# Antinociceptive Effect of Morphine Microinjections into the Dorsal Hippocampus in the Formalin-Induced Orofacial Pain in Rats

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## Abstract

In the present study, the effects of intra-hippocampal microinjections of morphine (an opioid agonist) and naloxone (an opioid antagonist) were investigated in the formalin-induced orofacial pain in rats. Orofacial pain was induced by subcutaneous injection of formalin (1 %, 50 µl) in the upper lip region and the time spent of face rubbing was measured in 3-min blocks for 45 min. Formalin induced a biphasic (first phase: 0-3 min; second phase: 15-33 min) pain response. Intra-hippocampal microinjections of morphine at doses of 2 and 4 µg significantly ($P < 0.05$) attenuated the first phase, and at doses of 1, 2 and 4 µg, morphine significantly ($P < 0.05$) suppressed both phases of formalin-induced orofacial pain response. Intra-hippocampal microinjections of naloxone (1 and 4 µg) non-significantly increased pain when used alone, and in pretreatment microinjection, naloxone (4 µg) reversed morphine (2 µg)-induced antinociception. These results indicate that at the level of hippocampus of the brain, morphine through a naloxone-reversible mechanism produced an antinociceptive effect confronting the pain induced by formalin in the orofacial region in rats.

**Key words:** Hippocampus, Morphine, Naloxone, Orofacial pain, Rats

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## Introduction

Recent evidences suggest that the hippocampal formation may have important roles in the central perception of pain. Microinjections of acetylcholine and pilocarpine into the dorsal hippocampus decreased the electrical activity of pain exciting neurons, while increased the pain-inhibiting neurons electrical activity in the sciatic nerve electrical stimulation model of nociception in rats.[1] Intra-hippocampal microinjection of MK801, a competitive NMDA receptor antagonist, suppressed the second phase of formalin-induced pain in rats.[2] Microinjection of histamine into the dorsal hippocampus produced antinociceptive effect in the first and second phases of formalin-induced orofacial pain in rats.[3]

Current consensus describes four types of opioid receptors: mu (μ), delta (δ), kappa (κ) and nociceptin/orphanin FQ peptide receptors.[4] These four receptors mediate many physiological effects of endogenous opioid system including behavior, pain and analgesia, stress and social status, tolerance and dependence, learning and memory, eating and drinking, alcohol and drugs of abuse, sexual activity and hormones secretion, mental illness and mood, seizures and neurological disorders, electrical activity of neurons, general activity and locomotion, gastrointestinal, renal and hepatic functions, respiratory control, thermoregulation and immunological responses.[5] Opioid receptors are distributed in the hippocampal formation and are involved in mediation of hippocampal functions including adult neurogenesis, the action of gonadal hormones, development of neonatal transmitter system and pain modulation.[6-8]

The orofacial region is one of the most densely innervated (by the trigeminal nerves) areas of the body, which focuses some of the most common acute, chronic and referred pains.[9] The orofacial formalin test was introduced by Clavelou et al.[10]

and was completed by Clavelou et al.[11], and thereafter has been frequently used with success in the brain modulation of orofacial pain.[3, 12-14]

In the present study, the effects of bilateral intra-hippocampal microinjections of morphine (an opioid receptors agonist) and naloxone (a non-selective opioid receptors antagonist) were investigated on pain response induced by subcutaneous (SC) injection of formalin in the upper lip region in rats.

## Materials and Methods

**Animals.** Sixty adult male Wistar rats, weighing 300–350 g were used in this study. Rats were maintained in polyethylene cages with food and water available *ad libitum* in a laboratory with controlled ambient temperature (22 ± 0.5°C) and under a 12 h light-dark cycle (lights on at 0700 h). Six rats were used in each experiment. Experiments were performed between 12:00 and 03:00 p.m. The experimental protocol was approved by the Veterinary Ethics Committee of the Faculty of Veterinary Medicine of Urmia University.

**Drugs.** Drugs used in the present study included morphine sulfate (Temad, Tehran, Iran) and naloxone hydrochloride (Sigma–Aldrich Inc., St Louis, MO, USA). The drugs were dissolved in normal saline 30 min before intra-hippocampal microinjection.

