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Post-Transcriptional Regulation of the Human Mu-Opioid Receptor (MOR) by Morphine-Induced RNA Binding Proteins hnRNP K and PCBP1

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Abstract

Expression of the mu-opioid receptor (MOR) protein is controlled by extensive transcriptional and post-transcriptional processing. MOR gene expression has previously been shown to be altered by a post-transcriptional mechanism involving the MOR mRNA untranslated region (UTR). Here, we demonstrate for the first time the role of heterogeneous nuclear ribonucleic acids (hnRNA)-binding protein (hnRNP) K and poly(C)-binding protein 1 (PCBP1) as post-transcriptional inducers in MOR gene regulation. In the absence of morphine, a significant level of MOR mRNA is sustained in its resting state and partitions in the translationally inactive polysomal fraction. Morphine stimulation activates the downstream targets hnRNP K and PCBP1 and induces partitioning of the MOR mRNA to the translationally active fraction. Using reporter and ligand binding assays, as well as RNA EMSA, we reveal potential RNP binding sites located in the 5'-untranslated region of human MOR mRNA. In addition, we also found that morphine-induced RNPs could regulate MOR expression. Our results establish the role of hnRNP K and PCBP1 in the translational control of morphine-induced MOR expression in human neuroblastoma (NMB) cells as well as cells stably expressing MOR (NMB1).

Opioid receptors (μ , δ , and κ) bind to opioid drugs or endogenous opioid ligands to affect analgesia, reward, autonomic reflexes, and endocrine/immune-regulation. Several studies have suggested that the mu-opioid receptor(MOR) plays a key role in mediating the major clinical effects of morphine, as well as in the development of morphine tolerance and physical dependence with chronic administration (Law et al., 2004). Morphine induces less receptor phosphorylation and internalization compare to [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO) and etorphine, but mainly activates protein kinase C (PKC) and subsequent signaling molecules that contribute to development of analgesic tolerance (Yan et al., 2014). Although opioid addiction is considered to be a complex process involving genetic/epigenetic, cellular, and molecular regulation of neuronal plasticity and drug-related

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contextual memory, opioid receptors are the major determinants in the addictive process because the receptors are the initiation sites of all opioid function (Zheng, 2012).

MOR gene expression has been shown to be regulated by a diverse group of substances (Wagley et al., 2013) and many regulatory elements have been shown to interact with MOR *cis*-acting elements and positively (Regan et al., 2016) or negatively (Gach et al., 2008) affect the expression of the MOR gene expression. Furthermore, gene expression can be regulated at multiple levels, including transcription, mRNA stability and translation. For gene expression to be established at an appropriate level, some of these regulatory processes must be under rapid and precise control. Because transcriptional regulation is not sufficient to determine the level of gene expression and its duration, post-transcriptional regulation is necessary for finely tuned gene expression (Takada et al., 2009). Nearly all of the mechanistic pathways discovered for such regulation involve the binding of proteins to coding or untranslated regions (UTRs) of mRNAs (Chaudhury et al., 2010). Post-transcriptional regulation of MOR requires its 5' - and 3' -UTR, and multiple *cis*-acting elements within the promoter and *trans*-acting RNA-binding proteins (RBPs), which together form functional ribonucleoprotein (RNP) complexes, have been identified (Keene, 2002; Kim et al., 2004, 2006; Wei et al., 2004; Choi et al., 2007, 2008a,b; Hwang et al., 2007; Song et al., 2007; Hwang et al., 2009; Song et al., 2010). mRNA localization is a complex process that begins with the recognition of *cis*-acting elements in nascent transcripts by *trans*-acting RBPs. After the RNP complex is established, it exits the nucleus and is joined by additional cytoplasmic factors to create a RNP granule. The RNP granule associates either with the cytoskeleton or with specific membranes, mitochondria and peroxisomes to facilitate mRNA delivery and, possibly, localized translation. Therefore, determination of RBP specificities is a critical step in the elucidation and analysis of mechanisms involved in co- and post-transcriptional gene regulation (Ray et al., 2009).

