Yin Yang 1 Phosphorylation Contributes to the Differential Effects of μ-Opioid Receptor Agonists on MicroRNA-190 Expression*

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μ-Opioid receptor regulates microRNA-190 (miR-190) in an agonist-dependent manner; fentanyl, but not morphine, decreases the miR-190 level in rat primary hippocampal neuron cultures and in mouse hippocampi. In this study, the correlation between the cellular content of miR-190 and the mRNA level of its host gene, talin2, suggested that fentanyl decreases the miR-190 level by inhibiting the transcription of talin2. Fentanyl-induced β-arrestin2-mediated ERK phosphorylation led to the phosphorylation of Yin Yang 1 (YY1). In addition, YY1 phosphorylation impaired the association of YY1 with the −208 to −200 region on the Talin2 promoter, and this association was essential for YY1 to stimulate the transcription of talin2. Thus, fentanyl decreased the transcription of talin2 and subsequently the cellular level of miR-190 by inducing YY1 phosphorylation. In contrast, because morphine induces ERK phosphorylation via the protein kinase C pathway, morphine did not induce YY1 phosphorylation and had no effect on the transcription of talin2 and the cellular content of miR-190. This study therefore delineates a signaling pathway that mediates the effects of fentanyl on miR-190 expression.

miR-190 expression is differentially regulated by μ-opioid receptor (OPRM1) agonists. Chronic treatment with fentanyl results in the down-regulation of miR-190 in rat primary hippocampal neuron cultures and mouse hippocampi (7). This down-regulation can be blocked when fentanyl-induced ERK phosphorylation is attenuated. However, morphine does not affect miR-190 expression, even though it also induces ERK phosphorylation (7). As reported previously, fentanyl induces ERK phosphorylation in a β-arrestin2-dependent manner, and the phosphorylated ERK translocates into the nucleus, whereas morphine utilizes a protein kinase C (PKC) pathway, and the phosphorylated ERK are retained in the cytosol (10). Knocking down β-arrestin2, but not using PKC inhibitor, attenuates the fentanyl-induced decrease in miR-190 expression, indicating the decrease in miR-190 expression results from β-arrestin2-mediated ERK phosphorylation (7). Thus, the different mechanisms utilized by the two agonists to activate ERK account for their different abilities to control miR-190 expression. Nevertheless, how phosphorylated ERK regulates the expression of miR-190 remains unknown.

An miRNA is initially transcribed as part of a much longer primary transcript (11). This primary miRNA transcript then undergoes extensive processing to generate an ~22-nucleotide mature miRNA. Although the processing of primary transcript is under tight regulation, the initial transcription is a potential checkpoint in the miRNA expression (12). Over 50% of mammalian miRNAs are located within the intronic regions of annotated genes (13), suggesting that the expression of these miRNAs might be controlled by the promoters of their host genes. Because miR-190 has potentially important central nervous system functions (7), the mechanism regulating its expression was investigated. miR-190 is conserved and located in the intronic regions of the talin2 gene in the mouse, rat, and human genomes (14). The transcript of talin2 is detected with higher levels in the heart and brain than in other tissues (15). The transcription regulation of talin2 could modulate the overall miR-190 level within the hippocampal neurons. It was therefore hypothesized that fentanyl regulates the cellular content of miR-190 at the transcriptional level. This study examined whether the Talin2 promoter controlled the expression of miR-190 and how it contributed to the fentanyl-induced decrease in miR-190 levels.

EXPERIMENTAL PROCEDURES

Cell Culture and Gene Expression—HEKHM cells were cultured in Eagle’s minimum essential medium supplemented
with 10% fetal bovine serum and 200 ng/ml G418 and transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instruction.

