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Morphine induces the apoptosis of mouse hippocampal neurons HT-22 through upregulating miR-181-5p

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Abstract. – OBJECTIVE: To elucidate the role of morphine in inducing apoptosis of mouse hippocampal neurons HT-22 by upregulating microRNA-181-5p (miR-181-5p).

MATERIALS AND METHODS: After treatment of different doses of morphine, changes in proliferative ability, apoptosis, and expression levels of miR-181-5p and MAPK1 in HT-22 cells were assessed through a series of functional experiments. Regulatory effects of miR-181-5p on morphine-induced phenotype changes of HT-22 cells were examined. The interaction between miR-181-5p and MAPK1, and their involvement in morphine-induced neuron apoptosis were explored by Luciferase assay and rescue experiments, respectively.

RESULTS: Morphine treatment markedly attenuated viability and proliferative ability in HT-22 cells, while apoptotic rate increased. MiR-181-5p was upregulated and MAPK1 was downregulated in HT-22 cells by morphine induction. Knockdown of miR-181-5p enhanced viability and proliferative ability, as well as reduced apoptosis in morphine-induced HT-22 cells. MiR-181-5p could specifically bind MAPK1 and negatively regulate its expression level. Knockdown of MAPK1 was able to reverse the regulatory effects of miR-181-5p on morphine-induced phenotype changes of HT-22 cells.

CONCLUSIONS: Morphine induces apoptosis of hippocampal neurons HT-22 by upregulating miR-181-5p to suppress the level of MAPK1.

Key Words:
Morphine, MiR-181-5p, Hippocampal neurons.

Introduction

Morphine, a powerful anesthetic, has been widely applied in the clinic for a long time. However, the side effects of long-term use of morphine should not be ignored. Morphine brings a strong euphoria, which in turn causes morphine dependence. Long-term misuse of morphine can result in cognitive dysfunction and uncontrolled behaviors[1]. In recent years, experimental evidence has shown that morphine penetrates the nervous system through the circulation of the body, thus causing dysfunction, senescence, and even death of nerve cells[2].

The hippocampus is composed of arranged neurons, which is divided into CA1, CA2, CA3, and CA4 subfields. Current researches mainly focus on overall, brain section and cellular levels of hippocampus[3], involving biological functions of learning and memory abilities[4], and pathological lesions such as epilepsy[5] and cerebral hypoxic injury[6]. Morphine neurotoxicity has been well concerned. It is reported that conditioned place preference (CPP) develops after morphine administration in rats. The interfering peptide GluR2-3Y markedly inhibits CPP formation and reconstruction after CPP regression, indicating that GluR2-3Y effectively blocks endocytosis of AMPARs containing the GluR2 subunit[7]. Guzman et al[8] showed decreased GSH level in weaned and adult rats after morphine administration, suggesting that morphine intake can cause oxidative stress. Morphine causes damage to the nervous system with varying degrees depending on different intake approaches, doses, time points of morphine administration, and brain regions that morphine affect[9].

MicroRNA (miRNA) is a non-coding, endogenous RNA consisting of 19-22 nucleotides. It is widely distributed in eukaryotic cells and regulates gene expressions through complementary base pairing[10,11]. MiRNAs have been identified to be involved in hippocampal neuronal injury[12]. MiR-181 is associated with inflammation and metabolism. Ye et al[13] reported that miR-181 is

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effective in regulating microglial activation and inflammation-induced neurotoxicity in persistent neuroinflammation. In addition, miR-181 is a vital regulator in vasculitis[14]. In squamous cell carcinoma of lung, upregulated miR-181b-5p facilitates the malignant progression[15]. This study investigated whether morphine can induce apoptosis of hippocampal neurons HT-22 by upregulating miR-181-5p.

