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Role of NMDA receptors blockade in the thalamic paraventricular nucleus in morphine dependent rat model of formalin-induced pain

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Abstract
Evidence shows that the N-methyl-D-aspartate (NMDA) antagonist MK801 reduces the development of morphine (Mor) tolerance. The paraventricular nucleus of the thalamus (PVT) comprises the highest levels of μ-opioid receptors in the thalamus and is involved in pain modulation. The present study examined whether blocking NMDA receptors by administration of MK801 in the PVT nucleus could affect the nociceptive behavioral manifestations caused by the formalin in Mor-dependent rats. Male Wistar rats weighing 250–300 g were dependent on Mor by subcutaneously (s.c.) injection (6, 16, 26, 36, 46, 56, and 66 mg/kg, 2 ml/kg) at an interval of 24 h for 7 days. Animals were randomized into four experimental groups in which the NMDA receptor antagonist, MK801 (20 mM in 0.1 ml), or its vehicle were injected into the PVT nucleus for 7 days before each Mor injection. On day 8, the formalin test was carried out. Results showed that repetitive Mor administration prompted antinociception in interphase and phase II of formalin test. Also, inhibition of NMDA receptors decreased formalin-induced nociceptive behaviors in all phases of the test in Mor-dependent rats. Our findings suggested that continuous co-administration of MK801 into PVT with Mor could enhance the antinociceptive effect of Mor and reduce the nociceptive behaviors prompted by formalin in Mor-dependent rats.

Keywords NMDA receptor · MK801 · Paraventricular nucleus · Morphine · Nociceptive behaviors · Formalin test

Introduction

The thalamus is one of the places that receive projections from multiple ascending pain pathways. This structure is involved in the processing of nociceptive information before conveying the information to different parts of the cortex [1, 34]. The PVT in humans and rats comprises the primary levels of μ-opioid receptors in the thalamus [10, 24] and comprises a high aggregation of thalamic fibers with the endogenous μ-opioid receptor ligands [36]. Following noxious stimulation, the initiation of c-fos expression has been revealed in the thalamic PVT nucleus [7, 8, 12]. Mor enhances basal PVT neuronal firing. The intrinsic excitability of PVT neurons is increased by Mor [27]. Initiation of the μ-opioid receptor activity in PVT diminishes physical pain and may also control social pain. The firing of PVT neurons is inhibited

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by the activation of μ-opioid receptors in this nucleus [5]. Furthermore, it is revealed that frequent Mor injection enhances the spontaneous firing of PVT neurons along with augmentation of PVT neuronal excitability and excitatory synaptic glutamatergic transmission [27].
It is known that the PVT consists primarily of glutamatergic neurons [9, 30]. Evidence has shown that NMDA receptors are implicated in nociceptive transmission in the thalamus [11, 33]. Though NMDA receptor subunits have been detected in the medial thalamus [6] and NMDA inactivation has a significant role in drug addiction, the inhibition of NMDA receptors on nociception in the PVT nucleus has not been established and needs further investigation. Therefore, our attention was attracted by the question of whether intra-PVT administration of NMDA antagonism has a significant effect on nociceptive behaviors in Mor-dependent rats.

Materials and methods

Animals

Male Wistar rats, weighing 250–300 g were purchased from the Iran University of Medical Sciences and kept in plexiglass breeding cages with free access to water and food. Animals were kept in a colony room at constant room temperature and 12 h dark and light cycles. All experiments were carried out at 7–9 a.m. to evade any bias induced by circadian rhythm.

Ethics declarations

The current research was achieved based on the ethical rules of Iran University of Medical Sciences Ethics Committee, Tehran, Iran, which is based on the NIH Guide for the Care and Use of Laboratory Animals.

Stereotaxic surgery and cannulation

The rats anesthetized by ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). then, bregma was identified as the coordinates taken from Paxinos and Watson's rat brain atlas by a stereotaxic device [31]. The coordinates for the PVT were 3.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 4.0 mm ventral to the skull, with the incisor bar set at 3.3 mm below the intraaural line [23]. Then, a stainless-steel guide cannula (23-gauge needle) was unilaterally placed at the depth of 1 mm above the PVT and was fixed by means of dental acrylic cement and two screws [2]. Animals were allowed to recover after the operation for 7 days.
Rats were randomly assigned to four groups ($n=32$): Group 1: Animals received a subcutaneous injection of saline at an interval of 24 h for 7 days (Sal, $n=8$). Group 2: Animals received a subcutaneous injection of Mor at an interval of 24 h for 7 days (Mor, $n=8$). Groups 3 and 4, animals received intra-PVT microinjection of MK801 (20 mM in 0.1 ml, $n=8$, MK. Mor), or its vehicle (Sal.Mor, $n=8$), respectively, for 7 days before each Mor injection.

