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Immuno-Opioid Interaction A Potential Connection

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Immuno-Opioid Interaction: A Potential Connection?

By

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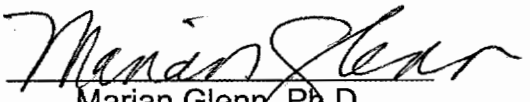

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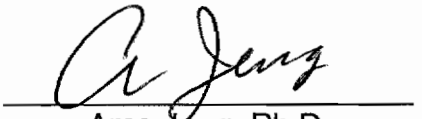

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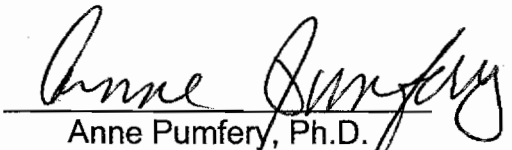

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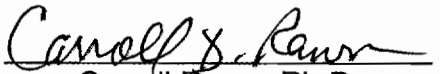

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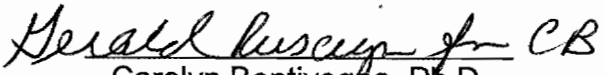

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Abstract

In a chronic morphine state the balance between the opioid system and the immune system is altered. This may result in an increased susceptibility to infection and disease. Prior work in our laboratory has shown that a desensitization of the hypothalamic-pituitary-adrenal axis occurs in the chronic morphine state resulting in increased serum levels of LPS-induced pro-inflammatory cytokine levels such as TNF-α, IL-1β and IL-6 in a rodent model. Based on these findings, I hypothesized that chronic morphine exposure alters the cytokine expression profile in the rat brain and that a potential immuno-opioid pathway may exist in which the mu-opioid receptor (MOR) may be modulated in the presence of pro-inflammatory cytokines. In a rodent model of morphine tolerance, there was an increase in the pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines. Using a human astrocytic cell line, IL-1β exposure up-regulated MOR, which was mediated through IL-1 receptor 1 IL-R1 both in the morphine naïve and chronic morphine state. These data suggest that the inflammatory response is elevated in the brain during morphine tolerance and that an immuno-opioid pathway may allow for the modulation of MOR in response to a pro-inflammatory stimulus.

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Introduction

The immuno-neuroendocrine modulation of the hypothalamic-pituitary-adrenal (HPA) axis during inflammation

The immune system and the nervous system express similar molecules such as cytokines and neurotransmitter receptors. The expression of similar molecules facilitates the bidirectional coordination between the two systems that are essential for managing the immuno-neuro response to stresses such as disease or inflammation (Chesnokova and Melmed, 2002). This cross-talk between the immune and the nervous systems allows for the modulation of the hypothalamic-pituitary-adrenal (HPA) axis and the two systems interact in order to maintain physiological homeostasis (Chesnokova and Melmed, 2002).

Activation of the HPA axis occurs in response to triggers such as stress, anxiety, inflammation and disease (Orth, 1995). Upon activation, corticotrophin-releasing hormone (CRH) is released from the paraventrivular nucleus (PVN) in the hypothalamus, which then stimulates the release of pro-opio-melanocortin (POMC) peptide from the anterior pituitary gland (Slominski *et al.*, 2005). POMC is then cleaved into adrenocorticotropic hormone (ACTH) which results in corticosterone production in rats or cortisol production in humans, by cells of the adrenal

zona fasciculata. Subsequently, corticosterone or cortisol, a potent glucocorticoid, is secreted into the bloodstream. The increase in glucocorticoid levels then negatively regulates the HPA axis through a feedback loop (Hadley, 1996).

Anterior pituitary hormone regulation, upon activation of the HPA axis, is proposed to be under three tiers of control (Melmed, 2001). In the first tier, the hypothalamic nuclei produce and release stimulatory and inhibitory hormones. These hormones pass through the blood system that connects the hypothalamus to the anterior pituitary and activate receptors located on the anterior pituitary cells. Receptor activation then results in hormone synthesis and secretion (Melmed, 2001). In the second tier of control, autocrine, paracrine or centrally derived factors such as cytokines directly regulate anterior pituitary cell function, by either working independently or in conjunction with hypothalamic hormones (Melmed, 2001). The third tier of control utilizes the production of anterior pituitary hormones which serves to increase the production of distally derived hormones, which then provide a negative feedback loop that suppresses further hormone secretion from the anterior pituitary (Melmed, 2001).

During inflammation, the activation of the HPA axis is an important protective mechanism as the induction of corticosteroids provides anti-

inflammatory properties by inhibiting the secretion of pro-inflammatory cytokines. Cytokines control the second and third tiers of the HPA axis. Excessive stimulation of the HPA axis results in immuno-suppression and increased susceptibility to infections, such as gram-negative bacterial infections (Melmed, 2001). In immunocompromised states, gram-negative bacterial infections can result in the development of sepsis, a systemic response to an infection that results in multi-organ system failure and possible death (Melmed, 2001).

Bacterial endotoxin and the inflammatory response

The bacterial endotoxin lipopolysaccharide (LPS) is a complex glycolipid component of the gram-negative bacterial cell wall and is a potent inducer of the inflammatory response (Schletter *et al*,, 1995). LPS is a commonly used model molecule to study the mechanisms behind the neuroendocrine responses to gram-negative bacterial infections (Turnbull and Rivier, 1999). LPS exposure induces an acute inflammatory response by binding to the serum glycoprotein LPS-binding protein and forming a complex which then binds to the CD14R, a toll-like receptor (TLR), expressed on macrophages, monocytes and neutrophils. The activation of CD14 then results in the release of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor

necrosis factor-alpha (TNF-α) (Chen *et al.*, 2005). The production of pro-inflammatory cytokines in turn induces the expression of endothelial-adhesion molecules, an early measure of inflammation, resulting in the facilitation of bacteria-induced leukocyte recruitment and ultimate initiation of an acute inflammatory response (Meng and Lowell, 1997; Fujihara *et al.*, 2003; Davenpeck *et al.*, 1998). The acute inflammatory response may, when in the presence of high doses of LPS, result in the development of endotoxic shock and sepsis (Meng and Lowell, 1997; Fujihara *et al.*, 2003).

Under normal conditions, the release of glucocorticoids serves to inhibit LPS-induced release of pro-inflammatory cytokines and leukocyte recruitment resulting in an inhibition of the activation of the inflammatory response (Davenpeck *et al.*, 1998). Repeated doses of non-lethal amounts of LPS, in rodent models, have been shown to desensitize the HPA axis as measured by a decrease in choline levels, a precursor to acetylcholine and a marker for cell loss and dysfunction, in the paraventricular nucleus of the hypothalamus (Chang *et al.*, 2005). The hypothalamic deregulation results in decreased levels of corticosterone and a subsequent inhibition of the anti-inflammatory response occurs (Chang *et al.*, 2005).

Initiation of the inflammatory response: leukocyte-endothelial adhesion (LEA) and leukocyte flow (flux)

Under normal physiological conditions, leukocytes are restricted to the central blood flow in the lumen of the blood vessel where they travel at a constant velocity along the blood vessel. Periodically, leukocytes come into contact with the endothelial lining; however, there is minimal adhesion of the leukocytes to the endothelial lining (Chang *et al.*, 2000). In response to an inflammatory stimulus, such as the endotoxin LPS, or pro-inflammatory cytokines, such as IL-1β, TNF-α or IL-6, there is an increase in the production of the cellular adhesion molecules present on the vascular endothelial cells and leukocytes. This increase in adhesion molecules results in adhesion of leukocytes to the endothelial lining also known as leukocyte-endothelial adhesion (LEA). As LEA increases, there is a concurrent decrease in the leukocyte flow (also referred to as flux) in the blood vessel wall, as the leukocytes adhere to each other on the endothelial lining, rather than flow through the lumen (House *et al.*, 2001). Subsequent to an increase in LEA and a decrease in flux, leukocytes extend pseudopodia and transmigrate through gaps between the endothelial lining of the blood vessel. Once through the endothelium, the leukocytes migrate into the underlying tissue to the source of inflammation or injury along a chemotatic gradient (House *et al.*, 2001).

The inflammatory response: the induction of pro-inflammatory cytokines and chemokines

Cytokines and chemokines are typically induced in response to injury or inflammation and are important mediators of the HPA axis. Cytokines consist of a large group of pleiotropic polypeptides that are constitutively expressed and serve as paracrine or autocrine regulators (Chesnokova and Melmed, 2002). Pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 activate the HPA axis by stimulating the paraventrivular nucleus (PVN) in the hypothalamus which results in the secretion of corticotrophin-releasing hormone (CRH). The increase in CRH results in an increase of cortisol secretion in a human model (Chesnokova and Melmed, 2002). In addition to activating the HPA axis, pro-inflammatory cytokines increase the expression of neurotransmitter systems such as opioid receptors suggesting that pro-inflammatory cytokines may play a role in the opioid pathway (Chesnokova and Melmed, 2002; Ruzicka and Akil, 1997).

Chemokines are a family of small chemotatic cytokines produced by macrophages and endothelium cells that are critical for the initiation of the immune response (Huang *et al.*, 2000; Aloisi *et al.*, 1992). Chemokines, such as interleukin-8 (IL-8), recruit leukocytes through a chemotatic gradient, across the endothelial, to the site of injury or

inflammation, an event that occurs concurrently with increased LEA and decreased flux (Rot *et al.*, 1996). Whereas cytokines are detectable in a normal physiological state, chemokines are typically not constitutively expressed and rather are seen in response to an inflammatory stimulus such as pro-inflammatory cytokines (IL-1β and TNF-α) (Zhang and Oppenheim, 2005; Aloisi *et al.*, 1992). A correlation between pro-inflammatory cytokines and chemokine levels has been observed and this correlation suggests that a convergent interaction between pro-inflammatory cytokines and chemokines occurs during injury or inflammation in the central nervous system (Zhang and Oppenheim, 2005; Aloisi *et al.*, 1992).

Interleukin-1 (IL-1)

The interleukin-1 (IL-1) family of pro-inflammatory cytokines is produced by a wide variety of cells such as glia, astrocytes, neurons, monocytes and endothelial cells (Vidal *et al.*, 1998; Dinarello, 2000). Members of the IL-1 family consist of IL-1α, IL-1β and IL-1 receptor antagonist (IL-1RA). IL-1α and IL-1β are derived from different genes, but bind to the same receptor and produce the same agonistic effects. IL-1RA is an endogenous receptor antagonist that does not elicit a signal transduction signal but rather binds to one of the IL-1 receptors and by

doing so blocks IL-1α or IL-1β from binding to the receptor (Vidal et al., 1998; Dinarello, 2000). The IL-1 family binds to one of two distinct receptors, interleukin-1 receptor 1 (IL-1R1) and interleukin-1 receptor 2 (IL-1R2). Both IL-1R1 and IL-1R2 are monomers and are members of the toll-like receptor family (TLR). Upon ligand binding and receptor activation of IL-1R1, accessory molecules, such as IL-1R accessory protein (IL-1RAcP) and myeloid differentiation factor 88 (MyD88), required for the initiation of the signaling cascade are recruited to the receptor. The signaling cascade is initiated and ultimately results in the activation of the transcription factor nuclear factor-kappa B (NF-κB) (Barksby *et al.*, 2007). Whereas IL-1R1 is a functional receptor, IL-1R2 is a decoy receptor that acts as a competitive inhibitor of IL-R1. Upon binding of IL-1 to IL-1R2, there is no recruitment of adaptor molecules and intracellular signaling is not initiated, rather IL-1R2 serves to regulate the amount of IL-1 that can be bound to IL-1R1 (Sunyer *et al.*, 1999).

The relationship of IL-1 and inflammation in the central nervous system is well characterized. During injury or inflammation, IL-1 expression activates the paraventricular nucleus (PVN) of the hypothalamus which results in the release of corticotrophin-releasing hormone (CRH) and subsequent activation of the hypothalamic-pituitary-adrenal (HPA) axis (Dinarello, 2000). In addition to activating the

secretion of CRH, IL-1 activates pro-inflammatory cytokines and chemokines, such as IL-6 and IL-8, respectively (Barton, 1997; Harada *et al.*, 1994).

As with an injury or inflammation, IL-1 expression increases in the rat brain and rat serum following exposure to LPS (Ocasio *et al.*, 2004; Chen *et al.*, 2005). *In vivo* studies in a rat model have indicated that in response to LPS induced IL-1, there is an increase in LEA, an early marker of inflammation, on the vascular endothelial cells and leukocytes (Ato *et al.*, 2002; Li *et al.*, 2002). Whereas the effects of IL-1 on the inflammatory response have been well characterized, the effects of IL-1 on the opioid system have not been studied as extensively. Early studies have shown that IL-1 increases the expression of opioid receptors in primary human glial cells (Ruzicka and Akil, 1997) and human brain microvascular primary cells (Vidal *et al.*, 1998) suggesting that this pro-inflammatory cytokine may posses neuromodulatory effects and may be an important component in the immune-opioid circuit.

Tumor necrosis factor-alpha (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic pro-inflammatory cytokine that is a member of the tumor necrosis factor (TNF) family. TNF-α plays a critical role in innate immunity, a non-specific immune response that provides early defense against a pathogen by activating macrophages to further secrete pro-inflammatory cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6), and chemokines, such as interleukin-8 (IL-8) (Popa *et al.*, 2007). TNF-α is also essential for the adaptive immune response, a highly specialized process that uses immunological memory to eliminate pathogens. During an adaptive immune response, TNF-α is secreted by CD4+ T helper-1 cells (Th1) which in turn induce either a cell mediated immune response or an antibody based immune response. The cell mediated immune response resulting in macrophage activation wherease the antibody based immune response results in activation of B lymphocytes that in turn secrete antibodies that specifically bind to pathogens (Kulmatycki and Jamali, 2005).

Activation of TNF-α is mediated through the binding to one of two receptors, tumor necrosis factor receptor type 1 (TNF-RI) or tumor necrosis factor receptor type 2 (TNF-RII) (Popa *et al.*, 2007). The two

receptors differ in their signaling pathways. TNF-RI activation results in the induction of apoptosis, through the binding of TNF receptor-associate death domain protein (TRADD) (Li *et al.*, 2002; Popa *et al.*, 2007). Upon ligand binding to TNF-RII, TRADD is not recruited but rather there is an activation of the transcription factors NF-κB and activator protein-1 (AP-1), which induces genes involved in acute and chronic inflammatory responses (Li *et al.*, 2002; Popa *et al.*, 2007).

In response to an inflammatory stimulus, TNF-α is secreted by macrophages and monocytes which activate the HPA axis resulting in the secretion of adrenal glucocorticoid (Xiao *et al.*, 2001). Additionally, TNF-α mediates the immune response by increasing leukocyte-endothelial adhesion (LEA) and enhancing leukocyte-endothelial permeability (Parrillo, 1990; 1993). This pro-inflammatory cytokine is a primary mediator of the LPS-induced immune response and increased levels of TNF-α have been observed in the rat serum and rat brain in the presence of LPS (Ocasio *et al.*, 2004; Chen *et al.*, 2005). Furthermore, LPS induced TNF-α in human plasma stimulates the release of pro-inflammatory cytokines, such as IL-1, and chemokines such as IL-8 (Eggesbo *et al.*, 1996).

TNF-α's immune properties are well characterized as is it's relationship with the opioid system. TNF-α is capable of inducing the expression of the mu-opioid receptor (MOR) in a human neuroblastoma cell line, SH-SY5Y, through the activation of TNF-RII, the pro-inflammatory signaling pathway of TNF-α (Zadina *et al.*, 1993; Kraus *et al.*, 2008). Additionally, treatment with anti-TNF-α decreases antinociception, pain mediated through the opioid receptor, in an *in vivo* rodent model of neuropathic pain and injury (Rittner *et al.*, 2005). Similarly, naltrexone, an antagonist to MOR, reduces LPS-induced TNF-α in an *in vivo* rodent model of sepsis suggesting that TNF-α may serve as an important element in the immune-opioid relationship (Greeneltch *et al.*, 2004).

Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine expressed by activated monocytes, macrophages and endothelial cells in inflamed tissues (Moller and Villiger, 2006). IL-6 is an important mediator in the acute phase of the immune response, an early phase where an increase in pro-inflammatory cytokines occurs, as it stimulates energy mobilization, which results in an induction of fever (Barton, 1997). IL-6 is induced by elevated levels of TNF-α and/or IL-1β and binds to the IL-6 receptor (IL-6R) (Barton, 1997).

The IL-6R is widely distributed throughout various cells such as lymphocytes, monocytes and endothelial cells. IL-6R is a cell surface cytokine receptor composed of a ligand binding chain and a signal transducing component, glycoprotein 130 (gp130). Upon ligand binding to the IL-6R, dimerization of gp130 and IL-6R occurs resulting in an activation of the Janus kinase (Jak) and signal transducer and activator of transcription (STAT) signaling pathway which induces the expression of inflammatory genes (Heinrich *et al.*, 2003; Barton, 1997).

Whereas IL-1β and TNF-α possess pro-inflammatory properties, IL-6 elicits both pro-inflammatory and anti-inflammatory effects (Moller and Villiger, 2006). The pro-inflammatory properties of IL-6 include its ability to induce the expression of adhesion molecules both in the endothelium and in leukocytes which result in an increase in leukocyte-endothelial adhesion (LEA) (Wong *et al.*, 2003). IL-6's anti-inflammatory properties arise from the cytokine's ability to induce the production of adrenocorticotropic hormone (ACTH) in the HPA axis which inhibits TNF-α and IL-1 synthesis, as has been observed in both *in vivo* and *in vitro* models (Barton, 1997). IL-6's anti-inflammatory aspects are seen in the presence of LPS, where with an elevation of IL-6 in the brain and serum in a rodent model, there is a subsequent inhibition of IL-1 and TNF-α synthesis (Van Meir, 1995; Chen *et al.*, 2005; Barton, 1997). This is

consistent with the clinical progression of sepsis in which IL-6 peak serum levels are delayed, as compared to TNF-α and IL-1β, and this delay is directly related to a decrease in TNF-α and IL-1β plasma levels (Boontham *et al.*, 2003).

IL-6's ability to serve as a pro-inflammatory or anti-inflammatory cytokine is well established. The ability of IL-6 to mediate the opioid pathway has also been examined. As with IL-1 and TNF-α, IL-6 can up-regulate the MOR in a human neuroblastoma cell line, SH-SY5Y (Borner *et al.*, 2004). IL-6 up-regulation of MOR has also been correlated with increased antinociception, opioid receptor mediated pain. Co-treatment with nalaxone, a MOR antagonist, and IL-6 decreased antinociception in an *in vivo* rodent model, suggesting that IL-6 may serve as a regulator of inflammatory pain and may be essential in the immuno-opioid pathway (Członkowski, *et al.*, 1993; Kraus *et al.*, 2008).

Interleukin-8 (IL-8)

IL-8, also known as C-X-C motif chemokine 8 (CXCL8), is a member of the chemokine family that is secreted during injury or inflammation (Mahajan *et al.*, 2002). IL-8 is produced by phagocytes, lymphocytes and endothelial cells in response to bacterial endotoxins such as LPS (Boontham *et al.*, 2003). A neutrophil chemotactic

polypeptide, IL-8's primary function is to increase leukocyte-endothelial adhesion (LEA), activate neutrophils to the site of inflammation and to release lysosomal enzymes, which aid in phagocytosis (Harada *et al.*, 1994; Rot *et al.*, 1996). IL-8 production is not constitutive but rather occurs in the presence of an inflammatory stimulus. In the normal physiological state, IL-8 levels are not detectable in plasma; however, exposure to an inflammatory stimulus, such as LPS, results in a rapid induction of IL-8 expression (Harada *et al.*, 1994). In addition to LPS induced IL-8 expression, pro-inflammatory cytokines, such as IL-1β and TNF-α also results in a rapid and massive induction of IL-8 (Aloisi *et al.*, 1992).

The relationship of IL-8 and the opioid system has also been established. Chronic morphine exposure has been shown to decrease IL-8 gene expression in human astrocytic cells (U87 MG) (Mahajan *et al.*, 2002). Treatment with β-funaltrexamine, an antagonist to MOR, results in a reversal of morphine induced IL-8 inhibition (Mahajan *et al.*, 2002). Additionally, IL-1β induced IL-8 up-regulates the kappa-opioid receptor (KOR) in a human epithelial model, Caco-2 cell line (Neudeck *et al.*, 2003). Treatment with nor-binaltorphimine, an antagonist to KOR, decreases IL-8 expression suggesting that an opioid antagonist that

alters chemotactic stimulus and chemokines such as IL-8 may play a part in the immuno-opioid circuitry (Neudeck *et al.*, 2003).

