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MiR-33-5p Regulates CREB to Induce Morphine State-dependent Memory in Rats: Interaction with the μ Opioid Receptor

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ABSTRACT

The aim of the present study was to examine the hypothesis that miR-33-5p attenuates morphine state-dependent (StD) memory via the μ opioid receptor by regulating cyclic AMP response element-binding protein (CREB). The effects of post-training morphine and morphine StD memory and their interaction with pre-test naloxone were evaluated using a single-trial inhibitory avoidance paradigm. Then, the hippocampal miR-33-5p gene and pCREB/CREB protein expression profiles were evaluated using quantitative real-time PCR and western blotting, respectively. We found that while post-training morphine and morphine StD memory respectively up- and down-regulate the miR-33-5p expression profile in the hippocampus, the reverse results are true for the expression of pCREB/CREB. Pre-test naloxone antagonized the response. Overall, our findings suggest that the expression levels of miR-33-5p in the hippocampus set the basis for morphine StD memory with low miR-33-5p enabling state dependency. The mechanism is mediated via miR33-5p and CREB signaling with the interaction of the μ opioid receptor. This finding may be used as a potential strategy for ameliorating morphine-induced memory-related disorders.

Keywords: miR-33-5p, CREB, state-dependent memory, opioids, Rats

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Introduction

MicroRNAs (miRNAs or miRs) have been introduced as essential epigenetic regulators of synaptic plasticity (1). MiRs are non-coding short-chain RNAs that regulate gene expression at the post-transcriptional level by interaction with target messenger RNAs. MiRs are involved in the regulation of synaptic plasticity, learning, and memory in the nervous system (2-4). While there are a huge number of

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studies on the role of miRNAs in learning and memory, their regulatory role in the context of state-dependent (StD) memory is still poorly understood. Jovasevic et al. showed that miR-33 mediates StD fear memory by targeting the GABA receptors (5). Besides, misregulation of miRNAs has been already reported in the pathophysiology of a variety of neuroplastic, learning, and memory disorders (6-9).

MiR-33 is highly expressed in the brain and involved in lipid metabolism, which is critical for neuronal actions (10). In rodents, the downregulation of miR-33 expression in the brain induces ATP-binding cassette transporter A1 (ABCA1) expression and ApoE lipidation and decreases the levels of intrinsic brain Amyloid beta (Aβ). The aberration of Aβ peptide metabolism is postulated to trigger pathogenic processes that can result in Alzheimer's disease (AD) (11). ABCA1 is a major cholesterol carrier that controls ApoE lipidation in the brain (12). Moreover, the pharmacologic blockade of cerebral miR-33 in an Alzheimer's animal model remarkably decreased cortical Aβ (13), signifying not only a potential cure for AD but also a broad verity of neural/cognitive disorders (10, 13-15).

The StD memory is a memory process by which the retrieval of an emotional memory is more efficient when learners are in the same state of learning as they were when the memory was formed (16). It is believed that in emotional memories, which are mainly dependent on an endogenous StD mechanism(17), the so-called "critical states" are the states after training that consolidation occurs and at the time of recall (17, 18). The drug-induced or exogenous state dependency has been proposed to mimic the endogenous mechanisms of acquisition and retrieval (18). Evidence shows that morphine (Mor) induces StD memory in inhibitory avoidance (IA) models (16, 19) using a single-trial paradigm (16). IA paradigms are among the well-known models of StD studies (20). Gamma-aminobutyric acid (GABA) receptors in the hippocampus and their associated miR-33 are essential for the induction of StD memory (21). GABA receptors also have a pivotal role in StD memory recall (22) and the involvement of GABA receptors has been reported in both memory consolidation and Mor-StD learning (23). Accordingly, interactions between GABA receptors and opioids in learning and memory are well documented in literature (20, 23-27). GABA and opioid systems have also been proposed to be linked via the μ opioid receptors (28). Considering that Mor-mediated changes via the μ opioid receptor in the hippocampus may be involved in the regulation of miR-33-5p and that miR33 is involved in StD, it is rational to hypothesize that miR-33-5p may also be involved in Mor-StD memory.

MiRNAs are also involved in the regulation of cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (29, 30) which is in turn involved in the consolidation of long-term memory (31). In addition to being a miRNA target, CREB also regulates miRNAs' transcription. For instance, miR-134 regulates long-term memory by altering the CREB mRNA and consequently the neurotrophic factor levels (32).

While we are currently in the preliminary steps of understanding the contributing mechanisms of miRNAs in opioid-related learning and memory functions, there is increasing proof for miRNAs' role in regulating opioid signaling (33-39). Such increased supporting data proposes that miRNAs are essential modulators of opioid-related cognitive functions (34, 35), and as a representative metabotropic receptor, μ opioid receptor signaling is mediated by adenylyl cyclase (40).

Given the vital significance of CREB for memory consolidation and the role of miR-33 in regulating CREB expression as discussed, it was of particular interest to hypothesize that in the second phase of our

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Mor-StD memory study, CREB expression would also be under the control of miR-33. Hence, the dual aims of the present study were to investigate (1) the possible alterations of hippocampal miR-33 and p-CREB/CREB levels following Mor-StD memory in the IA memory model; and (2) the involvement of μ opioid receptors in this process.

Materials and Methods

Animals

A total of 208 male Wistar rats in 26 experimental groups (n=8 per group, each aged 10-12 weeks and weighing 200-220 g) were used in the study. All the rats were housed in a polycarbonate cage inside a room at 24 ± 2 °C with a 12-h light/dark cycle. Unrestricted access to standard diet and water was provided for all the rats. All the study procedures were approved by the Animal Ethics Committee of Kashan University of Medical Sciences (committee registration code: IR. KAUMS. REC.1396.96). The study was in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH Publications No. 8023, revised Edition 1978).

Drugs

Morphine and naloxone were acquired from Temad (Tehran, Iran) and Sigma (England), respectively. All the drugs were dissolved in sterile saline and freshly prepared to the required volume and concentration on a daily basis. The post-train or pre-test intraperitoneal (i.p.) administrations of the drugs were performed after a 30-min interval from the training or testing session. Primary antibodies specific to phospho-CREB and β-actin as well as anti-rabbit HRP-conjugated secondary antibody were obtained from Cell Signaling Technology (New York, USA). Anti-CREB primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

IA test and study design

The IA experiments were carried out as previously described (41). The IA setup comprised two equal-sized chambers (20 × 20 × 30 cm^3) separated by a wall with a manually-adjustable guillotine door located in the middle (Technique Azma, Tabriz, Iran). The walls and floor of one chamber were white and lit with a 25 W electric bulb. The walls of the other chamber were dark with its floor consisting of parallel stainless-steel grids (3 mm in diameter), located 1 cm apart. Intermittent electric shocks (at the frequency of 50 Hz, duration of 3 s, and intensity of 1 mA) were applied to the grid floor of the dark chamber using an isolated stimulator (Technique Azma, Tabriz, Iran). The test consisted of a training session followed by a memory retention session carried out 24 h after the training. During the training session, each rat was gently placed in a white chamber and its latency to step through the guillotine door and enter the dark chamber with all four paws was measured. The rats were subjected to an electric shock following their complete entrance into the dark chamber with their four paws on the grid floor. The memory retention session was conducted in a similar manner to the training session, except that no electric shock was applied. The latency time (s) to enter the dark chamber was considered a criterion for measuring memory. A cut-off time of 300 s was used for latency time. Considering the effects of environmental conditions on synaptic plasticity (42), a uniform experimental condition was secured for all the rats.

