Contribution of the Nucleus Cuneiformis to the Antinociceptive Effects of Systemic Morphine on Inflammatory Pain in Rats

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ABSTRACT

Introduction: The role of midbrain reticular formation, which includes the nucleus cuneiformis (NCF), as a crucial antinociceptive region in descending pain modulation has long been investigated. In this study, we tried to highlight the role of NCF in morphine-induced antinociception in formalin-induced pain model in rats.

Methods: A total of 201 male Wistar rats weighing 260-310 g were used in this study. The effective dose of morphine in systemic administration (intraperitoneal; i.p.) was determined after a dose- and time-response protocol. In consequent groups, bilateral electrolytic lesion (500 μA, 30 sec) or reversible inactivation (lidocaine 2%) were used in the NCF before systemic administration of morphine, and then, the nociceptive test was immediately carried out.

Results: The results showed that administration of 6 mg/kg morphine, 30 min before the formalin test, is the best dose- and time-response set in these experiments. The obtained data also indicated that bilateral electrical destruction or reversible inactivation of the NCF significantly decreased antinociceptive responses of systemic morphine (6 mg/kg; i.p.) during the second phase of formalin test ($P<0.05$).

Discussion: Therefore, it seems that opioid receptors located in the NCF may be involved in modulation of central sensitization which occurred in inflammatory pain in rats.

1. Introduction

It is well established that systemic administration of morphine produces antinociception in part through the activation of supraspinal systems that inhibit spinal nociresponsive neurons through descending projections (Yeung & Rudy, 1980). The antinociceptive effects of morphine and related compounds on formalin-induced pain behaviors have already been demonstrated. Systemic morphine inhibited both the early and late phases of the formalin-induced licking responses, and this action was naloxone-sensitive (Dubuisson & Dennis, 1977; Oluyomi, Hart, & Smith, 1992) as well.

It has been shown that wide variety of brain regions including the frontal lobe, anterior cingulate cortex, insula, amygdala, hypothalamus, periaqueductal gray

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(PAG), nucleus cuneiformis (NCF), and rostral ventromedial medulla (RVM) are able to produce descending modulation to noxious stimuli (Tracey & Mantyh, 2007). Conversely, electrolytic lesion of the RVM or bilateral lesions of the dorsolateral funiculus markedly decreased the analgesic effect of systemic morphine in the tail-flick (TF) test, but not in the formalin test (Abbott & Melzack, 1982; Abbott, Melzack, & Leber, 1982; Ryan, Watkins, Mayer & Maier, 1985). Previous study showed that the antinociceptive response of morphine microinjected into the NCF was attenuated by lesion of the nucleus raphe magnus (NRM) in the TF test (A. Haghparast, Ordikhani-Seyedlar, & Ziaei, 2008). It provided strong support that descending pathways to the spinal cord play an important role in the analgesic effect of morphine (Abbott, Hong, & Franklin, 1996). In a previous study, which morphine analgesia was abolished in rats transected rostral to the pons, it was suggested that forebrain areas also participate in the analgesic effect of morphine in the formalin test (Matthies & Franklin, 1992). This could be plausible whereas higher brain centers interact in this test. In addition, formalin test was extensively utilized as a model of persistent pain such as postoperative hyperalgesia (Franklin, et al., 1990) in human. Despite the above evidence, very few evidences have shown the role of NCF in the morphine analgesic effect in the inflammatory pain.

The NCF is a nucleus with the nociceptive controlling action in which its role in other aspects such as human migraine has been recently disclosed (Moulton, et al., 2008). Evidences also implicated that the NCF is a mediator of morphine analgesia (A. Haghparast & Ahmad-Molaei, 2009; A. Haghparast, Gheitasi, & Lashgari, 2007; Rezvanipour, Haghparast, & Millan, 2006). Recent studies in our laboratory demonstrated that morphine application into the NCF depresses the rat TF reflex (A. Haghparast & Ahmad-Molaei, 2009; A. Haghparast, et al., 2008; A. Haghparast, Soltani-Hekmat, Khani, & Komaki, 2007). An electrophysiological study undertaken in this laboratory also revealed that subcutaneous injection of formalin into the plantar surface of one hind paw significantly increases the spontaneous activity of NCF neurons in rat (A. Haghparast & Ahmad-Molaei, 2009). Although it has been hypothesized that lack of function in some descending neurotransmitter systems are responsible for the central sensitization and pain chronification (Vanegas & Schaible, 2004); previous reports also demonstrated that some other structures such as NCF and PAG are possibly involved in the same processes in both animal and human (Ossipov, Lai, Malan, & Porreca, 2000; Zambreanu, Wise, Brooks, Iannetti, & Tracey, 2005). Furthermore, several studies suggest an opioid link in NCF-mediated analgesic responses (Haghparast & Ahmad-Molaei, 2009; Haghparast et al, 2007, 2008, 2010). Therefore, we tried to explore the contribution of this nucleus to systemic morphine-induced antinociception in formalin test as a model of persistent inflammatory pain in rats.

