ROLE OF METHADONE AND BUPRENORPHINE ON THE HIV-1-MEDIATED NEUROINFLAMMATORY RESPONSE

by

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Abstract

Currently over 35 million people are infected with human immunodeficiency virus (HIV) worldwide. Injection drug use is a major risk factor for the transmission of HIV. It is estimated that near 25% of HIV cases in the United States are directly related to injection drug use. Methadone and buprenorphine are commonly used opiate-substitution therapies that are frequently prescribed to HIV patients. Previous studies have indicated that methadone enhances HIV-1 replication in latently infected monocytes and microglia, raising a major concern that methadone and perhaps buprenorphine could play a role in peripheral HIV-1 infection and also in the pathogenesis of HIV-associated neurocognitive disorders (HAND). To address these questions, we chose to examine whether acute and chronic methadone exposure modulate HIV-1 infectivity in a divergent manner. Acute exposure of methadone exacerbates HIV-1 infection, cytokine release and NF-κB-related molecules. In contrast, chronic exposure to methadone exerts a lesser effect in these paradigms. Since the use of cART has not declined the prevalence of HIV-1 associated neurocognitive impairments (HAND), we explored the possible effects of methadone and buprenorphine on HIV-1 infection and HIV-1-Tat-induced glia activation and dysfunction. In vitro cell lines models revealed that methadone and buprenorphine increase HIV-1 infection and expression of CXCR4 and CCR5 receptors. Furthermore, the use of a more suitable in vitro primary mixed-glia model revealed a role of these drugs on the HIV-1-Tat-induced inflammatory response. Release of cytokines, such as RANTES and MCP-1, was enhanced after 8 hours of co-exposure of Tat and

methadone, but not co-exposure with buprenorphine. Conversely, after 24 hours of co-exposure with buprenorphine, but not methadone, increased the release of the mentioned cytokines. Moreover, a greater increase in reactive oxygen species, nitric oxide, and lipid peroxidation was seen after methadone treatment compared to buprenorphine treatment. Interestingly, co-exposure with Tat and buprenorphine, but not Tat and methadone, caused a reduction in glutamate uptake by astrocytes, which is crucial for neurotrophic support of neurons. Our findings reveal that methadone and buprenorphine modulates peripheral HIV-1 infection and HIV-Tat-induced glia dysfunction. Furthermore, this study supports the need to elucidate the broad mechanism in which these drugs exert a modest, but significant, modulation during HIV pathogenesis.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
BBB	Blood-brain barrier
cART	Combined antiretroviral therapy
CD4	Cluster of differentiation 4
CNS	Central nervous system
CXCR4	C-X-C chemokine receptor 4 (CD184)
DOR	Delta opioid receptor (δ-opioid receptor)
gp120	Glycoprotein 120
HAART	Highly active antiretroviral therapy
HAD	HIV-1 associated dementia
HAND	HiV-1 associated neurocognitive disorders
HIV-1	Human immunodeficiency virus type-1
HIVE	HIV-1 encephalitis
IDU	Injection drug users
KOR	Kappa opioid receptor (κ-opioid receptor)
MCP-1	Monocyte chemoattractant protein-1
MIP-1β	Macrophage inflammatory protein-1 beta (CCL4)
MOR	Mu opioid receptor (μ-opioid receptor)
mRNA	Messenger Ribonucleic acid
NF-κB	Nuclear factor kappa B

NOP	Nociceptin/orphanin FQ receptor (NOP)
R5-tropic	CCR5-tropic
RANTES	Regulated upon activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
Tat	Transactivator of transcription
TLR-4	Toll-Like Receptor 4
X4-tropic	CXCR4-tropic

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Chapter 1: Introduction and Background

History of the HIV epidemic

The 1980s marked the beginning of the HIV pandemic, with an unusual increase of opportunist infections and immune deficiency in healthy individuals (Gottlieb et al., 1981). The incidence of these clinical cases continued to increase rapidly and subsequently was termed Acquired immunodeficiency syndrome (AIDS) (MMWR Morbidity and mortality weekly report, (1982)). The diseased was transmitted by blood exposure, sexual contact, and mother-to-child transmission (Gallo, 2000). Researches and physicians raced to find the etiologic agent causing this deficiency, and in 1983 the AIDS retrovirus was successfully isolated (Barre-Sinoussi et al., 1983, Gallo et al., 1983), after which a single anti-retroviral therapy was implemented to treat the disease. Despite the newly discovered therapy against the retrovirus, at that time, the average survival time after receiving an AIDS diagnostic was from 10 to 13 months (Bacchetti et al., 1988).

After combined efforts from researchers and physicians, the successful introduction of highly active antiretroviral therapy (HAART) in 1996 substantially altered the disease course (Collier et al., 1996, Staszewski et al., 1996). Unlike previous anti-retroviral therapy, HAART consisted of various combinations of a reverse transcriptase inhibitor, a protease inhibitor and a nucleosides reverse transcriptase inhibitor. The effectiveness of this therapy involves the intervention of different stages in the HIV-1 life cycle. Aside from the success of the presently

termed combined antiretroviral therapy (cART) in reducing HIV-1 morbidity and mortality (Mocroft et al., 1998, Ray et al., 2010); AIDS is still considered a pandemic. Globally, almost 35 million people were living with HIV at the end of 2012; during that year, 3.2 million new HIV-1 diagnoses and 1.6 of AIDS deaths were reported (UNAIDS, 2013). HIV-1/AIDS continues to be one of greatest epidemic in society.

HIV-1 structure and cycle

HIV-1 belongs to the family of *Retroviridae*, subfamily *Lentivirinae* and genus *Lentivirus*. The genome of the retrovirus is composed of two copies of single-stranded ribonucleic acid (RNA) molecules. Viral RNA is reverse-transcribed to DNA by the viral reverse transcriptase after entering a host cell (Turner and Summers, 1999). HIV-1 possesses accessory proteins (nef, vpr, vif, vpu), structural proteins (gag, pol, env) and regulatory elements such as rev and tat (Ferguson et al., 2002). Early studies demonstrated that the CD4 antigen, principally expressed by T-lymphocytes and monocytes/macrophages, is an essential and specific receptor for HIV cellular entry (Dalgleish et al., 1984). In addition, HIV-1 utilizes various chemokine receptors, such as, chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 4 (CXCR4) as co-receptor for viral entry (Doranz et al., 1996, He et al., 1997).

The first step of the HIV replication cycle is fusion, starts with the viral surface glycoprotein gp120 interaction with cell receptors, after which, viral glycoprotein gp41 is directly inserted into the cell (Ferguson et al., 2002).

Following viral entry, viral RNA is transcribed into RNA by a reverse transcriptase enzyme that the virus carries (Pomerantz and Horn, 2003). Viral DNA can now be transported into the nucleus where viral enzyme integrase integrates it to the host cell DNA (Freed, 2001). Once the viral DNA is integrated to the host cell, the cell is infected for its lifetime. Successively, the transcription stage begins when proviral DNA is used as a template for the production new viral RNA (Stevenson, 2003), and new viral RNA is formed and transported out of the nucleus. Viral proteins and enzymes are translated from viral RNA, and assembled into a bud. After assembly is completed, the virus buds from the cell outer membrane and a new viral particle is release. A final maturation step allows the viral particle to become infectious (Sundquist and Krausslich, 2012).

HIV progression

Approximately four weeks following the infection, known as acute phase, the majority of HIV-infected individuals present flu-like clinical symptoms associated with high plasma viremia, fever, lymphadenopathy, myalgia, skin rash, headache, and diarrhea (Moir et al., 2011). Although during acute phase symptoms are considered minor, there is abrupt uninhibited viral replication, causing permanent damage to CD4+ lymphocytes. Almost one billion new HIV copies are produced each day in an acutely infected person (O'Brien and Hendrickson, 2013). Significant decrease in viral replication marks the beginning of the chronic phase. During this phase viral load eventually stabilizes, known as

viral set point, allowing immune cells to develop a cytotoxic response against the virus.

HAART is very efficient at maintaining viral load below detection, but it has no effect on the virus capacity to remain inactive or latent in numerous cells including lymphocytes, dendritic cells and glia (Saksena et al., 2010). Although there is a continuous renewal of both CD4+ and CD8+ T lymphocytes, (Montagnier, 2010), after several years, the virus gradually depletes CD4+ lymphocytes. CD4+ depletion is caused by persistent immune activation and direct HIV-induced cell death (Miedema et al., 2013). It takes numerous years after the primary infection to develop advanced HIV symptoms (Fauci, 1993) and consequently progress to AIDS. During AIDS stage, patients suffer from numerous opportunistic infections and chronic complications such as neurocognitive impairments, which lead to death.

HIV-1 Tat

Tat is a nonstructural viral protein composed of 86 to 104 amino acids, formed from two exons. Despite the high mutagenesis of HIV-1, Tat is a relatively a well-conserved protein (Debaisieux et al., 2012). HIV-1 Tat is produced early in the HIV-1 virus cycle and, when released by infected cells, not only Tat can interact with extracellular receptors, it can also be internalized by endocytosis (Chang et al., 1997, Liu et al., 2000). Tat-mediated direct toxicity lays on the capacity to modulate numerous cellular pathways, resulting in cellular injury (Eugenin et al., 2007, Kim et al., 2008), however, indirect toxicity seems to have

a more detrimental effects (Chang et al., 1997).

It was demonstrated that HIV-1 Tat possess structural motifs typical of the β-chemokine family. Tat not only has the ability to signal through β-chemokine receptors, but also attracts monocytes toward virus producing cells (Albini et al., 1998). Furthermore, HIV-1 Tat activates NF-**κ**B, which leads to the induction of nitric oxide production and cytokine release in glia and monocytes (Chen et al., 1997, Polazzi et al., 1999, Rayne et al., 2004). Furthermore, HIV-1 Tat indirectly potentiates NMDA-evoked increases in intracellular calcium concentration $[Ca2+]_i$ (Krogh et al., 2014). Additionally, HIV-1 Tat significantly impairs glutamate uptake by astrocytes (Zhou et al., 2004), consequently leading to excitotoxicity. Among other HIV-1 proteins, Tat has an important role in mediating central nervous system (CNS) effects in HIV-1-infected individuals (Nath et al., 1999). In-vitro studies using Tat are closely representative of HIV in-vitro infection. There is vast amount of information about Tat and glia interactions; therefore, all of the non-infectious studies presented will use Tat treatment exclusively.

HIV-1 infection in the central nervous system

HIV invasion of the CNS strikes at an early stage of the primary infection (An et al., 1999). There are several theories describing the mechanism used by the virus to infiltrate into the brain, however, it is widely accepted that the invasion occurs mainly through migration of infected monocytes and lymphocytes from the blood into the brain (Meltzer and Gendelman, 1992, Georgsson, 1994,

Nath, 1999). These infected monocytes subsequently differentiated into perivascular macrophages, and become the main source of virus replication and spread in the brain (Zhou and Saksena, 2013).

Perivascular macrophages, microglial cells and astrocytes can be actively, persistently or latently infected (Nath and Clements, 2011). Chemokines and cytokines play a crucial role hastening leukocyte transmigration and directing the microglia and macrophages movement to infection sites (Hazleton et al., 2010). Infected microglia release viral proteins and neurotoxins such as inflammatory cytokines, reactive oxygen species (ROS) and excess glutamate (Kaul et al., 2001, Wang et al., 2003). While microglia are the major brain-resident cells in the production of new virions and viral proteins, astrocytes can be extensively infected with HIV as well (Churchill et al., 2009). Infected astroglia also contribute to the release of neurotoxic and inflammatory molecules; however, one of the main consequences of HIV infection in astrocytes is the disruption of neurotrophic support to neurons (Nath et al., 1999, Hauser et al., 2006). Furthermore, these inflammatory and neurotoxic responses lead to an increase in neuronal injury and death (Achim et al., 1994),

HIV-associated neurocognitive disorders (HAND)

The use of combination antiretroviral therapy (cART) has drastically improved medical morbidity of HIV infection. Not only it has been successful in controlling viral infection but it has also decreased the incidence of HIV dementia (Sacktor et al., 2000). Although the more severe neurological deficits, particularly

associated with HIV encephalitis (HIVE) are almost inexistent, neuroinflammation still occurs to a significant extent (Zhou and Saksena, 2013). Therefore, HIV neurocognitive disorders are most closely associated to neuroinflammation rather than directly to viral load. Neuroinflammation levels between HIV-1 positive individuals, are still comparable to pre-HAART era (Anthony and Bell, 2008). Even though nowadays HIV dementia is more infrequent, HAND incidence remains high (Heaton et al., 2011), becoming a major concern in medical care of chronic HIV patients.

Around 40% of HIV patients suffer from a certain level of HAND (Sacktor, 2002). Among this population, patients may suffer from minor forms of neurocognitive abnormalities known as asymptomatic neurocognitive impairment (ANI), minor neurocognitive disorders (MNDs), considered as moderate deficits, and the most severe form termed HIV-1 associated dementia (HAD) (Ellis et al., 2007). Though these impairments hold different levels of severity, common deficits range from disturbance in memory learning, retrieval of new information, psychomotor slowing, attention deficits, and disturbances in executive functions (Grant, 2008).

Opiates and HIV: Interlinked epidemics

Opiate abuse and HIV are interconnected epidemics. Estimates indicate that injection drug users (IDUs) accounted approximately 25% of HIV cases in the USA in 2011 (CDC, 2012). Opiates abusers engaged risky behaviors leading to a faster spread of HIV. Aside from the concern of opiates-abuse associated

behaviors increasing HIV, these drugs can also increase the rate of viral replication (Peterson et al., 1994) and hasten the progression to AIDS (Donahoe and Vlahov, 1998). Opiates injecting drug users (IDU) are at increased risk of developing neuronal and cerebrovascular disorders (Smith et al., 2014), and a more rapid progression of HIV-mediated cognitive disorders due (Kumar et al., 2006). Furthermore, opiates abusers are more likely to develop HIV-1 encephalitis (HIVE) (Bell et al., 2002).

It seems that there is a strong association between opiates abuse and HIV-1 neuropathogenesis (Anthony and Bell, 2008, Dutta and Roy, 2012, Hauser et al., 2012). In vitro models have shown that opiates exacerbate HIV-1 Tat induced neuronal cell death (Gurwell et al., 2001, Hauser et al., 2006), and it is well defined that the presence of glia is critical for this consequences (Zou et al., 2011). Vast amount of evidence demonstrate that opiates aggravate glial dysfunction triggered by HIV infection in the brain (Stiene-Martin et al., 1993, Peterson et al., 1994, Nath et al., 2002, Kumar et al., 2004, El-Hage et al., 2005, Hauser et al., 2005, Turchan-Cholewo et al., 2006, Bruce-Keller et al., 2008, Hazleton et al., 2010). Glia show a synergistically increase of Tat-morphine mediated cytokines and chemokines production, such as MCP-1, RANTES, and IL-6 and intracellular calcium release (El-Hage et al., 2005). Not only opiates can exacerbate HIV-1-induce inflammation and neurotoxicity, these drugs can alter endogenous opioid peptide levels and modify the neural response to HIV, affecting the response of the nervous system to the virus (Nath et al., 2002).

Treatments for opiate dependence

Currently, a wide variety of treatments for illicit opiate dependence are available, including detoxification, residential/abstinence treatments and behavioral interventions (Fischer et al., 2002). Opioid maintenance treatment, based on oral pharmacotherapy, is the most generally used, especially by HIV-1 positive individuals. These treatments are associated with reductions in intravenous drug use and HIV risk behaviors, such as unsafe sexual conduct (Weber et al., 1990, Selwyn et al., 1992, Sorensen and Copeland, 2000, Stotts et al., 2009). Methadone, a full μ-opioid receptor agonist, has been the standard of care for illicit opioid dependence for the past 40 years (Mauger et al., 2014). A stabilization dose is achieved, normally between 60mg–120mg daily, and infrequently an increase in dose due to development of tolerance is needed (Bart, 2012). Estimates indicate that methadone long terminal elimination half-life is approximately 33 to 46 h (Wolff et al., 1997); therefore, methadone has longer effect than other opiates, reducing euphoria and withdrawal symptoms.

One major inconvenient of methadone treatment is that during the initial stages of therapy, patients must go to a licensed clinic every day to receive their dose of methadone (Jerry and Collins, 2013). Hence, the introduction of buprenorphine, an office-based maintenance treatment broadened the alternatives for these patients. Buprenorphine is a partial μ-opioid agonist and κ-opioid antagonist (Mello and Mendelson, 1985). Buprenorphine has a higher affinity for the μ-receptor than other opiates, such as heroin, thus, it reduces the

effects of full agonists (Wesson, 2004). Nowadays, FDA-approved Suboxone®, the combination of sublingual buprenorphine/naloxone, seems to be a logical first-line office-based maintenance treatment for opioid dependence (Wesson, 2004).

Methadone and buprenorphine interaction with the immune system

Opiate dependence treatments are associated with a significant reduction in drug- and sex-related HIV risk behaviors (Sullivan et al., 2008). Furthermore, theses treatments also contribute to the sustained adherence to highly active antiretroviral therapy (HAART) in HIV-infected IDUs (Roux et al., 2008). Although methadone maintenance therapy is the standard treatment for heroin addiction, and it is still the most effective method for addiction therapy (Toskulkao et al., 2010) the available evidence of the effect of methadone on the immune system is controversial. Some studies have shown that long-term methadone normalizes abnormalities in cellular immunity of heroin abusers (Novick et al., 1989). However, in vitro and in vivo studies demonstrated that methadone suppresses function of monocytes, neutrophils, NK cells and T-cells, and the production of IFN-γ (Peterson et al., 1989, Novick et al., 1991, Chuang et al., 1993, Choi et al., 1999). Furthermore, methadone activates HIV replication in acutely and latently infected human macrophages/microglia and PBMC (Li et al., 2002)**.**

Whereas methadone is the most widely used maintenance treatment for opioid addiction, the partial opioid agonist buprenorphine is a valid alternative to methadone-maintenance treatment of chronic heroin abuse (Neri et al., 2005).

The introduction of buprenorphine has broadened the number of agonist-based methods available to treat opioid dependence in HIV patients (Altice et al., 2006). Studies had suggested that buprenorphine has fewer interactions with antiretroviral medications (Gruber and McCance-Katz, 2010) and preserves more cognitive function (Mintzer et al., 2004) than methadone. Conversely, it was also demonstrated that buprenorphine induces potent dose-dependent, opioid receptor-mediated immunosuppressive effects (Carrigan et al., 2004). Despite methadone and buprenorphine benefits to intravenous drug users in terms of decreasing HIV risk behaviors there is no enough evidence whether they aggravate HIV infection-induced glial dysfunction.

Figure 1.1

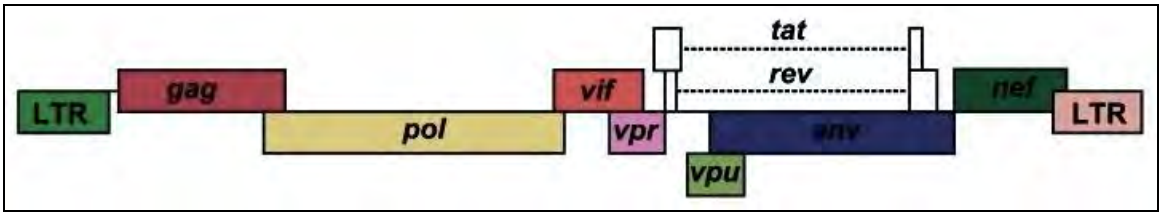

Figure 1.1 The HIV proviral genome consists of the structural genes gag, pol, and env. The regulatory genes are rev and tat and the accessory genes are nef, vpu, vif and vpr. Image taken from (Vlachakis et al., 2013).

Figure 1.2

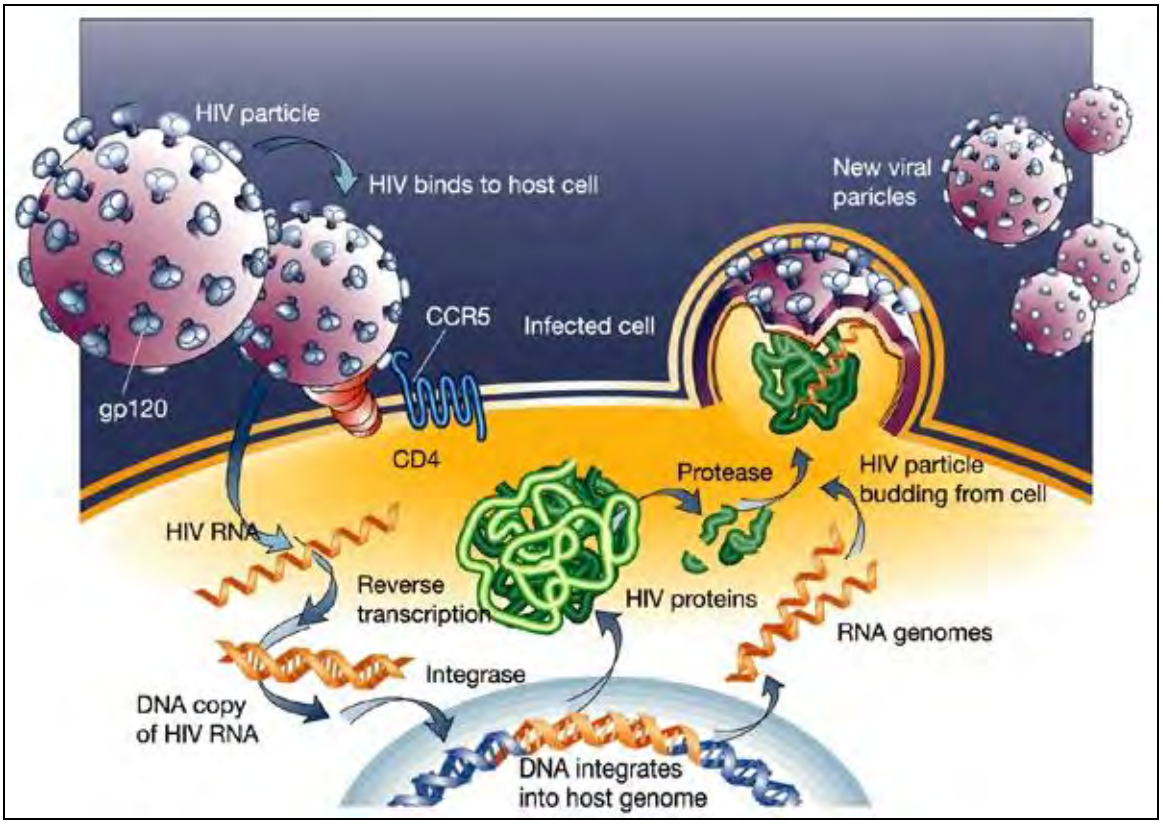

Figure 1.2 HIV-1 cycle starts with the fusion of the virus with the cell membrane. Following with the reverse transcription of the single-stranded viral RNA to double-stranded viral DNA. The viral DNA is integrated into cellular DNA and transcripted to viral messenger RNA. The mRNA is used to produce long chains of viral proteins. During assembly, HIV protease hydrolyzes the long chains of viral proteins into functional proteins. New virions are then assembled and bud off from the host cell. Image taken from (Weiss, 2003).

