Acute and chronic effects of morphine on Low-Mg^{2+} ACSF-induced epileptiform activity during infancy in mice hippocampal slices

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Abstract

Interaction of morphine and seizure is complex. Mouse brain hippocampal slices were used to estimate how acute and chronic morphine treatment alters the low-magnesium artificial cerebrospinal fluid (LM-ACSF)-induced seizure activity. Hippocampal slices were taken from the normal and morphine-treated mice. The normal mice received saline while the other group (morphine-treated mice) received morphine daily for 5 consecutive days. Saline/morphine administration was performed subcutaneously (s.c, 0.1 mL) at postnatal days 14-18. Hippocampal slices of all animals were perfused with LM-ACSF followed by different morphine concentrations (0, 10, 100, and 1000 μM) or naloxone (10 μM). Changes in the spike count were considered as indices for quantifying the seizure activity in the slices. In hippocampus of both groups perfused with 10 or 1000 μM morphine, epileptiform activity was suppressed while it was potentiated at 100 μM morphine. The excitatory effect of morphine at 100 μM was stronger in normal mice (acute exposure) than in dependent mice (chronic exposure). Naloxone suppressed the epileptiform activities in both groups. Suppressive effect of naloxone was more significant in morphine-treated mice than in normal mice. The seizure activity in morphine-dependent mice was more labile than that of normal mice. It can be concluded that morphine had a biphasic effect on LM-ACSF-induced epileptiform activities in both groups. The occurrence of seizure was comparable in acute and chronic exposure of morphine but strength of the effect was considerably robust in normal mice. The down regulation of opioid receptors in chronic exposure is likely to be responsible for these differences.

Keywords: Epilepsy; Infancy; Morphine; Neonatal; Seizure.

INTRODUCTION

Seizure activities are complex neurobehavioral complaints arising from abnormal neural excitability in various brain regions. In particular, the hippocampus has been associated in beginning, propagation, and cessation of seizures. The site of origin of ictal activity seems to be situated in the CA1 area, whereas interictal activity has been found in the CA2 and CA3 areas (1). The hippocampal formation is one of the most studied brain structure because of its multiple roles in learning and memory, Alzheimer disease, and epilepsy. Its inherent circuitry allows the initiation of synchronized neuronal discharges and the hippocampus is considered to have the lowest seizure threshold of any of the brain regions. Epilepsy is a prolonged neurological illness characterized by periodic seizures. Neural damage and genetic aberrations underlie this sickness (2,3). Throughout an epileptic seizure, nerve cells in the epileptogenic area initiate to discharge hypersynchronous electrical signals at an extremely high rate and in an unusual pattern (3,4). A seizure may begin in certain brain structures (*e.g.* amygdala) but the seizure can then spread to other area of the central nervous system (*e.g.* the cerebral cortex) (5,6). When a patient has developed epilepsy, distinct seizures can be triggered by a variety of circumstances.

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Several mechanisms have been assumed to account for the various types of seizures (3,7). Since pharmacological obstruction of GABA-mediated inhibition can elicit interictal discharges that may cause ictal events, an enduring yet controversial hypothesis is that epileptic seizures arises from decreased synaptic inhibition. Additional hypothesis is that amplification of the N-methyl-d-aspartate (NMDA) receptor contributes to epileptogenesis (8). Other investigators have revealed that strong seizure activity can occur without active chemical synapses *in-vitro*(3). Hippocampal slice preparations, that preserve synaptic circuits and cytoarchitecture, enable the exploration of functional and morphological features; thus these *in vitro* models are necessary for electrophysiological studies (9). The epileptiform activity *in vitro* is induced in the hippocampal slices by removing or reducing Mg^{2+} from the perfusion solution that is recognized to unblock the NMDA receptors (10). The ionic channel of NMDA receptor is extremely permeable to Ca^{2+} ions. Entry of Ca^{2+} can trigger many intracellular processes, including seizure like events (SLEs) (11). Opiates play an important role in the initiation and termination of seizures. Systemic administration of μ, δ, and κ opioid receptor agonists such as fentanyl, pentazocine, meperidine, and morphine persuades seizure protection (12). Biphasic effects may have been seen by morphine and fentanyl; they induce anticonvulsant effects with low doses whereas high doses persuade proconvulsant effects. Administration of naloxone (a general opiate antagonist) reduces postictal depression, a period of diminished seizure susceptibility instantly after a seizure that prevents seizure relapse (10). It is possible that a neonate/infant be exposed to opiate directly (prescription by physician) or indirectly (having addicted parents) at early stage of his/her life, then experience seizure (for any reasons) later in life. Hence, the main goal of this study was to compare the effect of acute and chronic exposure to morphine on low-magnesium artificial cerebrospinal fluid (LM-ACSF)-induced SLEs in combined with entorhinal/hippocampal slices of infant mice after exposure to additive doses of morphine during early infancy. Here, we studied the effects of different concentrations of morphine and naloxone on epileptiform activity induced by the absence of Mg^{2+} (blocker of NMDA-receptor complex) in the perfusion medium on brain slices of morphine-dependent and normal mice.