2. Methods

2.1. Animals

A total of 201 male Wistar rats weighing 260-310 g were used in this study. Animals were kept under standard laboratory conditions, with tap water and regular rat chow ad libitum. They were housed in a temperature controlled vivarium on a 12-h light-dark (7:00 h–19:00 h) cycle. All experiments executed with the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Shahid Beheshti University of Medical Sciences.

2.2. Formalin Test

Animals were placed individually in an open Plexiglass box (30 × 30 × 30 cm) with a mirror arranged in a 45º angle under the chamber to allow an unimpeded view of the animal's hind paws nociceptive response. Formalin 2.5% was made from 1 part formaldehyde (37%; Merck, Germany) and 13.8 part saline. After the surgical operation (7-days recovery period), 50 µl of a formalin (2.5%) solution was injected subcutaneously into the plantar surface of the right or left hind paw of the rat using a 29-gauge needle. Observations to determine nociceptive responses immediately began upon placing the rat into the box and continued for the next 60 min. The nociceptive behavior was used to quantify nociceptive effects of drugs by assigning weight to the pain-related behaviors (Coderre, Fundytus, McKenna, Dalal, & Melzack, 1993; Dubuisson & Dennis, 1977; Hasanein, Parviz, Keshavarz, & Javanmardi, 2007; Manning & Franklin, 1998). A nociceptive score was determined for each 5-min time block by measuring the amount of time spent in each of four behavioral categories: 0 is when the injected paw bears the animal's weight on the floor, 1 is when the animal lightly rests its injected paw on the ground, bearing only some of its weight, 2 is when the injected paw elevates and is not in contact with any surface, and 3 is when the animal licks, bites, or shakes the injected paw. Control rats, were injected subcutaneously in the same place of the right or left hind paw with 50 µl normal saline. 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.

Nociceptive score = $(t_0 \times 0) + (t_1 \times 1) + (t_2 \times 2) + (t_3 \times 3)/t_0 + t_1 + t_2 + t_3$

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

2.3. Stereotaxic Surgery

Rats were anesthetized with a cocktail of 100 mg/kg ketamine HCl and 10 mg/kg xylazine prior to surgery. Bilateral stainless steel guide cannulae (23-gauge needle, 9 mm in length and 0.6 mm outer diameter) were implanted directly overlying the NCF, using standard stereotaxic technique. Jeweler's screws were anchored to the skull and attached to the cannulae with dental acrylic. Stainless steel guide cannulae were bilaterally directed, in accordance with stereotaxic coordinates in atlas of Paxinos and Watson (Paxinos & Watson, 2005), to the NCF as AP= 8.2-8.5 mm caudal to bregma, Lat = ±1.9 mm lateral to midline, DV= 6.2-6.4 mm ventral from the skull surface (guide cannulae were aimed 1 mm above the appropriate injection place). They were sealed with occluding stylette in recovery period (7 days). During the recovery period, rats were handled daily to decrease stress associated with handling. This procedure habituated the animals to the microinjection procedure and reduced effects resulting from mechanical damage to neurons on the test day. Microinjections were made using 30-gauge injection cannulae inserted through and extending 1 mm beyond the tip of the guide cannulae. In the reversible inactivated animals, the lidocaine (0.3 µl/side) were injected into the NCF over 45 sec while the rat was awake and gently restrained. The injection cannulae remained in place for 60 additional seconds and then stylette were replaced to minimize backflow of the drug. In the non-reversible inactivated rats, electrolytic lesions (500 µA DC, 30 sec) were made by anodal microelectrode, at the same coordinates. The chemicals used were: morphine (Temad Co., Iran) and lidocaine HCl (Sigma-Aldrich, Germany). All agents were freshly dissolved in saline at the day of examination.

