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Association of morphine-induced analgesic tolerance with changes in gene expression of *GluN1* and *MOR1* in rat spinal cord and midbrain

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ABSTRACT

Objective(s): We aimed to examine association of gene expression of MOR1 and GluN1 at mRNA level in the lumbosacral cord and midbrain with morphine tolerance in male Wistar rats.
Materials and Methods: Analgesic effects of morphine administrated intraperitoneally at doses of 0.1, 1, 5 and 10 mg/kg were examined using a hot plate test in rats with and without a history of 15 days morphine (10 mg/kg) treatment. Morphine-induced analgesic tolerance was also assessed on days 1, 5, 10 and 15 of chronic morphine injections. Two groups with history of 15 days injections of saline or morphine (10 mg/kg) were decapitated on day 15 and their lumbosacral cord and midbrain were dissected for evaluating *MOR1* and *GluN1* gene expression.
Results: The results of the hot plate test showed that morphine (5 and 10 mg/kg) induced significant analgesia in naïve rats but its analgesic effects in rats receiving 15 days injections of morphine (10 mg/kg) was decreased, indicating tolerance to morphine analgesia. The results also showed that the *GluN1* gene expression in tolerant rats was decreased by 71% in the lumbosacral cord but increased by 110 % in the midbrain compared to the control group. However, no significant change was observed for the *MOR1* gene expression in both areas.
Conclusion: It can be concluded that tolerance following administration of morphine (10 mg/kg) for 15 days is associated with site specific changes in the *GluN1* gene expression in the spinal cord and midbrain but the *MOR1* gene expression is not affected.

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Introduction

Morphine, as one of the most effective analgesics, inhibits nociceptive signals by acting on mu-opioid receptors (MORs) located pre- or post-synaptically on neurons in the pain pathway (1-3). However, long term use of morphine induces analgesic tolerance limiting its efficacy that enforces using an escalating dose of the opioid to obtain a previous analgesic level (4, 5). Accumulating evidence also shows that chronic use of the opioid may result in a nociceptive sensitization commonly known as opioid-induced hyperalgesia, which is not overcome by increasing the dosage [for review see (6)]. Significant clinical challenges arise from morphine induced tolerance and hyperalgesia, and more effective pain treatment can be achieved when these conditions are recognized and managed (7). Pain signals originating from peripheral organs are sent via ascending primary sensory neurons to the dorsal horn of the spinal cord where the second order neurons receive and transmit the signals to supra-spinal sites (8-10). An endogenous descending pathway originating mainly from midbrain and medulla is also projected to the dorsal horn of spinal cord and modulates pain signals by facilitating and/or inhibiting transmission of nociceptive signals from the spinal cord neurons to supra-spinal sites (11, 12). A growing body of evidence has shown adaptations at cellular and molecular levels in the pain pathway after chronic use of morphine (13, 14). According to previous researches, desensitization mainly followed by phosphorylation of the MORs has been reported as a main cause of morphine tolerance (3).

Furthermore, N-methyl-D-aspartate subtype of glutamate receptors (NMDARs) have a main role in both transmission and modulation of pain signals (12, 15). In addition, Mao *et al* (1995) have shown that morphine analgesic tolerance and opioid-induced hyperalgesia might share common cellular mechanisms in part mediated through changes in NMDARs (16). It has been shown that NMDARs become activated during

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morphine administrations and their inhibition prevent the development of tolerance and opioid-induced hyperalgesia (16, 17). Recent data has also shown that several substrates including the NMDARs have emerged as potential modulators of opioid-induced hyperalgesia (18).

Incomplete understanding of the mechanisms involved in morphine-induced tolerance and hyperalgesia remained them as main areas of research in the field of pain control. Spinal cord and midbrain are key sites in transmission and modulation of pain (11, 12), and they are of particular interest for searching mechanisms of morphine-induced analgesic tolerance and/or hyperalgesia. Therefore, we aimed to examine changes in gene expressions of MOR1, a common subtype of MOR and GluN1 as an obligatory subunit of NMDAR, at mRNA level in rat lumbosacral portion of the spinal cord and midbrain to reveal their association with morphine-induced analgesic tolerance and/or hyperalgesia.

