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Dual regulation of *mu* opioid receptors in SK-N-SH neuroblastoma cells by morphine and interleukin-1 β : evidence for opioid-immune crosstalk

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Abstract

Treatment of SK-N-SH cells with morphine and interleukin-1 β (IL-1 β) produced dual regulation of the mRNA for the human *mu* opioid receptor (MOR) protein. Morphine produced a decrease in the MOR mRNA while IL-1 β increased it, as assessed by real-time quantitative PCR. These data were consistent with immunocytochemical studies of treated and untreated cells. Morphine-mediated down-regulation of MOR was blocked by naltrexone and IL-1 β -induced up-regulation of MOR was blocked by interleukin-1 receptor type 1 antagonist. Immune-opioid crosstalk was examined by IL-1 β and morphine co-treatment. These data are the first to show dual regulation of MOR in neuroblastoma cells.

Keywords

interleukin-1 β ; morphine; *mu* opioid receptor; SK-N-SH cells; opioid-immune crosstalk

1. Introduction

The pro-inflammatory cytokine, interleukin-1 β (IL-1 β) plays a major role in host defense and inflammation and is increasingly associated with inflammatory pain and opioid analgesia. In rodent models, peripheral administration of IL-1 β produced hyperalgesia (Ferreira et al., 1988; Oka et al., 1994; Watkins et al., 1994) and reduced morphine analgesia while contributing to morphine tolerance (Shavit et al., 2005). At the molecular level, the interaction of IL-1 β and the opioid system is shown by the finding that IL-1 β increased the levels of *mu* opioid receptor (MOR) mRNA in primary astrocytes (Ruzicka et al., 1996; Ruzicka and Akil, 1997) and in neural microvascular endothelial cells (Vidal et al., 1998). Other cytokines, including IFN α , TNF α , IL-4 and IL-6, also increase the expression of MOR in neuroblastoma (SH-SY-5Y) and peripheral immune cells (Börner et al., 2004, 2007;

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Kraus et al., 2001, 2006). These results and others show that cytokines interact with endogenous opioid systems but explicit molecular mechanisms remain elusive.

IL-1 β mediates its effects through the interleukin 1 receptor type 1 (IL1R1) protein, which is a member of the Toll-like/IL-1R1 (TIR) domain family of membrane receptors (O'Neill, 2002). Like the toll-like receptors, the IL1R1 receptor signals through a complex of accessory proteins and downstream signaling events including activation of the JAK-STAT, MAPK, and NF- κ B pathways (Allan et al., 2005; Hibi and Hirano, 1998; Park et al., 2005; Wan et al., 2008). The ultimate transcription factors from these pathways translocate to the nucleus and bind to regulatory regions on select genes to alter transcription. Genetic studies examining the structure of the human MOR gene regulatory regions found consensus sequences for cAMP response element (CRE, site of CREB regulation), activator-protein 1 (AP-1), and yin-yang-1 (YY-1) domains for transcriptional regulation (Wendel and Hoehe, 1998). Functional studies in cell lines show that transcription factors from the JAK-STAT, MAPK and NF- κ B signaling pathways alter *mu* opioid receptor gene transcription after cytokine stimulation (Kraus et al., 2001, 2003, 2006).

Recent studies from our lab provided evidence of opioid and immune crosstalk in the regulation of NF- κ B pathways using human astrocytes following treatment with opioid agonists and antagonists (Davis et al., 2007, 2008). These opioid effects on cytokine-stimulated signaling pathways did not appear to be mediated by a classical opioid receptor in that these effects were not blocked by the general opioid antagonist, naltrexone, and were similar with opioid agonist and antagonist treatment. To examine opioid-immune crosstalk in a cell line with high levels of opioid receptors, the present study used SK-N-SH cells which express abundant and functional *mu* and *delta* opioid receptors (Baumhaker et al., 1993). The SK-N-SH cells were immortalized from a neuroblastoma and are extensively used for studies of opioid receptor regulation and intracellular signaling (Baker et al., 2000; Bare et al., 1994; Baumhaker et al., 1993; Breivogel et al., 1997; Raut et al., 2007; Rubovitch et al., 2003; Sarne et al., 1998; Xu and Carr, 2001; Yu et al., 1986).

We now report, for the first time in a neuroblastoma cell line, that treatment with IL-1 β induces the increase of both MOR mRNA as assessed by real-time quantitative PCR and MOR protein levels as assessed by immunocytochemistry and confocal and epifluorescence imaging. Co-treatment of cells with IL-1 β and its receptor antagonist, IL-1RA, blocked the effect of IL-1 β on increasing MOR mRNA levels in cells. Treatment of cells with morphine decreased mRNA for MOR and these results were blocked by the general opioid antagonist, naltrexone. Co-treatment of cells with morphine and IL-1 β produced a complete blockade of morphine-induced down regulation of MOR mRNA and resulted in an increase in MOR expression comparable to IL-1 β alone. These results provide additional insights into the molecular mechanisms that are fundamental to opioid-immune crosstalk.