Induction of Mor dependence and formalin test

To induce Mor dependence, Mor was injected (6, 16, 26, 36, 46, 56, and 66 mg/kg, 2 ml/kg) for 7 days [18, 32]. On day 8, formalin (50 μL of 2%) was injected and nociceptive behaviors were observed and calculated for 30 min in a transparent plexiglass chamber. Injections were achieved at the same time during the experiments.

Intra-PVT microinjection

The non-NMDA receptor antagonist or dizocilpine hydrogen malate (MK801) (20 mM) solved in 0.1 ml sterile saline (5 μg solved in 1.0-μl sterile saline [18, 35]. The solution was divided into portions and then frozen in − 20 °C. MK801 and its vehicle were injected into PVT prior to each Mor injection through an injection cannula that was connected to hamilton syringes with volume of 1 μl by a 20 cm polyethylene tube (PE-20). A 30-gauge needle, the length of which was 1 mm longer than the guide cannula, was applied for injection. Drugs were microinjected for 60 s, and the microinjection needles were left at the site of injection for a further 60 s before being taken out [25].

MK801 is a selective non-competitive NMDA receptor antagonist. It inhibits NMDA-induced excitation by interacting with open ion channels associated with NMDA receptors [21, 22].

Histological verification

After each test, the correct placement of the cannula tips was verified histologically. For this purpose, animals were deeply anesthetized by urethane (1.5 g/kg, i.p.). Afterward, pontamine sky blue (2%, 0.2 µl) dye was microinjected into the PVT nucleus. Then rats were sacrificed, the brains were removed, and kept in a solution of 10% phosphate-buffered formalin for 24 h. The fixed tissues were sectioned into 300-µm-thick slices and injection sites were verified histologically by the rat brain atlas of Paxinos and Watson [31] (Fig. 1). Rats with misplaced cannula were excluded from the analysis.

Evaluation of nociceptive behaviors in Mor-dependent rat using formalin test

Formalin tests were achieved in the Plexiglas chamber (30 × 30 × 30 cm) with a mirror located below at a 45° angle to provide an unimpeded view of the animals' paws.

In the current research, initially, rats were acclimatized for 30 min in an acrylic observation chamber. Afterward, 10 to 20 min after the last injection of Mor, formalin (50 µl;s.c.; 2%) was injected subcutaneously through a 25-gauge needle into the plantar surface of the right hind paw.

The stable scores from formalin were ensured by inserting the needle 5 mm under the skin. Subsequently, each rat was immediately returned to the observation box, and behavioral recording was commenced. Pain behaviors were scored as

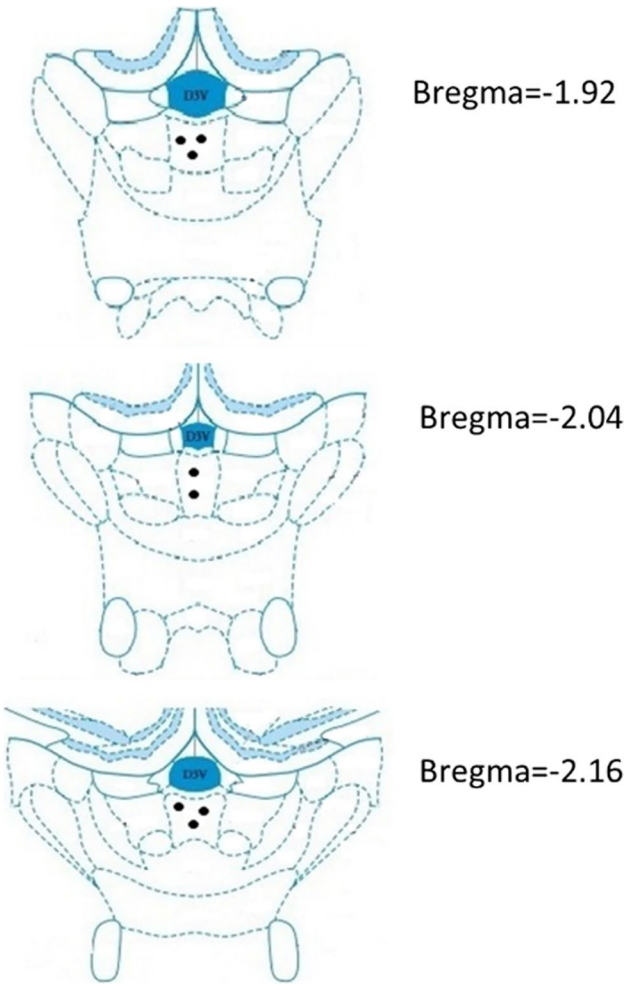

Fig. 1 Histological verification by pontamine sky blue (2%) injection site in the PVT nucleus according to the atlas of Paxinos and Watson. The black points show the injection sites in PVT