Materials and Methods

Subjects

In this study, we used 80 male Wistar rats weighing 250-300 g. The animals were kept in an animal house at a constant temperature (22±2 ˚C) under 12-hr light/dark cycle (light beginning at 7:00 a.m.). They had free access to food and water except for during experiments. Experimental groups consisted of either 7-8 rats for hot plate test or four rats in the gene expression study. 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.

Hot plate apparatus

A hot plate apparatus (Pooya-Armaghan Co., Iran) was used to assess pain behavior in rats. The time elapse between placement of each animal on the hot plate (52±1 °C) and licking one of the hind paws or first jumping was measured as an index of pain reaction latency. First, baseline latency was measured 30 min before each injection. Second, the animals were tested to measure test latency on the hot plate apparatus. A cutoff time of 80 sec was set according to our previous reports (19, 20). Some other investigators have also reported the cut-off time of 80-120 sec in their studies (21-23).

We checked the paws of the animals and no tissue damage was observed. Finally, the two measured latencies were converted to percentage maximum possible analgesic effect (%MPAE) using the following formula: %MPAE = [(test latency – baseline latency)/ (cut-off time – baseline latency)] × 100 (24, 25). In this test, a decrease in %MPAE means reduction of morphine analgesia.

Induction of morphine tolerance

Morphine sulfate was purchased from Temad (Temad Co., Tehran, Iran). Two groups of rats received saline (1 ml/kg) or morphine (10 mg/kg) for 15 days. Hot plate test of analgesia was performed during 15 days of morphine injections on days 1, 5, 10 and 15 to evaluate possible hyperalgesia and analgesic tolerance induced by repeated administrations of morphine.

Dissection of rat midbrain and the lumbosacral portion of the spinal cord for evaluating gene expression

On day 15 of the chronic injections of saline or morphine (10 mg/kg), each rat was sacrificed, whole brain was quickly removed from the skull and rat midbrain mostly including the periaqueductal gray matter (PAG) was immediately dissected on an ice-chilled sterile surface. The lumbosacral portion of the vertebrae was cut; a cold saline solution was injected with a 10 ml syringe into the vertebral canal from the end of the canal to protrude the lumbosacral spinal cord from the upper end. Then, each tissue was immediately moved into a tube in which it was submerged in an RNAlater RNA Stabilization Reagent (Qiagen, USA) and incubated overnight at 4 ˚C. After 24 hr the RNAlater solution was aspirated and tubes containing the tissues were stored at −70 °C until further analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

Seventy mg of midbrain and lumbosacral cord was weighed and submitted to total RNA extraction using a Trizol method according to our previous study (20). Quality of the extracted total RNAs were assessed with electrophoresis on 1% agarose gel to visualize sharp bands of 28 s and 18 s ribosomal RNA. The quantities of the total RNAs were also measured spectrophotometerically (Specord210, Analytic Jena, Germany). Synthesis of cDNA from the total RNAs was performed using a “Revert-Aide First Strand cDNA Synthesis Kit” according to manufacturer’s protocol (Thermo Scientific, USA). A semi-quantitative RT-PCR method was used to evaluate gene expression of the GluN1 subunit of NMDARs and the MOR1 subtype of MORs in the lumbosacral portion of the spinal cord and midbrain according to methods used by other investigators (26). Separate multiplex PCR were performed to amplify related cDNAs of the *β-actin* (as control) and the *GluN1* or *MOR1* genes (C1000 Thermal Cycler, BIO-RAD, USA). The accession numbers for the genes were NM-031144 for the *β-actin* gene, NM-017010 for the *GluN1* gene and NM-013071 for the *MOR1* gene. Primers were those that we used in our previous studies and had the following sequences: the *β-actin* forward primer, 5′-CTGGGTATGGAAT CCTGTGGC-3′; the *β-actin* reverse primer, 5′-AGGAGGAGCAATGATCTTGATC-3′; the *GluN1* forward

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primer, 5′-TGGCATCATCGGACTTCAG-3′; the *GluN1* reverse primer, 5′- TCTGGTGGACATCTGGTATC-3′; the *MOR1* forward primer, 5′- CAGGGGTCCATAGATTGCAC-3′ and the *MOR1* reverse primer, 5′- GAAGTGCCAG GAAACGGTC-3′ (20, 27).