Figure 1.3

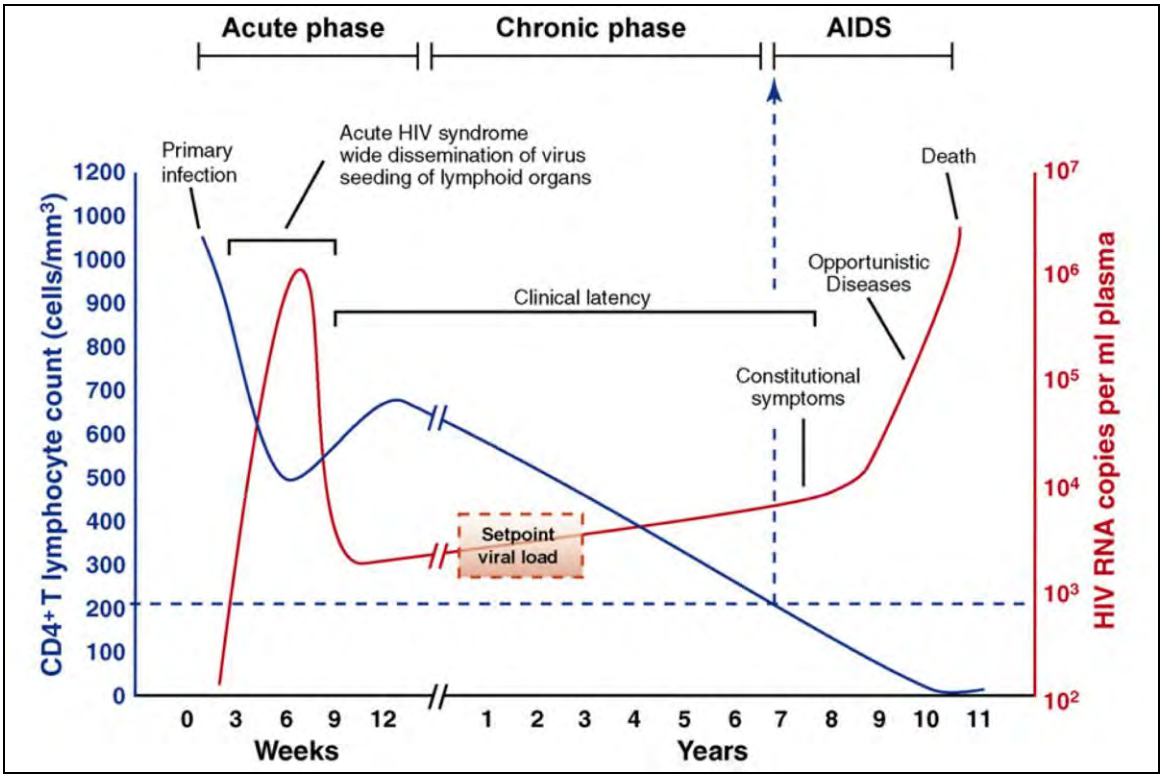

Figure 1.3 HIV pathology progression starts with the exposure to HIV-1 and the infection of the CD4+ T-lymphocytes. During the acute phase, the virus titer in circulating blood begins to rise and CD4+ lymphocytes decline by the infection. Although the Immune response is active, this stage shows the highest virus titer. During the chronic phase, the viral load reaches a plateau level, known as setpoint, and patients present minimal symptoms. After several years, the virus gradually decreases the CD4 lymphocytes levels to below 200 CD4+ cells/mm3. This stage is denoted as AIDS, the patients suffer from a variety of innocuous, opportunistic infections and chronic complications leading to death. Image taken from (O'Brien and Hendrickson, 2013).

Figure 1.4

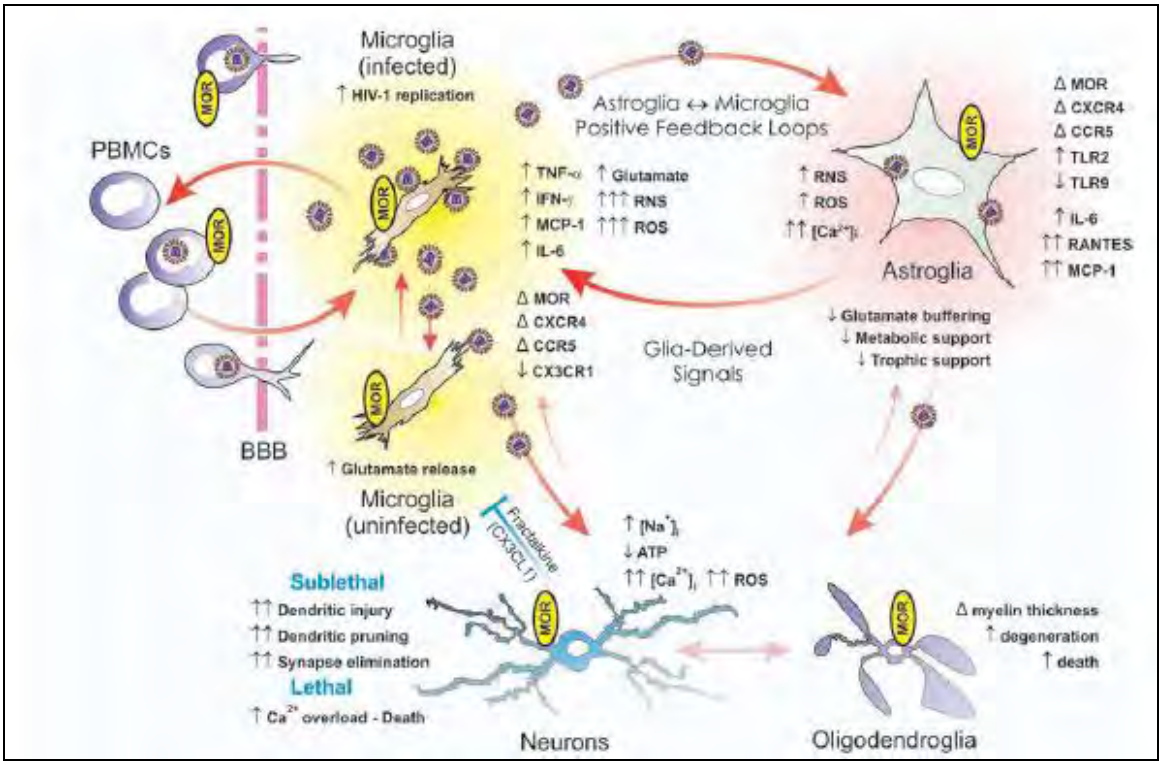

Figure 1.4 During HIV infection of the brain, infected peripheral monocytes infiltrate through the blood brain barrier. Monocytes differentiate into microglia and infect adjacent microglia. Infected microglia release viral particles, cytokines/chemokines and cytotoxic molecules. These inflammatory signaling alter astrocytes function of offering metabolic and trophic support to neurons. Microglia-astrocyte inflammatory responses lead to neuronal injury and death. Image adapted from (Hauser et al., 2012).

Chapter 2: Chronic and Acute Effect of Methadone on HIV-1 Infection and Cytokine Release in Human Peripheral Blood Mononuclear Cells

Introduction

Worldwide, approximately 40 million people are infected with HIV-1. With the increase of HIV-1 infection cases, the problem of drug abuse among HIV-1 patients has become a great concern. HIV-positive individuals with a history of IDU experienced higher rates of death and AIDS after starting cART, compared with individuals without a history of IDU (Murray et al., 2012). Although a number of studies have revealed that drug abusers have a lower survival rate (Selwyn et al., 1992, Lucas et al., 2006), reports indicate that effect of opiates drugs on the immune response is dependent on the length and condition exposure (Carpenter et al., 1995, Yeager et al., 1995, Avila et al., 2004), leading to paradoxical observations. For example, it has been shown that chronic use of opiates induces a state of tolerance; subsequently, withdrawal from the drug leads to decreased immune function (Govitrapong et al., 1998, Avila et al., 2004) and increased viral replication (Donahoe and Vlahov, 1998). Furthermore, opiates appear to contribute to a shift in the cytokine TH1/TH2 profile (Sacerdote et al., 2003). This shift in cytokine/chemokine patterns accelerates HIV-1 progression. Moreover, enhanced expression of MCP-1, RANTES and IP-10 may directly contribute to HIV-1-induced T cell depletion, which leads to immunosuppression, pathogenesis and progression to AIDS (Wetzel et al., 2000).

Methadone is widely used by HIV-infected opiates abusers, offering advantages such as the decrease in the frequency of injection and needle sharing (Gibson et al., 1999, Sorensen and Copeland, 2000, MacArthur et al., 2012). Methadone is a successful drug therapy for opiates abusers because of its slow onset and offset, which gives it low abuse liability and reinforcing effects (Kreek et al., 2010). Moreover, evidence has shown that long-term methadone treatment gradually down-regulates the expression of opiate receptors on immune cells, which may restore the immune response (Liu et al., 1999), therefore leading to a slow progression to AIDS (Weber et al., 1990, Novick et al., 1991). Methadone's interaction with the immune system is unclear. Several studies have demonstrated that methadone treatment is associated with lower CD4 cell percentage and lower CD4:CD8 T-cell ratio in HIV-1 positive or HIV-1 negative subjects (Carballo-Dieguez et al., 1994). Further studies showed that methadone treatment enhances HIV-1 infection in human monocytes (Li et al., 2002). The viral infection increased may be attributed to methadone's ability to activate μ-opioid receptor (MOR), inducing the CCR5 chemokine receptor on human lymphocytes, which facilitates viral entry (Suzuki et al., 2002).

It is well established that cytokines and chemokines play a crucial role in orchestrating the HIV-1-induced immune response (Poli and Fauci, 1992, Than et al., 1997). Early studies had demonstrated that TNF-α induced a significant increase in HIV reverse transcriptase activity (Folks et al., 1989). In addition, elevated levels of IL-6 is associated with HIV-1 infection in peripheral blood

mononuclear cells (PBMC) (Breen et al., 1990). The increase of theses cytokines activates the transcription factor, NF-κB (Osborn et al., 1989). NF-κB acts synergistically with the viral tat-III gene product to enhance HIV expression in T cells (Nabel and Baltimore, 1987). Therefore, an increase in cytokines and chemokines production will activate the NF-κB cascade, subsequently leading to an increase in HIV-1 load. We aimed to investigate whether acute or chronic methadone exposures divergently modulate HIV-1 infectivity, cytokine/chemokine secretion and inflammatory molecules in PBMC.

Materials and Methods

Human blood samples

A total of 20 blood and urine samples were collected from HIV-1 negative chronic methadone patients and 5 samples from HIV-1 negative non-drug abusers. The study was done with IRB approval from the Universidad Central del Caribe School of Medicine and all subjects gave informed consent before participating. Chronic methadone patients needed to show a history of injecting drug abuse and had to have been treated with a maintenance dose of methadone for at least one year. Patients and controls were pre-screened for the presence of chronic infections such as HIV-1 and Hepatitis C and presence of other abused drugs and were excluded from the study if any were present. In addition, CD4+ cell counts were performed to assure that individuals were in a similar immunological status (500-1000 cells/μl were considered normal ranges).

PBMC Isolation

PBMCs were obtained from HIV-1 negative chronic methadone patients and HIV-1 negative control individuals. Sixty milliliters of peripheral blood from each participant was obtained by venipuncture. Blood was centrifuged for 10 minutes at 1,600 RPM and plasma was removed. PBMCs were isolated by carefully layering whole blood on Ficoll-Histopaque (Sigma). Tubes were centrifuged at 1600 RPM for 25 min at 20^{o}C. Mononuclear cells were carefully collected from the visible clear interface. Cells were washed twice using phosphate buffer solution (PBS) and centrifuged at 1600 rpm for 10 min at 20^{o}C. After centrifugation, cells were resuspended in 1mL of RPMI 1640 and counted. Cell viability was determined before and after culturing by Trypan blue exclusion (Sigma). PBMC were stimulated with phytohemagglutinin (PHA) (1 µg/mL) at 37^{o}C, 5% CO_2 for 72 hours at 37^{o}C and then washed once with RPMI 1640 and plated in a density of 1x10^{6}/ mL.

HIV-1 Infection

PHA-stimulated PBMCs were cultured in absence and presence of methadone (1µM) for 3 days at 37^{o}C, 5% CO_2. After incubation, cells were acutely infected with the HIV-1_{SF2} strain. PBMCs were incubated in 900 µL of culture medium and 100 µL of HIV-1_{SF2} strain (1000 $TCID_{50}$/ml) for 2hr at 37^{o}C. After 2 hours, 1 million PBMCs were plated for each treatment and additional 2 mL of fresh media were added and incubated for 24 hours at 37^{o}C, 5% CO_2. After 24 hours, cells were washed with 2mL of phosphate buffer saline (PBS) to

remove unabsorbed virus. A total of 6 mL of fresh media and the respective treatments were added to the cells. Media was changed every 72 hours after infection and incubated for 6 days.

Cytokine Measurements

After 6 days of infection, supernatants were collected for the measurement of cytokines and chemokines by using multiplexed cytometric bead array (CBA) kits according with the manufacture (BD Bioscience, San Diego, CA). Briefly, supernatants (50 µL) were diluted and transferred to tubes containing 50 µL of mixed capture beads and phycoerythrin (PE) detection reagents. The mixed-capture was incubated for 3 h at room temperature in the dark, before adding 1 mL of wash buffer. Tubes were then centrifuged (200 x g for 5 min) and pellets were resuspended in wash buffer. After preparation, prepared samples were read in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Analysis and statistics were performed using the CBA software package (Becton-Dickinson, Mountain View, CA). Cytokine and chemokine concentrations were expressed as pg/mL.

Quantitative RT-PCR

Quantitative RT-PCR was performed following the manufacturer's instructions (ROCHE COBAS AmpliPrep/COBAS TaqMan HIV-1 Test). Supernatants samples were prepared automatically using the AmpliPrep/COBAS instrument with amplification and detection using the COBAS TaqMan Analyzer. An automated reverse transcription, PCR amplification and detection of HIV-1

target RNA and HIV-1 Quantitation Standard (QS) Armored RNA was performed. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon. The HIV-1 QS was added to each supernatant specimen and was carried through the specimen preparation, reverse transcription, PCR amplification and detection steps of cleaved dual-labeled oligonucleotide detection probes. The HIV-1 RNA concentration of the test specimens was calculated by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control.

NF-κB and Inflammatory Molecules

After 1 and 6 hours of treatment with HIV-1 Tat and/or methadone, cell lysates from acutely exposed PBMC were prepared by adding 1X Cell Lysis Buffer plus 1 mM phenyl-methylsulfonyl fluoride (PMSF) lysates were sonicated on ice. For measurement of NF-κB signaling molecules, a sandwich ELISA was performed following the manufacture's directions (Cell Signaling Technology, Beverly, MA). Briefly, samples were incubated for 2 hours at 37°C. After incubation, plates were washed 4 times following the addition of the detection antibody for 1 hour 37°C. Plates were washed once again and a horseradish peroxidase-linked secondary antibody was added, followed by another set of washes. Tetramethylbenzidine substrate was added for 10 minutes at 37°C followed by a stop solution. The colorimetric reaction was allowed to develop

color and absorbance was read at 450 nm. The magnitude of absorbance is proportional to the quantity of bound target protein.

Results

Acute methadone treatment elevates HIV-1 infection on human PBMC

To assess whether an acute methadone treatment increase HIV-1 infection, PBMCs from healthy donors were infected with HIV-1_{SF2} virus for 24 hours and pre-treated with naltrexone for 30 min before treatment with methadone for 6 days. An acute methadone treatment significantly increases HIV-1 replication on PBMC (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). Groups receiving naltrexone pre-treatment for 30 min before exposure to agonist or naltrexone alone did not show significant difference between controls. Moreover, naltrexone was able to reverse methadone increase in viral load (§$p < 0.05$ vs. MTD, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 5 experiments.

Acute methadone treatment elevates cytokine release on HIV-1 infected human PBMC

PBMCs from healthy donors were infected HIV-1_{SF2} virus and treated with MTD for 6 days, at day 6 culture media was collected for cytometric bead array assay. Unpaired t-test analysis showed that methadone significantly increased TNF-α and IL-6 release on HIV infected PBMCs compared to HIV alone groups (*$p < 0.05$ vs. HIV). Data is based on the mean ± SEM of n = 5 experiments.

Effect of acute methadone on NF-κB activation molecules expression on human PBMCs

To explore whether methadone increased on HIV-1 infectivity and cytokines release is dependent of NF-κB pathways, PBMC were treated with combinations of MTD, and/or HIV-1 Tat for 8 hours. Methadone alone and HIV-Tat groups showed a significantly increased p65 subunit of NF-κB expression when compared to control groups (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). In addition, co-exposure with Tat and methadone exacerbate the Tat-induced increase of this subunit (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). In the case of the NF-κB regulator, Phospho-IκBα (Ser32) expression, methadone treatment did not significantly increase protein levels of IκBα when compared to control. However, co-exposure with methadone significantly increased HIV-Tat effect on Phospho-IκBα (Ser32) expression (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n= 3 experiments.

Effect of chronic methadone treatment in HIV-1 infection on human PBMCs

PBMCs from chronic methadone users were infected HIV-1_{SF2} virus and treated with MTD or un-treated for 6 days, at day 6 culture media was collected for RT-PCR analysis. Unpaired t-test analysis showed no significant difference in viral load between groups that did not receive methadone treatment or groups treated with methadone. Data are based on the mean ± SEM of n= 20 experiments.

Effect of chronic methadone treatment in cytokines release on human PBMCs

PBMCs from chronic methadone users were infected HIV-1_{SF2} virus and treated with MTD or treated vehicle lacking of MTD for 6 days, at day 6 culture media was collected for cytometric bead array assay. Unpaired t-test analysis showed no significant difference in TNF-α and IL-6 release between groups that did not received methadone treatment and groups treated with methadone. Data are based on the mean ± SEM of n= 20 experiments.

Discussion

Methadone maintenance treatment programs remain central to the management of drug use in the HIV population (Durvasula and Miller, 2014). Although these treatments can prevent the psychosocial and physical morbidity of drug misuse (Weber et al., 1990), there is not enough evidence of the role of methadone use on HIV-1 infection. Herein, we show that methadone acute treatment increased HIV-1 infection in PBMCs. These observations correlate with the findings of Li and colleagues (2002) where methadone treatment activates viral replication in latently infected peripheral blood mononuclear cells from HIV-infected patients. Suggesting that not only methadone could activate HIV-1 replication from latency, it can also worsen a recently acute HIV-1 infection. Although we did not measure CCR5 expression, others have shown that this receptor is uprgulated by methadone and HIV-1 replication is activated (Suzuki et al., 2002). Increased expression of CCR5 facilitates viral entry, but it may also be

a convergence point for opiate-HIV-1 interactions (Podhaizer et al., 2012).

In addition to the increases in CCR5, we aimed to elucidate whether acute treatment of methadone exacerbated the release of pro-inflammatory cytokines. In vitro studies suggested that methadone suppresses chemokines such as IL-8, MIP-1β, and RANTES (Choi et al., 1999), which decrease the migration of neutrophils and monocytes to infection sites. With this suppression in the cellular immune response, the main antiviral mechanism available to cells gets weakened. In contrast, in vivo studies demonstrated that methadone stimulates immunologic hyperactivation of an immune system that was formerly inhibited by heroin, particularly, in the increase of plasma levels of TNF-α, IL-2β, and IL-1β (Neri et al., 2005). Our in-vitro studies show that methadone exposure increases TNF-α and IL-6 release in acutely infected PBMC. HIV-1 infection severity has been associated with the increases in these pro-inflammatory cytokines (Hazenberg et al., 2003, Naif, 2013, Van der Watt et al., 2014). It is important to note that HIV-1 immune pathogenesis is characterized by an imbalance in the cytokine profile, rather than the modulation of a specific cytokine (Clerici and Shearer, 1994).

The pathogenesis of HIV is also controlled by the cellular transcription factor, NF-κB (Maciaszek et al., 1994). HIV-1 Tat counteracts the post-activation turn off of NF-κB through direct interaction with IκB-α and p65, which enhances the DNA binding and transcriptional activity of the NF-κB complex (Fiume et al., 2012). Since NF-κB stimulates viral transcription and elongation (West et al.,

2001), we aimed to explore whether methadone's capacity to increase HIV-1 infection is due to an interaction trough this mechanism. We report that acute exposure of methadone increased the expression of the p65 subunit of NF-κB when compared to control. Moreover, co-exposure of HIV-Tat and methadone exacerbate Tat-induced increases in this subunit. The phosphorylated form of IκB is needed for the release of NF-κB from IκB, and this release subsequently leads to nuclear entry and activation of NF-κB (Ghosh and Baltimore, 1990). Although methadone treatment did not show an effect on phospho-IκBα (Ser32) expression, we observed a significant increase in its expression with Tat treatment. Furthermore, co-exposure with methadone exacerbates the HIV-Tat-induced increase in phospho-IκBα (Ser32) expression. This finding suggests that phospho-IκBα (Ser32) is not being ubiquitinated; subsequently p65 is not being translocated to the nucleus. Methadone-induced increase HIV-1 infection may be mediated by interactions with NF-κB signaling.

It has been suggested that prolonged methadone maintenance treatment is not associated with unexpected adverse effects (Novick et al., 1993). Although methadone treatment seems to offer a better outcome for patients (Fischer et al., 2002), there is not direct evidence whether the long-term effect of methadone could modulate HIV-1 infection. In vitro studies demonstrated an impaired responsiveness of lymphocytes to mitogens in methadone patients (Quagliata et al., 1977); however, a normal T-cell proliferative response to antigens in another cohort of long-term methadone users (Reddy et al., 1987) was also reported.

Herein, we show that PBMCs from chronic methadone users shows a less acute viral infection when compared to viral infection with acute methadone exposure in PBMCs from non-users. In addition, chronic exposure to methadone does not exacerbate the HIV-opiate-mediated TNF-α and IL-6 release as seen in PBMCs from healthy donors. Although we induced an abrupt withdrawal of methadone in PBMCs from chronic methadone users, we did not observe a significant change in viral load and cytokine release from controls when compared to a continuous methadone exposure in cells from the same donor. We concur with the suggestion of Liu and colleagues (1999) that chronic methadone treatment may normalize the immune response. Overall, our findings demonstrate that acute methadone exposure enhances HIV-1 infection through the release of pro-inflammatory cytokines and the activation of NF-κB. In contrast, a long-term methadone exposure seems to normalize the peripheral immune response and leads to a lower extent of HIV-1 infectivity.

Figure 2.1

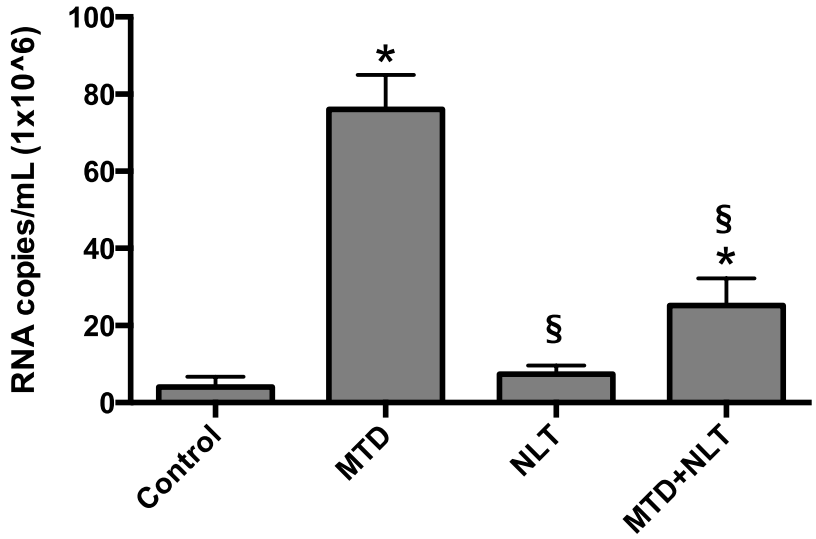

Figure 2.1 Effect of acute methadone treatment in HIV-1 infection on human PBMCs. Cultures infected with HIV-1_{SF2} virus and treated with combinations of NLT (pretreatment) and/or MTD for 6 days. Analysis showed that acute methadone treatment significantly increased HIV-1 replication (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). NLX treatment was able to reverse the increase on viral load (§$p < 0.05$ vs. MTD, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 5 experiments.

Figure 2.2

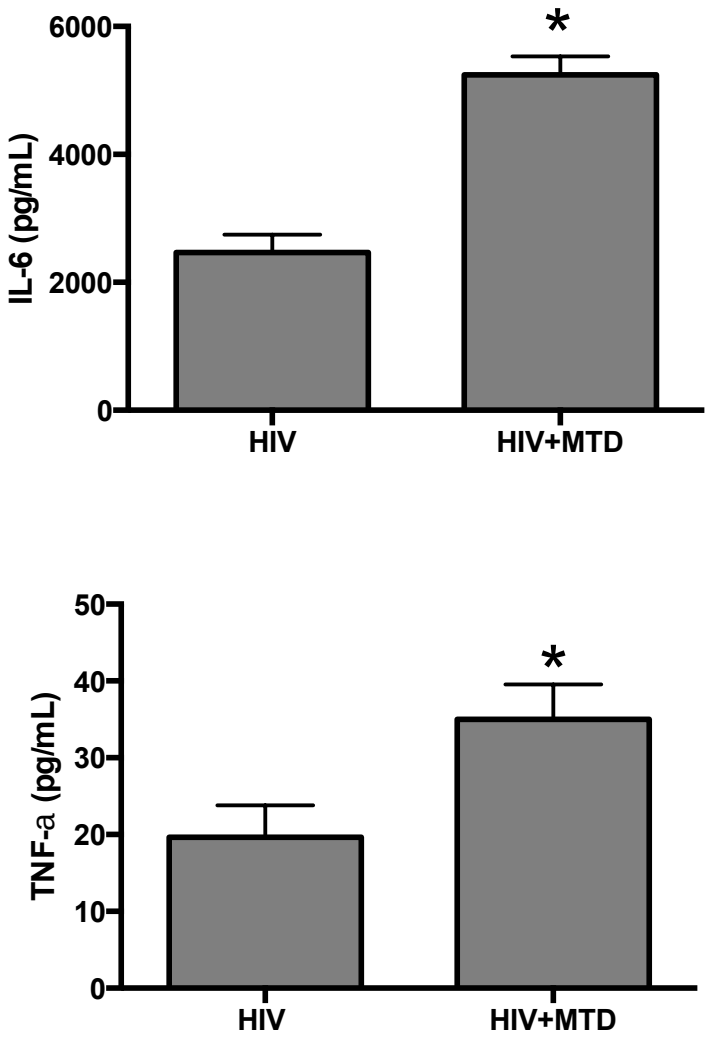

Figure 2.2 Effect of acute methadone treatment in cytokine release on HIV-1 infected PBMCs. Cultures were infected with HIV-1_{SF2} virus and treated with MTD for 6 days. Analysis showed that methadone significantly increased TNF-α and IL-6 release (*$p < 0.05$ vs. HIV, unpaired t-test). Data are based on the mean ± SEM of n = 5 experiments.

