Neuropharmacology and analgesia

N-methyl-D-aspartate receptors involved in morphine-induced hyperalgesia in sensitized mice

Shamseddin Ahmadia a,*, Hajar Golbaghi b, Ronak Azizbeigic c, Nabaz Esmailzadehd d

a Department of Biological Science and Biotechnology, Faculty of Science, University of Kurdistan, P.O. Box 66167-15145, Sanandaj, Iran
b Department of Physiology, Faculty of Veterinary Science, Islamic Azad University, Hamedan Branch, Hamedan, Iran
c Department of Biology, Faculty of Science, Islamic Azad University, Sanandaj Branch, Sanandaj, Iran
d Department of Statistics, Faculty of Science, University of Kurdistan, Sanandaj, Iran

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A B S T R A C T

The aim of this study was to investigate role of the N-Methyl-D-Aspartate (NMDA) receptors in the decrease of morphine analgesia in mice after nociceptive sensitization. We used a hot plate test to assess effects of morphine on pain behavior in male NMRI mice. All drugs were administered through an intraperitoneonal route. Sensitization schedule composed of 3-days pre-treatment of morphine (20 mg/kg) followed by 5-days washout. The results showed that morphine (5, 7.5, 10 and 15 mg/kg) induced a significant analgesia in normal mice. However, the analgesic effects of morphine significantly decreased at higher dose (15 mg/kg) in sensitized mice. Injections of either a competitive NMDA receptor antagonist, D-AP5 (0, 0.25, 0.5 and 1 mg/kg) or an NMDA receptor channel blocker (30, 60 and 120 mg/kg) alone had no effect on pain behavior. However, injections of D-AP5 (1 mg/kg), along with morphine over 3-days of the sensitization schedule, significantly prevented the decrease in the analgesic effect of the opioid at doses of 7.5 and 10 mg/kg on the hot plate test. Similarly, injections of MgSO4 (120 mg/kg), along with morphine over 3-days of the sensitization schedule, significantly prevented the decrease in analgesic effect of morphine at doses of 10 and 15 mg/kg. It can be concluded that NMDA receptors are influenced by morphine during the sensitization schedule, which in turn may affect morphine analgesia after the schedule. This may further support the potential effectiveness of NMDA blockade during repeated use of morphine for control of chronic pain.

1. Introduction

Opioid analgesics are still the most effective and frequently used pain relievers (Benyamin et al., 2008; Cunha et al., 2010; Somogyi et al., 2007). However, other than the common side effects associated with opioid analgesics, continuous morphine therapy has been shown to induce hyperalgesia (Chu et al., 2008). According to research, a state of nociceptive sensitization may be caused by exposure to opioids leading to opioid-induced hyperalgesia, which is believed to be different from tolerance in some aspects (Lee et al., 2011; Silverman, 2009). Since mechanisms behind this phenomenon remain elusive, study on associated neurochemical changes and mechanisms still need to be continued.

N-Methyl-D-Aspartate (NMDA) receptors have been shown to be involved in expression of morphine tolerance and dependence (Mori and Mishina, 1995). Gudehithlu et al. (1994) were the first to report that long-term treatment with morphine caused change of NMDA receptors in the rat brain (Gudehithlu et al., 1994). Later, it was found that the expression of NMDA receptors became upregulated in the brain of morphine-dependent rats (Koyuncuoglu et al., 1999). Mao (1999) also reported that intraperitoneal or intrathecal administrations of NMDA receptor antagonists during chronic morphine treatments resulted in the inhibition of morphine tolerance and dependence [for review see (Mao, 1999)]. This was in line with a later report by Bisaga et al. (2001) which indicated that glutamatergic signal transduction, mediated by NMDA receptors, were involved in the formation and maintenance of morphine dependence in humans (Bisaga et al., 2001).

Furthermore, NMDA subtype of glutamate receptors, located pre- and post-synaptically on dorsal horn neurons of the spinal cord, have a pivotal role in transmission of pain signals (Willcockson and Valtschanoff, 2008). It has been shown that NMDA receptors play an important role in more prolonged pain states to enhance, prolong and alter activity in nociceptive circuitry in the spinal cord where it seems to be responsible for hyperalgesia (Dickenson, 1997; Marvizon et al., 2002). According to the research, NMDA receptors antagonism by D-AP5 effectively attenuated analgesic tolerance to morphine

* Corresponding author. Tel.: +98 871 6660075; fax: +98 871 6622702.
E-mail addresses: sh.ahmadi@uok.ac.ir, shamseddin2009@yahoo.com (S. Ahmadi).

