Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study

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Abstract

The enigma of inter- and intra-individual variability mediated by the mu opioid receptor in response to opioid analgesic pharmaceuticals is not fully elucidated. MOR-1 is the heptahelical guanine nucleotide couple receptor GPCR targeted by prototypical opioids involved in pain mediation, tolerance development and subsequent addiction. Gaps in our understanding of the functional capacity of recently-identified MOR-1 alternatively-spliced variants still exist. The aim of this single dose, time-course, pilot study was to evaluate the effects of chronic opioid exposure on selected downstream molecular targets involved in intracellular processes (signal transduction, alternative splicing and protein phosphorylation) responsible for MOR-1 gene expression and morphine tolerance in human neuroblastoma (SH-SY5Y) cell cultures. Dual application of Phosflow® revealed modified activation patterns of pathway-specific, signaling proteins. Using qRT-PCR techniques, mRNA levels of MOR-1 alternatively-spliced variants were spatially, temporally and differentially upregulated by chronic opioids, indicating a tolerant state. The expression profiles for various gene coding transcription factors, as well as the cellular proliferation of SH-SY5Y cells, were temporally regulated. Taken together, the observed significant alterations in measured outcomes suggest a potential role for MOR-1 transcripts in elucidating posttranscriptional mechanisms of morphine tolerance.

Keywords: G-protein; mu Opioid Receptor; Opioid, Morphine, Naloxone, Tolerance, Withdrawal; Dependence; Addiction, Alternative Splicing; Differentiation; Posttranslational; Posttranscriptional; Gene Expression; Transcription Factor; Signal Transduction; Phosflow; Nanodrop; Vehicle; Agonist; Antagonist; Neuropeptide

Abbreviations

GPCR: g-protein Coupled Receptor; G-protein: Guanine Nucleotide Binding Protein; OPRM1: mu Opioid Receptor Gene; MOR-1: mu Opioid Receptor Subtype 1; ASV: Alternatively-Spliced Variant; Phosflow: Phosphorylation-Specific Flow Cytometry; qRT-PCR: Real-Time, Quantitative, Reverse Transcription Polymerase Chain Reaction; mRNA: Messenger Ribonucleic Acid; DNA: Deoxyribonucleic Acid; SH-SY5Y: Human Neuroblastoma Cells; RA: Trans-Retinoic Acid; cDNA: DNA Complementary to RNA; cAMP: Cyclic Adenosine 3',5'-Monophosphate; BACT: Beta Actin; MOR-1: mu Opioid Receptor Subtype 1; MOR-1A: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant A; MOR-1B1: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant B1; MOR-1B2: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant B2; MOR-1B3: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant B3; MOR-1B4: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant B4; MOR-1B5: mu Opioid Receptor Subtype 1, Alternatively Spliced Variant B5; MOR-

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**Introduction**

Morphine is the prototypical alkaloid analgesic that is derived from the opium poppy plant (*Papaver somniferum*). It is renowned for its proven efficacy in providing reprieve from various forms of physiological and psychological pain [1-5], relieving stress and insomnia, cough suppression and inducing a sense of well-being (euphoria) [5-7] in a manner that is proven to be more expedient than the implementation of behavioral or lifestyle modifications [8]. In addition, its high bioavailability (16 - 60%), rapid onset of action (20 - 30 minutes), extended duration of action (4 - 24 hr) and low organ toxicity [9] make it particularly attractive for palliative care [10], surgical sedation, supraspinal/spinal/peripheral analgesia, nociceptive pain [2,5], and more recently, neuropathic pain. However, the propensity for morphine to induce tolerance, bradycardia (respiratory depression), constipation (gastrointestinal immotility), itching [5], immunosuppression [11], physical dependence and a host of other undesirable adaptive changes [5] reportedly accounts for the reluctance of the medical community to prescribe it as first-line pharmacotherapy for chronic, non-malignant pain [12]. Identification of mechanisms to regulate opioid receptor activities [13] may provide useful targets for intervention of undesired side-effects of these drugs.

The opioid receptors belong to a superfamily of guanine nucleotide-binding protein (G-protein)-coupled receptors (GPCRs) that constitute ~3% of the human genome. There is growing interest in the studying the pharmacogenomics of the mu opioid receptor (MOR-1), which is encoded by the MOR-1 gene (OPRM1). MOR-1 is a 7-transmembrane GPCR that is chiefly responsible for the mediation of pain and is the primary target for analgesic drugs such as morphine. Gaps in the functional capacity of recently-identified MOR-1 alternatively-spliced variants still exist. Morphine is the prototypical agonist that binds to MOR-1. The medical decision to prescribe morphine for the management of moderate to severe chronic pain is complicated by the existence of polymorphisms in drug response to opioid pharmacotherapy. Under acute or chronic conditions, morphine induces pharmacological tolerance, avoidable only by abstaining from its use. Over the past three decades, the enigmatic development of morphine tolerance has been variably attributed to reversible modifications in receptor binding, signal transduction and neuropeptide mechanisms, but none of these theories fully explain the sustained tolerance that progresses to physical dependence and in some cases, opioid addiction [14-16].

Morphine elicits its pharmacological effects primarily through specific binding to the inhibitory G-protein-linked, mu opioid receptor subtype 1 (MOR-1) [17,18] of interneurons within the central nervous system. Opioid receptors are widely distributed [19] in the central neuroaxis (neocortex, thalamus, nucleus accumbens, hippocampus, amygdala) as well as in the peripheral nervous system (PNS) (myenteric neurons and vas deferens) (http://www.genecards.org). These receptors commonly bind endogenous opiate peptides, the enkephalins, with high affinity [20]. Morphine interacts with the heptahedral, transmembrane domains (7TM) of G-proteins, whose primary function is signal transduction [21,22,23]. The receptor-ligand interaction triggers the dissociation of the βγ subunit and association of the α-subunit with GTP-activated adenyl cyclase [24,25]. Subsequent inhibition of adenyl cyclase reduces intracellular cyclic adenosine monophosphate (cAMP) stores [26] by restricting protein phosphorylation activities required for signaling. Without functional activation (phosphorylation) of signaling cascades by cytosolic kinases, cellular function is diminished or eventually abolished [27]. Additional cellular actions of morphine include changes in voltage-gated ion channels that are directly coupled to G-proteins. Morphine facilitates the opening of potassium channels, leading to increased potassium ion conductance associated neuronal hyperpolarization and secondary reductions in cellular excitability. Moreover, there is a concomitant blockade of calcium channels which inhibits vesicular transport toward the nerve terminus and prevents neurotransmitter release (e.g. 5-HT, enkephalin, norepinephrine) [22,28]. The cumulative suppressive effect of morphine action suppresses firing of inhibitory interneurons [5]. Hence, all three core mechanisms of opioid action (i.e. receptor mechanisms, opioid peptides, and second messengers) are implicated in the development of morphine tolerance as a consequence of their natural progression.

The extent of human genetic variation is illuminated at the molecular level by the provision of a near-complete sequence of the human genome in 2001. One mechanism by which genetic variation occurs is through the process of alternative splicing, a common modulatory mechanism for enhancing the diversity of the transcriptome and proteome from bacteria to humans. Studies of the genomic architecture indicate that > 90% [29] of protein-coding human genes (142,634) undergo alternative splicing [30]. The pre-mRNA splicing process is a

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vital regulatory phase of the gene expression pathway that orchestrates the excision of non-coding introns and the subsequent ligation of relevant coding exons to form mature mRNA [31]. Alternative splicing is the discriminatory inclusion of different exons in mature mRNA, resulting in the production of different gene isoforms from a single gene, and by extension, different protein isoforms [32].

The overarching hypothesis governing this work is that alternatively-spliced variants are modulated by chronically administered opioid drugs, thereby individually or collectively contributing to these outcomes. Following a preliminary study and qRT-PCR optimization, five specific aims were proposed to address these concerns, and together these aims establish the human neuroblastoma (SH-SY5Y) cell line as a suitable model for these investigations due to their high expression of MOR-1 as well as six MOR-1 alternatively-spliced variants without the use of cloning technology. These variants display differential sensitivities to prototypical mu-opioid receptor ligands as well as differential gene expression. We also report a time-dependent perturbation of PI3K/Akt, CREB and Elk phosphorylation, as well as chronic morphine-induced up-and down-regulation of transcription factors and neurological disease genes. Collectively, these data connect opioid-induced stimulation of MOR-1 with progressive intracellular actions (Figure 1) that allow the chronological interaction of signal transduction, phosphorylation, transcription, post-transcriptional and posttranslational mechanisms, and thereby provide meaningful and relevant insight into the pharmacological actions of opioids at the cellular and molecular levels.

**Figure 1:** General scheme of research progression.
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In lieu of historical emphasis on cell-surface interactions of opioids, this pilot study explored the intracellular perspective, stratifying the pharmacogenomic assessment of chronically-exposed human cell cultures into cellular, molecular and transcriptomic levels. We report the simultaneous up-regulation of six MOR-1 alternatively-spliced variants in SH-SY5Y cells, disinhibition of G-protein signaling, as well as modulation of on-target and off-target transcription factor expression in a time-dependent and cell-specific manner. These findings are consistent with previous publications establishing the up-regulation of MOR-1 as a biomarker of tolerance. Additional research to further elucidate the role of these alternatively-spliced variants, as well as off-target transcription factors, in the development of sustained opioid tolerance. Given the progression of these cellular events beyond receptor binding, these data are likely to improve our understanding of the inter-individual and intra-individual variability observed in opioid pharmacotherapy.

Materials and Methods

General chemical reagents and pharmacologic agents

Isopropanol, chloroform, dosing chemicals (i.e. morphine, naloxone, DAMGO), distilled water, and consumable supplies were supplied by Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol (100%) was obtained interdepartmentally.

qRT-PCR chemicals

The DNAse treatment and removal kit were purchased from Ambion (Foster City, CA, USA). iQ™ SYBR Green Supermix and iScript cDNA Synthesis System were ordered from Bio-Rad Laboratories (Hercules, CA).

Phosflow chemicals and antibodies

Consumable flow cytometry supplies and initial supplies of fluorochrome (Annexin V-FITC, propidium iodide)-conjugated antibodies to phospho-proteins (PKC, PKA, Akt, Elk1, CREB) and assay buffers were gifts from BD Biosciences Pharmingen (San Diego, CA, USA).

Methods

Human neuroblastoma (SH-SY5Y) cell line

Human neuroblastoma (SH-SY5Y) cells are epithelial cells that were derived from the bone marrow of a metastasized tumor originating in the brain of a 4-year old girl. SH-SY5Y cells are stable neuroblasts that were thrice-cloned from the original SK-N-SH cell line [33]. The expression of mu opioid receptors in SK-N-SH cells was determined to be five times higher than that of delta opioid receptors [34], which is reproduced in SH-SY5Y subclones [34]. SH-SY5Y cells are generally considered to be a reproducible cell model for studying the biochemical correlates of opiate efficacy and tolerance [35]. Additionally, SH-SY5Y cells can express several distinct phenotypes, including immature neuroblast forms that can be induced to differentiate into mature neurons (Ross., et al. 1983) following treatment with retinoic acid (RA) [36]. It is advantageous to use an in vitro model of specialized nervous system cells (i.e. neurons) in this study because isolation of the effects of chemicals at the molecular level is complicated by the heterogeneity of in vivo nervous system networks within tissues.