Materials and Methods

Cell Culture and Treatment

HT-22 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. On the third day, 5 μmol/L Cytarabine was applied and complete medium was replaced 24 h later. HT-22 cells passaged to the fifth to tenth generation were collected for experiments. They were induced with 1, 10 or 100 μM morphine (No. H21021995, Shenyang No.1 pharmaceutical factory), respectively, for 48 h.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs were subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). U6 was used as internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta Ct}$. Primer sequences were as follows. MiR-181-5p, forward: 5′-GCGAACATTCAACGCTGTCGGTGAGT-3', reverse: 5'-CAGTGCGTGTCGTGGAGT-3'; U6, forward: 5'-GCTTCGGCACATATACTAAAAT-3', reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; MAPK1, forward: 5'-TTTCCTCTGGATCAGCGTGT-3', reverse: 5'-TGAGATGTCGGGGCTTCTTT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 3×10^3 cells per well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells inoculated in a 96-well plate (6×10^3 cells/well) were incubated with EdU solution in the dark. 30 min later, they were dyed with Hoechst 33342 for another 30 min. EdU-positive ratio was calculated by the number of EdU-positive cells to that of Hoechst 33342-labeled nuclei.

TUNEL

Cells were subjected to 30-min fixation in 4% paraformaldehyde, followed by 30-min incubation in H_2O_2 to inactivate endogenous enzyme. Cells were immersed in 0.2% Triton X-100 solution for 5 min to enhance cell membrane permeability, and further incubated with deoxynucleotide terminal transferase (rTdT) at 37°C for 1 h. The cell nucleus was stained brown. Each section was randomly selected for 5 fields for observation. Apoptotic rate was finally calculated (magnification 100×).

Transfection

Cells were inoculated in 6-well plates with 4×10^5 cells/well. Until cells were grown to 80% confluence, transfection was conducted using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced 24 h later.

Luciferase Assay

Binding sequences in 3'UTR of miR-181-5p and MAPK1 were predicted in Diana (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=site/index), miRDB (http://www.mirdb.org/miRDB/), and TargetScan (http://www.targetscan.org/). Cells inoculated in a 24-well plate were co-transfected with MAPK1-WT/MAPK1-MUT and NC/miR-181-5p mimics, respectively. 48 h later, cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

Western Blot

Total protein was extracted from cells or tissues using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemi-

luminescence (ECL) and analyzed by Image-Pro Plus (Silver Springs, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

Morphine Induction Suppressed Viability and Induced Apoptosis of Hippocampal Neurons

HT-22 cells were induced with 1, 10 or 100 μM morphine, respectively, for 48 h. Viability in HT-22 cells was markedly reduced following 10 and 100 μM morphine induction (Figure 1A). EdU assay results showed dose-dependently decreased EdU-positive ratio in morphine-induced HT-22 cells (Figure 1B, 1C). In addition, apoptosis was markedly stimulated by morphine induction in hippocampal neurons (Figure 1D, 1E).

Morphine Induction Upregulated MiR-181-5p in Hippocampal Neurons

MiR-181-5p was dose-dependently upregulated by morphine induction in HT-22 cells (Figure 2A). Subsequently, we tested transfection efficacy of miR-181-5p inhibitor and mimics, both of which displayed a satisfactory efficacy (Figure 2B). Knockdown of miR-181-5p markedly enhanced EdU-positive ratio (Figure 2C, 2D) and viability (Figure 2E) in HT-22 cells, and overexpression of miR-181-5p achieved the opposite trends. TUNEL-positive rate increased in HT-22 cells overexpressing miR-181-5p (Figure 2F, 2G). Notably, EdU-positive ratio increased (Figure 2H, 2J) and TUNEL-positive rate decreased (Figure 2I, 2K) in 100 μM morphine-induced HT-22 cells transfected with miR-181-5p inhibitor.