Heterogeneous nuclear ribonucleic acids (hnRNA)-binding protein (hnRNP) families are multifunctional: along with other RNP proteins, they participate in pre-mRNA processing such as splicing and are important determinants of mRNA export, localization, translation, and stability. In mammalian cells, hnRNP families are divided into two subsets: hnRNP K/J and poly(C)-binding protein (PCBP or alpha-complex proteins) 1–4 (Dreyfuss et al., 2002; Makeyev and Liebhaber, 2002). Their diverse functions arise from the capability of hnRNPs to bind both RNA and DNA containing poly(C) sequences. Both mouse and human MOR contain multiple poly(C) sequences in their respective 5' -UTR regions. Thus, hnRNP family members may play a role in MOR-mediated effects induced by morphine. Here, we demonstrate that morphine stimulates MOR translation in human neuroblastoma cells by stimulating the expression of hnRNP K and PCBP1, which induce translocation of MOR mRNA to the translationally active polysomal fraction. Thus, our results show that morphine induces differential mRNA partitioning for translational control of the MOR via these mediator RNPs (hnRNP K and PCBP1).

Materials and Methods

Plasmid construction

The mouse and human 5'-UTR containing luciferase fusion constructs uAUG (+) and huAUG (+) used here have been described previously (Song et al., 2007, 2010) (Fig. 5A). The mouse hnRNP K and PCBP1 clone used here have also been described previously (Choi et al., 2008b). To clone the human hnRNP K and PCBP1 genes, total RNA was isolated from human neuroblastoma (NMB) cells and treated with RNase-free DNase (Promega, Madison, WI). RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Valencia, CA). PCR was performed with primers designed using the gene sequence information for each protein: human hnRNP K (Gene ID: BC000355.2): 5'-CAGGTACCAGAATATGGAACTGAACA-3' (forward) and 5'-GACTCGAGGAATCCTTCAACATCTGCA-3' (reverse); human PCBP1 (Gene ID: NM_006196): 5'-CTAAGCTTTCGCCATGGATGCCGGTGT-3' (forward) and 5'-GACTCGAGGCTGCACCCCATGCCCTTC-3' (reverse). The PCR conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min, and 72°C for 10 min. The RT-PCR products were excised from a 1% agarose gel and purified using a QIAquick gel extraction kit (Qiagen). Purified products were subcloned into the pcDNA-4A vector using *KpnI* and *XhoI* sites created with the PCR primers. All constructs were confirmed by DNA sequencing.

Cell culture, DNA transfection, and reporter gene assay

MOR-negative mouse (NS20Y) and MOR-positive human (NMB) neuroblastoma cell lines were grown in Dulbecco's minimum essential medium and RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Kim et al., 2004). Cells stably expressing human MOR (NMB1) were cultured in RPMI 1640 medium with 10% heat-inactivated FBS, and 200 µg/ml G418 sulfate at 37°C in a humidified atmosphere of 5% CO₂. The transfection and reporter gene assays were carried out as described previously (Choi et al., 2007; Song et al., 2007).

Radioligand binding

Binding activity assays were carried out as described previously (Choi et al., 2006). Briefly, after transient transfection with the indicated constructs, confirmation of MOR expression was determined by a whole-cell binding assay using [³H] diprenorphine in 25 mM HEPES buffer, 5 mM MgCl₂ (pH 7.6). Specific binding was defined as the difference between the radioactivity bound to the cells in the presence and absence of 100 µM CTOP.

Quantification of human hnRNP K and PCBP1 transcripts by real-time PCR and reverse transcription (RT)-PCR

These assays were performed as described previously (Song et al., 2007). Specific primer pairs for PCR amplification were as follows: hnRNP K, 5'-AATGCCAGTGTTCAGTCCC-3' (forward) and 5'-AGGCCCTCTTCCAAGGTAGG-3' (reverse); PCBP1, 5'-CAGAGGTGAAAGGCTATTGG-3x2032; (forward) and 5'-GGCAGCAGAGCCAGTGATAG-3' (reverse).

