Interactions between Intracerebral Human Immunodeficiency Virus-1 Glycoprotein 120 and Systemic Heroin on Expression of Messenger Ribonucleic Acid (mRNA) of Inducible Nitric Oxide Synthase, Interleukin-1β, Tumor Necrosis Factor-α, and Cyclooxygenase-2 in Hippocampus and Cortex Brain Tissue of the Lewis Rat

Alison F. Wagner

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Approved by:

Donald T. Lysle

Mitchell J. Picker

Todd E. Thiele

Abstract

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(Under the direction of Donald T. Lysle)

Neurological complications caused by interactions of the immune system and the nervous system commonly occur in human immunodeficiency virus patients (HIV). Intravenous drug users, including heroin users, have increased rates of HIV-induced pathology in the brain. Glycoprotein 120 (gp120), a coat protein of HIV, has been implicated as a possible causative factor. These experiments examined an *in vivo* model of HIV infection in the brain by injecting gp120 into the lateral ventricles of Lewis rats with and without systemic heroin administration. The effects of gp120 and heroin were measured in expression of messenger RNA (mRNA) for the important proinflammatory mediators inducible nitric oxide synthase, interleukin-1β, tumor necrosis factor-α, and cyclooxygenase-2 in hippocampus and cortex tissue. The results of these experiments indicate that heroin may uniquely alter the acute effects of gp120 in

the rat brain on mRNA, allowing increased expression of interleukin-1β and inducible nitric oxide mRNA.

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List of Abbreviations and Symbols

α- alpha

β- beta

μ- mu

AIDS- acquired immunodeficiency syndrome

BSA- bovine serum albumin

COX-2 – cyclooxygenase-2

Gp120- glycoprotein 120

HIV- human immunodeficiency virus

HIVE- human immunodeficiency virus encephalitis

icv- intracerebroventricularly

IL-1β- interleukin 1beta

iNOS – inducible nitric oxide synthase

ip- intraperitoneal

mRNA- messenger ribonucleic acid

PCR- polymerase chain reaction

PGE2- prostaglandin E 2

RT-PCR- real-time polymerase chain reaction

sc- subcutaneously

TNF-α- tumor necrosis factor alpha

I. Introduction

HIV/AIDS and Neurological Complications: Clinical Data

During the 1980s, a puzzling condition that came to be known as acquired immunodeficiency syndrome (AIDS) began to become prevalent in the United States, particularly among intravenous drug users and homosexual men. The first signs of the HIV epidemic in the United States were reported in the Morbidity and Mortality Weekly Report (MMWR) of Centers for Disease Control (CDC) in June of 1981 (CDC, 1981). In 1983, it was discovered that AIDS was caused by a lentivirus, which is now known as human immunodeficiency virus (HIV) (Barre-Sinoussi et al., 1983). Today, 22 million people are known to have died from AIDS and AIDS-related complications, and 1 million more are infected with HIV in the United States alone as of 2006 (CDC, 2006).

Neurological complications are common among HIV patients. The terminology of these complications is complex and must be determined in order to understand the conditions fully (for review of terminology and diagnostic criteria, see Grant, Sacktor, & McArthur, 2005). These neurological complications are collectively referred to as neurocognitive impairments. HIV-associated dementia (HAD) indicates a severe dementia in which the patient is unable to function in daily life due to cognitive impairments. Mild cognitive motor disorder (MCMD) refers to deficits in 2 or more symptom areas that may affect

daily life. Included in these symptoms are neurocognitive decline, motor symptoms, and behavioral changes. Another term often used in the literature is HIV encephalitis (HIVE), which simply refers to the physiological evidence of pathology believed to be associated with neurocognitive impairments in HIV.

In the early years of the HIV epidemic, these symptoms were severe and death usually occurred within onset of dementia. Prevalence of dementia has decreased with the use of HAART, but the most drugs used in HAART do not cross the blood brain barrier (Flexner, 1998; Groothier & Levy, 1997). Today, with the use of highly active antiretroviral therapy (HAART), neurocognitive impairments are more commonly diagnosed as the milder MCMD, which may be a more prevalent problem with the increased lifespan of HIV/AIDS patients (Grant, Sacktor, & McArthur, 2005).

Neurocognitive impairments in HIV are associated with neuronal loss due to HIV infection of the brain (Everall, Luthert, & Lantos, 1991; Ketzler, Weis, Haug, & Budka, 1990). Clinical studies have not been able to clearly elucidate the relationship between viral load in the brain and dementia or other indicators of neurocognitive deficits. For instance, ribonucleic acid (RNA) levels of HIV were correlated with severity of dementia, but it was later found that levels of gp41, an HIV protein, are not correlated with severity of dementia in a postmortem analysis of patients with known dementia (Glass, Fedor, Wesselingh, & McArthur, 1995; Wesselingh, Glass, McArthur, Griffin, & Griffin, 1994). However, another study found that dementia was correlated with increased HIV positive cells of the brain regions associated with the symptoms

seen in the patients prior to death (Nuovo & Alfieri, 1996). Interestingly, HIV does not infect neurons directly, and apoptosis of neurons that is seen in HIV is not associated with productive HIV infection in the brain (Adle-Biasette et al., 1995). It is apparent that there might be a correlational relationship between HIV levels in the brain and severity of symptoms, but it is not consistent and other components may play a much bigger role in the pathogenesis of neurocognitive impairments in HIV.

One of the key components involved in causing neurocognitive impairment is the activation of the immune system in the brain. Interactions between the immune system and the central nervous system have taken a prominent role in the mechanisms of pathology in neurocognitive impairments in HIV. While HIV does not infect neurons directly, immune cells such as microglia/macrophages and astrocytes are well-known to be infected readily by HIV and are the likely culprits involved in bringing HIV into the brain (for review, see Eugenin & Berman, 2005). Activated microglia/macrophages are correlated with severity of impairment in patients diagnosed with HIV-associated dementia (Glass, Fedor, Wesselingh, & McArthur, 1995; review, Kaul & Lipton, 2006). Chronic activation of microglia may induce neurotoxic levels of release of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as upregulation of products involved in the arachadonic acid cascade such as cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) (for review of neurotoxic effects of microglial activation, Block & Hong, 2005; Brune, 2003; Marchetti & Abbracchio, 2005). Another product of microglial activation is

inducible nitric oxide (iNOS), which is responsible for release of large amounts of nitric oxide that can take on neurotoxic characteristics (Reynolds, Laurie, Mosley, & Gendelman, 2007; Snyder, 1993). A number of these molecules are present in high levels in patients with HIV, and particularly in HIV positive patients with neurocognitive impairments. For instance, one early study found increased levels of TNF-α and IL-1 in cerebrospinal fluid (CSF) and cortical tissue of HIV patients, but did not find an increase according to central nervous system symptoms (Tyor et al., 1992). However, later studies have found differences in proinflammatory activity in those HIV positive patients with neurological symptoms and those with none. Examination of RNA extracted from HIV positive patients diagnosed with dementia, vacuolar myelopathy (associated with motor symptoms), or neuropathy found increased levels of TNF-α, but there was no increase in TNF-α in HIV patients without neurological impairments (Wesselingh et al 1994). CSF samples of HIV patients with neurological disease show increased PGE2, iNOS, TNF-α, and IL-1 that is correlated with severity of neurological disease (Griffin, Wesselingh, & McArthur, 1994; Perrella, Carrieri, Guarnaccia, & Soscia, 1992; Vincent et al., 1999). HIV patients with encephalitis also show increased expression of IL-1β and iNOS (Zhau, Kim, Morgello, & Lee, 2001). Human astroglia and monocytes exposed to HIV *in vitro* also release toxic levels of IL-1β, TNF-α and products of the arachadonic acid cascade (Genis et al., 1992). The high levels of these markers of inflammation indicate a chronic condition in the brain of HIV patients with neurological symptoms that could very well lead to apoptosis.

Indeed, these markers are found in colocalized with HIV positive cells in the brain, indicating that productive infection of microglia and astrocytes are causing the release of the proinflammatory mediators from the immune cells (Nuovo & Alfieri, 1996; Zhau et al., 2001; Rostasy et al., 1999). However, apoptosis of neurons does not occur immediately adjacent to these HIV positive cells, nor does it occur immediately after infection; instead, apoptosis only occurs after a delay of at least 1-2 weeks of infection, and the lack of immediate localization indicates the apoptosis is caused by soluble factors released by the infected cells (Shi et al., 1996). These data indicate that apoptosis of neurons is caused indirectly through immune cell mediators.

Collectively, these data indicate an increased, chronic inflammatory response in brains of HIV positive patients that is correlated with neuron loss associated with neurological impairments. Unlike many of the hallmark symptoms of HIV/AIDS, neurological impairments in HIV are not associated with opportunistic infections, and evidence clearly points towards a fundamental part of HIV as being the stimulating factor of the cascade of actions that begins with activation of immune cells and ultimately induces apoptosis of neurons (for review, see Kaul & Lipton, 2001). There is substantial evidence indicating that some of the nine proteins made by HIV are able to induce activation of immune cells. Interestingly, actual productive infection by HIV is not necessary to produce cytokines such as IL-1 and TNF-α, supporting the possibility of HIV protein-induced immune activation (Merrill et al., 1992). Although many proteins of HIV are implicated in these processes, tat and gp120 are the most studied.

For a review on Tat, see King, Eugenin, Buckner, and Berman (2006). *In vitro* evidence from cultured human glial cultures produce IL-1, TNF-α, and nitric oxide when exposed to gp120, and gp120 induces death in human neuroblastoma cells and rat striatal neurons (Corasaniti et al., 1995; Koka et al., 1995; Singh et al., 2004). Oxidative stress is another factor that may induce apoptosis by gp120 (Jana & Pahan, 2004).

HIV/AIDS and Neurological Complications: Experimental Evidence (*In Vivo* and *In Vitro)*

Injection of gp120 into the lateral ventricles of rats has become a common experimental model of neurological effects of HIV in the brain (for review, see Brenneman, McCune, Mervis, & Hill, 1994; Corasaniti et al., 2001). Daily intravcerebroventricular injections of gp120 up to 14 days caused an increase in COX-2, PGE2, and IL-1β in brain tissue as well as neuronal apoptosis, which was reversed by COX-2 inhibitor indomethacin or an IL-1β converting enzyme inhibitor (Bagetto, et al., 1995; Bagetta, et al., 1996; Bagetta, et al., 1996; Bagetta et al., 1998; Bagetta et al., 1999; Barak et al., 2002; Maccorrone et al., 2000). Injection of gp120 into the rat striatum produces a decrease in brain-derived neurotrophic factor (BDNF) and glial-derived neurotrphic factor (GDNF) and a loss of dopaminergic neurons in the striatum and substantia nigra; pretreatment with a BDNF viral vector prevented neuronal loss (Nosheny, Bachis, Acquas, & Mocchetti, 2004; Nosheny, Bachis, Aden, DeBernardi, & Mocchetti, 2006; Nosheny et al., 2007). Gp120 also causes sickness behaviors such as fever, appetite loss and weight loss, and increased corticosterone

(Bagetta et al., 1999; Barak et al., 2002). Due to these sickness behaviors, it is difficult to test more complex behaviors in rats administered gp120, but Pugh et al. found that administration of gp120 after contextual fear conditioning but prior to testing showed impairment in freezing response when compared to cued fear conditioning, which was correlated with increased levels of IL-1β in the frontal cortex and hippocampus and prevented by IL-1β antagonists (Pugh et al., 2000). In addition to evidence gathered from intracerebral injections of gp120, a gp120 transgenic mouse developed to express gp120 only in astrocytes show the neuronal damage produced by gp120 selectively in the brain are similar to those seen in HIV patients with neurological disease (Toggas et al., 1994). Gp120 and the proinflammatory mediators induced by gp120 produce alterations in the blood brain barrier, which may also increase the viral load and potentiate inflammation and neurotoxicity further (for review, see Banks, Ercal, & Price, 2006).

Opiates and HIV

The relationship between HIV and progression to AIDS in opiate users has long been hotly debated due to known immunosuppressive effects of opiates and the increased risk of HIV infection in heroin user populations (for review, see Grant, Sacktor, & McArthur, 2005). However, the association between opiates and the development of neurological effects in HIV positive patients has been clearly supported. HIV encephalitis and neuropathy, both conditions closely related to HIV-induced neurocognitive impairments, show increased prevalence in intravenous drug users (Bell, Brettle, Chiswok, & Simmonds, 1998; Davies et al., 1997; Morgello et al., 2004). Interestingly, opiate abuse is the most

significant predictor of neuropathy among intravenous drug users (Morgello et al., 2004).