Figure 2.3

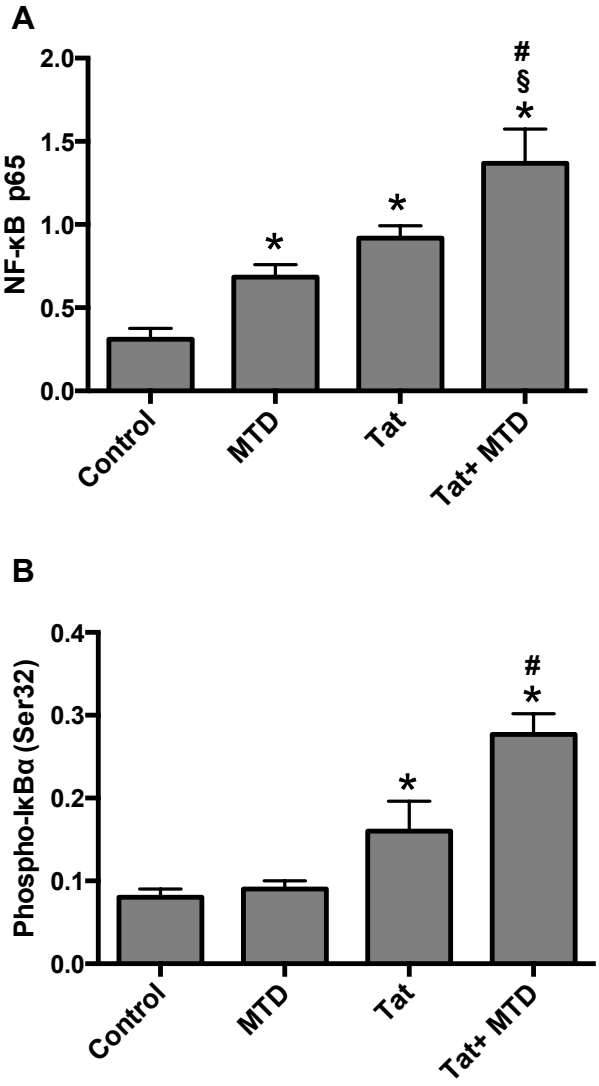

Figure 2.3 Effect of acute methadone on NF-κB activation molecules expression on human PBMCs. PBMCs were treated with combinations of MTD, and/or HIV-1 Tat for 8 hours. Methadone alone and HIV-Tat groups significantly increased p65 subunit of NF-κB expression when compared to control groups (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). Co-exposure with Tat and methadone exacerbate the Tat-induced increase of this subunit (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc) **(A)**. Methadone treatment did not significantly increase protein levels of IκBα when compared to controls. Co-exposure with methadone significantly increased the effect of HIV-Tat effect on Phospho-IκBα (Ser32) expression (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc test) **(B)**. Data are based on the mean ± SEM of n= 3 experiments.

Figure 2.4

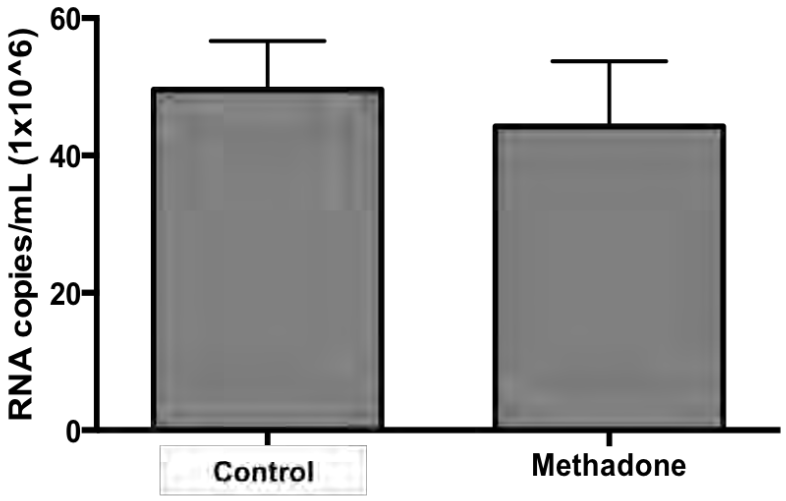

Figure 2.4 Effect of chronic methadone treatment in HIV-1 infection on human PBMC. Cultures were infected with HIV-1_{SF2} and treated with MTD for 6 days. Unpaired t-test analysis showed no significant difference in TNF-α and IL-6 release between controls and chronic methadone exposure groups. Data are based on the mean ± SEM of n = 20 experiments.

Figure 2.5

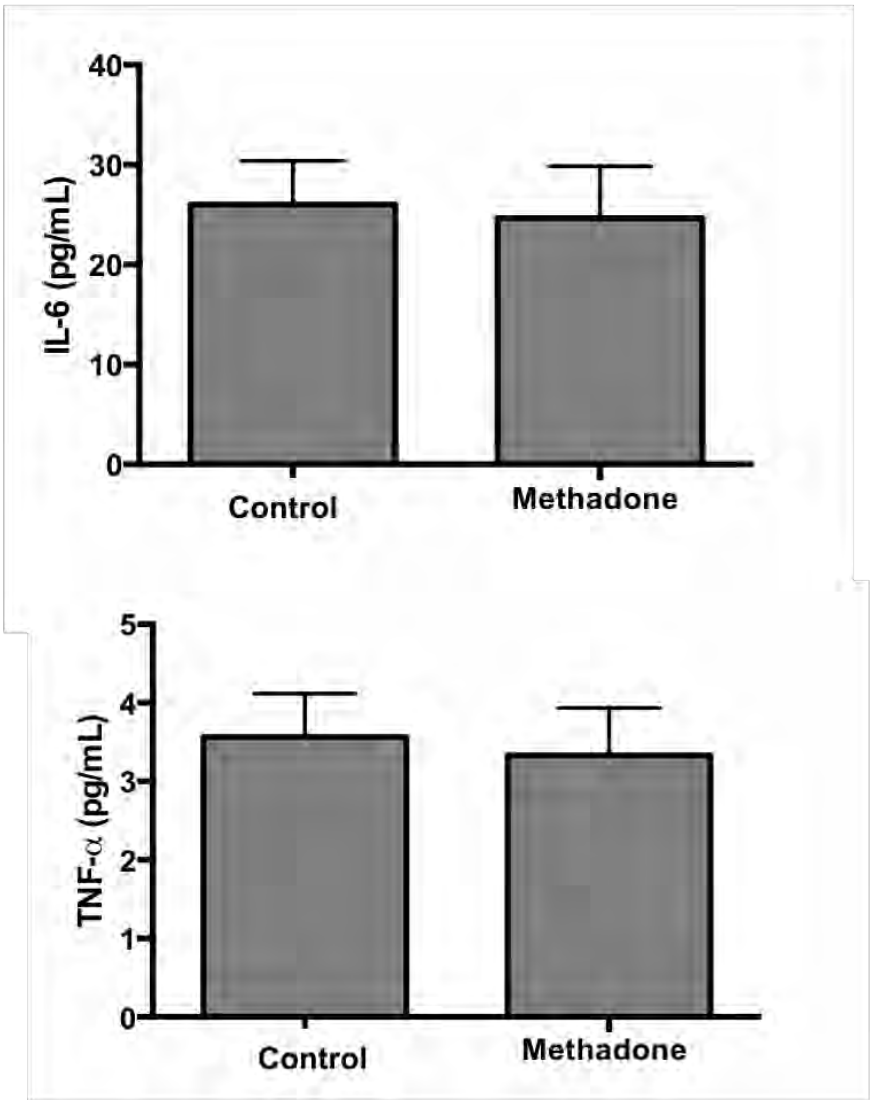

Figure 2.5 Effect of chronic methadone treatment in cytokine release on human PBMCs. Cultures were infected HIV-1_{SF2} virus and treated with MTD for 6 days. Unpaired t-test analysis showed no difference between HIV-1 viral load in controls and chronic methadone exposure groups. Data are based on the mean ± SEM of n = 20 experiments.

Chapter 3: Methadone and Buprenorphine Effect on Human Glial HIV-1 Activation and Infection

Introduction

The increase survival of HIV-1 infected individuals using cART therapy, has raised the prevalence of HAND (McArthur et al., 2010). Although antiretroviral therapy maintains viral load below detection, neuronal injury remains constant. This suggests that, in the absence of new viral replication, an additional mechanism must be involved in HAND pathogenesis. It is well established that glial activation due to HIV infection plays a key role in neurocognitive disorders (Wiley et al., 1986, Peterson et al., 1992, Hauser et al., 2005). Macrophages and microglial are critical reservoirs of HIV and they are the principal site of productive infection in the brain (Churchill and Nath, 2013). Microglial neuroinflammation is commonly associated with the production of reactive oxygen species (ROS) and NO-dependent reactive nitrogen species (RNS) (Kraft and Harry, 2011). The constant release of inflammatory molecules by this infected microglia alters astrocyte function. The role of astrocytes role in HIV-1 infectivity is more controversial. Although these cells have a low frequency of HIV infection (Nath et al., 1995); astrocytes are indispensable for brain homeostasis. Thus, the interaction between activated microglia, astrocytes, and neurons triggers the onset of neuronal dysfunction or apoptosis and the progression of CNS damage (Dutta and Roy, 2012).

A strong relationship exists between opiate usage and HIV-1 neuropathogenesis. Opiates have been shown to enhance HIV-1 replication in vitro (Peterson et al., 1994, Bell et al., 1998, Bell et al., 2002). Opiates exacerbate HIV-1's detrimental effects through interactions with glia (El-Hage et al., 2005, Zou et al., 2011), specially the enhancement of microglial activation (Arango et al., 2004). Early studies demonstrated that morphine potentiates TNF-α production in human microglia (Peterson et al., 1994). In addition, there is evidence that morphine dramatically enhances HIV-Tat-induced production of IL-6, IL-1β, IP-10, and iNOS in murine microglia (Bokhari et al., 2009). These inflammatory signals, specifically, the increase of TNF-α, IFN-γ and IL-1β induce neuronal apoptosis and death in vitro (Downen et al., 1999). Therefore, there is a strong connection between cytokine/chemokine release and microglia-mediated inflammation. In addition to the modulation of the glial inflammatory signaling, morphine elevates CCR5 and CXCR4 gene (Li et al., 2003, Steele et al., 2003), facilitating HIV entry to uninfected cells.

The role of opiate maintenance treatments in HAND pathogenesis is not well understood. Previous studies have demonstrated that methadone patients show high rates of psychiatric comorbidity and cognitive deficits compared to matched controls (Darke et al., 2000). Moreover, heroin abusers show verbal memory deterioration during the first week of methadone-aided withdrawal treatment (Curran et al., 1999). It was demonstrated that methadone disrupts acetylcholine release and consequently impairs verbal memory to a greater

extent than buprenorphine (Rapeli et al., 2007). However, other studies concluded that both buprenorphine and methadone patients performed poorly on visual memory tests compared to controls (Pirastu et al., 2006). While there are several studies implying that methadone and buprenorphine may modulate cognitive functions, there is not extensive evidence regarding the role of these drugs on HIV-1-glia-mediated inflammation. We aimed to elucidate whether methadone and buprenorphine increase viral infectivity and whether this interaction aggravates glia infection and activation.

Materials and Methods

Cell lines cultures

Human microglia cell line (CHME-5) kindly provided by Dr. Randall Davis, Oklahoma State University and human astrocytes cell line (U-87 MG) (ATCC, Manassas, VA) were cultured in Eagle's Minimum Essential Medium (EMEM; ATCC, Manassas, VA), which contained penicillin/streptomycin (100 U/mL and 100 µg/mL; Invitrogen) and 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). Cell were seeded and maintained until 70-80% confluence was reached.

Opiates treatment, HIV infection and Tat treatment

To measure HIV-1 infectivity, human glial were acutely infected with p24 50 pg/10^6 cells of a dual tropic (R4/R5) HIV-1_{SF2} strain obtained from National Institutes of Health AIDS Reagents originally isolated by Dr. Jay Levy, for 2 hours at 37°C in 1mL of culture media. After 2 hours of infection at 37ºC and 5% CO_2,

an additional 2 mL of media was added and cells were cultured for 24 hours. After 24 hours, cells were washed with phosphate buffered saline (PBS) to remove unabsorbed virus. Fresh culture medium and appropriate treatments were added. Cells were cultured for 6 days and media was exchanged with fresh media containing same reagents and treatments every 72 hours. Supernatants and cells were collected for further analysis. Aside HIV-1 infection measurements, all of the testing paradigms included HIV-1 Tat exclusively. For HIV-1 Tat_{1-86} (Immunodiagnostics, Inc.; Woburn, MA) treatment, human glial cells were seeded until confluence was reached. A total of 1.0 mL of fresh media was added and the cells were treated with methadone or buprenorphine (1uM) in the presence or absence of 100 nM of HIV-Tat for 6, 8 or 24 hours. After incubation time, supernatants and cells were collected for further analysis.

Quantitative RT-PCR

Quantitative RT-PCR was performed following the manufacturer's instructions (ROCHE COBAS AmpliPrep/COBAS TaqMan HIV-1 Test). Supernatant samples were prepared automatically using the AmpliPrep/COBAS instrument with amplification and detection using the COBAS TaqMan Analyzer. An automated reverse transcription, PCR amplification and detection of HIV-1 target RNA and HIV-1 Quantitation Standard (QS) Armored RNA was performed. The detection of amplified DNA is performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon. The HIV-1 QS was added to each

supernatant specimen and was carried through the specimen preparation, reverse transcription, PCR amplification and detection steps of cleaved dual-labeled oligonucleotide detection probes. The HIV-1 RNA concentration in the test specimens was calculated by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control.

Cytokines Quantification

Supernatants were used for the measurement of chemokines and TH1/Th2 cytokines by flow cytometry. Quantification was conducted by cytometric bead array (CBA) analysis (BD Biosciences, San Jose, CA) according to the manufacturer. Briefly, 50 µL of culture supernatants was diluted and transferred to tubes containing 50 µL of mixed capture beads and phycoerythrin (PE) detection reagents. Resulting solution was incubated at room temperature for 3 h in the dark. Samples were washed with buffer and centrifuged at 200 x g for 5 min. Resulting pellets were resuspended in wash buffer. Samples suspensions were read in a FACSCalibur flow cytometer (Becton-Dickinson). Analysis and statistics were performed by CBA software package (Becton-Dickinson).

Flow cytometry

CXCR4 and CCR5 immunoreactivity were detected by direct immunofluorescence in human glial cells by using flow cytometry. Human glial cells were washed in PBS/0.1% BSA buffer and incubated with allophycocyanin (APC)-conjugated anti-CXCR4 and Alexa-488 tagged anti-CCR5 antibodies in

permeabilization buffer (PBS/0.1% BSA/0.1% triton X) to detect surface and intracellular expression. Fluorescence was measured from 10,000-gated glial cells per treatment in each experiment using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). Autofluorescence was compensated by setting the detector voltage to the minimum level that discriminates between autofluorescence and specific immunofluorescence in both negative and positive controls.

Nitric oxide quantification

Nitric oxide (NO) production by glial cells was measured using the Griess Reagent System (Promega, Madison, WI), according with the manufacture. Briefly, in a 96-well plate, 50 µL of culture supernatant was dispensed into the well in triplicate. After adding all the culture supernatants, 50 µL of the sulfanilamide solution was added to all the samples and incubate for 10 minutes at room temperature, protected from light. After incubation, 50 µL of the *N*-1-napthylethylenediamine dihydrochloride (NED) solution was added to all the wells and incubate for an additional 10 minutes at room temperature, protected from light. Absorbance was measure within 30 minutes in the plate reader with filters between 520 nm and 550 nm. Concentration of NO was calculated based on the standard curve using known concentrations of nitrite.

Reactive oxygen species measurement

Reactive oxygen species were quantified by the cell-permeable dye, 2',7'-dichlorofluorescein diacetate (DCF-DA). Cells were incubated for 1 with DCF-DA in warm PBS according to the manufacturer's indications. Dichlorofluorescein (DCF) fluorescence was measured at an excitation wavelength (λ_{ex}) of 485 nm and an emission wavelength (λ_{em}) of 520 nm using a PHERAstar plate reader (BMG Labtech, Cary, NC). DCF-DA, when inside the cell is hydrolyzed by cytosolic esterases to its non-cell-permeable form (DCF) and which fluoresces when in contact with the ROS/RNS species. ROS levels were estimated by relative DCF fluorescence.

MDA Quantification

To assess lipid peroxidation levels, Thiobarbituric Acid Reactive Substances (TBARS) assay Cell Biolabs, San Diego, CA) was performed according to the manufacture's instructions. Briefly, human microglia cell lines were treated with combinations of MTD, BUP, and/or 100 nM of HIV-1 Tat for 1, 4, 8 and 24 hours, and cells were collected and resuspended with PBS. Cells were homogenized on ice and samples were transferred to separate microcentrifuge tubes. A SDS lysis solution was added and incubated at room temperature for 5 minutes. After incubation, TBA reagent was added and incubated at 95°C for 60 minutes. Samples were cooled on ice for 5 minutes and centrifuged at 3000 rpm for 15 minutes. Supernatants were removed and transferred to a 96-well plate. Absorbance was read at 532 nm.

Glutamate uptake quantification

Glutamate uptake was measured in human astrocytes cell line (U87), according to previous studies (Zou et al., 2011). Cells were pre-incubated for 60 min at 37°C with 500 µL of Hank's balanced salt solution (HBSS) alone or with combinations of 1uM methadone, buprenorphine, naloxone and/or 100 nM HIV-Tat. Glutamate at a concentration of 1 mM was added to each well. Sample supernatants were collected from individual wells at 0 min-240 min time points. Glutamate levels were quantified using a glutamate assay kit (BioVision, Mountain View, CA) according to manufacture's directions. Briefly, 10 µL to 50 µL of supernatant will be directly diluted in the assay buffer. 100 µL of reaction mix will be added to the samples and the Glutamate standard. The reaction will be incubated at 37ºC for 30 minutes. Optical density will be measured at 450 nm and glutamate concentrations will be calculated.

Real Time PCR

Cells were collected and total RNA was isolated using TRIzol reagent (Invitrogen). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen; CA) then 1ug of RNA was used to generate cDNA by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) according to the manufacture's instructions. After obtaining total cDNA of each sample a real-time PCR was performed with a volume of 20 µL containing SensiMix SYBR qPCR reagents (Bioline USA, MA) using a Corbett Rotor-Gene 6000 real-time PCR system (Qiagen, CA). PCR conditions consisted of an initial

hold step at 95°C for 10 min followed by 40 amplification cycles of 95 °C for 10 s, 58 °C for 30 s, and 72°C for 30s. Sequences of primers set used for the amplification of mu opioid receptor was 5'-CTTGGAACCCGAAAAGTCTC-3' as forward and 5'-TGCCATCTAAGTGGGACAAG-3' as reverse. This set of primers amplified a product of 187 bp. For human kappa opioid receptor amplification the set of primers used was: 5'-GAAACAAGCCCTGGTGAAAT-3' as forward and 5'-TGAGCCAAGACTGTGCTACC-3' as reverse, amplifying a product of 239bp. For the amplification of TLR-4 the set of primers used was 5'-CAGCTCTTGGTGGAAGTTGA-3' as a forward and 5'-GCAAGAAGCATCAGGTGAAA-3' as a reverse. This set of primers amplified a product of191bp. For GAPDH amplification, the sequences of primers used were 5'- CATGGCACCGTCAAGGCTGAGAA -3' as forward and 5'- CAGTGGACTCCACGACGTACTCA -3' as reverse. The specificity of the amplified products was verified by melting curve analysis and agarose gel electrophoresis.

Results

Methadone and buprenorphine modulation on human microglia HIV-1 infection

After HIV-1_{SF2} infection of human microglia cell lines for 24 hours, cells were treated with combinations of NLX (pretreatment), MTD, and BUP for 6 days. Methadone and buprenorphine significantly increase HIV-1 replication (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). Groups receiving a naloxone

pre-treatment for 30 min before opiate exposure did not show significant difference between controls. Moreover, naloxone was able to reverse the increase in viral load of methadone and buprenorphine co-exposure (§$p < 0.05$ vs. MTD or BUP, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Effect of methadone and buprenorphine treatment on CXCR4 and CCR5 co-expression in human microglia and astrocytes

An increase of CXC4 and CCR5 may facilitate viral entry to the cell (Kalinina et al., 2013). To evaluate methadone and buprenorphine effects on CXCR4 and CCR5 co-expression, cultures were treated with combinations of MTD, BUP, and/or HIV-1 Tat for 8 hours followed by flow cytometry analysis. Drug alone did not exert any effect in these receptors expression compared to controls. Moreover, CXCR4 and CCR5 co-expression did not show any change of expression with the interaction of Tat alone, or combination of Tat and methadone on both cell lines. However, co-exposure with Tat and buprenorphine significantly increase CXCR4 and CCR5 co-expression in human microglia and human astrocyte (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). We did not observed any significant difference between other groups. Data are based on the mean ± SEM of n = 3 experiments.

Effect of methadone and buprenorphine on lipid peroxidation in human microglia cells

High levels of MDA have been associated with oxidative stress and poor cytokines responsiveness in HIV infection. For the assessment of MDA levels in glia, cultures were treated with MTD, BUP, and/or HIV-1 Tat for 1, 4, 8 and 24h. Concentrations of malondialdehyde (MDA), which is a stable indicator of lipid peroxidation, were determined by TBARS assay. Shorter exposure of Tat, drugs alone, or co-exposure of Tat and drugs did not increased MDA levels. However, after 8 hours of exposure, methadone, co-exposure of Tat and methadone and co-exposure with Tat and buprenorphine significantly increased lipid peroxidation when compared to control and Tat alone groups (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc) (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). Interestingly, methadone alone showed the capacity of exacerbated MDA formation, while buprenorphine alone did not exert any significant difference when compared to control. However, it was unexpected to observe that Tat alone did not increased MDA production at any time of exposure. Data indicate the mean concentration ± SEM of n=3.

Effect of methadone and buprenorphine treatment on nitric oxide production by human microglia

To assess nitric oxide production microglia cells were treated with combinations of MTD, BUP, and/or HIV-1 Tat for 6 or 24 hours. One-way ANOVA and Tukey's post-hoc analysis did not show significant difference between

controls and opiates alone, Tat or co-exposure of Tat and methadone or buprenorphine at 6 hours. Treatment was increased to 24 hours to explore whether a longer exposure would affect methadone and buprenorphine effect on nitric oxide production. Although increasing the time of exposure to 24 hours intensified nitric oxide release, no significant differences were detected between controls and treatments. Data are based on the mean ± SEM of n = 3 experiments.

Methadone and buprenorphine effect on intracellular ROS production

To assess whether methadone and buprenorphine interact as well with ROS production, mixed-glia were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 1 and 6 h. Mean DCF relative fluorescence units (MFI) were used as an estimate of ROS. One-way ANOVA, Tukey's post-hoc analyses did not show a significant difference between control and any other treatment groups.

Methadone and buprenorphine effect on glutamate uptake by astrocytes

Glutamate uptake was measured by pre-treating human astrocytes with combinations of MTD, BPN, and/or HIV-1 Tat for 30 min. After pre-treatment, cells were challenged cells with 1 mM glutamate. Media was collected from each group at 0, 15, 30, 60 and 120 min for glutamate concentration quantification. Glutamate levels were assessed from 0 min to 120 min. After 15 minutes of incubation and through the duration of the assay, all treatments show a decrease in glutamate uptake compared to control (*$p < 0.05$ vs. control, repeated

measures ANOVA, Tukey's post-hoc). After 60 minutes of incubation, levels of residual glutamate were significantly higher Tat treatment groups compared with methadone or buprenorphine treatments groups (§$p < 0.05$ vs. drug alone, repeated measures ANOVA, Tukey's post-hoc), there was no significant difference between Tat and co-exposure of Tat and methadone or buprenorphine. After 120 minutes, co-exposure with Tat and methadone showed a decrease in glutamate uptake disruption when compared to Tat treatment and co-exposure with Tat and buprenorphine (¶$p < 0.05$ vs. Tat and Tat+ buprenorphine, repeated measures ANOVA, Tukey's post-hoc). Data shown as concentration of glutamate (mM) ± SEM. n = 3.

