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RESEARCH ARTICLE

Long non-coding RNA MEG3 attends to morphine-mediated autophagy of HT22 cells through modulating ERK pathway

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ABSTRACT

Context: Morphine is an alkaloid isolated from the poppy plants. The addiction of morphine is a very serious social issue. Some long non-coding RNAs (lncRNAs) have been proposed to engage in drug addiction.

Objective: Whether lncRNA maternally expressed gene 3 (MEG3) attended to morphine-mediated autophagy of mouse hippocampal neuronal HT22 cells was probed.

Materials and methods: HT22 cells were subjected to 10 μM morphine for 24 h. Cell autophagy was assessed by measuring LC3-II/LC3-I and Beclin-1 expression. qRT-PCR was carried out to measure MEG3 expression. SiRNA oligoribonucleotides targeting MEG3 (si-MEG3) was transfected to silence MEG3. The orexin1 receptor (OX1R), c-fos, p/t-ERK and p/t-PKC expressions were tested by western blotting. SCH772984 was used as an inhibitor of ERK pathway.

Results: Morphine elevated OX1R (2.92 times), c-fos (2.06 times), p/t-ERK (2.04 times) and p/t-PKC (2.4 times), Beclin-1 (3.2 times) and LC3-II/LC3-I (3.96 times) expression in HT22 cells. Moreover, followed by morphine exposure, the MEG3 expression was also elevated in HT22 cells (3.03 times). The silence of MEG3 lowered the Beclin-1 (1.85 times), LC3-II/LC3-I (2.12 times), c-fos (1.39 times) and p/t-ERK (1.44 times) expressions in morphine-treated HT22 cells. Inhibitor of ERK pathway SCH772984 further promoted the influence of MEG3 silence on morphine-caused Beclin-1 (1.97 times) and LC3-II/LC3-I (1.92 times) expressions decreases.

Conclusions: Up-regulation of MEG3 attended to the morphine-caused autophagy of HT22 cells might be through elevating c-fos expression and promoting ERK pathway activation. More experiments are also needed in the future to analyse the influence of other lncRNAs in drug addiction.

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Drug addiction; morphine; cell autophagy; LncRNA maternally expressed gene 3; orexin1 receptor

Introduction

Drug addiction is a chronic, relapsing neuropsychiatric disease manifested by uncontrolled drug-seeking and drug-taking (Yager et al. 2015; Koskela et al. 2017). It can be comprehended as a neuropathological process caused by drug stimulation, results in the construction of obsessive drug-seeking behaviour (Robbins and Everitt 2002). Due to the molecular mechanism related to drug addiction is very complex and mysterious (Kenny 2014; Sun et al. 2016), it believes that studying the biological mechanism of drug addiction is of great significance for the treatment and intervention of drug addiction.

Morphine is a very effective opiate analgesic drug widely used to ease both acute and chronic pain (Du 2018). However, morphine has become a commonly abused recreational drug due to its widespread availability and a high potential for addiction (Kim et al. 2016). Researching the pathogenesis of morphine addiction can provide new ideas and theoretical basis for elucidating the molecular mechanism of opioid addiction and clinical treatment. Earlier experimental studies have indicated that neuronal cell mitochondrial dysfunction happens in morphine addiction process, which in turn induces cell autophagy (Lixia et al. 2010; Cai et al. 2016; Su et al. 2017). Su et al. (2017) reported that compared to wild-type mice, mice specific knockout of autophagy-related 5 (Atg5) and Atg7 in dopaminergic neurons had lower sensitivities to constructing morphine-induced addictive behaviours. Orexin1 receptor (OX1R) is a G-protein-coupled receptor in central nervous system, which contributed to the establishment of morphine addiction (Baimel et al. 2015). *c-fos* is a proto-oncogene that expressed in central neurons after adverse stimulation (Dziopa et al. 2011). Studies in rats revealed that c-fos protein took part in the neurobiological responses to morphine in dopamine neurons (Dziopa et al. 2011). Besides, both ERK and PKC pathways have been found to participate in the morphine-mediated drug addiction (Liu et al. 2016; Pena et al. 2018).

