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Seton Hall University

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Endotoxin-Induced Inflammation and Morphine-Induced Conditioned Place Preference in the HIV-1 Transgenic Rat

By

Natasha F. Homji-Mishra

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Bioscience from the Department of Biological Sciences of Seton Hall University, May 2012

Approved By

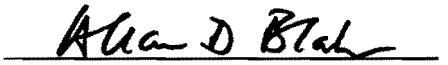
Sulie L. Chang, Ph.D.
MENTOR

Michael Vigorito, Ph.D.
COMMITTEE MEMBER

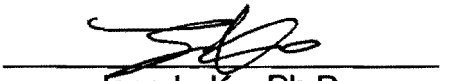
Marian Glenn, Ph.D.
COMMITTEE MEMBER

Tin-Chun Chu, Ph.D
COMMITTEE MEMBER

Constantine Bitsaktsis, Ph.D.
COMMITTEE MEMBER



Allan D. Blake, Ph.D.
DIRECTOR OF GRADUATE STUDIES



Jane L. Ko, Ph.D.
CHAIRPERSON,
DEPARTMENT OF BIOLOGICAL SCIENCES

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Contents

Abstract

HIV infection is a known co-factor in the increased incidence of sepsis which is a state of endotoxin tolerance (ET). During ET, with repeated exposure to non-pyrogenic amounts of endotoxin, there is an imbalance in pro- and anti-inflammatory cytokines when re-challenged with the endotoxin. We hypothesized that the ET state would be exacerbated in the HIV-1 transgenic (HIV-1Tg) rat, which has 7 of the HIV-1 viral proteins expressed without viral replication. Upon measuring the gene-expression we found that IL-1α, IL-1β, IFN-γ, Ccl2, 7 and 9 were upregulated to a greater extent in the spleen and cytokine receptors were modulated differently in the brain of the HIV-1Tg rat versus control in the ET state, indicating that the continuous presence of HIV-1 viral proteins alters the cytokine- and chemokine-profiles in the ET state. The expression of the μ-opioid receptor (MOR), which is known to mediate the rewarding and anti- nociceptive properties of its ligands like morphine, is modulated by inflammatory cytokines. As MOR is unregulated in the HIV-1Tg rat we hypothesized that rewarding properties of morphine may be enhanced in HIV-1Tg rat and tested this hypothesis using the conditioned preference placement (CPP) test. After developing a CPP, the rats with morphine paired with the white compartment of the CPP apparatus failed to show extinction after greater than 2 weeks of testing. Following extinction in rats with morphine paired with the black chamber, both drug and stress failed to induce a reinstatement of CPP. The morphine CPP procedure may sometimes result in

other forms of behavioural plasticity that does not conform to the Pavlovian/memory model.

Introduction

HIV-1 infection and the HIV transgenic rat model

Following infection with the Human Immunodeficiency Virus-1 (HIV-1), a burst of virus replication disseminates the virus to lymphoid organs. A robust cellular and humoral immune response ensues within a few weeks but the virus invariably escapes immune containment and results in chronic persistent infection leading to advancing clinical disease(Fauci 1996). The pathogenesis of HIV-1 infection results from the actions of HIV-1 viral proteins, including the envelope glycoprotein 120 (gp120), on targeted cells, such as macrophages and T-lymphocytes (Clapham and McKnight 2001; Cicala, Arthos et al. 2002; Jiang and Aiken 2006). Since 1996, highly active antiretroviral therapy, or HAART—has produced dramatic improvements in health and longevity for HIV-infected individuals (Palella, Delaney et al. 1998). HARRT regimens typically include two nucleoside (or nucleotide) analogue reverse transcriptase inhibitors (NRTIs) and either a protease inhibitor or a non-nucleoside reverse-transcriptase inhibitor (NNRTI) (Ellis, Calero et al. 2009). By inhibiting HIV replication at several stages in the viral life cycle, HAART markedly reduces the likelihood that drug resistance will develop (Ellis, Calero et al. 2009). Judicious use of antiretroviral therapy, by suppressing viral replication and partially restoring immune

function, can prevent opportunistic infections and markedly prolong survival with HIV (Ellis, Calero et al. 2009). However, HAART drugs are limited in their capacity to enter the CNS and other organs that are protected by tight endothelial barriers. Thus, in this post-HAART era, the clinical challenge is to identify the biological and physiological changes that occur due to the persistent presence of HIV-1 viral proteins in the host even when active viral replication is arrested (Jones and Perelson 2005; Vigano, Trabattoni et al. 2006).