MiR-181-5p Directly Bound MAPK1

A total of 439 shared target genes binding miR-181-5p were identified through analyzing Diana, miRDB, and TargetScan (Figure 3A). Protein level of MAPK1 was markedly downregulated after 100 μM morphine induction in HT-22 cells (Figure 3B). According to the predicted binding sequences in 3'UTR of MAPK1 and miR-181-5p, Luciferase vectors were constructed (Figure 3C). Overexpression of miR-181-5p markedly reduced Luciferase activity in wild-type MAPK1 vector

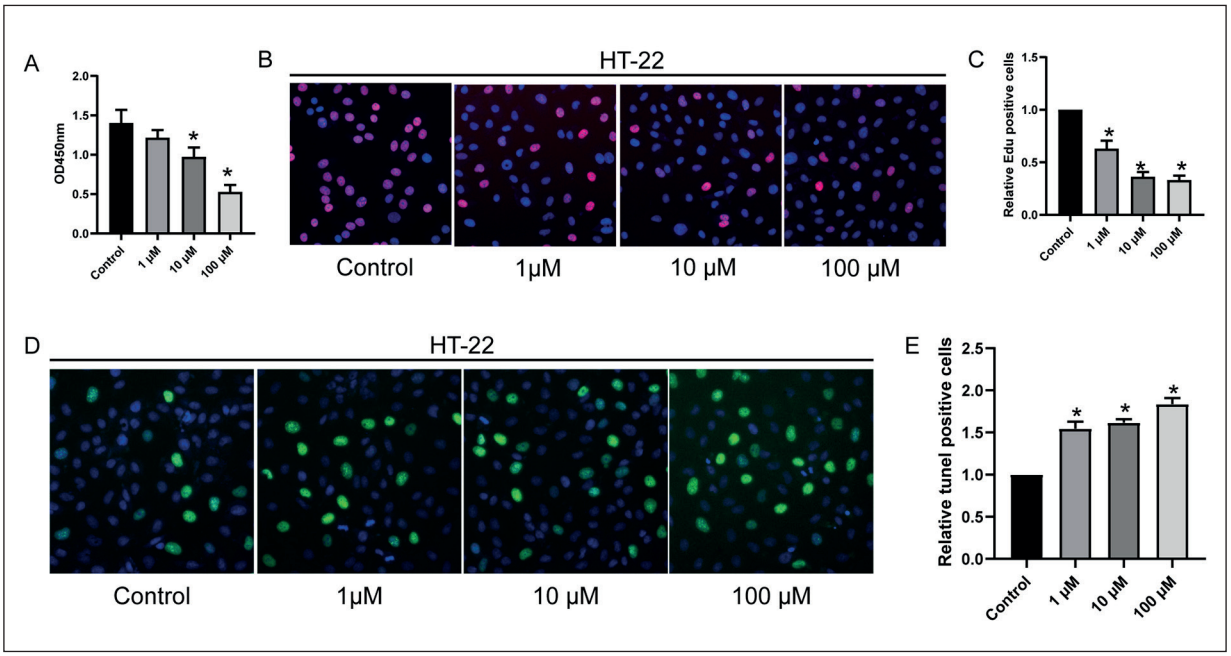

Figure 1. Morphine induction suppressed neuron viability and induced apoptosis. Viability (**A**), EdU-positive ratio (**B, C**) and TUNEL-positive rate (**D, E**) in HT-22 cells induced with 1, 10 or 100 μM morphine for 48 h (magnification: 200×).