Immunoblot analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium vanadate and 1× protease inhibitor cocktail; Roche) for 20 min at 4°C. After centrifugation, the supernatant was transferred to a new tube, and mixed with sample buffer. Approximately 50 µg of protein from each lysate was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Bioscience, Pittsburgh, PA) in transfer buffer (48 mM Tris-HCl, 39 mM glycine, and 20% methanol). Membranes were blocked in a solution of 5% dry milk and 0.1% Tween 20 in Tris-buffer (TBS-T). Primary antibody was added after 1 h of blocking, and membranes were incubated overnight at 4°C. After washing three times with TBS-T, secondary antibodies conjugated with alkaline phosphate were added, and membranes were incubated for 1 h and then washed three times with TBS-T. After developing, signals were detected using a Storm 860 PhosphorImager system (Amersham Biosciences). The intensity of individual bands was determined with ImageQuant analysis software (GE Healthcare). For determination of hnRNP K and PCBP1 activity, amounts of hnRNP K and PCBP1 were monitored by a polyclonal antibody for hnRNP K (abcam) and PCBP1 (Abnova) and normalized to internal controls surveyed with β-actin or Lamin A/C antibodies (Cell Signaling) used as cytosol and nuclear markers, respectively.

Preparation of nucleus and cytosol fractions

Cells were washed and scraped into PBS and pelleted by centrifugation. To prepare nuclear and cytosolic extracts, cells were lysed in NE-PER extraction reagent (Pierce) according to the manufacturer's protocol.

In vitro transcription/translation

RNAs were synthesized in vitro with the MEGAscript In Vitro Transcription kit (Ambion, Waltham, MA) according to the manufacturer's instructions. Equal amounts (1 µg) of DNA were added to the TnT Coupled Reticulocyte System (Promega) for in vitro translation and the proteins were labeled with the Transcend™-tRNA using the Transcend™ Non-Radioactive Translation Detection System (Promega) under conditions recommended by the manufacturer. In brief, reactions were incubated for 90 min at 30°C and analyzed by 10% SDS-PAGE. Gels were transferred to PVDF membrane and membranes were blocked in a solution of 0.5% Tween 20 in Tris-buffer (TBS-T). Streptavidin-alkaline phosphatase (AP) was added after 1 h of blocking, and membranes were incubated for an additional 1 h. After washing the membranes twice with TBS-T followed by twice with distilled water, the membranes were developed with Western Blue® stabilized substrate and washed twice with distilled water. Signal detection on the dried membrane was performed using a Storm 860 PhosphorImager system (Amersham Biosciences).

RNA electromobility shift analysis (REMSA)

RNA EMSA to detect direct binding of hnRNP K and PCBP1 to the human MOR 5'-UTR was performed using a LightShift Chemiluminescent RNA EMSA kit (Thermo Fisher

Scientific, Waltham, MA) according to the manufacturer's instructions. In brief, in vitro transcribed RNAs were biotinylated using the Pierce RNA 3' End Biotinylation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The gel shift assay was carried out using the LightShift Chemiluminescent RNA EMSA Kit (Thermo Fisher Scientific). Biotinylated wild-type RNA probes (20 fmol) were incubated 30 min at room temperature with in vitro translated hnRNP K and PCBP1 in a 20 μ l binding reaction containing 1 $\times$ binding buffer, 5% glycerol and 0.1 mg/ml tRNA. The samples were electrophoresed on a 4% native PAGE in 0.5 $\times$ Tris Borate EDTA, transferred to a positively charged nylon membrane (GE Healthcare) and crosslinked in a UV Stratilinker 1800 (Stratagene). Detection of biotin-labeled RNA probes was performed using the chemiluminescent nucleic acid detection module (Thermo Fisher Scientific).