Chronic morphine exposure and the inflammatory response

Various endogenous mechanisms exist in which the body responds to physiological pain. The dorsal horn of the spinal cord functions as one such endogenous mechanism as peripheral nerve impulses are modulated prior to being transmitted to the central nervous system (CNS) to evoke the perception of pain. The immune system, through the release of pro-inflammatory cytokines, interacts with the peripheral sensory nerve endings of the afferent nerves to inhibit antinociception, and decrease pain (Stein, 1995). Low basal levels of opioid receptors have been identified in the peripheral system on the dorsal horn of the spinal cord and upon exposure to an inflammatory stimulus an up-regulation of the opioid receptors is observed which assists in reducing antinociception (Stein, 1995). In addition to mediating pain, the activation of opioid receptors inhibits neuronal excitability resulting in an analgesic response. Additionally, opioid receptor activation increases the release of pro-inflammatory cytokines, such as IL-1β and TNF-α, which may facilitates the immune response in an effort to minimize the physiological pain (Stein, 1995).

Endogenous opioids such as endomorphin-1 and endomorphin-2, are released in the CNS as part of the inflammatory response and result in the development of fever (Patel *et al.*, 1996). Synthetic opioids, such as morphine or heroin, are associated with endocrine and immune regulation (Patel *et al.*, 1996; Sharp, 2003) and are well known for their analgesic effects (Kogel *et al.*, 2005). Opioids, both synthetic and endogenous, elicit their effects by binding to one of three opioid receptors, mu-opioid receptor (MOR), delta-opioid receptor (DOR) or the kappa-opioid receptor (KOR) (Mizoguchi *et al.*, 2002). The three opioid receptors are seven transmembrane domain G protein-coupled-receptors (GPCR). Upon stimulation of an opioid receptor, there is an activation of the G_i/G_o protein which results in the activation of adenylyl cyclase and there is an increase in the conversion of intracellular cyclic adenosine triphosphate (ATP) to intracellular cyclic adenosine monophosphate (cAMP), resulting in increased cAMP levels. Additionally, activation of the G_i/G_o protein results in an inhibition of calcium channels, activation of potassium channels and mitogen-activated protein kinases (MAPK) (Vidal *et al.*, 1998; Connor *et al.*, 2004). Opioids elicit their effects via the μ opioid receptor (MOR) and morphine has a high affinity for MOR, which is expressed in the CNS, in microglial cells as well as in immune cells,

exemplified by macrophages and T cells (Sedqi *et al.*, 1996; Tomassine *et al.*, 2004; Borner *et al.*, 2007).

Chronic morphine use results in addiction and morphine addicts have an increased incidence of viral hepatitis, bacterial pneumonias, endocarditis, tuberculosis, and infections of the central nervous system (Roy *et al.*, 2006; Kreek *et al.*, 2005). The mechanism by which morphine alters the immune system is complex as it can be an immunosuppressive as well as an immunostimulative. Morphine directly binds to the mu-opiate receptor (MOR) located on immune cells and upon doing so results in immunosuppression as determined by a decreasing the activity of natural killer cells, lymphocyte proliferation, antibody formation and macrophage phagocytosis (Liang-Suo *et al.*, 2002). Interestingly, whereas chronic morphine suppresses the function of the immune cells, chronic morphine exposure can stimulate the immune response indirectly by suppressing the HPA axis. The suppression of the HPA axis results by a desensitization of the PVN (Chang *et al.*, 1993; House *et al.*, 2001). This desensitization results in an inhibition of the cascade associated with the production of CRH and leads to suppression of the HPA axis, which results in a decrease in corticosterone, a glucocorticoid with immunosuppressive properties, of up to 20% (House *et al.*, 2001; Ocasio *et al.*, 2004).

In addition to suppressing the HPA axis, chronic morphine exposure has also been shown in the rate model to increase the production and activity of various cytokines, including IL-1β (Chang *et al.*, 1998), IL-6 (Zubelewicz *et al.*, 2000) and TNF-α (Kapasi *et al.*, 2000). Morphine treatment also potentiates LPS-induced serum levels of IL-1β, TNF-α and IL-6, suggesting that morphine exposure may intensify the effects of LPS (Ocasio *et al.*, 2004).

Human immunodeficiency virus (HIV) infection and the inflammatory response

As of 2007, 40 million individuals worldwide were infected with the human immunodeficiency virus (HIV) (World Health Organization, 2007). Of the HIV infected individuals, one-third of them are also addicted to opioids and among the opioid users there is an exacerbation of HIV disease progression and neurological complications such as meningitis or HIV-associated-dementia (HIVD) (World Health Organization, 2007; Turchan-Cholewo *et al.*, 2008). Neurological complications associated with HIV infection result from HIV entry into the brain where subsequent damage occurs. Autopsies from individuals with HIVD have shown an increase in microglial cells and astrocytes and a decrease in synaptic density and neuronal loss as compared to normal individuals (Turchan-Cholewo *et al.*, 2008). Although neuronal loss is observed in the brains

of HIVD individuals, neurons do not possess the appropriate chemokine receptors to sustain HIV infection. Rather HIV infected microglial cells and astrocytes produce oxide radicals, proteases and pro-inflammatory cytokines and chemokines that interfere with neuronal function resulting in neuronal loss (Speth *et al.*, 2005). The lack of receptors to sustain a HIV infection makes neurons a less ideal model to study HIV related illnesses. However, a more appropriate model may be microglial cells or astrocytes which posses the appropriate chemokine receptors, such as CXC chemokine receptor-4 (CXCR4) or CC chemokine receptor-3 (CCR3), to sustain HIV infections and are capable of being infected with HIV (Turchan-Cholewo *et al.*, 2008).

Microglial cells are the resident brain macrophages that are essential for the brain's defense against infectious agents (Speth *et al.*, 2005). Microglial cells, whose functions include phagocytosis, antigen presentation and the production and secretion of cytokines make up approximately 12% of the cells found in the CNS (Minagar *et al.*, 2002). Microglial cells can be found in one of three stages, the resting state which is typically found in the CNS of a normal adult, the activated non-phagocytic macrophage state seen in CNS inflammation and the phagocytic and reactive microglial cell, typically found during an infection (Minagar *et al.*, 2002). During CNS inflammation, activated microglial

cells produce chemoattractant chemokines, such as IL-8 as well as pro-inflammatory cytokines such as IL-1β and TNF-α, as part of the inflammatory response (Mahajan *et al.*, 2005; Liang *et al.*, 2008). In addition to possessing the ability to produce immuno-mediatory components, such as pro-inflammatory cytokines and chemokines, microglial cells also possess the MOR suggesting that a potential immuno-opioid relationship may exist in the brain (Chao *et al.*, 1997). The immuno-opioid relationship becomes evident with morphine use which activates the MOR in microglial cells and results in a suppression of chemotaxis, a process necessary for transmigration of leukocytes (Chao *et al.*, 1997) and phagocytosis (Peterson *et al.*, 1995). Whereas morphine treatment suppresses chemotaxis and phagocytosis, morphine treatment increases cytokine production, cytokine release (Peng *et al.*, 2000; Wetzel *et al.*, 2000) and the expression of functional cytokine receptors in microglial cells (Rogers and Peterson, 2003), a response that is consistent with chronic morphine use in *in vivo* rodent models (Ocasio *et al.*, 2004; Chang *et al.*, 1998). The relationship of the immuno-opioid pathway in the microglial cells has been studied extensively. Additionally, the role of the immuno-opioid relationship as it relates to HIV in microglial cells has also been examined. Recent *in vivo* work has shown that an inhibition of microglial cell migration occurs towards a chemokine

associated with HIV-infection, RANTES, in the presence of morphine. This decrease in microglial cell migration in the presence of an opioid suggests that MOR agonists such as morphine, may serve to minimize the inflammatory aspects of microglial cells infected with HIV, as is seen with HIVD (Hu *et al.*, 2000).

Whereas significant research has been conducted in the immuno-opioid relationship of microglial cells, minimal work has been done to date on this relationship in astrocytes, a CNS cell type capable of maintaining an HIV infection. Astrocytes are essential for brain development and neuronal function as they serve to guide neurons and axons during growth and development (Brack-Werner, 1999). Additionally, astrocytes assist in the maintenance of homeostasis and are an important component of the blood-brain-barrier (Speth *et al.*, 2005). Astrocytes surround the endothelial cells that make up the blood-brain-barrier and provide structural support to the endothelial cells (Speth *et al.*, 2005). In addition to supporting the blood-brain-barrier, astrocytes are also the main producers of cytokines in the brain and produce anti-inflammatory cytokines such as IL-6, pro-inflammatory cytokines, such as IL-1β, TNF-α and IL-6 and chemokines, such as IL-8 (Speth *et al.*, 2005).

The relationship of astrocytes and the immune system to an inflammatory response have been well established. Upon exposure of an

inflammatory stimulus, such as LPS and IL-1β, human astrocytes produce a variety of chemokines and cytokines. Astrocytes treated with IL-1β have an increase in levels of IL-6, an anti-inflammatory cytokine, and IL-8, a chemokine, two immune mediators responsible for leukocytosis, an increase in leukocytes associated with inflammation (Sharma *et al.*, 2007). The increase in IL-6 and IL-8 in response to an inflammatory stimulus may serve as a protective mechanism and may provide neuroprotection to the astrocytes and surrounding neurons (Sharma *et al.*, 2007).

Astrocytes are crucial cells during times of inflammation in the brain, as these cells are capable of surviving under inflammatory conditions and are resistant to death receptor mediated apoptosis (Farina *et al.*, 2007). Whereas the immunological aspects of astrocytes have been well established, the relationship of astrocytes and the immuno-opioid pathway has not been examined. Astrocytes are a crucial component of a healthy blood-brain-barrier and understanding how this cell type is involved in the relationship between the immune system and the opioid pathway may serve as a basis for therapeutic intervention in disease states such as HIVD.

Summary and Significance of the Project

Chronic opioid use, such as with morphine abusers, leads to the development of tolerance and addiction. Opiates bind to the opiate receptors located in a variety of cell types, such as immune cells, neurons and endothelial cells. Under normal physiological conditions, upon activation of the opiate receptors by endogenous opiates, there is a decrease in pain mediated through the opioid receptors, antinociception, and a subsequent decrease in pain occurs (Stein, 1995). This decrease in antinociception indicates that the opiate receptors serve to minimize pain during times of injury or inflammation (Stein, 1995).

The immune response to infection and/or injury consists of the release of both pro-inflammatory and anti-inflammatory cytokines which are released by various immune cells such as macrophages and function to restore homeostasis (Chesnokova and Melmed, 2002). In a chronic opioid tolerant state, the balance between the opioid system and the immune system is altered due to the increased levels of opioids. Chronic opioid use results in a desensitization of the opioid receptors. Additionally, chronic morphine use is immunosuppressive and its continuous use results in an increased susceptibility to infection (Roy *et al.*, 2006; Kreek *et al.*, 2005).

Prior work in our laboratory has shown that in a chronic morphine state there is a desensitization of the HPA axis which results in a decreased response to an inflammatory stimulus, such as LPS (House *et al.*, 2001). Additionally, our laboratory has shown that in response to an inflammatory stimulus, such as LPS, there is an increase in pro-inflammatory cytokines in the serum of a rat model (Ocasio *et al.*, 2004). This purpose of this study was to examine if a potential connection between the immune system and the opioid system exists during an acute or chronic inflammatory disease state, so to further our understanding of this immuno-opioid relationship.

The first aspect of this study examined if chronic morphine exposure could alter the pro-inflammatory cytokine expression profile (IL-1β, TNF-α and IL-6) in the rat brain in response to an inflammatory stimulus such as LPS. The objective of this study was to better understand the relationship of the immune system and the opioid system as it would relate to an acute inflammatory condition, such as sepsis. The second component of this study examined the expression of the MOR in the presence of a pro-inflammatory cytokine in order to determine if MOR serves as a potential convergence point of the immune-opioid relationship in the central nervous system. For this objective, a human astrocytic cell line, U87 MG, was used to examine the relationship of the

pro-inflammatory cytokine, IL-1β, and MOR. The objective of this study was to translate the rodent work into a more relevant clinical model. The U87 MG cell line was chosen in order to better understand the relationship of the immune system and the opioid system as it would relate to a chronic inflammatory condition, such as HIVD.

Materials and Methods

Materials

A complete list of all materials is located on Table 1, page 27. Briefly, cell culture reagents were obtained from GIBCO/Invitrogen (Carlsbad, CA). Lipopolysaccharide (LPS), 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), morphine and naloxone were purchased from Sigma (St. Louis, MO). TRIzol® reagents were obtained from Invitrogen (Carlsbad, CA) and all other reagents used for RNA extraction were obtained from Sigma (St. Louis, MO). DAPI (4'-6-Diamidino-2-phenylindole) was obtained from Pierce (Rockford, IL). Antibodies to human mu-opioid receptor (MOR) were obtained from Chemicon (Rosemont, IL). Radioimmunoassay (RIA) kits used to determine cyclic adenosine monophosphate (cAMP) levels were obtained from Amersham BioSciences (Piscataway, NJ) and RIA kits used to determine morphine serum levels were obtained from Diagnostic Products (Los Angelos, CA). Interleukin-1β (IL-1β) and interleukin-1 receptor antagonist protein (IL-1RAP) were obtained from R& D Systems (Minneapolis, MN).

Table 1: Materials

Material	Provider	Catalog #	Uses
2'-deoxynucleoside 5'-triphosphate (dNTP)	Invitrogen (Carlsbad, CA)	18427-013	Reverse transcription
4'-6-Diamidino-2-phenylindole (DAPI)	Pierce (Rockford, IL)	46190	Immuno-fluorescence staining
12-*O*-tetradecanoylphorbol-13-acetate (TPA)	Sigma-Aldrich (St. Louis, MO)	P-1585	Differentiation of HL-60 cells
Chloroform	Sigma-Aldrich (St. Louis, MO)	C2432	RNA isolation
Di-ethyl-propyl carbonate treated (DEPC) water	Ambion (Austin, TX)	AM9922	RNA isolation
Dimethylsulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO)	D-5879	Solvent used in reverse transcriptase polymerase chain reaction
Dithiothreitol (dTT)	Invitrogen (Carlsbad, CA)	Y00147	Reverse transcription
Dulbecco's Modified Eagle Media (DMEM)	Gibco/Invitrogen (Carlsbad, CA)	12100-046	Cell culture
Dulbecco's phosphate-buffered saline (PBS) (with Ca/Mg)	Gibco/Invitrogen (Carlsbad, CA)	14040-117	Immuno-fluorescence staining

Dulbecco's phosphate-buffered saline (PBS) (without Ca/MG)	Invitrogen (Carlsbad, CA)	14040-182	Cell culture
Earle's minimum essential medium with non-essential amino acids	Invitrogen (Carlsbad, CA)	41500-034	Cell culture
Ethanol (100%)	Sigma-Aldrich (St. Louis, MO)	C2432	RNA isolation
Fetal bovine serum (FBS)	Gibco/Invitrogen (Carlsbad, CA)	10437-028	Cell culture
First strand reaction buffer	Invitrogen (Carlsbad, CA)	10812-014	Reverse transcription
Formaldehyde	Sigma-Aldrich (St. Louis, MO)	F1635	Immunofluorescence staining
Forskolin	CalBioChem (San Diego, CA)	344270	Adenylyl cyclase activator used in cyclic adenosine monophosphate assay (cAMP)
Goat anti-rabbit IgG- fluorescein isothiocyanate (FITC)	Southern Biotech (Birmingham, AL)	4050-02	Immuno-fluorescence staining
Goat serum	Biomeda (Plovdiv, Bulgaria)	MS003	Immuno-fluorescence staining
Ham's nutrient mixture F12	Invitrogen (Carlsbad, CA)	21700-075	Cell culture
Harlan Spraque-Dawley rats	Harlan (Indianapolis, IN)	N/A	Morphine tolerance /intravital microscopy

Heparin	Baxter (Deerfield, IL)	NDC 0641-2440-41	Anticoagulant used for intravital microscopy experiments
HL-60 cell line	American Type Culture Collection (ATCC) (Rockville, MD)	CCL-240	Cell culture
Isobutylmethy-xanthine (IBMX)	Sigma-Aldrich (St. Louis, MO)	N/A	Forskolin-induced cAMP assay
Isopropyl alcohol	Sigma-Aldrich (St. Louis, MO)	19516	RNA isolation
Lipopolyscarradie (LPS)	Sigma-Aldrich (St. Louis, MO)	L-6511	Cell culture
Moloney murine leukemia virus (M-MLV) reverse transcriptase	Invitrogen (Carlsbad, CA)	18057-018	Reverse transcription
Morphine	Sigma-Aldrich (St. Louis, MO)	M-8777	Cell culture
Morphine sulfate pellets	National Institute of Drug Abuse (Rockville, MD)	N/A	Morphine tolerance
Naloxone	Sigma-Aldrich (St. Louis, MO)	N-7758	Cell culture
n-formyl-methionyl-leucyl-phenylalanine (FMLP)	Fluka (Switzerland)	47729	Intravital microscopy
Olympus FluoView ™ 1000	Olympus (Center Valley, PA)	N/A	Immuno-fluorescence staining
Paraformaldehyde	Sigma-Aldrich (St. Louis, MO)	P6148500G	Immuno-fluorescence staining

Penicillin and streptomycin sulfate	Invitrogen (Carlsbad, CA)	15070-063	Cell culture
Rabbit anti-mu-opioid receptor (MOR)	Chemicon (Rosemont, IL)	AB1580	Immuno-fluorescence staining
Random primer	Invitrogen (Carlsbad, CA)	58875	Polymerase chain reaction
Recombinant human interleukin-1 receptor antagonist protein (IL-1ra/IL-1RAP)	R&D Systems (Minneapolis, MN)	280-RA	Cell culture
Recombinant human interleukin-1 β (IL-1β)	R&D Systems (Minneapolis, MN)	201-LB-005	Cell culture
Radioimmuno-assay kit for cyclic adenosine monophosphate (cAMP)	Amersham Biosciences (Pisctaway, NJ)	RPA 509	cAMP
Radioimmuno-assay kit for morphine	Diagnostic Products (Los Angelos, CA)	N/A	Radio Immunoassay
Roswell Park Memorial Institute -1640 (RPMI-1640) media	Gibco/Invitrogen (Carlsbad, CA)	11875	Cell culture
Sodium bicarbonate	Gibco/Invitrogen (Carlsbad, CA)	11810-025	Cell culture
TaqMan® gene expression kit	Applied Biosystems (Foster City, CA)	RN00562055_M1	Polymerase chain reaction
TaqMan® universal master mix	Invitrogen (Carlsbad, CA)	4326708	Polymerase chain reaction

TRIzol® reagent	Invitrogen (Carlsbad, CA)	15596-026	RNA isolation
Trypsin-ethylene diamine tetraacetic acid (EDTA)	Invitrogen (Carlsbad, CA)	25200-072	Cell culture
U87 MG cell line	American Type Culture Collection (ATCC) (Rockville, MD)	HTB-14™	Cell culture
Vectashield	Vector Laboratories (Burlingame, CA)	H-1000	Immuno-fluorescence staining

Animals

Harlan Sprague-Dawley rats (adult male; 200–300 g) (Harlan, Indianapolis, IN) were housed in a temperature and humidity controlled environment with a 12 hour light:dark cycle. Animals were given free access to a standard rat diet and water. Animals were allowed to acclimate for five to seven days prior to any experimental procedures. Animal studies were conducted with the approval of the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University.