Cell culture

Cell culture and experimental treatments were performed as previously described [37] with modification. Briefly, human neuroblastoma (SH-SY5Y) cells (ATCC, Bethesda, MD) were maintained under sterile conditions in Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F-12 (1:1) (Gibco Laboratories, Grand Island, NY), supplemented with L-glutamine (2 mM), 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (5,000 U. penicillin and 5,000 µg streptomycin per ml) (Sigma-Aldrich, St. Louis, MO). The cells were maintained under standard conditions (37°C, 5% CO₂) in a humidified incubator.

Treatment of naïve (undifferentiated) SH-SY5Y cells

The measurement outcome (dependent variable) of these experiments is "gene expression", quantified as relative messenger RNA (mRNA) levels. The independent variables examined in this study are "treatment", "time" and "genotype". Four categories of treatments were used in the experiments, including: morphine (the prototypical, full opioid receptor agonist and strong analgesic); DAMGO (a MOR-selective, synthetic opioid peptide); naloxone (the prototypical, pure opioid receptor antagonist); or control (media alone). Individual treatments were administered to triplicate (n = 3) plates as independent experiments. MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR1-B5, MOR1-K1 and β-ACT genotypes (n = 8) were evaluated in each sample for each treatment at one time-point (24hr). Plated cells were randomly allocated to the respective treatment groups at or near confluency. A total of 12 samples (experimental units) were prepared for analysis.

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RA differentiation and treatment of SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells are celebrated as the first neuronal-derived cell line suitable for studying chronic opiate (morphine) effects [38]. Two unique properties of SH-SY5Y cells that auger well for their use in opioid research are their constitutive expression of the mu opiate receptor in measurable quantities [39-41] and the rare ability of this cell line to be induced to express the neuronal phenotype by addition of retinoic acid [35,38,42-44].

To stimulate differentiation of SH-SY5Y cells, all-trans retinoic acid (10 mM) dissolved in absolute ethanol was added to fresh culture medium at 70 - 80% confluence (~15 x 10^6 cells) (final concentration: 10 µM). The cells were exposed to RA for 48 hrs before all media was removed and refreshed. The cells were reintroduced to RA (10 µM) for a further 24-hr period. On harvesting, the SH-SY5Y cells were thrice washed with 1X PBS then stored at -70°C until analysis.

Stock solutions of morphine, DAMGO and naloxone (10 mM) were prepared according to manufacturer's instructions. For qRT-PCR, MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR1-B5, MOR1-K1 and β-ACT genotypes (n = 8) were evaluated in each sample at one of three time-points (n = 3), 24hr, 48hr and 72hr. Plated cells were randomly allocated to the respective treatment groups at or near confluence. A total of 27 treated plates (experimental units) were prepared for analysis.

Differentiated but untreated SH-SY5Y cells [experimental controls]

The measurement outcome (dependent variable) of these experiments is “gene expression”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Control (media alone) received no chemical treatment but a supplemental volume of media only. Triplicate control plates were used for each set of independent experiments. MOR-1, MOR1-A, MOR1-B1, MOR1-B2, MOR1-B3, MOR1-B5, MOR1-K1 and β-ACT genotypes (n = 8) were evaluated in each sample at one of three time-points (n = 3), 24hr, 48hr and 72hr. Plated cells were randomly allocated to the respective treatment groups at or near confluence. A total of 9 plates (experimental units) were prepared for analysis.

Phase contrast microscopy

The growth of undifferentiated controls, differentiated controls and treated, differentiated cultures of SH-SY5Y cells was observed by phase-contrast microscopy. Where possible, photographs were taken to document the progression of neuritogenesis under experimental conditions when cells were differentiated with RA.

Primer design

Sense and anti-sense oligonucleotide (primer) pairs (Table 1) were designed to recognize and amplify the specific region within the C-terminus of the MOR-1 gene where the alternatively-spliced variant is located. Invitrogen's OligoPerfect™ Designer online system was used to design forward and reverse oligonucleotide primers (Invitrogen, Carlsbad, CA), no more than 26 base pairs in length. Additional primers were custom designed to confirm by qRT-PCR the expression of transcription factors and signaling molecules detected on PCR Array (Table 2).

<table>
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<tr>
<th>Primer</th>
<th>Type</th>
<th>Sequence (5’ to 3’)</th>
<th>Bases</th>
</tr>
</thead>
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<tr>
<td>hMOR-1 1F</td>
<td>Forward (Sense)</td>
<td>ATGCCAGTGTCATCATTTAC</td>
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<tr>
<td>hMOR-1 1R</td>
<td>Reverse (Antisense)</td>
<td>GATCCTTGAAAGATTCTGTCTCT</td>
<td>23</td>
</tr>
<tr>
<td>hMOR-1A 1F</td>
<td>Forward (Sense)</td>
<td>CAGGTACGCACTCTAGTTAGG</td>
<td>25</td>
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<tr>
<td>hMOR-1A 1R</td>
<td>Reverse (Antisense)</td>
<td>TTCCCCCTCATCTCTCTCTCTC</td>
<td>20</td>
</tr>
<tr>
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<td>TCAAAAGTCACATCTCTCTCACTGTG</td>
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<tr>
<td>hMOR-1B1 1R</td>
<td>Reverse (Antisense)</td>
<td>GCCTCACTATCTTCTCTCTAGG</td>
<td>25</td>
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<tr>
<td>hMOR-1B2 1F</td>
<td>Forward (Sense)</td>
<td>AAAGAAGACTGTTAGTGTTGTTAGTA</td>
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<td>GCAACCCGAACTAGAGGTTTTG</td>
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<td>hMOR-1B3 1F</td>
<td>Forward (Sense)</td>
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<td>Reverse (Antisense)</td>
<td>GGTGGTTCTTTGCTGTTAGAGG</td>
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<tr>
<td>hMOR-1B5 1F</td>
<td>Forward (Sense)</td>
<td>GGAATTGAACTCCTGGTTTCGTC</td>
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<td>hMOR-1B5 1R</td>
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<td>AGGCTACCTGACCACTACAAA</td>
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<td>Reverse (Antisense)</td>
<td>GCAAGCTGTTTACGACGACAA</td>
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<tr>
<td>hMOR-1K1F</td>
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<td>CTGGTTAGAAAGTCCAGGGAACAA</td>
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<tr>
<td>hMOR-1K1R</td>
<td>Reverse (Antisense)</td>
<td>TGACCCTGTTGCTGCTGCTGACAGA</td>
<td>21</td>
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Table 1: Oligonucleotide primers used for qRT-PCR analysis.

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Table 2: Customized oligonucleotide primers for transcription factors analyzed by qRT-PCR.

<table>
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<th>Bases</th>
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<td>Forward (Sense)</td>
<td>GGTCG CTTTT GGGAT TACCT</td>
<td>20</td>
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<tr>
<td>APOE 1R</td>
<td>Reverse (Antisense)</td>
<td>TCCAG TTTCC AGTTG AGGCC</td>
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<tr>
<td>ARFIP 2F</td>
<td>Forward (Sense)</td>
<td>CTGGCA AGGCT GGAAT ATGAT</td>
<td>20</td>
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<tr>
<td>ARFIP 2R</td>
<td>Reverse (Antisense)</td>
<td>GCTCC AGCTG TTTCT GGTTC</td>
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<tr>
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<td>Forward (Sense)</td>
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<td>BAX 1R</td>
<td>Reverse (Antisense)</td>
<td>CACTG TGACC TGCTC CAGAA</td>
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<tr>
<td>EEF1A 1F</td>
<td>Forward (Sense)</td>
<td>CAGTT CACCT CCGAC GTCA</td>
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<td>EEF1A 1R</td>
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<td>Forward (Sense)</td>
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<tr>
<td>GABRD 2F</td>
<td>Forward (Sense)</td>
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<td>CATCCA TCCTG ACCGT GAGCC</td>
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<td>PACSIN 1R</td>
<td>Reverse (Antisense)</td>
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<tr>
<td>PRKCB 1F</td>
<td>Forward (Sense)</td>
<td>GGGAG TTCAT TGCAC CAGTT</td>
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<td>PRKCB 1R</td>
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<tr>
<td>RAB6A 1F</td>
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<td>SOX2 1F</td>
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<td>AACC GCAAG TGCAG AACTT</td>
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<td>SOX2 1R</td>
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<td>STAT5B 1F</td>
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<td>Reverse (Antisense)</td>
<td>CTGCT GTTGT GGTGC GACAA</td>
<td>20</td>
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</table>

RNA isolation

All procedures were performed according to manufacturer’s protocols. Trizol extraction (Invitrogen, Carlsbad, CA) and DNase treatment of total ribonucleic acid (RNA) were used to purify the sample for reverse transcription (Turbo DNA-free™ Kit, Ambion, Foster City, CA). The final concentration of the reaction mixture was 10 µg of RNA/50 µl DNase cocktail. Total RNA concentrations were checked before proceeding with the remaining procedures. Samples with Nanodrop™ A260/A280 absorbance ratios ≥ 1.8 and adequate RNA concentrations were selected for amplification by quantitative real-time polymerase chain reaction (qRT-PCR) using the Bio-Rad iCycler/MyIQ™ (Bio-Rad, Hercules, CA).

cDNA synthesis

First strand cDNA was reverse-transcribed from purified RNA using a 20 µl reaction mixture (iScript™ cDNA Synthesis Kit, Bio-Rad, Hercules, CA) containing 5 µl (1 µg) of RNA. Samples were incubated in the thermocycler for 30 minutes (25°C, 5 minutes; 42°C, 15 minutes, twice; 85°C, 5 minutes) before storage at -70°C.

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Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was amplified using iQ SYBR Green Supermix™ (Bio-Rad, Hercules, CA) with human forward/reverse primer sets for β-actin (housekeeping gene) and MOR-1 variants (MOR-1, MOR1A, MOR1B1, MOR1B2, MOR1B3, MOR1B5 and MOR1K1 genes (12.5 µl SYBR, 9.5 µl water, 1.5 µl primer, 1.5 µl cDNA). Optimization of the thermal profile at 95°C (5 minutes) was followed by a 2-step amplification and melt process over 40 cycles (95°C, 10 seconds; 55°C, 45 seconds). Cycling at 95°C (1 hr) was followed by 55°C (1 hr), and finally 55°C (10 seconds). The specificity of qRT-PCR was checked by examining melt curves generated for each set of triplicate control, treated and standard curve samples.

Standard curve

Relative gene expression levels were determined using the standard curve method. For each primer pair (forward and reverse), the amplification efficiency for each gene of interest was based on a four-point, 5-fold sample dilution series. Signal threshold cycle (Ct) values were log-transformed in order to extrapolate the level of MOR-1 variant mRNA relative to β-actin (reference gene). Relative expression of an individual gene of interest was defined as the percentage ratio of log-transformed Ct values for treated (Ct-treat) samples to the Ct value for β-actin, relative to controls (Ct-control).

RT² profiler PCR array

Expression profiles for neurodegeneration genes and for transcription factors were generated using commercially-available 96-well plates for Huntington Disease RT² PCR Array, or the Transcription Factors RT² PCR Array (SABiosciences Inc, USA), respectively. RA-Differentiated human neuroblastoma (SH-SYSY) cells were untreated (media only control) or dosed with chronic (48 hr) morphine and cDNA was prepared according to the manufacturer’s instructions.

