OPIOID INDUCED DIFFERENTIAL REGULATION

OF TOLL-LIKE RECEPTOR 4

By

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2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2015

OPIOID-INDUCED DIFFERENTIAL REGULATION

OF TOLL-LIKE RECEPTOR 4

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ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to my faculty advisor, Dr. Craig W. Stevens. Dr. Stevens graciously accepted me into his laboratory after already having completed the first two years of my graduate career. His time, effort, and guidance is greatly appreciated.

I would also like to thank Dr. Randall Davis for his technical expertise and guidance throughout this project. Dr. Davis guided me through several issues encountered in this project and I am grateful for his assistance.

I would further like to thank the other members of my committee, Dr. Robert Allen and Dr. Kent Teague for their service and advice.

Dr. Subhas Das served as an unofficial mentor during this project and for that, I thank him. Additionally, the support of the laboratory technicians, Kelly McCracken and Daniel Buck, was always invaluable.

This work is dedicated to the support of my mother, Phyllis Gail Dodson and to the memory of my father, Ronald Wayne Dodson.

Name: SUMMER DODSON

Date of Degree: JULY, 2015

Title of Study: OPIOID-INDUCED DIFFERENTIAL REGULATION OF TOLL-LIKE RECEPTOR 4

Major Field: BIOMEDICAL SCIENCES

Abstract: With the growing recognition that toll-like receptor 4 (TLR4) interacts with opioids, this research aims to characterize these interactions in neuro-immune function. We have used the HEK-Blue™-hTLR4 cell line to investigate opioid-induced TLR4 activity in the presence or absence of the TLR4 ligand lipopolysaccharide (LPS). Our results suggest that the opioids methadone, oxycodone, and buprenorphine significantly downregulate LPS-induced TLR4 activity while the effect of morphine was not significant. To further investigate the role of opioids and TLR4 in neuro-immune interaction, we used the human microglial cell line CHME-5. It was found that these cells robustly express TLR4 and the interleukin-1 receptor (IL-1R) but evidence regarding the human mu opioid receptor (hMOR) remains inconclusive. Additionally, morphine and methadone significantly upregulate TLR4 protein expression independently while methadone downregulates LPS-induced TLR4 protein expression. These findings have led to the conclusion that opioid-induced TLR4 activity and expression regulation may account in part for neuro-immune modulation by opioids.

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CHAPTER I

INTRODUCTION

Opioids are common medicines used to treat both acute and chronic (primarily cancer) pain (Jamison *et al*., 2000). Prescriptions for opioids to treat chronic noncancer pain have also been significantly increasing over the past twenty-plus years (Chou *et al*., 2009). Further illustrating the vast consumption of opioids in the United States (U.S.) is the fact that while the U.S. modestly comprises 4.6% of the global population, it accounts for the indisputable majority (80%) of the world's opioid consumption (Manchikanti *et al*., 2008). This increasing licit use of opioids, coupled with the fact that they are also commonly abused (National Institute on Drug Abuse (NIDA), 2011), underscores the importance and urgency to fully understanding the effects of these drugs.

The list of adverse effects from opioids is lengthy and includes nausea, vomiting, constipation, pruritus, immunosuppression, hyperalgesia, respiratory depression, and more (Chou et al., 2009; Vallejo *et al*., 2011, Trescot *et al*., 2008). Of particular importance is the impact that these drugs have on the immune system. Over the past couple of decades it has been proven that opioids modulate peripheral immunity (Gavériaux-Ruff *et al*., 1998; McCarthy *et al*., 2001; Mellon and Bayer, 1998; Sacerdote *et al*., 1997) and increase susceptibility to opportunistic infections (Roy *et al*., 2011). Even more recently it has been

found that the immune effects of opioids extend to central nervous system (CNS) immune function and may even contribute to neuroinflammation and neurodegeneration (Hutchinson *et al*., 2011). In corroboration of this opioid-immune interaction it has been shown by our laboratory that while morphine downregulates both human *mu*-opioid receptor (hMOR) mRNA and protein, the cytokine interleukin-1β (IL-1β) upregulates hMOR at both the nuclear and protein levels and even overrides the morphine induced downregulation (Mohan *et al*., 2010). Additional opioid-immune interactions at toll-like receptor 4 (TLR4) have also been identified (Stevens *et al.,* 2013; Hutchinson *et al.,* 2010). It is this latter effect of opioid immunomodulation that embodies the research herein as it is hypothesized that methadone and morphine differentially modulate hMOR and TLR4 receptor expression in microglial cells. Identifying receptor profile differences in opioid-immune interactions will provide a solid basis for clinically observed immunomodulation that may result from opioids via crosstalk (Sacerdote *et al*., 2008; Budd, 2006; Neri *et al*., 2005; Kreek, 1990). Lending credence to this theory is evidence that receptors do not always function alone (Albizu *et al*., 2010; Fuxe *et al.,* 2007; Weber *et al.,* 2005; Zoli *et al*., 1994). While it has not been found in the literature that heteromers between different receptor *classes* exist, it is likely that differential regulation of opioid and immune receptors may regulate opioid immunomodulation and the overwhelming body of evidence for it warrants investigation into all potential avenues for this phenomenon to occur. Clinicians are also acknowledging that by not fully understanding this effect of opioids, they are doing a disservice to their patients (Budd 2006; Welters 2003). This research will aid in identifying possible explanations for immunological changes that may be contributing to conditions such as Alzheimer's disease and Parkinson's disease, further identify potential

new ways to use opioids in modulation of neuroimmunopathologies, and ultimately elucidate a possible pathway for opioid immunomodulation.

SECTION 1.1

OPIOIDS

Brief history of opioids. The opium poppy plant, *Papaver somnifoerum* (**Figure 1**), is the source for opiate drugs such as morphine and codeine. While the first recorded use of opium to treat pain is debatable, it is of general acceptance that the Sumerians first isolated opium sometime in the late third millennium B.C. (Brownstein, 1993). Its use has also been identified in ancient Egyptian papyrus records (Trescot *et al*., 2008), dating to confirmed use as early as circa 1500 B.C., and quite probably 5-20 centuries before then (Smith, 1930). The Papyrus Ebers record of Egyptian medicine lists the poppy plant—berries, seeds, grain, and stalk—as a plant remedy (Bryan, 1930a). The berries were used in a paste to "drive out the pains that are in his [the god Ra] head" and the stalk was ironically used to treat constipation (Bryan, 1930b, 1930c). The analgesic properties of opium span throughout the years, with records indicating its use for pain in ancient Greece, Arabia, European medieval times, the Renaissance, and today, albeit primarily as morphine (Bonica, 1991). Many centuries passed before morphine was isolated from the poppy plant in, most likely, 1804 by the

Figure 1. Diagram of ***Papaver somnifoerum*** and its components. Courtesy of bioweb.uwlax.edu

German pharmacist Friedrich Wilhelm Adam Sertürner (Schmitz, 1985). The exact date is debatable, but a couple of Sertürner's publications that went unnoticed in 1805 indicate the discovery had occurred the previous year (Schmitz, 1985). In the late-1800's through the mid-1900's morphine became a standard in advanced cancer and hospice care (Clark and Graham, 2008). Today, morphine and other opioids are still used to treat cancer pain, chronic noncancer pain, and acute pain with continued success (Joranson *et al*., 2002; Chou *et al*., 2009).

1.1.1 Morphine

Morphine (**Figure 2**) is a natural opium alkaloid that is used to treat severe acute pain, as well as myocardial infarction, cancer, and noncancer pain (Brenner and Stevens, 2010; Olsen *et al*., 2006). While morphine synthesis was mastered in 1963 (Schmitz, 1985), it is somewhat difficult and continues to be obtained from the poppy plant (Gutenstein and Akil, 2006), helping to explain the U.S.'s mass consumption of the world's opium supply (Manchikanti *et al*., 2008).

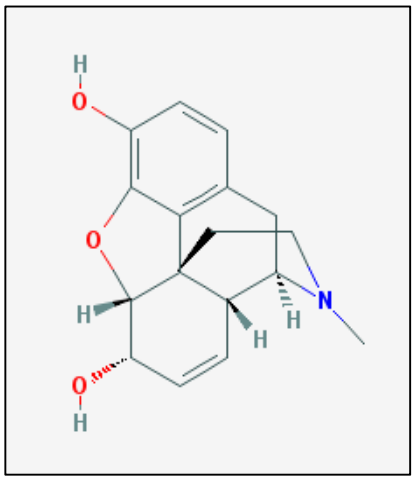


Figure 2. Molecular structure and chemical data for Morphine.
Chemical Formula: $C_{17}H_{19}NO_3$
Molecular wt: 285.34 g/mol
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5288826

Morphine is an opioid receptor agonist, primarily at the *mu* opioid receptor, MOR, with an affinity binding constant (K_i) of 1.168 nM (Volpe *et al*., 2011). Morphine is readily absorbed from the gastrointestinal tract and undergoes extensive first-pass metabolism (Vallejo *et al*., 2011). Morphine has 15-64% bioavailability after oral

administration (Inturrisi *et al*., 1984) with 25% bioavailability generally accepted in medicine and as a result, morphine is typically administered parenterally to avoid this hepatic first-pass metabolism (Gutenstein and Akil, 2006). Morphine can also be administered intrathecally or epidurally but because it is highly hydrophilic it can easily spread through the spinal fluid, causing latent respiratory depression by via supraspinal respiratory control centers (Gutenstein and Akil, 2006). Morphine more readily crosses the blood brain barrier (BBB) when it has not been ionized and is therefore, more lipophilic (Trescot, *et al*., 2008). P-glycoprotein (P-gp) is a primary transporter responsible for morphine efflux (Meineke *et al*., 2002). This has been shown in the case of intestinal P-gp, with quinidine blockade of the transporter resulting in elevated plasma morphine concentrations, but its role in brain efflux in humans remains debatable (Kharasch *et al*., 2003a). The typical half-life of morphine is roughly two hours (Stuart-Harris *et al*., 2000) and once in the system about one-third of the dose is bound by protein (Gutenstein and Akil, 2006) with plasma clearance ranging from 533 mL/min to 1256 mL/min in ill patients and 805 mL/min to 2590 mL/min in healthy volunteers (Meineke *et al*., 2002). Morphine is conjugated with glucuronic acid to form two primary metabolites: morphine-6-glucuronide (M6G) and morphine-3-glucuronide (M3G), of which M6G has even greater pharmacological activity than morphine and contributes to analgesia (Penson *et al*., 2000; Portenoy *et al*., 1992). M6G also has a longer half-life than morphine (Stuart-Harris *et al*., 2000) and fewer side effects (Lötsch *et al.,* 1996). M3G contributes little to analgesia, if at all (Penson *et al.,* 2000). Both of these metabolites are able to cross the BBB and are excreted by the kidney, with 90% of excretion occurring within 24 hours; however, traces of morphine can be found in bile because of enterohepatic recycling (Parker *et al*., 1980)

but this is not believed to have much of an effect on the pharmacodynamics of morphine (Lötsch *et al.,* 1996) as this is responsible for maintaining low concentrations of the glucuronide metabolites as opposed to a subsequent secondary increase in morphine levels (Osborne *et al*., 1990).

It should be noted that the opioid history of the patient, both physician-prescribed and illicit, should always be known when considering morphine or other opioid treatment. Pharmacokinetic and pharmacodynamic changes may occur as a result of pharmacological tolerance, in which a greater amount of the drug is required to achieve the desired analgesic outcome (Vallejo *et al.,* 2011). Chronic opioid use can also produce other adverse side effects such as the aforementioned immunomodulation, hormonal changes, abuse, addiction, and hyperalgesia (Manchikanti and Singh, 2008). Route of administration can also affect the pharmacological profile of morphine and should therefore also always be considered (Osborne *et al*., 1990).

1.1.2 Methadone

Methadone (**Figure 3**) is primarily prescribed to treat opioid addiction and is widely supported for this purpose within the medical community (Kreek, 2000). Methadone directly alters the same areas of the brain that produce addiction as well as normalizes hormonal imbalances associated with addiction (Kosten and George, 2002). It is also effective in treating chronic and cancer pain (Halpern, 1977). In recent decades, physicians have been increasingly

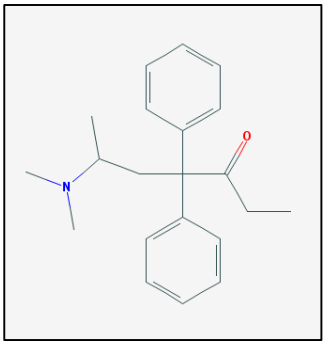


Figure 3. Molecular structure and chemical data for Methadone.
Chemical Formula: $C_{21}H_{27}NO$
Molecular wt: 309.45 g/mol
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4095

prescribing methadone to treat chronic noncancer pain and pain that does not respond to other treatments (Chou *et al*., 2009). This is primarily because it is inexpensive, is supported by insurance companies, and has a long half-life (Kuehn, 2012; Lugo *et al*., 2005). Unfortunately, this use is controversial as pain patients are more likely to self-administer than those in methadone maintenance therapy (MMT) and with either guided or improper use of methadone, it is still a respiratory depressant with potential prolonged QT side effects—both of which can lead to death (Modesto-Lowe *et al*., 2010). These side effects are likely aggravated by highly individual and variable pharmacokinetics (Fredheim *et al*., 2007; Chou *et al*., 2009).

Methadone is a synthetic opioid with relative potency to morphine (Davis and Walsh, 2001) and is typically used in clinical and laboratory situations as a racemic mix with the *l*- (aka. R-) enantiomer exhibiting agonist activity at MOR with a binding affinity constant (K_i) of 3.378 nM (Volpe *et al*., 2011). The *d*- (aka S) enantiomer expresses antagonist activity at the N-methyl-D-aspartate (NMDA) receptor (Davis and Inturrisi, 1999). Agonist activity has also been implicated at the *kappa* and *delta* opioid receptors (KOR and DOR, respectively) (Garrido and Trocóniz, 1999). The dual receptor activity at MOR and NMDA makes methadone particularly useful in pain management, regardless of its response to traditional opioid treatment. This is because the opioid receptor mediates transmission of the pain signal (Zubieta *et al*., 2001) and the NMDA receptor produces pro-nociceptive agents, such as substance P (Liu *et al*., 1997), making methadone an excellent choice for relief of non-relenting pain. Greater methadone DOR agonism relative to morphine is implicated in a weaker cross tolerance than when patients are maintained on more traditional opioids with a more similar structure to morphine (Lynch, 2005). As

far as activity is concerned, methadone has also been implicated as a 5-hydroxytryptamine (5-HT) and norepinephrine uptake inhibitor, albeit to a lesser extent for the latter (Codd *et al*., 1995). The effects on 5-HT and norphinephrine may also contribute to the non-opioid mediated analgesic properties of methadone as monoamine-producing descending neurons can inhibit transmission of the pain signal (Codd *et al.,* 1995; Dharmshaktu *et al*., 2012). The opioid-induced uptake inhibition of 5-HT helps explain the use of antidepressants in chronic pain such as migraine and diabetic neuropathy (Sharav *et al*., 1987; Dharmshaktu *et al*., 2012) and is considered to be a structural phenomenon as morphine—a phenanthrene containing an oxygen bridge—does not inhibit monoamine uptake while methadone—a phenanthrene without an oxygen bridge—does inhibit uptake (Codd *et al*., 1995).

The half-life of methadone is long and variable at roughly 15-40 hours (Lugo *et al*., 2005) with some reports of even 130 hours (Lynch, 2005). Methadone is a basic, lipophilic drug that can be administered intravenously (Fredheim *et al*., 2008). High oral and rectal bioavailability (Dale *et al*., 2004) and extensive tissue distribution have been observed in humans but the efficacy and viability in subcutaneous and epidural administration are questionable due to inflammation at the injection site (Davis and Walsh, 2001). As a result, the route of methadone administration may contribute to varying pharmacokinetic and pharmacodynamic effects on the patient as alterations in bioavailability can occur when comparing oral versus intramuscular administration (Lugo *et al*., 2005). Additionally, parenteral administration of methadone in addicts results in similar behavioral effects to parenterally administered morphine while oral doses of methadone do not produce behavioral changes (Gutenstein and Akil, 2006).

Methadone has a quick onset of action and is readily absorbed from the gastrointestinal tract, making it a great choice for oral dosing (Gutenstein and Akil, 2006; Trescot *et al*., 2008). It is extensively processed in the liver, primarily via oxidation by the cytochrome P450 enzyme CYP 3A4-induced N-demethylation, to the inactive metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (Karasch *et al*., 2004; Fredheim *et al*., 2007) and is excreted in the urine (Garrido and Trocóniz, 1999) with increased excretion in acidic urine as a result of the basic nature of the drug (Davis and Walsh, 2001; Inturrisi CE, 1976). Lack of drug accumulation in the urine of patients with renal failure indicates fecal excretion as well (Lynch, 2005). Hepatic clearance is minimal and varies depending on duration of treatment. For example, assuming a 1,500 mL/min hepatic blood flow rate in an opioid addict, the extraction ratio is twice that at the same rate in a chronic pain patient on methadone at 0.16 and 0.08, respectively (Garrido and Trocóniz, 1999), which is consistent with earlier findings that the liver only clears roughly 10% of free methadone as protein bound methadone is not processed as was indicated in chronic pain patients (Inturrisi *et al*., 1987).

Maximum concentrations of methadone in the brain are seen within 1-2 hours (Gutenstein and Akil, 2006) and a lack of active metabolites makes it appropriate to prescribe to patients with compromised hepatic function (Vallejo *et al*., 2011). Inactive metabolites for methadone may contribute to the decreased amounts of neurotoxicity with methadone when compared to other opioids (Trescot *et al*., 2008). However, a short analgesic period of 4-8 hours coupled with the long half-life may contribute to respiratory depression and death (Modesto-Lowe *et al*., 2010). Methadone enters the CNS with concentrations in cerebrospinal fluid (CSF) as high as 73% that of plasma with peak levels

reached after 3-8 hours in MMT patients (Rubenstein *et al*., 1978). The drug efflux transporter P-gp is used in intestinal methadone transport, as is the case with morphine, but its role in the BBB remains debatable and unclear (Fredheim *et al*., 2008; Karasch *et al*., 2004). Methadone is protein bound in tissue—including the brain—primarily to α_1-acid glycoprotein (AAG), whose expression increases with stress, cancer, and inflammatory states (Romach *et al*., 1981). Methadone binds to the plasma proteins albumin and *gamma* globulin (Olsen, 1973) with only about 12% remaining unbound in plasma (Inturrisi *et al*., 1987). Drug levels are maintained in the plasma at low concentrations once dosing has ceased due to a slow release from tissue stores (Dole and Kreek, 1973) with preferential storage in lipoproteins (Romach *et al*., 1981). Plasma binding may also influence this slow release. Protein bound methadone release increases with repeated dosing (Gutenstein and Akil, 2006) and is one potential reason why withdrawal from methadone is not as violent as from other opioids such as heroin or morphine.

Dosing of methadone is generally recommended to begin at 10% or less of the equianalgesic dose of other opioids, not to exceed 30-40 mg per day (Chou *et al*., 2009) to avoid drug accumulation and overdosing that can result from the long half-life of methadone (Trescot *et al*., 2008). Even opioid tolerant patients may not be completely cross-tolerant to the effects of methadone, which can at least in part be attributed to potential genetic variability (Modesto-Lowe *et al*., 2010; Fredheim *et al*., 2008) and greater DOR agonist activity when compared to morphine (Lynch, 2005). As a result, caution should be exercised when switching to methadone from other opioids due to the high inter-individual pharmacokinetic variabilities associated with the drug. Pharmacodynamic properties are similar to those of morphine but methadone may produce a longer miotic

effect than other opioids (Inturrisi, 1976). Side effects of dizziness, sleepiness, sweating, and an overall feeling of heaviness have been reported (Inturrisi *et al*., 1986) and methadone may also induce the cardiac arrhythmia Torsade de Pointes (Modesto-Lowe *et al*., 2010). Despite the safe use of methadone in MMT, it is still considered to be an addictive drug with potential for abuse in pain management (Gutenstein and Akil, 2006).

1.1.3 Oxycodone

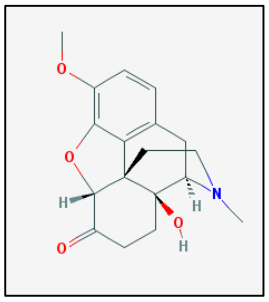


Figure 4. Molecular structure and chemical data for Oxycodone.
Chemical Formula: $C_{18}H_{21}NO_4$
Molecular wt: 315.36 g/mol
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=5284603

Oxycodone (**Figure 4**) is another opioid used in this study and was introduced to the medical community almost 100 years ago in 1917 (Klimas *et al*. 2013). The semi-synthesis of oxycodone from thebaine, a major constituent of opium, first occurred in Germany and has since been used in Europe, Canada, the United States, Australia, and Finland—where it is the parenteral drug of choice for acute pain (Kalso, 2005). Oxycodone was approved by the U. S. Food and Drug Administration as a sustained release analgesic, OxyContin®, in 1995 (Cicero *et al*., 2005). Routes of administration for oxycodone include oral (controlled and immediate release), rectal, intraspinal, and parenteral (Riley *et al*., 2008). The route of administration has been implicated in variable analgesia, which may be due to a differential activity of G-proteins resulting from different administration routes, as potent effects can be seen after subcutaneous delivery and poor analgesia may result after intrathecal administration (Lemberg *et al*., 2006). Oxycodone may be more potent than morphine in postoperative pain but the opposite may be the case for cancer pain (Kalso *et al*., 1991).

Oxycodone is an opioid receptor agonist at MOR (Kalso, 2005) with a binding affinity constant (K_i) generally accepted in the literature of ~20 nM at that receptor (Monory *et al*., 1999; Lalovic *et al*., 2006; Volpe *et al*., 2011), indicating that it has a weaker association at the recepetor than either morphine or methadone. It has been suggested that KOR may be responsible for the analgesic effects of oxycodone (Ross and Smith, 1997); however, this is debated in the literature (Kalso, 2005) as the K_i's for oxycodone at DOR and KOR are 958±499 nM and 677±326nM, respectively with a 95.7% relative affinity for MOR (Monory *et al*., 1999). Perhaps an observed increase in analgesia via KOR is a result of KOR interaction with another receptor such as MOR or TLR4. Regardless of the specific receptor mediating the analgesia, oxycodone is similar in analgesic potency to morphine (Pöyhiä and Seppälä, 1994).

Bioavailability of oxycodone is relatively high at about 60% with maximum concentrations occurring in the plasma at about one hour in healthy humans despite either intramuscular or oral administration (Pöyhiä *et al*., 1992). Oxycodone is metabolized in the liver via cytochrome P450 enzymes. CYP3A4 induces N-demethylation into the metabolite noroxycodone for roughly 46% of the dose while CYP2D6 induces O-demethylation for about 11% of the dose to produce the metabolite oxymorphone (Klimas *et al*., 2013). Small amounts of oxycodone may also be reduced to α- and β-oxycodol as has been indicated *in vitro* (Lalovic *et al*., 2006). According to a pharmacokinetic-pharmacodynamic model and GTPγS binding activity to G-proteins, both noroxycodone and oxymorphone undergo further metabolism to the active metabolite noroxymorphone (Klimas *et al*., 2013; Lalovic *et al*., 2006), which may further be reduced to α- and β-noroxycodol (Lalovic *et al*., 2006). It has not yet been clearly determined whether or not,

or to what extent, noroxymorphone or any of the other metabolites contribute to the central effects of oxycodone as there are conflicting reports about this matter in the literature (Kalso, 2005; Klimas *et al*., 2013; Lemberg *et al*., 2006). However, oxycodone as the parent compound *is* found in the brain [of rats] at concentrations twice that of drug in the plasma (Lalovic *et al*., 2006) and is therefore generally accepted as the source of oxycodone analgesia (Klimas *et al*., 2013). At least one study in rats indicates that three times as much oxycodone enters the brain than is cleared, suggesting a role for active influx across the BBB and/or increased brain tissue binding that is not seen with morphine despite similar lipophilicities (Boström *et al*., 2006). Liposolubility and protein binding of oxycodone is similar to that of morphine (Pöyhiä and Seppälä, 1994) but does not include interaction with P-gp (Boström *et al*., 2006). Oxycodone is hepatically metabolized via cytochrome P450 enzymes, thus genetic variations or drugs that interact with these enzymes will affect the pharmacokinetics of oxycodone (Lalovic *et al*., 2004; Klimas *et al*., 2013). The half-life for oxycodone is about 4 hours but can be longer in renal and/or liver failure (Kalso, 2005). Excretion of oxycodone is primarily via the kidney (Pöyhiä *et al*., 1992; Riley *et al.,* 2008).

Side effects of oxycodone are similar to those of other opioids, such as morphine, but are generally not as intense (Kalso, 2005; Riley *et al*., 2008). Oxycodone is also considered to be a drug with a high potential for abuse, comparable to that of morphine (Riley *et al*., 2008).

1.1.4 Buprenorphine

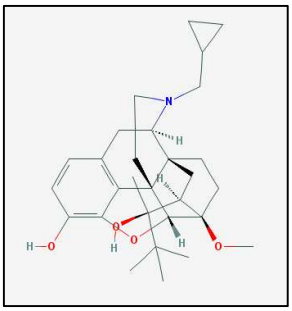


Figure 5. Molecular structure and chemical data for Buprenorphine.
Chemical Formula: $C_{29}H_{41}NO_4$
Molecular wt: 467.64 g/mol
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=644073&loc=ec_rcs

Buprenorphine (**Figure 5**) was designed in the 1970s to be an antinociceptive agent without narcotic properties, thus largely devoid of the unwanted side effects that accompany traditional MOR-agonist opioid analgesics (Cowan *et al*., 1977). Buprenorphine is structurally similar to the opioid antagonist diphrenorphine, only differing by one functional group (Cowan *et al*., 1977). While buprenorphine was designed as an analgesic alternative to morphine, early pharmacological data indicating a long half-life and less tolerance, toxicity, and abuse potential—relative to morphine—made it an ideal candidate to treat heroin addiction (Jasinski *et al*., 1978). Buprenorphine first came to the market as an analgesic in the United States in 1981 (Trescot *et al*., 2008) but it wasn't until 2002 when the U.S. Food and Drug Administration (FDA) labeled buprenorphine as a Schedule III drug and approved it to treat opioid dependency (Jones, 2004). As a result, buprenorphine is primarily used to detoxify patients from heroin and other opioid addictions and to maintain their sobriety with equal efficacy as methadone (Bickel *et al*., 1988). Buprenorphine may even be superior to methadone in detoxification (Ducharme *et al*., 2012); however, neither drug has been found to inherently *maintain* abstinence in lieu of other social support systems (Wright *et al*., 2011). Buprenorphine is an excellent alternative for addicted patients who are deterred from the methadone clinic and/or in-patient rehabilitation facilities because with the proper licensing, primary care physicians are now able to prescribe buprenorphine to treat opioid dependencies (Jones, 2004). Some clinicians and studies have recently

begun to promote and study buprenorphine in its initially intended role—as an analgesic (Berland *et al*., 2013; Davis, 2012; Gordon *et al*., 2010; Yamamoto *et al*., 2006).

Buprenorphine is a lipophilic drug and the clinically useful routes of administration include sublingual and transdermal, as these routes tend to be popular in research while abused routes of administration include intranasal, intravenous, and parenteral (Huestis *et al*., 2012; Middleton *et al*., 2011; Jasinski *et al*., 1978). It is prescribed in tablet form for sublingual treatment of opioid dependency either by itself (Subutex®) or in a 4:1 ratio of buprenorphine:naloxone (Suboxone®) in order to deter illicit use and abuse. While buprenorphine alone has abuse potential, albeit less than morphine, the naloxone in the formulation can precipitate withdrawal symptoms in opioid addicted individuals when it is crushed and injected (Jones, 2004) and also may induce withdrawal when 'snorted' as the bioavailability for intranasal naloxone was up to 30% in this study (Middleton *et al*., 2011). Individual on high opioid doses have a greater degree of physical dependency and are also likely to precipitate a withdrawal when taking buprenorphine alone because it has a higher affinity for MOR than other opioids of abuse (K_i=0.2157 nM, Volpe *et al*., 2011) and is more potent than morphine (Cowan *et al*., 1977), but possesses less intrinsic efficacy (Strain *et al*., 1995) because it is only a partial agonist at MOR (Martin *et al*., 1976).

Buprenorphine has a quick onset of action at 5 minutes after subcutaneous injection (Cowan *et al*., 1977) but it is about 1-1.2 hours after clinically relevant sublingual administration (Compton *et al*., 2006). Maximal plasma concentrations and brain MOR occupancy increase linearly with dose (Hestis *et al*., 2012) but there is a nonlinear association between MOR occupancy in the brain and plasma concentration, probably attributable to high affinity at MOR (Greenwald *et al*., 2003). At high, nontoxic doses the

efficacy of buprenorphine begins to decrease and can even pharmacodynamically present as an antagonist, indicating a ceiling effect and generating a bell-shaped dose-response curve indicative of multiple receptor interactions (Cowan *et al*., 1977). Indeed, buprenorphine has several molecular sites of action including agonist activity at opioid receptor like-1 (ORL-1), further contributing to antinociception; however, MOR agonism is primarily responsible for analgesia (Yamamoto *et al*., 2006). Furthermore, it is a KOR antagonist (Leander, 1987) which may contribute to its successful use in opioid dependency and abstinence programs as KOR is implicated in the neurobiology of drug abuse, affecting dopaminergic tone and modulating addictive behavior (Kreek, 1996).

These unique receptor activities contribute to the safety and utility of buprenorphine. Additionally, buprenorphine has a long duration of action (Jasinski *et al*., 1978), attributed to slow dissociation from MOR. This is supported with the finding that heroin cravings are suppressed for at least 28 hours with 50% MOR occupancy in the brain by buprenorphine (according to human *in vivo* PET imaging) but by 52 hours post dosing and 33% MOR occupancy, symptoms of withdrawal become evident (Greenwald *et al*., 2007). This data supports that there is sufficient BBB transport and CNS MOR is at least partially responsible for the sustained drug effects of buprenorphine.

Sublingual bioavailability of buprenorphine is about 70% in tablet form but deceases with liquid formulation (Harris *et al*., 2004). Equal doses in males and females show that females have higher amounts of circulating drug and metabolites than males (Moody *et al*., 2011). Extensive first pass hepatic metabolism occurs via phase I and phase II *N*-dealkylation and conjugation, and the drug is primarily excreted in feces as both free and conjugated metabolite (Cone *et al*., 1984). Minimal amounts (<15% of dose) of the

conjugated inactive metabolite norbuprenorphine are renally excreted with no free drug in the urine regardless of oral, sublingual, or subcutaneous administration (Cone *et al*., 1984). The half-life of buprenorphine is up to 28 hours after intravenous administration and is detected for up to 76 hours (Greenwald *et al*., 2007). Tolerance and cross-tolerance can occur with buprenorphine but at a slower rate compared to morphine (Cowan *et al*., 1977).

Potent MOR agonist activity makes buprenorphine a candidate for illicit abuse but its pharmacokinetics tend to be more predictable than those of methadone, contributing to its safety (Compton *et al*., 2006). In the past decade primary care physicians have been premitted to prescribe it to opioid-dependent individuals, increasing its availability to this population (Jones, 2004). While toxicological data is scarce, buprenorphine has been identified in some autopsies at both therapeutic and lethal levels but can rarely be determined as the sole cause of death because a benzodiazepine and/or other drugs are also involved in almost every case (Pirnay *et al*., 2004). Respiratory depression and other adverse physiological parameters such as lower heart rate and blood pressure are not pronounced with even high and/or accumulating concentrations of buprenorphine (Jasinski *et al*., 1978; Harris *et al*., 2004; Compton *et al*., 2005; Middleton *et al*., 2011). Other side effects are similar to morphine but are more tolerated—the exception is a longer and greater miotic effect with increasing doses of buprenorphine (Harris *et al*., 2004).

1.1.5 Δ^9-Tetrahydrocannabinol (THC)

While THC is not an opioid, the first set of preliminary experiments in this laboratory investigated the potential effects of this cannabinoid has on TLR4 activity. Because this was preliminary, only a brief discussion will occur on this drug. THC is the

major active compound in marijuana and is the focus of much debate both in the scientific and political communities. It has been found to be useful in treating many conditions, including pain, sometimes in conjunction with opioids in order to decrease the opioid dose (Lamarine, 2012).

The typical method of ingestion for marijuana is via inhalation; however, some other means of administration include oral and ophthalamic as it also has pharmacological use in treating glaucoma (Grotenhermen, 2003). THC binds to the cannabinoid receptors CB1 and CB2 with a relative equal affinity (Pertwee, 1999). Once in the system, it is primarily distributed throughout adipose tissue (Rawitch and Rohrer, 1979), where it can be stored for up to a month in chronic users (Grotenhermen, 2003). Chronic marijuana use is also associated with a decrease in cognitive skills, which suggests that neurotoxicity and/or neuroinflammation may be a contributing factor (Cutando *et al*., 2013). This finding helped initiate the possible involvement of TLR4 in THC-induced cognitive dysfunction.

SECTION 1.2

DRUG ABUSE, ADDICTION, DEPENDENCE, AND TOLERANCE

The importance in addressing drug abuse and addiction has two primary facets: 1) To illustrate the high prevalence of use that these drugs embody outside of clinical guidance and knowledge as a current medical and social issue, and 2) To address the doses chosen for use in the research as both abusers and addicts traditionally have higher plasma drug concentrations due to the phenomenon of opioid tolerance. These topics are of interest in this research because abuse, addiction, dependence, and tolerance are all mediated to some degree by MOR (Contet *et al*., 2004; Matthes *et al*., 1996).

1.2.1 Drug Abuse

Drug abuse is defined as the nonmedical use of a substance with the intent of achieving an altered mental or physical state (Hernandez and Nelson, 2010). Both prescription and illicit, or "street," opioids are commonly abused (Zacny *et al*., 2003). Many different drugs and drug types have abuse potential but for the purpose of this research, the focus will remain on opioids.

In the mid-1800's smoking opium was *en vogue* to get "high" beginning in the California west coast but after the hypodermic syringe was invented (c. 1856) and the subsequent discovery of heroin (1898), the trend became intravenous (IV) heroin (Ball, 1965). IV heroin abuse is still favored among some addicts, primarily in metropolitan areas where it is readily available and inexpensive (Cicero *et al*., 2005); however, a current and growing opioid trend is the abuse of prescription opioids, including pills such as

hydrocodone, oxycodone, and methadone and has reached epidemic levels (NIDA, Commonly Abused Prescription Drugs, 2011). The abuse of IV heroin and prescription pills is responsible for twice as many deaths as homicide/violent crimes or auto accidents in many cities—large and small—leading to the attention of the U.S. Attorney General (Johnson, 2014). Contributing to this is the fact that prescription drug abuse has increased over the past twenty years and may be attributed to greater availability from the increasing number of opioid prescriptions dispensed (NIDA, Research Report series, Prescription Drugs: Abuse and Addiction, 2011). Each of the drugs highlighted in the previous section are all frequently prescribed and often abused (Cicero *et al*., 2005).

The rise in these prescriptions is primarily due to two reasons: 1) greater recognition of and treatment for pain (Brennan *et al*., 2007) and 2) more patients receiving treatment for opioid dependency (Stein *et al*., 2012). Further adding to the number of prescriptions written is that both primary care physicians and specialized pain clinics are managing pain patients (Olsen *et al*., 2006). The prevalence of both legitimate and illegitimate pain patients exacerbates the burden on physicians to distinguish between the two and simultaneously maintain a high standard of care for all patients (Baldacchino *et al*., 2010).

1.2.2 Addiction

Addiction is a term commonly used in place of abuse and while the meanings are not mutually exclusive, they have distinct behaviors associated with them. While the abuser and the addict may both aim to achieve altered mental and/or physical states

(Hernandez and Nelson, 2010), the addict's behavior is more compulsive (Lutz and Kieffer, 2013; Fishbain *et al*., 1992). Risky addictive and substance abuse behaviors may present in those who recreationally abuse these drugs (McCabe and Cranford, 2012) and/or those on chronic opioid therapy (Chabal *et al*., 1997). Some pain patients may actually be 'pseudoaddicted' in that compulsive behaviors they might display result from undertreatment and subside once pain is effectively managed (Bell and Salmon, 2009). Ongoing work to reconcile definitions of abuse, addiction and dependence exist as the American Psychiatric Association (APA) has revised the criteria for these conditions in the Diagnostic and Statistical Manual of Mental Disorders for its fifth edition (DSM-5), released in May 2013 (Peer *et al*., 2013). This is especially important as debate continues around the prevalence of addiction as an iatrogenic condition, resulting from legally obtained prescription opioids, and how the physician should approach and treat pain patients with concurrent substance abuse disorders (Bailey *et al*., 2010).