Primary culture of hippocampal neurons from mice and rats were prepared as described previously (16). Treatments began on the 21st day after plating when the primary cultures were mature. Exogenous gene expression in these mature neurons was achieved by lentivirus infection. YYdn and YYup were generated by using the lentiviral miRNA Expression System (Invitrogen). YYdn was generated following the manufacturer’s instruction. It was constructed onto V5-DEST (Invitrogen) and expressed an miRNA that targets YY1 mRNA (5'-GAGCAUAGCUUGCCCUCAUA-3'). The sequence of this small RNA was designed by using the software provided by Invitrogen. Vector (control) was also constructed onto V5-DEST by inserting 5'-TGCTAGAGACCAGATTACCCGGGTGTAATGGT-CTCA-3' and expressed unrelated small RNA. YYup was generated by inserting the YY1 cDNA (a gift from Dr. Shi Yang, Harvard University) between the SpeI and XhoI sites of the vector, YYdn, and YYup, respectively, together with pLP1 and pLP2 and pLP-VSVG (Invitrogen). Titers of the viruses (respectively, 10^6 transducing units/ml) were determined in neuroblastoma N2A cells as described in the manufacturer’s instruction. Primary cultures were incubated with lentiviruses for 3 days before further analysis. Infection efficiency was at least 60%. The MEK1/2 inhibitor U0126 and the PKC inhibitor Ro-31-8425 were purchased from EMD Biosciences (San Diego).

**Immunoblotting and Immunoprecipitation**—Immunoblotting and immunoprecipitation were performed as described previously (10). Briefly, protein concentrations were determined by BCA assay (Pierce) to ensure that equal amounts of protein were loaded onto each lane. Membranes were developed using ECF substrate (GE Healthcare). The fluorescence intensity of each band was determined using a Storm 860 system and ImageQuant analysis software (GE Healthcare).

Cells were separated into three equal aliquots. One aliquot was used to prepare the nuclear extract by using the NE-PER nuclear and cytoplasmic extract kit (Pierce). The nuclear extract was mixed with antibody against YY1 for 6 h and then with protein G beads (Invitrogen) overnight at 4 °C. The proteins bound to the beads were extracted by using SDS-PAGE sample buffer after washing the beads twice with lysis buffer. Phosphorylation of YY1 was determined by measuring the amounts of phosphorylated amino acids in the immunoprecipitated YY1 using YY1 antibody. Another aliquot was used to measure the total YY1 and total NeuroD after nuclear extraction. The last aliquot was used for whole cell lysis using the same kit, in which the phosphorylated ERK and total ERK were determined.

The antibodies against phosphorylated ERK, total ERK, NeuroD, and YY1 were purchased from Cell Signaling (Danvers, MA). The antibody against phosphorylated amino acids was from Invitrogen. The second antibody with alkaline phosphatase-conjugated streptavidin was from Bio-Rad.

**Chromatin Immunoprecipitation**—After washing three times with PBS at 4 °C, cells were fixed with 1% formaldehyde in PBS for 15 min at 25 °C. A final concentration of 0.125 M glycine was used to terminate the fixation. Cells were resuspended in 400 μl (for 100-mm dish) of ChIP lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors). Samples were sonicated by using Sonicator Cell Disruptor model W-220F (Heat Systems-Ultrasonic, Inc., Plainview, NY) at the output level 6 for 120 s (15 s rest between 15-s sonication). After 5 min of centrifugation at 13,000 × g at 4 °C, the supernatant was incubated with the antibody against YY1 with 10% saved as input control. Then the samples were rotated in a cold room for 6 h. Protein G-agarose beads with salmon sperm DNA were added to the samples, which were then rotated for another 12 h. The protein G-agarose beads were then washed sequentially and twice individually with ChIP lysis buffer, high salt ChIP lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors), ChIP wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA), and TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). 75 μl of elution buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA) was added to the beads and incubated at 65 °C for 10 min. This step was repeated twice, and the collected elution buffer was incubated at 65 °C for another 6 h. The input controls were added with elution buffer to 150 μl and incubated at 65 °C for 6 h. DNA was then purified with QIAquick PCR purification kit (Qiagen, Valencia, CA) and used in further PCR analysis.

**Electrophoretic Mobility Shift Assay**—The −219 to −189 region on the rat Talin2 promoter was used as the probe (5’-GGAGTATATGTTGCAATTTCTCGAATTAGGA-3’), which was labeled with biotin by using biotin 3’ end DNA labeling kit (Pierce). A consensus binding sequence (5’-CTTCGAGTAAC-GCCATTTTGCAGGCAT-3’) of YY1 was used as a competitive probe (17). Nuclear protein was extracted by using the NE-PER nuclear and cytoplasmic extract kit (Pierce). The EMSA procedures were followed exactly as described in the LightShift chemiluminescent EMSA kit instructions (Pierce). Briefly, EMSA was performed using a 6% polyacrylamide gel and a positive charged nylon membrane (Pierce). Alkaline phosphatase-conjugated streptavidin was used to detect the biotin-labeled probe (Invitrogen).