Some HIV-1 viral proteins have been shown to affect the inflammatory response by altering the production of cytokines. For example, the HIV-1 Tat protein can alter the LPS-induced production of IFN-β and IL-6 in blood monocytes/macrophages(Yim HC 2009), and HIV-1 Vpr suppresses IL-12 production in human monocytes (Mirani M 2002). However, the effects of HIV-1 viral proteins on immune function during a state of ET has not been examined.

The HIV-1 transgenic (HIV-1Tg) rat model was developed with a functional deletion of the gag and pol genes in the HIV-1 genome. It is, however, under the control of the viral promoter and expresses seven of the nine HIV genes (Reid, Sadowska et al. 2001). Thus, there is no active viral replication but viral proteins are expressed in various organs' immune cells and in the circulation (Reid, Sadowska et al. 2001). Clinical signs include

wasting, mild to severe skin lesions, opaque cataracts, neurological signs, and respiratory difficulty (Reid, Sadowska et al. 2001). While it is well-known that HIV-1 affects the host target cells and causes the many pathological manifestations of AIDS, it has also been shown gp 120 and Tat, the HIV-1 viral proteins, can cause organ dysfunction independently (Klotman and Notkins 1996; Bruggeman, Dikman et al. 1997; van Maanen and Sutton 2003). It has been shown that, like HIV-1 infected patients, the HIV-1Tg rat is immunodeficient. LPS-induced leukocyte-endothelial adhesion (LEA) is greatly attenuated in the HIV-1Tg rat(Chang 2007). In addition, the HIV-1Tg rat shows signs of wasting and dies at a younger age even though there is no growth retardation and no sign of anorexia throughout its life span(Peng, Vigorito et al. 2010). It has been shown that prior to manifesting signs of wasting the expression of HIV viral proteins in various tissues changes(Peng, Vigorito et al. 2010). Reid and colleagues showed that spliced and unspliced viral transcripts were expressed in the spleen of HIV-1Tg rats(Reid, Sadowska et al. 2001) . Peng and colleagues showed that in the HIV-1Tg rats at 10-11 months of age the expression of Tat, gp120, nef, and vif viral protiens decreased significantly in the spleen compared to 2-3 months of age, but increased significantly in the spinal cord and cerebellum(Peng, Vigorito et al. 2010). These rats also have decreased alveolar macrophage expression of granulocyte-macrophage colony-

stimulating factor and impaired bacterial phagocytosis, which may increase their susceptibility to pneumonia(Joshi, Raynor et al. 2008). The HIV-1Tg rat has also been shown to develop neurological deficits including motor (Reid, Sadowska et al. 2001) and cognitive deficits(Lashomb, Vigorito et al. 2009). Brain AA incorporation and other markers of AA metabolism are upregulated in HIV-1 Tg rats (Basselin, Ramadan et al. 2011; Lund, Lucero et al. 2011). The HIV-1Tg rat model, thus, exhibits some of the characteristics of HIV-1 infected patients given HAART (Chang, Beltran et al. 2007; Peng, Vigorito et al. 2010; Basselin, Ramadan et al. 2011; Lund, Lucero et al. 2011).

Role of Inflammatory cytokines in HIV-1 infection and progression to AIDS

In HIV-1 infected individuals and persons with AIDS the immune system is dysfunctional at many levels. There is immunodeficiency induced due to loss of CD4-positive T helper cells (Lane, Masur et al. 1983) and hyperactivity as a result of B-cell activation (Holguin, O'Connor et al. 2004). Additionally, inflammatory cytokines are modulated in HIV-1 infected individuals by the actions of viral proteins, mainly gp120 (Holguin, O'Connor et al. 2004). Cytokine changes in HIV-1 infection have been assessed by a

variety of techniques, ranging from determination of cytokine gene expression at the mRNA level to secretion of cytokine proteins in vivo and in vitro. It has been shown that as soon as 5 days after detection of viremia, (at least 100 HIV RNA copies/ml), cytokines IFNα and IL-15 were elevated, followed by TNFα, CXCL10, and IFNγ, and then by IL-12 (Stacey, Norris et al. 2009). Increased mRNA and protein levels of anti-inflammatory cytokine, IL-10, are detected rather late in HIV infection, after the increased expression of proinflammatory cytokines(Stacey, Norris et al. 2009). Location of cytokine production in the very early phase of HIV infection is most likely local. However, after the virus disseminates, cytokines are produced by distant sites such as distal lymph nodes, gut-associated lymphoid tissue and genital draining(Haase 2010). Changes in cytokine levels in HIV-1 infected persons can affect the function of the immune system, and have the potential to directly impact the course of HIV disease by enhancing or suppressing HIV replication (Breen 2002). In particular, the balance between the pro-inflammatory cytokines IL1β, IL-6, and TNF-α, which up-regulate HIV expression, and IL-10, which can act both as an anti-inflammatory cytokine and a B-cell stimulatory factor, may play an important role in the progression to AIDS (Chen, Zhou et al. 2005). Pro-inflammatory cytokines, IL1-β, TNF-α, and IL-6, were shown to be up-regulated in spinal cord glia activated by gp-120 (Breen 2002). It has been shown that a non-pyrogenic dose of LPS induces a 7 to 38 fold increase in