1.2.3 Dependence

Dependence may occur with opioid use and is considered to be a combined cognitive, behavioral, and psychological condition resulting from substance use that does not subside despite significant problems resulting from drug use (Minozzi *et al*., 2012). Physical dependence is identified by unpleasant withdrawal symptoms that present upon drug abstinence (Trujillo and Akil, 1991a), at which point users tend to self-administer additional drug(s) to alleviate these symptoms (Lutz and Kieffer, 2013). This is when drug administration becomes necessary for the user to feel normal but the higher doses do not necessarily elicit a physiological response (Brenner and Stevens, 2010). This is likely due

to NMDA receptor-mediated neuroplasticity as neuroplasticity is the result of learned behavior (Trujillo and Akil, 1991b) and NMDA receptors have also been shown to influence dependence as well as antinociception and tolerance (Mao, 1999). Discontinuation of the drug results in withdrawal symptoms only relieved by more drug. Attempt to avoid withdrawal symptoms commonly leads to addiction (Lutz and Kieffer, 2013), which is believed to be at least the partial result of MOR activation because of its role in responding to endogenous opioids produced after addictive drug administration (Contet *et al*., 2004). Additionally, MOR knockout mice experience neither analgesia nor addiction—corroborating the receptor's role in these two conditions (Matthes *et al*., 1996). It quickly becomes clear that MOR is not solely associated with analgesia.

1.2.4 Tolerance

Tolerance refers to a decreased drug effect after continued use (**Figure 6a**); therefore, after tolerance develops, the dose must be increased in order for the drug to elicit a response (Trujillo and Akil, 1991a). Tolerance is identified pharmacologically as a right ward shift in the dose-response curve as shown in **Figure 6b**. Illustrating the concept of tolerance is the observation that plasma levels of morphine in a non-tolerant, or opioid naïve, person is 50 ng/mL, or 0.175 μM (Lötsch *et al*., 1996) while in an individual on constant pain management it can be as high as 1,440 ng/mL, or 5 μM (Ninković and Roy, 2011).

Cross-tolerance occurs when tolerance to one drug translates to another drug in the same class, as this is the idea behind opioid substitution therapy to wean a patient off of morphine, for example, by instead prescribing methadone (Brenner and Stevens, 2010).

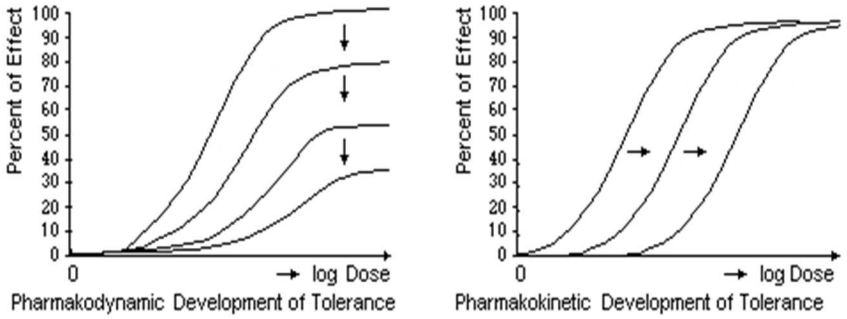


Figure 6. Pharmacological representation of tolerance. **a.** Pharmacodynamic attenuation of efficacy. **b.** Pharmacokinetic right-ward shift in dose response curve present in tolerance.
http://www.druglibrary.org/schaffer/heroin/ase/chap_3_6.htm

However, opioid cross-tolerance is incomplete—especially in the opioid tolerant patient taking MOR opioids—and the desired cinical outcome may take time to reach optimal effect as the doses are adjusted (Pasternak, 2005).

Several mechanisms are implicated in the development of tolerance. NMDA receptors are involved in neuroplasticity and learning and their involvement in tolerance has been studied. Antagonism of NMDA receptors attenuates tolerance to morphine (Trujillo and Akil, 1991b) contributing to the hypothesis that tolerance is at least in part, a neuroplastic phenomenon.

Further research has delineated that pharmacological tolerance can occur independent of physical dependency and that the mechanisms of tolerance most likely involve synaptic amplification at the connections between neurons, as concluded in a study using single neurons in a rat model (Christie *et al*., 1987). While whole neurons are certainly involved in tolerance, the contribution of MOR receptor density is somewhat

debated in the literature, as at least one study indicates that downregulation of MOR has no effect on pharmacological tolerance (Chan *et al*., 1997). In quasi-support for the lack of MOR density contributing to tolerance, is the research indicating that MOR agonists differentially regulate the receptor and that receptor sensitization is more likely to be involved than receptor density; however, this is agonist-dependent as morphine does not desensitize MOR, while methadone and buprenorphine do (Blake *et al*., 1997). The continued receptor activation by morphine may contribute to its addictive properties while the receptor desensitization by methadone and buprenorphine may help explain their effectiveness in treating dependence.

In agreement with both receptor density and desensitization contributing to tolerance is the finding that chronic morphine treatment does not induce MOR desensitization and endocytosis, while methadone does (Finn and Whistler, 2001). MOR is phosphorylated by G-protein receptor kinases (GRKs) which lead to internalization via a β-arrestin dependent mechanism at which point it can then be either recycled or degraded, reducing the number of receptors at the membrane (Finn and Whistler, 2001). Increasing the dose causes a higher concentration of drug at the synapse, allowing a greater probability that the comparatively few receptors present will bind the agonist and inhibit pain signal transmission—thus contributing to tolerance. Superactivation of the cAMP signaling pathway as a compensatory mechanism after continual inhibition by morphine was also suggested to be a marker for tolerance at the cellular level (Finn and Whistler, 2001). While dependence and tolerance are relatively simple pharmacological effects to conceptualize, their mechanisms are complex and a subject of continuous research.

SECTION 1.3

OPIOID RECEPTORS

The opioid receptors were first discovered in the 1970s. The seminal 1973 paper by Candace B Pert and Solomon H. Snyder in *Science* is generally regarded as the first time that a specific site for opioid binding was identified. They achieved this by homogenizing rat, guinea pig, and mouse brain and conducting a radioligand binding study on the tissue with tritiated naloxone to measure ligand displacement from the receptor (Pert and Snyder, 1973). Drugs characterized as potent opiates were able to displace naloxone binding at low concentrations, indicating an interaction at what was at the time deemed the "opiate receptor" (Pert and Snyder, 1973).

Following this research was the hypothesis that other opioid receptors exist. Martin *et al*., proved this in 1976 with experiments which induce chronic pain in a chronic spinal dog model and compared these effects to those seen in human. It was determined that there are three distinct yet structurally similar opioid receptors that each respond to different agonists in a differential manner. The first was called μ, for morphine; the second κ, for the agonist ketocyclazocine; and the third σ, for the agonist effects of SKF-10,047. While this last drug did not produce obvious physiological effects typical of other opioids, the behavioral effects were intense and antagonized by naltrexone—suggesting that it was an opioid receptor agonist (Martin *et al*., 1976). These receptor subtypes were expanded upon in 1977 by Lord *et al*., who corroborated the evidence for μ and κ receptors by comparing the potencies of different opioid agonists and using specific receptor antagonists to block individual receptors in the guinea pig ileum and mouse vas deferens, and by evaluating radioligand binding studies in guinea pig brain homogenates. This study identified a

different opioid receptor in the mouse vas deferens, which was called δ for vas *deferens* (Lord *et al*., 1977). These receptors (excluding σ) are now commonly referred to as MOR, DOR, and KOR in the literature for μ, δ, and κ opioid receptors, respectively.

Recent data indicates that the σ receptor is not actually an opioid receptor as it has no resemblance to a G-protein coupled receptor (GPCR) like the others, but is instead believed to be a ligand-regulated molecular signaling chaperone whose expression in nervous tissue and weak responsiveness to opioids convoluted its true function (Zamanillo *et al*., 2013). Recently, a fourth receptor called opioid receptor-*like* protein (ORL) has been identified and shares 60% homology to the other opioid receptors, which is the same percent homology that they share amongst each other; however, despite this homology, ORL does not bind opioids but is somehow involved in the pain response—contributing to the confusion, mystery, and current orphan receptor status surrounding this protein (Barlocco *et al*., 2000).

Bioinformatic analysis indicates that these receptors evolved as a result of gene duplication and that rapid and positive selection of the MOR gene may have had the evolutionary advantage of enhanced analgesic capacity in humans (Stevens *et al*., 2007). Subtypes of MOR, DOR, KOR, and ORL are alternative splice products of the parent receptors, with MOR having the greatest number of receptor subtypes (Stevens *et al*., 2007). The MOR variants may be responsible for the differential analgesic effects to opioids that have been clinically observed (Pasternak, 2005; Choi, 2006). Many currently identified splice variants have been shown to affect the potency and effectiveness of opioid analgesics and tend to exhibit their diversity at the intracellular carboxy c-terminal tail of the protein (Pasternak, 2005). The third intracellular loop has also been identified as a site

for G-protein interaction and subsequent initiation of signaling (Georgoussi *et al*., 2006). Additional MOR genetic polymorphisms have been hypothesized to influence susceptibility to addiction—primarily the N-terminal extracellular single nucleotide polymorphism (SNP) A118G allele, which changes the amino acid asparagine to aspartic acid and may augment agonist binding affinity thereby increasing the likelihood for addictive behavior (Stevens *et al*., 2007). However, this is controversial as conflicting studies indicate that further research is needed for a consensus on the role of genetic polymorphisms in addiction (LaForge *et al*., 2000). As the research presented here focuses on MOR, all subsequent discourse regarding opioid receptors will focus on MOR structure, function, and signaling unless otherwise noted.

1.3.1 μ Opioid Receptor (MOR)

MOR is an opioid receptor that mediates ascending and descending pain signals between the periphery and the central nervous system (CNS) (Zubieta *et al*., 2001). MOR also is involved in reward associated with social situations and addiction (Lutz and Kieffer, 2013). MOR is expressed throughout the CNS and the periphery. It has been detected in human tissue at high levels (12-20 x 10^6 mRNA copies/μg) in certain areas of the brain such as the cerebellum, nucleus accumbens, and caudate nucleus, and lower levels (2-8 x 10^6 mRNA copies/μg) in other areas such as the putamen, cerebral cortex, temporal lobe, hippocampus, substantia nigra, and spinal cord, with the lowest expression in the pancreas and small intestine (~2 x 10^4 mRNA copies/μg) (Peng *et al*., 2012). It is important to note that the higher levels of MOR expression include brain areas in the mesolimbic dopamine reward system, which is implicated in addiction (Contet *et al*., 2004). MOR mRNA is also

found in moderate levels in the dorsal root ganglion and adrenal gland (2-6 x 10^6 mRNA copies/μg) but is not detected in the human lung, spleen, kidney, heart, skeletal muscle, liver, or thymus (Peng *et al*., 2012). Furthermore, MOR expression has been identified in hippocampal GABAergic neurons, and functional protein can be induced in Jurkat T immune cells upon interleukin-4 (IL-4) or tumor necrosis factor-α (TNFα) cytokine stimulation (Börner *et al*., 2007), contributing to its role in immunomodulation. Further evidence for this is that the MOR transcript was also induced by IL-4 or TNFα in primary human T cells at comparable levels to the Jurkat T cells; however, for reasons not addressed by the authors the functional protein levels of MOR were only examined in the Jurkat T cells (Börner *et al*., 2007). MOR (and KOR) is also expressed in human microglia (Kettenmann *et al*., 2011).

MOR is a heterotrimeric membrane bound G-protein coupled receptor (GPCR) (Brenner and Stevens, 2010) with seven transmembrane passes (Traynor, 2012). GPCRs, including MOR, associate with α and βγ G-protein subunits. These subunits regulate adenylyl cyclase (AC) activity and can be generally divided into stimulatory (G_s) and inhibitory (G_i) proteins. In the case of MOR, the α subunit is coupled to G_i, which was initially termed N_i as it was being identified (Birnbaumer, 2007), and G_o proteins ($G\alpha_{i/o}$), that are activated upon agonist binding (Ueda *et al*., 1988; Laugwitz *et al*., 1993). There are three subtypes of α_i proteins (1, 2, and 3) and two splice variants of α_o proteins (A and B) and while it has been shown that MOR most efficiently couples to $G\alpha_{i3}$ than the other G-protein subtypes, this has no effect on ligand biased signaling (Traynor, 2012; Clark *et al*., 2006). Instead, research indicates that the β subunit of the βγ complex interacts with the C-terminal tail of MOR, contributing to differential agonist-stimulated signaling

(ligand bias), potentially employing monomeric Gα and the regulatory G-protein RGS4 in a signaling scaffold (Georgoussi *et al*., 2006). The β and γ subunits maintain a tight association with each other (Hildebrandt *et al*., 1984) and as a unit can inhibit adenylyl cyclase (AC) activity (**Figure 7**). This is rescued in the presence of G_s proteins (Smigel, 1985). Sodium also inhibits AC activity (Syed *et al*., 1987).

Prior to receptor activation, the βγ subunit is bound to the α subunit (Hildebrandt *et al*., 1984) and guanine diphosphate (GDP). After agonist binding the receptor undergoes a conformational change, allowing for the GDP to be exchanged for guanine triphosphate (GTP) at the α subunit and this Gα-GTP complex separates from the βγ subunit, initiating intracellular signaling (Traynor, 2012), binding to the calcium (Ca^{2+}) channel and inhibiting Ca^{2+} flow into the cell (**Figure 7**) (Al-Hasani and Bruchas, 2011). The reversal of GDP-GTP exchange is facilitated by inherent GTPase activity at a regulatory site (Cassel and Selinger, 1977) on the α subunit (Northup *et al*., 1983). Activated $G\alpha_i$-GTP then inhibits AC. It is important to acknowledge that AC activity can be influenced by other factors too, such as phospholipase C (PLC). PLC is a signaling molecule whose stimulation initiates phosphatidylinositol-4,5-bisphosphate generation of diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (Zuo, 2005). While PLC is commonly associated with and activated by the $G\alpha_q$ GPCR pathway, inhibition of PLC can block the inhibitory action that activated G_i proteins have on AC (Fan *et al.*, 1992). Inhibition of AC via MOR causes a decrease in cyclic adenosine monophosphate (cAMP) activity, which at least in part explains the analgesic effects of opioids (Collier and Roy, 1974) because cAMP has been shown to antagonize opioid antinociception (Ho *et al*., 1973). cAMP signaling also regulates MOR gene expression via cAMP response element-binding protein

(CREB) in fentanyl-treated PC-12 cells (Lee and Lee, 2003) and may therefore be involved in genetic modulation of MOR in other cell types and under other drug treatments as well.

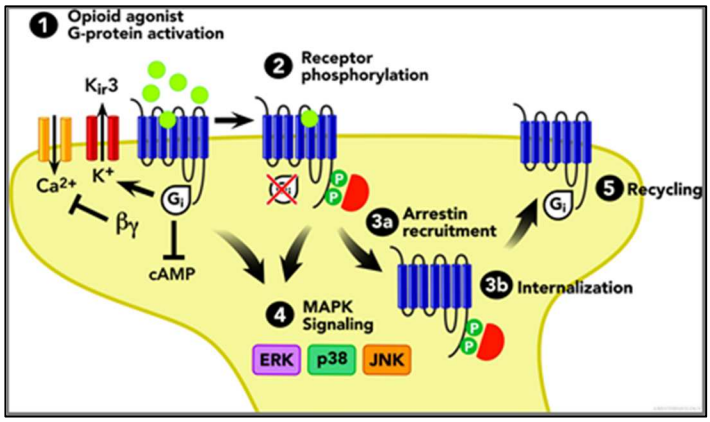


Figure 7. Summary of opioid receptor signaling. Illustrates all mechanisms addressed in Opioid Receptors section. "Biased agonism" suggests that different agonists at the same receptor can preferentially direct signaling in favor of one or more of the pathways outlined here. Arrows indicate activation steps; T-lines indicate inhibition; P = phosphorylation. Source: Al-Hasani and Bruchas, 2011.

MOR activation is also linked to ionic current and membrane polarization, which is what is primarily responsible for transmission of the pain signal from both spinal and supraspinal neurons (Brenner and Stevens, 2010). Inwardly-rectifying potassium (K^+) channels, primarily Kir3 (**Figure 7**), are opened by the Gα protein after receptor activation causes dissociation of the Gα from the βγ subunit (Al-Hasani and Bruchas, 2011). This increases neuronal K^+ currents and contributes to analgesia (North *et al*., 1987; Fan *et al*., 1992). Opening of CNS ATP-gated K^+ channels by K^+ channel openers induces antinociception by hyperpolarizing the cell; however, this is not antagonized by opioid antagonists, rather it has been suggested that changes in intracellular calcium may also modulate the K^+ channels (Welch and Dunlow, 1993). This is probable as it has been shown that opioids inhibit neuronal voltage-gated N-type Ca^{2+} currents (Seward *et al*., 1991) and this can be mediated by the βγ protein (**Figure 7**) (Al-Hasani and Bruchas, 2011). This inhibition of Ca^{2+} is generally accepted as the effect that GPCR activation has on the ion despite a few findings that MOR may stimulate Ca^{2+} channels (Porzig, 1990).

Taken together, the increase in K^+ conductance and resultant hyperpolarization attenuating Ca^{2+} flux involved in the action potential via MOR activation inhibits neurotransmitter release, at least in the locus coeruleus, contributing to the analgesic effect of MOR agonists (Duggan and North, 1984).

Illustrating the complexity of MOR molecular signaling are some seemingly contradictory findings on Ca^{2+}. For example, MOR agonism may also briefly open L-type Ca^{2+} channels to allow calcium influx, increasing the activity of PLC for a short time (Smart *et al*., 1995) which could be a $G\alpha_q$-independent mechanism for PLC activation and, as mentioned before, can inhibit AC (Fan *et al*., 1998). It has also been found that in differentiated NG108-15 rat neuroblastoma-glioma cells the DOR agonist DADLE stimulates PLC activity via $G_{i/o}$ activation leading to IP_3-mediated Ca^{2+} rlease from intracellular stores (Jin *et al*., 1994). Similar results were obtained in SH-SY5Y neuroblastoma cells where MOR was shown to induce IP_3 via morphine but this study implicated extracellular Ca^{2+} influx because both pre-incubation with a calcium channel blocker and use of calcium-free buffers blocked IP_3 formation (Smart *et al*., 1994). The role of these excitatory effects on Ca^{2+} in opioid-mediated analgesia is still not completely understood but are likely to be more pronounced in chronic opioid exposure (Jin *et al*., 1994).

MOR phosphorylation (**Figure 7**) is important as it initiates both mitogen-activated protein kinase (MAPK) signaling—via extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), or p38—and β-arrestin recruitment (Al-Hasani and Bruchas, 2011). Phosphorylated MOR increases the affinity for β-arrestin binding to the receptor, leading to desensitization and endocytosis via clathrin-coated pits (Lefkowitz, 1998). GRK

phosphorylation also uncouples the receptor from the G-proteins, discontinuing the G-protein mediated signaling (Zuo, 2005). This is not the only phosphorylation that mediates MOR desensitization as other mechanisms are suspected and being investigated (Williams *et al*., 2013). Regardless of how receptor internalization is initiated, once it has been endocytosed the receptors are packed in recycling vesicles and trafficked back to the plasma membrane (**Figure 7**) to repeat the process, as long as agonist is present (Roman-Vendrell *et al*., 2012).

It is important to reiterate that MOR desensitization is agonist dependent. For example, while the agonist DAMGO quickly desensitizes the receptor, morphine desensitization is much slower when it does occur and requires neither phosphorylation nor β-arrestin recruitment for desensitization (Chu *et al*., 2008). Instead, it is generally accepted that morphine does not induce receptor desensitization and internalization, but rather continues to signal in the presence of agonist, leading to an increase in cAMP activity believed to be the result of cellular adaptations, including upregulation of proteins, e.g., AC (Al-Hasani and Bruchas, 2011) or perhaps switching G-protein associations from $G_{i/o}$ to G_s proteins (Bian *et al*, 2012). The desensitization of MOR is believed to contribute to opioid tolerance (Zuo, 2005; Williams *et al*., 2013) and pharmacological manipulations exploiting endocytosis may help prevent tolerance from occurring with opioid treatment.

SECTION 1.4:

NEUROIMMUNITY AND NEUROINFLAMMATION

Inflammation occurs in response to infection and/or injury in both the periphery and the CNS (Matyszak, 1998). Similar to peripheral immunity, immune cells in the CNS are present to protect against infection and injury as they repair damaged tissue and remove dead cells. These neuroimmune cells are termed microglia and astrocytes and while they respond to infection and inflammation, they can also contribute to neuroinflammation. Oligodendrocytes are also considered glia, but express a more mechanical role, forming myelin sheaths around axons (Stoll and Jander, 2005) to aid in neuronal communication (Hughes, 2012). Astrocytes and microglia serve as central immune surveyors with microglia closely resembling peripheral macrophages, especially after they are activated by tissue infection or injury (Raivich *et al*., 1999). In fact, macrophages that have infiltrated the CNS are virtually indistinguishable from activated microglia (Yong, 2010).

Immune function in the CNS is quickly gaining interest as it is being recognized that neuroimmunity and neuroinflammation play a role in neurodegeneration, contributing to the etiology of conditions such as Alzheimer's disease (Rothwell and Hopkins, 1995; Stoll and Jander, 2005) and Parkinson's disease, with neuroinflammation as an underlying contributor in these two conditions (Calderón-Garcidueñas *et al*., 2013; Magrone *et al*., 2012). Highlighting the importance of CNS immunity is the fact that the response to various pathologies is highly conserved in the CNS, suggesting an evolved and meticulous orchestration for CNS maintenance and repair (Raivich *et al*., 1999). Taken together, this indicates that a thorough understanding of neuroimmunity will contribute greatly to the

development of therapies for many—if not all—of the neurodegenerative diseases plaguing society today.

1.4.1 Blood Brain Barrier (BBB)

The BBB plays a large role in neueroimmunity as it is the means through which peripheral immune cells (and other molecules) gain entry into the CNS. Infiltration of cells and endogenous or exogenous molecules via the BBB can influence microglia and other cells present in the brain, with greater migration occurring during inflammation (Zhou *et al*., 2007). The BBB is composed of tight junctions made of endothelial cells whose purpose is to provide a barrier, as the name suggests, between the central and peripheral systems and to help regulate capillary blood flow into the brain (Carvey *et al*., 2009). Other components of the BBB that make what is called the neurovascular unit (NVU) include the basement membrane, perivascular microglial cells, astrocytes, neurons, and a limited number of pericytes (Luissint *et al*., 2012). The BBB also has transporters, ion and drug pumps, and other machinery that help maintain brain homeostasis.

The BBB has traditionally been viewed as the protector of the CNS, keeping peripheral cells and drugs segregated, contributing to the "immune-privileged" view of the CNS (Matyszak, 1998). Research has shown that while this is true in that the BBB does serve as a physical interference between the periphery and CNS, it is more appropriately described as a gatekeeper letting in certain molecules to help maintain CNS function and aid in damage repair as necessary. Drug efflux pumps, such as P-gp mentioned in sections **1.1** and **1.2**, are important in reducing drug concentrations in the brain.

The gatekeeping function of the BBB is illustrated by the crossing of some lymphocytes and monocytes, the latter of which can transform into microglia once in the brain (Vitry *et al*., 2003). Activated T-cells also cross the BBB regardless of their antigen specificity under normal non-inflammatory and non-infectious conditions (Matyszak, 1998; Yong, 2010). Non-activated T-cells are not known to enter the CNS (Matyszak, 1998); however, recent research has identified a dural lymphatic system that directly links the peripheral lymphatic system to the CNS (Aspelund, *et al*., 2015). This indicates that the CNS in fact has a direct means of dispersing cellular waste that was not previously believed to exist.

Combined with the novel findings concerning the lymphatic system, it is known that the BBB increases in permeability in times of infection and inflammation and this is due at least in part to changes in expression of proteins that make up the tight juncions and Serine/Threonine and Tyrosine phosphorylation (Luissint *et al*., 2012). The increase in permeability is believed to allow a greater influx of peripheral immune cells to aid in the repair process (Raivich *et al*., 1999). Cytokines, such as TNFα and Interleukin-1β (IL-1β), have also been shown to compromise BBB integrity in models of neurodegeneration, such as MPTP-induced Parkinson's disease (Zhao *et al*., 2007). This permeability was directly attributable to neuroinflammation and not associated with the accompanying loss of dopaminergic neurons (Zhao *et al*., 2007). Understanding the BBB and its susceptibility to permeation in neuroinflammatory and neurodegenerative (most likely resulting from neuroinflammation) conditions illustrates the likelihood of increased drug concentrations entering the brain even when taken at prescribed doses and intervals.

1.4.2 Role of Microglia

Microglia were first officially recognized in the early 1900's by Dr. Pio del Rio-Hortega, although some of his predecessors suggested their existence as early as the 1890's

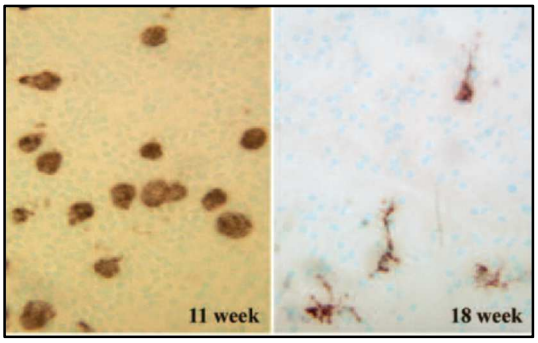


Figure 8. Human fetal microglial amoeboid phenotype at 11 weeks (left) and ramified human fetal microglial phenotype at 18 weeks (right). Image courtesy of Rock, *et al*., 2004.

(Rock *et al*., 2004). del Rio-Hortega suggested that these cells are evenly distributed in the CNS, morph, migrate, proliferate, and can even behave as phagocytes (Kettenmann, 2011). This remains an accurate description today; however, ideas regarding their origin have been debated over the years. It is now generally accepted that they originate from embryonic mesodermal cells that travel to the brain during development (Kettenmann *et al*., 2011; Rock *et al*. 2004). However, the theory that microglia originate as monocytes from bone marrow and travel to the brain to differentiate into microglia (Vitry *et al*., 2003) contributes to the idea that bone marrow transplants or transplantation of activated microglial cells may help in some neuropathologies (Prewitt *et al*., 1997). While research in this area is promising, the CNS immune response is very sensitive (Hughes, 2012), implicating that any changes in neuroimmune function may not have the intended outcome. As the CNS immune response is tightly regulated, any external modifications to microglia may have different results depending on the conditions surrounding the cells (Yong, 2010).

As can be seen in **Figure 8 above**, microglia have an amoeboid shape during early fetal development before beginning to resemble traditional resting microglia at about 18 weeks (Rock *et al*., 2004). Resting microglia represent about 20% of the cells in the adult human brain (Stoll and Jander, 2005). For many years it was thought that resting microglia have little purpose and simply wait for their cues to activate; however, it is now known that they are very active even when they are "resting." Pioneering research by Axel Nimmerjahn filmed microglia in their native, unresponsive state and found that they are very dynamic, extending and shortening their processes and moving them around in constant surveillance of their surroundings (Nimmerjahn *et al*., 2005). Interestingly, the microglial soma exhibited little movement, if any, while the ramified processes were active and even showed some evidence of clearing some metabolic cellular debris (Nimmerjahn *et al*., 2005). Manipulation of this behavior may be beneficial for neurodegenerative conditions such as Alzheimer's and Parkinson's diseases and it is also believed to contribute to normal neuronal maintenance, re-shaping the neurons and their environment while aiding in neuroplasticity and synaptic development (Hughes, 2012). Real-time videos of microglia in action are available at www.sciencemag.org/ci/content/full/1110647/DC1.

Another important and previously mentioned role for microglia is to respond to CNS injury and infection. Microglia react by becoming "activated," although as Nimmerjahn's research illustrates, they are already quite active even during times of quiescence. However, this "activated" state is accompanied by both phenotypical and reactive changes. Once presented with traumatic injury and/or infection, microglia morph in response to the stimuli (**Figure 9**). Microglia at rest are illustrated in **Figure 9a** and

present with a large soma and long ramified processes which are withdrawn upon activation (**Figure 9b).** Withdrawal of the processes makes them shorter and thicker and the size of the soma is increased (Stoll and Jander, 2005; Nimmerjahn *et al*., 2005). Upon complete activation they present in the amoeboid phenotype (**Figure 9c**) that was referred to in early embryogenesis (Rock *et al*., 2004). Microglia are known to respond quickly to their surroundings (Matyszak, 1998) and this is likely because they are always monitoring the extracellular milieu and the surrounding cells, including neurons, astrocytes, and oligodendrocytes. Their speed and efficiency allow them to evaluate the entire brain parenchyma every few hours (Nimmerjahn *et al*., 2005).

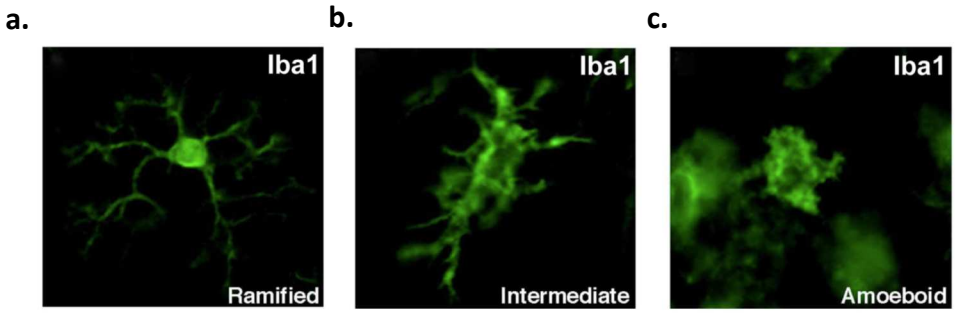


Figure 9. Distinct microglial phenotypes observed during activation. **a.** The resting, ramified microglia with long, thin processes. **b.** Intermediate microglia with shorter processes and a larger soma. **c.** The amoeboid phenotype. Cells are stained for the microglial marker Iba1+. Images from Ekdahl, 2012.

In addition to physical changes in the microglial response, several proteins are upregulated at both the mRNA and protein levels. Amyloidβ precursor protein (APP) is a large protein that is associated primarily with the early pathology of Alzheimer's disease and is quickly synthesized in activated microglia—but not in astrocytes or neurons—after non-lethal peripheral nerve injury; however, neurons may contribute to APP production in other forms of inflammation or trauma (Banati *et al*., 1993). Additional proteins whose expression is increased include (but are not limited to) cell adhesion molecules, cytokines

and their receptors such as interleukin-1 receptor (IL-1R), toll-like receptors (TLRs), and opioid receptors (MOR and KOR) (Rock *et al*., 2004). The fact that microglia express and regulate all of these receptors suggests that they are involved in the neuroimmunomodulatory effects of opioids.

1.4.2.1 CHME-5 Microglia

CHME-5 microglia are a transformed and immortalized human microglial cell line that will be used in this research. They were immortalized via transfection with the SV40 large T antigen in 1995 (Janabi *et al*., 1995). They respond to LPS and have been shown to express some cytokines such as IL-6, TNF, IL-1β (Atanassov *et al*., 1995; Lindberg *et al*., 2005). They are an ideal cell line to investigate neuro-immune interactions because they are simple to maintain in the laboratory and are of human origin; however, little characterization of these cells is present in the literature.

1.4.3 Role of Astrocytes

Astrocytes are named such because of their stellar physical appearance as the term "astro" references "star" (Seth and Koul, 2008). There are two main phenotypes of astrocytes that are predominantly expressed in the brain: 1) Fibrillary astrocytes that are found in the white matter and have long thin processes and 2) Protoplasmic astrocytes that are expressed primarily in the grey matter and have many more, yet shorter, processes (Raivich *et al*., 1999). These cells have historically been viewed as fillers of non-neuronal brain tissue and while they do physically support surrounding cells such as neurons (Raivich *et al*., 1999), recent advances have proven them to be greater contributors to

neuronal function (Lange *et al*., 2012). **Figure 10** illustrates some established primary astrocytic functions, which will be addressed below.

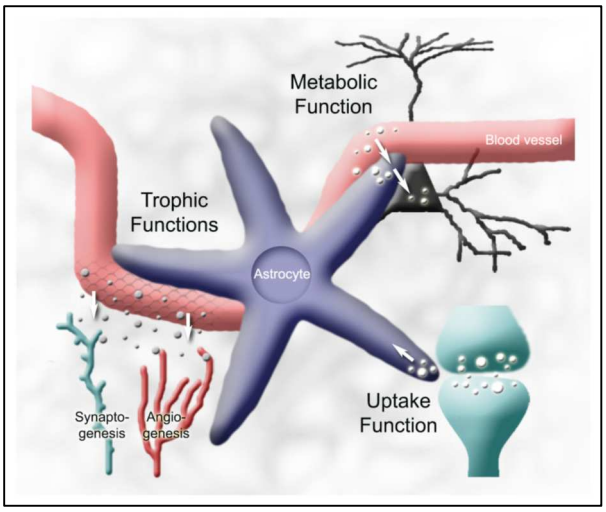


Figure 10. Established astrocytic functions include 1) angiogenesis, 2) synaptogenesis, 3) K^+ and neurotransmitter uptake, 4) establish and maintain BBB integrity, and 5) metabolic support for neurons. Image courtesy of Wang and Bordey, 2008.

Astrocytes contribute to neurogenesis, which is likely region and developmentally-age specific as spinal cord astrocytes do not promote neurogenesis while those in the hippocampus do and different stages of development may determine the ultimate fate of adult stem cells (Song *et al*., 2002). In a similar role, they also participate in helping the newly-formed neurons mature and develop synapses—a process known as synaptogenesis—by secreting trophic factors such as activity-dependent neurotrophic factor (ADNF) (Blondel *et al*., 2000). Astrocytes are also now known to respond to neurotransmitters such as gamma amino butyric acid (GABA) and glutamate via fluxuations in intracellular calcium (Seth and Koul, 2008). Additionally, they have been known to express several potassium (K^+) channels and contribute to K^+ buffering both passively and actively uptaking excess extracellular K^+ then likely releasing the stores when low K^+ levels are detected (Wang and Bordey, 2008). Providing further neuronal

support, astrocytes utilize the endfeet present at the terminus of their projections to take up glucose via the glucose transporter GLUT1, which they then store to release to neurons in times of hypoglycemia and/or heightened neuronal activity (Wang and Bordey, 2008).

In addition to direct neuronal support and communication, astrocytes participate in angiogenesis as well as modulation of blood vessel activity. Astrocytes produce and release epoxyeicosatrienoic acid (EET) as a product of cytochrome P450 epoxygenation of arachidonic acid and this has been shown to induce mitosis and modulate capillary endothelial cells in promotion of angiogenesis (Zhang and Harder, 2002). EETs also contribute to neurovascular coupling as the astrocytic product induces both vasodilative and vasoconstrictive dilations in blood vessels, at least in part due to increases in glial Ca^{2+}, although neither the intensity nor proximity of the signal was found to correlate to either dilations or constrictions as these are most likely mediated via nitric oxide (NO) (Metea and Newman, 2006).

The astrocytes' role at the BBB varies depending on developmental stage. In the process of development they may upregulate transporters and aid in the formation of tight junctions while in adults astrocytes are thought to maintain the tight junctions and help regulate vascular permeability (Wang and Bordey, 2008). This is evident in both the normal and pathological brain as astrocytes regulate BBB integrity in both conditions and BBB disruption is evident in virtually all neurodegenerative diseases (Seth and Koul, 2008). Thus astrocytes, like microglia, are also influenced by changes in the extracellular milieu and the delicate balance between them behaving as neuroprotectors and neuropathogenitors can be swayed either way.

SECTION 1.5

IMMUNE RECEPTORS

Several receptors contribute to immune function. These include, but are not limited to, the Toll-like receptors (TLRs), Interleukin receptors, and Tumor Necrosis Factor (TNF) receptor. The receptors Toll-Like Receptor 4 and Interleukin-1 recetpor will be discussed in detail in the upcoming sections; however, because the TNFα receptor is not in the research presented here but is a major immune receptor, it will be briefly highlighted.

1.5.1 Tumor Necrosis Factor (TNF)

TNF is named so because dating back to the 1890's it was discovered that tumors in cancer patients with concomitant bacterial infection would shrink; however, this would only occur with certain tumors and it was later found that its efficacy was increased in the presence of interferon (IFN) (Fiers, 1991). TNF is an overall name that refers to a superfamily of receptors and cytokines. The primary cytokines of interest are TNF and lymphotoxin (LT). (TNF and LT were previously known as TNFα and TNFβ, respectively.) TNFα is produced by several cell types, can be either membrane bound or soluble, and is mainly characterized as a pro-inflammatory cytokine whose activity is believed to contribute to the pathology of autoimmune disease (Idriss and Naismith, 2000). TNFα also has a dichotomous anti-inflammatory role and protects from infection by activating immune cells, inducing cell necrosis or apoptosis, and intriguingly, can even promote cell survival by activating nuclear factor kappa B (NFκB) (Idriss and Naismith, 2000). LT is only produced by T-lymphocytes, $CD4^+$ and $CD8^+$ cells, is solely membrane

bound, and has similar actions to TNF but has much less affinity for the receptors (Fiers, 1991).