The mechanism through which opiates may increase risk of developing neurocognitive impairments in HIV positive patients is still unclear. However, much evidence points towards an additive effect of opiates on increasing proinflammatory cytokines and oxidative stress. For instance, in intravenous drug users, microglia activation is increased in both HIV positive patients (compared HIV positive patients who were not intravenous drug users) and even in patients not infected with HIV (Arango, Simmonds, Brettle, & Bell, 2004; Bell et al., 2002).

The ability of opiates to be immunomodulatory is well known; however, the effects of opiates on the immune system vary wildly depending upon the conditions in which the drug is administered. Opiates given peripherally will decrease immune response of T and B lymphocytes as well as natural killer cells (Roy, Charbonauer, & Barke, 1999; West, Lysle, & Dykstra, 1997; West, Dykstra, & Lysle, 1998). The effects of opiates on proinflammatory cytokines is quite debatable as morphine has been shown to both increase and decrease IL-1 and TNF-α (for review, see Rogers & Peterson, 2003). Morphine may also influence conditions to produce an environment in which infections will overstimulate the immune system; for example, morphine downregulates antiapoptotic proteins and increases proapoptotic proteins (Emeterio, Tramulla, & Hurle, 2006). Other models of infection show that morphine alone does not usually produce cytotoxicity, but in combination with an immune system stimulator, morphine can

increase IL-1β, TNF-α, nitric oxide, and neuronal death (Chang, Felix, Jiang, & Fiala, 2001; Johnston et al., 2004; Kapasi, Gibbons, Mattana, & Singhai, 2000; review, Nath et al., 2002; Pacifi, diCarlos, Bacosi, Pichini, & Zuccaro, 2000). In sum, opiates produce a destabilizing effect on immune function which can have opposite effects depending on conditions. In particular, although opiates appear to suppress most peripheral immune functions, opiates can potentiate proinflammatory response in the central nervous system (for reviews, see Rogers & Peterson, 2003; Hauser et al., 2005).

In experimental models of HIV, opiates are also detrimental to neuronal health. Neuronal/glial cultures exposed to gp120 and morphine showed an increase in cell death compared to cultures exposed to gp120 alone, and morphine had no effect by itself (Hu, Sheng, Lokensgard, & Peterson, 2005). Exposure of neuroblastoma, gliablastoma, and HIV-exposed macrophages combined with morphine also showed an increase in cytotoxicity and signs of oxidative stress (Koutsilieri et al., 1997). Rhesus macaques dependent on morphine infected with simian immunodeficiency virus (SIV), a lentivirus similar to HIV that naturally infects non-human primates, showed a number of detrimental effects compared to those infected monkeys not dependent on morphine: increased replication rate of SIV, increased mutation of SIV and resistance to treatment, increased replication in the cerebral compartment (Chuang et al., 2005; Kumar et al., 2004).

Present Experiments

Given the evidence of opiate interactions with HIV in the brain, the following experiments were undertaken to evaluate the effects of intracerebroventricular (icv) injections of gp120 and subcutaneous (sc) injections of heroin. The purpose is to measure changes induced by gp120 and heroin alone and in combination in the expression of proinflammatory mediators at the messenger RNA (mRNA) in specific brain regions that are affected in HIV infection of the brain.

The first set of experiments (Experiments 1 and 2) were focused on chronic administration of gp120 and/or heroin for 7 or 8 days. The first experiment was based on the methods of Bagetta et al. (1996) and sacrificed animals 24 hours after last treatment. However, Bagetta et al. used immunohistochemistry to examine protein in the brain, while the present experiments were focused on mRNA levels. For this reason, we performed a second experiment to examine effects of chronic gp120 and heroin on mRNA so that the animals were sacrificed 6 hours after last treatment, which is a timepoint that has proven successful in capturing mRNA cytokine changes in our laboratory for another model (lipopolysaccharide) of infection. We hypothesized for these experiments that gp120 would increase mRNA expression of iNOS, IL-1β, TNF-α, and COX-2, and that heroin would increase gp120-induced effects further while having no effect by itself.

To further examine immediate effects of gp120 and heroin at various timepoints, the second set of experiments (Experiments 3 and 4) focused on acute effects of gp120 and heroin on brain mRNA of proinflammatory mediators. These experiments were important to help determine whether acute changes occur in mRNA expression of proinflammatory mediators that cannot be seen in a chronic model due to potential adaptive changes. In these experiments, gp120 and heroin were administered once and rats were sacrificed at 4, 6, and 8 hour in order to examine mRNA expression of iNOS, IL-1β, TNF-α, and COX-2 at various timepoints. It was expected that gp120 would induce increased expression of these important proinflammatory molecules and heroin would potentiate these effects in combination with gp120.

The last experiment (Experiment 5) examined the effects of glycosylated gp120 in three doses: 0, 100ng, and 1μg. The purpose of this experiment was to determine if glycosylation of gp120 has differential effects on expression of IL-1β and COX-2 as it has been shown that glycosylation may affect binding properties of gp120 (Li, Luo, Rasool, & Kang, 1993). Glycosylated gp120 was administered in the same manner as previous experiments and rats were sacrificed at 2 and 6 hours to provide two timepoints at which to evaluate mRNA expression of iNOS, IL-1β, and COX-2. It was anticipated that glycosylation of gp120 would induce higher levels of iNOS, IL-1β, and COX-2 than artificial cerebrospinal fluid controls, and there would be a dose-dependent increase in mRNA expression of these immune factors.

Collectively, these experiments examine the effects of chronic and acute gp120 and heroin on brain mRNA expression of the proinflammatory mediators iNOS, IL-1β, TNF-α, and COX-2. An additional goal of this experiment is to examine brain region (cortex versus hippocampus) differences in mRNA expression of these vital immune molecules using RT-PCR. Prior to these experiments, mRNA has not been measured in brain tissue in this laboratory. Successful measurement of brain tissue mRNA will provide a new avenue through which the neuroimmune functions may be measured.

II. Methods

All methods were approved by University of North Carolina at Chapel Hill's Institutional Animal Care and Use Committee. A total of 113 male Lewis rats (2-4 months of age) were used for these experiments. All animals were housed on a 12:12 light:dark cycle in temperature-controlled DLAM-approved facility. All experimental manipulations took place during the dark portion of the light cycle. Rats were allowed at least one week to habituate to the new environment prior to any experimental manipulations. Food and water were available ad libitum.

Surgical Procedures

Animals were anesthetized with a 1:1 mixture of ketamine/xylazine administered, i.p. Anesthesia was monitored throughout surgery with toe-pinch. Rats were implanted with a 28 gauge 4.5mm length stainless steel guide cannula (Plastics One, Roanoke, VA). Coordinates used for placement of unilateral left lateral ventricle were AP -0.90 and ML +1.5, DV flat to skull. Coordinates used for placement in bilateral lateral ventricles were AP -0.90, ML +1.5, DV flat to skull and AP -0.90, ML -1.5, DV flat to skull. Rats were allowed at least 5 days recovery time prior to any experimental procedures. Gp120IIIB was obtained from Immunodiagnostics (Woburn, MA), glycosylated gp120IIIB was obtained

from Advanced Biotechnologies (Columbia, MD) and diacetylmorphine (heroin) was obtained from National Institute of Drug Abuse (Bethesda, MD).

Injections

Microinjections of gp120 (100ng or 1µg) and vehicle were performed with 1µl, 5µl or 10µl Hamilton syringes at a volume of 1µl or 3µl. These injections were performed manually over one minute, and injector was left in place for 30 seconds more to allow for full dispersion.

Injections of heroin (1mg/kg or 3mg/kg) or saline were administered subcutaneously on the abdomen.

Chronic Administration of Gp120 and Heroin

Experiment 1. Rats (n=12) were injected daily with gp120 (100ng) or bovine serum albumin (BSA) in a volume of 1µl and heroin (1mg/kg) or saline daily for 7 days. Rats were sacrificed 24 hours after last injection.

Experiment 2. Rats (n=12) were given a higher dose of gp120 (1µg) or BSA in a volume of 1µl and heroin (1mg/kg) or saline daily for 8 days. Animals were then sacrificed 6 hours after last injections, based on previous mRNA analyses in the laboratory using other models of infection.

Acute Administration of Gp120 and Heroin

Experiment 3. Rats (n=28) were tested for acute responses to a one-time dose of gp120 (1µg) or BSA vehicle in a volume of 3µl and heroin (1mg/kg) or saline. Sacrifice was performed at 6 hours after injections.

Experiment 4. Rats (n=33) were administered a one-time injection of gp120 (1µg) or BSA in a volume of 3µl and heroin (1mg/kg) or saline. Sacrifice was performed at 4 or 8 hours after injections.

Acute Administration of Glycosylated Gp120

Experiment 5. Rats (n=25) were given injections of glycosylated gp120 diluted in artificial cerebrospinal fluid in doses of 0, 100ng, or 1µg. Animals were sacrificed 2 or 6 hours after microinjection treatment.

Sacrifice and Tissue Treatment

All rats were sacrificed using cervical dislocation to preserve immunological and neurochemical integrity. Brains were rapidly removed from the skull and dissected for hippocampus and cortex. Tissues were processed for extraction of RNA using TriReagent (Molecular Research Company, Cincinnati, OH). Complimentary DNA was created using the Clontech Advantage RT-for-PCR kit (Mountain View, CA). Polymerase chain reaction (PCR) was performed using Master Fast Start SYBR Green I kit (Roche, Indianapolis, IN) in a LightCycler 2.0 using LightCycler 4.0 software (Roche, Indianapolis, IN). Primer

and protocol information regarding the specific molecules examined (GAPDH, iNOS, IL-1β, TNF-α, COX-2) can be found in Table 1.

Table 1. RT-PCR primer sequences and treatment.

Target	Primer Sequence		Cycles	Anneal C°
GAPDH	3' gtgcagcgaactttattga	5' tccaaggagtaagaaaccc	30	58
iNOS	3' gatgttgtagcgctgtgtgtca	5' acaacaggaacctaccagctca	40	62
IL-1β	3' ctctgcttgagaggtgctgatgta	5' gaagctgtggcagctacctatgtct	40	63
TNF-α	3' cagccttgtcccttgaagagaacc	5' tactgaacttcggggtgattggtcc	40	63
COX-2	3' cagtattgaggagaacagatggg	5' tacaagcagtggcaaaggcc	40	62

Statistical Treatment

All statistics were analyzed using SPSS 15.0 (Chicago, Illinois). Effects with a p value of 0.05 or less indicate a significant effect. Trends or effects approaching significance were reported for effects with a p value between 0.05 and 0.075. Posthoc tests (Tukey) or *a priori* t-tests, if applicable, were run to determine group differences if the ANOVA indicated a significant main effect or interaction. Non-significant results are not reported. If rats were recorded as having abnormal behavioral responses and data from the same rat was identified as outliers as determined by Grubb's test, this data was removed from the dataset prior to analysis.