It is well known that not all genes in cells transcribed into mRNAs, some of them also transcribed into long non-coding RNAs (lncRNAs) (Jarroux et al. 2017). As a class of regulatory RNAs, lncRNAs have been discovered to engage in the modulation of numerous biological processes (Quinn and Chang 2016). Maternally expressed gene 3 (MEG3) is an lncRNA that attends to the modulation of cell autophagy (Pawar et al. 2016).

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However, most of the current studies only focus on the roles of MEG3 in cancer cell autophagy (Xia et al. 2018; Xu et al. 2018). Whether MEG3 engages in the morphine-caused neuronal cell autophagy remains unclear.

Herein, mouse hippocampal neuronal HT22 cells were exposed to morphine stimulation. The OX1R, c-fos, p/t-ERK and p/t-PKC expressions in HT22 cells, along with the HT22 cell autophagy were tested. What's more, the MEG3 expression, as well as the influence of MEG3 up-regulation on morphine-caused HT22 cell autophagy were probed. We believe that the outcomes of our research will offer experimental basis for comprehending the influence of lncRNAs on morphine-mediated drug addiction.

Materials and methods

Cell culture and treatment

Mouse hippocampal neuronal HT22 cells were received from Procell Life Science & Technology Co., Ltd. (CL-0595, Wuhan, China) and grown in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG, D5796, Sigma-Aldrich, St. Louis, MO, USA) including 10% (v/v) foetal bovine serum (FBS, 164210-500, Procell Life Science & Technology Co., Ltd.) and 1% (v/v) penicillin–streptomycin solution (PB180120, Procell Life Science & Technology Co., Ltd.) at 37 °C with 5% CO_2.

Morphine solution was received from Sigma-Aldrich (M-005, St. Louis, MO, USA). HT22 cells were exposed to 10 μM morphine for 24 h in our experiments. SCH772984 was received from MedChem Express (HY-50846, NJ, USA). 50 μM SCH772984 was added into the culture medium of HT22 cells to inhibit the ERK pathway.

Cell transfection

SiRNA oligoribonucleotides targeting MEG3 (si-MEG3) and siRNA negative control (si-NC) were received from Invitrogen (Carlsbad, CA, USA) and transfected into HT22 cells with the help of Lipofectamine™ 3000 Transfection Reagent (L3000-008, Invitrogen).

Quantitative reverse transcription PCR (qRT-PCR)

The MEG3 expression in HT22 cells was tested by qRT-PCR. Total RNAs were separated by RNAiso Plus (9108, Takara Biomedical Technology, Beijing, China). The cDNA was synthesized with the help of PrimeScript cDNA Synthesis kit (6210, Takara Biomedical Technology). Then, the MEG3 expression was measured by TaqMan Non-coding RNA assay (4426961, Applied Biosystems, Foster City, CA, USA) and compared to β-actin expression. Results were calculated using $2^{-\triangle\triangle Ct}$ method (Ish-Shalom and Lichter 2010).

Western blotting

Total proteins were separated from HT22 cells with the help of Cell Lysis Buffers (635656, Takara Biomedical Technology) including Protease Inhibitor Cocktail-ProteoGuard (635672, Takara Biomedical Technology). The concentration of total proteins was tested by TaKaRa CBA Protein Assay kit (T9300A, Takara Biomedical Technology). Then, proteins were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane (03010040001, Roche, Basel, Switzerland). Anti-OX1R antibody (sc-166111), anti-c-fos antibody (sc-271243), anti-t-ERK antibody (sc-514302), anti-p-ERK antibody (sc-81492), anti-t-PKC antibody (sc-8393), anti-p-PKC antibody (sc-377565), anti-Beclin-1 antibody (sc-48341), anti-LC3 antibody (sc-271625) and anti-β-actin antibody (sc-47778) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PVDF membrane was incubated with primary antibodies for 12 h at 4 °C. Subsequently, the PVDF membrane was incubated with Goat anti-rabbit (or anti-mouse) IgG-HRP (sc-2004, sc-2005) for 1 h at 20–25 °C. After that, the PVDF membrane was placed into the Bio-Rad ChemiDoc™ XRS system (Bio-Rad Laboratories, Hercules, CA, USA) and coated with Pierce™ ECL Western Blotting (32109, Thermo Fisher Scientific, Waltham, MA, USA). The signals of proteins were recorded and the intensities of bands were calculated with the help of Image Lab™ software (Bio-Rad Laboratories).