MATERIALS AND METHODS

Animals

Both male and female mice were used in the present study (n = 70, at postnatal day 14 (P14)). The mice were kept at 22 ± 2 °C under a 12/12-h light/dark cycle and were supplied food and water *ad libitum* (light on at 07:00 AM). All experimental events obeyed with the guidelines of the Declaration of Helsinki, and were approved by the Research and Ethics Committee at Urmia University of Medical Sciences. Pups were maintained in a single cage with their mothers at above-mentioned conditions. At P14, the infant mice were randomly allocated to a morphine (n = 35) or a saline/control group (n = 35). Morphine or saline administrations were performed subcutaneously (s.c, 0.1 mL). Normal mice were injected with saline, and the morphine-treated mice received cumulative doses of morphine sulfate daily for five consecutive days (2, 4, 8, 16, and 32 mg/kg) at 08:00 AM. Even though many previous investigations had used greater doses of morphine (> 32 mg/kg), such doses were avoided here because they led to considerable toxicity and mortality in this study and in our previous investigation (13).

Drugs and chemicals

All drugs used in the current study were purchased from Sigma-Aldrich (St. Louis, MO, USA), except morphine sulfate and naloxone which obtained from Food and Drug Division of Urmia University of Medical Sciences (Urmia, I.R. Iran). Drugs were dissolved in LM-ACSF to prepare morphine solutions at 0, 10, 100, and 1000 μM and naloxone at 10 μM. Throughout electrophysiological recordings, all drugs dissolved in LM-ACSF were perfused (9).

Slice preparation

At postnatal days 19-20 (P19-P20), the mice were anesthetized with ether inhalation and rapidly truncated and the brains were quickly detached and transferred into ice-cold 2-4 °C, constantly oxygenated with

95% O_2-5% CO_2. ACSF-composition in (mM) was as follows: 123 NaCl, 2.5 KCl, 1.5 $CaCl_2$, 2 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose (pH 7.35-7.45). The brain was glued to a slicer stage (Vibroslice, Campden instruments, UK) and flooded in cold oxygenated ACSF and sliced to 400 µm-pieces). The trials were done on horizontal hippocampal slices for LM-ACSF-induced SLEs. The slices were incubated for 90 min at 22-24 °C in continuously oxygenated normal ACSF (9).

Electrophysiological recordings

Micropipettes were pulled from glass capillaries (World Precision Instruments (WPI), Sarasota, FL, USA) using a one-stage pipette puller (Narishige, Japan). The micropipettes (2-6 MΩ) were filled with normal ACSF, and located in stratum pyramidale of the CA1 area. After 10 min of accommodation, recordings were initiated (1kHz sampling rate) in the recording chamber (interfaced type). An amplifier with a preamplifier (Science beam, Tehran, I.R. Iran; bandwidth 0.16-2 kHz, 2 k gain, and a built in 50 Hz notch filter) was used to obtain the electrophysiological activity. Recordings were achieved from the CA1 pyramidal layer of the hippocampus. LM-ACSF was applied to persuade SLEs and micropipette was positioned at locations yielding maximal evoked field potential amplitudes. Data were digitized with eTrace Analyzer software (Science beam, Tehran, I.R. Iran) and stored on a computer for offline analysis.