III. Results

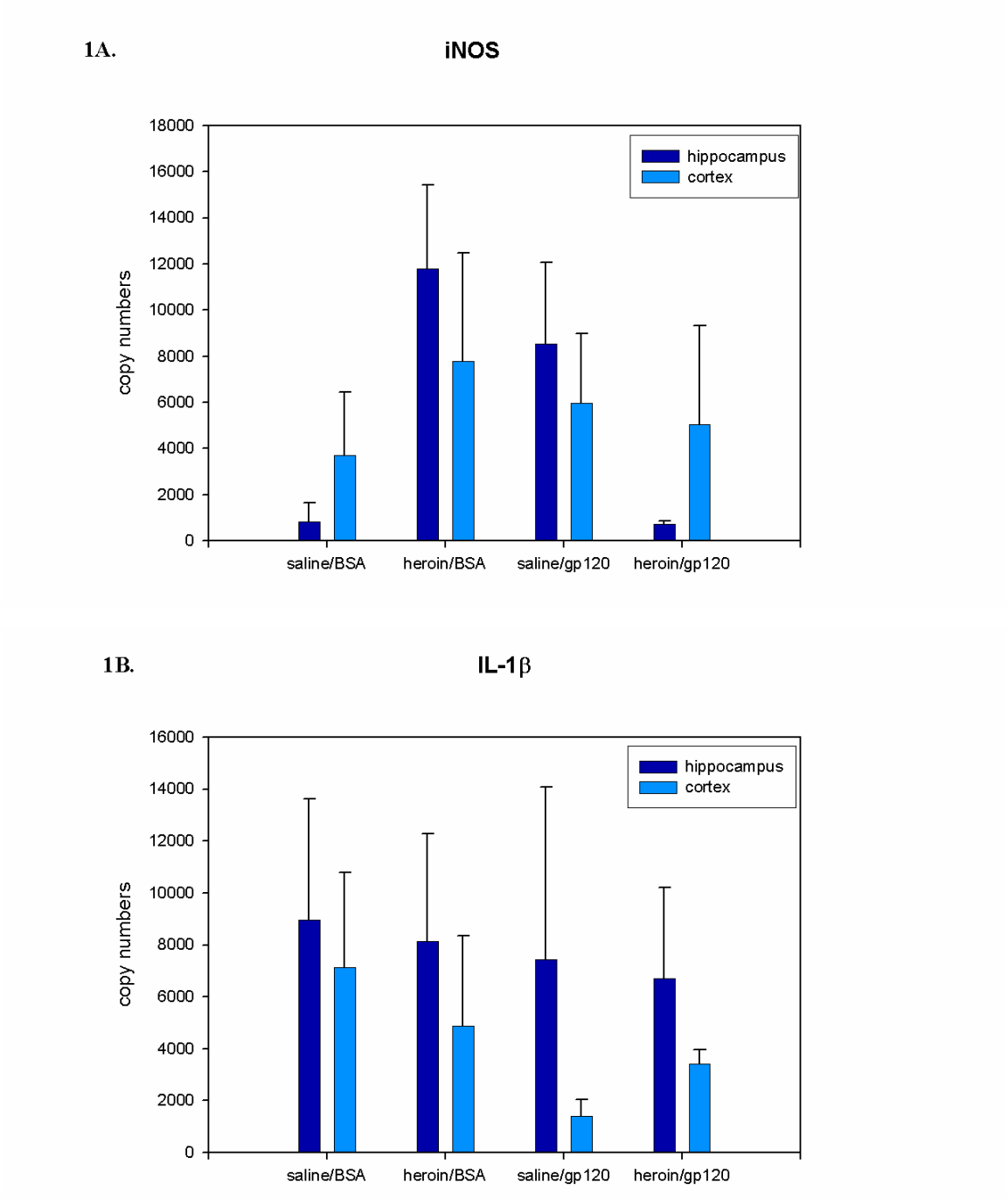
Housekeeping Gene

All samples had glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels quantified by RT-PCR and groups were determined to be not statistically different in GAPDH expression prior to the quantification of any other molecules. Any samples that showed evidence of primer dimers as determined by melting peak analysis were not included in analyses.

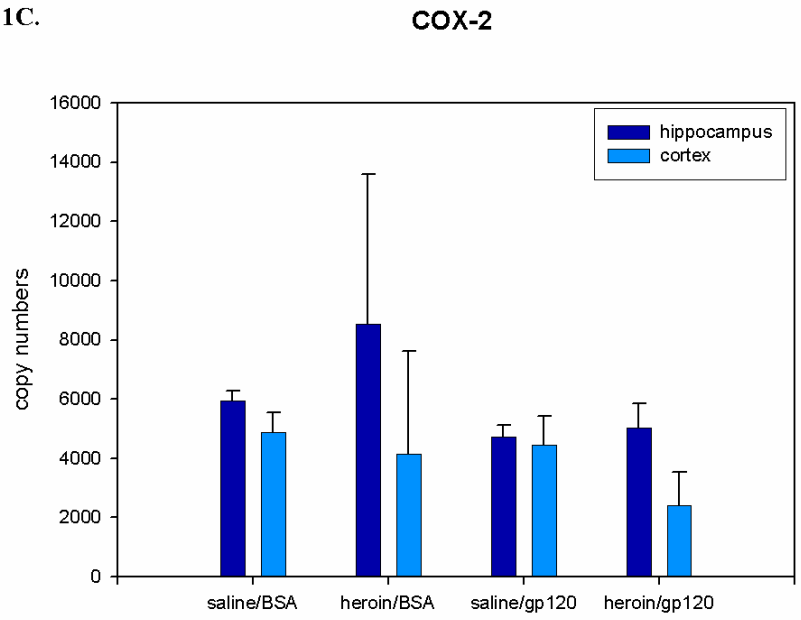
Chronic Administration of Gp120 and Heroin

Experiment 1. RT-PCR successfully detected iNOS, IL-1β, and COX-2 in the hippocampus and cortex samples. TNF-α was detected but was not measurable due to primer dimer contamination. Statistical analysis was run using a 2 (gp120) X 2 (heroin) X 2 (brain region) analysis of variance (ANOVA). The analysis yielded a significant interaction for gp120 X heroin on iNOS, $F(1, 12) = 7.199$, $p=0.020$ (Figure 1A). No significant effects of gp120, heroin, or brain region were found in IL-1β or COX-2 (Figures 1B & 1C). TNF-α analysis revealed high levels of primer dimers and was not used.

Figure 1. Mean (+/- SEM) mRNA levels of iNOS, IL-1β, and COX-2 as measured by RT-PCR. Rats were administered icv gp120 (100ng) or BSA and subcutaneous heroin (1mg/kg) or saline daily for 7 days and sacrificed 24 hours after last treatment.

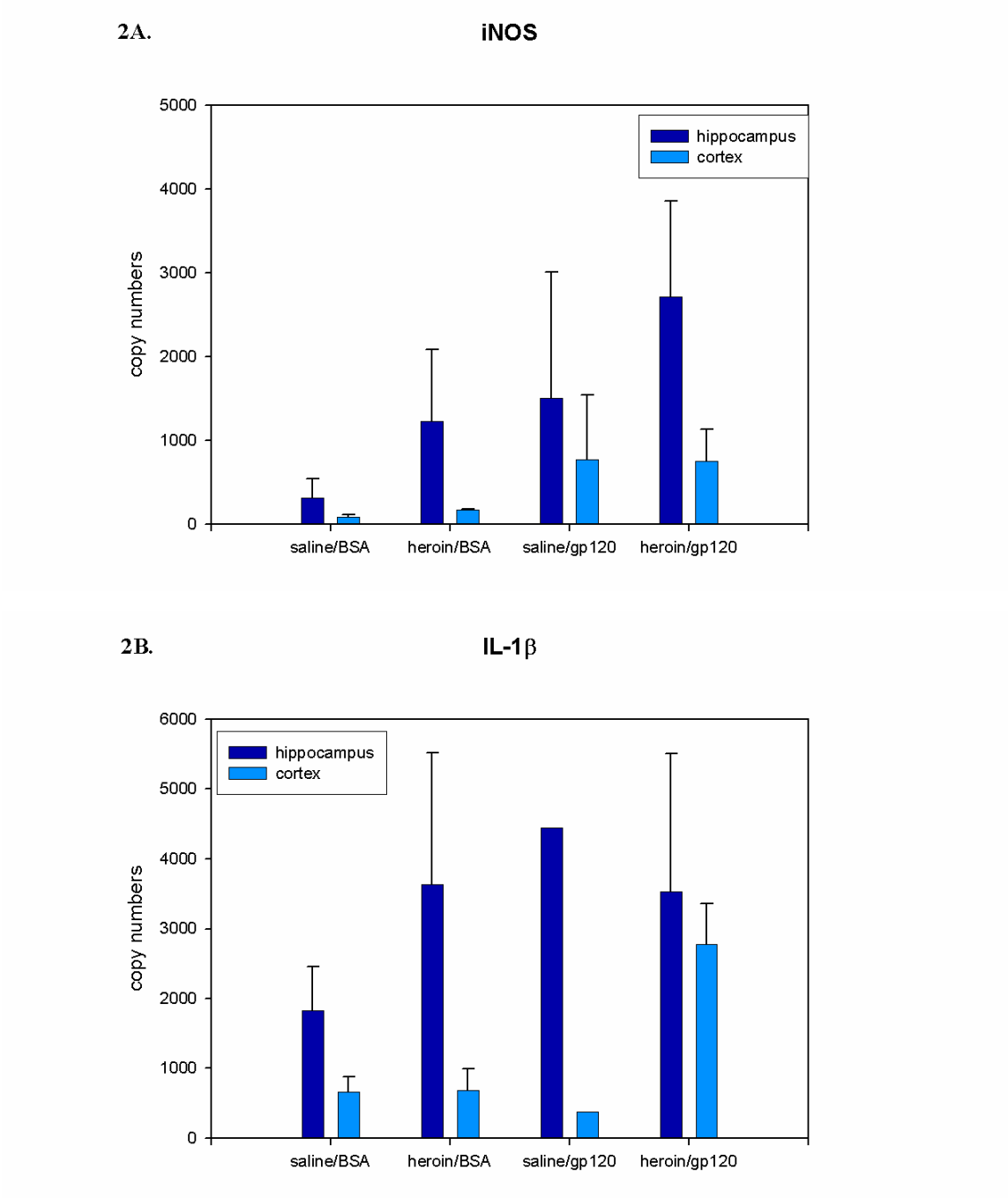


1C.

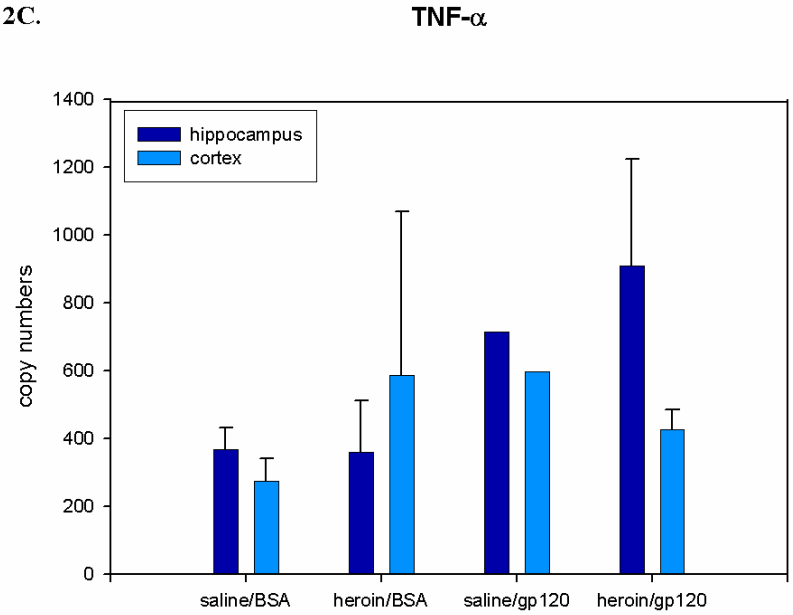


Experiment 2. Statistical analysis was run using a 2 (gp120) X 2 (heroin) X 2 (brain region) ANOVA. For this experiment, there were no significant effects of heroin or gp120 in iNOS (Figure 2A) or IL-1β (Figure 2B). However, the effect of gp120 on mRNA of iNOS approached significance, $F(1, 14) = 3.798$, $p=0.072$. Brain region differences were found in mRNA of IL-1β, $F(1, 12) = 4.799$, $p=0.049$, and approached significance in mRNA levels of iNOS, $F(1, 14) = 3.898$, $p=0.068$. Analyses did not reveal any significant effects of gp120, heroin, brain region, or any interactions of the three on TNF-α and COX-2 mRNA (Figures 2C & 2D).