Limitation

This segment of the research was limited by the use of a minimal number of samples or arrays per treatment group but offered a basic indication of neurodevelopment and transcription factor genes affected by chronic morphine exposure. This information was best used to inform our design of primers for transcription factors that were subsequently validated by qRT-PCR experiments.

Analysis of phosphorylation using BD Phosflow™

This experiment tested the hypothesis that chronic morphine alters the level of serine-residue phosphorylation in differentiated human neuroblastoma (SH-SYSY) cells. The measurement outcome (dependent variable) of these experiments is “level of phosphorylation”. The independent variables examined in this study are “treatment”, “time” and “genotype”. Treatments used in the experiment included morphine, DAMGO, naloxone, or control (media alone), each of which was administered to triplcate plates as independent experiments. Akt, ELK1, PKA and CREB genotypes were evaluated for each treatment at one of two time-points, 24hr and 48hr. Plated cells were randomly allocated to the respective treatment groups. A total of 24 samples (experimental units) as well as relevant assay setup controls were prepared for analysis.

To assess the degree of variability in serine residue phosphorylation after chronic opioid exposure, human neuroblastoma (SH-SYSY) cells were stimulated for 24 or 48 hours in the presence of morphine, DAMGO or naloxone (10 µM) or complete growth media (DMEM:F12 1:1, 2.5 mM L-glutamine, 10% FBS, penicillin/streptomycin) immediately after RA-withdrawal.

The intracellular levels of serine residue phosphorylation in cycling cells was measured by Phosflow technology using BD FACScalibur™ and C ellQuest Pro™ software (Version 6.0) according to manufacturer’s protocols. Briefly, SH-SYSY cell populations were harvested and centrifuged at 3000 RPM for 3 minutes prior to being washed with 1 ml of 1X phosphate buffered saline (PBS) solution. The resultant pellet was stabilized in BD Cytofix™ Fixation Buffer (BD Catalog #557870; 1:1) and overnight stored at 4°C. Next, the cells were permeabilized in 1 ml of ice-cold BD Phosflow™ Perm Buffer III (BD Catalog #558050) and incubated for 30 minutes on ice. The SH-SYSY cells were thrice washed with 3 ml stain buffer (1X PBS, 1% FBS, and 0.09% sodium azide) to block any non-specific action of the antibodies and centrifuged at 600 x g for 6 minutes. The cells were resuspended at a density of 5 x 10⁶ to 10 x 10⁶ cells/ml. An aliquot of 100 µl (1 x 10⁶ cells) was removed and incubated with phosphospecific antibody for 60 min protected from light under a sterile, ventilated hood. After a final wash, the cells were resuspended in 500 µl of stain buffer, passed through a 1cc syringe to eliminate clumps, and immediately analyzed by FACScalibur™. The signals detected in treated and untreated (control) phosphoprotein-specific samples were measured in units of ‘X mean’ fluorescence intensity.
Fluorometric analysis of SH-SY5Y viability by alamarBlue® assay

Cellular viability via the alamarBlue® assay measures both the metabolism capacity and viability of cells, which are biomarkers of cellular physiology in response to chemical cytotoxicity. The alamarBlue® solution (0.5 mg/ml) was prepared in 1X phosphate buffered saline (PBS), kept in a light-shielded eppendorf tube and gently pre-heated to 37°C prior to use. Working solutions of morphine sulfate in Hank’s Balanced Salt Solution (HBSS) were prepared from a 10 mM stock to achieve final well concentrations of 0, 0.1, 0.5, 1, 5, 10, 50 and 100 μM. Morphine treatments were added to 8 consecutive wells on triplicate plates in a ratio of 1:20 for a final well volume of 200 μL. Negative controls containing experimental medium only and positive controls that contained HBSS, water or growth media instead of morphine were included on each plate. Treated plates were incubated for 24 hrs at 37°C followed immediately by the addition of alamarBlue® reagent (10% v/v) to each well volume. All experiments outside the incubator were conducted under a sterile, vented hood using aseptic techniques. The plates were returned to a 37°C, 5% CO₂ humidified incubator for a further 3 hours to allow for color development per manufacturer’s instructions.

The settings for measuring fluorescence intensity were entered onto the microplate fluorometer (Model 7620 Version 5.02, Cambridge Technologies) to reflect excitation and emission wavelengths of 550 nm and 580 nm, respectively. Plates were carefully removed from the incubator and concealed under foil during transport to the fluorometer. Data from triplicate plates was collected immediately thereafter.

Quality assurance/control

Sample size determination

A sample size (n) of three (n = 3) was calculated using the stated equation and based upon the following assumptions:

\[ n = \left( \frac{z_{\alpha/2} \times s}{E} \right)^2 \]

Assuming,

- 80% power level (β = 0.2);
- Standard deviation (σ) between samples of 0.5;
- Difference (Δ) of 0.6 between treatment group means;
- False positive rate (α) of p < 0.05;
- 95% certainty that the true mean is captured within the interval comprising my results.

Assay optimization

Prior to expanding this study, selected parameters for the qRT-PCR assay were optimized, namely: volume (μL) of cDNA loaded per well (equivalent to RNA/cDNA concentration, μg); concentration of cDNA loaded per well (equivalent to relative dilution); and primer concentration (nM) (equivalent to primer volume loaded, μL). Four-point primer volume (2 - 5 μL) or sample dilution curves (1X - 8X, 1X - 27X, 1X - 64X, 1X - 125X, 1X - 1000X) and five-point RNA/cDNA template curves (0.2 - 1 μg) were tested. MOR-1 ASVs were tested for performance at each level. The specificity of qRT-PCR was checked by examining melt curves generated for each set of triplicate control, treated and standard curve samples.

Instrumentation

Prior to each use, the Nanodrop and analytical scale were sanitized and calibrated according to standard laboratory protocols.

Normalization of qRT-PCR data

Replicate samples should be run at least in triplicate assays, and the experiments repeated at least thrice. Relative gene expression is calculated based on the standard curve method (as above) and normalized by housekeeping and control genes. The data should be subjected to dual normalization based on the ratio of log base 2 equivalent values for target and control genes. Specifically, the ratios of target gene: housekeeping gene and target gene: control gene are computed.
Statistical analysis

The data (mean ± SEM) represent triplicate assays of samples obtained from three independent experiments. Statistical analyses were performed using GraphPad Prism™ Version 5. Statistical significance was assessed using one-way analysis of variance (ANOVA) at an alpha level of p < 0.05, followed by Tukey’s multiple comparison post-hoc tests.

Results

Model selection

Human neuroblastoma SH-SY5Y cells are celebrated as the first neuronally-derived cell line suitable for the in vitro study of chronic opiate (morphine) effects [38]. The high constitutive expression of the mu opiate receptor in SH-SY5Y [39-41] and the ability of this cell line to be induced to express the neuronal phenotype by addition of retinoic acid [35,38,43] are two unique properties that enable the SH-SY5Y cells to be widely used in opioid research. Based on our findings, the human neuroblastoma (SH-SY5Y) cell line as a suitable model for studying opioid receptor pharmacology and for elucidating the functional role of MOR-1B alternatively-spliced variants in naïve and differentiated SH-SY5Y cells.

The RA differentiation process elicits a neuronal phenotype in SH-SY5Y cells

Retinoic acid (RA) induced differentiation of native SH-SY5Y cells into cells morphologically classifiable as neuronal cells (Figures 2A-2D). Differentiation of adherent subpopulations of SH-SY5Y cells was checked microscopically. Morphological changes indicative of the neuronal phenotype were confirmed by the presence of dendritic formations, neurite outgrowths, and axonal extensions.

Figure 2A: Growth of monolayer (▼) and adherent (▲) control (non-RA-treated, undifferentiated) SH-SY5Y cells.

Figure 2B: Onset of neurite (▲) outgrowth 24 hr post-RA treatment of SH-SY5Y cells.

Citation: Alrena V Lightbourn. “Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study.” EC Pharmacology and Toxicology 7.5 (2019): 284-324.
Regulation of opioid-responsive MOR-1 ASVs by prototypical opioid receptor ligands in RA-treated SH-SY5Y cells

Based upon our present preliminary screen of mRNA extracted from RA-differentiated human neuroblastoma (SH-SY5Y) cells and analyzed by qRT-PCR using Bio-Rad Thermocycler/MyIQ® software, prototypical opioids induced notable morphological changes after 48 hours of opioid exposure (Figures 3A-3D). Moreover, there was evidence of differential expression of MOR-1 C-terminal alternatively-spliced variants (MOR-1A, MOR-1B1, MOR1-B2, MOR1-B3 and MOR1-B5) and N-terminal variant (MOR-1K1) after 48 hrs of treatment (Figures 4A-4C). A strong tolerogenic effect of aqueous morphine was indicated by highly significant reductions (p < .005) in all measured MOR-1 ASVs as follows: MOR-1A (62.4%), MOR-1B1 (61.9%), MOR1-B2 (66.4%), MOR1-B3 (60.6%), MOR1-B5 (55.0%), MOR-1K1 (72.2%), as compared to controls (Table 3). Naloxone only slightly antagonized MOR-1 ASV expression levels; hence, its effects were not statistically significant (p > .05) when compared to controls (Figure 4B). DAMGO induced MOR-1B5 mRNA expression (118.3%) among the C-terminal alternatively-spliced variants after 48 hrs of treatment (Figure 4C).

Citation: Alrena V Lightbourn. "Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study". EC Pharmacology and Toxicology 7.5 (2019): 284-324.
Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study

Figure 3A: Control (differentiated, untreated) nerve cells (std4_091410).

Figure 3B: Effect of morphine (10 µM) on nerve cell growth 48 hr post-treatment (pic5_090810) (std4_091410).

Figure 3C: Effect of naloxone (10 µM) on nerve cell growth 48 hr post-treatment (pic2_090810).

Citation: Alrena V Lightbourn. "Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study". EC Pharmacology and Toxicology 7.5 (2019): 284-324.
**Figure 3D:** Effect of DAMGO (10 µM) on nerve cell growth 48 hr post-treatment (pic5_090810).

**Figure 3A-3D:** Neuronal Response to Opioid Treatment. Morphologic changes in neuronally-differentiated SH-SY5Y cells treated with conventional prototypic opioids, morphine or naloxone, for 48 hours (Inverted Microscope, Phase Contrast Settings, 10X Objective).

**Figure 4A:** Tolerogenic effect of morphine (48 hr) on MOR-1 alternatively spliced variant expression in differentiated human neuroblastoma (SH-SY5Y) cells. Morphine induced a statistically significant ($p < .005$) reduction in MOR-1 ASV gene expression, which corresponds to the known and expected down-regulation of mu opioid receptors that typifies the development of morphine tolerance.
Figure 4B: Relative expression of MOR-1 spliced variants following 48-hr treatments naloxone in differentiated human neuroblastoma (SH-SY5Y) cells. Marginal decreases in MOR-1 ASV expression were induced by the naloxone. Though not statistically significant (p > .05), overall response is generally indicative of the antagonistic action of naloxone at the mu opioid receptor.