After the termination of the IA memory test, the hippocampi were rapidly removed as previously described (43) and put on an ice-cube. The hippocampi were rapidly frozen in liquid nitrogen and stored at -80 °C until later use. In parallel, the hippocampi were also removed from an intact group with no treatment (no manipulation). Subsequently, the evaluation of miR transcripts together with CREB protein expression levels was performed using quantitative real-time PCR and western blotting, respectively, on three separate randomly-chosen hippocampi per experimental group and method (Figure 1).

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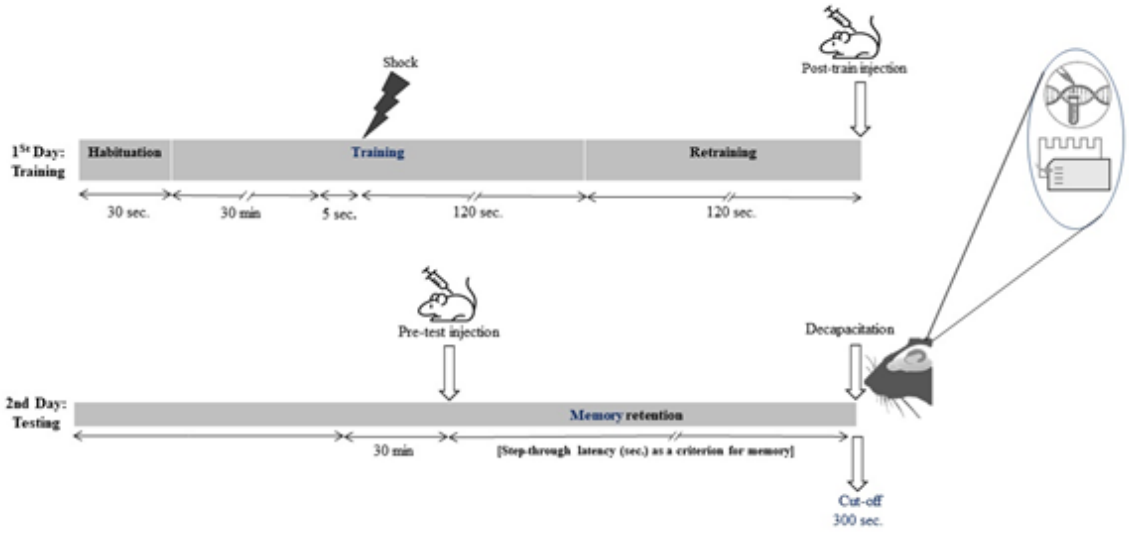

Fig. 1. The schematic timeline for experiments.

Behavioral experiments

Experiment 1

This experiment examined the effects of post-training intraperitoneal (i.p.) administration of different doses of Mor on IA memory retention. Four groups of the rats were used for this experiment. The control group received post-training and pre-test administration of saline (1 ml/kg), whereas the other three groups received post-training administration of Mor (2.5, 5, and 7.5 mg/kg). On the testing day, all the groups received pre-test saline (1 ml/kg) 30 min prior to the test.

Experiment 2

This experiment evaluated the effects of pre-test and post-training i.p. administration of Mor on IA memory retrieval. Four groups of rats were used for this experiment. On the training day, all the groups received post-training saline (1 ml/kg) or Mor (7.5 mg/kg) 30 min following the training session, whereas on the testing day, the groups received either pre-test saline (1 ml/kg) or Mor (2.5, 5, and 7.5 mg/kg) 30 min prior to the test.

Experiment 3

This experiment examined the effects of pre-test administration of naloxone on step-through latency of IA. Four groups of rats were used for this experiment similar to the previous tests. All the rats received post-training administration of saline (1 ml/kg/i.p.); however, they received pre-test administration of either saline (1 ml/kg/i.p.) or different doses of naloxone (0.5, 1 and 2 mg/kg, i.p.) 30 min prior to the test.

Experiment 4

This experiment examined the effects of pre-test co-administration of naloxone and Mor on step-through latency in IA. All the groups of rats received post-training administration of saline (1 ml/kg), while for the pre-test, they received pre-test administration of either saline (1 ml/kg) or co-administration of naloxone (0.5, 1 and 2 mg/kg, i.p.) and Mor (7.5 mg/kg, i.p.) 30 min prior to the test.

Experiment 5

This experiment examined the interactive effect of pre-test naloxone on step-through latency in the Mor-induced state of IA memory. All the rats received post-training administrations of saline (1 ml/kg) or Mor (7.5 mg/kg, i.p.), while the groups underwent pre-test administration of either saline (1 ml/kg) or co-

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administration of naloxone (0.5, 1, and 2 mg/kg, i.p.) and Mor (7.5 mg/kg, i.p.) 45 and 30 min prior to the test, respectively. The dosage and route of Mor (16) and naloxone (44) administration were chosen based on the results of previous studies.

Real-Time PCR

Total hippocampal miRNA was extracted using Hybrid-RTM miRNA Kit (GeneAll, Seoul, Korea) according to the manufacturer's instructions. The quantity and quality of the extracted miRNAs were evaluated on Nanodrop ND 3300 spectrophotometer device (NanoDrop technologies, USA). Following the miRNA extraction, cDNA synthesis was carried out for each sample specifically for both SNORD6 and miR-33-5p via stem-loop primer kit (ZistRoyesh, Tehran, Iran), and quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Ampliqon, Odense, Denmark) and LightCycler® 96 Instrument (Roche Life Science). The average threshold (Ct) was determined for miR-33-5p and normalized to SNORD6 (U6) as an internal normalization control gene. The $2^{-\Delta\Delta ct}$ method was used to calculate the relative expressions (45).