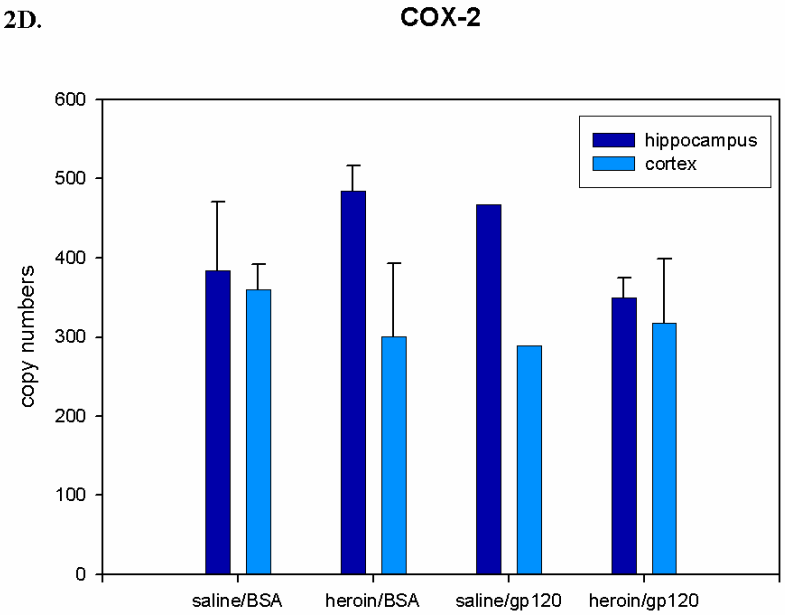
Figure 2. Mean (+/- SEM) mRNA levels of iNOS, IL-1β, TNF-α and COX-2 as measured by RT-PCR. Rats were administered icv gp120 (1μg) or BSA and subcutaneous heroin (1mg/kg) or saline daily for 8 days and sacrificed 6 hours after last treatment.



2C.


TNF-α
1400
1200
1000
800
600
400
200
0
copy numbers
hippocampus
cortex
saline/BSA
heroin/BSA
saline/gp120
heroin/gp120

2D.


COX-2
600
500
400
300
200
100
0
copy numbers
hippocampus
cortex
saline/BSA
heroin/BSA
saline/gp120
heroin/gp120

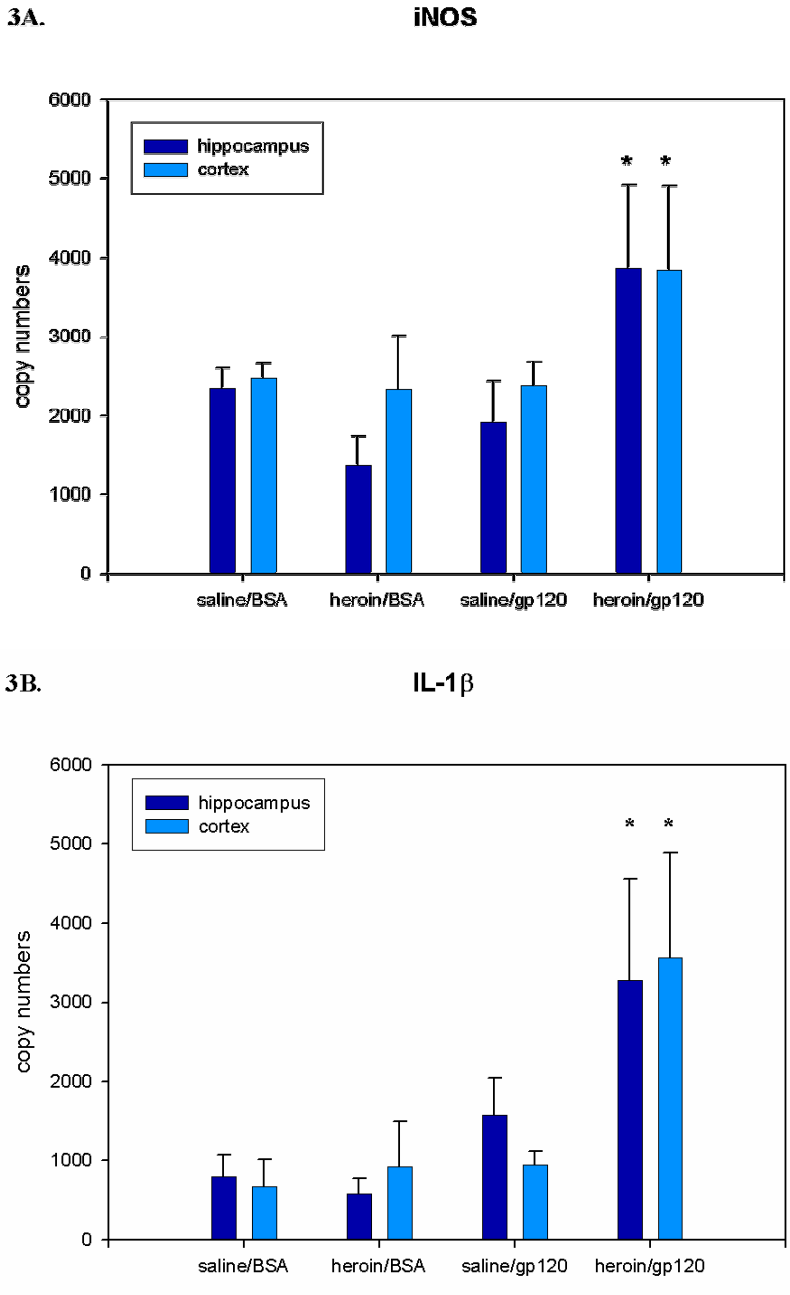
Acute Administration of Gp120 and Heroin

Experiment 3. Statistical analysis was run using a 2 (gp120) X 2 (heroin) X 2 (brain region) ANOVA. For this experiment, significant effects of gp120 were found in mRNA levels of both iNOS, $F(1, 34) = 4.387$, $p=.044$ (Figure 3A) and IL-1β, $F(1, 44) = 8.481$, $p=0.006$ (Figure 3B). An additional interactive effect between gp120 and heroin on iNOS mRNA was present, $F(1, 34) = 7.271$, $p=0.011$. Posthoc Tukey's tests showed iNOS mRNA measured in Group 4 (heroin/gp120) differed from Groups 2 (heroin/BSA) and 3 (saline/gp120), but not from Group 1 (saline/BSA). The heroin/gp120 group also showed significantly increased IL-1β mRNA compared to saline/BSA ($t(14.11) = -2.938$, $p=0.011$), heroin/BSA ($t(15.29) = -2.852$, $p=0.012$), and saline/gp120 ($t(15.169) = -2.333$, $p=0.034$) groups, which did not differ from each other. The effect of heroin on IL-1β mRNA approached significance, $F(1, 44) = 3.932$, $p=0.054$, as did the interaction of heroin and gp120, $F(1, 44) = 3.832$, $p=0.057$. The effect of gp120 approached significance in expression of TNF=α, $F(1, 44) = 3.485$, $p=0.069$ (Figure 3C). COX-2 mRNA was successfully measured and revealed only a trend towards brain region differences, $F(1, 41) = 3.688$, $p=0.062$.

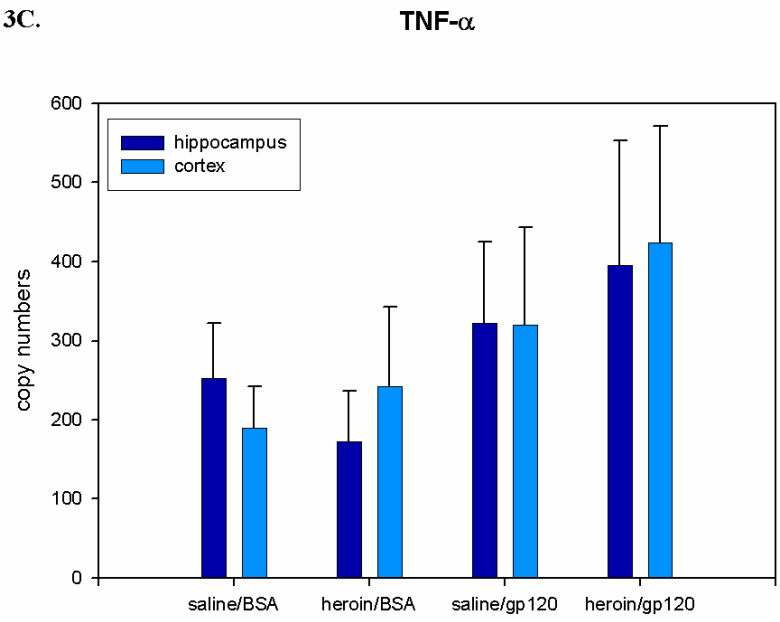
Experiment 4. Statistical analysis was performed using a 2 (gp120) X 2 (heroin) X 2 (time point) X 2 (brain region) ANOVA for each molecule. For this experiment, no significant effects of heroin or gp120 were found for iNOS (Figure 4A) or IL-1β (Figure 4B). There was, however, a significant difference shown

between brain regions for IL-1β, F (1, 35) = 10.842, p=0.002. TNF-α mRNA levels were not measurable due to primer dimers.

Figure 3. Mean (+/- SEM) mRNA levels of iNOS, IL-1β, TNF-α and COX-2 as measured by RT-PCR. Rats were administered icv gp120 (1μg) or BSA and subcutaneous heroin (1mg/kg) or saline once and sacrificed 6 hours after treatment.



3C.


TNF-α
hippocampus
cortex
copy numbers
600
500
400
300
200
100
0
saline/BSA
heroin/BSA
saline/gp120
heroin/gp120

3D.

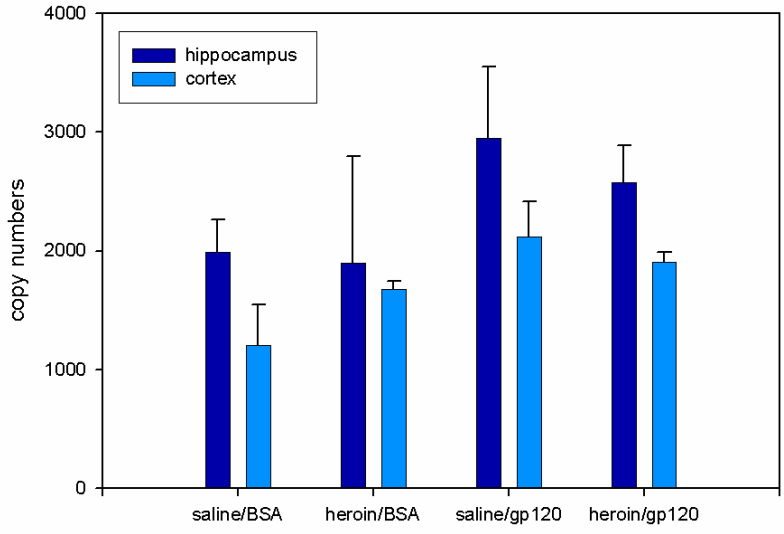
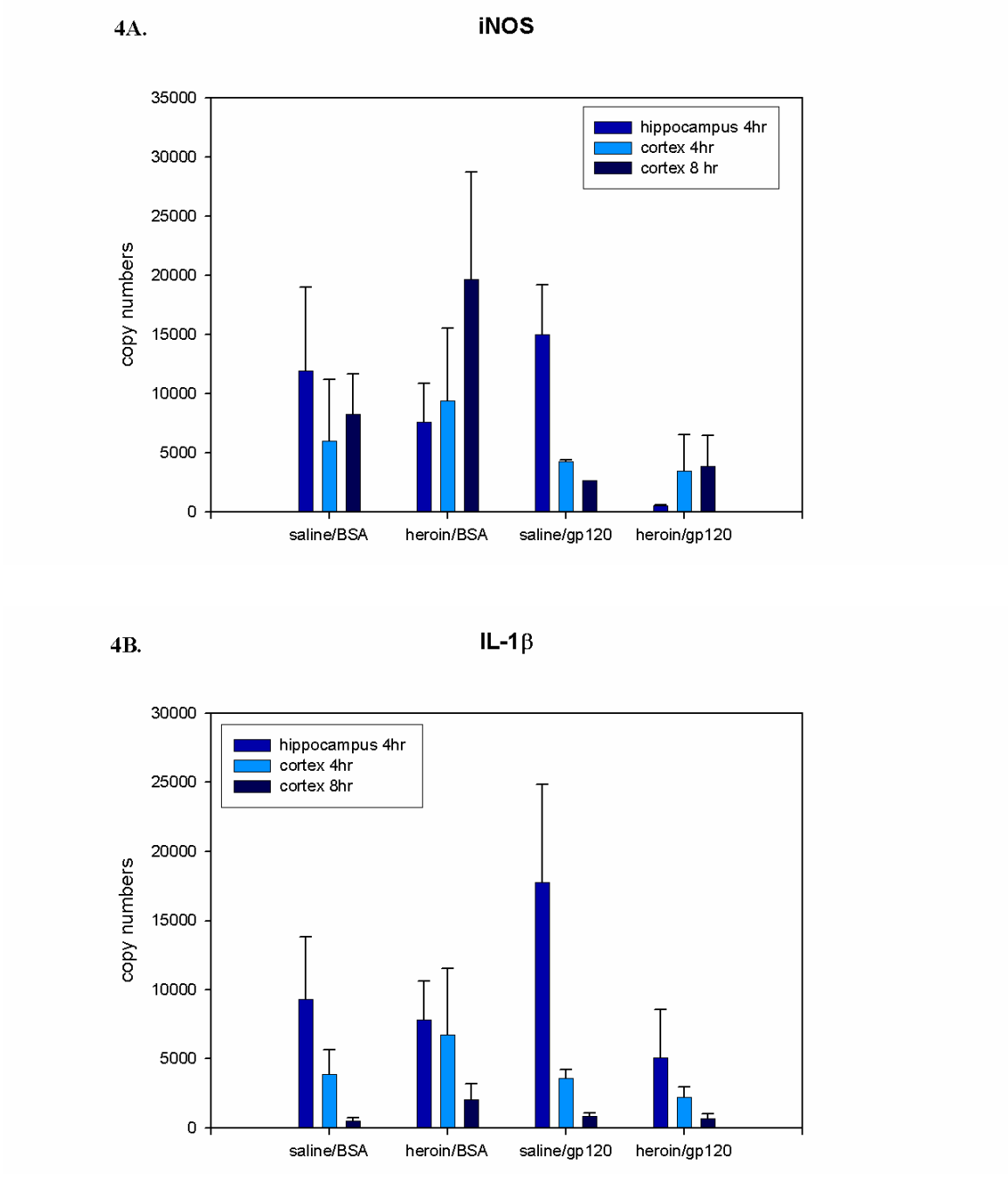

