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Inhibition of c-Jun NH_2-terminal kinase stimulates mu opioid receptor expression via p38 MAPK-mediated nuclear NF-κB activation in neuronal and non-neuronal cells

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ABSTRACT

Despite its potential side effects of addiction, tolerance and withdrawal symptoms, morphine is widely used for reducing moderate and severe pain. Previous studies have shown that the analgesic effect of morphine depends on mu opioid receptor (MOR) expression levels, but the regulatory mechanism of MOR is not yet fully understood. Several *in vivo* and *in vitro* studies have shown that the c-Jun NH_2-terminal kinase (JNK) pathway is closely associated with neuropathic hyperalgesia, which closely resembles the neuroplastic changes observed with morphine antinociceptive tolerance. In this study, we show that inhibition of JNK by SP600125, its inhibitory peptide, or JNK-1 siRNA induced MOR at both mRNA and protein levels in neuronal cells. This increase in MOR expression was reversed by inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway, but not by inhibition of the mitogen-activated protein/extracellular signal-regulated kinase (MEK) pathway. Further experiments using cell signaling inhibitors showed that MOR upregulation by JNK inhibition involved nuclear factor-kappa B (NF-κB). The p38 MAPK dependent phosphorylation of p65 NF-κB subunit in the nucleus was increased by SP600125 treatment. We also observed by chromatin immunoprecipitation (ChIP) analysis that JNK inhibition led to increased bindings of CBP and histone-3 dimethyl K4, and decreased bindings of HDAC-2, MeCP2, and histone-3 trimethyl K9 to the MOR promoter indicating a transcriptional regulation of MOR by JNK inhibition. All these results suggest a regulatory role of the p38 MAPK and NF-κB pathways in MOR gene expression and aid to our better understanding of the MOR gene regulation.

1. Introduction

Opiate drugs exert their effects through three major types of opioid receptors: mu, delta and kappa [1]. These receptors in the brain are activated by endogenous peptides such as enkephalins, dynorphins and endorphins, which are released by neurons. Opioid receptors can also be activated by exogenous alkaloid opiates, the prototype of which is morphine, which remains the most valuable painkiller in contemporary medicine. Although the three opioid receptor genes are highly homologous in their coding exons, their amino-terminal and caboxy-terminal ends are diverse, and these regions govern the unique ligand-binding and signal transduction properties of each receptor [2]. Experiments with transgenic and knockout mice have clearly demonstrated the role of mu opioid receptor (MOR) in morphine's pharmacological effects, including analgesia, physical dependence, and tolerance [3–5].

The mouse gene that encodes MOR (*oprm1*) is located on chromosome 10 and covers a length of 250 kilobases (kb). MOR transcription can start from the distal promoter (DP) or the proximal promoter (PP) [6]. PP transcripts are preferentially used in most tissues and cultured cells and account for most MOR activity. The DP and PP are both TATA-less and GC-rich, and they contain binding sites for multiple

Abbreviations: MOR, mu opioid receptor; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; NF-κB, nuclear factor-kappaB; DP, distal promoter; PP, proximal promoter; Oct-1, octamer-1; SOX, sry-related high-mobility-group box; PCBP, poly(C) binding protein; SP1, specificity protein 1; AP2, activator protein 2; CREB, cAMP response element binding protein; SAPK, stress-activated protein kinase; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; DRG, dorsal root ganglion; SP600125, anthra(1,9-cd)pyrazol-6(2H)-one; QNZ, 6-amino-4-(4-henoxyphenylethylamino)quinazoline; SB203580, 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole; LY294002, 2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-amino phenylthio]butadiene; PDTC, pyrrolidine dithiocarbonate; PI3-K, phosphoinositide 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; MeCP2, methyl-CpG-binding protein 2; H3dmK4, histone 3-dimethyl lysine 4; H3me3K9, histone 3-trimethyl lysine 9; HDAC, histone deacetylase; aceH, acetyl-histone; Brg1, Brm-related gene 1; NRSF, neuron-restrictive silencing factor; Dnmt1, DNA methyltransferase

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regulatory elements such as Oct-1 [7], IL-4 response element [8], SOX [9,10], PU.1 [11,12], PCBP [13], SP1 [14], AP2 [14–17], NF-κB [18], and CREB [19]. In addition to the DP and PP, the mouse gene also uses a TATA-containing promoter known as E11, located more than 10 kb upstream of the translation start site [1]. Several studies have shown that transcription of the MOR gene can be regulated by fentanyl, morphine, interleukin-1, lipopolysaccharide, protein synthesis inhibitors (cycloheximide, anisomycin and puromycin), dopaminergic drugs, histone deacetylase inhibitors, and demethylating agents [19,20]. However, the molecular events that lead to changes in MOR gene expression have just begun to be explored.

JNK (c-Jun NH_2-terminal kinase), a serine threonine protein kinase, is a member of the mitogen-activated protein kinase (MAPK) family and includes three genes, *jnk1, jnk2,* and *jnk3*. JNKs are a type of stress-activated protein kinase (SAPK), and can be activated by various cellular stresses such as heat shock, DNA damage, a rise in intracellular reactive oxygen species and calcium influx, neurodegeneration, and proinflammatory cytokines (such as tumor necrosis factor-alpha[TNF-α], interleukin-6 [IL-6], interleukin-1beta [IL-1β], interferon-gamma [IFN-γ]) [21]. JNKs have been implicated in processes such as oncogenic transformation, apoptosis, and neurodegeneration [22]. Of the three JNK members, JNK-3 is predominantly found in the brain and has different functions than JNK-1 and JNK-2. SP600125 (SP) is an anthrapyrazole and a reversible ATP-competitive inhibitor of JNK-1, JNK-2 and JNK-3; it has been successfully used *in vivo* and *in vitro* to block JNK activation [23].

Chronic morphine treatment has been shown to activate JNK in SH-SY5Y cells [24,25], T cells [26], and spinal cord [27]. In a rat model, single or chronic morphine injections induce JNK-3 mRNA in the frontal cortex and after cessation of morphine treatment, sustained elevation of JNK-3 mRNA expression occurs in the hippocampus and thalamus [28]. Moreover, MOR desensitization and acute analgesic tolerance to morphine and related opiates were blocked by JNK inhibition [27,29]. In L5-spinal nerve ligation pain models, transient JNK activation increases in dorsal root ganglion (DRG) neurons followed by a persistent activation in spinal astrocytes which contributes to the maintenance of neuropathic pain symptoms [21,30]. In these animal pain models, selective inhibition of JNK inhibits mechanical allodynia and heat hyperalgesia [30,31]. Collectively, these results suggest a role for JNK in the pharmacological effects of nociception and opioid systems. In our previous efforts to identify the signaling events in transcriptional activation of the MOR gene, we observed that SP treatment of P19 cells significantly increases MOR mRNA expression [20]. In this study, we investigate the molecular mechanism that leads to expression of the MOR gene upon JNK inhibition.

2. Materials and methods

2.1. Materials

SP600125 (SP), cell-permeable JNK inhibitor, and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from EMD Biosciences (San Diego, CA). 2,(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002 (LY)), wortmannin and 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126) were purchased from Cell Signaling Technology (Beverly, MA). 4-(4-flurophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)imidazole (SB203580 (SB)), actinomycin-D (act-D), and pyrrolidine dithiocarbonate (PDTC) were purchased from Sigma (St Louis, MO). Anti-MOR antiserum was generated in rabbits by injecting GST-fused MOR protein containing amino acids 340–398 of the MOR C-terminus. The specificity of the antiserum was confirmed in flow cytometry analysis of HEK 293 T cells and P19 cells stably expressing MOR. Anti-phospho-c-Jun, anti-phospho-SAPK/JNK, anti-JNK-1, anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-AKT, anti-AKT, anti-phospho p42/p44 MAPK, anti-p42/44 MAPK, anti-phospho-p65 (Ser 536), anti-phospho CREB, anti-phospho MSK1 (Thr 581) antibodies were obtained from Cell Signaling Technology (Beverly, CA). Anti-c-Jun, anti-c-fos, anti-p65, anti-phospho-p65 (Ser 276), and anti-p50 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho serine antibodies and anti-CREB were obtained from Millipore (Billerca, MA). Anti-histone-dimethyl lysine 4 and anti-histone-trimethyl lysine 9 antibodies were obtained from Abcam (Cambridge, MA). Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse IgG were supplied by BioRad (Hercules, CA). Alexa Fluor 488-conjugated goat anti-rabbit were purchased from Invitrogen (Carlsbad, CA). Other reagents for molecular studies were supplied by Sigma Chemicals (St. Louis, MO).

2.2. Cell culture and transfection

P19 cells were cultured and differentiated as described previously [32]. For treatments, 5×10^5 cells were seeded into each wells of a 6-well plate one day before treatment. Cells were treated for 6 h with SP (25 μM), SB (25 μM), LY (25 μM), U0126 (10 μM) and QNZ (10 nM), and total RNA was harvested for RT-PCR. For transfection, 1×10^5 cells were seeded in 12-well dishes and co-transfected the next day for 24 h with the MOR promoter construct and a one-fifth molar ratio of pCH110 (for β-galactosidase assay) using effectene transfection reagent (Qiagen, Valencia, CA) as described previously [10]. Cells were treated with SP for 12 h, and cell lysates were analyzed for firefly luciferase activity and β-galactosidase activity as described by the manufacturer's protocol (Promega and Tropix, respectively). Results were expressed as relative luciferase activity compared to the control cells. For siRNA transfection, cells were seeded as above and transfected on the following day with 50 nM of control siRNA, JNK-1 siRNA, JNK-2 siRNA (Santa Cruz, CA) or SignalSilence NF-κB p65 siRNA (Cell Signaling Technology) using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer's protocol. After 36 h of transfection, cells were treated as required and harvested for western blotting and RT-PCR. NMB neuroblastoma cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), and HEK 293 T cells were cultured in advanced DMEM medium containing 5% FBS supplemented with Glutamax (Gibco, Invitrogen). Cells were maintained at 37 °C in a humidified incubator and sub-cultured every 2–3 days as required.