Discussion

Due to the exacerbation of neurocognitive disorders in opioid abusers who are HIV-1 infected (Bell et al., 1996), it is of key importance to understand the underlying mechanisms by which opiates enhance these deficits. Since viral spread is the first tread for the initiation of HIV-mediated cognitive deficits, we explored whether methadone or buprenorphine modulate HIV-1 infection. Our findings suggest that methadone, and to a lesser extern buprenorphine modulate HIV infection in human glial cell lines. It is known that the upregulation of CXCR4 and CCR5 receptors lead to an increase in viral entry, and subsequently increase in viral load (Moore et al., 2004).Herein, we show that co-exposure of buprenorphine and Tat increases the co-expression of CXCR4 and CCR5, and contrary to previous experimental outcomes, showing that methadone increases

CCR5 expression in lymphocytes (Suzuki et al., 2002), we found that co-exposure to methadone and Tat did not change the co-expression of these receptors in human microglia and astrocytes. Taking together, this suggests that a convergent proinflammatory response and increased viral entry, rather than increased viral entry alone, drives the noted increase in viral replication.

It appears that HIV-induced cognitive pathogenesis can be explored with the understanding of the glial inflammatory signaling. Studies have suggested that opiates, in particular morphine exacerbate HIV-induced cytokine and chemokine release by glia (Turchan-Cholewo et al., 2009, Zhao et al., 2014). We show that co-exposure with methadone and HIV-Tat increased TNF-α by human microglia cells when compared to control and Tat alone. In the other hand, co-exposure with Tat and methadone significantly decreased TNF-α when compared to Tat alone. During these analyses, it was noted that cytokine release by the human glia cell lines was low and not specific HIV-Tat effect. Furthermore, we explored the effect of methadone and buprenorphine in the production of toxic molecule, however, we did not detect effect any effect of these drugs or HIV-Tat in our model. On the other hand, methadone seems to increase the levels in lipid peroxidation in both microglia and astrocytes.

Besides from the exacerbated production of cytokines and toxic molecules, activated microglia release glutamate to a greater extent, which cause a dramatic elevation of neuronal calcium levels and leads to loss of neurites and synapses (Barger and Basile, 2001). Therefore, we explored the

possible modulation of methadone and buprenorphine on astrocyte glutamate transport. Our results demonstrate that methadone and buprenorphine show a modest, but significant disruption of glutamate buffering. Although, at some time points buprenorphine shows a slight increased in the Tat-mediated glutamate buffering interference, none of the difference noted were significant. Interestingly, after 120 minutes, methadone significantly decreases Tat effect on glutamate uptake and glutamate levels were significant lower than Tat treatment and co-exposure of Tat and buprenorphine.

During this study, several experiments showed an inconsistent or inexistent HIV-opiate cellular response between assay replicates. Because some studies demonstrate that opiates effects, in particular buprenorphine, are dose-dependent (Sanchez et al., 2008, Eschenroeder et al., 2012), we repeated cytokines and infection assays with a range of opiates concentration, however positive effects were not noted at any dose tested. We determined that results discrepancies were not dose related. Furthermore, we speculated that a receptor-drug interaction did not occur, and explored the possibility of cellular receptors depletion. Real time PCR analyses did not show the expression of MOR and KOR in the human microglial cell line (CHME-5) or in a human astrocyte cell lines (U-87). Since it has been suggested that, in addition to MOR and KOR, opiate drugs can also activate various Toll-like receptor (TLR) complexes (Hutchinson et al., 2007, El-Hage et al., 2011), we were interested to examine whether the Toll-like receptor 4 complex (TLR-4) complex was driving

the responses observed. However, we did not detect TLR-4 mRNA in either the microglia or astroglia cell lines. Suspecting that the genetic expression for these receptors was diminished with the numerous cellular passages, experiments were repeated with a new astrocyte cell line prior passaging. Expression of mRNA from MOR, KOR and TLR-4 receptors was detected in the astrocyte cell line. Although our negative results prompted us to hypothesize that this phenomenon might occur in the human microglial cell line, we were unable to obtain fresh CHME-5 cells from the same source, and therefore we are unable to determine whether there is a difference in MOR, KOR or TLR-4 mRNA expression between passages. While the use of these cells might be convenient for preliminary studies, considerable precautions should be taken when extrapolating in-vitro results using these cell lines to what its seen in the CNS of HIV-1 patients. Our results are consistent with several studies that use human astroglial cell lines to explore HIV-glia interactions (Speth et al., 2000, Kehn-Hall et al., 2011, Sharma et al., 2011, Byrne et al., 2012, Zhao et al., 2014), however, we reported that the constant passage of U87 cells causes the depletion of MOR, KOR and TLR4 messenger RNA. If these cell lines are used in future experiments, we strongly recommend that meticulous genetic expression analyses between cellular passages and replicates experiments to assure the presence of the receptor of interest.

Figure 3.1

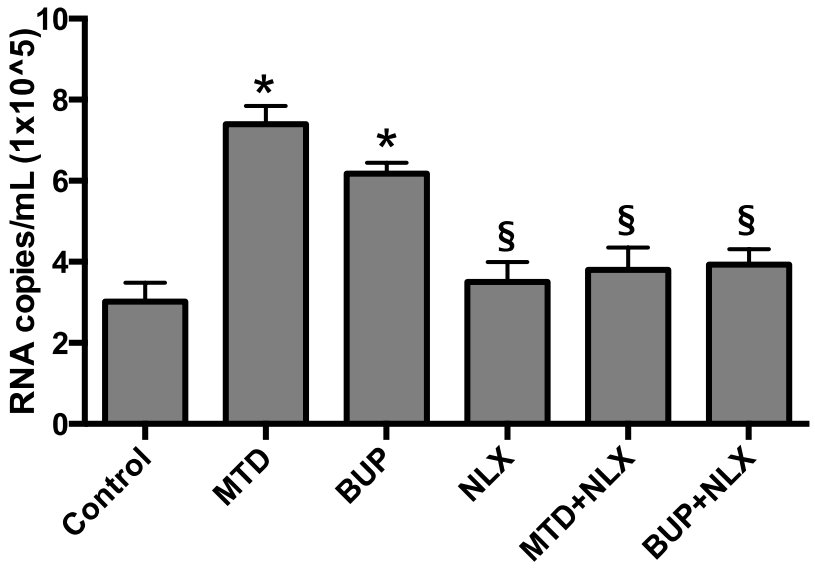

Figure 3.1 Effect of methadone and buprenorphine treatment on HIV-1 infection in human microglia. Cultures infected with HIV-1_{SF2} virus and treated with combinations of NLX (pretreatment), MTD, and BUP for 6 days. Analysis showed that methadone and buprenorphine significantly increased HIV-1 replication (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). Naloxone treatment was able to reverse the increases in viral levels (§$p < 0.05$ vs. MTD or BUP, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Figure 3.2

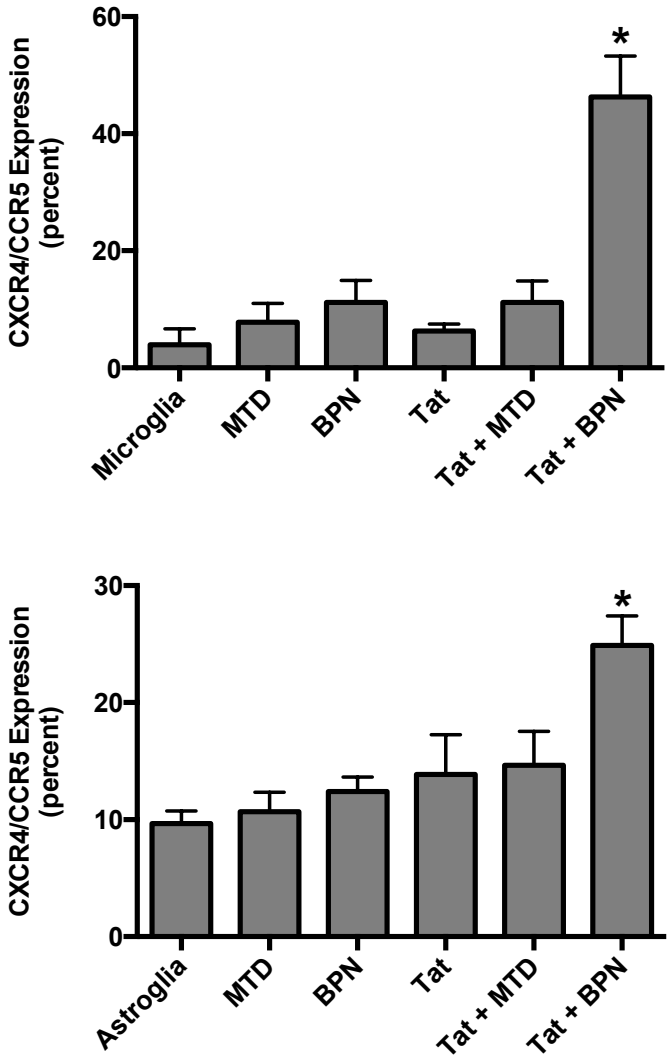

Figure 3.2 Effect of methadone and buprenorphine treatment on CXCR4 and CCR5 co-expression in human microglia and astrocytes. Cultures were treated with combinations of MTD, BUP, and/or HIV-1 Tat for 8 hours. Analysis showed that only co-exposure of Tat and buprenorphine significantly increased CXCR4 and CCR5 co-expression in human microglia (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc). There was no significant difference between other groups **(A)**. Astroglia treated with combination of Tat and buprenorphine showed a significantly increases of CXCR4 and CCR5 co-expression (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc) (**B)**. Data is based on the mean ± SEM of n = 3 experiments.

Figure 3.3

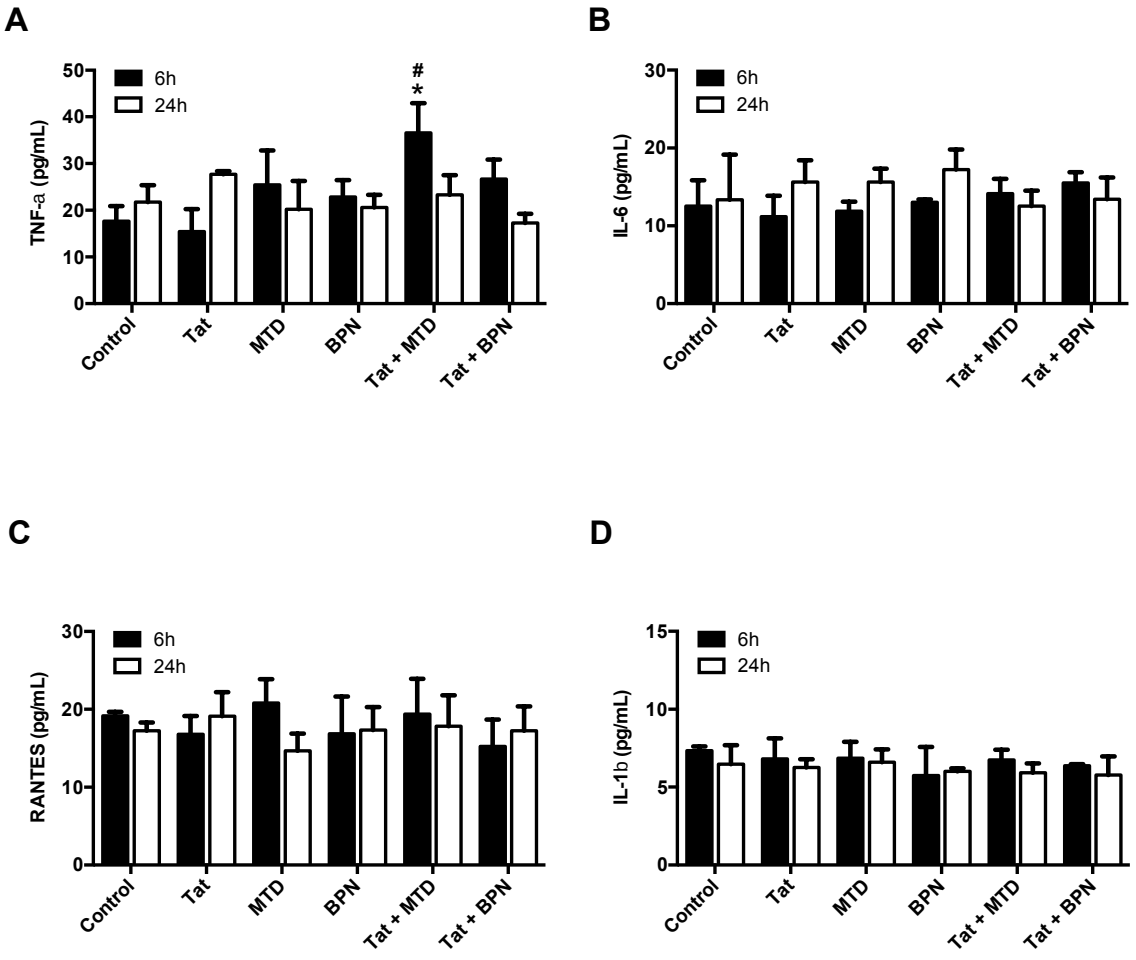

Figure 3.3 Effect of methadone and buprenorphine on neuroinflammatory signaling in human microglia. Cultures were treated MTD, BUP, and/or HIV-1 Tat for 6 and 24 h. Co-exposure with Tat and methadone showed a significant increase of TNF-α compared with control (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc) and Tat alone (# $p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc) **(A)**. Not other significant differences were observed in the release IL-6, RANTES or IL-1β. The duration of treatment did not have a significant effect. Data are based on the mean ± SEM of n = 3 experiments.

Figure 3.4

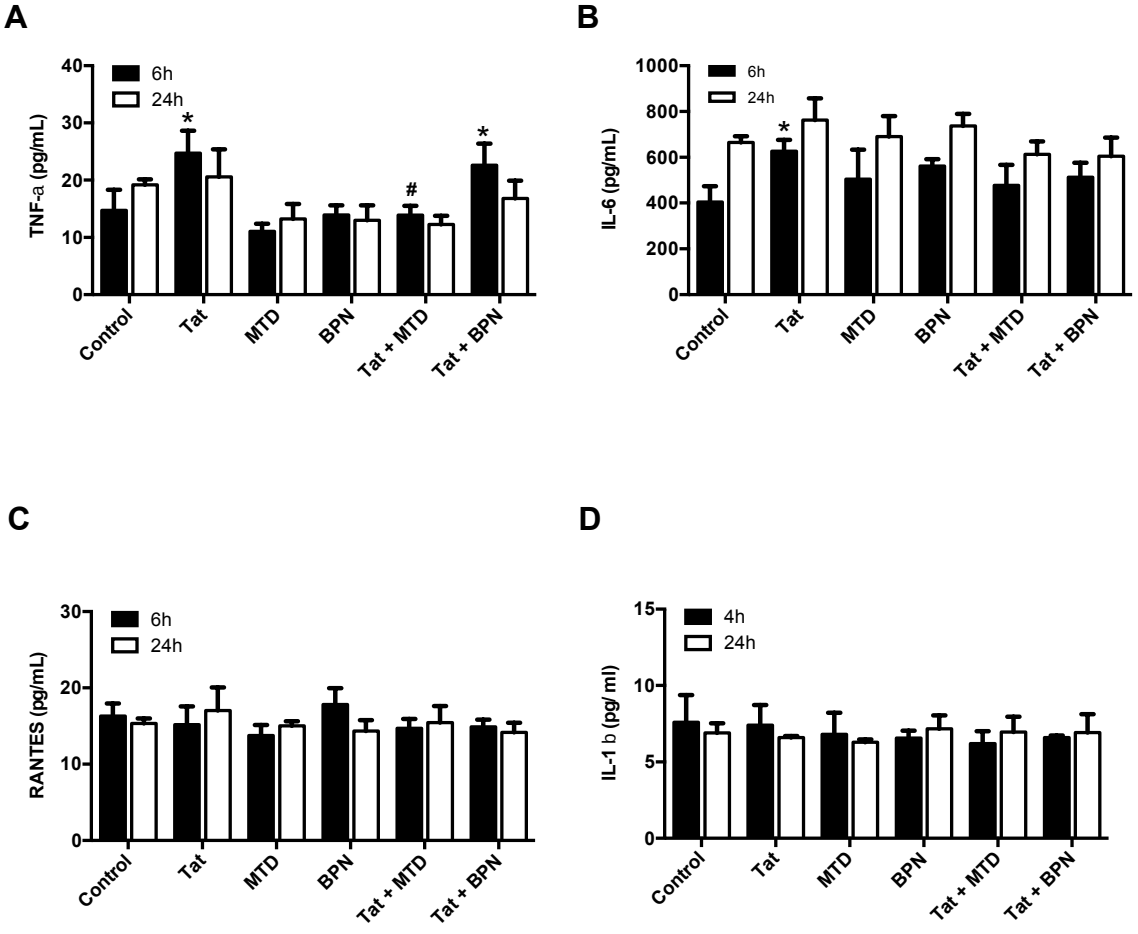

Figure 3.4 Effect of methadone and buprenorphine on neuroinflammatory signaling in human astrocytes. Cultures were treated with MTD, BUP, and/or HIV-1 Tat for 6 and 24 h. Tat alone or co-exposure with Tat and buprenorphine significant increased TNF-α levels when compared with controls (*p < 0.05 vs. control, one-way ANOVA, Tukey's post-hoc). Co-exposure with Tat and methadone significantly decreased TNF-α compared when to Tat alone (# p < 0.05 vs. Tat, one-way ANOVA, Tukey's post-hoc) **(A)**. Tat alone significantly increased IL-6 when compared with control (*p < 0.05 vs. controls, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Figure 3.5

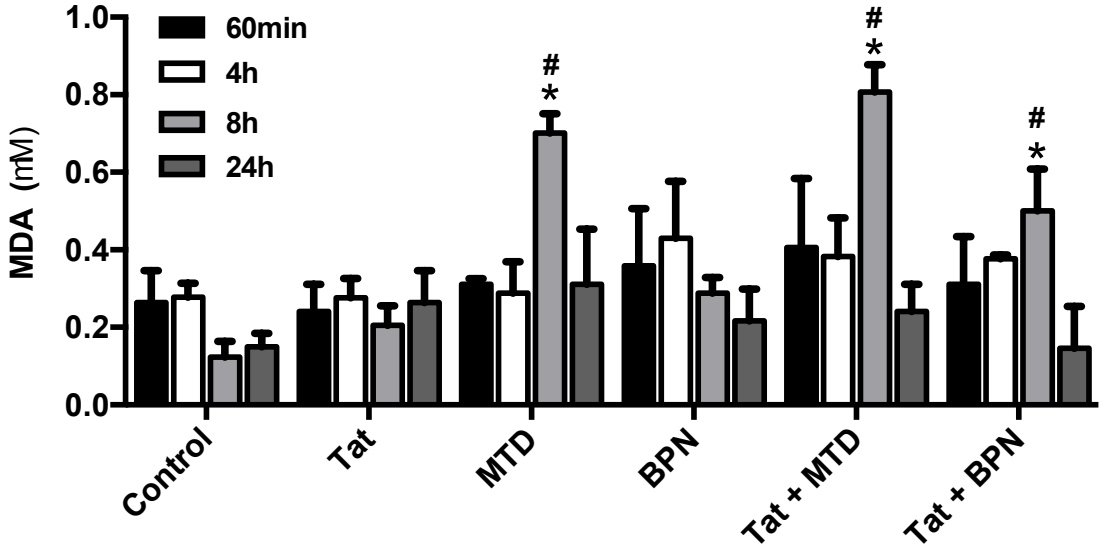

Figure 3.5 Effect of methadone and buprenorphine on lipid peroxidation in human microglia. Cultures were treated with MTD, BUP, and/or HIV-1 Tat for 1, 4, 8 and 24h. Concentrations of Malondialdehyde (MDA), which is a stable indicator of lipid peroxidation, were determined by TBARS assay. After 8 hours of exposure, methadone alone, co-exposure of Tat and methadone, or co-exposure with Tat and buprenorphine significantly increased lipid peroxidation when compared to controls or groups treated Tat alone (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc) (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Figure 3. 6

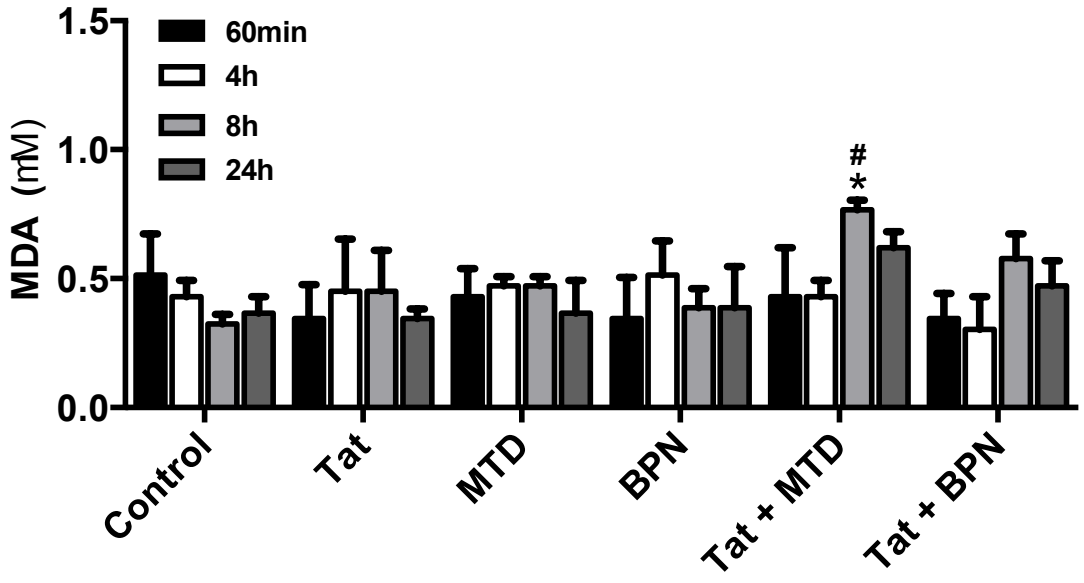

Figure 3.6 Effect of methadone and buprenorphine on lipid peroxidation in human astrocyte. Cultures were treated with MTD, BUP, and/or HIV-1 Tat for 1, 4, 8 and 24h. Concentrations of Malondialdehyde (MDA), which is a stable indicator of lipid peroxidation, were determined by TBARS assay. After 8 hours of exposure, methadone, co-exposure of Tat and methadone and co-exposure with Tat and buprenorphine significantly increased lipid peroxidation when compared to control and Tat alone groups (*$p < 0.05$ vs. control, one-way ANOVA, Tukey's post-hoc) (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Figure 3. 7

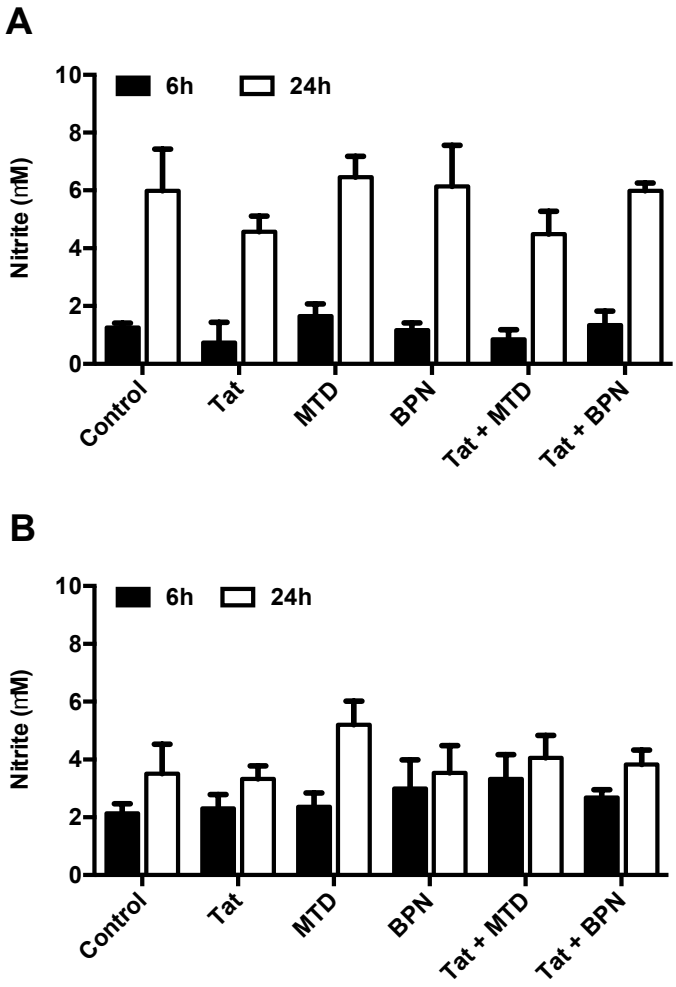

Figure 3.7 Effect of methadone and buprenorphine treatment in nitric oxide production on human glia. Cultures were treated with combinations of MTD, BUP, and/or HIV-1 Tat for 6 or 24 hours. Analysis did not show any significant difference between any treatments at any exposure time in human microglial **(A)** or human astroglial cell line **(B)**. One-way ANOVA and Tukey's post-hoc analysis, data are based on the mean ± SEM of n = 3 experiments.