2. Materials and Methods

2.1. Cell culture

The human neuroblastoma cell line (SK-N-SH, ATCC # HTB-11; American Type Culture Collection, Manassas, VA) was used in the assays to measure potential changes in MOR expression. SK-N-SH cells were maintained in Royal Park Memorial Institute-1640 growth media (RPMI; ATCC), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen) and 2 μ g/mL amphotericin B. SK-N-SH cells were grown as a monolayer in a humidifier incubator at 37°C, 5% CO₂ and 95% air in 100mm² round culture plates. Stock cultures were seeded at a cell density (1 $\times$ 10⁵/cm²) in order to provide 70–80% confluence at the time of passage. Stock plates were cultured for 5–6 days, with growth media changed every 48 h. Following 5–6 days of growth, SK-N-SH

cells were 70–80% confluent and were then passaged with 0.25% trypsin, seeded at 1×10^5 cells/well in 500 μ L/well of RPMI-1640 growth media (as supplemented in the stock plates) into 24-well culture plates (Corning, Lowell, MA). Experimental plates were cultured for 2–3 days in order to achieve 80–90% confluence at the time of treatment. All experiments were conducted between passages 6 and 10.

2.2. Morphine and naltrexone treatment

Following 80–90% confluency of SK-N-SH cells, morphine sulphate (Sigma Aldrich, St. Louis, MO) dose-response studies were done by removing growth media and replacing with fresh growth media containing the following final concentrations of morphine: 1, 10, and 100 μ M. SK-N-SH cells were either vehicle treated or incubated with morphine for 6, 12, and 24 h for each dose. As a negative control, cells were treated with an equivalent volume of vehicle in growth media. Quantitative real-time (qRT-PCR) experiments were completed from at least three independent experiments for each time point. In order to determine if opioid receptors were mediating morphine's effects, the general opioid antagonist, naltrexone (Sigma Aldrich), was coincubated with morphine in additional studies. The treatment groups were vehicle, morphine (10 μ M), naltrexone alone (10 μ M), and morphine (10 μ M) with naltrexone (10 or 100 μ M) for 6 and 24 h.

2.3. IL-1 β and IL-1RA treatment

To assess IL-1 β -induced changes in MOR expression in SK-N-SH cells, experiments were set up as follows: human recombinant IL-1 β (PeproTech, Rocky Hill, NJ) was made in RPMI-1640 to final concentrations of 1, 10 and 100 ng/mL and cells were exposed for 0, 6, 12 and 24 h. To assess IL-1RI-dependent regulation of MOR expression by IL-1 β , the IL-1RI type specific antagonist, IL-1RA (R&D systems, Minneapolis, MN) was used. The treatment groups were vehicle, IL-1 β (10 ng/mL), IL-1RA (10 ng/mL), and IL-1 β (10 ng/mL) with IL-1RA (10 or 100 ng/mL) for 6 and 24 h.

2.4 RNA isolation and cDNA synthesis

Total RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX) as detailed in the manufactures manual. Briefly, SK-N-SH cellular growth media was removed and replaced with guanidinium-based lysis buffer, plates were placed on ice and the cell lysate was mixed gently with an equal volume 64% ethanol and the lysate/ethanol mixture was pipetted into a glass-fiber filter mounted on a 1.5 mL RNase-free tube. Following the binding of RNA to glass fiber filters, filters were washed to remove contaminants, and then the RNA was eluted in a small volume (80 μ L) of elution solution. 1 μ g of total RNA was subjected to DNase-I treatment as instructed by the manufacturer (rDNase I; Ambion). Total RNA concentration was determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA) and A_{260}/A_{280} ratio (1.8–2.1) readings were recorded as described in the user manual. RNA integrity was determined by running 0.5 – 1 μ g of total RNA on a 1.5% agarose gel; RNA was mixed with one volume of NorthernMax-Gly sample loading dye (Ambion) and heated to 50°C for 30 min.; glyoxylating the RNA and preventing the formation of secondary structures. All RNA samples were run on 1.5% agarose gels to check for accidental degradation; 18s and 28s rRNA bands were visual determinants of the isolated RNA quality and integrity. Total RNA was aliquoted into 1.5 mL tubes and stored at –80°C. DNAase-I treated 1 μ g total RNA was reverse transcribed using the QuantiTect reverse transcriptase cDNA synthesis kit (Qiagen, Valencia, CA). A further step to remove genomic DNA from isolated RNA samples was carried out by using gDNA Wipeout Buffer 7x (Qiagen) to 1 μ g of RNA as instructed in the manufactures manual. One microgram of RNA was used for cDNA synthesis with Quantiscript reverse transcriptase (RT), which contained RNase-H activity, 5x Quantiscript RT buffer containing dNTPs and RT primer mix which was a blend of oligo-dT and random primers. cDNA was then synthesized in a

thermal cycler using the following cycling parameters: 2 min. at 42°C for genomic DNA elimination followed by 45 min. at 42°C and 3 minutes at 95°C to inactivate the Quantiscript RT. All RT-reactions were stored at -20°C until qRT-PCR was performed.

