SIGNALING PATHWAYS INVOLVED IN IL-1BETA-INDUCED REGULATION OF MOR EXPRESSION IN HUMAN NEURONS

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TABLE OF CONTENTS

Chapter **Page**

Chapter **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

κ (KOR)	Kappa opioid receptor
δ (DOR)	Delta opioid receptor
μ (MOR)	Mu opioid receptor
IL-1β	Interleukin-1 beta
IL-1α	Interleukin-1 alpha
IL-1R	Interleukin-1 receptor
IL-1RA	Interleukin-1 receptor antagonist
GPCR	G-protein coupled receptor
cAMP	Cyclic adenosine monophosphate
GRK	G-protein coupled receptor kinases
PTX	Pertussis toxin
CHO	Chinese hampter ovary cells
HEK	Human embryonic kidney cells
DRG	Dorsal root ganglia
CNS	Central nervous system
P2X/Y	Purinergic receptors
CX3CR1	Fractalkine receptor
TLR	Toll-like receptor
CCR	Chemokine receptor
PGE2	Prostaglandin E2

TNF	Tumor necrosis factor
NO	Nitrix oxide
iNOS	Inducible nitric oxide synthases
BDNF	Brain derived neurotrophic factor
NGF	Nerve growth factor
IL-4	Interleukin-4
IL-6	Interleukin-6
IFN-γ	Interferon gamma
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
ERK	Extracellular signal-regulation kinase
MCP-1	Monocyte chemotactic protein-1
LPS	Lipopolysaccaride
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerization chain reaction
qRT-PCR	Quantitative RT-PCR
6-OHDA	6-Hydroxydopamine

CHAPTER I

INTRODUCTION

The ability to alleviate pain through the use of substances derived from opium has been known for centuries. With the discovery of exogenous compound derived treatment of pain, extensive research has been conducted on the endogenous-opioid system (Terenius and Wahlstrom, 1975). The body's innate ability to produce, secrete and elicit opioid induced analgesia spurred on the interest for the discovery of opioid binding sites of both endogenous opioid peptide and exogenous opioid compounds. Since the early 1950's, researchers have postulated the existence of multiple opioid receptor types (Lasagna and Beecher, 1954; Veatch et al., 1964). Early studies in the 1970's using the dog analgesic model provided the first real evidence for the expression of multiple opioid receptor types, identifying three distinct opioid receptors located at the spinal and supraspinal levels (Mansour et al., 1994; Martin et al., 1976). These three receptors types were termed μ (for morphine), κ (after ketocyclazocine) and δ (after mouse van deferens) (Holtzman, 1980; Hughes et al., 1975; Lord et al., 1977). All three opioid receptors have been confirmed by molecular cloning and have been pharmacologically identified as μ (MOR), κ (KOR) and δ (DOR) (Chen et al., 1993a; Chen et al., 1993b; Evans et al., 1992; Kieffer et al., 1992; Raynor et al., 1994; Yasuda et al., 1993)

Chronic pain and other neuroinflammatory associated disorders usually persist for months and even years, therefore the use of opioids to alleviate chronic pain is common. However, long-term use of prescribed opioids to treat chronic pain often leads to opioid-

induced tolerance and addiction. Both exogenous opioids and endogenous opioids may contribute to the addictive properties of several drugs of abuse including opioids (e.g. morphine and heroin), psychostimulants (cocaine and amphetamine), alcohol, cannabinoids and nicotine (Bailey and Connor, 2005). Opioid receptor expression (transcription and translation) and function (G-protein coupling, phosphorylation opioid receptor internalization and recycling) have been postulated as playing a role in opioid-induced tolerance. By definition, opioid tolerance, results in pain patients requiring a higher dose of opioids to maintain a desired level of analgesia.

There has been tremendous interest in the endocytic trafficking of opioid receptors and their functional changes in response to acute and chronic opioid treatment. However less attention has been paid to understanding the various cellular factors that may contribute to the expression and function of opioid receptors, independent of opioid-ligand and receptor binding events. From the time when the discovery of opioid receptor expression on immune and glial cells became apparent (Chuang et al., 1995), extensive research has aimed at discovering the role opioids may have on the immune system. Studies on the effect of opioids on the immune system have been well-defined as immuno-suppressive, worsening the disease conditions associated with neuroinflammation and autoimmune disorders (Ballard et al., 2006; Bayer et al., 1990).

Extensive research has been conducted on studying the effects of opioids on the functions of the immune system, however little research has been conducted on understanding the role of immune or glia cells and their released cytokines on the expression and function of opioid receptors in neurons. Neuroinflammatory diseases are complex and involve an array of cell types, immune, glial and neuronal cells. Understanding the

interactions between these cell types will allow us to gain insight into various neuroinflammatory diseases.

The answers to the following questions should allow us to better understand the interactions between the neuro-immune system and opioids. Which immune cell derived factors affect the expression and function of opioid receptors? Are different opioid receptor types differentially targeted and/or affected? Is the expression of opioid receptor in different cell types affected?

The extent of this dissertation is to report our investigations on the possible mechanism(s) for the regulation of mu opioid receptor type (MOR) in a neuronal cell line (SK-N-SH) by morphine and the pro-inflammatory cytokine interleukin-1 beta (IL-1β).

CHAPTER II

REVIEW OF LITERATURE

2.1 The Anatomy of Pain

Pain is a complex physiological, behavioral and subjective response to nociceptive inputs. The term "nociception" is defined as detection of noxious stimuli or stimuli that can damage the tissue (Basbaum, 2000). Pain has two components which involve dedicated projection systems from spinal cord to higher centers in the brain (Basbaum, 2000). One component is a modality of *somatic* sensory perception. Somatic sensory perception permits the localization of pain and enables discrimination among different pain stimuli. The second component is *affective*. Which activates circuits in the brain that result in negative emotions.

2.1.1 Neuropathic Pain.

The ability of an animal to detect and react appropriately to an aversive stimulus is of fundamental importance to its survival (Clatworthy, 1998). Pain is the natural consequence of tissue injury and can serve as a biologically useful defense mechanism that warns against existing or imminent damage (Tsuda et al., 2005). In contrast to this nociceptive and acute pain, neuropathic pain (chronic, intractable pain due to nerve injury) serves no biological purpose and can be debilitating and cause extreme physical, psychological and social distress (Colburn et al., 1999). Therefore neuropathic pain fails to play a protective role in disease or injury, rather the feeling of chronic pain exists long after the healing process and eventually becomes the disease. Neuropathic pain typically develops after central or

peripheral nerve damage caused by trauma, surgery, cancer, diabetes or infections and usually persists long after the initiating event has healed (Woolf and Salter, 2000). Neuropathic pain can also develop after nervous system dysfunction (Merskey, 1994), as in multiple sclerosis (MS) (Svendsen et al., 2005) and amyotrophic lateral sclerosis (ALS) (Galer et al., 2000). Ironically, data suggests patients suffering from these diseases are not receiving the correct pharmacological therapy and further, that almost one quarter are receiving no treatment for pain (Berger et al., 2004). It was found that a large percentage of patients received a short-acting opioid (53.2%), and opioid of any class were most commonly used (53.9%) (Berger et al., 2004). The mechanism of opioid analgesics used to treat neuropathic pain is poorly understood. At best opioids provide partial relief and therefore are used inappropriately and in excess leading to the most common disease associated with opioid use - addiction (Colburn et al., 1999). Currently, there is no universally effective treatment for neuropathic pain that parallels the use of morphine for nociceptive pain (Hansson and Dickenson, 2005; Woolf, 2004; Woolf and Salter, 2000; Zimmermann, 2001).

2.2 Role of the Immune system in the modulation of Pain and Neuroinflammation

2.2.1 Pain modulation

Nerve injury and neuroinflammation are known to make changes to neurons at the molecular and cellular level, often resulting in neuronal plasticity and anatomical re-organization (Tsuda et al., 2005; Woolf, 2004; Woolf and Salter, 2000; Zimmermann, 2001). The cellular mechanisms that initiate and maintain long-term neuropathic pain are poorly understood, however there are various mechanisms in the nervous system that have been suggested to play a major role (Fig. 1). Some examples of these players include: changes in

neural behavior and /or chemical environment leading to increased activity and increase neuronal cell degeneration in surrounding areas of the initial injury (Devor et al., 1994; Waxman et al., 1999), changes in neurotransmitter levels through alterations in Ca^{2+} channels, (Furukawa et al., 2008; Matthews and Dickenson, 2001; Yamamoto and Sakashita, 1998) Na^{+} channels (Hains et al., 2003; Lindia et al., 2005), spinal neuroma hyper excitability (sensitization) (Huang et al., 2008) and increases in descending pain facilitation (Bee and Dickenson, 2008; Vera-Portocarrero et al., 2006).

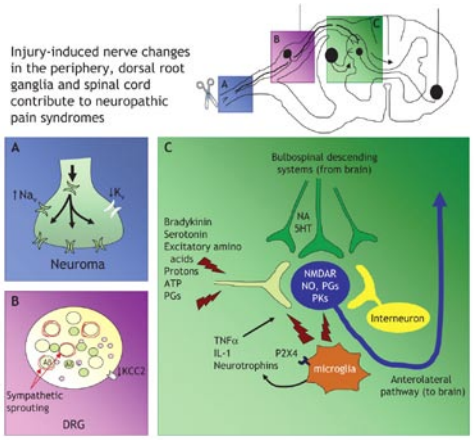

Fig. 1 Neuropathic pain arises following nerve injury. **A**: following nerve damage, axonal transport of Na^{2+} and K^{+} ions are enhanced and diminished respectively, resulting in neuron hypersensitivity. **B**: at the cell body of the peripheral afferent neurons within the DRG, sympathetic neuron sprouting occurs. **C**: Nerve injury induced multitude of cellular changes, eliciting hypertrophy of glial cells (microglia) within the grey matter of the spinal cord, releasing pro-nociceptive cytokines, e.g. IL-1. Released cytokines exacerbate nociceptive transmission, contributing to the sensitization and maintenance of neuropathic pain (Gilron et al., 2006).

2.2.2 Neuroinflammation

Many studies have concentrated on the role of the immune system and glial cells in neuropathic pain states associated with neurodegenerative diseases (Colburn et al., 1999; Ledeboer et al., 2005; McMahon et al., 2005; Moalem and Tracey, 2006; Song and Zhao, 2001; Watkins et al., 2007). The role of glial cells in pain and the neuroplasticity associated with neuropathic pain has been known for over 15 years (Muller, 1992) and it has been estimated that over 50% of all neuropathic pain clinical cases are associated with neuroinflammation (Said and Hontebeyrie-Joskowicz, 1992). Additionally, stimuli that initiate neuropathic pain also initiate the activation of microglia cells within the CNS, underscoring the significance of glial cells in neuropathic pain state.

The relationship between the immune system, glial cells and pain has been discussed in depth in a few review articles: (Romero-Sandoval et al., 2008; Watkins and Maier, 2002). As much as it may seem contradictory, neuropathic pain is defined clinically as "non-inflammatory" pain despite years of research on neuro-immuno modulatory cascades at the site of injury are central to the development of sensitization (Ledeboer et al., 2005; Machelska and Stein, 2000; Marchand et al., 2005; Moalem and Tracey, 2006; Omoigui, 2007; Raghavendra et al., 2002; Rittner et al., 2006; Saurer et al., 2008; White et al., 2005; Williams and Lambert, 2005). Although the clinical view of neuropathic pain is centered around and mediated by neurons, today accumulating suggests that spinal cord glial cells contribute to immuno-mediated pain enhancement (Moalem and Tracey, 2006).

2.2.3 Role of Microglia cells

Glial cells account for approximately 90% of the cells in the CNS and provide support and nutrients, maintain homeostasis, form myelin and participate in signaling in the CNS. Glial cells are divided into three groups: oligodendroglia, astroglia and microglia.

Microglia cells represent 5-10% of glial cells in the CNS, acting as sensors for a range of stimuli released in response to physiological changes, CNS trauma, ischemia and infections (Tsuda et al., 2005). Under basal conditions, microglia are in a resting, ramified form (Brierley and Brown, 1982) and undergo functional phenotypical changes that awaken microglia cells into a amoeboid reactive form. These changes in microglia phenotype have been linked to their change in function: from 'surveying' microglia in the 'resting' state to 'effector' microglia in the 'activated' state seen following disease and challenge.

Activated spinal microglia have been observed in various rodent models of chronic pain, including spinal nerve injury (Raghavendra et al., 2003; Sweitzer et al., 2001), peripheral inflammation (Bao et al., 2001; Chacur et al., 2004; Sun et al., 2006; Sweitzer et al., 1999; Watkins et al., 1997), peripheral tissue injury (Fu et al., 2006), spinal cord injury (Hains and Waxman, 2006), and bone cancer pain (Zhang et al., 2005). Microglia cell activation have no definitive marker (Guillemin and Brew, 2004), but studies have relied on OX-42 immunoreactivity, which labels complement receptor 3 (CR-3) to identify them. For example, increased levels of OX-42 in microglia have been measured in spinal cord injuries (SCI) (McMahon et al., 2005).

2.2.4 Consequences of microglia activation

Activated microglia produce numerous inflammatory mediators (IFMs), e.g. IL-1β, TNF-α, IL-6, PGE2, NO and BDNF, which are produced following spinal cord injury and have been implicated in pain facilitation (Arruda et al., 2000; Coull et al., 2005; DeLeo and Yezierski, 2001; Marchand et al., 2005; Sweitzer et al., 2001). Also the RNA and protein levels of microglia produced IFMs are elevated (DeLeo et al., 1997; Hashizume et al., 2000; Raghavendra et al., 2004b; Sweitzer et al., 1999).

2.2.5 Opioid analgesia and proinflammatory cytokines

Activated microglial cells release inflammatory mediators, the release of which can become imbalanced, toxic in disease and induce pain facilitation. Thus, there are multiple pathways that inflammatory mediators that are released from activated microglia which can induce pain facilitation and other neuro-excitatory substances. Several of these pathways might also be activated by morphine and therefore create a neuro-excitation opposing morphine induced analgesia. There is strong evidence that nerve injury activates spinal microglia and that their activation is involved in the induction and maintenance of neuropathic pain. Opioids, described in more detail in sections 2.5 through 2.8, are used clinically to treat neuropathic pain, but of recent there has been a negative association between the development of neuropathic pain and morphine –induced analgesia (Mayer et al., 1999). Possible explanation for the decrease in morphine analgesia include morphine-induced activation of microglia as indicated by the increased phosphorylation of extracellular signal-regulated kinase (ERK1/2) (Takayama and Ueda, 2005). Morphine also increases the release of NO (Stefano, 1998) and proinflammatory cytokines (Peterson et al., 1998). Intriguingly, microglia exposure to morphine or proinflammatory cytokines enhances their expression of MOR (Mahajan et al., 2002; Ruzicka et al., 1996). Therefore, it is natural to extend the question to how microglia released inflammatory mediators may be involved in reduced morphine analgesia.

The first link between glial cells and morphine-induced analgesia was reported when chronic morphine increased astrocyte activation in the spinal cord, where co-administration of fluorocitrate (a glial metabolic inhibitor) with morphine significantly attenuated both glial activation and morphine-induced analgesia *in vivo* (Song and Zhao, 2001). Therefore, work rapidly followed to extend and support these initial observations. Some of the work to

support the effect of chronic morphine on glial cells includes: (a) up-regulated TNF, IL-1 and IL-6 release in the spinal cord (Johnston et al., 2004; Raghavendra et al., 2002), (b) up-regulated TNF, IL-1 and IL-6 in microglia but not in neurons (Tai et al., 2006), and (c) reduced opioid analgesia in response to glial activation and increased release of proinflammatory cytokines (Raghavendra et al., 2004a). Interestingly and more importantly, reduced morphine analgesia was slowed or blocked by either inhibition of spinal inflammatory cytokines (Johnston et al., 2004; Raghavendra et al., 2002; Shavit et al., 2005) or by knocking out IL-1 signaling (Shavit et al., 2005). Furthermore, proinflammatory cytokines have been shown to oppose opioid-induced acute and chronic analgesia. Combined, Cui et al., (2006, 2008), Shavit et al., (2005) and Hutchinson et al., (2008) studies have concluded the following: (a) acute *in vitro* morphine-increased the release of proinflammatory cytokines and chemokines (IL-1β, IL-6, Fractalkine, MCP-1 and TNF-α), (b) chronic morphine and methadone administration in rats increased proinflammatory IL-1 mRNA expression in the lumbar dorsal spinal cord, (c) chronic morphine and methadone administration in rats increased proinflammatory mRNA expression (IL-1β, IL-6 and TNF-α) in the lumbar dorsal spinal cord, (d) ≤ 5 min after intrathecal opioid, endogenous, spinal IL-1 reduced (8-fold) morphine analgesia, (e) IL-1RA potentiated morphine analgesia in < 5 minutes, (f) p38 MAPK inhibitor, SB203580 potentiated morphine-induced analgesia (Hutchinson et al., 2008). This last study not only added to the work linking proinflammatory cytokines to reduced morphine analgesia but also showed that glial activation and proinflammatory cytokines contribute to opioid tolerance and opioid-induced hyperalgesia. It also showed that opioid actions are opposed by proinflammatory mediators following both acute and chronic administration. This and other studies, namely Shavit et al.,

(2005) suggest the potential therapeutic utility of targeting opioid-induced proinflammatory mediators to attenuate the development of opioid tolerance.

Mechanisms underlying proinflammatory induced regulation of morphine analgesia are likely to be complex. For example, IL-1 might inhibit binding of opioids to receptors expressed in guinea pig brain-enriched membrane preparations (Ahmed et al., 1985) or not as demonstrated in rat brain cells *in vitro* (Wiedermann, 1989). Whether IL-1 influences opioid binding in the spinal cord under normal, neuropathic or chronic morphine conditions remains unknown. Therefore, this dissertation will attempt to address how IL-1β regulates the expression of opioid receptors (MOR) *in vitro* and mechanistically provide a better understanding of MOR mRNA expression in response to both IL-1β and morphine treatment.

2.3 Neuro-Immune interactions

Until two decades ago, the CNS and immune system were thought to act independently. However, there have been numerous studies demonstrating that the immune system and CNS interact in a concerted manner. Supporting studies include the discovery of the synthesis of opioid peptides (Galin et al., 1990; Galin et al., 1991; Harbour et al., 1991; Lyons and Blalock, 1997) and the expression of their receptors in T cells (Wybran et al., 1979). Similar discoveries were also made in the CNS, where it is was found that the proinflammatory cytokine, IL-1, was endogenously expressed in neurons (Breder et al., 1988). IL-1 (IL-1α and IL-1β) was also expressed in the lymphoma cell line U937 as discovered by cDNA cloning (Nishida et al., 1987) and in neuroblastoma cell lines (Stephanou et al., 1992). Furthermore, identification of IL-1 receptor (IL-1RI) expression in T-cell (immune cell) (Sims et al., 1989; Sims et al., 1988) and later in the human

hypothalamus (Hammond et al., 1999) confirmed that the immune system and the CNS are undergoing a complex level of bidirectional communication.

2.3.1 Bi-directional communication between immune system and the CNS

There is no doubt that the immune system communicates with the CNS (Hosoi et al., 2002; Kleine and Benes, 2006), however the BBB still presents a physical barrier for this communication, so how do inflammatory mediators enter the CNS? Because the BBB's role is to exclude circulating proteins and macromolecules from the brain (e.g. albumin) it was thought that cytokines would also fit this description and be excluded from the brain, making the brain "immune privileged". It has been shown that the brain is highly immunologically active as seen in neuroinflammatory diseases (Rothwell et al., 1997). Several pathways that cytokines use to enter the CNS have been studied, these include: **(1) BBB dependent pathways** – It was demonstrated in the late 1980's (Banks et al., 1989) that IL-1 is actively transported through the BBB (Banks, 2005). Little evidence existed in support of this theory of cytokine transport through the brain. How does a peripheral immune stimulus, following peripheral insult, increase levels of IL-1 in the brain? This level of communication was first demonstrated in the mid-1990's, where the peripheral injection of IL-1 induced intense transcriptional (*c-fos*) activity in cells of the BBB (Brady et al., 1994). Later, *in situ* hybridization studies showed that cells of the BBB respond to peripheral immune stimulation by producing IL-1 (Quan et al., 1998). Thus, during systemic immune challenge, production of IL-1 by cells of the BBB may result in widespread IL-1 activity in the CNS. **(2) BBB independent pathways** – Cytokines have also been shown to relay inflammatory signals to the CNS by pathways that bypass the BBB. Two of the main pathways include: (a) *Circumventricular organs (CVOs)* – cytokines traveling in the blood to the CNS via CVOs in the brain occurs in capillary beds that do not form a BBB. Therefore CVOs are considered

portals for circulating cytokines to reach the CNS. However, the role of CVOs in cytokine entry to the CNS does not go without controversy. For example, lesions of organum vasculosm laminae terminalis (OVLT), a CVO close to the hypothalamic thermoregulatory center, suppressed i.p. LPS-induced fever (Blatteis et al., 1983) and in support, the removal of the area postrema (AP), a CVO close to the nucleus of the solitary tract (NTS), blocked IL-1-induced *c-fos* expression in the hypothalamic paraventricular nucleus (PVN) (Lee et al., 1998). In contrast, no change in *c-fos* expression in the PVN was found following AP lesion (Ericsson et al., 1997). (b) *Vagus nerve* - Since the early 1980's, studies have demonstrated that peripheral LPS-induced CNS effects can be blocked by vagotomy, and suggested that afferent vagal pathway innervating specific regions of brain as a key connection between peripheral and CNS immune responses (Pitterman et al., 1983). This was later confirmed when a vagotomy blocked the induction of IL-1β in the hypothalamus after peripheral injection of IL-1β and LPS (Hansen et al., 1998; Laye et al., 1995).

Nonetheless, via *in situ* hybridization, most of the IL-1β mRNA expression has been localized in the hypothalamus in non-neuronal cells (Buttini and Boddeke, 1995; Eriksson et al., 2000a; Quan et al., 1998; Yabuuchi et al., 1993). Even though the majority of cytokines act locally in a paracrine manner, in pathological conditions such as neuropathatic pain, cytokines can act in an endocrine manner.

Inflammatory cytokine IL-1β, which promotes or enhances neuroinflammatory processes, is often described as a prototypic cytokine and was originally identified because of its action in the brain: IL-1β was originally described as an "endogenous pyrogen" i.e. mediator of fever. Therefore, IL-1β has long been associated with the brain microenvironment. It and other cytokines are produced by endogenous brain cells – microglia and astrocytic cells, where they are up-regulated during peripheral damage, brain

trauma, CNS infection (neuroAIDS), ultimately effecting the homeostatic processes by de-regulating the normal protective roles into potent neurotoxic roles (Rothwell and Luheshi, 2000).

2.4 The Interleukin-1 (IL-1) Family

2.4.1 IL-1 Cytokine family

The IL-1 family, which is composed of at least 10 molecules has an important role in neuroinflammation and host defense. Following the identification of IL-1 as a endogenous pyrogen over 50 years ago, it became apparent that that IL-1 consisted of two ligands, IL-1α and IL-1β (March et al., 1985). Following the identification of IL-1α and IL-1β expression in macrophages, it became obvious that the two ligands where highly homologous and identical despite being the products of different genes. Both IL-1 cytokines are synthesized as a result of truncation of large precursor peptides inside cells such as lymphocytes and monocytes. Pro-IL-1α is biologically active and cleaved to its mature form by calpain and both forms of the cytokine remain intracellular unless released due to cellular death (Huising et al., 2004). By contrast, pro-IL-1β is biologically inactive and requires cleavage by caspase I (Thornberry et al., 1992) to produce an active 17 kDa protein that is released from the cells in a non-classical mechanism still unknown today. IL-1β expression in the CNS under basal conditions is minimal but becomes elevated following infections, insult and injury. For example IL-1β has been detected in the brain following kainic acid treatment, which is used to mimic a neuroexcitotoxic or epileptogenic event in the brain (Yabuuchi et al., 1993). The third member of the IL-1 is well characterized as the IL-1-receptor antagonist (IL-RA), which was discovered to be naturally occurring in human monocytes (Hannum et al., 1990). IL-1RA is expressed in the same cells as IL-1 and is present in three intracellular isoforms

(icIL-1RA1, icIL-1RA2 and icIL-1RA3) and one secreted form (sIL-1RA), which functions as a competitive antagonist by binding IL-1RI and preventing IL-1 intracellular signaling (Malyak et al., 1998). The role of IL-1RA in disease models of neuroinflammation has been one of neuroprotection (Liu et al., 2008; Loddick et al., 1997; Vogt et al., 2008), with its release being "activated microglia cell" dependent (Pinteaux et al., 2006) (See Fig. 2 illustrating members of the IL-1 family and its actions).

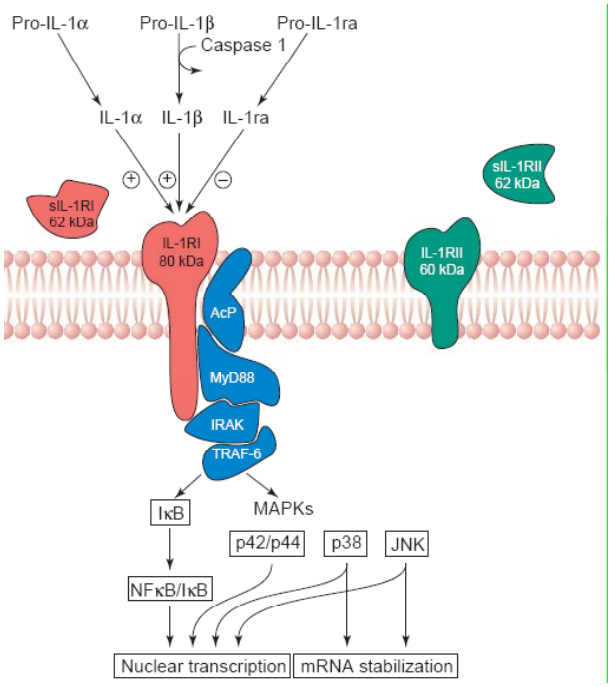

Fig. 2 Actions of IL-1: IL-1 family members include ligands (IL-1α and IL-1β), antagonist (IL-1RA) and the two receptors (Type I and II). (Rothwell and Luheshi, 2000).

2.4.2 IL-1 receptors and CNS expression

IL-1α and IL-1β exert similar biological effects by binding to membrane-bound receptors – type I (IL-1RI) and type II (IL-1RII). The IL-1RI, when bound with IL-1 ligands, associates with IL-1 receptor accessory protein (IL-1RAcP), forming a complex that allows intracellular signaling (Korherr et al., 1997). In contrast to IL-1RI, IL-1RII lacks the intracellular signaling domain important for downstream effector mechanisms. Therefore, the IL-1RII is considered a decoy receptor acting as a scavenger for the uptake of excess IL-1 (Bourke et al., 2003). A more recent study demonstrated an engaging relationship between the IL-1RI signaling protein (IL-1RAcP) and the IL-1RII and thus speculated that IL-1RII may function to reduce the amount of signaling IL-1RI-IL-1RAcP-agonist complexes when IL-1 is bound (Malinowsky et al., 1998). IL-1 receptors belong to the Toll-like receptor (TLR) superfamily. The IL-1 receptor and TLRs share cytosolic regions found to be conserved in all TLRs involved in triggering a complex series of signaling cascades that result in the activation of NF-κB as well as stress-activated mitogen-activated protein kinases (MAPKs) (Dunne and O'Neill, 2003). This then leads to the transcription of multiple inflammation-associated genes: chemokines (e.g. CXCL8), cytokines (IL-1, IL-6 and TNF) and adhesion molecules (E-selectin) (Subramaniam et al., 2004).

Both IL-1 receptor types have been cloned and are expressed in numerous tissues in the human body including the endothelial cells that make up the BBB (Boraschi et al., 1991). In addition to IL-1RI expression in the BBB, IL-1RI and IL-1RII are expressed throughout the brain in neurons and non-neuronal cell types (Ban et al., 1993; Pinteaux et al., 2002; Takao et al., 1990; Wong and Licinio, 1994; Yabuuchi et al., 1994a). Also, IL-1β induces mRNA expression of IL-1RI and IL-1RII in the rat brains following 15 and 120 minute treatments (Parker et al., 2000). In the case of IL-1RII, the expression pattern mirrors that of

IL-1RI, but the exact nature for the expression is still unknown and is in need of more exploration now that the human gene for IL-1RII has been cloned and expressed in recombinant *E.coli* BL21 (Hu et al., 2004). (See Fig. 3 illustrating members of the IL-1 family and their receptors).

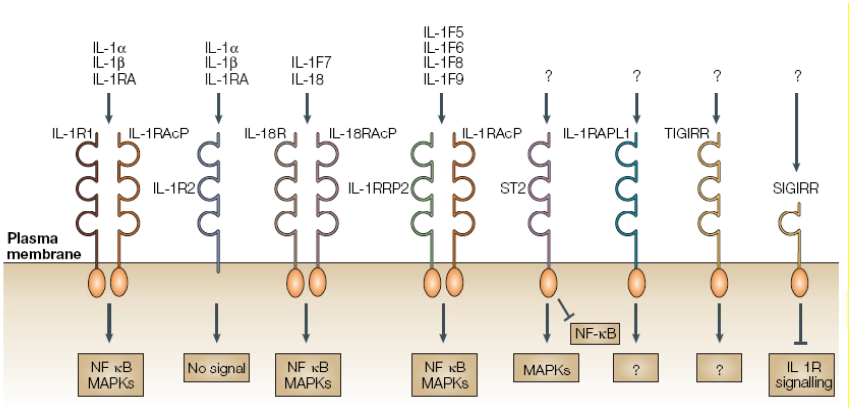

Fig. 3 The interleukin-family and receptors. (Allan et al., 2005).

2.4.3 Regulation of IL-1β

All members of the IL-1 cytokine family are expressed endogenously by brain cells. However IL-1 expression is at low levels in the healthy CNS. The specific cellular source of these proteins is unclear and little is known about regulation of the IL-1 system in the brain. Microglia, which express caspase-1, release IL-1β and express IL-1RI (Pinteaux et al., 2002; Touzani et al., 1999; Wang et al., 2008a), appear to be the early, primary source of IL-1 following experimental CNS injury, infections or inflammation (monocytyes and macrophages are the major peripheral sources of IL-1) (Davies et al., 1999). Astrocytes also produce IL-1, however their source is slightly delayed compared to microglia after an acute insult (Pearson et al., 1999). The IL-1RA expression is stimulated by the same cellular stimuli that result in production of IL-1, however IL-1RA expression occurs in neurons at a later time point (1-3 hr) compared to IL-1 expression (Loddick et al., 1997).

The expression of genes that encode IL-1 is induced by various proinflammatory stimuli, e.g. bacterial and viral products, cytokines (TNF), cellular injury and hypoxia. Most of these observations have been made with peripheral cells (macrophages), but given that microglia and macrophages have a common monocytic progenitor, it is probably safe to correlate these findings to the CNS. The promoter region of the IL-1β gene contains a TATA box, which is the TATA-binding protein (TBP) binding site, and a CAAT box, which controls the efficiency of the promoter for initiating transcription. On the IL-1β gene promoter, several regions have been identified for tissue-specific factors, including nuclear-factor-βA (NF-βA) and SP1, which determine cell-type-specific expression. The level of IL-1 gene transcription is also affected by factors that recognize and bind DNA regulatory sites, such as the cAMP-response element, activator protein 1 (AP1), NF-κB binding site, and the LPS-response enhancer site (Watkins et al., 1999). Lastly, several intracellular signals (e.g. caspase-1) can be stimulated by many extracellular stimulus (e.g. kainic acid) which function to increase or decrease the rate of IL-1β transcription. (Eriksson et al., 2000b; Liu et al., 2005).

2.4.3.1 Post-transcriptional regulation and cellular release of IL-1β

Translation of IL-1β is increased by epidermal growth factor (EGF), corticotrophin-releasing hormone and ICAM1, whereas dexamethasone inhibits IL-1β translation (Watkins et al., 1999), therefore, indicating that the expression of IL-1β protein can be regulated in response to exogenous compounds. Until recently, the post-translational regulation of IL-1β has not been studied in CNS disease and injury model. Neuronal injury can induce the release of pro-IL-1β from LPS-activated microglia *in vitro*, providing the overdue correlation between nerve damage alone and increased IL-1β release from microglia cells (Wang et al., 2008a).

The cellular release of IL-1β is dependent on the following two mechanisms: **(1)** P2X7 (purinergic receptor and ligand-gated ion channel) activation. P2X7 is activated by LPS, which induces the release of pro-IL-1β by ATP-dependent cleavage of mature IL-1β through a caspase-I-dependent mechanism and subsequent release (Le Feuvre et al., 2002). **(2)** The process of IL-1β release depends on calcium from intracellular stores and on the activation of phopholipase C (PLC) and phopholipase S (PLS) (Andrei et al., 2004; Brough et al., 2003). (See Fig. 4 illustrating the regulation of IL-1β production and action).

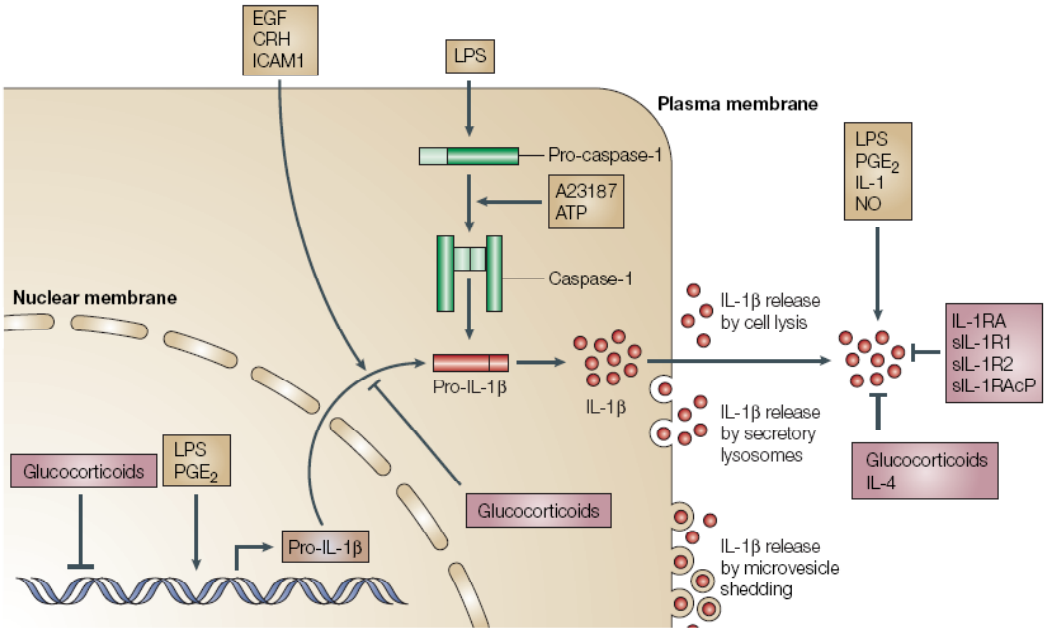

Fig. 4 Regulation of IL-1β production and action: diagram illustrating the regulation of expression of the gene encoding, cellular release of IL-1β and its biological activity. (Allan et al., 2005).

2.4.4 Overview of IL-1β's role in CNS injury and Neuronal diseases

The expression of both IL-1α and IL-1β are up-regulated within minutes at the mRNA level and within hours at the protein level in response to a neurotoxic stimulus. Most studies suggest that IL-1β signaling is harmful to the injured CNS as shown in different

models of brain injury (Boutin et al., 2001; Lu et al., 2005; Relton and Rothwell, 1992; Yamasaki et al., 1995) and infection (Brabers and Nottet, 2006; Das et al., 2008). Some however have reported a neuroprotective role of IL-1β and it appears to be dependent on the concentration of the cytokine and timing of the response relative to the insult (Carlson et al., 1999; Strijbos and Rothwell, 1995). In regards to IL-1β's neuroprotective role, it has been proposed as being IL-1RI-independent and dependent on the regulation of Na^+ and K^+ currents in neurons (Diem et al., 2003). Importantly, IL-1 alone, in the absence of additional CNS impairment, has been found not to be neurotoxic (Loddick and Rothwell, 1996; Relton and Rothwell, 1992; Shaftel et al., 2008; Yamasaki et al., 1995). Therefore, studies have concluded that the release of IL-1β following injury or insult is part of a protective response however, a response that ultimately goes awry in disease or chronic damage. An example of the common factor in disease associated with neuronal damage is the release and effects of the glutamate neurotransmitter. Glutamate is an abundant excitatory neurotransmitter present in the mammalian CNS and glutamate induced excitotoxicity is a key factor involved in ischemic cascades (Liao et al., 2008) in stroke and excitotoxicity via glutmate also has been implicated in diseases such as amyotrophic lateral sclerosis (Andreadou et al., 2008) and Alzheimer's disease (Griffith et al., 2008). In addition, evidence has emerged linking IL-1β and glutamate excitotoxicity (Jander et al., 2000; Liu et al., 2008), where IL-1β contributed to glutamate-induced damage following SCI, suggesting that blocking IL-1β may counteract glutamate toxicity.