Statistical analysis

Prism 6.0 software (GraphPad Software, San Diego, CA, USA) was carried out for statistical analysis. Results were represented as mean ± SD from three repeated experiments. p-Values were calculated via Student's t-test or ANOVA. Significant difference was set as $p < 0.05$.

Results

Morphine elevated OX1R, c-fos, p/t-ERK and p/t-PKC expressions in HT22 cells

Firstly, HT22 cells were exposed to 10 μM morphine for 24 h. The OX1R, c-fos, p/t-ERK and p/t-PKC expression were tested. Data in Figure 1(A) showed that 10 μM morphine exposure dramatically elevated the OX1R protein level in HT22 cells ($p < 0.01$). The c-fos protein level in HT22 cells was also increased after 10 μM morphine exposure (Figure 1(B), $p < 0.01$). Moreover, 10 μM morphine exposure notably enhanced the p/t-ERK and p/t-PKC expression rates in HT22 cells (Figure 1(C), $p < 0.05$ or $p < 0.01$). These outcomes illustrated that morphine could elevate OX1R and c-fos expression and activate ERK and PKC pathways in HT22 cells.

Morphine promoted HT22 cell autophagy

Then, followed by 10 μM morphine exposure, the Beclin-1 and LC3-II/LC3-I expression in HT22 cells were tested to assess cell autophagy. As displayed in Figure 2, 10 μM morphine exposure significantly elevated the Beclin-1 protein level and LC3-II/LC3-I expression rate in HT22 cells ($p < 0.01$), which illustrated that morphine could promote HT22 cell autophagy.

Morphine elevated MEG3 expression in HT22 cells

The MEG3 expression in HT22 cells after 10 μM morphine exposure was also measured. Result in Figure 3 presented that 10 μM morphine exposure noticeably enhanced the MEG3 expression in HT22 cells ($p < 0.01$), which implied that up-regulation of MEG3 caused by morphine might be associated with the influences of morphine on HT22 cell autophagy, as well as elevation of OX1R, c-fos, p/t-ERK and p/t-PKC expression.