2.5. Quantitative Real-Time PCR (qRT-PCR)

To quantify change in human MOR messenger RNA (mRNA), the SYBR green I dye chemistry was employed. All primers were developed using software from Integrated DNA Technologies (IDT); <http://scitools.idtdna.com/Primerquest/> and were designed to cross an intron to prevent amplification of genomic DNA. The annealing efficiency of the primer sets (Table 1) for the human MOR (GenBank NM_000914) and housekeeping gene (HKG), human GAPDH (GenBank NM_002056.3) were optimized using gradient RT-PCR. In order to determine the qRT-PCR efficiency (rate at which a PCR amplicon is generated) and the dynamic range for both MOR and GAPDH genes, tenfold dilution series of cDNA (standard curve) starting from 1000 ng to 1 ng were made for each assay (date not shown), as a result the correlation coefficient for MOR and GAPDH was 0.996 and 0.97 respectively. The qRT-PCR standard curve also determined the concentration of cDNA template to use for each gene (MOR and GAPDH). Each MOR and GAPDH qRT-PCR reactions consisted of 10 ng/ μ L (50 ng total cDNA mass) of cDNA template in 15 μ L total reaction volume which was run in duplicate in a 96 well qRT-PCR plates on a BioRad iCycler thermal cycler (BioRad, Hercules, CA). Each qRT-PCR contained 2X *Premix Ex Taq* SYBR Green I Master Mix (Takara, Madison, WI), consisting of *Eq Taq*TM Hot Start DNA polymerase, SYBR Green I, dNTP mix and Mg^{2+} , primer sets (25 nM final concentration) and PCR-grade water. The qRT-PCR amplification cycle for MOR was: 95°C for 10 s followed by 40 cycles of 95°C, 64°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles. The qRT-PCR amplification cycle for GAPDH was: 95°C for 10 s followed by 30 cycles of 95°C for, 60°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles. The threshold cycle (C_T) for both MOR (33.1 cycles) and GAPDH (26.5 cycles) remained constant between experiments. Expression of MOR mRNA was normalized with GAPDH to adjust for sample loading. Relative quantification of MOR expression was calculated using the comparative C_T method as described by Livak et al., (2001); formula, $2^{-\Delta\Delta C_T}$, yields relative fold change in MOR gene expression.

2.6. Immunocytochemistry (ICC) and imaging

SK-N-SH cells were seeded at 1×10^4 cells/well in 24-well culture plates on sterile 12 mm round glass coverslips (VWR, West Chester, PA) coated with poly-D-lysine. Following 1–2 days of culture to obtain 40–60% confluent cells, cells were washed 3x with 1X phosphate buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min., washed 2x, 5 min. each with 1X PBS at room temperature (RT) with gentle agitation. Coverslips were blocked using blocking buffer made up of normal goat serum (10%), normal horse serum (10%), fetal bovine serum (10%), bovine serum album (2%), PVP (polyvinyl pyrrolidone) in 1X PBS with Tween-20 (66%) for 1 h at RT with gentle agitation. To improve the signal-to-noise ratio, Image-iT signal enhancer (Invitrogen, Carlsbad, CA) was applied to each well (~200 μ L) and incubated at RT for 30 min. Human specific MOR primary antibody was applied to SK-N-SH cells at a concentration of 7.4 μ g/mL (1:50) (Abcam, Cambridge, MA) diluted in blocking buffer. Cells were incubated with diluted primary antibody overnight at 4°C with agitation. The cells were then washed with 1X PBS and incubated with Alexa-Fluor 488 secondary antibody (goat anti-rabbit IgG) (10 μ g/mL) (1:200) (Invitrogen) diluted in blocking buffer for 1h at RT with gentle agitation. SK-N-SH cells were counter stained with Hoechst DNA stain (2 μ g/mL) (1:5000) (Sigma Aldrich) in 1X PBS for 5 min. at RT in the dark following washing with 1X PBS. Coverslips were mounted onto microscope slides using Prolong gold antifade (Invitrogen). Microscope slides were allowed to cure in the dark

overnight at RT before analysis. Application of isotype-specific IgG and Hoechst to SK-N-SH cells, in the absence of human MOR antibody, served as a control for the specificity of the human MOR antibody stain. Human β -tubulin III antibody 0.34 μ g/ml (1:100) (Epitomics, Burlingame, CA) was applied to vehicle treated SK-N-SH cells as a control for the ICC protocol. Fluorophores were excited with UV light and immunostaining was observed using a Leica SP2 (Bannockburn, IL) confocal and a Nikon SMZ1000 (Melville, NY) epifluorescence microscope.