Polysomal fractionation and RNA immunoprecipitation (IP)

Polysomal fractionation was conducted as described previously (Wu and Bag, 1998). Following treatment, the cells were lysed in 200 μ l of polysomal buffer (10 mM MOPS, pH 7.2, 250 mM NaCl, 2.5 mM MgOAc, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and 50 μ g/ μ l cycloheximide). After removing the nuclei and cell debris by centrifugation at 12,000g for 10 min, the polysomes were pelleted by ultracentrifugation at 100,000g for 1 h in an 80 Ti rotor (Beckman, Brea, CA) at 4°C and resuspended in 200 μ l of polysomal buffer. The supernatant was collected as the post-polysomal fraction. Total RNA was isolated from polysomal and post-polysomal fractions with TRIzol reagent (Molecular Research Center Inc, Cincinnati, OH) and subjected to RT-PCR analysis for MOR mRNA.

For RNA immunoprecipitation, untreated control and morphine-treated cells were harvested by trypsinization and resuspended in 2 ml PBS, 2 ml nuclear isolation buffer (1.28 M sucrose; 40 mM Tris-HCl pH 7.5; 20 mM MgCl₂; 4% Triton X-100), and 6 ml water on ice for 20 min (with frequent mixing). Nuclei were pelleted by centrifugation at 2,500g for 15 min. Nuclear pellets were resuspended in 1.2 ml RNA immunoprecipitation (RIP) buffer (150 mM KCl, 25 mM Tris pH 7.4, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 9 μ g/ml leupeptin, 9 μ g/ml pepstatin, 10 μ g/ml chymostatin, 3 μ g/ml aprotinin, 1 mM PMSF, 100 U/ml SUPERASin; Ambion). Resuspended nuclei were split into three fractions of 400 μ l each (for mock and IP) and mechanically sheared using a Dounce homogenizer with 15–20 strokes. Nuclear membrane and debris were pelleted by centrifugation at 13,000 rpm for 10 min. An antibody to hnRNP K (Abcam) or PCBP1 (Cell Signaling) was added to the supernatant and incubated overnight at 4°C with gentle rotation. Samples were incubated with 40 μ l of protein G beads (Invitrogen) for 1 h at 4°C with gentle rotation. Beads were pelleted at 2,500 rpm for 30 sec, the supernatant was removed, and beads were resuspended in 500 μ l RIP buffer. This was repeated for a total of three RIP washes, followed by one wash in PBS. Beads were resuspended in 0.5 ml of Trizol. Coprecipitated RNAs were isolated, and RT-PCR for human MOR (forward, GATCATGGCCCTCTACTCCA; reverse TACTGGTGGCTAAGGCATCT) or human β -actin (forward, AATCTGGCACACACCTTCTAC; reverse, ATAGCACAGCCTGGATAGCAAC) was performed as previously described (Song et al., 2009).

Statistical analysis

The data were expressed as means $\pm$ SD. Statistical differences between values from two groups were determined using unpaired Student's *t*-test, whereas statistical differences between two or more groups were analyzed using analyses of variance (ANOVA) followed by Newman–Keuls (one-way) and Bonferroni posttests (two-way) post-hoc comparisons.

Results

Morphine induces expression of RNA binding proteins (RNPs) hnRNP K and PCBP1

To determine the effects of morphine on the expression of RNPs, we examined time-dependent morphine-induced RNP expression in NMB and NMB1 cells via immunoblotting. Expression levels of both RNPs were high in the NMB1 cells, which stably express MOR (Fig. 1A). The expression of PCBP1 peaked to 1.62 ± 0.2 -fold (NMB) and 2.04 ± 0.2 (NMB1) over control 30 min after 1 μ M of morphine treatment. Two-way ANOVAs on the PCBP1 levels from NMB and NMB1 cells treated with the morphine revealed significant cell lines $\times$ time interactions ($F_{4,24} = 6.66$). Indeed, the morphine induces higher expression of PCBP1 in both cell lines after 30 min ($P < 0.05$) treatment. The stimulatory effect declined after 30 min of treatment (Fig. 1A, left panel). We obtained similar results when morphine was used as an inducer of hnRNP K. The maximum expression of hnRNP K (1.43 ± 0.2 -fold in NMB and 1.51 ± 0.2 -fold in NMB1; Fig 1A, right panel) was observed 30 min after morphine treatment. Two-way ANOVAs on the hnRNP K levels from NMB and NMB1 cells treated with the morphine revealed significant cell lines $\times$ time interactions ($F_{4,16} = 11.44$). Indeed, the morphine induces higher expression of PCBP1 in both cell lines after 30 min ($P < 0.05$) treatment. Because maximal RNP expression after morphine treatment occurred at 30 min, concentration-dependent studies were carried out 30 min after the initiation of agonist treatment. Our immunoblotting data also show that maximum expression levels of the two RNPs induced by morphine are similar in NMB and NMB1 cells (1.2–1.3-fold for PCBP1 and 1.3–1.4-fold for hnRNP K with 1 μ M of morphine treatment; Fig. 1B). Two-way ANOVAs on the PCBP1 and hnRNP K levels from NMB and NMB1 cells treated with the morphine revealed significant cell lines $\times$ dose interactions ($F_{4,16} = 8.72$ and $F_{4,24} = 4.52$). Indeed, the morphine induces higher expression of PCBP1 and hnRNP K than untreated control in both cell lines with 1 μ M of morphine ($P < 0.05$) treatment.