2.4. Experimental Design

This study was constructed in two sections; in the first section, the 50% effective dose (ED50) of intraperitoneal (i.p.) administration of morphine was determined after a dose- and time-response protocol and then, in the second part, the selected systemic dose of morphine was administered after bilateral destruction (electrolytic lesion) or reversible inactivation (lidocaine 2%) of the NCF.

In the first stage of this experiment, a total of 15 groups (n = 7-8 rats in each group) containing one control (saline) group (1 ml/kg) and four experimental groups, who were treated with systemic doses of morphine (1, 3, 6 and 12 mg/kg/ml, i.p.) in 3 different times (15, 30 and 60 min) before the formalin test, were considered. At the end of this set of experiment, the ED50 value for antinociceptive effect of systemic morphine and the optimal time of injection before the formalin test were determined. In the second stage, the ED50 dose of morphine was used in experimental groups (see Table 1) in order to find out the effect of destruction or reversible inactivation of the NCF on systemic morphine-induced analgesia.

2.5. Statistical Analysis

The obtained results 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- or two-way ANOVA and were respectively followed by protected Tukey's or Bonferroni's test for multiple comparisons, as needed. P-values less than 0.05 were considered to be statistically significant.

2.6. Histological Verification

Upon the completion of the behavioral testing, experimental animals were deeply anaesthetized and perfused transcardially with heparinized saline followed by buffered formalin (10%) solution. Then, the brain was removed and stored in buffered formalin 10% prior to sectioning by using a vibratome and the sections were examined under a stereomicroscope. The most ventral point of the microinjector tips were mapped onto schematics of the appropriate plates using a rat brain atlas.

The locations of injection and lesion sites were determined according to the atlas (Paxinos & Watson, 2005). The data reported here are only from animals in which the placement of cannulae and lesion sites were histologically verified.

3. Results

In the present study, the saline control group considered as a group without any antinociceptive treatment, and the AUC of weighted pain scores in early and late phases were normalized by AUC values of respective saline control groups in all experimental 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. Additionally, there were no significant differences between saline microinjected groups versus morphine misplacement control.

3.1. Dose- and time-response effects of systemic administration of morphine on time-course of formalin-induced pain behaviors

Fig. 1 shows the dose- and time-response effects of different doses of morphine (1, 3, 6 and 12 mg/kg; i.p.) in three injection times on time-course of formalin-induced pain behaviors in early and late phases. Based on the data of these curves, the ED50 value was calculated using linear regression. Mean pain score values in Fig. 2 revealed significant differences in time-course of formalin-induced pain behaviors in early and late phases between various doses of morphine in each time (15, 30 and 60 min) as compared to respective saline control group. One-way ANOVA followed by Tukey's post-hoc test showed that different doses of systemic morphine, 15 min before the formalin test, could not induce antinociception except for the dose of 12 mg/kg. While administration of other doses of morphine, 30 and 60 min before the formalin test, could produce significant antinociceptive effects in early phase (Fig. 2A). Additionally, the antinociceptive effect of morphine appeared well in late phase of formalin test in all set injection times (Fig. 2B). However, one-way ANOVA revealed that systemic administration of different doses of morphine, 60 min before the formalin test induce antinociception only at two high doses (6 and 12 mg/kg) in late phase. Therefore, for evaluating the contribution of the NCF to morphine-induced antinociception, we chose the dose of 6 mg/kg for systemic administration 30 min before formalin test. It seems that this dose is near to ED50 of morphine and 30-min injection time is the best time for induction of antinociception in both early and late phases of formalin test.

3.2. Effects of bilateral electrolytic lesion of the NCF on analgesic response of systemic morphine

In the present study, the control (NCF sham lesion+Saline) group was considered as a group without any antinociceptive treatment and the AUC of weighted

Table 1. The Control and experimental groups of the study.