In vivo experiments

Intravital microscopy

Rats (three animals/experiment) were anesthetized with an intraperitoneal (i.p) injection of sodium pentobarbital (45 mg/kg) (Veterinary Laboratory, Lenexia, KS). Using electric hair clippers, the ventral fur of each animal was shaved and the animals were placed in the supine position on a warm isothermal heating pad. A tracheotomy was performed to facilitate respiration and a mid-sagittal incision was made in the abdomen. The animals were draped onto a transparent Plexiglass shelf exposing the intestines. Drip solution (1% Ringer's gelatin drip solution, 3.43 mM KCl, 22.85 mM $NaHCO_3$, 4.19 mM HEPES, 0.28 mM

$CaCl_2$) was maintained at 37°C and was added to the exposed intestines. The intestines were covered with gauze moistened with drip solution. A Nikon UM3 metallurgic microscope adapted for intravital microscopy was used to examine the animal's mesentery on a Panasonic TR-930A high-resolution video monitor, as indicated in Figure 1. Vessels ranging from 20 to 40 μm in length and width were selected using the video image shearing technique. Leukocyte-endothelial adhesion (LEA) and leukocyte flow (flux) were recorded using a video camera and videocassette recorder. LEA was determined as the number of leukocytes adhering to the endothelium (per 100 μm vessel length) for a 15 second interval throughout the duration of hemodynamic measurements (House and Lipowsky, 1987). Leukocyte flux was determined by counting the number of leukocytes rolling past a defined area of the vessel during a 15 second interval (House *et al.*, 2001).

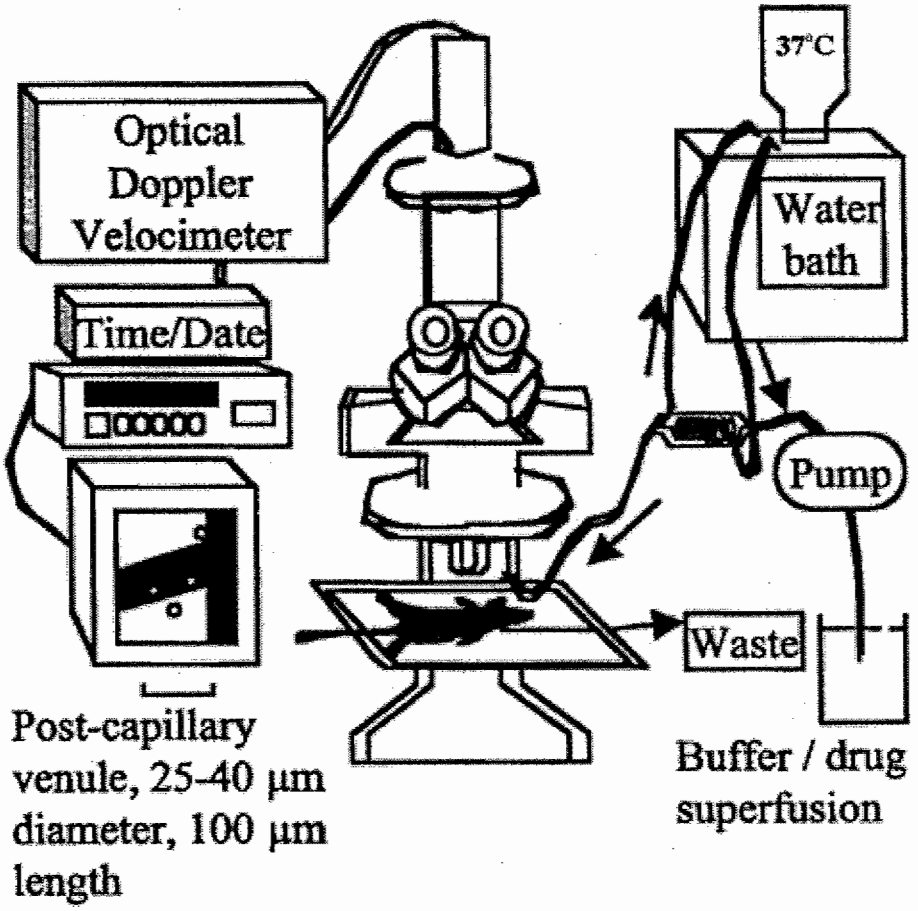

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Figure 1: A representation of an intravital microscopy setup

Intravital microscopy was used to examine the effects of inflammation as induced by n-formyl-methionyl-leucyl-phenylalanine (FMLP), a short acting bacterial chemoattractant, on leukocyte-endothelial adhesion (LEA) and leukocyte flux in an *in vivo* rodent model.

Induction of acute inflammation using FMLP, a short acting bacterial chemoattractant

Intravital microscopy was used to measure leukocyte-endothelial adhesion (LEA) and leukocyte flow (flux) in an *in vivo* rodent model (as described on pages 32-33). Inflammation was induced with a two minute topical application of 10^{-7} M n-formyl-methionyl-leucyl-phenylalanine (FMLP), a short acting bacterial chemoattractant (Fluka, Switzerland). FMLP was prepared prior to each intravital microscopy experiment by dissolving the FMLP powder into dimethylsulfoxide (DMSO). The stock solution was then dissolved in Ringer's lactate-gelatin drip solution. Final pH of the FMLP stock solution dissolved in the Ringer's drip solution was 7.4. Animals treated with Ringer's lactate-gelatin solution alone served as the control.

Establishment of a chronic morphine model

A chronic morphine model was created to examine the pro-inflammatory cytokine profile in a morphine tolerant animal using a two-plus-four morphine pellet regimen that produces morphine tolerance and dependence (Zadina *et al.*, 1995; Ocasio *et al.*, 2004). Adult male Harlan Sprague-Dawley rats were divided into two groups (ten animals/group) and were assigned to receive either two 75 mg pellets of morphine sulfate (National Institute of Drug Abuse, Rockville, MD) on Day 1 subcutaneously (s.c) followed by four 75 mg morphine pellets on Day 2 (s.c), or two pellets of placebo on Day 1 (s.c) followed by four pellets of placebo on Day 2 (s.c). Pellets (morphine or placebo) were placed (s.c.) on the ventral side of each animal. The pellets remained under the skin and did not enter the intraperitoneal cavity, which allowed for continuous treatment diffusion throughout the study (Zadina *et al.*, 1995; Ocasio *et al.*, 2004).

On Day 4, the animals were further divided into two groups. One group of animals was given 250 μg/kg lipopolysaccharide (LPS) (five animals/experiment) and the other group was given saline (five animals/experiment) via intraperitoneal (i.p.) injection. The animals were sacrificed on Day 5 at 16 hours following treatment with either LPS or

saline (Figure 2). A parallel study was conducted using a similar design as described above. However, on Day 5, rather than Day 4, the animals were administered 250 µg/kg LPS (five animals/experiment) or saline (five animals/experiment) (i.p.) and were sacrificed two hours following treatment with either LPS or saline. Sera and half brains were collected and stored at -80°C for further analysis.

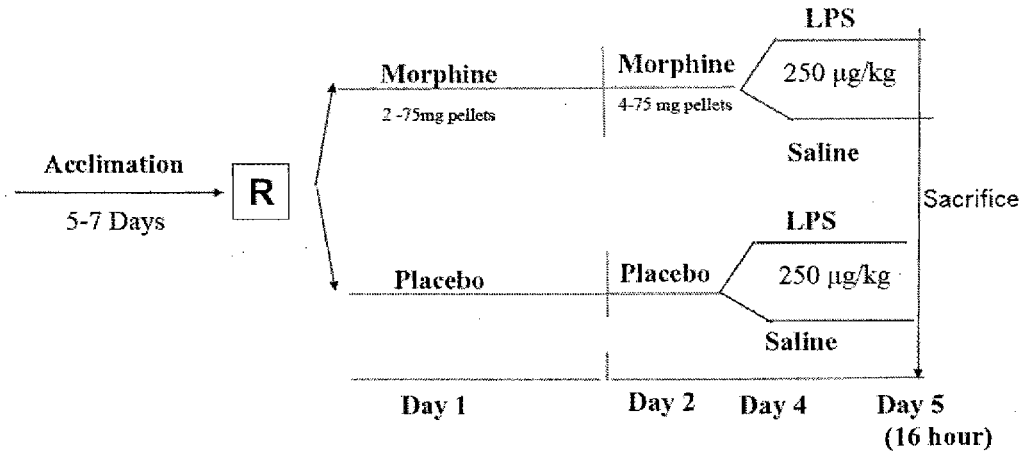

Figure 2: A schematic representation of morphine tolerance experiment

Rats were randomly assigned to either receive morphine or placebo pellets (ten animals/group). Animals in the morphine treatment group were implanted with two 75 mg pellets of morphine on Day 1 and four 75 mg pellets on Day 2. Animals were then injected intraperitoneally (i.p) with either LPS (250 µg/kg) (five animals/treatment) or saline (five animals/treatment) at Day 4. Animals were sacrificed 16 hours post-injection (at Day 5). A parallel study was conducted using a similar design; however, on Day 5, rather than Day 4, the animals were given 250 µg/kg LPS (five animals/treatment) or saline (five animals/treatment (i.p.) and sacrificed two hours following treatment with LPS or saline.

Cell culture

U87 MG cell line

Human glioblastoma cells (U87 MG) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA). U87 MG cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and were maintained in a humidified cell culture incubator 5% CO_2 at 37°C.

HL-60 cell line

HL-60 cells (human promyelocytic leukemia cells) were grown and maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) media containing 20% FBS and 1% penicillin and streptomycin sulfate. HL-60 cells were obtained from ATCC (Rockville, MD) and were maintained in a humidified environment of 5% CO_2 at 37°C.

NMB cell line

Human neuroblastoma cells (NMB cells) were a generous gift from Dr. Horace H. Loh's laboratory (University of Minnesota, MN). NMB cells were grown and maintained in RPMI-1640 media containing 10% FBS.

NMB cells were maintained in a humidified environment of 5% CO_2 at 37°C.

SH-SY5Y cell line

Human neuroblastoma cells (SH-SY5Y cells) were a generous gift from Dr. Robert Ross's laboratory (Fordham University, NY). SH-SY5Y cells were grown and maintained in a mixture of 45% Earle's Minimum Essential Medium with non-essential amino acids, 45% Ham's Nutrient Mixture F12 (Invitrogen, Carlsbad, CA) and 10% FBS with penicillin and streptomycin sulfate (100 µg/mL).

***In vitro* experiments**

Differentiation of HL-60 cells using 12-O-tetradecanoylphorbol-13-acetate (TPA)

When exposed to12-*O*-tetradecanoylphorbol-13-acetate (TPA), HL-60 cells differentiate into macrophage-like cells (Beltran *et al.*, 2006; Collins, 1987; Emiliani *et al.*, 1995; Kalf and O'Connor, 1993). HL-60 cells were cultured and maintained in a T-75 flask until approximately 60-80% confluent. Once the appropriate confluence was obtained, the cell culture media were removed and replaced with fresh media containing 16 nM TPA. Cells were returned to the cell culture incubator (5% CO_2 at 37°C). The cell culture media were replaced at Day 2 with fresh media

containing 16 nM TPA. In total, the cells were exposed to 16 nM TPA for four days to induce differentiation into macrophage-like cells.

Interleukin-1β (IL-1β) treatment

U87 MG cells ($2x10^5$ cells/well) were cultured in 6-well plates and were incubated at 5% CO_2 at 37°C until 60-80% confluent. Cells were then treated with 1 mL cell culture media containing either vehicle (cell culture media) or interleukin-1β (IL-1β) (20 ng/mL or 40 ng/mL) for 0, 3, 6, 12, 24 or 48 hours. The media were then aspirated and stored in 1.5 mL Eppendorf microcentrifuge tubes for the determination of IL-8 expression levels (as described on pages 51-52). TRIzol® (1 mL) was added to each well and using a pipetman the cells and TRIzol® were removed from each well and placed into a fresh set of 1.5 mL Eppendorf microcentrifuge tubes. The Eppendorf tubes containing cells in TRIzol® and the Eppendorf microcentrifuge tubes containing cell culture supernatant were then frozen and stored at -80°C for further analysis.

Co-treatment with interleukin-1β (IL-1β) and interleukin-1 receptor antagonist protein (IL-1RAP)

U87 MG cells ($2x10^5$ cells/well) were cultured in 6-well plates and were incubated at 5% CO_2 at 37°C until 60-80% confluent. Cells were removed from the incubator and cell culture media were aspirated. Cells

were then treated with 1 mL cell culture media containing either vehicle (cell culture media), IL-1β (20 ng/mL), interleukin-1 receptor antagonist protein (IL-1RAP) (400 ng/mL) + vehicle, IL-1RAP (400 ng/mL) + IL-1β (20 ng/mL), IL-1RAP (4,000 ng/mL) + vehicle or IL-1RAP (4,000 ng/mL) + IL-1β (20 ng/mL). IL-1RAP doses (400 ng/mL and 4,000 ng/mL) exceeded the manufacturer's recommendation of a 1:100 ratio of IL-1β to IL-1RAP needed for IL-1RAP to be effective. Cells were then incubated at 5% CO_2 at 37°C for 12 hours. Upon completion of incubation, the media were aspirated and 1 mL TRIzol® was added to each well. The cells in TRIzol® were transferred into 1.5 mL Eppendorf microcentrifuge tubes and the tubes were then frozen and stored at -80°C for further analysis.

Morphine up-regulation of the mu-opioid receptor (MOR) in a time dependent manner

U87 MG cells ($1.5x10^5$ cells/well) were cultured in 6-well plates and were incubated at 5% CO_2 at 37°C until 60-80% confluent. Cells were removed from the incubator and cell culture media were aspirated. Cells were then treated with fresh cell culture media (1 mL) containing either vehicle or 100 nM morphine (a dose that mimics chronic dependent conditions (Suarez-Roca H and Maixner W, 1992). Cells were incubated

at 5% CO_2 at 37°C for 45 minutes, 3, 6, 12, 24 or 48 hours. The media were aspirated and 1 mL TRIzol® was added to each well. The cell suspension in TRIzol® was transferred into 1.5 mL Eppendorf microcentrifuge tubes and the tubes were then frozen and stored at -80°C for further analysis.

Pre-treatment U87 MG cells with morphine followed by IL-1β treatment

U87 MG cells ($1.5x10^5$ cells/well) were cultured in 6-well plates and were incubated at 5% CO_2 at 37°C until 60-80% confluent. Cells were removed from the incubator and cell culture media were aspirated. Cells were treated with fresh cell culture media (1 mL) containing vehicle (cell culture media) or 100 nM morphine (a dose that mimics chronic dependent conditions (Suarez-Roca H and Maixner W, 1992). Cells were incubated at 5% CO_2 at 37°C for 24 hours. The media were aspirated and the cells were treated with fresh media containing either vehicle or IL-1β (20 ng/mL). Cells were incubated at 5% CO_2 at 37°C for 12 hours. The media were aspirated and 1 mL TRIzol® was added to each well. The cell suspension in TRIzol® were then transferred into 1.5 mL Eppendorf microcentrifuge tubes and the tubes were then frozen and stored at -80°C for further analysis.

Morphine radioimmunoassay (RIA) to confirm morphine serum levels

At the end of the morphine pelleting study, the animals were sacrificed and the sera were collected (as described on page 36-37). Sera were assayed for morphine levels in order to confirm that the animals were exposed to morphine upon the completion of the experiment. A radioimmunoassay (RIA) kit from Diagnostic Products Corporation (Los Angeles, CA) was used according to the manufacturer's instructions. The kit reagents and samples were removed from the -20°C freezer and placed on a shaker at 250 revolutions per minute (rpm) at room temperature. The reagents and samples remained on the shaker until fully thawed. Once the reagents were thawed, serum samples (25 μl) were added to each tube, with the exception of the controls. The positive control and normal control had 25 μl of positive reference standard (100 ng/mL of morphine) and normal control (cell culture media) added, respectively. Morphine tracer [^{125}I] (200 μl) was added to each tube, followed by 100 μl of primary antibody (rabbit anti-morphine serum in 0.1 M phosphate buffer in a 1:100 concentration). The samples were vortexed and 200 μl of secondary antibody (goat anti-rabbit antibody in 0.1 M phosphate buffer in a 1:100 concentration) was added to each sample. Samples were vortexed and

incubated at room temperature for 60 minutes. Upon completion of the incubation, the samples were centrifuged for 10 minutes at 1200 rpm at room temperature. The supernatant containing the unbound antigen was separated from the antibody-bound antigen by decanting the supernatant into a radioactive labeled container. A Wallac Wizard Gamma Counter 1470 was used to determine the quantity of radioactivity (counts per minute or cpm) for the standards as well as the sera samples (PerkinElmer, Waltham, MA). The mean cpm for each standard was then subtracted from the background non-specific binding reading and a standard curve was generated using Microsoft® Excel 2003. The standard curve was used to calculate the morphine sera levels from the animals in the morphine pelleting study (as described on page 36-37).

RNA isolation

Total RNA was extracted from harvested cells using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Grand Island, NY). Cell culture media were aspirated and 1 mL TRIzol® was added to each well. Cells were incubated at room temperature for approximately five minutes to allow for the TRIzol® to dissociate the cells. The cell mixtures were removed from the 6-well plates and were placed into labeled 1.5 mL Eppendorf microcentrifuge tubes. Chloroform (0.2

mL) was then added to each tube and the samples were vigorously mixed by hand for approximately 15 seconds. Samples were then incubated at room temperature for five minutes. The samples were then centrifuged at 1,200 rpm at 4°C for 15 minutes to allow the separation of the different phases. Upon completion of centrifugation, the upper aqueous phase, which contained the RNA, was carefully transferred into sterile 1.5 mL Eppendorf microcentrifuge tubes. The RNA was then precipitated with 0.2 mL isopropyl alcohol. Following the addition of the isopropyl alcohol, the samples were incubated at room temperature for ten minutes and then centrifuged at 12,000 rpm at 4°C for ten minutes. The supernatant was then discarded and the RNA washed with 1 mL of 75% ethanol. The samples were briefly vortexed and centrifuged at 4°C at 7,500 rpm for five minutes.

Upon completion of the centrifugation, the ethanol was gently discarded and the pellet dried. Once the RNA pellet dried, 20 μl di-ethyl-propyl carbonate (DEPC) treated water was added to each sample. The samples were then vortexed and centrifuged briefly. The samples were placed on ice and the concentration of RNA was measured at 260 nm and 280 nm using ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE).

Forskolin induced cyclic adenosine monophosphate (cAMP) accumulation assay

A forskolin induced cyclic adenosine monophosphate (cAMP) accumulation assay was conducted in U87 MG cells treated with and without morphine in order to assess the functionality of mu-opioid receptor (MOR) expressed in the U87 MG cell line. The forskolin induced cAMP accumulation assay was conducted as described by Yu *et al.* (2003). Briefly, U87 MG cells were grown and maintained in 6-well plates until approximately 60-80% confluent. The cell culture media were aspirated and replaced with fresh media containing 0.5 mM isobutylmethylxanthine (IBMX), a phophodiesterase inhibitor used to block the breakdown of cAMP. Cells were incubated at 5% CO_2 at 37°C for 30 minutes. Following incubation, the cell culture media were removed and replaced with fresh media containing vehicle, 75 µM forskolin (an adenylyl cyclase activator), 10 µM morphine alone, 10 µM morphine + 75 µM forskolin, or 10 µM morphine + 75 µM forskolin + 10 µM nalaxone (an antagonist to the mu-opioid receptor). The cells were incubated for ten minutes at 5% CO_2 at 37°C. The media were then aspirated and the cells were lysed with 0.1 N HCl. The cells were then frozen at -20°C until the RIA was performed. Parallel studies were

conducted using two opioid peptides with a high affinity for MOR, 10 μM endomorphin-1 or 10 μM endomorphin-2, in place of morphine.

Intracellular cAMP levels were measured from the thawed cell lysates using a commercially available RIA kit (Amersham Biosciences Inc., Piscataway, NJ). The kit reagents (cAMP buffer, cAMP standards, primary antibody and samples) were removed from the -20°C freezer and placed on a shaker at 250 rpm at room temperature. The samples remained on the shaker until fully thawed. Once thawed, the cAMP standards were prepared by adding 2 mL cAMP buffer to the cAMP standard (for a final concentration 1600 fmol/mL). The standards were then vortexed and allowed to incubate at room temperature for five minutes. Serial 1:2 dilutions of the cAMP standards were then conducted to generate the cAMP standards ranging from 1600 fmol to 25 fmol. The cAMP standards (100 μl) were aliquoted into 1.5 mL Eppendorf microcentrifuge tubes. Cell lysate samples (100 μl) were then aliquoted into the 1.5 mL Eppendorf microcentrifuge tubes. The cAMP buffer (100 μl), primary antibody (rabbit anti-succinyl cAMP serum in a 1:1000 concentration) (100 μl) and [^{125}I] cAMP tracer (100 μl), a radioactively labeled compound that competes with the unlabeled antigen for binding sites on the primary antibody, were added

to each tube. The samples were allowed to incubate overnight at room temperature.