Western blotting

Following the termination of the behavioral tests, some rats were deeply anesthetized by CO_2 inhalation and then immediately decapitated and their hippocampi (n=3) were isolated on ice over a short period of time and stored at -80 ◦C until further molecular experiments. The hippocampi of the rats were quickly weighed and homogenized at 3 times volume/weight of cold RIPA lysis buffer on ice containing a protease and phosphatase inhibitor cocktail (Sigma, USA). The lysates were centrifuged at 13,000 RPM for 35 min at 4 °C, and the protein-containing supernatants were collected. The concentration of the proteins was determined according to Bradford's method using BSA as a reference standard (46). Twenty μg of the protein lysates were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel and then transferred to PVDF membranes (Amersham Bioscience, USA). The blots were subsequently blocked in 5% BSA-TBST (0.1M Tris–HCl (pH8.5), 0.5% Tween-20, 1.5M NaCl) and then probed with primary antibodies (1/4000) overnight at 4 °C. The next day, after washing the blots with TBST, they were incubated for 110 min at room temperature with anti-rabbit IgG HRP-conjugated (1/10,000) as the secondary antibody. The membranes were developed using ECL select™ (Amersham Bioscience, USA) reagents. After visualization of the bands developed on a photographic film membrane, the intensity of immune-reactive polypeptides was analyzed. To normalize the loaded protein, blots were stripped in the stripping buffer containing 62.5 mM Tris–HCl (pH: 6.7), 100 mM 2-mercaptoethanol and 2% (w/v) SDS and then probed with anti-β-actin antibody (1/2,000). The quantification of the results was performed by a densitometric scan of the films, and the density of the bands was calculated by ImageJ software.

Bioinformatic analysis

To evaluate the predicted binding and targeting of rno-mir-33-5p microRNA to the CREB-1 of Rattus norvegicus, we used validated online bioinformatics microRNA target prediction tools such as TargetScan (http://www.targetscan.org/vert_72/), miRmap (https://mirmap.ezlab.org/app/) and microT_CDS (http://diana.imis.athena innovation.gr/DianaTools/index.php?r=microT_CDS/index) were used for miRNA selection. Target miRNAs appearing in these databases for CREB1 gene were selected and the miRNAs that negatively correlated with CREB1 were further screened computationally in RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). Among the miRNA items, rno-miR-33-5p was selected as one of the best complementary scores and the relationship between the regulation of CREB-1 gene expression and rno-miR-33-5p was analyzed.

Statistical analysis

After assessing the normality of the data using the Kolmogorov-Smirnov test, the results of the behavioral experiments were statistically analyzed using the parametric/nonparametric analysis of variance

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(ANOVA) depending on the experimental protocol. Sigmaplot software V.14.0 (SysTest Software, Inc.) was used for all the analyses and artwork. A $P < 0.05$ was considered statistically significant.

Results

The hippocampal miR-33 and p-CREB/CREB expression levels in Mor-induced memory impairment

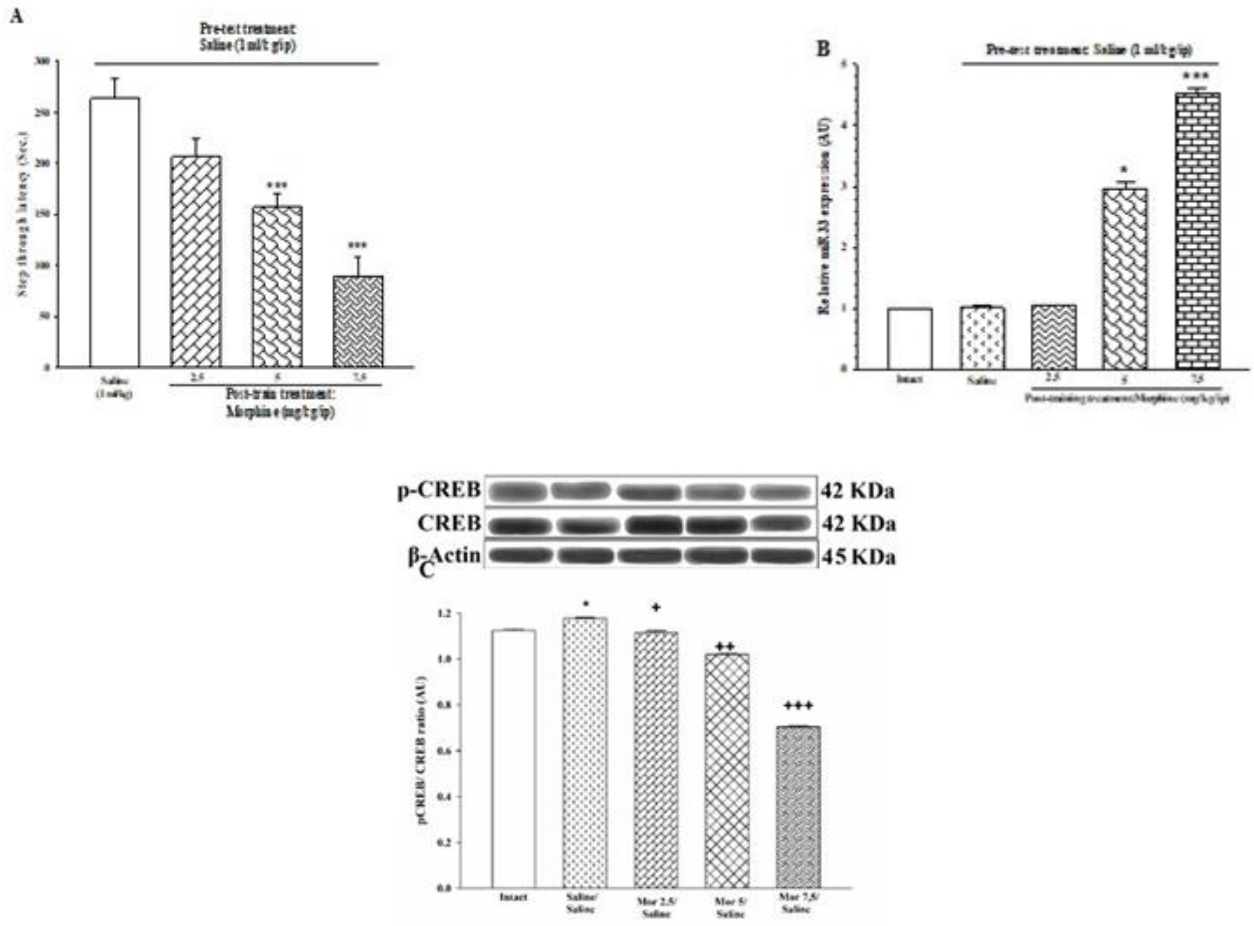

Fig.2. A. The effect of post-training administration of Mor on step-through latency in an inhibitory avoidance memory task. All animals received pre-test administration of saline (1 mL/kg/i.p.) and post-training administration of either saline (1 mL/kg/i.p.) or different doses of Mor (2.5, 5, and 7 mg/kg, i.p.). Each value denotes the median ± quartile for each experimental group (n=8)). ***$p < 0.001$ compared to the saline control group. B) The hippocampal miR-33 expression in Mor-induced memory impairment. The miR-33 expression level was evaluated following memory assessment using quantitative real-time PCR (on randomly chosen three rats per each group) in five experimental groups as follow: intact (group 1, no treatment and no-testing), saline (1 mL/kg/i.p.; group 2); the Mor (2.5, 5 and 7.5 mg/kg i.p.; groups 3, 4 and 5, respectively). Each value denotes the median ± quartile for each experimental group (n=3). *$p < 0.05$, and ***$p < 0.001$ compared to the saline control group. C) The hippocampal *p*-CREB/CREB expression in Mor-induced memory impairment. (The upper panel shows the immunoblotting profile). The analysis indicated a significantly decreased p-CREB/CREB level in the Mor (7.5 mg/kg, i.p.) group compared with the saline/saline group. *$p < 0.05$ compared to the intact group; $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ compared to the saline/saline group.