hnRNP K and PCBP1 up-regulate MOR expression

Our previous work showed that MOR expression is regulated at the translational level by the 5'-untranslated region (UTR) of MOR (Song et al., 2007, 2009, 2010). However, the post-transcriptional mechanisms responsible for MOR regulation are not well known and are the focus of this study. To test the potential involvement of agonist-induced RNPs in MOR translation activity, we first investigated the effects of hnRNP K and PCBP1 on MOR expression in human NMB and NMB1 cell lines at the transcriptional level (via real-time PCR) and translational level (via immunoblotting and binding activity assay) (Fig. 2).

Transcriptional and translational assays were performed with RNPs, MOR protein and RNAs from NMB and NMB1 cells transfected with varying amounts (0–2 μ g) of hnRNP K

and PCBP1 protein expression vectors. As shown in Figure 2b, the protein levels of hnRNP K and PCBP1 were increased by transfection with expression vectors compared to non-transfected cells (NMB; $F_{5,10} = 17.41$, NMB1; $F_{5,10} = 12.69$). Furthermore, hnRNP K and PCBP1 expression up-regulated MOR gene expression at the post-transcriptional level in both NMB (2.3-fold for PCBP1 and 2.9-fold for hnRNP K) and NMB1 (1.9-fold for PCBP1 and 2.8-fold for hnRNP K) cells (Fig. 2c). However, for both RNPs, expression resulted in up-regulation of their own RNA levels only, and did not affect MOR transcriptional level (Fig. 2a). Collectively, these results demonstrate the stimulating, or activating, effect of both RNPs on de novo synthesis of MOR protein.

To determine the cellular location of the RNPs, nucleus and cytosol fractions were separated after transfection of the expression vectors as described. In NMB cell ($F_{5,10} = 74.98$), the hnRNP K protein was translocated to the cytosol compared to non-transfected control cells but human MOR stably expressed NMB1 cell ($F_{5,10} = 39.02$) showed that only a slight portion of hnRNP K protein was translocated to the cytosol. Therefore, it is possible that over-expressed MOR in NMB1 could have little effect on translocation, but the cytosol fraction still contains a large amount of hnRNP K and regulates MOR expression. However, most PCBP1 protein (control and induced) was located in the cytosol (NMB; $F_{5,10} = 35.42$, NMB1; $F_{5,10} = 12.9$). Successful isolation of the nucleus from the cytosol fractions was demonstrated by the presence of lamin A/C immunoreactivities in the isolated nucleus fractions and β -actin in the isolated cytosol fractions (Fig. 2d).