Following the overnight incubation, 250 µl of Amelex-M secondary antibody in a 1:1500 dilution (cAMP horseradish peroxidase secondary antibody) was added to each tube and the samples were incubated for ten minutes at room temperature. The samples were then centrifuged at 1500 rpm for 15 minutes at room temperature. The supernatant was decanted into a radioactive labeled container and a Wallac Wizard Gamma Counter 1470 was used to determine the quantity of radioactivity as determined by cpm (PerkinElmer, Waltham, MA). The background non-specific binding reading was then subtracted from the counts per minute reading from each standard and unknown test samples. The standard readings were used to generate a standard curve using Microsoft® Excel 2003, which was used to determine the cAMP levels in each sample.

Immunofluorescent staining

U87 MG cells were plated in a chamber slide containing 500 µl of DMEM + 10% FBS per chamber. The slides were incubated at 37°C in 5% CO_2 until 60-80% confluent. The chamber slide was then removed from the incubator, the media aspirated and the cells washed twice with 1 x phosphate-buffered saline (PBS). The chamber slide was then dried

and immersed into fixing solution consisting of 4% paraformaldehyde / 2% formaldehyde solution in 1x PBS with Ca/Mg for 20 minutes at room temperature. The slide was then removed from the fixing solution and washed three times with 1 x PBS with Ca/Mg for five minutes at room temperature. The slide was dried and either 60 µl of primary antibody (rabbit anti-MOR in a 1:1000 dilution in 1 x PBS) was added to each well or 60 µl of 4'-6-diamidino-2-phenylindole (DAPI in a 1:1000 dilution in 1 x PBS) was added to the chamber slide. The slide was then incubated overnight at 4°C. Upon completion of the incubation, the slide was washed three times in 1x PBS with Ca/Mg. Secondary antibody (60 µl) (goat anti-rabbit IgG- fluorescein isothiocyanate [FITC] [1:1000]) was then added to each well of the chamber slide and the slide was incubated for 2 hours at room temperature. The slide was then washed three times in 1 x PBS with Ca/Mg. The slide was then dried and sealed with vectashield, which sealed a cover-slip to the each well. Clear nail polish was used to seal the edges of the slide and the slide was allowed to dry for three hours at room temperature. Confocal microscopy was used to visualize the chamber slide (Olympus FluoView™ 1000, Center Valley, PA).

Interleukin-8 (IL-8) cytokine assay

A 96- well multi-array platform from Meso-Scale Discovery (MSD) (Gaithersburg, MD) was used to determine IL-8 expression levels in the cell culture supernatant from the IL-1β dose response experiments (as described on page 41). All reagents used for the MSD® platform assay were obtained from Meso-Scale Discovery (Gaithersburg, MD). Briefly, U87 MG cells ($2x10^5$ cells/well) were cultured in 6-well plates and were incubated at 5% CO_2 at 37°C until 60-80% confluent. Cells were then treated with 1 mL cell culture media containing either vehicle or interleukin-1β (IL-1β) (20 ng/mL or 40 ng/mL) for 12 hours (as described on page 41). Upon completion of incubation, the cell culture media were removed and placed in 1.5 mL Eppendorf microcentrifuge tubes and stored at -80°C for further analysis.

Cell culture media were removed from the -80°C freezer and MSD reagents were removed from the 4°C refrigerator and brought to room temperature. MSD® calibrator solution (10,000 pg/mL) (a standard tissue culture media containing 10% FBS) was used to prepare a calibration curve (10,000, 2500, 625, 156, 39, 9.8, 2.4 pg/mL). Detection antibody solution was prepared by diluting the detection antibody mix to 1 µg/mL in 3 mL of antibody diluent. Once the cell culture media were fully thawed,

25 µl of each sample was aliquoted into a 96-well plate. Eight wells were used per sample per experiment. The plate was incubated at room temperature for one hour on an orbital shaker at 300 rpm. Upon completion of the incubation, 25 µl of detection antibody solution was added to each well of the 96-well plate. The plate was incubated at room temperature for one hour on an orbital shaker at 300 rpm. Upon completion of incubation, the plate was washed three times with PBS + 0.05% Polysorbate 20 (Tween-20). Read buffer (2 x) was then added to each well (150 µl). The plate was analyzed on a sector instrument and a standard curve was generated from the standards. The IL-8 expression levels for the cell culture supernatant were determined using the standard curve and Microsoft® Office Excel 2003.

Reverse transcription

One microgram of total RNA was reverse transcribed to synthesize the first-strand cDNA on a GeneAmp 2400 Thermocycler (Perkin Elmer, Waltham, MA) using 200 units of Moloney murine leukemia virus (M-MLV) reverse transcriptase, 10 mM dithiothreitol (DTT), 0.5 mM 2'-deoxynucleoside 5'-triphosphate (dNTP), 1X reverse transcriptase (RT) buffer, 5 ng/mL random primer and di-ethyl-propyl carbonate (DEPC) H_2O for a total reaction volume of 20 µl (Invitrogen, Grand Island, NY) (Table

2). In order to determine the presence of DNA contamination in the total RNA, a negative control was utilized. The negative control consisted of one microgram of total RNA, 10 mM DTT, 0.5 mM dNTP, 1X RTbuffer, 5 ng/mL random primer and DEPC H_2O for a total reaction volume of 20 µl.

Table 2: Reagents for reverse transcription

Reagents needed for 1x reverse transcription reaction. Volumes of the various reagents were adjusted based on the number of reactions performed.

Regents	Amount Needed for 1 reaction (μl)
First strand buffer	4
10 mM dithiothreitol (DTT)	2
0.5 mM 2'-deoxynucleoside 5' triphosphate (dNTP)	1
5 ng/mL random primer	0.1
200 units Moloney murine leukemia virus (M-MLV)	1

Thermal cycling conditions were 37°C for 60 minutes, followed by a ten minutes incubation period at 65°C. The reaction was then cooled on ice as previously described (Chen *et al.*, 2005).

Real-time polymerase chain reaction (real-time-PCR)

Reverse transcription coupled with real-time-polymerase chain reaction (PCR) was performed to determine human mu-opioid receptor (hMOR), human kappa-opioid receptor (hKOR), human delta-opioid receptor (hDOR), human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), rat TNF-α (rTNF-α), and rat GAPDH (rGAPDH) mRNA levels using conditions previously reported by Herpers *et al.* (2003) and Chen *et al.* (2005).

Total RNA (1 μg) was first reverse transcribed into cDNA using the procedures described above (section titled: reverse transcription, page 52). The cDNA (2 μl) was then amplified using real-time PCR in a 50 μl PCR master mix (1 μl cDNA, 25 μl of 2X TaqMan® universal master mix, 1 μl of each probe (200 nM), 1 μl of each primer (400 nM) and 22 μl of DEPC water) in an ABI Prism 7000® (Applied Biosystems, Foster City, CA). A negative control consisted of the above TaqMan® universal mastermix with DEPC water. All primers, except rTNF-α were custom made from Applied Biosystems and the sequences are listed in Table 3.

Table 3: The sense and anti-sense primers used for real time polymerase chain reaction (real-time PCR).

Human mu-opioid receptor (hMOR)	5'-TACCGTGTGCTATGGACTGAT-3' (sense)
	5'-ATGATGACGTAAATGTGAATG-3' (antisense)
	5'/56-FAM/CTTGCGCCTCAAGAGTGTCCGCA/3BHQ_1/-3' (probe)
Human kappa-opioid receptor (hKOR)	5'-CGTCTGCTACACCCTGATGATC-3' (sense)
	5'-CTCTCGGGAGCCAGAAAGG-3' (antisense)
	5'/56-ROX/TGCGTCTCAAGAGCGTCCGGC/3BHQ_2/-3' (probe)
Human delta-opioid receptor (hDOR)	5'-GCGGGAAAGCCAGTGACTC-3' (sense)
	5'-TGCCCTGTTTAAGGACTCAGTTG-3' (antisense)
	5'/56-JOE/AGGAGAGGAGCGGGACCTGTGGCT/3BHQ_1/-3' (probe)
Human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH)	5'-GGAAGCTCACTGGCATGGC-3' (sense)
	5'-TAGACGGCAGGTCAGGTCCA-3' (antisense)
	5'/56-FAM/CCCCACTGCCAACGTGTCAGTG/3BHQ_1/-3' (probe)
Rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH)	5'-GAACATCATCCCTGCATCCA-3' (sense)
	5'-CCAGTGAGCTTCCCGTTCA-3' (anti-sense)
	5'-CTTGCCCACACGCTTGGCAGC-3' (TaqMan® probe)

rTNF- α oligonucleotides were purchased from Applied Biosystems as part of a TaqMan® gene expression assay kit (Assay- on-Demand) and the sequences of this kit are proprietary.

GAPDH mRNA was amplified for normalization of the MOR mRNA levels as previously described (Lai *et al.*, 2003). The 5' end of the TaqMan® probe was labeled with 6-carboxyfluorescein (FAM), a fluorophore, and the 3' end was labeled with 4-(4'-dimethylaminophenylaso) benzoic acid (DABCYL), a quencher. The conditions for thermal cycling were as follows: initial denaturation at 95°C for ten minutes, followed by 40 cycles at 95°C for 18 seconds, then at 60°C for 1 minute. Data was collected at the end of each PCR run and amplification plots generated. Analysis of the results was performed using the ABI Prism 7000® Sequence Detection System from Applied Biosystems (Foster City, CA). Microsoft® Office Excel 2003 was used to quantify the hMOR, hKOR, hDOR, rTNF-α, rGAPDH and hGAPDH mRNA levels in the samples. All the PCR amplification reactions were performed in duplicate.

Reverse-transcriptase polymerase chain reaction (RT-PCR)

Reverse transcription was conducted using the methods indicated previously (section titled: reverse transcription, page 52). Upon completion of reverse transcription, PCR amplification was performed using 5 μl of reverse transcriptase product, 1x PCR buffer, 0.2 mM sense and anti-sense primers (appropriate upstream and downstream primers specific for rat IL-1β (rIL-1β), rat IL-6 (rIL-6), or rat β-actin (rβ-actin) (Fisher Scientific, Springfield, NJ), 0.2 mM dNTP, 1 unit TaqMan® DNA polymerase, 3 mM $MgCL_2$ and 0.5% DMSO in a 50 μl total reaction volume according to manufacture's instructions and as reported by Chen *et al.* (2005). A negative control which consisted of 1 x PCR buffer, 0.2 mM dNTP, 1 unit TaqMan® DNA polymerase, 3 mM $MgCL_2$ and 0.5% DMSO was used. The primers used are listed in Table 4. The reaction conditions were used according to the manufacture's instructions and are indicated in Table 5.

Briefly, the reaction conditions for rat β-actin were: 94°C for five minutes and 15 cycles of 94°C for 30 seconds, 53°C for 30 seconds, 72°C for 30 seconds followed by one extension cycle of 72°C for five minutes. The reaction conditions for rat IL-1 β were: 94°C for 3 minutes and 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45

seconds followed by one cycle of 72°C for 3 minutes. The reaction conditions for rat IL-6 were: 94°C for two minutes and 45 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 90 seconds followed by one extension cycle of 72°C for ten minutes. Final PCR products were run on a 2.0% agarose gel. rβ-actin, a housekeeping gene, was used to normalize the levels of rIL-1β and rIL-6.

PCR data analysis

Copy number method

PCR (either real-time RCR or reverse-transcriptase polymerase chain reaction) data was analyzed based either on copy number or comparative C_T values (ΔCT). Copy number was determined using the standard curve method to determine copy number of the selected mRNA. The ABI PRISM 7700 Sequence Detection System software utilized the appropriate standards (hMOR, hKOR, hDOR, etc.) to determine the standard curve of the amplification of the appropriate sample. Using the standard curve generated from the standards, the copy number of each sample was determined. The mean copy number was then determined for each treatment group. The copy number of each sample was then normalized against a housekeeping control (either GAPDH or β-actin) by dividing the copy number of each sample by the copy number of the

Table 4: The sense and anti-sense primers used for reverse-transcriptase polymerase chain reaction (RT-PCR).

rat β-actin (rβ-actin)	5'-AGA-CCT-CTATGC-CAA-CAC-AGT-3' (sense) (Yu *et al.*, 2003)
	5'-GAC-ACACCT-AAC-CAC-CGA-GAT-3' (anti-sense) (Yu *et al.*, 2003)
rat IL-1β (rIL-1β)	5'-CTC-CAT-GAG-CTT-TGT-ACA-AGG-3' (sense) (Okada *et al.*, 2002)
	5'-TGCTGA-TGT-ACC-AGT-TGG-GG-3' (anti-sense) (Okada *et al.*, 2002)
rat IL-6 (rIL-6)	5'-TCC-GCA-AGA-GAC-TTC-CAG-CCA-GTT-3' (sense) (Okada *et al.*, 2002)
	5'-GGC-AAA-TTT-CCT-GGT-TAT-ATC-C-3' (anti-sense) (Okada *et al.*, 2002)

Table 5: Reaction conditions for reverse transcriptase polymerase chain reaction (RT-PCR).

	Initial denaturing	Denaturing	Annealing	Extension	Final Extension
rβ-actin	94°C for five minutes	30 seconds at 94°C	30 seconds at 53°C	30 seconds at 72°C	five minutes at 72°C
		15 cycles			
rIL-1β	94°C for three minutes	30 seconds at 94°C	30 seconds at 60°C	45 seconds at 72°C	three minutes at 72°C
		35 cycles			
rIL-6	94°C for two minutes	30 seconds at 95°C	30 seconds at 60°C	90 seconds at 72°C	ten minutes at 72°C
		45 cycles			

housekeeping control (either GAPDH or β-actin) of the same sample in order to determine the total copies per μg total RNA. Microsoft® Office Excel 2003 was used to graph the copy numbers of the various treatment groups.

Comparative C_T method

The comparative C_T method is a method used to determine threshold cycles (C_T), the number of cycle that the fluorescence generated crosses a threshold (Mahajan *et al.*, 2005). C_T values were generated from the ABI PRISM 7700 Sequence Detection System software and mean C_T values were determined for each treatment group. The difference in C_T values (ΔC_T) was then determined by subtracting the mean C_T of test samples from the mean C_T of the reference RNA (GAPDH or β–actin, as appropriate). The equation is as follows: $\Delta C_T = C_T$ (test sample)$-C_T$ (GAPDH or β–actin). The ΔC_T for the test samples was then subtracted from the ΔC_T for the control sample to generate a $\Delta\Delta C_T$ as follows: $\Delta\Delta C_T = \Delta C_T$ (test sample)$-\Delta C_T$ (control). The $\Delta\Delta C_T$ measurements were used to calculate expression of the test sample relative to the control (GAPDH or β–actin) and normalized to the untreated control: relative expression (fold change)$=2^{-\Delta\Delta C_T}$. Microsoft®

Office Excel 2003 was used to graph the relative expression for the various treatment groups.

Statistics

Data are presented as the mean ± standard error. Statistical data were analyzed using either a one way analysis of variance (ANOVA), paired or unpaired Student's t-test, as appropriate. Statistical significance was considered at $p < 0.05$. Treatment groups were performed in either duplicate or triplicate per experiment and all experiments were repeated at least twice.

Results

The effects of a bacterial chemotatic peptide (FMLP) on leukocyte-endothelial adhesion and leukocyte flux in the rat mesentery: confirmation of a functional immune response

Two measures of a functional immune response are the quantification of leukocyte-endothelial adhesion (LEA) and leukocyte flow (flux) in the presence or absence of an inflammatory stimulus. Under normal physiological conditions, there is minimal leukocyte-endothelial adhesion (LEA) as leukocytes do not adhere to the endothelium but rather roll through the vessel. Exposure to a pro-inflammatory stimulus, such as the bacterial chemotatic peptide f-methyl-leucyl-phenylalanine (FMLP) results in an increase in the adhesion molecules located on the leukocytes and the endothelium. This increase in adhesion molecules leads to increased contact between the leukocytes and endothelium resulting in an increase in LEA and a decrease in leukocyte flow (flux) (House and Lipowsky, 1987; House et al., 2001; Ocasio *et al.*, 2004).

Prior to examining the pro-inflammatory cytokine profile in an *in vivo* rodent model exposed to an inflammatory agent, the functionality of the immune response as determined by LEA and leukocyte flux (flow) in the rat model was examined using intravital microscopy. Upon exposure

of 10^{-7} M FMLP, there was a significant increase in LEA (10.6±1.46 cells/100 μM /15 sec) as compared to the control (4.8±0.73 cells/100 μM /15 sec) in the rat mesentery (Figure 3). A significant decrease in leukocyte flux was observed in the presence of FMLP (6.2±1.39 cells/15 sec) as compared to the control drip solution (11.8±0.58 cells/15 sec) (Figure 4).

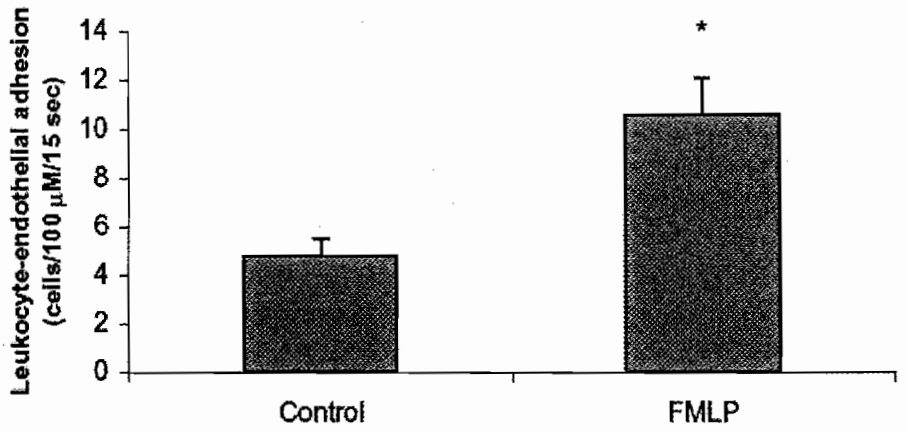

Figure 3: Leukocyte-endothelial adhesion (LEA) in the rat mesentery as determined by intravital microscopy.

Adult male rats (200-300 g) were anesthetized with sodium pentobarbital (45 mg/kg intrapertioneal injection). An incision was made in the animals' abdomen and the intestines exposed. Animals were first exposed to drip control solution for two minutes and intravital microscopy was used to observe the leukocyte-endothelial adhesion (LEA). Animals were then exposed to 10^{-7} M f-methyl-leucyl-phenylalanine (FMLP) for two minutes and LEA was measured.

This experiment was conducted three times and each experiment used three animals. The results were pooled from each experiment and analyzed. Data is indicated as mean $\pm$ standard deviation. Statistical significance of $p<0.05$ is depicted with an asterisk (*), as determined by Student t-test.

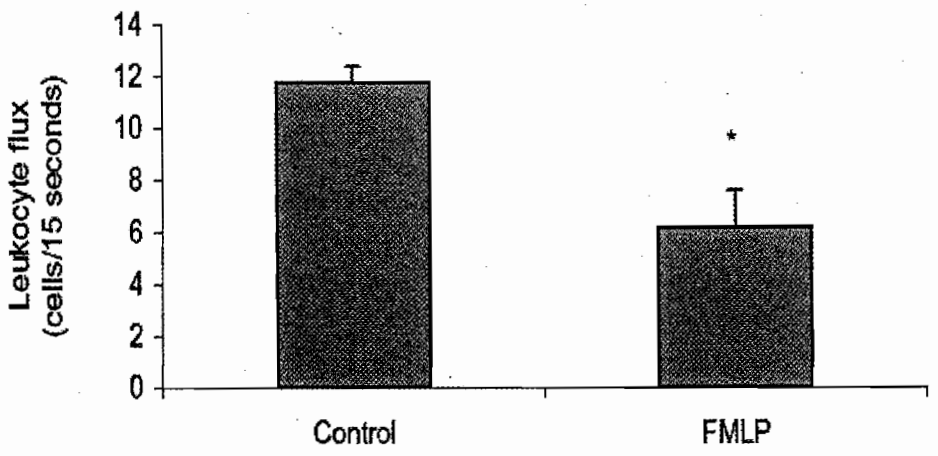

Figure 4: Leukocyte flux in the rat mesentery as determined by intravital microscopy.