Data analysis

Data were analyzed using SPSS software version 22. Distribution of data was checked; the data with non-normal distribution were analyzed by means of nonparametric tests. Two-dependent group comparison was done by Wilcoxon test and multiple dependent comparisons were performed by Friedman test. The findings were expressed as mean ± standard error of mean and $P < 0.05$ was considered significant.

RESULTS

Perfusion of hippocampal slices with LM-ACSF provoked status of repeated discharges, or status-like activity. Majority of recorded activities consisted of short 1-10 s discharges lasted for as long as the LM-ACSF perfusion remained low. These findings are illustrated in Figs. 1 to 8, where morphine (10, 100, and 1000 µM) and naloxone (10 µM) were mostly effective in potentiating and suppressing the SLEs when perfused with LM-ACSF on the hippocampal slices. Acute (normal mice) and chronic (morphine-dependent mice) use of morphine were done to determine the pattern of the seizure activity induced by LM-ACSF. In each of 48 different hippocampal slices, the SLEs were consistent and their morphologic appearance did not change. In slices of non-dependent mice (acute morphine), the SLEs started almost 5-10 min after perfusion of LM-ACSF and lasted for nearly 95-120 min in a steady and stable manner while in slices of morphine dependent mice (chronic morphine) SLEs started approximately 10-20 min after perfusion of LM-ACSF and continued for as long as magnesium remained low. Frequency of spikes and the effect of morphine on these spikes were considered and analyzed in the current study. Morphine at 10 and 1000 µM decreased frequency of spikes in both non-dependent (normal) and morphine-dependent mice ($P < 0.01$) (Figs. 1-4). Washout of slices for 10-20 min completely reversed these effects ($P < 0.01$). Addition of 10 µM naloxone to the LM-ACSF could not reverse such suppressing effects of morphine.

When morphine (100 µM) was applied, seizure activity was enhanced in spike count ($P < 0.01$) (Figs. 5 and 6). Naloxone at 10 µM slightly blocked such enhancement in seizure activity. There was a significant difference between acute and chronic morphine (normal slice and morphine-dependent slice) on SLEs when perfused with morphine at 100 µM ($P < 0.05$). Moreover, the SLEs in morphine-dependent mice were much more labile than those of normal mice.

Since naloxone is a general antagonist of opiate receptors, we also tested its effect on SLEs (Figs. 7 and 8). When naloxone at 10 µM was applied to slices perfused with LM-ACSF significantly attenuated seizure duration and frequency of spikes.
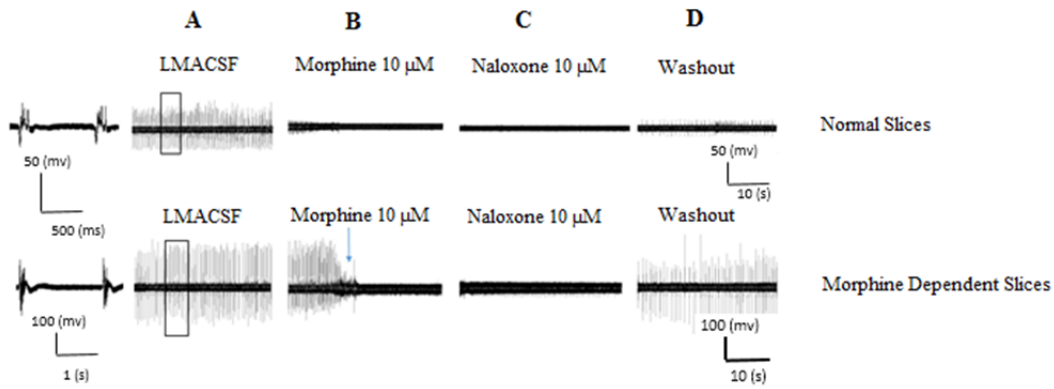
Fig. 1. Graphical illustration of the effects of LM-ACSF, morphine 10 µM, and naloxone on SLEs in the hippocampal slices of control and morphine-treated mouse. (A) The SLEs were induced by LM-ACSF and recorded in CA1 area; (B) morphine fully suppressed the LM-ACSF-induced SLEs; (C) naloxone did not recover the SLEs; and (D) washout with LM-ACSF reversed the effect of morphine and recovered the activities again. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
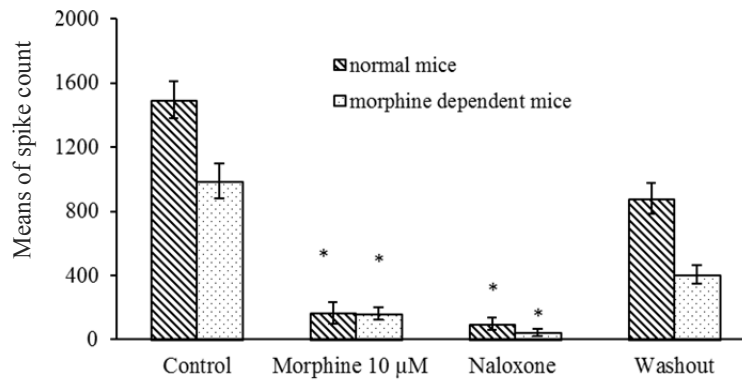