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(Bilsky et al., 1996; Wong et al., 1996), NMDA receptor antagonists appear to inhibit the neural plasticity underlying some forms of opiate tolerance, sensitization and physical dependence suggesting that NMDA receptors are involved in the development of changes in behavior induced by opioids (Trujillo, 2000). However, using NMDA antagonists may be limited because of their side effects of, for example, neuronal damage (Horvath et al., 1997). Magnesium ions (Mg$^{2+}$) are also NMDA receptor channel blockers at normal concentrations (Nikolaev et al., 2012). Some reports have shown that Mg$^{2+}$ may cause antinociceptive effects by itself and along with morphine in different models of pain assessment (Begon et al., 2002; Bujalska et al., 2008; Kroin et al., 2000). Therefore, the possibility investigated in the present study was whether NMDA receptors blockade by D-AP5 or Mg$^{2+}$ ions underlie adaptive changes due to sensitization to morphine and subsequent hyperalgesia induced by the opioid.

2. Materials and methods

2.1. Animals

Three hundred and forty adult male albino NMRI mice weighing 20–30 g (Pasteur Institute, Tehran, Iran) were used. They were kept in an animal house with a 12/12-h light/dark cycle (light on at 7:00 a.m.) and controlled temperature (22 ± 2°C). The animals were housed in groups of 10 in plexiglas cages with free access to food and water. Behavioral tests were performed during the light phase of the cycle, and each animal was tested once only. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (2011), prepared by the National Academy of Sciences’ Institute for Laboratory Animal Research.

2.2. Drugs

Morphine sulfate, an opioid agonist, was purchased from Temad (Tehran, Iran); D(-)-2-Amino-5-phosphopentanoic acid (D-AP5), a competitive NMDA receptor antagonist, was purchased from Ascent Institute for Laboratory Animal Research. In accordance with the Guide for the Care and Use of Laboratory Animals, magnesium sulfate (MgSO$_4$), an NMDA receptor channel blocker, was a gift from Merck (Germany). All drugs and saline used in this study were either lickable Mississippi cage solution before use, and injected through an intraperitoneal route at a volume of 1 ml/kg. The drug doses used in this study were based either on a pilot study or the previous literature data (Bujalska et al., 2009; Ulugol et al., 2002).

2.3. Hot plate test of analgesia

A hot plate test was used to assess pain behavior. On the day of testing, mice were acclimated to the testing environment for 30 min, then each animal placed in a glass square on a hot plate apparatus (Armaghan Co., Iran), with a set temperature of 55 ± 0.1°C. The time between the placements of each animal on the hot plate till they either licked their hind paws or first jump was recorded as a nociceptive response. A cutoff time of 80 s was defined as complete analgesia. First, the hot plate test was performed for each animal to access “baseline latency” prior to treatments. Second, mice were received saline or morphine treatments and 30 min later tested for “test latency”. Finally, the recorded latencies were converted to percentage maximum possible effect (%MPE) based on the following formula: %MPE = [(test latency – baseline latency)/(cut-off time – baseline latency)] x 100 (Keil and Delander, 1995; Ossipov et al., 1990).

2.4. Induction of nociceptive sensitization with pre-treatment of morphine in mice

A schedule of sensitization was used for eight days. First, a dose of 20 mg/kg of morphine was daily injected intraperitoneally for three consecutive days followed by five days of no drug treatment (washout). The control mice underwent a similar schedule but they received normal saline instead of morphine. On day 9 (one day after the schedule) the animals were tested for pain behavior on the hot plate apparatus.

2.5. Experimental design

2.5.1. Experiment 1: antinociceptive effect of morphine in normal and sensitized mice

To examine induction of nociceptive sensitization by morphine, one group of animals received morphine (20 mg/kg) for three days and each day, 30 min after morphine injection, hotplate test was performed to assess pain behavior. Then, on day 9 (one day after 5-days washout) hotplate test was also carried out to assess effects of morphine (20 mg/kg) on pain behavior. Ten other groups of animals were used in this experiment. Five groups received saline for three days followed by five days of no drug treatment and then on the test day (day 9) they received saline or different doses of morphine (5, 7.5, 10 and 15 mg/kg) 30 min before testing. The other five groups of the animals received morphine (20 mg/kg) for three days followed by five days drug free, and on the test day (day 9) they received saline or different doses of morphine (5, 7.5, 10 and 15 mg/kg) 30 min before the hot plate test.