Behavioral experiments

Dose-response of morphine on hot plate test in rats with no history of morphine treatment

Five groups of naïve animals received saline or different doses of morphine (0.1, 1, 5 and 10 mg/kg) to examine morphine analgesia on the hot plate test. For this purpose, first, each animal was examined for baseline latency and 30 min after injections of saline or morphine tested on the hot plate for evaluating test latency.

Dose-response of morphine on hot plate test in rats with a history of 15 days morphine pre-treatment

Five groups of animals with a history of 14 days injections of morphine (10 mg/kg) received saline or different doses of morphine (0.1, 1, 5 and 10 mg/kg) on day 15 to examine morphine analgesia on the hot plate test. For this purpose, on day 15 of the schedule each animal was examined for baseline latency and 30 min after receiving saline or morphine it was tested on the hot plate apparatus for evaluating test latency.

Evaluating gene expression of GluN1 and MOR1 in the lumbosacral cord and midbrain after induction of morphine tolerance

Multiplex PCRs were separately done for the *GluN1* and *MOR1* genes along with the *β-actin* gene. A process of PCR optimization was firstly done for each reaction. Thermal cycling for the *GluN1* and *β-actin* genes was initiated with a first denaturation step of 95 ˚C for 3 min, then was followed by 28 cycles of thermal cycling of 94 ˚C for 30 sec, 61 ˚C for 30 sec, 72 ˚C for 30 sec, and was finally followed by 10 min of a final extension step at 72 ˚C and was terminated at 4 ˚C. Thermal cycling for the *MOR1* and *β-actin* genes was done with similar conditions except that 29 cycles and 59 ˚C annealing temperature were used. Finally, the PCR products were analyzed on agarose gel electrophoresis (2%) and the bands were quantified with densitometry using Image J software.

Statistical analysis

The data of the hot plate test passed normality and equal variance tests so they were analyzed with one-way analysis of variance (ANOVA), one-way or two-way repeated measure ANOVA whenever it was appropriate. Then, after a significant F value, the Holm-Sidak's test was used for pairwise comparisons. The densitometry data for the *GluN1* and *MOR1* gene expression in each sample was normalized as a ratio of the *GluN1* or *MOR1* to the *β-actin*, and mean of data was set at 100% as relative gene expression of the *GluN1* or *MOR1* in the control group. The quantified data obtained for the gene expression in control and tolerant groups were analyzed with independent sample T-test. $P < 0.05$ was considered statistically significant level.

Results

Morphine dose-dependently induced analgesia in rats with no history of morphine treatment

The result of experiment 1 was analyzed with one-way ANOVA and revealed that single injection of different doses of morphine induced significant analgesia in rats [$F (4, 30) = 237.38$, $P < 0.001$].

Post hoc test showed that morphine at doses of 5 and 10 mg/kg induced significant analgesia ($P < 0.001$) on the hot plate test compared to the saline-treated group (Figure 1).

Repeated injections of morphine for 15 days induced analgesic tolerance

Figure 2A shows baseline latencies on hot plate test during 15 days of chronic injections of saline or morphine. The result of one-way repeated measure ANOVA showed no significant changes in baseline nociceptive pain threshold of the groups treated with saline [$F (3, 15) = 2.31$, $P > 0.05$], or with morphine [$F (3, 15) = 0.47$, $P > 0.05$].

The %MPAE of two groups was analyzed with a two-way repeated measure ANOVA to explore morphine-induced analgesic tolerance. Repeated injection was considered as factor A with two levels (saline or morphine), and days of hot plate test were defined as factor B with four levels (days 1, 5, 10 and 15).

