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Studies on the Regulation of Mu Opioid Receptor mRNA Expression in SHSY-5Y Human Neuroblastoma Cells

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by

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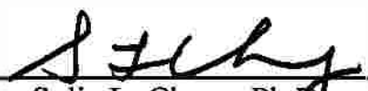

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Abstract

Prolonged exposure to morphine down-regulates mu opioid receptors (MOR) on both undifferentiated and differentiated (retinoic acid or phorbol ester treated) SHSY-5Y cells. However, morphine pretreatment does not alter MOR receptor affinity for morphine. To investigate the molecular basis for MOR regulation after exposure to its selective agonists, we have developed a quantitative competitive reverse transcriptase-polymerase chain reaction (QC-RT PCR) to quantify the expression of MOR in SHSY-5Y cells. Differentiation of SHSY-5Y cells with retinoic acid or phorbol ester up regulated MOR mRNA expression by 30 % and 78%, respectively. A 24 hours treatment with morphine (10 μM) down regulated MOR mRNA, an effect that was partially reversed by naloxone. However, after exposure to endomorphin-1, 2 for 24 hours, MOR mRNA expression is significantly increased in the differentiated and non-differentiated SHSY-5Y cells. Differences in intracellular cAMP accumulation are also observed in the differentiated and non-differentiated SHSY-5Y cells after the chronic exposure to morphine and endomorphin-1,-2. Taken together, a significant component of SHSY-5Y cellular adaptation during chronic morphine treatment may occur at the mRNA level and our data suggest different morphine or endomorphin-1, -2 mediated molecular events in the MOR receptor regulation.

1. Introduction

Opioid receptors are classified into three subtypes: mu, kappa, and delta, which was reinforced by the development of highly selective ligands for each class [Loh and Smith, 1990]. The genetic basis of the three opiate classes was identified with molecular cloning of mu, kappa, and delta opioid receptors [Chen et al., 1993; Wang et al., 1994; Evens et al., 1992; Meng et al., 1993]. Attention has now turned to receptor-mediated signaling pathways and receptor regulation. Opioid receptors belong to the G-protein-coupled receptor superfamily. Activation of opioid receptors stimulates inhibitory G proteins (G_i) that suppress the activity of adenylyl cyclase (AC), leading to decreased intracellular 3',5' cyclic adenosine monophosphate (cAMP) levels [Ueda et al., 1988]. Since mu opioid receptor (MOR) is thought to be the principal site of analgesic interaction [Raynor et al., 1995], studies of MOR offer potential molecular insight into the cellular mechanism of tolerance. The effect of chronic opiate administration through receptor regulation has turned out to be important to understand the molecular mechanism in the opioid receptor-mediated signaling pathway [Nestler et al., 1992].

Studies concentrating on the molecular basis of mu opioid receptor regulation use the SHSY-5Y cell line that is a human neuroblastoma cell line, a subclone of the SK-N-SH cell line [Kohl et al., 1980; Kuramoto et al., 1981]. It is of sympathetic adrenergic ganglial origin [Scott et al., 1986] and expresses both mu and delta opioid receptors in a ratio of 5:1 based on receptor binding studies [Yu et al., 1988]. The administration of retinoic acid (RA) or 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces a cellular differentiation of SHSY-5Y cells associated with a considerable reduction in proliferation

rate and an induction of neuritic processes [Kohring et al., 1998; Scott et al., 1986; Pahlman et al., 1990]. Pahlman et al. [Pahlman et al, 1990, 1995] have shown a series of biological and morphological differences between differentiated and non-differentiated SHSY-5Y neuroblastoma cells. It is also reported that the opioid receptor-mediated signaling pathway is associated with RA- and TPA-induced differentiation [Kohring et al., 1998]. Mu opioid receptor membrane density is enhanced in SHSY-5Y cells differentiated with either RA or TPA [Zadina et al., 1993, 1994]. Morphine, as well as the opiate peptides, endomorphin-1 and-2, are analgesic opiates selective for MOR. Both of them can induce tolerance in both differentiated and non-differentiated SHSY-5Y cells [Zadina et al., 1994, 1997; Harrison et al., 1999, 2000]. Down-regulation of MOR in SH-SY5Y cells was reported after exposure to 10 μM morphine [Zadina et al., 1993]. While chronic morphine treatment decreases MOR membrane density [Zadina et al., 1993], it may not involve internalization of MOR [Keith et al., 1996; Zhang et al., 1998]. On the other hand, endomorphin-1 and -2 bind to the MOR at the cell surface and cause rapid endocytosis of MOR [McConalogue et al., 1999]. However, the detailed molecular events underlying MOR regulation remain unclear.

In our current study, we evaluate MOR mRNA expression to delineate the molecular basis of MOR regulation in SHSY-5Y cells. A sensitive and precise method of quantitative competitive reverse transcriptase polymerase chain reaction (QC-RT-PCR) has been developed using primers derived from the human MOR sequence to measure MOR mRNA quantitatively. We concentrated on MOR mRNA expression following exposure to morphine, endomorphin-1, and -2, in the differentiated and non-differentiated SHSY-5Y cells. We also measured intracellular cAMP accumulation in the differentiated

and nondifferentiated SHSY-5Y cells after the chronic exposure to the morphine and the endomorphins. Taken together, our data suggest different morphine or endomorphins mediated molecular events in the MOR receptor regulation.

2. Materials and Methods

2.1 Chemicals and Peptides

All trans-retinoic acid (RA), 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), morphine sulfate, and naloxone were purchased from Sigma Chemical Co. (St. Louis, MO). Endomorphins-1 and-2 were obtained from Phoenix Pharmaceuticals (Mountain View, CA).

2.2 Cell Line and Culture Conditions

SHSY-5Y human neuroblastoma cells (passages 20-30) were cultured in 10 % EFN medium containing a 1:1 ratio of Eagle's Minimum Essential Medium and F-12 with 10 % fetal bovine serum (Gibco, Grand Island, NY). The cells were grown at 37° C in a humidified atmosphere containing 5% CO_2.

2.3 Differentiation and Drug Treatment

At about 65-75% confluence, SHSY-5Y cells were differentiated into a neuronal phenotype with RA or TPA as previously described (Zadina et al., 1993). Either RA (10 μMin 0.1% ethanol) or TPA (16 nM in 0.1 % ethanol) was added to the media every other day. For total RNA isolation, the cells were cultured in T25-cm^2/T75-cm^2 flasks. Cells were cultured in 24-well plates for cAMP accumulation assays. Test compounds were added to the media 24 h after the final treatment with the differentiating agent, and the cells were prepared 24 h later for either RNA isolation or cAMP assay. To avoid the metabolism of the administered peptides, the cells were switched to serum-free EFN medium that contains a 1:1 ratio of Eagle's Minimum Essential Medium and F-12 supplemented with insulin (bovine, 5 μg/ml), transferrin (human, 100 μg/ml),

progesterone (20 nM), putrescine (100 μM), and Na selenite (30 nM). Either endomorphin-1 or -2 was added with the last RA treatment for the last 24 hours of culture.