**Luciferase Reporter Assays**—The luciferase assay system was purchased from Promega (Madison, WI). Talin2 promoters of different lengths were inserted into the promoter region of luciferase in pGL3-Basic vector (PGL3BASIC, Promega). Mutations were prepared by using the site-direct mutagenesis kit from Stratagene, La Jolla, CA. The luciferase constructs and pRL-tk-luc, which encodes Renilla luciferase under the control of the thymidine kinase promoter, were transfected into HEK cells. Luciferase activities were determined using a Dual-Luciferase assay kit (Promega). Cells were extracted and assayed sequentially for firefly and Renilla luciferase activities. Firefly activities were normalized against Renilla luciferase activity and used to determine the luciferase activity.

**Real Time PCR**—mRNAs together with miRNAs were extracted with miRNeasy mini kit (Qiagen) and reverse-transcribed with miScript reverse transcription kit (Qiagen). Real time PCR was carried out by using the miScript SYBR Green.
YY1 Regulates miR-190 Expression

PCR kit (Qiagen). Primers used were as follows: Talin2, 5'-CAATCTTTGTCGTCCACCCCCAGAAGG-3' and 5'-GTCATTCACACTGCTGTCAGAACC-3'; NeuroD, 5'-CTCTGATGCATCTCCTACC-3' and 5'-AGGAGGGGCTGTTGCAATCACTGTTAGG-3'; YY1, 5'-CATCCTGACACCCAGGCTCCAGATGTC-3' and 5'-GCCAGTGAGCTTCCCGTTCAGC-3'; BACE1, 5'-CACAGGCTTCCCTCATTACCACCATCCTCCCTCCCAGCAGTTCGCC-3' and 5'-GGGCCCCCTCAGCTCGCCATTTGCCG-3'; DBH, 5'-CCTCAGGCTTCTCGTTCCCCAGCAGTTCGCC-3' and 5'-GGGTTCTTCCACTGCAGGAATGTCTTCTAGG-3'; glyceraldehyde-3-phosphate dehydrogenase, 5'-CTCTGACACCCAGGCTCCAGATGTC-3' and 5'-GCCAGTGAGCTTCCCGTTCAGC-3'; ChIP, 5'-AGGAGGGGCTGTTGCAATCACTGTTAGG-3' and 5'-GAAA-GTCATTCACTGCTCGTCAAGACC-3'; and miR-190, 5'-TGATATGTATATATAGGT-3'.

Statistical Methods—Experiments were repeated at least four times. Data were analyzed by one-way analysis of variance with Dunnett test as post hoc test to do comparisons, except for Figs. 1, C and D, 2B, 3C, and 4B in which the two-way analysis of variance and Bonferroni-test were used. The error bars and * presented the standard deviations and statistical significance, respectively.

RESULTS

Effects of Fentanyl on Talin2 mRNA Parallel Those on miR-190—If miR-190 is a product from the Talin2 transcript, a correlation between the Talin2 mRNA and miR-190 levels should exist. Hence, the Talin2 mRNA level was monitored by real time PCR in rat primary hippocampal cultures after treatment with fentanyl or morphine (Fig. 1A). A significant decrease in Talin2 mRNA was first observed 24 h after the initiation of fentanyl treatment and persisted for at least 72 h. In contrast, morphine did not alter Talin2 mRNA levels at any time point (Fig. 1A). Dose-dependent effects of agonists on the Talin2 mRNA level were also determined. Fentanyl decreased the Talin2 mRNA level at 1, 10, and 100 nM, although morphine did not result in a decrease at any concentration tested (Fig. 1B). Thus, there exists a direct correlation between Talin2 mRNA and miR-190 expression under these conditions (7).

To further evaluate the relationship between the agonist-induced decrease in Talin2 mRNA and miR-190 levels, primary hippocampal cultures from rats were treated with agonists after incubation with the nonselective opioid receptor antagonist naloxone or the OPRM1-specific antagonist Cys2-Tyr3-Orn7-Pen7-amide (CTOP). Both antagonists abolished the inhibitory effects of fentanyl on Talin2 mRNA (Fig. 1C). However, when δ-opioid receptor-specific antagonist H-Tyr-Tic[6(CH2)NH]Phe-Phe-OH was used, a fentanyl-induced decrease in Talin2 mRNA was still observed. Therefore, although δ-opioid receptor was present in the hippocampus, fentanyl decreases the level of Talin2 mRNA via the activation of OPRM1.