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Figure 2A presents the i.p. administration of Mor (2.5, 5 and 7.5 mg/kg) following successful training-impaired IA memory [H (3) = 18.23, P <0.001]. The post-hoc analysis showed that Mor administration at doses of 5 (P <0.001) and 7.5 mg/kg (P <0.001) decreased the step-through latency significantly compared to the saline control group. Mor maximal effect was seen at the dose of 7.5 mg/kg of administration.

Figure 2B shows the hippocampal overexpression of miR-33-5p following the post-training administration of Mor and indicates significant memory impairment compared with the saline control group ([H(4) = 24.41, (P <0.001). The post-hoc analysis showed that the administration of Mor at doses of 5 (P<0.05) and 7.5 mg/kg (P <0.001) increased miR-33-5p expression levels significantly compared to the saline control group.

Figure 2C shows the hippocampal p-CREB/CREB protein levels in Mor-induced memory impairment measured via western blot analysis. The densitometric analysis revealed significant changes in the hippocampal protein levels of p-CREB/CREB. The analysis indicated that IA memory impairment decreased hippocampal p-CREB/CREB levels compared to the saline control group [F (4, 10) =4286.32, (P= 0.01)].

The hippocampal miR-33 and p-CREB/CREB expression levels in Mor-StD memory

Figure 3A shows that the i.p. pre-test administration of Mor (2.5, 5 and 7.5 mg/kg) restored post-training Mor-induced IA memory impairment [H (3) = 20.55, P <0.001]. The post-hoc analysis showed that Mor administration at the dose of 7.5 mg/kg (P <0.001) significantly increased step-through latency compared to the saline control group. Mor maximal effect was seen at the dose of 7.5 mg/kg of administration.

Figure 3B shows the under-expression of hippocampal miR-33-5p, indicating successful memory retrieval compared to the saline control group ([F (4, 25) = 94.79, (P <0.001)]). The post-hoc analysis showed that the administration of Mor at doses of 5 (P <0.001) and 7.5 mg/kg (P <0.001) significantly increased miR-33-5p expression compared to the saline control group.

Figure 3C shows the hippocampal p-CREB/CREB protein levels in Mor-induced StD memory measured via western blot analysis. The densitometric analysis revealed significant changes in the hippocampal protein levels of p-CREB/CREB. According to the analysis, Mor-StD memory increased the hippocampal p-CREB/CREB levels compared to the saline control group ([H (4) =13.50, (P = 0.009).

The hippocampal miR-33 and p-CREB/CREB expression levels following the pre-test administration of naloxone in rats trained under saline administration

Figure 4A shows that the i.p. pre-test administration of naloxone per se (0.5, 1 and 2 mg/kg) had no effect on IA memory [H (3) =1.34, P = 0.72].

Figure 4B shows that the hippocampal expression levels of miR-33-5p in the rats receiving pre-test administration of naloxone prior to saline were not significantly different from the saline control group (H (4) = 7.82 (P = 0.09).

Figure 4C shows the hippocampal p-CREB/CREB protein levels in the pre-test naloxone-administered group measured via western blot analysis. The densitometric analysis revealed no significant changes in the hippocampal p-CREB/CREB compared with the saline control group (F (4, 10) = 0.797, P = 0.55).

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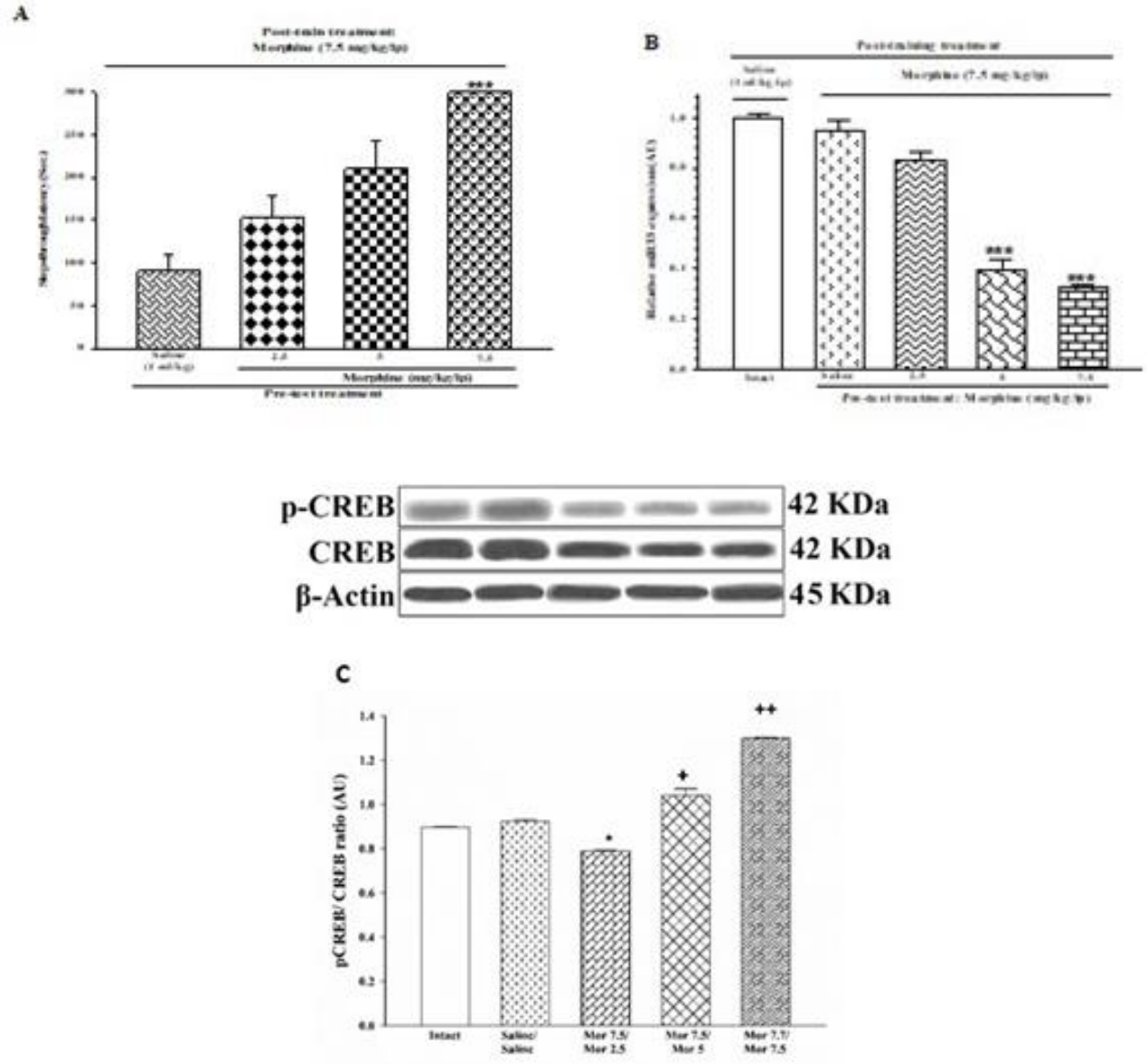