IL-1β and TNF-α in HIV-1Tgrats and only 1 to 2 fold increase in these cytokines in the F344 rats, whereas there was no significant difference observed in IL-10 levels in HIV-1Tg rats vs. F344 rats, suggesting that there is an imbalance of pro- vs. anti-inflammatory cytokine regulation with LPS administration in HIV infection (Chen, Zhou et al. 2005).

Endotoxin Tolerance

The bacterial endotoxin, lippopolysccharide (LPS), is a well-characterized glycolipid component of the gram-negative bacterial cell wall and is a potent inducer of the inflammatory response in animals (Schletter, Heine et al. 1995). LPS is model molecule commonly used to study the inflammatory responses caused by infection with bacteria, especially the induction and actions of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and anti-inflammatory cytokines like IL-10 (Turnbull and Rivier 1999). LPS and other Gram-negative bacterial pathogens primarily engage the Toll like receptor-4 (TLR-4), which employs signaling through two distinct adaptors – Myeloid differentation 88 (MyD88) and TIR domain-containing adapter protein inducing IFN-β(TRIF) (Biswas and Lopez-Collazo 2009). The MyD88 pathway leads to the activation of transcription factor NFκB which in the initial phase activates pro-inflammatory genes like TNFα, IL1β, IL6 and IL12β. The TRIF pathway

leads to the activation of transcription factors IRF3 and STAT1 which subsequently activate IFNβ and interferon-inducible genes like CCL5 and CXCL10, which ultimately leads to the induction of anti-inflammatory genes like IL-10 (Biswas and Lopez-Collazo 2009). These pathways are temporally segregated, MyD88 leads to the early activation of NFκB and the TRIF pathway is implicated in mediating the late-phase activation of NFκB (Biswas and Lopez-Collazo 2009). Crosstalk between the MyD88 and TRIF pathways exists and there are various negative regulators for each pathway that are modulated by one of both of these pathways forming a complex signaling network (Biswas and Lopez-Collazo 2009). It is the balance between the production of pro-inflammatory cytokines and chemokines and the subsequent production of anti-inflammatory cytokines that initiates a robust inflammatory response in the innate immune system cells like macropages and monocytes (Biswas and Lopez-Collazo 2009). A tight regulation of the initial production of the pro-inflammatory cytokines is required to protect against going into septic shock, an example of this regulatory mechanism is Endotoxin Tolerance (Biswas and Lopez-Collazo 2009). Exposure of the innate immune cells to minute amounts of endotoxin, like LPS, makes them refractory to subsequent endotoxin challenge establishing endotoxin tolerance. At a molecular level, during endotoxin tolerance, there is a decrease in the production of pro-

inflammatory cytokines with an endotoxin re-challenge when compared to the initial increase in pro-inflammatory cytokines with first exposure to the endotoxin. And an increase in anti-inflammatory cytokines is observed with an endotoxin re-challenge when compared to the initial anti-inflammatory cytokines levels observed with first exposure to the endotoxin (Biswas and Lopez-Collazo 2009) . A clinical example of endotoxin tolerance is sepsis. Following a systemic bacterial infection a dysregulated innate immune response leads to sepsis. Sepsis is biphasic with the first phase being of overt inflammation and a subsequent phase of "immunoconpromise", in which the innate immune cells show a endotoxin tolerant phenotype (Biswas 2009). The overt inflammation phase is called the systemic inflammatory response syndrome (SIRS) in which there is an overproduction of pro-inflammatory cytokines and organ failure(Adib-Conquy and Cavaillon 2009). The immunocompromised or 'endotoxin-tolerant' state of spesis is termed compensatory anti-inflammatory response syndrome or CARS. This is the state and is clinically correlated most with secondary infections and mortality (Adib-Conquy and Cavaillon 2009). Although, these two states of sepsis have been reported to be concomitant by some researchers, the failure of anti-inflammatory therapies in sepsis and the fact that most mortality is associated with patients in the immunocompromised state, supports the biphasic nature of spesis(Biswas

and Lopez-Collazo 2009). In sepsis patients blood monocytes have the expression of proinflammatory cytokines like TNFa, IL-6, IL-1a, IL-1b and IL-12, significantly diminished upon ex vivo LPS challenge as compared to that of monocytes from healthy individuals (Munoz, Carlet et al. 1991; Monneret, Venet et al. 2008; Draisma, Pickkers et al. 2009). At the same time, upregulated expression of anti-inflammatory cytokines like IL-10, TGFb and IL-1RA were reported in these cells (Cavaillon, Adrie et al. 2005; Monneret, Venet et al. 2008; Draisma, Pickkers et al. 2009).