2.7. Microscopy and imaging

The SK-N-SH expression of MOR protein was detected using a Leica SP2 (Bannockburn, IL), laser scanning confocal microscope (Oklahoma State University, Microscopy Laboratory, Stillwater, OK). As a negative control, human MOR primary antibody was replaced with equal volume of 1X PBS. Confocal microscopy was used to determine the expression, subcellular localization, and relative abundance of the MOR antigen in SK-N-SH cells in response to IL-1 β , morphine and naltrexone. To ensure consistency between samples from each set of analyzed experiments, a single user carried out each experiment. The images collected include confocal and epifluorescence images, which were collected using a Nikon SMZ1000 epifluorescence microscope housed at the OSU-Animal Sciences Department. Photomicrographs are representative of 3 independent experiments.

2.8. Statistical analysis

The delta C_T (ΔC_T) values from each experiment set (minimum, $n = 3$) were used to analyze significant differences between vehicle and stimulated groups. The ΔC_T values were used to perform statistical analysis to determine a concentration and/or time dependent and treatment dependent effects of morphine, naltrexone, IL-1 β and IL-1RA. Using the Statistical Analysis Software (SAS; Package 9.1), ΔC_T results were tested using 2 $\times$ 2 factorial Analysis of Variance (Two-way ANOVA) tables generated through PROC-GLM (General linear model) allowing the utilization of a complete randomized design. And P -values were analyzed through least square means (LSMEANS) difference of ΔC_T values from each treatment group. This model was used to determine a significant difference between vehicle vs. stimulated-dependent effects, and statistical differences determined at $P \leq 0.05$. Results are presented as least square means $\pm$ S.E.M were $n \geq 3$.

3. Results

3.1. Morphine induced the down-regulation of MOR expression

SK-N-SH cells were treated with 1, 10 and 100 μ M morphine for 6, 12 and 24 h (Fig 1A). Morphine treatment altered the expression of MOR mRNA in a time-dependent manner with no changes at any dose after 6 h, the highest dose significant at 12 h, and all three morphine doses decreasing MOR mRNA compared to untreated control cells at 24 h. At this last time point, the MOR mRNA levels were down-regulated 3.1-fold, 2.2-fold, and 1.5-fold by the three morphine doses (1, 10, and 100 μ M, resp.). Immunocytochemistry revealed that MOR protein expression was most pronounced at the cell membrane in untreated cells (Fig. 1B-panel *i*). MOR protein expression levels did not appear to be altered by 12 hr of morphine (10 and 100 μ M) treatment (data not shown). However, MOR protein levels were reduced by visual inspection following 24 h morphine exposure (Fig. 1B- panel *iv*).

The general opioid antagonist, naltrexone, given alone had no significant effect on the MOR mRNA levels compared to untreated control cells, but co-treatment of SK-N-SH cells with naltrexone (10 or 100 μ M) and morphine (10 μ M) for 6 and 24 h significantly blocked MOR mRNA down-regulation and restored mRNA levels to those no different than vehicle control cells (Fig. 1C).

3.2. IL-1 β induced the up-regulation of MOR expression

IL-1 β (10 and 100 ng/mL) significantly up-regulated MOR mRNA expression following 6, 12 and 24 h treatment compared to vehicle (Fig. 2A). Maximal effect of IL-1 β (10 and 100 ng/mL) treatment on MOR expression was seen after 6 h of treatment (4.4- and 5.4-fold increase respectively, compared to vehicle). After 12 h of IL-1 β treatment, MOR mRNA expression declined and by 24 h of IL-1 β treatment MOR mRNA expression was further reduced but still greater than that of the vehicle group. MOR protein expression was also up-regulated in response to 6 h IL-1 β (10 ng/mL) treatment (Fig. 2B-panel *iv*).

To assess the role of IL-1R1 in IL-1 β -induced up-regulation of MOR, the IL-1RI-specific antagonist, IL-1RA was used. IL-1 β (10 ng/mL) treatment for 6 and 24 h, significantly increased MOR mRNA expression (4.0- and 3.1-fold increase respectively, Fig. 2C). IL-1RA treatment alone did not alter the expression of MOR mRNA; however, IL-1 β induced up-regulation of MOR mRNA was significantly blocked by IL-1RA. Immunocytochemistry demonstrated that IL-1 β induced up-regulation of MOR protein is also attenuated by IL-1RA (Fig. 2D-panels *iv* and *v*).