COX-2
hippocampus
cortex
copy numbers
4000
3000
2000
1000
0
saline/BSA
heroin/BSA
saline/gp120
heroin/gp120

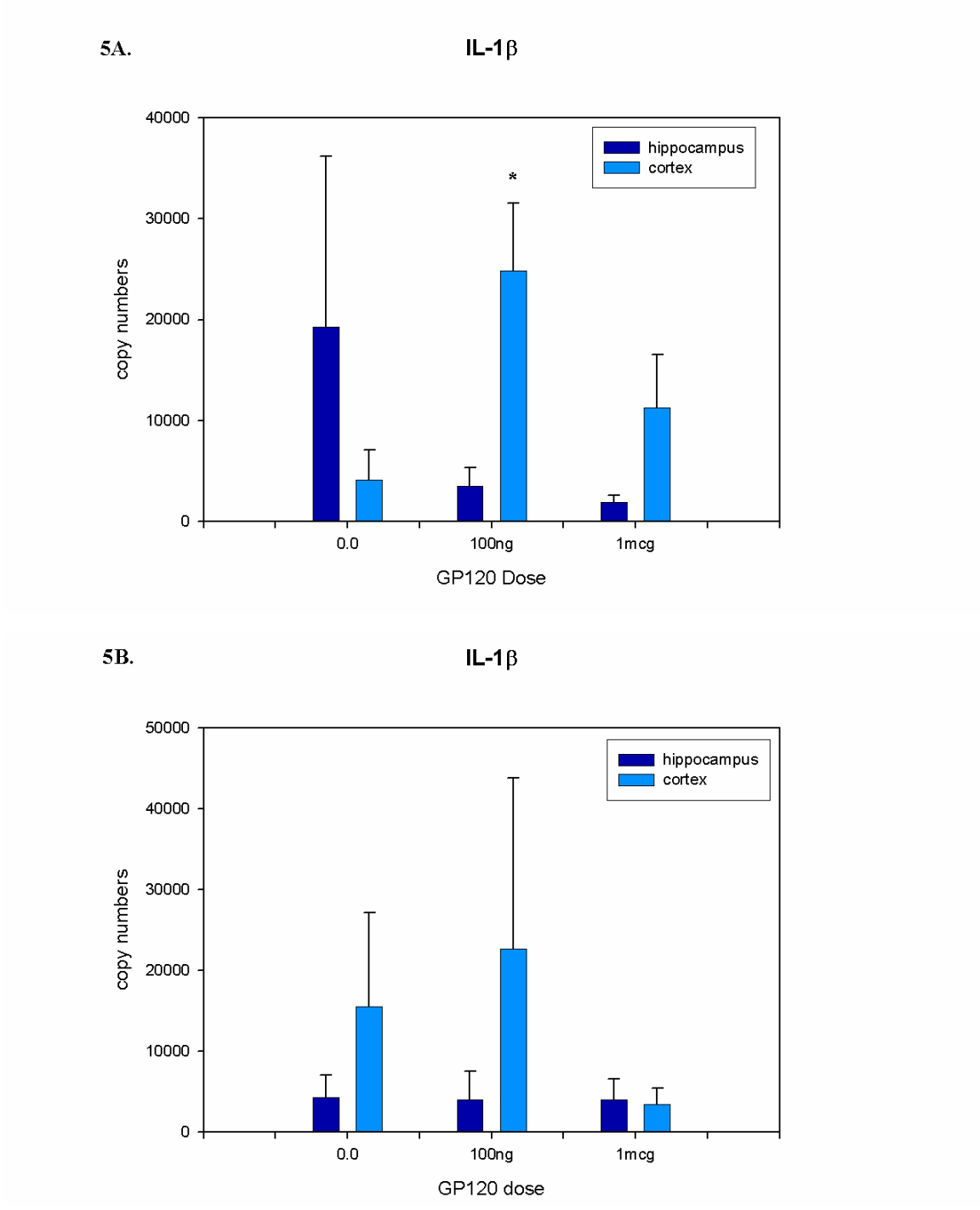
Figure 4. Mean (+/- SEM) mRNA levels of iNOS and IL-1β as measured by RT-PCR. Rats were administered icv gp120 (1µg) or BSA and subcutaneous heroin (1mg/kg) or saline once and sacrificed 4 or 8 hours after treatment.



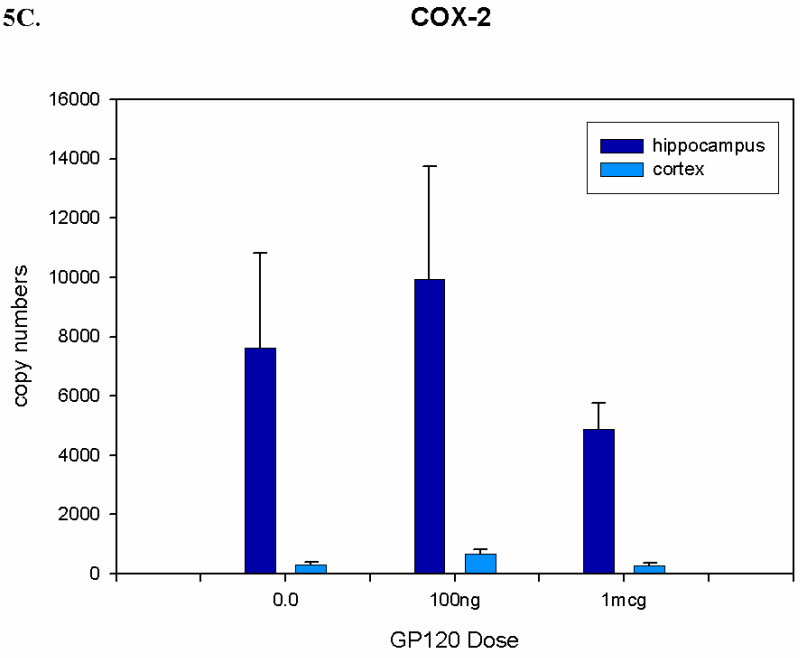
Acute Administration of Glycosylated Gp120

Experiment 5. Statistical analysis was run using a 2 (timepoint) X 2 (brain region) X 3 (gp120) ANOVA. The analysis revealed no significant effects of gp120 on IL-1β at 2 hours or 6 hours (Figure 5A & 5B) or COX-2 at 2 hours and 6 hours (Figure 5C & 5D). A significant brain region difference was detected for both COX-2, $F(1, 38) = 51.595$, $p=0.000$, and IL-1β, $F(1, 37) = 5.581$, $p=0.024$. TNF-α and iNOS were not measurable due to high levels of primer dimers. However, there was a significant difference between 0 and 100ng of gp120, $t(4.14) = -2.814$, $p=0.046$ at 2 hours for IL-1β as revealed by two-tailed t-test.

Figure 5. Mean (+/- SEM) mRNA levels of IL-1β and COX-2 as measured by RT-PCR. Rats were administered icv glycosylated gp120 (0, 100ng, or 1µg) sacrificed 2 (5A and 5C) or 6 (5B and 5D) hours after treatment.



5C.


COX-2
16000
14000
12000
10000
8000
6000
4000
2000
0
copy numbers
hippocampus
cortex
0.0
100ng
1mcg
GP120 Dose

5D.

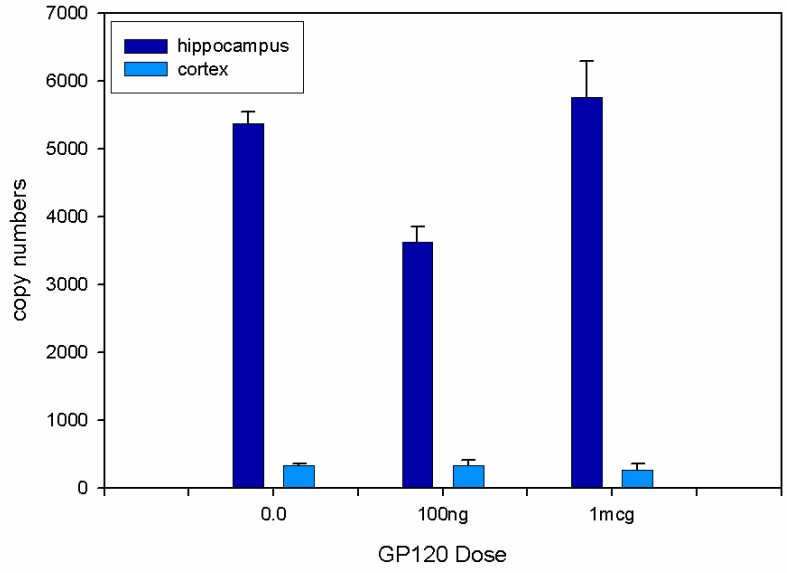

COX-2
7000
6000
5000
4000
3000
2000
1000
0
copy numbers
hippocampus
cortex
0.0
100ng
1mcg
GP120 Dose

Table 2. Means and Standard Error of Mean (SEM) for Experiment 1.
7 days of treatment, sacrificed 24 hours after last treatment.

Treatment			iNOS			IL-1β			TNF-α			COX-2	
		n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
saline/BSA	hippocampus	2	830.66	803.68	3	11875.11	5144.79	.	n/a		3	5933.33	339.23
	cortex	3	3589.98	2741.46	3	9464.22	3984.87				3	4873.33	670.36
heroin/BSA	hippocampus	2	11795	3641.67	2	8148.33	4118.33		n/a		3	8535	5065
	cortex	2	7794.1667	4699.17	2	4888.33	3474.5				3	4136	3474
saline/gp120	hippocampus	3	8540.56	3509.3	1	14073.33	n/a		n/a		3	4733	376.756
	cortex	3	5951.34	3044.68	1	2039.67	n/a				3	4443	986.6
heroin/gp120	hippocampus	3	716	143.27	2	6713.67	3499.67		n/a		3	5030	830.72
	cortex	2	5028.5	4298.17	3	3404.44	565.32				3	2406	1127.96

Table 3. Means and SEM for Experiment 2.
8 days of treatment, sacrificed 6 hours after last treatment.