A large number of studies have been conducted looking at the role IL-1β plays in cerebral ischemia, where both IL-1β mRNA and protein expression are increased following an ischemic insult (Buttini et al., 1994; Minami et al., 1992; Yabuuchi et al., 1994b). This up-regulation of IL-1β functions to induce neuronal cell death subsequent to cerebral ischemia

independent of the IL-1RI (Touzani et al., 2002) suggesting the existence of an additional signaling receptor or receptors for IL-1 in the brain. Experimental traumatic brain injury (TBI) is associated with rapid and prolonged up-regulation of IL-1β (Kamm et al., 2006; Lu et al., 2005; Nieto-Sampedro and Berman, 1987; Zhu et al., 2004). Furthermore, intercerebroventricular (i.c.v.) administration of IL-1RA caused a decrease in neuronal injury and improved functional outcome (Jones et al., 2005; Toulmond and Rothwell, 1995). Several lines of evidence indicate that increased expression of IL-1β contributes to tissue damage following spinal cord injuries (SCI). IL-1β mRNA and protein levels are increased at the lesion site as early as 1 hr following SCI in rats (Wang et al., 1997) and in humans (Yang et al., 2004). Subsequently it was found that neuronal damage elicited by IL-1β to be IL-1RI (Nesic et al., 2001) and p38 MAPK-dependent (Wang et al., 2005).

Due to the role of IL-1β in neuronal injuries, it has been recently implicated in having a substantial role in neurodegenerative disease such as multiple sclerosis (McFarland and Martin, 2007), Parkinson's disease (PD) (Ferrari et al., 2006) and Alzheimer's disease (AD) (Griffin et al., 2006; Shaftel et al., 2008). Its role in AD, however, is somewhat unclear as it has been reported to have a possible neuroprotective role rather than a neurodegenerative role as originally indicated (Tachida et al., 2008). Using a progressive 6-OHDA rat model of PD and evaluation by immunohistochemistry, ELISA and cell counting it became clear that levels of IL-1β were significantly elevated and identified as a mediator of dopaminergic (DA) neuronal loss. Administration of IL-1RA, resulting in significant reductions in TNF-α and IFN-γ levels without any effect on IL-1β levels, however, resulted in attenuation of DA neuron loss caused by LPS-induced sensitization of dopaminergic degeneration (Koprich et al., 2008). In respect to IL-1β, therefore, it has been proposed that IL-1β may have both a neurodegenerative and neuroprotective roles (Corasaniti et al., 2001).

2.4.5 Role of IL-1β in Neuroinflammatory Pain

Peripheral and central nerve injury is associated with an inflammatory response at the site of damage. This response includes increased levels of TNF-α, IL-1 and IL-6, concomitant with the development of hyperalgesia (Watkins and Maier, 2002) and these cytokines have been implicated in pain facilitation (DeLeo and Yezierski, 2001). Nerve injury is associated with the activation of spinal microglia cells, and therefore as a major source of proinflammatory cytokines, plays a crucial role in the development of neuropathic pain (Clatworthy, 1998; DeLeo et al., 2004; Raghavendra et al., 2003). Among the proinflammatory cytokines, IL-1β is particularly known to modulate pain sensitivity (Ren and Dubner, 2008).

Peripheral or central administration of IL-1β usually produces hyperalgesia (Ferreira et al., 1988; Oka et al., 1994; Watkins et al., 1994). Spinal administration of IL-1β produces thermal and mechanical hyperalgesia (Falchi et al., 2001; Tadano et al., 1999). To consolidate the role of IL-1β in neuropathic pain, neutralizing antibodies to the IL-1RI, genetic impairment of IL-1RI signaling and an IL-1RI-knockout (KO) mouse model all either reduced or completely attenuated IL-1β induced neuropathic pain (Sommer et al., 1999; Wolf et al., 2006; Wolf et al., 2003). The relationship between GPCR mechanisms (phosphorylation) and IL-1β-induced pain have not been studied, but recently it was demonstrated that the reduction of GRK2 expression in the spinal cord was dependent on IL-1β signaling as shown using a IL-1RI$^{-/-}$ model (Kleibeuker et al., 2008). In contrast to these studies, spinal i.t. administration of IL-1β during injury reduced inflammatory pain, and IL-1β was shown to have significant antinociceptive properties when administered to rats with peripheral inflammation (Souter et al., 2000). This study suggested the development of IL-1β like compounds or agonist with the aim of being able to reduce pain with minimal side

effects (Souter et al., 2000). The antinociceptive effects of IL-1β have shown to be opioid-mediated, as endogenous opioids are released from immune cells in response to IL-1β (Schafer et al., 1994).

2.4.6 Role of IL-1β in opioid mediated analgesia

Despite the wealth of knowledge about the mechanisms underlying the analgesic and hyperalgesic circuits, little is known about the interaction between them, particularly in relation to opioid analgesia. Interactions between analgesic and hyperalgesic circuits may contribute to the development of reduced opioid analgesia, which is the most prominent characteristic of repeated morphine administration and therefore constitutes a major clinical complication in chronic opioid treatment. The role and activation of glia and the production of proinflammatory cytokines, including IL-1β, have been implicated in the development of reduced morphine analgesia (Raghavendra et al., 2002; Song and Zhao, 2001).

It has been showne that IL-1β reduced opioid binding in specific regions of the guinea pig brain (Ahmed et al., 1985). Later, IL-1β was shown to increase the expression of MOR mRNA in primary astrocytes (Ruzicka and Akil, 1997; Ruzicka et al., 1996) and increase the expression of MOR mRNA in neural microvascular endothelial cells (Vidal et al., 1998). It was postulated (Min et al., 1994) and later discovered that there was the expression of a cytokine response element on the OPRM1 gene promoter, e.g. NF-IL6 binding site (Nuclear factor-IL6) (Im et al., 1999). In regards to opioid analgesia *in vivo*, the interaction between IL-1β and morphine was studied in the diabetic mice model, where it was shown that diabetic mice are less sensitive to morphine-induced analgesia, and this was found to be attenuated following i.c.v. administration of IL-1β (Gul et al., 2000). The role of proinflammatory cytokines and fractalkine in analgesia and tolerance was studied in mice and it was concluded that IL-1β is an endogenous regulator of morphine analgesia and is

involved in increasing pain sensitivity that occurs following chronic opioid administration (Johnston et al., 2004). Later, the role of IL-1β and morphine tolerance was studied in detail using IL-1RI KO mice. It was concluded following this study that IL-1β antagonized morphine analgesia (Shavit et al., 2005). The same group also suggested that polymorphisms of IL-1RA may contribute to the variation in postoperative morphine consumption (Bessler et al., 2006). In 2006, using a rat peripheral inflammation model, administration of Freud's complete adjuvant (FCA) (unilateral) induced the up-regulation of KOR mRNA in ipsilateral dorsal root ganglia (DRG) 12h after induction of local inflammation. More relevantly, FCA-induced up-regulation of KOR mRNA expression was mimicked by administration of IL-1β and was completely abolished by IL-1RA (Puehler et al., 2006). Also in this study, it was concluded that IL-RA prevented KOR mRNA as well as KOR up-regulation in response to FCA, suggesting that IL-1β is a specific mediator in the up-regulation of KOR in DRG. Spinal proinflammatory cytokines are powerful pain-enhancing signals that contribute to pain following peripheral injury (neuropathic pain). Recently, the increase in release of proinflammatory cytokines in the spinal cord has also been correlated to acute and chronic exposure to morphine both in *in vivo* and *in vitro* lumbar dorsal cord preparations (Hutchinson et al., 2008). To further elaborate, these studies demonstrated a novel finding that ≤5 min after intrathecal opioid administration, endogenous IL-1 reduced morphine analgesia. Also, Hutchinson et al., (2008) showed that intrathecal morphine analgesia was reduced 8-fold by spinal IL-1. Additional inhibitor studies revealed dependence on p38 MAPK activation and NO (Hutchinson et al., 2008). In support of Hutchinson et al., (2008), morphine-tolerant rats showed heightened IL-1β mRNA (2 hr post-LPS treatment) expression in the brain following LPS-induction (Staikos et al., 2008), suggesting that the

inflammatory response to bacterial infection may be potentiated in the brain when exposed to morphine.

The aim of this dissertation is to investigate the role of IL-1β and morphine on the mRNA regulation of MOR expression in SK-N-SH cells and to elucidate how and what signal transduction mechanisms regulate the expression of MOR by IL-1β and morphine. This study may provide an insight into the potential involvement of IL-1β in reduced morphine analgesia as measured following chronic morphine administration.

2.5 Opioids and opioid receptors

The use of opium and its derived compounds date back to the Sumerians some 4,000 B.C. There is general agreement that the Sumerians, who inhabited what is known today as Iraq, cultivated poppies and isolated opium from their seed capsules. It is believed that the first record of opium use was for religious rituals as an euphoriant. 'Medical' use became apparent when opium was being used to put people quickly and painlessly to death. The first known medical use of opium was recorded 1500 B.C. as a remedy to prevent excessive crying of children.

In 1806, a German pharmacist named Friedrich Serturner isolated the first active ingredient in opium and named it morphine after the Greek god of dreams, Morpheus. Pure morphine is a weak alkaloid base and was manufactured in large amounts following the invention of hypodermic syringe in the 1850s. Morphine began to be used for surgical procedures for postoperative and chronic pain. With the increased use of morphine and derivatives of opium, a considerable increase in opium abuse became obvious. In 1942, nalorphine, the first opioid antagonist was discovered, and was used initially to reverse the respiratory depression produced by morphine (Brownstein, 1993).

2.5.1 Opioid receptors

Following the opioid-mediated effects in mouse vas deferens, the opioid receptor in the mouse vas deferens was assigned the designation "*delta* opioid receptor" (DOR) (Lord et al., 1977; McKnight et al., 1984). The KOR and MOR assignments were based on differential analgesic properties *in vivo* (Martin et al., 1976). Dynorphin related peptides are derived from the prodynorphin precursor and they all have a high affinity for the KOR binding sites (Gillan et al., 1985; Goldstein et al., 1979).

The dissimilarities among the opioid peptides for opioid receptor binding sites revealed the existence of multiple opioid receptors as proposed earlier (Martin et al., 1976). Soon opioid receptors where characterized using selective radioactive ligands (Chang et al., 1979; Chang and Cuatrecasas, 1979); μ- (MOR) δ- (DOR) and ϰ- (KOR) classification of opioid receptors followed the discovery of opioid specific binding sites in neural tissue. In characterizing opioid receptors, it was discovered that opioid receptor binding was influenced by cations, i.e. Na^{+} ions, (Pert and Snyder, 1973a; Pert and Snyder, 1973b; Werling et al., 1986) and divalent cations, Ca^{2+}, $Mg^{2+,}$ and Mn^{2+} (Paterson et al., 1986)..

As the need for cations for opioid receptor binding became important, it also became apparent that opioid receptor binding is also regulated by nucleotides (Blume et al., 1979). Nucleotides, GTP, GDT and ITP all increase the dissociation of the agonist [^{3}H]-dihydromorphine as demonstrated in rat brain homogenates (Blume, 1978a; Blume, 1978b). Brain membrane based binding assays of opioid receptors characterized the mechanism of GTP binding to G-proteins (Childers and Snyder, 1980) eventually leading to a complete characterization of agonist stimulation of GTPase activity

2.6 Mu opioid receptor (MOR) type

The MOR type has an important role in mediating the analgesic effects of morphine and endogenous reward systems of the brain. The MOR1 type is thought to be involved in several opioid mediated effects, such as supraspinal analgesia, prolactin release and decrease in acetylcholine turnover (Pasternak and Wood, 1986). The MOR2 type is thought to be involved in respiratory depression, decreased dopamine turnover and the delayed gastrointestinal tract transit induced by opioids. The exact nature of the MOR types binding affinity to their respective ligands and how this may reflect MOR classification is still in dispute (Chen et al., 1993a; Raynor et al., 1994; Raynor et al., 1995; Thompson et al., 1993; Wang et al., 1994).

2.6.1 CNS expression of MOR

MOR is present both pre- and post-synaptically and is widely expressed in the spinal cord (Stevens et al., 1991), where the total opioid binding capacity of the spinal cord was 90% MOR specific. MOR is also expressed in the brain (Delfs et al., 1994), where i*n situ* hybridization of rat brain tissue revealed expression of MOR in the telencephalon, internal granular and glomerular layers of the olfactory bulb, the caudate putamen and nucleus accumbens (Delfs et al., 1994; Mansour et al., 1994; Minami et al., 1994). Anatomical and physiological studies have indicated that neurons of the medial thalamic nuclei express high levels of MOR mRNA and play a particular role in the transmission of nociceptive information (Albe-Fessard and Kruger, 1962; Kaelber et al., 1975). Furthermore, neurons in these nuclei are selectively depressed following morphine administration (Duggan and Hall, 1977; Nakahama et al., 1981). In the pons and the medulla, MOR mRNA is intensely expressed in the locus coeruleus, which is largely composed of noradrenergic neurons, suggesting that a large portion of noradrenergic terminals as well as cell bodies have MOR

expression (Delfs et al., 1994). In support of MOR expression in the noradrenergic neurons, DAMGO, a MOR selective agonist, inhibited the release of noradrenaline from slices of the rat cortex, hippocampus and cerebellum. Opioid analgesics such as morphine have several side effects (Vella-Brincat and Macleod, 2007). In humans, death from morphine overdose is commonly caused by respiratory arrest (Sternlo et al., 1998). Respiratory depression by opioids is caused by their direct effect on the brainstem respiratory centers (medullary nuclei; solitaries, ambiguous and parabrachial), where an intense population of MOR (MOR2 mRNA) are expressed (Pasternak et al., 1993

2.6.1.1 MOR binding properties

Cloned MOR expressed in transfected CHO cells has a strong affinity for morphine (Ki = 1.4), naloxone, (Ki = 3.9), [D-Ala^2, D-Leu^5] enkephalin (DADLE) (Ki = 6.4) and DAMGO (Ki = 0.87) (Kosterlitz, 1981). Several opioid antagonists bind to MOR with high affinity, including β-FNA (MOR-selective; beta-funaltrexamine), NTI (DOR-selective; naltrindole) and nor-BNI (KOR selective; nor-binaltorphimine).

2.7 Second messenger systems coupled to MOR

Mu opioid receptor (MOR) specific ligands bind to MOR expressed on cell membranes of neurons and non-neuronal cells which then initiate a physiological response. This interaction between opioids and MOR is characterized by the ability of opioids to bind their receptor (affinity) and the potential magnitude of the induced effect (efficacy). The role of G-proteins in opioid receptor-mediated signaling was evident early in opioid receptor history as discovery of opioid ligands binding to ORs was guanine nucleotide dependent (Blume, 1978a). Opioid receptors belong to the G-protein coupled, seven transmembrane region spanning super-family of receptors (GPCRs). Opioid receptors transduce their signals

through guanine nucleotide binding proteins (heterotrimeric G-proteins) composed of α, β and γ subunits (Childers, 1991). Activation of G-proteins involves association of the G-protein with opioid receptor, substitution of GTP for GDP followed by the hydrolysis of GTP by GTPase (Nestler and Aghajanian, 1997) (Fig. 5). The MOR couples to inhibitory G-proteins ($G_{i/o}$). G-protein activation following opioid receptor activation was studied using pertussis toxin (PTX), a protein derived from *Bordetella pertussis*, which interferes with the signal transduction by catalyzing the ADP-ribosylation of a cysteine side chain on the α-subunit of the inhibitory $G_{\alpha i}$-proteins indicating a $G_{\alpha i}/_{o}$- dependent MOR transduction. MOR activation leads to inhibition of adenylate cyclase (AC) (Collier and Roy, 1974a; Collier and Roy, 1974b), increased potassium conductance (North, 1986; North et al., 1987) and decreased calcium conductance (Hescheler et al., 1987). Presently there is no evidence of opioid receptors coupling to stimulatory G-protein (G_s). However, opioid receptors have been shown to stimulate AC in the rat olfactory tubercle through a cholera toxin-insensitive, G-protein, $G_{\beta\gamma}$-subunit mechanism (Onali and Olianas, 1991). Coupling through $G_{\beta\gamma}$-subunits has been proposed as the mechanism for opioid receptor mediated changes in potassium and calcium conductance, MAPK pathway, mobilization of intracellular calcium and activation of protein kinase C (PKC) (Williams et al., 2001). One of the controversial issues in opioid signaling is the ability of opioids to stimulate AC (Law et al., 2000). Stimulation of specific isoforms of AC by $G_{\beta\gamma}$-subunits may provide opioids alternative routes to increase intracellular cAMP levels (Chan et al., 1995; Tsu et al., 1995).

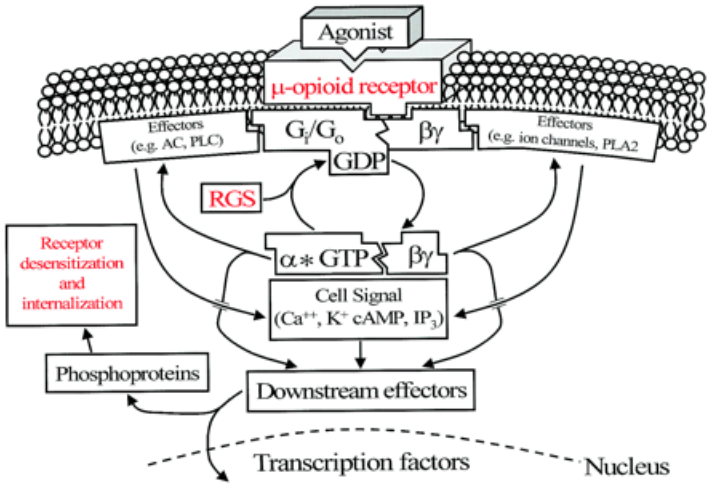

Fig. 5 MOR GPCR transduction and regulation (Taylor and Fleming, 2001).

The role of the G_α-subunits in opioid activity is important due to its intrinsic GTPase activity, making it a potential site of opioid receptor regulation. A family of 20 known protein isoforms called regulators of G-protein signaling (RGS proteins) were identified as having specific modulation of GTPase-activating proteins that function to accelerate the exchange of GTP for GDP on the α–subunits of $G_{i/o}$ and G_q proteins (Dohlman and Thorner, 1997). The extract role of RGS proteins in opioid receptor regulation and signaling is currently undergoing intense research as reviewed by Xie et al., 2005 (Brown and Sihra, 2008; Hooks et al., 2008; Xie and Palmer, 2005).

An overview of the cellular effects of MORs include the regulation of second messengers, were the activation of MOR causes the inhibition of AC activity (Chen et al., 1993a; Evans et al., 1992; Fukuda et al., 1993; Kieffer et al., 1992; Yasuda et al., 1993) and suppression of N-type (Tallent et al., 1994) and L-type (Piros et al., 1996) Ca^{2+} channels causing a transient decrease in the levels of intracellular Ca^{2+} (Johnson et al., 1994). Activation of MOR also increases phospholipase C (PLC) activity and the activation of inwardly rectifying K^+ channels (Henry et al., 1995), and the mitogen-activated protein

kinases ERK-1 and ERK-2 (Fukuda et al., 1996) (Fig. 7). The hyperpolarization of the cell's membrane potential by K^+ currents and the limiting of Ca^{2+} entry by suppression of Ca^{2+} currents are both mechanisms for opioid induced blockade of neurotransmitter release and pain transmission.

2.8 MOR receptor phosphorylation, desensitization, signaling and gene promoter structure; MOR gene activation and transcription

Pharmacological studies have concluded that MOR is the main site of action for morphine and other opioid-induced analgesia, tolerance and physical dependence (Law and Loh, 1999). Opioid receptors share high structural homology but also show distinct patterns of expression and functional profiles, their individual patterns of transcription are therefore likely to be determinants for different levels of opioid receptor expression in the PNS and CNS.

The MOR (MOR) promoter is a TATA-less type promoter with several potential binding sites for transcription factors (Law et al., 2004). MOR has been measured to be up-regulated following stimulation with endogenous mediators in neuronal cell lines. Examples of MOR up-regulating mediators include IL-1, IL-4, IL-6, TNF-α and IGF-1 (Bedini et al., 2008; Borner et al., 2004; Kraus et al., 2001; Kraus et al., 2003; Ruzicka and Akil, 1997; Ruzicka et al., 1996) and IFN-γ is a MOR down-regulator (Kraus et al., 2006).

2.8.1 MOR phosphorylation

Opioid receptors are members of the GPCR super-family. Lefkowitz et al., (1998) concluded that agonist-receptor configuration resulted in rapid receptor phosphorylation by protein kinases including G-protein-coupled receptor kinases (GRKs) and therefore promoting the association of β-arrestin, cellular proteins involved in receptor internalization.

The role of β-arrestin in GPCR regulation is generally two-fold: receptor uncoupling from respective G-proteins, therefore implicating a role in receptor desensitization (reduced or complete loss of receptor signaling); β-arrestin also initiates a series of events that result in receptor internalization (clathrin-coated vesicles). With respect to GPCR desensitization, initial steps include the rapid phosphorylation of the receptor as first demonstrated in HEK293 cells expressing MORs (Arden et al., 1995). The key protein involved in opioid receptor phosphorylation was initially found to be the GRKs as found in DOR and MOR expressing cells (Pei et al., 1995; Zhang et al., 1996). Following detailed correlation between agonist-induced phosphorylation of opioid receptors, extensive work was conducted with different opioids and phosphorylation was correlated to ligand efficacy. Morphine-induced the phosphorylation of MOR expressed in CHO cells (Yu et al., 1997) but failed to phosphorylate MOR expressed in HEK293 cells (Zhang et al., 1997; Zhang et al., 1998). Zhang et al., (1997) also found that morphine only induced MOR phosphorylation in the presence of over-expressed GRK2 in HEK293 cells, suggesting that morphine-receptor complex is a poor substrate for the GRKs. However this discrepancy could be a result of differences in the level of protein kinases in the CHO and HEK293 cell lines. More recently, amino acids residues responsible for the agonist-dependent MOR phosphorylation was concluded as being Ser and Thr residues in the C-terminus (El Kouhen et al., 2001).

Other kinases that might be involved in receptor phosphorylation include Ca^{2+}/calmodulin-dependent protein kinases II, PKA, and the ERK1/2. The role of CaM kinase has been narrowed down to the basal phosphorylation of MOR as indicated by CaM kinase inhibitor studies (Wang et al., 1996). The role of PKA in MOR phosphorylation is somewhat indirect. Back-phosphorylation studies in neuroblastoma cells (Chakrabarti et al., 1998) and animals treated with morphine (Bernstein and Welch, 1998) concluded a decrease

in PKA-induced phosphorylation of MOR. This reduction in PKA-mediated back-phosphorylation does not clearly indicate a role for PKA in MOR phosphorylation following morphine treatment. Similar results were mirrored with chronic DAMGO treated neuroblastoma cells (Chakrabarti et al., 1998).

A precise role of ERK1/2 of the MAPK family in MOR phosphorylation is more probable. Blockade of MEK using MAP kinase kinase (MEKK) inhibitor PD98059 resulted in inhibition of DAMGO's ability (2 hr pretreatment) to down-regulate MOR protein expressed in CHO cells (Polakiewicz et al., 1998a). The correlation between receptor phosphorylation by MAPK was not made clear in this study; however more recent studies have attempted to elucidate the role MAPK pathways in MOR phosphorylation, tolerance, desensitization, and internalization by opioids. These studies have generally concluded that phosphorylation acts like a "switch" in opioid tolerance and pharmacological interventions at one of these protein kinases may provide valuable strategies to improving opioid analgesia by attenuating tolerance to these drugs (Belcheva et al., 2005; Cui et al., 2008; Ferrer-Alcon et al., 2004; Schmidt et al., 2000; Trapaidze et al., 2000; Wang and Wang, 2006).

2.8.2 MOR internalization and down-regulation.

Following opioid agonist-induced activation of MORs, the cell terminates MOR signaling by the following two mechanisms: **1)** via G-protein-coupled receptor kinases (GKRs), which phosphorylate Ser- and Thr-residues on the C-terminus of MORs (El Kouhen et al., 2001). Phosphorylation of Ser- and Thr-residues increases the binding of β-arrestin to the C-terminal and results in MOR desensitization. **2)** Receptor desensitization is usually followed by receptor internalization into the cell's cytoplasm. Receptor internalization is a complex series of receptor trafficking events that eventually result in receptor re-sensitization on the cell's membrane. Receptor internalization events have been

correlated to increase opioid-induced tolerance, which from a clinical prospective is the diminished capacity of opioid analgesics to promote their analgesic properties following long-term, repeated use.

Agonist-induced receptor internalization and down-regulation has been demonstrated in clonal or recombinant cell lines expressing opioid receptors and neuroblastoma cells endogenously expressing the MOR types (Blanchard et al., 1982; Chang et al., 1982; Law et al., 1984; Law et al., 1994). It was also concluded that opioid agonist could only induce receptor down-regulation and that partial agonist and antagonist could not (Keith et al., 1998; Keith et al., 1996). In fact, opioid antagonist role in MOR internalization was the opposite to that measured using opioid agonist, increasing the expression of MOR *in vivo* (Rajashekara et al., 2003).

Agonist induced MOR down-regulation was demonstrated with 7315C pituitary cells, human neuroblastoma SHSY5Y, SK-N-SH and NMB cells (Baumhaker et al., 1993; Puttfarcken and Cox, 1989; Shapira et al., 1997; Zadina et al., 1993). Similar experiments in cloned MORs expressed in neuroblastoma Neuro2A and C6 glioma cells or fibroblasts were conducted (Arden et al., 1995; Chakrabarti et al., 1995; Yabaluri and Medzihradsky, 1997). In these and similar experiments, morphine consistently caused MOR protein down-regulation without MOR protein internalization. Following these studies the question still exists, why does morphine and not other opioids cause the down-regulation of MOR protein and not MOR protein internalization. One explanation for morphine-induced MOR down-regulation and not internalization could be due to receptor trafficking events and variations *in vitro* cell-culture models.

Trafficking of opioid receptors following agonist treatment became apparent when opioid receptors were found to co-localize with transferin after internalization (Keith et al.,

1996). Opioid receptor (MOR, KOR and DOR) internalization was blocked by dominant negative mutants of beta-arrestin and dynamin, confirming the role of these proteins in opioid receptor internalization (Murray et al., 1998; Whistler and von Zastrow, 1998; Zhang et al., 1998). The activation of the endocytic pathway appears to be agonist and receptor dependent, where DAMGO and not morphine treatment induced the internalization of MOR (Arden et al., 1995; Zhang et al., 1998). A common link between the lack of morphine induction of MOR internalization are the role of GRKs. G-protein coupled receptor kinases (GRKs) when over-expressed prevent morphine's ability to induce MOR internalization, therefore suggesting a unique mechanistic role of GRKs in activating the endocytic pathway.

2.8.3 MOR signal transduction

GPCRs regulate cellular events such as growth and differentiation by stimulating MAPK cascades. Three main MAPK cascades have been defined in mammals and these include the extracellular-signal-regulated kinases (ERKs), Jun N-terminal kinases (JNKs) and the p38 MAPKs. Mitogenic signals transmitted following MOR activation has been well defined to be carried out by MEK and ERK pathways (Fig. 6). ERK1 and ERK2 stimulation by opioids was first recorded when MORs where expressed in recombinant CHO cells (Li and Chang, 1996). Li et al., (1996) showed that stimulation of ERK1/2 was ligand selective, agonist dose-dependent and PTX sensitive ($G_{i/o}$ dependent).

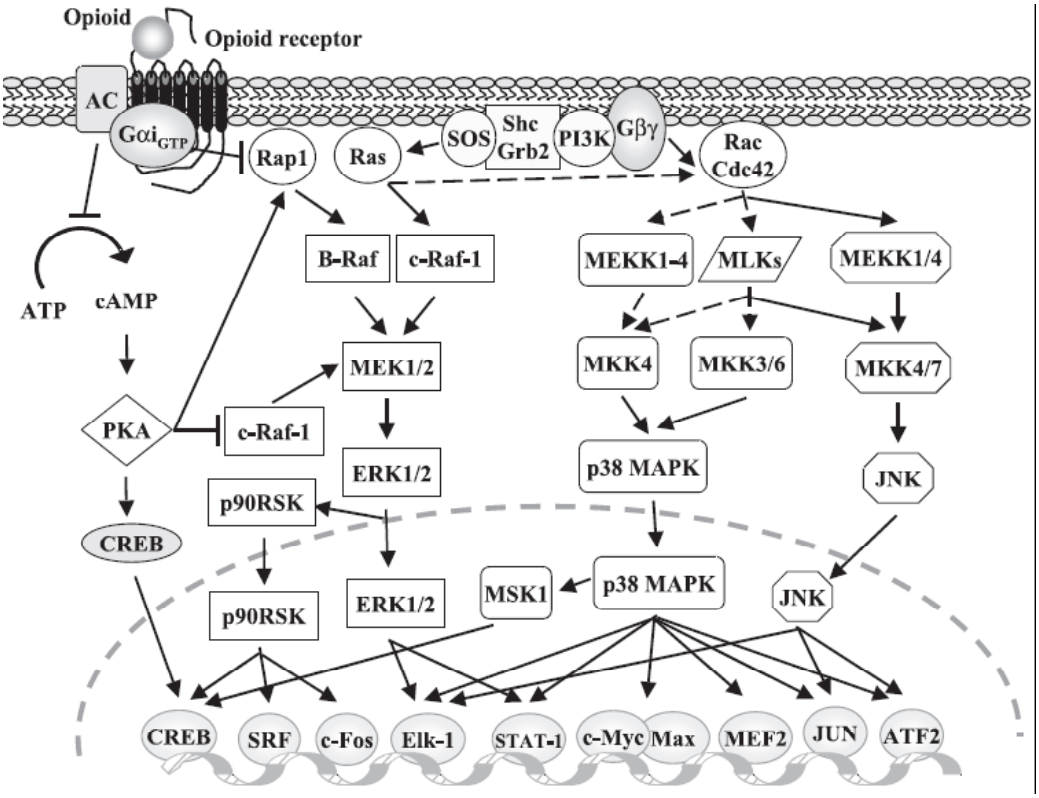

Fig. 6 Signaling pathways linked to the mitogen-activated protein kinases (MAPK) and transcription factors. Opioid receptors could use Gβγ to regulate MAPK cascade. AC, adenylyl cyclase; PKA, cAMP-dependent protein kinase; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; MEF2, myocyte enhancer factor 2; MEK or MKK, Mitogen-activated protein kinase kinase; MEKK, MAPK kinase kinase; MLK, mixed lineage kinase; p90 RSK, p90 ribosomal S6 kinase; STAT-1, signal transducer and activator of transcription 1; SRF; serum response factor; Elk-1, Eph-like kinase 1; ATF2, activating transcription factor 2; arrows, positive stimulation; blocked lines, inhibition; broken lines, interactions not well established. (Tso and Wong, 2003).

It has been proposed that opioid receptor desensitization following chronic opioid exposure is mediated by MAPK cascades. The role of MAPK activation, however, is not consistent between opioid receptor types. The DOR internalization following DOR agonist specific binding is dependent on activation of the MAPK cascades (Ignatova et al., 1999) but this was not the case for the KOR type internalization events following treatment with U50,488 (Li et al., 1999). The activation and expression patterns of ERK in rat brain before and after chronic morphine treatment showed increased ERK activity in hippocampus and increased ERK1/2 expression in the locus coeruleus and caudate/putamen after chronic morphine treatment. Apart from linking mitogenic events to opioid receptor regulation and expression, the stimulation of MAPK cascades has been associated to other facets of opioid receptor signaling. For example, the immunomodulatory and immunosuppressive effects of morphine on human lymphocytes is mediated by the activation of MAPK cascade (Chuang et al., 1997). In cell lines, tyrosine kinase activity has been stimulated following morphine treatment in SK-N-SH cells, where a 58 kDa protein was phosphorylated on tyrosine residues in a PTX-dependent manner (Nakano et al., 1994). The MOR and DOR proteins have different abilities to potentiate growth factor-induced cell proliferation in various cells types (Law et al., 1997). The differences between opioid receptors to potentiate growth factors may be related to the capacity of opioid receptors to regulate specific mitogenic signals. For example, very little is known about the role JNK and p38 MAPK play in opioid receptor signaling. This may prove to be important to our work, where the role of IL-1β in the regulation of MOR mRNA expression may be related to the capacity of IL-1RI -induced activation of mitogenic signals.

2.8.4 Regulation of MOR mRNA levels

Opioid receptors have a very high amino acid and structural homology; however they have distinct expression profiles. Thus, the pharmacological properties of opioid analgesics such as morphine depend on the regulation of opioid receptor expression in the CNS (Kim et al., 2004).

Therefore, in addition to opioid receptor regulation by phosphorylation and uncoupling events of G-proteins, opioid receptors are regulated at the transcriptional and translational level. Reduced levels of opioid protein expression following chronic opioid treatment have been proposed as a mechanism for the explanation of opioid tolerance. The logical explanation for the observed reduction in MOR protein would be due to the inhibition of the MOR gene transcription and therefore reduced basal opioid receptor mRNA level. MOR mRNA expression has been shown to not be affect following antagonist treatment, for example, when rats were chronically treated with naltrexone, MOR mRNA levels remain unchanged when measured using ribonuclease protection assay (RPA) (Castelli et al., 1997; Unterwald et al., 1995). A plausible explanation would be that chronic opioid administration in animals is difficult to maintain, however with the availability of drug-minipumps used to administer opioid continuously, this argument is experimentally inaccurate. The importance of understanding MOR mRNA to MOR protein translation remains to be studied; however, in order to further understand opioid gene expression, many experiments have used cell lines expressing opioid receptors to elucidate the affects of opioid treatment on opioid expression at the protein level only (Kim et al., 1995; Thorlin et al., 1997). These studies have failed to answer why the discrepancy between mRNA and protein expression levels of opioid receptors following chronic opioid treatment existed. Until

recently, experimental evidence has attempted to fill this void in opioid receptor expression studies.

2.8.5 MOR gene structure

Many years of research has allowed us to conclude that the regulation of opioid receptor mRNA is due to alterations in transcriptional activities of the receptor gene. This section will discuss the structure and the *cis*- and *trans*-elements that regulate the transcriptional activities. All three opioid receptors have multiple introns that span large distances in the chromosomal DNA and share a common genomic structure with the coding region being divided into three major exons. The human gene coding for MOR is present on the distal part of the long arm of chromosome 6. The MOR gene is more than 53k bp long, with exon splice junctions at the first intracellular loop (Arg^{95}), the second extracellular loop (Glu^{213}) and the cytoplasmic C-terminal region (Glu^{386}/Leu^{387}) a site at which most splice MOR variants occur at the cytoplasmic C-terminal regions (Kraus et al., 1995).

With the use of Rapid amplification of 5' complementary DNA ends (5'RACE) and RNase protection assays, multiple transcriptional start sites of opioid receptor gene have been identified. All opioid receptors have distal (DP) and proximal promoters (PP) (Augustin et al., 1995; Ko et al., 1997; Min et al., 1994). In most cases, transcriptional regulation of MOR is owed to the PP (95%) (Ko et al., 1997; Liu et al., 1999). The PP has been identified as specifically directing MOR transcription during murine development (Ko et al., 2002). The role of the DP in opioid receptor expression is as the start site of MOR transcriptional under inhibitory control. Truncation of the inhibitory control sites (-775 to -444 from the ATG start site) restores the distal promoter activity as shown by reporter gene assays. The location of DP sequence is around the center of a 34-bp negative *cis*-acting element and is both position- and promoter dependent (Choe et al., 1998; Liang et al., 1995).

2.8.6 MOR promoter and transcription factor binding sites

Opioid receptor genes contain no consensus TATA-box within the promoter regions (TATA-less promoters) allowing for the expression of opioid genes by multiple TATA-box related peptides or transcription factors. Therefore, opioid promoters are embedded within clusters of potential binding sites for different transcription factors, suggesting complex regulation of each gene promoter.

The transcription of the MOR gene starts at a cluster of four sites located between 291 and 268 base pairs before the start of the amino acid coding region (Choe et al., 1998; Ko et al., 1998; Liang et al., 1995; Min et al., 1994). In the 5' upstream region of the MOR gene, consensus binding sites have all been found for Sp1, AP2, AP1, glucocorticoid/mineralcorticoid response element, immune-cell-specific element PU-1, cytokine response elements NF-IL6 and NF-GMb, and the cAMP response elements.