The roles of protein kinases in regulating the Talin2 mRNA level were investigated. Inhibitors of protein kinase C (Ro-31-8425) did not block the inhibitory effects of fentanyl on Talin2 mRNA (Fig. 1C). However, U0126, which blocks ERK phosphorylation by inhibiting MEK1 and MEK2, prevented the fentanyl-induced decrease in Talin2 mRNA (Fig. 1C). These results are consistent with our earlier observations that miR-190 reduction by fentanyl can be blocked by naloxone, CTOP, and U0126 but not by Ro-31-8425 (7). Therefore, the miR-190 cellular level is directly correlated with the transcription of talin2.

Fentanyl-induced decrease in miR-190 expression results from the β-arrestin2-mediated ERK phosphorylation (7). Thus, the relationship between the decrease in Talin2 mRNA and β-arrestin2-mediated ERK phosphorylation was investigated. In addition, morphine induces ERK phosphorylation through the PKC pathway, whereas fentanyl functions in a β-arrestin2-dependent manner (10). Ro-31-8425 was used to block the morphine-PKC pathway, although primary hippocampal cultures from β-arrestin2−/− mice were used to test the fentanyl-β-arrestin2 pathway. In the primary hippocampal culture from wild type mice, fentanyl reduced Talin2 mRNA levels by 35 ± 4% (n = 4, Fig. 1D), which is similar to that induced by fentanyl in rat primary cultures (32 ± 7%, n = 4, Fig. 1A). This reduction could be blocked by U0126 but not by Ro-31-8425 (Fig. 1D). In the primary hippocampal culture from β-arrestin2−/− mice, fentanyl did not decrease Talin2 mRNA expression (Fig. 1D). This lack of fentanyl effect on Talin2 mRNA level parallels the absence of fentanyl effect on the miR-190 level in the β-arres-
Inhibitory Effects of Fentanyl

YY1 Regulates miR-190 Expression

The last 500 bp of the rat Talin2 promoter is 84% identical to that of mouse Talin2 promoter and 55% identical to that of the human Talin2 promoter. Regions longer than 20 bp that are conserved in mouse and rat Talin2 promoters were aligned with the human Talin2 promoter. Several conserved regions were then mutated individually in TLNP500, and their abilities to initiate luciferase expression were assessed. One of the mutants, TLNP500mu which has the −200 region on the rat Talin2 promoter is 84% identical to the human Talin2 promoter, exhibits reduced luciferase activity (Fig. 2B), indicating the −200 to −200 region functions as an activation domain for the Talin2 promoter. In addition, TLNP500mu did not respond to the fentanyl treatment, unlike its wild type counterpart (Fig. 2B). The −200 to −200 region is therefore essential for inhibitory effects of fentanyl.

To determine whether the inhibitory effects of fentanyl on Talin2 promoter activity in HEKHM cells depend on the ERK phosphorylation, U0126 was used to treat the cells for 3 h before agonist treatment. As indicated in Fig. 2B, U0126 attenuated the fentanyl-induced decrease in the luciferase expression from TLNP2000, TLNP1500, TLNP1000, and TLNP500. The results further demonstrated that the ERK pathway is used by fentanyl to decrease Talin2 transcription in HEKHM cells.

−200 to −200 Region on Talin2 Promoter Mediates the Inhibitory Effects of Fentanyl—The transcription of Talin2 is controlled by a promoter region about 2 kb long upstream of the translation initiation site (18). To examine whether fentanyl could regulate the activities of this promoter region, the promoter sequence from rat talin2 was cloned into the promoter region of a firefly luciferase reporter (PGL3BASIC) to generate TLNP2000 (Fig. 2A). To assess the functional region of this promoter, several deletions (TLNP1500, TLNP1000, and TLNP500) were also generated (Fig. 2A). These constructs were introduced into human embryonic kidney cells that stably expressed the hemagglutinin-tagged OPRM1 (HEKHM), and luciferase expression was determined. The full-length promoter enhanced the luciferase expression by 46 ± 8-fold (TLNP2000, n = 4) that from PGL3BASIC (Fig. 2B), demonstrating that the promoter was functional in HEKHM cells. TLNP500 resulted in the highest luciferase expression among these constructs, whereas the expression from TLNP1500 and TLNP1000 was lower than that from TLNP2000 (Fig. 2B).