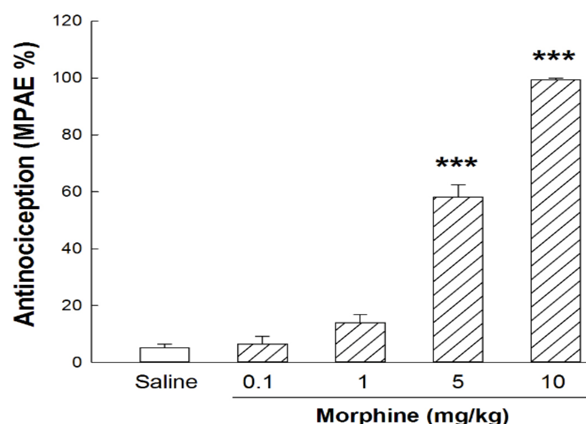

Figure 1. Analgesic effects of different doses of morphine on hot plate test in naïve rats. Five groups of rats were used. After measuring baseline latency on the test day, one group received saline (1 ml/kg) as control while the other groups received different doses of morphine (0.1, 1, 5 and 10 mg/kg), and 30 min later they were tested for test latency on the hot plate test. Each bar represents mean ± SEM related to %MPAE of the animals in each group. *** $P<0.001$ compared to the saline-treated group

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The results showed a significant main effect for factor A [F (1, 10) = 133.97, $P<0.001$], for factor B [F (3, 30)= 26.36, $P<0.001$], and for interaction of both factors [F (3, 30)= 30.59, $P<0.001$]. Post hoc test revealed that morphine at dose of 10 mg/kg on days 1, 5 and 10 of the injections induced a significant analgesia compared to the saline-treated group but its analgesic effect on day 15 of repeated injections was significantly decreased compared to previous days and was not significantly higher than %MPAE obtained for saline-treated group (Figure 2B).