2.4 Structure and Construction of Internal Standard Primers

Construction of the internal standard was accomplished by synthesizing two oligonucleotides of approximately 75 bp each containing sequences for the T7 promoter, the MOR target gene (mRNA), a random spacer, and a housekeeping gene (beta-actin). The rcRNA (reconstructed RNA of internal standard DNA) forward primer contained the T7 promoter, MOR mRNA forward primer, random sequence, and beta-actin forward primer. The rcRNA reverse primer contained the beta-actin reverse primer, random sequence, MOR mRNA reverse primer, and a poly (dT) tail. The MOR forward primer (5'-TAC-CGT-GTG-CTA-TGG-ACT-GAT-3') was from position 962, and the reverse primer (5'-ATG-ATG-ACG-TAA-ATG-TGA-ATG-3') was from position 1103 of the genomic MOR gene (Wang et al., 1994). The beta-actin forward primer (5'-AGA-CCT-CTA-TGC-CAA-CAC-AGT-3') was from position 2753 in exon 5, and the reverse primer (5'-GAC-ACA-CCT-AAC-CAC-CGA-GAT-3') was from position 3017 in exon 6. There is a 124 bp intron E between exons 5 and 6 (Nudel et al., 1983). Therefore, the beta-actin primers will generate products of 161 bp from mRNA and 285 bp from genomic DNA. All primers were synthesized and purified by Oligo, Etc. (Wilsonville, OR).

Reactions were conducted in a final volume 50 μl containing PCR buffer, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol upper and lower rcRNA primer, 200 ng genomic

DNA from the SHSY-5Ycells or cDNA, and 1.25 units Taq DNA polymerase (AmpliTaq, Perkin-Elmer, Norwalk, CT). The reactions were heated to 94° C for 3 min, and immediately cycled 30 times through a 10-s denaturing step at 94° C, a 30-s annealing step at 59° C, and a 45-s extension step at 72° C. Following the final cycle, a 5-min extension step at 72° C was included. The PCR products were diluted 1:100 in water, and 2 µl were re-amplified using the conditions stated above. The second amplification PCR products were pooled and purified using the Magic™ Prep DNA purification system (Promega, Madison, WI). The pooled PCR products were transcribed into RNA by the T7 promoter using the Riboprobe® Gemini II *in vitro* transcription system (Promega). The DNA templates were removed by digestion with DNase I following the transcription reaction. The rcRNA was extracted according to the procedure of the Riboprobe® Gemini II *in vitro* transcription system (Promega), and quantitated by absorbance at 260 nm. Figure 1 shows the general procedure of the construction of the internal standard and the diagram of QC-RT-PCR.

2.5 Quantitative Competitive RT-PCR

Total RNA was extracted from SHSY-5Y cells with TRIzol Reagents (GIBCO Invitrogen Life Technologies, Grand Island, NY). Competitive RT-PCR was carried out with the internal standard (IS) rcRNA as the competitor as described by Vanden Heuvel et al. (1993). For each sample, 6 aliquots of SH-SY5Y total RNA (50 ng each) were prepared, and a series of 1:2 dilutions, from 0.625 pg to 20 pg, of the rcRNA internal standard (231 bp) was added to these aliquots. Reverse transcription of RNA was performed in a final volume of 20 µl. The samples were incubated at 45° C for 30 min, and the reverse transcriptase was inactivated by heating to 99° C for 5 min. To these cDNA samples, a

PCR master mixture containing PCR buffer, 1 unit Taq DNA polymerase, and 10 pmol each of the MOR primers was added to bring the final volume to 50 µl. The reactions were heated to 94° C for 3 min and immediately cycled 30 times through a 20-s denaturing step at 94° C, a 20-s annealing step at 55° C, and a 20-s extension step at 72°C. Following the final cycle, a 5 min extension step at 72° C was included. Aliquotes (15 µl) of the PCR products were electrophoresed on a 3% NuSieve®/1 % agarose gel (FMC Bioproducts, Rockland, ME), visualized by ethidium bromide staining, and analyzed using the AlphaEase™ Stand Alone Software. Quantitation of the amount of MOR mRNA present was determincd as described (Vanden Heuvel et al., 1993; Gilliland et al., 1990). By titrating the unknown amount of MOR cDNA template against a dilution series containing known amounts of corresponding internal standard template, it should quantitate the amount of MOR mRNA. After electrophoresis, bands corresponding to Internal Standard and MOR cDNA were excised and a ratio of Internal Stand and MOR cDNA was calculated. As would be predicted of competitive templates, a plot of the ratio of internal standard to MOR cDNA versus the known concentration of input internal standard is linear when plotted on a log-log scale (figure 2). At the point where MOR and internal standard products are in equivalence (i.e. ration =1.0), the starting concentration of MOR prior to PCR is equal to the known starting concentration of the internal standard. Furthermore, we can obtain the amount of the MOR mRNA from the extracted cell samples based on the known concentration of the Internal Standard rcRNA. The QC-RT-PCR for the housekeeping gene, beta-actin, was also performed with the 285 bp internal standard as shown in Figure 2.

2.6 cAMP Accumulation Assay

The SHSY-5Y cells were sub-cultured in 24-well culture plates. For endomorphin-1 and -2 pretreatment, the 10% EFN growth medium was replaced with medium containing 10 μM of either endomorphin-1 or endomorphin-2, and the cells were incubated for 24 h. After treatment, the medium was removed and replaced with 0.5 ml of EFN medium containing 0.5 mM isobutylmethylxanthine, a phosphodiesterase inhibitor, to block the breakdown of cAMP in SH-SY5Y cells, and the cells were incubated for 30 min at 37° C. The culture medium was then removed and replaced with 0.5 ml of fresh medium with or without 25 μM forskolin. The cells were transferred to 37° C for 10 min. The medium was then removed, and the cells were rinsed once with 1 ml PBS. One-half milliliter of 0.1 N HCl was then added to lyse the cells, and the monolayers were frozen at -20° C. For determination of cAMP content, the monolayers were thawed, and the intracellular cAMP level from the cell lysate in each well was measured by radioimmunoassay (RIA) [Amersham Pharmacia, Piscataway, NJ].

2.7 Data Analysis

For MOR mRNA quantification, each treatment was performed in triplicate, and duplicate gel electrophoreses were carried out. The cAMP accumulation assays were performed in duplicate on triplicate samples. MOR and beta-actin mRNA expression of each sample was measured by QC-RT-PCR, and the MOR mRNA level in each sample was normalized to the beta-actin level as follows:

$$[mRNA_{MOR}]_{normalized} = [mRNA_{MOR}] / [mRNA_{beta\text{-}actin}]$$

All statistical data are presented as mean ± SD unless other wise noted and analyzed using one way ANOVA where appropriate. Statistical significance was considered $P<0.05$. (P value of each comparison is shown in the legends of each figure.