2.3. RT-PCR and real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) and analyzed by RT-PCR using the MOR gene-specific primers mMOR-S and mMOR-AS [32]. Semi-quantitative RT-PCR was performed in 200 ng–1 μg of total RNA using a Qiagen OneStep RT-PCR kit (Valencia, CA). Similar reactions were performed using β-actin as an internal control [32]. qRT-PCR was performed as described previously [33] using the same MOR primer set and the Quantitect SYBR Green RT-PCR kit (Valencia, CA). Relative mRNA expression was analyzed as described previously [32]. The number of target molecules was normalized against that obtained for β-actin, used as an internal control. The specificity of qRT-PCR primers was determined using a melt curve after the amplification to show that only a single species of PCR product resulted from the reaction. The PCR products were also verified on an agarose gel. The RT-PCR and qRT-PCR experiments were repeated at least three times to obtain statistical significance.

2.4. Western blotting and immunoprecipitation

Western blotting was performed as previously described [20]. 4×10^5 cells were seeded into each well of 6-well dishes and treated as required. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in buffer composed of 50 mM Tris–Cl,

150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitor cocktail, and phosphatase inhibitors. Cell lysates were clarified by centrifugation, and protein concentrations were determined using BCA protein assay (Thermo Scientific, Rockford, IL). 30 μg of each lysate was loaded into SDS-polyacrylamide gels and electrotransferred onto polyvinyldifluoride membranes. Membranes were blocked for 1 h in SuperBlock solution (Thermo Scientific, Rockford, IL) and then incubated with antibodies overnight at 4 °C with gentle shaking as suggested by the manufacturers. Membranes were washed 3× with T-TBS (Tris buffered saline containing 0.05% Tween-20) and incubated with alkaline-phosphatase conjugated secondary antibodies for 1 h at room temperature. Data were collected on a PhosphorImager with appropriate settings for each antibody. Band densities were analyzed using the ImageQuant software (GE Healthcare life sciences).

For nuclear and cytosolic protein preparations, cells were washed and collected by scraping in cold PBS. Cytosolic protein fraction was prepared by lysing the cells in hyopotonic lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, and protease inhibitor cocktail). After centrifugation, supernatants were collected as cytosolic protein fractions, and nuclear pellets were resuspended in hypertonic buffer solution (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail), vortexed vigorously, and stored on ice for 30 min. After removing insoluble particles by centrifugation, a BCA protein assay was performed to determine the protein yield. 20 μg of each fraction was used to perform western blotting as described above.

For immunoprecipitation reactions, 1 μg antibodies were incubated to protein G Dynabeads (Invitrogen,CA) for 4 h at 4 °C. The beads were washed thrice with PBS and added to 300 μg of nuclear extracts prepared as above, and incubated overnight on a rotating platform at 4 °C. On the following day, the beads were extensively washed and boiled in SDS-PAGE loading buffer. Eluted immunoprecipitates were separated on 8-10% SDS-PAGE gels and immunoblotting was carried out as described above.

2.5. Flow cytometry analyses

For flow cytometry analyses, 5×10^5 cells were cultured in 6-well plates and treated as required. Cells were then harvested, washed twice in cold PBS containing 0.5% FBS, fixed in 4% paraformaldehyde, and permeabilized in 5% FBS containing 0.2% Triton X-100 and 0.5% glycine. Cells were washed in PBS containing 0.1% horse serum and incubated on ice for 1 h with anti-MOR antibody (1:1000) in PBS containing 5% normal goat serum. Cells were washed and then incubated for 45 min with 1 μg of goat-anti-rabbit Alexa Fluor 488 in the same buffer condition as the anti-MOR antibody. Cells were washed twice, and 10,000 events were analyzed on a flow cytometer (FACScalibur, BD biosciences). For background subtraction, unstained cells and cells stained with secondary antibody were also included.

2.6. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation assays were performed as reported previously [34]. Briefly, chromatin complexes were crosslinked by the addition of 1% formaldehyde to treated cells. Cells were lysed (25 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP-40, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors) to isolate nuclei. Nuclei were re-suspended in sonication buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors) and sonicated (Vibra-cell Sonicator) to isolate chromatin. Purified chromatin was quantified, 1–5 μg of specific antibodies were added to the purified chromatin, and the mixture was on a rotating platform overnight 4 °C. Protein G Dynabeads (Invitrogen) pre-blocked with salmon sperm DNA and BSA were added, and incubation continued for 3 h at 4 °C to capture the immune complexes. The beads were extensively washed in series of low- and high-salt wash buffers and Tris-EDTA buffer. The immune complexes were eluted, decrosslinked, and treated with RNase and proteinase K. Finally, DNA was purified from the eluate by phenol–chloroform–isoamyl alcohol extraction and ethanol precipitation. Each immunoprecipitated DNA sample was analyzed by real-time qPCR using the specific PCR primers. ChIP assays were repeated at least three times for each antibody used.

2.7. Statistical analysis

Numerical values were presented as the mean ± S.E.M. For comparison between two samples, t-test analysis was performed. For multiple comparisons, analysis of variance with Bonferroni's *post hoc* test was used. *, $p < 0.05$ was considered statistically significant unless stated otherwise.

3. Results

3.1. JNK inhibition increases MOR gene expression.

In our previous study [20], we observed that treatment of MOR-negative P19 cells with JNK inhibitor SP600125 (SP) significantly increased MOR gene expression. To examine the role of various inhibitors on MOR gene expression, we treated P19 cells with SP, SB203580 (SB, a p38 MAPK inhibitor), LY294002 (LY, a PI3-K inhibitor), U0126 (a MEK1/2 inhibitor), and 6-amino-4-(4-phenoxyphenylethylamino) quinazoline) (QNZ, a NF-κB transcriptional activation inhibitor) for 6 h and analyzed MOR expression by RT-PCR (Fig. 1A). Only SP significantly increased the level of MOR mRNA (Fig. 1A, lane 2), whereas treatment with LY or QNZ caused minor changes in MOR levels (Fig. 1A, lanes 4 and 6). To determine whether SP's effect on MOR gene expression is a dose-dependent response, we treated P19 cells with various concentrations of SP for 6 h and analyzed the RNA by RT-PCR (Fig. 1B). At 10 μM SP, an ~4-fold increase in MOR expression was observed; the effect was clearer with 25 μM SP, which caused an ~7-fold increase (Fig. 1B). A time-course experiment with 25 μM SP showed an increase in MOR gene expression as early as 4 h (~3-fold, Fig. 1C). At later time points, MOR expression increased, and expression remained elevated until 48 h of treatment (data not shown). To test whether JNK inhibition by specific peptide inhibitor could also increase the MOR expression, P19 cells were incubated with increasing doses of JNK-inhibitory peptide (JIP) for 6 h and MOR gene expression was analyzed using qRT-PCR (Fig. 1D). A small increase (~1.7-fold) in MOR gene expression consistently occurred in cells treated with JIP compared with those treated with the control peptide (Fig. 1D). In order to identify the specific JNK isoform involved, we transfected P19 cells with siRNA against JNK-1, JNK-2 or both, and analyzed the MOR expression patterns (Fig. 1E, left panel). Knock-down of JNK-1 (Fig. 1E, right panel) showed a small increase in MOR expression (~1.7-fold, Fig. 1E left panel, lane 2) compared to the control (Fig. 1E left panel, lane 1), a result which was comparable to the MOR expression levels induced by JIP. However, JNK-2 knock-down did not increase the MOR expression levels, and knock-down of both JNK isoforms failed to further enhance the MOR expression level beyond that obtained with JNK-1 siRNA transfected cells (Fig. 1E, left panel, c.f. lanes 1, 2 and 4). In addition, when SP was treated to the JNK siRNA transfected cells, MOR expression levels were increased, which was comparable with SP-treated wild type P19 cells (data not shown). Collectively, these results suggested that the inhibition of JNK-1 can increase MOR expression in P19 cells, and SP may use other mechanisms in addition to JNK inhibition that leads to increased MOR expression.