Fig.3. A. The effect of pre-test and post-training administration of Mor (Mor- StD) on step-through latency in inhibitory avoidance memory (7.5 mg/kg, i.p.). One group of animals received pre-test and post-training administration of saline (1 mL/kg) and the other three groups received post-training administration of Mor (7.5 mg/kg, i.p.) and pre-test administration of different doses of Mor (2.5, 5, and 7 mg/kg, i.p.). Each value denotes the median ± quartile for each experimental group (n=8). ***$p < 0.001$ compared to the saline control group. B) The hippocampal miR-33 expression in Mor-StD memory. The miR-33 expression level was evaluated following memory assessment using quantitative real-time PCR (on randomly chosen three rats per each group) in five experimental groups as follow: intact (group 1, no treatment and no-testing), saline (group 2); the Mor-StD (2.5, 5 and 7.5 mg/kg) groups (groups 3, 4 and 4). Each value denotes the mean ±SEM for each experimental group (n=3). ***$p < 0.001$ compared to the saline control group. C) The hippocampal p-CREB/CREB expression levels in Mor-StD memory (the upper panel shows the immunoblotting profile). *$p < 0.05$ compared with the saline/saline group. $^{+}p < 0.05$ and $^{++}p < 0.01$ compared with the Mor 7.5/Mor 2.5 group, respectively.

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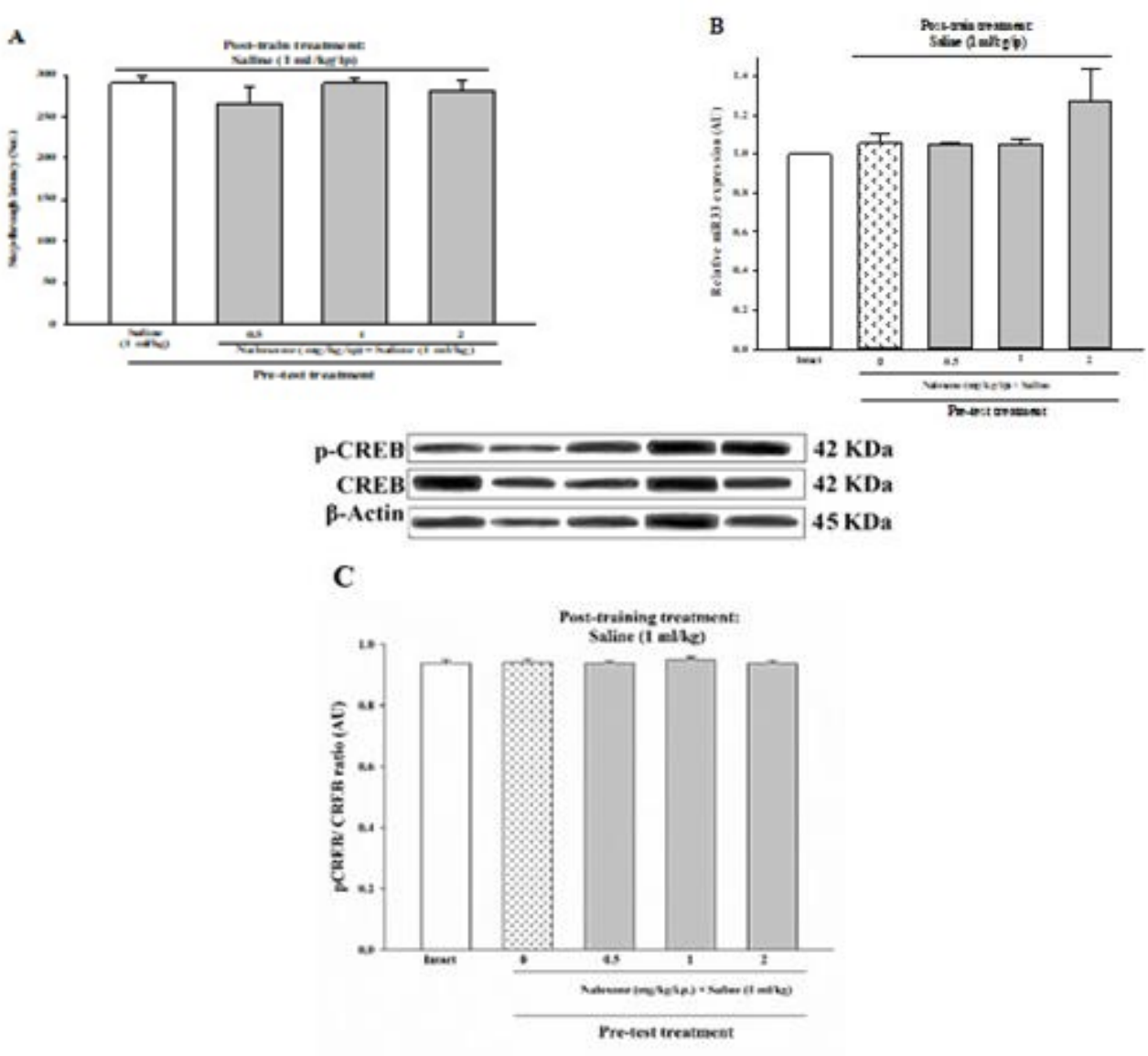

Fig.4. A. The effect of pre-test administration of naloxone on step-through latency in an inhibitory avoidance memory task. All the animals received post-training administration of saline (1 mL/kg/i.p.) and pre-test administration of either saline (1 mL/kg/ip) or different doses of naloxone (0.5, 1 and 2 mg/kg, i.p.). Each value denotes the median ± quartile for each experimental group (n=8). B) The hippocampal miR-33-5p expression levels in pre-test administration of naloxone. The miR-33 expression level was evaluated following memory assessment using quantitative real-time PCR (on randomly chosen three rats per each group) in five experimental groups as follow: intact (group 1, no treatment and no testing), saline (group 2); the co-administered naloxone (0.5, 1 and 2 mg/kg, i.p.) and saline groups (groups 3, 4 and 5). Each value denotes the median ± quartile for each experimental group (n=3). C) The hippocampal p-CREB/CREB expression levels in pre-test administration of naloxone (the upper panel shows the immunoblotting profile).