With the discovery of transcriptional and cytokine response elements (NF-IL6 binding sites) found at nucleotide -1481 (Im et al., 1999) (Fig. 7), it was postulated that the response elements present in opioid receptor promoter regions may have a role in the cytokine effects on opioid receptor gene expression through *cis-trans* interaction. Previous studies have shown a cytokine-mediated increase in MOR mRNA expression in astrocytes and neuroblastoma cell lines (Borner et al., 2004; Kraus et al., 2001; Puehler et al., 2006; Ruzicka et al., 1996; Vidal et al., 1998). However, the direct link between *cis*-acting elements and the up-regulation of MOR mRNA following cytokine treatment using reporter gene assays was not successfully established. Therefore, due to the lack of evidence for the role of NF-IL6 binding sites in cytokine-induced MOR mRNA expression, it has been concluded that NF-IL6 binding sites are nonfunctional (Im et al., 1999).

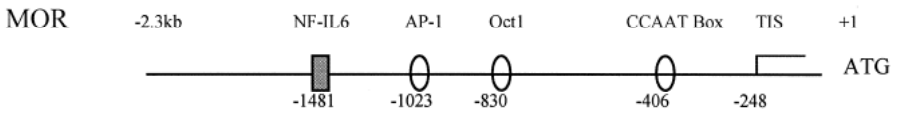

Fig. 7 Potential transcription binding sites in the MOR promoter region: transcriptional start codon (ATG) marked at +1. The MOR promoter region contain cytokine response element (NF-IL6) as well as other potential transcription binding sites (AP-1, Oct1) (Im et al., 1999).

Furthermore, other binding sites for transcription factors involved in cytokine stimulated MOR transcription have been recognized and experimentally verified to bind the predicted transcription factor: a STAT6 binding element at nucleotide -997 (Kraus et al., 2001), two AP-1 binding sites at nucleotides -2388 and -144 (Borner et al., 2002), three NF-κB binding sites at -2174, -577 and -207 (Kraus et al., 2003), a STAT1/3 element at -1583 (Borner et al., 2004) (Fig. 8).

2.8.7 MOR gene transcription is influenced by endogenous and exogenous compounds

Opioids **-** Several substances have been shown to modulate the expression of MOR mRNA and protein levels. Opioid ligand induced changes in the expression of their own receptors has provided an intriguing perspective, suggesting a feed-back loop engagement between MOR and its promoter. For example, DAMGO binding to MOR has been shown to enhance Sp1/Sp3 binding to its MOR gene promoter as recorded using a MOR luciferase promoter construct transfected in SK-N-SH cells (Xu and Carr, 2001).

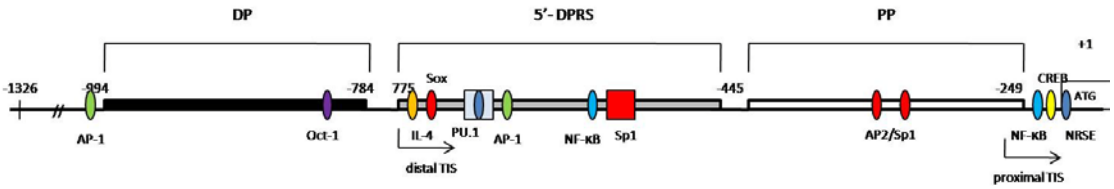

Fig. 8 Regulatory sequences of the MOR gene. The MOR gene transcription is controlled by two promoters, distal (DP) and proximal (PP) promoters. Transcription binding sites and factors represented by their individual colors. 5'-DPRS - 5'-distal promoter regulatory sequences. TIS - transcription initiation site. Activators: Oct-1, IL-4, Sox, Sp1, AP2, NF-$\varkappa$B, CREB. Repressors: PU.1 and NRSE (neuron restrictive silencer element). Figure constructed with data collected from (Choe et al., 1998; Hwang et al., 2003; Im et al., 1999; Kim et al., 2004; Ko et al., 2003; Ko et al., 1998; Ko et al., 1997; Liang and Carr, 1996; Liang et al., 1995; Rivera-Gines et al., 2006). (Adapted from (Law et al., 2004).

Also morphine and endomorphins (endogenous opioid peptide) have been found to modulate MOR gene promoter transcription in SH-SY5Y cells (sub-clone of SK-N-SH cells), where morphine down-regulated MOR gene transcription in both un-differentiated and differentiated cells and endomorphins-1 and -2 up-regulated MOR gene transcription in the same cell lines (Yu et al., 2003).

Non-opioids **-** Studies have postulated the modulation of the MOR transcript by peptides and agents not associated with opioids. Substance P (SP) has been associated with endogenous opioids since the 1980's, where the release of SP from the spinal dorsal horn has been found to be regulated by opioids (Aimone and Yaksh, 1989) and the discovery of the co-existence of opioid receptors and preprotachykinin A (PPTA), a SP precursor soon followed (Minami et al., 1995). Pain-induced release of SP, a neuropeptide present in primary

afferent neurons (PAF) for the transmission of nociceptive information (Otsuka et al., 1976), has been associated with the up-regulation of MOR expression and facilitation of an opiate-mediated intrinsic anti-nociceptive mechanisms (McLeod et al., 2000).

2.8.8 Up-regulation of MOR gene transcription by cytokines

It has been known for decades that the opioid system and immune system have a unique relationship, and this unique interaction was first discovered when mice where immunized with morphine and the level of morphine tolerance decreased (Meisheri and Isom, 1978). Addiction neurobiologists have studied the relationship between opioid addiction and its effect on the immune system. Therefore, the general consensus following 25 years of research is that opioids impact the immune system with immunosuppressive properties (Bayer et al., 1994; Bayer et al., 1990; Brown and Van Epps, 1985; Bryant and Roudebush, 1990; Bussiere et al., 1992; Bussiere et al., 1993; Carr et al., 1995; Chao et al., 1993; Clark et al., 2007; Coussons-Read et al., 1994; Davis et al., 2007; Eisenstein et al., 1993; Eisenstein et al., 1995; Gaveriaux-Ruff et al., 1998; Liu et al., 2006b; Nelson et al., 1997; Page, 2005; Portoles et al., 1995; Saurer et al., 2006; Shavit et al., 1986; Suzuki et al., 2003; Thomas et al., 1995; Tsai et al., 2000; Weber and Pert, 1989; Welters et al., 2000b; Welters et al., 2007; Zaki et al., 2006; Zou et al., 2007). As a potent immuno-modulator, the effects of morphine on the immune system have been summarized in Table 1 and is detailed in a review article by (Sacerdote, 2008).

Several cytokines have been shown to up-regulate MOR gene transcription in neuronal cell lines, primary cell cultures and immune cells expressing MOR and therefore this direction of interaction between the immune system and opioid receptors implicates the involvement of cytokines in the efficacy of analgesia induced by opioids. Studies using pro-inflammatory cytokines, IL-4 and IL-6 showed that MOR mRNA expression was

significantly up-regulated in SH-SY5Y cells through the activation of STAT6 (Kraus et al., 2001) and STAT3 (Borner et al., 2004) respectively.

Table 1 Summary of morphine induced immuno-modulatory effects.

Immuno-modulatory effects of morphine exposure
1. Lymphoid organ atrophy
2. Reduced Natural Killer (NK) cell activity
3. Reduced Macrophage (MΦ) activity
4. Reduced MΦ secretion of IL-1, IL-6 and TNF-α
5. Decreased CD4/CD8 double positive cells
6. Increased IL-4 expression
7. Decreased IFN-γ expression
8. Reduced T-cell secretion of IL-2
9. Phosphorylation of MAPK in lymphocytes

Interestingly, IL-4 and TNF-α treated immune cell (Jurkat T cells, and Raji B cells) induced an otherwise dormant MOR gene expression (MOR mRNA in the immune cells was 15 to 200 times less than those in primary cortical and SH SY5Y neuronal cells) (Borner et al., 2007). In contrast, interferon-γ (IFN-γ) treatment in immune and neuronal cells by the same group repressed MOR gene transcription, shutting down MOR expression, preventing IL-4 up-regulated MOR in Jurkat T cells (Kraus et al., 2006). Other cytokines such as TNF-α (Kraus et al., 2003) and IL-1β have been studied as being cytokines that up-regulate MOR in neurons and endothelial cell lines (Puehler et al., 2006; Ruzicka et al., 1996; Vidal et al., 1998).

The inter-relationship between the immune system and nervous system is complicated. And therefore, the relationship between opioid-induced analgesia, opioid

receptors and immune modulators is not very well understood, leaving a gap in our understanding of the complicated inter-relationship between the two biological systems. An improved, in depth understanding of the process involved in pain transmission, inflammation, immuno-modulation and analgesia will allow us to better tailor analgesia drugs by finding novel potential therapeutic targets. The answers to these questions are beyond the scope of this dissertation. However the aim is to provide the small missing piece to a puzzle, filling a gap in our knowledge about the relationship between neuroinflammatory mediators and opioid analgesia by studying the effects of IL-1β and morphine on the expression of MOR in neurons.

2.9 The role of NF-κB in MOR expression

Nuclear factor-κB (NF-κB) is one of the most diverse and critical transcription factors. NF-κB is a key downstream molecule that may directly or indirectly transmit receptor-mediated upstream signals to the nucleus, resulting in the regulation of NF-κB-dependent genes. NF-κB is composed of homo- and heterodimers of five members of the Rel family, including NF-κB1 (p50), NF-κB2 (p52), RelA, RelB, and c-Rel. The p50/p65 complex is the most common functional heterodimer found in cells. The NF-κB signaling pathways is as follows: cytokines and other released immune mediators bind to their receptors to activate IKK; activated IKK complex which consists of IKKα and IKKβ and IKKγ [NEMO] subunits, then phosphorylate IκBα, which is bound to the NF-κB p50/p65 heterodimer in an inactive state, mostly in the cytoplasm; phosphorylated IκBα is released from the complex and undergoes proteasome-dependent degradation; freeing NF-κB, which then translocates to the nucleus to induce the expression of target genes (Fig 9). For a complete review refer to (Hayden and Ghosh, 2004)

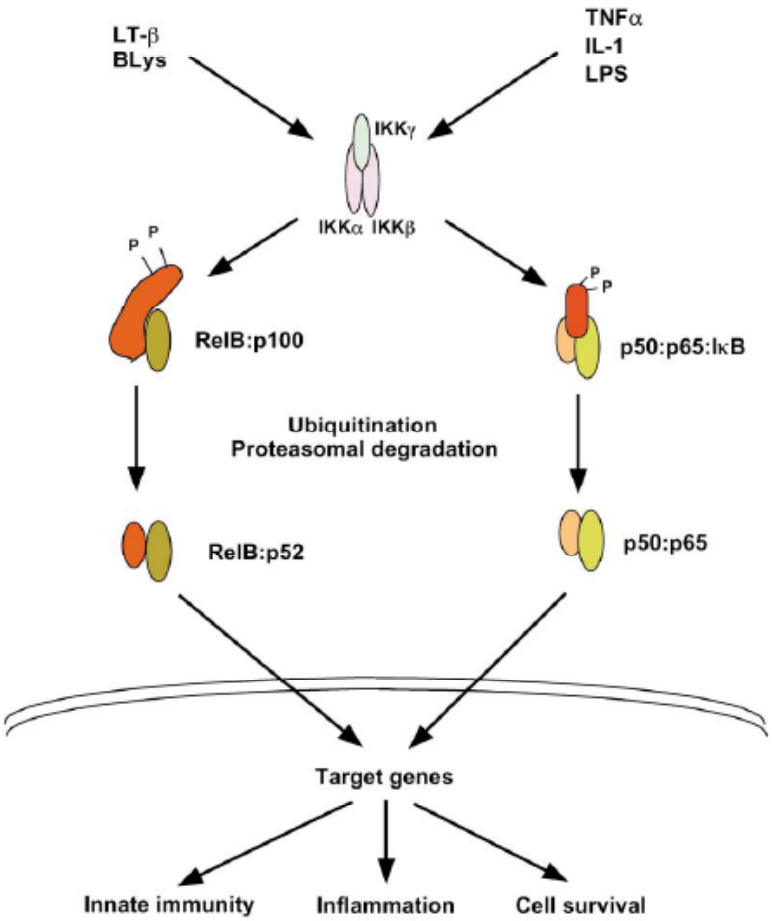

Fig. 9 Alternative and classical pathways of NF-κB signaling (Hayden and Ghosh, 2004)

GPCR mediated immune responses are translated through NF-κB, where it is involved in mediating down-streaming signaling pathways (Ye, 2001). Many studies have linked NF-κB involvement in opioid-induced immunosuppressive actions (Carr et al., 1995; Murphy, 2003; Welters et al., 2000a), and less so to the expression of opioid receptors in neurons and immune cell types expressing opioid receptors (Kraus et al., 2003; Ledeboer et al., 2005). Moreover, inhibition of NF-κB by pyrrolidine dithiocarbamate (PDTC) attenuated the effect of opioid withdrawal (Capasso, 2001). Therefore, these studies indicate that NF-κB may play a key role in opioid-mediated neuronal expression of opioid receptors.

2.10 Overview of SK-N-SH human Neuroblastoma cells

The SK-N-SH Neuroblastoma cells represent immature peripheral neurons that frequently express several morphological phenotypes (Yu et al., 1988). Human neuroblastoma, one of the most common early childhood solid tumors, is thought to arise from neural crest cells during embryonic development. The SK-N-SH cell line was established in culture in December 1970 from a bone marrow biopsy of a four year-old girl (Biedler et al., 1973). This cell line is composed of two morphologically distinct cell types, neuroblastic "N" and epithelial-like "S" cells. "N" cells are characterized as small and round with loosely adherent cell bodies consisting of neurite-like processes that contain noradrenergic biosynthesis enzymes as well as an uptake mechanism for norepinerphrine. "N" cells also express opioid receptors (Sadee et al., 1987). "S" cells are larger and flattened cells, resembling epithelial and highly substrate-adherent (Ciccarone et al., 1989). The SK-N-SH cells were further subcloned into the lines SH-SY5Y (neuroblast-like cells having catecholamine biosynthetic enzymes) and SH-EP (Ciccarone et al., 1989; Sadee et al., 1987).

The SK-N-SH cells are multi-potential with regards to neuronal enzyme expression. The cell line has enzymes required for the biosynthesis of several neurotransmitters such as choline acetyltransferase and tyrosine hydroxylase. In particular, SK-N-SH cells have a high activity of dopamine-β-hydroxylase and very low activity of glutamic acid decarboxylase, the enzyme responsible for the conversion of glutamate to GABA (Amano et al., 1972; West et al., 1977).

In a monolayer culture, SK-N-SH cells have a population-doubling time of 44 hr (Biedler et al., 1973). The total number of opioid-binding sites was estimated at 70,000 sites/cell based on saturation binding studies (Biedler et al., 1973; Yu et al., 1986). SK-N-SH cells express abundant MOR proteins (~50,000 binding sites/cell) and fewer DORs

(~10,000 binding sites/cell). Binding and function of these receptors have been well characterized. The opioid receptor binding sites on SK-N-SH cells closely resemble MOR and DOR binding sites in human and rodent brain (Yu et al., 1986). The SK-N-SH cells can be differentiated to a neuronal phenotype (~70%) with all the morphological and biochemical characteristics of neurons (Pahlman et al., 1984). Immature neuroblast forms can be induced to differentiate into mature neurons, which are marked by extensive neurite outgrowth (Ross et al., 1983).

Using the differentiating agent retinoic acid (RA) on SK-N-SH cells, it has been found that the neuroblast-like cells transform into large, flattened cells resembling epithelial or fibroblast cells (Sidell et al., 1983). RA is known to be one of the most potent agents inducing neuronal differentiation in many Neuroblastoma cell lines by influencing growth and morphogenesis.

In SK-N-SH cells, RA has been found to increase the protein expression of opioid receptors (Zadina et al., 1993) and therefore, RA-differentiated SK-N-SH cells can serve as a model to study opioid-induced analgesia efficacy and tolerance *in vitro* (Yu and Sadee, 1988).

The effect of prolonged exposure to MORs to opioid agonist has been studied in SK-N-SH cells. Chronic activation of MORs by morphine lead to partial desensitization of the receptors and upon activation of the opioids agonist, an overshoot in the production of cAMP measured (Wang and Gintzler, 1994; Yu et al., 1988). In SK-N-SH cells, it was found that desensitization occurs predominantly in the first 6 hr following agonist treatment. There were fewer G-proteins being activated, whereas the number of MORs did not change significantly. The MOR proteins returned to near normal levels after desensitization, but at lower receptor numbers (Breivogel et al., 1997).

2.11 Why is it important to elucidate the role of IL-1β on MOR expression?

The importance of understanding the effects of IL-1β on MOR expression is multiple. For example, decreased MOR expression following IL-1β exposure may result in reduced opioid analgesia and a result in increased opioid receptor down-regulation. Since opioid receptor genes are important to the development of cells, decreased MOR expression may result in attenuated cellular development, resulting in the disturbance of various physiological, MOR-dependent processes. Conversely, we may find that IL-1β stimuli increase MOR expression in SK-N-SH cells. Consequently, IL-1β-induced increase in MOR expression could result in an increase of potent, harmful effects of opioid (i.e. respiratory failure); however such effects would require a prolonged exposure to IL-1β at high concentrations. At present there are important gaps in our knowledge of how IL-1β affects MOR expression in the CNS *in vitro*. Gaps in our knowledge include: **a)** does IL-1β affect MOR expression? **b)** Does the level of IL-1β exposure of SK-N-SH cells expressing MOR differ (i.e., acute *vs.* chronic)? **c)** Does IL-1β impact the degree and rate of opioid-induced MOR mRNA expression? **d)** Are the effects of IL-1β on MOR expression dependent on specific intracellular signaling kinases? (I.e. NF-κB MEK/ERK/p38MAPK). This dissertation will address these basic gaps in our knowledge of morphine-IL-1β interactions on the expression of MOR.

2.12 Hypothesis and specific aims

The hypothesis for the dissertation is two-fold:

1 - IL-1β and morphine INCREASE/DECREASE the expression of MOR in SK-N-SH cells respectively.

The first hypothesis will be studied with the following specific aims:

a) Determine a time and dose-dependent effect of morphine and IL-1β treatment on MOR expression in neurons

b) Determine MOR and IL-1RI dependent mechanism

2 - Manipulation of signaling pathways will <u>alter</u> the effects of IL-1β and morphine on MOR expression in SK-N-SH cells.

The second hypothesis will be studied with the following specific aim:

a) Elucidate the role of signaling kinases and NF-κB on the expression of MOR by using signal inhibitors – SN50 (NF-κB), PD98059 (MEK1/2) and SB203580 (p38 MAPK).

CHAPTER III

RESEARCH DESIGN AND METHODS

3.1 SK-N-SH cell culture and materials

The human neuroblastoma SK-N-SH cells were obtained from ATCC (Cat # HTB-11). SK-N-SH cells were cultured in RPMI-1640 growth media (GM) (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin (Invitrogen). SK-N-SH cells were grown as a monolayer at 37°C in a 5% CO_2 atmosphere incubator in 100mm^2 round culture plates. The cells were seeded approximately 1x10^5 cells/mL as stock plates for 5-6 days, with growth media changed every two days. Following 5-6 days of growth, SK-N-SH cells were 70-80% confluent and were then passaged with 0.25% trypsin and seeded at 1x10^5 cells in 500 µL/well of RPMI-1640 growth media (GM) in 24-well culture plates (Corning). All experiments were conducted between passages 6 and 10.

3.2 Molecular cloning and bioinformatics of MOR in SK-N-SH cells

3.2.1 Isolation of RNA

Total RNA was isolated using the RNAqueous-4PCR column based kit (Ambion, Austin TX).When the SK-N-SH cells became 70-80% confluent, GM was removed and replaced with 1 mL guanidinium lysis solution and placed on ice for 2-3 minutes. Cells were then lysed vigorously by pipetting the cell lysate, disrupting the plasma membrane and

releasing their cytoplasmic and nuclear contents. The cell lysate was mixed gently with an equal volume 64% ethanol. 700 µL of the lysate/ethanol mixture was pippetted onto a silica-based filter mounted 1.5 ml RNase-free collection tube. The Silica-based filter selectively and quantitatively binds mRNA and larger ribosomal RNA (28s and 18s rRNA). The total volume of the lysate/ethanol mix was 2 mL, and the maximum volume of each silica-filter is 700 µL, the lysate/ethanol mix was added three times followed by a 30 second centrifuge of each lysate/ethanol mix at 12,000 xg on a table-top micro-centrifuge. After each centrifuge step, flow-through into the 1.5 mL tube was discarded and the filter cartridge was re-used for subsequent spins. The filter was then washed to remove residual DNA, protein and other contaminants; 700 µL of wash solution #1 was pippetted into the filter and centrifuged at 12,000 xg for 30 seconds. The flow-through was discarded and the same filter was washed with wash solution #2 (500 µL). This step was repeated again with wash solution #2 with the flow-through discarded after each wash step. The RNA was eluted in nuclease-free water containing trace amounts of ethylenediaminetetraacetic acid (EDTA) to chelate heavy metals.RNA was therefore eluted from the filter into a clean RNase-free 1.5 mL tube and strip-eluted using the provided elution buffer (EB). The EB was heated at 80°C before being used to elute the RNA. The RNA elution step was performed in two-steps (stripping), first, 60 µL of the heated EB was applied to the middle of the filter and centrifuged at 12,000 xg for 30 seconds, secondly 20 µL of the EB was pippetted into the center of the filter cartridge and centrifuged again. Total volume of elute containing total RNA was approximately 80 µL. 1 µg of total RNA was subjected to RNase-I treatment as instructed by the manufacturer (Ambion). Total RNA was aliquoted into 1.5 mL tubes and stored at -80°C. Total RNA concentration was determined using the Nanodrop-1000 spectrophotometer (Thermo Scientific) and A_{260}/A_{280} ratio readings were recorded as

described in the user manual. RNA integrity was determined by running 0.5 - 1 μg of total RNA on a 1.5% agarose gel; total RNA samples were subjected to 50°C temperature for 30 min mixed with a denaturing dye (Ambion) prior to running on 1.5% agarose gels. 18s and 28s rRNA bands were visual determinants of the isolated RNA quality and integrity.

3.2.2 cDNA synthesis

Approximately 1 μg of DNAase-I treated total RNA was used for cDNA synthesis using the QuantiTect reverse transcriptase cDNA synthesis kit (Qiagen, Valencia, CA). Another step to remove genomic DNA contamination from isolated RNA samples was carried out by adding 2μL of gDNA Wipeout Buffer 7x (Qiagen) to 1μg of RNA and heating the mix form 3 minutes at 42°C. A reverse transcription master mix was prepared on ice containing the template (RNA), Quantiscript reverse transcriptase (RT), which contain a 50 kDa RNase inhibitor protein, Quantiscript RT buffer, 5x, which contain dNTPs, RT primer mix, which is a blend of oligo-dT and random primers. cDNA was then synthesized in a thermal cycler (M.J.) using the following cycling parameters: 45 min at 42°C followed by 3 min at 95°C to inactivate the Quantiscript RT. All RT-reactions were stored at -20°C until RT-PCR was performed.

3.2.3 RT-PCR of SK-N-SH cDNA with opioid receptor family degenerate primers

PCR was set-up by combining 100 ng of cDNA, 100 pmol each of the Ra and Rb opioid receptor family degenerate primers (Li et al., 1996), was mixed with the Advantage 2 PCR Kit reagents to a final reaction volume of 50 μL (Clontech, Mountain View, CA), made up with PCR-grade ddH_2O. The reaction was run in a M.J. thermocycler programmed to run the following PCR steps: 94°C for 3 min; and 94°C for 1 min, 55°C for 1 min, 72°C for 40 sec, cycling for 30 cycles. 10 μL of the PCR product was run on 1.5% agarose gel containing 1 μg/mL Ethidium Bromide (EB) to visualize the162bp fragment using a UV gel box. The

162bp PCR product, as per the designed primers, spans a boundary between the 1st intracellular loop (IL1) and the 2nd (TM2) and 3rd transmembrane (TM3) regions. The 162bp PCR product reflected a highly conserved region of opioid receptors. The opioid family degenerate primer set were designed using human, rat and mouse cDNA cloned opioid receptor (MOR, DOR and KOR) coding sequences. The nucleotide sequences of the degenerate primers are as follows:

Ra: 5' – GAA(AG)AC(GATC)GC(GATC)AC(GATC)AA(TC)AT(TCA)TA - 3'

(Tm = 52°C)

Rb: 5' – GT(AG)AACAT(AG)TT(AG)TA(AG)TC – 3'

(Tm = 41°C)

The amplified PCR product was cloned into a pCR4-TOPO vector, and was submitted for BLASTn analysis against the NCBI non-redundant database and BLAST 2 sequence analysis. BLAST 2 sequence analysis tool produces the alignment of two given sequences using BLAST engine for local alignment (Tatusova and Madden, 1999).

3.2.4 Full-length cDNA PCR amplification of MOR in SK-N-SH cells

The following primers were designed using the Primer3 online tool to anneal across the 5' and 3' untranslated regions (UTRs) in order to PCR-amplify the entire coding region:

Forward: 5' - TCTTCAGCCATTGGTCTTCC - 3' (Tm = 72°C)

Reverse: 5' - GGAGCAGTTTCTGCTTCCAG - 3' (Tm = (72°C)

The PCR reaction was run in a M.J. thermocycler programmed to run the following PCR steps: 94°C for 60 sec; and 94°C for 30 sec, 68°C for 30 sec, 72°C for 90 sec, cycling for 35 cycles and final extension 72°C for 3 min. 10 µL of the PCR product was run on 1.5% agarose gel containing 1 µg/mL EB to visualize an approximate ~1.5 kb PCR product.

3.2.5 Cloning and sequencing of RT-PCR products

PCR products were cloned into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Standard reagents from the kit were combined with 4 μL of the PCR product and the ligation reaction was incubated for 15 min at room temperature. TOP10 (*E. Coli*) chemically competent cells (Invitrogen) were transformed with 2 μL of the ligation reaction and incubated on ice for 30 min. The competent cells were heat shocked at 42°C for 30 sec and incubated in 1 mL of S.O.C (0.5% Yeast extract, 2.0% tryptone, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM $MgSO_4$, 20mM glucose) medium for 1 hr at 37°C, placed horizontal in a shaking incubator set at 225 rpm. S.O.C is used as a cell growth medium to ensure maximum transformation efficiency. Transformed *E. coli* cells cultured in S.O.C medium were plated on Luria-Burtani (LB medium) agar plates by pipetting 75 μL and 100μL of now transformed and spread onto LB agar plates containing 100 μg/mL ampicillin and incubated overnight at 37°C. Approximately 16 hr later, individual colonies (minimum six) were picked and further cultured overnight in 2 mL of LB containing 100 μg/mL of ampicillin (per liter of LB: 10g tryptone, 5g yeast extract). The next morning, high-copy plasmid DNA was purified from the *E. coli* cells cultred in LB medium.The QIAprep Spin Miniprep Kit and the protocol was followed as outlined in the kit (Qiagen) to purify approximately 10μg of plasmid DNA. Following the isolation of plasmid DNA, 5 μL of each clone was digested with 0.5U of *EcoRI* (New England Biolabs) for 1 hr at 37°C in a water-bath. The digest(s) were run on 1.5% agarose gel to verify the product size. Clones containing the gene insert of the correct size were submitted to the OSU Molecular Biology CORE Facility in Stillwater, OK for verification of the DNA sequence of cloned MOR.

3.2.6 Bioinformatics of cloned SK-N-SH MOR seqeunce

3.2.6.1 Determination of MOR consensus sequence

The SeqMan II software (v. 5.03 DNASTAR, Madison, WI) was used to edit the MOR sequence and identify the MOR open reading frame. Full-length MOR sequences from three-independent PCR reactions were sequenced and aligned to compare the consensus sequence of MOR expressed in SK-N-SH cells.

3.2.6.2 BLASTn of cloned MOR sequence

Nucleotide and amino acid sequence homologous to human MOR were identified using (nucleotide) BLASTn program on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3 Drugs

The opioid agonist morphine sulphate (1-100 μM) (MS) and the opioid antagonist naltrexone (10-100 μM) were purchased from Sigma Aldrich, USA. Human recombinant Interleukin-1β (IL-1β; 10-100 ng/mL) was obtained from PeproTech, Rocky Hill, NJ and its receptor antagonist, (IL-1RA; 10-100 ng/mL) from R&D systems, Minneapolis, MN. Signaling protein kinase inhibitors - p38MAPK inhibitor, (SB203580; 1 μM) (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5(4-pyridyl) 1H-imidazole) (Sigma); MEK1/2 inhibitor, (PD98059; 50 μM) (2'-amino-3'-methoxyflavone) (Sigma); and NF-κB inhibitor, SN50; 10 and 50 μM) (Biomol).

3.4 Quantification of MOR gene expression using Quantitative real-time PCR (qRT-PCR)

3.4.1 RNA isolation

SK-N-SH cells were grown in 24-well cell culture plates seeded at $1x10^5$ cells/ml for 1-2 days. Total RNA was isolated using the RNAqueous-4PCR column based kit (Ambion, Austin TX). Following 70-80% confluence of the cells, GM was removed and replaced with 0.5 mL lysis buffer and placed on ice for 2-3 minutes. RNA was isolated as detailed in section 3.2.1.

3.4.2 cDNA Synthesis

Total RNA underwent reverse transcription (RT) using the QuantiTect RT kit, dedicated for synthesis of qRT-PCR quality cDNA (Qiagen) following the manufacture's protocol as detailed in section 3.2.2.

3.4.2.1 Reverse transcription (RT)-PCR: Semi-quantitative

PCR was carried out for MOR (800 and 106bp), IL-1RI (123bp) and GAPDH (163bp). The PCR cycling parameters were: 94°C for 30 sec; and 94°C for 30 sec, 62°C for 30 sec, 72°C for 90 sec, cycling for 35 cycles and final extension 72°C for 2 min. These PCR experiments were carried out to identify and confirm the expression of MOR, IL-1RI and GAPDH by SK-N-SH cells. The primer sets used for both RT-PCR (MOR and IL-1RI) and qRT-PCR (MOR and GAPDH) experiments are in Table 2.

3.4.3 Quantitative real-time PCR (qRT-PCR)

All qRT-PCR primer sets (Table 2) were designed to span an intron/exon boundary to prevent amplification of genomic DNA. The efficiency of each primer set (MOR and GAPDH) was checked using gradient RT-PCR, the most efficient primer melting (annealing) temperature (T_m) was determine by performing six-independent RT-PCR with six different

annealing temperatures (°C). The RT-PCR products were visualized on a 1.5% EtBr (10mg/mL) agarose gel, the PCR products that cooresponded to the length of the gene amplified, which was determined using a 1kB DNA ladder. If a clear single band, without any secondary PCR products, of the correct base-pair (bp) size was visualized, the annealing temperature (T_m) that produced this PCR product was chosen. In order to determine the qRT-PCR efficiency (rate at which a PCR amplicon is generated, i.e. if a PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay has 100% efficiency) for both MOR and GAPDH genes, a relative cDNA standard curve was carried out.

Table 2. Genes of interest, primers and their sequences used for RT-PCR and qRT-PCR.

Target	Primer Sequence (5'→ 3')	Amplicon Size	GenBank™ Accession
MOR	**Fwd:** (100 – 123) TCAGCCAGGACTGGTTTCTGTAAG **Rev:** (926-905) CAGTACCAGGTTGGATGAGAGA	**800**	**NM_000914**
MOR	**Fwd:** (590 - 609) CTTCAGCCATTGGTCTTCCT **Rev:** (689 - 668) CAGTACCAGGTTGGATGAGAGA	**106**	**NM_000914**
IL-1RI*	**Fwd:** (1542-1562) CTGGTCAGGGGACTTTACACA **Rev:** (1664-1643) GCTGGTGACAGTAACTGGTGTT	**123**	**NM_000877**
GAPDH^	**Fwd:** (222 - 243) CAACTACATGGTTTACATGTTC **Rev:** (402- 385) GCCAGTGGACTCCACGAC	**163**	**NM_002056.3**

***-IL-1RI** primer sequence obtained from:

http://pga.mgh.harvard.edu/primerbank/index.htmk: PrimerBank ID 4504659A3.

^ - GAPDH primer sequences obtained from Kraus et al., 2003. **MOR** primer sets were designed using Integrated DNA Technologies (IDT) PrimerQuest tool.

The relative cDNA standard curve was set-up using stock cDNA (standard) (1 μg) that was serial diluted into 1, 10, 100 and 1000 ng of final cDNA mass (mass represents the proportion of RT reaction and the mass units (ng) cancel-out in relative quantitation calculations). The standard curve was produced using the BioRad, *icyler* thermocycler. The slope of the standard curve was used to estimate qRT-PCR amplification efficiency, when the qRT-PCR standard curve reactions were completed, the qRT-PCR standard curve was graphically represented as a semi-log regression line plot of CT vs. log of input nucleic acid. A standard curve of -3.32 indicated a PCR with 100% efficiency. The qRT-PCR standard curve also allowed us to determine the concentration of cDNA template to use for each gene (MOR and GAPDH). Each qRT-PCR contained 10 ng/μL (50 ng total cDNA mass) of cDNA template in 15 μL total reaction volume which was run in duplicate in a 96 well q-RT-PCR-plate.

Each qRT-PCR contained 2X Premix *Ex Taq* SYBR Green I Master Mix (Takara), (which contained Eq Taq™ Hot Start DNA polymerase, buffer, dNTP mix, Mg2+ and SYBR green I), primer sets (25 nM each) and PCR-grade water. The SYBR green I fluorescent dye was used for real-time detection of double-stranded DNA. The qRT-PCR amplification cycle for MOR was: 95°C for 10 s followed by 40 cycles of 95°C, 64°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles. The qRT-PCR amplification cycle for GAPDH was: 95°C for 10 s followed by 30 cycles of 95°C for, 60°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles. The thresholds for both MOR (33.1 cycles) and GAPDH (26.5 cycles) remained constant between experiments. The GAPDH gene was chosen as our

endogenous reference gene and to normalize against the amplification of the MOR gene to calculate fold change.

Following qRT-PCR, quantification of MOR expression was made by setting the threshold cycle (C_T) in the geometric amplification phase of the plot. Relative quantification of MOR expression was calculated using the comparative C_T method as described by Livak et al., (2001). The amount of MOR (fold change), normalized to GAPDH (endogenous reference gene) and relative to the calibrator (untreated) was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula. The raw C_T output values from the calibrator (untreated) and treated groups were averaged and these averaged C_T values were used to calculate the ΔC_T values: ($MOR_{(target)} - GAPDH_{(reference)}$). Calculation of $\Delta\Delta C_T$ values were carried out using the mean ΔC_T values as an arbitrary constant to subtract from all other ΔC_T mean values. The $\Delta\Delta C_T$'s were calculated using the ΔC_T's values: ΔC_T treated - ΔC_T untreated. The formula, $2^{-\Delta\Delta CT}$, yields relative fold change MOR gene expression.

3.4.4 Statistical analysis of qRT-PCR data

The ΔC_T values from each experiment set (minimum, n=3) were used to analyze significant differences between un-stimulated and stimulated groups. The ΔC_T values were also used to perform statistical analysis to determine a concentration and/or time-dependent and treatment dependent effects with IL-1β and morphine. Using the Statistical Analysis Software (SAS; Package 9.1), ΔC_T results were tested using 2X2 factorial Analysis of Variance (2-way ANOVA) tables generated through PROC-GLM (General linear model) allow the utilization of a complete randomized design. And p-values were analyzed through least square means (LSMEANS) difference of ΔC_T values from each treatment group. This model was used to determine a significant difference between unstimulated - *vs.* stimulated-

dependent effects, and statistical differences determined at $p \leq 0.05$. Error bars show ± S.E.M were $n = \geq 3$.

3.4.5 Human MAPK qRT-PCR array

The human MAPK qRT-PCR gene array kit was used to elucidate the role of MAPK in the expression of MOR in response to morphine and IL-1β. A 96-well plate spotted with primers specific to 84 MAPK pathway genes was purchased from SABiosciences. In order to keep experimental conditions consistent, the protocol used to synthesized cDNA in section 3.4.3 was followed. 50 ng (mass) of cDNA was pippetted into each well spotted with one human gene-specific MAPK primer set. The qRT-PCR reaction master mix used was the same as that used in section 3.4.3.

The 96-well PCR-array plate is set-up with 84-human MAPK pathway focused genes (wells A1 - G12), 5 housekeeping genes (HKG) (wells H1 - H5), genomic DNA control gene (well H6), RT control genes (wells H7 - H9) and positive PCR control genes (wells H10 - H12) as illustrated in Fig. 10.