The receptors for these cytokines are TNFR1 and TNFR2. Both of these receptors are expressed on a variety of immune cells (Fiers, 1991) as well as neurons and glia (Shen *et al*., 2012). Their extracellular domains are about 30% homologous but the intracellular domains are completely distinct (Barbara *et al*., 1996) with different intracellular signaling events for each receptor (Shen *et al*., 2012). TNFR1 has been found to activate both serine/threonine mitogen activated protein (MAP) kinases and protein kinase C (PKC), the activation of both resulting in phospholipase A2 (PLA2) mediated arachadonic acid (AA) release and formation of prostaglandins and leukotrienes (Barbara *et al*., 1996). TNFR2 is also activated by one common serine/threonine kinase as well as the kinase casein kinase 1 (CK-1) that is unique to TNFR2; however, both receptors associate with other proteins such as the TNF receptor-associated factors (TRAF1 and TRAF2), TNF receptor-associated protein (TRAP), TNFR1-associated death domain protein (TRADD), and receptor-interacting protein (RIP) (Barbara *et al*., 1996). These proteins will be further addressed as they also interact with the immune receptor TLR4. Receptor density at the membrane does not impact the cellular effect; however, soluble receptor proteins act as a decoy to bind soluble TNFα and regulate TNF receptor activity (Fiers, 1991). Etanercept is a commercially available decoy drug that binds soluble TNFα to reduce inflammation (Shen *et al*., 2012).

1.5.2 Toll-Like Receptor 4 (TLR4)

The toll like receptors (TLRs) are a relatively new receptor family, with the discovery of the first gene coding for a toll receptor in 1979. The toll receptor gene was identified in the German lab of Christiane Nüsslein-Volhard and Eric Wieschaus while conducting studies on *Drosophila melanogaster* segmenting patterns (Nüsslein-Volhard and Wieschaus, 1980). When they were evaluating mutant phenotypes, one of them displayed a completely ventral phenotype thus illustrating the importance of the gene in question and surprising the scientists. As Nüslein-Volhard recalls…(see below)

"*Probably I just shouted: "Toll!" in a conversation with Eric Wieschaus…when he and I scored the fixed embryos together on a discussion microscope…[this] is the essential basis for our Nobel award."*
– Christiane Nüsslein-Volhard, Personal communication to *The FASEB Journal*, May 14, 2010.

Although Nüsslein-Volhard had initially planned on calling the gene "ventral" after the most obvious result of the discovery, the first word shouted, "Toll!"—meaning "crazy, curious, amazing, cool, awesome"—was the name that stuck (Weissmann, 2010). However, the term Toll is not to be found in the seminal 1980 paper. It finally was published as such from their lab in 1985 (Anderson *et al*., 1985) and has changed the scientific community forever.

Basic research was first done on TLRs prior to the identification of TLR4. It was found that the Toll gene codes for a transmembrane protein exhibiting typical extracellular, transmembrane, and intracellular domains, and also has a leucine rich domain characteristic

of a receptor (Hashimoto *et al*., 1988). About a decade later it was determined that both *Drosophila* Toll and a very closely related human protein both signal the innate immune response via NF-κB, in a nearly identical manner as IL-1R, and all display homologous cytoplasmic domains (Medzhitov *et al*., 1997). This was the first human Toll protein identified and it was named toll-like receptor 4 (Ostuni *et al*., 2010)—"toll-like" due to its high sequence homology to *Drosophila* Toll.

Ten additional TLRs have been identified and can be divided into two groups: those that reside on the plasma membrane and respond to extracellular ligands (TLRs 1, 2, 4, 5, 6, and 11) and those that reside in the endolysosomal compartments and respond to bacterial and viral nucleic acids (TLRs 3,7,8, and 9) (Barton and Kagan, 2009). TLR10 is only expressed in humans and signals via similar TLR4 adaptor proteins (Hasan *et al*., 2005) and TLR11 only in mice (Ostuni *et al*., 2010). TLR4, like the other TLRs, stimulates the innate immune response upon ligand binding (Means *et al*., 2000). The immunological role of toll receptors, as acknowledged by their discoverer, has begun to overshadow the initial discovery of dorsal-ventral patterning in *Drosophila* (Weissmann, 2010), despite the fact that both roles are pivotal in the life of the organism.

1.5.3 TLR4 in Neurological and Other Diseases

TLR4 is now widely known for its varying roles and scientists are beginning to uncover several beneficial and deleterious effects due to a TLR4 response. Exemplifying the Dr. Jekyll and Mr. Hyde behavior is the fact that while TLR4 may be involved in adult neurogenesis, it readily contributes to neurotoxicity (Trudler *et al*., 2010). One mechanism for this is via TLR4-induced inflammation in the CNS where TLR4 activity induces

microglial activation and it has been shown specifically that *TLR4* induction of microglial activation is required for neurodegeneration (Lehnardt *et al*., 2003). Not surprisingly, TLR4 activity is implicated in several pathological conditions. One of these is Alzheimer's disease, where TLR4-induced microglial activation is necessary for amyloidβ (Aβ)-induced neurotoxicity and is supported by evidence of increased TLR4 expression at Aβ plaques in human post-mortem brain tissue (Walter *et al*. 2007). On the other hand, TLRs 2, 4, and 9 may all also participate in Aβ uptake in a mouse model of Alzheimer's, providing a beneficial clearing of the Aβ deposits; however, while some research does support this—primarily for TLRs 2 and 9 (Tahara *et al*., 2006)—more evidence indicates that the pro-inflammatory mediation is the greater TLR4 contribution in the pathology of Alzheimer's (Trudler *et al*., 2010; Buchanan *et al*., 2010).

Experimental autoimmune encephalitis (EAE) is a research model in rodents used to study the human disease multiple sclerosis (MS) and it has been found that either without the TLR4 adaptor protein myeloid differentiation factor 88 (MyD88) or in double knockout mice lacking TLR4 receptor expression, development of EAE was attenuated (Aravalli *et al*., 2007). TLR4 is also likey involved in the pathogenesis of Parkinson's disease (PD), which is characterized by dopaminergic neuronal loss in the substantia nigra pars compacta and loss of associated projections into the striatum, as there is an upregulation of TLR4 and cluster differentiation factor 14 (CD14) mRNA and protein in this brain region evident in the mouse model of PD (Panaro *et al*., 2008).

While TLR4 contributes to a variety of neurodegenerative diseases, a pathological hallmark of TLR4 activity is sepsis, where its activation is responsible for the acute systemic inflammation that damages otherwise healthy cells in humans and oftentimes

leads to their death (Buchanan, *et al*., 2010). Recent evidence indicating that systemic inflammation and infection also contribute to neurodegenerative disease, as is indicated by several clinical observations of rheumatoid arthritis patients on chronic non-steroidal anti-inflammatories (NSAIDs) being more resistant to development of Alzheimer's and there is also a quick cognitive decline in neurodegenerative patients after having a systemic infection (Cunningham, 2013). CNS inflammation is exacerbated from chronic inflammatory conditions such as atherosclerosis, smoking, diabetes, and liver disease (Cunningham, 2013)—and as has already been highlighted—TLR4-activated microglia are primary culprits in neurodegeneration and inflammation (Lehnardt *et al*., 2003). As neuronal targets for neurodegenerative conditions have yielded small successes, the investigation into new drugs targeted at microglia is a viable alternative with the potential for effective therapeutics in a variety of illnesses (Buchanan *et al*., 2010).

1.5.4 The TLR4 Complex

1.5.4.1 LPS and Other TLR4 Ligands

The TLR4 receptor complex is large and several proteins contribute to its formation for ligand binding to initiate the intracellular signaling cascade. The most widely recognized ligand for TLR4 is lipopolysaccharide (LPS), which is a cell wall component of Gram-negative bacteria (Ostuni *et al*., 2010). LPS is comprised of three main parts: an O-polysaccharide chain that is attached to a core (the outer core is attached to the O-polysaccharide chain and is comprised of mainly hexose sugars while the inner core is attached to a lipid A moiety and is mostly characterized by its composition of more unique sugars such as heptose) that is followed by the lipid A moiety (Erridge *et al*., 2002). The

lipid A moiety is a hydrophobic portion of the molecule that binds to the cells and is primarily responsible for the toxic effect of LPS, which is modulated by the number and length of acyl chains on the molecule and their phosphorylation state (Rietschel *et al*., 1994). At least two acyl chains are necessary to bind to the receptor; however, lipid A binding alone is insufficient to elicit a response (Rietschel *et al*., 1993). Illustrating he variations of LPS activity is the lipid A analogue, eritoran, which is in clinical trials to treat septic shock and sepsis-related cardiac dysfunction (Wittebole *et al*., 2010) as a TLR4 antagonist (Buchanan *et al*., 2010). In addition, a highly conserved link exists between lipid A and the inner core that consists of a phosphorylated diglucosamine backbone joined to a minimum of one unique sugar, typically 3-deoxy-D-manno-octulosonic acid (Kdo), (Erridge *et al*., 2002). While Kdo may be at least part of the pathogen associated molecular pattern (PAMP) that is recognized by TLR4, it is recognized in the literature that the conserved PAMP in LPS is the hydrophobic lipid A portion (Rietschel *et al*., 1993; Park *et al*., 2011). Furthermore, while the lipid A moiety is responsible for the endotoxic activity of LPS, this may be modulated via the inner core Kdo component (Rietschel *et al*., 1993). Despite seemingly limitless variations of LPS structure via different sugars, numbers and lengths of acyl chains, etc., (Jin and Lee, 2008) conserved PAMPs—such as the Kdo sugar that is not typically found in humans and the inner core of LPS—are responsible for the diverse array of pathogens that initiate a TLR4 response.

It is now known that TLR4 has an arsenal of ligands other than LPS, including endogenous danger molecules such as heat sock proteins, natural products such as curcumin and resveratrol, and endogenous and exogenous opioids (Buchanan *et al*., 2010).

comprehensive, yet seemingly ever growing, list of TLR4 ligands and their action on TLR4 function is presented in **Table 1**.

1.5.4.2 LPS Binding Protein (LBP)

As was mentioned above, the receptor complex to recognize a ligand (notable LPS) is quite large and several proteins interact to activate TLR4. The first step for TLR4 recognition of LPS is via LBP. Soluble LBP binds to the lipid A moiety of LPS with a high affinity at the NH_2-terminus of LBP (Iovine *et al*., 2002), removing LPS from the bacterial membrane (Schumann *et al*., 1990; Wright *et al*., 1990). The LPS/LBP complex then binds to another accessory protein, cluster differentiation factor 14, at the COOH-terminus of LBP (Iovine *et al*., 2002) before activating TLR4; however, LBP alone is not sufficient to elicit a TLR4 response (Wright *et al*., 1990).

Table 1. TLR4 ligands and their interactions with the receptor.[a]

TLR4 Ligand	Interaction with TLR4 Receptor or Signaling
Lipopolysaccharide (LPS) and LPS derivatives	Outer cell wall component of gram-negative bacteria; potent initiator of TLR4 signaling. LPS structure varies with bacterial species
Curcumin	Polyphenol found in the plant Curcuma longa. Inhibits TLR4 by binding MD-2.
Cinnamaldehyde (3-phenyl-2-propenal)	Anti-inflammatory, inhibits ligand-induced TLR4 oligomerization and downstream signaling
Ethanol	Appears to redistribute TLR4 complexes on the cellular membrane by preventing receptor association and/or dimerization in the lipid raft
E5564 (eritoran)	LPS analogue clinically tested for sepsis; inhibits TLR4 signaling
Opioids	Both opioid stereoisomers alter downstream TLR4 signaling. Opioid agonists (e.g. morphine) have different effects than antagonists (e.g. naloxone)
TAK-242 (Ethyl(6*R*)-6-[*N*-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate)	Clinically tested cyclohexene derivative, selectively inhibits intracellular signaling by TLR4
Paclitaxel (Taxol)	Widely used cancer therapeutic, reported to inhibit MD-2, thereby knocking down TLR4 activity which was found to correlate with drug efficacy
Resveratrol (*trans*-3,5,4-trihydroxystilbene)	Antioxidant reported to inhibit TLR4 signaling; found in the skin of grapes, it is known for anti-inflammatory and anti-carcinogenic effects
Statins Amyloid-β 42 peptide	Statin drugs influence TLR4-mediated cytokine expression through a Rho-protein feedback mechanism The peptide hallmark of Alzheimer's pathogenesis, appears to activate TLR4 directly and also through signals from damaged neurons (e.g. 4-hydroxynonenal)
Extracellular matrix proteins (Biglycan, Fibrinogen, Fibronectin, Tenascin C)	Negatively charged glycoproteins are reported to activate TLR4 signaling
Fatty acids	Fatty acids are reported to regulate TLR4 receptor dimerization and recruitment into lipid rafts
Heat-shock proteins (HSP) 60, 70, 90	Released from dead or dying cells. HSP60 mediates neurodegeneration via TLR4. HSP90 may influence TLR4 pain amplification. LPS contamination is a common problem in HSP studies
Polysaccharides	Heparin sulfate and endogenous hyaluronic acid fragmentation products may activate dendritic cells and macrophages through TLR4

[a] **Table 1** was reproduced from the review by Buchanan, *et al*., 2010. For complete references for each of the studies indicating the ligands' activity at TLR4, please refer to the referenced article.

1.5.4.3 Cluster Differentiation Factor 14 (CD14)

CD14 can be either a slightly smaller soluble molecule (sCD14) or membrane-bound (mCD14), attaching to the membrane via a glycosylphosphatidylinositol (GPI) anchor (Haziot *et al*., 1988). CD14 was identified as a pattern recognition receptor as it was found to respond to gram-negative, gram-positive, and mycobacteria with sCD14 responsible for mediating the response for cells lacking mCD14 (Pugin *et al*., 1994). Despite the initial idea that CD14 is the LPS receptor—and it's true, LPS can bind to it—because it lacks a transmembrane and intracellular domain it cannot be solely responsible for intracellular signaling (Haziot *et al*., 1988). Studies have shown that CD14, like TLR4, contains several leucine rich repeats (LRR) (Wright *et al*., 1990). An amino-terminus hydrophobic pocket on CD14 binds the lipid chains of LPS while the area next to the pocket aids in LPS transfer from CD14 to the next protein in the receptor complex, myeloid differentiation factor 2 (Kim *et al*., 2005).

1.5.4.4 TLR4 Receptor Dimerization

MD-2 is necessary for TLR4 dimerization. The TLR4/MD-2 dimer complexes with another TLR4/MD-2 dimer upon agonist ligand binding, creating a heteromer before initiation of intracellular signaling (Park *et al*., 2009). The signaling cascade is believed to result from a conformational change that occurs after MD-2 binding (Akashi *et al*., 2000) and receptor complex dimerization, allowing TLR4 proteins to orient themselves such that the intracellular receptor domains are exposed, allowing recruitment of additional signaling molecules (Manalavan *et al*., 2011). Without MD-2 mediated dimerization, TLR4 activation does not occur (Gay *et al*., 2006). Although other inhibitory mechanisms exist

(see **Table 1**), a common TLR4 antagonistic theme is for the ligand to bind to MD-2, thereby preventing receptor dimerization and subsequent activation (Park *et al*., 2011).

1.5.4.5 The TLR4 Protein

TLR4 is a type 1 transmembrane protein, with an extracellular domain for ligand binding, one transmembrane helical pass, and an intracellular domain for signaling (Manavalan *et al*., 2011; Gay *et al*., 2006). It is primarily expressed on immune cells such as monocytes, macrophages, dendritic cells, T cells, T helper cells, B cells, (Medzhitov *et al*., 1997) and microglia (Kettenmann *et al*., 2011). The extracellular portion of the receptor has a characteristic horseshoe shape resulting from the LRR domains (Kim *et al*., 2007). While TLR4 is primarily expressed on the cell membrane (Jiang *et al*., 2000), as this is necessary for LPS signaling, it can also be found in the Golgi apparatus, where it appears to be stored in unstimulated cells (Husebye *et al*., 2006).

Shortly after activation (~40 minutes) TLR4 internalization then occurs via clathrin-coated pits; however, after prolonged LPS stimulation clathrin-independent mediated endocytosis occurs (Husebye *et al*., 2006). TLR4 quickly and readily recycles back and forth between the plasma membrane and Golgi, along with colocalized CD14 (Espevik *et al*., 2003) and LPS (Thieblemont and Wright, 1999). However only aggregated LPS (LPS micelles bound to LBP that form in the absence of sCD14) is internalized with colocalized CD14 after LPS stimulation while monomeric LPS is internalized after binding to mCD14, but dissociates from mCD14 after endocytosis (Vasselon *et al*., 1999). This suggests that MD-2 must also internalize and localize to the Golgi, but studies illustrating that LPS, CD14, MD-2, and TLR4 are all transported there simultaneously as a complex

have not been found. Importantly, endocytosis of TLR4 may be necessary to attenuate signaling as it is required for ubiquitination and trafficking to the lysosome for degradation; however, it has been suggested that the TLR4 complex continues to signal intracellularly until it has been transferred to the luminal side of the endosome (Husebye *et al*., 2006). This receptor-mediated intracellular signaling may be important in the TRIF-dependent signaling cascade (Tanimura *et al*., 2008), which has delayed kinetics when compared to the MyD88-dependent pathway. How these findings may contribute to TLR4 signaling and disease is unknown.

1.5.4.6 The TIR Domain

The intracellular domain of TLR4 has a high homology to that of the interleukin-1 receptor (IL-1R) (Medzhitov *et al*., 1997). The two immune receptors utilize the same adaptor proteins and signaling sequence, as is alluded to in the name of the Toll-interleukin receptor (TIR) domain.

It is believed that a conformational change resulting from receptor oligomerization activates signaling by positioning the TIR domains such that they can interact and expose certain areas to recruit the appropriate adaptor molecules (Barton and Kagan, 2009). According to a computer model, the TIR interface is also believed to serve as a scaffold for assembly of the adaptor proteins (Miguel *et al*., 2007). The TIR domains tend to vary in size, with conserved residues hiding in a hydrophobic core and non-conserved residues present at the first TIR-TIR interface likely determining adaptor recruitment specificity to activate the different signaling pathways (Xu *et al*., 2000), similar to T-cell activation (Mills 2011). Two more TIR-TIR interfaces also interact: the second one mediates the

receptor TIR domain with the TIR domain of the adaptor protein myeloid differentiation factor 88 (MyD88) and the third interface mediates the TLR4 TIR domain with the other adaptor molecules—this third one is important for signaling specificity (Xu *et al*., 2000). A protruding area of the TIR domain termed the BB loop is believed to be responsible for this specificity in adaptor protein interactions with the TIR domain (Miguel *et al*., 2007). The BB loop may also play a role in receptor dimerization (Gay *et al*., 2006). In accordance for the requirement for ligand binding and receptor dimerization under normal conditions the TIR domains have a low affinity for each other. This is exemplified in the absence of an agonist, when their association is only facilitated by receptor overexpression or ligand binding (Xu *et al*., 2000).

1.5.4.7 The TIR Domain-Containing Adaptor Molecules

The five TIR domain-containing adaptor molecules that interact with the TIR domain of TLR4 are MyD88, MyD88-adaptor like (Mal, also referred to as TIRAP), TIR-domain-containing adaptor protein inducing interferon (IFN)-β (TRIF, also referred to as TICAM1), TRIF-related adaptor molecule (TRAM, also referred to as TICAM2), sterile α-and huntingtin-elongation-A subunit TOR (HEAT) (Ohnishi *et al*., 2009), and armadillo-motif containing protein (SARM) (Watters *et al*., 2007). All of these proteins promote TLR4 signaling except for SARM. Little literature exists for SARM, except that it is a negative regulator to TLR4 TRIF-dependent signaling (Carty *et al*., 2006).

1.5.4.8 MyD88

MyD88 lacks a transmembrane region, is highly conserved among species (e.g., 81% amino acid homology between humans and mice (Bonnert *et al*., 1997)), and is a soluble protein that is recruited to the TIR domain of all TLRs except for TLR3 (Kawai and Akira, 2007). Like TLR4, it has a TIR domain, a small intermediate domain (ID) (Watters *et al*., 2007), and also a death domain (DD) that is unique in that it does not appear to induce apoptosis, but rather directly upregulates transcription of IL-8 (Bonnert *et al*., 1997). In the cytoplasm, MyD88 exists as a dimer, interacting with itself via the ID and DD domains (Ohnishi *et al*., 2009). Once recruited to the receptor, it is considered to be a "signaling adaptor" in that it functions to link activated TLR4 to the other proteins involved in the signaling pathway (Kagan and Medzhitov, 2006). MyD88 also has three distinct sites, two of which are located on opposite sides of the molecule and are important for binding it to Mal via its TIR domain, although it does not directly associate with TLR4 (Ohnishi *et al*., 2009). MyD88 typically requires an association with Mal to initiate signaling; however, it can also act independently of Mal if it is attached to a phosphatidylinositol 4,5-bisphosphate (PIP2) dense region of the cell membrane (Kagan and Medzhitov, 2006). Furthermore, MyD88 is also known to have a shorter splice variant with deletion of the ID, known as $MyD88_s$, that acts as a negative regulator of NFκB activation by removing the domain responsible for phosphorylation of the next molecule in the signaling cascade, IL-1R-associated kinase (IRAK) (Janssens *et al*., 2002).

1.5.4.9 Mal

Mal is an adaptor molecule that links TLR4 to MyD88 (Horng *et al*., 2001) via their TIR domains (Ohnishi *et al*., 2009). Tyrosine phosphorylation of Mal is necessary for its signaling (Watters *et al*., 2007). The TIR domain also is the interface for interaction between Mal and protein kinase C-δ (PKCδ), which potentiates signaling to MAPKs and NFκB (Watters *et al*., 2007). Mal is found attached to the plasma membrane via PIP2, similar to MyD88 signaling in lieu of Mal, providing evidence for a possible phosphoinositide role in TLR4 signaling (Kagan and Medzhitov, 2006). Despite the high sequence and signaling homology between IL-1R and TLR4, when comparing the two receptors, Mal is only used in TLR4 signaling as it is not required for IL-1R (Horng *et al*., 2002). Mal is additionally suggested to directly signal to TRAF6 via a putative TRAF6 binding domain that is not found in MyD88 (Mansell *et al*., 2004). This mechanism is believed to be at least partially responsible for the activation of MAP kinases JNK and P42/P44 as well as activate NFκB p65, perhaps potentiating the MyD88-induce pro-inflammatory response (Mansell *et al*., 2004).

1.5.4.10 TRIF

TRIF is another TIR domain-containing adaptor molecule and is responsible for the TRIF-dependent signaling cascade. TRIF is essential for interferon regulatory factor 3 (IRF3) translocation to the nucleus and induction of interferon-inducible genes, such as IFNβ (Yamamoto *et al*., 2003a). Although TRIF is more traditionally linked to a viral response via IRF3 in response to TLR3 ligands, a delayed pro-inflammatory cytokine profile mimicking that of MyD88-dependent signaling is also generated and this late phase

NFκB activation may somehow serve to stimulate transcription of the interferon-inducible chemokine gene, IP-10 (Kawai *et al*., 2001). The NFκB-induced transcription of IP-10 is an example of redundant cytokine production between the MyD88-dependent and TRIF-dependent signaling pathways.

1.5.4.11 TRAM

TRAM colocalizes with TLR4 on the plasma membrane and is also found in the Golgi apparatus (Rowe *et al*., 2006). TRAM, like Mal, is an adaptor molecule connecting TLR4 to the signaling mediator, TRIF (Oshiumi *et al*., 2003). However, unlike Mal, TRAM does not have to specifically associate with a PIP2 lipid domain (Kagan *et al*., 2008). While it colocalizes with TLR4, this may or may not be via a TIR-domain containing mechanism as there is conflicting research on how TRAM and TLR4 physically interact (Oshiumi *et al*., 2003; Kagan *et al*., 2008). However, the BB loop in the TIR domain of TRAM has been determined as necessary for signaling via TRIF, (Rowe *et al*., 2006), consistent with the role of the BB loop in the TIR domains of other proteins such as TLR4 (Miguel *et al*., 2007). TRAM also undergoes myristoylation, a fatty acid modification, which targets it to the membrane where it can associate with the GPI-anchored protein CD14, as CD14 has been identified as a requisite for TRAM-mediated signaling (Jiang *et al*., 2005; Rowe *et al*., 2006; Kagan *et al*., 2008). TRAM and TRIF colocalize at the plasma membrane and transfer to the endosome/lysosome after stimulation (Tanimura *et al*., 2008). TLR4 continues to signal via the TRIF-dependent pathway from endosomes, and curiously, does so mostly from this location when signaling through TRIF (Kagan *et al*., 2008). Perhaps the association with CD14 is only necessary

for signaling at the plasma membrane, as the literature illustrates CD14 internalization then localization at the Golgi, not endosomal/lysosomal locations.

1.5.5 The TLR4 Signaling Cascades

Prior to the discussion on the signaling cascades, please note that while the research presented here focuses primarily on microglia, the studies elucidating the signaling pathways have mostly been performed in epithelial cells and macrophages.

TLR4 signaling involves two different cascades: the MyD88 dependent and the MyD88 independent—herein after referred to as the TRIF dependent—pathways. Early TLR4 research identified that first of all, MyD88 quickly stimulated transcription of pro-inflammatory cytokines via NFκB—just like it did when it associated with IL-1R—but it did not take long before researchers noticed that the same NFκB-induced transcription occurred in the absence of MyD88—albeit it with delayed kinetics (Kawai *et al*., 1999). Either a greater affinity between TLR4 and Mal (Miguel *et al*., 2007) or sequential signaling via MyD88 first then endosomal/lysosomal signaling via TRIF (Kagan *et al*., 2008) are potential explanations for the different kinetics. Furthermore, type 1 interferons could also be produced via TLR4, which occurs independent of MyD88 (Oshiumi *et al*., 2003). These observations led to the identification of the TRIF-dependent signaling pathway. Both the MyD88-dependent pathway and the TRIF-dependent pathway are depicted in **Figure 11**.

1.5.5.1 The MyD88-Dependent Pathway

After ligand binding and receptor complex formation the MyD88-dependent signaling pathway begins with membrane-bound Mal associating with TLR4 via their TIR domains (Horng *et al*., 2001). MyD88 is then recruited to the TLR4-Mal complex, where it also interacts with a TIR domain of Mal (Ohnishi *et al*., 2009). MyD88 then recruits a serine/threonine kinase IL-1 receptor-associated kinase (IRAK), typically IRAK4, via the DDs of the two proteins (Wesche *et al*., 1997a; Kawai and Akira, 2007). IRAK has an N-terminal DD, a kinase domain with 15 conserved kinase residues, and a C-terminus, allowing for multiple phosphorylation sites (Yamin and Miller, 1997).

MyD88 has a high affinity for unphosphorylated IRAK4, which autophosphorylates at least three times after recruitment by MyD88 (Wesche *et al*,. 1997a; Yamin and Miller, 1997). IRAK then phosphorylates IRAK1 (Watters *et al*., 2007), which in turn phosphorylates and mediates tumor necrosis factor receptor-associated factor 6 (TRAF6) and transforming growth factor-β-activated kinase binding protein 2 (TAB2) movement from the membrane to the cytosol (Qian *et al*., 2001). TAB2 connects TRAF6 to TAK1 (Dauphinee and Karsan, 2006). TRAF6 is necessary for production of pro-inflammatory cytokines (Tanimura *et al*., 2008).

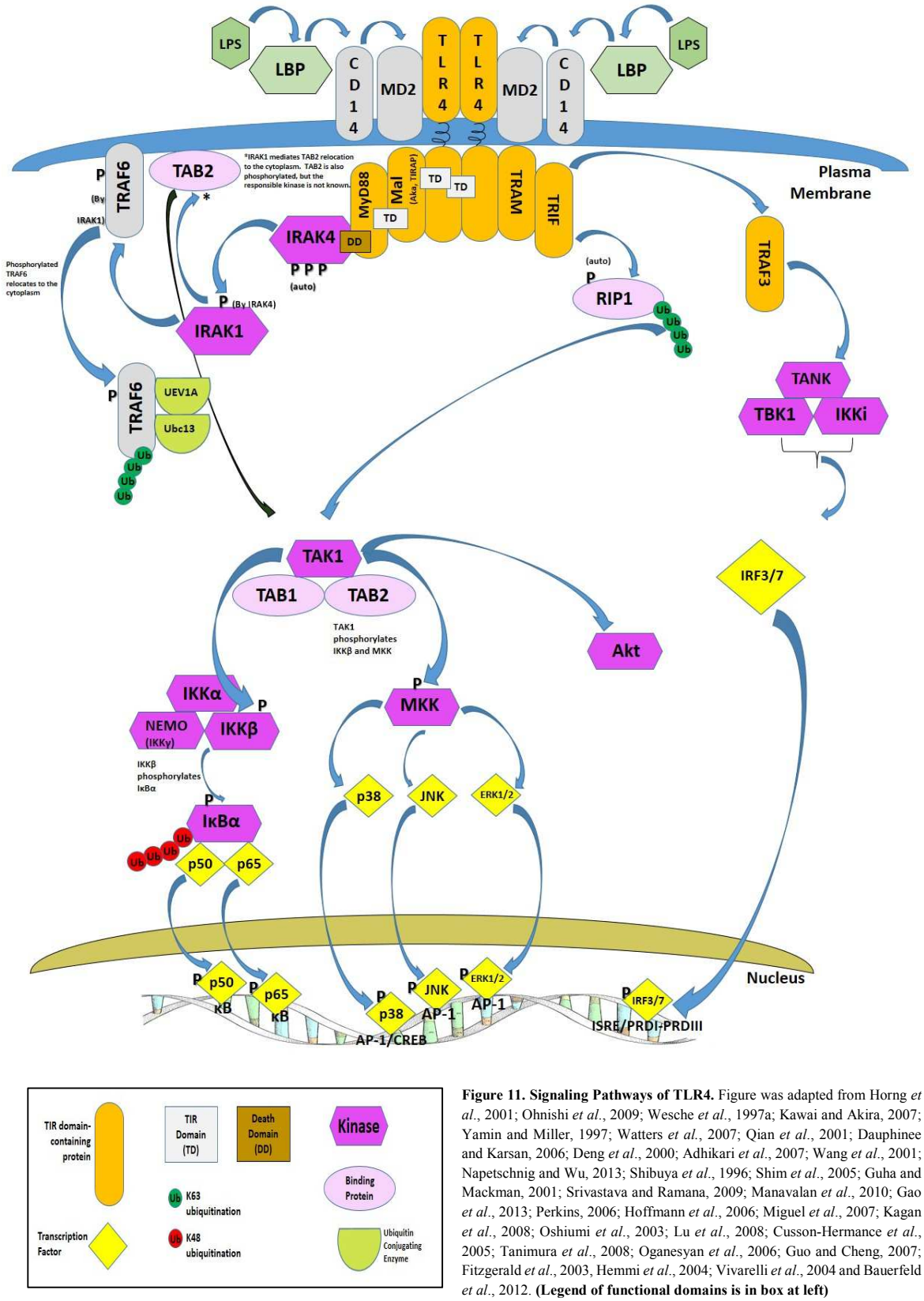


Figure 11. Signaling Pathways of TLR4. Figure was adapted from Horng *et al*., 2001; Ohnishi *et al*., 2009; Wesche *et al*., 1997a; Kawai and Akira, 2007; Yamin and Miller, 1997; Watters *et al*., 2007; Qian *et al*., 2001; Dauphinee and Karsan, 2006; Deng *et al*., 2000; Adhikari *et al*., 2007; Wang *et al*., 2001; Napetschnig and Wu, 2013; Shibuya *et al*., 1996; Shim *et al*., 2005; Guha and Mackman, 2001; Srivastava and Ramana, 2009; Manavalan *et al*., 2010; Gao *et al*., 2013; Perkins, 2006; Hoffmann *et al*., 2006; Miguel *et al*., 2007; Kagan *et al*., 2008; Oshiumi *et al*., 2003; Lu *et al*., 2008; Cusson-Hermance *et al*., 2005; Tanimura *et al*., 2008; Oganesyan *et al*., 2006; Guo and Cheng, 2007; Fitzgerald *et al*., 2003, Hemmi *et al*., 2004; Vivarelli *et al*., 2004 and Bauerfeld *et al*., 2012. **(Legend of functional domains is in box at left)**

TRAF6 also has a RNG domain with E3 ubiquitin ligase activity (Kawai and Akira, 2007) and is K63 polyubiquinated via UEV1A-mediated Ubc13 ubiquitination (Deng *et al*., 2000). K63 ubiquitination results from an optimal structure allowing a 'donor' ubiquitin covalently linked to Ubc13 to be transferred to a UEV1A ubiquitin 'acceptor site at K63 (Hofmann and Pickart, 1999). K63 ubiquitination serves a signaling function and does not target the protein for proteasomal degradation (Adhikari *et al*., 2007). The K63 chain present on TRAF6 yields TAK1 activation, via an unknown mechanism, allowing TAK1 to autophosphorylate inhibitory κB kinase (IKK) β, thereby activating the IKK complex (Wang *et al*., 2001; Napetschnig and Wu, 2013). TAB1 in another protein that associates with TAB2 and TAK1 and enhances TAK1 kinase activity (Shibuya *et al*., 1996). TAK1 is the point at which the pathway can diverge into either NFκB- and/or mitogen activated protein kinase (MAPK)-dependent transcriptional activation.

1.5.5.2 MAPK Activation

In the MAPK pathway branch, TAK1 phosphorylates MAPKs such as mitogen activated kinase kinase 6 (MKK6) to activate Jun N-terminal kinase (JNK) and p38 kinase (Want *et al*., 2001). JNK activation activates the transcription factors c-Jun, ATF-2 (c-Jun and ATF-2 comprise the AP-1 complex (Shim *et al*., 2005)), and ETS domain-containing protein, Elk-1, while p38 phosphorylates the transcription factors activating transcription factor 2 (ATF-2), Elk-1, C/EBP homologous protein (CHOP), myocyte-specific enhancer factor 2C (MEF2C), Sap1a, MNK1/2, map kinase-activated protein kinase 2 (MK2), MSK1, and PRAK either directly or indirectly (Guha and Mackman, 2001). The

extracellular signal-regulated kinase (ERK 1/2) is also activated leading to the activation of the transcription factor Elk-1 in monocytes (Guha and Mackman, 2001).

1.5.5.3 NFκB Activation

In the NFκB pathway branch, after TAK1 autophosphorylates in the presence of TAB1, it activates the IKK complex (Napetschnig and Wu, 2013), which consists of IKKα, IKKβ, and IKKγ (IKKγ is a scaffolding protein, also known as NFκB essential modulator, NEMO) (Srivastava and Ramana, 2009). This kinase complex phosphorylates the inhibitory κB (IκB) protein IκBα that binds to the NFκB dimer and maintains its (primarily) cytoplasmic residency (Manavalan *et al*., 2010). In actuality, despite an apparent cytoplasmic steady-state location, these complexes travel back and forth between the nucleus and the cytoplasm because only the nuclear localization signal (NLS) for p65 is hidden by the IκBα protein while the NLS and nuclear export sequence (NES) for p50 remains exposed (Hayden and Ghosh, 2008). This has been specifically shown for NFκB p50:p65 dimers, which are involved in TLR4 signaling as are measured in LPS-induced microglial activation (Gao *et al*., 2013). Once IκBα is phosphorylated on a conserved serine residue by IKKβ in the IKK complex, it is K48-linked polyubiquitinated by an E3 ubiquitin ligase complex and quickly targeted for proteosomal degradation (Perkins, 2006) thereby releasing the NFκB p50 and p65 transcription factors. It has been found that at least p65 requires activation via phosphorylation in the cytoplasm prior to translocation to the nucleus (Guha and Mackman, 2001) where it binds to the κB consensus sequence sites located in the promoter region of genes and initiates transcription (Hoffman *et al.*, 2006) of pro-inflammatory cytokines such as IL-1β and TNFα (Gao *et al*., 2013). Under typical

circumstances this is regulated via additional transcription of IκB proteins and targeting of the DNA-bound dimers as well as other posttranslational modifications that decrease the affinity of the dimers for transcriptional coactivators, thereby terminating the response (Hayden and Ghosh, 2008).