Fig. 2. Effect of morphine 10 µM on LM-ACSF-induced SLEs in normal and morphine-treated infant mouse. Applying morphine decresed spikecount number, and naloxone (10 µM) couldn't reverse the supressive effect of morphine while washout with LM-ACSF recovered the SLEs in both groups. In each group morphine was applied to at least six brain slices. * Indicates significant differences ($P < 0.01$) *vs* the control stage. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
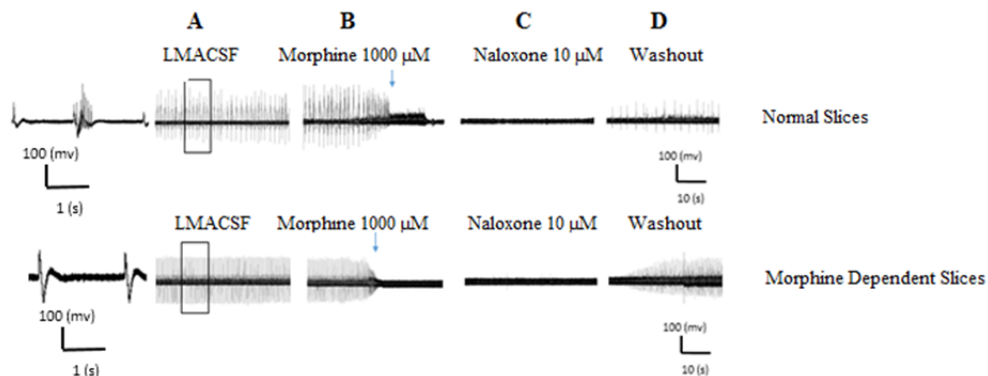

Fig. 3. Graphical illustration of the effect of LM-ACSF, morphine 1000 µM, and naloxone on SLEs in the hippocampal slices of normal and morphine-treated mouse. (A) The SLEs were induced by LM-ACSF and recorded in CA1 area; (B) morphine fully suppressed the LM-ACSF-induced SLEs; (C) naloxone couldn't recover the activity; (D) washout with LM-ACSF reversed effect of drugs and restored the activity again. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
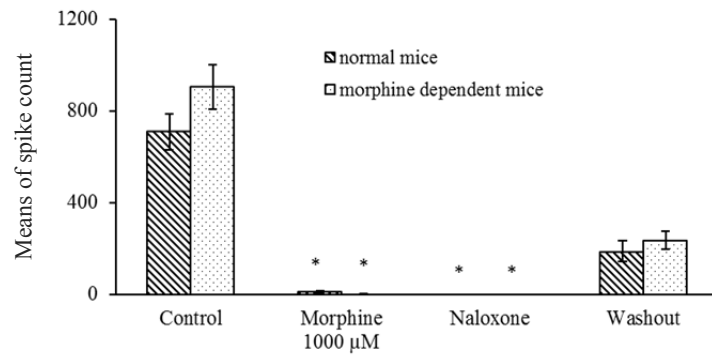
Fig. 4. Effects of morphine (1000 µM) on SLEs in normal and morphine-treated imature mouse. Morphine decresed spikecount number and naloxone (10 µM) didn't reversed the supressive effect of morphine while washout with LM-ACSF restored the activity. In each group, data were obtained at least from 6 slices. * Indicates significant differences ($P < 0.01$) in comparison with the control and washout. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
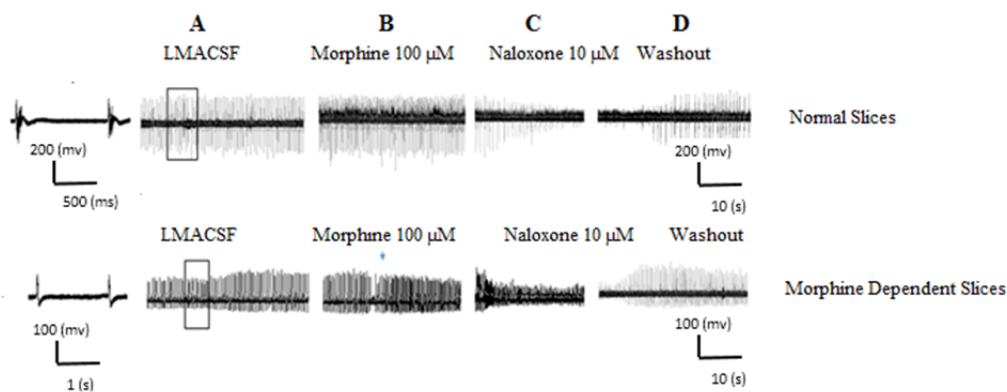