The qRT-PCR data was analyzed using the web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). Raw C_T values were calculated into fold-change using the comparative $\Delta\Delta C_T$ method and a scatter plot of the MAPK genes was generated. Other analysis options included multi-group plots and cluster-grams, howver, the PCR array analysis software required one control reaction and at least 3-independent treatment reactions in order to perform any statistical significance of thresholds, however only two-independent experiments and one-control experiment were performed and therefore no statistical analysis were conducted.

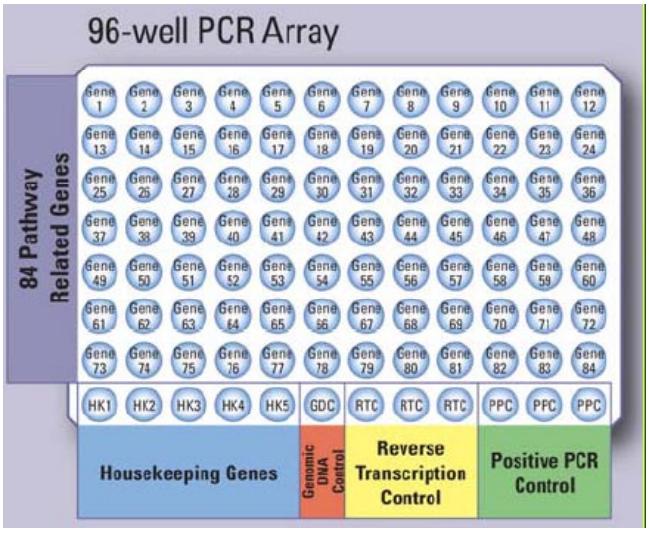

Fig. 10 A 96-well qRT-PCR-array plate layout of 84-genes of the human MAPK pathway. (www.SABioscience.com)

3.5 Cytotoxicity (MTT) assay

To assess cell viability, the MTT assay was performed (Carmichael et al., 1987). Cells were incubated for 1 hr in GM containing 0.55 mg/mL 3-[4,5-Dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide (MTT). Then cells were washed three times with 1x PBS, then dissolved using 1 mL DMSO and absorbance measured at 492 nm using a BIO-TEK HT spectrophotometer (OSU-CHS). MTT assays were conducted to determine whether the signaling inhibitors were cytotoxic to the SK-N-SH at the selected concentrations before being used to treat the SK-N-SH cells in the qRT-PCR experiments.

3.5.1 Statistical analysis

Data is presented as mean ± S.E.M of duplicate measures from 3-independent experiments. One-way Analysis of Variance (ANOVA) followed by Newman-Kuel's post-hoc multiple comparison tests was used to test for significance between treatment groups.

3.6 Immunocytochemistry (ICC)

3.6.1 Coverslip preparation

12 mm round glass coverslips (VWR) were coated with poly-L-lysine for 1 hr at room temperature. Coverslips were rinsed with sterile H_2O (3x-5 min each). Coverslips were dried and sterilized under UV light for at least 4 hr.

3.6.2 Immunocytochemistry (ICC)

ICC was used to detect basal levels and changes in MOR protein expression following morphine and IL-1β treatment. This fluorescence detection protocol was kindly provided by Dr. K.E. Miller, Oklahoma State University-Center for Health Sciences with help from Ernest Mathew Hoffman. SK-N-SH cells were seeded at $1x10^4$ cells/well on poly-L-lysine treated 12 mm glass coverslips placed in 24-well culture plates. Following 1-2 days of culture to obtain 40-60% confluent cells, cells were washed 3x with 1x PBS (phosphate buffered saline), fixed with 4% paraformaldehyde (PF), washed 2x with 1x PBS, blocked using blocking buffer made up of normal goat serum (NGS; 10%), normal horse serum (NHS; 10%), fetal bovine serum (FBS; 10%), bovine serum album (BSA; 2%), PVP (polyvinyl pyrolidone), phosphate buffered saline Tween-20 (PBS-T; 66%) for 1 hr. The primary antibody (Rabbit anti-Goat; Abcam) was used to detect the peptide sequence specific to the 2nd extracellular loop of MOR; (CTLTFSHPTWYWENLLK). Cells were blocked with blocking buffer over night at 4°C. Primary anti-body was diluted 1:50 in blocking buffer, and was applied to the cells overnight at 4°C while being gently rocked. The cells were then washed with 1x PBS and incubated with Alexa-Fluor 488-secondary antibody (Goat anti-rabbit) (10 μg/mL) (Invitrogen) made in 1x PBS for 1hr at room temperature. After washing with 1x PBS, cells were counter stained with Hoechst DNA stain (1 μg/mL) (Sigma) in 1x PBS for 5 min at room temperature (RT) in the dark. Coverslips were washed

using 1x PBS and mounted up-side down onto microscope slides using Prolong (Invitrogen) (adhesive/anti-bleaching agent). Microscope slides were allowed to cure overnight at RT in the dark at room before analysis.

3.6.3 Laser scanning confocal microscope

The SK-N-SH expression of MOR protein was detected using a Leica SP2, Laser scanning confocal microscope (Oklahoma State University, Microscopy Laboratory, Stillwater, OK). As a negative control, primary antibody was replaced with equal volume of 1x PBS. The aim of this study was not to quantify the expression of MOR protein, and therefore no confocal microscope settings were recorded. However, an effort was made to ensure consistency between samples from each set of analyzed experiments. A single user was used for each experiment. The images collected include confocal and epifluorescence images. Epifluorescence images were collected using a fluorescence microscope housed at the Animal Sciences Dept. OSU-Stillwater, OK.

3.7 SK-N-SH cell treatment schedule and methodology

Morphine: To evaluate the change in MOR expression in SK-N-SH cells, dose-response studies were conducted using the following final concentrations of morphine in SK-N-SH growth media (GM): 1, 10, and 100 μM. Exposure times were 0, 6, 12, 24 hr for each dose. A negative control, cells were treated with an equivalent volume of water in growth media (GM). After treatment, the cells were lysed in 500 μL of lysis/binding solution to start the total RNA isolation and cDNA synthesis (section 3.4.1) for each treatment group. For each time point, at least three independent experiments were completed. Cells were plated in duplicate wells for each concentration of morphine using a 24-well cell-culture plate.

IL-1β: In order to record IL-1β-induced change in MOR expression, experiments were set up similarly to that described for morphine treatment above. IL-1β was added to GM at: 1, 10 and 100 ng/mL and cells were exposed for: 0, 6, 12, 24hr (Kraus et al., 2006). The concentrations used for morphine and IL-1β were based on a survey of literature describing cytokine exposure on opioid receptor expression and results from preliminary data.

Naltrexone: In order to determine MOR-dependent regulation of MOR mRNA expression by morphine, naltrexone (MOR-antagonist) was used to antagonize morphine's effect on MOR expression. SK-N-SH cells were pre-treated (1 hr) with naltrexone (10 and 100 μM) prior to morphine (10 μM) treatment. 10 μM naltrexone treatments alone were also used. Following treatment for 6 and 24 hr, opioid exposure was stopped with the replacement of GM-containing opioids with 0.5 mL lysis buffer and cells were collected for RNA isolation.

IL-1RA – In order to assess IL-1RI-dependent regulation of MOR expression by IL-1β, the IL-1RI type specific antagonist, IL-1RA was used. SK-N-SH cells were pre-treated (1 hr) with IL-1RA (10 and 100 ng/mL) prior to IL-1β treatment (10 ng/mL). Cells were exposed to the treatments for 6 and 24 hr followed by RNA collection.

Morphine and IL-1β – To elucidate the interaction between morphine and IL-1β and their effects on MOR expression, the following two experiments were conducted: (1) – SK-N-SH cells were treated with morphine (10 μM) for 6 and 24 hr followed by IL-1β (10 ng/mL) treatement for another 6 and 24 hr; (2) – SK-N-SH cells were treated with morphine (10 μM) and IL-1β (10 ng/mL) concurrently for 6 and 24 hr.

Signaling Inhibitors:

The following signaling inhibitors were all checked for their cytotoxic effects on SK-N-SH cells at three different concentrations using the MTT assay before being used for qRT-PCR experiments to measure changes in MOR expression.

PD98059 – MEK1/2 inhibitor, was used to elucidate the role of MAPK family member, MEK1/2 on MOR expression. PD98059 has been shown to be a specific inhibitor of MEK1/2 (Alessi et al., 1995), and MEK1/2 has been implicated in having a key role in morphine tolerance and withdrawal in rats (Asensio et al., 2006). SK-N-SH cells were pre-treated with PD98059 (50 μM) followed by treatment with either vehicle control (water), IL-1β (10 ng/mL), morphine (10 μM) or IL-1β (10 ng/mL) + morphine (10 μM) concurrently for 6 hr.

SB203580 – p38 MAPK inhibitor, was used to elucidate the role of MAPK family member, p38 MAPK on MOR expression. SB203580 is a α- and β-p38 MAPK specific inhibitor (Alessi et al., 1995). SB203580 is a pyridinylimidazole compound that was originally prepared as an inflammatory cytokine synthesis inhibitor. Subsequently, the compound was found to be a selective inhibitor for p38 MAPK. SB203580 inhibits the catalytic activity of p38 MAPK by competitively binding in ATP pockets (Lee et al., 1999). SK-N-SH cells were pre-treated with SB203580 (1 μM) followed by treatment with either vehicle control (water), IL-1β (10 ng/mL), morphine (10 μM) or IL-1β (10 ng/mL) + morphine (10 μM) concurrently for 6 hr.

SN50 – NF-κB transcription factor inhibitor. SN50 peptide was used to elucidate the role of NF-κB in MOR expression. SN50 is a cell-permeable inhibitor peptide ($C_{129}H_{23}N_{36}O_{29}S$), which masks the nuclear localization sequence (NLS), preventing NF-κB-p50 from translocating into the cell nucleus. The N-terminal of the peptide confers cell permeability and remaining sequence inhibits NF-κB translocation (Boothby, 2001). SK-N-

SH cells were pre-treated with SN50 (10 and 50 μM) followed by treatment with either vehicle control (0.001% DMSO), IL-1β (10 ng/mL), morphine (10 μM) or IL-1β (10 ng/mL) + morphine (10 μM) concurrently for 6 hr.

CHAPTER IV

RESULTS

Part I:

Cloning and Expression of MOR in SK-N-SH cells

4.1. SK-N-SH cells express an opioid receptor type

In the first set of experiments, a 162bp fragment corresponding to the opioid receptor gene was amplified. The degenerate primers used were designed to span highly conserved regions of the opioid receptor family (Li et al., 1996). SK-N-SH cells are widely used as a neuronal model for the study of MOR expression and function (Agarwal and Glasel, 1993; Bare et al., 1994; Baumhaker et al., 1993; Bennett and Ratka, 2003; Breivogel et al., 1997; Cheng et al., 1997; Raut et al., 2006; Raut et al., 2007; Rubovitch et al., 2003; Sadee et al., 1988; Yin et al., 1997).

As shown in Fig. 11, RT-PCR has been used to successfully amplify opioid receptor fragments in SK-N-SH cells.

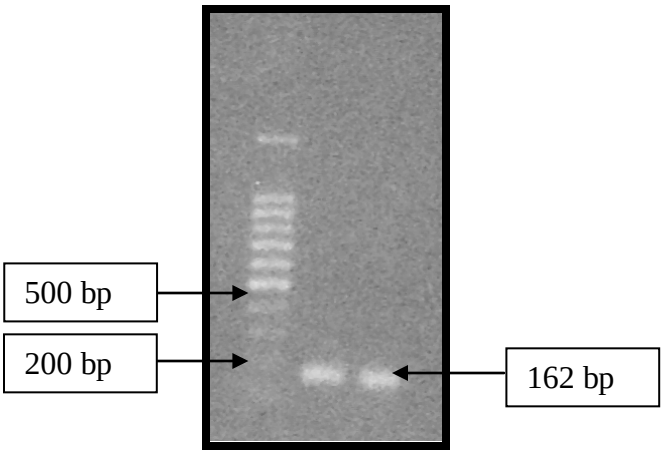

Fig. 11 Two-degenerate PCR products amplified from SK-N-SH cDNA. These PCR product represent a highly conserved opioid receptor region which were generated using a set of opioid receptor family degenerate primers. Products were run on a 1.5% agarose (1 μg/mL EtBr) gel. **Lane 1** – 1Kb DNA ladder (BioRad), **Lanes 2-3** – independent PCR products, each yielding an 162 bp DNA fragment.

Following PCR, the fragments were cloned into a pCR4-TOPO vector, and subsequent sequencing revealed a 162bp fragment as shown in Fig. 12. When submitted for BLASTn analysis against the NCBI non-redundant database, the cloned sequence had the highest identity (97%) to the human DOR type with an E value of 5e-72 (GenBank™ Accession: NM_000911.3) as shown in Fig. 12. This confirms the expression of an opioid receptor (OR) type by SK-N-SH cells (Yu et al., 1986).

5'/GTGAACATGTTGTAGTAGTCGATGGAGAGCACAGCCTTGCAGAGCAGCTC GCCGAAGGGCCACGTCTCCATCAGGTACTTGGCACTCTGGAAAGGCAGCGTG CTGGTGGCCAGCGCATCGGCTAAGGCCAGGTTGAAGATGTAGATATTAGTC GCCGTCTTA/3'

Fig. 12 162bp PCR amplified and cloned in pCR4-TOPO vector and sequenced.

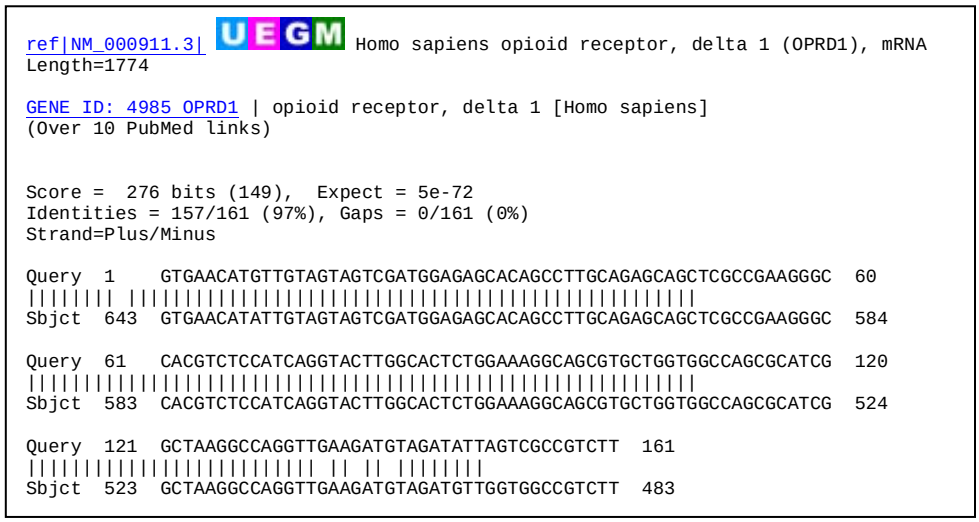

Fig. 13 BLASTn alignment of 162bp PCR product cloned into pCR4-TOPO vector and sequenced; matched human DOR type with an E-value of 5e-72.

However, because we are interested in the expression of MOR in SK-N-SH cells, the 162bp fragment shown in Fig. 13 was also subjected to BLAST 2 sequence analysis. This tool produces the alignment of two given sequences using BLAST engine for local alignment (Tatusova and Madden, 1999). BLAST 2 sequence analysis results provide an E-value (expected-value) of 7e-14 and a 75% identity to full-length MOR coding sequence (GenBank™ Accession: NM_000914.2) (alignment/data not shown). Therefore SK-N-SH cells also express an OR other than DOR, which confirms the expression of two OR types by SK-N-SH cells (Yu et al., 1986).

4.2 SK-N-SH cells express full-length MOR type

Following verification of OR expression, primers were designed and to PCR amplify full-length MOR type from SK-N-SH cDNA. Fig. 14 depicts the PCR amplification of a

>1.5 kb fragment representing the mRNA expression of full-length MOR by SK-N-SH as verified previously (Bare et al., 1994).

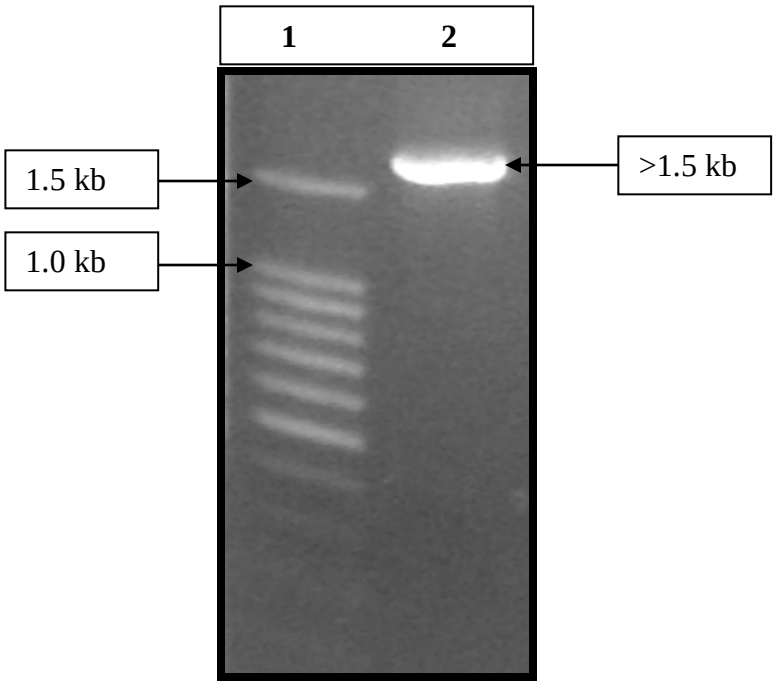

Fig. 14 PCR reaction of SK-N-SH cDNA used to amplify the full-length MOR expresseds. Primers designed annealed to 5' and 3' UTR-coding border, yielding a >1.5 kb product. Product was run on a 1.5% agarose gel. **Lane 1** – 1Kb DNA ladder (BioRad), **Lane 2** – PCR product yielding an >1.5 kb DNA fragment.

After cloning, *EcoRI* digest was performed to verify the size of cloned insert into pCR4-TOPO cloning vector. As shown in Fig. 15, 8 colonies were picked and cultured overnight. The plasmid was purified and digested, yielding two products. The more important of the two products was the ~1.4 kb MOR as this reflects the correct insert size excised from the plasmid vector. It was not only important to amplify MOR mRNA but to also verify the MOR sequence.

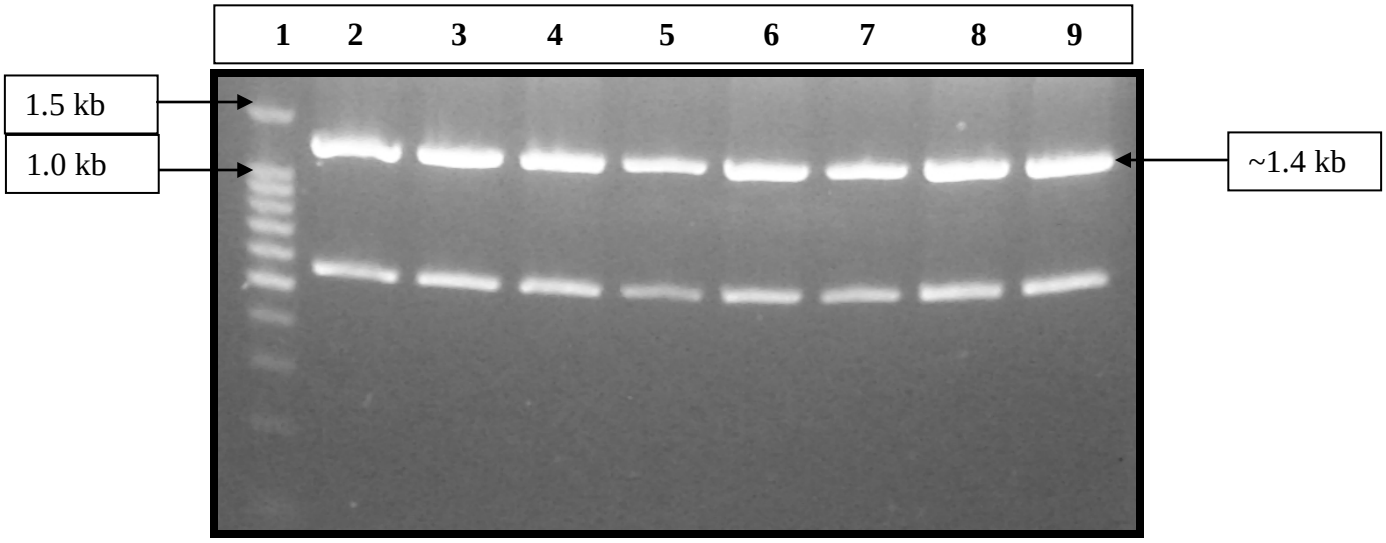

Fig. 15 PCR cloning of full-length MOR PCR product (Fig. 14) obtained from SK-N-SH cDNA. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit protocol described in the kit (Qiagen). Following isolation, 5μL of each clone was digested with 0.5U of *EcoRI* (New England Biolabs) for 1 hr at 37°C. The digests were run on 1.5% agarose gel to verify the ~1.4 kb MOR product. **Lane 1** – 1Kb DNA ladder (BioRad), **Lanes 2-8** – PCR cloned MOR fragment digested from plasmid DNA.