Figure 3.8

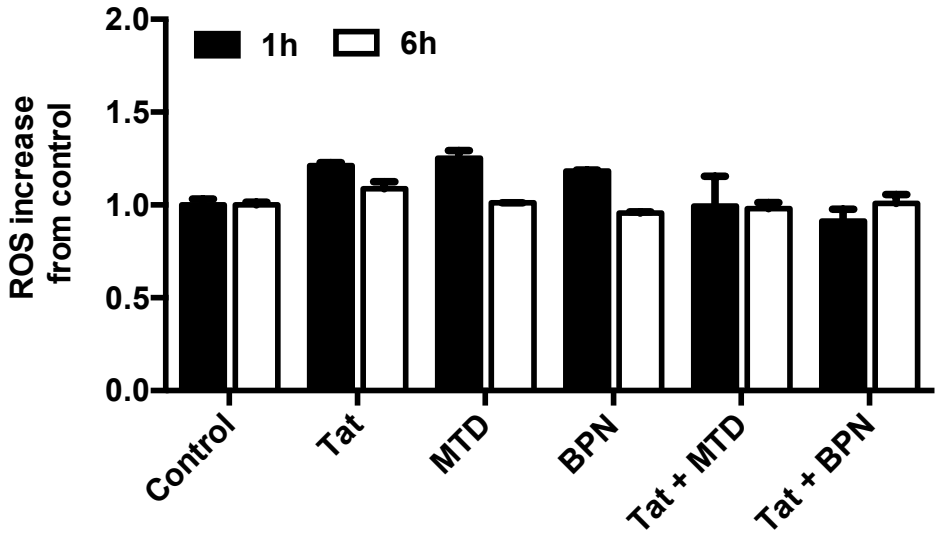

Figure 3.8 Methadone and buprenorphine effect on intracellular ROS production by human microglia. Cultures were treated MTD, BUP, and/or HIV-1 Tat for 1 and 6 h. Mean DCF relative fluorescence units (MFI) were used as an estimate of ROS. One-way ANOVA and Tukey's post-hoc analysis did not show significant differences between treatments. Data is based on the mean ± SEM of n = 3 experiments.

Figure 3. 9

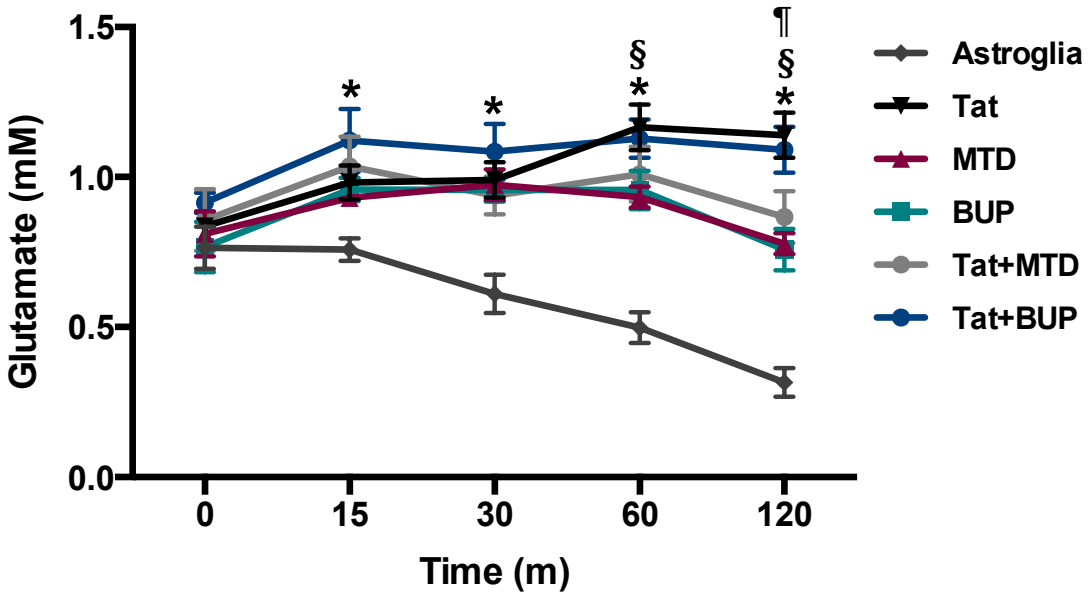

FIGURE 3.9 Methadone and buprenorphine effect on glutamate uptake by human astrocytes. Cultures were pre-treated with combinations of MTD, BPN, and/or HIV-1 Tat for 30 min, and then challenged with 1 mM glutamate. Glutamate levels were assessed from 0 min to 120 min. After 15 minutes, all treatments show a decrease in glutamate uptake compared to control (*$p < 0.05$ vs. control, repeated measures ANOVA, Tukey's post-hoc). At 60 minutes Tat-treated groups were significantly different from groups receiving drug alone (§$p < 0.05$ vs. drug alone, repeated measures ANOVA, Tukey's post-hoc). After 120 minutes, co-exposure to Tat and methadone caused a decrease in glutamate uptake disruption when compared to Tat by itself or following co-exposure to Tat and buprenorphine (¶$p < 0.05$ vs. Tat and Tat+ buprenorphine, repeated measures ANOVA, Tukey's post-hoc). Data are based on the mean ± SEM of n = 3 experiments.

Figure 3. 10

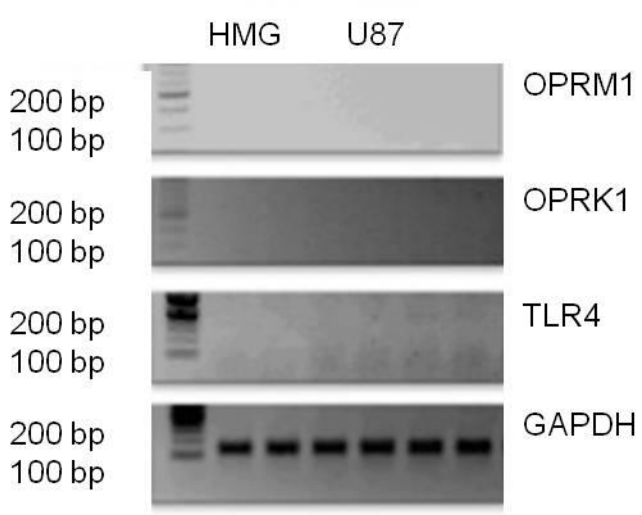

Figure 3.10 MOR, KOR and TLR4 mRNA expression on human microglia and astrocytes. PCR products amplified using primer sets for the indicated MOR, KOR and TLR4 in a human microglia cell line (HMG) and in a human astroglial cell line (U87). Microglial or astroglial cell lines did not show MOR, KOR or TLR4 expression. Samples were loaded in triplicates. GAPDH served as a loading control.

Figure 3.11

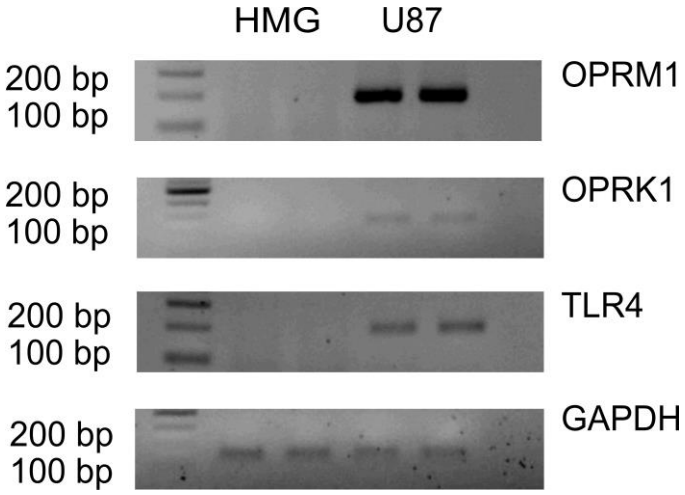

Figure 3.11 MOR, KOR and TLR4 mRNA expression on human microglia and astrocytes. PCR products amplified using primer sets for the indicated MOR, KOR and TLR4 in lower passage human microglia cell line (HMG) and new human astrocyte cell line (U87). New U87 showed MOR, TLR4 and to a lesser level KOR expression. Samples were loaded in duplicates. Note the expected relative sizes of the bands. GAPDH served as a loading control.

Chapter 4: Methadone and Buprenorphine Modulate HIV-Tat-Mediated Glial Neuroinflammatory Response

Introduction

Cognitive function is impaired in around 40% of HIV-1 patients (Cysique et al., 2004). Patients with cognitive deficits may suffer of behavioral abnormalities, motor dysfunction, and in worst scenario, dementia (Ramesh et al., 2013). Since individuals using cART show low levels of HIV-1, it is important to consider the direct and indirect effects of HIV-1 Tat in the etiology of the pathology. After treatment, protease inhibitors from cART inhibit the cleavage of the HIV gag-pol polyprotein, preventing the formation infectious virus; however, these protease inhibitors do not prevent the formation of early viral proteins, such as Tat (Nath and Steiner, 2014). Therefore, while viral replication is restrained, HIV infected cells continue producing and releasing Tat. Interactions of HIV-Tat in the brain incite a positive feedback interaction between microglia and astrocytes. Infected macrophages induce nearby astrocytes to upregulate secretion of macrophage-specific chemokines, leading to the release of toxic molecules and lesion formation (Ramesh et al., 2013).

HIV-infected individuals who are also opiate drug show more severe neurocognitive and pathological abnormalities (Bell et al., 1998, Bell et al., 2002). Studies coincide that one of the most important mechanisms leading to neuroinflammation is the interaction of multiple abused drugs with glia (Nath et

al., 2002, Hauser et al., 2006). Evidence has shown that morphine exacerbates SIV-induced increases of CCR5 receptor, nitric oxide synthase and proinflammatory cytokines, such as, TNF-α, IL-1b, and IL-6, in murine microglia (Bokhari et al., 2009). Moreover, it is established that morphine increases the HIV-1-Tat-induced cytokine and chemokine release in astrocytes (El-Hage et al., 2005, El-Hage et al., 2006). Furthermore, additional studies have shown that morphine and HIV-1 synergistically increase intracellular calcium release and inhibit glutamate buffering by astrocytes (El-Hage et al., 2005, Zou et al., 2011).

There is no clear evidence whether methadone and buprenorphine also interaction with the glial function. Limited studies suggest that methadone intensifies glia activation through the increase of TLR-4 and the expression of cytokines (Hutchinson et al., 2008). Furthermore, our observations in human glial cell-lines imply that both opiate maintenance treatment modulate several HIV glial response. However, there is the need to explore these interactions in a more suitable model. Using an in vitro mixed-glia model in which the proportion of astroglia and microglia are closely related to a typical brain environment, allows the inspection of the HIV-1-induced inflammatory response via glial crosstalk. Although numerous studies have described the HIV-Tat-mediated neuroinflammatory pathogenesis, the effects of methadone and buprenorphine in this context has not been considerably elucidated. Therefore, the central aim of this work was to examine whether methadone and buprenorphine contribute to the dysregulation of glial function using a mixed-glia model.

Materials and Methods

Primary Mixed Glia Cultures

Mixed glial cultures were prepared from the striata of post-natal day 0 - 1 (P0 - P1) imprinting control region (CD1) mouse pups (ICR; Charles River Laboratories, Boston, MA). Approximately eight pups were decapitated, brains were removed from the skull and lastly the striatum was dissected away from the cortex. Tissue was minced and incubated in trypsin (2.5 mg/mL; Sigma-Aldrich Co., St. Louis, MO) and DNase (0.015 mg/mL; Sigma-Aldrich Co.) in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen Corp., Carlsbad, CA) containing glucose (27 mM; Sigma-Aldrich Co.), Na_2HCO_3 (6 mM; Invitrogen), and penicillin/streptomycin (100 U/mL and 100 µg/mL; Invitrogen) for 30 min. at 37°C and 5% CO_2 / 95% air with intermediate manually mixing every 10 minutes.

Tissue was centrifuged at 1000 x g for 5 -10 min, resuspended in 10 mL of media containing DMEM with the previously mentioned supplements with the addition of 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT). Tissue was triturated using 5mL pipette and filtered through 100 µm nylon mesh pores (BD Falcon; Bedford, MA) and then centrifuged at 1000-x g as the first centrifugation, resuspended in 3 mL of medium. Tissue was triturated with a 5 mL pipette, filtered through a 40 µM cell strainer and plated at 1.5 - 2.0 × 10^5 cells/cm^2 on poly-L-lysine coated cell culture plates. Cell density depended of the type of assay to be conducted. Medium was exchanged 24h after plating and subsequently every 3 for approximately 10 to 14 days until. Each experiment

used cells from a single culture pooled from multiple pups, and was considered as a separate *n*.

Cytokines and chemokines quantification

For the measurement of cytokines and chemokines, conditioned medium from treated cells was used and assayed by ELISA for IL-1b, IL-10, IL-6, TNF-α, MCP-1 and RANTES (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions. Briefly, plates coated with cytokines antibody were blocked with 1% bovine serum albumin (BSA) in PBS. After 4 washes, each plate was incubated with culture supernatants and a series of dilution standards for a minimum of 2 hours. Following 4 washes, detection antibody was added to each plate and incubated for 2 hours. Horseradish peroxidase (1:200) was added to each plate and was incubated for 20 min. Tetramethylbenzidine substrate (BD Pharmingen, San Diego, CA) was added to the reactions and color optical density was measured at 450 nm using a PHERAstar F5 plate reader (PerkinElmer, Inc; Waltham, MA) following the termination of the reaction.

Glutamate uptake quantification

Glutamate uptake was measured in human astrocytes cell line HTB-14 cells according to previous studies (Zou et al., 2011). Cells were pre-incubated for 60 min at 37°C with 500 µL of Hank's balanced salt solution (HBSS) alone or with combinations of 1 µM methadone, buprenorphine, naloxone and/or 100 nM HIV-Tat. Glutamate in a concentration of 1mM was added to each well. Sample supernatants were collected from individual wells at 0min-240min time points.

Glutamate levels were quantified with glutamate assay kit (BioVision, Mountain View, CA) according to manufacture's directions. Briefly, 10 µL to 50 µL of supernatant will be directly diluted in the assay buffer. 100 µL of reaction mix will be added to the samples and the glutamate standard. The reaction will be incubated at 37ºC for 30 minutes. Optical density will be measured at 450 nm and glutamate concentrations will be calculated.

Nitric Oxide

To determine nitric oxide production by glial cells we used the Griess Reagent System (Promega, Madison, WI), according with the manufacturer. Briefly, in a 96-well plate, 50 µL of culture supernatant will be dispensing into the well in triplicate. After adding all the culture supernatants, 50 µL of the sulfanilamide Solution will be added to all the samples and will be incubate for 10 minutes at room temperature, protected from light. After incubation, 50 µL of the *N*-1-napthylethylenediamine dihydrochloride (NED) solution will be added to all the wells and will be incubate for 10 minutes at room temperature, protected from light. Absorbance will be measure within 30 minutes in the plate reader with filters between 520 nm and 550 nm. Concentration of NO was calculated based on the standard curve using known concentrations of nitrite.

Reactive Oxygen Species

Reactive oxygen species were quantified by the cell-permeable dye, 2',7'-dichlorofluorescein diacetate (DCF-DA). Cells were incubated for 1 with DCF-DA in warm PBS according to the manufacturer's indications. After incubation, cells

were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Dichlorofluorescein (DCF) fluorescence was measured at an excitation wavelength (λ_{ex}) of 485 nm and an emission wavelength (λ_{em}) of 520 nm using a PHERAstar plate reader (BMG Labtech, Cary, NC). DCF-DA is hydrolyzed by cytosolic esterases on the cells to a non-cell-permeable form (DCF). Fluoresce is detected when DCF is in contact with ROS/RSN produced by the cell. ROS levels were estimated by relative DCF fluorescence.

MDA Quantification

To assess lipid peroxidation levels, Thiobarbituric Acid Reactive Substances (TBARS) assay was performed according to the manufacture's instructions (Cell Biolabs, San Diego, CA). Briefly, mixed-glia were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h and cells were collected and resuspended with PBS. Cells were homogenized on ice and samples were transferred to separate microcentrifuge tubes. A SDS lysis solution was added and incubated at room temperature for 5 minutes. After incubation, TBA reagent was added and incubated at 95°C for 60 minutes. Samples were cooled on ice for 5 minutes and centrifuge at 3000 rpm for 15 minutes. Supernatants were removed and transferred to a 96-well plate. Absorbance was read at 532nm.

Results

Effects of 8 hours of HIV-1 Tat and/or buprenorphine and methadone on cytokine release

TNF-α, IL-6, RANTES, MCP-1 production was assessed in mixed-glia after 8 of treatment with naloxone (pre-treatment), HIV-1 Tat (100 nM) and/or buprenorphine and methadone (1.0 μM) (Fig. 4.1). For MCP-1, treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). None of opiates alone revealed significance from control. Co-exposure to HIV-1 Tat and methadone, but not buprenorphine, significantly increased MCP-1 release compared to Tat alone ($p < 0.05$). Naloxone (1.0 μM) was able to decrease MCP-1 release ($p < 0.05$) when added with Tat and methadone but naloxone did not block combined Tat and buprenorphine-induced increases in MCP-1. For RANTES, treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). None of opiates alone revealed significance from control. Co-exposure to HIV-1 Tat and methadone, but not buprenorphine revealed significant difference to Tat alone treatment. Naloxone (1.0 uM) was able to decrease RANTES release ($p < 0.05$) when co-exposed to Tat and methadone but no effect was noted for co-exposure of Tat and buprenorphine. For IL-6, all treatments were significantly different from control ($p < 0.05$) except for the opiate alone treatments. Co-exposure of HIV-1 Tat and opiates drugs did not revealed significant difference to the Tat alone treatment. For TNF-a,

treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). Tat and methadone, but not buprenorphine, significantly decreased TNF-a release compared to Tat alone ($p < 0.05$). Naloxone (1.0 μM) was able to significantly reverse this effect ($p < 0.05$).

Effects of 24 hours of HIV-1 Tat and/or buprenorphine and methadone on cytokine release

TNF-α, IL-6, RANTES, MCP-1 production was assessed in mixed-glia after 24 of treatment with naloxone (pre-treatment), HIV-1 Tat (100 nM) and/or buprenorphine and methadone (1.0 μM). For MCP-1, treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). None of opiates alone revealed significance from control. Co-exposure to HIV-1 Tat and buprenorphine, but not methadone, significantly increased MCP-1 release compared to Tat alone ($p < 0.05$). Naloxone (1.0 μM) was able to decrease MCP-1 release ($p < 0.05$) when added with Tat and buprenorphine but naloxone did not block combined Tat and methadone-induced increases in MCP-1. For RANTES, treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). None of opiates alone revealed significance from control. Co-exposure to HIV-1 Tat and buprenorphine, but not methadone revealed significant difference to Tat alone treatment. Naloxone (1.0 μM) was able to decrease RANTES release ($p < 0.05$) when co-exposed to Tat and buprenorphine but no effect was noted for co-exposure of Tat and methadone. For IL-6, all treatments were significantly

different from control ($p < 0.05$) except for the opiate alone treatments. Co-exposure of HIV-1 Tat and opiates drugs did not revealed significant difference to the Tat alone treatment. For TNF-α, treatments with Tat alone, and combined with buprenorphine or methadone were significantly different from control ($p < 0.05$). Co-exposure of HIV-1 Tat and opioid drugs did not revealed significant difference to the Tat alone treatment.

Effect of methadone and buprenorphine effect on glutamate uptake by astrocytes

To assess whether methadone and buprenorphine and/or Tat impair the ability of astroglia within the mixed-glial cultures to uptake glutamate, we challenged the cells with an excess of glutamate (1 mM) and measured the glutamate remaining in the media for 240 min. Glutamate levels remained high through the first 15 minutes of measurement, there was no different between each treatments. After 15 min, glutamate levels were hastily decreased in control and opiates alone groups. During 30 minutes time point throughout 240 min, glutamate was depleted in the control group and glutamate uptake was significantly diminished in Tat alone, and Tat combined with buprenorphine or methadone groups compared to control group ($p < 0.05$). None of the opiates alone groups revealed significance from control. Co-exposure of HIV-1 Tat and opiates drugs did not revealed significant difference in glutamate levels compared to the Tat alone treatment. Although, Tat and buprenorphine group buffering was significantly ($p < 0.05$) higher than Tat and methadone group.

Methadone and buprenorphine effect on nitric oxide production

During inflammatory processes in the CNS, nitric oxide production is one of the mechanisms in which activated microglia and astrocyte affect neuron viability (Bal-Price and Brown, 2001). To measured nitric oxide production, cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Concentrations of nitrite, which is one of two primary stable breakdown products of nitric oxide was measured by a colorimetric reaction. Tat, and co-exposure of Tat and methadone or buprenorphine significantly increase nitrite production when compared to control (*$p < 0.05$ vs. Control and drugs alone, one-way ANOVA, Tukey's post-hoc). However, either methadone or buprenorphine did not increase the HIV-Tat-mediated nitric oxide production.

Methadone and buprenorphine effect on intracellular ROS production

Evidence demonstrated that morphine exacerbate Tat-induced ROS production in mixed-glial culture model (Zou et al., 2011). To assess weather methadone and buprenorphine interact as well with ROS production, mixed-glia were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Mean DCF relative fluorescence units (MFI) were used as an estimate of ROS. Methadone or buprenorphine alone did not show a significant increase on ROS production when compared to control. Tat lone groups showed an increase on ROS production compared to control and drugs alone groups (*P

< 0.05 vs. control and drugs alone, one-way ANOVA, Tukey's post-hoc). Methadone shows the capacity to increase Tat-induced ROS production (#p < 0.05 vs. Tat, one-way ANOVA, Tukey's post-hoc), while buprenorphine did not show significant interaction with Tat effect. Pre-treatment with naloxone was able to reverse the effect of co-exposure with methadone and Tat (§p < 0.05 vs. Tat +MTD, one-way ANOVA, Tukey's post-hoc).

Methadone and buprenorphine effect on lipid peroxidation

Levels of malondialdehyde (MDA), a lipid peroxidation product, and indicator of oxidative stress are found to be higher in HIV-1 the periphery of HIV-1 positive individuals and patients suffering from neuroinflammatory diseases (Sonnerborg et al., 1988, Romero et al., 1998, Gil et al., 2003). To measure MDA levels, mixed-glia were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Concentrations of malondialdehyde (MDA), were determined by ELISA. Analyses show that Tat alone and combination of Tat and drugs significantly increase MDA levels compared to controls and drugs alone groups (*p < 0.05 vs. Control and drugs alone, one-way ANOVA, Tukey's post-hoc). Methadone significantly exacerbates Tat-induced increase MDA levels compared (#p < 0.05 vs. Tat, one-way ANOVA, Tukey's post-hoc), while buprenorphine did not show any significant difference when compare to Tat. Pre-treatment with naloxone significantly decrease the effect of methadone on Tat-induced MDA levels (§p < 0.05 vs. Tat +MTD, one-way ANOVA, Tukey's post-hoc).

Discussion

The use of pharmacologic treatments for opiates abuse in individuals living with HIV/AIDS have not been consistently studied (Durvasula and Miller, 2014). While opiate abuse drugs seem to modulate neurocognitive deficits due to HIV (Nath et al., 2002, Hauser et al., 2005, Kumar et al., 2006), it is imperative to investigate the effect of opiates substitution treatments in these disorders. Our results suggest that there are differences between methadone and buprenorphine modulation on HIV-Tat-induced glia signaling. Herein, we report that methadone treatment for 8 hours increased the HIV-Tat-induced release of MCP-1 and RANTES. Interestingly, after a 24-hour exposure of methadone, the increase in these cytokines seems to reverse and levels are not significant different from Tat. On the other hand, within this 24-hour exposure time, buprenorphine shows an increase in the release of MCP-1 and RANTES. Studies have demonstrated that increase in the production of the chemokine RANTES contributes to the Tat-induced inflammatory signaling, including the exacerbation of MCP-1 expression (El-Hage et al., 2008a). MCP-1 levels are elevated in the CNS of patients with AIDS dementia (Wesselingh et al., 1993), suggesting that this chemokine plays an important role in this pathogenesis. Astrocytes are a major source of MCP-1 (Bethel-Brown et al., 2012), and increased release of MCP-1 has been related to BBB disruption and the migration of uninfected microglia and astrocytes to sites of HIV-infected leukocyte infiltration and active infection (Eugenin et al., 2006). Others have shown that morphine potentiates

Tat-induced cytokine release by increasing $[Ca^{2+}]_i$, which increases NF-κB activation and cytokine production (El-Hage et al., 2008b). In accordance with these observations and our current studies, we suggest that methadone and buprenorphine could also play a role on these mechanisms.