follows: 0 = the injected paw was not favored, 1 = the injected paw had little or no weight placed on it, 2 = the injected paw was increased and not in contact with any surface, and 3 = the injected paw was licked or bitten. Recording of nociceptive behaviors began immediately after formalin injection (time 0) and was continued for 60 min. The length of licking/biting the formalin-injected hind paw during each phase was assessed by a digital time-out stopwatch as an indicator of the pain response. In all groups, the behavioral response of rats during the first phase, interphase, and second phase were separately measured. The behavioral assessment was achieved just once for each animal, i.e., the formalin was never injected into the same animal twice [29, 37].

Injection of formalin induces a biphasic nociceptive and active response, including an early phase (0–5 min), quiescent interphase (5–20), and a second long-lasting phase (20–60 min). To confirm stable scores from formalin, it was required to be sure that the needle was inserted through the skin and run for 5 mm under the skin. Afterward, each rat was directly returned to the observation box, and behavioral recording started. Nociceptive behaviors were scored as follows: 0, the injected paw was not favored;1, the injected paw had little or no weight placed on it; 2, the injected paw was raised up and not in contact with any surface; and 3, the injected paw was licked or bitten. Recording of pain behaviors commenced immediately after formalin injection (time 0) and was sustained for 60 min.

Data analysis

Data were expressed as mean ± SEM and analyzed using unpaired two-tailed Student's *t* test for comparison of two groups by prism software. The defined level of statistical significance was $p < 0.05$.

Results

To study the effect of tolerance to the analgesic effect of Mor, we used the formalin test, the nociceptive score was measured in different phases (phases I, II, and interphase) of the formalin test.

To examine the impact of Mor in the induction of tolerance formalin-induced pain was used. The nociceptive score was evaluated in each phase of the pain evoked by formalin (phase I, interphase, and II). Mor failed to alter pain behaviors evoked by formalin in phase I. In interphase and phase II, Mor could reduce the nociception (analyzed by unpaired *t* test, Fig. 2).

We found that the pain behaviors created by formalin in Mor-treated rats decreased by the chronic application of MK801 in phase I ($p < 0.05$), interphase ($p < 0.01$), and phase II ($p < 0.0001$) (analyzed by unpaired *t* test, Fig. 3).

These results propose that NMDA receptors are important mediators of the development of long-lasting, non-associative Mor effect. The increment of Mor's antinociceptive influences by MK801 recommends the exciting likelihood that the NMDA receptor activity may exert nociception.

Discussion

The current results displayed that the pain behavior in rats who received repeated Mor failed to have any significant difference compared to the saline-treated rats in phase I of the formalin test. This suggests that the absence of analgesia caused by the long-standing application of Mor in the current study might result from the development of tolerance in phase I. The pain behavior in rats who received repeated Mor exhibited significant reduction compared to the saline-treated rats in interphase and phase II of the formalin test that was consistent with our previous study [19].

Our findings also showed that long-term injection of MK801 into the PVT significantly decreased the nociceptive behaviors in all phases of the formalin test in Mor-dependent rats. Consistent with our study, previous studies have shown that NMDA receptors have a considerable role in the development and expression of opioid physical dependence [3]. It has been demonstrated that MK801 blocks Mor dependence and inhibits the behavioral symptoms of the Mor abstinence syndrome [22].

The reduction of nociceptive behaviors observed in MK801-treated animals during the experiment is consistent with previous ideas that this drug blocks the development of opiate dependence. These results illustrate that the