**Hippocampal cannulation.** For intra-hippocampal cannulation, each rat was anaesthetized with a mixture of ketamine (80 mg kg$^{-1}$) and xylazine (10 mg kg$^{-1}$) injected intraperitoneally (IP), 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. Two 23-gauge, 12 mm stainless-steel guide cannulas were bilaterally implanted into the right and left dorsal hippocampus. The tip of cannulas was aimed at the following coordinates: 3.6 mm posterior to the

bregma, 2.4 mm left and right sides of the midline and 2.7 mm below the top of the skull.[15] The cannulas were then fixed with dental acrylic cement (Acropars, Tehran, Iran) to three preplaced screws in the skull. A 12 mm stylet was inserted to each cannula to keep them patent prior to injection. At least 14 days were allowed for recovery from the surgery.

**Intra-hippocampal microinjection.** For intra-hippocampal microinjections of normal saline (control), morphine (0.5, 1, 2 and 4 μg), naloxone (1 and 4 μg) and naloxone (4 μg) before morphine (2 μg), a 30-gauge, 12 mm injection needle was attached to a 30 cm polyethylene tube fitted to a 5 μl Hamilton syringe. Then, the rat was placed on a wooden plate for a period of 15 min, thereafter the stylet was withdrawn, and the injection needle was inserted into the guide cannula. The volume of the drug solution to be injected into each dorsal hippocampus 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-hippocampal microinjections of naloxone and morphine were performed 10 and 5 min before SC injection of formalin into the upper lip, respectively. The drug doses used here were closer to other investigations in which the used dose ranges were reported 1-5 μg for morphine and 1-4 μg for naloxone.[16, 17]

**Orofacial formalin test.** Orofacial formalin test was performed according to the method described by Erfanparast *et al*.[3] and Raboisson and Dallel. [9] Each rat was placed in plexiglass observation chamber (30 × 30 × 30 cm) with a mirror mounted at 45° beneath the floor to allow an unobstructed view of the orofacial region. After a 30-min adaptation period, 50 μl of 1 % diluted formalin solution was SC injected into the left side of upper lip just lateral to the nose using a 29-gauge injection needle. Immediately following formalin injection, the rat was returned into the observation chamber. The time each animal spent face rubbing with ipsilateral forepaw was recorded (using a stopwatch), in consecutive 3-min bins over a period of 45 min, and was considered as an index of nociception. SC injection of formalin induced a stereotyped response characterized by two well distinct phases. In the present study, data collected between 0 to 3 min post-formalin injection represented the first (early) phase and data collected between 15 to 33 min after injection of formalin represented second (late) phase.[3]

**Cannula verification.** At the end of each experiment, 0.25 μl of methylene blue was injected into the each side of hippocampus Animals were killed with the high dose ether, and perfused intracardially with physiological saline followed by 10 % formalin solution. The brains were removed and placed in the formalin (10 %) solution. At least 24 h later, the brains were sectioned coronally (50-100 μm) and viewed under a loop to localize the injection site.[3, 15] The results obtained from rats with guide cannula outside the hippocampus were eliminated from the data analysis.

**Statistical analysis.** To evaluate significant differences among intra-hippocampal treated groups, one-way analysis of variance (ANOVA) and Duncan's test were applied. All values are expressed as the mean ± S.E.M. A value of $P < 0.05$ was considered statistically significant.

## Results

The placements of the tip of the cannulas in the dorsal hippocampus of rats are shown in Fig. 1. The rat brain section was modified from the atlas of Paxinos and Watson[17] (Fig. 1A). The location of the cannula tip placements in the dorsal hippocampus was confirmed with intra-dorsal hippocampus injection of methylene blue (Fig. 1B).
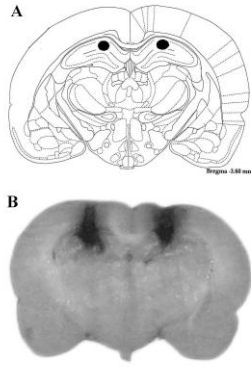
**Fig 1.** Verified section was taken from the atlas of Paxinos and Watson[15] (A). The black circles represent the cannulas tip placements (A). Location of the injection cannula tips in the dorsal hippocampus of all rats included in the data analysis (B).