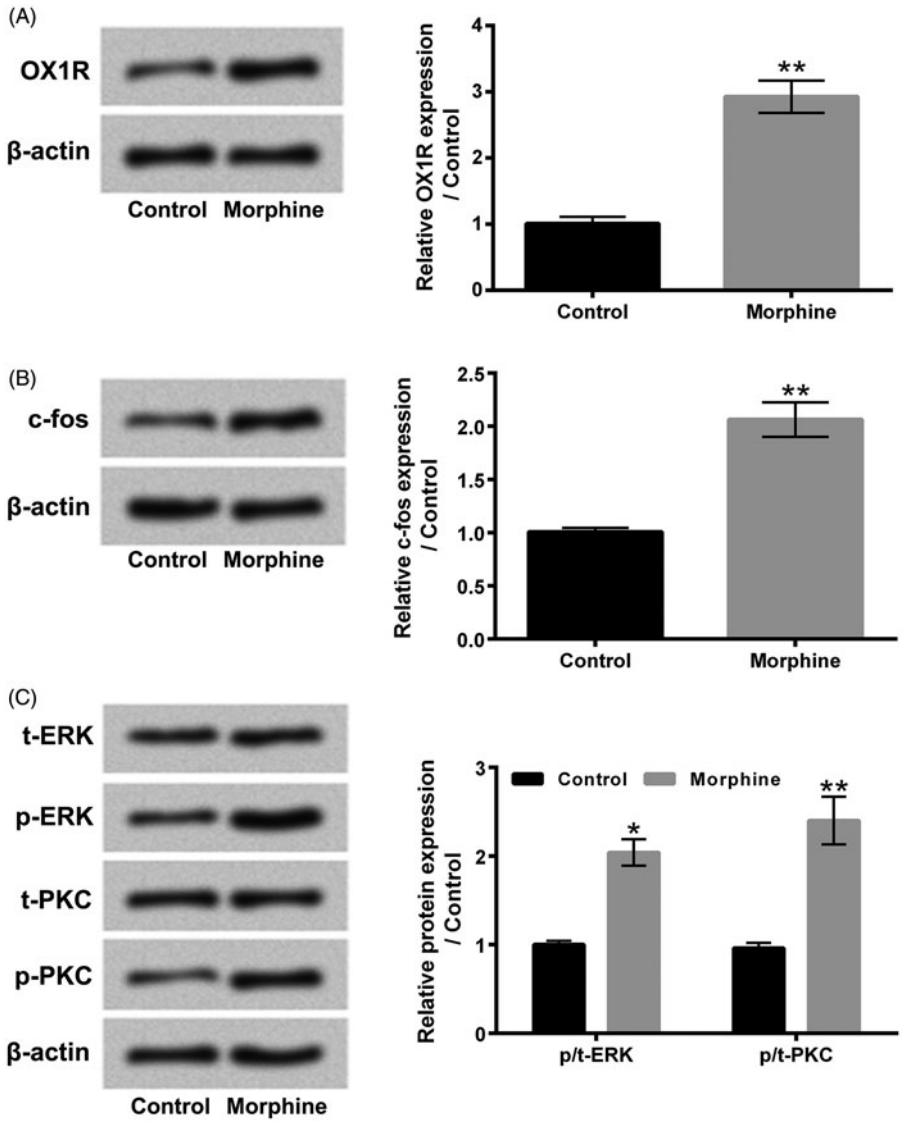

Figure 1. Morphine elevated OX1R and c-fos expressions and activated ERK and PKC pathways in HT22 cells. HT22 cells were exposed to 10 μM morphine for 24 h. The protein levels of OX1R (A), c-fos (B), t-ERK, p-ERK, t-PKC and p-PKC (C) were tested by western blotting. OX1R: Orexin1 receptor. $^{*}p<0.05$, $^{**}p<0.01$.

MEG3 attended to the influence of morphine on HT22 cell autophagy

In order to probe the influence of MEG3 up-regulation on morphine-caused HT22 cell autophagy, si-MEG3 was transfected into HT22 cells. Figure 4(A) showed that the MEG3 expression in HT22 cells was dramatically declined by si-MEG3 transfection ($p<0.01$). By contract with morphine + si-NC group, the Beclin-1 protein level and LC3-II/LC3-I expression rate in HT22 cells were both lowered in morphine + si-MEG3 group (Figure 4(B), $p<0.05$ or $p<0.01$). These outcomes illustrated that up-regulation of MEG3 attended to the influence of morphine on HT22 cell autophagy.

MEG3 took part in the influences of morphine on c-fos expression and ERK pathway

Further experiments were carried out to investigate whether MEG3 took part in the morphine-caused elevations of OX1R, c-fos, p/t-ERK and p/t-PKC expression in HT22 cells. Data in Figure 5(A) presented that there was no significant difference of OX1R protein level in morphine + si-NC group and morphine + si-MEG3 group. Figure 5(B) displayed that morphine-caused elevation of c-fos expression was notably weakened by si-MEG3 transfection ($p<0.05$). Besides, morphine-caused enhancement of p/t-ERK expression in HT22 cells was remarkably mitigated by si-MEG3 transfection (Figure 5(C), $p<0.05$), while the p/t-PKC expression in HT22 cells was not changed between transfection with si-NC and transfection with si-MEG3. These outcomes illustrated that up-regulation of MEG3 also took part in the influences of morphine on c-fos and p/t-ERK expressions in HT22 cells.