1.5.5.4 TRIF-Dependent Pathway

As mentioned above, the TRIF-dependent pathway generates a slower response to agonist binding. This is potentially due to a lower affinity of TRAM for the receptor dimer because it is not situated as close to the receptor as Mal (Miguel *et al*., 2007). Supporting this theory is that after the TLR4-MyD88-Mal complex is internalized and myD88-Mal is removed from the receptor, the TIR domain is exposed for the lower affinity TRAM to bind to the TIR domain of TLR4—most likely on the early endosomes resulting from the endocytosed receptor complex (Kagan *et al*., 2008), since the two adaptor proteins likely bind sequentially to the same TIR domain (Miguel *et al*., 2007). In summary, membrane localized TRAM binds to the TIR domain of TLR4, recruiting TRIF to the complex (Oshiumi *et al*., 2003). Then the C-terminus of TRIF interacts with the serine/threonine kinase receptor-interacting protein 1 (RIP1) via its Rip homotypic interaction motif (RHIM) (Lu *et al*., 2008). RIP1 then auotphosphorylates followed by K63-linked polyubiquitination, which is recognized by TAB2 in the TAK1/TAB1/TAB2 complex (Cusson-Hermance *et al*., 2005), after which the pathway mimics the MyD88-dependent cascade to NFκB and MAPK activation via TAK1 phosphorylation of MKK and IKKβ, mediating late phase MAPK and NFκB activation, respectively (Lu *et al*., 2008).

While the TRIF-dependent pathway can lead to pro-inflammatory cytokine production, it is more traditionally associated with the production of Type 1 interferons. In this cascade, TRIF and TRAM recruit TRAF3 (Tanimura *et al*., 2008), which is required for induction of Type 1 interferons (Oganesyan *et al*., 2006). TRAF3 then interacts with the non-canonical IKK members, TANK-binding kinase 1 (TBK1, aka NFκB activating kinase or TRAF2 associated kinase) and inducible IκB kinase (IKKi, aka IKKε), which interact with the TRAF family member-associated NFκB activator, TANK (Guo and Cheng, 2007). IKKi and/or TBK1 then, either via direct or indirect mechanisms, phosphorylate and activate the transcription factor IRF3, which homodimerizes and translocates to the nucleus (Fitzgerald *et al*., 2003) to induce transcriptional activation of the IFNβ gene and IFN-induced genes by binding to IFN-stimulated response elements (ISRE)/IRF-binding elements (Hemmi *et al*., 2004).

1.5.5.5 Other TLR4 Intracellular Effects

Other cytosolic effects that can also occur via TLR4 include PI3 kinase activation. PI3 kinases can be activated via LPS stimulation, which then phosphorylates lipids such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate, and then goes on to activate Akt (Dauphinee and Karsan, 2006), also known as protein kinase B (PKB). Akt also can be activated by associating with RIP1 via TAK1, activating the PI3 kinases to phosphorylate Akt and aid in cell survival and proliferation (Vivarelli *et al*., 2004). Activation of both the PI3 kinase and Akt pathways via LPS-stimulated TLR4 has been found to increase the pro-inflammatory cytokines TNFα and IL-1, reactive oxygen

species, and nitric oxide in murine macrophages, likely by decreasing phosphatase activity, thereby maintaining signal kinase activity (Bauerfeld *et al*., 2012).

1.5.6 Interleukin-1

The interleukin family of cytokines was first described in the 1970's (Gery and Handschumacher, 1974), with the associated membrane-bound receptor's identification occurring about a decade later (Dower *et al*., 1985). As the area was just in its infancy, the nomenclature was highly varied with the cytokine currently identified as interleukin-1 (IL-1) studied under the names of mitogenic protein (MP), helper peak-1 (HP-1), T cell – replacing factor III (TRF-III), T cell-replacing factor$_{M\phi}$ (TRF$_M$), B cell-activating actor (BAF), B cell differentiation factor (BDF), and more commonly, lymphocyte-activating factor (LAF). Fortunately, after the Second International Lymphokine Workshop in 1979 several scientists agreed that all of these terms described one factor that seemed to primarily communicate between leukocytes and although other biological activities had also been recognized and acknowledged, the term *Interleukin* for 'between leukocytes,' was proposed with interleukin-1 being the first and encompassing the seven aforementioned factors into one term (No Authors Listed, 1979; Dinarello, 1984).

1.5.6.1 Interleukin-1α and Interleukin-1β

IL-1 is a generic term for the two cytokine agonists for the interleukin-1 receptor (IL-R), IL-1α, and IL-1β (Sims, 2002). IL-1α and IL-1β were shown to bind to the same receptor via radioligand binding studies, which further identified a high affinity of the cytokines for the receptor (albeit IL-1α has a lower affinity than IL-1β), a low number of

receptors present per cell, and merely a picomolar concentration needed to elicit a response (Matsushima *et al*., 1986; Kilian *et al*., 1986). This efficient combination for biological activity highlights the evolutionarily conserved importance of IL-1 in host defense. However, despite the fact that IL-1α and IL-1β bind to the same receptor and elicit the same response (Kilian *et al*., 1986), they only share about 27% homology (Gubler *et al*., 1986). The conserved regions shared by both molecules, primarily in their C-terminal domains, are responsible for IL-1 activity (March *et al*., 1985; Matsushima *et al*., 1986). Both IL-1 proteins have molecular weights of 15,000-17,000 daltons (Gery and Handschumacer, 1974; Mizel, 1979) after they are proteolytically cleaved from the larger precursor proteins, proIL-1α and proIL-1β (March *et al*., 1985). The IL-1β converting enzyme (ICE) is responsible for cleaving IL-1β into its active form, while other proteases process IL-1α (Dinarello, 1998). IL-1α and IL-1β also differ in that IL-1α tends to remain intracellular while IL-1β is found in circulation after initiation of the immune response (Dinarello, 1985). This is thought to be because IL-1β acts as a hormone in that it induces fever, fibrosis, etc., while IL-1α may function more on an autocrine level communicating between macrophages and lymphocytes (Lepe-Zuniga *et al*., 1985). A membrane-bound form of IL-1 has also been identified that may be important for specific T-cell activation (Kurt-Jones *et al*., 1985). Several cells and cell lined produce and respond to IL-1, such as monocytes, macrophages, keratinocytes, epithelial cells, astrocytes, and microglia (Kettenmann *et al*., 2011; Dinarello, 1984).

1.5.6.2 Biological Activity of IL-1

IL-1 is a pro-inflammatory cytokine that has several biological actions. Under normal circumstances IL-1 does not tend to circulate; however, concentrations of IL-1 are elevated in times of infection or inflammation (Dinarello, 1984). Prior to the 1979 conference, IL-1 was often referred to as lymphocyte-activating factor, or LAF, due to its mitogenic activity in stimulating T-cells by initiating IL-2 synthesis and release (Gery *et al*., 1972). IL-1 also induces specific neutrophil degranulation that is dependent on calcium and magnesium, leading to the release of lactoferrin—which sequesters iron in serum—from neutrophils (Klempner *et al*., 1978). This explains infection- and inflammatory-induced anemia. IL-1 also contributes to fibroblast proliferation in graft-vs-host disease and other fibrous conditions (Schmidt *et al*., 1982). The peripheral roles of IL-1 are not limited to those mentioned here.

In the CNS, IL-1 increases the synthesis of prostaglandin E_2 in the hypothalamus, thereby increasing the temperature set point in the host resulting in fever (Dinarello, 1984). As a result, IL-1 is recognized as a pyrogen. IL-1 has also been found to induce slow-wave sleep; however, despite the fact that the same cytokine produces sleep and fever, co-administration of an anti-pyrogenic and IL-1 suppresses fever but not the increase in slow-wave sleep associated with IL-1 (Krieger *et al*., 1984). IL-1 in the brain, with activated microglia as a likely source (Yao *et al*., 1992), contributes to astroglial proliferation after injury (Giulian and Lachma, 1985). Interestingly, activated microglia primarily produce IL-1α (Yao *et al*., 1992). Despite this, IL-1β is a microglial activator and is directly associated with Alzheimer's disease. IL-1β-activated microglia phagocytose amyloid plaques in Alzheimer's but an IL-1β sustained upregulation of p38 MAPK results in

excessive tau phosphorylation in a mouse model (Ghosh *et al*., 2013), suggesting that IL-1β may have a *Jekyll and Hyde* role in Alzheimer's disease. In Parkinson's disease, IL-1 is a likely microglia activator in both the nigrostriatal system and the olfactory bulb, where the disease first manifests prior to motor dysfunction, as shown in MPTP-treated mice and in the post-mortem brains of Parkinson's patients (Vroon *et al*., 2007). However, because microglia are still activated in an IL-1 knockout mouse model of Parkinson's, it may have a differential regulation on microglia in different brain regions (Vroon *et al*., 2007) or have more prominent impacts in disease initiation than in disease progression. As is demonstrated by its roles in several varying conditions, IL-1 is clearly an important cytokine in both the immune response and in the pathology of disease.

1.5.7 The IL-1 Receptors: Type I and Type II IL-1R

TLR4 and the interleukin 1 receptor (IL-1R) share cytoplasmic domains and a high sequence homology (Medzhitov *et al*., 1997). While the agonist ligand for IL-1R, the cytokine IL-1, was described in the 1970's to be important to innate immunity before the identification of hTLR4 by Medzhitov *et al*., in 1997, IL-1R is thought to be an evolutionarily newer protein in comparison as it more closely connects innate to adaptive immunity by contributing to T helper cell and B cell proliferation and is only found in vertebrates (Boraschi and Tagliabue, 2006; Martin and Wesche, 2002). The IL-1R genes likely resulted from gene duplication events (McMahan *et al*., 1991) that occurred when birds and mammals diverged (Sims *et al*., 1994).

IL-1R was identified in 1985 with a molecular weight of ~80,000 daltons (Dower *et al*., 1985). Soon after it was discovered that both IL-1α and IL-1β bind to the same

receptor (Matsushima *et al*., 1986; Kilian *et al*., 1986). As was mentioned above, IL-1 activity is found in a variety of cells, mostly of lymphatic origin, but it has also been identified in the brain. While IL-1R is expressed throughout the brain, the highest concentrations tend to be associated with groups of neurons such as those in the olfactory bulb, the granule cells of the dentate gyrus, the pyramidal cells in the hippocampus, and neurons in the oculomotor nucleus; however, differing patterns imply a likelihood that not all neurons associate with IL-1R (Farrar *et al*., 1987; Farrar *et al*., 1988). The high levels of IL-1R in the olfactory bulb support the work of Vroon *et al.* suggesting that IL-1 participates in the early stages of Parkinson's disease.

A second IL-1R receptor exists, termed Type II IL-1R—the first is commonly referred to in the literature as Type I IL-1R, or simply IL-1R. These receptors are members of the immunoglobulin-like domain-containing (Ig) family of receptors (Sims, 2002). However, only the Type I IL-1R has the highly conserved cytoplasmic TIR domain (Gabay *et al*., 2010). They both have an extracellular portion responsible for ligand binding that has three Ig domains, a single transmembrane pass, and a cytoplasmic portion that differs between the Type I and Type II receptors (McMahan *et al*., 1991). While Type I IL-1R has a cytoplasmic domain of ~215 amino acids, the Type II IL-1R consists of only 29 amino acids (McMahan *et al*., 1991), lacks a TIR domain (Gabay *et al*., 2010), and does not signal to NFκB (Stylianou *et al*., 1992). Type II IL-1R exists as both membrane-bound and soluble forms and functions as a "decoy receptor" in that it sequesters extracellular IL-1 as a way to regulate levels of the circulating cytokine (Colotta *et al*., 1993). Its high affinity for IL-1β and low affinity for IL-1α and the endogenous antagonist IL-1Ra carefully helps balance the plasma concentration of IL-1β, underscoring the necessity to control IL-1β

plasma expression (Boraschi and Tagliabue, 2006). Recombinant soluble Type II IL-1R has been used in at least one phase 1 human clinical trial and was shown to be effective in inhibiting the late-phase response to allergens in volunteers (Sims *et al*., 1994).

Both IL-1 receptors bind IL-1α, IL-1β, and the endogenous antagonist for the receptors, IL-1Ra despite a low homology between Type I and Type II IL-1R in their ligand binding domains of 28% (McMahan *et al*., 1991). The crystal structure of Type I IL-1R bound to IL-1β has been solved. It suggests that van der Waals interactions between side chains of the ligand and the receptor are responsible for a large portion of the binding energy associated with the complex (Vigers *et al*., 1997). Crystal structure analysis of IL-1R complexed with IL-1Ra shows that the first two Ig domains are rigidly connected while the second and third Ig domains have flexible linders, with IL-1Ra binding in between domains 1 and 2, which are very similar (Sims *et al*., 1988). These two domains likely function as a single portion of the molecule (Schreuder *et al*., 1997). Furthermore, while domains 1 and 3 are also similar, domains 2 and 3 are very different (Sims *et al*., 1988). Domain 3 is necessary for agonist binding and, overall, polar interactions between IL-1R and IL-1Ra likely contribute to their strong association (Schreuder *et al*., 1997).

1.5.7.1 The Interleukin 1 Receptor Accessory Protein

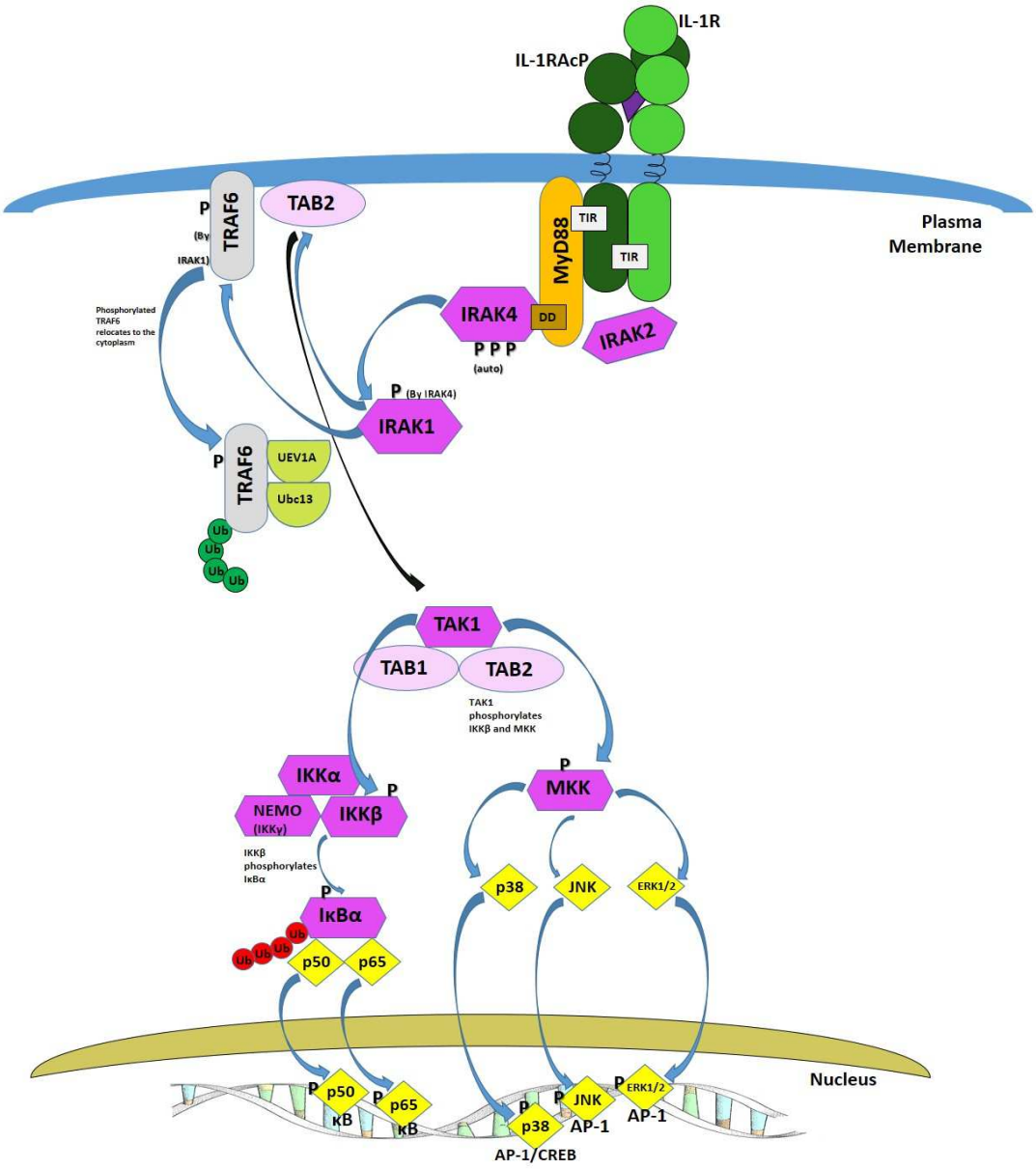
The interleukin 1 receptor accessory protein (IL-1RAcP) serves positive and negative regulatory roles for IL-1R. It is structurally similar to IL-1R with three conserved Ig domains, a cytoplasmic portion that is only 25% homologous to the receptor, and a protein kinase C acceptor site (KSRRL) with unknown function (Greenfeder *et al*., 1995). This protein maintains high levels in the brain regardless of the presence of inflammatory

mediators (Greenfeder *et al*., 1995). It has been suggested that IL-1RAcP envelops the IL-1R:IL-1 complex, not directly binding to IL-1 or IL-1R, but by interacting with a few sites at the cytokine-receptor interface (Boraschi and Tagliabue, 2006). This IL-1RAcP interaction with IL-1R enhances the affinity ofIL-1 for the receptor and may be required for the receptor to elicit a full signal (Wesche *et al*., 1997b), fulfilling its more popular role as a positive regulator for IL-1R. A soluble splice variant of IL-1RAcP (sIL-1RAcP) can inactivate IL-1β bound to IL-1R and also increase the affinity of both IL-1α and IL-1β for Type II IL-1R, further regulating IL-1 activity (Gabay *et al*., 2010). Another isoform of IL-1RAcP has been identified only in the CNS and is referred to as IL-1RAcPb. This isoform may provide an additional, untraditional negative regulatory function in that it does not either directly antagonize or sequester IL-1 but rather inhibits signal adaptor recruitment of MyD88 and IRAK, thereby greatly altering and diminishing the signal (Smith *et al*., 2009). Gene transcription is not completely eradicated though, as low transcription levels of interleukins were detected at questionable significance (Smith *et al*., 2009).

1.5.8 Interleukin-1 Signal Transduction

Figure 12 illustrates the signal transduction pathway for IL-1R. Signaling via IL-1R generally only occurs via Type I IL-1R (Stylianou *et al*., 1992) and is virtually identical to that of the MyD88-dependent pathway in TLR4. The primary difference between signaling via IL-1R vs. TLR4 is that the receptor complex is much smaller and a few proteins are different. It begins with IL-1 binding to IL-1R, which then recruits IL-1RAcP to establish the high affinity complex (Brikos *et al*., 2007). IL-1R and IL-1RAcP may

interact with each other, presumably via their TIR domains (Martin and Weshce, 2002) but may just slightly connect at the cytokine-receptor interface as mentioned in **Section 5.4.6.** MyD88 and IRAK4 are recruited to the receptor complex and stably associate with it while IRAK1 also associates with the complex via MyD88 but this association is transient (Cao *et al*., 1996; Brikos *et al*., 2007). IRAK2 may also briefly associate with the receptor complex as it coprecipitates with IL-1R and MyD88, although not simultaneously (Muzio *et al*., 1997), perhaps serving a brief structural or recruitment purpose. Because MyD88 has a high affinity for unphosphorylated IRAK4 (Wesche *et al*., 1997a), that association is stronger. IRAK4 then autophosphorylates (Yamin and Miller, 1997) before phosphorylating IRAK1 (Brikos *et al*., 2007), after which IRAK4 is proteolytically degraded (Yamin and Miller, 1997). It is possible that IRAK1 is inhibited by the protein Tollip until the receptor is activated, thereby dissociating Tollip from IRAK1 (Martin and Wesche, 2002). Regardless, after IRAK1 is phosphorylated by IRAK4, IRAK1 recruits TRAF6 and TAB2 from the membrane to the cytosol (Qian *et al*., 2001) as separate proteins. TRAF6 then K63 polyubiquitinates via UEV1A-mediated Ubc13 ubiquitination (Deng *et al*., 2000), which acts as a signal that is recognized by TAK1 (part of the TAK1/TAB1/TAB2 complex), which becomes activated via an unknown mechanism, and autophosphorylates itself before phosphorylating IKKβ (Wang *et al*., 2001; Napetschnig and Wu, 2013; Martin and Wesche, 2002). It is a this point that the NFκB and MAP kinase pathways bifurcate and continue to signal as described in **Sections 5.5.2** and **5.5.3.**



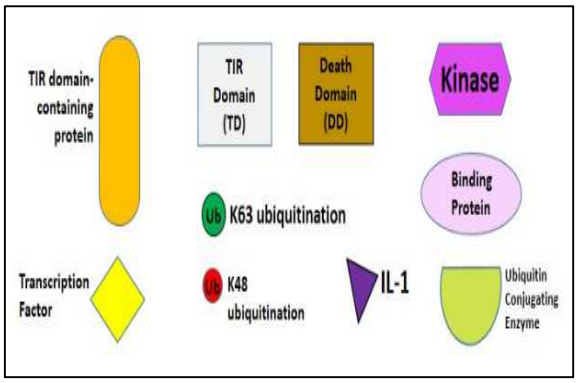


Figure 12. Signaling Pathways of IL-1R. Figure was adapted from Sims *et al*., 1988; Vigers *et al*., 1997; Greenfeder *et al*., 1995; Boraschi and Tagliabue, 2006; Stylianou *et al*., 1992; Brikos *et al*., 2007; Martin and Wesche, 2002; Cao *et al*., 1996; Muzio *et al*., 1997; Wesche *et al*., 1997a; Yamin and Miller, 1997; Qian *et al*., 2001; Deng *et al*., 2000; Wang *et al*., 2001; Napetschnig and Wu, 2013; Akhikari *et al*., 2007; Shibuya *et al*., 1996; Shim *et al*., 2005; Guha and Mackman, 2001; Srivastava and Ramana, 2009; Manavalan *et al*., 2010; Gao *et al*., 2013; Perkins, 2006; and Hoffmann *et al*., 2006. **Legend is shown in box at right.**

1.5.8.1 Other Intracellular Events via IL-1R

As is the case for TLR4, IL-1R activation has been implicated in activating PI3 kinases, leading to activation of Akt (Marin and Wesche, 2002). Additionally, at least one study in primary murine astroglia indicate that IL-1β also activates PKC, leading to the release of phospholipase A_2 and increased prostaglandin E_2 production (Molina-Hlgado *et al*., 2000). More importantly, as was the initially-thought signaling cascade, IL-1R can also cause an increase in adenylate cyclase activity and cAMP (O'Neill *et al*., 1990a). The intriguing aspect of this is that while sensitivity to pertussis toxin is traditionally associated with inhibitory G-proteins, in the case of IL-1R a pertussis toxin-sensitive G-protein is responsible for the observed increases in adenylate cyclase activity and cAMP (O'Neill *et al*., 1990a; Chedid *et al*., 1989). This initial observation regarding the intracellular activity of IL-1R is a potential link between the G-protein coupled receptor MOR to the immune receptors TLR4 and IL-1R.

SECTION 1.6

OPIOID NEUROIMMUNE CROSSTALK

As mentioned at the beginning of this document, the observations that opioid use directly affects the immune system have been made for over one hundred years. Recent advances in science and technology are finally allowing scientists to investigate for opioid-immune effects. Each sub-category in this section briefly highlights some of these possibilities.

1.6.1 Opioid Receptors are Expressed and Regulated in Immune Cells

The finding that opioid receptors are expressed in both peripheral immune cells, such as T and B lymphocytes, monocytes/macrophages (Chuang *et al*., 1995), and in immune cells of the CNS such as microglia and astrocytes (Kettenmann *et al*., 2011; Ruzicka *et al*., 1996) supports the hypothesis for a molecular mechanism in opioid immunomodulation. What is interesting is that this appears to be a direct exogenous drug effect, as some opioid receptors—such as μ_3—present on peripheral immune cells and neurons and readily bind exogenously-administered opioids but have a low affinity for endogenous opioids (Makman, 1994). This indicates that the endogenous opioids do not necessarily have the same detrimental effects on the immune system that drugs like morphine do, which makes sense as those with healthy immune systems also have endogenous opioids circulating. Additionally, while the μ_3 opioid receptor present on inactive thymocytes has a relatively low affinity for morphine, exposure of the cells to IL-1 enhances morphine binding (Bidlack *et al*., 2006)—indicating a direct link between

opioid expression and immune activity via IL-1. This is further exemplified in the CNS, where treatment of SK-N-SH neuroblastoma cells with IL-1β increases MOR expression; however, concomitant treatment with the IL-1R antagonist IL-1Ra completely inhibited this indicating that IL-1R activity is modulating MOR expression (Mohan *et al*., 2010).

1.6.2 Opioids Modulate Immune Activity via NFκB

Pioneering research in opioid immunomodulation found that morphine dose-dependently affected TNFα and IL-6 production in macrophages as low doses increased and high doses decreased synthesis of these cytokines (Roy *et al*., 1998). This occurred via NFκB and because the low dose of morphine was reversed by naloxone it is likely mediated via an opioid receptor; however, the inhibitory effects of the high dose could not be reversed by naloxone indicating that a different receptor must be mediating those effects (Roy *et al*., 1998). Recent work out of our laboratory and the independent laboratories of Linda Watkins and Mark Hutchinson suggests that opioid treatment affects the activity of the immune receptor TLR4. This research has found that opioid treatment in a reporter cell system (HEK-Blue^{TM}-hTLR4) activates the TLR4 signaling cascade, as measured by increases in NFκB activity (Stevens *et al*., 2013; Hutchinson *et al*., 2010). More prominently, the opioids morphine and fentanyl produced a 50-80% inhibition of LPS-activated TLR4 signaling, suggesting that there may be a dual regulatory role for opioid-induced immunomodulation (Stevens *et al*., 2013). Because TLR4 and IL-1R signal to NFκB (Marin and Wesche, 2002) and they are both expressed in microglia (Kettenmann *et al*., 2011), the possibility that opioids regulate expression of these receptors is heightened

given that opioids regulate TLR4 and IL-1R in a neuroblastoma cell line (Stevens *et al*., 2013; Mohan *et al*., 2010).

1.6.3 Morphine and Methadone Differentially Modulate the Immune System

The immunomodulatory effects of morphine and other opioids have been observed for both the peripheral (McCarthy *et al*., 2001) and CNS (Hutchinson *et al*., 2011) immune systems. Morphine is generally accepted as being immunosuppressive (Brown *et al*., 1974; Gavériaux-Ruff *et al*., 1998; Budd, 2006) and is known to increase susceptibility to opportunistic infections (Roy *et al*., 2011). Methadone, although also an opioid, is known to rescue immune function in heroin addicts (Sacerdote *et al*., 2008). This could be because of the normalization of natural killer cell activity resulting from the long half-life of methadone (Kreek, 1990) and/or other unknown mechanisms. The fact that these two drugs possess similar analgesic effects yet have opposing immunologic actions indicates that the two drugs likely crosstalk with the immune system differently.

1.6.4 IL-1R Interacts with G-Proteins

As was introduced in **Section 5.8.1**, the early research into IL-1R signaling identified the involvement of G-proteins as it activates pertussis toxin-sensitive G-proteins, but IL-1R only contains one transmembrane pass instead of seven like a traditional GPCR (Chedid *et al*., 1989). As MOR also signals via pertussis toxin-sensitive G_i proteins, the possibility that they could share some intracellular signaling molecules increases the

opportunistic probability that crosstalk between the two receptors exists. While the sensitivity to pertussis toxin implies that inhibitory G_i proteins are involved, it has also been found that IL-1 increases the activity of cAMP and DAG in certain or stimulated cells (O'Neill *et al*., 1990a). This is contrary to MOR, which decreases cAMP. As these findings oppose traditional G_i-protein signaling, such as that in MOR, the likelihood that these events occur via an indirect mechanism becomes greater. The cells may have to share a pool of G-proteins. Other unknown intracellular effects could also affect the activity of them to generate non-traditional responses. While the generally accepted signaling cascade for IL-1R does not involve G-proteins, the fact that under certain conditions G-proteins can become activated via IL-1R (O'Neill *et al*., 1990b) provides further evience for possible crosstalk between MOR and IL-1R.

1.6.5 MOR, TLR4, and IL-1R are All Recruited to and Signal from Lipid Rafts

Lipid rafts are membrane microdomains comprised of cholesterol and sphingolipids that have been found to be important gathering domains for receptor localization and for signaling scaffolds to form and initiate an intracellular signaling cascade (Simons and Toomre, 2000). MOR preferentially localizes in a lipid raft in the absence of agonist and remains there during G-protein signaling upon morphine stimulation; however, etorphine binding causes MOR to move to non-raft domains and recruits β-arrestins to signal (Zheng *et al*., 2008). This localized membrane association may therefore contribute to the phenomenon of ligand bias (Zheng *et al*., 2008), where different agonist ligands at the same receptor generate different responses.

TLR4 also is recruited to and signals from lipid rafts. TLR4 only dimerizes and initiates the signaling cascade after recruitment into the lipid raft; however, while LPS and saturated fatty acids initiate TLR4 lipid raft recruitment the polyunsaturated fatty acid docosahexaenoic acid (DHA, aka Omega-3) disrupts lipid raft formation and subsequent signaling as the adaptor molecules are also recruited to the raft (Wong *et al*., 2009). This indicates that different membrane lipid components may regulate TLR4 signaling. Additionally, both TLR and IL-1R are recruited into lipid raft domains of astrocytes after LPS or IL-1β treatment (Blanco *et al*., 2008), providing evidence that even in the CNS these microdomains are important in initiating the innate immune response. Most importantly, all three of the receptors—MOR, TLR4, and IL-1R—are recruited to the lipid raft upon agonist binding (the exception being MOR's association with non-lipid membrane portions after etorphine treatment (Zheng *et al*., 2008)). This means that these three receptors may be in physical proximity, creating an opportunity for receptor-receptor interactions between these proteins. Additional research is needed to determine whether or not they are all present in the same lipid raft.

1.6.6 MOR, TLR4, and IL-1R All Homo-and/or Heterodimerize

The concept of receptor heteromerization has taken years to come to acceptance in the scientific community. The idea that receptors do not function solely as monomers but can form dimers with another of the same protein to form a homomer or dimers with a different protein to form a heteromer is becoming widely accepted (Ferré *et al*., 2009). MOR, TLR4, and IL-1R all form homo- and/or heteromers. For example, MOR can form a MOR-MOR homomer or a MOR-DOR heteromer (van Rijn *et al*., 2010). The responses

of these dimers is not necessarily the same as it is for the monomer as in the case of the MOR-DOR heteromer research indicates that MOR is recycled back to the membrane while DOR is degraded (van Rijn *et al*., 2010). Of particular interest is the heteromer consisting of MOR and the chemokine receptor CCR5. Treatment of Chinese hamster ovary (CHO) cells with either the MOR agonist DAMGO or the CCR5 agonist RANTES (CCL5) induced cross-desensitization, indicating that the pharmacokinetics of one receptor can influence the other (Chen *et al*., 2004). It is important to note that in the case of the MOR-CCR5 heteromer both of the receptors are GPCRs. However, both MOR and CCR5 interact with G_i proteins and since they are likely sharing an intracellular store (Chen *et al*., 2004), a receptor-receptor interaction would produce more efficient use of the proteins for signaling. Furthermore, interactions between different classes of molecules has also been identified. It is well documented that opioids affect lymphocyte chemotaxis and other immune functions via indirect crosstalk mechanisms (Zheng and Oppenheim, 2005; McCarthy *et al*., 2001). The MOR-CCR5 heteromer is a prime example that receptor oligomerization can extend beyond heteromers that have the same general function and more importantly, that MOR can dimerize with an immune receptor.

TLR4, as discussed in **Section 5.4.4**, requires receptor dimerization for induction of the signaling cascade. IL-1R also forms a heterodimer receptor complex as it interacts both extracellularly and intracellularly with IL-1RAcP (Martin and Wesche, 2002). Type I IL-1R also forms aggregate clusters upon IL-1 stimulation in CHO-K1 cells, indicating that direct receptor-receptor interactions likely occur (Guo *et al*., 1995). This is likely a required, but not independently responsible step, for signal transduction to occur via IL-1R (Guo *et al*., 1995). While direct and simultaneous interactions between MOR, TLR4, and

IL-1R may be unlikely, the fact that all three of these receptors oligomerize at least to some extent indicates that receptor-receptor interactions among these proteins remains a distinct possibility.

SECTION 1.7

SUMMARY

Research in opioid and immune receptor functions continues to indicate that MOR, TLR4, and even IL-1R are not functionally mutually exclusive. Opioid immunomodulation is likely occurring, at minimum, via TLR4 and the vast similarities between TLR4 and IL-1R indicate that IL-1R may also be involved. Expression and activity of these proteins may help explain some of these findings. While much of the literature has focused on the peripheral immune system, it is becoming increasingly clear that similar immunologic activities via microglia and astrocytes are also in the CNS. As **Section 6** discusses the commonalities between MOR, TLR4, and IL-1R, the possibility for these receptors to interact at the plasma membrane in an as of yet unidentified an unconventional manner warrants investigation.

There is clear evidence described throughout this document indicating that MOR, TLR4, and IL-1R all have a common link: opioids. The quest remains as to what extent, how, where, and why opioids influence these immune receptors and to identify potential receptor interactions between all three. The drugs morphine and methadone are commonly used in today's culture—both in medical practice and illicit use on the streets. As the July 2012 DAWN report states, there were 72.6 heroin and 137.4 narcotic pain reliever

emergency department visits per population of 100,000 in 2012, and this is a growing trend. Routine morphine administration in emergency trauma and surgery, coupled with continued heroin use increases the likelihood for narcotics misuse. Increases in methadone popularity for pain management and continued success with methadone in methadone maintenance therapy programs also contributes to these emergency visits. Factor in the documented deleterious immunological effects of opioids and it quickly becomes an intellectual responsibility to experimentally elucidate potential answers to the mysteries surrounding neuroimmunomodulation and opioid drug use.

CHAPTER II

METHODS

2.1 Cell Culture

2.1.1 ***Human Embryonic Kidney 293 (HEK-Blue™-hTLR4)*** cells are stably co-transfected to overexpress hTLR4 and the accessory proteins CD14 and MD-2. HEK-Blue™-hTLR4 cells are also stably transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene that renders a proprietary detection media blue upon NFκB p65 binding in the κB promoter region of the DNA, indicating that TLR4 has been activated. This cell line has therefore been designed to study TLR4 activity by measuring the blue intensity of the detection media whereby greater absorbance values linearly correlate to the SEAP reporter gene activity.

Once out of cryogenic storage, the cells are grown in growth media for two passages before being passaged at least once more into a selection media before being used in assays. Growth media consists of DMEM, 4.5 g/L glucose, 10% (v/v) fetal bovine serum (FBS), 50 U/mL penicillin, 50 µg/mL streptomycin, 100 µg/mL Normocin™, and 2 mM L-glutamine. Selection media is comprised of the growth media supplemented with 1X HEK-Blue™ Selection antibiotics, a proprietary solution. Cells are fed or split every 2-3 days, until they've reached 70-80% confluency, and used in passages 20 and below. The HEK-

Blue™-hTLR4 cells, proprietary media, and antibiotics were all obtained from InvivoGen. LPS K12 was the chosen form of LPS for use in this cell line because the cells lack TLR2 expression, which LPS K12 binds to as well as to TLR4. This allowed for certainty that only TLR4 was being activated because LPS can stimulate TLR2 too. LPS K12 is also more economical than TLR4 specific LPS, so it was used whenever possible.

2.1.2 ***CHME-5*** cells are a human microglial cell line. CHME-5 microglial cells were a gift from Marc Tardieu, Laboratoire de Neurovirologie et Neuroimmunologie, Université Paris-Sud, France. They were maintained in Growth Media, which consists of DMEM, 11.5% FBS, and 1.1% of each L-glutamine, penicillin/streptomycin, and Amphotericin-B. They were fed every 2-3 days with Growth Media and passaged once per week for either maintenance or to seed an experiment. Treatments were given once the cells had reached 60-80% confluency at which time the Growth Media was replaced with serum free media. Passages 20 and under were used for experiments. LPS O111:B4 was used in this cell line because it is specific for TLR4.

2.1.3 ***CHO-hMOR-pIRES*** are Chinese hamster ovary cells that were stably transfected to express the hMOR receptor. The pIRESneo polycistronic expression vector (Clontech) method was used to insert hMOR into the cell line which was derived from a pcDNA3.1-hMOR construct (University of Missouri-Rolla cDNA Resource Center). They were transfected in Dr. Stevens laboratory by Chris Brasel in 2007. The vial taken out of cryogenic freezing was labeled C1.3 p14 6.3.7 indicating a passage of 14 and freezing date of June 3, 2007. These cells were maintained in complete Growth Media which was made of F-12K growth media, 10% FBS, 100 U/mL penicillin/100 mg/mL streptomycin, and

600-800 µg/mL geneticin, also known as G418 sulfate (Mediatech, Inc). Passages under 20 were used for a positive hMOR control.