3.3. IL-1 β blocked morphine-induced down-regulation of MOR expression

To identify interactions between IL-1 β and morphine on the expression of MOR, SK-N-SH cells were pretreated (6 and 24 h) with morphine followed by treatment with IL-1 β for 6 and 24 h. IL-1 β treatment (for 6 and 24 h), following morphine pre-treatment, significantly up-regulated the expression of MOR mRNA (2.93- and 2.53-fold increase) compared to morphine treatment alone (2.10- and 1.55-fold decrease, respectively, Fig. 3A). Similarly, when cells were co-treated concurrently with IL-1 β and morphine (no morphine pretreatment) for 6 h and 24 h, morphine-mediated down-regulation of MOR mRNA was significantly blocked by IL-1 β with a increase in MOR mRNA levels (3.14- and 1.52- fold increase, respectively) compared to morphine exposure alone (0.55 and 2.28-fold decrease, Fig. 3B).

Immunocytochemistry experiments showed that IL-1 β also prevented morphine-mediated decrease in MOR protein expression (Fig. 4-panel *vi*). As determined by epifluorescence microscopy, the expression of MOR protein was represented by punctate-round structures dispersed irregularly across the cell.

4. Discussion

Opioid agonists, such as endogenous opioid peptides and exogenous morphine, activate opioid receptors not only on neurons in the central nervous system, but also opioid receptors present on peripheral and central immune cells (Binder et al., 2004; Janson and Stein, 2003; Stein and Lang, 2009). Likewise, immune system substances, such as the major pro-inflammatory cytokine, IL-1 β , act on peripheral and central immune cells, but also on central nervous system neurons (Allan et al., 2005; O'Neil, 2002; Pinteaux et al., 2002). The colocalization of functional receptors from opioid and immune systems on the same cell types and the interconnectedness of molecular pathways in a single cell provide the basis for bidirectional opioid-immune crosstalk. The present study examined a single aspect of opioid-immune interactions by assaying the levels of mRNA for the *mu* opioid receptor and the expression of MOR protein in SK-N-SH neuroblastoma cells after treatment with morphine and IL-1 β .

4.1. Down-regulation of MOR expression by morphine

Present results show that treatment with morphine produced down-regulation of the mRNA for the *mu* opioid receptor and decreased MOR expression in SK-N-SH cells, confirming

previous studies showing decreased radioligand binding of opioid ligands (Baumhaker et al., 1993; Horner and Zadina, 2004) or MOR mRNA down-regulation (Yu et al., 2003) in the same or other neuroblastoma cell lines after chronic morphine exposure. The decrease of MOR mRNA was not observed until morphine exposure of the cells continued for at least 6 h, suggesting that the acute effects of morphine must trigger some longer term process that results in MOR down-regulation. The effect of morphine treatment to the cells was mediated by classical *mu* opioid receptors as co-treatment with the general opioid antagonist, naltrexone, blocked morphine's effect of reducing MOR mRNA levels (Fig 1C). However, as the cell line used expresses both *mu* and *delta* opioid receptors and morphine can mediate its effects through both types of opioid receptors (Baumhaker et al., 1993), it is not certain whether morphine induced MOR down-regulation was initiated by *mu* or *delta* opioid receptors, or a combination of both. Further studies are needed with type-selective opioid antagonists, such as β -FNA which irreversibly binds only to *mu* opioid receptors, to fully determine the initial receptor event of morphine-induced MOR down-regulation. Finally, *in vitro* results cannot fully explain *in vivo* data as chronic morphine administration to rodents has shown no change, up-regulation or down regulation of MOR mRNA levels in various brain regions (Harrison et al., 1998; Law et al., 2000).

Immunocytochemistry (ICC) experiments were done to use a second method to assess alterations of *mu* opioid receptors in response to morphine. The expression pattern of MOR appeared localized to the cell membrane as limited fluorescence was detected in the cytoplasm. Decreased signal of MOR at the cell membrane was detected in cells treated with morphine, consistent with the observed decrease in MOR mRNA. Previous studies have not examined the expression of MOR protein in SK-N-SH cells using ICC, however other ICC studies have shown changes in MOR protein expression in other cell-lines transiently or stably expressing MOR (Madsen et al., 2000).

The precise molecular chain of events leading from initial opioid receptor signaling to the observed decrease in levels of opioid receptor mRNA is not known. Changes in CREB phosphorylation were noted in neuroblastoma cells after acute and chronic morphine treatment (Bilecki et al., 2004) and the human gene for MOR has consensus sequences in regulatory regions for CREB binding (Wendel and Heohe, 1998). The *mu* opioid selective agonist, DAMGO, increased transcription factor SP-1 binding to the human gene for MOR in SK-N-SH cells which may also auto-regulate MOR mRNA expression (Xu and Carr, 2001). Further studies are needed to directly link opioid agonist stimulation with molecular events that regulate *mu* opioid receptor gene expression.