Fig. 5. Graphical illustration of the effects of LM-ACSF, morphine 100 µM and naloxone on SLEs in the hippocampal slices of normal and morphine-treated mouse. (A) The SLEs were induced by LM-ACSF and recorded in CA1 area; (B) morphine 100 µM augmented the SLEs that induced with LM-ACSF; (C) naloxone 10 µM attenuated the SLEs; and (D) washout with LM-ACSF reversed the effect of naloxone 10 µm and induced the epileptiform activity again. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
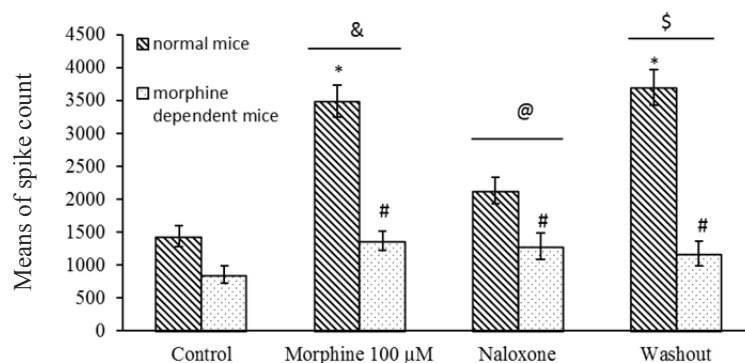