Experimental and control groups		Saline	Morphine		
		IP*	MI**	IP*	n#
Control	Intact	-	-	-	8
	Saline-control	+	-	-	8
	Sham-lesion+Saline	+	-	-	8
Electrolytic Destruction of the NCF	Sham-operated	-	-	-	8
	Sham-lesion+Morphine	-	-	+	8
	Lesion+Saline	+	-	-	8
	Lesion+Morphine	-	-	+	8
Reversible Inactivation of the NCF	Saline-Control	+	+	-	7
	Morphine-Control	-	+	+	8
	Inactivated+Saline	+	-	-	7
	Inactivated+Morphine	-	-	+	8

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* Intraperitoneal injection
** Microinjection into the nucleus cuneiformis (NCF)
# Number of animals

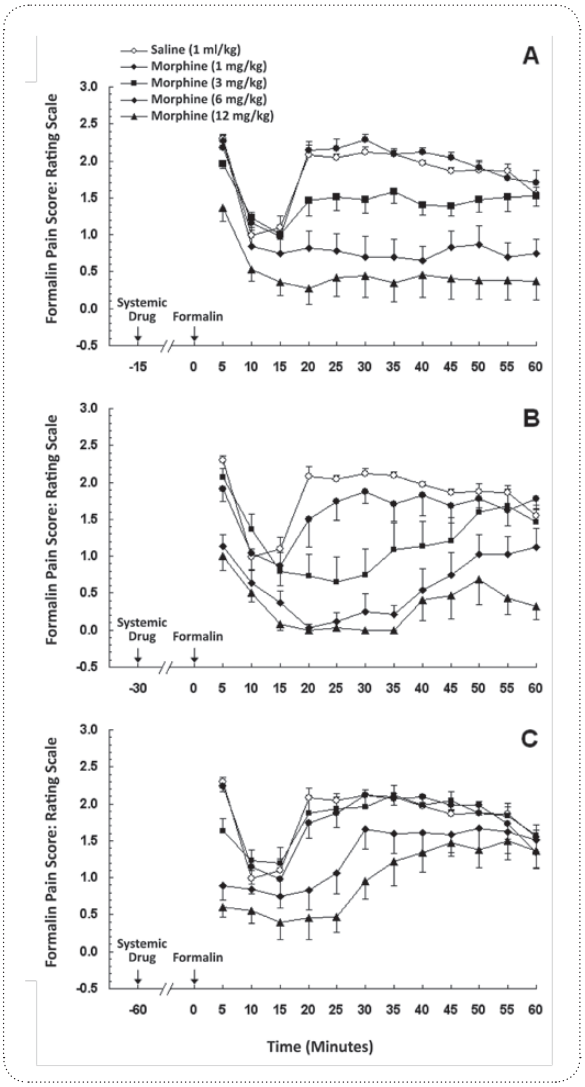

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Figure 1. Time-course of formalin-induced pain behaviors after intraperitoneal administration of saline (1 ml/kg) or different doses of morphine (1, 3, 6 and 12 mg/kg) when injected (A) 15 min (B) 30 min and (C) 60 min before the formalin test. The dose of 6 mg/kg of morphine at 30 min before the formalin test was determined as the 50% effective dose in this protocol. Each point is the mean ± SEM for 7-8 rats.

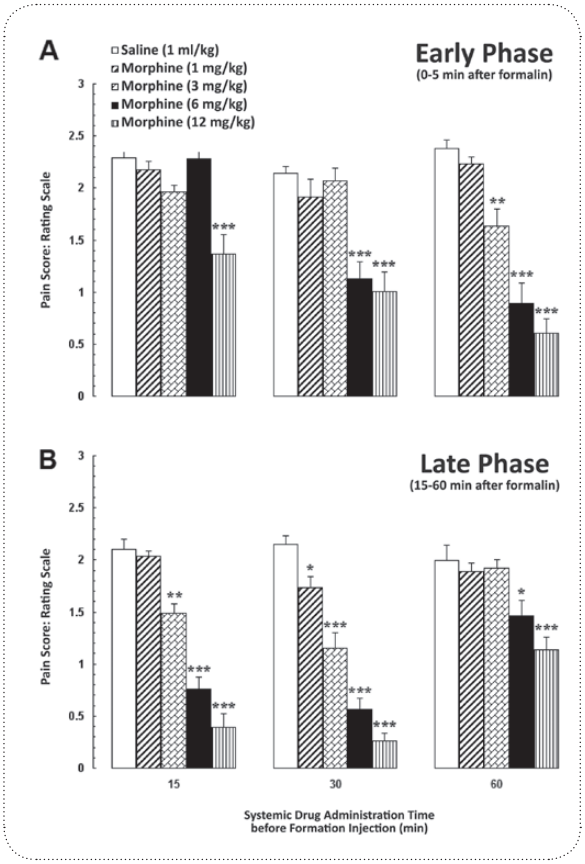

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Figure 2. Effects of different doses of morphine, when administered 15, 30 and 60 min before the injection of formalin, on mean pain scores in (A) early and (B) late phases of the formalin test. Each point is the mean ± SEM for 7-8 rats.
* P<0.05; ** P<0.01; *** P<0.001 compared to respective saline group