3. Results

3.1 Effects of RA and TPA on MOR mRNA Expression

Both RA and TPA have been shown to induce differentiation of SHSY-5Y cells (Scott et al., 1986; Kohring and Zimmermann, 1998; Pahlman et al., 1990, 1995). To determine if MOR mRNA expression is altered during differentiation, SHSY-5Y cells were treated with either RA (10 μMin 0.1% ethanol) for 6 days orTPA (16 nM in 0.1% ethanol) for 4 days to induce differentiation. MOR mRNA levels were then examined and compared to undifferentiated cells cultured in either 0.1 % ethanol (EtOH) or vehicle (growth medium). The MOR mRNA level increased 1.3 fold after RA-induced differentiation (Fig. 3A) and 1.7 fold after TPA-induced differentiation (Fig. 3B) when compared to undifferentiated SHSY-5Y cells.

3.2 Effects of Morphine With and Without Naloxone Co-treatment on MOR mRNA Expression in SHSY-5Y Cells

To determine morphine's effects on the transcriptional regulation of the MOR, we examined MOR mRNA levels after chronic exposure to morphine in both undifferentiated SHSY-5Y cells and in SHSY-5Y cells induced to differentiate with RA orTPA. We observed that chronic morphine treatment (10 μM) for 24 h significantly decreased MOR mRNA levels in undifferentiated cells (0.1 % EtOH + vehicle treated) by 47%, and that co-treatment with the opioid receptor antagonist, naloxone, completely blocked morphine's effects (Fig 4A). Chronic morphine treatment also significantly decreased MOR mRNA expression in both RA- and TPA-differentiated SHSY-5Y cells by 11%, and 70%, respectively, compared to vehicle (Fig. 4B,C) These results are consistent with the binding assay data previously reported by Zadina et al. (1993). Co-

treatment with morphine and naloxone (10uM) partially blocked morphine's effects in TPA-differentiated SHSY-5Y cells (Fig. 4C), but did not block morphine's effects on MOR mRNA expression in RA-differentiated SHSY-5Y cells (Fig. 4B).

3.3 Effects of Endomorphin-1 and -2 on MOR mRNA Expression in SHSY-5Y Cells

Since endomorphins-1 and -2, two endogenous tetra peptide opiates, have been shown to have high selectivity and affinity for the MOR (Zadina et al., 1997), we next investigated whether treatment with either endomorphin-1 or -2 would affect MOR mRNA regulation. Endomorphin-1 and -2 increased MOR mRNA levels in undifferentiated SHSY-5Y cells 3.7 fold and 2.5 fold, respectively, when compared to vehicle (Fig. 5A), and also increased MOR mRNA levels in RA-differentiated SHSY-5Y cells (1.8 fold and 2.3 fold, respectively) when compared to vehicle (Fig. 5B). Co-treatment with naloxone and either endomorphin-1 or -2 completely blocked endomorphin's effects in undifferentiated SHSY-5Y cells (Fig. 5A). Interestingly, while naloxone completely blocked endomorphin-2's effects in RA-differentiated SHSY-5Y cells (Fig. 5B), it only partially blocked the effects of endomorphin-1.

3.4 The Effects of Morphine, Endomorphin-1 and -2, and Naloxone on Forskolin Stimulated cAMP Accumulation in SHSY-5Y Cells

It has been reported that chronic exposure to morphine or to endomorphin-1 or -2 can affect adenylyl cyclase activity (Avidor-Reiss et al., 1995; Monory et al., 2000). To investigate if the changes in MOR mRNA expression observed following morphine or endomorphin treatment were associated with a change in adenylyl cyclase, we examined forskolin-stimulated cAMP levels in undifferentiated SHSY-5Y cells chronically treated with morphine or with enodmorphin-1 or -2. Chronic morphine treatment resulted in a

20.1 fold up-regulation of cAMP production after forskolin stimulation as compared to only a 9.5 fold increase after vehicle treatment when compared to cAMP levels with forskolin alone (Fig. 6A). Conversely, after endomorphin-1 and -2 treatments, forskolin-induced cAMP levels were observed to be about 4.7 fold, which is significantly lower than the 8.9 fold cAMP levels after vehicle treatment when compared to cAMP levels with forskolin alone. Co-treatment with the opioid antagonist, naloxone, inhibited morphine's and endomorphin-1 and -2's effects on forskolin-stimulated cAMP accumulation, returning the stimulated cAMP levels to vehicle control levels. These results suggest that while morphine pretreatment results in a compensatory up-regulation of adenylyl cyclase activity, the endomorphins serve to suppress adenylyl cyclase activity.

4. Discussion

First, in order to establish the baseline levels of MOR mRNA expression in non-differentiated and differentiated SHSY-5Y neuroblastoma cells, we measured the MOR mRNA expression in SHSY-5Y cells with or without RA/TPA treatment. We observed that RA/TPA-induced differentiation of SHSY-5Y cells significantly increases MOR mRNA expression (Fig. 3.). To understand the differentiated phenotypes of SHSY-5Y cells induced by RA and TPA, several observations have suggested that RA did not differentiate SHSY-5Y cells sympathetically as TPA. For example, Pahlman et al. [Pahlman. et al., 1990, 1995] have shown that the biologically active phorbol ester TPA induces morphological and biochemical changes, i.e. extension of long processes, increased number of neurosecretory granules and higher concentration of noradrenalin. On the other hand, they found that RA-treated cells developed a slightly higher choline acetyltransferase activity, suggesting that they switch to a cholinergic phenotype. Moreover, many studies have been done on transcriptional regulation events of RA-induced differentiation in different cell types, some of which are related to the MOR related signaling pathway, which will be discussed later. Although the differentiated phenotypes of SHSY-5Y cells induced by RA/TPA are not exactly same as each other, they still show similar effects on the MOR mRNA expression.

Secondly, we investigated morphine's effects on the differentiated and nondifferentiated SHSY-5Y cells. It has been shown by several studies that opioid agonists that demonstrate equivalent abilities to activate MOR signaling exhibit remarkable differences in their ability to functionally desensitize MOR and induce MOR

internalization. Unexpectedly, morphine cannot perform MOR sequestration in some systems [Keith et al., 1996, Zhang et al., 1998]. However, the detailed molecular events underlying morphine's specific regulation of the MOR remain unclear. It has been reported by Zadina JE [Zadina et al., 1993, 1994] that chronic morphine treatment will down regulate MOR expression in SHSY-5Y neuroblastoma cells. Previous studies [Zadina et al., 1993] have also shown that the membrane density of MOR in SHSY-5Y cells was reduced, reaching the lowest level 24 hours after exposure to morphine. To investigate if the down regulation of MOR is involved in the transcriptional level, we measured MOR mRNA expression level after exposure to morphine for 24 hours. We found that the chronic morphine treatment decreased MOR mRNA expression in the differentiated and nondifferentiated SHSY-5Y cells, as shown in figure 4. The reduction in mRNA levels may be one of the mechanisms underlying the reduction of MOR binding sites following chronic treatment with morphine.