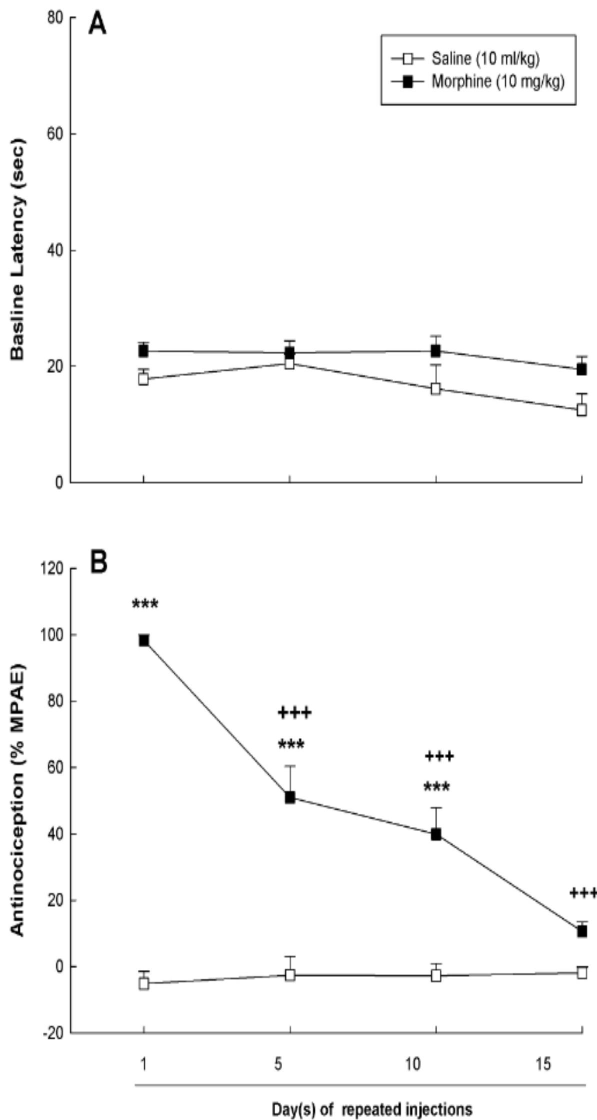

Figure 2. Baseline latency (Panel A) and %MPAE (Panel B) during 15 days of chronic morphine treatment. Two groups of rats were used. One group received saline (1 ml/kg) as control while the other group received morphine (10 mg/kg) for 15 days. The animals in each group were tested on days 1, 5, 10 and 15 of the injections to explore morphine-induced hyperalgesia and tolerance. Each point in panel (A) represents mean ± SEM of baseline latencies in each experimental group but in panel (B) it represents %MPAE of each group on the respective day. *** $P<0.001$ compared to the saline-treated group on the respective day. +++ $P<0.001$ compared to the morphine-treated group on the first day of the injections

Analgesic effect of morphine at different doses was decreased on the hot plate test in rats with history of 14 days morphine pre-treatment

The result of experiment 3 was analyzed with one-way ANOVA and revealed that in rats with a history of 14 days morphine pre-treatment, single injection of different doses of morphine induced significant analgesia [F (4, 30) = 5.76, $P < 0.001$].

However, post hoc test revealed that morphine at doses of 5 and 10 mg/kg induced significant analgesia ($P < 0.05$). In addition, post hoc test showed that morphine at doses of 0.1 and 1 mg/kg induced no significant analgesia or hyperalgesia on the hot plate test compared to control group that received saline (Figure 3).

The gene expression of GluN1 and MOR1 in the lumbosacral portion of the spinal cord after induction of morphine tolerance

Figure 4 shows the *GluN1* (panel A) and the *MOR1* (panel B) gene expression in the lumbosacral portion of the spinal cord.

Analysis of the results with independent samples T-test revealed that the *GluN1* gene expression in the lumbosacral portion of the spinal cord at mRNA level was significantly decreased by 71 % ($P < 0.01$) on day 15 of the repeated injections of morphine but no significant change was observed for the *MOR1* gene expression (Figure 4 A and B).

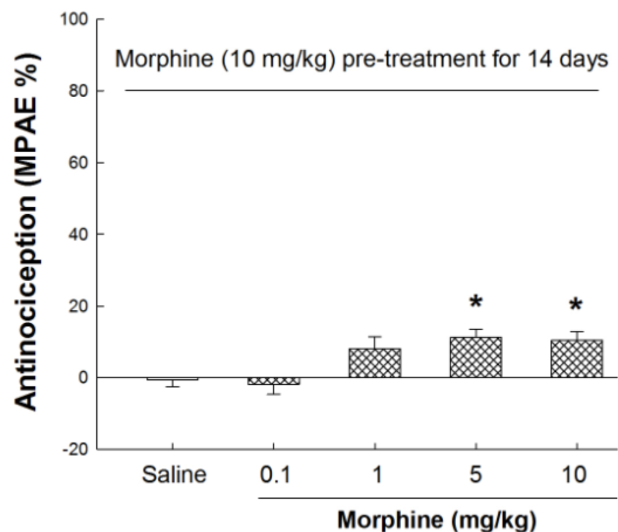

Figure 3. Analgesic effects of different doses of morphine on hot plate test in tolerant rats. Five groups of rats with a history of 14 days injection of morphine (10 mg/kg) were used. On day 15, first, baseline latency was recorded for each rat, then one group of the animals received saline (1 ml/kg) while the other groups received different doses of morphine (0.1, 1, 5 and 10 mg/kg), and 30 min later all groups were tested on the hot plate to examine test latency. Each bar represents mean ± SEM related to %MPAE of each group. * $P<0.05$ compared to the saline-treated group