Adult male rats (200-300 g) were anesthetized with sodium pentobarbital (45 mg/kg intrapertioneal injection). An incision was made in the animals' abdomen and the intestines were exposed. Animals were first exposed to drip control solution for two minutes and intravital microscopy was used to observe leukocyte flux (the rolling of white blood cells past a pre-defined point over a 15 second duration). Animals were then exposed to 10^{-7} M f-methyl-leucyl-phenylalanine (FMLP) for two minutes and leukocyte flux was measured.

This experiment was conducted three times and each experiment used three animals. The results were pooled from each experiment and analyzed. Data is indicated as mean $\pm$ standard deviation. Statistical significance of $p<0.05$ is depicted with an asterisk (*), as determined by Student t-test.

Serum morphine levels as determined by radioimmunoassay: a determination of continuous morphine exposure

Subcutaneous implantation of 75 mg morphine pellets in a two-plus-four pellet regimen is a protocol often used to induce morphine tolerance and dependence in a rodent model (Zadina *et al.*, 1996; Ocasio *et al.*, 2004). To establish an *in vivo* rodent model of continuous morphine exposure, animals (ten animals/group) were implanted with either morphine pellets or placebo pellets (s.c.). The morphine group received two 75 mg pellet (s.c) on Day 1 followed by four 75 mg pellet on Day 2 (s.c.). The placebo group received two and four placebo pellets on Day 1 and 2, respectively (s.c). On Day 4, animals were given either lipopolysaccharide (LPS) (250 μg/kg) (i.p.) (five animals/treatment) or saline (five animals/treatment) and were sacrificed 16 hours post-injection on Day 5. LPS was used to induce inflammation.

To confirm that the animals implanted with morphine pellets at Day 1 and Day 2 were exposed to morphine throughout the duration of the experiment, morphine levels in the rat sera were determined using radioimmunoassay (RIA) on Day 5, following sacrifice. Sera samples of morphine treated animals confirmed that this group of animals was exposed to morphine levels throughout the duration of the experiment.

Sera levels for animals implanted with morphine (M) and treated with saline (S) (M+S; 5,086.2 ±431 μg/mL) were similar to those of animals implanted with morphine (M) and treated with LPS (M+LPS; 250 μg/kg) (4,979±435 μg/mL) at 16 hours post-injection. Placebo (P) implanted animals had no detectable amounts of morphine in their sera, regardless of being treated with either saline (S) or LPS (250 μg/kg). There was no significant difference in the sera level of the morphine implanted rats (M) following LPS injection (250 μg/kg) (M+LPS; 4,979±435 μg/mL) compared to that in the saline (S) treated animals (M+S; 5,086.21±431 μg/mL) (Figure 5).

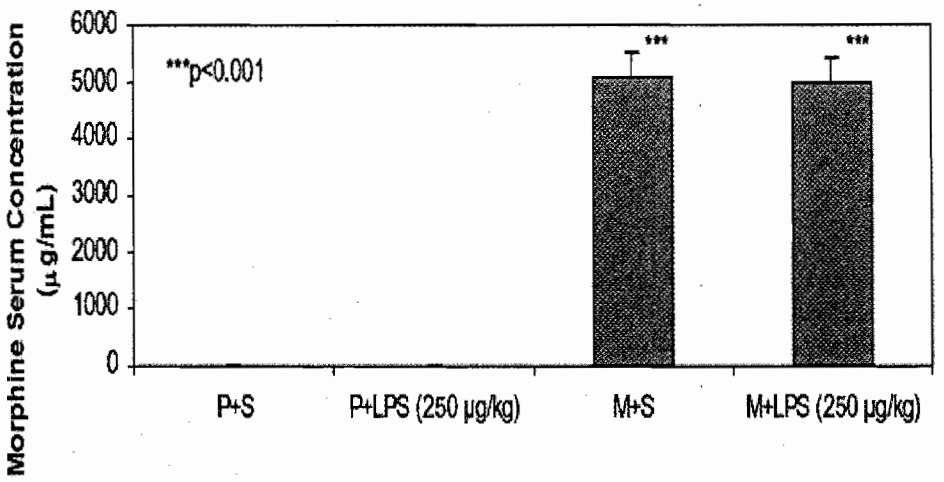

Figure 5: Serum morphine levels as determined by radioimmunoassay.

Adult male rats (N=20/experiment) (200-300 g) were implanted with either two-plus-four 75 mg pellets morphine (M) (n=ten) or placebo (P) (n=ten) on Day 1 and Day 2 (s.c), respectively. On Day 4, animals were then exposed (i.p) to either saline (S) or lipopolysaccharide (LPS) (250 µg/mL) and at 16 hours post-injection, the animals were sacrificed at Day 5. Sera were obtained from all the animals participating in the morphine pelleting study and radioimmunoassay was used to determine morphine sera levels. This experiment was conducted twice with similar results.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. p=non-significant (NS) for M+S group as compared to M+LPS group and ***p<0.0001 for P+S as compared to M+S and P+LPS as compared to M+LPS, respectively

IL-1β expression in the brain of morphine-tolerant rats

Serum levels of IL-1β have been examined in the presence of either morphine or LPS. An increase in IL-1β serum levels occurs in a morphine tolerant rodent model (Chang *et al.*, 1998). Additionally, morphine exposure increases LPS-induced levels of IL-1β in rat serum (Ocasio *et al.*, 2004). Whereas the serum IL-1β levels been established, the expression profile of LPS induced IL-1β in the brain of a morphine tolerant animal has yet to be examined. In order to evaluate the expression of LPS-induced IL-1β in the brain of a morphine-tolerant rat, total RNA was extracted from the brain of rats implanted (s.c.) with morphine (M, ten animals/treatment) or placebo (P, ten animals/treatment) and then treated (i.p.) with either LPS (250 μg/kg) (M+LPS, M+S, five animals/treatment) or saline (P+LPS, P+S, five animals/treatment). IL-1β mRNA expression was assayed using RT-PCR and the housekeeping gene β-actin was used to normalize the levels of IL-1β. Rats implanted with placebo (P) and treated with saline (S) were used as a control.

At two hours following LPS (250 μg/kg) or saline injection, there were significantly higher IL-1β mRNA levels in the brains of the P+LPS and M+LPS rats (1.5$\pm$0.5 and 2.63$\pm$0.6, respectively) than in the P+S and

M+S animals (0.03±0.02 and 0.02±0.01, respectively). Furthermore, IL-1β mRNA levels in the M+LPS rats (2.63±0.6) were significantly enhanced compared to the P+LPS (1.5±0.5) group. By 16 hours following LPS or saline (i.p), the IL-1β mRNA levels in the brains of both the P+LPS and M+LPS rats had declined (0.32±0.02 and 0.28±0.03, respectively), but were still significantly higher than in the P+S (0.18±0.01) and M+S (0.15±0.01) animals, respectively. The IL-1β mRNA levels in P+S and M+S animals were comparable at two hours post-injection and 16 hours post-injection (Figure 6).

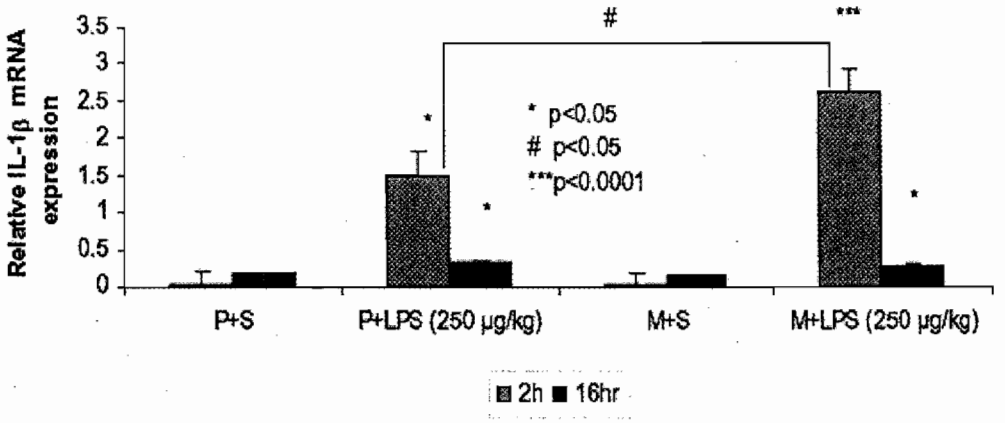

Figure 6: The effects LPS treatment on expression levels of IL-1β mRNA in a morphine tolerant rat brain.

Adult male rats were implanted with either two-plus-four 75 mg morphine pellets (M) or placebo pellets (P). Animals were administered with either saline (S) or lipopolysaccharide (LPS) (250 µg/mL) and were sacrificed either two hours or 16 hours post-injection. IL-1β mRNA expression in the rat brain was determined using RT-PCR and β-actin was used to normalize the IL-1β levels. This experiment was conducted twice with similar results and N=five animals/group/experiment. Error bars are present for all groups; however, the error bars for placebo implanted group injected with saline (P+S) and the morphine implanted group injected with saline (M+S) are too small ($<\pm 0.01$) to be observed.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. * $p<0.05$ as compared to corresponding control (P+S as compared to P+LPS and M+S as compared to M+LPS), ***$p<0.001$ as compared to corresponding control (P+S as compared to P+LPS and M+S as compared to M+LPS), # $p<0.05$ between groups (P+LPS and M+LPS).

IL-6 expression in the brain of morphine-tolerant rats

IL-6 levels in rat serum are increased in the presence of morphine as well as in the presence of LPS (Chang *et al.*, 1998; Ocasio *et al.*, 2004). To date; however, the LPS induced IL-6 mRNA expression profile in the brain of a morphine-tolerant rat has yet to be examined. The IL-6 mRNA expression levels in the brains of morphine-tolerant rats were examined at two hours and 16 hours following LPS or saline treatment using semi-quantitative RT-PCR and β-actin was used to normalize the levels of IL-6 mRNA. Animals implanted with placebo (P) and treated with saline (S) were used as a control.

At two hours post-treatment, the IL-6 mRNA levels in the brains of both the P+LPS (20.3±0.05) and M+LPS (10.29±0.05) rats were significantly higher than in the P+S (1.05±0.01) and M+S (0.05±0.03) animals, respectively. In contrast to the IL-1β mRNA expression as described in the above section (pages 70-72), the IL-6 mRNA levels in the brains of the M+LPS (10.29±0.05) rats were significantly lower than in the P+LPS group (20.3±0.05). By 16 hours following post-treatment, the IL-6 mRNA levels in the brains of the P+LPS (0.31±0.02) and M+LPS (0.25±0.03) rats had declined to levels similar to that of the P+S (0.29 ±0.04) and M+S (0.22±0.02) animals, respectively (Figure 7).

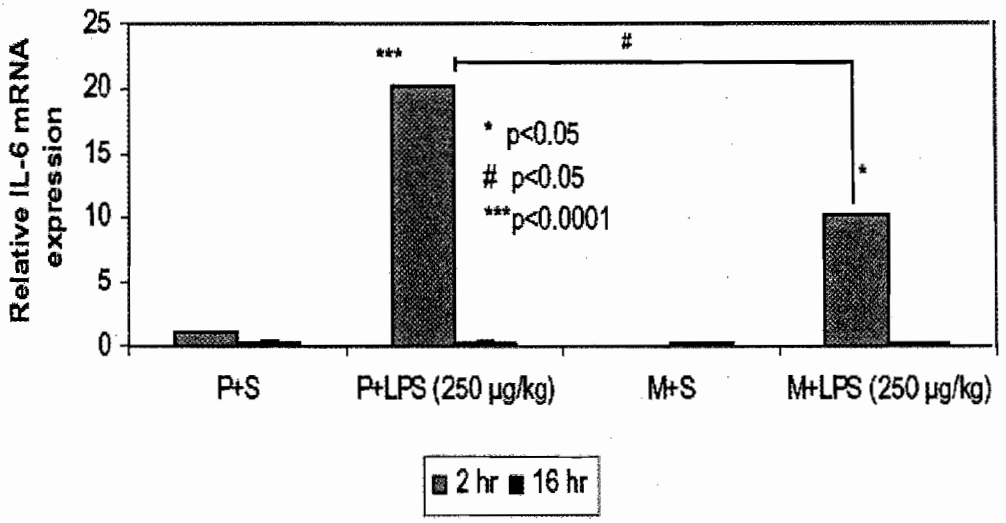

Figure 7: The effects LPS treatment on expression levels of IL-6 mRNA in a morphine tolerant rat brain.

Adult male rats were implanted with either two-plus-four 75 mg morphine pellets (M) or placebo pellets (P). Animals were administered with either saline (S) or lipopolysaccharide (LPS) (250 µg/mL) and were sacrificed either two hours or 16 hours post-injection. IL-6 mRNA expression in the rat brain was determined using semi-quantitative PCR and β-actin was used to normalize the IL-6 levels. This experiment was conducted twice with similar results and N=five animals/group/experiment. Error bars are present for all groups; however, the error bars are too small ($< \pm 0.05$) to be observed.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. * $p<0.05$ as compared to corresponding control (P+S as compared to P+LPS and M+S as compared to M+LPS), ***$p<0.001$ as compared to corresponding control (P+S as compared to P+LPS and M+S as compared to M+LPS), # $p<0.05$ between groups (P+LPS and M+LPS).

TNF-α mRNA expression in the brain of morphine-tolerant rats

Similar to IL-1β and IL-6, TNF-α serum levels are also increased in the presence of morphine alone or in combination with LPS in the rat model. Yet as with IL-1β and IL-6, the relative expression of LPS induced TNF-α mRNA in the brain of a morphine tolerant *in vivo* model has not yet been examined (Chang *et al.*, 1998; Ocasio *et al.*, 2004). The expression profile of TNF-α mRNA in the brains of morphine-tolerant rats injected with LPS was assayed with real-time PCR and the housekeeping gene GAPDH was used to normalize the TNF-α mRNA levels. Rats implanted with placebo (P), then treated with saline (S) were used as a control.

At two hours following LPS or saline treatment, there was a significant increase in TNF-α mRNA expression in the M+LPS group (0.816±0.17) as compared to the P+LPS (0.52±0.04) group. A decrease in TNF-α mRNA expression was observed in the M+S group (0.27±0.01) as compared to P+S (0.46±0.14) group. No difference was seen between the P+S (0.46 ± 0.14) and the P+LPS (0.52±0.04) groups. At 16 hours following LPS or saline treatment, the mRNA levels of TNF-α in both the P+LPS (1.82±0.21) and M+LPS (3.58±0.47) rats were significantly higher than that in the P+S (1.00±0.06) and M+S animals

(1.09±0.16) respectively. The TNF-α mRNA level in the M+LPS rats was significantly higher than that in the P+LPS animals (Figure 8).

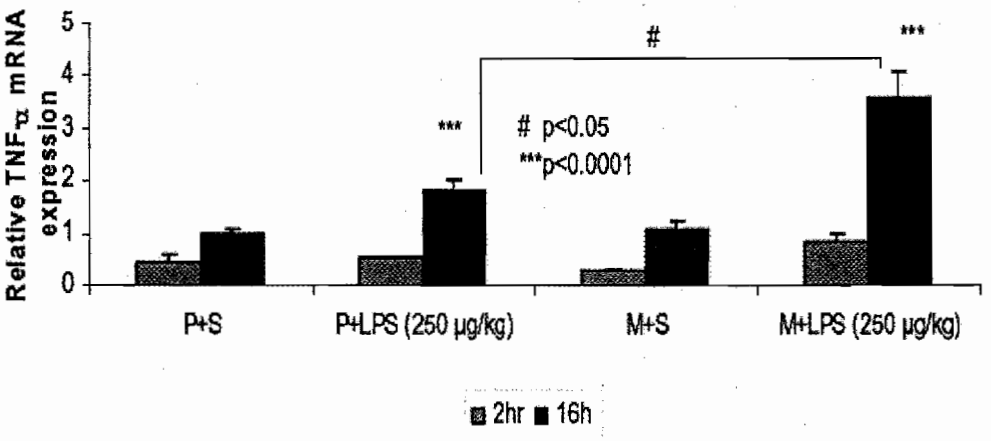

Figure 8: The effects of morphine and LPS on TNF-α mRNA expression in rat brain

Adult male rats were implanted with either two-plus-four 75 mg morphine pellets (M) or placebo pellets (P). Animals were administered either saline (S) or lipopolysaccharide (LPS) (250 µg/mL) and were sacrificed either two hours or 16 hours post-injection. TNF-α mRNA expression in the rat brain was determined using real-time PCR and GAPDH was used to normalize the TNF-α levels. This experiment was conducted twice with similar results and N=five animals/group/experiment. Error bars are present for all groups; however, the error bars are too small ($\leq \pm 0.05$) to be observed on the P+LPS and M+S groups at two hours post treatment.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. ***$p<0.001$ as compared to corresponding control (P+S as compared to P+LPS and M+S as compared to M+LPS), # $p<0.05$ between groups (P+LPS and M+LPS).

Basal level expression of the human opioid receptors in immune and neuronal cell lines

LPS induced pro-inflammatory cytokine mRNA expression levels are altered in the brain of morphine tolerant animals as compared to animals not in a morphine state (as indicated in the above results on page 70-77). This change in the pro-inflammatory cytokine profile may suggest that a potential immuno-opioid interaction exists in the brain when exposed to an inflammatory stimulus, such as LPS. In order to translate the rodent work into a more clinically applicable model, the U87 MG cell line, a human astrocytic cell line was examined to better understand the immuno-opioid relationship in a human model. Prior to examining this relationship, the expression of the opioid receptors in the U87 MG cell line were characterized as compared to immune cell lines (TPA-differentiated and undifferentiated HL-60) (Beltran *et al.*, 2006) and neuronal cell lines (SH-SY5Y, NMB) with known opioid receptor expression levels (Choi *et al.*, 2005). Real-time PCR was used to determine the number of copies (copy number) of each opioid receptor sub-type (hMOR, hDOR, hKOR). GAPDH was used to normalize the copy number of hMOR, hDOR and hKOR.

All of the cell lines examined possessed basal levels of all three opioid receptors, although the levels of expression of each opioid receptor varied between cell lines. SH-SY5Y neuroblastoma cells had the highest expression of hMOR ($1.63x10^7 \pm 0.41x10^7$copies of hMOR/μg of total RNA) as compared to the TPA-differentiated HL-60 cells ($6.06x10^5 \pm 0.30\ x10^5$ copies of hMOR/μg of total RNA), undifferentiated HL-60 cells ($1.43x10^5 \pm 0.67\ x10^5$ copies of hMOR/μg of total RNA), NMB cells ($9.5x10^4 \pm 4.8\ x10^4$copies of hMOR/μg of total RNA) and U87 MG cells ($3.43x10^5 \pm 0.67\ x10^5$ copies of hMOR/μg of total RNA).

NMB cells expressed the highest levels of hDOR ($3.68x10^7 \pm 0.48\ x10^7$ copies of hDOR/μg of total RNA) as compared to the TPA-differentiated HL-60 cells ($1.94x10^6 \pm 0.3\ x10^6$ copies of hDOR/μg of total RNA), undifferentiated HL-60 cells ($1.26x10^6 \pm 0.06\ x10^6$ copies of hDOR/μg of total RNA), SH-SY5Y cells ($1.67x10^7 \pm 0.41\ x10^7$ copies of hDOR/μg of total RNA) and U87 MG cells ($7.86\ x10^5 \pm 0.06\ x10^5$ copies of hDOR/μg of total RNA).

TPA-differentiated HL-60 cells had the highest expression of hKOR ($2.18x10^7 \pm 0.30\ x10^7$ copies of hKOR/μg of total RNA) as compared to HL-60 cells ($5.55x10^6 \pm 0.14\ x10^6$ copies of hDOR/μg of total RNA), NMB cells ($1.17x10^7 \pm 0.48\ x10^7$ copies of hDOR/μg of total RNA),

SH-SY5Y cells ($2.25x10^4 \pm 0.32 x10^4$ copies of hDOR/μg of total RNA) and U87 MG cell ($1.21x10^5 \pm 0.13 x10^5$ copies of hDOR/μg of total RNA) (Figure 9).