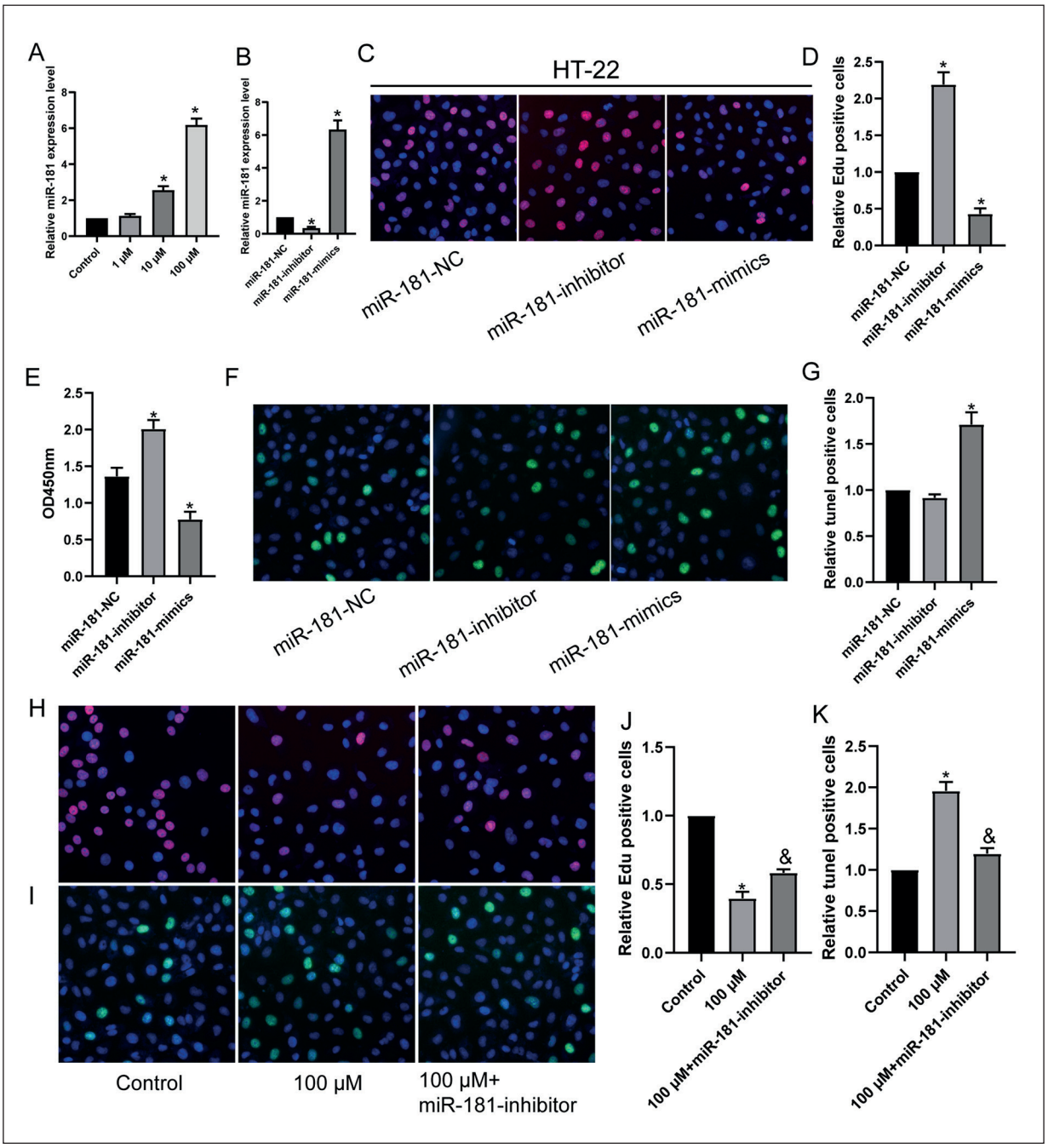

Figure 2. Morphine induction upregulated miR-181-5p in hippocampal neurons. **A,** MiR-181-5p level in HT-22 cells induced with 0, 1, 10 or 100 μM morphine for 48 h. **B,** Transfection efficacy of miR-181-5p inhibitor and mimics in HT-22 cells. **C, D,** EdU-positive ratio in HT-22 cells with overexpression of knockdown of miR-181-5p (magnification: 200×). **E,** Viability in HT-22 cells with overexpression of knockdown of miR-181-5p. **F, G,** TUNEL-positive rate in HT-22 cells with overexpression of knockdown of miR-181-5p (magnification: 200×). **H, J,** EdU-positive ratio in 100 μM morphine-induced HT-22 cells with overexpression of knockdown of miR-181-5p (magnification: 200×). **I, K,** TUNEL-positive rate in 100 μM morphine-induced HT-22 cells with overexpression of knockdown of miR-181-5p (magnification: 200×).

rather than mutant-type MAPK1, verifying the binding between miR-181-5p and MAPK1 (Figure 3D). In addition, both mRNA and protein levels of MAPK1 were negatively regulated by miR-181-5p (Figure 3E, 3F).