Clinical Relevance of Endotoxin Tolerance with Respect to HIV-1 Infection

There is an association between chronic HIV infection and elevated plasma endotoxin levels. Innate immune responses, which are dysregulated in ET, are also altered in HIV infection (Lester, Yao et al. 2008). There is increased expression and responsiveness of TLR in chronic HIV-1 infection. The mRNA expression of TLR 6 , 7 and 8 was elevated in chronic untreated HIV-1 infection and TLR 2, 3 and 4 were additionally elevated in advanced disease (CD4 cell count < 200 cells/ml) in peripheral blood mononuclear cells(Lester, Yao et al. 2008). This TLR expression alteration may perpetuate innate immune dysfunction and activation that underlies

HIV pathogenesis (Lester, Yao et al. 2008). The cytokines/chemokines modulated during ET play a role in HIV infection, replication (not applicable for our model) and pathogenesis. Toll-like receptor 4 (TLR4) mediates gram-negative bacteria activated signaling and significant changes in this receptor's level is directly correlated with HIV infection (Lester, Yao et al. 2008). TNF-α upregulation leads to HIV-induced cytotoxicity (Westendorp, Shatrov et al. 1995). Enhanced levels of Ccl2 in HIV-1 patients have been associated with HIV-1-associated dementia (Ansari, Heiken et al. 2007). The Center for Disease Control (CDC) has identified HIV-1 infection as a major reason for the increase in incidence of sepsis (Angus, Linde-Zwirble et al. 2001). Opportunistic infections are a common feature in HIV-1 positive patients, who have a compromised immune status (Levy 1998). ET leads to a similar immunosuppressed state. Identifying the mechanism by which ET affects an already immune-compromised system, observed with HIV-1 infection, could provide valuable information of clinical relevance. We hypothesized that the continuous presence of HIV-1 viral proteins alters the systemic immune response to bacterial endotoxins in terms of pro- and anti-inflammatory cytokine and chemokine expression, and that this altered immune response is exacerbated when the animal is in an ET state. Specifically, we hypothesized that the production of pro-inflammatory cytokines is diminished and anti-inflammatory cytokine production is

enhanced in the HIV-1Tg rat rendered endotoxin tolerant. To test this hypothesis, we examined the expression of an array of cytokines, chemokines, and their receptors in the serum, spleen, and brain of an endotoxin tolerant HIV-1Tg rat model in response to an LPS challenge.

Morphine, and HIV infection

Morphine is a derivative of opium, which is the juice of the opium poppy, *Papver somniferum.* Morphine was first used and still is used as a pain-killer in the management of diseases like cancer and acquired immunodeficiency syndrome (AIDS). When ingested, morphine can produce a feeling of euphoria and a state of well-being; this makes it a popular choice to be used as a recreation drug which is often abused. Approximately one third of the AIDS cases in the USA have been suggested to be related to the use of injected addictive drugs, such as morphine, making it a very significant co-factor in contracting human immunodeficiency virus type 1 (HIV-1) (Steffen, Blattler et al. 2001; Banerjee, Strazza et al. 2011). The reason for higher addiction rates to morphine among HIV-1 infected individuals may be to over-come the depression and stress due to the disease or that morphine addicted persons may be more prone to risky sexual behaviour and in turn be more prone to contracting HIV-1 infection (Beyrer 2007; Shetty 2011).

Conversely, HIV-1 infected persons are more prone to be addicted to morphine than the general population(Koulierakis, Gnardellis et al. 2000). Thus, there may exist a bidirectional and synergistic relationship between morphine abuse and the contraction of HIV-1 infection.

Most research in this field has been directed towards studying the effects of drugs of abuse on HIV-1 viral infection and there is a paucity of studies exploring the possible bidirectional feedback that may exist between drugs of abuse and the persistence of viral proteins in HIV infection.