We also investigated the effects of endomorphin-1, -2 on the MOR mRNA expression of the SHSY-5Y cells after exposure to these opiate peptides for 24 hours. The isolated peptides endomorphin-1 and endomorphin-2 have been suggested to be endogenous ligands for the mu-opioid receptor [Zadina et al., 1997]. Endomorphic analgesia was effectively blocked by naloxone [Fischer et al., 1999, Goldberg et al., 1998]. Endomorphin-1, -2 bind MOR at the cell surface and cause rapid endocytosis with identical potency, similarly to DAMGO, an MOR-specific peptide agonist [McConalogue et al., 1999]. Endocytosis and trafficking of the MOR induced by endomorphin-1, -2 may mediate receptor desensitization and down-regulation, mechanisms that regulate cellular responsiveness to ligand stimulation [Bohm et al.,

1997]. However, endomorphin -1, -2 significantly increased the MOR mRNA expression level, but did not reduce the MOR mRNA expression as chronic morphine treatment, as shown in figure 5. One possible reason is that the opiate peptide-associated signaling pathway can increase transcriptional factor binding activity, such as Sp transcription factors [Ko et al., 1998], which may be involved in the MOR gene transcription.

From this study, the transcriptional regulation of MOR gene is shown to be a major mechanism that regulates MOR protein synthesis. The down regulation in receptor density could be due to a reduction in MOR mRNA levels. To shed light on the details of the molecular mechanisms that control MOR mRNA transcription, Loh et al. [Min et al., 1994; Ko et al., 1998] has investigated the genomic structure and the transcriptional regulation factor of promoter of MOR gene. The putative promoter region contains consensus sequences for AP-1 and AP-2 transcription factors and potential cAMP response elements, in addition to a CCAAT box and SP1/SP3 site [Min et al., 1994; Xu et al., 2001 b]. Interestingly, it has been reported that treatment with MOR specific ligands, such as DAMGO, results in enhanced binding of SP1/Sp3 to the proximal promoter of human MOR gene [Xu et al., 2001 a].

Although the agonist mediated pathway that regulates MOR mRNA transcription remains to elucidated, the RA-induced enhancement of MOR mRNA expression could be related to a series of signaling pathways and gene expression regulations, as shown by several other studies. For example, RA-induced differentiation resulted in increased levels of the inhibitory G proteins $G_i\alpha 1$ and $G_i\alpha 2$, and reduced amounts of $G_s\alpha$, while the basal adenylyl cyclase activity was slightly increased [Ammer et al., 1994]. Moreover, exposure of mice to RA resulted in significant enhancement in the expression level of

cAMP response element binding protein (CREB) in murine branchial arches [Taylor et al., 1995]. As has been reported, the RA-initiated signaling pathway is mediated by the cAMP-dependent transcriptional regulation. This pathway requires specific nuclear transcriptional factors, such as CREB-binding protein (CBP/p300) and p300/CBP-associated factor (p/CAF), which exhibits strong histone acetyltransferase (HAT) activity [Korzus et al., 1998; Kawasaki et al., 1998]. Furthermore, Inturrisi et al has recently reported that RA-induced differentiation also enhanced the MOR mRNA expression, which is associated with higher expression level of *c-fos* mRNA as well as increased AP-1 DNA binding in the SHSY-5Y neuroblastoma cells [Jenab et al., 2002]. Based on the above mentioned results, the effects of RA-induced differentiation on transcriptional factors are speculated to be involved in the up-regulation of the MOR mRNA expression. Moreover, we can speculate about a possible feedback mechanism between receptor activation and receptor regulation, as shown in Figure 7.

Because adenylyl cyclase is involved in the agonist mediated regulation of MOR, we wanted to further compare the morphine and endomorphin-1, -2 associated MOR mediated signaling pathway by detecting adenylyl cyclase activity of SHSY-5Y cells after exposure to morphine and endomorphin-1, 2. As has previously been shown by Blake et al., [1998] we also found that the chronic morphine treatment could cause an adaptive sensitization of adenylyl cyclase, and result in a compensatory upregulation of cAMP production (Fig. 6). However, chronic treatment with opioid peptides endomorphin-1, -2 doesn't induce the upregulation of forskolin stimulated cAMP accumulation, but reduces adenylyl cyclase activity (Fig. 6).

Our current studies indicated that the effects of morphine and endomorphin-1,-2 on MOR receptor regulation are mediated by distinct molecular events. To delineate these events, further investigation is needed on the specific transcriptional factors and activities involved in MOR regulation that correlate with the differential effects of morphine and endomorphin-1,-2.

5. Conclusion

1. Differentiation with either RA or TPA did not change expression of beta-actin mRNA in SHSY-5Y cells, but increased MOR mRNA expression in SHSY-5Y cells. Differentiation seems to alter how naloxone reverses morphine's regulatory effects on MOR mRNA in these cells.

2. Chronic morphine treatment decreased MOR mRNA expression in both non-differentiated and differentiated SHSY-5Y cells with either RA or TPA. Morphine has been reported not to induce MOR endocytosis in many systems, a reduction in MOR mRNA following chronic treatment with morphine may be one of the mechanisms underlying the reduction of MOR binding sites following chronic treatment with morphine.

3. Treatment with endomorphin-1 or -2 is known to induce MOR internalization. However, each of these two opioid peptide agonists increased MOR mRNA level in both non-differentiated SHSY-5Y cells and the cells differentiated with RA. Naloxone co-treatment completely reversed the effects of endomorphin-1,-2 in non-differentiated, but not in RA-differentiated SHSY-5Y cells.

4. Forskolin induction of intracellular cAMP levels was enhanced by chronic treatment with morphine in SHSY-5Y cells without differentiation. This is consistent with previous report on supersensitization of MOR activity by chronic treatment with morphine. In contrast, SHSY-5Y cells treated with either endomorphin-1 or -2 reduced significantly forskolin induction of cAMP levels.

In summary, our data suggest that morphine and endomorphins-1 and -2 have differential effects on MOR receptor regulation in the SHSY-5Y neuroblastoma cells.