The silence of MEG3 suppressed HT22 cell autophagy through inactivating ERK pathway

Finally, the inhibitor of ERK pathway SCH772984 was added into experiment to analyse the roles of ERK pathway in morphine-caused HT22 cell autophagy. Figure 6 showed that 50 μM SCH772984 incubation significantly declined the Beclin-1 protein level and LC3-II/LC3-I expression rate in morphine-treated HT22 cells ($p<0.01$), which illustrated that ERK pathway played important role in HT22 cell autophagy. In addition, by contrast

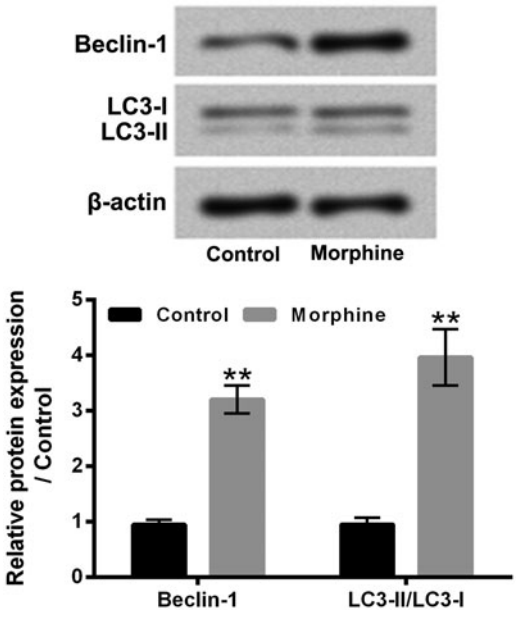

Figure 2. Morphine promoted HT22 cell autophagy. HT22 cells were exposed to 10 μM morphine for 24 h. The Beclin-1 and LC3 protein levels were tested by western blotting. $^{**}p < 0.01$.

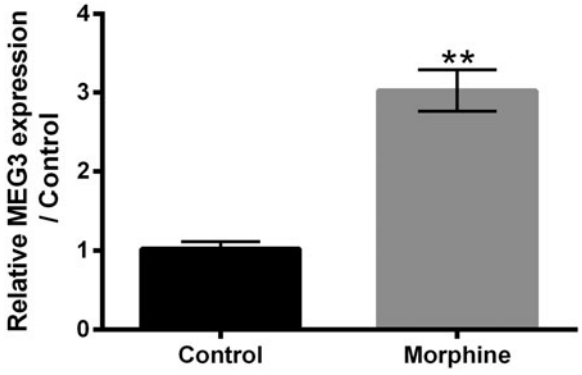

Figure 3. Morphine enhanced MEG3 expression in HT22 cells. HT22 cells were exposed to 10 μM morphine for 24 h. The MEG3 expression was tested by qRT-PCR. MEG3: LncRNA maternally expressed gene 3. $^{**}p < 0.01$.

with morphine + si-MEG3 group, the Beclin-1 protein level and LC3-II/LC3-I expression rate in HT22 cells were further declined in morphine + si-MEG3 group + SCH772984 group ($p < 0.05$), which illustrated that up-regulation of MEG3 took part in the influence of morphine on HT22 cell autophagy might be achieved by promoting ERK pathway activation.

Discussion

Some lncRNAs have been found to engage in neuroplasticity via modulating protein dynamics in the synapse (Sartor et al. 2012; Bohnsack et al. 2019). Given that addiction can be regarded as a process of synaptic plasticity (Luscher and Malenka 2011), it is worthy believing that identifying the roles of lncRNAs in this process will provide novel insights for addiction research. In the current research, we further confirmed the influences of morphine on OX1R, c-fos, p/t-ERK and p/t-PKC expressions in mouse hippocampal neuronal HT22 cells, as well as HT22 cell autophagy. We proposed that morphine-stimulated HT22 cells could be utilized as an *in vitro* cell model of drug addiction.

