned that the normalized percentage of decrease in AUCs, as an analgesic index, in Saline+Morphine and Naloxone+Morphine are significantly different from the baseline in both early [F(3,30)=6.546, P=0.0018] and late [F(3,30)=15.87, P<0.0001] phases of formalin test (Fig. 3B).

4. Discussion

The purpose of this study was to evaluate the involvement of opioid receptors located in the CnF in morphine-induced antinociceptive responses during formalin test. The major findings of this paper are as followed (1) bilateral microinjection of morphine into the CnF dose-dependently produced analgesia in both early and late phases of formalin test (2) morphine-induced antinociceptive effect was significantly attenuated by bilateral pre-microinjection of opioid receptor antagonist naloxone in the same site (3) bilateral naloxone microinjected into the CnF could not significantly decrease the analgesic responses elicited by systemic administration of morphine in the formalin test. The first observation is consistent with our previous studies (Haghparast et al., 2007a, 2007b) whereby microinjection of morphine into the CnF dose-dependently resulted in significant antinociceptive effect in tail-flick test as a model of acute pain. It demonstrates that opioid receptors within the CnF are involved in morphine-induced antinociception in acute pain. The present data also coupled with the previous studies indicate that the direct application of morphine into other structures involved in pain modulation, such as the PAG (Manning and Franklin, 1998) and NRM (Dickenson et al., 1978), elicits analgesia in the formalin test. Our results are also in harmonized with the data from previous studies suggesting that CnF's having opioid receptors involved in pain modulation (Behbehani and Zemlan, 1986; Beitz, 1990; Bernard et al., 1989; Zemlan and Behbehani, 1988).

On the other hand, analgesic response following morphine administration into the CnF is antagonized by naloxone pre-applied to the same site. Additional evidence shows that activation of μ-opioid receptor produces inhibition of the formalin-induced nociceptive behaviors. Morphine, primarily μ-opioid receptor agonist, decreases both the acute and chronic phases of the formalin-induced nociception (Guhring et al., 2001; Shannon and Lutz, 2002; Wettstein and Grouhel, 1996). However, the last observation in our study shows that morphine analgesia following systemic administration was not eliminated by bilateral microinjection of naloxone into the CnF. Several studies demonstrated antinociceptive-like effects of systemic morphine and related compounds on formalin-evoked behaviors and showed that inhibition of both the early and late phases of formalin-induced licking response, was naloxone-sensitive (Dubuisson and Dennis, 1977; Oluyomi et al., 1992). For instance, systemic morphine produced almost complete analgesia in the second phase of the formalin test and bilateral microinjection of the quaternary opioid antagonist naloxone into the PAG (Manning and Franklin, 1998). It seems that other sites of brainstem including PAG (Manning and Franklin, 1998) and NRM (Dickenson et al., 1978) have prominent roles in mediating antinociceptive effect following systemic morphine administration. According to Behbahani and Zemlan study (1986) that have mentioned the involvement of acetylcholine in the interaction between the CnF and NRM, and role of glutamatergic receptors located in the NRM on antinociceptive effect of morphine microinjected into the CnF (Haghparast et al., 2007b), we suggested that the opioid receptors located on cell body of the CnF projecting neurons to the NRM act, in part, in morphine-induced antinociception (pain modulation) indirectly. In addition, considering the reduction of analgesic response of local morphine administration in the CnF following the electrolytic lesion of NRM (Haghparast et al., 2008) and our current results, we also approve the hypothesis proposed by Bernard et al.(Bernard et al., 1989) that CnF is a relay for the modulation of pain processes.

In conclusion, the present study along with other aforementioned investigations demonstrated that the opioid receptors located in the nucleus cuneiformis are affected by local administration of morphine, but following systemic administration of morphine, their role is masked by other brainstem sites including the periaqueductal gray and the nucleus raphe magnus as essential areas in pain modulatory system.

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