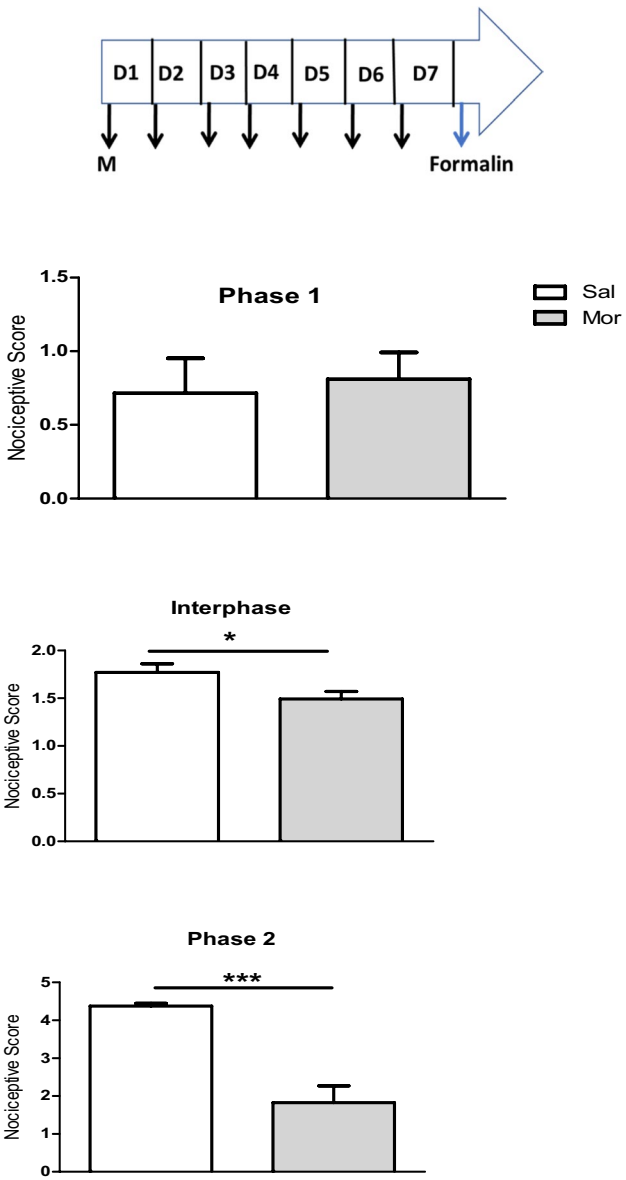

Fig. 2 Formalin-induced nociceptive behaviors following the infusion of Mor. Upper schematic plan demonstrates the experimental protocols used for assessment of nociceptive behaviors in Mor-dependent rats. Bar chart for injection of Mor (Mor) in the formalin-induced pain represents mean of the nociceptive score in each phase: phase 1 (minutes 0–5), interphase (minutes 5–20), and phase 2 (minutes 20–60). Recording of nociceptive behaviors began immediately for 60 min after formalin injection (50 µl, s.c.; 2%) into the hind paw (minute 0). Data are expressed as mean ± SEM. *$p < 0.05$ in comparison with saline (Sal) group, $n = 8$ per group

development of opiate dependence, similar to other kinds of plasticity [17, 20] comprises NMDA receptor activation. Gutstein et al. demonstrated that MK801 attenuates the development of Mor dependence at spinal sites [14]. Therefore, NMDA receptors are largely implicated in opiate-induced plasticity and the development of opiate dependency [28]. Current findings propose that NMDA-type glutamate receptor-mediated neurotransmission exhibits important impact on the antinociception induction following continuing opioid administration. In previous studies, it has been shown that the activation of NMDA receptors has been accompanying with hyperalgesia, neuropathic pain, and reduced functionality of opioid receptors [4]. Hyperalgesia may induced by augmented spinal neuron sensitization, resulting in an increment of pain [16]. Furthermore, in another study, the essential role of NMDA receptor in the central sensitization of spinal cord dorsal horn has been demonstrated [15]. This assumption is consistent with evidence that showed an augmentation of intracellular calcium concentration by NMDA receptor activation. Increased intracellular calcium and calcium-calmodulin dependent kinases activity can cause uncoupling of receptor-G-protein implicated in sensitization of mu-opioid receptor [13]. Furthermore, the continued analgesia by co-use of Mor and an NMDA antagonist demonstrated the prolongation of the analgesic effect of an opioid [26].