The HEKHM cells transfected with TLNP2000 were treated with morphine or fentanyl for 36 h. A lower expression of luciferase was detected in fentanyl-treated cells, when compared with that in control cells (Fig. 2B). However, morphine treatment did not affect the luciferase expression (Fig. 2B). This decrease (29 ± 2%, n = 4) was similar to the fentanyl-induced decrease in the Talin2 mRNA level in the primary cultures (36 ± 8%, n = 4; Fig. 1A), suggesting the pathway through which fentanyl controls Talin2 transcription exists in the HEKHM cells. Because the luciferase expression from cells transfected with TLNP1500, TLNP1000, and TLNP500 was also reduced by fentanyl (Fig. 2B), the last 500 bp of Talin2 promoter contains a functional domain that mediates the inhibitory effects of fentanyl.

To determine whether the inhibitory effects of fentanyl on Talin2 promoter activity in HEKHM cells depend on the ERK phosphorylation, U0126 was used to treat the cells for 3 h before agonist treatment. As indicated in Fig. 2B, U0126 attenuated the fentanyl-induced decrease in the luciferase expression from TLNP2000, TLNP1500, TLNP1000, and TLNP500. The results further demonstrated that the ERK pathway is used by fentanyl to decrease Talin2 transcription in HEKHM cells.

Yin Yang 1 (YY1) Regulates the Activity of Talin2 Promoter—The −200 to −200 region on the rat Talin2 promoter is conserved in mouse and human talin2 genes and contains a binding site for YY1 (Fig. 2A). YY1 is a highly conserved and ubiquitously expressed transcription activator/repressor and binds to the consensus DNA recognition sequence 5′-Ctg/a(G/t)(C/t/a)CATN(T/a)(T/g/c)-3′ (19). To study the role of YY1 in talin2 regulation, YYup and YYdn plasmids were prepared as described under “Experimental Procedures.” YYup overexpressed YY1 cDNA, whereas YYdn suppressed the expression of YY1 by expressing an miRNA-like small RNA targeting YY1 mRNA. Introduction of YYup or YYdn plasmids into the HEKHM cells significantly modulated the mRNA and protein levels of YY1 (Fig. 3, A and B).
YY1 Regulates miR-190 Expression

The two constructs were then transfected into the HEKHM cells together with PGL3BASIC, TLNP500, or TLNP500mu. YYup increased the luciferase expression from TLNP500, whereas YYdn decreased it (Fig. 3C). However, the luciferase expression from PGL3BASIC and TLNP500mu was not affected by YYup and YYdn (Fig. 3C). Hence, YY1 activates Talin2 promoter at the −208 to −200 region.

YY1 Contributes to the Inhibitory Effects of Fentanyl—To examine the role of YY1 on fentanyl-induced talin2 transcription inhibition, HEKHM cells were co-transfected with TLNP500 and a control vector. Cells were then treated with PBS (control), 10 nM fentanyl, or 1 μM morphine for 36 h. Fentanyl, but not morphine, induced a 32 ± 4% (n = 4) decrease in the luciferase activity in cells transfected with the TLNP500 construct (Fig. 4A). This decrease was plotted as the 2nd bar in Fig. 4B.

Then fentanyl-induced decrease was determined in HEKHM cells transfected with YYup + TLNP500 or YYdn + TLNP500 and treated with the agonist accordingly. As summarized in Fig. 4B, the fentanyl-induced inhibition of luciferase activity was enhanced to 60 ± 10% (n = 4) when YYup was co-transfected with TLNP500, whereas the inhibition was decreased to 12 ± 7% (n = 4) when YYdn was co-transfected with TLNP500. Thus, by regulating the YY1 level, the fentanyl-induced inhibition of the Talin2 promoter activity can be modulated. This suggests that YY1 mediates the inhibitory effects of fentanyl on talin2 transcription.

When PGL3BASIC and TLNP500mu were used to transfected HEKHM cells instead of TLNP500, no decrease in the luciferase expression was detected after fentanyl treatment (Fig. 4B). In addition, when the HEKHM cells transfected with TLNP500 were pretreated with 2 μM U0126 prior to agonist treatment, the fentanyl-induced decrease in the luciferase activity was not observed (Fig. 4B). Therefore, fentanyl-induced decrease in Talin2 promoter activity is the direct result of agonist-induced ERK phosphorylation.