When submitted for BLASTn analysis against the NCBI non-redundant database, the cloned full-length MOR sequence had an E-value of 0.0 and a 100% identity for the human MOR type (GenBank™ Accession: NM_000914.2) as shown in Fig. 16. This confirmed the expression of the MOR type by SK-N-SH cells.

```
>  ref|NM_000914.2|  Homo sapiens opioid receptor, mu 1 (OPRM1), transcript variant
MOR-1, mRNA
Length=1891

 GENE ID: 4988 OPRM1 | opioid receptor, mu 1 [Homo sapiens]
(Over 100 PubMed links)


 Score = 2045 bits (1107),  Expect = 0.0
 Identities = 1107/1107 (100%), Gaps = 0/1107 (0%)
 Strand=Plus/Plus

Query  1     CCCCAGCACCCAGCCCCGGTTCCTGGGTCAACTTGTCCCACTTAGATGGCAACCTGTCCG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305   CCCCAGCACCCAGCCCCGGTTCCTGGGTCAACTTGTCCCACTTAGATGGCAACCTGTCCG  364

Query  61    ACCCATGCGGTCCGAACCGCACCGACCTGGGCGGGAGAGACAGCCTGTGCCCTCCGACCG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365   ACCCATGCGGTCCGAACCGCACCGACCTGGGCGGGAGAGACAGCCTGTGCCCTCCGACCG  424

Query  121   GCAGTCCCTCCATGATCACGGCCATCACGATCATGGCCCTCTACTCCATCGTGTGCGTGG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425   GCAGTCCCTCCATGATCACGGCCATCACGATCATGGCCCTCTACTCCATCGTGTGCGTGG  484

Query  181   TGGGGCTCTTCGGAAACTTCCTGGTCATGTATGTGATTGTCAGATACACCAAGATGAAGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485   TGGGGCTCTTCGGAAACTTCCTGGTCATGTATGTGATTGTCAGATACACCAAGATGAAGA  544

Query  241   CTGCCACCAACATCTACATTTTCAACCTTGCTCTGGCAGATGCCTTAGCCACCAGTACCC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545   CTGCCACCAACATCTACATTTTCAACCTTGCTCTGGCAGATGCCTTAGCCACCAGTACCC  604

Query  301   TGCCCTTCCAGAGTGTGAATTACCTAATGGGAACATGGCCATTTGGAACCATCCTTTGCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  605   TGCCCTTCCAGAGTGTGAATTACCTAATGGGAACATGGCCATTTGGAACCATCCTTTGCA  664

Query  361   AGATAGTGATCTCCATAGATTACTATAACATGTTCACCAGCATATTCACCCTCTGCACCA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  665   AGATAGTGATCTCCATAGATTACTATAACATGTTCACCAGCATATTCACCCTCTGCACCA  724

Query  421   TGAGTGTTGATCGATACATTGCAGTCTGCCACCCTGTCAAGGCCTTAGATTTCCGTACTC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  725   TGAGTGTTGATCGATACATTGCAGTCTGCCACCCTGTCAAGGCCTTAGATTTCCGTACTC  784

Query  481   CCCGAAATGCCAAAATTATCAATGTCTGCAACTGGATCCTCTCTTCAGCCATTGGTCTTC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  785   CCCGAAATGCCAAAATTATCAATGTCTGCAACTGGATCCTCTCTTCAGCCATTGGTCTTC  844

Query  541   CTGTAATGTTCATGGCTACAACAAAATACAGGCAAGGTTCCATAGATTGTACACTAACAT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  845   CTGTAATGTTCATGGCTACAACAAAATACAGGCAAGGTTCCATAGATTGTACACTAACAT  904

Query  601   TCTCTCATCCAACCTGGTACTGGGAAAACCTGCTGAAGATCTGTGTTTTCATCTTCGCCT  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  905   TCTCTCATCCAACCTGGTACTGGGAAAACCTGCTGAAGATCTGTGTTTTCATCTTCGCCT  964

Query  661   TCATTATGCCAGTGCTCATCATTACCGTGTGCTATGGACTGATGATCTTGCGCCTCAAGA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  965   TCATTATGCCAGTGCTCATCATTACCGTGTGCTATGGACTGATGATCTTGCGCCTCAAGA  1024

Query  721   GTGTCCGCATGCTCTCTGGCTCCAAAGAAAAGGACAGGAATCTTCGAAGGATCACCAGGA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1025  GTGTCCGCATGCTCTCTGGCTCCAAAGAAAAGGACAGGAATCTTCGAAGGATCACCAGGA  1084

Query  781   TGGTGCTGGTGGTGGTGGCTGTGTTCATCGTCTGCTGGACTCCCATTCACATTTACGTCA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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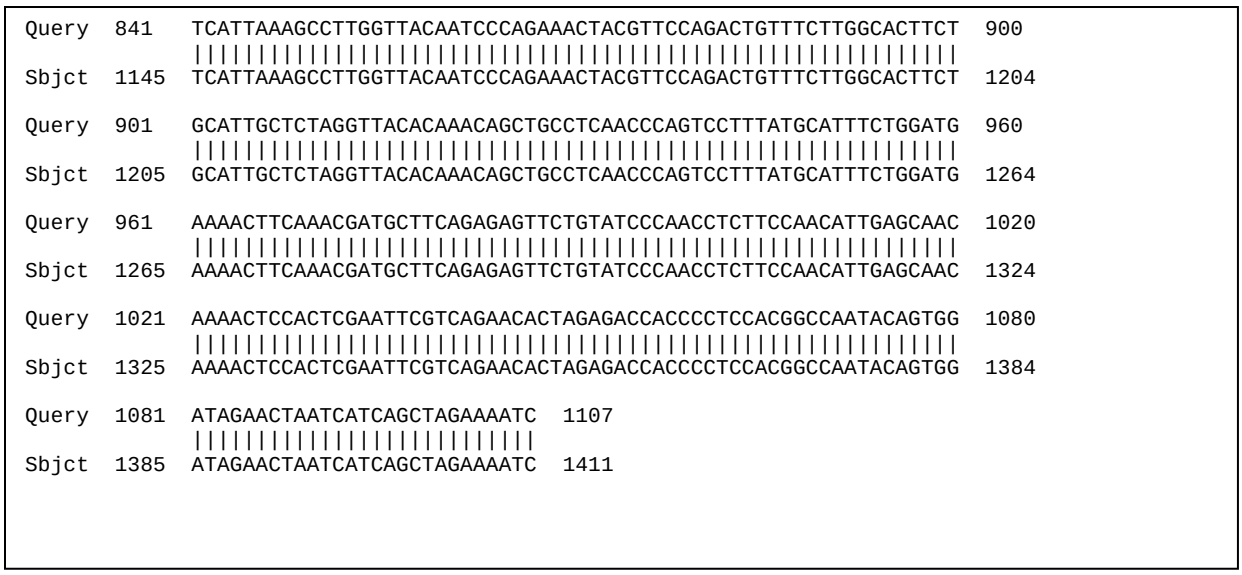


Fig. 16 BLASTn alignment of sequenced full-length MOR in SK-N-SH cells. Homo sapiens opioid receptor, mu 1 (OPRM1) had an E-value of 0.0 and 100% nucleotide identity to MOR expressed in SK-N-SH cells.

4.3 SK-N-SH cells express MOR, IL-1RI and GAPDH

In preparation for real-time qRT-PCR of MOR in SK-N-SH, optimum primer sets were designed to verify the expression of MOR, the IL-1β receptor, IL-1RI and the house-keeping gene, GAPDH. (See Table 2, Page 58).

As shown in Fig. 17, SK-N-SH cells express MOR (800 and 106 bp), IL-1RI (123 bp) and GAPDH (163 bp) genes. The RT-PCR cycling parameters included: 94°C for 120 s, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, 35 cycles followed by a final extension of 72°C for 120 s. Expression of these genes verified the suitability of the *in vitro* SK-N-SH cell line for qRT-PCR studies following treatment with IL-1β and morphine.

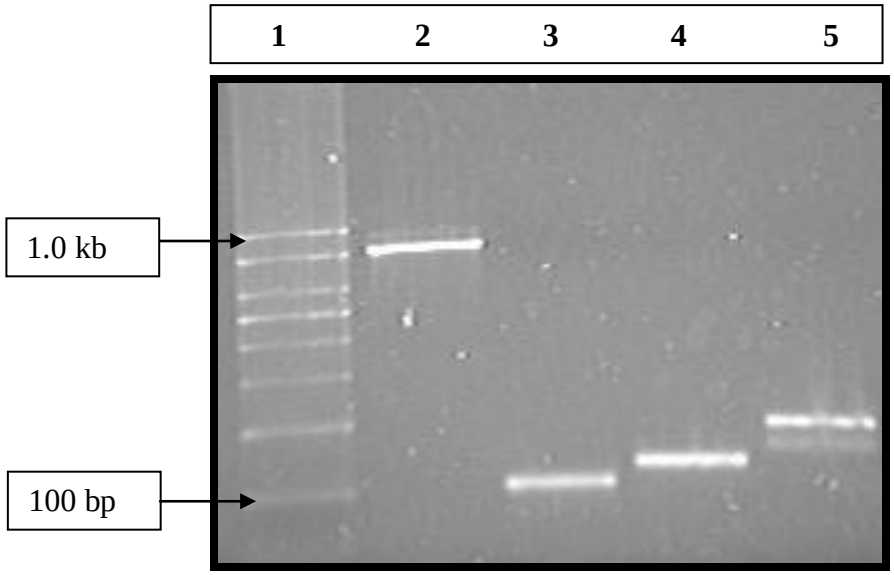


Fig. 17 PCR reaction products of SK-N-SH cDNA used to amplify MOR, IL-1RI and GAPDH expressed by SK-N-SH cells. Product were run on a 1.5% agarose gel**. Lane 1** – 1Kb DNA ladder, **Lane 2** – MOR (800 bp), **Lane 3** – MOR (106 bp), **Lane 4** – IL-1RI (123 bp) and **Lane 5** – GAPDH (163 bp).

4.4 Optimization of qRT-PCR for genes MOR and GAPDH

Once the expression of MOR and GAPDH was confirmed using RT-PCR (Fig. 17), to determine the optimal annealing temperature (T_m) for both genes, gradient PCR was performed. For the MOR gene, six T_m's, each 2°C apart were chosen based on the original T_m suggested by the primer synthesis company (IDT). The T_m's studies were 56°C, 58°C, 60°C, 62°C, 64°C and 66°C. The RT-PCR cycling parameters used were the same as those used in section 4.2, with T_m's adjusted accordingly. Given that the T_m of 64°C produced the most robust MOR product, this T_m remained constant in all experiments (Fig. 18).

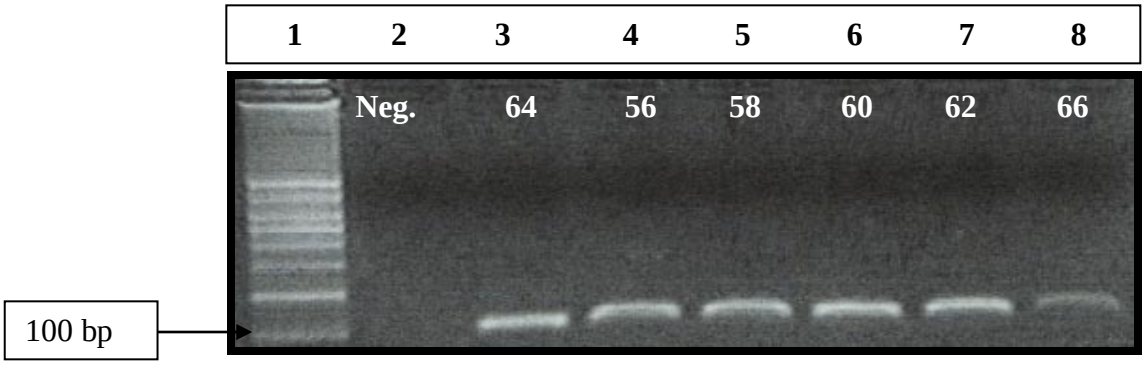


Fig. 18 Gradient PCR reaction of SK-N-SH cDNA used to amplify MOR (106 bp). Products were run on a 1.5% agarose gel. **Lane 1** – 1Kb DNA ladder**, Lanes 2** – Negative (NTC: non-template control), **Lanes 3 - 8**: respective annealing tempertures: 64, 56, 58, 60, 62 and 66 °C.

In order to determine the optimum concentration of cDNA template to use for qRT-PCR amplification of MOR, a relative standard curve was performed. Stock cDNA (~1000 ng/µL) was serial diluted in RNase-free water to provide: 1000, 100, 10 and 1 ng/15 µL (reaction volume) of cDNA. The qRT-PCR parameters did not differ from those already stated in section 3.5.5. Briefly: 95°C for 10 s followed by 40 cycles of 95°C for, 64°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles.

The results from the log amplification (Fig. 19) and standard curve (Fig. 20) showed that the optimal cDNA template amount to use for efficient MOR expression was between 10 and 100 ng. The standard curve (dynamic range) (Fig. 20) was used to accurately provide a range of cDNA amounts that would allow us to chose a cDNA amount (50 ng) that would be between a lower (10 ng) and a higher (100 ng) amount of cDNA. A final cDNA amount for MOR gene qRT-PCR was chosen as 50 ng. Therefore, stock cDNA was diluted into

10ng/µL using RNase-free water and 5µL of this diluted cDNA was used in a 15 µL final volume qRT-PCR mix.

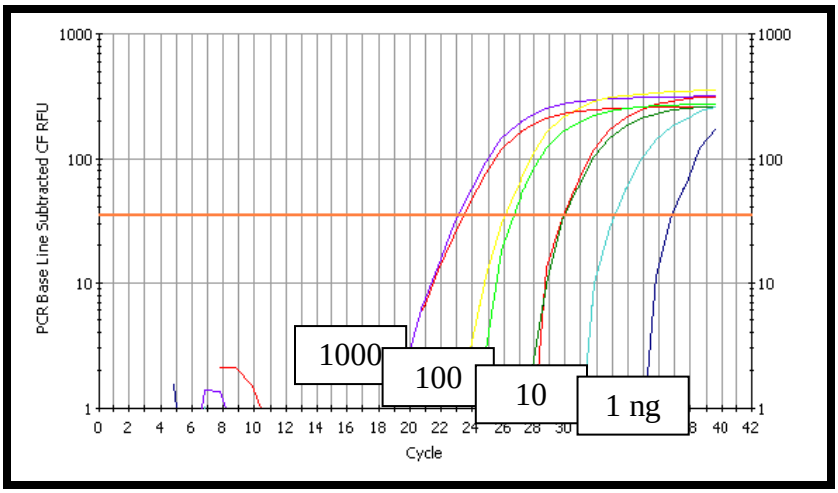


Fig. 19 MOR qRT-PCR amplification plot illustrates the amplification of MOR with four different amounts of cDNA template: 1000, 100, 10 and 1 ng. Horizontal bar – Cycle threshold (C_T) parameter set by thermocycler.

In addition to the amplification plot, the standard curve study for MOR generated a semi-log regression line plot of CT values *vs.* log of output nucleic acid (amplification). Within this plot, PCR efficiency was determined by the slope. If the slope = -3.32, then the qRT-PCR reaction was 100% efficient. The slope for the MOR gene was -3.333, reflecting 99.6% qRT-PCR efficiency (Fig. 20).

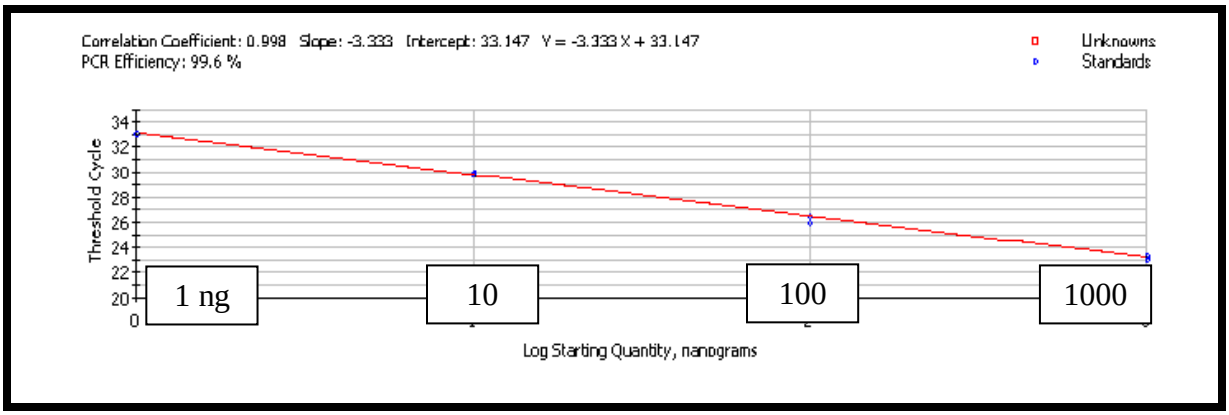


Fig. 20 MOR qRT-PCR standard curve representing 99.6% PCR efficiency.

The optimal annealing temperature (T_m) for the GAPDH was determined similarly as described for MOR. For the GAPDH gene, six T_m's, each 2°C apart were chosen based on the original T_m suggested by the primer synthesis company (Invitrogen). The T_m's chosen were 56°C, 58°C, 60°C, 62°C, 64°C and 66°C. The RT-PCR cycling parameter used was the same as those used in section 4.2 with T_m's adjusted accordingly. From the PCR gel depicted in Fig. 21, an annealing temperature of 60°C was chosen for the qRT-PCR of GAPDH and this remained constant in all experiments.

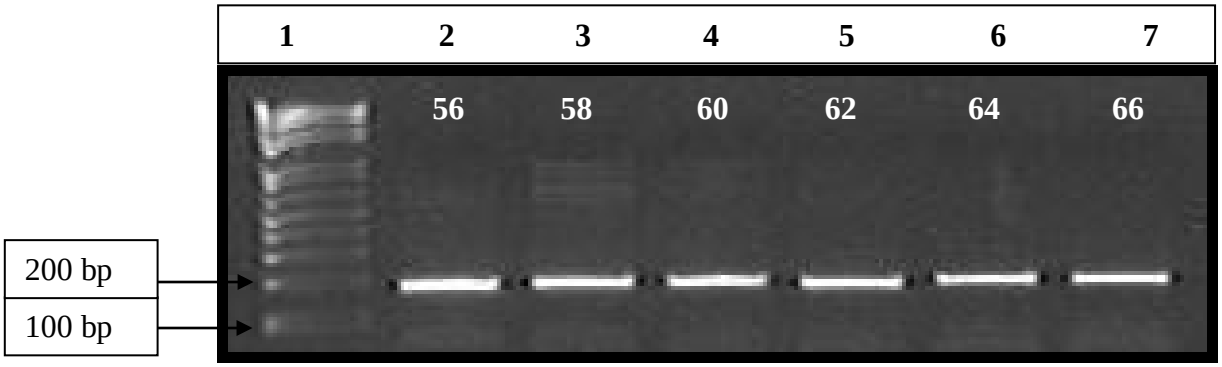


Fig. 21 Gradient PCR reaction of SK-N-SH cDNA used to amplify GAPDH (163 bp). Products were run on a 1.5% agarose gel. **Lane 1** – 1Kb DNA ladder, **Lanes 2 - 7**: respective annealing temperatures: 56, 58**,** 60, 62, 64 and 66 °C.

In order to determine the optimum concentration of cDNA template to use for qRT-PCR amplification of GAPDH, a relative standard curve was performed. Stock cDNA (~1000 ng/µL) was serial diluted in RNase-free water into the following amounts of cDNA: 1000, 100, 10 and 1 ng. The qRT-PCR parameters did not differ from those already stated in section 3.5.5. Briefly: 95°C for 10 s followed by 30 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 20 s. This was followed by 94°C for 60 s, 55°C for 60 s and for the melt curve analysis 55°C for 30 s for 80 cycles.

The results from both the log amplification plot (Fig. 22) and standard curve (Fig. 23) showed that the optimal cDNA amount to use for GAPDH expression was between 10 and 100 ng of cDNA. The concentration of cDNA template used for the GAPDH gene was 10 ng/µL.

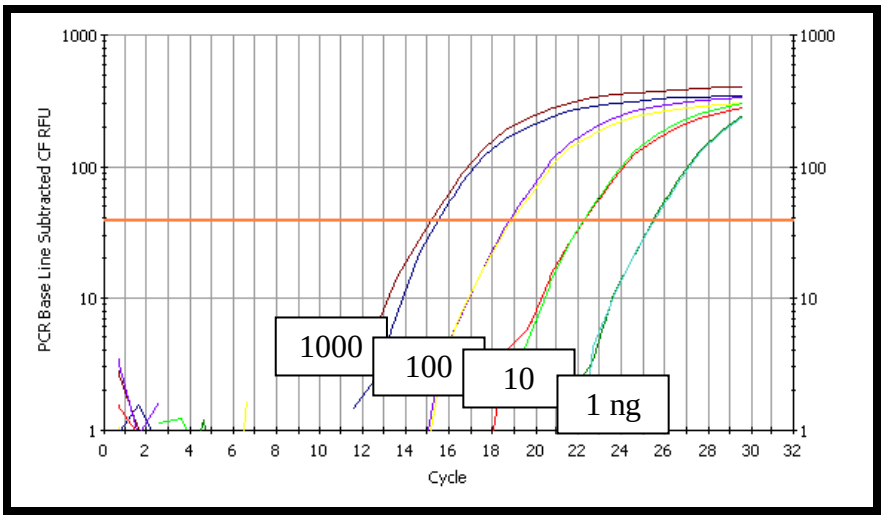


Fig. 22 GAPDH qRT-PCR amplification plot illustrates the amplification of GAPDH with four different amounts of cDNA template: 1000, 100, 10 and 1 ng. Horizontal bar – Cycle threshold (C_T) parameter set by thermocycler.

In addition to the amplification plot, the standard curve study for GAPDH generated a semi-log regression line plot of C_T values *vs.* log of output nucleic acid (amplification). Within this plot, PCR efficiency is determined by the slope. The slope for GAPDH was -3.396, reflecting 97% qRT-PCR efficiency (Fig. 23).

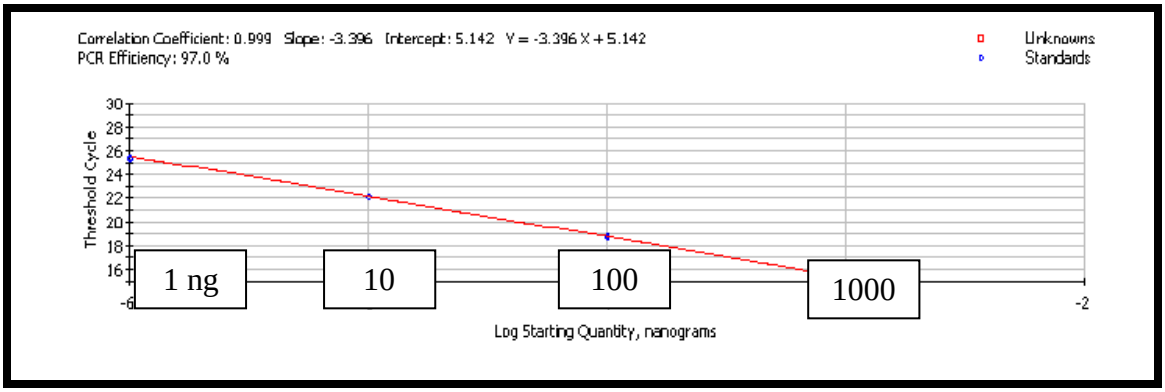


Fig. 23 GAPDH qRT-PCR standard curve: representing 97% PCR efficiency

4.5 Effect of cell-passage number on the expression of MOR in SK-N-SH cells

During the preliminary studies, it was determined that the expression of MOR by SK-N-SH cells varied considerably. Therefore a set of experiments were conducted to measure basal transcript mRNA levels of MOR between passages 4 and 13. A measure of MOR expression was taken from each passage independently (n=2 for each passage). As shown in Fig. 24, the expression of MOR changed between passages 4 and 13. The optimum basal expression of MOR in SK-N-SH was between passages 6 and 10. Therefore all qRT-PCR and ICC experiments were conducted between passages 6 and 10 to keep the measure of expression of MOR mRNA and protein consistent among all studies.

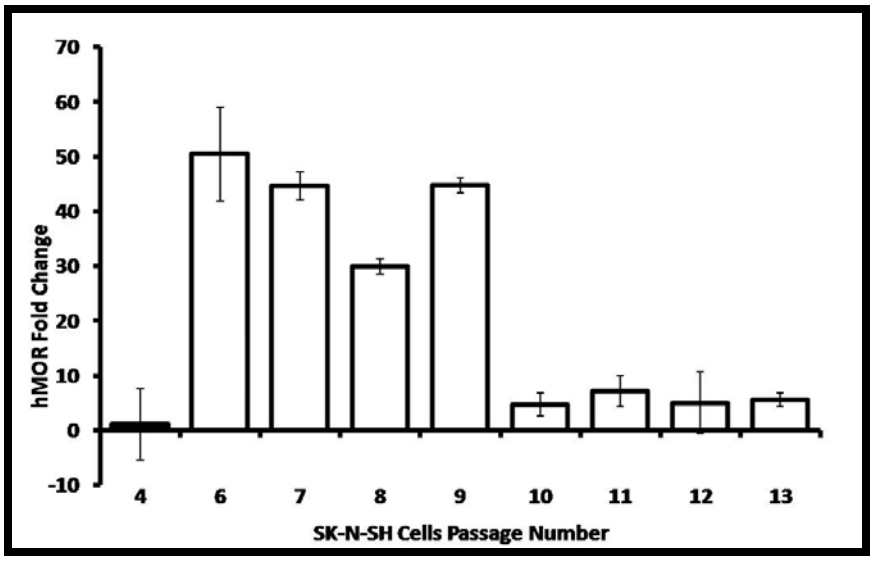


Fig. 24 Effect of SK-N-SH cell passage on MOR mRNA expression. SK-N-SH cells were seeded at $2.0x10^6$ cells/well in a 24-well plate and cultured until 70-80% confluent. The expression of MOR was normalized against passage 4(calibrator). Data represents duplicate measure from triplicate wells obtained from two independent experiments for each passage. Data shown are mean ±S.E.M of ΔC_T values.

Part II:

Effect of morphine and IL-1β on MOR expression in SK-N-SH cells

4.6 Morphine-induced down-regulation of MOR expression

SK-N-SH cells were treated with three different concentrations of morphine for 6, 12 and 24 hr. As shown in Fig. 25, morphine time dependently down-regulated the expression of MOR as measured using qRT-PCR. Significant differences when compared to unstimulated (US) showed that morphine down-regulated the expression of MOR when treated with 10 (-1.06 fold) and 100μM (-1.79 fold) for 12 hr ($p<0.05$). Similarly, MOR expression was down-regulated when SK-N-SH cells were treated with 1 (-3.08 fold), 10 (-2.19 fold) and 100μM (-1.52 fold) for 24 hr ($p<0.05$).

Immunocytochemistry (ICC) experiments using confocal microscopy were used to visualize change in MOR protein expression. The down-regulation of MOR mRNA in response to 24 hr of 1, 10 and 100μM morphine exposure was also visualized for the expression of MOR protein (Fig. 26). MOR protein was down-regulated in response to morphine treatment as visualized following 24 hr treatment. In contrast, MOR mRNA levels were significantly down-regulated at 12 and 24 hr exposure times in response to all three concentrations of morphine.

Fig. 25 Morphine treatment down-regulated MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) morphine sulphate (MS) (1, 10 and 100μM) for 6, 12 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three-five independent experiments. Statistical analysis was carried out using a Two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS means). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a Two-way ANOVA. # $p \leq 0.05$ vs. US.

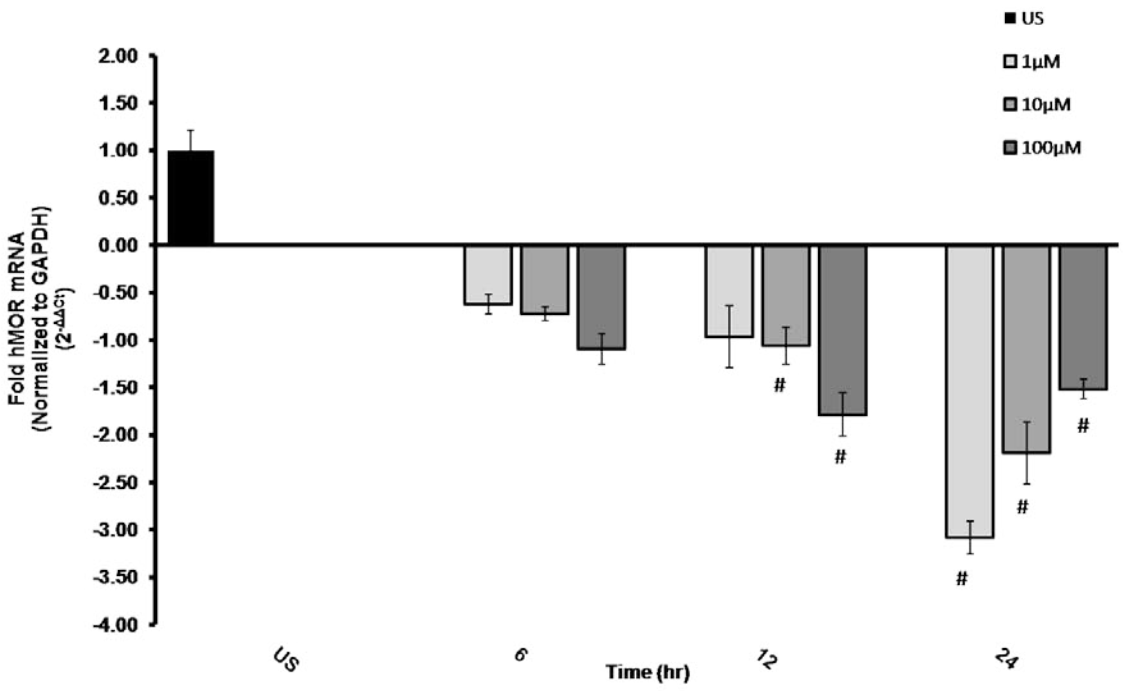
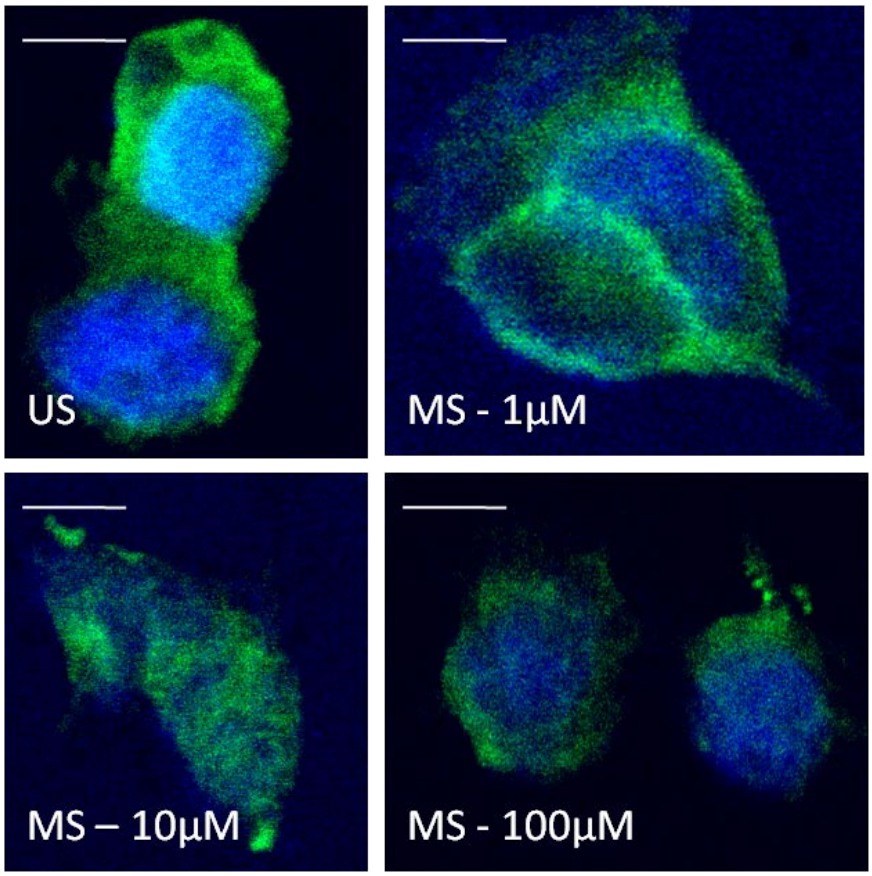

US
1μM
10μM
100μM
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
-3.50
-4.00
Fold hMOR mRNA
(Normallized to GAPDH)
(2-ΔΔCt)
#
US
6
12
24
Time (hr)

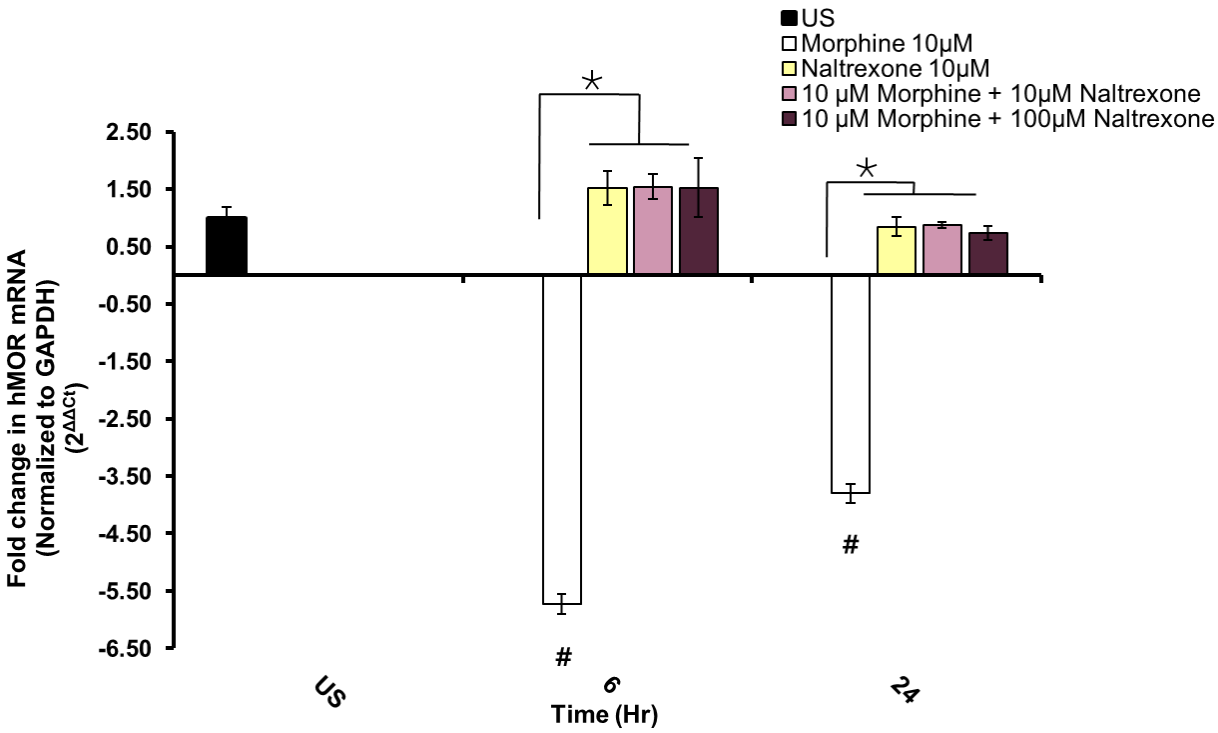
Fig. 26 Morphine treatment decreased the expression of MOR protein expression in SK-N-SH cells. SK-N-SH cells were cultured on sterile glass coverslips in a 24-well culture plate at 37°C for 2 days in GM and cultured with or without (un-stimulated (US)) morphine sulphate (MS) (1, 10 and 10μM) for 24 hr. The cells were washed with 1x PBS, fixed with 4% paraformaldehyde, blocked with blocking buffer for 1 hr before being treated with MOR-primary antibody (Rabbit anti-Goat) (1:50) (Abcam) made in blocking buffer overnight at 4°C. Cell were washed with 1x PBS and incubated with Alexa-Fluor 488-secondary antibody (Goat anti-rabbit) (Invitrogen) (10 μg/mL) made in 1x PBS for 1hr at room temperature. After washing with 1x PBS, cells were counter stained with Hoechst (DNA stain) (1 μg/mL) (Sigma) in 1x PBS for 5 min. at room temperature. Coverslips were washed using 1x PBS and mounted up-side down onto microscope slides using Prolong (Invitrogen). Images analyzed using confocal microscopy. 50μm


US
MS - 1µM
MS – 10µM
MS - 100µM

4.7 Morphine down-regulates MOR in MOR-dependent manner

To identify the role of MOR in morphine-induced down-regulation of MOR, the MOR-specific antagonist, naltrexone was used. Naltrexone treatment alone had no significant effect on the expression of MOR. Morphine treatment for 6 and 24 hr down-regulated (-5.73 and -3.8 fold respectively) the expression of MOR mRNA. Co-exposure of SK-N-SH cells with naltrexone (10 and 100μM) and morphine (10μM) resulted in less down-regulation of MOR mRNA expression. Co-exposure (morphine and naltrexone) for 6 and 24 hr resulted in a 1.54 and 1.53 and 0.87 and 0.74 fold change in MOR expression respectively (Fig. 27), this was a significantly different ($p \leq 0.05$) from the effect measured on MOR expression following the 6 and 24 hr morphine exposure. Morphine-induced MOR down-regulation was reduced by naltrexone; therefore these results indicate that morphine's effect on MOR expression is MOR-dependent.

Fig. 27 Naltrexone co-treatment blocked morphine-induced down-regulation MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated (US)) morphine (10μM), naltrexone (10μM), morphine (10μM) + naltrexone (10μM) and morphine (10μM) + naltrexone (100μM) for 6 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a Two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a Two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. morphine alone.


US
Morphine 10μM
Naltrexone 10μM
10 μM Morphine + 10μM Naltrexone
10 μM Morphine + 100μM Naltrexone
Fold change in hMOR mRNA
(Normalized to GAPDH)
(2^ΔΔCt)
2.50
1.50
0.50
-0.50
-1.50
-2.50
-3.50
-4.50
-5.50
-6.50
*
*
#
#
US
6
24
Time (Hr)

4.8 IL-1β-induced up-regulation of MOR expression

As illustrated in Fig. 28, IL-1β significantly up-regulated MOR mRNA expression in a concentration-dependent manner when compared to un-stimulated. IL-1β (10 and 100 ng/mL) up-regulated MOR mRNA expression following 6 (4.35 and 5.4 fold), 12 (3.84 and 4.10 fold) and 24 hr (2.45 and 2.56 fold) exposure. IL-1β (10 and 100 ng/mL) exposure for 6 hr resulted in the most significant up-regulation of MOR expression, suggesting a time-dependent mechanism for IL-1β's effect on MOR mRNA expression.

ICC experiments indicated that MOR protein expression was also up-regulated by IL-1β treatment. As shown in Fig. 29, IL-1β (10 ng/mL) up-regulated MOR protein expression following 6 hr exposure. However, IL-1β (10 ng/mL) exposure for 12 hr did not up-regulate MOR protein expression, indicating a time-dependent effect on MOR protein expression, because the largest change in MOR protein expression visualized was at only 6 hr.

Fig. 28 IL-1β treatment up-regulated MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated (US)) human recombinant IL-1β cytokine (IL-1β) (10 and 100ng/mL) SK-N-SH for 0.15, 6, 12 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three-four independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US.

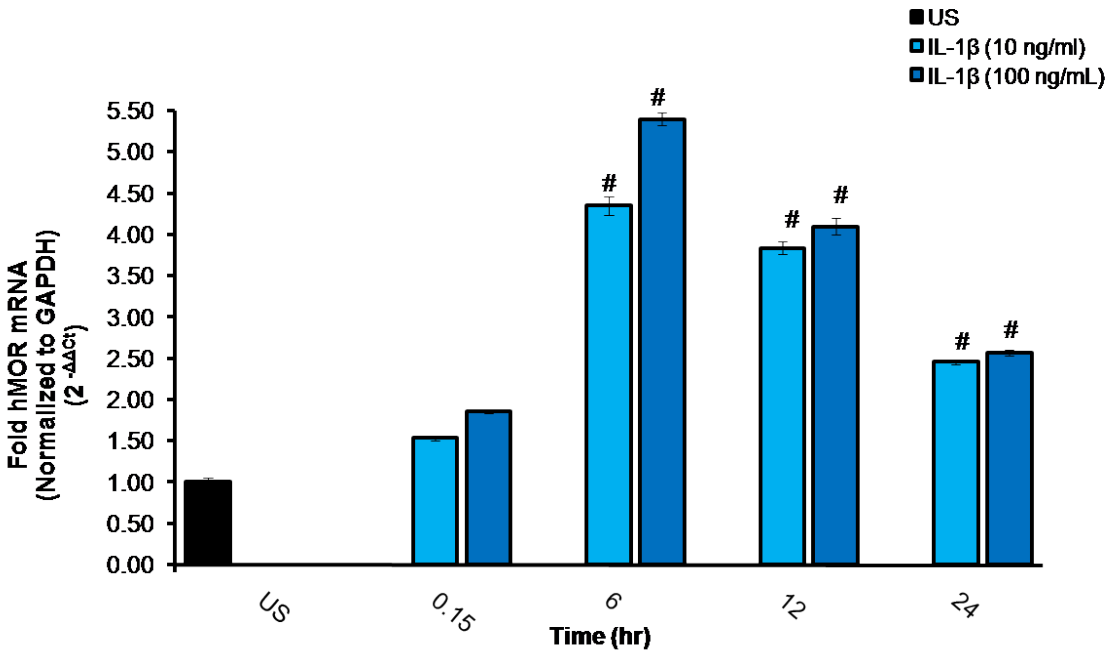
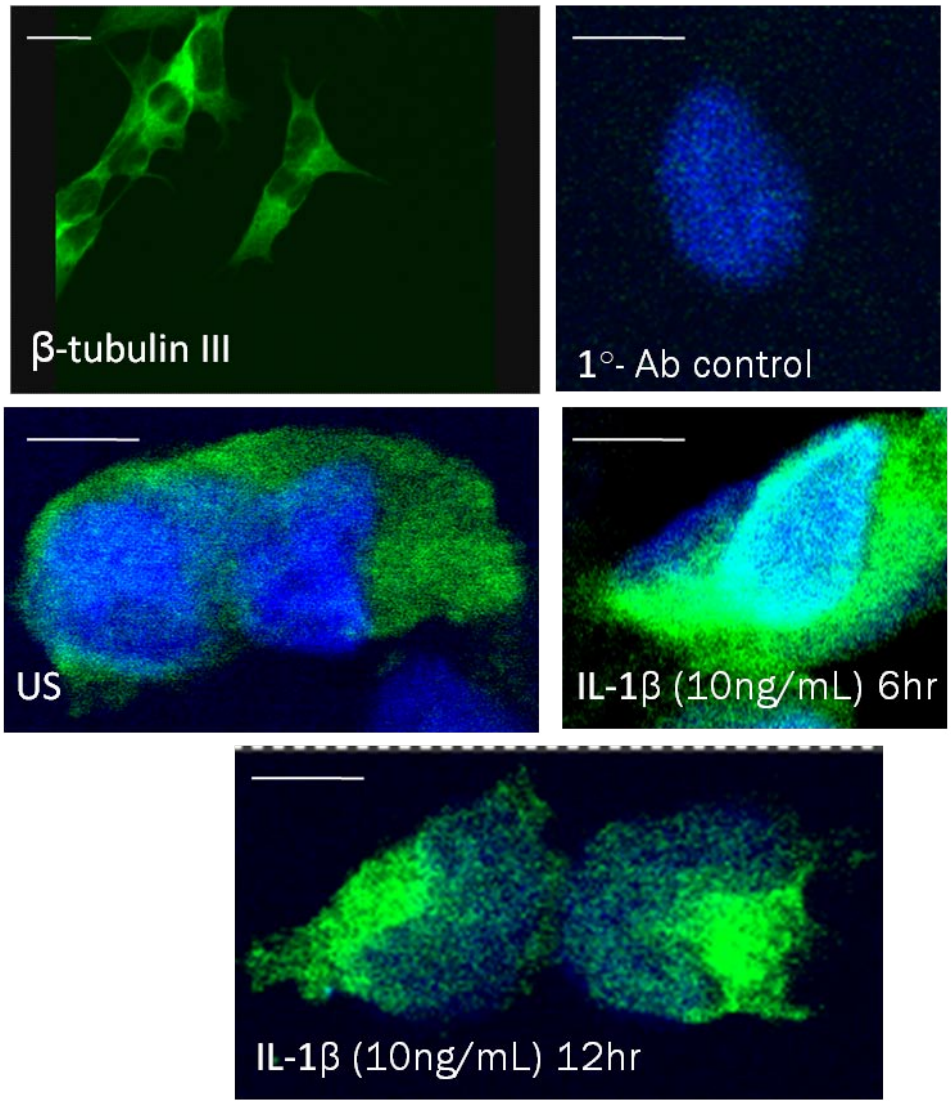

US
IL-1β (10 ng/ml)
IL-1β (100 ng/mL)
Fold hMOR mRNA
(Normalized to GAPDH)
(2 -ΔΔCt)
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
#
US
0.15
6
12
24
Time (hr)

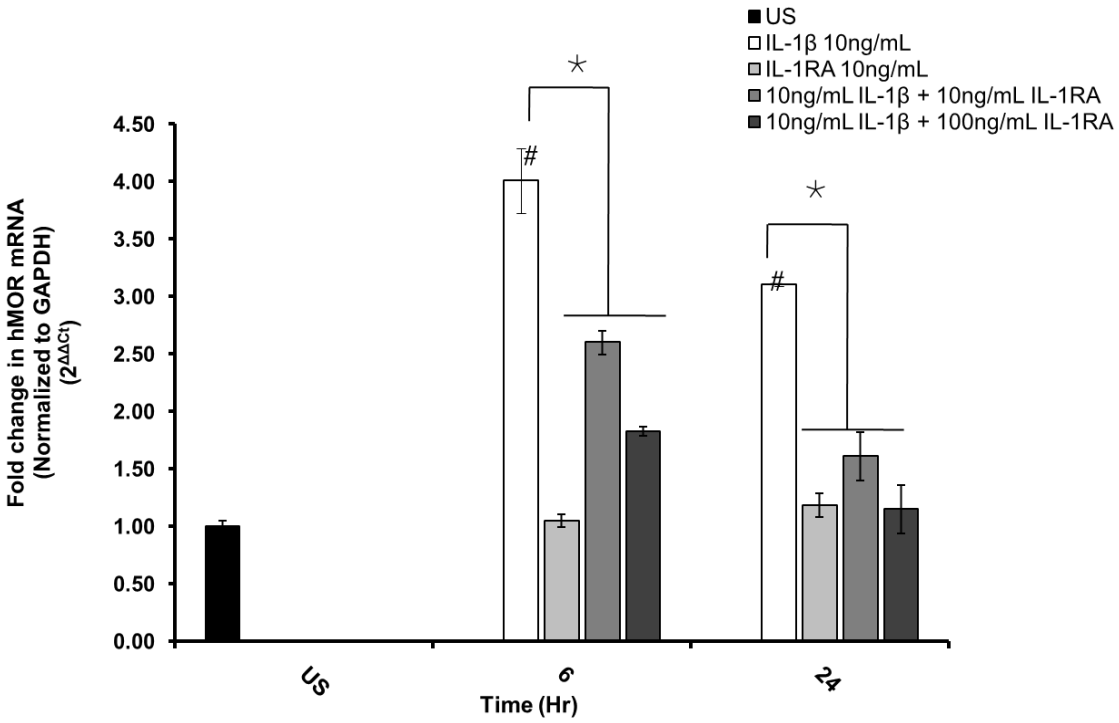
Fig. 29 IL-1β treatment up-regulated MOR protein expression in SK-N-SH cells. SK-N-SH cells were cultured on sterile glass coverslips in a 24-well plate at 37°C for two days in GM and cultured with or without (un-stimulated (US)) IL-1β (10 ng/mL) for 6 and 12 hr. The cells were washed with 1x PBS, fixed with 4% paraformaldehyde, blocked with blocking buffer for 1 hr before being treated with 1x PBS (1° Ab. control), Anti-MOR RabMAb. (1:50; Abcam), anti-β-tubulin III RabMAb. (1:100; Epitomics) made in blocking buffer overnight at 4°C. Cell were washed with 1x PBS and incubated with Alexa-Fluor 488-secondary antibody (Goat anti-rabbit; Invitrogen) (10 μg/mL) made in 1x PBS for 1hr at room temperature. After washing with 1x PBS, cells were counter stained with Hoechst (DNA stain) (1 μg/mL) (Sigma) in 1x PBS for 5 min. at room temperature. Coverslips were washed using 1x PBS and mounted up-side down onto microscope slides using Prolong (Invitrogen). Images analyzed using confocal microscopy. 18μm —— 50μm ——


β-tubulin III
1°- Ab control
US
IL-1β (10ng/mL) 6hr
IL-1β (10ng/mL) 12hr

4.9 IL-1RI-dependent up-regulation of MOR expression by IL-1β

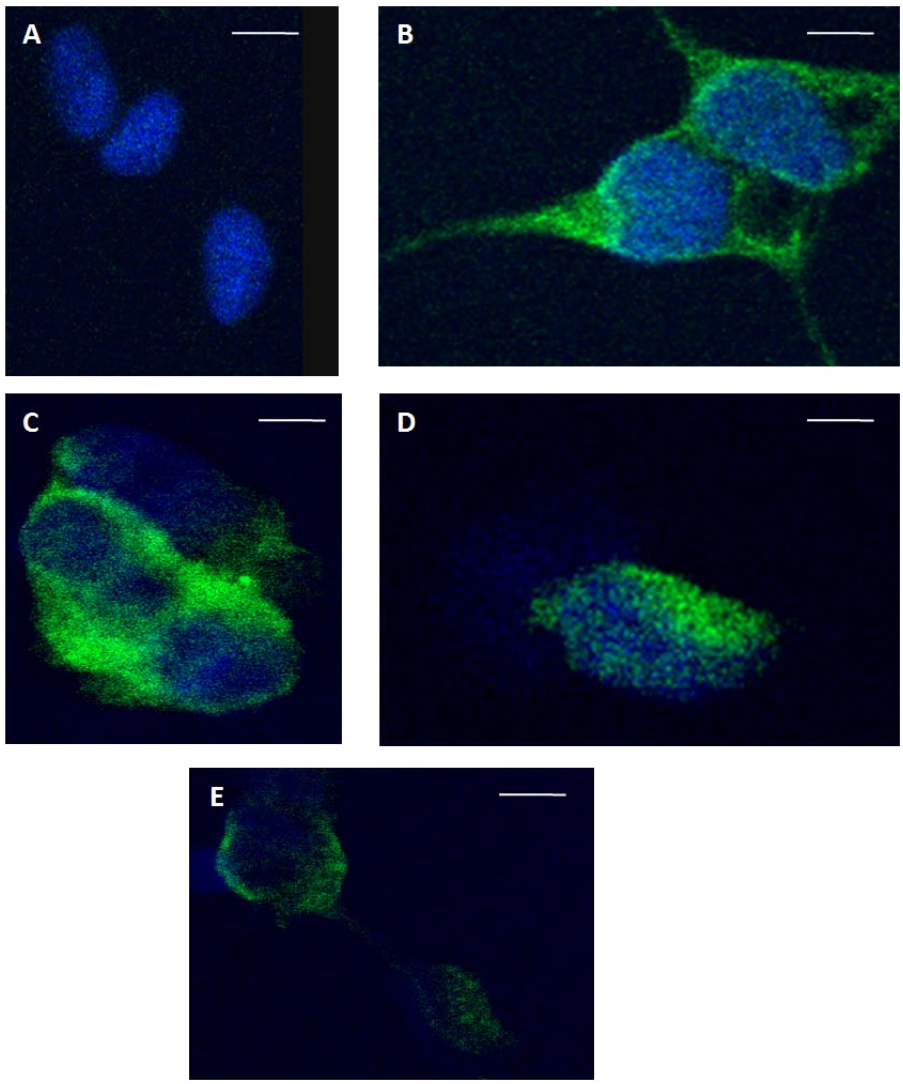
IL-1β treatment for 6 (4.01 fold) and 24 hr (3.10 fold) up-regulated the expression of MOR. IL-1RA treatment alone did not alter the expression of MOR; however the co-treatment with IL-1β (10 ng/mL) and IL-1RA (10 and 100 ng/mL) resulted in the significant blockade of MOR mRNA up-regulation by IL-1β. Co-treatment of SK-N-SH cells with IL-1RA (10 and 100 ng/mL) and IL-1β (10 ng/mL) for 6 and 24 hr blocked IL-1β-induced up-regulation of MOR expression. The 6 hr co-treatment resulted in a 2.6 (IL-1β (10 ng/mL) + IL-1RA (10 ng/mL)) and 1.83 (IL-1β (10 ng/mL) + IL-1RA (100 ng/mL)) fold change in MOR expression and the 24 hr co-treatment resulted in 1.6 (IL-1β (10 ng/mL) + IL-1RA (10 ng/mL)) and 1.15 (IL-1β (10 ng/mL) + IL-1RA (100 ng/mL)) fold change in MOR expression. The response elicited by IL-1RA on MOR mRNA expression ensured an IL-1RI-dependent mechanism for the up-regulation of MOR expression by IL-1β (Fig. 30).

Fig. 30 IL-1RA treatment blocked IL-1β induced up-regulation of MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated (US)) IL-1β (10 ng/mL), IL-1RA (10 ng/mL), IL-1β (10 ng/mL) + IL-1RA (10 ng/mL) and IL-1β (10 ng/mL) + IL-1RA (100 ng/mL) for 6 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. IL-1β alone.


US
IL-1β 10ng/mL
IL-1RA 10ng/mL
10ng/mL IL-1β + 10ng/mL IL-1RA
10ng/mL IL-1β + 100ng/mL IL-1RA
Fold change in hMOR mRNA (Normalized to GAPDH) (2^ΔΔCt)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
#
#
US
6
24
Time (Hr)

Using qRT-PCR to elucidate an IL-1RI-dependent mechanism for IL-1β-induced up-regulation of MOR expression (Fig.30), ICC experiments showed that IL-1β (10 ng/mL) treatment for 6 hr can also up-regulate MOR protein expression (Fig. 31.C). Co-treatment of SK-N-SH cells with IL-1β (10 ng/mL) and IL-1RA (10 ng/mL) for 6 (Fig 31.D) and 24 hr (Fig 31.E) induced a decrease in MOR protein expression. The greatest decrease in MOR protein expression was evident in the 24 hr IL-1β (10 ng/mL) and IL-1RA (10 ng/mL) co-treatment experiment (Fig 31.E). The decrease in MOR protein expression in response to IL-1β (10 ng/mL) and IL-1RA (10 ng/mL) co-treatment indicates an IL-1RI-dependent regulation of MOR expression by IL-1β.

Fig. 31 IL-1RA treatment blocked IL-1β's up-regulation of MOR protein expression in SK-N-SH cells. SK-N-SH cells were cultured on sterile glass coverslips in a 24-well plate at 37°C for two days in GM and cultured with or without (un-stimulated (US)) IL-1β (10 ng/mL) and IL-1β (10 ng/mL) and IL-1RA (10 ng/mL) for 6 and 24 hr. The cells were washed with 1x PBS, fixed with 4% paraformaldehyde, blocked with blocking buffer for 1 hr before being treated with 1x PBS (1° Ab. control), Anti-MOR RabMAb. (1:50; Abcam) made in blocking buffer overnight at 4°C. Cell were washed with 1x PBS and incubated with Alexa-Fluor 488-secondary antibody (Goat anti-rabbit; Invitrogen) (10 μg/mL) made in 1x PBS for 1hr at room temperature. After washing with 1x PBS, cells were counter stained with Hoechst (DNA stain) (1 μg/mL; Sigma) in 1x PBS for 5 min. at room temperature. Coverslips were washed using 1x PBS and mounted up-side down onto microscope slides using Prolong (Invitrogen). **A** – 1°antibody control; **B** – un-stimulated for 6 hr; **C** – IL-1β (10 ng/mL) for 6 hr; **D** – IL-1β (10 ng/mL) + IL-1RA (10 ng/mL) for 6 hr; **E** - IL-1β (10 ng/mL) + IL-1RA (10 ng/mL) for 24 hr. 50μm ——


A
B
C
D
E

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

As illustrated in Fig. 32, IL-1β (10 ng/mL) exposure for 6 and 24 hr induced the up-regulation (3.1 and 1.4 fold) of MOR and morphine (10 μM) exposure for 6 and 24 hr induced the down-regulation (-2 and -1.6 fold) of MOR expression in SK-N-SH cells. Experiments were set-up to study any interactive relationship between IL-1β and morphine and the effect this may have on MOR expression. Therefore, when IL-1β (10 ng/mL) exposure followed morphine (10 μM) exposure for 6 and 24 hr each, morphine-induced down-regulation of MOR expression was blocked. In the experiments where IL-1β (10 ng/mL) exposure for 6 and 24 hr followed morphine (10 μM) exposure, there was a 2.9 and 2.5 fold up-regulation of MOR expression, there was a significant ($p \leq 0.05$) up-regulation of MOR expression when compared to morphine exposure alone.

Following the analysis of the first experiment illustrated in Fig. 32 and to further elucidate the role of the interactive relationship between IL-1β and morphine on MOR expression, co-treatment experiments were performed. SK-N-SH cells were co-treated with IL-1β (10 ng/mL) and morphine (10 μM) for 6 and 24 hr. Treatment of IL-1β (10 ng/mL) and morphine (10μM) together (competition) resulted in the blockade of morphine-induced down-regulation of MOR expression (Fig. 33). IL-1β and morphine co-treatment for 6 and 24 hr resulted in a 3.14 and 1.52 fold up-regulation of MOR expression respectively. In comparison to morphine (10 μM) alone for 6 and 24 hr, which resulted in a -0.6 and -2.3 fold down-regulation of MOR expression, co-treatment with IL-1β and morphine significantly up-regulated the expression of MOR ($p \leq 0.05$)?

The IL-1β-induced blockade of morphine-induced down-regulation of MOR expression as illustrated in Fig 33 was also repeated in ICC experiments. When compared to the morphine (10μM) (Fig. 34.D) and IL-1β (10 ng/mL) (Fig. 34.E) exposure alone for 6 hr,

MOR protein expression was up-regulated when co-treated with IL-1β and morphine (Fig. 34.F). The expression of both MOR mRNA and protein levels in response to IL-1β and morphine co-treatment was up-regulation (Fig. 33 and 34.F). An epiflouresence microscope was used to capture images of MOR protein expression in these experiments. As illustrated in Fig. 34, MOR protein is represented as punctate-round structures dispersed irregularly across the cell. Therefore IL-1β and morphine co-treatment for 6 hr induced an increase in MOR protein expression when compared to morphine (Fig. 34.D) and IL-1β (Fig. 34.E) treatment alone.

Fig. 32 IL-1β treatment blocked morphine-induced down-regulation of MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated (US)) IL-1β (10 ng/mL), morphine (10μM) and IL-1β (10 ng/mL) following treatment with morphine (10μM) for 6 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. morphine alone.

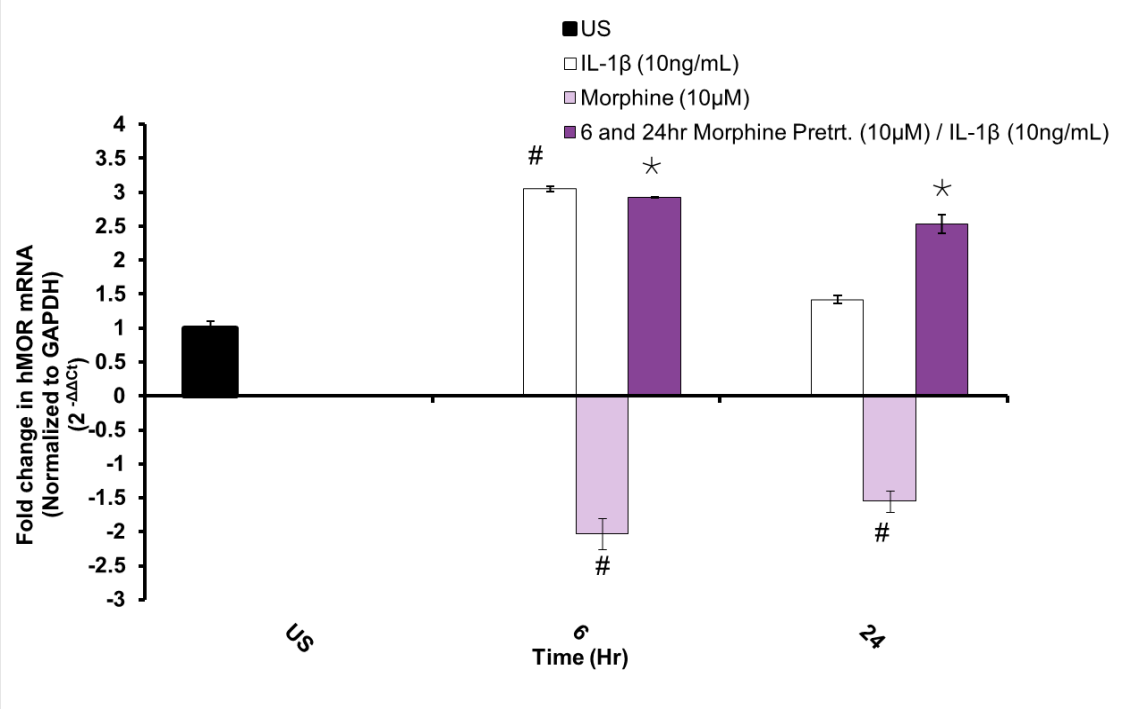

US
IL-1β (10ng/mL)
Morphine (10μM)
6 and 24hr Morphine Pretrt. (10μM) / IL-1β (10ng/mL)
Fold change in hMOR mRNA (Normalized to GAPDH) (2 -ΔΔCt)
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
#
#
#
US
6
24
Time (Hr)

Fig. 33 IL-1β treatment blocked morphine-induced down-regulation of MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) IL-1β (10 ng/mL), morphine (10μM) and IL-1β (10 ng/mL) and morphine (10μM) co-treatment for 6 and 24 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. morphine alone.