Treatment			iNOS			IL-1β			TNF-α			COX-2	
		n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
saline/BSA	hippocampus	3	313.57	230.99	3	1830.33	629.87	3	367.32	64.03	3	384.33	86.19
	cortex	3	89.63	23.68	3	659.77	224.74	3	275.48	66.57	3	360.33	31.93
heroin/BSA	hippocampus	3	1230.03	857.77	3	3628.22	1885.62	3	359.1	152.89	2	484.5	32.5
	cortex	3	168.33	14.45	3	388.01	305.55	3	585.33	483.67	3	301	92.34
saline/gp120	hippocampus	2	1508.48	1501.52	1	4446.67	n/a	1	714.67	n/a	1	468	n/a
	cortex	2	770.37	769.63	1	373.33	n/a	1	597.67	n/a	1	289	n/a
heroin/gp120	hippocampus	3	2717.33	1137.85	3	3534.33	1975.37	3	910	314.21	3	350	24.5
	cortex	3	747.33	386.04	3	2773.33	582.64	3	427.922	58.1	3	317	81.15

Table 4. Means and SEM for Experiment 2.
1 day of treatment, sacrificed 6 hours after last treatment.

Treatment			iNOS			IL-1β			TNF-α			COX-2	
		n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
saline/BSA													
	hippocampus	6	2341.89	262	6	799.72	270.05	6	252.62	70.05	6	2771.67	373.47
	cortex	6	2478.72	186.88	6	676.07	340.3	6	189.28	53.36	6	2391	558.88
heroin/BSA													
	hippocampus	5	1374.8	361.45	6	573.06	200.76	6	172.74	63.31	4	2739.75	615.27
	cortex	3	2331.78	387.85	6	930.56	566.04	6	242.03	101.14	6	2251.67	294.18
saline/gp120													
	hippocampus	4	1915.42	526.86	7	1575	473.13	7	321.67	103.37	6	3225	311.44
	cortex	4	2374	306.19	7	946.9	175.67	7	320.08	123.82	7	2852.71	301.83
heroin/gp120													
	hippocampus	5	3862.07	1059.08	7	3277.05	1287.05	7	358.29	158.08	7	3677.14	652.08
	cortex	5	2833.93	1083.23	7	3562.14	1326.48	7	371.89	147.55	7	2298.57	315.28

Table 5. Means and SEM for Experiment 4.

1 day of treatment, sacrificed 4 hours after last treatment.

Treatment			iNOS			IL-1β	
		n	Mean	SEM	n	Mean	SEM
saline/BSA							
	hippocampus	4	11934.00	7092.71	4	9329.00	4505.02
	cortex		5984.50	5215.50	3	3874.67	1803.59
heroin/BSA							
	hippocampus	3	7630.00	3228.51	3	7840.00	2783.17
	cortex		9422.50	6111.56	4	6759.50	4810.50
saline/gp120							
	hippocampus	4	1512.50	4150.42	4	17755.00	7070.90
	cortex		4232.50	153.21	4	3612.50	599.02
heroin/gp120							
	hippocampus	4	506.75	93.82	5	5096.40	3463.73
	cortex		3468.60	3037.71	5	2217.32	1223.80

1 day of treatment, sacrificed 8 hours after last treatment.

Treatment			iNOS			IL-1β	
		n	Mean	SEM	n	Mean	SEM
saline/BSA							
	cortex	2	8260.00	3440.00	4	511.65	256.24
heroin/BSA							
	cortex	3	19666.00	9079.26	4	2062.50	1125.37
saline/gp120							
	cortex	1	2670.00	n/a	4	843.25	247.93
heroin/gp120							
	cortex	2	3845.00	2605.00	4	660.50	360.22

Table 6. Means and SEM for Experiment 5.

1 day of treatment, sacrificed 2 hours after last treatment.

Treatment			IL-1β			COX-2	
		n	Mean	SEM	n	Mean	SEM
0.0 gp120							
	hippocampus	4	19298.58	16895.65	4	7622.50	3181.33
	cortex	4	4113.70	2993.02	4	313.78	76.80
100ng gp120							
	hippocampus	4	3521.00	1817.16	4	9935.00	3795.49
	cortex	4	24817.50	6719.97	4	664.25	147.62
1µg gp120							
	hippocampus	4	1893.25	660.21	4	4860.00	911.33
	cortex	4	11305.00	5188.55	4	256.53	83.78

1 day of treatment, sacrificed 6 hours after last treatment.

Treatment			IL-1β			COX-2	
		n	Mean	SEM	n	Mean	SEM
0.0 gp120							
	hippocampus	4	4239.25	2766.92	4	5375.00	166.86
	cortex	4	15567.50	11587.59	4	339.25	15.00
100ng gp120							
	hippocampus	4	3988.83	3509.36	4	3630.00	229.56
	cortex	4	22623.25	21140.07	4	332.75	82.41
1µg gp120							
	hippocampus	4	4006.40	2527.02	4	5764.00	522.54
	cortex	4	3401.60	1972.22	4	262.82	100.18

IV. Discussion

Significant and Marginal Effects

The results of these experiments show a collection of interesting effects of gp120, heroin, and brain regions on mRNA expression of the proinflammatory mediators iNOS, IL-1β, TNF-α, and COX-2. These data established a precedent to perform RT-PCR on immune mediators in the brain, which has never been done before in this laboratory. The data also help to establish experimental protocols in determining effects of chronic and acute administration of gp120 and heroin alone and together on expression of immune mediators in the brain.

Experiment 1 was modeled after the findings that 7 days of gp120 icv injections caused an increase in IL-1β and COX-2 in when rats were sacrificed 24 hours after the last treatment (Bagetta et al., 1998; Bagetta et al., 1999). However, Bagetta et al. used immunohistochemistry to stain for the protein of IL-1β and COX-2; the present experiment examined the expression of IL-1β and COX-2 messenger RNA. The changes in mRNA expression may very well occur at different time points than that of protein, and it is likely that with this study, the time point of sacrificing 24 hours after last treatment simply missed the appropriate time for measuring mRNA expression. Given the interaction effect of

gp120 and heroin to decrease expression of iNOS, it may be possible that adaptive changes had already occurred in this group, and mRNA of iNOS was being downregulated to adapt to a previous increase prior to the 24 hour sacrifice timepoint. These effects may also been seen in IL-1β with less variability and a larger sample size. However, these experiments did not address this possibility, and this may be a potential avenue of future research, in order to determine if perhaps heroin alters the timeline of gp120-induced changes in iNOS expression in mRNA.

Brain tissue of rats administered gp120 or BSA and heroin or saline for 8 days and sacrificed 6 hours after the last treatment showed a significant difference only in expression of IL-1β in brain regions. This time point was chosen because previous studies in our laboratory have found effects in mRNA of other tissues after a systemic immune challenge. In particular, cortex tissue showed an increase when gp120 and heroin were combined, but the hippocampus did not show this effect. These results are intriguing, and may represent a mechanism through which heroin may acutely disrupt potential adaptive changes affecting IL-1β mRNA after chronic gp120 exposure. There were also a few notable trends in the analysis of iNOS mRNA. The marginally significant effects of gp120 and heroin suggest that with a higher sample number, these effects may play out to be significant.

The aim of the acute gp120 and heroin experiments were to observe any effects that occur immediately after the first injection of gp120 and heroin. These effects were important to assess in light of our findings of possible adaptations occurring in chronic gp120 and heroin: no effect at 24 hours after last treatment and increases only in heroin and gp120-treated rats when sacrificed 6 hours post-treatment. These findings suggested that heroin may either increase the length at which gp120 has an effect, or may alter it and increase it altogether.

Experiment 3, in which rats were administered gp120 or BSA and heroin or saline once and sacrificed 6 hours later, showed significant effects and a few important trends. INOS presented the most robust findings, with a main effect of gp120 and an interaction between gp120 and heroin being clearly seen. Posthoc tests revealed that the effects lie primarily in increased expression of iNOS in group 4 (gp120/heroin). The significant effect of gp120 and the marginally significant effect of heroin on expression of IL-1β mRNA also indicate the importance of heroin on the acute effects of gp120. As seen in iNOS, IL-1β mRNA levels for group 4 (gp120/heroin) are significantly different from all other treatments. The fact that gp120 by itself was not altered may represent an alternate pathway through which heroin may have unique effects on the proinflammatory mediators; that is, gp120 may primarily affect proinflammatory proteins through other pathways (such as through infiltrating lymphocytes from the peripheral or stores of uncleaved protein), but when combined with heroin, may also affect mRNA expression. The trend of TNF-α mRNA expression to increase with the administration of heroin is something that will be examined

more closely in future studies. TNF-α is one of the most prominent cytokines implicated in neurological complications of HIV (Tyor et al., 1993; Wesselingh et al., 1994), and it is not clear from these results if heroin also plays a role in this important molecule as is seen in iNOS and IL-1β.

To further examine the time course of the acute effects of heroin and gp120, gp120 or BSA and heroin or saline were administered once to rats with sacrifice occurring 4 or 8 hours after treatment. The lack of significant effects of heroin or gp120 on IL-1β or iNOS could be explained by a number of factors. The most obvious explanation would be that the increases seen in Experiment 3 are transient and are most measurable at 6 hours post-treatment. Considering the very specific time frame in which mRNA increases are seen in other models of inflammation, increases easily seen only at 6 hours would not be surprising. In order to assess smaller effects, a larger sample size would be required to fully evaluate the specific time frame in which gp120 and heroin affect mRNA expression of proinflammatory mediators.

It has been reported that the posttranscriptional modification of glycosylation is necessary for gp120 to bind to human CD4 receptors (Li et al., 1993). However, this same report indicates that inflammatory responses can be induced without the binding of the CD4 receptor. In order to address these concerns, the last experiment in this series administered glycosylated gp120 in 0, 100ng, and 1μg doses. This experiment was undertaken in order to assess whether glycosylated gp120 differed in its effects on expression of iNOS, IL-1β, TNF-α, and COX-2 mRNA. However, due to primer dimer complications, iNOS

and TNF-α were not measurable. IL-1β showed a significant increase at a dose of 100ng for brain tissue collected two hours after treatment; however, this effect was not seen at 6 hours. However, the variability in the 100ng/6 hour treatment was substantial and may have obscured any significant effects. Administration of 1μg of glycosylated gp120 showed no effects on IL-1β mRNA expression at either 2 hours or 6 hours. The reasons for this are unclear, but consistent with previous experiments (Experiments 3 & 4) in which (unglycosylated) gp120 at the same dose did not cause any increases in IL-1β mRNA by itself at 4, 6, or 8 hours post-treatment. It would seem, then, that glycosylation of gp120 may not necessary in this particular model of HIV effects in the brain (gp120 administered icv).

While none of the experiments showed effects of gp120 or heroin on COX-2, there were some very interesting findings regarding mRNA expression of COX-2 in the brain. Brain region differences showing increased levels of expression of COX-2 in the hippocampus were seen in 3 of the 5 experiments (Experiments 3-5), and the experiments that did not reach significantly different expression levels between brain regions consistently had higher means of COX-2 expression in the hippocampus compared to the cortex (Experiments 1-2). While no effects of heroin or gp120 were seen in COX-2, these data are important for establishing parameters for future studies.

Potential Explanations for Marginal Effects

There are a number of significant results in these experiments, as well as a number of trends (p=0.05 to p=0.075) that are reported. These trends are reported due to their importance when considered in context. For experiments measuring immunological effects in the past, this laboratory has often used samples of 4 to achieve sufficient statistical power. It was based on this knowledge that the experiments were designed with samples of 3 or 4- it was expected that these relatively low sample numbers would be able to show the robust effects expected in these experiments. However, there were a number of factors that may have caused a low sample size to become a confound in the statistical analysis.

The method of detection used in these experiments, SYBR Green I, has been useful in the past in this laboratory for detecting tissues taken from peripheral organs such as spleen, lung, and liver. However, measuring expression of mRNA in brain tissue is something new altogether for this laboratory, and problems with primer dimer complications were encountered. The primer dimers are most likely due to low levels of these mediators present in the brain, and are not due to contamination because diluted purified standards also show primer dimers in the molecules that are prone to this (specifically, iNOS and TNF-α). This is a problem that has been encountered in other laboratories for other molecules, and one method that has been successfully used to solve the problem of primer dimers is the use of the Universal Probe Library (UPL) method of detection for RT-PCR (Bakker, 2006). While the SYBR Green I

method of detection causes fluorescence to be measured for any double-stranded DNA, the UPL method of detection utilizes the fluorescence of only sequence specific probes that are bound, thus eliminating primer dimers from the analysis (Bakker, 2006; manufacturer information). The laboratory is currently transitioning to using UPL for RT-PCR with the expectations that this will solve the primer dimer problems and allow for more precise and greater statistical analysis. Despite the difficulty with primer dimers, the results from these experiments were sufficient to provide data that will serve as a reference for future studies in our laboratory.