YY1 Regulates miR-190 Expression in the Primary Hippocampal Cultures—Our initial observations indicated that fentanyl attenuates the miR-190 level in primary hippocampal neuron cultures (7). Hence, we decided to examine whether the fentanyl-induced decrease in miR-190 levels and the talin2 transcription (Fig. 1) in hippocampal neurons are mediated by YY1, paralleling the HEKHM observation. Lentiviruses (YYup-vir and YYdn-vir) were then constructed and used to regulate YY1 expression in the rat primary hippocampal cultures. As indicated in Fig. 5, A and B, YYup-vir increased the levels of YY1 mRNA (412 ± 87%, n = 4) and YY1 protein (187 ± 12%, n = 4),...
YY1 Regulates miR-190 Expression

Fentanyl-induced ERK Activation Leads to YY1 Phosphorylation—Fentanyl-induced decrease in talin2 transcription and miR-190 levels could reflect the alteration in the YY1 level within the hippocampal neurons. However, when YY1 expression in rat primary hippocampal cultures was examined after agonist treatment, no significant change in the YY1 mRNA or protein level was identified during the 3-day treatment with 1 μM morphine or 10 nM fentanyl (Fig. 6, A–C). Thus, treatment with OPRM1 agonists does not affect YY1 expression in the hippocampal neurons.

YY1 undergoes extensive post-translational modifications, including phosphorylation, acetylation, and caspase-dependent cleavage (20). Because it has been suggested that YY1 phosphorylation modulates its ability to bind DNA (17), phosphorylated YY1 was quantified after agonist treatment. The phosphorylation of YY1 began to increase 10 min after the initiation of fentanyl treatment, peaked between 30 and 60 min, gradually decreased after 60 min, and returned to the basal level by 48 h (Fig. 6B). Fentanyl-induced ERK phosphorylation was also monitored. The amount of phosphorylated ERK reached its highest level within 10 min and returned to the basal level after 1 h (Fig. 6A). Thus fentanyl-induced ERK phosphorylation precedes fentanyl-induced YY1 phosphorylation. Although morphine similarly induced ERK phosphorylation, it did not increase YY1 phosphorylation (Fig. 6A).

In primary hippocampal cultures from wild type mice, 1 h of fentanyl treatment induced significant YY1 phosphorylation, which was not affected by the PKC inhibitor but was attenuated by pretreating the primary cultures with 2 μM U0126. However, no increase in YY1 phosphorylation was observed in primary cultures from β-arrestin2−/− mice (Fig. 6C). Therefore, YY1 phosphorylation results from fentanyl-induced β-arrestin2-mediated ERK phosphorylation.

YY1 Phosphorylation Impairs Its Binding to Target DNA—YY1 has been reported to enhance the transcription of β-site precursor protein-cleaving enzyme 1 (BACE1) and DBH in

whereas YYdn-vir had the opposite effects (mRNA, 43 ± 13%, n = 4; protein, 56 ± 11%, n = 4).

Similar to our observation in HEKHM cells, consistent changes on Talin2 mRNA and miR-190 in the primary hippocampal neuron cultures were observed after modulating YY1 expression with the lentiviruses. YYup-vir increased Talin2 mRNA and miR-190 content are similarly regulated by YY1 in hippocampal neurons. To demonstrate that YY1 also regulates the miR-190 function as predicted, the expression of NeuroD, a previously identified target of miR-190 (7), was monitored in the hippocampal neurons. YYup-vir infection decreased the levels of NeuroD mRNA (42 ± 7%, n = 4) and NeuroD protein (61 ± 5%, n = 4), whereas YYdn-vir infection increased both the mRNA (181 ± 8%, n = 4) and protein (153 ± 8%, n = 4) of NeuroD in the primary hippocampal neuron cultures.

FIGURE 6. Fentanyl induces YY1 phosphorylation. A and B, primary hippocampal cultures from rat were treated with 1 μM morphine (A) or 10 nM fentanyl (B) for the indicated times. Phosphorylated YY1, phosphorylated ERK, total YY1, and total ERK were determined by immunoblotting (IB) as described under “Experimental Procedures.” The results were normalized against those at 0 h in each group. IP, immunoprecipitation. C, primary hippocampal cultures from rats were treated as in A. The mRNA levels of YY1 were determined. D, primary hippocampal cultures from wild type mice were treated with 1 μM morphine or 10 nM fentanyl for 1 h after a 3-h pretreatment with PBS, 2 μM U0126, or 4 μM Ro-31-8425. Primary hippocampal cultures from β-arrestin2−/− mice were treated with 1 μM morphine or 10 nM fentanyl for 1 h. The phosphorylated YY1, total YY1, and total ERK were determined as in A. The results were compared with “control” in each group, and * presented the statistical significance.
YY1 Regulates miR-190 Expression