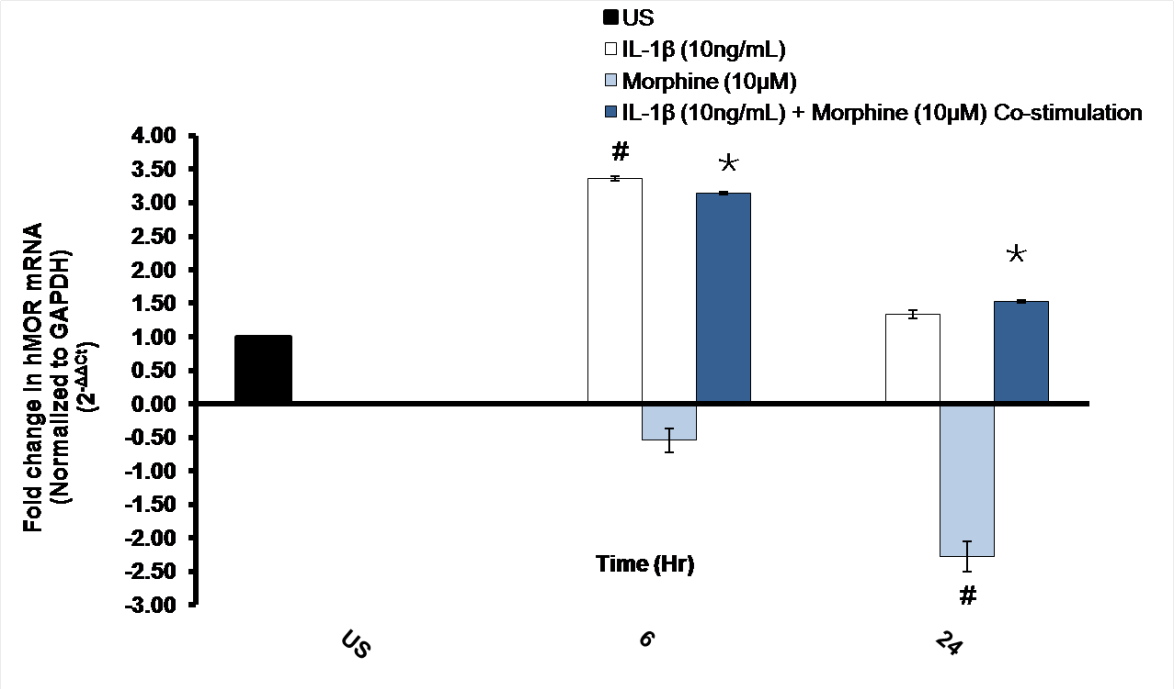
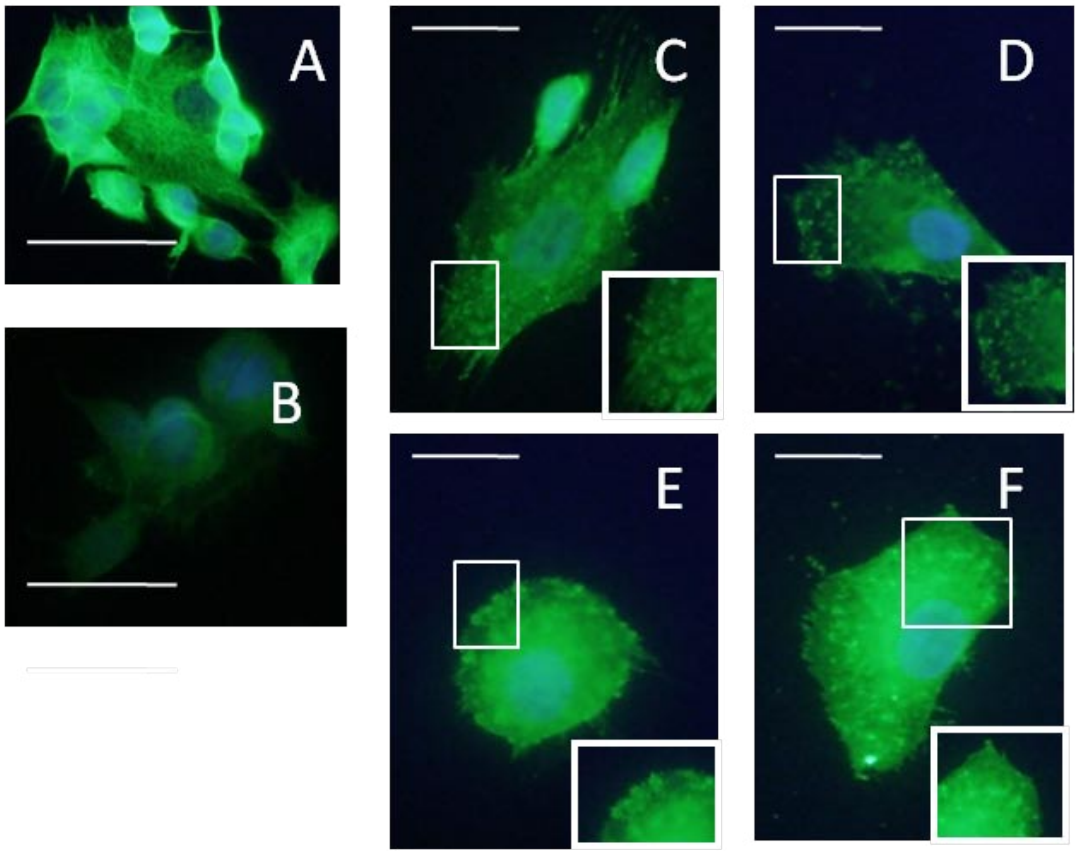

US
IL-1β (10ng/mL)
Morphine (10μM)
IL-1β (10ng/mL) + Morphine (10μM) Co-stimulation
Fold change in hMOR mRNA (Normalized to GAPDH) (2-ΔΔCt)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
-2.50
-3.00
#
*
*
#
Time (Hr)
US
6
24

Fig. 34 Co-treatment with IL-1β and morphine up-regulated MOR protein expression in SK-N-SH cells. SK-N-SH cells were cultured on sterile glass coverslips in a 24-well plate at 37°C for two days in GM and cultured with or without (un-stimulated; US) morphine (10 μM), IL-1β (10 ng/mL) and morphine (10 μM) and IL-1β (10 ng/mL) together for 6 hr. The cells were washed with 1x PBS, fixed with 4% paraformaldehyde, blocked with blocking buffer for 1 hr before being treated with 1x PBS (1° Ab. control), Anti-MOR RabMAb. (1:50; Abcam), Anti-β-tubulin III RabMAb. (1:100; Epitomics) made in blocking buffer overnight at 4°C. Cell were washed with 1x PBS and incubated with Alexa-Fluor 488-secondary antibody (Goat anti-rabbit; Invitrogen) (10 μg/mL) made in 1x PBS for 1hr at room temperature. After washing with 1x PBS, cells were counter stained with Hoechst (DNA stain) (1 μg/mL; Sigma) in 1x PBS for 5 min. at room temperature. Coverslips were washed using 1x PBS and mounted up-side down onto microscope slides using Prolong (Invitrogen). **A** – anti-β-tubulin III; **B** – 1°Ab. control; **C** – unstimulated; **D** – Morphine (10 μM); **E** - IL-1β (10 ng/mL); **F** - Morphine (10 μM) and IL-1β (10 ng/mL) co-treatment for 6 hr. 200μm ——— 100μm ——


A
B
C
D
E
F

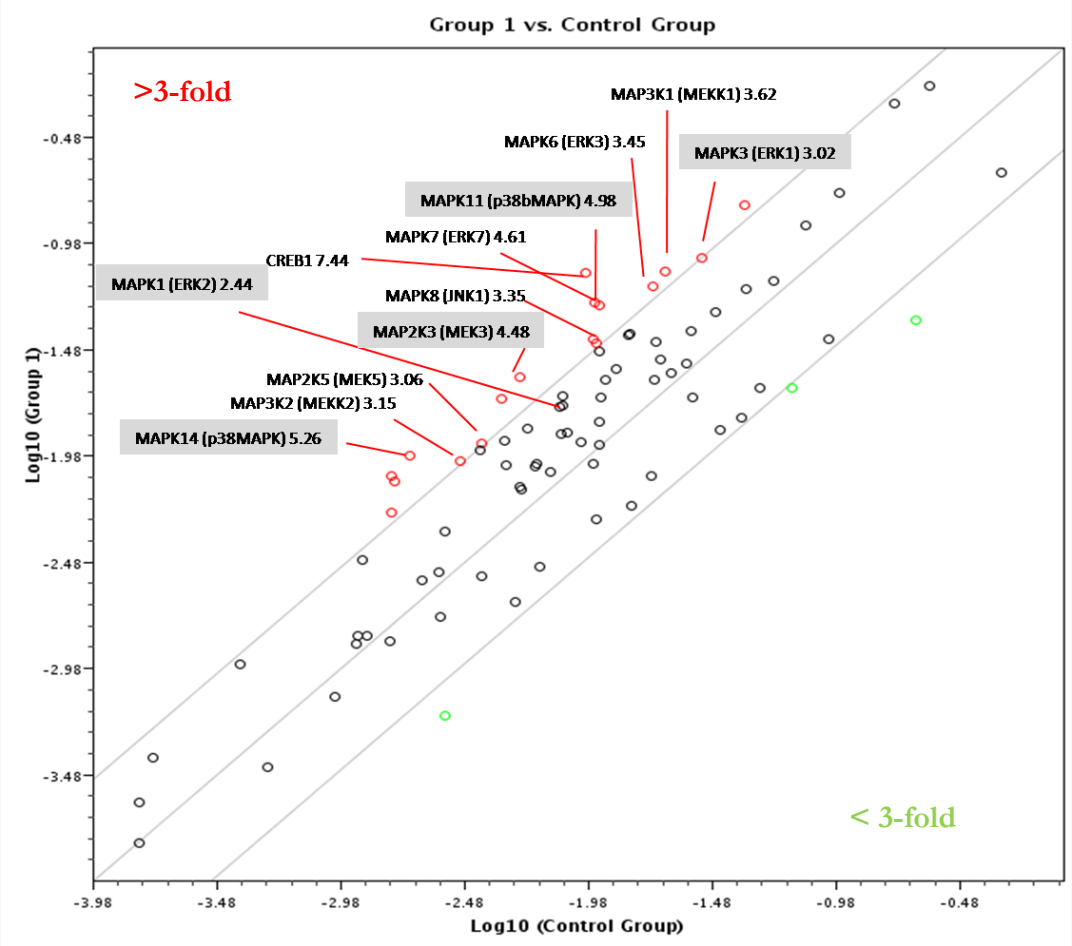
Part III:

Signal transduction mechanism involved in the regulation of MOR expression in SK-N-SH cells treated with morphine and IL-1β

4.11 Morphine (10 μM) induced up-regulation of MAPK genes

Using a 96-well plate spotted with 84-MAPK gene specific primers, it was elucidated that morphine up-regulated the expression of 18 MAPK genes. Fig. 35 illustrates that SK-N-SH cells, when treated with 10 μM morphine for 6 hr, induced the up-regulation of MAPK genes ≥ 3-fold, for example. Among the genes up-regulated were MAPK3 (ERK1; 3.0 fold); MAPK1 (ERK2; 2.4 fold); MAPK6 (ERK3; 3.4 fold); MAP2K3 (MEK3; 4.5 fold); MAPK11 (p38 MAPK; 5.0 fold) and MAPK14 (p38 MAPK; 5.3 fold).

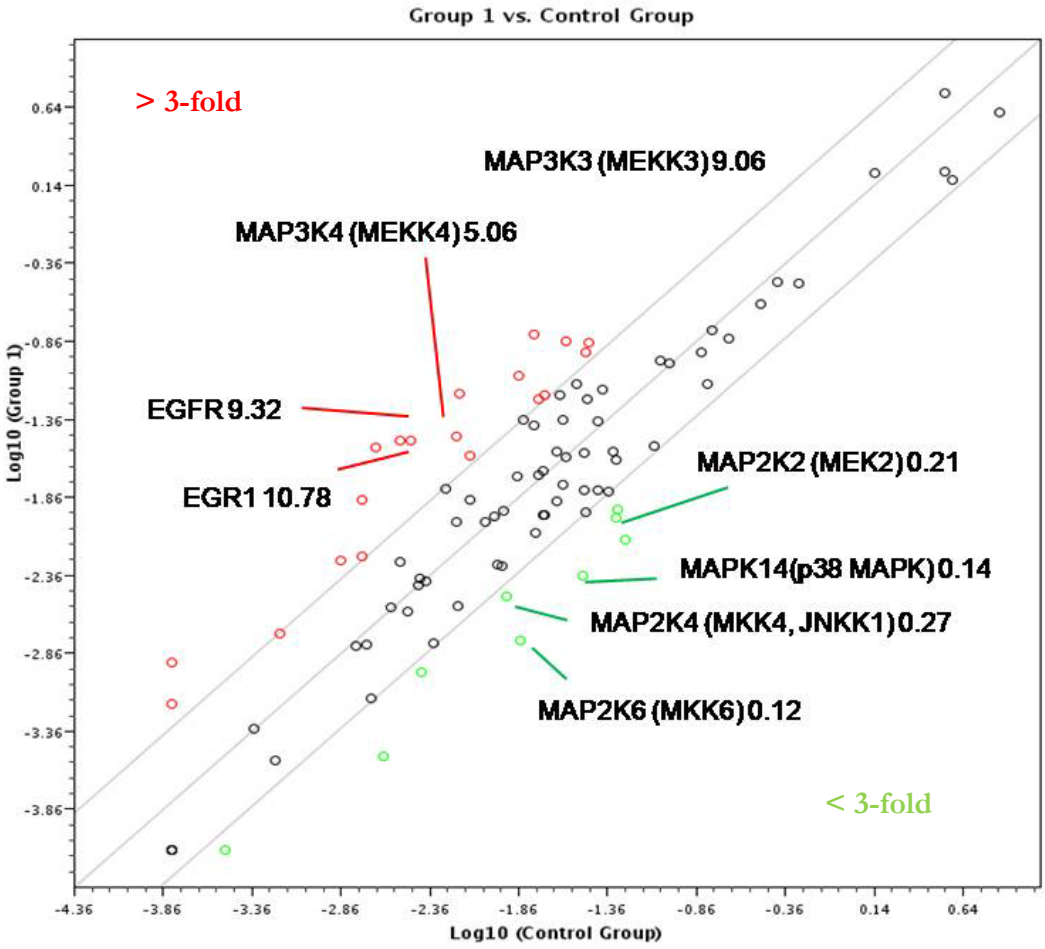
Fig. 35 Morphine treatment up-regulated the expression of human MAPK genes in SK-N-SH cells. Using a 96-well qRT-PCR plate, 84 wells, each representing a specific MAPK gene was treated with the qRT-PCR master mix containing 50 ng of total cDNA. Cells were treated with 10 μM morphine for 6 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the three endogenous reference genes RP13A (Ribosomal protein L13a), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and ACTB (Actin Beta). In order to calculate fold changes from average ΔC_T values, one un-treated (control) and two independent morphine-treated experiments were performed. Using the online data analysis web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php), specific genes of interest were selected and plotted against un-treated (control) group vs. treated group (Log10 control group vs. Log10 morphine treated group).


Group 1 vs. Control Group
>3-fold
MAP3K1 (MEKK1) 3.62
MAPK6 (ERK3) 3.45
MAPK3 (ERK1) 3.02
MAPK11 (p38bMAPK) 4.98
MAPK7 (ERK7) 4.61
CREB1 7.44
MAPK1 (ERK2) 2.44
MAPK8 (JNK1) 3.35
MAP2K3 (MEK3) 4.48
MAP2K5 (MEK5) 3.06
MAP3K2 (MEKK2) 3.15
MAPK14 (p38MAPK) 5.26
< 3-fold
Log10 (Group 1)
Log10 (Control Group)
-0.48
-0.98
-1.48
-1.98
-2.48
-2.98
-3.48
-3.98

4.12 IL-1β (10 ng/mL) induced up-regulation of MAPK genes

There is limited evidence for the role of MAPK pathways in regulation of MOR mRNA or protein following treatment with cytokines such as the pro-inflammatory cytokine, IL-1β. Using a 96-well plate spotted with 84-human p38 MAPK gene specific primers, we elucidated that IL-1β up-regulated and down-regulated the expression of MAPK genes. Fig. 36 illustrates that when SK-N-SH cells were treated with IL-1β (10 ng/mL) for 6 hr, the following MAPK genes were up-regulated ≥ 3-fold: MEEK3 (9.0); MEKK4 (5.0); and conversely down-regulated the following MAPK genes ≤ 3-fold: MEK2 (-3.0); p38 MAPK (-3.1).

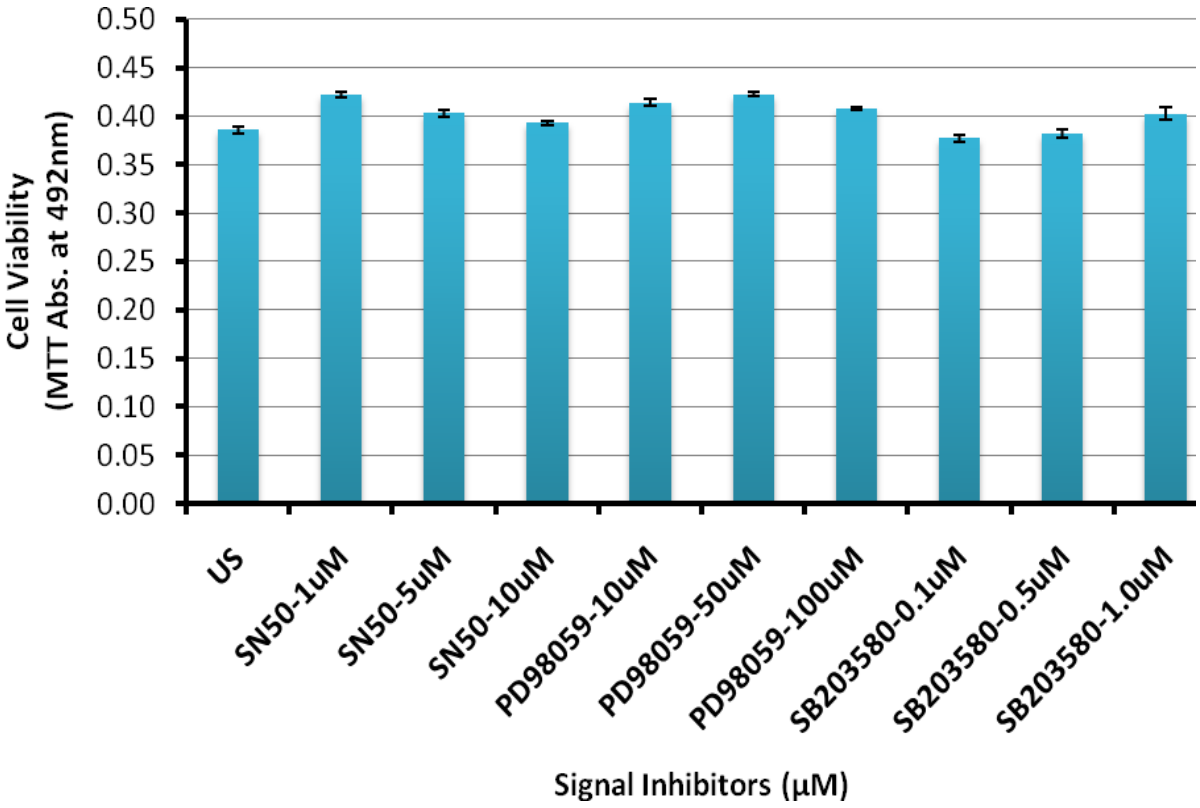
Fig. 36 IL-1β treatment differentially regulated the expression of human MAPK genes in SK-N-SH cells. Using a 96-well qRT-PCR plate, 84 wells, each representing a specific MAPK gene was treated with the qRT-PCR master mix containing 50 ng of total cDNA. Cells were treated with 10 μM morphine for 6 hr. Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the three endogenous reference genes RP13A (Ribosomal protein L13a), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and ACTB (Actin Beta). In order to calculate fold changes from average ΔC_T values, one un-treated (control) and two independent morphine-treated experiments were performed. Using the online data analysis web portal (http://www.SABiosciences.com/pcrarraydataanalysis.php), specific genes of interest were selected and plotted against un-treated (control) group vs. treated group. (Log10 control group vs. Log10 morphine treated group).


Group 1 vs. Control Group
> 3-fold
MAP3K3 (MEKK3) 9.06
MAP3K4 (MEKK4) 5.06
EGFR 9.32
EGR1 10.78
MAP2K2 (MEK2) 0.21
MAPK14(p38 MAPK) 0.14
MAP2K4 (MKK4, JNKK1) 0.27
MAP2K6 (MKK6) 0.12
< 3-fold
Log10 (Group 1)
Log10 (Control Group)
0.64
0.14
-0.36
-0.86
-1.36
-1.86
-2.36
-2.86
-3.36
-3.86
-4.36

4.13 Cytotoxicity of signaling inhibitor – MTT assay

In order to determine if the signaling inhibitors, SN50, PD98059 and SB203580 were cytotoxic to SK-N-SH cells, MTT assays were performed. The cytotoxic effects of three different concentrations of each inhibitor were measured using MTT assays. MTT assays showed the all concentrations of the signaling inhibitors were non-cytotoxic (Fig. 37).

Fig. 37 Signal inhibitors SN50, PD98059 and SB203580 pre-treatment (1hr) followed by IL-1β (10 ng/mL) treatment for 6 hr in the same cell-culture well resulted in no cytotoxic effects. . SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated (US)), SN50 (1, 5 and 10 μM), PD98059 (10, 50 and 100 μM) and SB203580 (0.1, 0.5 and 1.0 μM) for 1 hr and then treated with IL-1β (10 ng/mL) for 6 hr. MTT assay were performed (Carmichael et al., 1987) following incubation of cell in GM with 0.55 mg/mL 3-[4,5-Dimethylthiazol-2-yl]-2,5,-diphenyltetrazolium bromide (MTT) for 1 hr. Cells were then washed three times with 1x PBS, and cell lysate was dissolved using 1 mL DMSO and absorbance measured at 492 nm using a BIO-TEK HT spectrophotometer. One-way analysis of Variance (ANOVA) followed by Newman-Kuel's post-hoc multiple comparison tests were used to test for significance between treatments groups performed using GraphPad Prism 4. Error bars show ± S.E.M of triplicate wells from three independent experiments.

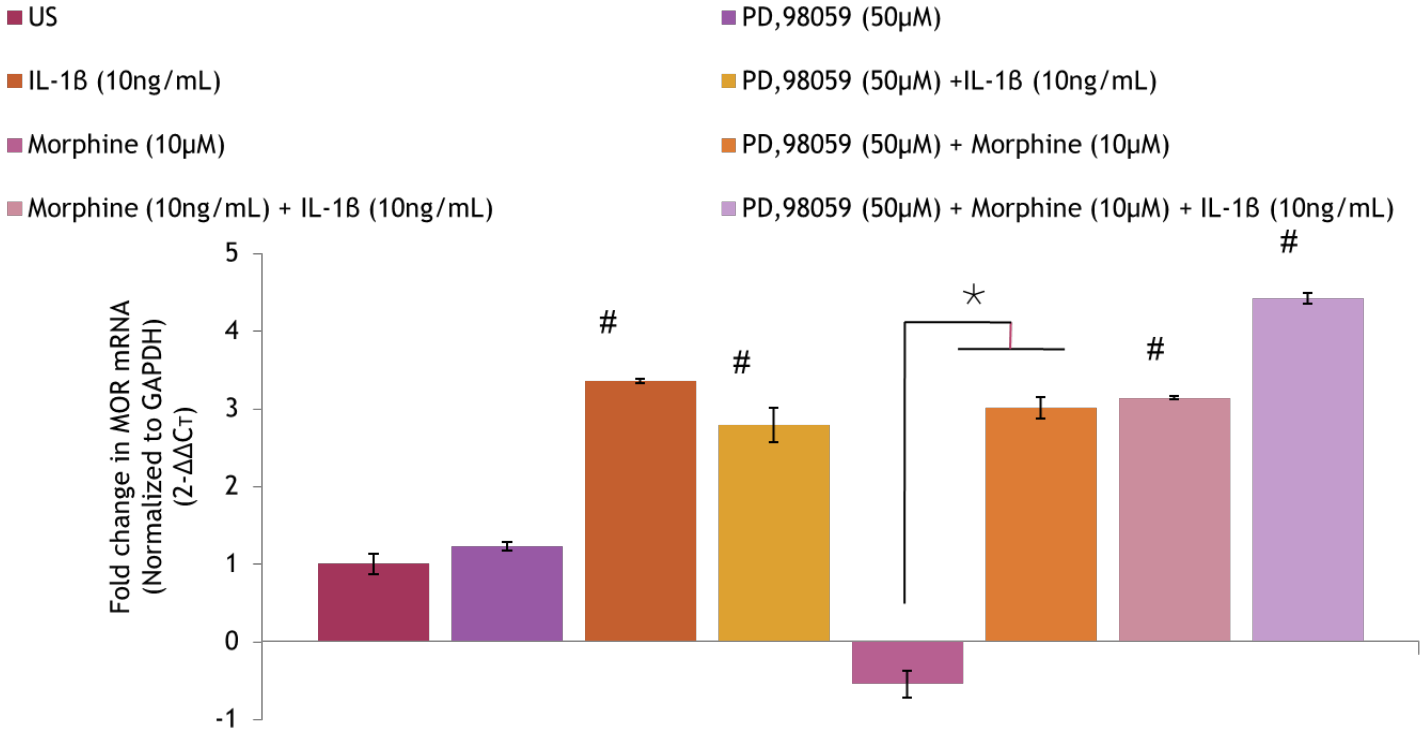

0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Cell Viability
(MTT Abs. at 492nm)
US
SN50-1uM
SN50-5uM
SN50-10uM
PD98059-10uM
PD98059-50uM
PD98059-100uM
SB203580-0.1uM
SB203580-0.5uM
SB203580-1.0uM
Signal Inhibitors (μM)

4.14 MEK1/2-dependent down-regulation of MOR expression by morphine

In order to elucidate the role of MEK1/2 in the regulation of MOR expression in SK-N-SH cells, PD98059, a MEK1/2 specific inhibitor was used (Alessi et al., 1995). SK-N-SH cells were pretreated with PD98059 (50 μM) or vehicle control (water) for 1 hr (PD98059 wash not washed out) before being treated with IL-1β (10 ng/mL), morphine (10 μM) and IL-1β (10 ng/mL) and morphine (10 μM) co-treatment. Fig. 38 illustrates a MEK1/2-dependent regulation of MOR expression following morphine treatment. Morphine (10 μM) treatment for 6 hr down-regulated MOR expression (-0.55-fold). However, in the presence of PD98059, the down-regulation of MOR expression caused by morphine was not evident and significant up-regulation of MOR expression (3.01-fold) was measured when the cells were treated with PD98059 and morphine. IL-1β (10 ng/mL) and morphine (10 μM) co-treatment for 6 hr up-regulated (3.14-fold) MOR expression, however in the presence of PD98059, the effect of IL-1β (10 ng/mL) and morphine (10 μM) co-treatment on MOR expression was exacerbated, increasing the expression of MOR to 4.42 fold (Fig. 38).

In contrast to the role of MEK1/2 in the down-regulation of MOR expression by morphine, MEK1/2 inhibition had an insignificant effect on IL-1β-induced up-regulation of MOR expression. IL-1β (10 ng/mL) for 6 hr induced MOR up-regulation (3.36-fold) and in the presence of the PD98059, IL-1β-induced MOR regulation remained unchanged (2.79-fold) (Fig. 38). The effect of PD98059 treatment for the duration of the experiment (7 hr) resulted in no significant change in MOR expression.

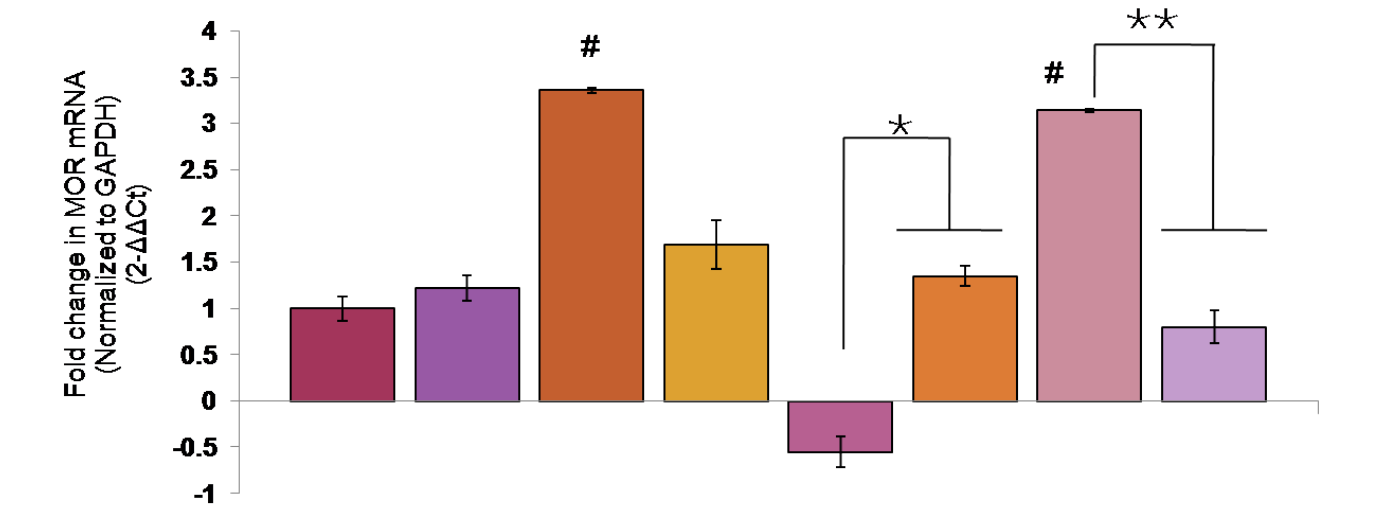
Fig. 38 PD98059 treatment significantly blocked morphine-induced down-regulation of MOR expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) PD98059 (50 μM) IL-1β (10 ng/mL), IL-1β (10 ng/mL) and PD98059 (50 μM), morphine (10 μM), morphine (10 μM) and PD98059 (50 μM), IL-1β (10 ng/mL) and morphine (10 μM) co-treatment and IL-1β (10 ng/mL) and morphine (10 μM) co-treatment with PD98059 (50 μM). Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. morphine alone.


US
IL-1β (10ng/mL)
Morphine (10μM)
Morphine (10ng/mL) + IL-1β (10ng/mL)
PD,98059 (50μM)
PD,98059 (50μM) +IL-1β (10ng/mL)
PD,98059 (50μM) + Morphine (10μM)
PD,98059 (50μM) + Morphine (10μM) + IL-1β (10ng/mL)
Fold change in MOR mRNA (Normalized to GAPDH) (2-ΔΔCT)
5
4
3
2
1
0
-1
#
#
*
#
#

4.15 p38 MAPK-dependent down-regulation of MOR expression by morphine

Treatment with SB203580 significantly blocked morphine and IL-1β + morphine co-treatment effects on MOR expression (Fig. 39). Morphine (10 μM) treatment for 6 hr down-regulated MOR expression (-0.6 fold), however in the presence of SB203580 (1 μM), morphine-induced down-regulation of MOR expression was blocked. SB203580 and morphine treatment resulted in a significant up-regulation of MOR expression (1.4-fold) when compared to morphine treatment alone. IL-1β (10 ng/mL) treatment for 6 hr significantly up-regulated MOR expression (3.1 fold), and in presence of SB203580 (1 μM), IL-1β-induced up-regulation of MOR expression was reduced to only 1.7 fold; however this change in MOR expression in response to SB203580 and IL-1β treatment was not statistically significant. In the case of IL-1β (10 ng/mL) and morphine (10 μM) co-treatment for 6 hr, MOR expression was significantly up-regulated (3.2 fold) when to compared to un-stimulated. In the presence of SB203580 (1 μM), IL-1β (10 ng/mL) and morphine (10 μM) co-treatment induced up-regulation of MOR was significantly blocked, reducing the effect of IL-1β (10 ng/mL) and morphine (10 μM) co-treatment on MOR expression to only 0.6 fold (Fig. 39).

Fig. 39 SB203580 treatment significantly blocked the effect of Morphine and IL-1β and morphine co-treatment on MOR mRNA expression in SK-N-SH cells. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) SB203580 (1 μM), IL-1β (10 ng/mL), IL-1β (10 ng/mL) and SB203580 (1 μM), morphine (10 μM), morphine (10 μM) and SB203580 (1 μM), IL-1β (10 ng/mL) and morphine (10 μM) co-treatment and IL-1β (10 ng/mL) and morphine (10 μM) co-treatment with SB203580 (1 μM). Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US; * $p \leq 0.05$ vs. morphine alone.


US
IL-1β (10ng/mL)
Morphine (10μM)
Morphine (10ng/mL) + IL-1β (10ng/mL)
SB,203580 (1μM)
SB,203580(1μM) +IL-1β (10ng/mL)
SB,203580 (1μM) + Morphine (10μM)
SB,203580 (1μM) + Morphine (10μM) + IL-1β (10ng/mL)
Fold change in MOR mRNA (Normalized to GAPDH) (2-ΔΔCt)
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
#
*
#
**

4.16 NF-κB-sensitive down-regulation and up-regulation of MOR expression by morphine and IL-1β respectively

SK-N-SH cells treated with 10 μM SN50 alone and when treated with IL-1β, morphine and IL-1β and morphine co-treatment did not significantly change the regulation of MOR expression, indicating that the expression of MOR is insensitive to the activation of NF-κB-p50 (Fig. 40). However, in order to elucidate the role of NF-κB-p50 on MOR expression, SK-N-SH cells were treated with 50 μM of SN50 (Fig. 41). Compared to unstimulated, morphine (10 μM) treatment for 6 hr significantly down-regulated MOR expression (-2.03 fold). However, in the presence of SN50 (50 μM), morphine-induced down-regulation of MOR expression was significantly exacerbated to -5.06 fold (Fig. 41). IL-1β (10 ng/mL) treatment for 6 hr significantly up-regulated MOR expression (3.22 fold), but in the presence of the SN50 (50 μM), the effect of IL-1β on MOR expression was significantly attenuated, decreasing the fold change in MOR to just -0.33 fold (Fig. 41). SN50 (10 and 50 μM) treatment did not significantly alter IL-1β and morphine co-treated up-regulation MOR expression in SK-N-SH cells (Fig. 40 - 41).

Fig. 40 SN50 (10 μM) treated SK-N-SH cells failed to block the effects of IL-1β, morphine and IL-1β and morphine co-treatment on MOR mRNA expression. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) SN50 **(10 μM),** IL-1β (10 ng/mL), IL-1β (10 ng/mL) and SN50 **(10 μM),** morphine (10 μM), morphine (10 μM) and SN50 **(10 μM),** IL-1β (10 ng/mL) and morphine (10 μM) co-treatment and IL-1β (10 ng/mL) and morphine (10 μM) co-treatment with SN50 (10 μM). Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p \leq 0.05$ vs. US

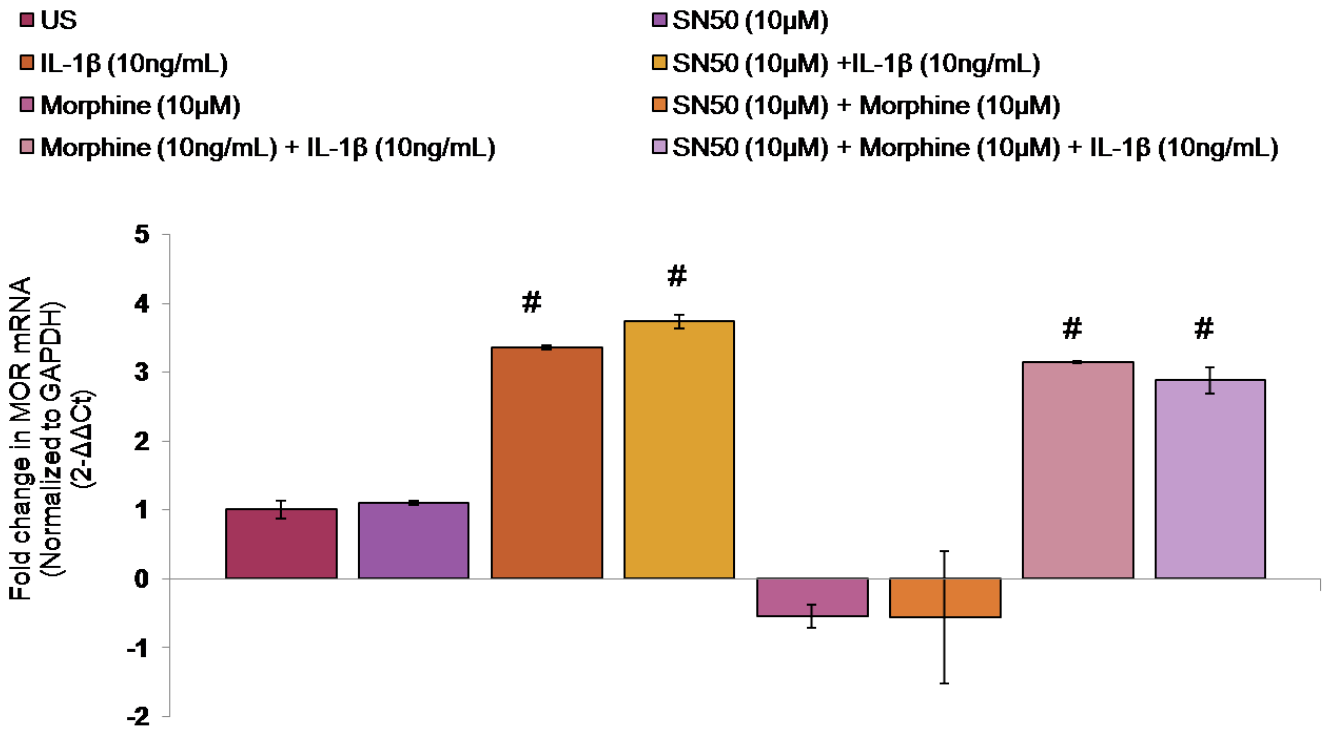
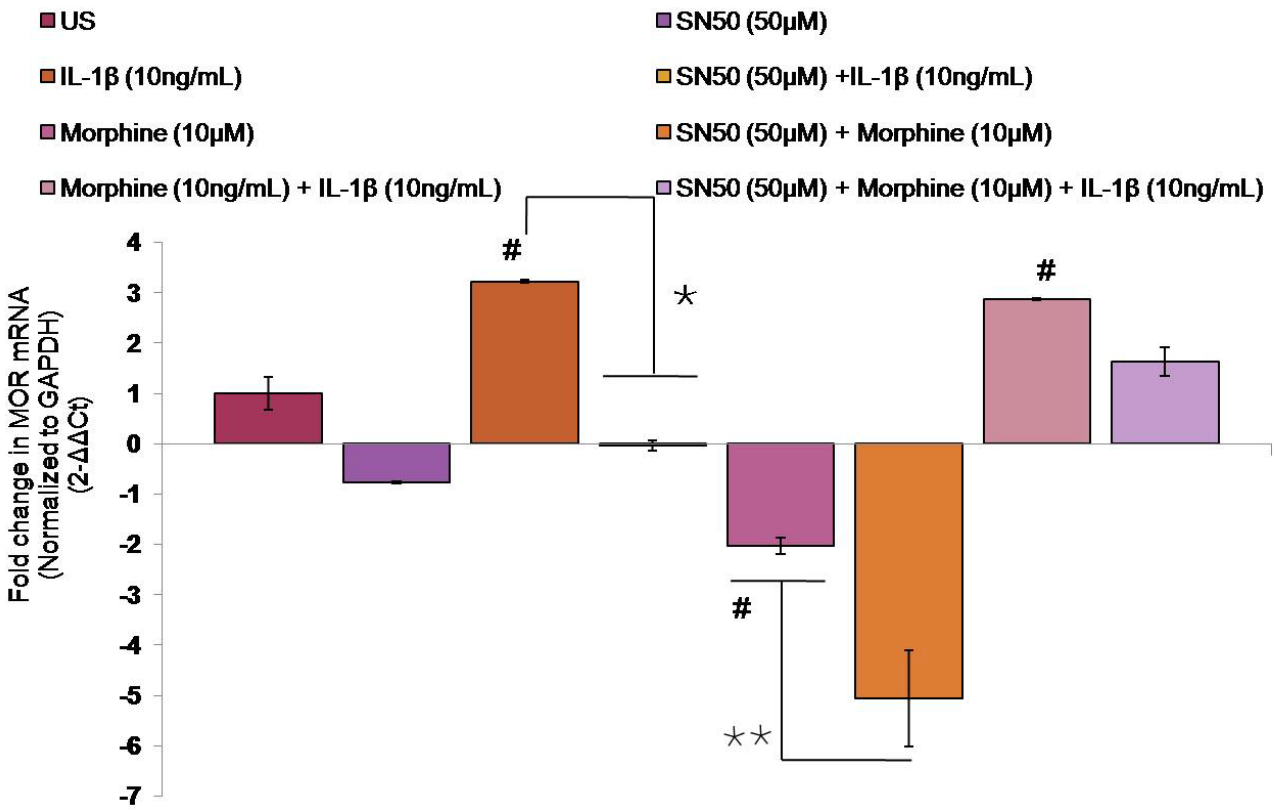

US
IL-1β (10ng/mL)
Morphine (10μM)
Morphine (10ng/mL) + IL-1β (10ng/mL)
SN50 (10μM)
SN50 (10μM) +IL-1β (10ng/mL)
SN50 (10μM) + Morphine (10μM)
SN50 (10μM) + Morphine (10μM) + IL-1β (10ng/mL)
Fold change in MOR mRNA (Normalized to GAPDH) (2-ΔΔCt)
5
4
3
2
1
0
-1
-2
#
#
#
#

Fig. 41 SN50 (50 μM) treated SK-N-SH cells significantly blocked the effects of IL-1β and morphine treatment on MOR mRNA expression. SK-N-SH cells were cultured in 24-well plates at 37°C for 3-4 days in GM and cultured with or without (un-stimulated; US) SN50 (50 μM), IL-1β (10 ng/mL), IL-1β (10 ng/mL) and SN50 (50 μM), morphine (10 μM), morphine (10 μM) and SN50 (50 μM), IL-1β (10 ng/mL) and morphine (10 μM) co-treatment and IL-1β (10 ng/mL) and morphine (10 μM) co-treatment with SN50 (50 μM). Fold change in MOR mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ arithmetic formula normalized to the endogenous reference gene GAPDH. Data represent mean ± S.E.M (error bars) of duplicate measures from triplicate wells from three independent experiments. Statistical analysis was carried out using a two-way ANOVA, with pair wise (p-values) differences (PDIFF) determined from least square means (LS mean). SAS was used to perform a PROC-GLM analysis as the preferred procedure for doing a two-way ANOVA. # $p<0.05$ vs. US; * $p\leq0.05$ vs. IL-1β alone; ** $p\leq0.05$ vs. morphine alone.


US
IL-1β (10ng/mL)
Morphine (10μM)
Morphine (10ng/mL) + IL-1β (10ng/mL)
SN50 (50μM)
SN50 (50μM) +IL-1β (10ng/mL)
SN50 (50μM) + Morphine (10μM)
SN50 (50μM) + Morphine (10μM) + IL-1β (10ng/mL)
Fold change in MOR mRNA (Normalized to GAPDH) (2-ΔΔCt)
4
3
2
1
0
-1
-2
-3
-4
-5
-6
-7
#
#
#
*
**

CHAPTER V

DISCUSSION

Part I:

5.1 Cloning and expression of MOR in SK-N-SH cells

Our data demonstrated that SK-N-SH cells express full-length human MOR. Using cDNA as the template to verify the expression of full-length MOR and PCR-cloning followed by sequencing resulted in a ~1.4 kb consensus sequence, which included 1,107 bp open reading frame (ORF) encoding a 388 amino acid protein as previously published (Bare et al., 1994). The verification of MOR expression by SK-N-SH cells was initially important to allow progress onto qRT-PCR measured changes in MOR transcript following treatment with morphine and IL-1β.

To measure the level of function of MOR in SK-N-SH cells was equally important as the expression of MOR, however, our study did not seek to measure the functionality of MOR in SK-N-SH cells because the functionality of MOR has been extensively studied in these and other neuroblastoma cell lines (Ammer and Schulz, 1996; Baker et al., 2000; Baumhaker et al., 1993; Breivogel et al., 1997; Cheng et al., 1997; Horner and Zadina, 2004; Raut et al., 2006; Raut et al., 2007; Rubovitch et al., 2003; Sarne et al., 1998; Yu et al., 1990; Zadina et al., 1993). Results from these studies are important to our study because they verified our use of SK-N-SH cells as an *in vitro* model to study MOR transcript expression. As an example, one such study recorded reduced MOR binding in undifferentiated SK-N-

SH cells when treated with 100 nM etorphine (Baumhaker et al., 1993). Also, increased G-protein activation in SK-N-SH cells was measured following treatment with DAMGO (Breivogel et al., 1997) and the classic inhibitory effect of MOR stimulation on PLC activity in SK-N-SH cells was prevented by pretreating the SK-N-SH cells with pertussis toxin (PTX) (Rubovitch et al., 2003). Therefore, due to the large number of studies that have researched MOR function in neuroblastoma cell lines in response to opioids, an attempt was not made to measure changes in MOR function in response to morphine or IL-1β. However, if needed, a complete study could be completed using ligand binding assay protocols established in the labs of Dr. C. W. Stevens.

5.2 Expression and significance of IL-1RI in SK-N-SH cells

The expression of IL-1RI in the CNS is still regarded relatively novel, because the CNS was considered 'immune privileged' before the discovery of IL-1RI expression in the brains of mice (Takao et al., 1990) and humans (Hammond et al., 1999). In our study we discovered that SK-N-SH cells express the IL-1β receptor, IL-1RI. The discovery of IL-1RI expression is novel; to our knowledge there is no evidence of IL-1RI expression among human neuroblastoma cells types to date. The confirmation of IL-1RI expression was important to our hypothesis. We hypothesized that IL-1β would up-regulate the expression of MOR, therefore in order to measure changes in MOR expression in response to IL-1β treatment, it was important to know if SK-N-SH cells expressed an IL-1β receptor, proving a binding site for IL-1β existed in our model. However, if SK-N-SH cells did not express IL-1RI at high levels, experiments could have been conducted to elucidate the IL-1RI-independent mechanism of IL-1β-induced expression of MOR in SK-N-SH cells.

The discovery of IL-1RI expression in SK-N-SH cells makes our study on understanding the effects of IL-1β on MOR expression more relevant to neuro-inflammatory diseases where IL-1β has been implicated as a major factor in pain modulation. For example, IL-1β is involved in cellular apoptosis associated with neurodegenerative disease such as Alzheimer's disease (Griffin et al., 2006; Shaftel et al., 2008; Tachida et al., 2008), Parkinson's disease (Ferrari et al., 2006; Kim and Joh, 2006; Koprich et al., 2008; McGeer et al., 2002), multiple sclerosis (Ferrari et al., 2004; Schrijver et al., 2003) and CNS damage, including that elicited by neuroAIDS (Ferrari et al., 2004; Kaul and Lipton, 2006; Liu et al., 2008; Minami et al., 1992; Nesic et al., 2001; Rothwell, 2003; Rothwell and Luheshi, 2000; Schrijver et al., 2003; Sommer et al., 1999; Wang et al., 1997). With the result of IL-1RI expression by SK-N-SH cells, one can assume that the IL-1β-IL-1RI interaction that occured in our model may also occur in neuroinflammatory diseases. Therefore if studied precisely, it could result in the discovery of a unique therapeutic target that would not only reduce neuroinflammation but also enhance the analgesic properties of opioids frequently administered to severe neuroinflammatory diseases patients, such as those suffering from multiple sclerosis.

Part II:

5.3 Regulation of MOR expression in SK-N-SH cells treated with morphine

Using qRT-PCR, MOR mRNA expression was not significantly down-regulated following morphine treatment for 6 hrs; however MOR mRNA expression was significantly down-regulated following 12 and 24 hr treatment at all three morphine concentrations. These findings are consistent with Zadina et al., (1993) studies performed using SH-SY5Y cells (SK-N-SH cell sub-clone). It was concluded that chronic morphine exposure decreased receptor number but not the affinity of MOR in a time-dependent manner and naltrexone up-regulated MOR expression (Zadina et al., 1993). Morphine-induced down-regulation of MOR mRNA showed that the expression levels of MOR in SK-N-SH cells were sufficient to measure changes in mRNA transcript using qRT-PCR. The use of shorter morphine exposure times, 15 and 60 minutes (data not shown) resulted in no significant change in MOR expression compared to unstimulated cells and therefore were not studied further. The lack of response in MOR mRNA expression to morphine (15 and 60 minutes) treatment can be possibly explained by the following reasons: exposure times were probably insufficient to measure change in MOR mRNA levels using qRT-PCR. Also for morphine-MOR binding events to be at equilibrium, 15 and 60 minutes morphine exposure may not have been long enough. Therefore 15 and 60 minutes morphine exposure in SK-N-SH cells may have not been enough to activate signaling cascades (extranuclear) that would regulate the rate and pattern of MOR gene expression as frequently measured to occur in hormonal receptor systems (Madak-Erdogan et al., 2008).

The significant down-regulation of MOR mRNA expression in response to 24 hr morphine exposure supported our hypothesis. However, the expression of MOR protein in response to morphine treatment was less predictable. For example, MOR protein down-

regulation in response to morphine occurred in a non-concentration-dependent manner as seen using ICC. A possible explanation for the lack of correlation between MOR mRNA and protein expression could be explained by the lower expression levels of MOR protein at the cell membrane, which would affect opioid-receptor binding of morphine. Previous studies have employed the growth factor retinoic acid (RA) to induce MOR protein expression (Ammer and Schulz, 1994; Pahlman et al., 1984; Sidell et al., 1983). In preliminary studies (data not shown), RA (10μM) treatment for 5 days induced a 10-fold increase in MOR mRNA expression. Protein levels were not measured, however other studies using ligand binding assays have conclusively shown an increase in MOR density and opioid-specific binding affinities in SK-N-SH cells (Baumhaker et al., 1993). The main aim of this project was to measure MOR mRNA levels and therefore RA stimulation was omitted from all qRT-PCR experiments.

Fig. 27 shows that naltrexone antagonized morphine-induced down-regulation of MOR mRNA expression. The effects of naltrexone were neither time nor concentration dependent; however naltrexone antagonized morphine-MOR binding, confirming a MOR-dependent down-regulation of MOR by morphine. The lack of a time or concentration-dependent effect of naltrexone on morphine-induced MOR down-regulation suggests a non-graded MOR-response system. There was statistically no difference between the effects elicited on MOR gene expression when SK-N-SH cells were co-treated with naltrexone and morphine for 6 and 24 hrs. This suggests that morphine-induced MOR down-regulation is not an efficient system in SK-N-SH cells and can be easily blocked by naltrexone. In comparison to our results, the effects of chronic administration (7 days) of naltrexone on MOR expression levels in the rat brain significantly changed MOR binding (protein) without any change in mRNA levels (Castelli et al., 1997). This study by Castelli et al., (1997),

conducted *in vivo*, supports the results obtained from our naltrexone treated SK-N-SH cells, where naltrexone treatment alone for 6 and 24 hrs failed to significantly up-regulate MOR mRNA levels. However, to the contrary, morphine-induced down-regulation of MOR mRNA expression in a rat PNS model of chronic morphine treatment showed that naloxone-induced withdrawal (non-selective opioid antagonist) restored the MOR mRNA to control levels in the DRG (Meuser et al., 2003). With these studies and perhaps more, the effects of naltrexone on MOR mRNA expression are inconsistent between *in vivo* and *in vitro* models. This suggests a significant difference between the models when studying MOR gene expression. Therefore it would be ideal to conduct MOR gene expression studies in both *in vivo* and *in vitro* models with consistent experimental conditions.

In conclusion, our results showed that naltrexone blocked morphine-induced down-regulation of MOR mRNA expression, elucidating a MOR-dependent mechanism without up-regulation MOR mRNA expression.

5.4 Regulation of MOR expression in SK-N-SH cells treated with IL-1β

IL-1β (10 and 100 ng/mL) treatment of SK-N-SH cells for 0.15, 6, 12 and 24 hrs resulted in the concentration dependent up-regulation of MOR expression. Our results were similar to those recorded using RNA dot blot experiments using rat brain primary astrocytes treated with IL-1β (Ruzicka et al., 1996), however this study showed a greater increase (70-80%) in MOR expression, suggesting that rat brain primary astrocytes expressing MOR are more sensitive to IL-1β than our SK-N-SH neurons, which may not be an ideal model for measuring MOR gene expression *in vitro*. An improved *in vitro* model would be to utilize primary brain neurons and microglial cells, the co-culture of neuron and microglia cells