To assess whether MOR gene expression in response to SP can be observed in other cell types, we treated NMB neuroblastoma cells for 6 h with various concentrations of SP and performed semi-quantitative RT-PCR analysis (Fig. 2A). Indeed, a small increase in MOR gene expression occurred in cells treated with 10 μM or 25 μM SP. To further analyze the effect of SP on MOR expression in primary neurons, we treated

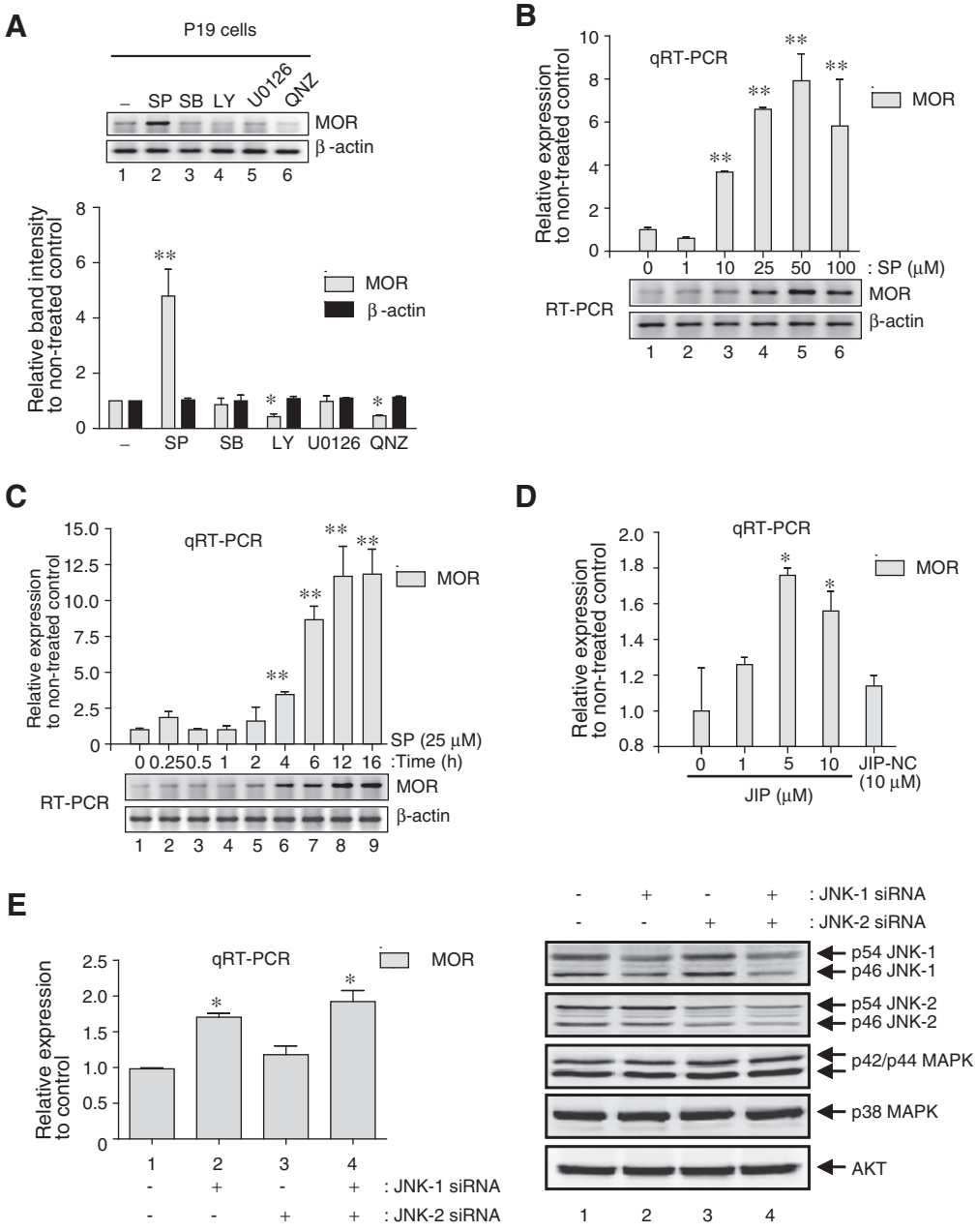

Fig. 1. JNK inhibition increases MOR gene expression in P19 cells. (A) P19 cells were incubated with SP (25 μM), SB (25 μM), LY (25 μM), U0126 (10 μM) or QNZ (10 nM) for 6 h. RNA was extracted and semi-quantitative RT-PCR was performed to determine the levels of MOR. β-actin was used as internal control. (histogram) The MOR and β-actin signals were quantified and plotted as a relative fold change compared to untreated control. (B) P19 cells were treated with increasing concentrations of SP for 6 h as indicated. RNA was extracted, reverse transcribed, and analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel). (C) Quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) was performed as described above in RNA samples from P19 cells treated with SP for various lengths of time. (D) P19 cells were treated with increasing concentrations of JNK-inhibitory peptide (JIP) and negative control peptide (JIP-NC) for 6 h, RNA was extracted and quantitative real-time RT-PCR was performed as described above. (E) (left panel) P19 cells were transfected with siRNA against JNK-1, JNK-2 or both for 36 h and MOR expression was analyzed by quantitative real-time RT-PCR. (right panel) P19 cells were transfected with siRNA as indicated for 36 h, and immunoblotting was performed to analyze the expression levels of JNK-1, JNK-2, p42/p44 MAPK, p38 MAPK and AKT. Error bars represent the range of standard errors, and asterisks above the histograms indicate statistically significant findings (*, $p < 0.05$; **, $p < 0.01$). Results are representative of three separate experiments.

hippocampal neurons from newborn rats with increasing concentrations of SP for 6 h and performed qRT-PCR (Fig. 2B). A dose-dependent response to SP occurred, and 10 μM SP caused an ~2-fold increase in MOR gene expression. These results show that JNK inhibitor SP causes an increase in MOR gene expression in neuronal cells, although the changes are smaller than in P19 cells.

SP has previously been shown to suppress Cdk1 and induce endoreplication directly from G2 phase, resulting in polyploid cells [35]. Another study showed that SP induces defective cytokinesis and enlargement of P19 cells [36]. These results could be used to argue that the SP-induced increase in MOR expression is caused by the presence of increased DNA content and increased stability of the MOR mRNA. To rule out these possibilities, NMB cells (MOR-positive cells) were treated with actinomycin-D (act-D, a transcription inhibitor) in the presence or absence of SP, and the amount of MOR mRNA remaining at various time points was estimated. As shown in Fig. 2C, the degradation pattern of MOR mRNA followed a similar course in the presence or absence of SP (*c.f.* lanes 5, 6, 7 and 11, 12, 13). Because

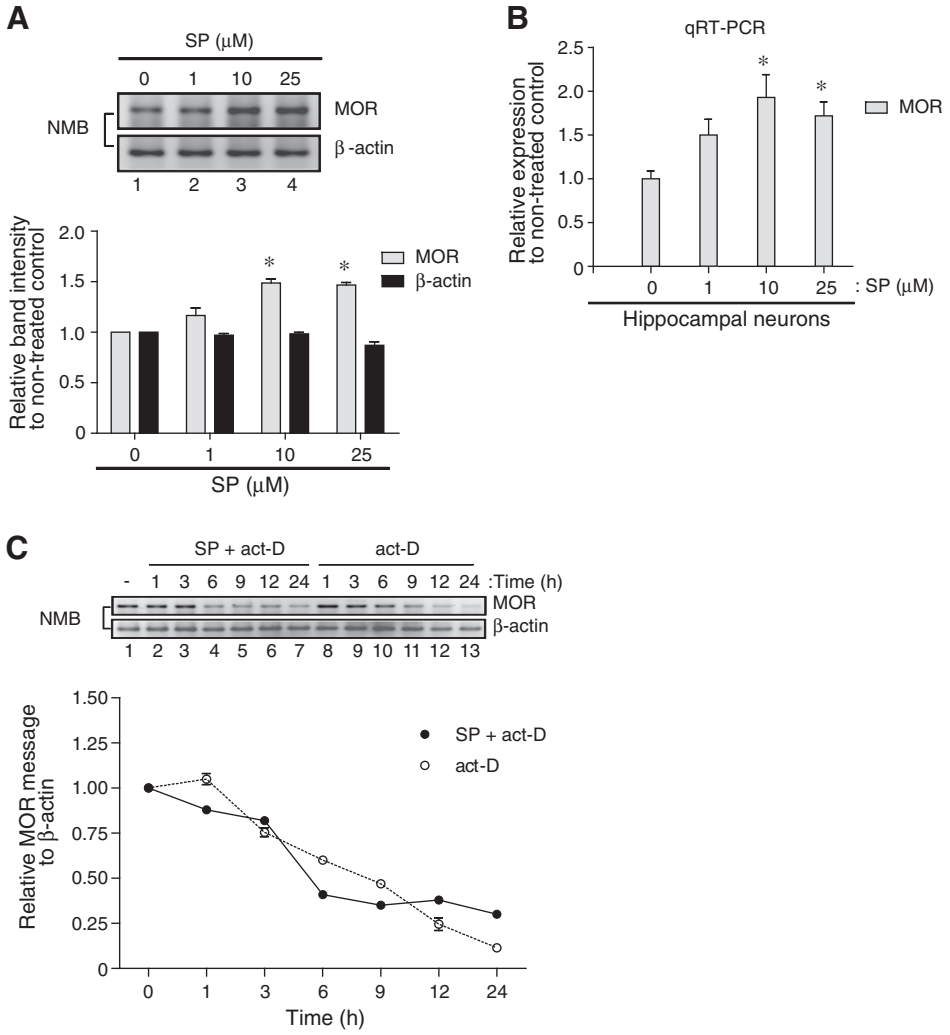

Fig. 2. JNK inhibition activates MOR transcription in neuronal cells. (A) (gel) NMB cells were treated with increasing concentrations of SP for 6 h and semi-quantitative RT-PCR was performed to examine MOR expression. (histogram) The relative band intensities were calculated and plotted as a fold change compared to untreated control. (B) Rat primary hippocampal neurons were treated with increasing concentrations of SP for 6 h, RNA was extracted, and quantitative real-time RT-PCR was performed to determine MOR expression relative to β-actin control. (C) (gel) NMB cells were treated with act-D in the presence or absence of SP for various lengths of time, RNA was extracted, and semi-quantitative RT-PCR was performed to determine the levels of MOR. (graph) The MOR signal relative to β-actin was quantified and plotted as relative fold change compared to β-actin. Error bars represent the range of standard errors, and asterisks above the histograms indicate statistically significant findings (*, $p < 0.05$). A representative result of three independent experiments is shown.

MOR RNA stability was not changed with act-D and SP co-treatment of NMB cells, and because SP could increase expression of MOR in P19 cells as early as 4 h (Fig. 1C), as opposed to the 24 h needed to attain polyploid cells, it appears that SP-mediated MOR increase was due to active transcription of the MOR gene.