4.2. Up-regulation of MOR expression by interleukin-1 β

The pro-inflammatory cytokine IL-1 β increased the levels of MOR mRNA after 6, 12 and 24 h incubation (Fig 2A). At the highest dose of 100 ng/ml and exposure for 6 h, IL-1 β induced about a 5.5-fold increase in receptor mRNA. By 24 h, this dose of IL-1 β induced only a 2.5-fold increase in receptor mRNA. Thus, unlike the morphine-induced down-regulation of receptor mRNA above, the maximal effect of IL-1 β -induced MOR mRNA up-regulation appeared after a shorter period of exposure. Like the delay in effect in morphine-treated cells, a significant increase was not observed until 6 h of treatment suggesting a longer-term process is required after the initial binding of IL-1 β to its target receptor, IL1-R1. These findings support previous studies that showed IL-1 β up-regulated the expression of MOR in astrocytes and microvascular endothelial cells (Ruzicka et al., 1996; Ruzicka and Akil, 1997; Vidal et al., 1998),

The role of IL1-R1 was confirmed by the concurrent treatment of IL-1 β with the IL1-R1 antagonist, IL1-RA (Fig. 2). This IL1-R1 receptor-selective antagonist is an endogenous peptide that is released in response to IL-1 β stimulation and is thought to limit the pro-

inflammatory effects of IL-1 β (O'Neil, 2002). IL1-RA completely prevented the increase in MOR mRNA when cells were concurrently treated with 10 ng/ml of IL-1 β and IL1-RA.

As with morphine-treated cells above, ICC studies in cells treated with IL-1 β were also done. Increased immunostaining was evident in the cells treated with IL-1 β alone consistent with the observed increased levels of MOR mRNA detected with qRT-PCR.

As a pro-inflammatory cytokine, IL-1 β is released in pathological conditions and mediates a cascade of immunological responses by acting on IL-1RI. IL-1 β induces numerous transcription factors from pathways including JAK-STAT, MAPK, and NF- κ B (Allan et al., 2005; Hibi and Hirano, 1998; Park et al., 2005; Wan et al., 2008). Activation of any or all of these transcription factors may induce the up-regulation of opioid receptor genes, given the presence of consensus response elements in the human MOR gene (Im et al., 1999; Kraus et al., 2003; Vidal et al., 1998; Wendel and Hoehe, 1998).

4.3. Effect of morphine and interleukin-1 β co-treatment on MOR expression

Results of the present studies show that neuroblastoma cells co-treated with morphine and IL-1 β increased their levels of MOR mRNA (Fig 3). IL-1 β -induced increases in receptor mRNA occurred when cells were treated first with morphine (pretreatment condition) then exposed to IL-1 β and also occurred when cells were exposed concurrently to morphine and IL-1 β . Thus IL-1 β not only blocked morphine-induced down-regulation of MOR mRNA but increased mRNA levels to the same degree as IL-1 β treatment alone. This suggests that the mechanisms of receptor mRNA down-regulation by morphine are secondary to the mechanisms of receptor mRNA up-regulation by IL-1 β , with IL-1 β playing a greater role in the regulation of MOR mRNA transcription. The dominance of IL-1 β effects over morphine effects on receptor mRNA levels could be due to greater activation by IL-1 β of common signaling pathways or related to the finding above that maximal IL-1 β -induced up-regulation occurred with less treatment time than maximal morphine-induced down-regulation of receptor mRNA. The dominance of receptor mRNA up-regulation may also reflect a more basic hypothesis that the *mu* opioid receptor gene is biased towards up-regulation (in number and types of positive enhancers to promoters, response elements, etc) compared to down-regulation of gene transcription. Further dose-response studies with morphine and IL-1 β are needed to examine their interaction in this cell line.

As with morphine- and IL-1 β -treated cells above, ICC studies in cells treated with IL-1 β and morphine were also done (Fig. 4). Increased immunostaining was evident in the cells treated with IL-1 β and morphine consistent with the observed increased levels of MOR mRNA detected with qRT-PCR.

4.4. Interleukin-1 β and morphine crosstalk

As there is no evidence for IL-1 β binding directly to *mu* opioid receptors and in light of present finding that IL1-R1 antagonist blocks the action of IL-1 β , it is likely that the opioid-immune interaction does not occur at the membrane receptor level. As noted above, IL-1 β acting on its own receptor, IL-1RI, initiates a complex signaling cascade that results in the generation of numerous transcription factors including JAK-STAT, MAPK, and NF- κ B pathways (Allan et al., 2005; Hibi and Hirano, 1998; Park et al., 2005; Wan et al., 2008). Morphine and other opioids acting at opioid receptors also initiate signal transduction pathways that results in the generation of numerous transcription factors, including those associated with JAK-STAT, MAPK, NF- κ B, AP-1, and SP-1 (Bilecki et al., 2004; Börner et al., 2002; Kraus et al., 2003; Xu and Carr, 2001). As highlighted above, it is likely that IL-1 β -induced MOR mRNA up-regulation via increases in transcription factors that bound to the promoter regions of the human MOR gene. Other cytokines including IL-4, IL-6 and

TNF- α also up-regulate MOR expression in studies using neuroblastoma and peripheral immune cells (Börner et al., 2004, 2007; Kraus et al., 2006). This suggests that the observed IL-1 β effects in the present study may not be unique to this particular pro-inflammatory cytokine but a shared property of a number of other cytokines and chemokines that signal through common intracellular pathways.