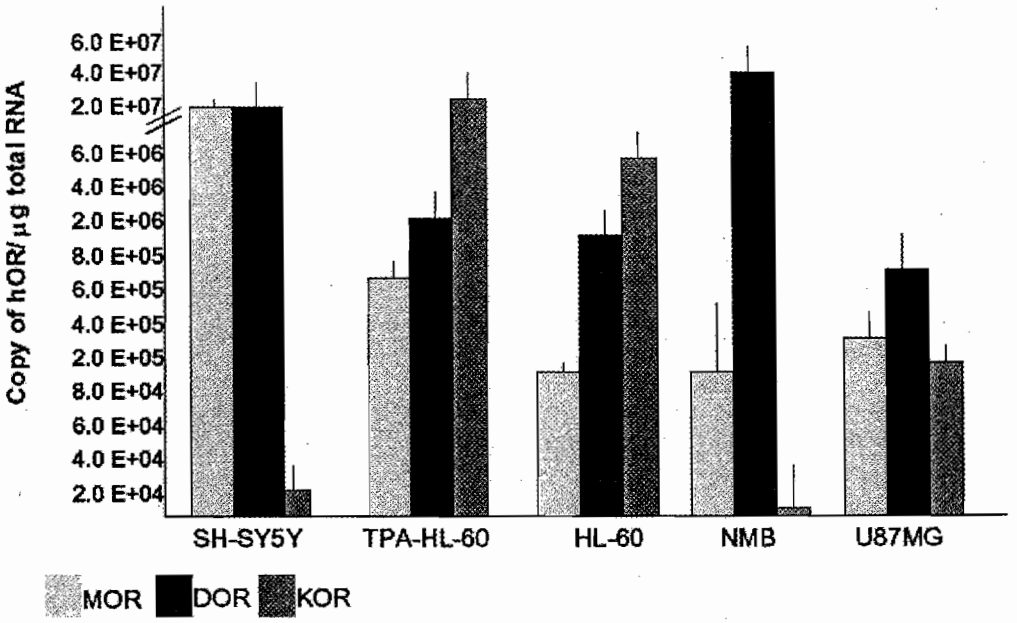

Figure 9: The basal expression levels of hMOR, hDOR, hKOR mRNA as determined using real-time PCR in various neuronal or immune cell lines.

Expression (copy number) of basal levels of the human opioid receptors, hMOR, hDOR and hKOR in the SH-SY5Y, HL-60 (TPA differentiated and undifferentiated), NMB and U87 MG astrocytic cell lines were determined using real-time PCR. GAPDH was used to normalize the levels of hMOR, hDOR and hKOR in each cell line.

Data is indicated as mean $\pm$ standard deviation. This experiment was conducted twice with similar results and each cell line has an n=3 T-75 flasks/ experiment.

Immunofluorescence staining of human MOR (hMOR) in U87 MG cell line

Immunofluorescence staining was used to further characterize the basal expression of hMOR in the U87 MG cell line. U87 MG cells stained with goat anti-rabbit IgG (1:1000) alone served as a negative control (Figure 10A). 4'-6-Diamidino-2-phenylindole (DAPI) (1:1000) staining was used to stain the nucleus as is shown in blue (Figure 10B). Immunofluorescence staining with rabbit anti-MOR as indicated with the green stain (1:1000) shows that basal levels of hMOR are expressed in the U87 MG cell line in the cell membrane of these astrocytic cells (Figure 10C & 10E). A superimposed image (Figure 10D) indicates the position of hMOR as compared to the nucleus, as stained in blue.

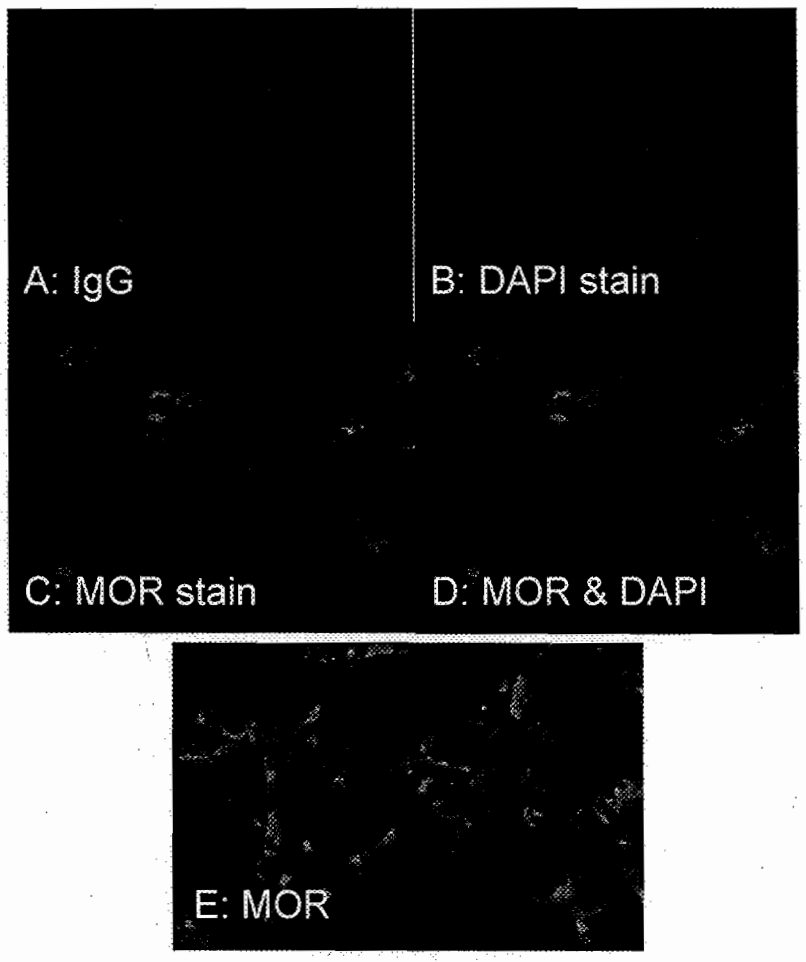

Figure 10: Immunofluorescence staining of human mu-opioid receptor (hMOR).

Immunofluorescence staining was used to visualize the basal expression level of human mu-opioid receptor (hMOR). (A) U87 MG cells stained with goat anti-rabbit IgG (1:1000) alone which served as a negative control. (B) U87 MG cells stained with 4'-6-Diamidino-2-phenylindole (DAPI) (1:1000). (C&E) U87 MG cells stained with rabbit anti-MOR (1:1000) (D) A superimposed image of Figures B and C to show location of MOR. This experiment was conducted twice with similar results.

Functionality of human mu-opioid receptor (hMOR) expressed in U87 MG cell

The mu-opioid receptor (MOR) is a G-inhibitory protein coupled seven transmembrane receptor that upon activation initiates a signaling cascade that inhibits adenylyl cyclase which in turn results in decreased intracellular cAMP levels (Vigano *et al.*, 2003). In order to determine the functionality of hMOR expressed in the U87 MG cell line, a forskolin induced cAMP assay was conducted as described by Yu *et al.* (2003). As expected, forskolin (75 μM), an adenylyl cyclase activator, resulted in significantly increased levels of cAMP (6.0±0.25 pmol/μg protein) as compared to untreated control cells (0.1±0.005 pmol/μg protein). U87 MG cells treated with morphine (10 μM), a MOR agonist, had similar cAMP levels (0.3±0.05 pmol/μg protein) to the control (0.1±0.005 pmol/μg protein). U87 MG cells treated with morphine (10 μM) and forskolin (75 μM) had a significant decrease in cAMP levels (1.4±0.075 pmol/μg protein), as compared to forskolin treated cells (6.0±0.25 pmol/μg protein). The addition of a MOR antagonist, naloxone (10 μM) resulted in an increase in cAMP levels (4.5±0.075 pmol/μg protein), similar to the forskolin treated cells (6.0±0.25 pmol/μg protein) (Figure 11).

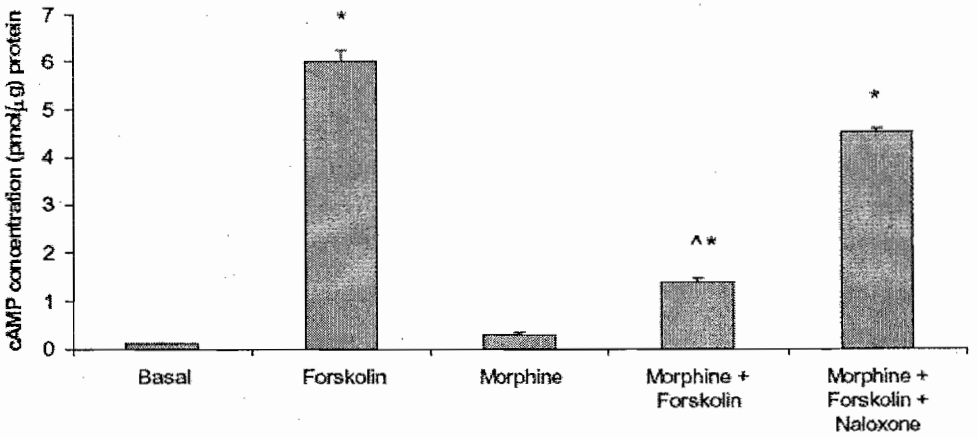

Figure 11: The effects of morphine and nalaxone, a mu-opioid receptor antagonist, on forskolin-induced cAMP levels.

Functionality of hMOR expressed in U87 MG cells was determined using a forskolin-induced cAMP accumulation assay. cAMP accumulation levels were determined in basal (untreated) U87 MG cells and in U87 MG cells treated with forskolin alone (75 μM), morphine alone (10 μM), forskolin (75 μM) + morphine (10 μM) or forskolin (75 μM) + morphine (10 μM) + naloxone (10 μM). This experiment was conducted twice with similar results.

During each experiment, each treatment group was repeated three times. Data is indicated as mean $\pm$ standard deviation. Error bars are present for all groups; however, the error bars for basal and morphine treatments are too small ($\leq \pm 0.05$) to be observed.

One-way ANOVA was used *$p<0.05$ as compared to basal treatment and ^$p<0.05$ as compared to forskolin treatment alone.

Endomorphin-1 and endomorphin-2 are potent endogenous MOR agonists (Zadina *et al.*, 1997). A parallel experiment was conducted using endomorphin-1 and endomorphin-2 in place of morphine to further understand the functionality of the hMOR expressed in U87 MG. A similar pattern was observed with endomorphin-1 and endomorphin-2 as was observed with morphine (Figure 12).

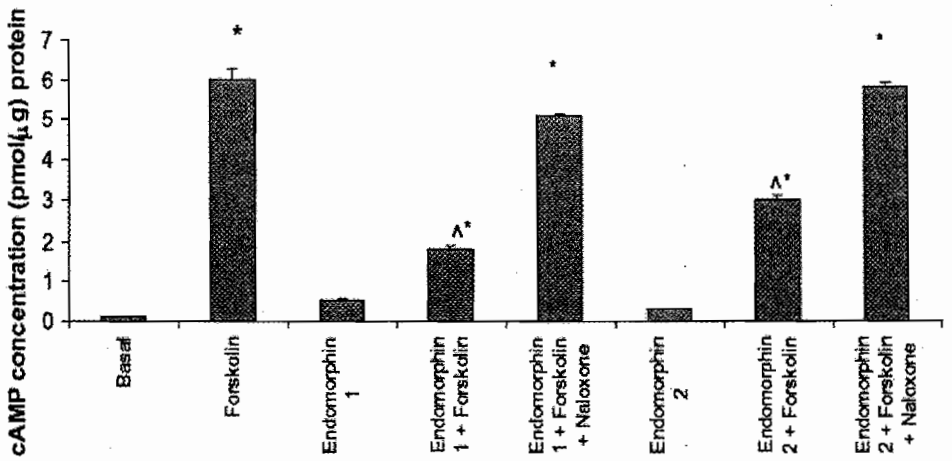

Figure 12: The effects of two endongenous mu-opioid receptor agonists, endomorphin-1 and endomorphin-2 and nalaxone, a mu-opioid receptor antagonist, on forskolin-induced cAMP levels.

Functionality of hMOR expressed in U87 MG cells was determined using a forskolin-induced cAMP accumulation assay. cAMP accumulation levels were determined in basal (untreated) U87 MG cells and in U87 MG cells treated with forskolin alone (75 μM), endomorphin-1 alone (10 μM), endomorphin-2 alone (10 μM), fosrkolin (75 μM) + endomorphin-1 (10 μM), forskolin (75 μM) + endomorphin-2 (10 μM), forskolin (75 μM) + endomorphin-1 (10 μM) + naloxone (10 μM) or forskolin (75 μM)+ endomorphin-2 (10 μM) + naloxone (10 μM). This experiment was conducted twice with similar results.

During each experiment, each treatment group was repeated three times. Data is indicated as mean $\pm$ standard deviation. Error bars are present for all groups; however, the error bars for basal, endomorphin-1 and endomorphin-2 treatments are too small ($<\pm 0.05$) to be observed.

One-way ANOVA was used *$p<0.05$ as compared to basal treatment and ^$p<0.05$ as compared to forskolin treatment alone.

Up-regulation of hMOR, hDOR, and hKOR with Interleukin-1β (IL-1β)

The ability of a pro-inflammatory cytokine to induce an opioid receptor has been shown previously as IL-1 exposure results in an induction of the MOR in human brain microvascular endothelial cells (Vidal *et al.*, 1998). In order to determine the effects of IL-1β on the opioid receptors in an astrocytic cell line, U87 MG cells were treated with IL-1β (20 ng/mL or 40 ng/mL) for 12 hours. The expression of hMOR, hDOR and hKOR were determined using real-time PCR and GAPDH was used to normalize the expression of hMOR, hDOR and hKOR. The comparative C_T method was used to calculate expression of hMOR, hDOR and hKOR relative to GAPDH expression. Cells treated with control were assigned relative expression of 1.0 and all treatment groups were compared to the control.

A significant up-regulation of hMOR in a dose dependent manner was seen in the U87 MG cells treated with IL-1β at 20 ng/mL and 40 ng/mL for 12 hours (5.35±0.44 and 14.5±0.32, respectively) (Figure 13). U87 MG cells treated with IL-1β at 20 ng/mL had an increase in hDOR as compared to the control treated cells, although not significant. However, U87 MG cells treated with 40 ng/mL IL-1β had a significant increase in hDOR, as compared to the cells treated with control (1.74±0.24 and

1.0±0.00, respectively) (Figure 14). A significant up-regulation of hKOR was observed in a dose dependent manner with 20 ng/mL and 40 ng/mL IL-1β (1.79±0.27 and 2.09±0.27, respectively), as compared to the control (1.0±0.00) (Figure 15).

A time-course experiment was then conducted to examine if the up-regulation of hMOR in the U87 MG cells was time dependent. IL-1β (20 ng/mL) enriched media were added to U87 MG cells for 0, 3, 6, 12, 24 and 48 hours. Cells treated with cell culture media for the above mentioned times were used as controls. IL-1β up-regulation of hMOR was seen as early as three hours (1.81±0.26); however, the levels of hMOR expression were not significant as compared to the control (1.0±0.00). Significant increases in hMOR expression were observed at six hours (3.95±0.38), 12 hours (5.35±0.26), 24 hours (4.23±0.24) and 48 hours (3.53±0.39) as compared to the control (1.0±0.00) (Figure 16).

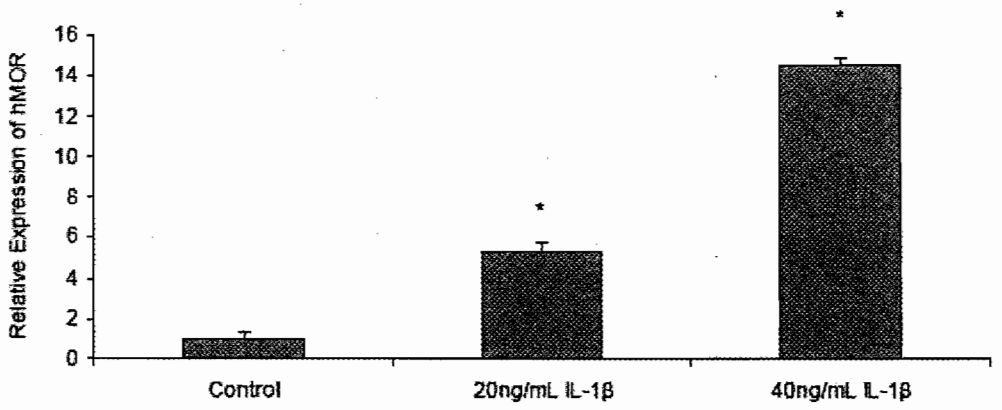

Figure 13: The effects of IL-1β on hMOR expression in U87 MG cells.

U87 MG cells were treated with either control (cell culture media) or IL-1β (20 ng/mL or 40 ng/mL) for 12 hours. Real-time PCR was used to determine the levels of hMOR and GAPDH was used to normalize the hMOR levels. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean ± standard deviation. Statistical significance as determined by Student t-test. * $p<0.05$ as compared to untreated control cells.

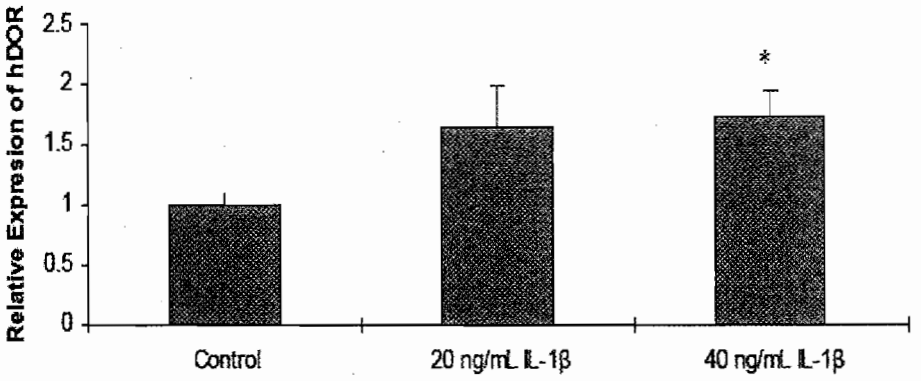

Figure 14: The effects of IL-1β on hDOR expression in U87 MG cells.

U87 MG cells were treated with either control (cell culture media) or IL-1β (20 ng/mL or 40 ng/mL) for 12 hours. Real-time PCR was used to determine the levels of hDOR and GAPDH was used to normalize the hDOR levels. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. * $p<0.05$ as compared to untreated control cells.

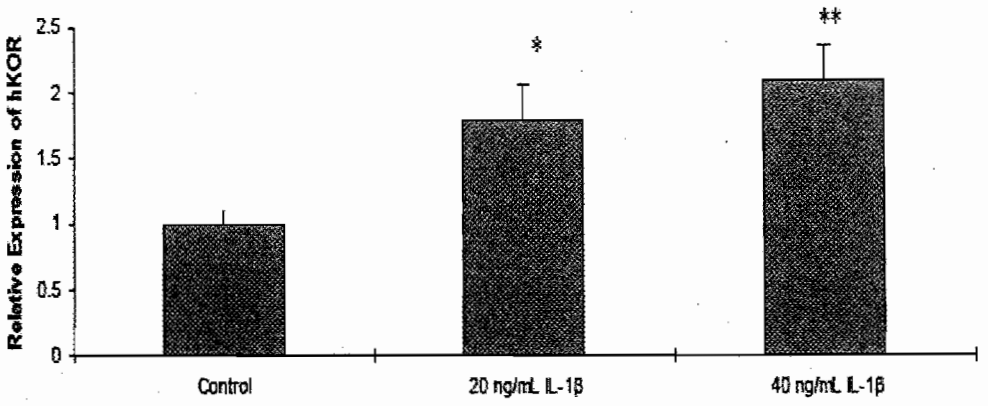

Figure 15: The effects of IL-1β on hKOR expression in U87 MG cells.

U87 MG cells were treated with either control (cell culture media) or IL-1β (20 ng/mL or 40 ng/mL) for 12 hours. Real-time PCR was used to determine the levels of hKOR and GAPDH was used to normalize the hKOR levels. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean $\pm$ standard deviation. Statistical significance as determined by Student t-test. * $p<0.05$ as compared to untreated control cells and ** $p<0.001$ as compared to untreated control cells.