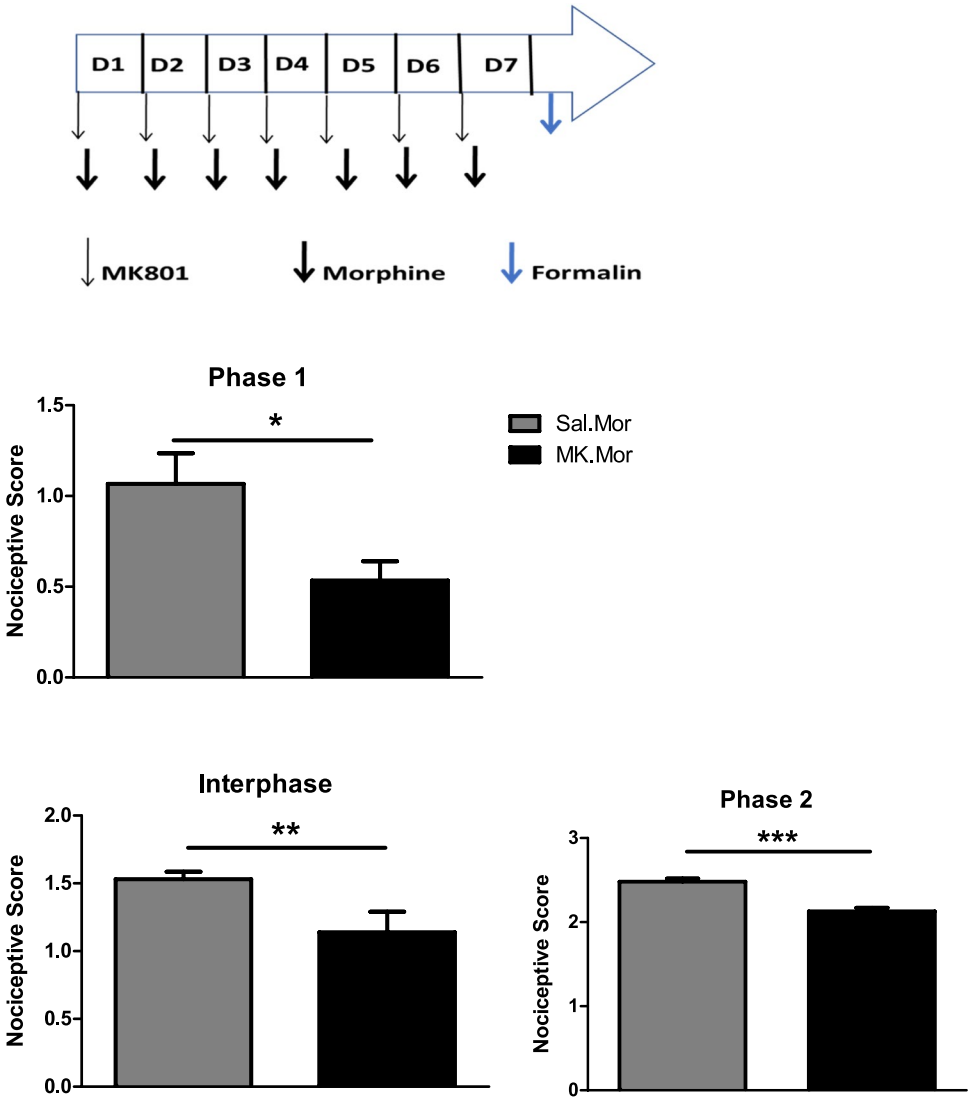

Fig. 3 Formalin-induced nociceptive behaviors following infusion of MK801. Upper schematic design shows the experimental protocols used for measuring nociceptive behaviors following MK801 injection in Mor-dependent rats. The mean of the nociceptive score for injection of MK801 in the formalin test in each phase: phase 1 (minutes 0–5), interphase (minutes 5–20), and phase 2 (minutes 20–60). Recording of the nociceptive behaviors began immediately after formalin (50 µl, s.c.; 2%) injection into the hind paw (time 0) and was continued for 60 min. Data are analyzed by unpaired *t* test and expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, *** $p < 0.0001$ in comparison with vehicle, $n = 8$ per group

Conclusion

In conclusion, current findings demonstrated that NMDA receptors in the PVT nucleus seem to act either directly or indirectly on the signaling pathways of Mor to exhibit a reasonable path for the development of Mor dependence and demonstrate an innovative potential therapeutic goal in the treatment of pain. Therefore, it may be concluded that NMDA receptors has the capability to block the non-associative opiate tolerance at the thalamus level. Indeed, MK801 might have changed the development of dependence and enhance the analgesic effect of Mor when administered along with each Mor injection. Nevertheless, additional in vitro and in vivo studies are required to clarify how the NMDA receptors play a role in pain modification.

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Author contribution FS, MKA, and MF were responsible for the study concept and design. FS performed the experiments. MKA was responsible for provision of study materials and equipment, study validation, and supervision. MKA and MF assisted with data analysis and interpretation of findings. MKA drafted the manuscript. All authors critically reviewed content and approved final version for publication.

Data availability Data will be made available upon request.

Declarations

Competing interests The authors declare no conflict of interest.

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