A broader picture of IL-1 β and opioid system crosstalk is shown by *in vivo* studies using rodent models. Administration of IL-1 β antagonized morphine analgesia, and chronic morphine treatment increased IL-1 β expression, implying a role for this cytokine in morphine tolerance (Hutchinson et al., 2008; Shavit et al., 2005). Additionally, the expression of spinal IL-1 β mRNA and protein was up-regulated by chronic intrathecal administration of morphine and methadone (Hutchinson et al., 2008). Blockade of IL-1 β with IL-1RA, or use of IL-1 β gene knock-out mice, resulted in enhanced morphine analgesia (Hutchinson et al., 2008; Shavit et al., 2005), suggesting the involvement of signaling cascades common to both IL-1RI and MOR. Although it is not clear how the *in vitro* effects of IL-1 β -induced up-regulation of MOR expression translates to reducing morphine analgesia *in vivo*, it has been suggested that morphine-induced IL-1 β expression allows for auto-regulation of *mu* opioid receptors to replace those internalized and destroyed by ligand occupation (Börner et al., 2002).

Finally, recent evidence from our laboratory has shown a unique anti-inflammatory role of opioid agents, where both chemokine and iNOS expression were inhibited in human astroglial cells following treatment with β -funaltrexamine, a *mu* opioid receptor-selective antagonist (Davis et al., 2007, 2008). Both opioid agonists and antagonists produced an inhibition of chemokine and iNOS expression and the effects were not blocked by the general opioid antagonist, naltrexone. Morphine and other opioids also interact at the toll-like receptor-4 (TLR4) which results in the increase of IL-1 β , an effect not blocked by naltrexone (Lewis et al., 2010). These above studies and others highlight the finding that opioid-immune crosstalk may also involve opioid sites of action distinct from the classical *mu* opioid receptor examined in the present studies.

In summary, the present studies are the first to show dual regulation of MOR expression by morphine and IL-1 β in a neuroblastoma cell line. Down-regulation of MOR mRNA was observed after morphine treatment and up-regulation after IL-1 β treatment. Pretreatment or co-treatment with morphine in addition to IL-1 β treatment produced a elevation of MOR mRNA equal to that induced by IL-1 β alone. These findings were consistent with images obtained from immunocytochemical studies of treated cells labeled with anti-MOR antibodies. Overall, these studies contribute to the growing evidence for opioid-immune crosstalk that may be mediated by common intracellular signaling pathways.

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Fig. 1.

(A) Time-course of MOR mRNA expression in SK-N-SH cells following morphine treatment. Cells were cultured in growth media with or without (vehicle) morphine sulphate (1, 10 and 100 μ M) for 6, 12 and 24 h. (B) Effects of morphine on MOR protein expression in SK-N-SH cells. Cells were cultured in growth media with either vehicle or morphine sulphate (1, 10 and 100 μ M) for 24 h. The cells were treated with anti-MOR RabMAb (1:50) followed by Alexa-Fluor 488-secondary antibody (goat anti-rabbit) (1:200). Cells were counter stained with Hoechst (1:5000). Vehicle (i); morphine- 1 μ M (ii), 10 μ M (iii), 100 μ M (iv). Scale bar = 50 μ m. (C) Effect of Naltrexone co-treatment on morphine-induced down-regulation of MOR mRNA expression in SK-N-SH cells. Cells were cultured in either

vehicle, morphine (10 μ M), naltrexone (10 μ M), morphine (10 μ M) + naltrexone (10 μ M) and morphine (10 μ M) + naltrexone (100 μ M) for 6 and 24 h. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean $\pm$ S.E.M of duplicate measures from triplicate wells from 3–5 independent experiments. Statistical analysis was carried out using a Two-way ANOVA, with pair wise (*P*-values) differences determined from least square means (LSMEANS). #*P* $\leq$ 0.05 *vs.* vehicle; * *P* $\leq$ 0.05 *vs.* morphine alone.

Fig. 2.