The hippocampal miR-33 and p-CREB/CREB expression levels following the pre-test administration of naloxone in rats trained under Mor administration

Figure 5A shows that the i.p. pre-test administration of naloxone (0.5, 1 and 2 mg/kg) prior to Mor (7.5 mg/kg) restored IA memory [H (3) = 21.73, P < 0.001 for all the groups]. The post-hoc analysis showed

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that naloxone administration at doses of 0.5 ($P < 0.001$), 1 ($P < 0.01$) and 2 mg/kg ($P < 0.05$) significantly increased step through latency compared with the saline control group. Maximal restorative effect was seen with naloxone at the dose of 2 mg/kg of administration.

Figure 5B shows the under-expression of hippocampal miR-33-5p in the rats receiving pre-test administration of naloxone prior to Mor compared with the saline control group (H (4) = 21.56 ($P = 0.001$ for all the groups).

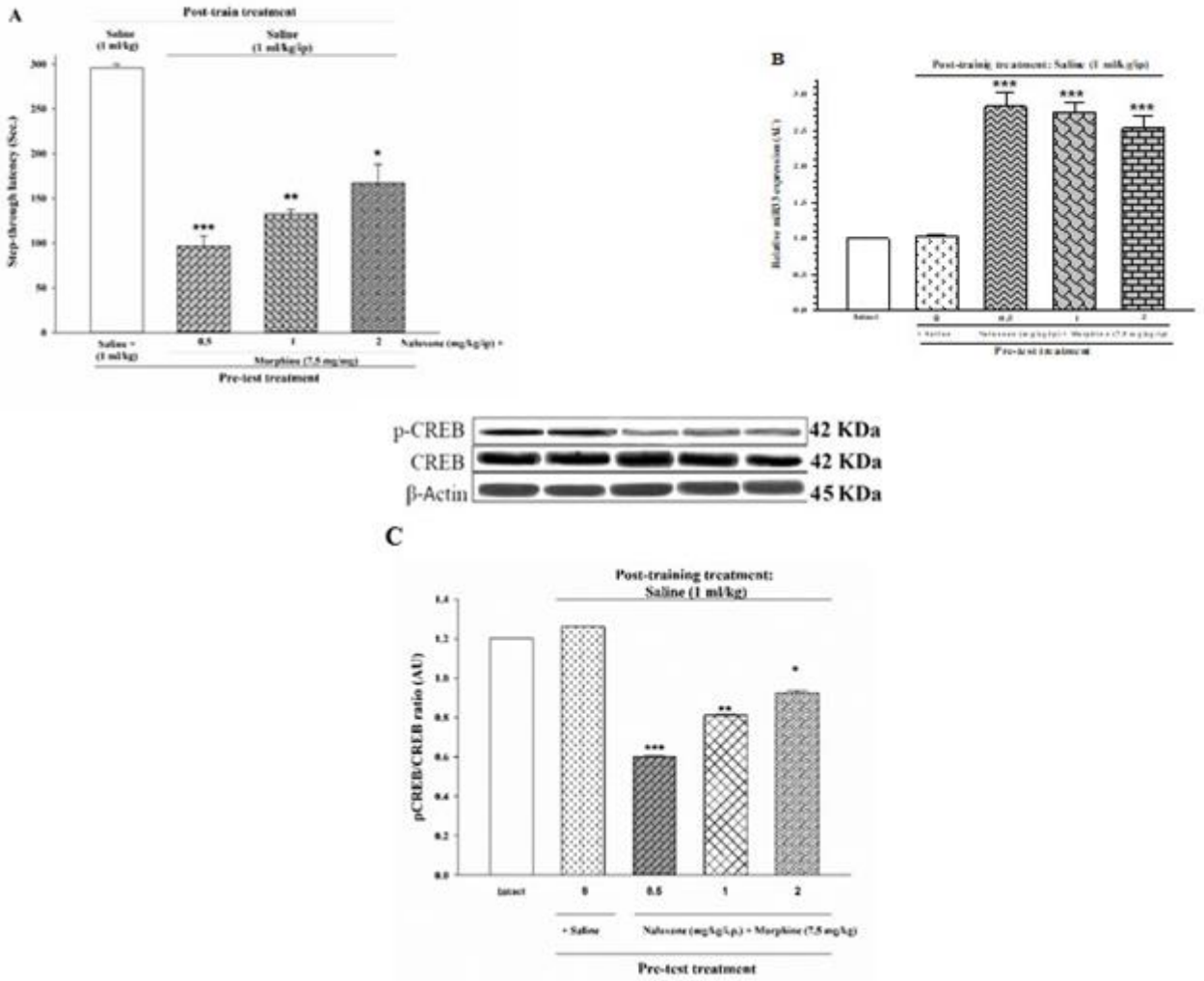

Fig.5. A. The effect of pre-test co-administration of naloxone and Mor on step-through latency in IA memory task. All the groups of animals received post-training administration of saline (1 mL/kg) and pre-test administration of either saline (1 mL/kg, i.p.) or co-administration of naloxone (0.5, 1 and 2 mg/kg, i.p.) and Mor (7.5 mg/kg, i.p.). Each value denotes the median ±quartile for each experimental group (n=8). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to the saline control group. B) The hippocampal miR-33 expression in pre-test co-administration of naloxone and Mor. The miR-33-5p expression levels was evaluated following memory assessment using quantitative real-time PCR (on randomly chosen three rats per each group) in five experimental groups as follow: intact (group 1, no treatment and no-testing), saline (group 2); the naloxone (0.5, 1 and 2 mg/kg, i.p.)/Mor mg/kg, i.p. groups (groups 3, 4 and 5). Each value represents the median ±quartile for each experimental group (n=3). ***$p < 0.001$ compared to the saline control group. C) The hippocampal p-CREB/CREB expression levels in pre-test co-administration of naloxone and Mor (the upper panel shows the immunoblotting profile). *p < 0.05, **p < 0.01, and ***p < 0.001, compared to the saline control group.

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Figure 5C shows the hippocampal p-CREB/CREB protein levels in pre-test naloxone and Mor co-administered group measured via western blot analysis. The densitometric analysis revealed significant changes in hippocampal p-CREB/CREB compared with the saline control group [F(4,10) = 9091.53, (P <0.001).