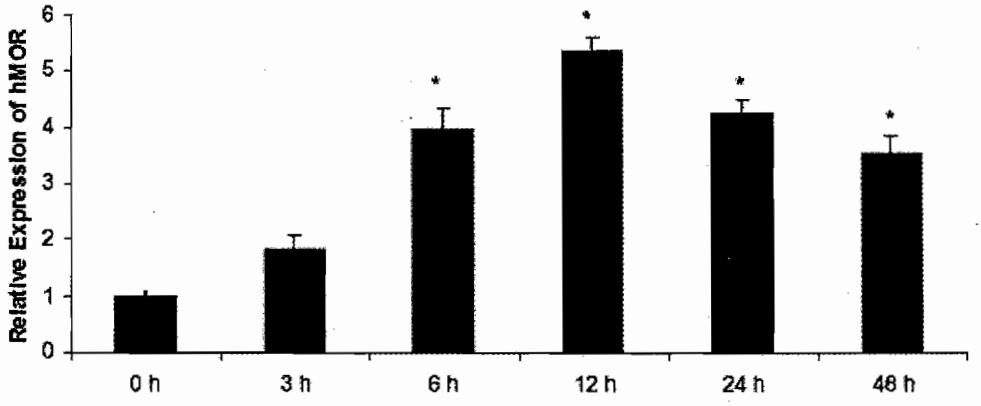

Figure 16: The time-course effects of IL-1β on hMOR expression in U87 MG cells.

U87 MG cells were treated with either control (cell cuture media) or IL-1β (20 ng/mL) for 0, 3, 6, 12, 24 and 48 hours. Real-time PCR was used to determine the levels of hMOR and GAPDH was used to normalize the hMOR levels. Each time-point was adjusted by the appropriate time-point control. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean $\pm$ standard deviation. Student t-test was used to determine signficance and *$p<0.05$ as compared to 0 hours (control).

IL-8 expression correlates with increased expression levels of the opioid receptors

IL-1 regulates expression of chemokines such as IL-8 and a correlation between IL-1 exposure and increased levels of IL-8 expression has been established as a measure of IL-1 receptor functionality (Sunyer *et al.*, 1999;Gkoumass *et al.*, 2007). In order to confirm the functionality of the IL-1 receptor in the U87 MG cell line, the expression levels of IL-8 in U87 MG cells treated with IL-1β (20 ng/mL or 40 ng/mL) for 12 hours were examined. Cell culture supernatant was removed and IL-8 expression levels were determined using the MSD® 96-well multi assay platform system.

U87 MG cells treated with IL-1β (20 ng/mL and 40 ng/mL) had significant increases in IL-8 expression ($1.1x10^3 \pm 3.7$ ng/mL and $1.1 x10^3 \pm 5.8$ ng/mL, respectively) as compared to the control (354 ± 15.0 ng/mL). U87 MG cells treated with 20 ng/mL IL-1β had comparable levels of IL-8 expression ($1.1 x10^3 \pm 3.7$ ng/mL) as compared to U87 MG cells treated with 40 ng/mL IL-1β ($1.1 x10^3 \pm 5.8$ ng/mL) (Figure 17).

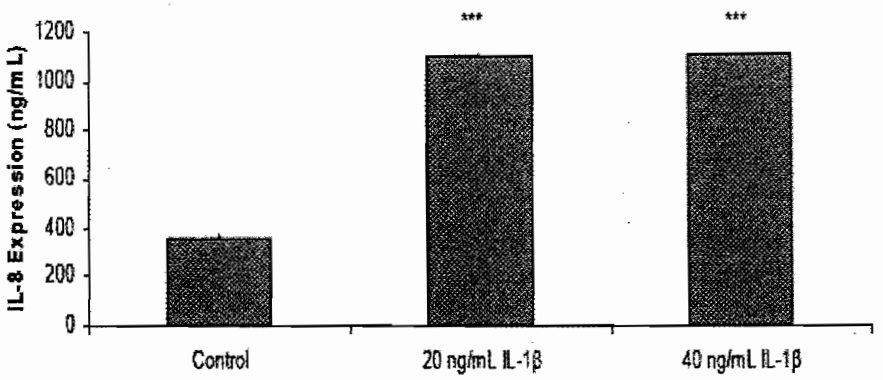

Figure 17: The effects of IL-1β on IL-8 expression in U87 MG cells.

U87 MG cells were treated with either control (cell culture media) or IL-1β (20 ng/mL or 40 ng/mL) for 12 hours. Following the 12 hours, the cell culture media were aspirated and assayed for IL-8 levels. This experiment was conducted twice with six samples/treatment. Error bars are present for all groups; however, the error bars are too small ($<\pm 50$) to be observed.

Data is indicated as mean $\pm$ standard deviation. Student t-test was used to determine signficance ***$p<0.001$, as compared to control untreated cells.

IL-1β up-regulation of hMOR is reversible with IL-1 receptor antagonist protein (IL-1RAP)

IL-1β exerts its biological effect through the binding of interleukin-1 receptor 1 (IL-1R1) and the use of an antagonist to this receptor, interleukin-1 receptor antagonist protein (IL-1RAP) has been shown to result in a decrease in IL-1β effects (Hagihara *et al.*, 2004). IL-1RAP was used to examine whether IL-1β up-regulation of hMOR is mediated through IL-1R1. U87 MG cells were treated with either vehicle (cell culture media), IL-1β (20 ng/mL) alone or in combination with IL-1RAP (either 400 ng/mL or 4,000 ng/mL) for 12 hours. A 1:100 ratio of IL-1β to IL-1RAP has been shown to be necessary for IL-1RAP to be effective (Hagihara *et al.*, 2004). Real-time PCR was used to determine the levels of hMOR and GAPDH and expression of hMOR was normalized to GAPDH expression. The comparative C_T method was used to calculate expression of hMOR and cells treated with control were assigned relative expression of 1.0 and all treatment groups were compared to the control with regard to expression levels of hMOR.

U87 MG cells treated with IL-1β (20 ng/mL) had an increase in hMOR (3.8±0.05) as compared to the U87 MG cells treated with control (1.0±0.0) (Figure 18). U87 MG cells treated with IL-1RAP (400 ng/mL) +

vehicle had a significant decrease in hMOR expression (0.16±0.64), as compared to the control (1.0±0.0) and as compared to IL-1β treatment (3.8±0.05). The co-treatment of IL-1RAP (400 ng/mL) + IL-1β (20 ng/mL) (0.36±0.27) also resulted in a significant decrease of hMOR, as compared to vehicle treated control (1.0±0.0) and IL-1β (20 ng/mL) (3.8±0.05).

U87 MG cells treated with higher concentrations of IL-1RAP (4,000 ng/mL) + vehicle resulted in no difference in hMOR expression as compared to the vehicle (0.89±0.24); however, there was a significant decrease as compared to IL-1β treated cells (3.8±0.05). The co-treatment of IL-1RAP (4,000 ng/mL) + IL-1β (20 ng/mL) resulted in a significant decrease in hMOR (0.43±0.38) as compared to control treated cells (1.0±0.0) and IL-1β (20 ng/mL) (3.8±0.05) (Figure 18).

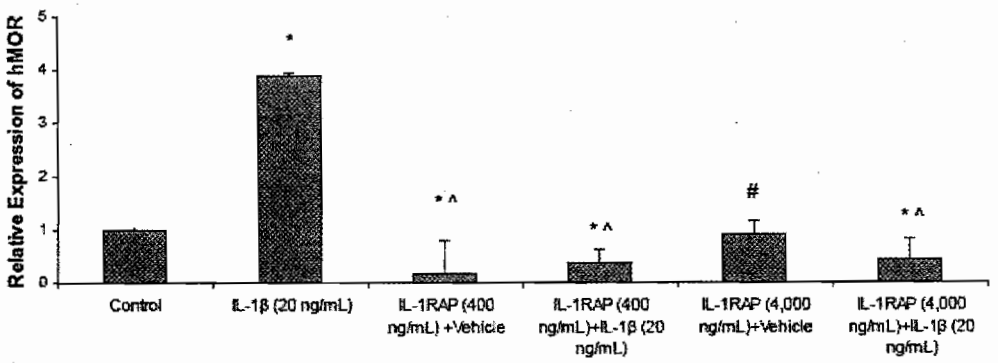

Figure 18: The effects of IL-1 receptor antagonist protein (IL-1RAP) on IL-1β up-regulation of hMOR in U87 MG cells.

U87 MG cells were treated with control, IL-1β (20 ng/mL), IL-1 receptor antagonist protein (IL-1RAP) (400 ng/mL) + vehicle, IL-1RAP (400 ng/mL) + IL-1β (20 ng/mL), IL-1RAP (4,000 ng/mL) + vehicle or IL-1RAP (4,000 ng/mL) + IL-1β (20 ng/mL) for 12 hours. Real-time PCR was used to determine the levels of hMOR and GAPDH was used to normalize the hMOR levels. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean $\pm$ standard deviation. Student t-test was used to determine signficance *$p<0.05$ as compared to control, ^ $p<0.001$ as compared to IL-1β (alone) and # $p<0.01$ as compared to IL-1β (alone).

The effects of morphine pre-treatment on IL-1β up-regulation of hMOR expression in U87 MG cells.

Chronic exposure to morphine results in a desensitization of the mu-opioid receptor (Conner *et al.*, 2004). To further examine the immuno-opioid relationship, IL-1β's ability to potentially up-regulate a desensitized hMOR after chronic morphine treatment was examined. U87 MG cells were first treated with morphine (100 nM) (a dose that mimics chronic dependent conditions (Suarez-Roca H and Maixner W, 1992) for either 0, 45 minutes, 3, 6, 12, 24 or 48 hours to examine the time-course of desensitization of the hMOR (as determined by a decrease in MOR mRNA) in this astrocytic cell line. Copy numbers of hMOR and GAPDH were determined using real time PCR. GAPDH was used to normalize hMOR levels.

U87 MG cells treated with 100 nM morphine for 45 minutes had a significant decrease in the copy number of hMOR ($2.5x10^4 \pm 3.7x10^3$ copies of hMOR/μg total RNA) as compared to the control ($9.4 x10^3 \pm 3.2 x10^3$ copies of hMOR/μg total RNA). However, hMOR was significantly increased at three hours of morphine treatment ($1.9 x10^6 \pm 2.9 x10^4$ copies of hMOR/μg total RNA) as compared to the control ($9.4 x10^3 \pm 3.2 x10^3$ copies of hMOR/μg total RNA). Expression level of hMOR decreased at 6, 12, 24 and 48 hours of morphine treatment ($7.0 x10^5 \pm 2.9 x10^4$ copies

of hMOR/μg total RNA at 6 hours; 4.4 $x10^5 \pm 1.4\ x10^4$ copies of hMOR/μg total RNA at 12 hours; 2.2 $x10^5 \pm 2.3\ x10^4$ copies of hMOR/μg total RNA at 24 hours; and 2.9 $x10^5 \pm 9.3\ x10^4$ copies of hMOR/μg total RNA at 48 hours), as compared to control (9.4 $x10^3 \pm 3.2\ x10^3$ copies of hMOR/μg total RNA) (Figure 19).

Time-course experiments indicated that a desensitization of hMOR occurred as indicated with a decrease in the copy number of hMOR as early as six hours of morphine treatment and this decrease in copy number of hMOR continued through 48 hours morphine treatment, the last time-point assessed. In order to examine IL-1β's ability to restore hMOR levels after being desensitization, U87 MG cells were treated with morphine (100 nM) for 24 hours, a time-point in which a decrease in hMOR expression was seen, as compared to three hours. Upon completion of the morphine treatment, cell culture media containing morphine was removed and fresh media containing either vehicle or IL-1β (20 ng/mL) was added to the U87 MG cells. The cells were incubated for 12 hours and real-time PCR was used to measure hMOR and GAPDH expression levels. GAPDH was used to normalize hMOR expression. The comparative C_T method was used to calculate the expression of hMOR and cells treated with control were assigned a relative expression

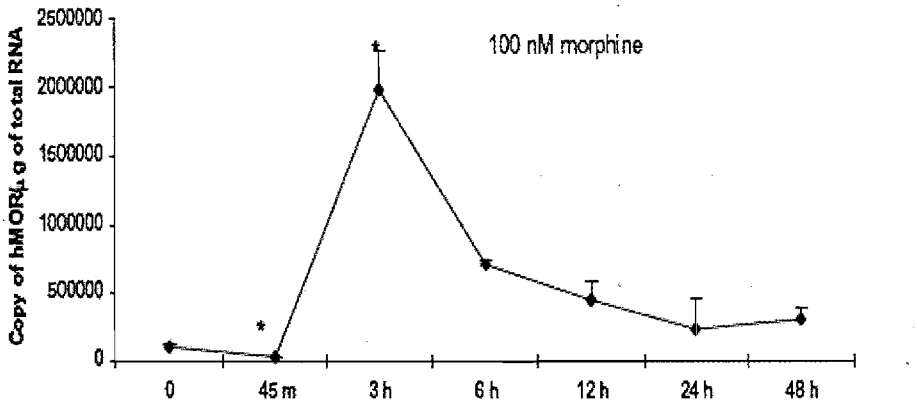

Figure 19: The effects of morphine on hMOR expression in U87 MG cells.

U87 MG cells were treated with either vehicle (cell culture media) or morphine (100 nM) for 0, 45 minutes, 3, 6, 12, 24 or 48 hours. Real-time PCR was used to determine the copy number of hMOR and GAPDH. GAPDH levels were used to normalize the hMOR levels. Each time-point was adjusted by the appropriate time-point control. This experiment was conducted twice with similar results and each treatment group was repeated three times/experiment.

Data is indicated as mean $\pm$ standard deviation. Student t-test was used to determine signficance and * $p<0.05$ as compared to 0 hours (control).

of 1.0. All treatment groups were compared to the control with regard to expression levels of hMOR.

U87 MG cells treated with IL-1β (20 ng/mL) had similar levels of hMOR (0.9±0.15) as compared to the vehicle treated group (1.0±0.8) (Figure 20). This variation in IL-1β up-regulation of hMOR was possibly due to the over confluency of the cells. Upon completion of the 36 hours incubation (24 hours pre-treatment followed by 12 hours treatment), the cells were approximately 90-100% confluent. Prior experiments which showed IL-1β up-regulation of hMOR used U87 MG cells that were approximately 60-80% confluent at the conclusion in the incubation. Cells treated with 100 nM morphine + vehicle had significant increases in hMOR levels (2.94±0.35) as compared to the control (1.0±0.0). Cells treated with 100 nM morphine followed by IL-1β for 12 hours (20 ng/mL) had a significant increase in hMOR (7.32±0.24) as compared to the control (1.0±0.0) as well as compared to the cells treated with 100 nM morphine + vehicle (2.94±0.35).

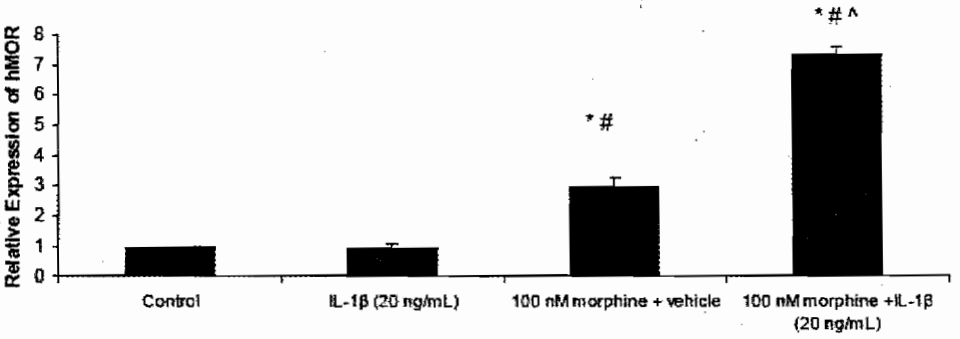

Figure 20: The effects of morphine pre-treatment on IL-1β up-regulation of hMOR expression in U87 MG cells.

U87 MG cells were pre-treated with either vehicle (cell culture media) or morphine (100 nM) for 24 hours. Following pre-treatment, media were aspirated and fresh cell culture media containing either vehicle or IL-1β (20 ng/mL) was added to the cells and the cells were incubated for 12 hours. Real-time PCR was used to determine expression of hMOR and GAPDH. Levels of hMOR were normalized to GAPDH. This experiment was conducted one time with six wells/treatment.

Data is indicated as mean $\pm$ standard deviation. Student t-test was used to determine signficance * $p<0.05$ as compared to the control, # $p<0.05$ as compared to IL-1β (alone) and ^ $p<0.001$ as compared to the control.

Discussion

The inflammatory response is an essential component of the immune system and is a complicated process that can be activated by various stimuli including bacterial components, such as lipopolysaccharide (LPS) and cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) or interleukin-6 (IL-6) (House *et al.*, 2001; Boontham *et al.*, 2003). Upon activation of the inflammatory response, an alteration in the expression of adhesion molecules present in the vascular endothelium as well as in leukocytes occurs. This amplification of adhesion molecules results in an increase in leukocyte-endothelial interaction (LEI) which includes an increase in leukocyte-endothelial adhesion (LEA) and a decrease in leukocytes rolling through the mesentery (flux). Upon activation of LEI, there is a subsequent initiation of various immune responses which result in cytokine release, phagocytosis and intracellular degradation which serve to restore homeostasis (Ocasio *et al.*, 2004, Chang *et al.*, 2001).

The bacterial chemoattractant n-formyl-methionyl-leucyl-phenylalanine (FMLP) is routinely used to examine the functionality of the immune response in *in vivo* rodent models. Prior to examining the effects

of morphine on the pro-inflammatory cytokine expression profile in a rat model of inflammation, the functionality of this model's immune system was examined using FMLP and intravital microscopy, a microscope that allows the user to examine internal biological processes in a live animal. Upon application of topical FMLP, there was a significant increase in LEA (Figure 3, page 67) and a significant decrease in leukocyte flux (Figure 4, page 68) indicating that an increase in adhesion molecules had occurred. The rodent model's response to FMLP (as assessed by LEA and flux) indicated that the model had a functional immune response and could be used to examine the effects of morphine on pro-inflammatory cytokine expression levels in an inflammatory state.

Prior work in our laboratory has used FMLP and intravital microscopy to examine the effects of morphine on the hypothalamic-pituitary-adrenal (HPA) axis (House *et al.*, 2001). Under normal physiological conditions, pro-inflammatory cytokines, such as IL-1β, activate the HPA axis by stimulating the production of corticotrophin-releasing hormone (CRH) in the paraventricular nucleus (PVN) of the hypothalamus. A subsequent secretion of adrenocorticotropic hormone (ACTH) from the adrenal pituitary gland occurs which in turn results in the production of corticosterone from the adrenal cortex. Corticosterone is a glucocorticoid that has anti-inflammatory properties and is involved in the

regulation of the HPA axis by providing a negative feedback loop and countering the inflammatory respone (Hadley, 1996; Slominski *et al.*, 2005). In the morphine tolerant state, a desensitization of neuronal activation of PVN occurs which modifies CRH expression and subsequently results in decreased plasma corticosterone levels (Chang *et al.*, 1993; House *et al.*, 2001). Upon exposure to an inflammatory stimulus, such as LPS, in a morphine tolerant state, an increase in LEA is observed, which may be an indirect result of decreased plasma corticosterone levels (House *et al.*, 2001). In addition to desensitizing the HPA axis, systemic morphine administration disrupts the blood-brain-barrier by decreasing the viability of the vascular and brain microvascular endothelial cell monolayer (Liu *et al.*, 2004). This alteration in the blood-brain-barrier may serve as the cellular mechanism by which bacterial endotoxins such as LPS, when given systemically, enter into the brain compartment resulting in inflammation and a subsequent increase in pro-inflammatory cytokines in the brain tissue.

LPS, a bacterial surrogate, is a potent and well studied activator of the immune response. Exposure to LPS in large doses results in endotoxin shock and sepsis; however, the use of LPS in non-lethal doses has proven a valuable tool in understanding the mechanisms associated with inflammation. Unlike FMLP, which is topically administered, LPS can

be given systemically to elicit an immune response and mimics a gram-negative bacterial infection.

Our laboratory, along with other laboratories, has previously shown that LPS exposure results in a significant increase in IL-1β, TNF-α and IL-6 in the serum and brain in a rodent normal model (Ocasio *et al.*, 2004; Chen *et al.*, 2005; Qin *et al.*,2008; Datta and Opp, 2007). However, in order to test the hypothesis that chronic morphine exposure alters the pro-inflammatory cytokine profile in the brain, the expression profile of LPS induced IL-1β, TNF-α, and IL-6 was examined in the brain of a morphine tolerant rat. A two-plus-four morphine pellet implantation regimen was used to create a morphine tolerant animal model (Ocasio *et al.*, 2004). Morphine serum levels were assayed using radioimmunoassay at day five (16 hours post-LPS injection). Morphine serum levels confirmed that the animals, which were implanted with morphine pellets at days one and two, still had significant morphine levels in their serum upon sacrifice, indicating that the animals were morphine tolerant (Figure 5, page 71). This increase in serum morphine levels was consistent, although higher, with prior morphine pelleting studies in mice, which showed a 30 fold increase in serum morphine levels in animals implanted with morphine, as compared to placebo implanted animals (Levier *et al.*, 1993). The increase in morphine serum levels in this

experiment was higher due to higher doses of morphine being studied. Additionally, as expected, there was no difference in morphine serum levels in the morphine implanted animals injected with either saline or LPS.