(A) Time-course of MOR mRNA expression in SK-N-SH cells following IL-1 β treatment. Cells were cultured in growth media with or without (vehicle) human recombinant IL-1 β (10 and 100 ng/mL) for 0.15, 6, 12 and 24 h. (B) Effects of IL-1 β on MOR protein expression in SK-N-SH cells. Cells were cultured in growth with either vehicle or IL-1 β (10 ng/mL) for 6 and 12 h. *i*: anti- β -tubulin III RabMAb; *ii*: anti-MOR RabMAb control (1:50); *iii*: Vehicle; *iv*: IL-1 β (10 ng/mL) 6 h; *v*: IL-1 β (10 ng/mL) 12 h. (C) Effect of IL-1RA treatment on IL-1 β induced up-regulation of MOR mRNA expression in SK-N-SH cells. Cells were cultured in either vehicle IL-1 β (10 ng/mL), IL-1RA (10 ng/mL), IL-1 β (10 ng/mL) + IL-1RA (10 ng/mL) and IL-1 β (10 ng/mL) + IL-1RA (100 ng/mL) for 6 and 24 h. (D) Effect

of IL-1RA treatment on IL-1 β -induced up-regulation of MOR protein expression in SK-N-SH cells. Cells were cultured in growth media with either vehicle, IL-1 β (10 ng/mL) and IL-1 β (10 ng/mL) and IL-1RA (10 ng/mL) for 6 and 24 h. *i*: 1 $^{\circ}$ antibody control; *ii*: Vehicle for 6 h; *iii*: IL-1 β (10 ng/mL) for 6 h; *iv*: IL-1 β (10 ng/mL) + IL-1RA (10 ng/mL) for 6 h; *v*: IL-1 β (10 ng/mL) + IL-1RA (10 ng/mL) for 24 h. For all ICC experiments, cells were treated with anti-MOR RabMAb. (1:50), anti- β -tubulin III RabMAb (1:100) followed by Alexa-Fluor 488-secondary antibody (goat anti-rabbit) (1:200). Cells were counter stained with Hoechst (1:5000). Scale bar = 18 μ m and 50 μ m respectively. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean $\pm$ S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a Two-way ANOVA, with pair wise (*P*-values) differences determined from least square means (LSMEANS). # *P* $\leq$ 0.05 *vs.* vehicle; * *P* $\leq$ 0.05 *vs.* IL-1 β alone.

Fig. 3.

(A) Effect of IL-1 β treatment on morphine-induced down-regulation of MOR mRNA expression in SK-N-SH cells. Cells were cultured in growth media with either vehicle, IL-1 β (10 ng/mL) and morphine (10 μ M) alone and IL-1 β (10 ng/mL) for 6 and 24 h, following pre-treatment with morphine (10 μ M) for 6 and 24 h. (B) Effect of IL-1 β and morphine co-treatment on the expression of MOR mRNA in SK-N-SH cells. Cells were in growth media with either vehicle, IL-1 β (10 ng/mL) and morphine (10 μ M) alone and IL-1 β (10 ng/mL) + morphine (10 μ M) co-treatment for 6 and 24 h. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean $\pm$ S.E.M (error bars) of duplicate measures from

triplicate wells from three independent experiments. Statistical analysis was carried out using a Two-way ANOVA, with pair wise (*P*-values) differences determined from least square means (LSMEANS). # $P \leq 0.05$ vs. vehicle; * $P \leq 0.05$ vs. morphine alone.

Fig. 4.

Changes in MOR protein expression in SK-N-SH cells following co-treatment with IL-1 β and morphine. Cells were cultured in growth media with either vehicle, morphine (10 μ M), IL-1 β (10 ng/mL) alone and morphine (10 μ M) + IL-1 β (10 ng/mL) for 6 h. *i*: anti- β -tubulin III; *ii*: 1 $^{\circ}$ Ab. control; *iii*: Vehicle; *iv*: Morphine (10 μ M); *v*: IL-1 β (10 ng/mL); *vi*: Morphine (10 μ M) and IL-1 β (10 ng/mL) co-treatment for 6 h.. Cells were treated with anti-MOR RabMAb. (1:50), anti- β -tubulin III RabMAb (1:100) followed by Alexa-Fluor 488-secondary antibody (goat anti-rabbit) (1:200). Cells were counter stained with Hoechst (1:5000). Scale bars = 200 μ m — and 100 μ m —.

Table. 1

MOR and GAPDH primer sets used quantitative RT-PCR (qRT-PCR).

Target	Primer Sequence (5'→ 3')	Amplicon Size	GenBank™ Accession
MOR	Fwd: (590 – 609)n- CTTCAGCCATTGGTCTTCCT Rev: (689 - 668) - CAGTACCAGGTTGGATGAGAGA	106	NM_000914
GAPDH[^]	Fwd: (222– 243) - CAACTACATGGTTTACATGTTC Rev: (402- 385) - GCCAGTGGACTCCACGAC	163	NM_002056.3

[^] GAPDH primer sequences obtained from Kraus et al., (2003).