2.5.2. Experiment 2: effects of combination treatments of D-AP5 and morphine during 3-days followed by 5 days wash out on pain behavior in mice

First, we examined effects of different doses of D-AP5 (0, 0.25, 0.5 and 1 mg/kg) by itself on pain behavior. Second, ten groups of mice were divided into two sets of normal and sensitized mice. Five groups of the normal mice received morphine (20 mg/kg) plus saline, but the groups of the sensitized mice received morphine plus D-AP5 (1 mg/kg) during 3-days of the sensitization schedule. On the test day, the groups of both sets received saline or morphine at the different doses (5, 7.5, 10 and 15 mg/kg), 30 min before the hot plate test.

2.5.3. Experiment 3: effects of combination treatments of MgSO$_4$ along with morphine during 3-days followed by 5 days wash out on pain behavior in mice

First, we examined effects of different doses of MgSO$_4$ (0, 30, 60 and 120 mg/kg) by itself on pain behavior. Five groups of normal mice used in experiment 2 which received morphine (20 mg/kg) plus saline during 3-days of the sensitization schedule were also used as control groups in this experiment. Five other groups of sensitized mice received morphine plus MgSO$_4$ (120 mg/kg) during 3-days of morphine sensitization. On the test day, they received saline or morphine at different doses (5, 7.5, 10 and 15 mg/kg), 30 min before the hot plate test.

2.6. Statistical analysis

All data were presented as mean ± S.E.M. of %MPE related to ten animals in each experimental group. The results of Shapiro–Wilk test for normality of data revealed that data was normal (P > 0.05). One-way repeated measure ANOVA, one-way ANOVA, and two-way ANOVA were used for analyzing data where appropriate. Following a significant F-value, post-hoc Tukey’s test was performed to assess paired groups comparisons. P < 0.05 was
considered a statistically significant level throughout (IBM SPSS Statistics 22, New York, USA).

3. Results

3.1. Morphine induced analgesia in normal mice but it caused hyperalgesia in sensitized mice

A one-way repeated measure ANOVA was conducted to compare %MPE on days 1, 2 and 3 of morphine injections as well as day 9 (one day after 5-days washout). There was a significant effect of days of hotplate test on %MPE [Wilks’ Lambda = 0.034, F (3, 7) = 65.33, P < 0.001]. Post-hoc Tukey’s test revealed that %MPE on day 9 compared to day 1, 2 or 3 significantly (P < 0.001) decreased. This may support a nociceptive sensitization induced by 3-days injections of morphine (20 mg/kg) followed by 5-days wash out (data not shown as a figure).

Two-way ANOVA revealed that there is a significant interaction between sensitization (as factor A) and morphine (as factor B) on %MPE [F (4, 90) = 5.1, P < 0.01]. Post-hoc Tukey’s test revealed that sensitization to morphine caused a decrease in morphine analgesia (or hyperalgesia) with a significant difference in dose of 15 mg/kg compared to the results of the same dose in normal mice (Fig. 1).

Fig. 1. Analgesic effects of morphine in normal and sensitized mice. Five groups of mice received 3 days pre-treatments of saline but the other five groups received morphine (20 mg/kg), and each group was allowed to spend 5 days wash out. On the hotplate test day (day 9), five groups of normal mice and the other five groups of sensitized mice received saline or different doses of morphine (5, 7.5, 10 and 15 mg/kg), 30 min before the hotplate test. Each point represents mean ± S.E.M. of %MPE related to 10 mice in each group.

3.2. Co-administration of D-AP5 along with morphine during 3-days of morphine sensitization prevented morphine-induced hyperalgesia in sensitized mice

One way ANOVA showed that different doses of D-AP5 by itself had no significant effect on %MPE [F (3, 36) = 0.62, P > 0.05] (data not shown as a figure). On the other hand, analysis of the results of the combined treatment of D-AP5 and morphine during 3-days of morphine sensitization by two-way ANOVA revealed that there is a significant interaction between D-AP5 (as factor A with two levels) and morphine (as factor B with five levels) on %MPE [F (4, 90) = 4.24, P < 0.01]. Post-hoc Tukey’s test revealed that co-administration of D-AP5 plus morphine during 3-days of the sensitization schedule prevented morphine hyperalgesia as revealed by an increase in morphine analgesia in groups that received pre-treatments of morphine plus D-AP5 compared to groups with pre-treatments of morphine plus saline especially at doses of 7.5 and 10 mg/kg on the test day (Fig. 2).