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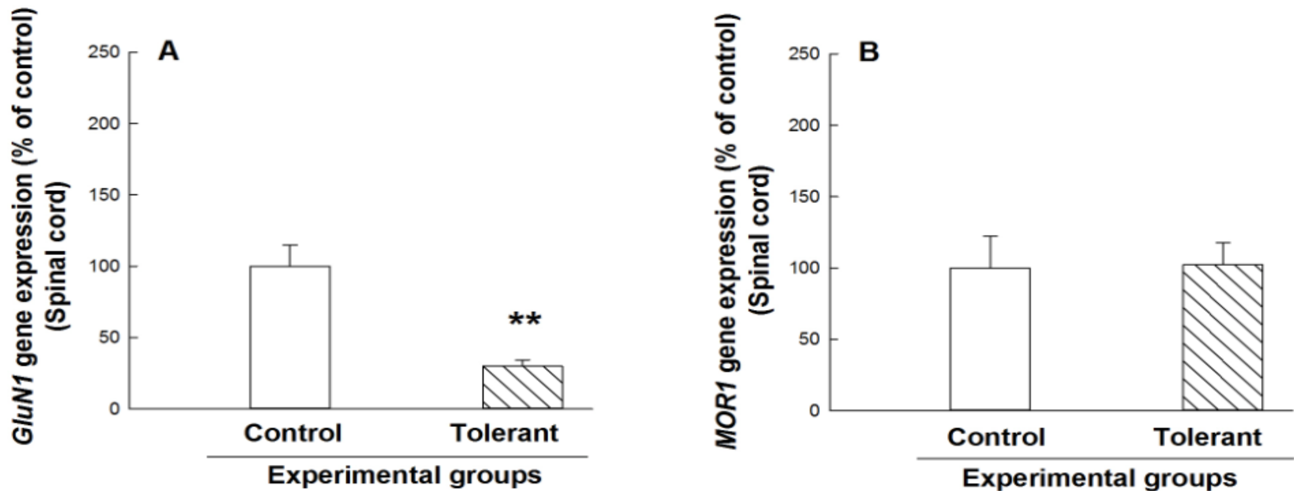

Figure 4. Relative gene expression of the *GluN1* (panel A) and the *MOR1* (panel B) in the lumbosacral cord in control and tolerant groups. Each bar represents mean ± SEM of quantified data related to the expression of the respective genes in each group. ** P<0.01 compared to the saline-treated control group

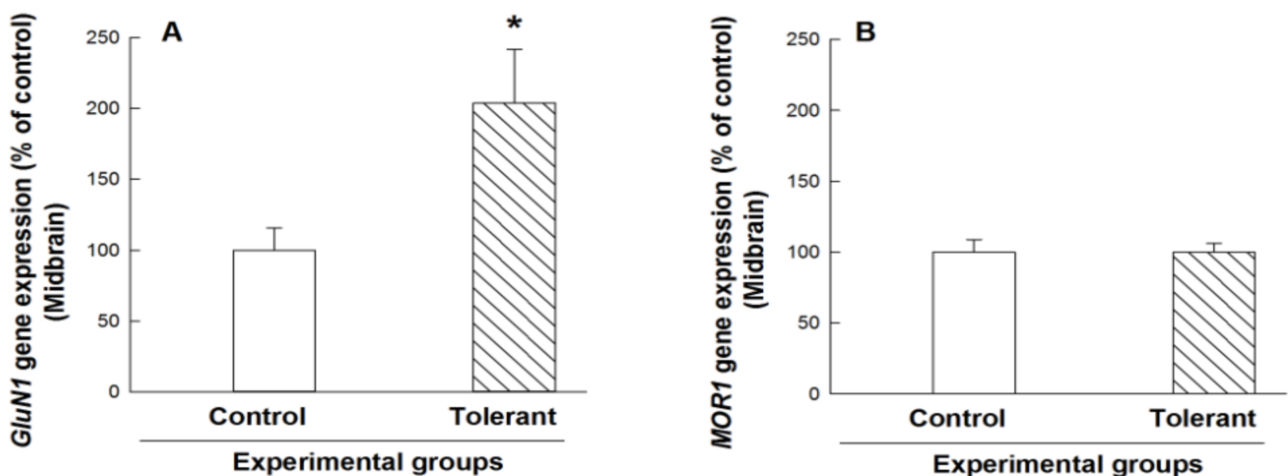

Figure 5. Relative gene expression of the *GluN1* (panel A) and the *MOR1* (panel B) in the midbrain of control and tolerant groups. Each bar represents mean ± SEM of quantified data related to the expression of the respective genes in each group. * $P < 0.05$ compared to the saline-treated control group