Figure 4C: Effect of DAMGO (D-Ala2,N-MePhe4,Gly-ol5]enkephalin) (48 hr) on MOR-1 alternatively-spliced variant mRNA levels in differentiated human neuroblastoma (SH-SY5Y) cells. A trend toward augmentation of mRNA levels was noted for all C-terminal variants (MOR-1A1, -B1, -B2, -B3, and -B5), which was in contrast to the reduced expression at the N-terminus variant, MOR-1K1. DAMGO significantly increased MOR-1B5 expression (p < .005), as compared to controls.
Constitutive expression of MOR-1 ASVs in naïve SH-SY5Y cells (24 Hrs)

Under optimized conditions, the effect of chronic (24hr) opioid exposure in naïve SH-SY5Y cells was observed (Figure 5). Prototypical opioids differentially regulate MOR-1 ASV expression. Morphine reduced gene expression of one ASV while increasing mRNA of the remaining six. The expression of MOR-1B5 mRNA was decreased by < 1% when compared with control values (p > .05). There was a marginal increase (2.5-8.0%) in MOR-1, MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1K1 mRNA levels above controls (p > .05). Following DAMGO treatment, five ASVs were down-regulated and two were up-regulated. Specifically, MOR-1A (112.0 ± 6.4%) and MOR-1B3 (121.88 ± 18.81%, p < .05) mRNA were higher than controls (p > .05). MOR-1 (87.05 ± 9.9%), MOR-1B1 (96.54 ± 1.6%), MOR-1B2 (98.73 ± 1.4%), MOR-1B5 (96.89 ± 4.1%) and MOR-1K1 (93.13 ± 7.7%) mRNA levels were 1.3-12.9% below controls (p > .05). The responses to naloxone in naïve cells were also negligible and statistically insignificant (p > .05). Following naloxone treatment, five ASVs were up-regulated and two were down-regulated. Naloxone diminished MOR-1 (96.84 ± 1.1%) and MOR-1B1 (97.22 ± 0.33%) expression while negligibly increasing MOR-1B3 (100.3 ± 2.6%) and MOR-1B5 (100.6 ± 1.8%). MOR-1A (104.9 ± 1.9%), MOR-1B2 (106.3 ± 1.8%) and MOR-1K1 (106.5 ± 1.8%) increased slightly above controls (p > .05).

Table 3: Expression of Mu opioid receptor subtype 1 (MOR-1) alternatively-spliced variants (ASV) following 48Hr-treatment with conventional opioids.

<table>
<thead>
<tr>
<th>MOR-1 Variant</th>
<th>Morphine</th>
<th>Naloxone</th>
<th>DAMGO</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR-1A1</td>
<td>62.40%</td>
<td>95.60%</td>
<td>106.90%</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1B1</td>
<td>61.90%</td>
<td>90.80%</td>
<td>106.60%</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1B2</td>
<td>66.40%</td>
<td>90.60%</td>
<td>100.70%</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1B3</td>
<td>60.60%</td>
<td>86.30%</td>
<td>108.20%</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOR-1B5</td>
<td>55.00%</td>
<td>92.10%</td>
<td>118.30%</td>
</tr>
<tr>
<td>(p &lt; 0.0005)</td>
<td></td>
<td>(p &lt; 0.05)</td>
<td></td>
</tr>
<tr>
<td>MOR-1K1</td>
<td>72.20%</td>
<td>91.80%</td>
<td>97.70%</td>
</tr>
<tr>
<td>(p &lt; 0.005)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Opioid-induced (24Hr) mRNA levels of MOR-1 ASVs in RA-differentiated SH-SY5Y cells

Under optimized conditions, chronic (24hr) opioids significantly modulate C-terminus MOR-1 variant expression in differentiated SH-SY5Y cells. A summary of opioid-induced gene expression levels (24Hr), as a percentage of control values, is presented in table 4. Briefly, MOR-1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was significantly enhanced by acute (24hr) morphine (p < .05) and naloxone (p < .001) over RA-control levels (Figure 6A). MOR-1A expression was significantly enhanced by acute (24hr) morphine (p < .001), DAMGO (p < .01) and naloxone (p < .001) over RA-control levels (Figure 6B). MOR-1B1 expression was significantly increased by acute (24hr) DAMGO (p < .05) and naloxone (p < .05) over RA-control levels (Figure 6C). MOR-1B2 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was significantly augmented by acute (24hr) morphine (p < .05) and naloxone (p < .001) over RA-control levels. Compared to naloxone-induced mRNA levels, MOR-1B2 expression after morphine or DAMGO were significantly lower (p < .01) (Figure 6D). MOR-1B3 expression was significantly amplified by acute (24hr) DAMGO (p < .05) and naloxone (p < .001) over RA-control levels. Compared to naloxone, the level of mRNA induced by morphine (p < .01) or DAMGO (p < .001) was significantly reduced (Figure 6E). MOR-1B5 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was significantly enhanced by acute (24hr) naloxone (p < .05), as compared to RA-control levels (Figure 6F). The expression of MOR-1K1 in the N-terminus of RA-differentiated human neuroblastoma (SH-SY5Y) cells was insignificantly enhanced by acute (24hr) morphine, DAMGO, or naloxone (p > .05) when compared to RA-control levels (Figure 6G).

<table>
<thead>
<tr>
<th>MOR-1 Variant</th>
<th>Percent Change in Relative Gene Expression after 24Hr-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morphine</td>
</tr>
<tr>
<td>MOR-1</td>
<td>130.3 ± 7.3</td>
</tr>
<tr>
<td>MOR-1A</td>
<td>139.4 ± 7.2</td>
</tr>
<tr>
<td>MOR-1B1</td>
<td>109.9 ± 1.6</td>
</tr>
<tr>
<td>MOR-1B2</td>
<td>109.6 ± 2.3</td>
</tr>
<tr>
<td>MOR-1B3</td>
<td>109.5 ± 5.0</td>
</tr>
<tr>
<td>MOR-1B5</td>
<td>115.0 ± 4.1</td>
</tr>
<tr>
<td>MOR-1K1</td>
<td>106.8 ± 6.5</td>
</tr>
</tbody>
</table>

Table 4: Effect of opioid (24Hr) exposure on Mu opioid receptor subtype 1 (MOR-1) variant mRNA levels.

Figure 6A: MOR-1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was significantly enhanced by chronic (24hr) morphine (p < .05) and naloxone (p < .01) over RA-control levels.
Figure 6B: MOR-1A expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly enhanced by chronic (24hr) morphine (p < .001), DAMGO (p < .01) and naloxone (p < .001) over RA-control levels.

Figure 6C: MOR-1B1 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly enhanced by chronic (24hr) DAMGO (p < .05) and naloxone (p < .05) over RA-control levels.

Figure 6D: MOR-1B2 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly enhanced by chronic (24hr) morphine (p < .05) and naloxone (p < .001) over RA-control levels. Compared to naloxone-induced mRNA levels, MOR-1B2 expression after morphine or DAMGO were significantly lower (p < .01).
Figure 6E: MOR-1B3 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly enhanced by chronic (24hr) DAMGO (p < .05) and naloxone (p < .001) over RA-control levels. Compared to naloxone, the level of mRNA induced by morphine (p < .01) or DAMGO (p < .05) was significantly reduced.

Figure 6F: MOR-1B5 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly enhanced by chronic (24hr) naloxone (p < .05), as compared to RA-control levels.

Figure 6G: The expression of MOR-1K1 in the N-terminus of MOR-1 in RA-differentiated human neuroblastoma (SH-SYSY) cells was not significantly enhanced by chronic (24hr) morphine, DAMGO, or naloxone (p>.05) when compared to RA-control levels.

Citation: Alrena V Lightbourn. “Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study”. EC Pharmacology and Toxicology 7.5 (2019): 284-324.
Chronic (48- or 72Hr) opioid effects on MOR-1 ASV expression in differentiated SH-SY5Y cells

Forty-eight (48)-hour study

Data summarizing the effect of prototypical opioids on differentiated SH-SY5Y cells at 48Hrs (Table 5) are presented as the mean ± SEM. Briefly, significant variability in posttranscriptional expression of MOR-1 alternatively-spliced variants (ASVs) was observed at the 48Hr time point. Active but less stable activity than at the earlier time point (24Hrs) may suggest a switch to adaptation mode due to prolonged perturbation of the cellular environment in the presence of prototypical opioids. With the exception of MOR-1 and the MOR-1B5 variant, which were fairly robust at 48Hrs despite the chemical turbulence, the remaining ASVs appear to be more tightly regulated.

<table>
<thead>
<tr>
<th>MOR-1 Variant</th>
<th>Morphine</th>
<th>DAMGO</th>
<th>Naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR-1</td>
<td>144.2 ± 50.0</td>
<td>129.3 ± 12.1</td>
<td>163.3 ± 28.8</td>
</tr>
<tr>
<td>MOR-1A</td>
<td>113.9 ± 8.0</td>
<td>98.2 ± 6.2</td>
<td>117.7 ± 18.1</td>
</tr>
<tr>
<td>MOR-1B1</td>
<td>105.0 ± 3.9</td>
<td>91.8 ± 5.9</td>
<td>108.3 ± 3.5</td>
</tr>
<tr>
<td>MOR-1B2</td>
<td>112.1 ± 10.2</td>
<td>103.2 ± 7.7</td>
<td>123.0 ± 11.1</td>
</tr>
<tr>
<td>MOR-1B3</td>
<td>117.3 ± 15.2</td>
<td>104.0 ± 9.8</td>
<td>124.9 ± 20.3</td>
</tr>
<tr>
<td>MOR-1B5</td>
<td>141.0 ± 9.5</td>
<td>134.9 ± 6.9</td>
<td>153.4 ± 13.8</td>
</tr>
<tr>
<td>MOR-1K1</td>
<td>110.1 ± 12.6</td>
<td>107.1 ± 6.6</td>
<td>113.8 ± 15.6</td>
</tr>
</tbody>
</table>

Table 5: Effect of opioid (48Hr) exposure on Mu opioid receptor subtype 1 (MOR-1) variant mRNA levels.

MOR-1 mRNA levels increased 44.2 ± 50%, 29.3 ± 12.1% and 63.3 ± 28.8% above control (100%) values following morphine, DAMGO and naloxone, respectively (Figure 7A). Morphine (13.9 ± 8.0%) and naloxone (17.7 ± 18.1%) comparably increased the expression of MOR-1A over control levels, while DAMGO slightly lowered these effects by 1.8% (Figure 7B). Similar effects were observed for MOR-1B1, in which morphine and naloxone respectively produced mRNA levels roughly 5 and 8.2% higher than controls. A slight reduction in MOR-1B1 levels was seen after DAMGO treatments (91.8 ± 5.9%) (Figure 7C).