pain scores, in both early and late phases in all experimental groups, were normalized by AUC values in control group. In this set of experiments, animals received saline (1 ml/kg; i.p.) or morphine (6 mg/kg; i.p.), 30 min before the formalin test. Two-way ANOVA for repeated measures over time, followed by Bonferroni's test for obtained pain score values shown in Fig. 3A, revealed a significant difference in time-course of formalin-induced pain behaviors between NCF sham lesion+Morphine and NCF lesion+Morphine groups [treatment main effect: $F(3,336)=84.21$, $P<0.0001$; time main effect $F(11,336)=6.298$, $P<0.0001$; treatment×time interaction effect: $F(33,336)=1.359$, $P=0.0957$]. On the other hand, the Tukey's multiple comparison test for normalized AUC values in Fig. 3B showed that administration of morphine in sham lesion group significantly increased the antinociception by the percentage of decrease in normalized AUC values in both early [$F(3,31)=5.204$, $P<0.01$] and late phases [$F(3,31)=41.24$, $P<0.0001$]. Nevertheless, this figure showed that normalized decrease percentages of AUCs, as analgesic index in bilateral electrolytic lesion of the NCF that received systemic saline (NCF lesion +Saline), are not significantly different from the baseline in both early and late phases of formalin test. Furthermore, data obtained in this experiment indicated that the bilateral electrolytic lesion in the NCF could significantly decrease the morphine-induced antinociception in the late ($P<0.05$; Fig. 3B), but not early phase in NCF lesion +Morphine group as compared to the control group.

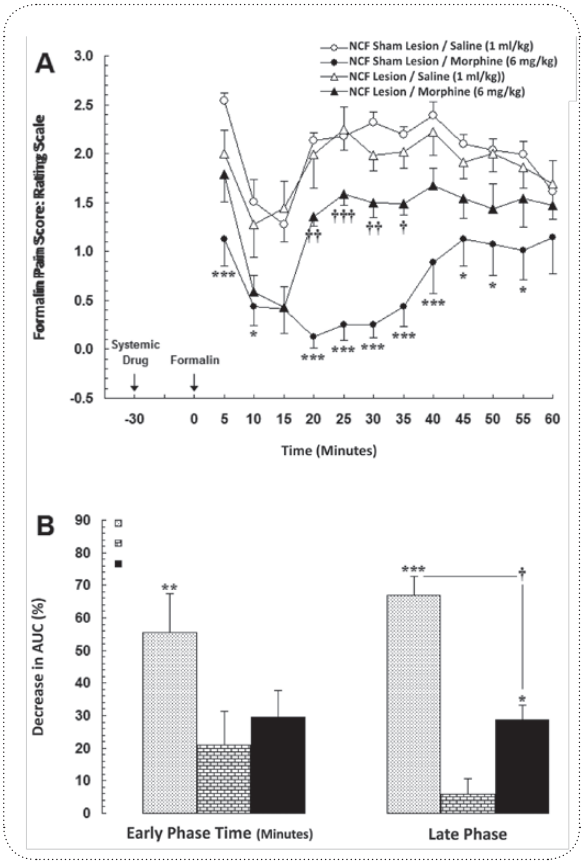

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Figure 3. (A) Effect of electrolytic lesion of the nucleus cuneiformis (NCF) on antinociception induced by systemic administration of morphine (ED50; 6 mg/kg, i.p.) during formalin test. Lesions were bilaterally made into the NCF and morphine was administered 30 min before formalin test. Each point is the mean ± SEM for 8 rats. (B) The percentage of reduction (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 NCF sham lesion+Saline group

† P<0.05; †† P<0.01; ††† P<0.001 compared to NCF sham lesion+Morphine (morphine control) group

3.3. Effects of bilateral reversible inactivation of the NCF on analgesic response of systemic administration of morphine