Knockdown of MAPK1 Reversed the

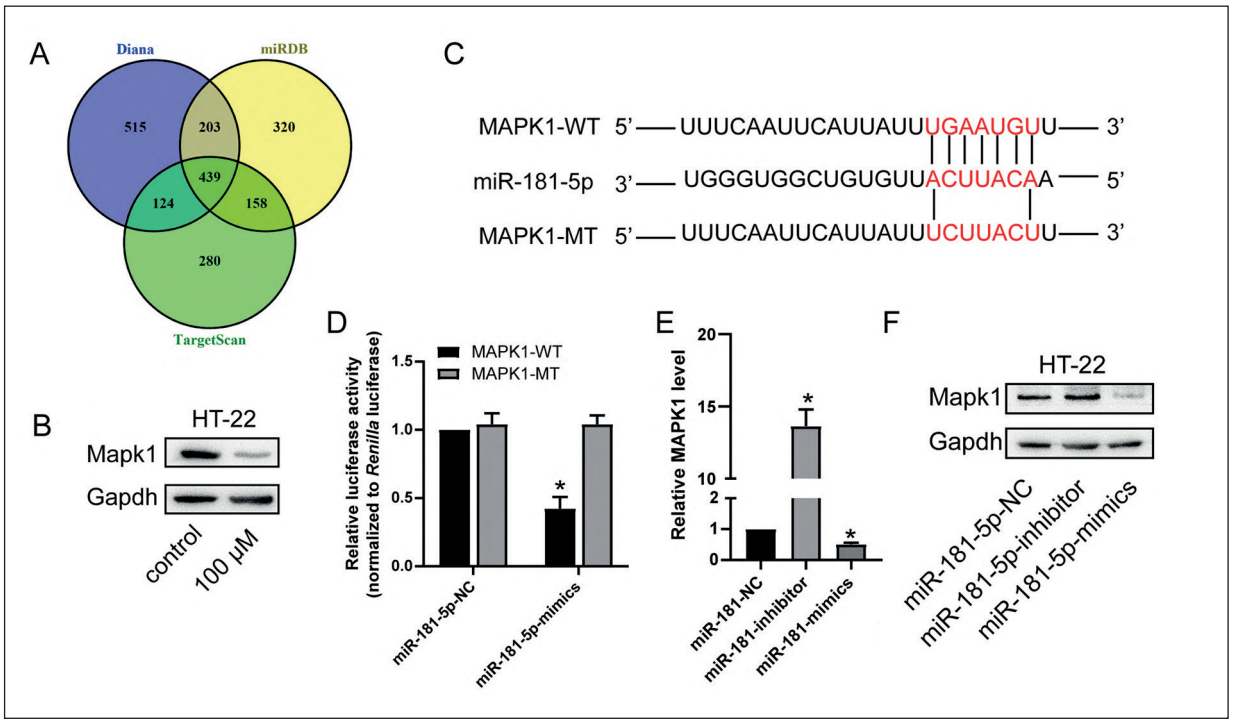

Figure 3. MiR-181-5p directly bound MAPK1. **A,** 439 shared target genes binding miR-181-5p were identified through analyzing Diana, miRDB, and TargetScan. **B,** Protein level of MAPK1 in HT-22 cells treated with 0 or 100 μM morphine for 48 h. **C,** Predicted binding sequences in 3'UTR of MAPK1 and miR-181-5p. **D,** Luciferase activity in HT-22 cells co-transfected with MAPK1-WT/MAPK1-MUT and NC/miR-181-5p mimics. **E-F,** The mRNA (**E**) and protein (**F**) levels of MAPK1 in HT-22 cells with overexpression of knockdown of miR-181-5p.