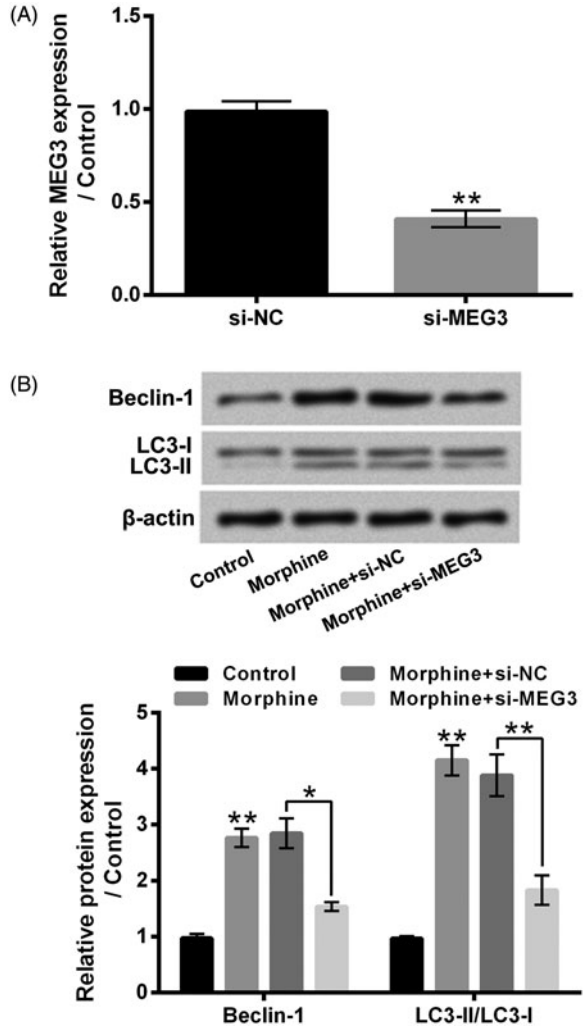

Figure 4. MEG3 took part in the influence of morphine on HT22 cell autophagy. (A) Followed by si-NC or si-MEG3 transfection, the MEG3 expression in HT22 cells was tested by qRT-PCR. (B) Followed by 10 μM morphine treatment and/or si-MEG3 transfection, the Beclin-1 and LC3 protein levels in HT22 cells were tested by western blotting. MEG3: LncRNA maternally expressed gene 3. $^{*}p < 0.05$, $^{**}p < 0.01$.

More importantly, we discovered that morphine elevated the MEG3 expression in HT22 cells. Up-regulation of MEG3 attended to the influence of morphine on HT22 cell autophagy might be achieved by promoting ERK pathway activation.

Morphine is one of the most abundant alkaloids isolated from the poppy plants (Du 2018). Although morphine has strong analgesic activity, the addiction of morphine also is a very serious social issue (Kim et al. 2016; Antolak et al. 2017). Many efforts have been made to explore the molecular basis of morphine addiction and some molecules and cellular biological processes have been found. OX1R, c-fos, ERK and PKC pathways are all discovered to join in morphine addiction. For example, Erami et al. (2012) reported that suppression of OX1R decreased the progress of morphine tolerance and physical dependence in rats. Siahposht-Khachaki et al. (2018) indicated that exposure to morphine produced an elevation of c-fos expression in nucleus accumbens, prefrontal cortex and hippocampus of Wistar rats. In addition, Liu et al. (2016) illustrated that ERK pathway contributed to the maintenance of morphine-mediated memory in hippocampus. Pena et al. (2018) pointed out that followed by morphine stimulation, PKC pathway in mouse neuroblastoma cells was activated. As an intracellular protein self-degradation