Because the treatment of P19 cells with SP significantly increased MOR at the transcript level, we wanted to examine whether the protein was also increased. P19 cells were treated with SP (25 μM), and MOR expression patterns were analyzed by flow cytometry. P19 cells expressed low levels of MOR protein, which were increased by SP treatment (Fig. 3A). The mean fluorescent intensity (corresponding to the MOR signal level) was increased by ~3-fold in SP-treated cells. These results confirm that SP treatment increases both MOR mRNA and protein expression.

3.2. p38 MAPK is involved in the increased expression of MOR by SP.

It has been demonstrated that JNK inhibition by SP can activate CREB *via* the p38 MAPK-dependent pathway, leading to increased expression of CREB responsive genes [37]. SP increases the transcriptional activation of p21 in an ERK-dependent pathway that causes increased phosphorylation and binding of SP1 to the p21 promoter [38]. In cerebellar granular cells, SP protects cells from serum/potassium withdrawal-induced apoptosis, a phenomenon that is mediated through phosphorylated (activated) AKT [39]. To examine the involvement of these signaling pathways in MOR transcriptional regulation, we treated P19 cells with 25 μM of SP for various lengths of time and performed western blotting with anti-phospho-p38 MAPK, anti-phospho-AKT, and anti-phospho-ERK 1/2 antibodies. SP treatment increased the levels of phospho-p38 MAPK as soon as 15 min, and it remained elevated until 2 h of SP treatment, whereas the levels of phospho-AKT were unchanged (Fig. 4A). Interestingly, the activation of ERK 1/2 followed a biphasic response. At 15 min post-SP treatment, a decrease in the ERK 1/2 phosphorylation occurred followed by a gradual increase over the 2 h treatment. These results suggested that SP treatment is able to modulate p38 MAPK and ERK 1/2 pathways in P19 cells. Since we could not detect phospho-JNK or phospho-c-jun (downstream of JNK pathway) in P19 cells; as a control for SP's activity, HEK 293 T cells were treated with SP and immunoblotting was performed to detect changes in the levels

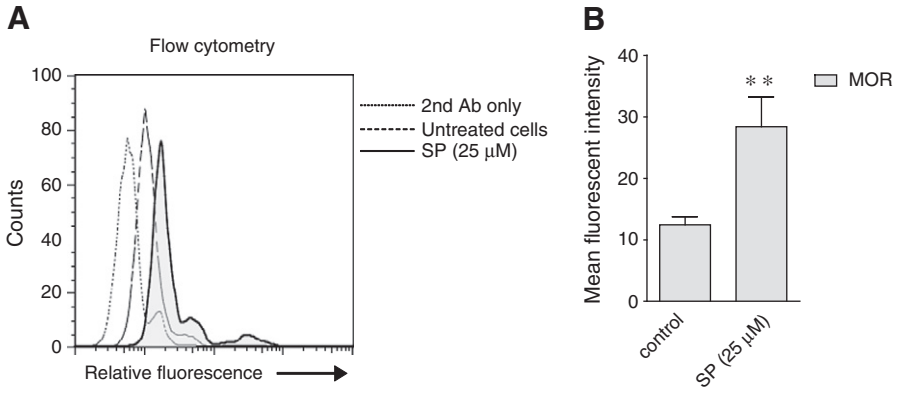

Fig. 3. SP increases MOR protein expression in P19 cells. (A) P19 cells were left untreated or treated with SP for 12 h, and expression of MOR protein was analyzed by flow cytometry. Unstained cells were used to gate true cell population, and P19 cells stained only with second antibody were used to subtract background fluorescence. (B) The geometric mean fluorescent intensities in untreated and SP-treated P19 cells were quantified and plotted as a bar graph (**, $p < 0.01$). Error bars represent the range of standard errors, and a representative of three separate experiments is shown.

of phospho-c-jun. As expected, the levels of phospho-c-jun was reduced by SP treatment of HEK 293 T cells (data not shown). In the next set of experiments, we pretreated P19 cells with SB, wortmannin (a PI3-K/AKT inhibitor), U0126, and act-D followed by 6 h of SP treatment in order to identify whether p38 MAPK, PI3-K/AKT, ERK pathways transcriptionally regulate MOR gene expression in response to SP treatment. RT-PCR analyses (Fig. 4B) showed that SB completely inhibited the SP-mediated increase in MOR (~80% reduction in MOR expression level compared to SP alone, Fig. 4B, lanes 2 and 3), and wortmannin also showed a small decrease in MOR levels (~30% reduction compared to SP alone, Fig. 4B, lane 4). U0126 treatment had little effect suggesting that ERK pathway is not implicated in this response. Treatment with act-D also reversed the increase in MOR mRNA, confirming that the response to SP treatment involves an increase in transcription. When we pretreated the cells with a range of SB concentrations (2.5 μM–25 μM), a dose-dependent reduction of MOR expression occurred, and 5 μM SB returned MOR expression to near baseline level (Fig. 4C, lanes 1, 2 and 4).

As stated earlier, JNK inhibition by SP can activate CREB *via* a p38 MAPK-dependent pathway leading to an increased expression of CREB responsive genes [37]. Indeed, the MOR proximal promoter contains a CREB binding site that is conserved in mouse, rat and human MOR genes [19]. To determine if CREB is activated in the nucleus of P19 cells, we performed immunoblots with nuclear protein from SP-treated P19 cells. CREB was not activated by SP treatment, although it was highly activated by 1 h of 10 μM forskolin treatment as a positive control (Fig. 4D, last lane). Additional RT-PCR experiments with forskolin and SP in P19 cells did not show an increase in MOR expression levels; instead, forskolin treatment alone showed a slight decrease in MOR mRNA (data not shown), confirming that CREB is not involved in regulation of MOR in P19 cells.

3.3. NF-κB is involved in the increased expression of MOR upon JNK inhibition.

To find the region of MOR promoter and the downstream factors of p38 MAPK pathway that mediate the MOR expression in P19 cells in response to SP treatment, we performed luciferase assays with MOR promoter construct. Upon SP treatment, activity of the MOR promoter construct containing the core promoter region (−450 bp/+1) increased about 2.5-fold compared to the control (Fig. 5A, lower panel). The binding sites for transcription factors in the core promoter region of MOR gene (−450 bp/+1) contains NRSE [34,40], CREB [19], NF-κB [18], Sp1/iGA [14], AP2/SP1 [15], and PCBP [13] elements (Fig. 5A, upper panel). To further delineate whether NF-κB is responsible for increased MOR expression in response to SP treatment, we pretreated P19 cells with SB, U0126, and NF-κB inhibitors QNZ and PDTC and then performed the luciferase assay. The increased activity of the MOR promoter upon SP treatment could be reduced by SB, QNZ and PDTC pretreatment, but not by U0126 pretreatment (Fig. 5A, lower panel). This result suggested that p38 MAPK and NF-κB factors are important for MOR expression in response to SP treatment. To further identify the role of NF-κB for MOR gene expression in response to SP treatment, we pretreated P19 cells with various doses of QNZ (1 nM − 20 nM) and performed RT-PCR analysis. A dose-dependent decrease in MOR expression occurred, and 10 nM QNZ significantly inhibited MOR expression (Fig. 5B, c.f. lane 5 with lane 2, ~60% decrease in expression compared to SP alone). Similar results were obtained with a different NF-κB inhibitor, PDTC (data not shown), which further support our observation that NF-κB is an important downstream factor in SP-mediated MOR expression.

The classical pathway of NF-κB activation involves the phosphorylation and ubiquitin-mediated degradation of IkappaBalpha proteins followed by increased nuclear translocation of NF-κB proteins and increased transcriptional activation of responsive genes [41]. To analyze whether SP treatment activates the NF-κB in this fashion, we analyzed the extracts of SP-treated cells against phospho-IkappaBalpha antibodies. However, we could not observe increase in the phosphorylation of IkappaBalpha beyond the basal level (Supplementary Fig. A). Also, the analysis of the NF-κB subunit proteins, p65 and p50, in the cytosolic and nuclear proteins of SP-treated cells did not show significant changes in either cell compartments upon SP treatment (Fig. 5C). Of note, significant amounts of p65 and p50 proteins were constitutively present in the nuclear fraction of P19 cells under non-stimulated conditions (Fig. 5C). Since the transactivation ability of NF-κB is increased by phosphorylation of p65 subunit, we analyzed whether SP treatment changes the phosphorylation status of p65 subunit of NF-κB. Using two known phospho-specific p65 antibodies (S536 and S276), we performed western blots with protein extracts from the cytosolic and nuclear fraction of SP-treated cells. As shown in Fig. 5D, we did not observe any phosphorylation of p65 in the cytosolic pools but the phosphorylation of phospho-p65 (Ser 536) increased in the nuclear fraction upon SP treatment; and it could be abrogated by SB pretreatment (Fig. 5D). Analysis of the phosphorylation of p65 at another residue, Ser 276 also showed a small increase upon SP treatment which was not decreased by SB pretreatment (Fig. 5D, lower panel). These results suggested that the SP-mediated increase in phosphorylation of p65 (Ser 536 and Ser 276) specifically occurs inside the nucleus, and that the Ser 536 phosphorylation of p65 is dependent on p38 MAPK activity.