Immunosuppression associated with Morphine Abuse and HIV infection

Chronic morphine abuse has been well-documented to result in severe immune consequences. Pulmonary infections, caused by Mycobacterium, Staphylococcus, Streptococcus, Haemophilus, and other bacteria, are among the most common diagnoses of opiate abusers (Risdahl, Khanna et al. 1998). Other serious diseases caused by microbial pathogens in opiate users are AIDS (HIV), endocarditis (Staphylococcus, Enterococcus, Pseudomonas, Klebsiella, Serratia, and Candida), abscesses and cellulitis (Staphylococcus, Streptococcus, Haemophilus, Enterobacter, Pseudomonas, Klebsiella, Clostridium, Candida, and others), hepatitis A, B,

and C (hepatitis A, B, and C viruses), sexually transmitted diseases, and skeletal infections (Staphylococcus and Pseudomonas) (Risdahl, Khanna et al. 1998). Studies concerning the effects of addictive drugs on immunocompetence have taken on a greater urgency with the onset and explosive expansion of the worldwide pandemic of AIDS caused by HIV, which results in the collapse of the immune system, rendering individuals highly susceptible to opportunistic microorganisms that normally would not cause life-threatening infections. Intravenous (i.v.) drug abusers become highly susceptible to HIV not only because of contaminated shared needles but also because of associated immunosuppression caused by opioids (Friedman, Ompad et al. 2003). Studies over the past few decades have shown that opiates markedly affect many immune responses, both in vivo and in vitro, in animal models such as rodents and mice, and even in monkeys and swine, including altered resistance following challenge infection with a microorganism. Morphine is known to supress the functionality of both innate and adaptive immune cells (Friedman and Eisenstein 2004; Dinda, Gitman et al. 2005; Roy, Wang et al. 2006). Morphine suppresses pro-inflammatory signal in dendritic cells, macrophage activation and phagocitosis(Wang, Ma et al. 2011; Wang, Ye et al. 2011). TLR signalling and production of pro-inflammatory cytokines is significantly modulated by morphine(Roy, Ninkovic et al. 2011) .

Several opiate receptors have been identified on cells of the nervous system and immune system of animals and humans, with μ, κ, and γ subtypes predominating (Cabral 2006). These classical opiate receptors, similar to cannabinoid receptors, are G-protein-coupled seven transmembrane molecules. Opiates directly ligate μand γ opiate receptors as well as non-classical opiate-like receptors on immune cells, and also bind to similar receptors on CNS cells. Opioids have been reported to have direct (House, Thomas et al. 1996; McCarthy, Wetzel et al. 2001) as well as indirect effects (Chang, Wu et al. 1998; Roy, Charboneau et al. 2001) on the immune system through the mediation of μ, κ, and δ opioid receptors as well as non-classical opioid-like receptors. MOR ligands can mimick chemoattractants (direct effect) (Simpkins, Taylor et al. 1984; Grimm, Ben-Baruch et al. 1998; Grimm, Ben-Baruch et al. 1998) and can inhibit chemotaxis of cytokines and chemokines by (indirect effect) (Grimm, Ben-Baruch et al. 1998). Peterson and colleagues proposed that MOR ligands "can be viewed as cytokines operating both within somatic immune tissues and the CNS" (Peterson, Molitor et al. 1998). Treating astroglial cells with IL-1β increased MOR mRNA expression by 70-80% (Ruzicka, Thompson et al. 1996); simultaneous treatment with IL-1α and IL-1β increased MOR expression in primary cell cultures of human brain microvesicular endothelial cells (Vidal, Patel et al. 1998). These studies were among the

first to report the link between cytokines and MOR and opened up the field of neuroimmunology, showing that the immune system interacts with the CNS by modulating the expression of neurotransmitter receptors which mediate the effects of addictive substances. CCR5 and CXCR4 are the main HIV-1 co-receptors, which cooperate sequentially with CD4 to facilitate virus entry into target cells. A possible mechanism for increased susceptibility to HIV-1 with morphine abuse is provided by studies that show that the expression of CCR5 and CXCR4 in CD3(+) lymphoblasts and CD14(+) monocytes is increased 3-5 folds by DAMGO, a selective MOR agonist(Guo, Li et al. 2002; Wang, Barke et al. 2005). By enhancing CXCR4 and CCR5 expression, opioids increase HIV-1 infection(Steele, Henderson et al. 2003). Additionally, it has been shown by several studies that morphine and other MOR agonists increase the expression of CCR5 and CXCR4 on glial cells(Miyagi, Chuang et al. 2000; Steele, Henderson et al. 2003; Mahajan, Aalinkeel et al. 2005; Mahajan, Schwartz et al. 2005; Burbassi, Aloyo et al. 2008; Happel, Steele et al. 2008; Bokhari, Yao et al. 2009). It has been postulated that morphine induces the expression of these chemokine receptors by the modulation of cytokines , like IL-12 and TNF-α, which are known to stimulate chemokine receptor expression(Hu, Chao et al. 2000).