In this section, animals received saline (0.3 µl/side) or lidocaine 2% (0.3 µl/side) into the NCF after administration of morphine (6 mg/kg; i.p.) or saline (1 ml/kg; i.p.), just before formalin injection, and formalin test was carried out immediately. Two-way ANOVA for repeated measures over time, followed by Bonferroni's test for obtained pain score values [treatment main effect: $F(3,309)=59.54$, $P<0.0001$; time main effect: $F(11,309)=6.724$, $P<0.0001$; treatment×time interaction $F(33,309)=1.734$, $P<0.01$], revealed that there was a significant difference in pain scores between two groups of Saline+Morphine and Lidocaine+Morphine at the late phase. However, the increase of formalin-induced pain behaviors in Lidocaine +Morphine group was not significant in both phases compared to the baseline saline control (Saline+Saline) group (Fig. 4A). Nevertheless, as shown in Fig. 4B, the normalized decrease percentages of AUCs as analgesic index in NCF-inactivated animals that received systemic saline (Lidocaine+Saline) showed the hyperalgesic responses in both early and late phases of formalin test; however, it is not significantly different from the baseline. Furthermore, data obtained in this experiment indicated that the bilateral reversible inactivation of NCF could significantly decrease the morphine-induced antinociception compared to Saline+Morphine group only in the late phase of the formalin test ($P<0.05$; Fig 4B).

4. Discussion

The findings of the present study were: (1) systemic administration of morphine, dose- and time-dependently, attenuated the formalin-induced pain behaviors in early and late phases and (2) neither electrolytic nor reversible inactivation of NCF had no effect on baseline formalin-induced pain sensitivity. However, (3) both electrolytic lesion and reversible inactivation of the NCF significantly decreased antinociceptive responses of systemic administration of morphine.

There is some evidence that the specific areas involved in chronic nociception in animals are the midbrain PAG and adjacent NCF, the parabrachial nucleus in the rostral pons, and the RVM (Suzuki, Morcuende, Webber, Hunt, & Dickenson, 2002; Urban & Gebhart, 1999; Williams & Beitz, 1993). One of the neighboring areas of the NCF, which has long been known to be a major site of descending pain modulation, is the PAG and its sub-regions involving in pain modulatory effects through the opioid receptors (Dostrovsky & Deakin, 1977; Manning & Franklin, 1998; Smith, Monroe, & Hawranko, 1994; Wiedenmayer & Barr, 2000). It has been demonstrated that action by morphine at the CNS region/regions may be followed by release of endogenous opiates in the PAG (da Costa Gomez & Behbehani, 1995), which is somehow causally linked to the development of analgesia. Moreover, direct application of morphine into the PAG elicits analgesia in the formalin test (Manning & Franklin, 1998), suggesting that the PAG is the site of action of morphine in the modulation of persistent