Additionally, the strain from which the gp120 used in these experiments is derived from is important to consider. HIV gp120 binds to human CD4 receptors, which causes a conformational change in the gp120 which allows it to bind to one of the chemokine receptors, CCR5 or CXCR4 (Deng et al., 1996; Feng, Broder, Kennedy, & Berger, 1996). Which chemokine receptor the gp120 preferentially binds to is how the strain is described; that is, those that bind to CCR5 are called R5 and those that bind to CXCR4 are called X4. Most HIV patients are infected with R5 strains, but up to 50% of these patients go on to develop X4 strains, which are associated with progression to AIDS, increased neurotoxicity, and increased depletion of T cells (Gorry, Churchill, Crowe, Cunningham, & Gabuzda, 2005; Moore, Kitchen, Pugach, & Zack, 2004; Kaul & Lipton, 2004; Philpott, 2003). The gp120 used in this study is gp120IIIB, which is derived from a primarily X4 strain of HIV. It was felt that an X4-derived gp120 would be more appropriate, as cognitive deficits and related neuroinflammation is

usually seen at the later stages of AIDS that correlate with the development of X4 HIV (Dunfee et al., 2006; Philpott, 2003; van de Bovenkamp, Nottet, & Pereira, 2002). Although R5 strains are usually present in the brain first, it is X4 that is more commonly associated with cognitive impairments (for review, Dunfee et al., 2006).

Coreceptor Alterations Induced by Opiates: A Potential Mechanism for Interactions of Gp120 and Heroin

The mechanism through which opiates and gp120 may interact to produce increase inflammatory mediators is not clear at this point in time. One possible explanation is that opiates may alter receptor expression of the gp120 coreceptors. Experimental studies have shown that opiates such as morphine and DAMGO upregulate CCR5 expression and downregulate natural ligands to CCR5, which may be due to heterodimerization of the μ-opioid receptor and CCR5 (Chen et al., 2004; Suzuki, Chang, Yau, Doi, & Chuang, 2002; for review, see Vallejo, de Leon-Casasola, & Benyamin, 2004). Infection of SIV in rhesus macaques is mediated through CCR5, and those monkeys exposed to morphine show increased replication rate, mutation and resistance to AZT, increased expression of CCR5, increased susceptibility to SIV, and decreased natural ligands of CCR5 (Chuang et al., 2005; Guo, et al., 2002; Miyagi et al., 2000). The evidence for CXCR4 and opiate interactions is less compelling, with some results indicating morphine causes an upregulation in replication of only R5, although *in vitro* human immune cells treated with μ agonist DAMGO upregulated

both CCR5 and CXCR4, and pretreatment with DAMGO increased replication of both types of HIV strains (Guo et al., 2002; Steele, Henderson, & Rogers, 2003).

Future Directions

A future study to follow up on these experiments will examine the effects of opiates in combination with gp120 on the expression of CXCR4 and CCR5 in order to further examine the mechanism through which opiates alter gp120-induced effects in the brain.

Another future study would involve a comparison of protein and mRNA effects. Recently published data has emerged that shows no effect of intracerebroventricular injection of gp120 (100ng) on mRNA of IL-1β in adult rats, which is consistent with our results (Abraham et al., 2008). Most published data using the icv gp120 model in rats has only measured protein using immunohistochemistry (Bagetta et al., 1998; Bagetta et al., 1999). However, immunohistochemistry is notoriously difficult to quantify. To better address the differences between mRNA expression and protein expression, Western blots and available Enzyme-Linked Immunosorbent Assay (ELISA) assays will be employed to measure protein in specific brain regions. Preliminary evidence is currently being measured in our laboratory indicates that although TNF-α mRNA expression was not measurable (due to primer dimers) when rats were given icv 100ng gp120 and sacrificed at 2 or 6 hours, TNF-α protein expression in the hippocampus and cortex as measured by an ELISA shows significant increases

with gp120 administration. This is encouraging evidence for more effects being drawn out through protein analysis.

Summary

In summary, these experiments show a potentially unique interaction of heroin and gp120 on the mRNA expression of proinflammatory mediators such as iNOS and IL-1β that occur approximately 6 hours after treatment with heroin and gp120 in both chronic and acute models using icv administration of gp120. These data provide evidence that heroin and gp120 interact in a novel way that has not previously been in examined in mRNA in the brain.

References

Adle-Biassette, H., Levy, Y., Colombel, M., Poron, F., Natchev, S., Keohane, C., & Gray, F. (1995). Neuronal apoptosis in HIV infection in adults. *Neuropathology and Applied Neurobiology*, 21, 218-227.

Arango, J., Simmonds, P., Brettle, R.P., & Bell, J.E. (2004). Does drug abuse influence microglial response in AIDS and HIV encephalitis? *AIDS*, 18 (suppl 1), S69-S74.

Bagetta, G., Corasaniti, M.T., Aloe, L., Berliocchi, L., Costa, N., Finazzi-Agro, A., & Nistico, G. (1996). INtracerebral injections of human immunodeficiency virus type 1 coat protein gp120 differentially affects the expression of nerve growth factor and nitric oxide synthase in the hippocampus of rat. *Proceedings of the National Academy of Science*, 93 (2), 928-933.

Bagetta, G., Corasaniti, M.T., Berliocchi, L., Navarra, M., Finazzi-Agro, A., & Nistico, G. (1995). HIV-1 gp120 produces DNA fragmentation in the cerebral cortex of rat. *Biochemical and Biophysical Research Communications*, 211 (1), 130-136.

Bagetta, G., Corasaniti, M.T., Berliocchi, L., Nistico, R., Giommarioli, A.M., Malorni, W., Aloe, L., & Finazzi-Agro, A. (1999). Involvement of interleukin-1β in the mechanism of human immundeficiency virus type 1 (HIV-1) recombinant protein gp120-induced apoptosis in the neocortex of the rat. *Neuroscience*, 89 (4), 1051-1066.

Bagetta, G., Corasaniti, M.T., Malorni, W., Rainaldi, G., Berliocchi, L., Finazzi-Agro, A., & Nistico, G. (1996). The HIV-1 gp120 causes ultrastructural changes typical of apoptosis in the rat cerebral cortex. *Neuroreport*, 29 (7), 1722-1724.

Bagetta, G., Corasaniti, M.T., Paoletti, A.M., Berliocchi, L., Nistico, R., Giammarioli, A.M., Malorni, W., & Finazzi-Agro, A. (1998). HIV-1 gp120 apoptosis in the rat neocrotex involves enhanced expression of cyclo-oxygenase type 2 (COX-2). *Biochemical and Biophysical Research Communications*, 244, 819-824.

Bakker, O. (2006). To probe or not to probe: Comparison of qPCR assays using the LightCycler® 2.0 instrument and either SYBR Green I intercalculation or the Universal Probe Library as detection format. *Biochemica*, 3, 8-10.

Banks, W.A., Ercal, N., & Price, T.O. (2006). The blood-brain barrier in neuroAIDS. *Current HIV Research*, 4, 259-266.

Barak, O., Weidenfeld, J., Goshen, I., Ben-Hur, T., Taylor, A.N., & Yirmiya, R. (2002). Intracerebral HIV-1 glycoprotein 120 produces sickness behavior and pituitary-adrenal activation in rats: Role of prostaglandins. *Brain, Behavior, & Immunity*, 16, 720-735.

Barre-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozebaum, W., & Montagnier, L. (1983). *Science*, 220, 868-871.

Bell, J.E. & Gray, F. (2005). Brain pathology in HIV-1-infected drug abusers. The Neurology of AIDS, 2nd edition, Oxford University Press Inc., Oxford, NY, 393-404.

Bell, J.E., Arango, J.C., Robertson, R., Brettle, R.P., Leen, C., & Simmonds, P. (2002). HIV and drug misuse in the Edinburgh cohort. *Journal of Acquired Immune Deficiency Syndrome*, 31, S35-S42.

Bell, J.E., Brettle, R.P., Chiswick, A., & Simmonds, P. (1998). HIV encephalitis, proviral load and dementia in drug users and homosexuals with AIDS. Effect of neocortical involvement. *Brain*, 121, 2043-2052.

Block, M.L. & Hong, J.S. (2005). Microglia and inflammation-mediated neurodegeneration: Multiple triggers with a common mechanism. *Progress in Neurobiology*, 76, 77-98.

Brenneman, D.E., McCune, S.K., Mervis, R.F., & Hill, J.M. (1994). Gp120 as an etiologic agent for NeuroAIDS: neurotoxicity and model systems. *Advanced Neuroimmunology*, 4 (3), 157-165.

Brune, B. (2003). Nitric oxide: NO apoptosis or turning it ON? *Cell Death and Differentiation*, 10, 864-869.

Center for Disease Control (1981). Pneumocystic Pneumonia—Los Angeles. *Morbidity and Mortality Weekly Report*, 30, 1-3.

Center for Disease Control (2006). Twenty-five years of HIV/AIDS—United States, 1981-2006. *Morbidity and Mortality Weekly Report*, 55, 585-589.

Chang, S.L, Felix, B., Jiang, Y., & Fiala, M. (2001). Actions of endotoxin and morphine. *Advances in Experimental Medicine and Biology*, 493, 187-196.

Chen, C., Li, J., Bot, G., Szabo, I., Rogers, T.J., & Liu-Chen, L.Y. (2004). Heterodimerization and cross-desensitization between the μ-opioid receptor and the chemokine CCR5 receptor. *European Journal of Pharmacology*, 483, 175-186.

Chuang, R.Y., Suzuki, S., Chuang, T.K., Miyagi, T., Chuang, L.F., & Doi, R.H. (2005). Opioids and the progression of simian AIDS. *Frontiers in Bioscience*, 10, 1666-1677.

Corasaniti, M.T., Bagetta, G., Rotiroti, D., & Nistico, G. (1998). The HIV envelope protein gp120 in the nervous system: Interactions with nitric oxide, interleukin-1β and nerve growth factor signalling, with pathological implications *in vivo* and *in vitro*. *Biochemical Pharmacology*, 56, 153-156.

Corasaniti, M.T., Maccarrone, M., Nistico, R., Malorni, W., Rotiroti, D., & Bagetta, G (2001). Exploitation of the HIV-1 coat glycoprotein, gp120, in neurodegenerative studies *in vivo*. *Journal of Neurochemistry*, 79, 1-8.

Corasaniti, M.T., Melino, G., Navarro, M., Garaci, E., Finazzi-Agro, A., & Nistico, G. (1995). Death of cultured human neuroblastoma cells induced by HIV-1 gp120 is prevented by NMDA receptor antagonists and inhibitors of nitric oxide and cyclooxygenase. *Neurodegeneration*, 4 (3), 315-321.

Corasaniti, M.T., Rotiroti, D., Nappi, G., & Bagetta, G. (2003). Neurobiological mediators of neuronal apoptosis in experimental neuroAIDS. *Toxicology Letters*, 139, 199-206.

Davies, J., Everall, I.P., Weich, S., McLaughlin, J., Scaravilli, F., & Lantos, P.L. (1997). HIV-associated brain pathology in the United Kingdom: an epidemiological study. *AIDS*, 11, 1145-1150.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., DiMarzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., & Landau, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature*, 381, 661-666.

Dunfee, R., Thomas, E.R., Gorry, P.R., Wang, J., Ancuta, P., & Gabzuda, D. (2006). Mechanisms of HIV-1 neurotropism. *Current HIV Research*, 4, 257-278.

Emerterio, E.P., Tramullas, M., & Hurle, M.A. (2006). Modulation of apoptosis in the mouse brain after morphine treatment and morphine withdrawal. *Journal of Neuroscience Research*, 83, 1352-1361.

Eugenin, E.A. & Berman, J.W. (2005). Mechanisms of viral entry through the blood-brain barrier. The Neurology of AIDS, 2nd edition, Oxford University Press Inc., Oxford, NY, 147-154.

Feng, Y., Broder, C.C., Kennedy, P.E., & Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G-protein-coupled receptor. *Science*, 272, 872-877.