2.1.4 *SK-N-MC* cells are also a human neuorblastoma cell line that was given from Santa Cruz Biotechnology to be used a positive control for measuring hMOR. They were shipped in a 1 mL vial and were already prepared for use therefore plating and Growth Media were not necessary.

2.2 Drugs

All drugs were obtained from commercial chemical suppliers. Morphine sulfate salt pentahydrate, methadone hydrochloride, oxycodone hydrochloride, buprenorphrine hydrochloride, and (+)-naloxone hydrochloride were all obtained from Sigma Aldrich, USA. LPS-EK (aka LPS K12) and ultra-pure biotin-LPS from E. coli O111:B4 were provided from Invivogen. (-)-delta 9-THC was obtained from Cerilliant, USA in 1 mg/mL vials and stored in 1 mL of methanol.

2.3 SEAP Reporter Assay

The SEAP reporter assay uses the HEK-Blue™-hTLR4 cells, as these cells were designed for this assay. SEAP is a GPI-anchored protein that is commonly used as a reporter. In this system SEAP is fused to five NFκB and AP-1 promoter binding sites which are activated downstream of TLR4. Once SEAP is activated, alkaline phosphatase is secreted into a proprietary detection media, turning it blue. Darker shades of blue

indicate increased TLR4 activity and the absorbance is measured with a spectrophotometer. Cells were plated in detection media at a density of 50,000 cells/well in 96-well plates and immediately treated with drugs and/or LPS and incubated for 18 hours at 37°C. The plates were then read at 592 nm.

2.4 MTT Toxicity Assay

3-[4,5 dimethylthiazol-2-y]-2,5 diphenyltetrazolium bromide (MTT) was used to determine overall toxicity in vitro by measuring formation of an insoluble blue formazan product. Viable cells are able to convert MTT to formazan while non-viable cells are not. Adherent cells and non-adherent cells had to be treated differently in this assay. The intensity of the blue solution created by the viable cells was directly proportional to cell viability (Riss 2013). Toxicity was considered when a treatment created significant difference from control values as determined via a one-way ANOVA.

2.4.1 MTT in HEK-Blue™-hTLR4 cells

After the cells were treated according to experimental protocol (**Section 2.3**), a 0.5 mg/mL sterile MTT in phosphate buffered saline (PBS) solution was added to each well at $1/9^{th}$ of the total volume in the well. The cells were then incubated for 45 minutes at 37°C at which time the viable cells took up the MTT and converted it to formazan. The contents of each well were then transferred to microcentrifuge tubes because the HEK-Blue™-hTLR4 cells do not adhere to the bottom of the plates. They were then centrifuged in an

Eppendorf 5417R centrifuge for 10 minutes at 5,974 rcf, 4°C. The supernatant was aspirated and the pellet resuspended in 1 mL of dimethyl sulfoxide (DMSO), which was added in 500 µL increments because it was difficult to get the pellet to solubilize. The DMSO solution was transferred to a new 24-well plate and spectrophotometrically read in a plate reader at an absorbance of 492 nm.

2.4.2 MTT in CHME-5 Microglia

After the cells were treated according to experimental protocol a 0.5 mg/mL sterile MTT in PBS solution was added to each well at $1/9^{th}$ of the total volume in the well. The cells were then incubated for 45 minutes at 37°C. The media was then aspirated and the cells were solubilized with DMSO on a plate rocker for 15 minutes to release and solubilize the formazan product. Because the CHME-5 microglia adhere to the plates, they did not need to be transferred to microcentrifuge tubes and centrifuged. The lysing of the cells turned the DMSO solution blue from the formazan in the viable cells. The optical absorbance was then read at 492 nm.

2.5 Protein Extraction

Whole cell lysates were collected from 6-well plates (unless otherwise noted) after drug treatment and corresponding incubation. The cells were washed one time with 1 mL PBS in a sterile hood then incubated on ice for 30-45 minutes in 200 µL RIPA buffer supplemented with 10 µL/mL Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO)

which inhibits serine proteases, aminopeptidases, cysteine proteases, and acid proteases. The lysates were then collected in microcentrifuge tubes and vortexed before being centrifuged in an Eppendorf 5417R centrifuge for 10 minutes at 10,621 rcf, 4°C. The supernatants were collected and stored at -80°C.

2.6 BCA Protein Quantitation

Total cell lysates were retrieved from the -80°C freezer and thawed on ice. A bovine serum albumin (BSA) standard (Sigma Aldrich, St. Louis, MO)was prepared at concentrations of 2 - 16 µg/mL. 10 µL of each standard was pipetted in triplicate into a 96-well plate. Samples were diluted 1:6 then 10 µL was pipetted in triplicate into the plate. The volume of proprietary reagents A and B (ThermoFisher, Waltham, MA) was calculated according to the following equations:

Reagent A: (# wells)*(0.200 mL), then round up to the nearest whole number and add 2 = volume of reagent A

Reagent B: (volume of reagent A)/50 = volume of reagent B

The reagents were mixed together to obtain the working reagent.

200 µL of the working reagent was pipetted into each well via a multichannel pipettor then the plate was incubated for 1 hour at 37°C/5% CO_2. Absorbance was immediately read on a spectrophotometer at 570 nm and these values were used to calculate mg/mL of protein.

2.7 Western Blot

12% polyacrylamide gels were made 1-2 days prior to running the western blot. Whole cell lysate extracts were thawed on ice and 50 µg or 100 µg of protein per sample was pipetted into a reaction tube, as was determined by a BCA protein analysis (**section 2.6**). 10 µL of loading dye containing sodium dodecyl sulfate (SDS) was added to the protein then the reactions were placed in a thermocycler for 15 minutes at 99°C. The electrophoresis chambers were prepared and the gels inserted into conductive holders. The chambers were filled with 1× Running Buffer before the combs creating the wells were removed and the samples loaded. The gels were run at 100V for the first 10 minutes to get the protein through the stacking gel and then the voltage was increased to 150V for about an hour and a half. Typically, the loading dye would run out of the gel by then but it took that much time to get cleaner and crisper bands.

Sponges and filter paper were soaked in chilled 1× Transfer Buffer containing methanol for about 5-10 minutes prior to transferring the gels to polyvinyldifluoride (PVDF) membranes. To activate the membranes, they were soaked for 20-30 seconds in methanol prior to assembling the transfer apparatus. The gel and membrane were placed in the transfer apparatus such that the current flowed from negative to positive, allowing for the proteins to transfer from inside the gel and onto the PVDF membrane. The chamber was completely filled with cold 1× Transfer Buffer with an ice pack also in the chamber. Ice was packed around the chamber and the transfer was done at 100V for one hour. The membranes were then blocked in 5% milk in tris-buffered saline with Tween 20 (TBST) for two hours at room temperature on a rocker before primary antibodies were added. This was to minimize non-specific antibody binding. TLR4 mouse monoclonal IgG_1 (Santa

Cruz Biotechnology SC-293072), MOR-1 goat polyclonal IgG (Santa Cruz Biotechnology SC-7488), and IL-1R rabbit polyclonal IgG (Santa Cruz Biotechnology SC-688) were made at a 1:200 dilution while β-tubulin rabbit polyclonal IgG (Santa Cruz Biotechnology #SC9104) primary antibody was used at 1:500. All antibodies were made in 5% milk in TBST. Once the primary antibody was added, the membranes were incubated at 4°C overnight on a plate rocker. The next morning they were placed on a rocker at room temperature for 30 minutes to facilitate any additional binding. Six washes in TBST for ten minutes each were done at room temperature on the plate rocker before adding the secondary antibodies. Rabbit anti-goat IgG-AP (Santa Cruz Biotechnology SC-2771) was used at 1:2000 dilution for MOR-1, anti-rabbit IgG AP-linked antibody (Cell Signaling #7054S) was used at 1:1000 for IL-1R and β-tubulin while anti-mouse IgG AP-linked antibody (Cell Signaling #7056S) was used at 1:1000 for TLR4. Secondary antibodies were also made in 5% milk in TBST. The membranes incubated with the secondary antibodies for two hours at room temperature on a plate rocker before being washed six times for ten minutes each in TBST. Antibodies were saved and re-used up to 4 times before being discarded. Membranes were stripped in Resore™ Western Blot Stripping Buffer (ThermoScientific, Rockford, IL) by decanting the secondary antibody then incubating the membrane for 15 minutes on a plate rocker at room temperature. The membranes were stripped and re-probed up to 3 times. β-tubulin was always used to normalize the bands. The membranes were visualized in ECF substrate using a Blue2 (488) laser on a Typhoon Scanner then quantified using Image J software.

2.8 RNA Extraction

RNA was isolated from 6-well plates (unless otherwise noted) in the results. All working areas and equipment were thoroughly wiped down with RNase Zap (Life Technologies, Carlsbad, CA). Cells were rinsed one time with 1 mL of PBS in the cell culture hood. In the fume hood, cells were incubated for 5 minutes in 0.5 mL of Trizol (Life Technologies, Carlsbad, CA). Lysates were collected in clear RNase-free microcentrifuge tubes. 100 µL of RNA only chloroform was added to each tube followed by a vortex and 10 minute incubation at room temp. Tubes were centrifuged for 10 minutes at 10,621 rcf, 4°C then the top, clear layer was carefully removed and transferred to new RNase-free microcentrifuge tubes. 250 µL of RNase/DNase/protease free isopropanol was added to each tube after which they were inverted 10-15 times to mix with a 10 minute incubation at room temperature immediately following. Next, they were centrifuged in an Eppendorf 5417R centrifuge for 15 minutes at 20,817 rcf, 4°C. The supernatant was carefully pipetted out and discarded, leaving the RNA pellet. The pellets were carefully washed once with 400 µL RNase Free 70% ethanol and then incubated for 10 minutes on ice with the lids open to dry the pellet and remove excess ethanol. 40 µL of RNase/DNase free water was pipetted into each tube then they were placed on a heat block for 10 minutes at 65°C with the lids closed to dissolve the pellet. Samples were quickly vortexed and quick spun to collect all RNA at the bottom of the tubes. They were placed on ice and the RNA was quantified on the nanodrop. 2 µg of RNA was calculated for each sample and pipetted into 200 µL RNase free microcentrifuge tubes with 1 µL of DNase buffer, 2 µL of DNase at 1 U/µL, and the difference up to 10 µL with RNase free water. They were vortexed and quickly centrifuged then incubated for 15 minutes at room temperature. 1 µL

of 25 nM EDTA was then added to the samples to inactivate the DNase and the samples were vortexed and quickly centrifuged again before being placed in the thermocycler for 10 minutes at 65°C. Samples were stored at -20°C until cDNA was made.

2.9 cDNA

The 2 µg RNA after the samples had been DNase treated (**section 2.8**) were thawed on ice. 15 µL of RNase/DNase free water was added to each tube. This increased the total volume to 26 µL. 6 µL of reaction mix 1 was then added to the tubes.

Reaction Mix 1: 10 mM dNTP mix (Promega, Madison, WI) at 2 µL/tube
+ 10x RT Random Hexamers (GeneLink™, Hawthorne, NY) at 4 µL/tube = 6 µL of mix 1 per cDNA reaction

Each tube was then vortexed and quickly centrifuged to mix. Reactions were incubated in the thermocycler at 65°C for 5 minutes while reaction mix 2 was made.

Reaction Mix 2: 10x RT Buffer (New England BioLabs, Ipswich MA) at 4 µL/tube
+ rRNasin RNase Inhibitor (Promega, Madison, WI) 10,000 units at 40 units/ µL
+ Reverse Transcriptase (Applied Biosystems, Foster City, CA) at 20 units/tube
+ RNase/DNase free water at 2.75 µL/tube
+RNase/DNase free water to increase the total volume per reaction to 8 µL

8 µL of reaction mix 2 were pipetted into each reaction tube, then vortexed and quickly centrifuged again before placing the reactions in the thermocycler at 25°C for 15 minutes

followed by 42°C for one hour, followed by 95°C for 5 minutes. Samples were stored at -20°C.

2.10 RT Polymerase Chain Reaction (PCR)

PCR amplification of genes of interest was accomplished individually. Either 50 ng or 100 ng of cDNA was amplified depending on the run. The PCR reaction mixtures included 10 µL of 2x GoTaq Polymerase, 1 µL of sense primer, 1 µL of anti-sense primer, and 3 µL of nuclease free water. All primers were obtained from Integrated DNA Technologies (IDT Coraville, IA) and primers used include TLR4, hMOR, IL-1R, GAPDH, and β-actin. The reaction mix combined with the appropriate volume of cDNA template went in the thermocycler initially for 2 minutes at 95°C to denature the cDNA. It then went through up to 35 cycles of 94°C for 15 seconds to melt it, 55°C to anneal, and 72°C to extend before sitting for 5 minutes at the 72°C for a final extension time. These samples were stored at -20°C if they were not immediately analyzed by gel electrophoresis.

Electrophoresis of PCR products was performed in a 2% agarose gel. 500 mL solutions of 2% agarose were made by adding 10 g of agarose (MidSci, St. Louis, MO) to 50 mL 10x Tris Borate EDTA (TBE) buffer and 450 mL of water. This was heated in the microwave in 1 minute intervals until all of the agarose was completely dissolved and had boiled for about a minute. Once liquefied, the agarose solution was left to cool for about 15 minutes before 50 µL of 10 mg/mL ethidium bromide was added. The gel was then poured into a gel cast with the combs inserted and allowed to polymerize for at least 30 minutes. The chamber was then filled with 1x TBE until it covered the gel. A 100 kbp

ladder was loaded followed by the amplified samples. Either GAPDH or β-actin was used as a loading control. The gels were run at 150-170V for 20-30 minutes, or until a 2.5 cm-3 cm band separation was detected. The gels were imaged on a Kodak Molecular Imaging camera and software.

2.11 Statistics

CHME-5 data was analyzed using Statistica software (StatSoft, Inc., 2014). Either a one-way or two way ANOVA was used to calculate statistical significance as appropriate. Significance was defined at $p<0.05$. A Fisher's least significant difference (LSD) post-hoc test was used to determine significant differences in the CHME-5 data. All error bars are indicative of the standard error of the mean (SEM). HEK-Blue™-hTLR4 data was analyzed using GraphPad Prism and also used either a one-way ANOVA or two-way ANOVA with significant differences being defined at $p<0.05$. Post-hoc tests are detailed in the results section (**Chapter III**).

CHAPTER III

RESULTS

3.1 THC-Induced TLR4 Activity in HEK-Blue™-hTLR4 Cells

The first set of experiments performed in the laboratory involved the effect of (-)-delta9-tetrahydrocannibinol (THC) on TLR4 activity. Cells were plated at a density of 50,000 cells/well in 96 well plates then treated with THC at concentrations ranging from 0.00003-3 mM and LPS at concentrations from 0.001-1000 ng/mL immediately prior to a 0-24 hour incubation at 37°C/5%CO_2. This was a quick, preliminary time-course study and was performed in duplicate; therefore, no statistics were performed. **Figure 13** illustrates the gradual increase in TLR4 activity induced by LPS that begins at about 12 hours and is maximized at around 24 hours. Interestingly, THC in the presence of LPS appears to have an inhibitory effect on TLR4 activity, with the greatest effect at 0.3 mM. As **Figure 14** indicates, the 3 mM treatment of THC was toxic to the cells either with or without any concentration of LPS but the methanol used to keep THC in solution was not. The asterisk indicating toxicity is relative to control values.

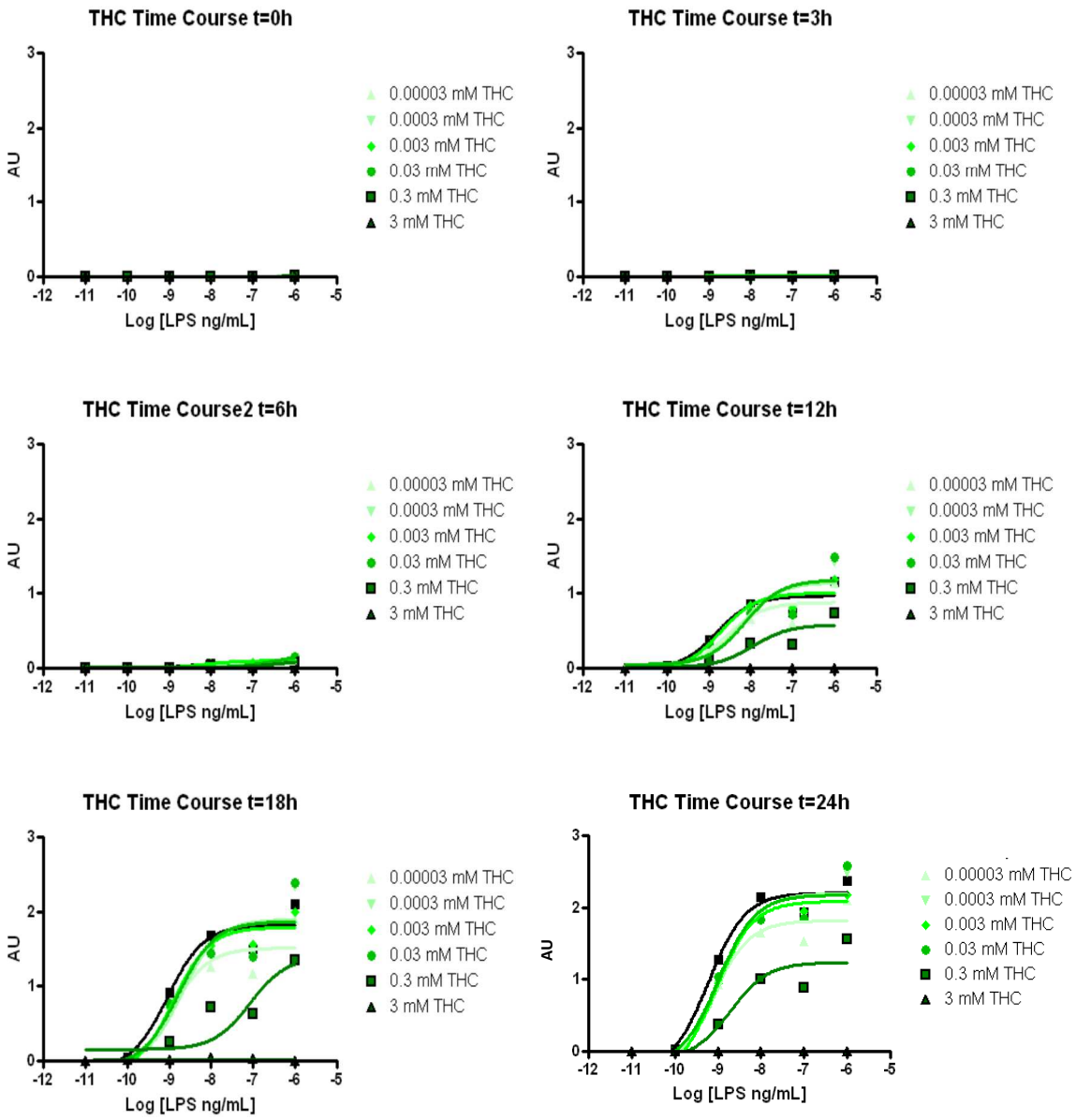


Figure 13. Time course of LPS-induced TLR4 activity inhibited by THC in HEK-Blue™-hTLR4 cells. Cells were treated with 100 ng/mL LPS ± THC for 0-24 hours. Activity was determined via the SEAP reporter assay. A dose dependent decrease in activity is most likely to occur at 24 hours. Absorbance units (AU) are on the y-axis, N=2.

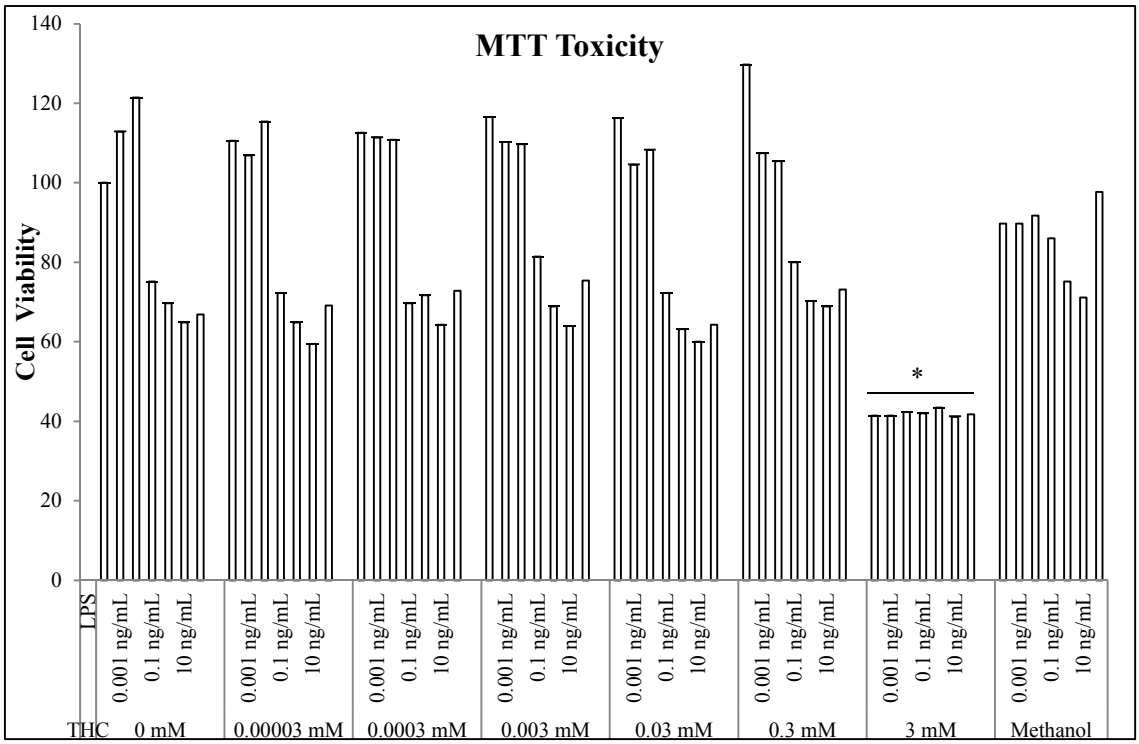


Figure 14. MTT Toxicity of THC ± LPS in HEK-Blue™hTLR4 Cells. This assay was performed once to gain an idea of THC toxicity in the HEK-Blue™hTLR4 cells; therefore, toxicity was visually interpreted as opposed to being determined via one-way ANOVA. *Presumed toxic doses of THC.

3.2 Opioid-Induced TLR4 Activity in LPS-Induced HEK-Blue™-hTLR4 Cells

These experiments were designed to determine the effect of morphine, methadone, oxycodone, and buprenorphine on TLR4 activity using the HEK-Blue™-hTLR4 SEAP reporter assay. LPS was also included both as a control and in simultaneous treatment with the given opioid. The drugs chosen in this section include morphine and oxycodone, because they are drugs of choice for abuse, are known to be addictive and are immunosuppressive (Brown *et al*., 1974; Gavériaux-Ruff *et al*., 1998; Budd, 2006). Methadone and buprenorphine were also included because of their success in treating opioid addiction and the improved immune function associated with their use in treating addiction (Sacerdote *et al*., 2008). Experiments were completed a minimum of three times in triplicate. **Figure 15b**, **15c**, and **15d** illustrates that methadone ($F_{5,342}$, $p<0.001$), buprenorphine ($F_{5,342}$, $p<0.001$), and oxycodone ($F_{5,331}$, $p<0.001$) all significantly inhibit LPS-induced activation of TLR4. Morphine had no significant effect on TLR4 activity despite an apparent inhibitory trend with increasing concentrations of the drug (**Figure 15a**). This is consistent with previous data from this laboratory showing maximal TLR4 activation with LPS only and a decrease in TLR4 activation with simultaneous treatment with morphine (Stevens *et al.,* 2013). The Stevens *et al.* study found statistical significance in this inhibition whereas the current one did not. The inhibition is further observed by decreases in E_{max}, as shown in **Tables 2-5**.

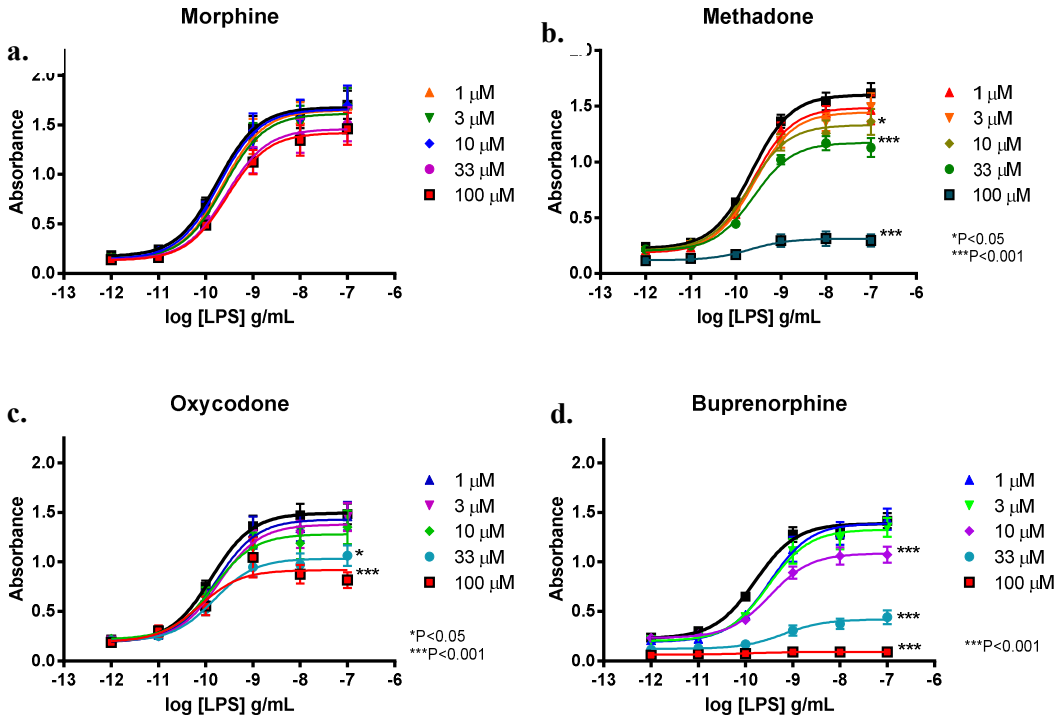


Figure 15. Opioid inhibition of LPS-induced TLR4 activation in HEK-Blue™hTLR4 Cells. Cells were treated for 18 hours with LPS ± drug. **a. Methadone** significantly inhibits LPS-induced TLR4 activation at 10 μM ($p<0.05$), 33 and 100 μM ($p<0.001$). **b. Buprenorphine** significantly inhibits LPS-induced TLR4 activation at 10, 33, and 100 μM ($p<0.001$). **c. Morphine** has no significant effect on LPS activation of TLR4. **d. Oxycodone** significantly inhibits LPS-induced activation of TLR4 at 33 μM ($p<0.05$), and 100 μM ($p<0.001$). (N=3 in triplicate, analyzed via 2-way ANOVA to determine any drug-LPS interactions followed by a Bonferroni post hoc test to identify those interactions. Data points represent mean values +/- SEM.

This was colorimetrically measured by the activation of NFκB, upon which releases the blue alkaline phosphatase into the culture media in the presence of a proprietary detection media. Note that while all four drugs do have inhibitory trends, as illustrated by the decreases in E_{max}, they inhibit LPS-induced TLR4 activation to different degrees. Buprenorphine has the greatest effect at 33 μM, followed by oxycodone and methadone at the same dose. **Tables 2-5** highlight the EC_{50}, E_{max}, and 95% E_{max} confidence intervals for the four drugs in the presence of LPS. The EC_{50} for morphine and methadone are very close to each other at 10 μM and 33 μM, which were the doses highlighted in subsequent experiments.

a. Methadone EC_{50} and E_{max} values for TLR4 Activation in HEK-Blue™hTLR4 Cells.

	EC_{50}	EC_{50} (nM)	E_{max}	95% Confidence Interval
LPS Only	2.26E-10	22.6	1.603	1.525-1.681
1 µM	2.39E-10	23.9	1.486	1.394-1.579
3 µM	2.64E-10	26.4	1.447	1.352-1.542
10 µM	1.98E-10	19.8	1.332	1.229-1.434*
33 µM	2.49E-10	24.9	1.174	1.105-1.243***
100 µM	2.06E-10	20.6	0.312	0.252-0.371***

b. Buprenorphine EC_{50} and E_{max} values for TLR4 Activation in HEK-Blue™hTLR4 Cells.

	EC_{50}	EC_{50} (nM)	E_{max}	95% Confidence Interval
LPS Only	1.61E-10	16.1	1.387	1.314-1.460
1 µM	3.15E-10	31.5	1.389	1.268-1.509
3 µM	3.06E-10	30.6	1.327	1.212-1.443
10 µM	3.27E-10	32.7	1.086	1.004-1.168***
33 µM	6.34E-10	63.4	0.042	0.353-0.487***
100 µM	1.25E-10	12.5	0.091	0.078-0.105***

c. Morphine EC_{50} and E_{max} values for TLR4 Activation.

	EC_{50}	EC_{50} (nM)	E_{max}	E_{max} 95% Confidence Interval
LPS Only	1.77E-10	17.7	1.675	1.542-1.809
1 µM	2.29E-10	22.9	1.648	1.471-1.824
3 µM	2.41E-10	24.1	1.612	1.420-1.804
10 µM	1.91E-10	19.1	1.656	1.470-1.842
33 µM	2.72E-10	27.2	1.458	1.296-1.621
100 µM	2.82E-10	28.2	1.419	1.269-1.570

d. Oxycodone EC_{50} and E_{max} values for TLR4 Activation.

	EC_{50}	EC_{50} (nM)	E_{max}	E_{max} 95% Confidence Interval
LPS Only	1.34E-10	13.4	1.494	1.386-1.601
1 µM	1.69E-10	16.9	1.429	1.280-1.577
3 µM	1.88E-10	18.8	1.378	1.236-1.519
10 µM	1.47E-10	14.7	1.281	1.122-1.439
33 µM	1.49E-10	14.9	1.034	0.929-1.139*
100 µM	7.35E-11	7.40	0.917	0.779-1.055***

Table 2. EC_{50} and E_{max} values for TLR4 Activation in HEK-Blue™ hTLR4 Cells. Tabular representation of **Figure 15**. Cells were treated for 18 hours with LPS ± drug. **a. Methadone** significantly inhibits LPS-induced TLR4 activation at 10 µM ($p<0.05$), 33 and 100 µM ($p<0.001$). **b. Buprenorphine** significantly inhibits LPS-induced TLR4 activation at 10, 33, and 100 µM ($p<0.001$). **c. Morphine** has no significant effect on LPS activation of TLR4. **d. Oxycodone** significantly inhibits LPS-induced activation of TLR4 at 33 µM ($p<0.05$), and 100 µM ($p<0.001$). N=3 in triplicate, analyzed via 2-way ANOVA with Bonferroni post hoc test. Data points represent mean values +/- SEM.

3.3 MTT Toxicity in HEK-Blue™-hTLR4 Cells

In MTT toxicity studies, a one-way ANOVA found that methadone ($F_{5,30}$=4.551, p<0.01) and buprenorphine ($F_{5,30}$=9.098, p<0.01) were both toxic at the highest dose of 100 μM (**Figure 16a** and **16b**). This explains the 100 μM "inhibitory effect" of LPS-induced TLR4 activation as a toxic phenomenon for these two drugs. Morphine and oxycodone had no observed toxicity (**Figure 16c** and **16d**). According to a one-way ANOVA LPS however, had significantly different effects at the three highest doses when compared to control ($F_{6,35}$=10.13, p<0.01) (**Figure 16e**). This indicates that some toxicity may be occurring at the doses of 1, 10, and 100 ng/mL; however, it was presumed to be even across all treatment groups therefore the doses were continued to be used in this study. Together, **Figures 15 and 16** indicate that at certain doses, methadone, buprenorphine, and oxycodone are all inhibitors of LPS-induced TLR4 activation.

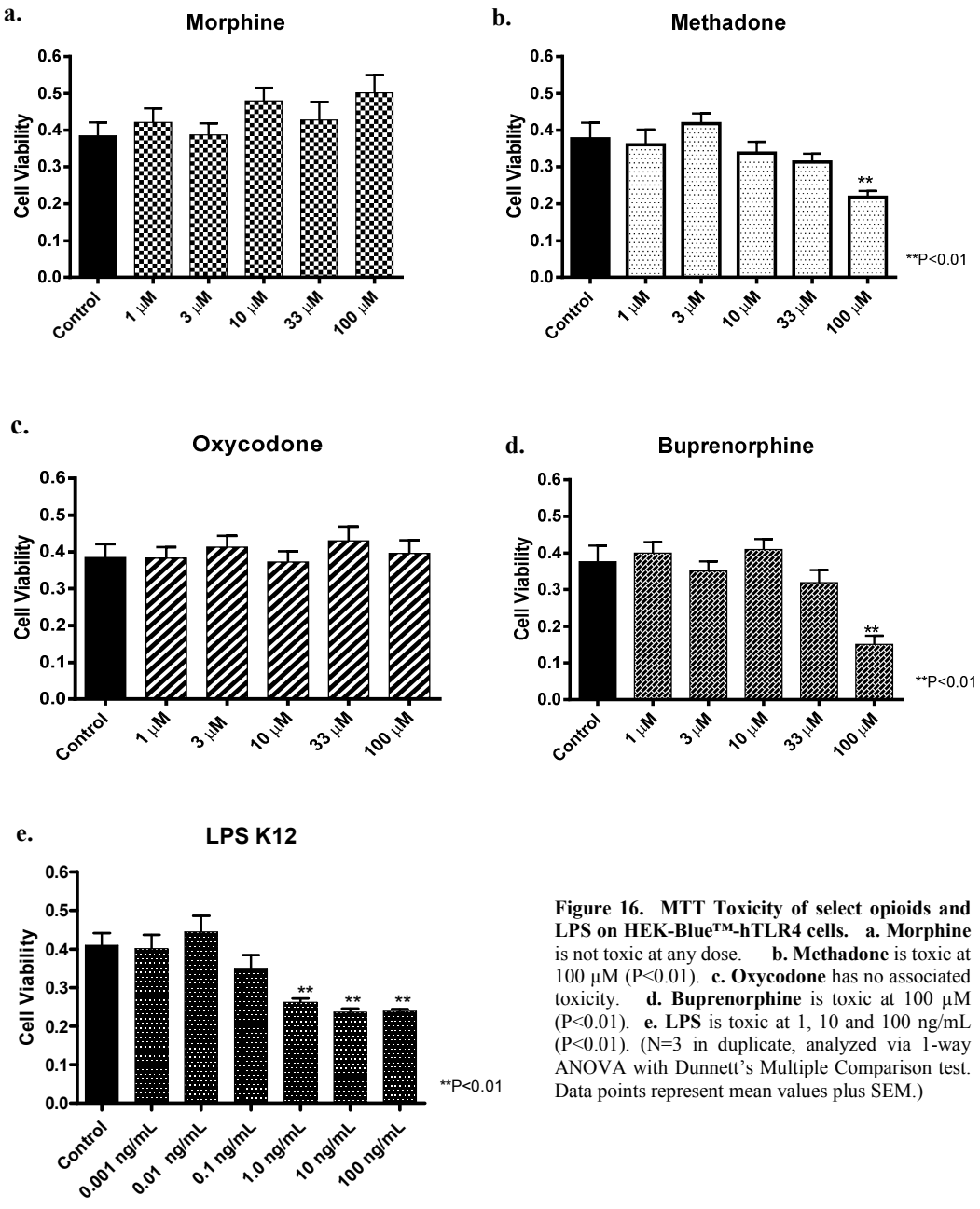


Figure 16. MTT Toxicity of select opioids and LPS on HEK-Blue™-hTLR4 cells. a. Morphine is not toxic at any dose. **b. Methadone** is toxic at 100 μM (P<0.01). **c. Oxycodone** has no associated toxicity. **d. Buprenorphine** is toxic at 100 μM (P<0.01). **e. LPS** is toxic at 1, 10 and 100 ng/mL (P<0.01). (N=3 in duplicate, analyzed via 1-way ANOVA with Dunnett's Multiple Comparison test. Data points represent mean values plus SEM.)