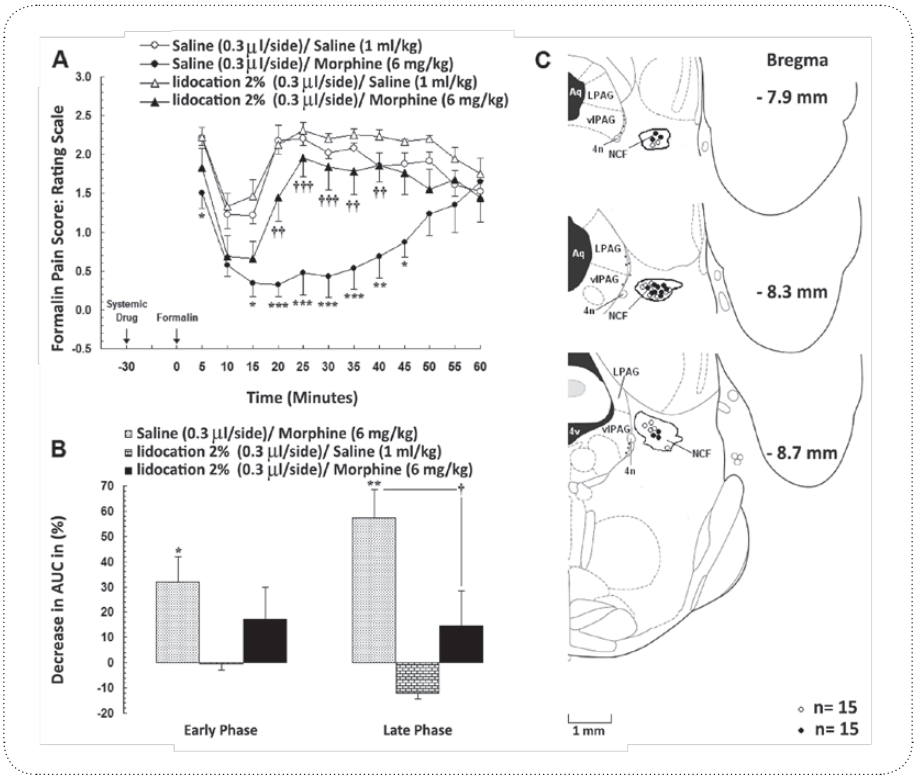

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Figure 4. (A) Effect of reversible inactivation (lidocaine 2%) of the nucleus cuneiformis (NCF) on antinociception induced by systemic administration of morphine (ED50; 6 mg/kg, i.p.) during formalin test. Morphine was systemically administered 30 min before formalin test and lidocaine was bilaterally injected into the NCF, 28-29 min after the morphine, just before the formalin test. 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. (C) Coronal schematic sections showing the locations of microinjections of lidocaine (●) and saline (○) in the NCF during the formalin test. 4n, trochlear nerve; 4v, 4th ventricle; Aq, aqueduct; LPAG, lateral periaqueductal gray; NCF, nucleus cuneiformis; vlPAG, ventrolateral periaqueductal gray.
* P<0.05; ** P<0.01; *** P<0.001 compared to Saline+Saline (saline control) group
† P<0.05; †† P<0.01; ††† P<0.001 compared to Saline+Morphine (morphine control) group

inflammatory pain. It has been reported that intra-PAG injection of the µ-opioid antagonist, blocked an opioid receptor-mediated antinociception in the rat in hot-plate test. In addition, injection of naloxone into the PAG blocked the action of local opioid, resulting in attenuation of analgesia (Manning & Franklin, 1998). Several studies have also shown that PAG lesions reduce morphine analgesia in rats (Bouhassira, Villanueva, & Le Bars, 1992; Dostrovsky & Deakin, 1977; McGaraughty, Farr, & Heinricher, 2004). With respect to the similarities between the NCF and PAG areas in ultrastructural (Gioia & Bianchi, 1987b) and functional (Gioia & Bianchi, 1987a) characteristics and their anatomical projections to the same regions such as NRM (A. Haghparast, et al., 2008; A. Haghparast, Soltani-Hekmat, et al., 2007; Haws, Williamson, & Fields, 1989; Hudson & Lumb, 1996; Jiang & Behbehani, 2001), it was suggested that the same mechanisms in NCF may involve in descending pain modulation (A. Haghparast, et al., 2008). Notably, (A. Haghparast & Ahmad-Molaei, 2009) it was shown that there is a functional connection between some parts of PAG (dorsolateral sub-region) and this region, which involves in the antinociceptive responses of morphine microinjected into the NCF in formalin-induced pain behaviors. Furthermore, NCF has a similar cytoarchitecture to the PAG and projects directly to the RVM, which exerts bidirectional control of dorsal horn nociceptive neurons (Fields, Basbaum, & Heinricher, 2006; Zambreanu, et al., 2005).

In previous studies, we also showed the importance of NCF in local morphine-induced analgesia through the NRM (A. Haghparast, Gheitasi, et al., 2007; A. Haghparast, et al., 2008). In line with the present investigation, sole lesion of NCF was not effective on baseline pain threshold. The relative failure of effectiveness of NCF lesions, but not concurrently with systemic administration of morphine, on preventing the antinociception suggests that the NCF may not initially participate in triggering of the antinociception and/or it might be compensated by other pathways. We suppose that it may be involved as a functional unit in the mediation of morphine-induced analgesia in a complexity of neural connections. On the other hand, our findings in the present study indicate that the NCF participates in the morphine-induced analgesia, whereas this analgesic effect is significantly reduced following the electrolytic and reversible inactivation of the NCF in the formalin test. Therefore, this function of NCF would be mostly through mechanisms involving the opioid receptors located in this region. These results confirm some reports that opioidergic system directly acts in NCF descending pain modulatory system (A. Haghparast & Ahmad-Molaei, 2009; A. Haghparast, Gheitasi, et al., 2007; A. Haghparast, et al., 2008) but it still need more investigations.

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