Figure 7A: MOR-1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was not significantly augmented by chronic (48hr) morphine, DAMGO, or naloxone when compared to RA-control levels (p > .05).
MOR-1B2 mRNA levels was increased by morphine (112.1 ± 10.2%) and naloxone (123.0 ± 11.1%), but only marginally so by DAMGO (103.2%) when compared to controls (Figure 7D). Up-regulation of MOR-1B3 was observed after morphine (117.3 ± 15.2%), DAMGO (104.0 ± 9.8%) and naloxone (124.9 ± 20.3%) relative to controls. MOR-1B3 expression was not significant (p > .05) following opioid treatment (Figure 7E). One-way ANOVA detected statistically significant increases in the magnitude of morphine- (141.0 ± 9.5%, p < .05) and naloxone-induced (153.4 ± 13.8%, p < .05) up-regulation of MOR-1B5 expression, compared to controls (Figure 7F). The levels of MOR-1K in morphine-, DAMGO- and naloxone-stimulated cells were 110.1 ± 12.6%, 107.1 ± 6.7% and 113.8 ± 15.6%, respectively (Figure 7G).

**Figure 7B:** MOR-1A expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was not significantly enhanced by chronic (48hr) morphine, DAMGO, or naloxone over RA-control levels (p > .05).

**Figure 7C:** MOR-1B1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was only marginally altered by chronic (48hr) morphine, DAMGO, or naloxone (p > .05), as compared to RA-control levels.

Citation: Alrena V Lightbourn. "Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study". *EC Pharmacology and Toxicology* 7.5 (2019): 284-324.
Figure 7D: MOR-1B2 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was not significantly modified by chronic (48hr) morphine, DAMGO, or naloxone (p > .05) when compared to RA-control levels.

Figure 7E: MOR-1B3 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was not significantly greater in chronic (48hr) morphine-, DAMGO-, or naloxone-treated samples (p < .05), as compared with RA-control levels.

Figure 7F: MOR-1B5 expression in RA-differentiated human neuroblastoma (SH-SYSY) cells was significantly increased following exposure to chronic (48hr) morphine (p < .05) or naloxone (p < .05), as compared to RA-control levels.
With the exception of DAMGO-induced down-regulation of MOR-1A and MOR-1B1, all other variants for all chronic (48Hr) treatments were consistently up-regulated over control levels following stimulation with prototypical mu agonists (Table 5).

**Seventy-two (72)-hour study**

Data summarizing the effect of prototypical opioids on differentiated SH-SY5Y cells at 72Hrs, by gene, are presented as the mean ± SEM (Table 6). To further assess the effect of prolonged opioid exposure on MOR-1 variant expression, the mRNA levels of SH-SY5Y cells treated for 72 hrs were examined. Overall, dramatic increases in the amount of gene expression after naloxone treatments were observed and found to be statistically significant. In many cases, prolonged opioid action intensified the effects of the ligands to produce mRNA levels higher than those observed at both 24 and 48 hrs. For instance, although differences in the level of MOR-1K1 were negligible (100.08 ± 29.0%), increases in variant expression following naloxone ranged from 2.5- to 14-fold over control levels (Figure 8G, Table 6). All cells treated with DAMGO experienced induction of their MOR-1 variant mRNA levels, ranging from 11.1-170% above control levels. The action of morphine was less consistent, however: There were considerable reductions in expression of MOR-1A1 (81.1 ± 4.5%, Figure 8B), MOR-1B1 (73.6 ± 8.3%, Figure 8C) and MOR-1K1 (55.0 ± 18.6%, Figure 8G) versus marginal to modest changes in MOR-1B5 (102.9 ± 27.5%, Figure 8F), MOR-1B2 (132.5 ± 2.1%, Figure 8D), MOR-1B3 (136.8 ± 12.3%, Figure 8E) and MOR-1 (252.8 ± 35.1%, Figure 8A) levels.

<table>
<thead>
<tr>
<th>MOR-1 Variant</th>
<th>Percent Change in Relative Gene Expression after 72Hr-Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morphine</td>
</tr>
<tr>
<td>MOR-1</td>
<td>252.8 ± 35.1</td>
</tr>
<tr>
<td>MOR-1A</td>
<td>81.1 ± 4.5</td>
</tr>
<tr>
<td>MOR-1B1</td>
<td>73.6 ± 8.3</td>
</tr>
<tr>
<td>MOR-1B2</td>
<td>132.5 ± 2.1</td>
</tr>
<tr>
<td>MOR-1B3</td>
<td>136.8 ± 12.3</td>
</tr>
<tr>
<td>MOR-1B5</td>
<td>102.9 ± 27.5</td>
</tr>
<tr>
<td>MOR-1K1</td>
<td>55.0 ± 18.6</td>
</tr>
</tbody>
</table>

Table 6: Effect of opioid (72Hr) exposure on Mu opioid receptor subtype 1 (MOR-1) variant mRNA levels.

**Figure 7G:** MOR-1K1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was not significantly enhanced by chronic (48hr) morphine, DAMGO, or naloxone (p > .05), as compared to RA-control levels.

_Citation:_ Alrena V Lightbourn. "Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study". _EC Pharmacology and Toxicology_ 7.5 (2019): 284-324.
Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study

**Figure 8A:** MOR-1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was substantially and significantly (p < .001) up-regulated after chronic (72hr) naloxone as compared to controls, while comparatively marginal increases were observed after morphine and DAMGO exposure.

**Figure 8B:** MOR-1A expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was significantly enhanced by chronic (72hr) naloxone (p < .05) over RA-control levels, while morphine down-regulated MOR-1A.

**Figure 8C:** MOR-1B1 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was substantially and significantly increased by chronic (72hr) naloxone (p < .001) over RA-control levels, while morphine-induced levels were down-regulated.

**Citation:** Alrena V Lightbourn. “Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study”. *EC Pharmacology and Toxicology* 7.5 (2019): 284-324.
Figure 8D: MOR-1B2 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was substantially and significantly enhanced by chronic (72hr) naloxone (p < .001) over RA-control levels, while comparatively marginal increases were observed after morphine and DAMGO exposure.

Figure 8E: MOR-1B3 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was substantially and significantly enhanced by chronic (72hr) naloxone (p < .001) over RA-control levels, while comparatively marginal increases were observed after morphine and DAMGO exposure.

Figure 8F: MOR-1B5 expression in RA-differentiated human neuroblastoma (SH-SY5Y) cells was substantially and significantly up-regulated by chronic (72hr) naloxone (p < .001) over RA-control levels.

Citation: Alrena V Lightbourn. “Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study”. EC Pharmacology and Toxicology 7.5 (2019): 284-324.
A comparison of the posttranscriptional regulation of MOR-1 variants in differentiated SH-SY5Y cells revealed a consistent trend at both 48- and 72hr time-points, i.e., expression of all MOR-1 variants exceeded control levels in differentiated cells (Tables 5 and 6). However, the level of MOR-1 expression induced by morphine (Figure 9A), DAMGO (Figure 9B), and naloxone (Figure 9C) at 72 hrs was substantially higher than that of the alternatively-spliced variant forms at 48hrs (Figure 7A-7C).

Regulation of MOR-1 variant by chronic opioids at 72 hrs produced substantial statistically significant differences in their gene expression profile. The level of MOR-1 after morphine treatment was highly significant (p < .001) when compared to controls, and significantly different from the individual alternatively-spliced variant responses: MOR-1A1 (p < .001), MOR-1B1 (p < .001), MOR-1B2 (p < .01), MOR-1B3 (p < .01), MOR-1B5 (p < .001), MOR-1K1 (p < .001) (Figure 9A). Greater variability existed among variant responses induced by DAMGO, yet all variant mRNA levels were higher than control values (Figure 9B). Similar to the changes seen after morphine, naloxone up-regulation of MOR-1 at 72 hrs was greatly enhanced (>14-fold). The naloxone-induced levels of MOR-1B2 and MOR-1B3 were significantly higher than controls (p < .001), and all variant mRNA levels were statistically significantly lower than the level by MOR-1 (p < .001) (Figure 9C).
Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study

Citation: Alrena V Lightbourn. “Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study”. *EC Pharmacology and Toxicology* 7.5 (2019): 284-324.

PhosFlow™: Phospho-specific determination of signaling and transcription proteins

**Effect of chronic (24Hr) opioids on protein phosphorylation**

After 24hr morphine treatments, phosphorylation of Akt (pAkt at S473) (98.37 ± 1.7%), PKA (pPKA at S114) (96.67 ± 6.0%), and CREB (pCREB at S133) (96.93 ± 4.6%) were reduced relative to untreated controls. Phospho-ELK1 (pELK at S383) levels (101.99 ± 0.7%) were increased compared to control values (100%) (Figure 10A). Chronic (24hr) DAMGO had the opposite effects in differentiated SH-SYSY cells, increasing pAkt (S473) (100.74 ± 1.6%), pPKA (S114) (104.68 ± 13.7%), and pCREB (S133) (119.11 ± 8.02%), while decreasing pELK (S383) levels (97.39 ± 0.8%) compared to controls (Figure 10B). Naloxone administration for 24hrs produced increases in pAkt (102.40 ± 3.6%), pPKA (106.58 ± 14.8%) and pELK (100.16 ± 1.3%), but attenuated CREB phosphorylation (100.16 ± 1.3%) as compared to controls (Figures 10C).

**Effect of chronic (48Hr) opioids on protein phosphorylation**

Following 48hr morphine treatments, pAkt (S473) (123.94 ± 5.32%, p < .01), pPKA (S114) (103.45 ± 1.9%), and pELK (S383) (102.84 ± 2.6%) were increased relative to untreated controls. pCREB (S133) levels (97.16 ± 20.3%) were decreased compared to control values (100%) (Figure 10A). Chronic (48hr) DAMGO decreased pPKA (S114) (99.40 ± 2.7%), pCREB (S133) (61.96 ± 7.8%, p < .001) and pELK (S383) levels (89.77 ± 7.7%) while increasing intracellular pAkt (S473) (108.13 ± 0.9%) levels, as compared to controls (Figure 10B). Naloxone administered for 48hrs enhanced signalling protein levels [pAkt (112.23 ± 4.7%) and pPKA (108.02 ± 5.7%)] but the phosphorylation of transcription factors [pELK (95.30 ± 4.2%) and pCREB (62.61 ± 7.6%, p < .001)] were reduced when compared to controls (Figures 10C).

Figure 9A-9C: Comparison (by genotype) of chronic (72Hr) effects of morphine (A), DAMGO (B), or naloxone (C) on MOR-1 variant expression in RA-differentiated human neuroblastoma (SH-SYSY) cells. Significant differences between treated and vehicle controls groups determined by one-way ANOVA are denoted as: *P < .05, **P < 0.01, and ***P < 0.001. Significant differences from MOR-1 are denoted: ###P<0.001.
Figure 10A-10C: Comparison of posttranslational modifications (i.e., phosphorylation) of signaling and transcription proteins, by treatment, after chronic (24- or 48Hrs) opioid exposure: (A) morphine, (B) DAMGO, or (C) naloxone. Data represent the mean ± SEM (n = 3). Statistical significance set at p < .05 is denoted here as: **p < .01, ***p < .001 (treated versus control); ## p < .001, ### p < .001 (inter-sample comparisons).