Mechanisms of morphine immunomodulation

Although the specific mechanisms of the changes induced in the immune system by morphine are not fully understood, the initial step is mediated through the MOR, which is expressed on immune cells including macrophages and lymphocytes as well as in the central nervous system (CNS) (House, Thomas et al. 1996; Chang, Wu et al. 1998; McCarthy, Wetzel et al. 2001; Roy, Charboneau et al. 2001). Expression of the MOR can be modulated in both the immune system and neuronal cells by various cytokines. IL-1 was the first cytokine that was demonstrated to increase the mRNA level of MOR in endothelial cells (Vidal, Patel et al. 1998). IL4 was shown to induce MOR transcripts in human primary blood cells, immune cell lines, and dendritic cells (Kraus, Borner et al. 2001). IL6 induced only induced MOR mRNA and not the μ opioid receptor in a human neuroblastoma cell line (Borner, Kraus et al. 2004). TNF-α was shown to induce MOR gene transcription in primary human T lymphocytes, Raji B cells, U937 monocytes, primary human polymorphonuclear leukocytes, and mature dendritic cells (Kraus, Borner et al. 2003). Morphine also exerts an indirect effect on immune cells through MORs located in the CNS. Several studies indicate that morphine acts on central MORs and activates the hypothalamic–pituitary–adrenal (HPA) axis resulting in the release of glucocorticoids from the adrenal cortex (Gottlieb, Schroff et al. 1981).

These studies indicate that cytokines and chemokines modulate the effects of MOR ligands like morphine but these MOR ligands also have an effect on the function of these immune molecules. Studies indicate that that morphine use increases the progression of HIV-1 infection to AIDS through its interaction with cytokines, chemokines, and their receptors (McCarthy, Wetzel et al. 2001; Hahn, Jagwani et al. 2010; El-Hage, Dever et al. 2011). Morphine phosphorylates CXCR1 and CXCR2 receptors and induces heterogeneous desensitization of these receptors thus inhibiting the migration of CXC1 and CXC2 to their receptors (Grimm, Ben-Baruch et al. 1998). On the other hand, chemokines and their receptors, which are also HIV-1 co-receptors, have been implicated in the neuropathogenesis of HIV-1 infections. Astrocytoma cell line U87 treated with morphine showed significant down-regulation of IL-8 gene expression, whereas expression of the IL-8 receptor CXCR2 was reciprocally up-regulated (Mahajan, Schwartz et al. 2002). Furthermore, treatment of normal human astrocyte (NHAs) with morphine suppressed IL-8 and macrophage-inflammatory protein-1β gene expression, whereas expression of their receptor genes, CCR3 and CCR5, was simultaneously enhanced (Mahajan, Schwartz et al. 2002). These morphine-induced effects on U87 and NHA cells were reversed by the MOR antagonist β -funaltrexamine. Morphine also enhanced the constitutive expression of the MOR on astroglial cells (Mahajan, Schwartz

et al. 2002). These results suggest that morphine has the potential to increase HIV-1 co-receptor expression, promoting viral trafficking and binding on infected cells as well as increasing CNS disease progression, and increasing susceptibility to HIV infection. Opioid receptor selective agonists have been shown to modulateinfection by HIV-1 and HTLV-1 by *in vitro* syncytia formation (Nyland, Specter et al. 1998). Morphine promoted the survival of CEMx174 cells in early stages of simian immunodeficiency virus (SIV) infection in vitro, a model system that has been used to study HIV infection. Cell cycle analysis indicated that, in response to the treatment with morphine, SIV-infected cells were accumulated in the C1 phase. The investigators suggested that the calcium–PKC–MAPK cascade was involved in morphine prolonged survival of cells in the early stages of virus infection(Li, Hao et al. 2004). In an HIV-1 transactivator of transcription (TAT)-transgenice mouse model morphine was shown to have a major inhibitory effect on mitogen-induced IL-2 production. This decrease of IL-2 production correlated with reduced percentages of CD4+ and CD8+ splenic lymphocytes (Garza, Prakash et al. 1996). These studies indicate that morphine affects the responsiveness of immune cells to infectious agents and their interaction with the HIV *in vitro*.

Upregulation of the Mu-opioid receptor in HIV-1Tg rat brain may enhance propensity for addiction

The persistent presence of HIV viral proteins may contribute to the dysregulation of both the dopaminergic and opioidergic pathways, and may lead to reward and addictive behaviors. Expression of the dopamine receptor (D1R) is increased in HIV-1Tg rats and methamphetamine-induced behavior sensitization is more profound in the HIV-1Tg rats. HIV-1Tg rats showed less behavior sensitization to nicotine in comparison to F344 (Midde, Gomez et al. 2011). Both these studies suggest that that the presence of HIV proteins in the HIV-1Tg rat altered its sensitivity to an addictive drug. The μ opioid receptor (MOR) which is expressed in the central nervous system cells and in the immune system cells, may contribute to the mediation of the analgesic and rewarding actions of opioids such as morphine (Fowler and Fraser 1994; Chang, Beltran et al. 2007). Exposure of peritoneal macrophages of F344 rats to serum from HIV-1Tg induced MOR-upregulation, which was abolished by addition of gp120 antibody to the serum (Chang, Beltran et al. 2007). HIV-1Tg rats when treated with morphine showed significantly longer tail flick latencies compared to control F344 rats, indicating that there is an increased potency of morphine's anti-nociceptive properties in the HIV-1Tg rats (Chang 2011).