Morphine regulates polysomal partitioning of MOR mRNA

There are several uncharacterized mechanisms that can be considered for the effects of morphine on MOR translational control. We examined the association of MOR mRNAs with polysomal fractions and their translation efficiency in MOR-negative (NS20Y; $F_{6,12} = 21.2$) and MOR-positive (NMB; $F_{6,12} = 22.1$ and NMB1; $F_{6,12} = 32.1$) cells. The three cell types were treated with morphine for 30 min and cell extracts were fractionated into polysomal and post-polysomal fractions from which mRNAs were isolated. The mRNAs were then subjected to semiquantitative RT-PCT of MOR and β -actin (Fig. 3). In the MOR-negative cells, MOR mRNA was not present in either the polysomal or post-polysomal fractions, but was present in the morphine-treated total fraction. Interestingly, in the human cell lines after morphine stimulation, MOR mRNA levels in the polysomal fraction were increased. Therefore, we suggest that morphine treatment triggered the more abundant human MOR mRNAs to partition into the polysomal fractions. As a control, we monitored the distribution of β -actin mRNA in each fraction, which was not significantly altered after morphine treatment. These data provide evidence for morphine-stimulated de novo human MOR synthesis in human neuroblastoma cells. During stimulation, the vast majority of MOR mRNA partitions to the polysomal fraction for synthesis of MOR proteins. These effects are more evident in NMB1 cells, which stably express MOR.

hnRNP K and PCBP1 proteins interact with human MOR mRNA

To determine whether hnRNP K and PCBP1 proteins bind human MOR mRNA after morphine treatment, we conducted an RNA pull-down assay (Fig. 4). The association of MOR mRNA with the RNPs increased after morphine treatment in both NMB ($F_{5,10} =$

11.62, $P < 0.001$) and NMB1 ($F_{5,10} = 13.12$, $P < 0.001$) cells. As a negative control, the associated β -actin was also monitored, and remained unchanged after morphine treatment. Thus, our results demonstrate that hnRNP K and PCBP1 bind to human MOR mRNA. Our previous reports showed that translational control of MOR mRNA usually involves its 5'-untranslated region (UTR) (Song et al., 2007, 2009, 2010, 2012, 2013). To further demonstrate that the human MOR 5'-UTR is regulated by hnRNP K and PCBP1 proteins, we transiently co-transfected reporter constructs containing the 5'-UTR of mouse (uAUG +) or human MOR (huAUG +) into NS20Y and NMB cells (Fig. 5). Our results indicate that up-regulation of hnRNP K (2.21 ± 0.2 -fold) and PCBP1 (1.76 ± 0.1 -fold) increases the activity of the 5'-UTR of MOR in human cells in the reporter and ligand binding assays (Fig. 5A, B). In contrast, the reporter carrying the mouse MOR 5'-UTR failed to respond to stimulation with either RNP. These results are consistent with the polysomal partitioning data in Figure 3. Furthermore, we performed REMSAs using biotin-labeling methods (Fig. 6). The REMSA experiments were carried out using a biotin-labeled in vitro transcribed probe, Biotin-huAUG (+), and unlabeled in vitro translated hnRNP K and PCBP1 proteins (Fig. 6A; figure shows biotin-labeling of both proteins). Our results demonstrate that both RNPs bind the target huAUG (+) probe (see Lane 2 vs. Lane 1; Fig. 6B). A 10-fold molar excess of unlabeled huAUG (+) RNA transcript (Fig. 6B, Lane 3) completely inhibited the complex formation. Collectively, data from the co-transfection, ligand binding, MOR specific activity and REMSA assays confirm that both RNPs bind to the human 5'-UTR region of human MOR and regulate its activity.

Morphine-stimulated human MOR synthesis is more efficient in NMB1 cells due to the abundant amounts of MOR mRNA partitioned to the polysomal fraction for the synthesis of MOR proteins. Therefore, our results indicate that both induced RNPs more efficiently bind to the polysomal fraction of MOR mRNA and regulate its activity.