Time-dependent alterations in protein phosphorylation

A shift in the level of phosphorylation of signal transduction proteins (PKA, Akt) and transcription factors (CREB, ELK1) at specific serine residues was observed and found to be significantly different following exposure to mu opioid receptor (MOR-1) ligands for 48 hrs versus 24 hrs (Figure 11). As compared to controls, the morphine-reduced pAkt levels at 24 hrs (98.37 ± 1.7%) were significantly enhanced after 48hr exposures (123.91 ± 5.3%, p < .0001). The increases in pCREB (119.11 ± 8.0%) induced by DAMGO at 24 hrs were noticeably reversed after 48hr treatments (61.96 ± 7.8%, p < .001). In addition, naloxone-mediated reductions in pCREB at 24 hrs (88.43 ± 3.2%) were further attenuated after 48 hrs (62.61 ± 7.6%, p < .05).
Figure 11: Temporal effects of chronic opioids on phosphoprotein signals in differentiated human neuroblastoma (SH-SY5Y) cells, by gene. Data represent the mean ± SEM (n = 3). Statistical significance set at p < .05 is denoted as:

** p < .01, *** p < .001 (treated versus control); # p < .01, ## p < .001 (inter-sample comparisons).

The chronic (24hr) effect of morphine or DAMGO on the phosphorylation of PKA were opposite to the effects elicited on pELK levels. Morphine reduced PKA while increasing ELK1 phosphorylation. In comparison, DAMGO increased pPKA while decreasing pELK. Moreover, there were distinct differences in chronic opioid effects elicited at 48 hrs. Morphine increased both PKA and ELK1 phosphorylation, while DAMGO decreased pPKA and pELK levels. Chronic naloxone-augmented pPKA levels after 24hrs were slightly lowered at 48hrs, and enhancements in pELK levels after 24hrs were somewhat reduced following 48hr exposures. With the exception of PKA whose activation was strongly induced by naloxone at 24 hrs, the postranslational modification (i.e. phosphorylation) of PKA and ELK1 were more intensely induced after 48hrs. Also, while pCREB and pELK were both reduced by DAMGO or naloxone at 48 hrs, opposing regulation of these proteins was observed in all 24hr experiments (morphine, DAMGO and naloxone) and following chronic (48hr) morphine studies.

Screening analysis of signaling and transcription proteins by RT2 profiler PCR array

The effect of chronic (48hr) morphine, the most vulnerable time-point observed in this series of assays, was screened using PCR Array of proteins expressed in neurological disease and transcription factors. Transcription factors (Table 7) and genes highly expressed in Huntington’s disease (Table 8) are summarized below. Given existing limitations to this assay, detected responses were further restricted to those genes that were up- or down-regulated at least 2-fold over control levels. Chronic (48 hrs) morphine up-regulated the following transcription factors: TBP, FOXA2, ETS1, MYC, GATA2, but down-regulated STAT5B, as compared to controls. Among neurological disease genes, PLOD2 alone was down-regulated ≥2-fold. In contrast, 13 genes were up-regulated (APOE, ARF1P2, BAX, EEF1A2, GABRD, LPL, PACSIN1, PRKCB, RAB6A, RILP, SOX2), including two reference genes (B2M and HGDC).
### Table 7: Expression profile of transcription factors following chronic (48 Hr) morphine treatments: PCR array versus qRT-PCR.

<table>
<thead>
<tr>
<th>Unigene</th>
<th>GeneBank</th>
<th>Symbol</th>
<th>Description</th>
<th>Gene Name</th>
<th>Fold Change (PCR Array)</th>
<th>Fold Change (qRT-PCR)</th>
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<tbody>
<tr>
<td>Hs.654439</td>
<td>NM_000041</td>
<td>APOE</td>
<td>Apolipoprotein E</td>
<td>AD2/LDLQ5/LPG/MGC1571</td>
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<td>Hs.75139</td>
<td>NM_012402</td>
<td>ARFIP2</td>
<td>ADP-ribosylation factor interacting protein 2</td>
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<tr>
<td>Hs.624291</td>
<td>NM_004324</td>
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<td>BCL2-associated X protein</td>
<td>BCL2L4</td>
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<td>Not analyzed</td>
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<tr>
<td>Hs.433839</td>
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<td>EEF1A2</td>
<td>Eukaryotic translation elongation factor 1 alpha 2</td>
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</tr>
<tr>
<td>Hs.113882</td>
<td>NM_000815</td>
<td>GABRD</td>
<td>Gamma-aminobutyric acid (GABA) A receptor, delta</td>
<td>EIG10/EJMG/EFSP5/MGC45284</td>
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<td>Not analyzed</td>
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<td>Hs.180878</td>
<td>NM_002375</td>
<td>LPL</td>
<td>Lipoprotein lipase</td>
<td>HDLCQ11/LIPD</td>
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<tr>
<td>Hs.520087</td>
<td>NM_020804</td>
<td>PACS1N</td>
<td>Protein kinase C and casein kinase substrate in neurons 1</td>
<td>KIAA1379/DPI</td>
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<td>1.9</td>
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<td>Hs.460355</td>
<td>NM_002738</td>
<td>PRKCB</td>
<td>Protein kinase C, beta</td>
<td>MGC41878/PKCB-PRI/PRKCB1/PRKCB2</td>
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<tr>
<td>Hs.503222</td>
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<td>RAB6A</td>
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<td>Hs.533497</td>
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<td>Rab interacting lysosomal protein</td>
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<tr>
<td>Unigene</td>
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<td>Symbol</td>
<td>Description</td>
<td>Gene Name</td>
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<td>Fold Change (qRT-PCR)</td>
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<td>Hs.518438</td>
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<td>Hs.477866</td>
<td>NM_182943</td>
<td>PLOD2</td>
<td>Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2</td>
<td>LH2/TLH</td>
<td>-2.6</td>
<td>Not analyzed</td>
</tr>
</tbody>
</table>

### Table 8: Expression profile of Huntington's disease genes following chronic (48 Hr) morphine treatments: PCR array versus qRT-PCR.

<table>
<thead>
<tr>
<th>Unigene</th>
<th>GeneBank</th>
<th>Symbol</th>
<th>Description</th>
<th>Gene Name</th>
<th>Fold Change (PCR Array)</th>
<th>Fold Change (qRT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hs.369438</td>
<td>NM_005238</td>
<td>ETS1</td>
<td>V-ets erythroblastosis virus E26 oncogene homolog 1 (avian)</td>
<td>ETS1-EWSR2, FLJ10768</td>
<td>2.3</td>
<td>1.0</td>
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<tr>
<td>Hs.155651</td>
<td>NM_021784</td>
<td>FOXA2</td>
<td>Forkhead box A2</td>
<td>HNF3B, MGC19087, TCF3B</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Hs.367725</td>
<td>NM_032638</td>
<td>GATA2</td>
<td>GATA binding protein 2</td>
<td>FLJ45948, MGC2306, NFE1B</td>
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<td>Not analyzed</td>
</tr>
<tr>
<td>Hs.202453</td>
<td>NM_002467</td>
<td>MYC</td>
<td>V-myc myelocytomatosis viral oncogene homolog (avian)</td>
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<tr>
<td>Hs.595276</td>
<td>NM_012448</td>
<td>STAT5B</td>
<td>Signal transducer and activator of transcription 5B</td>
<td>STAT5</td>
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<td>0.9</td>
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<tr>
<td>Hs.590872</td>
<td>NM_003194</td>
<td>TBP</td>
<td>TATA box binding protein</td>
<td>GTF2D, GTF2D1, HDL4, MGC117320, MGC12654, MGC126054, MGC126055, SCA17, TFIIID</td>
<td>2.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Citation: Alrena V Lightbourn. "Biochemical and Molecular Modulation of Mu Opioid Receptor Signal Transduction, Phosphorylation, Transcription and Posttranscriptional Gene Expression in Human Neuroblastoma Cells: A Translational Basic Sciences Pilot Study". EC Pharmacology and Toxicology 7.5 (2019): 284-324.
qRT-PCR validation of genes altered on RT^2 profiler PCR array

The expression of ETS1, FOXA2, STAT5B, TBP, ARFIP2, LPL, PACSIN1, RAB6A, RILP and SOX2 seen in PCR array experiments was confirmed in qRT-PCR. STAT5B was down-regulated in both experiments but not with the same magnitude. ARFIP2, LPL and RAB6A were up-regulated in PCR array but down-regulated in qRT-PCR. Up-regulation of ETS1 in PCR array contrasted with negligible change in gene expression in RT-qPCR. FOXA2, TBP, PACSIN1, and SOX2 were up-regulated in both PCR array and RT-qPCR, though to varying extents (Table 7 and 8). Morphine-induced up-regulation of PAC, and down-regulation of RILP and LPL expression in RT-qPCR were each highly statistically significant (***p < .001, two-way ANOVA) compared to controls, and significant (###p < .001) differences were also noted between PAC and the other genes under analysis.

Cell Viability

Neuronally-differentiated human neuroblastoma (SH-SY5Y) cells treated with morphine sulfate (0, 0.1, 0.5, 1, 5, 10, 50 or 100 μM) for 24, 48 or 72 hours were assayed fluorometrically using the alamarBlue® reagent. A sharp reduction in cellular viability was observed between 24 and 48 hours, with slight recovery detected at the 72-hr time-point. Growth response to morphine was approximately linear between concentrations of 0.1 μM and 10 μM (Figure 14).

Figure 12: RT-qPCR Gene Expression Profile of Transcription Factors and Neurological Disease Genes Following Chronic (48 Hr) Morphine. Statistical significance determined by two-way analysis of variance (ANOVA): ***p < .001, compared to controls; ###p < .001 compared to PAC.

Figure 13: GPCR Signaling Pathways. G-protein coupled receptors (GPCRs) couple with a diverse guanine nucleotide binding protein (G-protein) signal transduction pathways that converge on a variety of kinase cascades. Multiple target proteins may be activated through this mechanism to produce multiple and varied cellular responses.

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Figure 14: Dose-response curve for chronic morphine administered to human neuroblastoma (SH-SY5Y) cells.

Neuronally-differentiated human neuroblastoma (SH-SY5Y) cells treated with morphine sulfate (0, 0.1, 0.5, 1, 5, 10, 50 or 100 μM) for 24, 48 or 72 hours were assayed fluorometrically using the alamarBlue® reagent as described in the Materials and Methods section. Data shown are mean ± SEM, percentage of control (bars, n = 8). Statistical analysis by repeated measures (mixed model) ANOVA followed by Bonferroni post-hoc tests for significant differences between treatment groups (α < .05).

Discussion

The pharmacological significance of newly-identified mu opioid receptor (MOR-1) alternative splice variants has not been characterized relative to drug response mechanisms and may inform the issue of morphine tolerance. Recent indications of a potentially functional role for MOR-1 variants [45] raise prospects for a more specific contribution to the development of morphine tolerance. My research therefore focused on the molecular characterization of MOR-1 variants to elucidate mechanisms of morphine tolerance. Consideration of whether the human neuroblastoma (SH-SY5Y) cell line is a good model for investigating MOR-1 splice variants, whether morphine tolerance could be observed in this model at the sub-receptor level, as well as factors that contribute to the regulation of human MOR-1 alternatively spliced variants helped to frame the design of the research.

SH-SY5Y Cells as Model for MOR-1 ASV Expression

Based on our findings, the human neuroblastoma (SH-SY5Y) cell line as a suitable model for studying opioid receptor pharmacology and for elucidating the functional role of MOR-1B alternatively-spliced variants in naïve and differentiated SH-SY5Y cells. Morphological changes indicative of the neuronal phenotype were confirmed by the presence of dendritic formations, neurite outgrowths, and axonal extensions. These preliminary data demonstrate that both naïve and RA-differentiated SH-SY5Y cells simultaneously express all tested, alternatively-spliced isoforms of the mu opioid receptor subtype 1 (MOR-1).