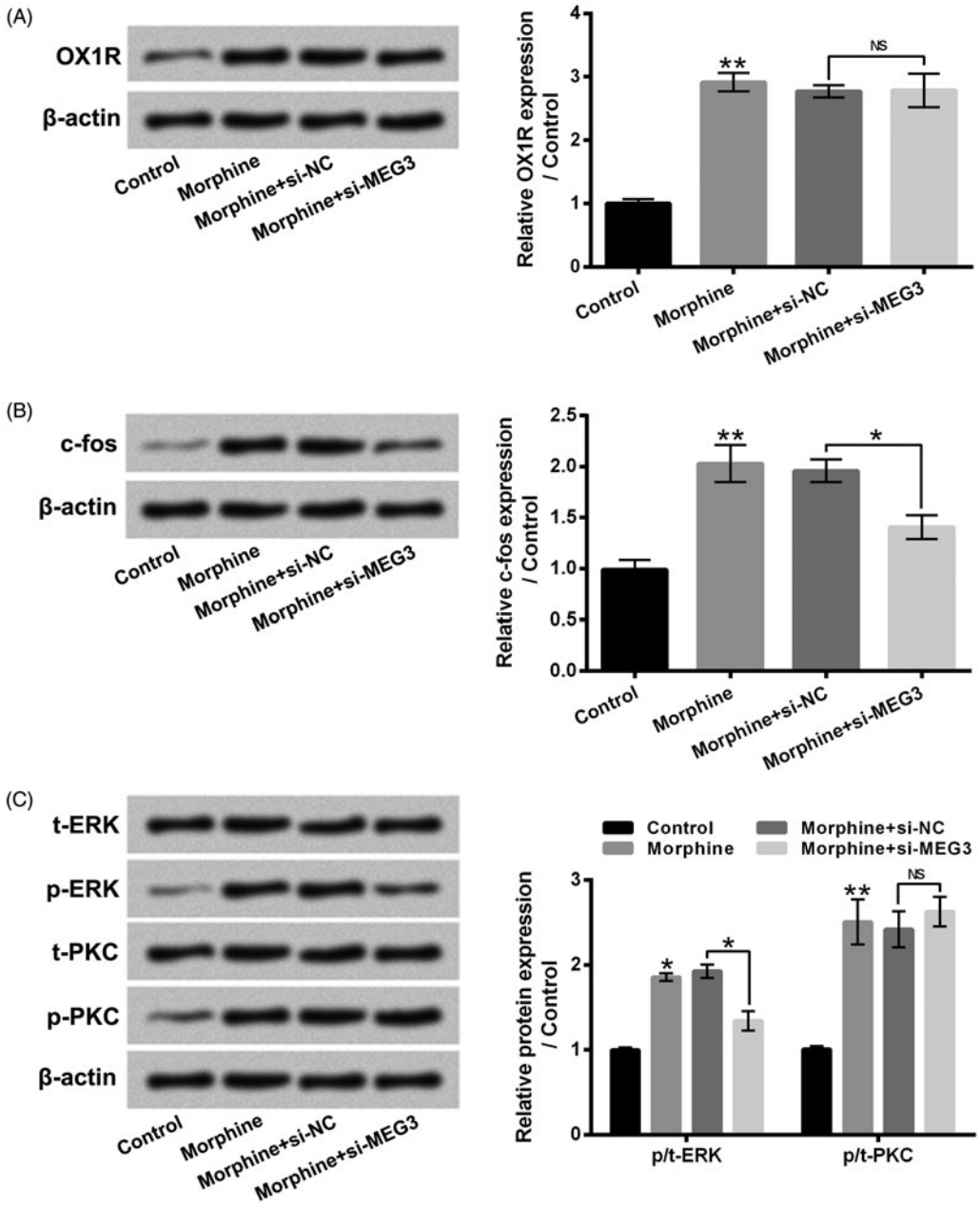

Figure 5. MEG3 took part in the influences of morphine on c-fos expression and ERK pathway. Followed by 10 μM morphine treatment and/or si-MEG3 transfection, the protein levels of OX1R (A), c-fos (B), t-ERK, p-ERK, t-PKC and p-PKC (C) in HT22 cells were tested by western blotting. OX1R: Orexin1 receptor; MEG3: LncRNA maternally expressed gene 3. NS: No significant. $^{*}p<0.05$, $^{**}p<0.01$.

process capable of upholding cellular metabolism, cell autophagy also has been demonstrated to take part in morphine-mediated drug addiction (Lixia et al. 2010; Su et al. 2017). In the cell autophagy process, the LC3-I binds to phosphatidylethanolamine to form LC3-II, which joins in the formation of autophagosome membranes (Pugsley 2017). Beclin-1, also known as Atg6, is a key regulator of cell autophagy (Booth et al. 2014). Herein, we found that the OX1R, c-fos, p/t-ERK and p/t-PKC expressions in HT22 cells were all increased after morphine stimulation. The LC3-II/LC3-I and Beclin-1 expressions were also elevated in HT22 cells after morphine stimulation. Therefore, we proposed that morphine-stimulated HT22 cells could be utilized as an *in vitro* cell model of morphine addiction to explore the influence of MEG3 on morphine addiction.