Fig. 2. Effects of co-administration of D-AP5 and morphine over 3-days of sensitization on hyperalgesia induced by the opioid. Five groups of the animals received morphine (20 mg/kg) plus saline, but the other five groups received morphine plus D-AP5 (1 mg/kg) during 3-days morphine sensitization. On the test day (day 9), each group received saline or morphine (5, 7.5, 10 and 15 mg/kg), 30 min before the hotplate test. Each point represents mean ± S.E.M. of %MPE related to 10 mice in each group. **P < 0.01 compared to saline group with pre-treatment of morphine plus D-AP5, *P < 0.05 compared to respective morphine treated group with pre-treatment of morphine plus saline.

3.3. Co-administration of MgSO4 along with morphine during 3-days of morphine sensitization prevented morphine-induced hyperalgesia in sensitized mice

One way ANOVA showed that different doses of MgSO4 by itself had no significant effect on %MPE [F (3, 36) = 0.54, P > 0.05] (data not shown as a figure). Analysis of the results of the combined treatment of MgSO4 and morphine during 3-days of morphine sensitization on effects of morphine on %MPE on the hotplate test day by two-way ANOVA revealed that there is a significant interaction between MgSO4 (as factor A with two levels) and morphine (as factor B with five levels) on %MPE [F (4, 90) = 4.48, P < 0.01]. Post-hoc Tukey’s test revealed that co-administration of...
MgSO₄ along with morphine during 3-days of the schedule of sensitization prevented morphine hyperalgesia on the hot plate test, as revealed by an increase in morphine analgesia in groups with pre-treatments of morphine plus MgSO₄ compared to groups with pre-treatment of morphine plus saline especially at doses of 10 and 15 mg/kg (Fig. 3).

4. Discussion

The results of the present experiments showed that morphine induced analgesia in normal mice, but its analgesic effect was decreased in mice that received pre-treatment of 3-days morphine followed by 5-days wash out. Decreasing the analgesic effect of morphine after a pro-nociceptive process induced by chronic use of the drug is a serious problem in pain control. The process known as hyperalgesia means an enhanced response to a normally painful stimulus, which is often referred to as opioid-induced hyperalgesia or nociceptive sensitization induced by the opioid (Mao et al., 1995; Silverman, 2009). The effects of morphine are mediated primarily through activation of the mu-opioid receptors (Chen and Pan, 2006). In support of this claim, it has been shown that the analgesic, rewarding, and withdrawal-induced aversive effects of morphine are eliminated in mice disrupted for the mu-opioid receptor (Matthes et al., 1996; Sora et al., 1997).

According to some previous research, a regimen of 3-days morphine followed by 5-days wash out induced sensitization to morphine (Rezayof et al., 2013; Zarrindast and Rezayof, 2004). Since we used a similar regimen of morphine treatment, one possibility for hyperalgesia in the present research may be due to an increase in pain perception resulted from a process of sensitization to morphine in mice. It has been also reported that opioid-induced hyperalgesia causes insensitivity to opioids (Garnier et al., 2003). One may propose that mu-opioid receptors and/or subsequent signal transductions in peripheral and central neurons of the pain pathways may undergo changes during the process of sensitization, which finally may result in subsequent hyperalgesia. However, multiple signal transduction systems including protein kinases A and C, and nitric oxide are engaged once mu-opioid receptors are activated by morphine (Freye and Latasch, 2003; Koch and Hollt, 2008; Liu and Anand, 2001). However, changes in other receptors in addition to NMDA receptors may also account for morphine-induced hyperalgesia.

Nociceptors send their axons to the dorsal horn of the spinal cord where their terminals synapse onto second order neurons. Glutamate is one of the main neurotransmitters in the pain pathways, and NMDA receptors are densely located on pre- and post-synaptic sites in the spinal cord dorsal horn (Almeida et al., 2004). However, since the NMDA receptor’s channel is plugged by normal physiological levels of magnesium, the NMDA receptor and its channel does not participate in normal activity in pain circuits, but is brought into play under certain conditions (Dickenson, 1997). A possible mechanism for hyperalgesia induced by the sensitization schedule used in this study may be due to hypersensitivity in nociceptors to the thermal stimulus, which in turn sends more signals, removes the voltage-dependent magnesium block of the NMDA receptors more rapidly allowing calcium influx into the post-synaptic second order neurons in the pain pathway. This in turn will activate calcium sensitive intracellular signaling cascades that lead to the phosphorylation of the NMDA and other receptor/ion channels, which may initiate prolonged increases in the excitability of spinal cord neurons (Woolf and Salter, 2000). In support of this possibility, NMDA receptors have also shown to be a key player in central pain hypersensitivity (Inturrisi, 2005).