The expression of MOR-1 ASVs in human neuroblastoma (SH-SY5Y) cells was differentially regulated in response to developmental cues activated by all-trans retinoic acid. Together, these findings demonstrate the developmental effect of retinoic acid on the posttranscriptional expression of certain MOR-1 variants, a response typically mediated through opioid interaction with MOR-1, 7-transmembrane (7TM) receptors. These data are also consistent with existing literature suggesting that in vitro treatment of cell cultures with all-trans retinoic acid induces neuronal differentiation [46-48]. An earlier report by Kasmi and Mishra [49] also demonstrated that SH-SY5Y cells express the classic mu opioid receptor. The combination of MOR-1 expression, as well as its capacity to assume the neuronal phenotype on RA-stimulation, suggested that these cells would likely also express alternatively-spliced variants. Taken together, these data suggest that

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differentiated SH-SY5Y cells are a suitable model for investigating the influences of opioids, or other structurally-similar compounds, on the expression of MOR-1 alternatively-spliced isoforms. The differential expression of these isoforms may be both time-dependent and ligand-specific.

**Preliminary (48Hr) test of MOR-1 ASV-ligand interactions**

To evaluate the responsiveness of OPRM1 splice variants to regulation by opiate receptor ligands, we compared and contrasted the levels at which morphine (specific MOR-1 agonist), naloxone (non-selective MOR-1 antagonist), or DAMGO \([\text{D-Ala}^2,\text{MePhe}^4,\text{Gly}[\text{ol}]^5]\) enkephalin (synthetic opioid peptide, specific MOR-1 agonist) regulates the expression of MOR-1 alternatively-spliced variants following chronic (48 hr) exposure. We measured C-terminal variants (MOR-1A1, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B4 and MOR-1B5), N-terminal variant (MOR-1K1) and housekeeping gene, beta-actin. From these data, we sought to identify any correlation between the development of morphine tolerance and the regulation of human MOR-1 alternatively-spliced variants by chronically administered (48hr) morphine-like compounds. Tolerance can be induced through acute or chronic administration of morphine. Since gene regulation is a slower process, differential induction of gene expression and ligand-specificity were expected to be more pronounced, thereby facilitating discrimination between key variants that could be targeted for further investigation.

The preliminary data from this study are consistent with previous studies indicating that activation of the mu opioid receptor stimulates cellular proliferation and growth \([50,51]\). The patterns observed in agonist responses appear to parallel the open literature, in that, morphine characteristically down-regulates, while DAMGO up-regulates, MOR-1 receptor expression. Additionally, these data demonstrate the spatial (C-terminal vs. N-terminal) and temporal nature of alternative splicing of the OPRM1 gene, similar to Steitz., et al. \([52]\) and Konig and Muller \([53]\). It has been suggested that alternative splicing may down-regulate gene expression through transcript decay \([54,55]\). Given the differential expression observed in this experiment, the extent to which the rate of transcript degradation directly influences MOR-1 ASV expression may be further explored by expanding the experimental time-course.

Of note are the lack of equivalence in drug-induced responses between samples, the partial selectivity of clusters within samples, and the overall difference in the intensity of responses of C-terminal variants versus N-terminal variants under assay conditions. On chronic exposure to prototypical agonist (morphine), C-terminal variant expression sharply declined, compared to a smaller shift in the level of N-terminal MOR-1K1 expression. There was no appreciable difference in the level of C-terminal versus N-terminal response following treatment with a prototypical MOR-1 antagonist. This agonist-driven pattern may suggest a ligand-dependent role in the activation of N-terminal versus C-terminal promoters. Taken together, the ability of chronically-administered mu opioid receptor ligands to regulate RNA alternative splicing mechanisms is an indication of the potency, efficacy, long-term and far-reaching effects of these compounds on potentially new therapeutic target sites. These data also suggest a potential role for alternative splicing as a control mechanism in the development and/or progression of morphine tolerance.

**Regulation of MOR-1 ASVs after 24Hrs**

To study the effects of chronic opioids on the posttranscriptional expression of MOR-1 variants, naïve or differentiated SH-SY5Y cells were exposed to morphine, DAMGO, or naloxone for 24 hours. Aqueous morphine has previously shown diminished capacity to elicit a strong (2- to 4-fold) MOR-1 ASV expression response in RA-differentiated under unoptimized conditions in a 24hr assay \([37]\). However, morphine is soluble in both water (64 mg/mL) and alcohol (1.8 mg/mL), per manufacturer’s product information sheet at the time of study.

The present evaluation demonstrates that both naïve and RA-differentiated SH-SY5Y cells measurably express MOR-1 ASVs, and that these variants are differentially regulated by \(*\text{all-trans*} retinoic acid, which triggers certain developmental cues. Studies by Wei and Loh \([56]\), and later Wu and colleagues \([57]\), draw attention to the transcriptional-level repression of posttranscriptional expression by the mouse MOR-1 3’-untranslated region (UTR). In contrast, retinoic acid enhances the posttranscriptional expression of certain MOR-1 variants \([58]\), a response typically mediated through opioid interaction with MOR-1, 7-transmembrane (7TM) receptors \([59]\). Taken together,
these data confirm the suitability of RA-differentiated SH-SY5Y cells for investigating the influences of opioids or other structurally similar compounds, and more importantly, for the expression of MOR-1 alternatively-spliced isoforms. As such, the differential expression of these isoforms may be both time-dependent and ligand-specific.

The 24-hr study data align with receptor binding studies which show that MOR-1 and MOR-1A mRNA species are more abundant than that observed for MOR-1B in rat CNS [60]. They are also consistent with MOR-1 variant pharmacokinetics studies which indicate a greater efficacy of morphine than DAMGO, and higher morphine activity at MOR-1 and MOR-1A than at MOR-1B [61]. Differences in the abundance of individual mRNA species may reflect changes in the transcription machinery, possibly resulting from altered posttranslational modifications such as protein phosphorylation [62]. Taken together, these data represent novel findings that MOR-1 variants display differential sensitivities to chronic (24 hr) opioids in both naïve and RA- differentiated SH-SY5Y cells.

**MOR-1 posttranscriptional outcomes after 48- or 72Hrs**

To evaluate the effects of chronic opioids on the posttranscriptional gene expression of MOR-1 in differentiated SH-SY5Y cells exposed to a MOR-1 agonist, morphine or DAMGO, or naloxone (MOR-1 antagonist) for 48 or 72 hours. These study data are supported by prior reports that the degree to which narcotic receptors (e.g. MOR) are sensitive to opioids depends on the abundance of receptors [63], which in the present study, is indicated by the mRNA species (gene expression) of individual variants which direct receptor protein synthesis. A loss of maximal inhibition of adenylate cyclase in RA-differentiated SH-SY5Y cells is considered to be indicative of tolerance due to chronic (72 hrs) morphine (10 µM) [35,64]. Chronic morphine also increases all G-protein subunits and thereby induces G-protein up-regulation. These changes have been suggested to potentially occur due to de novo protein (receptor) synthesis [65] and may explain the receptor up-regulation seen in the current investigation. Antagonist-induced up-regulation of opioid receptors is well-documented [66], producing functional opioid receptor super-sensitivity. These reports are consistent with our findings of naloxone-induced MOR-1 variant up-regulation.

**Implications of serine phosphorylation along the MOR-1 signaling pathway**

The current study shows that mu opioid receptor (MOR-1) agonists acting through various GPCR pathways to differentially regulate the phosphorylation of serine residues on signaling and transcription proteins involved in MOR-1 gene expression. Chronic (24hr) morphine significantly attenuated the phosphorylation of PI3K-dependent Akt, while DAMGO and naloxone dramatically suppressed cAMP/PKA-dependent activation of CREB. The observed divergent regulation of MOR-1 pathways on development of morphine tolerance suggests the involvement of multiple epigenetic mechanisms in the regulation of the OPRM1 gene in response to chronic opioid signals. Moreover, GPCR coupling to either stimulatory (G s), or inhibitory (G i) guanine nucleotide binding protein (G-protein) [67], may induce diverse receptor conformations and activate effector systems through AMP-PKA cascades [68,69] along different, but often converging, signaling pathways [70]. Ligand selectivity with respect to the choice of interacting with one of multiple G-proteins contributes to the diversity of MOR-1 responses and individual variability, but is not fully understood [67,71,72]. Further research into the potential regulatory role of protein kinases on the crosstalk between kinase-mediated pathways may also clarify these mechanisms.

It is well-recognized that opioid receptors become phosphorylated upon prolonged stimulation. Several serine residues exist along the heptahelical mu opioid receptor (MOR-1), namely: S263, S268, S270, S357, S358, S365 and S377 (www.phosphosite.org). Of these serine residues, five of them were phosphorylated in either low throughout or high throughout assays (i.e. S263, S268, S270, S365 and S377). These phosphorylation sites increase in value as they have been associated with certain physiological effects in the modified MOR-1 protein. For example, S263-p and S377-p are associated with altered receptor internalization. S263-p is important for altered receptor sensitization. S377-p also plays a role in biological processes such as regulation of signaling pathway. Not surprisingly, certain threonine residues on MOR-1 are also phosphorylation sites that produce desensitization or internalization of the receptor. Phosphoproteins measured in our experiments included CREB (S133), ELK1 (S365), Akt1 (S473) and PKA (S144). In its altered state, the S133 residue on CREB induces activity, regulates molecular association, and phosphorylates the protein. Phosphorylation of CREB at S133 has the following effects on biological processes: inhibition of apoptosis, alteration or induction of cellular differentiation, induction of cell growth, alteration of cell motility, altered or

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induced transcription, and inhibition of translation. On ELK1, S383-p alters or induces transcription, induces protein activity, triggers intracellular protein localization, regulated molecular association, and modifies protein conformation. Effects on protein modification and on biological processes triggered by S473 are extensive. Phosphorylation at Akt1(S473) affects apoptosis, carcinogenesis, cell cycle, cell growth, cell motility, cytoskeletal reorganization and transcription. It also triggers protein degradation, induction of enzymatic activity, intracellular localization, regulation of molecular association, phosphorylation, and induction of protein activity. Lastly, neither of the human PKA isoforms, nor mouse, rat or cow listings in the PhosphoSitePlus™ database was specifically modified at the S114 site.

It was also noted that certain signaling pathways might be associated with a particular p-serine, suggesting a need for optimization of phosphoprotein selection, which is a limitation of phosphorylation experiments in our study. By extension, it may be that activity at the serine residues measured in the present study represent novel sites that become disinhibited due chronic opioid exposure. Discovering biological and physiological functions associated with off-target serine residues may help to clarify the role of these residues in complex cellular communication and further explain phenomena associated with opioid addiction and mechanisms of morphine tolerance.

**Phosphospecific determination of signaling and transcription proteins**

In the present study, chronic (24 or 48 hrs) opioids differentially regulated the phosphorylation of serine residues on signaling proteins (PKA, Akt) as well as that of downstream transcription factor targets (CREB, ELK1) in differentiated SH-SY5Y cells, as detected by Phosflow™ immunomonitoring techniques [73-76]. In particular, the amplification of the posttranslational modifications of Akt (S473) and CREB (S133) were more pronounced after 48 hr- versus 24hr opioid agonist exposures.