Genis, P., Jett, M., Benton, E.W., Boyle, T., Gelbard, H.A., Dzenko, K., Keane, R.W., Resnick, L., Mizrachi, Y., Volsky, D.J., et al. (1992). Cytokines and arachadonic metabolites produced during human immunodefiency virus (HIV) infected

macrophage-astroglia interactions: implications for the neuropathogenesis of HIV disease. *Journal of Experimental Medicine*, 176, 1703-1718.

Glass, J.D., Fedor, H., Wesselingh, S.L., & McArthur, J.C. (1995). Immunocytochemical quantitation of human immunodeficiency virus in the brain: correlations with dementia. *Annals of Neurology*, 38, 755-762.

Gorry, P.R., Churchill, M., Crowe, S.M., Cunningham, A.L., & Gabuzda, D. (2005). Pathogenesis of macrophage tropic HIV-1. *Current HIV Research*, 3, 53-60.

Grant, I., Sacktor, N., & McArthur, J. (2005). HIV neurocognitive disorders. The Neurology of AIDS, 2nd edition, Oxford University Press Inc., Oxford, NY, 359-374.

Griffin, D.E., Wesselingh, S.L., & McArthur, J.C. (1994). Elevated central nervous system prostaglandins in human immunodeficiency virus-associated dementia. *Annals of Neurology,* 35, 592-597.

Guo, C.J., Li, Y., Tian, S., Wang, X., Douglas, S.D., & Ho, W.Z. (2002). Morphine enhances HIV infection of human blood mononuclear phagocytes through modulation of beta-chemokines and CCR5 receptor. *Journal of Investigative Medicine*, 50, 435-442.

Hauser, K.F., El-Hage, N., Buch, S., Berger, J.R., Tyor, W.R, Nath, A., Bruce-Keller, A.J., & Knappe, P.E. (2005). Molecular targets of opiate drug abuse in neuroAIDS. *Neurotoxicity Research*, 8, 63-80.

Hu, S., Sheng, W.S., Lokensgard, J.R., & Peterson, P.K. (2005). Morphine potentiates HIV-1 gp120-induced apoptosis*. Journal of Infectious Diseases*, 191, 886-889.

Jana, A. & Pahan, K. (2004). Human immunodeficiency virus type 1 gp120 induces apoptosis in human primary neurons through redox-regulated activation of neutral sphingomyelinase. *Journal of Neuroscience*, 24 (43), 9531-9540.

Kapasi, A.A., Gibbons, N., Mattana, J., & Singhal, P.C. (2000). Morphine stimulates mesangial cell TNF-α and nitrite production. *Inflammation*, 24, 463-476.

Kaul, M. & Lipton, S.A. (2004). Signaling pathways to neuronal damage and apoptosis in human immunodeficiency virus type 1-associated dementia: chemokine receptors, excitotoxicity, and beyond. *Journal of Neurovirology*, 10 (suppl 1), 97-101.

Kaul, M. & Lipton, S.A. (2006). Mechanisms of neuronal injury and death in HIV-1 associated dementia. *Current HIV Research*, 4, 307-318.

Kaul, M., Garden, G.A., & Lipton, S.A. (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature*, 410, 988-994.

King, J.E., Eugenin, E.A., Buckner, C.M., & Berman, J.W. (2006). HIV tat and neurotoxicity. *Microbes & Immunity*, 8, 1347-1357.

Koka, P. He, K., Zack, J.A., Kitchen, S., Peacock, W., Fried, I., Train, T., Yashar, S.S., & Merrill, J.E. (1995). Human immunodeficiency virus 1 envelope proteins induce interleukin 1, tumor necrosis factor alpha, and nitric oxide in glial cultures derived from fetal, neonatal, and adult human brain. *Journal of Experimental Medicine*, 182 (4), 941-951.

Koutsilieri, E., Gotz, M.E., Sopper, S., Sauer, U., Demuth, M., ter Meulen, V., & Riederer, P. (1997). Regulation of glutathione and cell toxicity following exposure to neurotropic substances and HIV-1 *in vitro*. *Journal of Neurovirology*, 3 (5), 342-349.

Kumar, R., Torres, C., Yamamura, Y., Rodriguez, I., Martinez, M., Staprans, S., Donahoe, R.M., Kraiselburd, E., Stephens, E.B., & 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.

Li, Y., Luo, L., Rasool, N., & Yong Kang, C. (1993). Glycosylation is necessary for the correct folding of HIV gp120 in CD4 binding. *Journal of Virology*, 67 (1), 584-588.

Marchetti, B. & Abbracchio, M.P. (2006). To be or not to be (inflamed)- is that the question in anti-inflammatory drug therapy of neurodegenerative disorders? *TRENDS in Pharmacological Sciences*, 26, 517-525.

Merrill, J.E., Koyanagi, Y., Zack, J., Thomas, L., Martin, F., & Chen, I.S. (1992). Induction of interleukin-1 and tumor necrosis factor alpha in brain cultures by human immunodeficiency virus type 1. *Journal of Virology*, 66 (4), 2217-2225.

Miyagi, T., Chuang, L.F., Doi, R.H., Carlos, M.P., & Torres, J.V. (2000). Morphine induces gene expression of CCR5 in human CEM x174 lymphocytes. *The Journal of Biological Chemistry*, 225, 31305-31310.

Moore, J.P., Kitchen, S.G., Pugach, P., & Zack, J.A. (2004). The CCR5 and CXCR coreceptors- central to understanding the transmission and *pathogenesis of human immunodeficiency virus type 1 infection. AIDS* Research and Human Retroviruses, 20, 111-126.

Nath, A., Hauser, K.F., Wojna, V., Booze, R.M., Maragos, W., Prendergast, M., Cass, W., & Turchan, J.T. (2002). Molecular basis for interactions of HIV and Drugs of Abuse. *Journal of Acquired Immune Deficiency Syndrome*, 31, S62-S69.

Nosheny, R., L., Ahmed, F., Yakovlev, A., Meyer, E.M., Ren, K., Tessarollo, L., & Mocchetti, I. (2007). Brain-derived neurotrophic factor prevents the nigrostriatal degeneration induced by human immunodeficiency virus-1 glycoprotein 120 *in vivo*. *European Journal of Neuroscience*, 25, 2275-2284.

Nosheny, R.L., Bachis, A., Acquas, E., & Mocchetti, I. (2004). Human immunodeficiency virus type 1 glycoprotein gp120 reduces the levels of brain-derived neurotrophic factor *in vivo*: potential implication for neuronal cell death. *European Journal of Neuroscience*, 20, 2857-2864.

Nosheny, R.L., Bachis, A., Aden, S.A., DeBernardi, M.A., & Mocchetti, I. (2006). Intrastriatal administration of HIV-1 gp120 reduces glial cell-line derived neurotrophic factor levels and causes apoptosis in the substantia nigra. *Journal of Neurobiology*, 66, 1311-1321.

Nuovo, G.J. & Alfieri, M.L. (1996). AIDS dementia is associated with massive, activated HIV-1 infection and concomitant expression of several cytokines. *Molecular Medicine*, 2, 358-366.

Pacifici, R., di Carlo, S., Bacosi, A., Pichini, S., & Zuccaro, P. (2000). Pharmacokinetics and cytokine production in heroin and morphine-treated mice. *International Journal of Immunopharmacology*, 22, 603-614.

Perrella, O., Carrieri, P.B., Guarnaccia, D., & Soscia, M. (1992). Cerebrospinal fluid cytokines in AIDS dementia complex. *Journal of Neurology*, 239, 387-388.

Philpott, S.M. (2003). HIV-1 coreceptor usage, transmission, and disease progression. *Current HIV Research*, 1, 217-227.

Pugh, C.R., Johnson, J.D., Martin, D., Rudy, J.W., Maier, S. F., & Watkins, L.R. (2000). Human immunodeficiency virus-1 coat protein gp120 impairs contextual fear conditioning: a potential role in AIDS related learning and memory impairments. *Brain Research*, 861, 8-15.

Reynolds, A., Laurie, C., Mosley, R.L., & Gendelman, H.E. (2007). Oxidative stress and the pathogenesis of neurodegenerative disorders. *International Review of Neurobiology*, 82, 297-325.

Rogers, T.J. & Peterson, P.K. (2003). Opioid G-protein-coupled receptors: signals at the crossroads of inflammation. *TRENDS in Immunology*, 24, 116-121.

Rostasy, K., Monti, L., Yiannoutsos, C., Kneissl, M., Bell, J., Kemper, T.L., Hedreen, J.C., & Navia, B.A. (1999). Human immunodeficiency virus infection, inducible nitric oxide synthase expression, and microglial activation: pathogenic relationship to the acquired immunodeficiency syndrome dementia complex. *Annals of Neurology*, 46, 207-216.

Roy, S., Charboneau, R.G., & Barke, R.A. (1999). Morphine synergizes with lipopolysaccharide in a chronic endotoxemia model. *Journal of Neuroimmunology*, 95, 107-114.

Shi, B., De Girolami, U., He, J., Wang, S., Lorenzo, A., Busciglia, J., & Gabuzda, D. (1996). Apoptosis induced by HIV-1 infection of the central nervous system. *Journal of Clinical Investigation*, 98, 1979-1990.

Singh, I.N., Goody, R.J., Dean, C., Ahmad, N.M., Lutz, S.E., Knappe, P.E., Nath, A., & Hauser, K.F. (2004). Apoptotic death of striatal neurons induced by human immunodeficiency virus-1 Tat and gp120: differential involvement of caspase-3 and endonuclease G. *Journal of Neurovirology*, 10, 141-151.

Snyder, S. (1993). Janus faces of nitric oxide. *Nature*, 364, 577.

Steele, A.D., Henderson, E.E., & Rogers, T.J. (2003). μ-Opioid modulation of HIV-1 coreceptor expression and HIV-1 replication. *Virology*, 309, 99-107.

Suzuki, S., Chang, L.F., Yau, P., Doi, R.H., & Chuang, R.Y. (2002). Interactions of opioids and chemokine receptors: oligomerization of mu, kappa, and delta with CCR5 on immune cells. *Experimental Cell Research*, 280, 192-200.

Toggas, S.M., Masliah, E., ROckenstein, E.M., Rall, G.F., Abraham, C.R., & Mucke, L. (1994). Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature*, 367, 188-193.

Tyor, W.R., Glass, J.D., Griffin, J.W., Becker, P.S., McArthur, J.C., Bezman, L., & Griffin, D.E (1992). Cytokine expression in the brain during acquired immunodeficiency syndrome. *Annals of Neurology*, 31, 349-360.

Vallejo, R., de Leon-Casasola, O., & Benyamin, R. (2004). Opioid therapy and immunosuppression. *American Journal of Therapeutics*, 11, 354-365.

van de Bovencamp, M., Nottett, H.S.L.M., & Pereira, C.F. (2002). Interactions of human immunodeficiency virus-1 proteins with neurons: possible role in the development of human immunodeficiency virus-1 associated dementia. *European Journal of Clinical Investigation*, 32, 619-627.

Vincent, V.A.M., DeGroot, C.J.A., Lucassen, P.J., Portegies, P., Troost, D., Tilders, F.J.H., & Van Dam, A.M. (1999). NOS expression apoptotic cell death in brains of AIDS and AIDS dementia patients. *AIDS*, 13, 317-326.

Vincent, V.A.M., DeGroot, C.J.A., Lucassen, P.J., Portegies, P., Troost, D., Tilders, F.J.H., & Van Dam, A. (1999). Nitric oxide synthase expression and apoptotic cell death in brains of AIDS and AIDS dementia patients. *AIDS*, 13, 317-326.

Wesselingh, S.C., Glass, J., McArthur, J.C., Griffin, J.W., & Griffin, D.E. (1994). Cytokine dysregulation in HIV-associated neurological disease. *Advances in Neuroimmunology*, 4, 199-206.

West, J.P., Dykstra, L.A., & Lysle, D.T. (1998). Differential tolerance to morphine's immunmodulatory effects following continuous administrations. *Drugs and Alcohol Dependence*, 53, 31-38.

West, J.P., Lysle, D.T., & Dykstra, L.A. (1997). Tolerance development to morphine-induced alterations of immune status. *Drug and Alcohol Dependence*, 46, 147-157.

Zhao, M., Kim, M., Morgello, S., & Lee, S.C. (2001). Expression of inducible nitric oxide synthase, interleukin-1 and caspase-1 in HIV-1 encephalitis. *Journal of Neuroimmunology*, 115, 182-191.