To further verify that p65 is involved in the upregulation of MOR gene expression in response to SP treatment; we employed knock-down

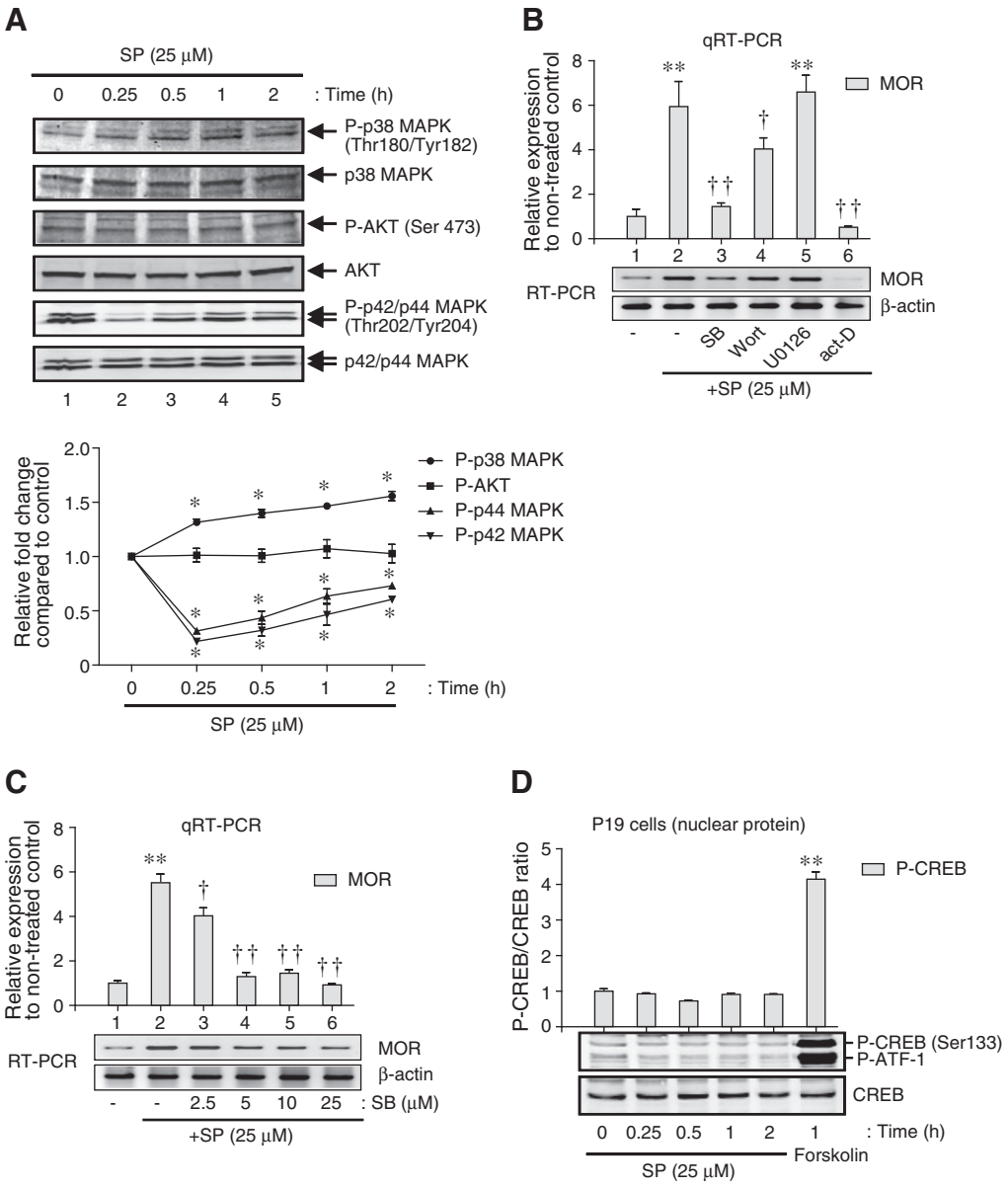

Fig. 4. p38 MAPK is involved in MOR mRNA expression by SP. (A) (gel) P19 cells were treated with 25 μM SP for various time points as indicated and immunoblotting was performed with anti-phospho-p38MAPK, anti-p38 MAPK, anti-phospho-AKT, anti-AKT, anti-phospho-p42/p44 MAPK, and anti-p42/p44 MAPK antibodies. (graph) The pixel densities obtained for phospho-p38 MAPK, phospho-AKT, phospho-p44 MAPK and phospho-p42 MAPK was plotted against the pixel densities obtained for p38 MAPK, AKT, p44 MAPK and p42 MAPK respectively. (B) P19 cells were pretreated for 1 h with 25 μM SB, 200 nM wortmannin, 10 μM U0126, or 5 μg/ml act-D and then treated for 6 h with 25 μM SP. RNA was extracted, reverse transcribed, and analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) as described in Fig. 1. (C) P19 cells were pretreated for 1 h with various concentrations of SB as indicated, followed by 6 h of SP treatment. Total RNA was analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel). (D) P19 cells were treated with 25 μM SP for various lengths of time, nuclear protein was extracted, and immunoblotting was performed with anti-p-CREB (ser 133) and anti-CREB antibodies. Pixel densities were quantified and plotted as fold change compared to untreated control (histogram). Results shown are representative of three independent experiments; error bars indicate the range of standard errors. Asterisks above the histograms indicate statistically significant findings compared to control (**, $p < 0.01$), daggers indicate significant findings compared to SP treatment (†, $p < 0.05$; ††, $p < 0.01$).

strategy using siRNA. P19 cells were transfected with control siRNA or p65 specific siRNA for 36 h and then treated with SP for 6 h (Fig. 5E). RNA and total cell lysates were then extracted to perform RT-PCR for MOR expression patterns and immunoblotting to assess p65 knock-down. As shown in Fig. 5E left panel, transfection of p65 siRNA abolished the SP-induced MOR gene expression by ~50% compared to the control siRNA transfected cells (c.f. lane 2 with lane 4), a result which reflected the ~50% reduction of p65 proteins in the siRNA transfected cells (Fig. 5E, right panel). As a control for specific activity of the p65 siRNA, the p50 subunit of NF-κB was analyzed by immunoblotting, and was found unaffected.

In a previous study [42], it has been shown that the MAPK nuclear kinase, mitogen-and stress-activated protein kinase 1 (MSK1), induces NF-κB p65 serine 276 phosphorylation upon IL-1β treatment, as well as MAPK inhibition abolishes binding of p65, of its coactivator CBP and of MSK1 to the κB intronic enhancer site of the stem cell factor gene. Our results also indicated a minor increase in S276 phosphorylation of p65 by SP treatment and that knock-down of p65 by siRNA abolishes the MOR gene expression by SP (Fig. 5E), we tested whether MSK1 is involved in the MOR gene activation. Therefore, P19 cells were pretreated with increasing concentrations of H89 (a MSK1-PKA inhibitor) followed by SP treatment for 6 h. Semi-quantitative and quantitative RT-PCR

results showed that inclusion of H89 indeed decreased the SP-induced MOR gene expression in a dose-dependent fashion (Fig. 6A). To analyze whether activated/phosphorylated MSK1 and p65 interacted in the nucleus of P19 cells, the nuclear fractions of SP-treated cells were immunoprecipitated with anti-p65 antibody and subjected to immunoblotting with anti-phospho-MSK1 antibody. As shown in Fig. 6B, phosphorylated MSK1 and p65 had a minimal interaction under non-stimulated conditions (lane 1), and the interaction increased after 1 h of SP treatment (lane 2) which increased further at 2 h and 4 h post-SP treatment (lanes 3 and 4) suggesting that p38 MAPK/MSK1 pathway may also be involved in the MOR gene expression upon SP treatment.

Because the MOR promoter in un-stimulated P19 cells resembles a closed chromatin structure [32], we analyzed several epigenetic tags and co-activators of NF-κB that are changed in the active MOR promoter upon SP treatment (Fig. 6C). Binding of CBP (a co-activator of NF-κB) to the MOR promoter increased upon SP treatment along with the increased histone 3-dimethyl lysine 4 (a hallmark of active transcription), while showing decreased bindings of histone 3-trimethyl lysine 9 (a hallmark of repressive transcription), HDAC-2, and MeCP2 (protein that binds to methyl CpG islands and blocks transcription) [32,33] at the MOR promoter region. These results suggest that SP treatment, along with the activation of p38 MAPK and NF-κB activation, is able to modify the chromatin structure to favor the transcriptional activation of MOR gene.

3.4. Two major factors, p38 MAPK and NF-κB are also associated with MOR gene expression in neuronally differentiating P19 cells

Neuronal differentiation of P19 has been used as an excellent model to investigate the MOR gene expression [32,33,43,44]. During retinoic acid induced neuronal differentiation of P19 cell, MOR gene expression starts to increase by 2 days after plating (P19-AP2D), and maximum expression is observed at 4 days (P19-AP4D), when the cells differentiate into neuronal cells [32,33]. As reported previously, we observed a similar pattern of MOR expression (~70-fold and ~200-fold on day 2 and day 4, respectively, compared with undifferentiated P19 cells) [33]. Therefore, we used this model to examine whether the p38 MAPK and NF-κB pathways are important for MOR gene expression during neuronal differentiation of P19 cells. We treated differentiating P19 cells at day 2 (P19-AP2D) with various inhibitors and examined MOR levels the following day by semi-quantitative RT-PCR and real time RT-PCR. We chose longer treatment times because the half-life of MOR mRNA as shown by act-D treatment in NMB cells was ~9 h (Fig. 2C, lanes 1 and 11). As expected, treatment with SB and NF-κB inhibitors QNZ and PDTC significantly decreased MOR levels