Morphine attenuated the phospho-activation of PKA, Akt and CREB after 24 hr opioid exposures. These findings were expected since MOR-1 is known to regulate the cAMP-dependent transduction of signals in SH-SY5Y cells [64], resulting in the ligand-induced activation of the MAP kinase signal transduction cascade [77-80]. Moreover, modulation of kinases involved in cAMP-related signaling cascades [81] may directly affect gene transcription and protein synthesis mediated by transcription factors (i.e. CREB) that are essential for these processes.

Our findings are consistent with previous reports that morphine induces tolerance at the level of a single cell [82,83]. In the present study, chronic (24 hr) morphine (10 μM) inhibited the phosphorylation of PKA, Akt and CREB. The development of morphine tolerance has previously been reported in SH-SY5Y cells following treatment with morphine (1 - 10 μM) lasting ≥ 12 - 48 hrs [Yu et al., 1988] or 10 μM morphine for 12 - 48 hrs [64]. In studies by Oldfield and associates [60], MOR-1 was desensitized in HEK293 cells at receptor saturation concentrations (10 μM morphine or 10 μM DAMGO) via patch clamp recordings.

In the present study, the observed reductions in the phosphorylation of PKA, CREB and Akt following chronic (24 hr) opioid exposure suggest at least partial loss of function by MOR-1 to stimulate G-protein mediated signal transduction [84]. A reduction in PKA-mediated MOR phosphorylation was reportedly associated with the induction of tolerance in mice chronically administered a morphine pellet coupled with morphine sulfate injections [85]. The contribution of cAMP-dependent PKA to the development and maintenance of opioid tolerance [86,87] is presumably of lesser consequence and shorter term than the widespread and extended tolerance induced by PKC [88,89] that promotes caveola-dependent internalization and endosomal recycling of MOR-1 [90-92]. Consequently, the observed inhibitory effects of morphine (24 hr) on PKA and CREB phosphorylation in our study were anticipated, as these changes commonly occur through the \( G_m \)-coupled mu opioid receptor. In contrast, ELK1 may be regulated by \( G_m \)-G\(_{αs}\), or RAS-signaling pathways, all of which cooperatively or independently subserve GPCR – and hence, mu opioid receptor function. The interaction of \( G_{αs} \) subunit with MOR-1 enhanced PI3K-dependent phosphorylation of Akt; however, these effects would also be independent of \( G_m \)-complex. Taken together, our findings highlight the complexity of MOR-1 signaling and the potential for divergent cellular and molecular adaptations [93-95].

The phosphorylation and activation of the mammalian cyclic AMP response element (CRE) binding (CRE) protein have been implicated in the development of opioid tolerance and dependence [81]. The upstream phosphorylation of MOR-1 signaling targets along the Akt/CREB pathway is essential for the downstream activation of CREB. The addition of phosphate at serine-residue 133 (S133) is...
essential for its dimerization along its cAMP response element (CRE) DNA-binding site and for initiation of its transcriptional activity [96]. Hence, the phosphorylation of CREB is a rate-limiting step in mu opioid receptor gene (OPRM1) expression. In our study, the strong inhibition of CREB phosphorylation by naloxone was consistent with its known rapid, selective and specific antagonism of MOR-1. On the other hand, the modest increase in CREB phosphorylation after chronic (24hr) DAMGO (a synthetic, enkephalin analog that is selective for MOR-1) was in sharp contrast to nearly halved pCREB levels after 48 hrs. Several studies have shown that outcomes of chronic opioid signaling may result in attenuation of mu opioid effects via the cAMP/PKA pathway [85,93] or amplification of such effects via the PI3K/Akt pathway [93,97], although the outcomes may also vary depending upon the opioid administered and the strength of the signal. Other studies have also shown that the diminution of pCREB after chronic (48hr) DAMGO occurs downstream of the highly efficacious induction of MOR-1 endocytosis and internalization [98] mediated by beta-arrestin and PKC phosphorylation [99,100].

The observed up-regulation of PKA is consistent with the actions of long-term morphine via the induction of AC [101]. Smaller increases in PKA were observed along with significant downstream elevations in pCREB. These findings are consistent with studies stating that hypersensitization of AC isoforms by chronic opioids leads to hypertrophy of the cAMP pathway and activation of CREB in mice [102], COS cells transfected with MOR [103], SH-SY5Y cells [64] and other cell types [104,105]. The down-regulation of ELK-1 seen in the current studies is consistent with repeated reports that repeated exposure to morphine inhibits protein synthesis through altered activation by extracellular signal-related kinase (ERK1/2)-ELK1 signaling. These changes were also accompanied by decreases in protein phosphorylation in mouse brain [106]. The significant elevation of PKC in PCR Array, compared to low levels of pPKA, were consistent with literature citing prevalence of PKC in the functional uncoupling of G-proteins from receptors. These events partly depend on the phosphorylation of active and inactive receptors by PKC and PKA, which may lead to receptor desensitization [107].

MOR-1 couples with a diverse array of G-protein subtypes \( G_{y} G_{i/o} G_{q} \) [68,70,108,109], which act as regulatory macromolecules within distinct signal transduction pathways (cAMP-PKA, PI3K/Akt, DAG-PKC, PLC/IP3/CaMK), frequently initiating the activity of assorted membrane-bound or intracellular effectors that evoke sundry responses [70,110]. The complexity of these protein-protein interactions broaden the scope of inter-individual and intra-individual variability possible following chronic opioid exposure. In our studies, we report signaling switches between GPCR pathways that is temporally regulated. While several studies support the apparent switching of G-protein mediated signal transduction following chronic opioid exposure [111-113], progress toward understanding related time-dependent adaptations is still progressing [114-116].

Screening analysis of signaling and transcription proteins by RT² profiler PCR array

Assessment of PCR array outcomes

The transcriptional and epigenetic regulation of opioid receptor genes has been extensively studied in mice and humans [117]. On limited screening, we identified by PCR several transcription factors and neurological disease genes that were stimulated in SH-SY5Y cells after 48hr morphine treatments. The contributions of many of these molecules are not clear. However, given the vital role of transcription factors in the initiation of OPRM1 transcription and gene expression, further follow-up is warranted. These molecules may provide - through their interaction with DNA - mechanistic insight into both the selectivity and sensitivity of MOR-1 alternatively-spliced variant expression.

Conclusion

The enigma of inter- and intra-individual variability in response to opioid analgesic pharmaceutical agents is not fully elucidated. This project was uniquely designed to engage an academic research constituency in the conversation and examination of integrated approaches to forge a path toward translational science in a basic science environment, while incorporating the use of innovative biotechnology (qRT-PCR, PCR Array and Phosflow™) and new insights gained from the Human Genome (Transcriptome) Project. The methods applied move beyond the interrogation of membrane receptor levels to explore changes in the intracellular activity and downstream responses through signal transduction pathways, protein phosphorylation, gene transcription, posttranscriptional modifications (via alternative splicing) and posttranslational modifications of transcription factors following chronic opioid exposure of a human cell line.

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This study confirmed our hypothesis that chronic opioids modulate posttranscriptional and posttranslational molecules involved in the regulation of opioid receptor function, implicating various signaling proteins and transcription factors in the expression of alternatively-spliced variant forms. Our study demonstrated the suitability and utility of the human neuroblastoma (SH-SY5Y) cell line as a model for studying opioid receptor pharmacology. The comparative expression of MOR-1B alternatively-spliced variants in naïve and differentiated SH-SY5Y cells was indicative of potential variant-specific mechanisms of action. Both N-terminus variant, MOR-1K1, and C-terminus variants (MOR-1, MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B5) were differentially sensitive to prototypical mu-opioid receptor ligands (morphine, DAMGO, or naloxone) after chronic (24-, 48-, or 72hr), in vitro exposure.

The application of novel Phosflow™ technology allowed discrimination of important intracellular and nuclear proteins that contribute to MOR-1 signal transduction and are altered after chronic exposures. To our knowledge, the observed time-dependent changes in PKA, Akt, CREB and Elk phosphorylation following 3 chronic opioids in SH-SY5Y cells have not been previously articulated. These data may provide clues to better understand the impact of chronic opioids on critical functional proteins that contribute to GPCR signaling and MOR-1 gene regulation.

The identification of a series of transcription factors (FOXA2, TBP, PACSIN1, and SOX2 were up-regulated and STAT5B was down-regulated in both PCR Array and RT-qPCR; PKC highly elevated) provides preliminary evidence of key proteins affected by chronic morphine exposure that may serve as the basis for future studies to reveal their relevance to MOR-1 variant pharmacology as well as the transcriptional regulation of OPRM1.

Collectively, these data connect opioid-induced stimulation of MOR-1 with progression of intracellular actions that allow the chronological interaction of signal transduction, phosphorylation, transcription, post-transcriptional and posttranslational mechanisms, and thereby provide meaningful and relevant insight into the pharmacological actions of opioids at the cellular and molecular levels. The unique design of this study facilitated the simultaneous determination of up-regulation of six MOR-1 alternatively-spliced variants in a time-dependent and cell-specific manner: The genomic and transcriptomic analyses expand our knowledge of the biochemical and molecular basis of opioid tolerance beyond the typical receptor binding approach to capture involvement of proteins that were previously not prescribed a prominent role in the development of morphine tolerance. Whether recently identified alternatively-spliced variants have any definitive functional role in outcomes mediated by the mu opioid receptor subtype 1 (MOR-1) is still undecided. However, in keeping with the present study objectives, we have expressed six MOR-1 alternatively-spliced variants (ASVs: MOR-1A, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B5 and MOR-1K1) in naïve and differentiated human neuroblastoma SH-SY5Y cells, confirming the utility of this model for in vitro pharmacodynamic investigations. Our findings also demonstrate the differential posttranscriptional expression of MOR-1 and these six ASVs variants in vitro in naïve and differentiated human neuroblastoma SH-SY5Y cells exposed for 24-, 48-, or 72hrs to: morphine (10 µM) - a MOR-1-specific agonist; DAMGO (10 µM) - a MOR-1-specific, synthetic peptide (enkephalin) analog; or naloxone (10 µM) - a nonselective, competitive MOR-1 antagonist. A drastic reduction in MOR-1 variant expression and SH-SY5Y cell viability at the 48hr time-point, followed by supersensitivity and up-regulation of the cells at 72hrs, indicate cellular crisis on prolonged exposure, the activation of compensatory mechanisms at the gene level, and tolerance. The posttranslational modification of signaling proteins (PKA, Akt) and nuclear transcription factors (CREB, Elk1) after chronic (24- or 48hrs) opioids suggests that opioid regulation of these intracellular phosphorylation events through divergent signaling pathways and/or crosstalk may have a functional role in OPRM1 alternatively-spliced variant gene expression. More comprehensive signal mapping and transcriptomic analyses are necessary to further delineate the contribution of transcription factors affected by chronic opioid exposure that have been identified in these studies. Together, these findings suggest a future potential for enhancing our understanding of the contribution of MOR-1 alternatively-spliced variants to the development of morphine tolerance in human subjects.

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Conflict of Interest

The author knows of no financial interest or any conflict of interest relative to this article.

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