Upon confirmation that the rodent model had a functional immune system and was in a morphine tolerant state, the first objective of this project was to examine the ability of chronic morphine exposure to alter the pro-inflammatory cytokine expression profile (IL-1β, TNF-α and IL-6) in the rat brain in response to an inflammatory stimulus (LPS). Prior work in our laboratory has shown that exposure to LPS results in peak levels of IL-1β and IL-6 two hours post-LPS injection in the normal rat brain and serum (not exposed to morphine). By 16 hours post-LPS injection, the levels of IL-1β and IL-6, although still elevated when compared to non-LPS treatment, were much lower than those at two hours post-LPS injection (Ocasio *et al.*, 2004; Chen *et al.*, 2005). This time-course of LPS induced IL-1β and IL-6 indicates that both IL-1β and IL-6 may serve as important cytokines in the initiation of the inflammatory response both in the central nervous system as well as systemically. Upon activating the inflammatory response, the levels of LPS induced IL-1β and IL-6 then gradually decrease.

In the morphine tolerant animal exposed to LPS, the expression profile of IL-1β in the brain followed a similar expression pattern as was seen in the brain and serum of animals treated with LPS alone (Ocasio *et al.*, 2004; Chen *et al.*, 2005). Animals implanted with either placebo or morphine and injected with LPS had an increased expression of IL-1β at two hours post-LPS injection, as compared to animals injected with saline. However, a significant increase in LPS induced IL-1β was observed in the morphine tolerant group as compared to the placebo treated animals, indicating that morphine exposure potentiates the LPS induced IL-1β (Figure 6, page 74). This increase in LPS induced IL-1β in the morphine tolerant state suggests that chronic morphine exposure intensifies the inflammatory response in the brain and may potentially result in damage to sensitive brain cells upon frequent exposure to inflammatory agents.

Whereas LPS induced IL-1β expression was potentiated in the morphine tolerant model, the levels of IL-6, a cytokine that possess both pro-inflammatory and anti-inflammatory properties, were suppressed in the morphine tolerant state. At two hours post-LPS injection, both placebo treated and morphine treated animals had peak levels of IL-6 mRNA in the brain, a result that was consistent with previous reports of LPS induced levels of IL-6 in rat serum and brain (not in a morphine

tolerant state) (Ocasio *et al.*, 2004; Chen *et al.*, 2005). However, the morphine tolerant group had a decrease in LPS induced IL-6, as compared to placebo treated group injected with LPS (Figure 7, page 76). This decrease in LPS induced IL-6 in the morphine tolerant state is consistent with IL-6's expression profile. Prior studies have reported that an inverse relationship exists between IL-6 and IL-1β and TNF-α. With an increase in IL-6 levels, there is a subsequent decrease in the expression of IL-1β and TNF-α (Van Meir, 1995; Chen *et al.*, 2005; Barton, 1997). As chronic morphine exposure resulted in an increase in LPS induced IL-1β levels, the inverse relationship between IL-6 and IL-1β remains intact and there is a decrease in LPS induced IL-6 levels. The decrease in LPS induced IL-6 levels in the morphine tolerant state, suggests that a decrease in anti-inflammatory cytokines, such as IL-6 may serve to further potentiate the inflammatory response in the morphine tolerant brain.

Whereas peak concentration of IL-1β and IL-6 both in the serum and in the brain occur at two hours post-LPS injection both in morphine naïve (Ocasio *et al.*, 2004; Chen *et al.*, 2005) and morphine treated rodents (Staikos *et al.*, 2008), TNF-α levels vary between serum and brain. In a rodent model, LPS induced TNF-α levels in the brain increase at two

hours; however, peak concentrations were not observed until 16 hours post-injection. In contrast, serum levels of TNF-α peaked two hours post-injection and by 16 hours there was little detectable TNF-α observed (Ocasio *et al.*, 2004; Chen *et al.*, 2005). In the morphine tolerant animal exposed to LPS, the expression profile of TNF-α in the brain follows a similar expression pattern as is seen with LPS treatment alone in the rat brain. LPS induced TNF-α levels at two hours post-injection were very low in all treatment groups; however, at 16 hours post-LPS injection, there was in increase in TNF-α levels observed in both the placebo and morphine treated animals (Figure 8, page 79). Similar to the expression profile of LPS induced IL-1β, LPS induced TNF-α was increased in the morphine group indicating that a potentiation of pro-inflammatory cytokines occurs in the brain of a morphine tolerant rodent model. The delay in TNF-α seen upon LPS exposure, both with and without morphine exposure, suggests that TNF-α is not an immediate cytokine necessary for the initiation of the LPS induced inflammatory response in the central nervous system. Rather TNF-α may serve as a pro-inflammatory mediator downstream to the initial LPS induced inflammatory response. The increase in LPS induced TNF-α in the morphine tolerant state further suggests that there is an amplification of the inflammatory response in the

chronic morphine brain which may result in subsequent damage if upon repeated exposure to inflammatory agents.

When exposed to an inflammatory challenge such as LPS, in the normal physiological state, there is an activation of the HPA axis which results in the secretion of corticosterone. Corticosterone's anti-inflammatory properties then inhibit the secretion and activation of various pro-inflammatory cytokines. Chronic morphine exposure reduces corticosterone levels by desensitizing the paraventricular nucleus (PVN) of the hypothalamus (Ocasio *et al.*, 2004). This desensitization of the PVN results in a reduction of corticosterone which minimizes corticosterone's anti-inflammatory abilities, as observed in the decrease in LPS induced IL-6 in the morphine tolerant animals and increasing pro-inflammatory cytokines with IL-1β and TNF-α. The increase in LPS-induced IL-1β and TNF-α expression and inhibition of IL-6 expression in the brain of morphine tolerant rats suggests that the inflammatory response to bacterial infection in the brain may be potentiated in the morphine tolerant state. This data supports the hypothesis that chronic morphine exposure alters the pro-inflammatory cytokine profile in the brain of a rodent model exposed to an inflammatory stimulus, LPS.

The immuno-opioid relationship as examined by the effects of cytokine expression in a morphine tolerant state is evident with the

alteration in LPS induced cytokine expression profile as described above. Similarly, this relationship is also seen when immune cells such as TPA-differentiated cells are exposed to LPS resulting in an up-regulation of the mu-opioid receptor (MOR) (unpublished data-not shown and data was conducted by Linda Staikos-Byrne during a laboratory rotation in the fall 2004 under Dr. Chang's supervision). Both the alteration in cytokine profile in a morphine state and the increase in MOR in the presence of an inflammatory agent suggest that a modulatory mechanism between the nervous and immune systems occurs during acute inflammatory disease states as with gram-negative bacterial infections (as demonstrated with LPS exposure).

The first objective of this project examined the immuno-opioid relationship in a model of acute inflammatory disease. The second aspect of this work was to translate the animal work into a human cell culture model in order to examine a more clinically relevant model. The second objective examined the relationship of the immune-opioid interaction in a chronic inflammatory disease model, such as human immunodeficiency virus associated dementia complex (HIVD). Due to the nature of HIV, the use of an *in vivo* model to mimic HIVD was limited. Although our laboratory currently has access to an HIV transgenic rat model, the second portion of this work examined a human model so to be

more clinically applicable. Therefore, the focus of this work used a human astrocytic cell line, U87 MG. The U87 MG cell line was selected due to the nature of HIVD, which affects astrocytes. Additionally, astrocytes are the most abundant brain cell and autopsies of HIVD infected individuals have revealed an increase in astrocyte activation (Turchan-Cholewo *et al.*, 2008). Upon activation of astrocytes in response to neurodegenerative injury or inflammation as with HIVD, astrocytes produce a large majority of the cytokines (Farina *et al.*, 2007). In addition to being a relevant model of HIVD, the immuno-opioid relationship in astrocytes has yet to be examined and this interaction in this cell type may serve to better understand chronic inflammatory disease states of the CNS, such as HIVD.

Prior to examining the relationship of the immune system and the opioid system in the U87 MG cell line, the characteristics of this cell line were examined. A recent study has shown that the U87 MG cell line possess basal levels of IL-1R1, the functional receptor for the IL-1 pro-inflammatory cytokine family (Beskina *et al.*, 2007); however, the characterization of the opioid receptors in this cell line is unknown. Therefore, prior to examining the immuno-opioid relationship in an astrocytic cell line, the basal expression levels and functionality of the opiate receptors were examined. Using real-time PCR, the basal levels

of the opioid receptors were evaluated. The U87 MG cell line possess moderate basal expression of MOR, DOR and KOR as compared to cell lines with known opioid receptor expression levels such as immune cell lines (HL-60 cells [TPA-differentiated and un-differentiated]) and neuronal cell lines (NMB cells and SH-SY5Y cells) (Beltran *et al.*, 2006; Choi *et al.*, 2005; Borner *et al.*, 2007) (Figure 9, page 83). The basal expression of the opioid receptors in the U87 MG cell line indicates that this cell line may be used to study the opioid pathway. Although the basal expression levels of opioid receptors in the U87 MG cell line were unknown up until this point, the opioid receptor expression of the comparator cells were known and expression levels are consistent with previous reports (Beltran *et al.*, 2006; Choi *et al.*, 2005; Borner *et al.*, 2007).

As future experiments would utilize morphine to activate the opiate receptor, the basal protein level of MOR, the receptor in which morphine binds to, was also examined. Immunofluorescence staining indicated that the U87 MG cells possesses basal protein levels of MOR and may serve as an adequate model to examine the relationship of the immune system and the opiate system (Figure 10, page 85). The final aspect of the characterization of the MOR in the U87 MG cell line was to determine the functionality of the MOR expressed in this astrocytic cell line. A forskolin-induced cAMP assay using a synthetic ligand, morphine and two

endogenous ligands, endomorphin-1 and endomorphin-2, examined the functionality of the MOR expressed in the U87 MG cell line. U87 MG cells treated with both forskolin and morphine had a significant decrease in cAMP concentration, as compared to forskolin treated cells. This decrease in cAMP levels was reversed with the addition of naloxone, a MOR antagonist and indicates that MOR expressed in the U87 MG cell line is functional (Figure 11, page 87). A similar trend was observed when U87MG cells were exposed to endomorphin-1 or endormorphin-2, two endogenous opioid peptides that have a higher affinity to MOR as compared to morphine (Figure 12, page 89).

With the characterization of the MOR in the U87 MG cell line complete, the relationship of the immune system and the opioid system was examined in order to test the hypothesis that a pro-inflammatory cytokine can modulate the expression of an opioid receptor. Upon treating U87 MG cells with IL-1β (20 ng/mL or 40 ng/mL), a significant up-regulation of MOR, DOR and KOR was observed at varying concentrations of IL-1β. Although all three types of opioid receptors were significantly up-regulated when U87 MG cells were exposed to IL-1β, there was a drastic increase in MOR (Figure 13, page 92) levels as compared to DOR (Figure 14, page 93) and KOR (Figure 15, page 94) expression. The basal expression levels of MOR, DOR and KOR were

relatively similar in the U87 MG cell line and therefore the basal expression level may not have been a reason for the substantial differences between IL-1β up-regulation of MOR, DOR and KOR. Rather this difference in up-regulation of the various opioid receptors may indicate that the MOR is a more potent mediator of the immuno-opioid relationship than DOR and KOR. These findings are consistent with prior work that examined the relationship of IL-1β and the opiate receptors in the central nervous system. Vidal *et al.* (1998) showed that IL-1 can induce MOR expression in human cell culture model of the blood brain barrier (neural microvascular endothelial cells). Similarly, studies with primary human neuronal and glial cells from various adult brain regions have shown that IL-1β treatment increases the MOR and the fold increase of MOR is variable, depending on the region of the brain. Brain regions with higher concentrations of glial cells were more apt to have a larger increase in MOR expression (Ruzicka *et al.*, 1996).

In order to further understand IL-1β's ability to up-regulate MOR, a time course was performed. IL-1β up-regulation of MOR was observed as early as 3 hours of IL-1β exposure with peak levels occurring at 12 hours of IL-1β treatment and remaining elevated until 48 hours, the last time point examined (Figure 16, page 95). This data indicates that IL-1β

up-regulation of MOR occurred rather quickly and with increased exposure, an increase in IL-1β up-regulation of MOR was observed. Although prior published literature in this area did not examine a time-course of IL-1β up-regulation but rather examine the relationship of IL-1β and the MOR at one time-point, 24 hours of IL-1β treatment, the trend is similar in that there is a significant up-regulation of MOR with increased IL-1β treatment (Ruzicka *et al.*, 1996; Vidal *et al.*, 1998). This significant up-regulation of MOR in response to a pro-inflammatory cytokine over time may serve as a mechanism by which the opioid system functions to reduce the pro-inflammatory effects of the pro-inflammatory cytokines and may serve as a mechanism of immunosuppression.

Previous studies have used IL-8 expression as a surrogate marker to confirm the functionality of the IL-1 receptor and/or TNF-α receptors (Sunyer *et al.*, 1999; Gkoumass *et al.*, 2007). In order to confirm that the IL-1β up-regulation of MOR was mediated through the IL-1 receptor, IL-8 levels were measured in the cell culture supernatant of cells treated with IL-1β. U87 MG cells treated with IL-1β had a significant increase in IL-8 expression levels although a dose response was not observed, as compared to cells treated with control (Figure 17, page 97). Whereas prior work did show a dose-response of IL-1β mediated IL-8 in human

osteoclast cells and human bronchial cells, this relationship was not examined in the U87 MG cell line, until now (Sunyer *et al.,* 1999; Gkoumass *et al.*, 2007). The failure to observe a dose response may be a result of either an over-saturation of IL-8 with the doses of IL-1β chosen or the 12 hours treatment time frame may not have been ideal for the determination of IL-8 expression. Regardless, IL-8 expression in the U87 MG cells treated with IL-1β was significantly increased as compared to untreated cells. IL-1 induced IL-8 has been shown to be mediated through the signaling of IL-1R1 in human endothelial cells (Banda *et al.*, 2005), osteoclasts (Sunyer et al., 1999) and mononuclear cells (Porat *et al.*, 1992). The IL-1β up-regulation of IL-8 expression and the IL-1β up-regulation of MOR in the U87 MG cell line indicate that IL-1R1 may serve as a convergent point for both IL-8 and MOR in the U87 MG cell line. This is a unique aspect in that the activation of MOR has been shown to be immunosuppressive in immune cells (Chao *et al.*, 1997) whereas IL-8 is an essential chemokine released by phagocytes and lymphocytes during acute inflammation (Boontham *et al.*, 2003; Harada *et al.*, 1994). This dual nature of IL-1R1 may serve as a potential component to restore homeostasis by counterbalancing the pro-inflammatory aspects of IL-1β during the inflammatory response.

The relationship of IL-1R1 and IL-1β up-regulation of IL-8 has been characterized previously (Banda *et al.*, 2005; Sunyer et al., 1999; Porat *et al.*, 1992), yet the relationship of IL-1R1 and IL-1β up-regulation of MOR was previously unknown. In order to directly examine IL-1R1's role in the IL-1β up-regulation of MOR, an IL-1 receptor antagonist protein (IL-1RAP) was used in conjunction with IL-1β. A ratio of 1:20 and 1:200 of IL-1β to IL-1RAP was used to block IL-1R1. U87 MG cells treated with the higher ratio of IL-1β to IL-1RAP (1:20) had a significant decrease in MOR up-regulation, both in the absence and presence of IL-1β. However, U87 MG cells treated with a lower ratio of IL-1β to IL-1RAP (1:200) had no difference in MOR expression as compared to the control, yet a significant decrease in IL-1β up-regulation of MOR was observed (Figure 18, page 100). This decrease in IL-1β up-regulation of MOR in U87 MG cells treated with a 1:200 ratio of IL-1β to IL-1RAP indicated that IL-1R1, a toll like receptor, mediates the IL-1β up-regulation of MOR in the U87 MG cell line (Figure 18, page 100). Additionally, the inability of the higher concentrations of IL-1RAP to block IL-1β up-regulation of MOR may indicate the ratio of IL-1β to IL-1RAP used may have been too high and resulted in an over-saturated the U87 MG cells.

As IL-1R1's relationship to IL-1β up-regulation of MOR was determined, both indirectly through the elevation of IL-8 expression levels and directly through the use of an antagonist to the IL-1R1, the relationship of IL-1β and the MOR was then examined. Upon chronic morphine exposure, a desensitization of the MOR occurs (Conner *et al.*, 2004). Additionally, in the brain, there is an increase in the pro-inflammatory cytokines and a decrease in the anti-inflammatory cytokines, when exposed to LPS (Conner *et al.*, 2004; Staikos *et al.*, 2008). As IL-1β up-regulated MOR in the U87 MG cell line under normal (non-morphine) conditions, the ability of IL-1β to up-regulate a desensitized MOR in a chronic morphine state was examined. This experiment was conducted to provide insight into the mechanism by which astrocytes, upon exposure to a pro-inflammatory stimulus, respond to while in a compromised chronic morphine state.

Prior to examining IL-1β's ability to up-regulate a desensitized MOR, a time-course was conducted to determine the levels of desensitization of MOR in the U87 MG cell line. In the presence of morphine, MOR expression levels significantly increased at three hours post exposure; however, by six hours post exposure, a desensitization of MOR was observed, as indicated by a decrease in mRNA levels of MOR,

which continued until 48 hours of morphine exposure, the last time-point examined (Figure 19, page 104). As the expression levels of MOR were similar between 24 hours and 48 hours of morphine treatment, 24 hours of morphine exposure was used for future experiments to desensitize the MOR in U87 MG cells.

The ability of IL-1β to up-regulate a desensitized MOR was examined by pre-treating U87 MG cells with morphine for 24 hours followed by IL-1β (20 ng/mL). Interestingly, U87 MG cells desensitized with morphine and treated with vehicle had an increase in MOR up-regulation as compared to U87 MG cells not exposed to morphine. A potential explanation for this increase in MOR expression without exposure to the pro-inflammatory stimulus, IL-1β, is that upon the completion of the 24 hour morphine desensitization, the media containing morphine was aspirated and replaced with fresh media that did not contain morphine. This removal of morphine enriched media may have precipitated cellular morphine withdrawal which results in MOR up-regulation (Tomassini *et al.*, 2004). However, U87 MG cells desensitized with morphine and treated with IL-1β resulted in a significant up-regulation of MOR as compared to morphine treated cells not exposed to IL-1β (Figure 20, page 106). This data indicates that an up-regulation of

MOR occurs in the chronic morphine state as well as in human astrocytic cells not exposed to morphine and supports the hypothesis that a pro-inflammatory cytokine is able to modulate the opioid receptors, both in a normal state as well as in a morphine desensitized state. To date, the ability of morphine to desensitize the opiate receptors has been well established, as has the ability of IL-1β to up-regulate the MOR in neural microvascular endothelial cells, neuronal cells and glial cells (Ruzicka *et al.*, 1996; Vidal *et al.*, 1998; Conner *et al.*, 2004). Yet, to date, there is no other literature describing the ability of a pro-inflammatory cytokine to regulate an opiate receptor.

This work to date suggests that morphine alters the immune response and affects the expression levels of LPS induced cytokines in the brain. Additionally, pro-inflammatory cytokines, such as IL-1β, are shown to affect the opioid-dependent pathways by up-regulating the expression of the opioid receptors. This relationship between the nervous system and the immune system may be a potential convergence point in which the body functions to restore homeostasis upon exposure to an inflammatory stimulus. This work suggests that upon exposure to an inflammatory stimulus, in an uncompromised disease state, an activation of pro-inflammatory cytokines occurs that stimulates the expression of MOR, which functions to suppress the inflammatory

response and to restore homeostasis. Similarly, exposure to an inflammatory stimulus, results in a further up-regulation of MOR which may also result in a suppression of the inflammatory response. This work will serve to better understand the molecular pathways associated with neuronal inflammation and may be used as a basis for the development of therapeutic agents to treat inflammatory conditions such as gram-negative bacterial infections or HIVD.

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