Regulatory Effects of MiR-181-5p on Hippocampal Neurons

To explore the involvement of MAPK1 in morphine-induced phenotype changes of HT-22 cells, si-MAPK1 was constructed (Figure 4A, 4B). Knockdown of miR-181-5p enhanced morphine-induced viability and EdU-positive ratio declines in HT-22 cells, which were partially reversed by knockdown of MAPK1 (Figure 4C). Furthermore, decreased apoptosis in HT-22 cells with miR-181-5p knockdown was abolished by transfection of si-MAPK1 (Figure 4D, 4E).

Discussion

With the increased demand for comfortable medical care, morphine has been widely used in clinical application. Morphine is able to induce neuronal apoptosis and activate apoptogenic factors[16,17]. Heidari et al[18] suggested that morphine enhances synaptic plasticity in rat hippocampal CA1 subfield and promotes synaptic growth. As a vital part of the central nervous system, hippocampus is responsible for learning, memory, and cognitive functions. Therefore, increased apoptosis of hippocampal neurons is the major reason of cognitive dysfunction[19]. Consistently, our findings uncovered that morphine induction inhibited proliferation and stimulated apoptosis of hippocampal neurons.

MiRNA is encoded by nuclear DNA in eukaryotic cells. Through acting on the 3' untranslated region (3'UTR), miRNAs downregulate gene expressions by inhibiting mRNA transcription or directly degrading them. Meanwhile, they are capable of regulating endogenous gene expressions by activating mRNA transcription[20]. MiRNAs are involved in neuroinflammation by mediating relevant pathways and inflammatory factors[21,22]. Wang et al[23] has shown that miR-146a is upregulated in the rat epilepsy model and hippocampus of patients with epilepsy.

In this paper, we found that miR-181-5p was upregulated in HT-22 cells following morphine induction. Knockdown of miR-181-5p enhanced viability and proliferative ability, as well as reduced apoptosis in morphine-induced HT-22 cells, while overexpression of miR-181-5p yielded the opposite results. In addition, knockdown of