![Figure 7](image-url)

**FIGURE 7. YY1 phosphorylation impairs its association with the −208 to −200 region.** A, primary hippocampal cultures from rats were treated with 1 μM morphine or 10 nM fentanyl for 36 h with or without a 3-h pretreatment with 2 μM U0126. Mor, Fent, and U stand for morphine, fentanyl, and U0126, respectively. The mRNA levels of BACE1 and DBH were determined by real time PCR. The results were normalized against those in control samples in each group. B, primary cultures were treated as in A, except that the agonist treatment was limited to 1 h. The binding of YY1 to the −288 to −138 region in the rat Talin2 promoter was determined by real time PCR after ChIP assay. No Ab (without YY1 antibody) was used to confirm the binding is specific to YY1. Real time PCR performed for the inputs was used to confirm equal amounts of samples were used. The results were normalized against those in control samples in each group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The results were compared with “con-vir” in each group, and * presented the statistical significance. C, EMSA was performed as described under “Experimental Procedures.” L-Probe stands for the biotin-labeled probe. NE stands for the nuclear extract from the untreated primary hippocampal cultures. YY1 Ab stands for the antibody against YY1. C-Probe stands for the competitive probe. Shift represents the binding of YY1 to L-Probe. Supershift presented the association among YY1 antibody, YY1, and C-Probe. The results were normalized against that in lane 3. D, primary cultures were treated as in B. The nuclear extracts were used to perform the EMSA assay. The results were normalized against that in lane 1.

neuronal cells (21, 22). As shown in Fig. 7A, fentanyl decreased the mRNA levels of BACE1 and DBH in the primary hippocampal neuron cultures. The decrease was attenuated by U0126 pretreatment. Thus, ERK-dependent YY1 phosphorylation correlated with the reduced ability of YY1 to function as a transcriptional activator.

Although three different mechanisms have been proposed to describe how YY1 functions as a transcriptional activator (23), all of them require the binding of YY1 to the promoters of target genes. In addition, YY1 phosphorylation has been suggested to modulate its DNA binding ability (17). Thus, the fentanyl-induced YY1 phosphorylation could alter the binding of YY1 to the Talin2 promoter. The association between YY1 and the −208 to −200 region on the rat Talin2 promoter was measured by ChIP. Primers were used to amplify the −288 to −138 region in the cross-linked and immunoprecipitated YY1. As indicated by the real time PCR results in Fig. 7B, the binding of YY1 to the −208 to −200 region was reduced after fentanyl treatment in concordance with the increased YY1 phosphorylation by ERK, suggesting that YY1 phosphorylation impaired its association with target DNA.

EMSA was also performed to confirm the results obtained from the ChIP assay. An oligodeoxynucleotide probe corresponding to the −219 to −189 region of the rat Talin2 promoter was biotinylated and used in the studies. When the nuclear extract containing YY1 was added to the reaction system, a shift of the biotinylated probe was observed (Fig. 7C, lane 2). The mobility shift was the result of YY1 binding to the biotinylated probe because antibody against YY1 caused a supershift (Fig. 7C, lane 3). When an unlabeled competitive probe containing the consensus DNA recognition sequence of YY1 was added (17), the mobility shifts of the biotinylated probe were blocked (Fig. 7C, lane 4). These results show that YY1 binds to the rat Talin2 promoter.

The binding of YY1 to the −208 to −200 region was further measured using nuclear extracts of the primary cultures treated with agonists and U0126. Morphine did not affect the YY1 binding to the biotinylated probe, whereas fentanyl decreased the YY1 binding to the biotinylated probe by 47 ± 11% (n = 4). In addition, this decrease was blocked by treating the primary hippocampal neuron cultures with U0126 prior to fentanyl exposure (Fig. 7D). Again, the EMSA confirms our results with ChIP that fentanyl reduced the miR-190 level by regulating talin2 transcription via the control of YY1 phosphorylation state.