As MOR is up-regulated in the HIV-1Tg rat and it has also been shown that the anti-nociceptive properties of morphine are enhanced in the HIV-1Tg rat we wanted to investigate if the rewarding and addictive properties of morphine are also enhanced in the HIV-1Tg rat.

Craving for and relapse to a drug after a period of abstinence are the salient features of addiction (Koob and Nestler 1997). In drug addiction there is a very significant behavioral change in that that the addict has decreased ability to control drug seeking behaviour(Kalivas and Volkow 2005). In laboratory animals relapse following abstinence is modeled using the reinstatement procedure. Animals are first trained to associate a behavior (self administration) or a place (CPP) with a drug. Then a period of extinction is introduced whereby the behavior or place is no longer followed by the drug and responding returns to baseline. The effects of various stimuli are then introduced to look for evidence of the return of responding or reinstatement. Reinstatement of drug seeking behavior, or relapse, is caused in the laboratory by re-introduction to the drug (drug-primed) , exposure to stress (stress-primed) or re-introduction of the stimuli which was previously associated with drug use (cue-primed) (Shalev, Grimm et al. 2002; Kalivas 2003). Re-introduction to previous stimuli/environment associated with drug-use increases the urge to use drugs (Kalivas and Volkow 2005). These motivational properties of the conditioned stimuli

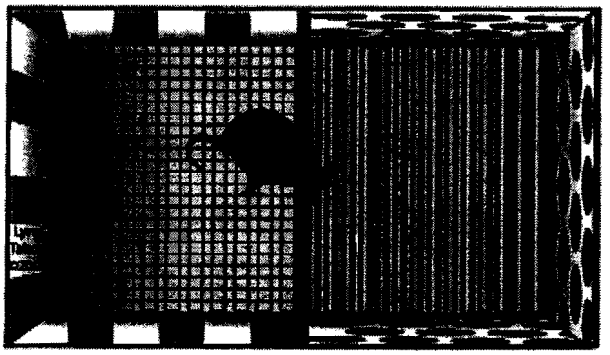
(associated with drug) use can persist over a very long period of time, even after years of abstinence the conditioned stimuli can evoke drug-re-instatement (Kalivas and Volkow 2005).

Conditioned Place Preference

The conditioned place preference (CPP) is a commonly used test in animals to measure the rewarding/addictive properties of a drug by measuring the motivational salience of drug-paired cues, by means of classical conditioning (Tzschentke 1998; Tzschentke 2007). In the CPP procedure, there is repeated pairing of an unconditioned stimuli (test-drug) with a conditioned stimulus (distinct environment) and of a control vehicle with another environment (Tzschentke 1998). The drug-paired environment gains motivational significance and the animal shows approach behavior to that environment and a preference for it over the vehicle-paired environment in the drug-free state(Tzschentke 1998). After the establishment of a CPP the animal is given free access to both environments for several sessions in the absence of the drug until the animal ceases to display a statistically significant preference to the drug-paired environment (extinction)(Tzschentke 1998). Extinction is evidence that the preference is a learned association(Rutten K 2011). Extinguished associations are not permanently lost and can be re-expressed. After

extinction exposure to a drug-prime or to a stressor can be studied for their ability to re-instate the extinguished CPP response (Tzschentke 1998). Reinstatement procedures cause the previous associations to be re-expressed. It has been clearly demonstrated that stress causes a greater propensity towards drug-seeking behavior, which causes a greater chance to become addicted (Donovan, Padin-Rivera et al. 2001; Ouimette, Coolhart et al. 2007) and puts the subject at a greater risk for relapse (Dewart, Frank et al. 2006). This phenomenon has been noted in animals as well. In rats, an increase in locomotor activity (behaviour response to drug) was noted in morphine-treated rats pre-expose to mild repeated foot-shock(Leyton and Stewart 1990). Exposure to foot-shock also re-instated morphine-induced CPP in rats (Wang, Luo et al. 2000).