would be ideal from the because neurons would abundantly express MOR and microglia would be an abundant source of IL-1β if stimulated with LPS or morphine. The use of astrocytes by Ruzicka et al., (1996 and 1997) to measure the effects of IL-1β on MOR expression was interesting because it has been published that astrocytes express less MOR than DOR and KOR (Ruzicka et al., 1995). Also, it was demonstrated that IL-1β reduced MOR binding in neural microvascular endothelial cells (NMVEC) (Ahmed et al., 1985; Wiedermann, 1989), suggesting that the effects of IL-1β on MOR expression in neurons may be inconsistent because in our study IL-1β up-regulated the expression of MOR in SK-N-SH neurons. As an example for the measure of inconsistency and perhaps the level of complexity involved in measuring MOR expression regulated by IL-1 in neurons, Vidal et al., (1998), found that when NMVEC were treated with IL-1α and IL-1β independently, low basal levels of MOR expression did not change. However, when NMVEC were co-treated with IL-1α and IL-1β, MOR expression levels were up-regulated, suggesting a possible synergistic effect of IL-1α and IL-1β on MOR expression. Vidal et al., (1998) study was one of three studies; the other studies were by Ahmed et al., (1985) and Ruzicka et al., (1996) that first provide evidence of a relationship between opioid and cytokine actions, highlighting a possible role of proinflammatory cytokines as factors that may modulate opioid-dependent pathways in neuroinflammatory diseases.

Recent *in vivo* studies have concluded IL-1β as an anti-opioid analgesia pro-inflammatory mediator (Hutchinson et al., 2008; Shavit et al., 2005). Shavit et al., (2005) demonstrated for the first time that morphine-induced analgesia was extended in mice strains genetically impaired in IL-1 signaling. From *in vitro* studies discussed earlier (above), we hypothesized that IL-1β would cause the up-regulation of MOR expression in SK-N-SH cells, but if our hypothesis was based on *in vivo* studies, we would have hypothesized that IL-

1β would cause the down-regulation of MOR expression in SK-N-SH cells because the logic mechanism for IL-1β-induced anti-analgesic effects would be at the MOR level. However, because our research model was *in vitro*, it would be an unlikely correlation between *in vivo* and *in vitro* models when studying MOR mRNA expression in response to IL-1β exposure.

More recently, other pro-inflammatory cytokines, IL-4, TNF and IL-6 have been studied as having the ability to affect the expression of MOR expression in neurons and immune cells (Borner et al., 2004; Borner et al., 2007; Kraus et al., 2001; Kraus et al., 2003; Kraus et al., 2006; Ruzicka and Akil, 1997; Ruzicka et al., 1996). Studies completed by Volker Hollts group, which measured MOR expression in response to cytokines in neurons and immune cells greatly complements our work. For example, IL-6, time-dependently increased MOR gene expression and MOR-specific binding with the use of ^{3}H-DAMGO in SH SY5Y cells. With the use of decoy oligonucleotides this group also found that IL-6-induced MOR up-regulation was dependent on transcription factor signal transducers and activators of transcription 1 (STAT 1 and STAT 3) (Borner et al., 2004). The role of IL-6 in nociception was supported in an earlier study where IL-6 had antinociceptive affects in animal model of peripheral inflammation (Czlonkowski et al., 1993).

Studies on IL-1β effects on MOR protein expression are limited at best, therefore our study is perhaps the first to demonstrate that IL-1β regulates the expression of MOR protein. The greatest up-regulation in MOR mRNA (Fig. 27) and protein (Fig. 28) expression was measured following treatment with IL-1β (10 ng/mL) for 6 hrs. IL-1β (10 ng/mL) exposure for 6 hrs consistently caused the up-regulation of both MOR mRNA and protein expression. From a mechanistic perspective, there is an improbable simultaneous event occurring where both mRNA and protein expression are up-regulated in response to IL-1β treatment for 6 hrs. In respect to the Central Dogma (Crick, 1970), the expression of

MOR protein would be expected to up-regulated following MOR mRNA up-regulation. However there are many factors that may influence the rate of translation from mRNA to protein. For example, the factors that may influence the translation of proteins would include mRNA shuttling, mRNA stability, post-translational events, protein stability and cytoplasmic shuttling of MOR into the cell membrane. In our study, mRNA and protein levels were up-regulated following IL-1β (10 ng/mL) exposure for 6 hrs. One possible explanation for the up-regulation of both MOR mRNA and MOR protein expression following exposure to IL-1β would be the signaling cascades that are activated following activation of IL-1β specific receptor, IL-1RI. For example, an obvious question would be to ask if IL-1β-induced signaling is more robust and faster than morphine-induced. The answer(s) to such a question would help explain why the effect of IL-1β on MOR protein up-regulation is more obvious than the MOR protein down-regulation induced by morphine.

The importance of showing the effects of IL-1β on MOR expression is multi-fold. Firstly, it demonstrated that IL-1β affects the expression of MOR at both the mRNA and protein level, complementing studies performed on other pro-inflammatory cytokines, such as IL-4, IL-6 and TNF showed that IL-1β was not the only pro-inflammatory cytokine that could induce MOR expression (Borner et al., 2004; Kraus et al., 2001; Kraus et al., 2003). Secondly, IL-1β-induction of MOR expression in SK-N-SH cells shows that our studies may also compliment earlier studies performed to measure the activity of a cytokine response element (NF-IL-6) on the opioid receptor promoter (Min et al., 1994), supporting the idea that pro-inflammatory cytokines such as IL-1β may regulate the expression of MOR in neurons by increasing the activity of NF-IL-6. In contrast to data from Volker Hollts group and our data, Im et al., (1999) showed that the cytokine response element, NF-IL-6 was nonfunctional in immune cells treated with IL-6, IL-1α and IL-1β, where no significant

increase in MOR expression was measured in U266 and RAW264.7 cells transfected with MOR. The lack of NF-IL-6 activity in immune cells may not translate into neurons. Im et al., (1999) does highlight the need to study other possible mechanisms that cytokines may modulate the expression of MOR. Herein, we aimed to discover a signal transduction pathway protein kinase that might be responsible for the modulation of MOR in response to IL-1β in SK-N-SH cells.

In order to determine the role of IL-1RI, SK-N-SH cells were treated with IL-1RA and IL-1β together for 6 and 24 hrs. Results from these experiments showed that up-regulation of MOR by IL-1β was IL-1RI-dependent. When co-treated with IL-1β, IL-1RA (10 and 100 ng/mL) attenuated the up-regulation of MOR expression induced by IL-1β at both concentrations of IL-1RA and exposure times (Fig. 29). IL-1RA (10 ng/mL) treatment alone showed no significant effect on MOR expression. In conclusion, results show that up-regulation of MOR expression by IL-1β to be occurring in IL-1RI-dependent manner. These were important experiments because it demonstrated that the expression of IL-1RI, as verified using RT-PCR to be functional and responsive to IL-1β binding and that its effects on MOR expression are in part occurring thru IL-1RI.

5.5 Regulation of MOR expression in SK-N-SH cells treated with morphine-IL-1β together

In an attempt to elucidate any complimentary or synergistic effects of IL-1β and morphine treatment on MOR expression, IL-1β + morphine co-treatment experiments were conducted (Fig. 31 and Fig. 32). IL-1β + morphine co-treatment significantly ($p \leq 0.05$) attenuated morphine-induced down-regulation of MOR mRNA expression; therefore IL-1β blocked morphine-induced MOR down-regulation.

It can be interpreted from the IL-1β + morphine co-treatment experiments that IL-1β-induced up-regulation of MOR expression is occurring more robustly. Also the effects of IL-1β on MOR expression are perhaps occurring faster than morphine's effect on MOR mRNA down-regulation. Further studies may need to be completed to elucidate the pathways responsible for IL-1β induction of MOR expression. For example, the classical IL-1β signaling pathway includes the JAK/STAT pathway (Hibi and Hirano, 1998; Park et al., 2005; Yu et al., 2006). As a stress-induced pathway, the JAK/STAT pathway could translate the binding of IL-1β to the IL-1RI onto the MOR promoter quicker than that translated by morphine-MOR binding. Plausible explanations for the signaling delay following morphine-MOR binding could be due to mechanisms followed by all GPCRs; multiple G-proteins are phosphorylated (activated) before down-stream protein kinases (e.g. MAPK) can be phosphorylated. Therefore, depending on the stimuli, a step-wise activation of signaling proteins by GPCRs could delay the down-stream effects as measured at the mRNA level. To the contrary, morphine-induced MAPK activation has been measured to occur from anything as little as one minute to over two hours following morphine treatment (Connor et al., 2004). Another, important caveat in response to these studies is that morphine can induce the internalization (5 minutes) and recycling (30 minutes) of MOR faster than its down-regulation (2 hrs), suggesting that the gene expression of MOR is a relatively slow process that occurs after desensitization, internalization and recycling. From our suggestive data, it would be beneficial to conduct further experiments that would measure the effect of IL-1β on MOR internalization and recycling and correlate these results to the effects of IL-1β on MOR expression. To date the answer to this question has not been comprehensively studied.

Immunocytochemistry (ICC) experiments were conducted to visualize changes in MOR protein expression in response to morphine, IL-1β and IL-1β + morphine together (Fig. 33). The expression pattern of MOR proteins appears to be localized to the cell membrane as limited fluorescence was detected in the cytoplasm. Increased fluorescence was evident in the cells co-treated with IL-1β + morphine, demonstrating up-regulation of MOR protein expression. Previous studies have not demonstrated the expression of MOR protein in SK-N-SH cells using ICC. To highlight an *in vitro* model technicality, ICC studies have been carried out to see changes in MOR protein expression using cell-lines transiently or stably expressing MOR (Madsen et al., 2000). It can be misleading to use *in vitro* models stably or transiently expressing MOR and using ICC studies to observe changes in MOR expression. For example CHO (Chinese hamster ovary) cells expressing MOR provide a disproportionate amount of fluorescence. However, cell-lines expressing MOR in this manner (i.e. CHO-MOR) make ideal models for the measure of MOR binding properties and could be used to measure IL-1β-induced changes in MOR binding, internalization and recycling (Brasel et al., 2008). Our ICC experiments are important to the field of opioid receptor pharmacology because the results in our study provide a relatively accurate *in vitro* model to visualize changes in an endogenously expressing MOR protein in SK-N-SH cells.

The aim of our study was not to measure MOR internalization in SK-N-SH cells, but as part of our discussion, it is important to relate our ICC studies to MOR internalization in response to morphine and IL-1β treatment. Generally, neurons endogenously expressing MOR are localized to the plasma membrane (Keith et al., 1998). However, it is also true that MOR is expressed in cells cytoplasm. For example, surprisingly, >70% of immunogold-labeled MOR was found in the cytoplasm of dendrites of C1 adrenergic neurons in the rat rostral ventrolateral medulla (RVM) (Drake et al., 2005). In regards to opioid-agonist

induced MOR internalization, DAMGO and etorphine caused significant internalization of MOR, but morphine did not (Keith et al., 1998). To compare this to our ICC studies, MOR staining was reduced in response to morphine (10 μM) for 6 hrs (Fig. 33). This difference in morphine effect on MOR protein expression on the plasma membrane could be due to differences in models, with the expression patterns of MOR being a major source of inconsistency. On a similar note, the efficiency of MOR staining in our ICC experiments could have been affected by the differential sub-cellular distribution of MOR in endogenously expressing MOR neurons; the expression and distribution of MOR in such neurons can be less predictable than cells transfected with MOR (i.e. CHO-MOR) (Wang et al., 2008c).

As a determinant of MOR internalization, the results obtained when measuring changes in MOR staining using ICC and results obtained when using binding assay techniques are very different. Many would argue that using the latter technique to measure MOR internalization would have been a more accurate determinant of morphine-induced MOR internalization. However, since our ICC experiments were conducted in triplicate with appropriate antibody controls, decreased staining of MOR visualized in our ICC experiments is most likely caused by morphine treatment. However, this conclusion would have been greatly supported if ICC experiments were completed with naltrexone + morphine treatments. The results from naltrexone + morphine studies would have helped confirm the changes in MOR staining to have been morphine-dependent as naltrexone co-treatment could have blocked the morphine-induced decrease in MOR staining as seen in our MOR mRNA expression studies.

Part III:

Signal transduction mechanism involved in the regulation of MOR expression in SK-N-SH cells treated with Morphine and IL-1β

5.6 Differential regulation of human p38 MAPK genes by morphine and IL-1β

Due to current evidence supporting the role of MAPK signaling in the development of various forms of neural plasticity associated with chronic opioid use, our aim was to study the expression patterns of human p38 MAPK genes. Therefore, to study 84 human p38 MAPK genes, a qRT-PCR array was set up to identify key p38 MAPK genes regulated by morphine and IL-1β. The experiments revealed an opposite effect on the regulation of the p38 MAPK gene in response to morphine and IL-1β treatment. Treatment of SK-N-SH cells with morphine up-regulated p38 MAPK gene greater than three-fold (Fig. 34) and IL-1β treatment down-regulated the p38 MAPK gene by greater than three-fold (Fig. 35). In support of our qRT-PCR array studies, previous studies have attempted to elucidate the role of p38 MAPK in response to IL-1β activation. A number of studies have correlated the role of spinal microglia p38 MAPK to MOR desensitization, internalization and decreased morphine antinociception (Cui et al., 2006; Cui et al., 2008; Gilhotra et al., 2007; Law et al., 2004; Liu et al., 2006a). For example, Cui et al., (2006) demonstrated that chronic morphine treatment induced the activation of spinal microglia and enhanced their p38 phosphorylation; because when inhibited with a specific p38 inhibitor (SB203580), morphine tolerance was significantly attenuated. More recently, Cui et al., (2008) demonstrated that inhibition of microglia activation with minocycline blocked morphine tolerance by inhibiting p38 MAPK activation. Also, by the same group, Lui et al., (2006a) showed the increased activation of p38 MAPK in spinal microglia was mediated by neuronal nitric oxide synthase

(nNOS), which when inhibited, attenuated both morphine tolerance and p38 activation together. The discussion of microglia activation and morphine tolerance is important due to the enhanced cellular relationship that occurs during morphine tolerance; however the expression of MOR in neurons and the role of p38 MAPK are more relevant to our studies. For example, Ma et al., (2001) demonstrated that chronic morphine exposure increased the phosphorylation of p38 in DRG neurons, suggesting that levels of phosphorylated p38 may contribute to the development tolerance to opioid-induced analgesia.

Many studies have provided evidence that supports changes or abnormalities in MAPK signaling involvement in morphine–induced MOR expression and function both *in vivo* and *in vitro* (Ferrer-Alcon et al., 2004; Hutchinson et al., 2008; Ignatova et al., 1999; Kramer and Simon, 2000; Law et al., 2000; Ma et al., 2001; Mouledous et al., 2004; Nakano et al., 1994; Ortiz et al., 1995; Polakiewicz et al., 1998a; Schmidt et al., 2000; Schulz et al., 2004; Schulz and Hollt, 1998; Trapaidze et al., 2000; Wang and Wang, 2006). For example, Mouledous et al., (2004) found that *mu* agonist treatment induced ERK activation acutely or after withdrawal of chronic opioids in one glial cell line and not in three neuronal cell lines. Mouledous et al., (2007) extended this study *in vivo* and found that the ERK signaling cascade is not involved in the development or expression of opioid tolerance and dependence as discovered using an ERK kinase inhibitor, SL327. In contrast to Mouledous et al., (2004; 2007) studies, Schmidt et al., (2000) found that DAMGO treated HEK-MOR cells resulted in the rapid stimulation and transient (3-5 min) activation and nuclear translocation of MAPK. To elucidate the role of MAPK, HEK-MOR cells were exposed to the MAPK inhibitor PD98059, which, not only prevented MAPK activation but also inhibited homologous desensitization of MOR. Therefore, in summary, the role of MAPK signaling in morphine analgesia has been determined as important; however limited evidence

supports the role of MAPK in regulation of MOR expression.

In regards to IL-1β signaling, it was found that the amount of IL-1β produced decreased in LPS-stimulated cells when treated with a p38 MAPK inhibitor (Lee et al., 1994). More importantly, the p38 MAPK pathway is known to have a key role in stabilizing inflammatory response proteins (post-transcriptional) and promotes their translation (Clark et al., 2003; Kracht and Saklatvala, 2002; Kumar et al., 2003). In regards to opioid receptor gene regulation, a cytokine response element has been discovered; however it is unresponsive to IL-1β (Im et al., 1999). The lack of promoter level regulation of MOR expression by IL-1β supports the need to explore other mechanistic pathways responsible for IL-1β regulation of MOR expression. Until recently, evidence correlating the role MAPK genes (i.e. p38 MAPK) in the regulation of opioid receptor by pro-inflammatory cytokines has been limited. However, two groups have concluded that decreased morphine antinociception can be blocked when p38 MAPK is inhibited (Cui et al., 2006; Cui et al., 2008; Hutchinson et al., 2008). For example, Hutchinson et al., (2008) found that when lumbar dorsal spinal cord *in vitro* was treated with morphine, a significant increase in the release of proinflammatory cytokine and chemokine resulted. And for the first time, it was shown that spinal proinflammatory cytokines rapidly (5 minutes) opposed systemic and intrathecal opioid analgesia, causing reduced pain suppression. Hutchinson et al., (2008) also documented that the opposition of analgesia mediated by proinflammatory cytokines cannot be accounted for by an alteration in spinal morphine concentrations and that the acute anti-analgesic effects of proinflammatory cytokines occurred in a p38 mitogen-activated protein kinase and nitric oxide dependent manner. On the same note, but in an independent study, intrathecal (i.t.) IL-1β caused the time-dependent up-regulation of phosphorylated p38 MAPK protein expression in rat spinal cords administered with IL-1β. This study also found

that IL-1β also increased iNOS expression (Sung et al., 2005). If put together, Sung et al., (2005) study suggests that p38 MAPK plays a pivotal role in i.t. IL-1β-induced spinal sensitization and nociceptive signal transduction and if IL-1β can induce p38 MAPK, can it play a role in decreased opioid analgesia as documented by Huchinson et al., (2008).

Recent studies support the role of proinflammatory cytokines in the development of decreased morphine analgesia *in vitro* and *in vivo* in response to IL-1β (Hutchinson et al., 2008). However, to the contrary, our study showed that IL-1β-induced the up-regulation of MOR expression. Increased MOR expression is not normally associated with decreased morphine analgesia and therefore the following question exists - is IL-1β-induced up-regulation of MOR occurring through the similar MAPK pathways as those activated by morphine-induced down-regulation of MOR? In support for IL-1β induced signaling, IL-1β release *in vitro* (Wang et al., 2008b) and IL-1β-induced neural apoptosis is dependent on p38 activation after spinal cord injury (Wang et al., 2005). However, no evidence exists for the role of IL-1β-induced MAPK signaling in the regulation of MOR expression. If IL-1β up-regulation of MOR is MAPK dependent as proven the case for morphine regulation of MOR (see sections 5.7 and 5.8), what will happen to the regulation of MOR expression if their respective MAPKs are inhibited? Another important study would be to understand the mechanism involved in IL-1β-induced down-regulation of p38 MAPK expression (Fig. 35) and how down-regulated p38 MAPK may play a role in MOR expression. From our data, it can be suggested that IL-1β-induced MOR up-regulation is occurring more quickly and robustly than morphine-induced down-regulation of MOR expression (Fig. 32 and Fig. 33). A plausible explanation for the rate of MOR expression by IL-1β and morphine could be due to the activation of p38 MAPK. The threshold for p38 MAPK activation could be different for IL-1β and morphine, where elements that control activation threshold,

subcellular location, and p38 docking in ribosomal protein kinases may be differentially affected by IL-1β and morphine (Tomas-Zuber et al., 2001). Therefore, in summary, it seems that p38 MAPK is a convergence point for IL-1β and morphine-induced signaling pathways that regulate MOR expression in SK-N-SH cells.

5.7 MEK1/2-dependent down-regulation of MOR expression by morphine

In order to elucidate the role of MAPK signaling on the expression of MOR mRNA in response to morphine and IL-1β, the up-stream MAPK signaling protein kinase, MEK1/2 was inhibited using MEK1/2-specific PD98059 (50μM) inhibitor (Fig. 38). MEK1/2 was found to be crucial to the regulation of MOR expression in response to morphine.

As an example of increased MAPK signaling in response to morphine exposure, Ma et al., (2001) studied the intracellular signal transduction pathways involved in morphine-induced increases in CGRP- and SP-IR in vivo and vitro (DRG neurons). From this study, it was found that chronic morphine increased the phosphorylation states of MAPK, ERK, JNK, p38 and CREB both *in vivo* and *in vitro*. Thereby, suggesting that increased MAPK activation plays a major role in morphine-induced increase in spinal CGRP and SP levels, contributing to the development of tolerance to opioid-induced analgesia (Ma et al., 2001). More recently, a study was conducted to elucidate the exclusive role of MEK1/2 following acute, chronic and morphine withdrawal in rat brains. Results from this study concluded that acute (2 hrs) morphine exposure increased MEK1/2 in both the cerebral cortex and corpus striatum by 50-70% and chronic (5 days) morphine exposure failed to significantly increase the phosphorylated state of MEK1/2. However, in morphine-tolerance rats, naloxone-precipitated withdrawal (2-6 hrs) induced robust increases in MEK1/2 phosphorylation in both the cortex (27-49%) and striatum (83-123%); however, spontaneous opioid withdrawal

(24 hrs) in morphine-dependent rats did not alter MEK1/2 activity in the brain (Asensio et al., 2006). The role of MEK1/2 signaling in regulation of MOR expression *in vitro* has previously been shown to be key to the functional modulation of MOR (Polakiewicz et al., 1998a). For example, in an attempt to study intracellular signaling mechanisms triggered by morphine in CHO-MOR cells, Polakiewicz et al., (1998), identified morphine-induced MOR internalization to be dependent on MEK1/2 phosphorylation. Overall, studies on the role of MEK1/2 in the regulation of opioid pharmacology *in vitro* are limited. However contrary to studies stated above, a key study concluded decreased activated (phosphorylated) MEK levels in the pre-frontal cortex samples of human opioid addicts (Ferrer-Alcon et al., 2004). Therefore, if put together with our results, it may be correct to suggest that MEK contributes to the actions of opioids, reducing their analgesic effects following chronic morphine exposure (Polakiewicz et al., 1998a).