Furthermore, alteration of cytokines profile may disrupt additional glial functions. It is known that activated microglia secrete TNF-α that may exert direct neurotoxicity effects; however, a more detrimental effect of TNF-α expression in the brain is its capacity to stimulate extensive microglial glutamate release (Takeuchi et al., 2006). Furthermore, HIV-1 Tat and morphine have been shown to exacerbate this release (Gupta et al., 2010). Although astrocytes play a crucial role in buffering glutamate excess in the brain, TNF-α expression potentially contributes to the disruption of glutamate uptake (Zou and Crews, 2005, Boycott et al., 2008). Our data reveal that co-exposure of Tat and methadone significant decrease TNF-α release. These observations correlate with our glutamate uptake studies, in which methadone treatment decreased Tat-induced disruption of glutamate uptake. On the other hand, buprenorphine increased HIV-Tat-induced glutamate buffering disruption. Although this model allows for the measurement of residuals glutamate levels, it does not distinguish whether these changes are due to glutamate release or glutamate uptake disruption. Previously, Zou and colleagues (2011) used this paradigm and showed that the presence of glutamate transporter inhibitors eliminate Tat and morphine effect on glutamate levels, suggesting that Tat and morphine

specifically inhibit glutamate transport, rather than enhancing glutamate release. Taken together, our data shows that buprenorphine increased Tat-induced glutamate uptake interference, while methadone decreased this Tat-induced disruption.

Oxidative stress induction by activated glia may also control intracellular inflammatory signaling and lead to neurotoxicity (Droge, 2002). Furthermore, several studies have demonstrated that morphine increases Tat-related oxidative stress generation (Hauser et al., 1998, Turchan-Cholewo et al., 2009). We assessed whether methadone or buprenorphine aggravate Tat-mediated oxidative stress. Nitric oxide (NO) produced by glial cells have been involved in the neuropathogenesis of HIV (Kong et al., 1996). We did not observe an exacerbation of Tat-induced nitric oxide production by either methadone or buprenorphine. On the other hand, ROS quantification shows that methadone exacerbates Tat-induces ROS production; this increment was reversed by naloxone. Buprenorphine did not show modulation in the production of ROS. It is possible that these observations resulted from the divergent agonist interactions with MOR. MOR antagonists own specific differences in the ability to activate phospholipase D2 (PLD2), which cause MOR internalization (Koch et al., 2005). MOR agonists that activate PLD2 and further induce MOR endocytosis, such as methadone, strongly induce NADH/NADPH-mediated ROS synthesis, while agonists that do not induce PLD2 activation and do not cause MOR endocytosis, such as buprenorphine, fail to activate ROS synthesis (Koch et al., 2009).

Furthermore, studies in rhesus macaques showed a significant relationship between morphine-dependent SIV/SHIV infection severity and the rate and extent of MDA increase (Perez-Casanova et al., 2007). Correlating with these observations, our in-vitro paradigms showed that methadone significantly increases Tat-dependent exacerbation of MDA levels; this increased was reversed by the pre-treatment with naloxone, however, buprenorphine did not showed a significant increase in Tat-dependent MDA production.

Taken together, we show that methadone modulates a rapid HIV-Tat response of cytokine release, while buprenorphine modulates a more delayed inflammatory signaling. Furthermore, methadone show a significant exacerbation in Tat-induced oxidative stress, however, buprenorphine interacts in the interference of glutamate uptake. We show basic differences in the effect of opiate dependence treatment on the HIV-Tat-induced inflammatory response. Our studies suggest that both methadone and buprenorphine modulate the Tat-induced glial inflammatory response in a different manner. Further studies are needed to elucidate the specific mechanisms involved in these interactions.

Figure 4.1

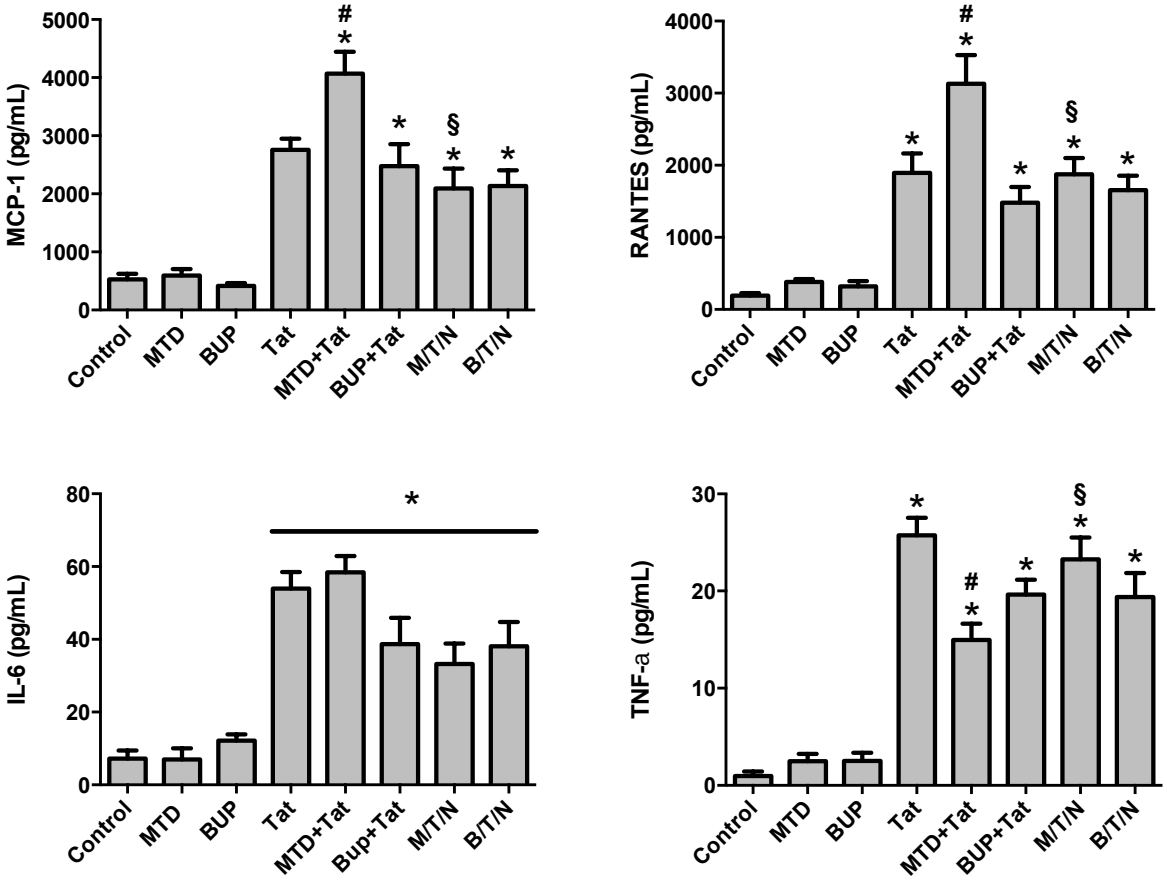

Figure 4.1. Effect of methadone and buprenorphine on neuroinflammatory signaling following 8-hour exposure. Cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Co-exposure with Tat and methadone show a significant increase of MCP-1 **(A)** and RANTES **(B)** release compared with Tat alone (# $p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc), while buprenorphine did not show significant difference when compared to Tat. Pre-treatment with naloxone reverse Tat and methadone co-exposure effect (§$p < 0.05$ vs. Tat + MTD, one-way ANOVA, Tukey's post-hoc). Tat alone, Tat and co-exposure with Tat and drugs significantly increased IL-6 **(C)** when compared to control and drugs alone (*$p < 0.05$ vs. Control and drugs alone, one-way ANOVA, Tukey's post-hoc), while co-exposure with Tat and drugs did not show any significant increase when compared to Tat alone. Methadone co-exposure with Tat significant decreased Tat-induced TNF-α **(D)** release (# $p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc). Data are shown as mean concentration ± SEM. n=3.

Figure 4.2

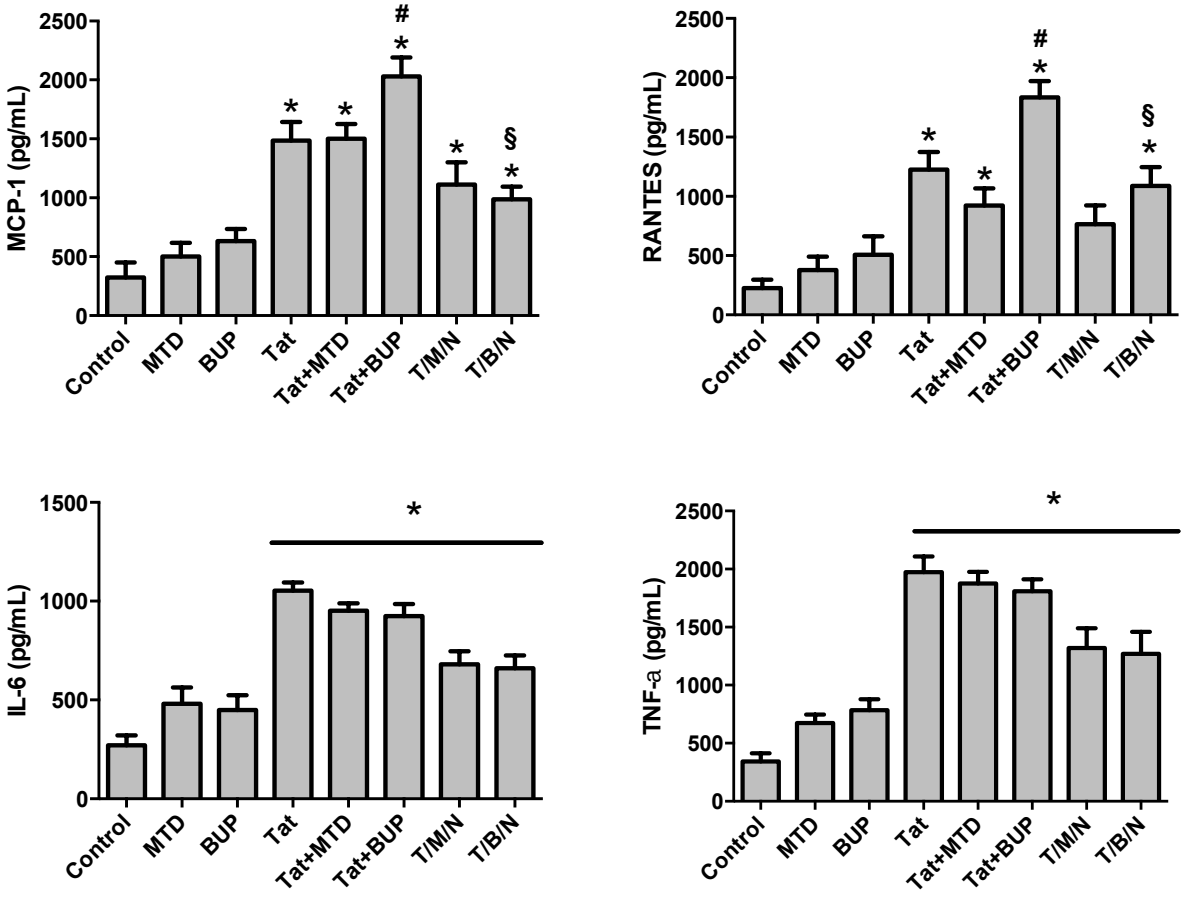

Figure 4.2. Effect of methadone and buprenorphine on neuroinflammatory signaling following 24-h of exposure. Cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Co-exposure with Tat and buprenorphine show a significant increase of MCP-1 (A) and RANTES (B) release compared with Tat alone (# $p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc), while methadone did not show significant difference when compared to Tat. Pre-treatment with naloxone reverse Tat and buprenorphine co-exposure effect (§$p < 0.05$ vs. Tat + BUP, one-way ANOVA, Tukey's post-hoc). Tat alone, Tat and co-exposure with Tat and drugs significantly increased IL-6 (C) and TNF-α (D) when compared to control and drugs alone (*$p < 0.05$ vs. Control and drugs alone), while co-exposure with Tat and drugs did not show any significant increase when compared to Tat alone. Data are shown as mean concentration ± SEM. n=3.

Figure 4.3

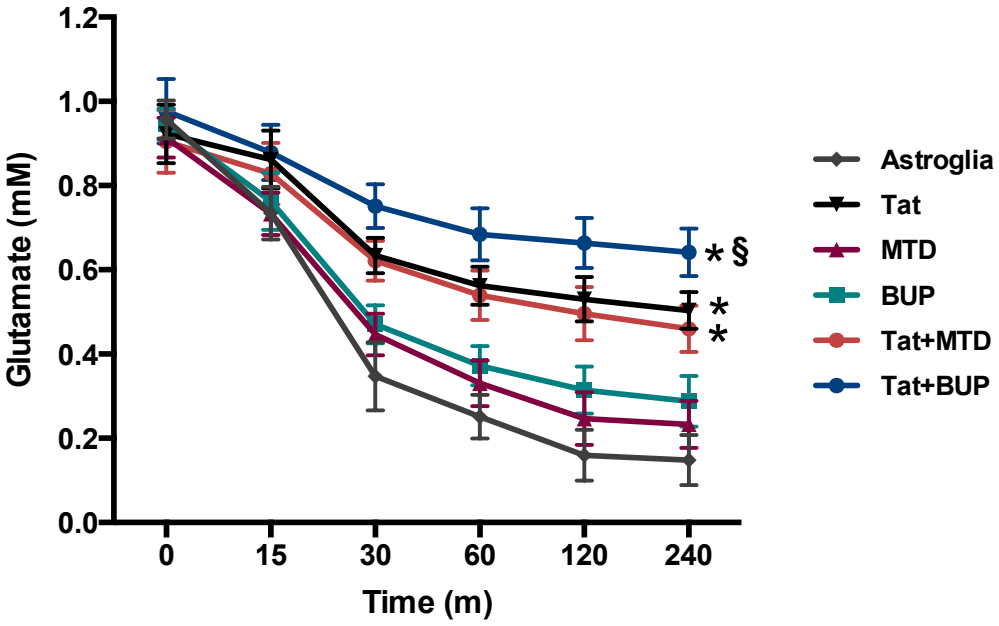

Figure 4.3. Methadone and buprenorphine disruption on glutamate uptake by astrocytes. Cultures were pre-treated with combinations of MTD, BPN, and/or HIV-1 Tat for 30 min, and then challenged with 1 mM glutamate. Glutamate levels were assessed from 0 min to 240 min. Tat alone and combination of Tat and methadone or buprenorphine significantly decreased glutamate buffering compared to control and drug alone groups (*$p < 0.05$ vs. Control and drugs alone, repeated measures ANOVA, Tukey's post-hoc), Although co-exposure of Tat and buprenorphine decreased glutamate uptake, this effect was not significant from Tat alone. Moreover, disruption of glutamate buffering by co-exposure of Tat and buprenorphine was significant higher than co-exposure of Tat and methadone (§$p < 0.05$ vs. Tat + MTD repeated measures ANOVA, Tukey's post-hoc). Data shown as concentration of glutamate (mM) ± SEM of n=4.

Figure 4.4

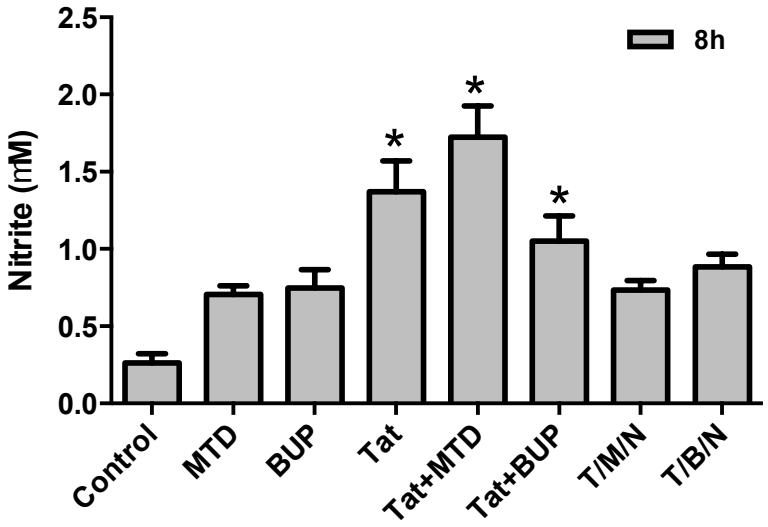

Figure 4.4. Methadone and buprenorphine effect on nitric oxide production. Cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Concentrations of nitric oxide were determined by Griess reaction. Tat alone and co-exposure of Tat and drugs significantly increased nitric oxide production when compared with control (*$p < 0.05$ vs. control and drugs alone, one-way ANOVA, Tukey's post-hoc). Data indicate the mean concentration ± SEM of n=3.

Figure 4.5

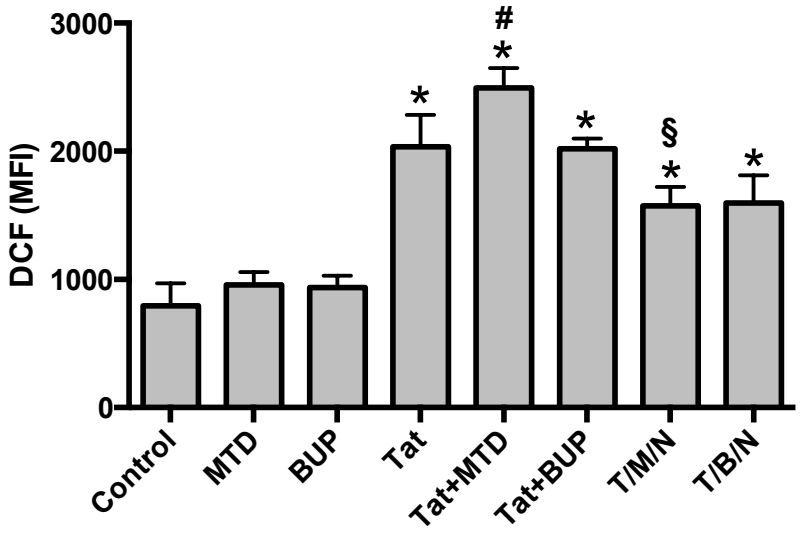

Figure 4.5. Methadone and buprenorphine effect on intracellular ROS production. Cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Mean DCF relative fluorescence units (MFI) were used as an estimate of ROS. Tat alone, and co-exposure with drugs increased ROS production when compared to control and drugs alone (*$p < 0.05$ vs. control and drugs alone, one-way ANOVA, Tukey's post-hoc). Co-exposure of Tat and methadone was significant different when compared to Tat alone (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc), Naloxone pre-treatment significantly decreased methadone effect on Tat-induced ROS production (§$P < 0.05$ vs. Tat +MTD, one-way ANOVA, Tukey's post-hoc). Data indicate the mean concentration ± SEM of n=3.

Figure 4.6

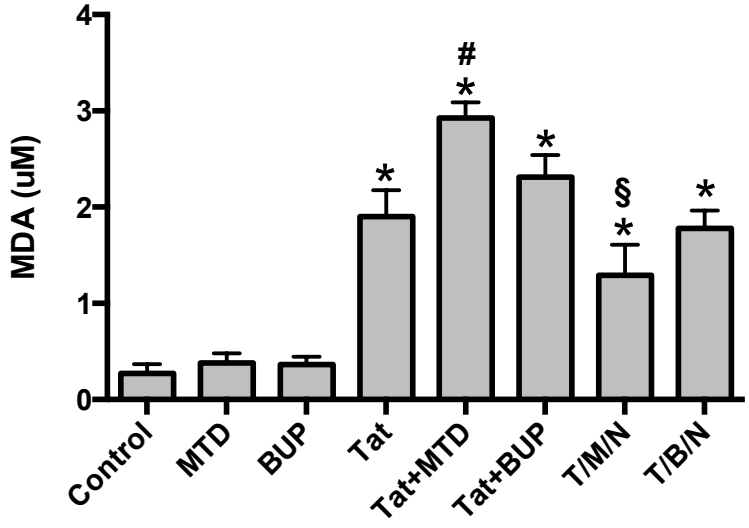

Figure 4.6. Methadone and buprenorphine effect on lipid peroxidation. Cultures were treated with combinations of NLX (pretreatment), MTD, BUP, and/or HIV-1 Tat for 8 h. Concentrations of Malondialdehyde (MDA), were determined by TBARS assay. Tat alone, and co-exposure with drugs increased ROS production when compared to control and drugs alone (*$p < 0.05$ vs. control and drugs alone, one-way ANOVA, Tukey's post-hoc). Co-exposure of Tat and methadone was significant different when compared to Tat alone (#$p < 0.05$ vs. Tat, one-way ANOVA, Tukey's post-hoc), Naloxone pre-treatment significantly decreased methadone effect on Tat-induced lipid peroxidation (§$P < 0.05$ vs. Tat +MTD, one-way ANOVA, Tukey's post-hoc). Data indicate the mean concentration ± SEM of n=3.

Chapter 5: Conclusions

The goal of these studies was to assess the role of methadone and buprenorphine on HIV-1-induced inflammatory responses in the CNS. Since glia are the principal resident CNS that is infected by HIV-1 and a critical site of opiate drug action in the context of HIV infection (Zou et al., 2011). HIV-opiate interactions were assessed in glia in these studies. The use of methadone and, more recently, buprenorphine to treat opiate-abusing HIV patients is prevalent. Therefore, it is imperative to understand the interaction that these drugs may have with the HIV-induced inflammatory response. Since HIV-1 infection is primarily spread from the periphery, and CD4+ lymphocytes are critical target cells for viral replication. The first effort of this work was to discern between the possible immunomodulation of an acute and chronic methadone exposure in peripheral mononuclear blood cells. Since studies in peripheral cells show that methadone enhances the activation and replication of HIV in latently infected PBMC from HIV patients (Li et al., 2002), we were interested in exploring whether methadone was capable of exacerbating an acute HIV infection, rather than a chronic and latent infection.

Our findings reveal that acute methadone treatment intensifies viral replication in PBMC from healthy donors. Moreover, corresponding with these observations, we show that methadone increases the release of inflammatory cytokines TNF-α and IL-6, which are strongly related to HIV infection and

immune activation in the periphery (Duh et al., 1989, Michihiko et al., 1989, Breen et al., 1990, Roy et al., 1998, Roux et al., 2000). In addition to the release of cytokines, NF-κB expression and activation play an essential role on HIV-1 infectivity (Baeuerle and Baltimore, 1996, Pitha, 2011). We show that methadone exacerbates the Tat-induced increase in the expression of p65 subunit of NF-κB and phospho-IκBα (Ser32). These molecules are tightly related to the activation and transport into the nucleus and activation of NF-κB.

Furthermore, we aimed to explore these testing paradigms on PBMC from chronic methadone users. Studies revealed that patients on long-term, stabilized methadone-maintenance treatments did not show significantly different NK cell activity and absolute B- and T-cell subsets compared to normal control subjects (Novick et al., 1989). We demonstrate that ex vivo chronic methadone exposure shows lesser levels of viral load and cytokine release when compared with acute methadone treatments in healthy donors. Moreover, contrary of what we expected, an abrupt withdrawal of chronic exposure did not show increase in HIV-1 infection or cytokines release in PBMC, suggesting that immunological activation is gradually and not abruptly achieved after methadone withdrawal.

Despite that the introduction of cART has substantially controlled HIV-1 replication, neuroinflammatory pathology is still prevalent among HIV-1 patients (Sacktor, 2002). Furthermore, it has been shown that opiate abuse hastens these deficits (Hauser et al., 2012). Therefore, we extend our analysis to a human glial cell lines models. This examination reveals that methadone and buprenorphine

significantly increase HIV-1 infection in human microglia. Buprenorphine showed a stronger interaction with the expression of CXCR4 and CCR5 and in glutamate buffering by astrocytes. Although, our cell line model did not allow us to clearly detect the effects of methadone and buprenorphine on the inflammatory signaling orchestrated by cytokines and oxidative stress, this model was useful for gaining preliminary insights into basic glial metabolism and the pattern of expression of other surface receptors.

In the murine mixed-glia model, a more evidently HIV-Tat-opiate interaction was observed. Methadone modulates Tat-induced cytokines release, while a longer exposure normalized the cytokine release profile. In contrast, a shorter exposure of buprenorphine did not exert effect in the Tat-related cytokine signaling, however, longer exposure exacerbate the Tat-induced cytokine increase. Furthermore, Methadone seems to exacerbate Tat-induced oxidative stress levels, while this response is affected to a lesser extend by buprenorphine. Conversely, our glutamate uptake assessments show that co-exposure of Tat with buprenorphine exerts a more pronounced disruption of glutamate uptake when compared to co-exposure with methadone. Extracellular levels of glutamate are tightly controlled by glutamate transporters and glutamine synthase in astrocytes (Schousboe et al., 1997), an interference in glutamate transport leads to excess levels of extracellular glutamate and cause neuronal injury. These observations suggest that even though methadone increases the production of neurotoxic molecules, buprenorphine's modulation on glutamate

uptake and cytokines release seems to be more related to the exacerbation of Tat-induced neurotoxicity.