**Modulations on miR-190 Expression and Talin2 mRNA Are Delayed Effects of Fentanyl Treatment—** The decrease in miR-190 and Talin2 mRNA levels and the increase in NeuroD mRNA were first observed 24 h after the initiation of fentanyl treatment (Fig. 1A) (7). Thus, to confirm that these modifications were the delayed effects of fentanyl treatment or due to the chronic fentanyl treatment, the opioid receptor antagonist, naloxone, was used to displace fentanyl from the receptor after 1 h of fentanyl treatment. The levels of miR-190, Talin2 mRNA, and NeuroD mRNA were measured after the primary cultures were incubated with naloxone for an additional 23 h. Consistent with previous experiments, 1-day fentanyl treatment decreased miR-190 expression and the mRNA level of Talin2 but increased the mRNA level of NeuroD. Naloxone incubation had no influence on these effects of fentanyl. In addition, the phosphorylation of YY1 was measured 5 h after naloxone treatment, and no influence of naloxone on YY1 phosphorylation was identified (Fig. 8B). Thus, the abilities of fentanyl to modulate the expression of miR-190 and NeuroD were not due to the chronic treatment but were the delayed effects of acute treatment.

**DISCUSSION**

A model of how OPRM1 agonists influence miR-190 expression was proposed (Fig. 9). Morphine induces ERK phosphory-
YY1 Regulates miR-190 Expression

YY1 not only regulates cell proliferation, differentiation, and survival but also plays critical roles in the central nervous system. Knock-out of yy1 in mice leads to embryonic lethality, whereas a small number of yy1 heterozygotes display neuronal defects (34). YY1 can function both as a transcription activator and as a repressor. YY1 is a known phosphoprotein, but how phosphorylation influences its DNA binding and function is unclear (17, 35). Our current studies indicate that YY1 activates talin2 transcription, which is in turn inhibited by fentanyl- and ERK-dependent phosphorylation of YY1. Reduced talin2 promoter binding by YY1 under conditions of increased YY1 phosphorylation was observed, suggesting that phosphorylation prevents the association between YY1 and its binding site.

The relationship between YY1 phosphorylation and its DNA binding ability is not clear, as both positive and negative results have been reported (17, 35). However, because these results were generated by using potato acid phosphatase, the phosphorylation of all eight conserved Ser/Thr residues on YY1 was affected. Because of the different locations of these Ser/Thr residues, the phosphorylation on these amino acids may influence the DNA binding ability of YY1 differentially. In addition, even the phosphorylation on the same site may have different contributions under distinct conditions. In this study, although the site of ERK-mediated YY1 phosphorylation has not been identified, the ERK-mediated phosphorylation correlates with impaired association between YY1 and rat Talin2 promoter.

How ERK activation leads to YY1 phosphorylation requires additional studies. ERK may phosphorylate YY1 directly, or the kinase itself may be one of the downstream targets of ERK.
YY1 Regulates miR-190 Expression

Interestingly, morphine induced ERK phosphorylation but not YY1 phosphorylation. Morphine and fentanyl induce ERK phosphorylation via different pathways, resulting in different subcellular locations of phosphorylated ERK (10). Fentanyl, but not morphine, induced the nuclear translocation of phosphorylated ERK. Thus, the nuclear translocation of phosphorylated ERK is critical for the YY1 phosphorylation. In addition, the abilities of β-arrestin to scaffold ERK (36), to translocate into nucleus (37), and to bind YY1 (38) suggest that ERK-mediated YY1 phosphorylation may require the involvement of β-arrestin.

As proposed previously, agonist-dependent signaling can lead to modulation of gene expression (10, 39, 40), which was further supported by the agonist-dependent regulation on YY1 phosphorylation, Talin2 transcription, and miR-190 expression. In addition, both YY1 and miR-190 have multiple targets, and thus their involvement may not be limited to the aspects reported here. For example, by regulating YY1 phosphorylation and the transcription of BACE1, fentanyl may have an influence on Alzheimer disease (41). By modulating the mRNA level of Pax6, which is also an miR-190 target, fentanyl may have different effects on neurogenesis from morphine (42). Such divergent regulation at multiple levels points to the complexity in the agonist activation of G protein-coupled receptors. This study suggests that not only the dogma that agonists activate a specific G protein-coupled receptor resulting in the same intracellular signal and responses is challenged, but also the pathway-dependent regulation of transcription activities and subsequent cascades indicates a significant obstacle in future drug development, in particular when the prolonged and repeated use of the drug is required.

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