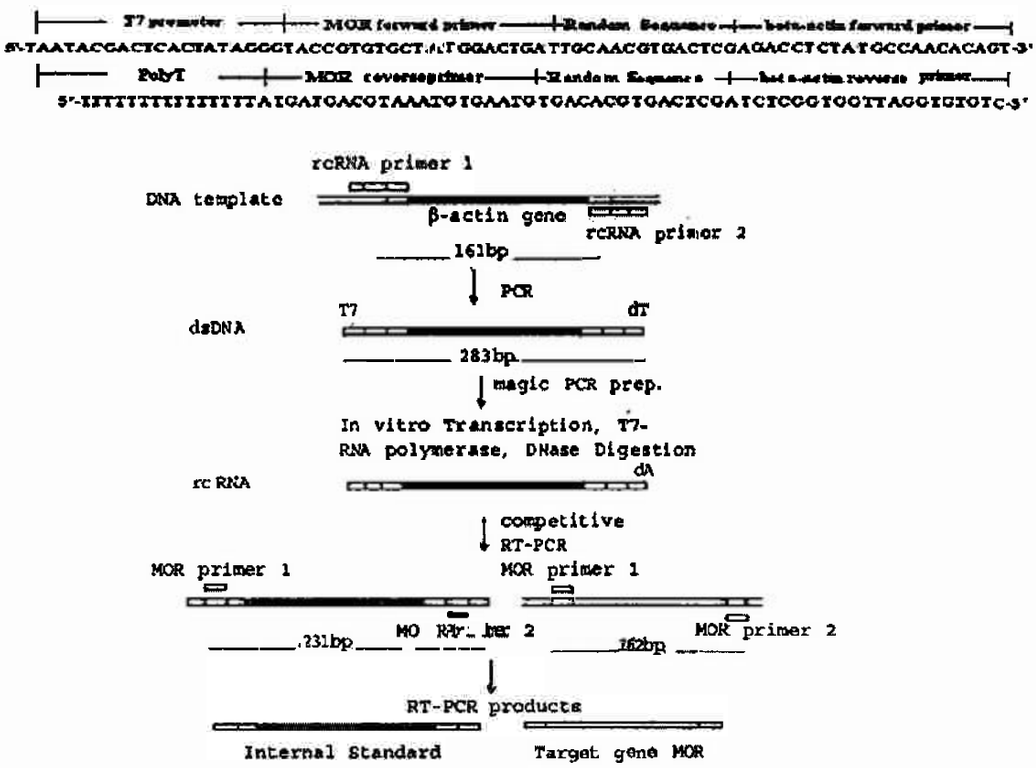

Figure 1. The internal standard (IS) primer sequence and structure. Flow chart for construction of the rcRNA internal standard and QC-RT-PCR.

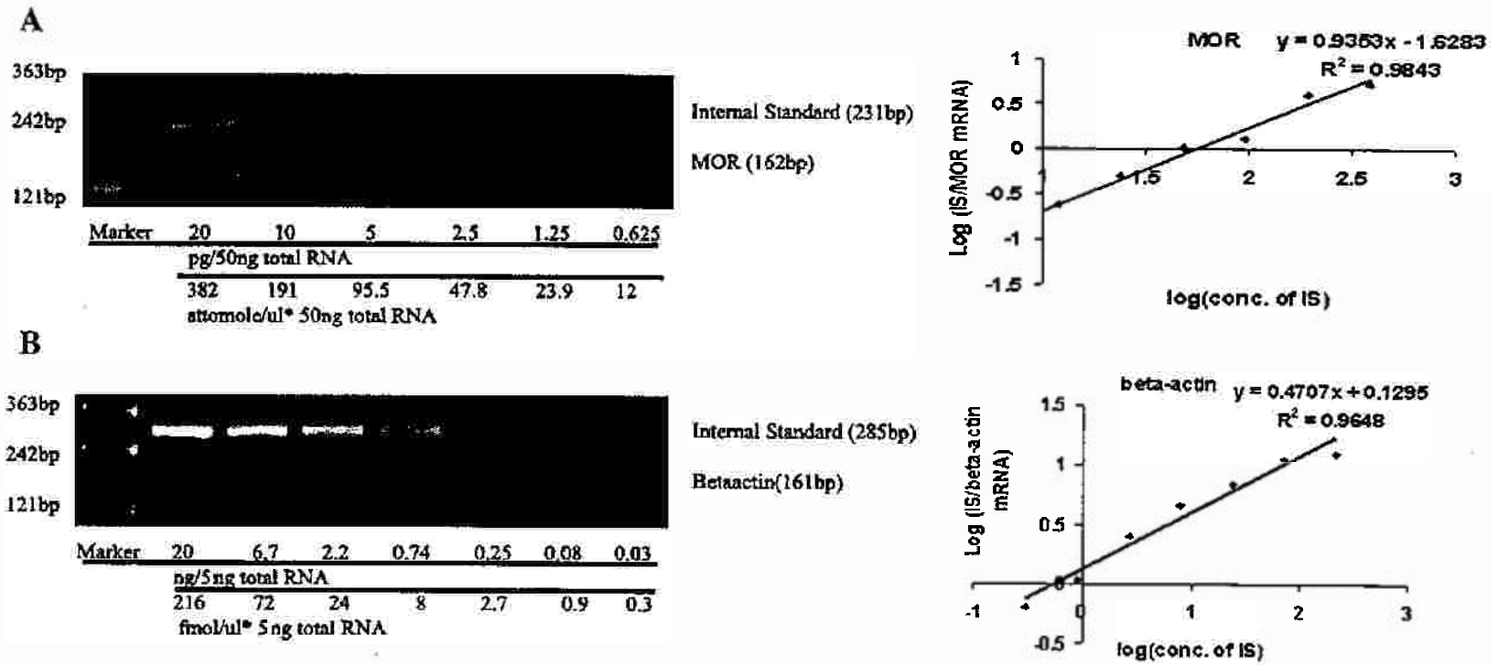

Figure 2. Quantification of MOR mRNA in SH-SY5Y cells using QC-RT-PCR. (A) A (left) is a representative QC-RT-PCR gel (2% Nusieve/1% agarose) showing the PCR co-amplification of MOR cDNA with decreasing amounts of IS cDNA (internal standard). A (right) shows calculation of MOR mRNA expression: The log [IS]/[MOR] ratio was calculated for each aliquot of series of IS rcRNA dilution and plotted against the log[molarity of IS]. The concentration of the MOR mRNA was determined by the ratio of 1(log [IS]/[MOR]=0). (B) B (left) is a representative QC-RT-PCR gel (2% Nusieve/1% agarose) showing the PCR co- amplification of beta-actin cDNA with decreasing amounts of IS cDNA. B (right) shows a plot of ratio of beat-actin to IS cDNA versus the concentration of IS. The IS was synthesized from genomic DNA (285 bp).