Intra-hippocampal microinjection of morphine at a dose of 0.5 μg was without effect, whereas at a dose of 1 μg, morphine significantly reduced the intensity of second phase of formalin-induced orofacial pain ($F_{(4,25)} = 9.925$, $P < 0.05$). Both the firs ($F_{(4,25)} = 5.469$, $P < 0.05$) and the second ($F_{(4,25)} = 9.925$, $P < 0.05$) phases of pain were significantly suppressed when morphine was intra-hippocampally microinjected at doses of 2 and 4 μg (Fig. 2).

Intra-hippocampal microinjections of naloxone at a dose of 1 and 4 μg alone non-significantly increased the intensity of pain in the first ($F_{(2,15)} = 0.361$, $P > 0.05$) and second ($F_{(2,15)} = 0.582$, $P > 0.05$) phases of formalin-induced orofacial pain (Fig. 3).



**Fig 3.** Effects of intra-hippocampal microinjection of naloxone in the formalin-induced orofacial pain in rats, n = 6 rats in each group.

Intra-hippocampal microinjection of naloxone (4 μg) before morphine (2 μg) significantly reversed the first ($F_{(2,15)} = 3.765$, $P < 0.05$) and the second ($F_{(2,15)} = 7.058$, $P < 0.05$) phases of antinociception induced by morphine in the formalin-induced orofacial pain (Fig. 4).
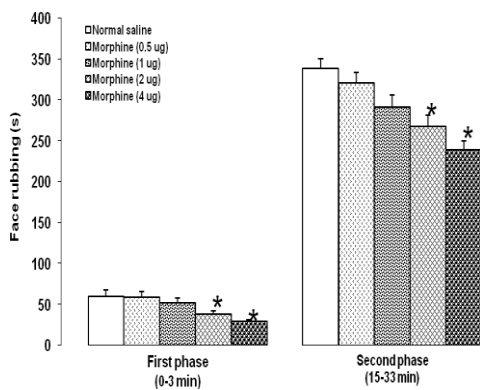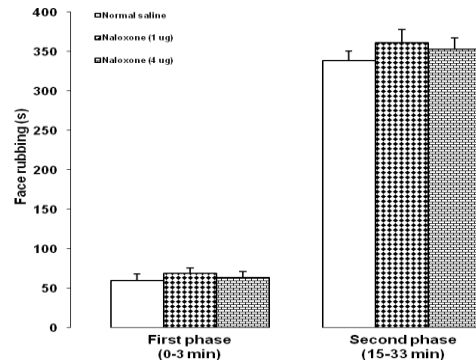


**Fig 2.** Effects of intra-hippocampal microinjection of morphine in the formalin-induced orofacial pain in rats. *$P < 0.05$ as compared with normal saline (control) group, n = 6 rats in each group.
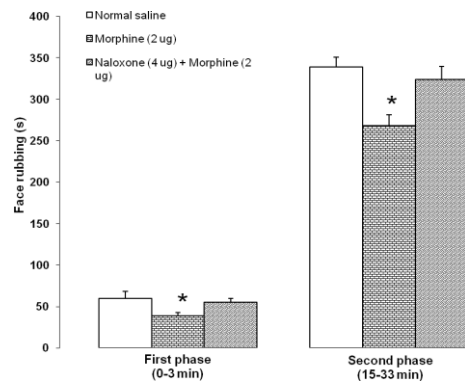


**Fig 4.** Effects of intra-hippocampal microinjection pretreatment with naloxone on the morphine-induced antinociception in the formalin-induced orofacial pain in rats. *$P < 0.05$ as compared with normal saline (control) and naloxone (4 μg) plus morphine (2 μg) groups, n = 6 rats in each group.

Significant differences were not observed among intact, hippocampal cannulated, hippocampal microinjected saline normal groups in the first ($F_{(2,15)} = 0.128$, $P > 0.05$) and second ($F_{(2,15)} = 0.0285$, $P > 0.05$) phases of formalin-induced orofacial pain (Fig. 5).
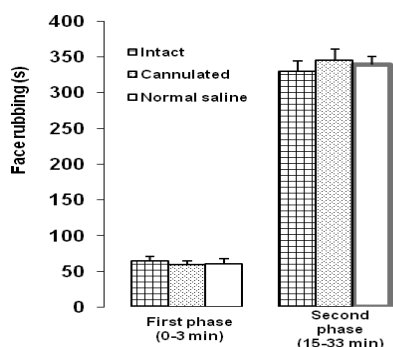


**Fig 5.** Comparison of the first and second phases of formalin-induced orofacial pain in intact, hippocampal cannulated and intra-hippocampal microinjected saline normal in rats. n = 6 rats in each group.