Previous studies reported that the expression of several lncRNAs were changed in the brains of addicted humans (Michelhaugh et al. 2011; Feng and Nestler 2013). What's more, some lncRNAs have been demonstrated to contribute to neuroplasticity (Sartor et al. 2012; Bohnsack et al. 2019). These findings provide great motivation for studying the influences of lncRNAs on drug addiction. MEG3 has been proved to take part in the modulation of cell autophagy, especially in brain cells (Xu et al. 2018; Zhao et al. 2018). Su et al. (2019) indicated that MEG3 might participate in the methamphetamine-mediated addiction in mice. In this study, we discovered that morphine exposure elevated the MEG3 expression in HT22 cells. Moreover, the silence of MEG3 relieved the morphine-stimulated HT22 cell autophagy through declining the Beclin-1 and LC3-II/LC3-I expressions. Besides, we also discovered that the silence of MEG3 lowered the c-fos and p/t-ERK expressions in morphine-treated HT22 cells, but had no significant influence on OX1R and p/t-PKC expressions. Inhibitor of ERK pathway SCH772984

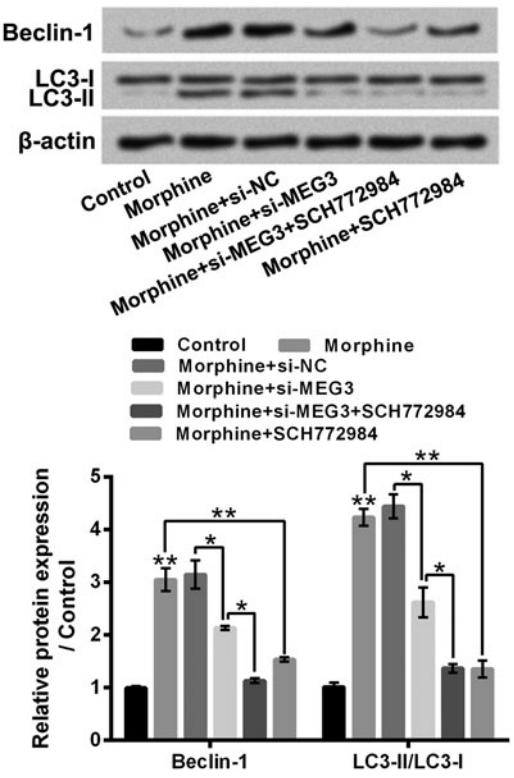

Figure 6. The silence of MEG3 suppressed HT22 cell autophagy through inactivating ERK pathway. Followed by 10 μM morphine treatment and/or si-MEG3 transfection or SCH772984 incubation, the Beclin-1 and LC3 protein levels in HT22 cells were tested by western blotting. MEG3: LncRNA maternally expressed gene 3. $^{*}p < 0.05$, $^{**}p < 0.01$.

further promoted the influence of MEG3 silence on morphine-caused HT22 cell autophagy. Considering that c-fos and ERK pathway also have been found to join in the regulation of cell autophagy (Wu et al. 2018; Fung and Liu 2019), the findings of our research suggested that up-regulation of MEG3 attended to the influence of morphine on HT22 cell autophagy might be achieved by elevating c-fos expression and promoting ERK pathway activation.

Conclusions

Taken together, the influence of MEG3 on morphine-mediated mouse hippocampal neuronal HT22 cell autophagy was investigated in this research. Up-regulation of MEG3 attended to the morphine-caused autophagy of HT22 cells might be through elevating c-fos expression and promoting ERK pathway activation. We propose that not only one lncRNA, MEG3, take part in the morphine-mediated drug addiction. More experiments are also needed in the future to analyse the influence of other lncRNAs in drug addiction.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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