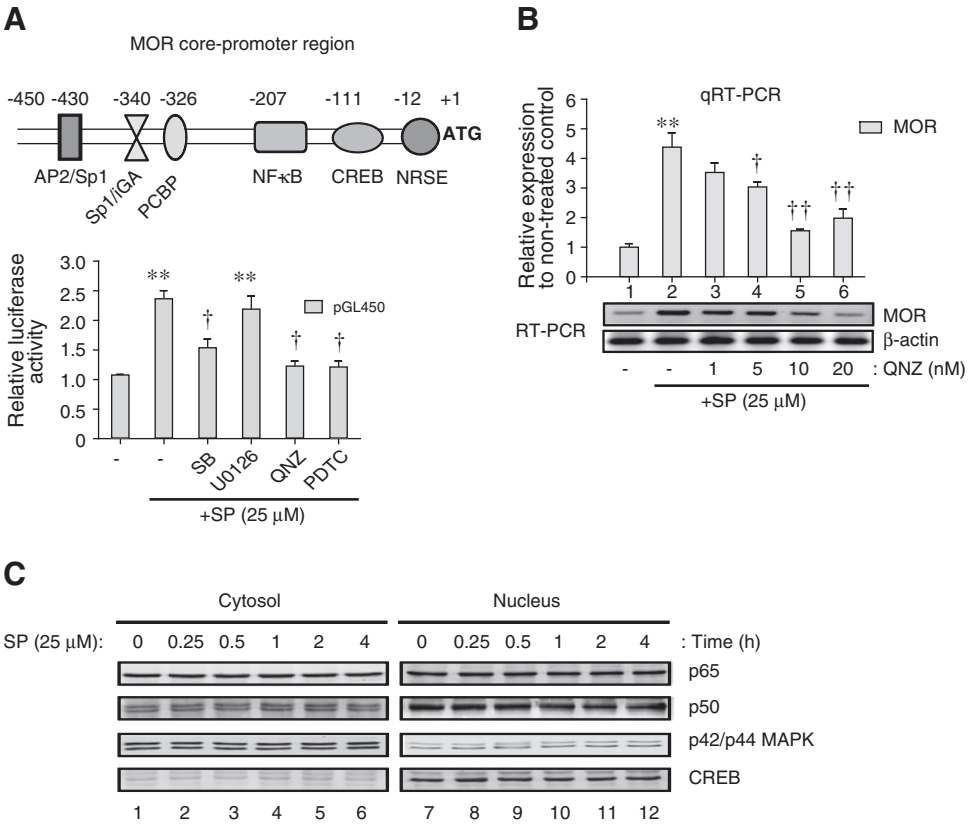

Fig. 5. NF-κB is involved in MOR mRNA expression by SP. (A) (upper panel) Schematic representation of the transcription factor binding sites in the 450 bp region (minimal promoter) of the MOR gene (references are provided in text). Numbers indicate base pair location of binding site starting position respective to the start codon, shown as +1. Lower panel; P19 cells were transfected with MOR minimal promoter (pGL450) construct for 24 h and treated as indicated. Relative luciferase activites were calculated compared to that of untreated control (B) P19 cells were pretreated for 1 h with various concentrations of QNZ followed by 6 h of SP treatment. Total RNA was analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) as described in Fig. 1. (C) P19 cells were treated with SP for indicated times; cytosolic and nuclear protein fractions were collected and immunoblotted with anti-p65 and anti-p50 antibodies. Anti-CREB and anti-p42/p44 MAPK were used as loading controls for nuclear and cytosolic proteins respectively. (D) (upper panel) Cytosolic and nuclear protein fractions from P19 cells treated with SP, either alone or in combination with SB were used for immunoblotting with anti-phospho-p65 antibodies as indicated. Each blot was then reprobed with anti-p65, anti-p42/44 MAPK and anti-CREB antibodies as control. (lower panel) The pixel densities for levels of phospho-p65 Ser 536 and Ser 276 were quantified and plotted against the pixel densities obtained for p65 in the nuclear fraction (histogram) (E) (left panel) P19 cells were transfected with control siRNA or p65 specific siRNA for 36 h followed by treatment with SP for 6 h. Total RNA was extracted and analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) as described in Fig. 1. (right panel) Cell lysates from siRNA transfected cells were analyzed by immunoblotting for expression of p65, p50 and β-actin. In histogram, the signal intensity of p65 was quantified against the signal intensity obtained for β-actin. Results shown are representative of three independent experiments. Asterisks indicate statistically significant findings compared to control (*, $p < 0.05$; **, $p < 0.01$), daggers indicate significant findings compared to SP treatment (†, $p < 0.05$; ††, $p < 0.01$), and error bars represent the range of standard errors.

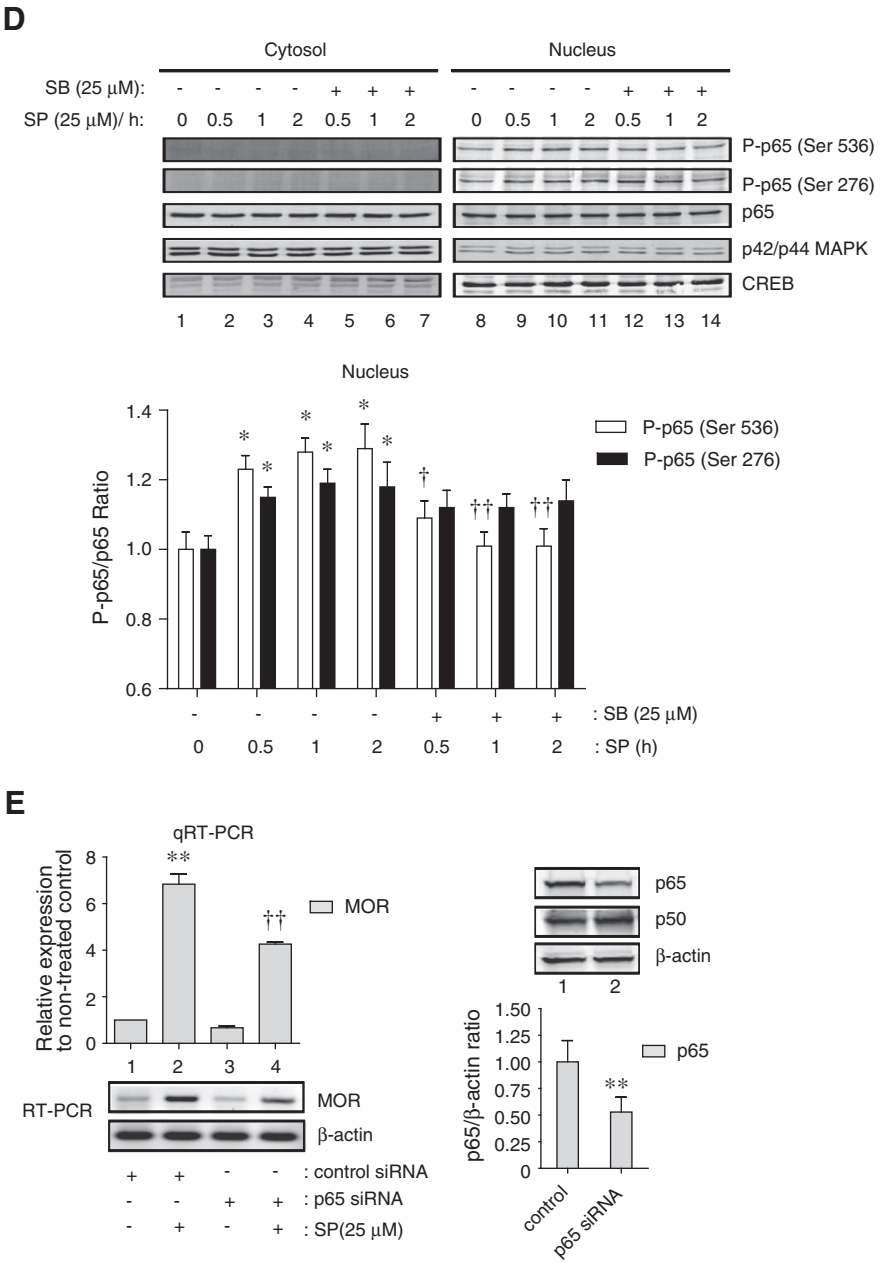

Fig. 5 *(continued).*

(~50–60% decrease compared with the control cells; Fig. 7B, lanes 1, 3, 6 and 7), whereas wortmannin and U0126 treatments showed no effect (Fig. 7B, lanes 1, 4 and 5). These results are consistent with those obtained with undifferentiated P19 cells, where SB and NF-κB inhibition significantly inhibited MOR expression induced by SP (Figs. 4B, C, 5B and E). However, SP treatment in the differentiating neurons led to only a small increase in MOR expression (~2-fold) (Fig. 7B, lanes 1 and 2, 16 h) compared with the ~12-fold increase in undifferentiated P19 cells (Fig. 1C, lanes 1 and 9, 16 h) suggesting that the p38 MAPK and NF-κB pathways are already activated in the differentiating neuronal cells, and thus, SP's effect on MOR expression in these cells may be less noticeable. Again, this result is consistent with SP's reduced potency in NMB neuroblastoma cells (Fig. 2A) and rat primary hippocampal neurons (Fig. 2B), cells types that constitutively express MOR gene. Indeed, the immunoblot analysis of active MAPKs in differentiating P19 cells showed that p38 MAPK is activated by day 1 after plating and continues to increase further as the cells begin to differentiate (Fig. 7C). In addition to the p38 MAPK, activation of ERK1/2, AKT and JNK pathways were also observed in differentiating P19 cells with the maximal activation observed at days 1, 2 and 3 respectively after plating (Fig. 7C). As a control for the neuronal differentiation of cells, the expression of neuronal specific HuB/HuD proteins were analyzed and found to be continually increased after plating (Fig. 7C).