The gene expression of GluN1 and MOR1 in the midbrain after induction of morphine tolerance

Figure 5 shows the quantitative densitometry data related to the *GluN1* (panel A) and the *MOR1* (panel B) gene expression in the midbrain. Analysis of the results with independent samples T-test revealed that the *GluN1* gene expression in the midbrain at mRNA level was significantly increased by 110 % ($P < 0.05$) on day 15 of the repeated injections of morphine. However, similar to the results obtained for the lumbosacral portion of the spinal cord, no significant change was observed for the *MOR1* gene expression in the midbrain (Figure 5 A and B).

Discussion

Morphine is still an excellent choice for controlling acute and chronic pain. However, decrease in its analgesic effect due to chronic use of the opioid remains as a major unresolved problem in the clinical management of pain (28). Morphine-induced analgesic tolerance and hyperalgesia are two different processes with some similarities that may underlie loss of analgesic efficacy after chronic morphine treatment (6, 29, 30). A growing body of evidence suggests that different mechanism other than desensitization and/or internalization of MORs might underlie morphine tolerance and hyperalgesia supporting this notion that other intracellular events may play a crucial role in the

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long-term changes elicited by chronic exposure to the drug (31, 32).

The results of hot plate test in the present study showed that a single injection of morphine at doses of 5 and 10 mg/kg induced significant analgesia in naïve rats but its analgesic effects in rats receiving 15 days injections of morphine (10 mg/kg) was decreased as revealed by significant decrease in MPAE%, supporting induction of tolerance to morphine analgesia. Different investigators have reported morphine-induced analgesic tolerance after different regimen of chronic treatment of the opioid (16, 19, 33). Many studies have shown that morphine tolerance is likely due to morphine-induced hyperalgesia (4, 7, 30). However, in the present experiments no significant changes was observed in baseline latencies during 15 days of the injections of saline or morphine, suggesting that the decrease in morphine analgesia was not resulted from opioid-induced hyperalgesia. On the contrary, Mao *et al* (1995) reported that chronic intrathecal morphine administration progressively reduces baseline nociceptive pain thresholds (30). It is possible that induction of morphine analgesic tolerance and/or hyperalgesia depends on different factors including route of morphine administration, duration of repeated injections, doses of the opioid, animal subjects and finally methods that have been used for evaluating analgesia. It is also possible that different authors have defined different levels for induction of morphine-induced analgesic tolerance that may in turn influence the subsequent reported results in their studies. Morphine activate peripheral, spinal, and supra-spinal opioid receptors (5, 34). Both peripheral and central changes in pain pathway may be account for tolerance to morphine analgesia after repeated injections of the drug (4, 35). We have only done hot plate test of analgesia to monitor induction of morphine-induced analgesic tolerance but other investigators have also reported induction of analgesic tolerance with tail-flick test after repeated injections of morphine (16, 30).

Cellular changes associated with opioid-induced tolerance and hyperalgesia have been identified at many anatomic sites including afferent neurons, the spinal cord, brainstem, cortex and the descending modulatory pathway (7). However, molecular data at gene expression level is still incomplete in these regions. Changes in gene expression, or the production of new mRNAs, are not the only critical adaptive events in brain. Rather, changes in translation rates or stability of existing mRNAs, or modification of existing proteins may be also critical for induction of tolerance and dependence to drugs (36). Therefore, a search for changes in gene expression will identify interesting genes and their products involved in specific process. Besides the well-known involvement of the MORs in mediating the effects of morphine, it has been shown that the NMDARs are also involved in morphine-induced analgesic tolerance and locomotor sensitization in rats (37). The GluN1 subunit is the main subunit of the NMDA receptors which is an obligatory subunit to form the receptors. It has been shown that complete deletion of the obligatory GluN1 subunit of the NMDA receptors in hippocampal slice cultures completely eliminates NMDA receptors (38). Therefore, we examined the *GluN1* gene expression, as the most important part of these receptors, to follow changes in these receptors at transcription or mRNA levels after induction of morphine analgesic tolerance.