Fig. 6. Effects of morphine 100 µM on SLEs in normal and morphine-treated imature mouse. In both groups morphine 100 µM incresed spikecount number and naloxone 10 µM decresed the effect of morphine whereas washout with LM-ACSF induced the epileptiform activity. In each group, experiments were performed at least on 6 slices. * Compared to control stage in normal mice ($P < 0.001$); # compared to control stage in morphine-dependent mice ($P < 0.01$); & significant difference between normal and morphine-dependet mice at stage morphine 100 µM ($P < 0.01$) was observed; @ specifies significant difference ($P < 0.05$) between normal and morphine-dependet mice at stage naloxone, and $ indicates morphine-dependet mice significantly differ ($P < 0.01$) from normal group at washout stage. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
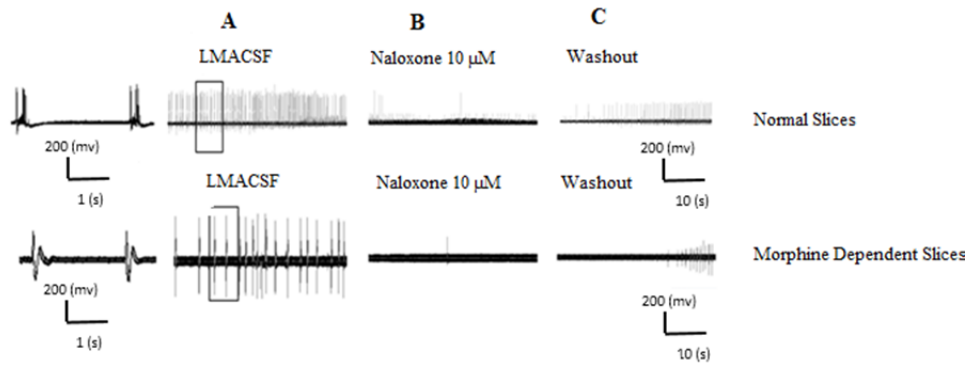
Fig. 7. Graphical illustration of the effects of LM-ACSF and naloxone on SLEs in the hippocampal slices of normal and morphine-treated mouse. (A), The SLEs were induced by LM-ACSF and recorded in CA1 area; (B) naloxone fully suppressed the LM-ACSF-induced seizure activity; and (C) washout with LM-ACSF reversed the effect of naloxone and restored the activity. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.
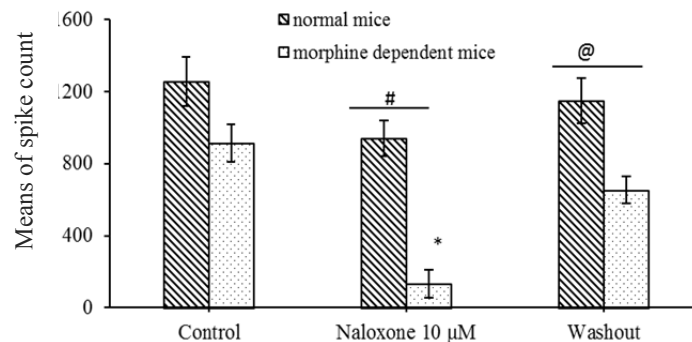

Fig. 8. Effect of naloxone 10 μM on epileptiform activity in normal and morphine-treated imature mouse. Naloxone decresed spikecount number, and washout with LM-ACSF reversed the effect. In each group, experiments were performed at least on 6 slices. * Compared to the control and washout stages in morphine-dependent mice ($P < 0.01$); # significant difference between normal and morphine-dependet mice at stage naloxone at 10 μM ($P < 0.05$) was observed, and @ indicates morphine-dependet mice are significantly different from normal mice at stage washout ($P < 0.05$). SLEs in morphine-dependent mice were more labile (small error bar) than those in normal mice. LM-ACSF, low-magnesium artificial cerebrospinal fluid; SLEs, seizure like events.

DISCUSSION

In the current study, the effects of morphine and naloxone were investigated on SLEs induced by LM-ACSF in normal and morphine-dependent infant mice. Newborn mice were used because the neurological development, synapse formation and expansion take place mainly in this period and are equivalent to the third trimester of human fetus, and on the other hand are physiologically in immature state. Therefore, chronic exposure to morphine expected to cause changes in the nervous system that cause or prevent epileptic activity involved (14,15). Pro-seizure and anti-seizure effects of drugs were examined by assessing overall suppression of SLEs. This study is the first to

demonstrate that morphine at 10 and 1000 μM reduced and at 100 μM enhanced seizure activity in the hippocampal slices of saline- and morphine-treated infant mice. Similar observation have been confirmed *in vivo* in animal models of epilepsy when morphine at greater doses was administered by various routes (2). The enhancing effect of morphine at 100 μM on SLEs was stronger in normal mice compared to morphine-treated mice. It may be related to down regulation of opioid receptors in morphine-dependent mice. In this connection, prolonged use of morphine (or other opiate receptor agonists) can cause significant down regulation of corresponding receptors that is one of the involving mechanisms of tolerance to these drugs (16). Meanwhile, it is stated that morphine-

pretreated rats display faster achievement of seizure behavior. Evaluation of the postictal seizure suppression following a completely kindled seizure revealed that morphine-exposed rats has a diminished sensitivity to new kindling stimulations (17). Also it is mentioned that chronic exposure to morphine in early life stage in rats could change their vulnerability to pentylenetetrazole-induced seizure in an age-specific style (18,19).