While methadone exerts its effects primarily through interactions with MOR (Regan et al., 2012), buprenorphine shows divergent effects by agonist interactions with MOR and antagonist interactions with KOR (Robinson, 2006, George and Day, 2007). It has been suggested that MOR activation by opiates leads to an increase of HIV-1 infectivity (Peterson et al., 1990, Chao et al., 1996b), whereas KOR activation decreases HIV-1 expression in human CD4+ lymphocytes, macrophages and microglia (Chao et al., 1996a, Chao et al., 2001, Peterson et al., 2001). Our findings on HIV-1 infectivity correlate with these observations and we report that buprenorphine exerts a more modest effect on HIV-1 infection compared to methadone. However, it is essential to keep in mind that the neuroinflammatory signaling does not need constant viral production.

Buprenorphine has a high affinity combined with a slow dissociation from the μ-receptor, which results in milder withdrawal symptoms upon discontinuation compared to methadone (Amass et al., 2004). Although this capacity is advantageous for the overall withdrawal state in the patient, a longer interaction with the μ-receptor could lead to more rapid μ-receptor activation. In addition to the pronounced receptor affinity that buprenorphine possess, another important aspect that needs to be noted is that buprenorphine can exert a strong dose-dependent effect. High-dose buprenorphine maintenance produces near maximal μ-opioid receptor occupation (Greenwald et al., 2003). Novel studies in

oligodendrocytes show that low concentrations of buprenorphine activate the high affinity MOR, whereas at high concentrations buprenorphine can occupy MOR and the low affinity nociceptin/orphanin FQ receptor (NOP) (Eschenroeder et al., 2012). In addition, buprenorphine seems to alter myelination and axonal growth in the developing rat brain in a dose-time dependent manner (Sanchez et al., 2008). Buprenorphine's capacity to interact not only with, more commonly known MOR and KOR, but also with delta opioid receptor (DOR) and opioid receptor-like (ORL-1) (Lutfy et al., 2003, Wee et al., 2012) makes difficult the understanding of its mechanism of action.

It is clear that methadone and buprenorphine treatment decrease HIV risk behaviors among opiate abusers (Longshore et al., 1993, Metzger et al., 1998, Gowing et al., 2006, Gowing et al., 2008, Sullivan et al., 2008). Therefore, methadone and buprenorphine treatments are currently a safer option compared to the use of drug of abuse. However, the data presented in this dissertation correlates with previous evidence, which established that these maintenance treatments possess the ability to modulate HIV-1-mediated inflammatory response in the periphery. In addition, we demonstrate that methadone and buprenorphine exacerbate HIV-Tat-induced glia dysfunction. Furthermore, this supports the need to elucidate the broad mechanism in which these drugs exert a modest, but significant, modulation during HIV pathogenesis. This may be impetus for the improvement of treatment interventions for the increasing population of opiate abusing, HIV-infected individuals.

References

1. (1982) Update on acquired immune deficiency syndrome (AIDS)--United States. MMWR Morbidity and mortality weekly report 31:507-508, 513-504.

2. Achim CL, Wang R, Miners DK, Wiley CA (1994) Brain viral burden in HIV infection. Journal of neuropathology and experimental neurology 53:284-294.

3. Albini A, Ferrini S, Benelli R, Sforzini S, Giunciuglio D, Aluigi MG, Proudfoot AE, Alouani S, Wells TN, Mariani G, Rabin RL, Farber JM, Noonan DM (1998) HIV-1 Tat protein mimicry of chemokines. Proc Natl Acad Sci U S A 95:13153-13158.

4. Altice FL, Sullivan LE, Smith-Rohrberg D, Basu S, Stancliff S, Eldred L (2006) The potential role of buprenorphine in the treatment of opioid dependence in HIV-infected individuals and in HIV infection prevention. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 43 Suppl 4:S178-183.

5. Amass L, Ling W, Freese TE, Reiber C, Annon JJ, Cohen AJ, McCarty D, Reid MS, Brown LS, Clark C, Ziedonis DM, Krejci J, Stine S, Winhusen T, Brigham G, Babcock D, Muir JA, Buchan BJ, Horton T (2004) Bringing buprenorphine-naloxone detoxification to community treatment providers: the NIDA Clinical Trials Network field experience. The American journal on addictions / American Academy of Psychiatrists in Alcoholism and Addictions 13 Suppl 1:S42-66.

6. An SF, Groves M, Gray F, Scaravilli F (1999) Early entry and widespread cellular involvement of HIV-1 DNA in brains of HIV-1 positive asymptomatic individuals. Journal of neuropathology and experimental neurology 58:1156-1162.

7. Anthony IC, Bell JE (2008) The Neuropathology of HIV/AIDS. International review of psychiatry (Abingdon, England) 20:15-24.

8. Arango JC, Simmonds P, Brettle RP, Bell JE (2004) Does drug abuse influence the microglial response in AIDS and HIV encephalitis? AIDS (London, England) 18 Suppl 1:S69-74.

9. Avila AH, Alonzo NC, Bayer BM (2004) Immune cell activity during the initial stages of withdrawal from chronic exposure to cocaine or morphine. Journal of neuroimmunology 147:109-113.

10. Bacchetti P, Osmond D, Chaisson RE, Dritz S, Rutherford GW, Swig L, Moss AR (1988) Survival patterns of the first 500 patients with AIDS in San Francisco. The Journal of infectious diseases 157:1044-1047.

11. Baeuerle PA, Baltimore D (1996) NF-kappa B: ten years after. Cell 87:13-20.

12. Bal-Price A, Brown GC (2001) Inflammatory neurodegeneration mediated by nitric oxide from activated glia-inhibiting neuronal respiration, causing glutamate release and excitotoxicity. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:6480-6491.

13. Barger SW, Basile AS (2001) Activation of microglia by secreted amyloid precursor protein evokes release of glutamate by cystine exchange and attenuates synaptic function. Journal of neurochemistry 76:846-854.

14. Barre-Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vezinet-Brun F, Rouzioux C, Rozenbaum W, Montagnier L (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220:868-871.

15. Bart G (2012) Maintenance medication for opiate addiction: the foundation of recovery. Journal of addictive diseases 31:207-225.

16. Bell JE, Arango JC, Robertson R, Brettle RP, Leen C, Simmonds P (2002) HIV and drug misuse in the Edinburgh cohort. Journal of acquired immune deficiency syndromes (1999) 31 Suppl 2:S35-42.

17. Bell JE, Brettle RP, Chiswick A, Simmonds P (1998) HIV encephalitis, proviral load and dementia in drug users and homosexuals with AIDS. Effect of neocortical involvement. Brain : a journal of neurology 121 (Pt 11):2043-2052.

18. Bell JE, Donaldson YK, Lowrie S, McKenzie CA, Elton RA, Chiswick A, Brettle RP, Ironside JW, Simmonds P (1996) Influence of risk group and zidovudine therapy on the development of HIV encephalitis and cognitive impairment in AIDS patients. AIDS (London, England) 10:493-499.

19. Bethel-Brown C, Yao H, Hu G, Buch S (2012) Platelet-derived growth factor (PDGF)-BB-mediated induction of monocyte chemoattractant protein 1 in human astrocytes: implications for HIV-associated neuroinflammation. Journal of neuroinflammation 9:262.

20. Bokhari SM, Yao H, Bethel-Brown C, Fuwang P, Williams R, Dhillon NK, Hegde R, Kumar A, Buch SJ (2009) Morphine enhances Tat-induced activation in murine microglia. Journal of neurovirology 15:219-228.

21. Boycott HE, Wilkinson JA, Boyle JP, Pearson HA, Peers C (2008) Differential involvement of TNF alpha in hypoxic suppression of astrocyte glutamate transporters. Glia 56:998-1004.

22. Breen EC, Rezai AR, Nakajima K, Beall GN, Mitsuyasu RT, Hirano T, Kishimoto T, Martinez-Maza O (1990) Infection with HIV is associated with elevated IL-6 levels and production. Journal of immunology (Baltimore, Md : 1950) 144:480-484.

23. Bruce-Keller AJ, Turchan-Cholewo J, Smart EJ, Geurin T, Chauhan A, Reid R, Xu R, Nath A, Knapp PE, Hauser KF (2008) Morphine causes rapid increases in glial activation and neuronal injury in the striatum of inducible HIV-1 Tat transgenic mice. Glia 56:1414-1427.

24. Byrne LS, Peng J, Sarkar S, Chang SL (2012) Interleukin-1 beta-induced up-regulation of opioid receptors in the untreated and morphine-desensitized U87 MG human astrocytoma cells. Journal of neuroinflammation 9:252.

25. Carballo-Dieguez A, Sahs J, Goetz R, el Sadr W, Sorell S, Gorman J (1994) The effect of methadone on immunological parameters among HIV-positive and HIV-negative drug users. The American journal of drug and alcohol abuse 20:317-329.

26. Carpenter GW, Breeden L, Carr DJ (1995) Acute exposure to morphine suppresses cytotoxic T-lymphocyte activity. International journal of immunopharmacology 17:1001-1006.

27. Carrigan KA, Saurer TB, Ijames SG, Lysle DT (2004) Buprenorphine produces naltrexone reversible alterations of immune status. International immunopharmacology 4:419-428.

28. Chang HC, Samaniego F, Nair BC, Buonaguro L, Ensoli B (1997) HIV-1 Tat protein exits from cells via a leaderless secretory pathway and binds to extracellular matrix-associated heparan sulfate proteoglycans through its basic region. AIDS (London, England) 11:1421-1431.

29. Chao CC, Gekker G, Hu S, Sheng WS, Shark KB, Bu DF, Archer S, Bidlack JM, Peterson PK (1996a) kappa opioid receptors in human microglia downregulate human immunodeficiency virus 1 expression. Proceedings of the National Academy of Sciences of the United States of America 93:8051-8056.

30. Chao CC, Gekker G, Sheng WS, Hu S, Peterson PK (2001) U50488 inhibits HIV-1 expression in acutely infected monocyte-derived macrophages. Drug and alcohol dependence 62:149-154.

31. Chao CC, Hu S, Peterson PK (1996b) Opiates, glia, and neurotoxicity. Advances in experimental medicine and biology 402:29-33.

32. Chen P, Mayne M, Power C, Nath A (1997) The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. The Journal of biological chemistry 272:22385-22388.

33. Choi Y, Chuang LF, Lam KM, Kung HF, Wang JM, Osburn BI, Chuang RY (1999) Inhibition of chemokine-induced chemotaxis of monkey leukocytes by mu-opioid receptor agonists. In vivo (Athens, Greece) 13:389-396.

34. Chuang LF, Killam KF, Jr., Chuang RY (1993) Opioid dependency and T-helper cell functions in rhesus monkey. In vivo (Athens, Greece) 7:159-166.

35. Churchill M, Nath A (2013) Where does HIV hide? A focus on the central nervous system. Current opinion in HIV and AIDS 8:165-169.

36. Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, Gorry PR (2009) Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. Annals of neurology 66:253-258.

37. Clerici M, Shearer GM (1994) The Th1-Th2 hypothesis of HIV infection: new insights. Immunology today 15:575-581.

38. Collier AC, Coombs RW, Schoenfeld DA, Bassett RL, Timpone J, Baruch A, Jones M, Facey K, Whitacre C, McAuliffe VJ, Friedman HM, Merigan TC, Reichman RC, Hooper C, Corey L (1996) Treatment of human immunodeficiency virus infection with saquinavir, zidovudine, and zalcitabine. AIDS Clinical Trials Group. The New England journal of medicine 334:1011-1017.

39. Curran HV, Bolton J, Wanigaratne S, Smyth C (1999) Additional methadone increases craving for heroin: a double-blind, placebo-controlled study of chronic opiate users receiving methadone substitution treatment. Addiction 94:665-674.

40. Cysique LA, Maruff P, Brew BJ (2004) Prevalence and pattern of neuropsychological impairment in human immunodeficiency virus-infected/acquired immunodeficiency syndrome (HIV/AIDS) patients across

pre- and post-highly active antiretroviral therapy eras: a combined study of two cohorts. Journal of neurovirology 10:350-357.

41. Dalgleish AG, Beverley PC, Clapham PR, Crawford DH, Greaves MF, Weiss RA (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312:763-767.

42. Darke S, Sims J, McDonald S, Wickes W (2000) Cognitive impairment among methadone maintenance patients. Addiction 95:687-695.

43. Debaisieux S, Rayne F, Yezid H, Beaumelle B (2012) The ins and outs of HIV-1 Tat. Traffic (Copenhagen, Denmark) 13:355-363.

44. Donahoe RM, Vlahov D (1998) Opiates as potential cofactors in progression of HIV-1 infections to AIDS. Journal of neuroimmunology 83:77-87.

45. Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell 85:1149-1158.

46. Downen M, Amaral TD, Hua LL, Zhao ML, Lee SC (1999) Neuronal death in cytokine-activated primary human brain cell culture: role of tumor necrosis factor-alpha. Glia 28:114-127.

47. Droge W (2002) Free radicals in the physiological control of cell function. Physiological reviews 82:47-95.

48. Duh EJ, Maury WJ, Folks TM, Fauci AS, Rabson AB (1989) Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. Proceedings of the National Academy of Sciences 86:5974-5978.

49. Durvasula R, Miller TR (2014) Substance Abuse Treatment in Persons with HIV/AIDS: Challenges in Managing Triple Diagnosis. Behavioral medicine (Washington, DC) 40:43-52.

50. Dutta R, Roy S (2012) Mechanism(s) involved in opioid drug abuse modulation of HAND. Current HIV research 10:469-477.

51. El-Hage N, Bruce-Keller AJ, Knapp PE, Hauser KF (2008a) CCL5/RANTES gene deletion attenuates opioid-induced increases in glial CCL2/MCP-1 immunoreactivity and activation in HIV-1 Tat-exposed mice.

Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 3:275-285.

52. El-Hage N, Bruce-Keller AJ, Yakovleva T, Bazov I, Bakalkin G, Knapp PE, Hauser KF (2008b) Morphine exacerbates HIV-1 Tat-induced cytokine production in astrocytes through convergent effects on [Ca(2+)](i), NF-kappaB trafficking and transcription. PloS one 3:e4093.

53. El-Hage N, Gurwell JA, Singh IN, Knapp PE, Nath A, Hauser KF (2005) Synergistic increases in intracellular Ca2+, and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. Glia 50:91-106.

54. El-Hage N, Podhaizer EM, Sturgill J, Hauser KF (2011) Toll-like receptor expression and activation in astroglia: differential regulation by HIV-1 Tat, gp120, and morphine. Immunological investigations 40:498-522.

55. El-Hage N, Wu G, Wang J, Ambati J, Knapp PE, Reed JL, Bruce-Keller AJ, Hauser KF (2006) HIV-1 Tat and opiate-induced changes in astrocytes promote chemotaxis of microglia through the expression of MCP-1 and alternative chemokines. Glia 53:132-146.

56. Ellis R, Langford D, Masliah E (2007) HIV and antiretroviral therapy in the brain: neuronal injury and repair. Nat Rev Neurosci 8:33-44.

57. Eschenroeder AC, Vestal-Laborde AA, Sanchez ES, Robinson SE, Sato-Bigbee C (2012) Oligodendrocyte responses to buprenorphine uncover novel and opposing roles of mu-opioid- and nociceptin/orphanin FQ receptors in cell development: implications for drug addiction treatment during pregnancy. Glia 60:125-136.

58. Eugenin EA, King JE, Nath A, Calderon TM, Zukin RS, Bennett MV, Berman JW (2007) HIV-tat induces formation of an LRP-PSD-95-NMDAR-nNOS complex that promotes apoptosis in neurons and astrocytes. Proceedings of the National Academy of Sciences of the United States of America 104:3438-3443.

59. Eugenin EA, Osiecki K, Lopez L, Goldstein H, Calderon TM, Berman JW (2006) CCL2/monocyte chemoattractant protein-1 mediates enhanced transmigration of human immunodeficiency virus (HIV)-infected leukocytes across the blood-brain barrier: a potential mechanism of HIV-CNS invasion and NeuroAIDS. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:1098-1106.

60. Fauci AS (1993) Multifactorial nature of human immunodeficiency virus disease: implications for therapy. Science 262:1011-1018.

61. Ferguson MR, Rojo DR, von Lindern JJ, O'Brien WA (2002) HIV-1 replication cycle. Clinics in laboratory medicine 22:611-635.

62. Fischer B, Rehm J, Kirst M, Casas M, Hall W, Krausz M, Metrebian N, Reggers J, Uchtenhagen A, van den Brink W, van Ree JM (2002) Heroin-assisted treatment as a response to the public health problem of opiate dependence. European journal of public health 12:228-234.

63. Fiume G, Vecchio E, De Laurentiis A, Trimboli F, Palmieri C, Pisano A, Falcone C, Pontoriero M, Rossi A, Scialdone A, Fasanella Masci F, Scala G, Quinto I (2012) Human immunodeficiency virus-1 Tat activates NF-kappaB via physical interaction with IkappaB-alpha and p65. Nucleic acids research 40:3548-3562.

64. Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, Fauci AS (1989) Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proceedings of the National Academy of Sciences of the United States of America 86:2365-2368.

65. Freed EO (2001) HIV-1 replication. Somatic cell and molecular genetics 26:13-33.

66. Gallo RC (2000) The path to the discoveries of human retroviruses. Journal of human virology 3:1-5.

67. Gallo RC, Sarin PS, Gelmann EP, Robert-Guroff M, Richardson E, Kalyanaraman VS, Mann D, Sidhu GD, Stahl RE, Zolla-Pazner S, Leibowitch J, Popovic M (1983) Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science 220:865-867.

68. George S, Day E (2007) Buprenorphine in the treatment of opioid dependence. British journal of hospital medicine (London, England : 2005) 68:594-597.

69. Georgsson G (1994) Neuropathologic aspects of lentiviral infections. Annals of the New York Academy of Sciences 724:50-67.

70. Ghosh S, Baltimore D (1990) Activation in vitro of NF-[kappa]B" by phosphorylation of its inhibitor I[kappa]B". Nature 344:678-682.

71. Gibson DR, Flynn NM, McCarthy JJ (1999) Effectiveness of methadone treatment in reducing HIV risk behavior and HIV seroconversion among injecting drug users. AIDS (London, England) 13:1807-1818.

72. Gil L, Martinez G, Gonzalez I, Tarinas A, Alvarez A, Giuliani A, Molina R, Tapanes R, Perez J, Leon OS (2003) Contribution to characterization of oxidative stress in HIV/AIDS patients. Pharmacological research : the official journal of the Italian Pharmacological Society 47:217-224.

73. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Fan PT, Wolf RA, Saxon A (1981) Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. The New England journal of medicine 305:1425-1431.

74. Govitrapong P, Suttitum T, Kotchabhakdi N, Uneklabh T (1998) Alterations of immune functions in heroin addicts and heroin withdrawal subjects. The Journal of pharmacology and experimental therapeutics 286:883-889.

75. Gowing L, Farrell M, Bornemann R, Sullivan L, Ali R (2008) Substitution treatment of injecting opioid users for prevention of HIV infection. The Cochrane database of systematic reviews Cd004145.

76. Gowing LR, Farrell M, Bornemann R, Sullivan LE, Ali RL (2006) Brief report: Methadone treatment of injecting opioid users for prevention of HIV infection. Journal of general internal medicine 21:193-195.

77. Grant I (2008) Neurocognitive disturbances in HIV. International review of psychiatry (Abingdon, England) 20:33-47.

78. Greenwald MK, Johanson CE, Moody DE, Woods JH, Kilbourn MR, Koeppe RA, Schuster CR, Zubieta JK (2003) Effects of buprenorphine maintenance dose on mu-opioid receptor availability, plasma concentrations, and antagonist blockade in heroin-dependent volunteers. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:2000-2009.

79. Gruber VA, McCance-Katz EF (2010) Methadone, buprenorphine, and street drug interactions with antiretroviral medications. Current HIV/AIDS reports 7:152-160.

80. Gupta S, Knight AG, Gupta S, Knapp PE, Hauser KF, Keller JN, Bruce-Keller AJ (2010) HIV-Tat elicits microglial glutamate release: role of NAPDH oxidase and the cystine-glutamate antiporter. Neuroscience letters 485:233-236.

81. Gurwell JA, Nath A, Sun Q, Zhang J, Martin KM, Chen Y, Hauser KF (2001) Synergistic neurotoxicity of opioids and human immunodeficiency virus-1 Tat protein in striatal neurons in vitro. Neuroscience 102:555-563.

82. Hauser KF, El-Hage N, Buch S, Berger JR, Tyor WR, Nath A, Bruce-Keller AJ, Knapp PE (2005) Molecular targets of opiate drug abuse in neuroAIDS. Neurotoxicity research 8:63-80.

83. Hauser KF, El-Hage N, Buch S, Nath A, Tyor WR, Bruce-Keller AJ, Knapp PE (2006) Impact of opiate-HIV-1 interactions on neurotoxic signaling. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 1:98-105.

84. Hauser KF, Fitting S, Dever SM, Podhaizer EM, Knapp PE (2012) Opiate drug use and the pathophysiology of neuroAIDS. Current HIV research 10:435-452.

85. Hauser KF, Harris-White ME, Jackson JA, Opanashuk LA, Carney JM (1998) Opioids disrupt Ca2+ homeostasis and induce carbonyl oxyradical production in mouse astrocytes in vitro: transient increases and adaptation to sustained exposure. Experimental neurology 151:70-76.

86. Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, Lange JM, Hamann D, Prins M, Miedema F (2003) Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS (London, England) 17:1881-1888.

87. Hazleton JE, Berman JW, Eugenin EA (2010) Novel mechanisms of central nervous system damage in HIV infection. HIV/AIDS (Auckland, NZ) 2:39-49.

88. He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, Busciglio J, Yang X, Hofmann W, Newman W, Mackay CR, Sodroski J, Gabuzda D (1997) CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature 385:645-649.

89. Heaton RK, Franklin DR, Ellis RJ, McCutchan JA, Letendre SL, Leblanc S, Corkran SH, Duarte NA, Clifford DB, Woods SP, Collier AC, Marra CM, Morgello S, Mindt MR, Taylor MJ, Marcotte TD, Atkinson JH, Wolfson T, Gelman BB, McArthur JC, Simpson DM, Abramson I, Gamst A, Fennema-Notestine C, Jernigan TL, Wong J, Grant I, Group C, Group H (2011) HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors. Journal of neurovirology 17:3-16.

90. Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR (2007) Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence, and reward. TheScientificWorldJournal 7:98-111.

91. Hutchinson MR, Coats BD, Lewis SS, Zhang Y, Sprunger DB, Rezvani N, Baker EM, Jekich BM, Wieseler JL, Somogyi AA, Martin D, Poole S, Judd CM, Maier SF, Watkins LR (2008) Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. Brain, behavior, and immunity 22:1178-1189.

92. Jerry JM, Collins GB (2013) Medication-assisted treatment of opiate dependence is gaining favor. Cleveland Clinic journal of medicine 80:345-349.

93. Kalinina OV, Pfeifer N, Lengauer T (2013) Modelling binding between CCR5 and CXCR4 receptors and their ligands suggests the surface electrostatic potential of the co-receptor to be a key player in the HIV-1 tropism. Retrovirology 10:130.

94. Kaul M, Garden GA, Lipton SA (2001) Pathways to neuronal injury and apoptosis in HIV-associated dementia. Nature 410:988-994.

95. Kehn-Hall K, Guendel I, Carpio L, Skaltsounis L, Meijer L, Al-Harthi L, Steiner JP, Nath A, Kutsch O, Kashanchi F (2011) Inhibition of Tat-mediated HIV-1 replication and neurotoxicity by novel GSK3-beta inhibitors. Virology 415:56-68.

96. Kim HJ, Martemyanov KA, Thayer SA (2008) Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:12604-12613.

97. Koch T, Seifert A, Wu DF, Rankovic M, Kraus J, Borner C, Brandenburg LO, Schroder H, Hollt V (2009) mu-opioid receptor-stimulated synthesis of reactive oxygen species is mediated via phospholipase D2. Journal of neurochemistry 110:1288-1296.

98. Koch T, Widera A, Bartzsch K, Schulz S, Brandenburg LO, Wundrack N, Beyer A, Grecksch G, Hollt V (2005) Receptor endocytosis counteracts the development of opioid tolerance. Molecular pharmacology 67:280-287.

99. Kong LY, Wilson BC, McMillian MK, Bing G, Hudson PM, Hong JS (1996) The effects of the HIV-1 envelope protein gp120 on the production of nitric oxide and proinflammatory cytokines in mixed glial cell cultures. Cellular immunology 172:77-83.