The present results showed that D-AP5, a competitive NMDA receptor antagonist and MgSO₄, a NMDA receptor channel blocker, alone had no significant effects on pain behavior in the hotplate test. We also examined NMDA receptor antagonism during the 3-days schedule of injections of morphine on the hyperalgesia induced by the opioid in the hotplate test. The results showed that co-injections of D-AP5 and morphine during 3-days of pre-treatment followed by 5-days wash out caused reinstatement of the analgesic effect of morphine in the hot plate test compared to the animals that received saline plus morphine during the sensitization schedule. The results also revealed that combination treatments of MgSO₄ along with morphine for 3-days followed by 5-days wash out, abolished morphine-induced hyperalgesia in the hot plate test compared to the animals that received saline plus morphine during the sensitization schedule. These results support the hypothesis that NMDA receptors are affected by morphine, which in turn influence analgesic effects of morphine.

Zhao and Joo (2006) characterized subpopulation of dorsal horn neurons that displayed enhanced NMDA receptor function after chronic morphine exposure (Zhao and Joo, 2006). It has been recently reported that pre-synaptic NMDA receptor activity at primary afferent terminals increases by chronic morphine and potentiates glutamatergic input to spinal dorsal horn neurons through protein kinase C (Zhao et al., 2012), which may explain hyperalgesic effect of morphine after its chronic use. Co-administration of NMDA receptor antagonists along with morphine may influence morphine effects on primary afferent neurons and spinal dorsal horn neurons to reduce the hyperalgesia induced by the opioid. Recent evidence have shown that glutamate could be released within the sensory ganglion, and the somata of primary sensory neurons as well as satellite ganglionic cells express functional glutamate receptors at their surfaces (Dupont Jasmin et al., 2013; Kung et al., 2013). It has also been reported that selective knockdown of NMDA receptors in primary...
afferent neurons decreases pain during phase 2 of the formalin test (McRoberts et al., 2011). These findings together suggest that glutamatergic transmission within the ganglionic cells could impact nociceptive threshold (Kung et al., 2013). The finding of the present results suggest that similar to the situation following nerve injury, peripheral and intraganglionic glutamatergic transmission can be a factor in morphine-induced hyperalgesia (Kung et al., 2013). Since pre-treatments with MgSO4 along with morphine had similar effects as D-AP5 plus morphine, one could suppose that NMDA receptors may have a pivotal role in control of pain perception. However, involvement of the other subtypes of glutamate receptors cannot be excluded, and clarification of their involvement needs more investigations. In support of this suggestion, it has recently been shown that peripheral nerve injury decreases the expression of metabotropic glutamate receptor subtype 7 in dorsal root ganglion neurons, which contributes to increased nociception (Li et al., 2012).

It has been reported that Mg2+ crosses the blood-brain barrier by active transport (Oppelt et al., 1963). Intravenous injection of Mg2+ can rapidly enter the cerebrospinal fluid, and peripheral injections of Mg2+ could also reach the spinal cord and brain in an appropriate time (Morris, 1992). However, some investigators have suggested that higher doses of Mg2+ are required to reach a sufficiently high concentration in the central nervous system. We do not know to what extent the injected amount of magnesium in the present experiments could reach the spinal cord and brain tissue, but our data suggested that significant effects are achievable with the administered dose of MgSO4 along with morphine. However, future studies will be needed to measure free magnesium levels in the cerebrospinal fluid. According to reports, the repeated pattern of magnesium administration via the systemic route seems to be essential for blocking NMDA receptors. Therefore, we administered it along with morphine during 3-days of repeated injections. We did not inject MgSO4 on the hotplate test day, so time was not a limiting factor to reach an effective concentration in the spinal cord and brain. D-AP5 is a competitive NMDA receptor antagonist, which specifically binds to NMDA receptor, and Mg2+ ions can block NMDA receptor channels. However, it has been reported that opioid receptor agonists may affect binding of NMDA receptor antagonists and vice versa (for review see [Mao, 1999]), Therefore, it is possible that effects of D-AP5 and Mg2+ on hyperalgesia induced by morphine after nociceptive sensitization have resulted, at least partly, from their impacts on receptors other than NMDA receptors. However, clarification of this suggestion needs more experiments.

4.1. Conclusions

Since the decrease of analgesia induced by morphine was resolved by co-administrations of D-AP5 and MgSO4 in sensitized mice, it could be concluded that NMDA receptors have been affected by morphine during repeated pre-treatments of the sensitization schedule, and therefore, mediated analgesic effect of morphine in sensitized mice. The obtained results may further support the hypothesis in favor of potential effectiveness of NMDA blockade during repeated use of morphine for control of chronic pain.

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