The role played by MEK1/2 in IL-1β-induced up-regulation of MOR was insignificant and could easily be further proven to be so following further experiments with inhibitors of protein kinases specific to the IL-1β-IL-1RI induced pathways, i.e. JAK/STAT. From our earlier study; IL-1β + morphine co-treatment attenuated morphine-induced down-regulation of MOR expression. The MEK1/2 inhibitor, PD98059 did not significantly inhibit IL-1β + morphine co-treatment induced up-regulation of MOR expression. Therefore, as a result, it can also be concluded that IL-1β-induced up-regulation of MOR expression is occurring via MEK1/2-independent pathway. Because there are lonely three published studies that have measured changes in MOR expression in response to IL-1β *in vitro* (Ruzicka and Akil, 1997; Ruzicka et al., 1996; Vidal et al., 1998), it is difficult to correlate our MAPK signaling results to these studies, but IL-1β-induced signaling has been associated with the increase of expression of other receptors (Brechter et al., 2008; Zhang et

al., 2007a; Zhang et al., 2007b). The role of MEK1/2 in IL-1β-induced expression of MOR, though performed in different models, seems to be occurring independent of MEK1/2.

5.8 p38 MAPK-dependent down-regulation of MOR expression by morphine

In order to elucidate the role of p38 MAPK signaling on the expression of MOR in response to morphine and IL-1β, p38 MAPK was inhibited using SB203580. It was found that the role of p38 MAPK is crucial to the regulation of MOR mRNA expression in response to morphine and IL-1β treatment (Fig. 39).

Evidence in support of the role of p38 MAPK and its relationship with morphine and IL-1β was recently explored (Hutchinson et al., 2008), where the acute anti-analgesic effects of IL-1β and similar pro-inflammatory cytokines were measured and occurred in a p38 MAPK-dependent manner. In comparison to our study, Cui et al., (2006) found that morphine activated spinal microglia cells had increased p38 immunoreactivity which was exclusively restricted in the activated spinal microglia and not in astrocytes or neurons, suggesting that the increased expression or activity of p38 in response to morphine exposure in neurons is not important. Moreover, in the same study, SB203580 pre-treatment, significantly attenuated tolerance to morphine analgesia assessed by tail flick test (Cui et al., 2006). The role of microglia cells was recently further studied, which supported the role of p38 MAPK as a factor that is central to the (anti/pro)-analgesic properties of pro-inflammatory cytokines (Hutchinson et al., 2008). This study extended previous work that concluded the ultra low dose of morphine induced glial activation, via a p38 dependent mechanism (Wu et al., 2006), and that activated p38 opposed morphine analgesia. Therefore, Hutchinson et al., (2008) co-administered SB20358 and morphine intrathecally, and given that p38 activation is well documented to lead to proinflammatory cytokine production and

release (Svensson et al., 2003; Svensson et al., 2005; Wu et al., 2006), this study would predict a likely involvement in p38 activation in opposing morphine analgesia. Co-administration of SB203580 and morphine in rats potentiated tailflick and hindpaw analgesia compared to morphine alone (Hutchinson et al., 2008).

Our study showed that IL-1β up-regulated the expression of MOR mRNA, however it would be difficult to correlate increased MOR expression with increased anti-analgesic effects of IL-1β as measured by Hutchinson et al., 2008. Decreased analgesic effects of morphine is usually associated with MOR desensitization or internalization, which can be measured following chronic opioid exposure (Bernstein and Welch, 1998). However, recent evidence suggests that opioid receptor internalization could in fact reduce opioid tolerance *in vivo* (Koch and Hollt, 2008). The change in MOR number in response to chronic opioid treatment has long been speculated to directly contribute to receptor desensitization and the development of opioid tolerance; but there is limited literature that supports this idea. First of all, receptor down-regulation after chronic opioid exposure has only been clearly shown *in vitro* (Baumhaker et al., 1993; Yabaluri and Medzihradsky, 1997; Zadina et al., 1993), whereas *in vivo* studies are highly variable (Zadina et al., 1995). In addition, multiple lines of evidence suggest that MOR down-regulation depends on the efficacy of the opioid agonist; but this appears to be non essential for the development of opioid tolerance (Nishino et al., 1990; Trafton and Basbaum, 2004; Yoburn et al., 1993). In summary, these studies do not support a direct correlation between receptor internalization/down-regulation and desensitization that is characteristic of tolerance. In fact, it has been demonstrated that receptor down-regulation requires higher doses and longer exposure times than receptor desensitization (Puttfarcken and Cox, 1989; Puttfarcken et al., 1988).

IL-1β-induced expression of MOR could play a more pro-analgesic role. Evidence suggest that cytokines such as TNF-α, IL-1β and IL-6 induce the release of endogenous opioids from immunocytes, which when bound to opioid receptors expressed in PNS and CNS, induce analgesia (Kapitzke et al., 2005). Moreover, cytokines such as TNF, IL-4, IL-6 and IL-1β contribute to pro-inflammation-induced pain (Borner et al., 2004; Kraus et al., 2001; Schafer et al., 1994; Zollner et al., 2008). Therefore the opposing effects of cytokines on pain transmission highlight the need for further studies on the specific factor(s) that contribute to achieving the balance between the pro-inflammatory and anti-nociceptive roles of IL-1β. Overall, these findings constitute a new concept of intrinsic pain control that involves mechanisms traditionally used by the immune system for mounting a host response to fight pathogens.

In regards to neuroinflammation, the expression of MOR by pro-inflammatory cytokines such as IL-1β may prove to be important in diseases conditions such as multiple sclerosis (MS) and Parkinson's disease where the pain associated with these diseases is often treated with opioids. For example in MS, patients often experience increased pain that is relatively insensitive to opiate treatment, and until recently the mechanistic basis for this increased nociception was poorly understood. Using a Theiler's murine encephalomyelitis virus (TMEV) model of MS to examine possible changes in spinal cord opioid receptor mRNA over the course of disease progression, TMEV infection caused a significant decrease in MOR mRNA expression analyzed by qRT-PCR in both male and female mice at 90, 150 and 180 days post-infection (Lynch et al., 2008). Lynch et al., (2008) study suggests that increased nociception experienced by MS patients may be due to the decreased expression of opioid receptors; however, what is the role for IL-1β in nociception experienced by MS patients. Several studies have implicated IL-1β as the key factor in the

development of MS (de Jong et al., 2002; Luomala et al., 2001; Schrijver et al., 1999; Schrijver et al., 2003) and a few key studies have suggested that IL-1 antagonizes morphine analgesia and contributes to morphine tolerance (Bessler et al., 2006; Shavit et al., 2005). Therefore overall, changes in MOR expression in neurons by cytokines may exacerbate the symptoms of neuroinflammatory diseases such as MS contributing to increased nociception by decreasing the anti-nociceptive properties of opioids. Also in AD where indirectly, increased levels of IL-1β in AD (Shaftel et al., 2008) may increase the expression of MOR in neurons, providing increased binding sites for endogenous opioids (e.g. enkephalin) which have been implicated in contributing to neuronal and behavioral impairments in a transgenic mouse model of AD (Meilandt et al., 2008).

5.9 NF-κB-sensitive down-regulation and up-regulation of MOR expression by morphine and IL-1β respectively

In order to elucidate the role of NF-κB, using a NF-κB inhibitor peptide, SN50, demonstrated that IL-1β-induced up-regulation and morphine-induced down-regulation of MOR expression in SK-N-SH cells was NF-κB-dependent (Fig. 41). In summary, the role of NF-κB in the regulation of MOR expression by IL-1β, morphine and IL-1β + morphine together was measured to be important at the higher of two concentrations of SN50 (50 μM) used (Fig. 41).

Previous research has demonstrated that the activation of GPCRs in leukocytes and neurons can result in the activation or inhibition of NF-κB through various down-stream effector pathways, including the cAMP/PKA/CREB, P13K/Akt/IKK and PLC/PKC/IKK signaling pathways (Ye, 2001). The phosphorylation of NF-κB via effector pathways activated by MOR stimulation is still unclear. It has been shown that acute opioid

treatment activates P13K/Akt signaling (Polakiewicz et al., 1998b), which is known to activate NF-κB (Brunet et al., 2001). GPCRs expressed in endothelial and epithelial cells have been documented to regulate the transcription and contribute to the expression of cytokines, adhesion molecules, and growth factors that are essential for extravasation of leukocytes and tissue repair. NF-κB is one of the most important transcription factors responsible for the expression of proinflammatory genes. Recent studies highlighted in a review article (Fraser, 2008) show that GPCRs utilize several different pathways to activate NF-κB . These pathways differ from the ones induced by classic cytokines in that they are initiated by heterotrimeric G-proteins, but they converge at IκB (phosphorylation) and induce the nuclear translocation/modification of the NF-κB proteins. GPCR-induced NF-κB activation provides an effective means for local expression of cytokines and growth factor genes due to the wide distribution of these receptors. From our results in neurons and other studies (Kraus et al., 2003) in immune effector cells, it can be suggested that due to similarities in signaling pathways between cytokine receptors and opioid receptors in both cell types, both pathways converge at NF-κB proteins to modulate the expression of MOR. For example, Kraus et al., (2003), demonstrated that when stimulated with TNF, B cells (Raji cells) expression of MOR increased to qRT-PCR detectable levels in a time-dependent manner. Also, using decoy oligonucleotides against AP-I and NF-κB, Kraus et al., (2003) concluded that TNF-induced expression of MOR in B cells is NF-κB-dependent. Also, in the same study and more relevant to our neuronal *in vitro* model, they demonstrated in SH SY5Y neurons (which constitutively express MOR), when treated with TNF, multiple NF-κB binding elements are activated in the MOR gene promoter. Therefore the presence of multiple NF-κB binding elements on the MOR gene most probably indicates the importance of NF-κB in MOR expression. In support of Kraus et al., (2003) studies, the relationship

between NF-κB, opioids and opioid receptors was studied using DAMGO (MOR-specific agonist). When primary rat cortical cultures were treated with DAMGO, the DNA binding activity of NF-κB increased (Hou et al., 1996). A more recent study showed that morphine treatment caused increased NF-κB promoter activation in NT2-N neurons and that the substance P (SP) antagonist CP-96345 abolished this activation; substance P activates NF-κB and therefore morphine may activate NF-κB via SP up-regulation (Wang et al., 2004). Also, in a recent attempt to detect MOR expression in a human breast cancer cell line (MCF-7), MOR expression was down-regulated by opioid agonists and up-regulated by opioid antagonists. The authors of this study proposed that the opioid-induced regulation of MOR mRNA expression to be mediated by the reduced binding of NF-κB binding elements to promoter regions on the MOR gene (Gach et al., 2008). Therefore, collectively, these studies heavily suggest that MOR expression is in part NF-κB-dependent.

Until more recently, MOR mRNA expression had not been measured to be significantly repressed following treatment with a NF-κB inhibitor (Philippe et al., 2006). Using a NF-κB inhibitor peptide, SN50, we demonstrated that IL-1β-induced up-regulation of MOR expression in SK-N-SH cells was NF-κB-dependent (Fig. 41). However, from the two concentrations of SN50 (10 and 50 μM) used, the higher concentration (50 μM) showed significant changes in MOR expression (Fig. 41). Therefore, these data suggest that IL-1β activates NF-κB, contributing to the expression of MOR in our neural model, but the role of NF-κB is not central to MOR expression in SK-N-SH cells as demonstrated by the lack of sensitivity to NF-κB inhibition by SN50. In contrast, a previous study has demonstrated that NF-κB is key to the expression of MOR in non-neuronal cell lines (Kraus et al., 2003), implicating that NF-κB is central to IL-1β-induced expression of MOR in immune cells. The central role of NF-κB in MOR expression in immune cells makes more sense than in

neurons because many functions and the development of immune cells are mediated members of the NF-kappaB/Rel transcription factor family (Bendall et al., 1999).

Although our study and other studies (Borner et al., 2004) suggest that the role of NF-κB in the regulation of MOR expression to be unsatisfactorily defined in neurons, our study found that when NF-κB is inhibited, morphine-induced down-regulation of MOR expression was exacerbated. In support of the insufficient role of NF-κB in MOR expression in neurons, the exacerbated down-regulation MOR expression by morphine in the presence of SN50 (NF-κB inhibitor) may be due to nonspecific inhibitory effects on the nuclear translocation of NF-κB-p50. Because the inhibitory effects of SN50 were only measured following the use of a higher concentration (50 μM) it is likely that in SK-N-SH neurons, the impact of NF-κB is less sensitive than MEK and p38 MAPK on MOR expression. Also, at higher concentrations (≥50 μM), SN50 has been documented to also inhibit the NLS of STAT, AP-1 and NFAT transcription factors in immune cells *in vitro* (Torgerson et al., 1998). The role of NF-κB in the regulation of MOR expression may be dependent on the cell line or the *in vivo* model. To further elucidate the role of NF-κB in MOR expression, the use of small interfering RNAs (siRNA) would help narrow the precise role NF-κB heterodimers. Also, the use of siRNA treatment would eliminate unspecific inhibition of other kinases and transcription factors that may modulate the expression of MOR in response to morphine or IL-1β treatment.

Studies have concluded that chronic opioid treatment activates AC, which then leads to the activation of CAMP/PKA/CREB signaling and inhibition of NF-κB in macrophages (Roy et al., 1998), neutrophils and monocytes (Welters et al., 2000b) and in T cells (Wang et al., 2003). Because these studies were performed in non-neural cells lines it would be difficult to relate these findings to neurons. However, in an attempt to correlate these findings to

neurons, chronic opioid-induced inhibition of NF-κB may help explain why when NF-κB was inhibited in our studies by SN50 (50 μM), morphine-induced MOR down-regulation was exacerbated, and this decrease in MOR expression may contribute to the development of reduced opioid analgesia frequently associated by chronic opioid exposure. Research has shown that chronic morphine exposure results in immunosuppression and data has correlated this suppression in the immune system to the inhibition of NF-κB (Bonnet et al., 2008; Eisenstein et al., 1995; Roy et al., 1998; Welters et al., 2000b). In regards to IL-1β regulation, inhibition of NF-κB (despite inhibition of the IL-1β gene transcription) results in enhanced processing of pro-IL-1β, (Ghosh and Karin, 2002). Increased storage of pro-IL-1β prepares microglia cells to release IL-1β in response to neuroinflammation. In respect to MOR expression, it could be suggested that increased levels of pro-IL-1β in response to NF-κB inhibition provides an innate response mechanism, which results in the release of active IL-1β. Therefore increased levels of IL-1β in the neuroinflammatory environment may regulate the expression of MOR in neurons. In support of this theory, it is now established that immunocytes-derived endogenous opioids induce analgesia by activating peripheral opioid receptors at late stages of inflammation (Cabot et al., 2001; Kapitzke et al., 2005; Stein et al., 1990). In this scenario, IL-1β could contribute to maximizing the analgesic effect of endogenous opioids by increasing the expression of MOR in neuronal cells. However, the studies stated above and used to support our theory for the role of IL-1β in MOR expression are opposite to recent data which demonstrated that proinflammatory cytokines (TNF and IL-1β) opposed opioid analgesia *in vivo* (Hutchinson et al., 2008). Therefore from the inconsistency between the data collected on the effects of IL-1β on MOR expression *in vivo* and *in vitro models*, it proves that the role of IL-1β on opioid analgesia remains to be

elucidated and that a multi-cellular (e.g. neurons, immune and glial) approach both *in vivo* and *in vitro* would significantly improve our insight on the role of IL-1β in opioid analgesia.

To put the role of NF-κB in perspective, studies in the last eight years on NF-κB activation in opioid functions and opioid receptor gene expression in both immune cells and neuronal cells have provided some insight into the connection between these two families of molecules (Chen et al., 2007; Kraus et al., 2003; Wang et al., 2004; Welters et al., 2000a; Welters et al., 2000b; Ye, 2001). However further mechanistic studies would benefit our understanding of how NF-κB activity is regulated via opioid receptor signaling and how NF-κB signaling controls opioid gene expression (a feedback control mechanism) in both immune and neuronal cells. Understanding the molecular mechanism involved in NF-κB signaling in opioid function and opioid receptor gene expression would shed some light in to the development of new interventions for immune system-related diseases and opioid tolerance.

Fig. 42 helps illustrate and depict an overview of the cellular mechanisms studied and involved in the regulation of MOR expression by morphine and IL-1β. Fig. 42 shows that chronic opioid administration may act through MOR expressed on microglia cells which then results in the production and secretion of cytokines. Once released, cytokines may then act on postsynaptic neurons to decrease or increase opioid analgesia by modulating the expression of MOR, which is mediated through the activation of protein kinases and transcription factors (i.e. NF-κB).

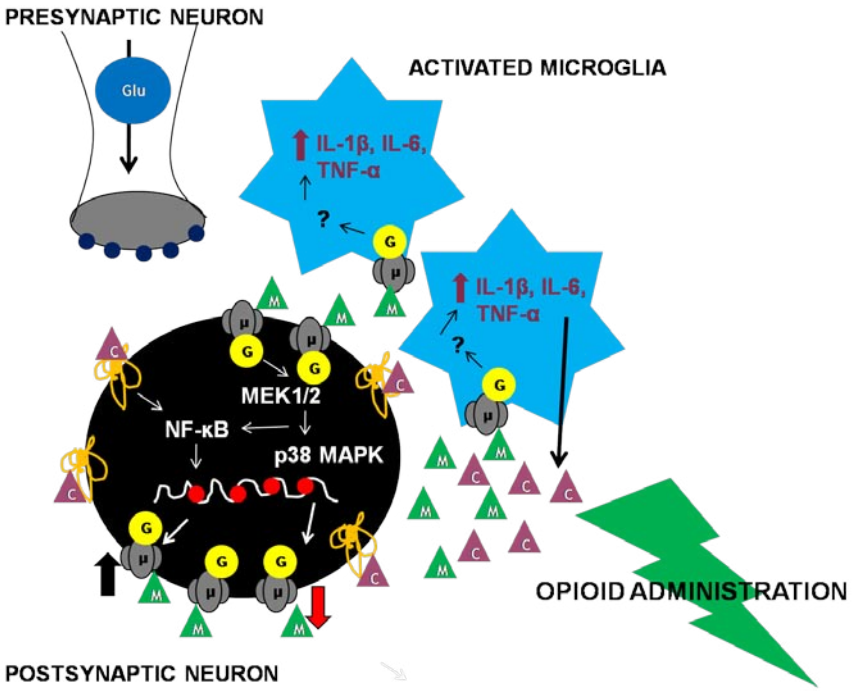


Fig. 42 *Proposed mechanism involved in the regulation of MOR in neurons by morphine and IL-1β (Adapted from DeLeo et al., 2004).*

CHAPTER VI

CONCLUSION

This study has reviewed the role of IL-1β as a proinflammatory mediator known to be released from microglia cells in the CNS in response to injury and contributing to neuropathic pain. In response to neuropathic pain, anti-nociceptive properties of opioids fail to provide efficient and sufficient analgesia. With reduced opioid-analgesia, opioid tolerance and eventual dependence are highly correlated events. Our study aimed to elucidate the role of IL-1β on the expression of MOR in SK-N-SH cells in order to discover a unique immuno-pharmacological relationship. We found that not only did IL-1β induce the expression of MOR but did so in a NF-κB-dependent manner. Morphine induced MOR down-regulation was MAPK-dependent as evident from our MEK1/2 and p38 MAPK inhibitor studies.

Overall, the relationship between immune mediators such as IL-1β and the genes exclusively expressed in the PNS, CNS and immune system such as opioid receptors is complex. Our study discovered and supported novel findings, such as the expression of IL-1RI in human neuroblastoma cells and the ability of IL-1β to out-compete the regulation of MOR expression by morphine. The results from the IL-1β + morphine competition studies opened new avenues for discovery, which would help answer why IL-1β had a more robust and faster effect on MOR expression than morphine.

In order to understand the role of IL-1β on the expression of MOR, further studies would need to be performed and would include multimodal approaches: 1 – use of

primary CNS cell cultures, i.e. ventral tegmental area (VTA) neurons; 2 – use of primary CNS/microglia cell cultures; 3 – use of an *in vivo* neuroinflammatory model; 4 – measure change in cytokine release in response morphine administration; 5 – conduct experiments to elucidate NF-κB-p50 DNA binding sites using siRNA against specific binding sites and 6 – determine the role of microRNAs on the expression of MOR in response to morphine and IL-1β *in vivo*. Studying the role of microRNAs on MOR expression would provide a detailed insight into MOR mRNA stability and how morphine treatment would affect mRNA stability.

Taken together, *in vivo* studies measuring neuroimmune and opioid analgesic interactions suggests that the neurons, in concert with pro-inflammatory cytokines contribute to the hypersensitivity and decreased efficacy of opioid in chronic neuropathic pain states. Our study showed that IL-1β up-regulates the expression of MOR mRNA expression in neurons *in vitro*. Because *in vitro* and *in vivo* data on the relationship of proinflammatory cytokine and MOR expression and opioid analgesia have opposing results, an understanding of the relationship between the individual components interacting to modulate pain transmission and analgesia is far from complete in both the *in vivo* and *in vitro* models. A more comprehensive study would certainly highlight key components of the neuroimmune system that mediates opioid analgesia, shedding some light into the development of new interventions for neuropathic related pain states.

To review, the challenge from the perspective of pain is to exploit the knowledge developed from understanding the complex layers of the neuro-immmuno system that would aid the discovery of novel analgesic strategies. With the success of anti-cytokine based treatment (i.e. anti-TNFα- and IL-1-neutralizing antibodies) for the treatment of pain (Schafers et al., 2001; Sommer et al., 2001; Sommer et al., 1999), it might also be important,

but highly creative to seek the discovery of a compound that would be based on opioids but combine the anti-TNFα- and IL-1-neutralizing antibodies effect on pain. The use of such a hybrid compound to treat pain would have both anti-nociceptive effects without the side effects and tolerance associated with opioids, making this compound an ideal and eventually normal for treating neuropathic pain in the future.

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VITA

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Candidate for the Degree of
Doctor of Philosophy

Thesis: SIGNALING PATHWAYS INVOLVED IN IL-1BETA-INDUCED REGULATION OF MOR EXPRESSION IN NEURONS.

Major Field: Molecular Pharmacology

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Institution: Oklahoma State University **Location**: Tulsa, Oklahoma

Title of Study: SIGNALING PATHWAYS INVOLVED IN IL-1BETA-INDUCED REGULATION OF MOR EXPRESSION IN NEURONS.

Pages in Study: 160 Candidate for the Degree of Philosophy

Major Field: Molecular Pharmacology

Scope and Method of Study:
The scope of this thesis was to study the effects of pro-inflammatory cytokine, IL-1β on the expression of MOR in SK-N-SH neurons in vitro. Used real-time PCR to measure changes in mRNA expression of MOR and immunocytochemistry (ICC) to see changes in MOR proteins levels as captured using Confocal microscopy.

Findings and Conclusions:
Our finding concluded an IL-1β-dependent up-regulation of MOR expression at both the mRNA and protein levels. Also, the effects of IL-1β on MOR expression were measured to be more robust than that induced by morphine, implicating a signaling pathway dependent process. Further studies using signaling inhibitors alluded to the role of MEK1/2 and p38 MAPK in the regulation of MOR expression following morphine treatment and an NF-κB-dependent mechanism for the regulation of MOR expression following IL-1β treatment.

Further studies to elucidate the precise role of IL-1β on the expression of MORs would be multiple-modal: 1 – primary CNS cell culture; 2 – primary CNS/microglia cell culture; 3 - neuroinflammatory, *in vivo* studies; 4 – measure cytokines released in response morphine administration, measure changes in mRNA and protein levels of MOR in the CNS of *in vivo* models; 5 – elucidate the role of microRNAs on the expression of MOR mRNA.

Overall, the relationship between the immune mediators such as IL-1β and the genes exclusively expressed in the CNS such as opioid receptor is complex and multimodal. Our study discovered and supported novel findings, such as the expression of IL-1RI in human Neuroblastoma cells and IL-1β ability to out-compete the regulation of MOR expression by morphine. Results from these IL-1β-morphine competition studies have opened new avenues for discovery, which would help answer why IL-1β had a more robust and 'faster' affect on MOR expression.

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