4. Discussion

MOR gene expression has been shown to be regulated by fentanyl, morphine, IL-1, lipopolysaccharide, protein synthesis inhibitors (such as cycloheximide, anisomycin and puromycin), demethylating agents, histone deacetylase inhibitors and dopaminergic drugs such as cocaine and haloperidol [19,20]. With such a diverse group of substances regulating the MOR gene, many regulatory elements have been shown to interact at the MOR promoter and positively or negatively affect the expression of the MOR gene (see Introduction for

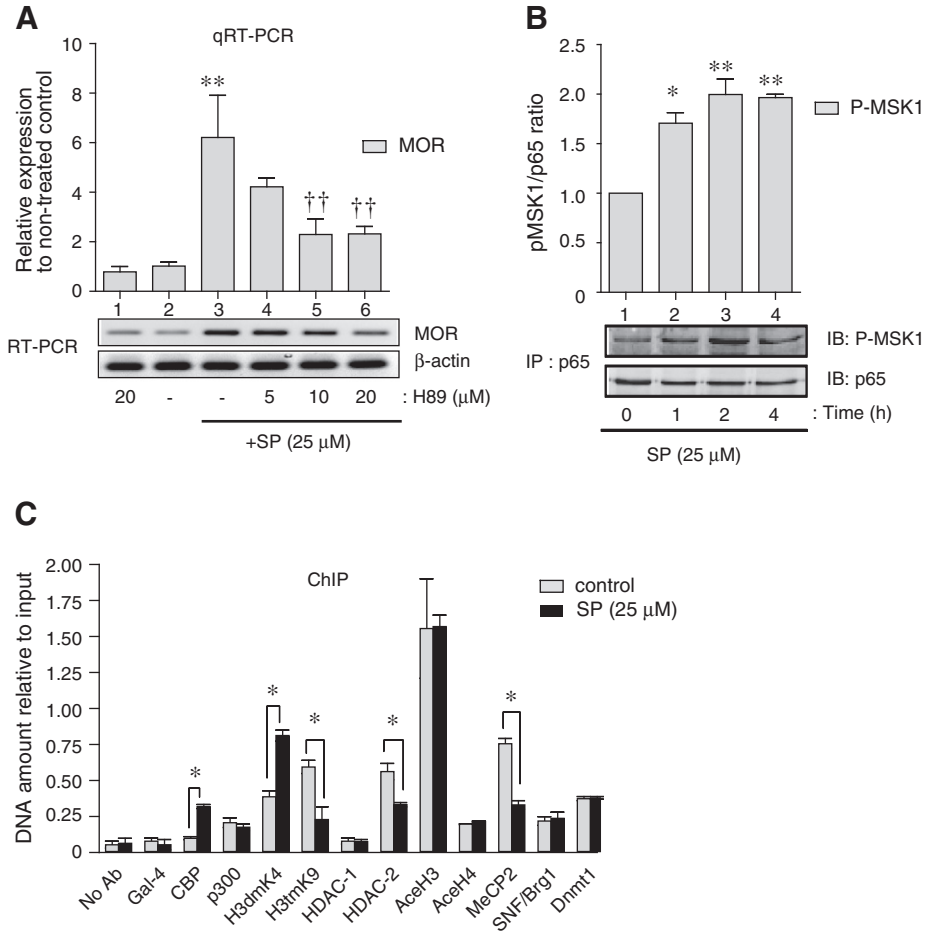

Fig. 6. (A) P19 cells were pretreated for 1 h with various concentrations of H89 followed by 6 h of SP treatment. Total RNA was analyzed by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) as described in Fig. 1. (B) (gel) Nuclear protein fractions from P19 cells treated with SP for various lengths of time were immunoprecipitated with anti-p65 antibody. Immunoprecipitated protein from each fraction was used for immunoblotting with anti-phospho-MSK1 antibodies. Each blot was then reprobed with anti-p65 as control. The pixel densities obtained for phospho-MSK1 was normalized against the pixel densities obtained for p65 and represented as pMSK1/p65 ratio in the histograms. (C) P19 cells were treated with SP for 4 h, chromatin was extracted, and ChIP experiments were performed as described in the Materials and methods section with antibodies as indicated to assess the binding of co-activators of NF-κB, and epigenetic markers known to regulate MOR gene expression. Results shown are representative of three independent experiments. Asterisks indicate statistically significant findings compared to control (*, $p < 0.05$; **, $p < 0.01$), daggers indicate significant findings compared to SP treatment (†, $p < 0.05$; ††, $p < 0.01$), and error bars represent the range of standard errors.

details). In this study, we show the transcriptional regulation of the MOR gene by JNK inhibition. In P19 embryonal carcinoma cells, NMB neuroblastoma cells, and primary hippocampal neurons, JNK inhibition by SP600125 led to increased MOR expression. This increase was reversed by inhibition of the p38 MAPK pathway. The use of synthetic inhibitors and activators of multiple pathways suggests an important role of NF-κB in upregulation of the MOR gene. Corresponding to the findings in undifferentiated P19 cells, MOR transcription was regulated by p38 MAPK and NF-κB pathways in P19 cells undergoing retinoic acid induced neuronal differentiation.

P19 cells can be terminally differentiated into neuronal cells by retinoic acid treatment, a process that closely resembles neurogenesis of mammalian CNS cells [45]. Analysis of the activation of MAPK pathways during P19 neuronal differentiation has shown that activation of the ERK and p38 MAPK pathways occur at day 1 and day 2 after plating, respectively, but the JNK pathway is not activated until 7 days after the induction of differentiation [45–47]. In addition, the p38/MEF2 pathway has been shown to prevent cell death during neuronal differentiation [45]. MOR gene expression during P19 neuronal differentiation occurs 2 days after plating, when the p38 MAPK pathway is also activated [33,47]. Our data, showing that inhibition of p38 MAPK by SB could reduce MOR transcript levels in differentiating P19 cells (Fig. 7B, lanes 1 and 3), correlates with the activation kinetics of p38 MAPK and MOR gene expression. The ERK pathway is not involved in MOR expression during neuronal differentiation of P19 cells, because inhibition of the MEK pathway had no effect on MOR expression. Also, neuronal differentiation of P19 cells occurs with extensive chromatin remodeling accompanied by the dissociation of MeCP2 from, and association of Brg1 and BAF155 with, the proximal promoter [33]. At present, it is uncertain whether signaling through the p38 MAPK pathway is involved in the epigenetic changes that occur at the MOR promoter during neuronal differentiation.

In a recent *in vivo* study, nerve growth factor was shown to increase the number of phosphorylated p38 MAPK immunoreactive neurons expressing MOR in dorsal root ganglia, increase peripherally directed axonal transport of MOR, and increase significant potentiation, as well as enhance efficacy in fentanyl- and buprenorphine-induced dose-dependent antinociception [48]. The expression of MOR gene in P19 cells by protein synthesis inhibitors is mediated through the activation of the PI-3K and p38 MAPK pathways, and inhibiting p38 MAPK decreases the constitutively expressed MOR expression levels in NMB neuroblastoma cells [20]. These results are consistent with the findings of our study, which show that MOR expression induced by SP treatment of P19 cells is blocked by p38 MAPK inhibition (Fig. 4B, and C). Although the JNK