3.4 Opioid-Induced Inhibition of TLR4 Activity in HEK-Blue™-hTLR4 Cells

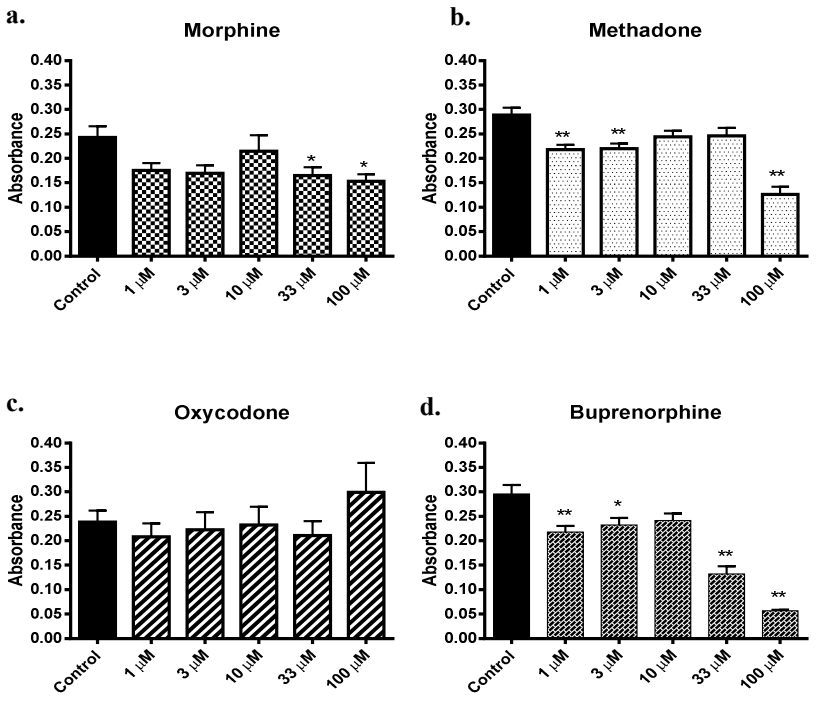


Figure 17. Opioid effect on non- LPS-stimulated TLR4 activity.

a. Morphine significantly inhibits TLR4 activation at 33 and 100 μM with no LPS stimulation ($P<0.05$). **b. Methadone** significantly inhibits TLR4 activation at 1, 3, and 100 μM ($P<0.01$). **c. Oxycodone** has no significant effect on TLR4 activity. **d. Buprenorphine** significantly inhibits TLR4 activation at 3 μM ($P<0.05$), 1, 33 and 100 μM ($P<0.01$). (N=3 in triplicate, analyzed via 1-way ANOVA with Dunnett's Multiple Comparison test. Data points represent mean values plus SEM.)

In **Figure 17**, the effect of these opioids on non-LPS stimulated TLR4 is illustrated. Morphine at 33 and 100 μM ($F_{5,57}$=2.978, $p<0.05$); methadone at 1 and 3 μM ($F_{5,57}$=13.69, $p<0.01$); buprenorphine at 1, 3 and 33 μM ($F_{5,57}$=29.39, $p<0.05$); all independently inhibit TLR4 activity according to a one-way ANOVA indicating a drug effect on TLR4 activity. The significant difference for 100 μM methadone and buprenorphine is due to toxicity and is therefore not considered to affect TLR4 activity (**Figure 17a, 17b and 17c**). Oxycodone treatment has no significant effect on TLR4 activity (**Figure 17d**).

3.5 Methadone-Induced 6- and 18-Hour hMOR Protein Expression in HEK-Blue™-hTLR4 Cells

This set of experiments was designed to focus on the relative expression levels of hMOR in response to methadone ± LPS treatment. Methadone was selected to identify changes in transcript because of the immune rescue that has been observed in those on MMT (Sacerdote *et al*., 2008). TLR4 was not investigated because it is overexpressed in this cell line; however, it was used as an additional positive control when looking at the mRNA (**Figure 19**). Both mRNA and whole cell lysate extractions were analyzed for comparison; however, only the whole cell lysate is shown here because of difficulties in getting the RT-PCR to work. Troubleshooting efforts included increasing the concentration of cDNA for hMOR from 50 ng (5 µL) to 100 ng (10 µL) per reaction. Making new heavily concentrated cDNA allowed for further increases in cDNA concentration by up to 400 ng to be added per reaction in an attempt to get hMOR to amplify without altering the total reaction volume. This yielded a decent melting curve for hMOR but still no amplification occurred until 29-30 cycles indicating that the primers were good but something else might be interfering with the reaction. It is possible that the hMOR transcript was too low to readily detect. It was at this point that it was decided to focus on hMOR protein.

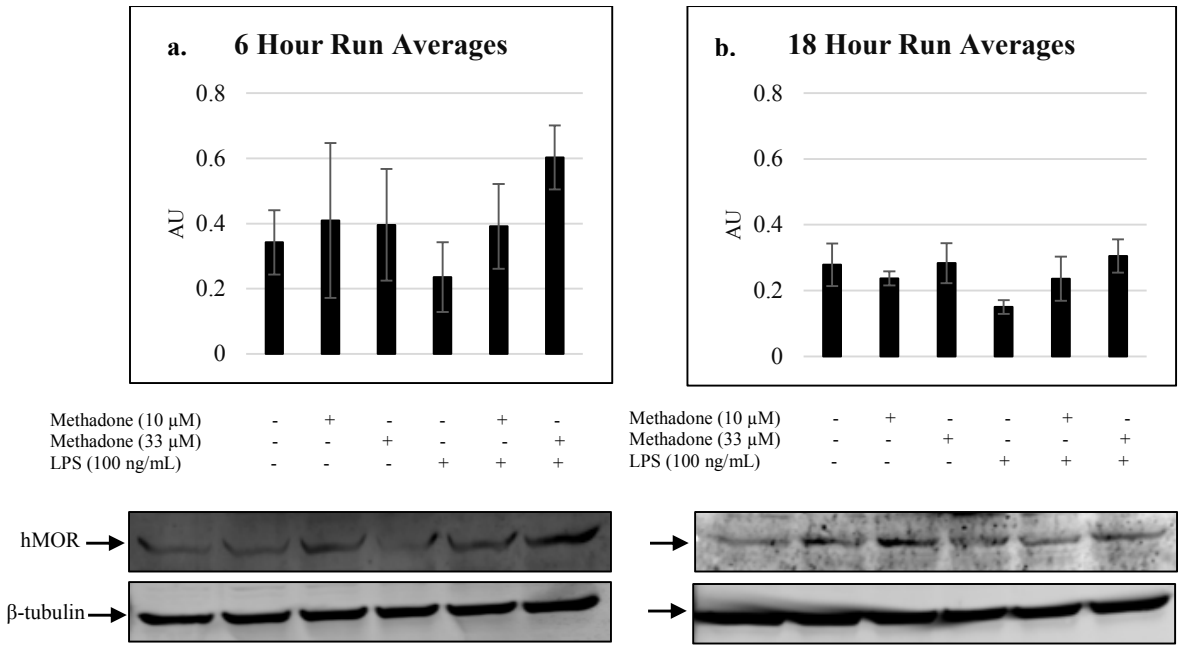


Figure 18. hMOR Western Blots of 6h and 18h Methadone ± LPS Treatments in HEK-Blue™-hTLR4 Cells. a. Six hour quantification of the data, N=4. **b.** Eighteen hour quantification of the data, N=5. Representative western blots with corresponding β-tubulin loading controls are shown beneath each graph. A two-way ANOVA run on each data set did not detect a main LPS effect or an LPS-methadone interaction. *Arrows point to 50 kDa protein.*

Figure 18 shows that there is no statistical difference in hMOR protein expression after either 6 or 18 hours of methadone exposure with or without LPS. The LPS control appears to have a lower hMOR protein expression than all of the groups at both time points but a two-way ANOVA testing the potential for interactions between methadone and LPS did not detect statistical significance. This was despite the apparent trend of increasing protein expression with increasing concentrations of methadone. It is possible that a more sensitive and quantitative technique may detect a difference; however, the data presented here indicates that regardless of methadone and/or LPS treatment there is no change in hMOR protein expression in this cell line.

3.6 Message Levels of hMOR Transcript in the Presence or Absence of Methadone in HEK-Blue™-hTLR4 Cells.

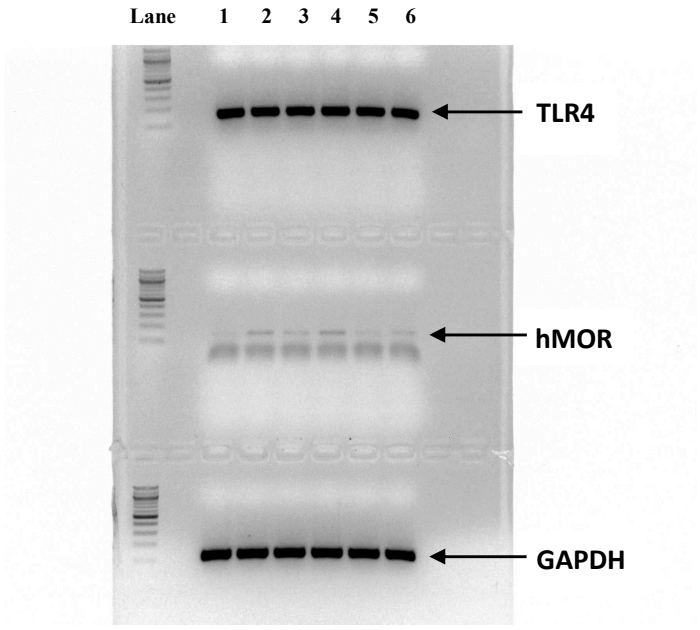


Figure 19a. End point Polymerase Chain Reaction (PCR) of TLR4 (top), hMOR (middle), and GAPDH (bottom) in HEK-Blue™-hTLR4 cells with methadone and/or LPS treatment for 18 hours.

Figures 19a and **19b** illustrate a preliminary run (N=1) highlighting the potential effect of methadone and/or LPS treatment on hMOR mRNA. Either methadone and/or LPS remained on the cells for 18 hours. This time point was chosen because it is the incubation time recommended prior to reading the absorbance when determining TLR4 activation as in the SEAP assay. hMOR transcript appears to be upregulated in response to LPS or 100 µM methadone but the bands are light and additional runs are needed before a conclusion can be made. The low intensity bands may be due to the 18 hour time point, as **Figure 17** suggests a relatively greater protein presence than the mRNA at the same time.

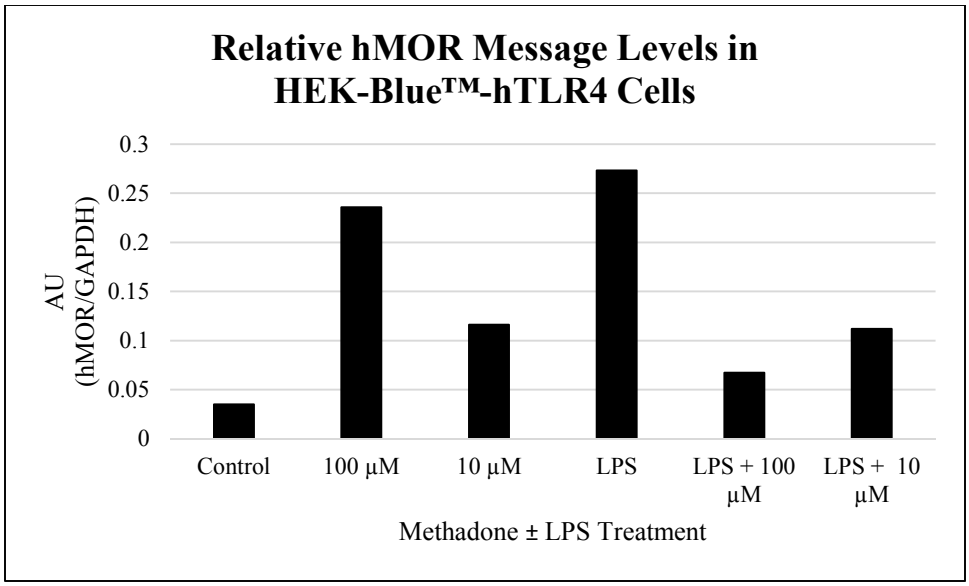


Figure 19b. Relative hMOR mRNA levels in HEK-Blue™-hTLR4 cells after methadone ± LPS treatment. Cells were treated with LPS (100 ng/mL) and/or methadone at the doses indicated on the graph for 18 hours. N=1

3.7 Detecting hMOR

hMOR started out relatively easy to detect in the HEK-Blue™-hTLR4 cell line. The CHME-5 cell line is a different story. Many attempts to optimize the detection of hMOR were made; however, conclusive results were not achieved. Technical support from the vendor, Santa Cruz Biotechnology, did not lend any success in this endeavor as **Figure 20** illustrates the lack of hMOR protein expression in the SK-N-MC positive control cell lysate that the vendor provided. This immediately led to the notion that the antibody may have gone bad. This section highlights the struggles faced with this protein, using every available method to troubleshoot it. In order to maintain some consistency, the primary hMOR antibody was always used at a 1:200 dilution. All other western blot conditions were maintained as described in **Section 2.7** unless otherwise noted in this section.

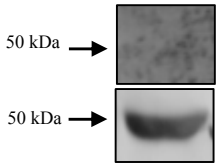


Figure 20. hMOR Western Blot of SK-N-MC Positive Control Cell Lysate. 100 µg of protein was loaded into the gel. β-tubulin loading control is shown beneath. N=3

As hMOR was detectable in the HEK-Blue™-hTLR4 cell line but not readily seen in the CHME-5 microglia, the CHME-5s were treated with 10 µM of the opioid antagonist naloxone. Naloxone upregulates hMOR (Unterwald, 2011; Unterwald 1995); therefore, treatment with naloxone would theoretically upregulate protein expression of the receptor for further analysis. Cells were treated for 1 or 18 hours in this attempt to upregulate hMOR. **Figure 21** illustrates that this was unsuccessful. Similarly, there was not a significant effect on TLR4 at either time point; however, TLR4 remained detectable (**Figure 22**).

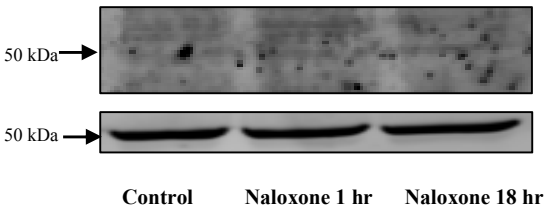


Figure 21. hMOR Western blot of CHME-5 microglia treated with 10 µM naloxone. β-tubulin controls are shown below.

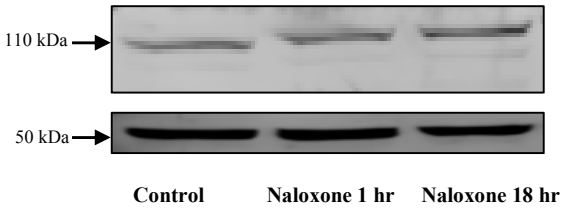


Figure 22. TLR4 Western blot of CHME-5 microglia treated with 10 µM naloxone. β-tubulin controls are shown below.

Figure 23 graphically depicts the apparent inability of naloxone treatment to upregulate hMOR protein expression. While TLR4 is robustly expressed in this cell line, hMOR is not and does not even appear to respond to naloxone. As microglia have been shown to express hMOR (Kettenmann *et al*., 2011) and the initially proposed project intended to look at the interaction between TLR4 and hMOR, every attempt to study the potential interaction was made.

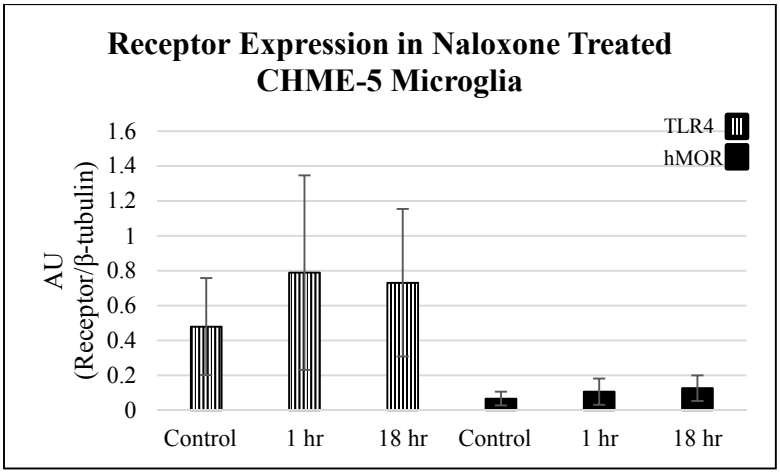


Figure 23. TLR4 and hMOR protein expression in naloxone treated CHME-5 microglia. Qualitative representation receptor expression. Control and 18 hr experiments were repeated for an N=3, the 1 hr was repeated twice, contributing to the large amount of error. Cells were treated with 10 μM naloxone. Receptor protein/β-tubulin is shown on the y-axis. Data are presented ± SEM.

In order to investigate the possibility that the hMOR antibody had gone bad, the CHO-hMOR-pIRES cell line was used as another positive control. C. Brasel stably transfected this cell line to express hMOR (Brasel *et al*., 2008); therefore, if the antibody is still functional then hMOR should be detected. As is illustrated in **Figure 24**, hMOR was not detected in the transfected cell line with any of the opioids used. 50 μg of CHO-hMOR-pIRES was loaded into lane 1.

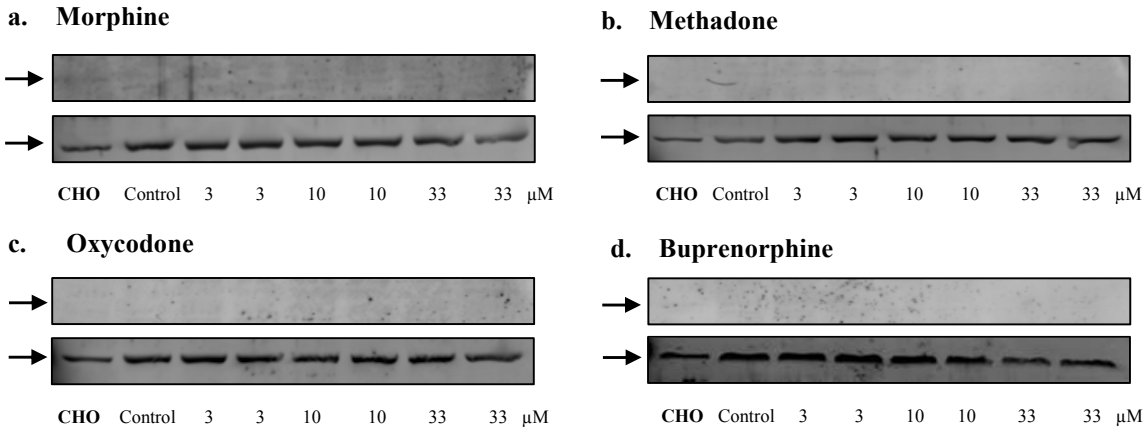


Figure 24. hMOR western blots in CHME-5 microglia and CHO-pIRES cell lines. CHO-pIRES and control cells were not treated with any dose of opioid. Opioid doses range from 3-33 μM. Treatments lasted 18 hours. No hMOR protein was detected. β-tubulin controls are shown beneath each blot. *Arrows indicate 50 kDa protein.*

Because the antibody did not detect hMOR in the transfected line, a couple of potential explanations arise. First of all, the transfection could no longer be effective after being in cryogenic storage for 7 years (date on vial: 06.03.2007, date taken out: 06.23.2014). However, this is not very likely to happen as the purpose of a stable transfection is to incorporate the gene of interest (in this case, hMOR) into the genome so that the next generations of cells also express the gene. The second, and more likely explanation, is again, that the antibody has degraded. IgG aggregates can form over time in antibodies. These aggregates are too large to bind to the protein in question, rendering the antibody ineffective. Because this antibody has worked a couple of years before both in the HEK-Blue-hTLR4™ cell line and in another investigator's research using the same CHME-5 cell line (**Figure 16**), the probability that this is the case is reasonable. Economic factors prohibited purchase of a new hMOR antibody which is why every available effort was made to optimize the antibody currently available.

To test the possibility that the antibody had degraded and large IgG aggregrates were inhibiting binding to the positive controls, the primary antibody was quick spun for 10-15 seconds immediately prior to being added to the membrane. This was to pull down any aggregates that may have formed; however, if this was done for too long then all or most of the IgGs might pull down which would inactivate the antibody. The primary antibody was added at a ratio of 1:200 then incubated on a plate rocker at 4°C for 4 days. This extended incubation time was in an attempt to maximize protein-antibody binding to compensate for any IgGs pulled down and/or if too many IgGs had already aggregated for the antibody to bind regardless of centrifugation and incubation time. The secondary antibody was added after the 4 day incubation and the western blot was completed from this point according to **Section 2.7**. This extended effort to isolate the cause for the blank hMOR membranes (**Figure 24**) confirmed the ability of naloxone to upregulate the protein when compared to control; however, the bands are light indicating low expression levels (**Figure 25**).

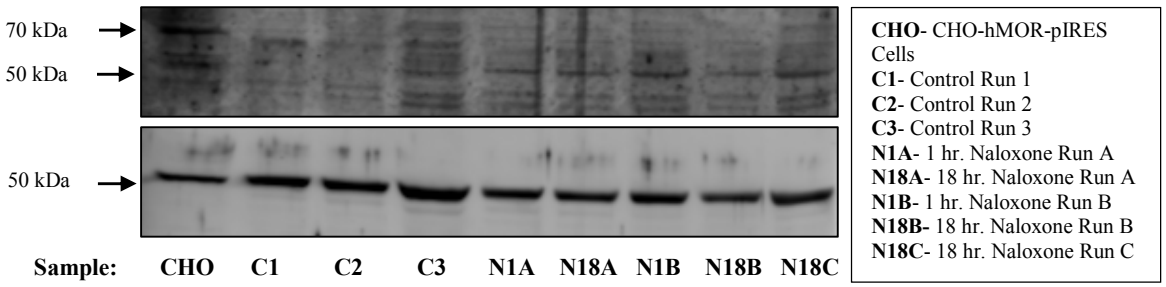


Figure 25a. hMOR Western blot of centrifuged and extended incubation time of CHO-hMOR-pIRES and CHME-5 microglia under control and 10 μM naloxone treatment at 1 and 18 hours. All samples other than CHO are CHME-5 microglia. 50 μg of protein was loaded into each well. Faint bands were visualized in naloxone-treated CHME-5 cells at 50 kDa. Faint bands were also visualized at 50 kDa and 70 kDa in the CHO-hMOR-pIRES cells. β-tubulin loading controls are shown beneath.

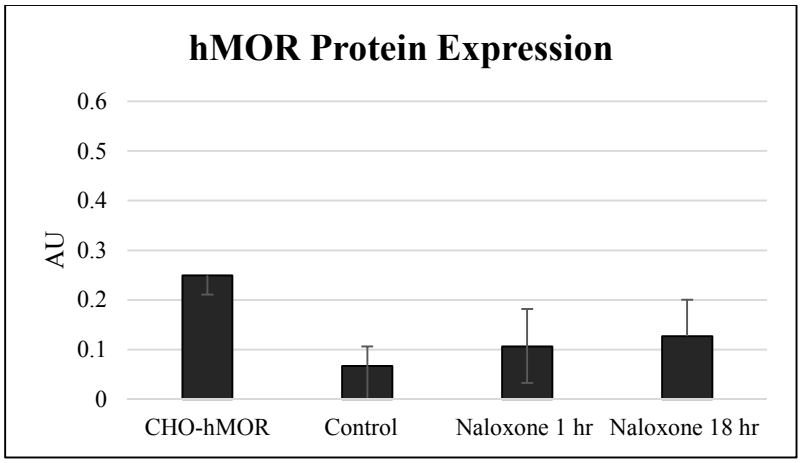


Figure 25b. Quantification of hMOR western blot in 25a. The 1 hour runs were duplicated. Receptor protein/β-tubulin is shown on the y-axis. The 18 hour runs were repeated for N=1-3.

This data is inconclusive as to whether or not hMOR is expressed in the CHME-5 microglia. hMOR protein expression in the CHO-hMOR-pIRES cell line does not appear to have robust expression of the protein either. Furthermore, naloxone treatment appears to upregulate hMOR as was previously hypothesized but additional western blots are necessary to confirm this. The naloxone bands are darker than the control bands in **Figure 25a**, indicating more protein, but as is illustrated in **Figure 25b** it was not statistically significant. These experiments were not all performed at an N=3 due to time restrictions on the project. Only the control and naloxone 18 hour runs could have statistics run making it possible that with more replicates significance might exist. The others were not further replicated because they were simply trial runs to see if hMOR could be detected in an hMOR control. All other data compares control to 18 hour treatments. The CHO-hMOR-pIRES cells primarily have two bands, one at 50 kDa and one at 70 kDa. According to the manufacturer of the antibody, it recognizes 16 different isomers so these two bands are most likely both isomers of hMOR that are represented in the cell line. The paper that

described the transfection of and used this cell line did not perform a western blot for comparison (Brasel *et al*., 2008).

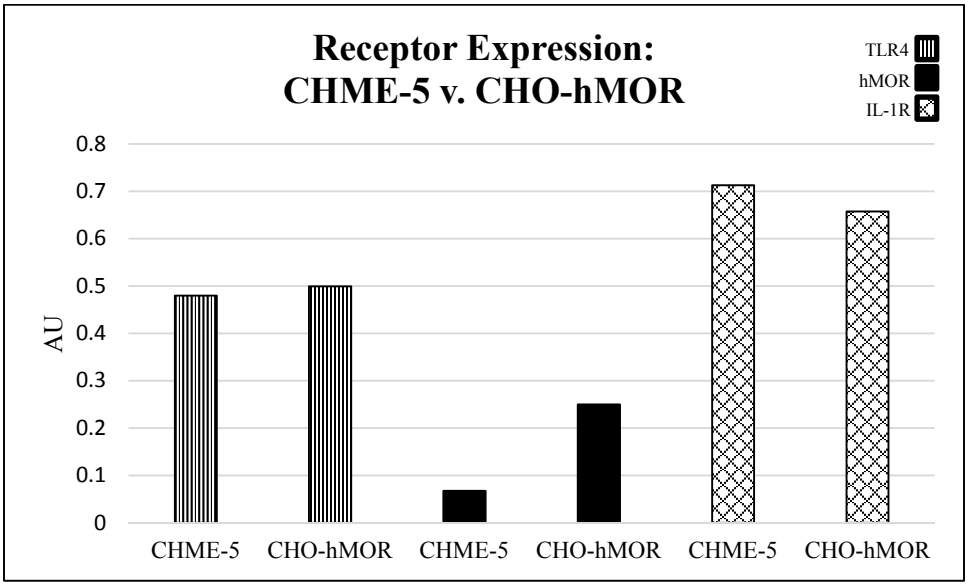


Figure 26. Qualitative receptor comparison of CHME-5 microglia to CHO-hMOR-pIRES cells. Cells were untreated and extracted to determine what proteins are present in the cell lines. The CHME-5 microglia and CHO-hMOR pIRES cells have comparable expression of TLR4 and IL-1R while the CHO-hMOR pIRES line more abundantly expresses hMOR. Receptor protein/β-tubulin is shown on the y-axis.

The immune receptors TLR4 and IL-1R were readily identifiable but hMOR was more difficult to detect and definitive data as to the presence of hMOR protein these cell lines is lacking. Given a fresh antibody it is believed that the protein would be better detected. The HEK-Blue™-hTLR4 reportedly does not express hMOR (Hutchinson MR et al. 2010); however, (**Figure 16**) shows that it can be found in this cell line. If the fresh antibody is able to detect hMOR in a cell line that allegedly does not express it, it is reasonable to entertain the concept that fresh antibody may more reliably detect hMOR in the CHO-hMOR-pIRES and CHME-5 microglial cell lines.

3.8 Receptor Expression in CHME-5 Microglia

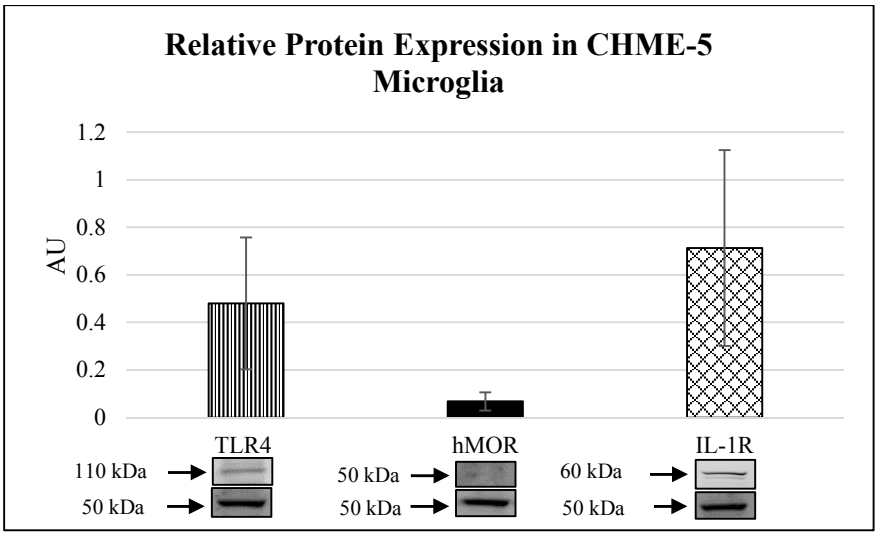


***Figure 27. CHME-5 Receptor Characterization.** CHME-5 cells were plated but not treated prior to extraction. Protein expression was determined via western blot. The corresponding β-tubulin control is shown beneath each image. Receptor protein/β-tubulin is shown on the y-axis. Error bars represent ± SEM.

*The western blots presented here were run and quantified after troubleshooting the problems in getting the hMOR primary antibody to work.

This experiment was a pilot run to determine which receptors are expressed in the CHME-5 microglia. Despite the relative greater abundance of IL-1R compared to TLR4 in **Figure 27**, TLR4 was chosen for further analysis because the western blot was more specific. Only one band was visible for TLR4 whereas more than one band could be seen for IL-1R. These were likely isomers of the receptor. The band shown in **Figure 27** is the most solid band and is at the expected weight.

Characterizing the receptors present in this cell line was a difficult task because of hMOR (**Section 3.5**) but ultimately TLR4 and IL-1R protein is expressed but inconclusive results regarding hMOR protein expression leaves that to be determined.

3.9 Pilot Dose-Responses

This set of experiments was done to determine an optimal dose of the drugs morphine and methadone to achieve maximal hMOR and TLR4 protein expression. As such, western blots were performed and analyzed to determine their relative expression in the presence of 3-33 μM morphine or methadone or LPS at 0.01-100 ng/mL. Different antibody concentrations and amounts of protein were added in an attempt to optimize both loading and drug concentration.

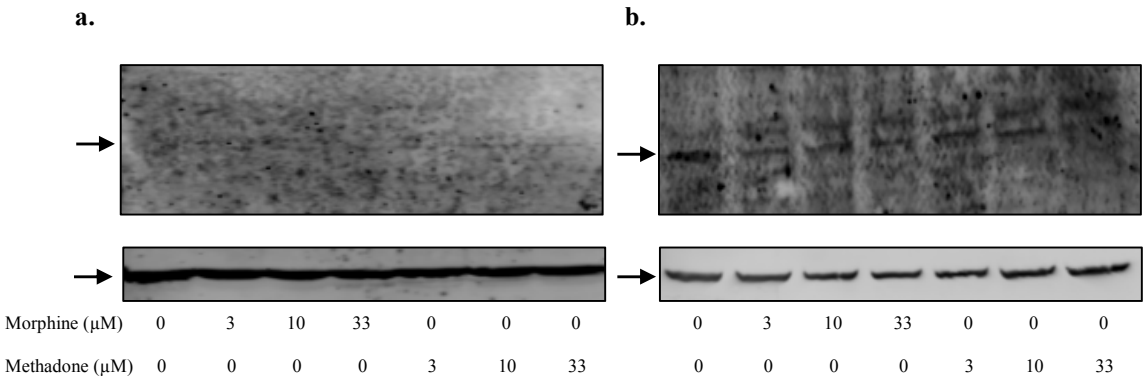


Figure 28. hMOR western blot of morphine and methadone pilot dose response in CHME-5 microglia. Cells were treated for 18 hours with 3-33 μM of drug. Faint bands can be seen in **b** at 50 kDa whereas virtually nothing is visualized in **a**. β-tubulin control shown beneath. 100 μg of protein was loaded into **a**; 50 μg of protein was loaded into **b**. Images were contrast optimized with Adobe Photoshop. *Arrows indicate 50 kDa protein.*

As can be seen in **Figure 28**, the hMOR response is inconsistent. These three runs were plated, treated, and whole cell lysates were extracted on separate days. Protein in **Figures 28a** and **29a** were loaded at 100 μg and the primary antibody was used at a 1:200 dilution for hMOR because of past issues at trying to detect the hMOR protein; however, virtually none was detected despite the high amount of protein loaded and antibody used. The relative quantities of protein loaded can be see when comparing **Figures 28a,** which

shows no hMOR protein and **29a**, in which the TLR4 protein is beginning to smear and the bands are much thicker than those in **Figure 29b**. Because of this failed attempt to identify the protein, the blots in **Figure 34b** were run on the same day and loaded with 50 µg of protein with fresh antibody prepared for each membrane at a 1:2000 dilution. Considering that this is a dilute mix of antibody, it still does not make sense that one membrane would show the protein while the other does not. Also eliminating the dilute antibody solution as the explanation for not detecting the protein is the fact that in **Figure 28a** the primary antibody was at a 1:200 dilution, with the blot run on a different day, and the protein was still only present as an extremely faint band at 50 kDa. **Figure 28b** illustrates a slight amount of hMOR protein at 50 kDa, with the most in the control lysate, while **Figure 28a** does not have any indication of hMOR protein at the same weight in either control or treated cells. The same membranes were stripped and then probed for TLR4 in **Figure 29** which suggests that TLR4 protein is constitutively expressed in the CHME-5 microglia given the robust control bands in the first lane.

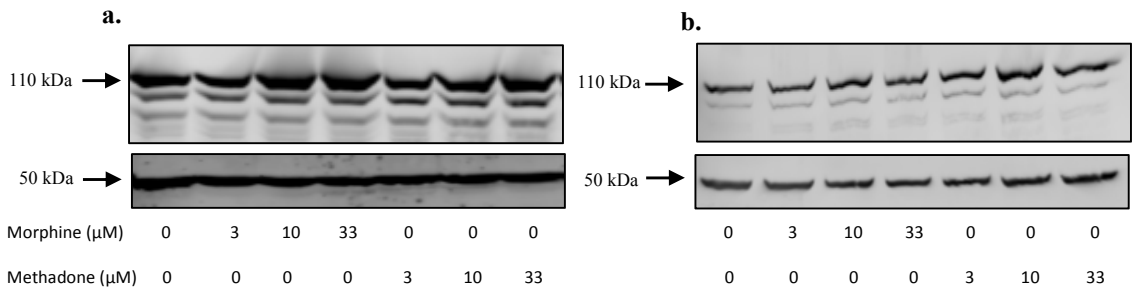


Figure 29. TLR4 western blot of morphine and methadone pilot dose responses in CHME-5 microglia. Cells were treated with 3-33 µM of drug for 18 hours. β-tubulin control shown beneath. 100 µg of protein was loaded into **a;** 50 µg of protein was loaded into **b**. 100 µg saturated the system while 50 µg yielded more distinct bands.

These images and the corresponding β-tubulin controls eliminate the possibility of a poor gel to membrane transfer process. If the protein had not properly transferred to the membrane then neither TLR4 nor β-tubulin would have been detected. This indicates that while hMOR may be present in the CHME-5 microglia, it is not easily detectible using the current methods.

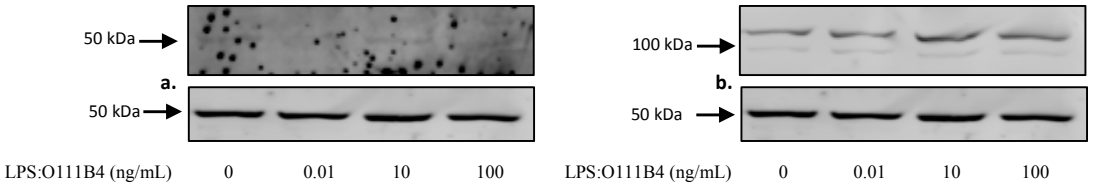


Figure 30. Western blot of hMOR (a.) and TLR4 (b.) in a pilot dose-response run to LPS:O111B4 (0-100 ng/mL) in CHME-5 microglial cells. Cells were treated for 18 hours. 50 µg of protein was loaded into the gel. hMOR could not be seen (**a**) but TLR4 was visible (**b**). There did not appear to be a change in protein expression in response to dose.