The results of the present research revealed that chronic morphine treatments decreased mRNA level of the *GluN1* gene in the lumbosacral spinal cord but increased it in the midbrain. These different changes in the *GluN1* gene expression shows a site specific pattern of changes in its gene expression after morphine tolerance in different regions of the pain pathway including lumbosacral cord and midbrain. Zhu *et al* (2003) also reported that morphine tolerance is associated with the down-regulation of the GluN1 at mRNA level in the spinal cord dorsal horn (39). However, some studies examining the *GluN1* gene expression have also reported either increase or no changes in its mRNA level in some brain areas after chronic treatment of morphine (39-42). These later cited reports also support the idea that changes in gene expression of NMDARs after morphine tolerance might show a site-specific process. The site-specific pattern of changes in the gene expressions may result from special pattern of afferents and interneurons in the lumbosacral cord and midbrain. Different nuclei in the midbrain are involved in pain transmission and modulation; however, the main part of our dissected tissue of the midbrain was included the periaqueductal gray matter (PAG) that is an important site for pain modulation. Therefore, it is possible to attribute main part of the increase in the *GluN1* gene expression to the PAG in the midbrain. Furthermore, examining these changes in each nucleus of the midbrain is also an interesting suggestion that we propose for future works.

Some investigators have reported that chronic opioid treatment potentiates presynaptic but impairs postsynaptic NMDARs activity in the spinal cord (35). These investigators have argued that protein kinase C-mediated increases in NMDAR activity at nociceptive primary afferent terminals in the spinal cord contribute critically to the development of analgesic tolerance and opioid-induced hyperalgesia (35). Therefore, it can be proposed that increases in NMDAR activity in the lumbosacral neurons during 15 days of repeated injections of morphine might reduce the *GluN1* gene expression. On the other hand, an increase in the *GluN1* gene expression in the midbrain may result from decreases in NMDARs activity that may finally leading to dysregulation of descending pathways controlling pain transmission. Our present results also showed that the *MOR1* gene expression in the lumbosacral spinal

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cord and midbrain was not affected after 15 days of chronic morphine treatments. In support of our results, Ammon-Treiber and Holt (2005) reported that chronic exposure to opioid agonists or antagonists does not alter mRNA expression of MORs. Therefore, these investigators suggested that physiological responses such as tolerance and dependence may involve post-transcriptional modifications affecting synthesis of new MORs, recycling of internalized receptors and function of existing receptors in the membrane (43).

In recent years, functional cross-regulation between MOR and NMDAR has been also shown to be implicated in the transmission and modulation of nociceptive signals (44, 45). In addition, it has been reported that chronic morphine treatment disrupts interactions between MOR and NMDAR complex to stimulate the activity of NMDAR, then induces the phosphorylation and uncoupling of the MOR via protein kinases, finally result in development of morphine-induced analgesic tolerance (45). It is also possible that the *MOR1* gene expression at mRNA level has not been affected by repeated exposure to morphine in the tolerant rats but their association with NMDAR may be affected due to a possible change in NMDAR, which may underlie, at least partly, morphine-induced analgesic tolerance. Taken together, it is possible that molecular changes related to NMDAR and MOR at transcriptional and/or post-transcriptional level to be associated with morphine-induced analgesic tolerance.

Conclusion

In summary, morphine-induced analgesic tolerance may have different associations with gene expression of *GluN1* and *MOR1* in the lumbosacral portion of the spinal cord and midbrain. One may propose that morphine-induced analgesic tolerance may influence NMDARs at transcriptional level but MORs are not influenced or may be affected at post-transcriptional level. According to some researches, transcript and protein levels for many genes do not correlate well (46); however, there are some reports indicating that transcript levels of the GluN1 subunit are correlate with its protein levels (47, 48). Therefore, it can be proposed that changes in the *GluN1* gene expression in the present study may importantly affect the amount of NMDA receptors in the lumbosacral cord and midbrain, which in turn influence morphine analgesia.

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