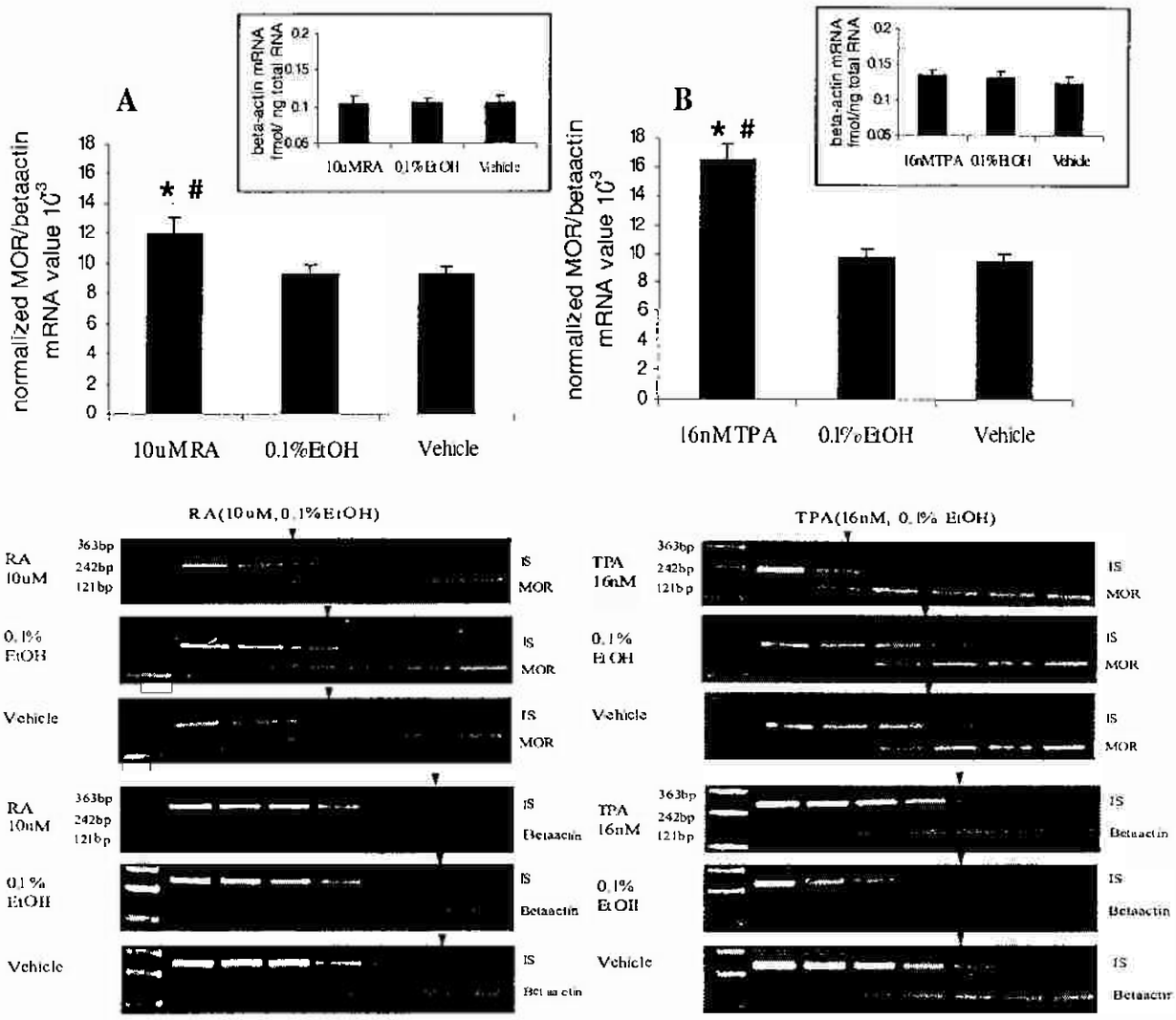

Figure 3. Effects of RA and TPA on MOR mRNA expression in SHSY-5Y cells. (A) RA (10 μM in 0.1% EtOH) and (B) TPA (16 nM in 0.1% EtOH)-induced differentiation of SHSY-5Y cells significantly increased MOR mRNA expression compared to vehicle- or 0.1% EtOH-treated undifferentiated SHSY-5Y cells by approximately 30% and 70%, respectively. One way ANOVA, Turkey post test. *, # $p< 0.01$ compared to 0.1% EtOH and vehicle*, respectively. The ethidium bromide stained gels show the competitive amplification of the MOR and the IS (231 bp) in the upper three lanes with each treatment, and the competitive amplification of beta-actin and the genomic IS (285 bp) in the lower three lanes. The arrows indicate the approximate positions where competition between the MOR or beta-actin and their respective IS is balanced.