Figure 30 illustrates the relative abundancies of hMOR and TLR4 protein in response to LPS. The membrane was first probed for hMOR (**Figure 30a**), then stripped and tested for TLR4 (**Figure 30b**), then stripped a third time to normalize for β-tubulin expression. Multiple attempts at different loading concentrations and antibody dilutions for hMOR were made to optimize the doses for morphine, methadone, and LPS but as can be seen in **Figures 28-30** there is virtually no change in either hMOR or TLR4 protein expression in response to these treatments. No statistical analysis was performed on this pilot dose-response as no attempt was successful in detecting a change in protein expression in either receptor investigated. It is possible that another endpoint in a more sensitive assay, such as an ELISA, would have identified changes in protein expression to

determine optimal doses. Instead, subsequent assays incorporated all of the doses of the opioids and the highest dose of 100 ng/mL LPS, which is frequently seen in the literature.

3.10 MTT Toxicity of Opioids in CHME-5 Microglia

As is observed in **Figure 31**, no toxicity is associated with any of the concentrations used in this study. Although buprenorphine appears to be slightly toxic, it was not statistically significant (p=0.07) according to a one-way ANOVA.

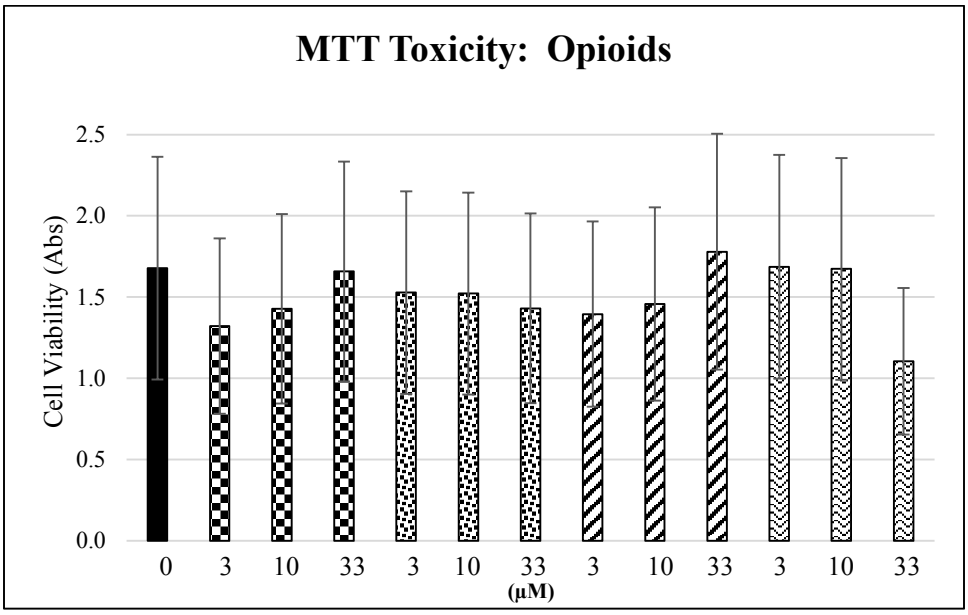


Figure 31. MTT Toxicity of opioids in CHME-5 microglia. MTT absorbance at 492 nm. Cells were treated with opioids (3-33 µM) for 18 hours. Error bars represent ± SEM. One-way ANOVA indicated no significance. N=3

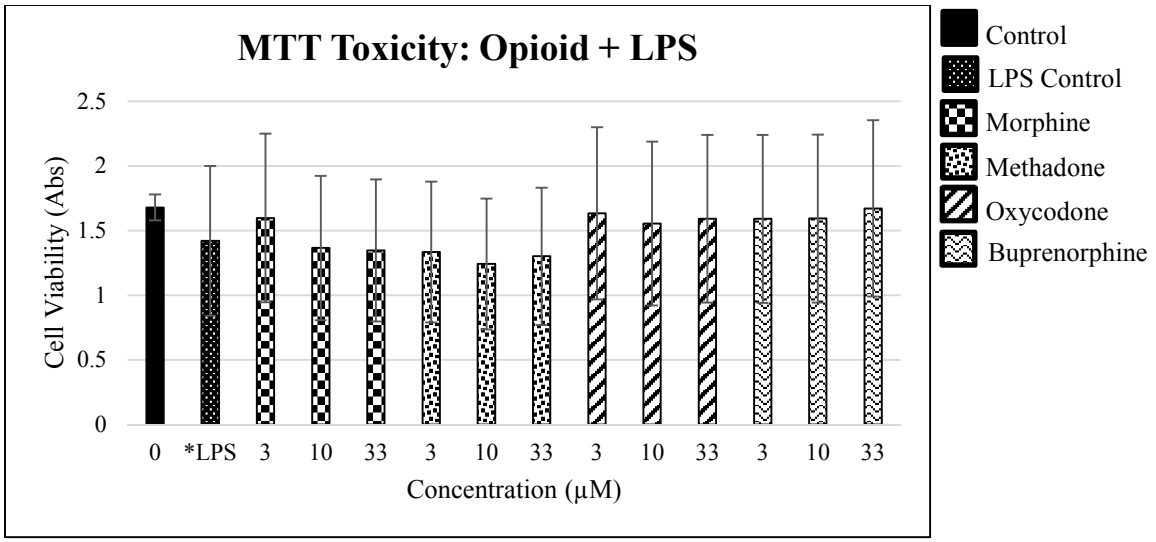


Figure 32. MTT Toxicity of opioids in the presence of LPS in CHME-5 microglia. MTT absorbance at 492 nm. *LPS was used at 100 ng/mL LPS O111:B4 in every treatment except control. Opioid treatment from 3-33 μM was given with the LPS for 18 hours. Error bars represent ± SEM. Data were analyzed via a two-way ANOVA which found no significant differences.

Additionally, no toxicity is associated with the combined treatment of morphine, methadone, oxycodone or buprenorphine with LPS (**Figure 32**).

3.11 TLR4 Expression in Response to Opioids in CHME-5 Microglia

In the experiments investigating the effects of the individual opioids on TLR4 protein expression the cells were treated with the drug then incubated at 37°C for 18 hours prior to whole cell lysate extraction. In the experiments with LPS the CHME-5 microglia were treated with the opioid and LPS simultaneously then incubated at 37°C for 18 hours. The concentration of LPS remained constant in all experiments at 100 ng/mL. All of the experiments in this section were repeated for an N=4 in duplicate.

3.11.1 Morphine

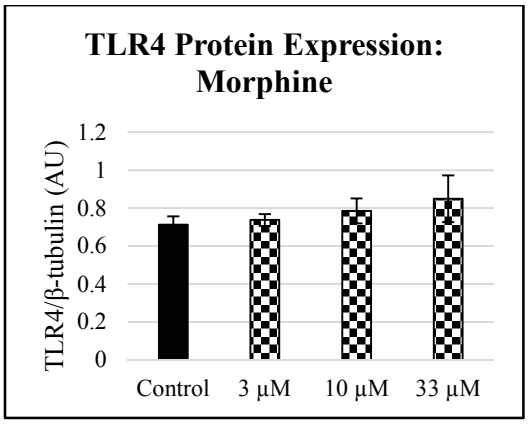


Figure 33. Morphine effect on TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with morphine, 3-33 μM. TLR4 western blot and β-tubulin normalization is below. Data analyzed via one-way ANOVA. N=4

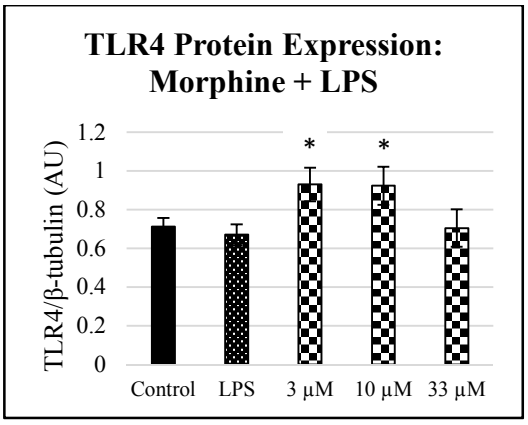
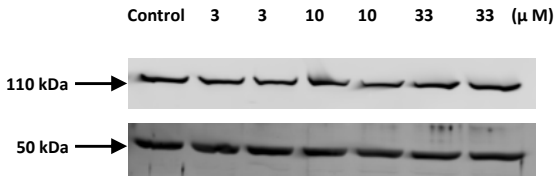
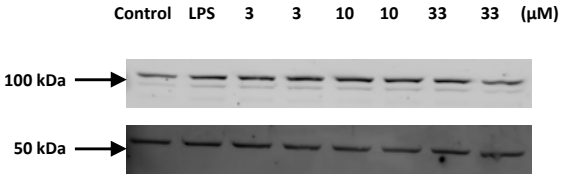


Figure 34. Morphine + LPS effect on TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 100 ng/mL LPS and 3-33 μM morphine. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA with a Fisher's LSD post-hoc test. (*p<0.05) N=4





Morphine had no significant influence on TLR4 protein expression in the microglial cell line (**Figure 33**). A one-way ANOVA was conducted to compare the effect of morphine on TLR4 protein expression in the presence of LPS and it was determined that morphine at 3 and 10 μM significantly upregulated TLR4 protein expression when simultaneously treated with LPS ($F_{4,64}$=2.62, p<0.05) (**Figure 34**). The subsequent decrease observed at 33 μM indicates that morphine may have a ceiling effect at doses above 10 μM. All experimental conditions were compared to control.

3.11.2 Methadone

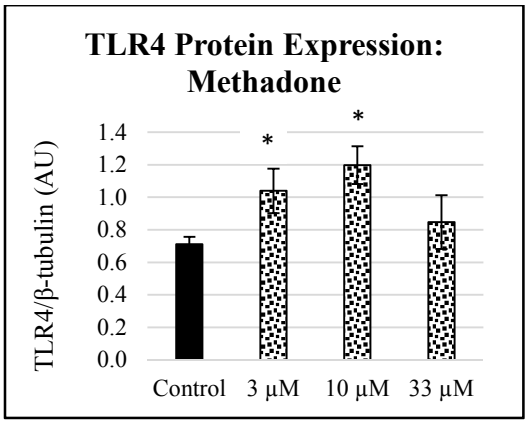


Figure 35. Methadone induced TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 3-33 μM methadone. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA with a Fisher's LSD post-hoc test. (*p<0.05) N=4

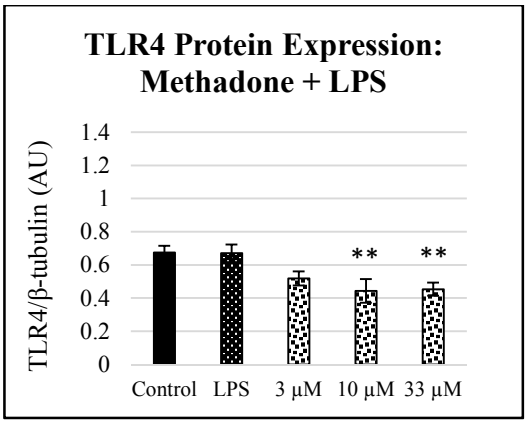
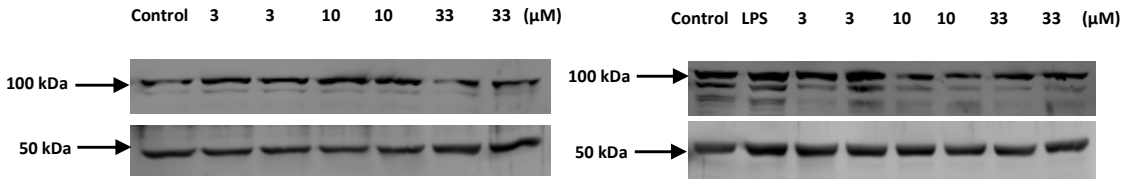


Figure 36. Methadone + LPS induced TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 100 ng/mL LPS and 3-33 μM meethadone. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA with a Fisher's LSD post-hoc test. (**p<0.01) N=4



A one-way ANOVA was again conducted to compare the effect of methadone on TLR4 protein expression and found that methadone significantly upregulated TLR4 protein expression at 3 and 10 μM ($F_{3,48}$=6.65, p<0.01) (**Figure 35**). It was additionally found that in the presence of LPS methadone at 10 and 33 μM significantly downregulated TLR4 protein expression ($F_{4,61}$=4.40, p<0.01) (**Figure 36**). This indicates that LPS is having a main effect on TLR4 protein expression in the presence of methadone.

3.11.3 Oxycodone

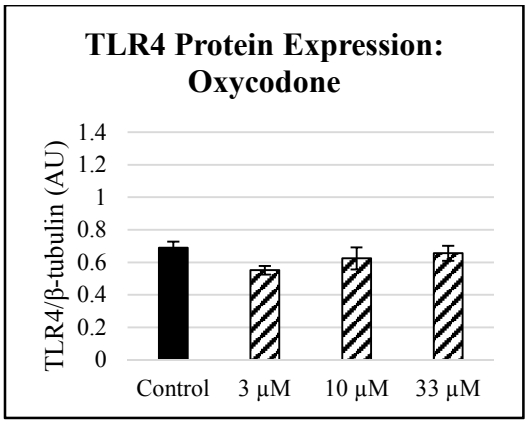


Figure 37. Oxycodone induced TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 3-33 µM oxycodone. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA. N=4

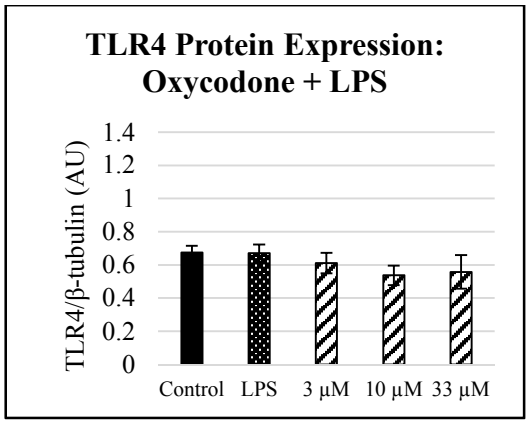
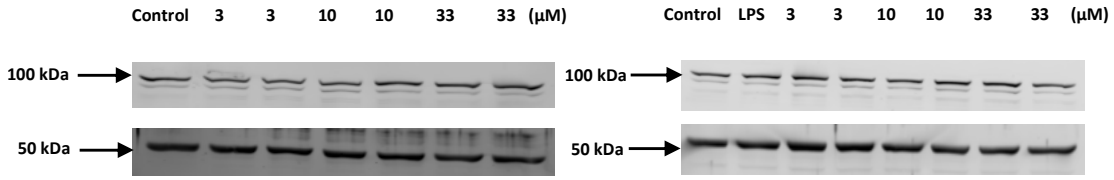


Figure 38. Oxycodone + LPS induced TLR4 protein expression in CHME- microglia. Cells were treated for 18 hours with 100 ng/mL LPS and 3-33 µM oxycodone. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA. N=4



No concentration of oxycodone either with or without 100 ng/mL LPS affected TLR4 protein expression in the CHME-5 microglia (**Figures 37** and **38**). This indicates that oxycodone has no effect on TLR4 protein expression in this cell line.

3.11.4 Buprenorphine

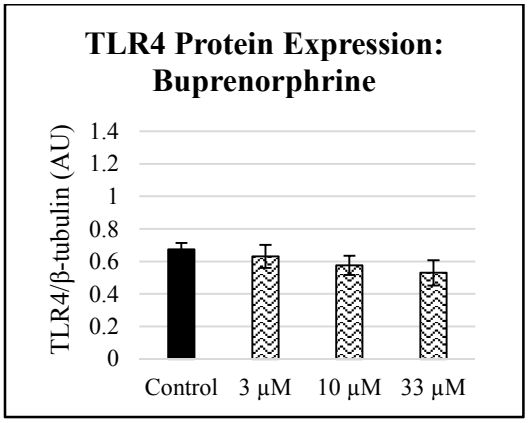


Figure 39. Buprenorphine induced TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 3-33 µM buprenorphine. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA. N=4

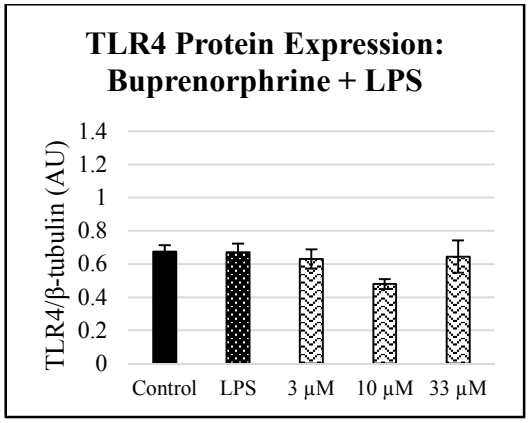
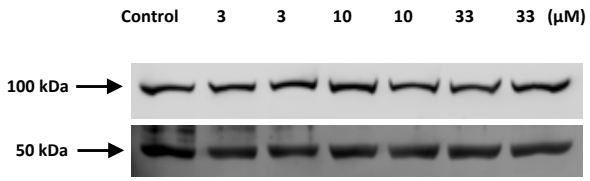
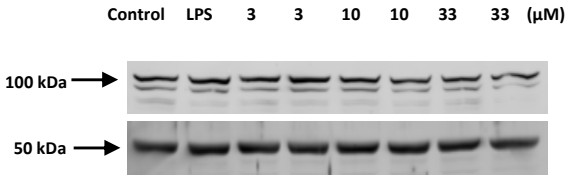


Figure 40. Buprenorphine + LPS induced TLR4 protein expression in CHME-5 microglia. Cells were treated for 18 hours with 100 ng/mL LPS and 3-33 µM buprenorphine. TLR4 western blot and β-tubulin normalization is shown below. Data analyzed via one-way ANOVA. N=4





As with oxycodone, no concentration of buprenorphine either with or without 100 ng/mL LPS significantly changed TLR4 protein expression in the CHME-5 microglial cell line (**Figures 39** and **40**).

3.11.5 Drug Comparison

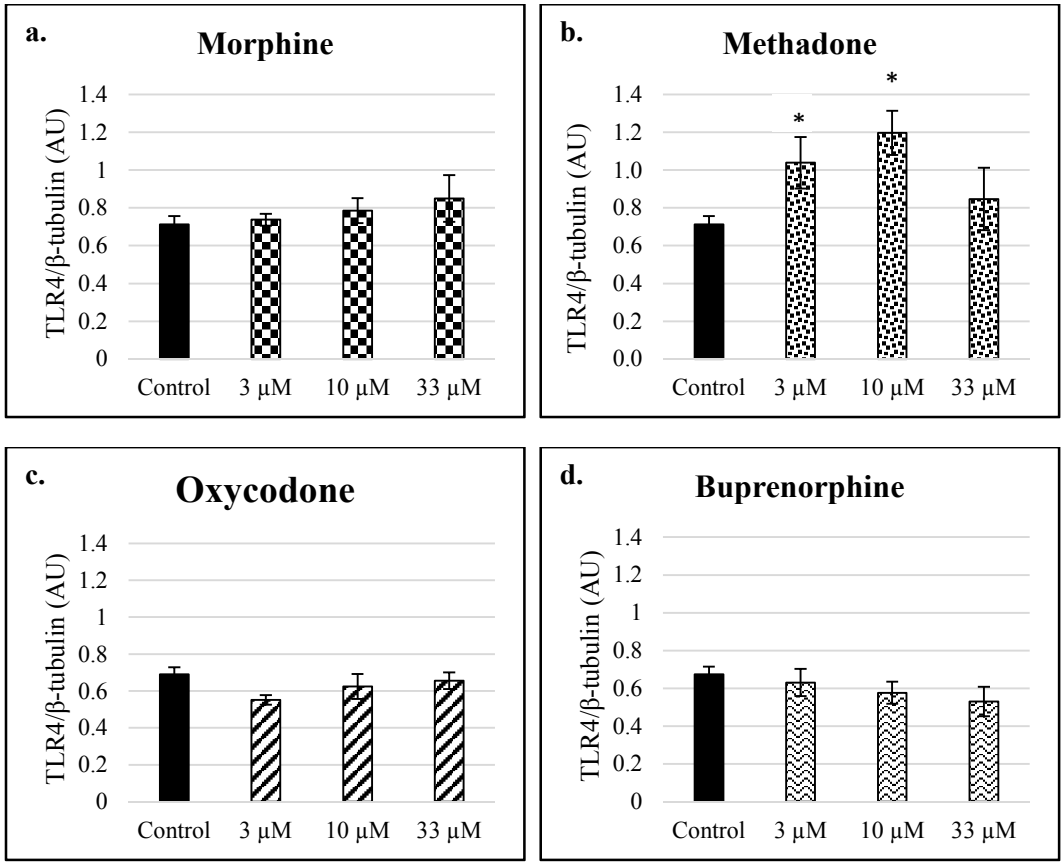


Figure 41. Opioid comparison of TLR4 protein expression in CHME-5 microglia. Cells were treated with the opioid for 18 hours at concentrations of 3-33 μM. Methadone upregulated TLR4 protein expression at 3 μM and 10 μM (*$p<0.05$). Analyzed via one-way ANOVA with Fisher's LSD post-hoc.

When comparing the opioids used in this research to each other it can be seen in **Figure 41b** that methadone upregulates TLR4 protein expression and is the only drug that significantly affects TLR4 in this microglial cell line ($F_{3,48}$=6.65, $p<0.01$). **Figures 41a, 41c,** and **41d** indicate that there is no difference between any of the opioids or doses when compared to control TLR4 levels.

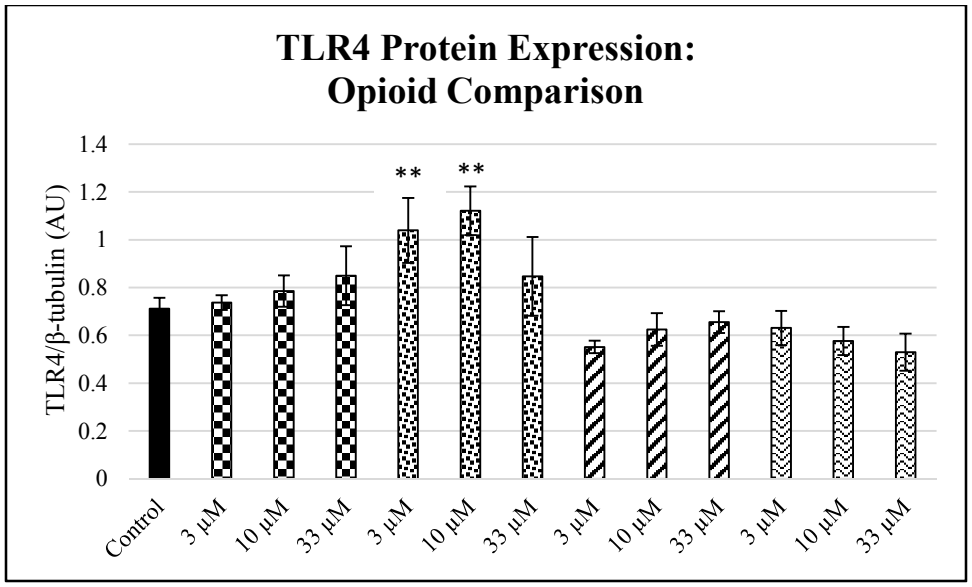


Figure 42. Simultaneous opioid comparison of TLR4 protein expression in CHME-5 microglia. This graph is a compilation of the **Figure 41** for visual comparison on one graph. Cells were treated with the opioid for 18 hours at concentrations of 3-33 μM. Methadone upregulated TLR4 protein expression at 3 μM and 10 μM (*$p<0.05$). Each drug group was individually analyzed via one-way ANOVA with Fisher's LSD post-hoc.

When simultaneously comparing the opioid data, a more significant effect of methadone at 3 μM and 10 μM was detected ($F_{3,48}$=6.65, $p<0.01$) (**Figure 42**). Methadone is the only opioid which independently and significantly regulates TLR4 protein expression in the CHME-5 microglia.

3.11.6 Opioid with LPS Comparison

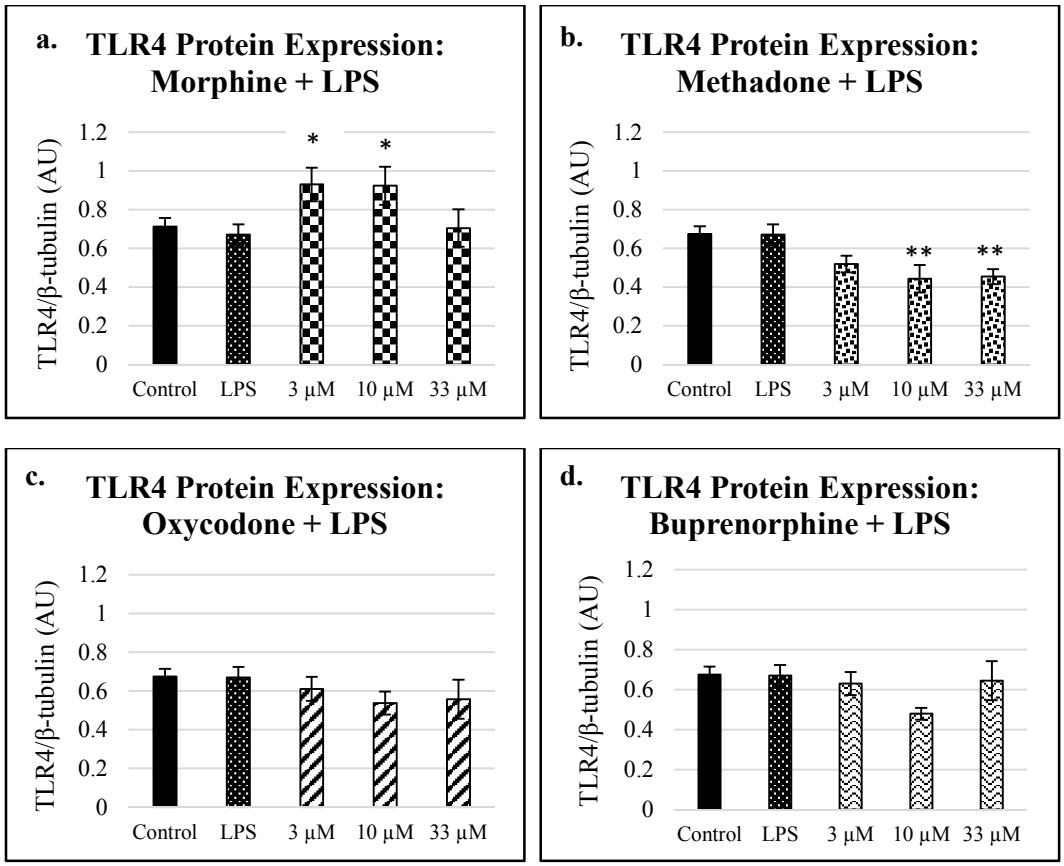


Figure 43. Opioid comparison of TLR4 protein expression in the presence of LPS in CHME-5 microglia. Cells were treated with 100 ng/mL LPS and the opioid for 18 hours at concentrations of 3-33 μM. Morphine + LPS upregulated TLR4 protein expression at 3 μM and 10 μM ($F_{4,64}$=2.62, p<0.05) while methadone + LPS downregulated it at 10 μM and 33 μM ($F_{3,48}$=6.65, p<0.01). Analyzed via one-way ANOVA with Fisher's LSD post-hoc.

When comparing the opioids used in this research under simultaneous LPS treatment, it can be seen in **Figure 43a** and 4**3b** that both morphine and methadone significantly affect TLR4 protein expression in this microglial cell line. However, they have opposite effects in that morphine with LPS upregulates the protein expression while methadone downregulates it. Neither oxycodone nor buprenorphine (**Figures 43c** and **43d**) have any significant effect when in the presence of LPS when compared to control expression levels.

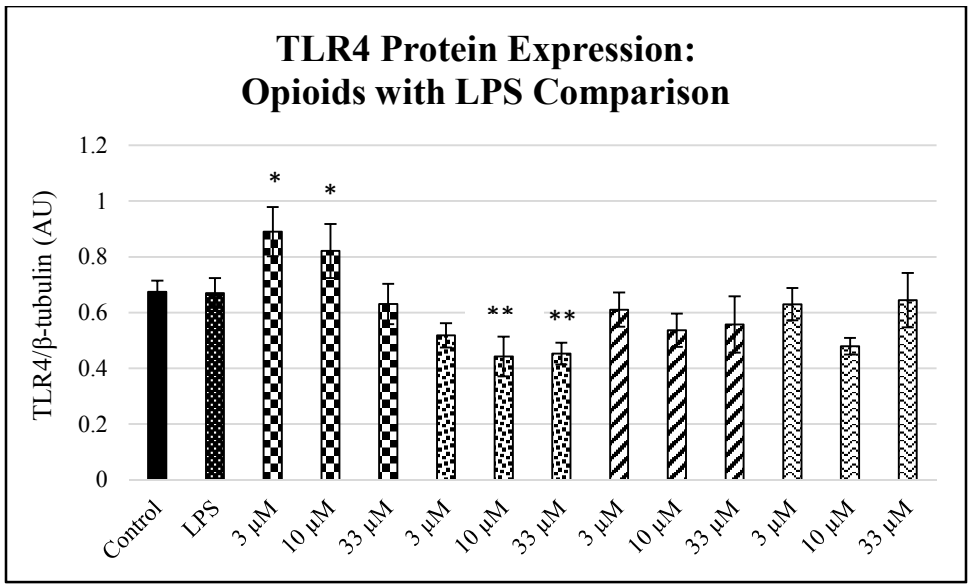


Figure 44. Simultaneous opioid with LPS comparison of TLR4 protein expression. This graph is a compilation of the **Figure 43** for visual comparison on one graph. Cells were treated with the opioid at concentrations of 3-33 μM + 100 ng/mL LPS for 18 hours. Morphine + LPS upregulated TLR4 protein expression at 3 and 10 μM ($F_{4,64}$=2.62, p<0.05) while methadone + LPS downregulated it at 10 μM and 33 μM ($F_{3,48}$=6.65, p<0.01). Each drug group was individually analyzed via one-way ANOVA with Fisher's LSD post-hoc.

When visually comparing all of the LPS treatments to control it is seen that TLR4 protein expression is upregulated in the case of morphine at 3 and 10 μM ($F_{4,64}$=2.62, p<0.05) while methadone downregulated the protein ($F_{3,48}$=6.65, p<0.01).

3.11.7 Comparison of Opioid and Opioid with LPS Treatments

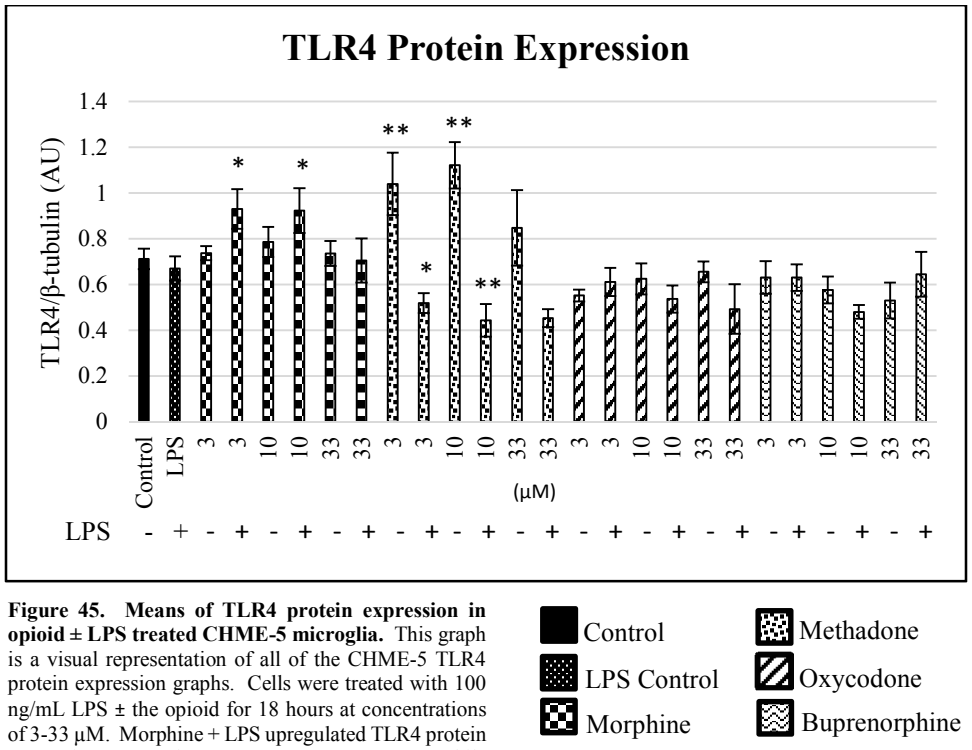


Figure 45. Means of TLR4 protein expression in opioid ± LPS treated CHME-5 microglia. This graph is a visual representation of all of the CHME-5 TLR4 protein expression graphs. Cells were treated with 100 ng/mL LPS ± the opioid for 18 hours at concentrations of 3-33 μM. Morphine + LPS upregulated TLR4 protein expression at 3 and 10 μM ($F_{4,64}$=2.62, p<0.05) while methadone + LPS downregulated it at 3 and 10 μM ($F_{3,48}$=6.65, p<0.01). Methadone alone upregulated TLR4 protein expression at 10 and 33 μM ($F_{3,48}$=6.65, p<0.01). Data were analyzed in independent drug groups (opioid only or opioid + LPS) via a one-way ANOVA with a Fisher's LSD post-hoc test.

Figure 45 is a complete compilation of all of the data for the CHME-5 microglia. It provides a direct relative comparison of TLR4 protein expression of all groups individually tested. It simultaneously represents **Figures 33-40**. It illustrates that morphine at 3 and 10 μM with LPS upregulates TLR4 ($F_{4,64}$=2.62, p<0.05). Methadone treatment alone also upregulates TLR4 at 3 and 10 μM ($F_{3,48}$=6.65, p<0.01). These were the only upregulatory effects detected. This compilation of the individual drug comparisons, which were analyzed via individual group one-way ANOVAs as indicated in **Figures 33-40**, does indicate a main LPS effect in methadone treated CHME-5 microglia

at 10 μM. At 10 μM methadone upregulated TLR4 protein expression while at the same dose in the presence of LPS TLR4 protein expression was downregulated.

Oxycodone and buprenorphine overall appear to decrease TLR4 protein expression but no treatments were significant. Methadone is the only opioid that independently upregulated TLR4 protein expression (**Figure 45**). The downregulatory effect of LPS in the methadone treated cells and upregulatory effect of LPS in morphine treatments suggests that LPS is a main effect in this system regarding TLR4 protein expression. LPS treatment alone did not have an effect when compared to control.

3.12 PCR Based Sequencing of TLR4 Primers

While PCR was not a large portion of this project, it did encompass designing primers for TLR4. The band for TLR4 was very crisp in end-point PCR and having the sequence verified for the laboratory was helpful for future experiments. Control CHME-5 mRNA extract was used to make cDNA then combined and concentrated to 100 ng to run the gel. This was to ensure enough material to send out for sequencing. The sequences from each primer are shown in **Figure 47**. Running the sequences through BLAST yielded positive alignment with TLR4. Because the PCR gel was clean and crisp (**Figure 46**), the gel did not have to be extracted. 30 μL of 7.5 ng/mL PCR product and 10 μL of each primer at 5 pmole/μL was sent to Stillwater for the sequencing.

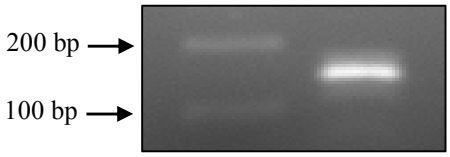


Figure 46. TLR4 PCR gel. Untreated CHME-5 microglial cells were run on a 2% agarose gel. The sample was concentrated to 7.5 ng/mL and sent for sequencing with the primers used in this experiment.

Forward Sequence: H06_SDhTLR4-TLR4F.seq

GAATTGGTTTTGGCAGCTATAGCTTCTTCGTTTCCCAGAACTGCAGGTGCTGGATTTATCCAGGTGTGAAATCCAGA

CAATTGAAGATGGGGCATATCAGAGCCTAAACCACCTCTTAAAGCCTAGCCACCTCTCCTCTAATACTCCGAACTGA

GTTCTGGGACATGAAGAGCTATACTTCTAATTGCCTCAGGGATTAAAGCTCGGTCGGTTTTC

Reverse Sequence: A07_SDhTLR4-TLR4R.seq

GCAGTTGGTAGTGTCTGGATTGACACCTGGATAAATCCAGCACCTGCAGTTCTGGGAAACTGAAGAAGCTATAGCTG

CCTAGATGCCTCAGGGGATTAAAGCTCAGGTCCAGGTTAGGAAGCTCAGGTCCAGGTTAGGTTAACCCCAACGGAAT

CGGGACCTGAACCAGCTGGCCCCAAGAGCCTCCGCCTCAAGCCC

Figure 47. TLR4 forward and reverse sequences. The sequences shown above were returned after sending the samples to a laboratory at Oklahoma State University in Stillwater, OK for analysis. BLAST alignment confirmed their specificity for TLR4.