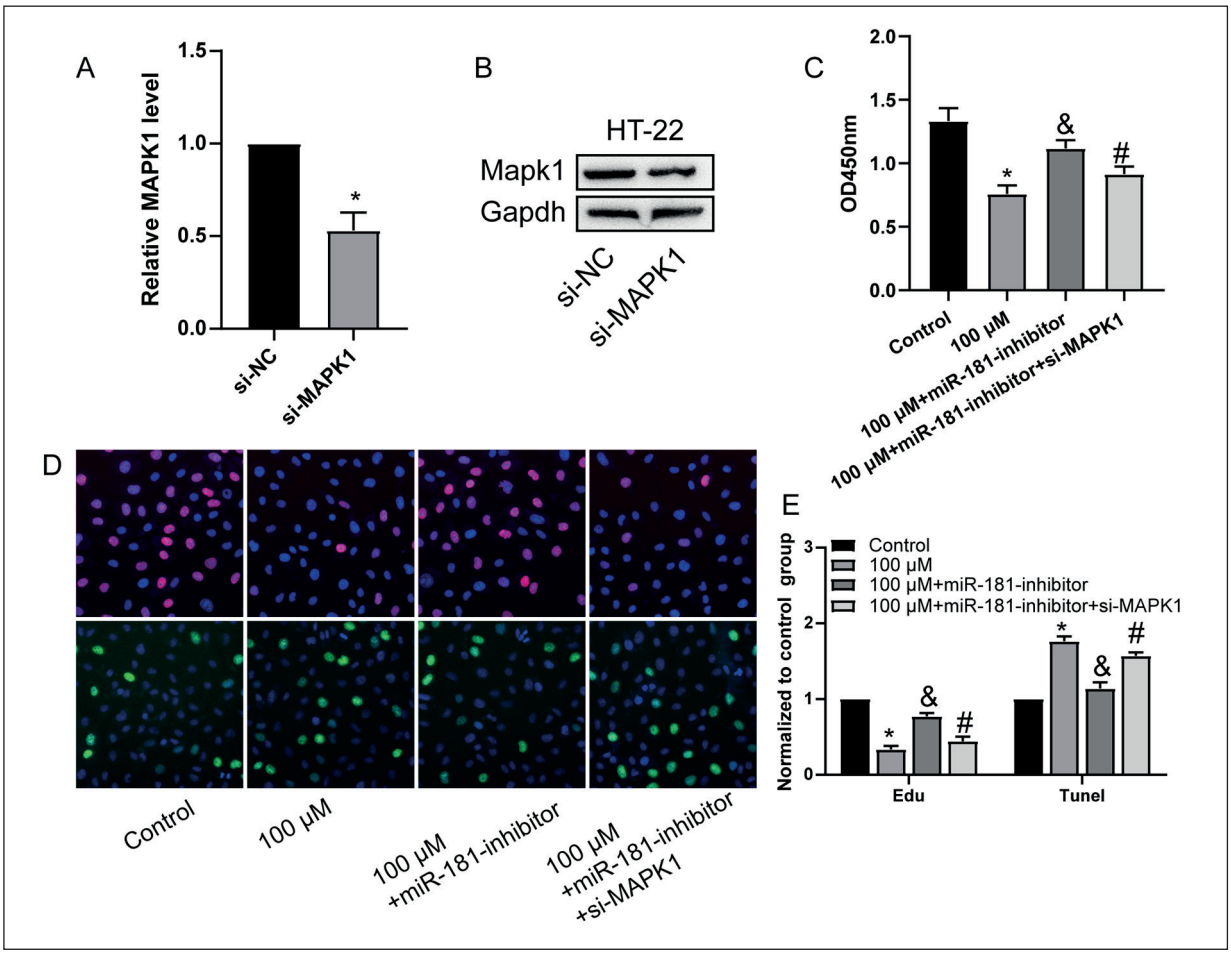

Figure 4. Knockdown of MAPK1 reversed the regulatory effects of miR-181-5p on hippocampal neurons. **A, B,** The mRNA (**A**) and protein (**B**) levels of MAPK1 in HT-22 cells transfected with si-NC or si-MAPK1. **C,** Viability in co-transfected HT-22 cells following 100 μM morphine induction for 48 h. **D, E,** EdU-positive ratio and TUNEL-positive rate in co-transfected HT-22 cells following 100 μM morphine induction for 48 h (magnification: 200×).

miR-181-5p could rescue the effects of morphine on inducing apoptosis and proliferation decline. These results showed that miR-181-5p may be a target of morphine. To further explore the mechanism of miR-181-5p in morphine-induced effect on hippocampal neurons, potential target of miR-181-5p was explored. As previously reported, miRNAs exert biological functions by targeting the 3'-UTR of target mRNAs[24,25].

In our study, we found that MAPK1 was identified to be a potential target gene of miR-181-5p. The subsequent Luciferase assay showed that miR-181-5p could bind the 3'-UTRT of MAPK1. Wang et al[26] showed that dexmedetomidine protects against oxygen-glucose deprivation/reoxygenation injury-induced apoptosis *via* the p38 MAPK/ERK signaling pathway. Besides, miR-423-5p contributes to malignant phenotypes and temozolomide chemoresistance in glioblastomas[27]. It is indicated that MAPK1 is involved in regulating apoptosis. In our research, we found that knockdown of MAPK1 was able to reverse the regulatory effects of miR-181-5p on morphine-induced phenotype changes of HT-22 cells.

In summary, this is the first time to demonstrate the role of miR-181-5p in morphine-induced apoptosis in HT-22 cell line. MiR-181-5p may be a therapeutic target in the future.

Conclusions

These results indicated that morphine induces apoptosis of hippocampal neurons HT-22 by upregulating miR-181-5p to suppress the level of MAPK1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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