The hippocampal miR-33 and p-CREB/CREB expression levels following the pre-test administration of naloxone in rats under Mor-StD memory

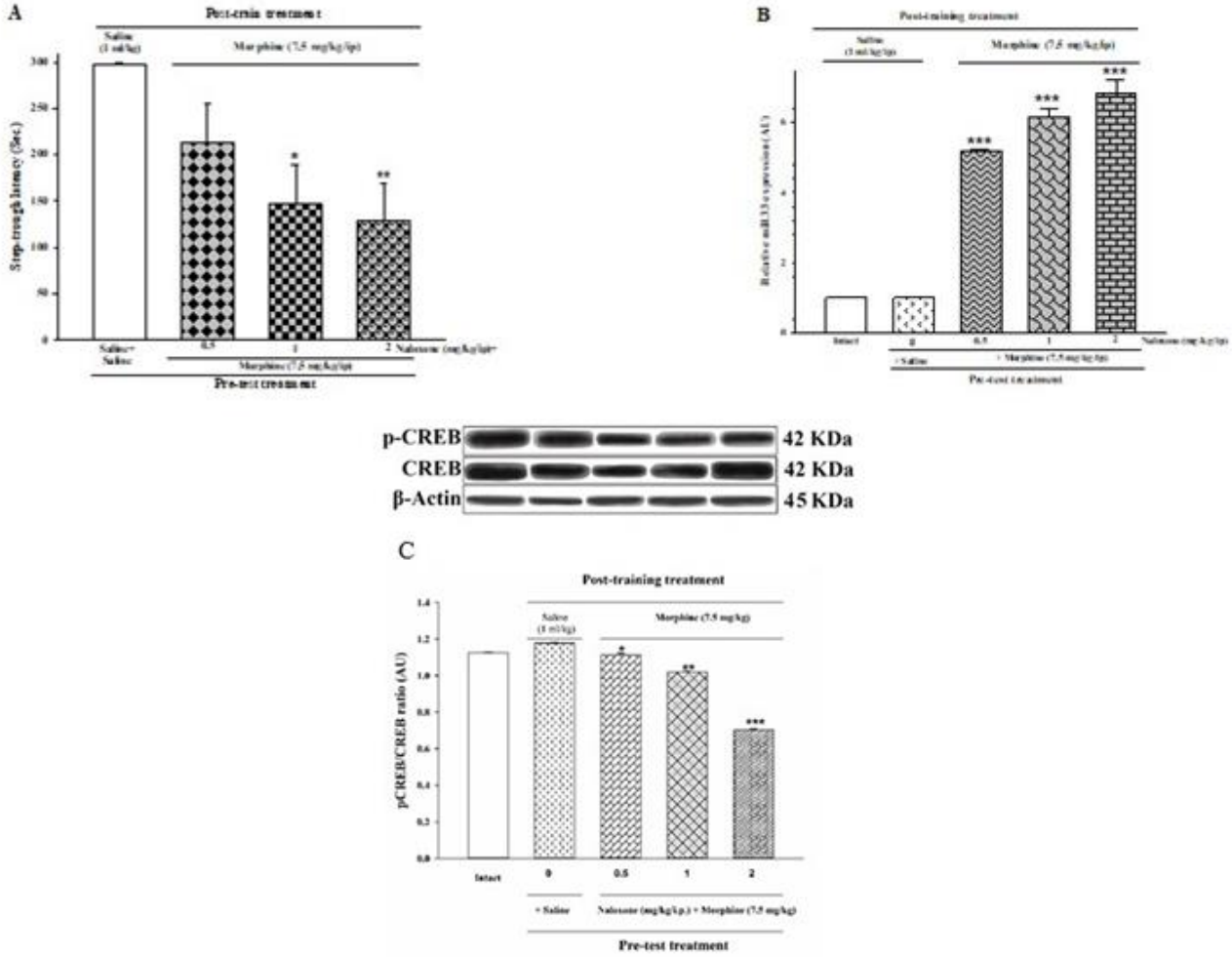

Fig.6. A. The effect of pre-test administration of naloxone on step-through latency in Mor-StD memory. All the rats received post-training administration of either saline (1 mL/kg, i.p.) or Mor (7.5 mg/kg, i.p.) and pre-test administration of either saline (1 mL/kg, i.p.) or co-administration of naloxone (0.5, 1 and 2 mg/kg, i.p.) and Mor (7.5 mg/kg, i.p.). Each value denotes the mean ±SEM for each experimental group (n=8). *p < 0.05, **p < 0.01 compared to the saline control group. B) The hippocampal miR-33-5p expression level in pre-test administration of naloxone in Mor-StD memory. The miR-33 expression level was evaluated following memory assessment using quantitative real-time PCR (on randomly chosen three rats per each group) in five experimental groups as follow: intact (group 1, no treatment and no-testing), saline (1 mL/kg, i.p.; group 2); the naloxone (0.5, 1 and 2 mg/kg i.p.)/Mor-StD groups (groups 3, 4 and 5). Each value denotes the median ± quartile for each experimental group (n=3). ***p < 0.001 compared to the saline control group. C) The hippocampal p-CREB/CREB expression levels in pre-test administration of naloxone in Mor-StD memory (the upper panel shows the immunoblotting profile). *p < 0.05, **p < 0.01 and ***p < 0.001 compared to the saline control group.

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Figure 6A shows that the i.p. pre-test administration of naloxone (0.5, 1 and 2 mg/kg) decreased Mor -StD memory (7.5 mg/kg) [$F(3, 28) = 4.47$, $P = 0.01$]. The post-hoc analysis showed that naloxone administration at doses of 1 ($P < 0.05$) and 2 mg/kg ($P < 0.01$) significantly decreased StD memory compared with the saline control group. Naloxone maximal effect was seen at the dose of 2 mg/kg of administration.

Figure 6B shows the over-expression of hippocampal miR-33-5p in Mor-StD memory compared with the saline control group ($H(4) = 25.78$ ($P < 0.001$ for all the groups)

Figure 6C shows the hippocampal p-CREB/CREB protein levels in the pre-test administration of naloxone under Mor-StD memory measured via western blot analysis. The densitometric analysis revealed significant changes in the hippocampal levels of *p*-CREB/CREB. The analysis indicated that IA memory impairment decreased the p-CREB/CREB levels compared with the saline control group [$F(4, 10) = 2486.32$, $P < 0.001$).

Discussion

In the current study, we investigated the alterations of miR-33-5p transcripts and CREB protein expression levels in Mor-StD memory. We also studied the miR-33-5p and CREB interaction with μ opioid receptors in the rat hippocampus**.** The experiments described above resulted in three main findings: Mor-StD memory induces miR-33-5p down-regulation and CREB up-regulation in the hippocampus, and this regulation in expression levels is mediated through μ opioid receptors.

In line with our earlier investigation revealing the impairment of IA memory following the post-training administration of Mor (16), the findings of our first series of behavioral experiments revealed the up-regulation of hippocampal miR-33-5p expression level following post-training administration of Mor. We also demonstrated that the re-administration of Mor on the test day reverses the memory impairment induced by post-training administration of Mor. This observation is best known as Mor-StD memory (16). The findings of our second series of experiments also revealed that the hippocampal miR-33-5p level was down-regulated following Mor-StD memory. Although a variety of Mor-regulated miRNAs has also been reported to affect learning, memory, and plasticity function by modulating the translation of μ opioid receptor mRNA (33, 36, 39), most such results have been derived from studies on the effect of Mor on dependence and addictive behaviors. As neuronal networks involving addictive behaviors correspond broadly to those that process learning and memory (47), addiction models could also be considered learning and memory models. Hence, StD memory has been implicated in the persistence of drug dependence, because being in a drug state can be a reasonable approach for optimal retrieving of information acquired while the animal is in a drug state (48).