100. Kraft AD, Harry GJ (2011) Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. International journal of environmental research and public health 8:2980-3018.

101. Kreek MJ, Borg L, Ducat E, Ray B (2010) Pharmacotherapy in the treatment of addiction: methadone. Journal of addictive diseases 29:200-216.

102. Krogh KA, Wydeven N, Wickman K, Thayer SA (2014) HIV-1 protein Tat produces biphasic changes in NMDA-evoked increases in intracellular Ca concentration via activation of Src kinase and nitric oxide signaling pathways. Journal of neurochemistry.

103. Kumar R, Orsoni S, Norman L, Verma AS, Tirado G, Giavedoni LD, Staprans S, Miller GM, Buch SJ, Kumar A (2006) Chronic morphine exposure causes pronounced virus replication in cerebral compartment and accelerated onset of AIDS in SIV/SHIV-infected Indian rhesus macaques. Virology 354:192-206.

104. Kumar R, Torres C, Yamamura Y, Rodriguez I, Martinez M, Staprans S, Donahoe RM, Kraiselburd E, Stephens EB, Kumar A (2004) Modulation by morphine of viral set point in rhesus macaques infected with simian immunodeficiency virus and simian-human immunodeficiency virus. Journal of virology 78:11425-11428.

105. Li Y, Merrill JD, Mooney K, Song L, Wang X, Guo CJ, Savani RC, Metzger DS, Douglas SD, Ho WZ (2003) Morphine enhances HIV infection of neonatal macrophages. Pediatr Res 54:282-288.

106. Li Y, Wang X, Tian S, Guo CJ, Douglas SD, Ho WZ (2002) Methadone enhances human immunodeficiency virus infection of human immune cells. The Journal of infectious diseases 185:118-122.

107. Liu JG, Liao XP, Gong ZH, Qin BY (1999) Methadone-induced desensitization of the delta-opioid receptor is mediated by uncoupling of receptor from G protein. European journal of pharmacology 374:301-308.

108. Liu Y, Jones M, Hingtgen CM, Bu G, Laribee N, Tanzi RE, Moir RD, Nath A, He JJ (2000) Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. Nature medicine 6:1380-1387.

109. Longshore D, Hsieh S, Danila B, Anglin MD (1993) Methadone maintenance and needle/syringe sharing. The International journal of the addictions 28:983-996.

110. Lucas GM, Griswold M, Gebo KA, Keruly J, Chaisson RE, Moore RD (2006) Illicit drug use and HIV-1 disease progression: a longitudinal study in the era of highly active antiretroviral therapy. American journal of epidemiology 163:412-420.

111. Lutfy K, Eitan S, Bryant CD, Yang YC, Saliminejad N, Walwyn W, Kieffer BL, Takeshima H, Carroll FI, Maidment NT, Evans CJ (2003) Buprenorphine-induced antinociception is mediated by mu-opioid receptors and compromised by concomitant activation of opioid receptor-like receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:10331-10337.

112. MacArthur GJ, Minozzi S, Martin N, Vickerman P, Deren S, Bruneau J, Degenhardt L, Hickman M (2012) Opiate substitution treatment and HIV transmission in people who inject drugs: systematic review and meta-analysis. Bmj 345:e5945.

113. Maciaszek JW, Talmage DA, Viglianti GA (1994) Synergistic activation of simian immunodeficiency virus and human immunodeficiency virus type 1 transcription by retinoic acid and phorbol ester through an NF-kappa B-independent mechanism. Journal of virology 68:6598-6604.

114. Mauger S, Fraser R, Gill K (2014) Utilizing buprenorphine-naloxone to treat illicit and prescription-opioid dependence. Neuropsychiatric disease and treatment 10:587-598.

115. McArthur JC, Steiner J, Sacktor N, Nath A (2010) Human immunodeficiency virus-associated neurocognitive disorders: Mind the gap. Annals of neurology 67:699-714.

116. Mello NK, Mendelson JH (1985) Behavioral pharmacology of buprenorphine. Drug and alcohol dependence 14:283-303.

117. Meltzer MS, Gendelman HE (1992) Mononuclear phagocytes as targets, tissue reservoirs, and immunoregulatory cells in human immunodeficiency virus disease. Current topics in microbiology and immunology 181:239-263.

118. Metzger DS, Navaline H, Woody GE (1998) Drug abuse treatment as AIDS prevention. Public health reports 113 Suppl 1:97-106.

119. Michihiko S, Yamamoto N, Shinozaki F, Shimada K, Soma G, Kobayashi N (1989) Augmentation of in-vitro HIV replication in peripheral blood mononuclear cells of AIDS and ARC patients by tumour necrosis factor. Lancet 1:1206-1207.

120. Miedema F, Hazenberg MD, Tesselaar K, van Baarle D, de Boer RJ, Borghans JA (2013) Immune Activation and Collateral Damage in AIDS Pathogenesis. Frontiers in immunology 4:298.

121. Mintzer MZ, Correia CJ, Strain EC (2004) A dose-effect study of repeated administration of buprenorphine/naloxone on performance in opioid-dependent volunteers. Drug and alcohol dependence 74:205-209.

122. Mocroft A, Vella S, Benfield TL, Chiesi A, Miller V, Gargalianos P, d'Arminio Monforte A, Yust I, Bruun JN, Phillips AN, Lundgren JD (1998) Changing patterns of mortality across Europe in patients infected with HIV-1. EuroSIDA Study Group. Lancet 352:1725-1730.

123. Moir S, Chun TW, Fauci AS (2011) Pathogenic mechanisms of HIV disease. Annual review of pathology 6:223-248.

124. Montagnier L (2010) 25 years after HIV discovery: prospects for cure and vaccine. Virology 397:248-254.

125. Moore JP, Kitchen SG, Pugach P, Zack JA (2004) The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS research and human retroviruses 20:111-126.

126. Murray M, Hogg RS, Lima VD, May MT, Moore DM, Abgrall S, Bruyand M, D'Arminio Monforte A, Tural C, Gill MJ, Harris RJ, Reiss P, Justice A, Kirk O, Saag M, Smith CJ, Weber R, Rockstroh J, Khaykin P, Sterne JA (2012) The effect of injecting drug use history on disease progression and death among HIV-positive individuals initiating combination antiretroviral therapy: collaborative cohort analysis. HIV medicine 13:89-97.

127. Nabel G, Baltimore D (1987) An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326:711-713.

128. Naif HM (2013) Pathogenesis of HIV Infection. Infectious disease reports 5:e6.

129. Nath A (1999) Pathobiology of human immunodeficiency virus dementia. Seminars in neurology 19:113-127.

130. Nath A, Clements JE (2011) Eradication of HIV from the brain: reasons for pause. AIDS (London, England) 25:577-580.

131. Nath A, Conant K, Chen P, Scott C, Major EO (1999) Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. The Journal of biological chemistry 274:17098-17102.

132. Nath A, Hartloper V, Furer M, Fowke KR (1995) Infection of human fetal astrocytes with HIV-1: viral tropism and the role of cell to cell contact in viral transmission. Journal of neuropathology and experimental neurology 54:320-330.

133. Nath A, Hauser KF, Wojna V, Booze RM, Maragos W, Prendergast M, Cass W, Turchan JT (2002) Molecular basis for interactions of HIV and drugs of abuse. Journal of acquired immune deficiency syndromes (1999) 31 Suppl 2:S62-69.

134. Nath A, Steiner J (2014) Synaptodendritic injury with HIV-Tat protein: What is the therapeutic target? Experimental neurology 251:112-114.

135. Neri S, Bruno CM, Pulvirenti D, Malaguarnera M, Italiano C, Mauceri B, Abate G, Cilio D, Calvagno S, Tsami A, Ignaccolo L, Interlandi D, Prestianni L, Ricchena M, Noto R (2005) Randomized clinical trial to compare the effects of methadone and buprenorphine on the immune system in drug abusers. Psychopharmacology 179:700-704.

136. Novick DM, Ochshorn M, Ghali V, Croxson TS, Mercer WD, Chiorazzi N, Kreek MJ (1989) Natural killer cell activity and lymphocyte subsets in parenteral heroin abusers and long-term methadone maintenance patients. The Journal of pharmacology and experimental therapeutics 250:606-610.

137. Novick DM, Ochshorn M, Kreek MJ (1991) In vivo and in vitro studies of opiates and cellular immunity in narcotic addicts. Advances in experimental medicine and biology 288:159-170.

138. Novick DM, Richman BL, Friedman JM, Friedman JE, Fried C, Wilson JP, Townley A, Kreek MJ (1993) The medical status of methadone maintenance patients in treatment for 11-18 years. Drug and alcohol dependence 33:235-245.

139. O'Brien SJ, Hendrickson SL (2013) Host genomic influences on HIV/AIDS. Genome biology 14:201.

140. Osborn L, Kunkel S, Nabel GJ (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proceedings of the National Academy of Sciences of the United States of America 86:2336-2340.

141. Perez-Casanova A, Noel RJ, Jr., Rivera-Amill V, Husain K, Kumar A (2007) Morphine-mediated deterioration of oxidative stress leads to

rapid disease progression in SIV/SHIV-infected macaques. AIDS research and human retroviruses 23:1004-1007.

142. Peterson PK, Gekker G, Brummitt C, Pentel P, Bullock M, Simpson M, Hitt J, Sharp B (1989) Suppression of human peripheral blood mononuclear cell function by methadone and morphine. The Journal of infectious diseases 159:480-487.

143. Peterson PK, Gekker G, Hu S, Anderson WR, Kravitz F, Portoghese PS, Balfour HH, Jr., Chao CC (1994) Morphine amplifies HIV-1 expression in chronically infected promonocytes cocultured with human brain cells. Journal of neuroimmunology 50:167-175.

144. Peterson PK, Gekker G, Hu S, Schoolov Y, Balfour HH, Jr., Chao CC (1992) Microglial cell upregulation of HIV-1 expression in the chronically infected promonocytic cell line U1: the role of tumor necrosis factor-alpha. Journal of neuroimmunology 41:81-87.

145. Peterson PK, Gekker G, Lokensgard JR, Bidlack JM, Chang AC, Fang X, Portoghese PS (2001) Kappa-opioid receptor agonist suppression of HIV-1 expression in CD4+ lymphocytes. Biochemical pharmacology 61:1145-1151.

146. Peterson PK, Sharp BM, Gekker G, Portoghese PS, Sannerud K, Balfour HH, Jr. (1990) Morphine promotes the growth of HIV-1 in human peripheral blood mononuclear cell cocultures. AIDS (London, England) 4:869-873.

147. Pirastu R, Fais R, Messina M, Bini V, Spiga S, Falconieri D, Diana M (2006) Impaired decision-making in opiate-dependent subjects: effect of pharmacological therapies. Drug and alcohol dependence 83:163-168.

148. Pitha PM (2011) Innate antiviral response: role in HIV-1 infection. Viruses 3:1179-1203.

149. Podhaizer EM, Zou S, Fitting S, Samano KL, El-Hage N, Knapp PE, Hauser KF (2012) Morphine and gp120 toxic interactions in striatal neurons are dependent on HIV-1 strain. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 7:877-891.

150. Polazzi E, Levi G, Minghetti L (1999) Human immunodeficiency virus type 1 Tat protein stimulates inducible nitric oxide synthase expression and nitric oxide production in microglial cultures. Journal of neuropathology and experimental neurology 58:825-831.

151. Poli G, Fauci AS (1992) The effect of cytokines and pharmacologic agents on chronic HIV infection. AIDS research and human retroviruses 8:191-197.

152. Pomerantz RJ, Horn DL (2003) Twenty years of therapy for HIV-1 infection. Nature medicine 9:867-873.

153. Quagliata F, Melton R, Soika I (1977) Decreased response to mitogens and impaired capping ability of lymphocytes in narcotic addiction. In: CLINICAL RESEARCH, vol. 25, pp A513-A513: SLACK INC 6900 GROVE RD, THOROFARE, NJ 08086.

154. Ramesh G, MacLean AG, Philipp MT (2013) Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. Mediators of inflammation 2013:480739.

155. Rapeli P, Fabritius C, Alho H, Salaspuro M, Wahlbeck K, Kalska H (2007) Methadone vs. buprenorphine/naloxone during early opioid substitution treatment: a naturalistic comparison of cognitive performance relative to healthy controls. BMC clinical pharmacology 7:5.

156. Ray M, Logan R, Sterne JA, Hernandez-Diaz S, Robins JM, Sabin C, Bansi L, van Sighem A, de Wolf F, Costagliola D, Lanoy E, Bucher HC, von Wyl V, Esteve A, Casbona J, del Amo J, Moreno S, Justice A, Goulet J, Lodi S, Phillips A, Seng R, Meyer L, Perez-Hoyos S, Garcia de Olalla P, Hernan MA (2010) The effect of combined antiretroviral therapy on the overall mortality of HIV-infected individuals. AIDS (London, England) 24:123-137.

157. Rayne F, Vendeville A, Bonhoure A, Beaumelle B (2004) The ability of chloroquine to prevent tat-induced cytokine secretion by monocytes is implicated in its in vivo anti-human immunodeficiency virus type 1 activity. Journal of virology 78:12054-12057.

158. Reddy MM, Englard A, Brown D, Buimovici-Klien E, Grieco MH (1987) Lymphoproliferative responses to human immunodeficiency virus antigen in asymptomatic intravenous drug abusers and in patients with lymphadenopathy or AIDS. The Journal of infectious diseases 156:374-376.

159. Regan PM, Dave RS, Datta PK, Khalili K (2012) Epigenetics of micro-opioid receptors: intersection with HIV-1 infection of the central nervous system. Journal of cellular physiology 227:2832-2841.

160. Robinson SE (2006) Buprenorphine-containing treatments: place in the management of opioid addiction. CNS drugs 20:697-712.

161. Romero FJ, Bosch-Morell F, Romero MJ, Jareno EJ, Romero B, Marin N, Roma J (1998) Lipid peroxidation products and antioxidants in human disease. Environmental health perspectives 106 Suppl 5:1229-1234.

162. Roux P, Alfieri C, Hrimech M, Cohen EA, Tanner JE (2000) Activation of transcription factors NF-kappaB and NF-IL-6 by human immunodeficiency virus type 1 protein R (Vpr) induces interleukin-8 expression. Journal of virology 74:4658-4665.

163. Roux P, Carrieri MP, Villes V, Dellamonica P, Poizot-Martin I, Ravaux I, Spire B (2008) The impact of methadone or buprenorphine treatment and ongoing injection on highly active antiretroviral therapy (HAART) adherence: evidence from the MANIF2000 cohort study. Addiction 103:1828-1836.

164. Roy S, Cain KJ, Chapin RB, Charboneau RG, Barke RA (1998) Morphine modulates NF kappa B activation in macrophages. Biochemical and biophysical research communications 245:392-396.

165. Sacerdote P, Limiroli E, Gaspani L (2003) Experimental evidence for immunomodulatory effects of opioids. Advances in experimental medicine and biology 521:106-116.

166. Sacktor N (2002) The epidemiology of human immunodeficiency virus-associated neurological disease in the era of highly active antiretroviral therapy. Journal of neurovirology 8 Suppl 2:115-121.

167. Sacktor NC, Skolasky RL, Lyles RH, Esposito D, Selnes OA, McArthur JC (2000) Improvement in HIV-associated motor slowing after antiretroviral therapy including protease inhibitors. Journal of neurovirology 6:84-88.

168. Saksena NK, Wang B, Zhou L, Soedjono M, Ho YS, Conceicao V (2010) HIV reservoirs in vivo and new strategies for possible eradication of HIV from the reservoir sites. HIV/AIDS (Auckland, NZ) 2:103-122.

169. Sanchez ES, Bigbee JW, Fobbs W, Robinson SE, Sato-Bigbee C (2008) Opioid addiction and pregnancy: perinatal exposure to buprenorphine affects myelination in the developing brain. Glia 56:1017-1027.

170. Schousboe A, Sonnewald U, Civenni G, Gegelashvili G (1997) Role of astrocytes in glutamate homeostasis. Implications for excitotoxicity. Advances in experimental medicine and biology 429:195-206.

171. Selwyn PA, Alcabes P, Hartel D, Buono D, Schoenbaum EE, Klein RS, Davenny K, Friedland GH (1992) Clinical manifestations and predictors of disease progression in drug users with human immunodeficiency virus infection. The New England journal of medicine 327:1697-1703.

172. Sharma A, Hu XT, Napier TC, Al-Harthi L (2011) Methamphetamine and HIV-1 Tat down regulate beta-catenin signaling: implications for methampetamine abuse and HIV-1 co-morbidity. Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology 6:597-607.

173. Smith DB, Simmonds P, Bell JE (2014) Brain viral burden, neuroinflammation and neurodegeneration in HAART-treated HIV positive injecting drug users. Journal of neurovirology 20:28-38.

174. Sonnerborg A, Carlin G, Akerlund B, Jarstrand C (1988) Increased production of malondialdehyde in patients with HIV infection. Scandinavian journal of infectious diseases 20:287-290.

175. Sorensen JL, Copeland AL (2000) Drug abuse treatment as an HIV prevention strategy: a review. Drug and alcohol dependence 59:17-31.

176. Speth C, Joebstl B, Barcova M, Dierich MP (2000) HIV-1 envelope protein gp41 modulates expression of interleukin-10 and chemokine receptors on monocytes, astrocytes and neurones. AIDS (London, England) 14:629-636.

177. Staszewski S, Miller V, Rehmet S, Stark T, De Cree J, De Brabander M, Peeters M, Andries K, Moeremans M, De Raeymaeker M, Pearce G, Van den Broeck R, Verbiest W, Stoffels P (1996) Virological and immunological analysis of a triple combination pilot study with loviride, lamivudine and zidovudine in HIV-1-infected patients. AIDS (London, England) 10:F1-7.

178. Steele AD, Henderson EE, Rogers TJ (2003) Mu-opioid modulation of HIV-1 coreceptor expression and HIV-1 replication. Virology 309:99-107.

179. Stevenson M (2003) HIV-1 pathogenesis. Nature medicine 9:853-860.

180. Stiene-Martin A, Mattson MP, Hauser KF (1993) Opiates selectively increase intracellular calcium in developing type-1 astrocytes: role of calcium in morphine-induced morphologic differentiation. Brain research Developmental brain research 76:189-196.

181. Stotts AL, Dodrill CL, Kosten TR (2009) Opioid dependence treatment: options in pharmacotherapy. Expert opinion on pharmacotherapy 10:1727-1740.

182. Sullivan LE, Moore BA, Chawarski MC, Pantalon MV, Barry D, O'Connor PG, Schottenfeld RS, Fiellin DA (2008) Buprenorphine/naloxone treatment in primary care is associated with decreased human immunodeficiency virus risk behaviors. Journal of substance abuse treatment 35:87-92.

183. Sundquist WI, Krausslich HG (2012) HIV-1 assembly, budding, and maturation. Cold Spring Harbor perspectives in medicine 2:a006924.

184. Suzuki S, Carlos MP, Chuang LF, Torres JV, Doi RH, Chuang RY (2002) Methadone induces CCR5 and promotes AIDS virus infection. FEBS letters 519:173-177.

185. Takeuchi H, Jin S, Wang J, Zhang G, Kawanokuchi J, Kuno R, Sonobe Y, Mizuno T, Suzumura A (2006) Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. The Journal of biological chemistry 281:21362-21368.

186. Than S, Hu R, Oyaizu N, Romano J, Wang X, Sheikh S, Pahwa S (1997) Cytokine pattern in relation to disease progression in human immunodeficiency virus-infected children. The Journal of infectious diseases 175:47-56.

187. Toskulkao T, Pornchai R, Akkarapatumwong V, Vatanatunyakum S, Govitrapong P (2010) Alteration of lymphocyte opioid receptors in methadone maintenance subjects. Neurochemistry international 56:285-290.

188. Turchan-Cholewo J, Dimayuga FO, Gupta S, Keller JN, Knapp PE, Hauser KF, Bruce-Keller AJ (2009) Morphine and HIV-Tat increase microglial-free radical production and oxidative stress: possible role in cytokine regulation. Journal of neurochemistry 108:202-215.

189. Turchan-Cholewo J, Liu Y, Gartner S, Reid R, Jie C, Peng X, Chen KC, Chauhan A, Haughey N, Cutler R, Mattson MP, Pardo C, Conant K, Sacktor N, McArthur JC, Hauser KF, Gairola C, Nath A (2006) Increased vulnerability of ApoE4 neurons to HIV proteins and opiates: protection by diosgenin and L-deprenyl. Neurobiology of disease 23:109-119.

190. Turner BG, Summers MF (1999) Structural biology of HIV. Journal of molecular biology 285:1-32.

191. Van der Watt JJ, Wilkinson KA, Wilkinson RJ, Heckmann JM (2014) Plasma cytokine profiles in HIV-1 infected patients developing neuropathic symptoms shortly after commencing antiretroviral therapy: a case-control study. BMC infectious diseases 14:71.

192. Vlachakis D, Tsiliki G, Pavlopoulou A, Roubelakis MG, Tsaniras SC, Kossida S (2013) Antiviral Stratagems Against HIV-1 Using RNA Interference (RNAi) Technology. Evolutionary bioinformatics online 9:203-213.

193. Wang Z, Pekarskaya O, Bencheikh M, Chao W, Gelbard HA, Ghorpade A, Rothstein JD, Volsky DJ (2003) Reduced expression of glutamate transporter EAAT2 and impaired glutamate transport in human primary astrocytes exposed to HIV-1 or gp120. Virology 312:60-73.

194. Weber R, Ledergerber B, Opravil M, Siegenthaler W, Luthy R (1990) Progression of HIV infection in misusers of injected drugs who stop injecting or follow a programme of maintenance treatment with methadone. Bmj 301:1362-1365.

195. Wee S, Vendruscolo LF, Misra KK, Schlosburg JE, Koob GF (2012) A combination of buprenorphine and naltrexone blocks compulsive cocaine intake in rodents without producing dependence. Science translational medicine 4:146ra110.

196. Weiss RA (2003) HIV and AIDS in relation to other pandemics. Among the viruses plaguing humans, HIV is a recent acquisition. Its outstanding success as an infection poses immense scientific challenges to human health and raises the question "What comes nest?". EMBO reports 4 Spec No:S10-14.

197. Wesselingh SL, Power C, Glass JD, Tyor WR, McArthur JC, Farber JM, Griffin JW, Griffin DE (1993) Intracerebral cytokine messenger RNA expression in acquired immunodeficiency syndrome dementia. Annals of neurology 33:576-582.

198. Wesson DR (2004) Buprenorphine in the treatment of opiate dependence: its pharmacology and social context of use in the U.S. Journal of psychoactive drugs Suppl 2:119-128.

199. West MJ, Lowe AD, Karn J (2001) Activation of human immunodeficiency virus transcription in T cells revisited: NF-kappaB p65 stimulates transcriptional elongation. Journal of virology 75:8524-8537.

200. Wetzel MA, Steele AD, Eisenstein TK, Adler MW, Henderson EE, Rogers TJ (2000) Mu-opioid induction of monocyte chemoattractant

protein-1, RANTES, and IFN-gamma-inducible protein-10 expression in human peripheral blood mononuclear cells. Journal of immunology (Baltimore, Md : 1950) 165:6519-6524.

201. Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MB (1986) Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. Proceedings of the National Academy of Sciences of the United States of America 83:7089-7093.

202. Wolff K, Rostami-Hodjegan A, Shires S, Hay AW, Feely M, Calvert R, Raistrick D, Tucker GT (1997) The pharmacokinetics of methadone in healthy subjects and opiate users. British journal of clinical pharmacology 44:325-334.

203. Yeager MP, Colacchio TA, Yu CT, Hildebrandt L, Howell AL, Weiss J, Guyre PM (1995) Morphine inhibits spontaneous and cytokine-enhanced natural killer cell cytotoxicity in volunteers. Anesthesiology 83:500-508.

204. Zhao L, Pu SS, Gao WH, Chi YY, Wen HL, Wang ZY, Song YY, Yu XJ (2014) Effects of HIV-1 tat on secretion of TNF-alpha and IL-1beta by U87 cells in AIDS patients with or without AIDS dementia complex. Biomedical and environmental sciences : BES 27:111-117.

205. Zhou BY, Liu Y, Kim B, Xiao Y, He JJ (2004) Astrocyte activation and dysfunction and neuron death by HIV-1 Tat expression in astrocytes. Molecular and cellular neurosciences 27:296-305.

206. Zhou L, Saksena NK (2013) HIV Associated Neurocognitive Disorders. Infectious disease reports 5:e8.

207. Zou JY, Crews FT (2005) TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. Brain research 1034:11-24.

208. Zou S, Fitting S, Hahn YK, Welch SP, El-Hage N, Hauser KF, Knapp PE (2011) Morphine potentiates neurodegenerative effects of HIV-1 Tat through actions at mu-opioid receptor-expressing glia. Brain : a journal of neurology 134:3616-3631.