## Discussion

The present study shows that the SC injection of formalin into the upper lip produced a distinct biphasic pattern in the face rubbing performed by ipsilateral forepaw. The SC injection of formalin 1 % and 1.5% into the upper lip induced a biphasic pattern in the face rubbing in rats.[3,11] During the orofacial formalin test, two distinct phases due to different mechanisms of nociception produces, the first phase is associated to direct stimulation of C-nociceptors, whereas the second phase reflects integration between nociceptors and spinal and brainstem signaling.[18] Face rubbing with the ipsilateral forepaw due to formalin injection into the upper lip, has been mentioned as a specific nociceptive response.[9] However, nociceptive behavior obtained from the present study is in agreement with other investigations.[3,9,11,19]

In the present study, intra-hippocampal microinjection of morphine produced an antinociceptive effect in the orofacial formalin test in rats. Moreover, intra-hippocampal microinjection of naloxone alone did not change the intensity of pain, but in pretreatment microinjection, naloxone reversed the antinociception induced by morphine. These indicate that hippocampal opioid receptors may be involved in pain modulation. Trigeminal nerve relays the sensory information including pain arising from orofacial structures to the spinal cord and brain regions and nuclei such as spinal trigeminal nucleus, solitary tractus nucleus, periaqueductal grey, thalamus, hippocampus, caudate putamen and cerebral cortex.[20] Morphine acts through mu-opioid receptors, and naloxone is a competitive antagonist of mu-, kappa- and sigma-opioid receptors with higher affinity for the mu-opioid receptors.[21] Morphine and naloxone have been frequently used to explore the role of endogenous opioid system in peripheral, spinal and supra-spinal trigeminal pain and analgesia mechanisms. Administration of morphine simultaneously with formalin reduced the early and late phases of formalin-induced facial pain in rats.[22] Local injection of naloxone completely reversed the antinociceptive effect of morphine.[22] Morphine, through a naloxone-reversible mechanism, reduced face grooming induced by capsaicin in the orofacial region in rats.[23] Cervicomedullary intrthecal injection of naloxone antagonized morphine-induced antinociception in the orofacial formalin test in rats.[24] ICV injection of morphine produced an antinociceptive effect in a rabbit model of SC injection of formalin in the ear skin.[25] Microinjection of morphine into the subnucleus oralis, the rostral division of the spinal cord trigeminal nucleus, reduced face rubbing induced by SC injection of formalin in the upper lip in rats.[26] Naloxone reversed the antinociceptive effect of morphine at the

subnucleus oralis level.[26] Morphine, when microinjected into the nucleus raphe magnus, nucleus reticularis paragigantocellularis and ventral central gray of brain, produced analgesic effects in the orofacial formalin (chemical) and thermal pain tests in rats, respectively.[14,27]

Various neurotransmitters and neuromodulators are involved in the hippocampal effects in pain and analgesia. Intra-hippocampal microinjections of cholinergic (pilocarpine), GABAergic (musimol) and opioidergic (morphine) agents produced antinociception in electric shock-induced pain response in guinea pigs.[8] By intra-dentate gyrus microinjection of atropine, the involvement of a muscarinic cholinergic system in the processing of noxious information in the hippocampal formation was confirmed.[28] Glutamatergic, serotonergic and histaminergic agents, when used by intra-hippocampal and intra-dentate gyrus routes of administrations, produced antinociception in the formalin test in rats.[2,3,29,30] Thus, the neurotransmitters such as acetylcholine, GABA, serotonin, histamine and opioids can modulate pain by influencing the mechanisms of pain perception in the hippocampal levels.

In conclusion, the results of the present study indicate that the activation and inhibition of endogenous opioid system in the hippocampus may influence perception of pain originating from orofacial structures.

## Acknowledgments

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