Accordingly, there is growing evidence supporting the modulatory role of miRs in opioid physiology. Namely, the administration of Mor affected miRNA expression and thereby altered mRNA regulation in different paradigms of addiction-related learning behaviors (35, 36, 39, 49-51), and the chronic administration of Mor reduced the expression of miR-105 in the mPFC(52). Since in all of these instances, miRs regulate Mor-dependent gene expression and addictive functions, it may be concluded that changes in miR expression secondary to Mor administration may adversely impact learning and memory. In line with other research fields in which numerous miRs are found which could be involved in gene regulation, we

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assume that in opioid studies, including research fields of learning and memory and μ opioid receptor regulation, there may be more miRNAs awaiting to be discovered.

Generally, in line with our study, indicating the role of miRNAs in learning and memory, many studies have reported the up-regulation of some miRNAs in learning, memory, and plasticity impairments. For instance, the up-regulation of miR-134 in the hippocampus resulted in significant impairment in long-term contextual fear-conditioning (32), miR-132 down-regulation was believed to be involved in recognition memory and synaptic plasticity in the perirhinal cortex (53), both hippocampus-specific miR-324-5p and the ventral striatum-specific miR-24 were shown to be down-regulated in a spatial memory model (54), hippocampal miR-132 was significantly up-regulated in Morris water maze and the administration of miR-132 inhibitor into the hippocampus of adult mice was shown to lead to a significant increase in escape latency(55), and memory acquisition and stability were both shown to be increased by miR-980 inhibition (56). Surprisingly, in spite of compelling evidence supporting the involvement of miR in plasticity and learning memory functions (see above), to our knowledge, only Jovasevic's study explored the miR-33 function in StD memory, demonstrating the role of miR-33-5p overexpression in impaired StD-conditioned fear memory (5). Considering the scarcity of literature on the relationship between StD memory and miRs and the fact that we are still at the beginning of learning about the mechanisms of miR-related fear memories (21), the first novel feature of this study is that it included an analysis of miRs in Mor-StD mechanism.

The notion that fear-inducing memories can be StD (5), reminds us of some similar features between StD and conditioning models. StD memory has been demonstrated to be involved in the reinforcement of learning (57) and IA memory (16) under various psychoactive drugs in rodent models. The vital role of miRNA-induced functions in associative learning has been established using different paradigms signifying the specific contributions of distinct miRNAs in different memory phases. The role of different miRs in fear learning-related behaviors has been reported in several studies. For instance, the role of miR-134 in long-term contextual fear-conditioning (32), miR-182 in fear learning in the amygdala (58), miR-132 in fear conditioning in the hippocampus (59), miR-34a in the consolidation of fear in the amygdala (60), miR-92 in contextual fear conditioning in autism (61) and antagonizing miRs' overexpression in impaired fear memory have been reported (60, 61). Since these mRNAs were related to proteins involved in the formation of a broad range of memories, as Konopka's extrapolated, the majority of reports described the miRs as the inhibitors of memory-related genes (62).

The mentioned studies showed that miRNA down-regulation improves some types of learning and memory; however, there are inconsistencies in these studies. To point out, selective down-regulation of hippocampal miR-146a in mice resulted in severe memory impairment (9); miR-132/-212 double-knockout mice showed significant cognitive deficits in a broad range of memory models (63); inhibition of hippocampal miR-9-3p caused learning and memory impairment (64)**;** spatial learning induced the down-regulation of miR-335-5p (3), and the Lym-miR-137 was up-regulated following single-trial conditioning (65).

We suggest that these discrepancies can be best explained first by assuming that different memory paradigms are associated with the expression of distinct miRs. In addition, considering the results including the up-regulation of the pre-frontal cortex (PFC) miR-10 and hippocampal miR-1 and miR-10, the down-

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regulation of amygdala miR-1 and miR-26 in memory impairment by scopolamine (66), as well as the regional differences in the post-transcriptional mechanisms of spatial memory in the two brain regions (54), it may be assumed that regional-specific effects exist for miRNAs' expression under memory formation. Moreover, a spatial and temporal pattern of miRNA expression is also indicated in both physiological and pathological conditions (4).

Besides, it is of note that the function of miRs on downstream genes may have a role in the expression profile of the CREB gene. Among the proteins involved in learning and memory processes, CREB plays a well-established role in synaptic plasticity and memory (51). Fear conditioning response significantly relies on hippocampal cAMP-dependent protein kinase (PKA) mechanism (67-69). Since CREB needs to be activated to intervene in downstream genes transcription (70), phosphorylated CREB (p-CREB) may be associated with memory formation via a similar process (71). In several studies on memory, a reciprocal relationship has been established between some miRs and the expression of CREB (30). Reportedly, these miRs regulate the expression of memory-related proteins (such as CREB), and these proteins in turn control other molecular pathways involved in memory (72). It has also been suggested that the up-regulation of the p-CREB/CREB ratio in the hippocampus increases the neuronal excitability required for memory consolidation (73). In addition, the hippocampal p-CREB/CREB level was also up-regulated in drug StD memory (74). In clear agreement, our study showed the up-regulation of p-CREB/CREB in the Mor-StD group; however, it was decreased in the post-training Mor group. Therefore, the inclusion of the p-CREB/CREB level in the Mor-StD mechanism is another novelty of the current study.

Empirical evidence demonstrated that memory impairment induced by Mor at different stages of learning and memory is improved by the μ opioid receptor antagonist naloxone, signifying the participation of μ opioid receptors in the event(75-77). The functional interactions between miRNAs and the opioidergic system have been mentioned in previous studies (33-39). In the present study, while the administration of naloxone per se showed no major effect, the co-administration of naloxone and Mor, either prior to the test or in StD memory, reversed the process. Obviously, since the antagonizing μ opioid receptor-related miRs are thought to contribute to Mor mechanism through its receptor (33), it may be supposed that the interrelated function of miR-33-5p and CREB is mediated via the μ opioid receptor.

To summarize, our study postulated that miR-33-5p might regulate CREB and be altered by a combination of Mor-StD and naloxone administration acting through the μ receptors. To our surprise, our results did not show the up-regulation of miR-33-5p in intact and control groups. While some miRs are necessary for conditioning and extinction of fear response (58, 78), it may be assumed that miR-33-5p does not influence fear conditioning under normal conditions. On the basis of these findings, we conclude that hippocampal miR-33 expression establishes the foundation for Mor–StD memory, with low miR-33-5p levels facilitating the state dependency. By giving an explanation of possible molecular mechanisms linking miR-33-5p manipulation to StD memory, our study may provide a new direction for future treatment of Mor-induced learning and memory disorders by miR expression.

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