CHAPTER IV

DISCUSSION

Because TLR4 is critical to both the peripheral and central innate immune systems, it is maintained under strict regulation. When the appropriate stimuli are elicited under normal conditions, primarily acute inflammation or infection, a healthy immune response results (Carty and Bowie, 2011). In these individuals TLR4 is tightly regulated, always primed and ready to respond to pathogenic insult leading to TLR4 activation of the immune system. In other situations, continued pro-inflammatory cytokine production can lead to tissue damage and sepsis as well as other chronic inflammatory conditions such as auto-immune disease (Biswas and Lopez-Collazo, 2009). Once the pathogen is cleared, the agonist for TLR4 is no longer present; therefore, TLR4 stops signaling. However, endotoxin tolerance can cause TLR4 to stop signaling in times of continued agonist exposure (Banerjee *et al*., 2013). The exact mechanism for this is unknown. As immune compromise has been identified with opioid use (McCarthy *et al*., 2001; Hutchinson *et al*., 2011), it is important to investigate the possibility that TLR4 is involved.

Further illustrating the importance of TLR4 regulation is that some genetic mutations in the TLR4 transcript negatively affect otherwise healthy persons. In the TLR4 genetic mutations Asp299Gly and Thr399Ile (Lorenz *et al*., 2002), the protein either does not respond at all or minimally responds to the conserved pathogen associated molecular

patterns on TLR4 ligands and these otherwise healthy people are rendered increasingly susceptible to opportunistic infections (Roy *et al*., 2011). Either in these genetically induced hyporesponsive situations or when TLR4 expression and/or activity are altered for extended periods of time pathogenesis of some type is likely to ensue. An example of this is in sepsis where the aforementioned mutations are linked to greater susceptibility to infection and death from septic shock (Lorenz *et al.,* 2002). Sepsis is a condition that can be described by excessive transcription and release of proinflammatory cytokines such as IL-1 and TNFα which leads to multiple accompanying symptoms such as fever and tachycardia (Bone, 1991). LPS mimics sepsis in experimental models and because LPS is a potent agonist for TLR4 (Chow *et al*., 1999), this receptor is a primary contributor to the deadly infection. As mentioned above, opioids also increase susceptibility to infection (Roy *et al*,. 2011) thereby compounding immunosuppression when being used by those with hyporesponsive TLR4 function.

On the other hand, an increase in TLR4 protein expression and activity may be a root cause for some chronic inflammatory conditions. Increased activation of TLR4 by LPS has been directly shown to contribute to neurodegeneration as TLR4 mutant mice were immune to the deleterious neurodegenerative effects of LPS (Lehnardt *et al*., 2003). The link between increased TLR4 expression and neurodegeneration has also been analyzed via microarray analysis, which indicates that TLR4 and MyD88 gene expression is increased in the hippocampus of aging and Alzheimer's disease populations (Cribbs *et al*., 2012). Additionally, increased expression of TLR4 has been found in both microglia and astrocytes near lesions caused by multiple sclerosis (MS) and other neurodegenerative conditions (Bsibsi *et al*., 2002). As TLR4 is linked to microglial activation (Jack *et al*.,

2005), upregulation of TLR4 protein expression in a microglial cell line identified in this research may contribute to neurodegeneration if an individual is under simultaneous morphine treatment (**Section 4.1.1**).

Further exacerbating and linking surgery to TLR4 activity is the finding that morphine causes gram-negative (and other) bacterial overgrowth in the intestines of rats having undergone central venous and subcutaneous infusion lines (Kueppers *et al*., 1993). Additionally, when morphine is given, it acts as a chemoattractant for the gut bacteria *P. aeruginosa* (Babrowski *et al*., 2012). *P. aeruginosa* is beneficial for intestinal mucous in healthy conditions; however, morphine exposure switches the bacteria to suppress the intestinal mucous in mice via an unknown mechanism (Babrowski, *et al*., 2012). While these studies did not directly address TLR4, *P. aeruginosa* is a gram-negative bacterial ligand for TLR4 (**Table 1**), making it possible to consider that TLR4 overactivation during and after surgery may occur as the two TLR4 ligands, bacteria and morphine, migrate together. Additionally, recent evidence has identified non-canonical opioid-TLR4 interactions (Stevens *et al*., 2013) which further implies that the findings of morphine as a chemoattractant for *P. aeruginosa* may be TLR4 mediated.

As far as the central nervous system is concerned, cranial and spinal surgeries may further expose TLR4-expressing microglia to bacteria, although the risk may be lower than previously thought (McClelland III and Hall 2007; Dashti *et al*., 2008; Shiono *et al*., 2012). Microglia are the cells with the highest expression of toll-like receptors and as microglia are the primary immune surveyors of the CNS they are instrumental in host CNS immune defense (Carty and Bowie, 2011). When the microglia withdraw their long ramifications and activate in response to a stimulus, there is an increase of proinflammatory cytokine

release mediated by TLR4 (Hines *et al*., 2013). Additionally, as glial activation is also linked to the development of pain and even hyperalgesia (Watkins *et al*., 2001), this could be a non-dopaminergic stimulus leading to abuse as illicit users of opioids typically become addicted via dopaminergic reinforcement (Koob and Volkow, 2010). In the situation that Watkins *et al,* 2001 describes, the drugs prescribed to control the pain may also contribute to the pain, resulting in a cyclical pattern of use that could quickly form from trying to control the pain. Given the many implications of changes in TLR4 expression and activation, it can be seen that TLR4 regulation has both beneficial and detrimental implications. The greater understanding of some of the opioid-mediated changes in TLR4 protein expression identified in this research has implications to help better understand several neuropathologies.

4.1 THC-Induced TLR4 Activity in HEK-Blue™-hTLR4 Cells

This initial preliminary experiment was primarily done in order to gain familiarity with the HEK-Blue™-hTLR4 cells and the SEAP assay. The HEK-Blue™-hTLR4 cells overexpress TLR4 and are an excellent way to study TLR4 activity via activation of SEAP upon NFκB activation. Because THC was already in the laboratory and the investigator has a great interest in natural products, the idea that THC might influence TLR4 activity became an ideal means to satisfy both goals.

THC is the active compound in marijuana and has both medicinal and recreational properties. In the past few years some states have approved the use of marijuana for medical purposes and on January 1, 2014, Colorado set the national stage and began

allowing legal sales of it for recreational use (Steinmetz, 2013). Research has also shown that cannabis has anti-inflammatory properties that are at least in part due to THC (Gertch *et al*., 2008). However, as with most issues in science and inflammation, other research has shown that chronic marijuana use may contribute to microglial-induced inflammation in the brain and cognitive dysfunction (Cutando *et al*., 2013).

Because TLR4 is an important receptor in inflammation, a In the 24-hour time course, the gradual increase in TLR4 activity induced by both LPS and LPS + THC as was expected because LPS is a TLR4 agonist (Buchanan *et al.*, 2010). This indicates that in the presence of THC, earlier timepoints have no effect on LPS-induced TLR4 activity; however, after 24 hours the THC appears to inhibit LPS-induced activity dose-dependently. This THC-induced inhibition of TLR4 activity indicates that THC may be modulating TLR4 after continued treatment. THC could be interacting with CD14 or MD-2 in a manner similar to the natural products curcumin and paclitaxel (Taxol) (**Table 1**). THC is also a large organic molecule and may be sterically hindering the LPS-TLR4 interaction. Because the THC and LPS were not toxic to the cells, the inhibition of TLR4 activity is not likely attributable to toxicity.

Other variables might also be involved in this interaction, such as dose and chronic versus acute use. The apparent dose-dependent decrease in TLR4 activity at 24 hours supports this notion. Individuals undergoing chronic marijuana use tend to present with slower cognitive and reaction skills to which neuroinflamation may contribute (Cutando *et al*., 2013). This may be at least partially due to a decrease in TLR4 activity. Additionally, users who frequently smoke cannabis likely have higher concentrations of THC in their system as THC is a lipophilic compound and is stored in adipose tissue (Rawitch *et al*.,

1979). As a greater inhibition is observed at higher doses of THC, the decrease in TLR4 activity at these doses may indicate a suppressed immune function. Further research into the effects of THC on TLR4 activity, expression, and mechanism of action would assist in determining specific causes for THC-induced inhibition of TLR4 activity.

4.2 Opioid- TLR4 Activity in LPS-Induced HEK-Blue™-hTLR4 Cells

The inhibition of LPS-induced TLR4 activity may be explained by a potential opioid binding pocket associated with TLR4 as is suggested by Stevens *et al*, 2013. This is further indicated by the finding that the opioids by themselves tend to inhibit TLR4 activity when compared to unstimulated cells. This inhibition may have similar possible mechanisms to that postulated for THC such as steric hindrance or interaction with the accessory proteins CD14 or MD-2 but because previous work in this lab indicates a specific binding area for opioids to TLR4 it is also possible that ligand bias may be contributing to the decrease in TLR4 activity which may help explain why the different opioids inhibit TLR4 activity to different degrees. This could result in an intracellular signaling event that could inhibit TLR4 such as inhibition of NFκB. By inhibiting NFκB, activation of TLR4 would not allow for NFκB p65 translocaction into the nucleus to induce transcription of pro-inflammatory cytokines. While these are merely speculative mechanisms, additional research is needed to actually determine *how* TLR4 activity is being inhibited in this cell line.

The activity associated with the unstimulated cells indicates that TLR4 may have constituitive activity. Whether or not this is associated with the overexpression of TLR4 in the HEK-Blue™-hTLR4 cells is unclear but remains a distinct possibility as TLR4 is

capable of self-activation via physical contact with the TIR domains when overexpressed (Xu *et al*., 2000). In this data, the morphine, methadone, and buprenorphine all inhibited TLR4 activity while oxycodone had no effect. Together, this data indicates that opioids inhibit TLR4 both in the presence and absence of LPS which may help explain the phenomenon of opioid-induced immunosuppression. If TLR4 is inhibited by the opioids, then by definition it would not be able to respond to any pathogenic invasion. The fact that oxycodone did not affect TLR4 activity indicates that it may be a better choice for pain control as it leaves the protein in a position to respond to pathogens.

4.3 Methadone-Induced 6- and 18-Hour hMOR Protein Expression in HEK-Blue™-hTLR4 Cells.

While both hMOR protein and RNA were isolated, only the protein was studied due to the difficulties in getting the RT-PCR to work that were addressed in **Section 3.3**. However, the western blot data indicates that with increasing doses of methadone the trend is an increase in hMOR expression either with or without LPS (**Figure 16**). Because this cell line overexpresses TLR4, any changes in TLR4 expression would not be detected. The changes in hMOR expression were interesting because the literature is inconclusive regarding methadone-induced hMOR expression and indicates that methadone downregulates hMOR in human neonatal mononuclear cells (Chavez-Valdez *et al.*, 2013) while another study found that methadone increased hMOR expression in peripheral blood lymphocytes (Vousooghi *et al*., 2009). These discrepancies could be due to the different cell types and ages (neonatal cells versus adult cells) that were investigated. An increase in hMOR expression from methadone may initially provide an increase in analgesia but as

was reviewed in **Sections 1.1.2** and **1.2.4**, tolerance is likely to occur. The fact that the studies aforementioned are in disagreement indicates that further research needs to be done in this area; however, in conjunction with the current study all cells discussed are immune cells and this reaffirms opioid-immune interactions.

4.4 MTT Toxicity in HEK-Blue™-hTLR4 Cells

In the HEK-Blue™-hTLR4 cell line, the majority of the doses of drugs used are not toxic to the cells. The highest doses of methadone and buprenorphine were toxic as were higher doses of LPS; however, even higher doses are used in the literature (Shanmugam *et al.,* 2012) even in the HEK-Blue™-hTLR4 cell line (Hutchinson *et al*., 2010a; Stevens *et al*., 2013). Overexpression of TLR4 in this ell line might also contribute to these toxic results as LPS-induced activation of TLR4 may overstimulate the cells.

4.5 Opioid-Induced Effect on TLR4 Activity in HEK-Blue™-hTLR4 Cells

When the opioids were the only treatment on the cells, the same inhibitory trend was seen as when the cells were simultaneously treated with LPS. Despite toxicity of the highest doses of methadone and buprenorphine, the higher doses of morphine and the lower doses of methadone and buprenorphine inhibited TLR4. The difference in the doses of morphine and methadone is intriguing because the two drugs have opposite immunomodulatory effects as was discussed in **Section 1.6.3**. Morphine is considered to be immunosuppressive (Brown *et al*., 2011; Magazine *et al.*, 1996, Gavériaux-Ruff *et al*., 1998; Budd, 2006) while methadone restores the immune function of opioid abusers

(Sacerdote *et al*., 2008). Perhaps the high doses of heroin used during abuse are partially to blame and the decrease in TLR4 activity is responsible for their increased susceptibility to opportunistic infections (Roy *et al*., 2011). Knowing that in MMT programs higher beginning doses of methadone or buprenorphine are required to help abstain from opioid abuse due to opioid tolerance (**Section 1.2.4**) (Modesto-Lowe *et al*., 2010), this data suggests that methadone and buprenorphine may help modulate TLR4 activity at high doses by not having a significant impact on TLR4 activity whereas morphine does at the same doses. More research would need to be done on TLR4 activity in patients in MMT programs in order to identify more specific reasons for immune rescue in maintenance therapy programs.

4.6 Message Levels of hMOR Transcript in the Presence or Absence of Methadone in HEK-Blue™-hTLR4 Cells

The endpoint PCR shows that despite the Hutchinson *et al*., 2010a article stating that the HEK-Blue™-hTLR4 cell line does not express hMOR, there does appear to be an upregulation of hMOR mRNA by methadone either with or without LPS. Granted hMOR is not highly expressed, but it is observed. When comparing hMOR to TLR4 transcript in this cell line, one is visually incapable of detecting a difference in the relative abundancies of TLR4 because TLR4 is over expressed in this system. This means that the likelihood of the opioids directly interacting with TLR4 is high; however, this illustrates that a potential role of hMOR in these experiments cannot be completely excluded.

4.7 Detecting hMOR

What quickly became perplexing is the difficulty encountered when it pertained to

detecting hMOR, both at the mRNA and protein level (the issues with the mRNA and troubleshooting attempts were addressed in **Section 3.3**). As is seen in **Figure 17**, hMOR protein is present in the HEK-Blue™-hTLR4 cell line. However, at one point hMOR ceased showing up on the western blots. A positive control, SK-N-MC cell lysate, was ordered from Sigma Aldrich to test whether or not the antibody had quit working (**Figure 20**). The antibody did not detect any hMOR in the positive control. Furthermore, attempts to upregulate the protein by using an hMOR antagonist (naloxone) also apparently failed to upregulate the protein to detectable levels. It was quickly suspected that the antibody may have degraded as TLR4 was still visible on the blot and the β-tubulin loading controls confirm that the western blot gel was run and transferred correctly to the membranes. An additional positive control that was available in the lab, the CHO-hMOR-pIRES cell line which was stably transfected to express hMOR, also yielded negative results regarding hMOR protein expression. As is seen in **Figure 24**, no opioid treatment in the CHME-5 microglia nor the control CHO-hMOR-pIRES cells were showing any hMOR. Again, the β-tubulin present shows that the western blots were performed correctly. Finally, as a last attempt to prove that the antibody was the cause for the unsuccessful hMOR western blots, the primary antibody was quickly pulse-spun down (about 10 seconds) prior to being added to the membranes. This was to pull down any IgG aggregates that might have formed and inhibit the antibody from binding to any protein on the membrane. A second and simultaneous change was in the length of time that the primary antibody was incubated with the membrane at 4°C. Instead of overnight, this incubation period was increased to 4 days in an attempt to maximize antibody binding affinity to the protein. While faint hMOR bands were detectable in the CHO-hMOR-pIRES cells and the naloxone treated CHME-5

cells, it cannot be ascertained as to whether or not hMOR is truly present in these cell lines (**Figure 25**). Furthermore, the issues in obtaining a positive control lends doubt as to hMOR protein expression in these cell lines. Knowing that the western blot technique was successful with a positive β-tubulin loading control suggests that the antibody was the issue in these assays.

The hMOR bands apparently observed at around 70 kDa and 50 kDa in the positive control CHO-hMOR-pIRES cells are likely due to two different isomers present in that cell line. According to the manufacturer, the antibody used can detect up to 16 different isomers of hMOR and considering that the CHO-hMOR-pIRES cells were transfected, it's possible that the transfected template for hMOR was different than one already present. When comparing receptor expression of the two cell lines they have comparable protein expression of TLR4 and IL-1R but the control CHO-hMOR-pIRES cells appear to express more hMOR than the control CHME-5 microglia.

4.8 Receptor Expression in CHME-5 Microglia

The CHME-5 microglia are a human microglial cell line that has been transformed to be immortal. They were established and transfected in France in 1995 using the SV40 large T antigen (Janabi *et al*., 1995). Even after transformation, the cells maintained the characteristics of primary human microglia (Janabi *et al*., 1995). The CHME-5 cells have also been referred to as CHME-3 although it is the same cell line. They are ideal to study the effects of drugs in microglial cell culture as they reproduce quickly and are easy to maintain. Few studies have been conducted to characterize these cells but they have been shown to produce IL-6, IL-1β, and TNFα in the presence of up to 1μg/mL LPS (Atanassov

et al., 1995; Lindberg *et al*., 2005) and upon ammonium acetate treatment—which also caused TNFα and IL-1β secretion but IL-1β to a lesser extent (Atanassov *et al*., 1995). Interestingly, treatement with 1 μM morphine has also stimulated IL-6 secretion as well as upregulated TLR 2, 4, and 9 mRNA expression (Dutta *et al*., 2012); however, morphine-induced upregulation of TLR4 protein in the current study using the same cell line was only found to be significant in the simultaneous presence of LPS (**Figures 33** and **34**). Stimulation with a human coronavirus OC43 failed to produce mRNA for TNFα, monocyte chemoattractant protein-1 (MCP-1) or IL-6 (Edwards *et al*., 2000). The CHME-5 microglia were also found to present the antigens KIM-7, EBM-11, OKM-1, 25F9, and the Fc receptors CD16 and CD32 similar to macrophages (Macouillard-Poulletier de Gannes *et al*., 1998). The CHME-5 microglia respond well to heat shock as evidenced by upregulation of the heat shock protein hsp70 (Macouillard-Poulletier de Gannes *et al*., 1998). Despite their similarity to macrophages, they have not been shown to successfully phagocytose cellular debris (Janabi *et al*., 1995; Macouillard-Poulletier de Gannes *et al*., 1998).

Given the lack of literature on the CHME-5 microglia little characterization has been done and none on the protein expression of immune and opioid receptors. This, combined with the high cost and low availability of primary microglia makes the CHME-5 cells ideal to investigate microglial effects of opioids and characterize the presence or absence of certain receptors (e.g., hMOR) in a human microglial cell line. This will aid other scientists in determining whether or not this is a good model for their area of research, especially if they aim to use a human cell line in opioid neuro-immune interactions.

Therefore, untreated CHME-5 cells were probed for TLR4, hMOR, and IL-1R.

Because microglia are the immune surveyors of the CNS (Matyszak, 1998) and there is a definite opioid-immune interaction (Roy *et al*., 2011), it was hypothesized that TLR4, hMOR, and IL-1R would all be present in the CHME-5 cell line. This was correct for TLR4 and IL-1R; however as was highlighted in **Section 4.7**, the presence of hMOR cannot be determined based on the data collected. This indicates that the CHME-5 microglia make an excellent candidate for investigating CNS immune function but might not be the best choice for investigating opioid receptor-mediated activities. Further characterization of receptor expression will help identify additional uses for this cell line.

4.9 Pilot Dose-Responses

A pilot dose-response experiment was run in an attempt to identify the optimal doses of opioids and LPS for use in subsequent experiments using the CHME-5 microglial cell line. As was determined in **Section 4.7**, hMOR protein expression is not a viable endpoint to quantify. Western blots were stripped and probed for TLR4, which was more robustly present (**Figure 29**). Unfortunately, copious amounts of TLR4 protein made this difficult to quantify and was not found to have a dose-response after methadone or morphine treatment. Ultimately, neither the expression endpoint of hMOR nor TLR4 was useful in identifying an optimal opioid dose. Differences in the amount of protein at different doses could not be detected. Furthermore, LPS-treated CHME-5 microglia did not show a difference in protein expression of hMOR or TLR4 either (**Figure 30**). As a result, in order to be able to better compare CHME-5 opioid data with the previous experiments done in the HEK-Blue™-hTLR4 cell line, the same doses from the HEK-Blue™-hTLR4 experiments were used in the CHME-5 cells. A different endpoint or more

quantitative method may have yielded better results. For example, the quantative western blot using the Protein Simple Simon machine to try to better measure the proteins or performing an ELISA to measure NF-κB activation might have been a better option. Alternatively, there might not be a dose response to the opioids used in this study. This is something that could be investigated further.

4.10 MTT Toxicity of Opioids in CHME-5 Microglia

In order to verify that the treatments given to the CHME-5 microglia were not toxic, an MTT toxicity assay revealed that doses were not toxic. This was true for both the opioid treatments when they were made independently of LPS and when simultaneous treatment with LPS was given. These findings allowed for the interpretation of the data to be truly representative of the drugs' effects that will be addressed in **Section 4.11**.

4.11 TLR4 Expression in Response to Opioids in CHME-5 Microglia

As the following discussion highlights, select opioids modulate TLR4 protein expression both independently and in the presence of the endotoxin LPS.

4.11.1 Morphine

Although morphine has been shown to activate and upregulate TLR4 in some studies, it did not affect this protein in the CHME-5 microglia; however, in the presence of LPS, upregulation of TLR4 protein expression indicates that when in the presence of an infection (simulation by the bacterial endotoxin LPS) morphine may cause the protein

expression to be induced, allowing for the opportunity to overactivate. While this experiment did not measure TLR4 or microglial activity, LPS is a well-known agonist for TLR4, upon which overactivation can lead to inflammation resulting from excessive cytokine production (Biswas and Lopez-Collazo 2009). For example, experiments in the HEK-Blue™-hTLR4 cells showed that TLR4 activity is increased by LPS (**Figure 15**); however, even in this system when the opioids were included with LPS, the maximum activity of LPS alone appeared to decrease although statistical significance was not achieved when morphine was included in the experiment. While this is not in complete agreement with the work of the Wang lab, in collaboration with the laboratories of M. Hutchinson and L. Watkins, their work indicates that morphine activates TLR4 by interacting with MD-2 (Wang *et al*., 2012). The work of Stevens et al. (2013) also indicates that morphine potentially activates TLR4 but any increase in activity was minimal and the interaction was not necessarily at MD-2. Despite the exact site of activation and ligand binding, and while morphine alone did not change TLR4 protein expression in the CHME-5 microglia, TLR4 is upregulated by morphine with LPS may indicate that in this human microglial cell line the two treatments potentiate each other to generate a greater expression of TLR4. This could lead to a potential associative scenario that increased TLR4 expression could be indicative of increased TLR4 activity because there is more protein present. Evidence is now beginning to accumulate indicating that TLR4 may be responsible for microglial activation (Sun *et al*., 2015). Microglial activation leads to the synthesis of Aβ-amyloid precursor protein (APP) and as excess APP is associated with Alzheimer's disease, active microglia may contribute to this pathogenesis (Banati *et al*., 1993). While it is unknown whether or not the TLR4 in the CHME-5 microglia are active,

it remains a possibility that should be investigated. Therefore, the upregulation of TLR4 that occurs with methadone and morphine in the presence of LPS could be the genesis of impaired CNS immunity if it is verified that the upregulated TLR4 is indeed active. Coupled with LPS, the increase in TLR4 protein expression may contribute to a greater incidence of opportunistic infection in those on opioids (Roy *et al*., 2011) if the protein is verified to be active after the treatments of morphine + LPS or methadone. While this experiment cannot corroborate these possibilities, it could help explain some of the immunological and proinflammatory effects of morphine, specifically in the case of opportunistic infection (Roy *et al*., 2011).

4.11.2 Methadone

It has been known for several years that methadone modulates and even rescues immune function in opioid abuse (Kreek 1990, Zajícová *et al*., 2004, Sacerdote *et al*., 2008). Methadone had the most pronounced effect on TLR4 protein expression in this set of experiments. The significant upregulation of TLR4 protein expression by methadone and drastic downregulation in the presence of LPS has several implications. This data supports the research done in 1995 by Thomas *et al*., which highlighted differences in immunomodulation by diacetylmorphine, aka heroin, and methadone. It was found that IL-6 production by murine macrophages was decreased after methadone and LPS treatment. Additionally, in human whole blood samples isolating peripheral blood mononuclear cells (PBMCs), methadone was similarly found to decrease IL-6 levels after stimulation by a monoclonal antibody (Boland *et al*., 2013). Because TLR4 activation and

signaling leads to transcription of IL-6, it is reasonable to consider that like in the CHME-5 microglia, TLR4 downregulation might be at least partially responsible for the cytokine decrease identified in the Boland *et al.,* study. In a rat model, TLR4 mRNA downregulation was also found to ease pain associated with bone cancer (Lan *et al*., 2010), but it is not clear whether or not there was less protein. Taken together, the TLR4 downregulation by methadone in the presence of LPS identified in this research may explain cytokine decreases identified in previous studies. Alternatively, it may also increase the likelihood for infection due to the lack of TLR4 protein ready to respond, especially if LPS is already present in the system.

The upregulation of TLR4 by methadone is seemingly contradictory to the negative effects of increased TLR4 expression discussed in **Section 4**. As methadone has been shown to rescue immune function of opioid dependent individuals (Sacerdote *et al*, 2008), it seems more logical to think that methadone would downregulate the protein. It does downregulate TLR4 with LPS present but perhaps methadone is acting at a different part of the TLR4 protein complex that could initiate a different response. A co-immunoprecipitation experiment could identify which proteins, if any, are interacting. If this is the case then once LPS is simultaneously present the LPS may work with the methadone to inhibit TLR4 expression. Or perhaps methadone is inhibiting LPS as was indicated in the HEK-Blue™-hTLR4 experiments described in **Section 4.2**. This is intriguing as they are both referred to as agonists at TLR4 (Hutchinson *et al*., 2010a) but once they are combined, they diminish its expression. This is the opposite of the combined upregulation of TLR4 by morphine and LPS discussed in **Section 4.11.1**. This could be structurally based as morphine is a larger molecule than methadone. This indicates that

opioids might be having a more pronounced effect on TLR4 protein regulation because despite LPS being constant, the opioid is the variable contributing to opposing regulatory effects. The possible explanations for the differences between morphine and methadone in the presence of LPS will have to be further investigated.

4.11.3 Oxycodone

Oxycodone had no significant effect on TLR4 protein expression even in the presence of LPS. While oxycodone may suppress the immune system-particularly in cases of abuse-as a general rule it is not considered to be an immunosuppressant. This is corroborated by a study of infection in human cancer patients given either morphine or oxycodone for pain that found the only relationship between the drugs is that those given morphine were significantly more likely to develop infections than those who were given oxycodone (Suzuki *et al*., 2013). In comparison with the morphine data in **Section 4.11.1**, morphine in the presence of LPS upregulates TLR4 protein expression and perhaps this is in part the reason that the morphine treated patients were more susceptible to infection. Because cancer patients are already immunocompromised, it's possible that the unresponsiveness of TLR4 protein expression to oxycodone with or without LPS may partially explain the fewer infections in the oxycodone treated patients. While the study did not investigate specific cytokine profiles or TLR4 activation, the lack of effect that oxycodone had on TLR4 protein expression may contribute to the safer immune profile associated with this drug.

4.11.4 Buprenorphine

Like methadone, buprenorphine is used to treat opioid dependency and has also been shown to rescue immune function in opioid abusers (Sacerdote *et al*., 2008). The downregulation of TLR4 protein expression in the presence of LPS identified in this research supports these findings. As buprenorphine is considered to be a safe drug based on its pharmacology, such as a ceiling effect on respiratory depression (Pergolizzi *et al*., 2010) and virtually no effect on the immune system (Canneti *et al*., 2013), the lack of effect of buprenorphine on TLR4 protein expression is not surprising. This research corroborates the aforementioned studies and provides a possible explanation for the safer immune profile of buprenorphine by not affecting TLR4 protein expression.

4.11.5 Opioid Comparison (Compilation of Sections 3.11.5-3.11.7)

When simultaneously comparing morphine, methadone, oxycodone, and buprenorphine it is quite obvious that morphine and methadone elicit the greatest effects on TLR4 protein expression (**Figure 45**). More interestingly is the dynamic opposite effect that morphine and methadone both have in the presence of LPS. This could explain some differences in opioid-induced immunomodulation. While all four of the drugs used in this study are opioids, they all have unique stuructures, pharmacokinetics, and pharmacodynamics as addressed in **Section 1.1**. It is possible that this is a reason for the differences in TLR4 regulation. The difference that LPS has in regulating TLR4 when combined with the different opioids may also be attributable to the physical and

pharmacological properties of the drugs. How they interact with the protein has yet to be determined but could occur directly at TLR4, CD14, MD-2, or even during intracellular signaling. It's possible that the opioids may interact with LPS itself or that there may be a TLR4-hMOR interaction. Additional research is necessary to determine which, if any, interaction is occurring.

The upregulation of TLR4 by methadone was not expected. Methadone was expected to downregulate the protein because of the immunologic rescue effect that it has on drug abusers (Riss *et al.,* 2012). The immune rescue associated with methadone led to the idea that TLR4 is downregulated in opioid use because without a TLR4 response there would be very little to no reaction to infection (Roy *et al*., 2011). While there is no evidence that excessive TLR4 protein expression is deleterious, it is known that excessive TLR4 activation can lead to detrimental effects such as sepsis (Biswas and Lopez-Collazo, 2009); therefore, additional research needs to be done to determine if the increases in TLR4 protein expression identified in this study are active or not. While there are generally positive effects associated with less TLR4 as discussed in **Section 4**, the fact that methadone upregulated the protein was surprising. Perhaps this is unique to the CHME-5 microglia as they are an immortalized cell line and may respond differently to the same stimuli than normal cells.

This experiment also was not mimicking chronic drug abuse; therefore, the initial effect of methadone on TLR4 protein expression might be to upregulate it. If the cells were chronically treated with morphine prior to methadone exposure the results might be very different. What is even more surprising is that when the microglia were simultaneously treated with methadone and LPS the protein expression was significantly decreased at all

concentrations of methadone. Because this data of methadone with LPS illustrates the downregulation of TLR4 protein expression and LPS is a well characterized TLR4 agonist (Chow *et al*., 1999), this treatment was expected to upregulate it—but it did the opposite. Likewise, morphine appeared to upregulate TLR4 protein expression, but with LPS it was downreglated. Why would morphine and LPS upregulate TLR4 but methadone and LPS downregulate it? The two drugs are having different interactions with the receptor but exactly what that interaction is remains to be determined. Additionally, the 18 hour treatment might contribute to this as at 18 hours of sole LPS treatment did not alter TLR4 protein expression when compared to control (**Figure 45**). The effect of LPS may have been moot at this point. However, as heroin abusers are more likely to succumb to infection, both because of lifestyle (Kaushik *et al*., 2011) and opioid use (Roy *et al*., 2011), the inclusion of LPS in the treatment is a more realistic model.

The lack of change in TLR4 potein expression from oxycodone and buprenorphine treatments was not surprising as these two drugs are not considered to have as much of an immunosuppressive effect (Suzuki *et al*., 2012, Pergolizzi *et al*., 2010). The question remains as to why? All opioids used in this study were used at the same concentration, yet they varied in the results. While morphine and methadone are potent MOR agonists (Volpe *et al*., 2011), oxycodone has less potency at MOR and more at KOR but very similar analgesic potency (Kalso 2005, Volpe *et al*., 2011, Pöyhiä and Seppälä 1994) and buprenorphine is a relatively potent agonist at MOR, but is also a KOR and ORL-1 agonist (Greenwald *et al*., 2007, Compton *et al*., 2006, Yamamoto *et al*., 2006). No pharmacokinetic studies have yet been done on these drugs at TLR4 as their non-canonical interaction with this receptor has only recently been identified (Stevens *et al*., 2013, Wang

et al., 2012, Hutchinson *et al*., 2010) and the lack of hMOR in the CHME-5 microglia further corroborates these findings. Additionally, it is possible that these drugs have varying agonist potencies at TLR4. Again, varying molecular structures may also have an effect on how and where they interact with TLR4. These potential interactions could also affect how the drugs regulate TLR4 because opioids have been shown to modulate immune activity via NFκB (Hutchinson *et al*., 2010). More research needs to be done in order to determine these possibilities.

4.12 PCR Based Sequencing TLR4 Primers

TLR4 was readily and reliably detectable in the CHME-5 cells. Because of this, the cell line was ideal to use to sequence the TLR4 primers desgned in the lab. Therefore, CHME-5 microglial RNA extracts were used to sequence the amplicons. The primer sequences received for TLR4 were short, but this allowed for certainty in the TLR4 product. A BLAST analysis confirmed alignment with TLR4, thereby verifying the primers for future use.

CHAPTER V

CONCLUSION

As opioids continue to be used and abused, the effects that they have on the immune system remain a necessary investigation. This research found that the TLR4 overexpressing HEK-Blue™-hTLR4 cells are an excellent means to study TLR4 activity. The cells grow quickly and the SEAP assay is straightforward. Using this cell line, it was determined that TLR4 activity is decreased by opioids with or without simultaneous LPS treatment. This finding of opioid-induced inhibition of LPS-stimulated TLR4 activity supports recent literature that opioids have non-canonical interactions via TLR4 (Stevens *et al*., 2013) and further supports the Davis *et al* 2015 study which found that the opioid antagonist β-funaltrexamine inhibits NF-κB signaling in astrocytes. The very low amounts of hMOR detected in this cell line are not believed to be interacting in this system but additional research using an opioid antagonist to ensure that hMOR is not signaling would affirm this hypothesis.

Further investigating TLR4 in neuro-immune interactions, the use of CHME-5 micoglial cells were found to robustly express TLR4 and IL-1R protein expression. hMOR protein expression in this cell line could not be determined because a positive control band could not readily be established. However, this is the first time that the CHME-5 microglia

have been characterized to identify immune and opioid receptor protein expression. This research provides novel information regarding a transformed human microglial cell line. Further studies to confirm the lack of hMOR are needed but the data found in this research concludes that the CHME-5 microglia are ideal to study non-canonical neuro-immune interactions and protein expression because they lack hMOR protein expression.

Additionally, this characterization of the CHME-5 microglia contributes to the scientific community because primary human microglia are very expensive and relatively difficult to obtain and culture. This research provides data for those aiming to study human microglia at a more economical cost—especially given recent budget cuts to the National Institutes of Health and decreased funding for research. Rodent, primarily murine, microglial cell lines have been readily studied in the literature and are not directly applicable to humans. While the CHME-5 are not primary microglia, they do provide a solid preliminary foundation to apply for funding to obtain primary human microglia for research.

Given these findings, in the absence of hMOR the opioids morphine and methadone were found to significantly upregulate TLR4 protein expression in the CHME-5 microglial cells. When in the presence of LPS, methadone downregulated TLR4 protein levels. The downregulation of protein expression induced by methadone and LPS could be a synergistic effect of the two treatments. Neither oxycodone nor buprenorphine had a great impact on TLR4 protein expression with or without LPS. Whether or not this is neuroprotective or a not remains to be determined. These data support the work of Davis *et al*., Stevens *et al*., Wang *et al.*, and Hutchinson *et al*., suggesting that there is an opioid-TLR4 interaction. While this study did not evaluate opioid-induced TLR4 activity in

microglia, the protein reglation of TLR4 via opioids indicates that there is more occurring than previously thought. The structurally different opioids in opioid-induced regulation of TLR4 provides potential pharmacological targets to minimize opioid-induced immunosuppression. It is also possible that the different opioids produce different cytokine profiles by themselves and/or in the presence of LPS that could be attributable to potential ligand bias. As TLR4 is associated with microglial activation and microglial activation is associated with apoptosis (Sun *et al*., 2015), keeping TLR4 tightly regulated may also contribute to neuroprotection. Determining if and how TLR4 is activated, as well as to what extent, will also help identify specific ligands for this purpose.

In summary, this is the first research to document receptor protein characterization and opioid neuroimmune interactions in a human microglial cell line. While this data is novel, a few items remain to be addressed. Further studying the TLR4 pathway in these cells would add insight into whether or not the protein identified in this research is active. Additionally, looking at the RNA that was collected would verify whether the changes in expression are regulated via transcript or perhaps the protein is being released from intracellular stores in the situations where TLR4 is upregulated. This additional information will help pinpoint additional molecular targets other than the TLR4 receptor itself in order to pharmacologically regulate it to the advantage of the individual.

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