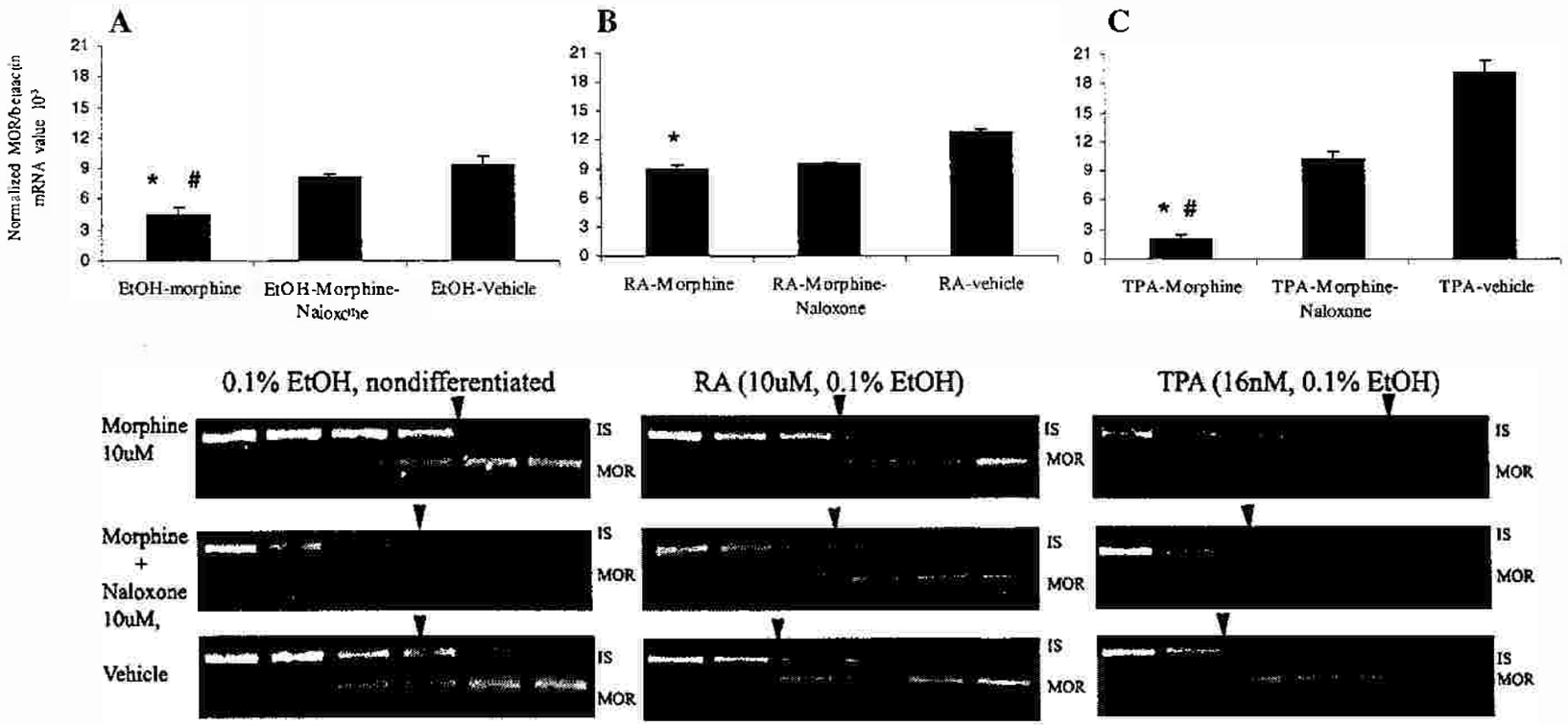

Figure 4. Effects of chronic morphine, with and without naloxone, on MOR mRNA levels in undifferentiated and RA- and TPA-differentiated SHSY-5Y cells. Chronic morphine treatment (10 μM for 24 h) inhibited MOR mRNA expression in (A) undifferentiated, (B) RA-differentiated, and (C) TPA-differentiated SHSY-5Y cells. Co-treatment with morphine and naloxone (10 μM each for 24 h) completely blocked morphine's effects in the undifferentiated SHSY-5Y cells, and partially blocked morphine's effects in TPA-differentiated SHSY-5Y cells, but not in RA-differentiated SHSY-5Y cells. One way ANOVA, Post test Turkey * $p<0.001$ compared to vehicle; # $p<0.001$ compared to naloxone co-treatment. The gels show the competitive PCR amplification of the MOR. The arrows indicate the position where competition between the MOR and the IS RNA template is balanced. QC-RT-PCR of beta-actin for each sample revealed similar levels of this housekeeping gene for each sample analyzed (data not shown).

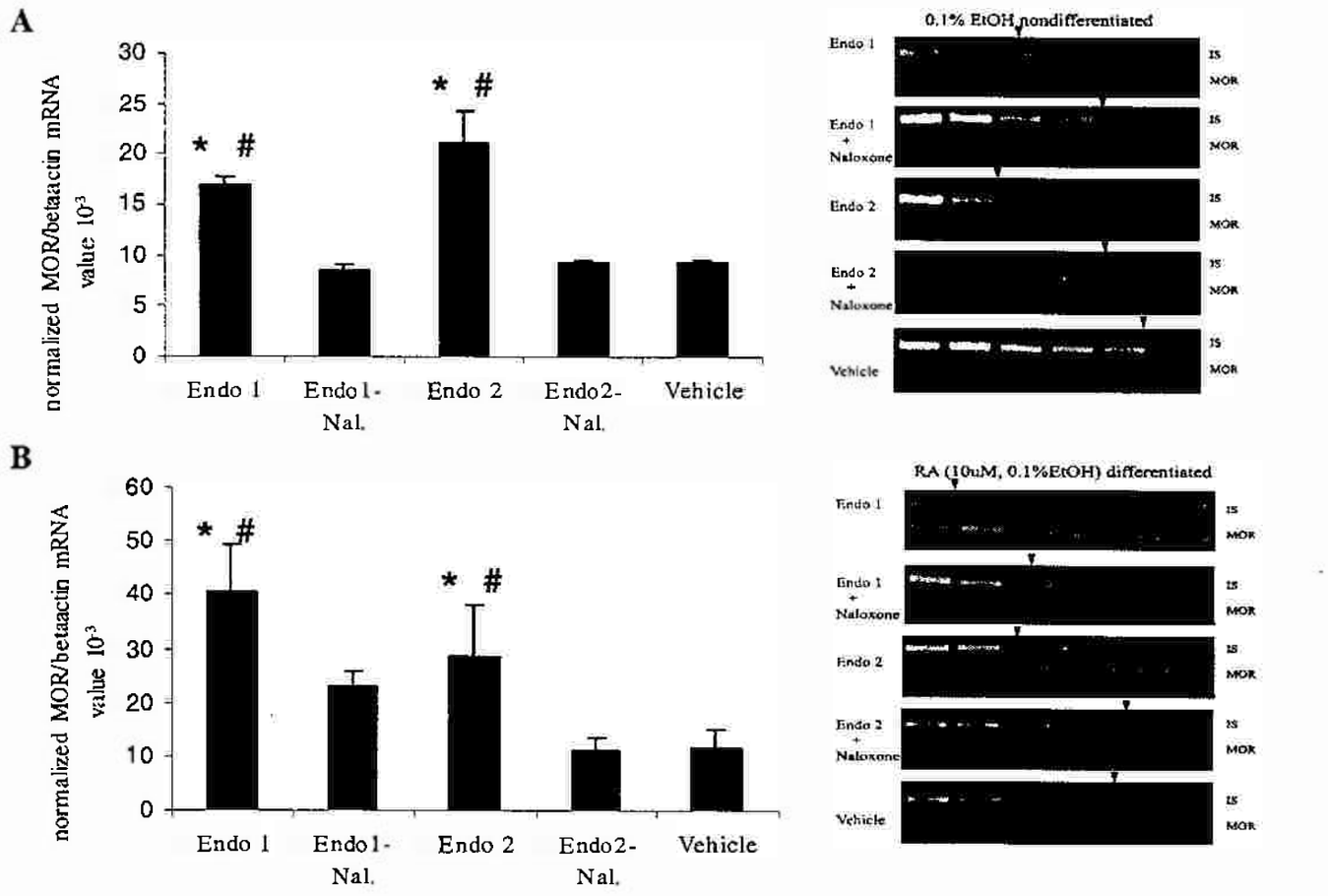

Figure 5. Effects of endomorphin-1 and -2 on MOR mRNA expression in SHSY-5Y cells. Endomorphin-1 and -2 (10 μM of each for 24 h) increased MOR mRNA expression in (A) undifferentiated SHSY-5Y cells and (B) RA-differentiated SHSY-5Y cells. One way ANOVA, post test Turkey* $p<0.001$ compared to vehicle; # $p<0.01$ compared to naloxone co-treatment. Student's t-test, two tailed. The gels show the competitive amplification of the MOR and IS. The arrows indicate the position where the competition is balanced.