Meanwhile, findings of this study confirmed a biphasic actions of morphine on SLEs, as reported in previous *in vivo* animal experiments (20-22); that is, a low and a high morphine concentrations established anti-seizure effects but intermediate morphine levels potentiated the seizure. The present study used an *in vitro* low Mg^{2+} model of SLEs in mice brain slices. Low Mg^{2+} causes neural hyperexcitability which leads to seizure initiation by reducing Mg^{2+} blockade of Ca^{2+} channels (2). In fact all concentrations of morphine could not provoke SLEs in the current study at normal level of Mg^{2+}. Our assumptions from the low Mg^{2+} model may be applicable to those cases when morphine persuades and/or suppresses seizure activities in the clinical circumstances. This model of epilepsy displays seizure-like activities comparable to electroencephalographic aberrations in epileptic patients with partial complex seizures and partial status epilepticus (23). It is documented that a low serum Mg^{2+} level may lead to seizures, and restoring Mg^{2+} level can stop and control convulsions linked with febrile convulsion as well as eclampsia (3). The molecular and cellular mechanism of this outcome in our study is possibly not connected to the direct stimulation of opioid receptors since addition of naloxone 10 µM to the perfusing solution did not restore the anti-seizure effect of morphine. It is likely that the mechanism of such action is inhibition of glutamatergic excitatory pathways and augmentation of GABAergic activity (22). Opiates appear to be involved in generating seizure activity in the hippocampus; they may be secreted by seizure activity inside the limbic system to extend the period of postictal suppression and thus stop or attenuate the seizures repetition during this period. Similar

to our study, low doses of morphine demonstrated an anti-seizure effect in seizure models induced by GABA-transmission blockers and greater doses of morphine augmented the susceptibility of subjects to the similar seizure models (24,25). Some reports specify that opiates can also have direct excitatory effects on intracellular signaling pathways including stimulation of adenylyl cyclase, potentiating calcium entry and elongation of action potential duration (25,26). In addition, it is likely that NO may persuade anti-seizure effect of morphine by increasing GABAergic tone (8,27). Our result showed that prolonged exposure to morphine postponed the beginning of LM-ACSF-induced SLEs. Collectively, it seems that the administration of systemic morphine without extra convulsant handlings triggers at least two epileptogenic mechanisms; one is mediated by specific opiate receptors, while the other is not. The endogenous opioids and i.c.v. morphine stimulate only a specific system (reversible by naloxone). There is no evidence to indicate that tolerance will be developed to the seizure activity of systemically administered morphine. In this study, significant differences of 10 and 1000 µM concentration of morphine on SLEs between normal and morphine-dependent infant mice were not observed. This assumption is supported by a previous work in dog, rabbit, and cat (28-30). Another study has addressed this issue in the rat. Tolerance was induced in rats by subcutaneous implant of pellets of morphine over 8 days. No augmented electrographic spiking happened in these rats compared to normal rats. But, when morphine administered to these rats, and withdrawal was then caused by naltrexone, more seizures were seen in the tolerant rats compared to the non-tolerant normal rats (20). In most of the cases, naloxone can antagonize the anticonvulsant effects of morphine and related opioids. For instance, the anticonvulsant effects of morphine on audiogenic seizures in mice could be reversed with 1 mg/kg of naloxone, while in this study 10 µM concentration naloxone could not reversed anticonvulsant effects of morphine at 10 and 1000 µM and itself induced anticonvulsant effects. It appeared that

suppressive effect of naloxone 10 μM on SLEs was stronger in morphine-dependent mice than that in normal mice (Fig. 8). There are some evidence that naloxone might have anti-seizure effects. For instance, the minimum dose of naloxone tried to reverse the pentylenetetrazole-induced seizure was 5 mg/kg in the rat, and this dose was fully effective (31). Also, naloxane increases the inhibitory effect of verapamil on the seizure induced by pentylenetetrazole kindling (32) Likewise, naloxone reversed the morphine-induced augmentation in recurrent seizures subsequent to a single injection of pentylenetetrazole (33).

CONCLUSION

It can be concluded that morphine exhibited a biphasic effect on LM-ACSF-induced epileptiform activities in acute (normal mice) and chronic (morphine-dependent mice) groups. The pattern of effect was similar in acute and chronic exposure of morphine nevertheless the extent of the effect was considerably robust in normal mice. It is likely that down regulation of opioid receptors in chronic exposure was responsible for these differences.

ACKNOWLEDGMENTS

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