Discussion

Opioid receptor agonists such as morphine are strong, highly effective analgesic agents for the treatment of severe acute, surgical, and cancer pain, but their specific regulatory mechanisms are still unknown. Here, we provide several lines of evidence that MOR post-transcriptional events are controlled by RNA-binding proteins (RBPs) and their *cis*-acting element in the untranslated region (UTR) (Song et al., 2007, 2009, 2010, 2012, 2013). Many other studies also have suggested that RBPs indirectly couple transcriptional and subsequent post-transcriptional steps by interacting with their target transcripts (Maniatis and Reed, 2002). The hnRNP proteins are multifunctional: along with other RNP proteins, they participate in pre-mRNA processing events such as splicing, and are important determinants of mRNA export, localization, translation, and stability (Dreyfuss et al., 2002; Choi et al., 2009). However, the precise transport and translation patterns of localized mRNAs are dictated by the combination of *cis*-acting elements present on the RNA molecule. These elements are recognized by trans-acting factors (either RBPs or non-coding RNAs) (Besse and Ephrussi, 2008). This study demonstrates that the RNPs hnRNP K and PCBP1 play a functional role in modulating the synthesis of hMOR.

We provide evidence for the post-transcriptional regulation of functional MOR expression by the RNPs hnRNP K and PCBP1. Our results from the human neuroblastoma cell lines NMB and NMB1 (NMB-MOR) demonstrate that morphine treatment results in an up-regulation of hnRNP K and PCBP1 proteins, and this phenomenon induces MOR protein expression. However, our quantitative results indicate that MOR mRNA expression levels are not regulated by hnRNP K or PCBP1, suggesting that transcriptional control of MOR is not involved in the morphine-mediated signaling pathway. Furthermore, upon morphine treatment, hnRNP K and PCBP1 lead to differential subcellular partitioning of MOR mRNA. Without stimulation, a significant portion of MOR mRNA remains in the total fraction. After stimulation by morphine, the majority of MOR mRNA is translocated to the polysomal fraction for activation of MOR translation.

While the 5'-UTR of MOR is sufficient for its translational regulation by morphine or by the RNPs, it is not clear how the 5'-UTR of MOR responds to morphine or RNP signaling. However, in the case of unstimulated cells, a portion of MOR mRNA partitions in the inactive fraction, but after activation, the RNPs trigger translocation of this MOR RNA pool to the polysomal fraction. Collectively, our data showed that morphine-stimulated MOR mRNA levels in the polysomal fraction were increased only in MOR positive (NMB and NMB1) cells but not in those which were MOR negative (NS20Y) cell. Because the polysomal fraction increased only in MOR-positive cell, hnRNP K and PCBP1 proteins may regulate MOR protein levels by interacting with the MOR 5'-UTR.

The close partnership between transcriptional and post-transcriptional control of gene expression is a theme of growing importance for development, during which it is used to fine-tune protein expression by directing it to where it is needed within the cell and by coupling it more directly with local signaling cascades (Liu and Szaro, 2011).

In summary, we demonstrate that the RNPs hnRNP K and PCBP1 upregulate MOR protein synthesis in response to morphine treatment by increasing the actively translated polysomal fraction of MOR mRNA. Future experiments will seek to characterize other hnRNP families' role in the MOR expression including knock-down assay.

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Fig. 1.

Time- and concentration-dependent expression of the RNA binding proteins (RBPs) hnRNP K and PCBP1. (A) Neuroblastoma cells were treated with vehicle or morphine (1 μ M) at various time points (5, 10, 30, and 60 min), and RBP expression was analyzed by western blotting. Morphine induced expression of PCBP1 protein (left panel, $n = 4$) and hnRNP K protein (right panel, $n = 3$) in NMB and NMB1 cells. (B) Cells were incubated with different concentrations of morphine (10 μ M, 1 μ M, 100 nM, and 10 nM) for 30 min and whole lysates were analyzed by western blotting. Morphine induced expression of PCBP1 protein

(left panel, $n = 3$) and hnRNP K protein (right panel, $n = 4$) in NMB and NMB1 cells. The data represent the mean $\pm$ SD. Statistical significance was assessed using two-way ANOVA followed by Bonferroni posttests post-hoc comparisons. *Statistically significant ($P < 0.05$) compared with control (untreated cells, 1).