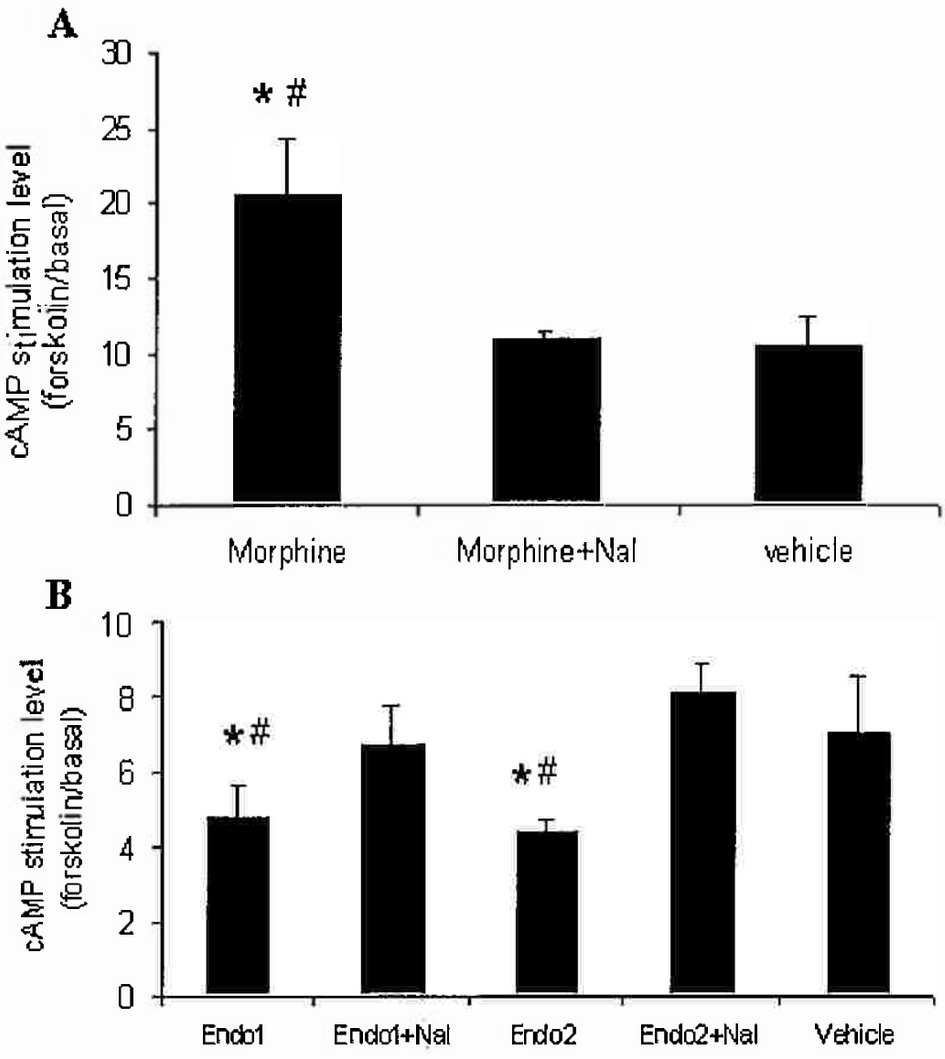

Figure 6. Effects of morphine and endomorphins-1 and -2 on forskolin-induced intracellular cAMP accumulation in SHSY-5Y cells. (A) Chronic morphine treatment significantly increased forskolin-stimulated cAMP levels. (B) Both endomorphin-1 and -2 decreased forskolin induction of cAMP accumulation. Co-treatment with naloxone completely abolished the agonists' effects on forskolin induction of cAMP levels. One way ANOVA, post test Turkey* $p< 0.01$ compared to vehicle;# $p< 0.01$ compared to naloxone co-treatment.

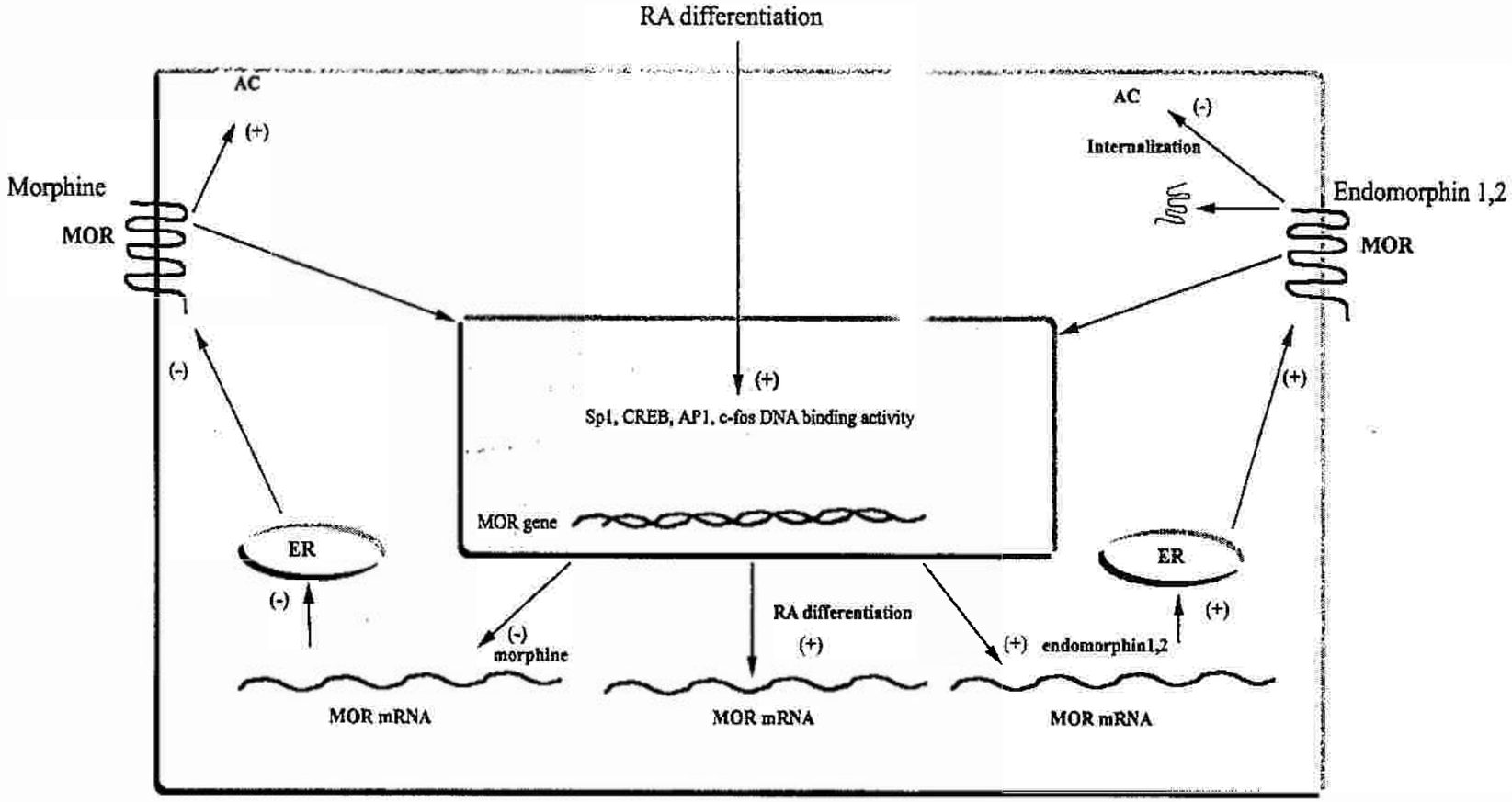

Figure 7. Regulation of MOR mRNA expression. ER: endoplasmic reticulum, AC: Adenylyl cyclase, MOR: mu-opioid receptor, + means enhancement effects, - means reduction. → means observed effects,

6. Reference

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