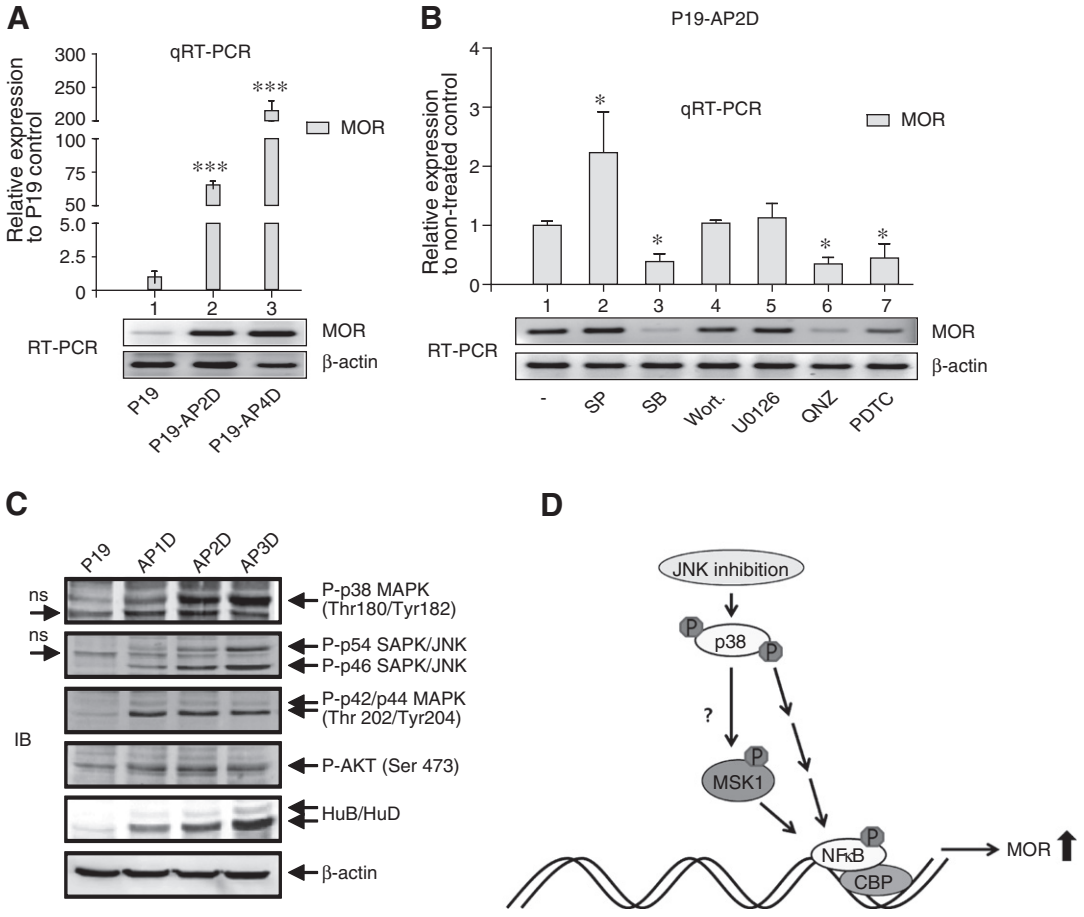

Fig. 7. p38 MAPK and NF-κB regulate the expression of MOR in differentiating neurons. (A) P19 cells were induced to differentiate in presence of 0.5 μM retinoic acid for 4 days. Cell aggregates were triturated and replated on tissue culture plates; RNA was extracted at 2 days (AP2D) and 4 days (AP4D) after plating, and quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel) were performed to determine MOR mRNA expression. Undifferentiated P19 cells served as control. (B) Differentiated P19 cells at day 2 were treated with 25 μM SP, 25 μM SB, 200 nM wortmannin, 10 μM U0126, 10 nM QNZ, or 100 μM PDTC. RNA was extracted and MOR expression was determined by quantitative real-time RT-PCR (histogram) and semi-quantitative RT-PCR (gel). (C) Total cell lysates from P19 cells seeded on tissue culture plates after retinoic acid treatment were collected each day for 3 days. Immunoblotting was performed with anti-phospho-p38 MAPK, anti-phospho-SAPK/JNK, anti-phospho-p42/p44 MAPK, and anti-phospho-AKT antibodies as indicated. Anti-HuB/HuD immunoblot was included to show proper neuronal differentiation and anti-β-actin was used to show uniform protein loading across samples. Results are representative of 3 separate experiments and error bars represent the range of standard errors. Asterisks indicate statistically significant findings compared with control (*, $p < 0.05$; ***, $p < 0.001$). (D) Schematic representation of events that leads to SP mediated increase in MOR expression in P19 cells. JNK inhibition by SP activates p38 MAPK which increases the phospho-NF-κB p65 subunit at the nucleus and leads to an increased transcriptional activation of MOR gene.

and p38 MAPK pathways can potentially synergize to induce AP1 transcriptional activity, several studies have shown antagonism between these pathways [49]. For example, genetic ablation or chemical inhibition of p38 MAPK has been shown to activate JNK [49,50], and JNK inhibition by SP has been shown to activate p38 MAPK [37]. Therefore, the opposing regulation of these two pathways observed in our system should exist at the level of an upstream MAP3K which is supported by the fact that upstream kinase mediated phosphorylation of p38 MAPK is not inhibited by SB [51]. Correspondingly, SB pretreatment had no effect on the phosphorylation status of p38 MAPK elicited by SP treatment (supplementary Fig. B). In a different study, TNF-α-mediated human MOR gene expression in immune and neuronal cells involved NF-κB binding; independent of AP-1 activity [18]. JNK activation, an important event for AP-1 mediated transcription, involves dimers of the c-Jun and fos family of transcription factors. In line with this concept, our results showing the p38 MAPK- and NF-κB- dependent expression of the MOR gene upon JNK inhibition agree with the findings that NF-κB but not AP-1, is involved in the regulation of human MOR gene. It has been reported that in multiple myeloma cells, SP can induce NF-κB activation in a dose-dependent manner, associated with phosphorylation of IkappaB kinase alpha (IKKalpha) and degradation of IkappaBalpha [52]. Contrary to this finding, we observed that the phosphorylation of IkappaBalpha did not increase beyond baseline level upon SP treatment (Supplementary Fig. A). In addition, the NF-κB subunit p65 protein is constitutively present (Fig. 5C), and p38 MAPK-dependent phosphorylation of p65 specifically increased in the nucleus of P19 cells upon SP treatment (Fig. 5D), suggesting a different mode of NF-κB activation in our system. Since p38 MAPK does not contain a consensus phosphorylation site within p65; it, therefore, seems probable that other secondary kinases may be involved in the p65 phosphorylation. One of them may be the PI-3K/AKT. Although the levels of activated AKT did not change upon SP treatment (Fig. 4A), the level of MOR expression was reduced by ~30% in wortmannin pretreated cells (Fig. 4B), and PI-3K/AKT inhibition by LY treatment reduced the basal MOR expression levels in P19 cells (Fig. 1A) suggesting PI-3K/AKT as one of the possible candidates that manifest the NF-κB transactivation activity in P19 cell system. In support of this notion, previous reports have shown that the transactivation ability of p65 is stimulated by the cross-talk between PI-3K/AKT/p38 MAPK pathways [53,54]. Alternatively, nuclear kinase of MAPKs, MSK1 may be considered another kinase that regulated p65 transactivation. As shown in Fig. 4A, besides sustained increase of p38 MAPK activation, ERK activation was gradually increased after initial downregulation upon SP treatment. Both of these kinases are well-known to activate nuclear kinase MSK1 [42,55],

which in turn phosphorylates p65 at Ser 276 to enhance its transactivation potential. In addition, MSK1 is also known to phosphorylate histone proteins that aids in the modification of chromatin structure and facilitate the binding of transcription factors [56]. Although we did not observe a dramatic increase in Ser 276 phosphorylation by SP treatment, the interaction of p65 with active MSK1 was found to be increased in the nucleus of SP-treated cells (Fig. 6B). It may be possible that the basal phosphorylation of p65 at Ser 276 associated with the histone modifications by MSK1 co-operated for MOR gene expression. Currently, we are performing experiments to determine whether activated MSK1 is able to modify chromatin structure at the MOR gene promoter and increase MOR expression. Apart from this, it is also possible that post-translational modification of p65 such as acetylation, and p65 phosphorylation by kinases such as protein kinase A (PKA), protein kinase C ζ (PKCζ), casein kinase 2 (CK2), and ribosomal S6 kinase (RSK1) at different serine residues such as Ser 311, Ser 529 [57] may have accounted to increase MOR gene expression. Nonetheless, the definitive role of p65 in MOR gene expression upon SP treatment of P19 cells is established by the fact that knock-down of p65 expression blocked MOR gene expression (Fig. 5E). Among the six putative NF-κB binding sites on the human MOR gene promoter, three sites located at −2194, −557 and −207 positively regulate MOR expression in response to TNF-α [18]. However, results from the luciferase assay in our system using the core-promoter region (−450 bp/+1) indicated an ~2.5-fold activation (Fig. 5A, lower panel), suggesting that the proximal NF-κB site is enough to increase the MOR gene expression in response to SP treatment. Although it is not analyzed in this study, it is possible that transcription factors such as SOX-18, SP1, and PCBP1 (which were shown to be dependent on the AKT and p38 MAPK pathways in response to cycloheximide treatment in P19 cells) bound to a different region of the MOR promoter and co-operated to increase MOR expression upon JNK inhibition [20]. Nevertheless, the expression of the MOR gene by JNK inhibition is important in the context of pain perception and opioid antinociception, as discussed below.

Among the MAPK family members, ERK and p38 MAPK have well-documented roles in regulating neuronal plasticity and nociceptive pathway *via* glial and neuronal mechanisms [21,58]. In recent years, JNK activation, apart from its known involvement in cell proliferation, differentiation and inflammatory responses, has gained an attention towards nociception and opioid systems [49]. For example, transient activation of JNK can be seen in DRG neurons followed by a persistent activation in the spinal cord after spinal nerve ligation [21]. Also, NMDA receptor-dependent phosphorylation of ERK, p38 MAPK and JNK in the spinal cord and DRG occurs in rats with streptozotocin-induced diabetes, and the mechanical hyperalgesia associated with diabetic neuropathic pain can be reversed by blocking MAPK or with an NMDA antagonist [59]. Additionally, chronic morphine treatment activates JNK in SH-SY5Y cells [24,25], T cells [26], and spinal cord [27]. In a rat model, single or chronic morphine injections induce JNK-3 mRNA in the frontal cortex, and following cessation of morphine treatment, sustained elevation of JNK-3 is found in the hippocampus and thalamus, where the chronic morphine treatment previously have had no effect [28]. Moreover, receptor signaling is disrupted by activated JNK in response to long-acting κ opioid antagonist [60] as well as MOR desensitization, and acute analgesic tolerance to morphine and related opiates is blocked by JNK inhibition [27,29]. Collectively, these results indicate that JNK activation has a negative role in neuropathic pain prognosis and opioid pharmacology. JNK inhibition, therefore, seems to have two positive effects on opioid therapy and neuropathic pain management. First, in the case of morphine tolerance observed by receptor densensitization and downregulation, a continuous supply of newly synthesized receptors by JNK inhibition may transmit adequate antinociceptive signals. In support of this notion, a recent study demonstrated that acute morphine tolerance can be avoided by inhibiting JNK [29]. Second, based on the observation that JNK is activated in neuropathic pain models and that JNK inhibition can reverse pain hypersensitivity, blocking JNK activation is likely to exert positive effects on long-term pain management. It remains to be determined, how/whether intrinsic pain response factors are modulated by JNK.

In conclusion, we demonstrate that JNK inhibition stimulates MOR gene expression and that this stimulation is blocked by inhibitors of p38 MAPK and NF-κB pathways. The results of our study suggest that because inhibition of p38 MAPK and NF-κB contribute to the downregulation of MOR expression levels in differentiating neuronal cells, strategies to activate p38 MAPK and NF-κB in the neurons, possibly by including JNK inhibitor in an opioid treatment regimen, could be used to increase receptor numbers and the effectiveness of opioid treatment.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.02.017.

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