Fig. 2.

hnRNP K and PCBP1 regulate MOR expression. MOR expression levels were analyzed in hnRNP K- or PCBP1-transfected NMB (A) and NMB1 (B) cells. (a) Relative MOR and RNP transcripts levels in hnRNP K or PCBP1-transfected cells. Relative mRNA levels were determined as the ratio of MOR/ β -actin and hnRNP K or PCBP1/ β -actin. (b) Expression of the RNPs was determined by western blot assay of cells transfected with 1–2 μ g of RBP expression construct. Relative protein levels were determined as the ratio of hnRNP K or PCBP1 to β -actin as described in Materials and Methods section. (c) MOR binding in cells

transfected with 1–2 μg of RBP expression construct. Relative MOR binding level was determined as the ratio of specific MOR/total binding. $*P < 0.05$. (Experiments were repeated a minimum of three times.) (d) Fractionation of NMB and NMB1 cells before and after hnRNP K or PCPB1 transfection. All experiments were carried out independently and at least in triplicate. Protein expression was quantified using densitometry (lower panels in each figure). Data represent the mean $\pm$ SD. Statistical significance was assessed using one-way ANOVA followed by Newman–Keuls post-hoc comparisons. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with untransfected control. β -actin and lamin were used as cytoplasmic and nuclear markers, respectively.

Fig. 3. Morphine stimulates MOR mRNA association with polysomes. Following morphine treatment, the polysomal and post-polysomal cellular fractions were isolated from NS20Y, NMB, and NMB1 cells as described in Materials and Methods section. Total RNA from both fractions was used for RT-PCR to quantify mRNAs. (A) Schematic of the isolation of translationally active and inactive mRNAs. (B) Abundance of MOR and β -actin mRNAs in polysomal (P) versus post-polysomal (S) fractions, as well as total mRNA pool (T), with (+) or without (-) morphine. PCR products are shown after 1.5% agarose gel electrophoresis (n

= 3). mRNA expression was quantified using densitometry (lower panels in each figure). The values indicate the mean $\pm$ SD. Statistical analysis was carried out using one-way ANOVA followed by Newman–Keuls post-hoc comparisons. ** $P < 0.01$, *** $P < 0.001$ versus untreated control. (Neg), negative control without RT enzyme.

Fig. 4.

RNA immunoprecipitation of hnRNP K or PCBP1 with MOR or β -actin mRNA in NMB and NMB1 cells with (+) or without (-) morphine treatment. mRNA precipitated by anti-hnRNP K or anti-PCBP1 was analyzed by semiquantitative RT-PCR. Amplified products are shown after 1.5% agarose gel electrophoresis ($n = 3$). mRNA expression was quantified using densitometry (lower panels in each figure). The values indicate the mean $\pm$ SD. Statistical significance was assessed using a one-way ANOVA followed by Newman-Keuls post-hoc comparisons. *** $P < 0.001$ compared with untreated and no pulled-down control. K, hnRNP K; 1, PCBP1.

Fig. 5.

Human MOR expression levels in hnRNP K or PCBP1-transfected NS20Y and NMB cells. (A) Graphical representation of relative luciferase expression determined by luciferase assay (Promega) after co-transfection of the constructs shown (uAUG [+] or huAUG [+]) with each RBP expression construct (plus K, 2 μ g of hnRNP K expression construct; plus 1, 2 μ g of PCBP1 expression construct). Relative LUC activity levels were determined as the ratio of LUC/LacZ as described in Materials and Methods section. (B) MOR binding in cells transfected with 2 μ g of RBP expression constructs. Relative MOR binding levels were determined as the ratio of specific MOR/total binding. * $P < 0.05$ (Experiments were repeated a minimum of three times.)

Fig. 6. Interaction of the RNPs with huAUG (+) RNA derived from the human MOR 5'-UTR. (A) hnRNP K and PCBP1 proteins labeled in vitro with Transcend™ tRNA (arrows). M, molecular weight markers. (B) REMSAs were performed using biotin-labeled huAUG (+) as a probe with non-labeled in vitro translated RNP proteins. Lane 1, probe alone; Lane 2, in vitro translated RNP with labeled probe; Lane 3, plus self-competitor. Filled arrow indicates the protein-RNA complexes, arrow head indicates non-specific band, and empty arrow indicates free probe.