Conditioned Place Preference Apparatus



Morphine-induced CPP has been studied using F344 rats (Gagin, Kook et al. 1997; Grakalic, Schindler et al. 2006; Davis, Roma et al. 2007). There exists a strain-based sensitivity to morphine-induced CPP; F344 rats are more sensitive to morphine and acquire morphine-induced CPP at lower doses and with fewer conditioning cycles than Lewis rats (Davis, Roma et al. 2007). In this study we have hypothesized that the HIV-1Tg animal would be more sensitive to the morphine associated cues than the F344 rat due to the up-regulation of MOR in the presence of HIV-1 proteins. As the unbiased CPP procedure used in the present study is not favorable for establishing differences in rates of acquisition due to potential "ceiling" effects (Roma and Riley 2005). We investigated the difference between HIV-1Tg and control, F344 rats, in the vulnerability to re-expression of motivational salience of morphine paired cues by reinstatement procedures. A greater motivational salience of morphine paired cues would be expected

to result in greater reinstatement of a preference for the drug-paired environment. In addition we investigated the effectiveness of a nonspecific stressor (uncontrollable foot-shock) in establishing a reinstatement effect.

Summary and significance of work:

There were an estimated 48,100 new HIV infections in 2009, per the CDC (Prejean, Song et al. 2011) and the estimated number of people living world-wide with HIV was 43 million in 2010, per the world health organization (WHO). The CDC has identified the increasing rate of HIV infection as a major reason for the increased incidence of sepsis, in which the patients are in a state of ET. During ET there is an imbalance of pro- and anti-inflammatory cytokine expression leading to an abnormal immune response when re-exposed to endotoxins like LPS. We hypothesized that the persistence of viral proteins alters the systemic immune response to bacterial endotoxins in terms of pro- and anti-inflammatory cytokine and chemokine expression, and that this altered immune response is exacerbated in an endotoxin tolerant state. Specifically, we hypothesized that the production of pro-inflammatory cytokines is diminished and anti-inflammatory cytokine production is enhanced. Taking advantage of the HIV-1Tg rat model, we examined the effects of LPS induced endotoxin tolerance by measuring the gene expression of 84 cytokines, chemokines and their receptors in the brain and spleen in response to an LPS challenge. This research will help better understand the mechanisms underlying the increased susceptibility of HIV-1 patients to spesis. It provides information on specific inflammatory cytokine, chemokine and their

receptors' genes that are modulated during ET in the HIV-1Tg rat. This information can be further used to study the inflammatory cytokine, chemokine and their receptors' gene-profiles in other models including humans.

Another aspect of the project was to examine the effect of morphine associated motivational salience in the HIV-1Tg rat. Inflammatory cytokines are known to modulate MOR expression and the progression of HIV-1 infection to AIDS. Also, MOR is upregulated in the HIV-1Tg rat (Chang, Beltran et al. 2007). We hypothesized that the HIV-1Tg animal would be more sensitive to the morphine associated cues than the F344 rat due to the up-regulation of MOR in the presence of HIV-1 proteins and altered cytokine profiles. We investigated the difference between HIV-1Tg and control, F344 rats, in terms of extinction of CPP and the vulnerability to re-expression of motivational salience of morphine paired cues by reinstatement procedures. A greater motivational salience of morphine paired cues would be expected to result in greater resistance to extinction (Rutten K 2011) and/or greater reinstatement of a preference for the drug-paired environment. In addition we investigated the effectiveness of a nonspecific stressor (uncontrollable foot-shock) in establishing a reinstatement effect. This research provides salient information on

exploring the bi-directional link that may exist between morphine-abuse and HIV-1.

Materials and Methods

Animals used for gene expression and protein analysis

Adolescent male Fisher 344/NHsd Sprague-Dawley background HIV-1 transgenic (HIV -1Tg) rats and age-matched Fisher /NHsd 344 (F344) control rats were purchased from Harlan Laboratories (Indianapolis, IN), and were delivered on post-natal day 28. The animals were group-housed immediately upon arrival, and stayed in group cages during the experiment. The animals were maintained in an environment of controlled temperature (21-22° C) on a 12-h light/12-h dark illumination cycle, with lights-on set at 7:00 AM. Food and tap water were provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

Lipopolysaccharide (LPS) administration for gene expression and protein analysis

Dosing solutions of LPS were prepared in saline. In our preliminary studies using Harlan Sprague Dawley rats, we found that two intraperitoneal (i.p) injections with a non-pyrogenic dose (250 μg/kg ea) of LPS, administered 9-12 hrs apart, was the lowest dosage regimen that would cause endotoxin tolerance and inhibit the production of IL-1β, TNF-α, and IL-6 in response to

a subsequent challenge with a significantly higher dosage of LPS [1, 4, 8, 16, or 32 mg/kg] (data not shown). In this study, HIV-1Tg and F344 rats (n = 12 ea, 19-20 wks old) were randomly assigned to four experimental groups (n = 3 animals/group). At 8:00 AM and 5:00 PM on Day 1, Groups 1 and 2 received two i.p. injections of 250 μg/kg LPS each (LL); Groups 3 and 4 received two i.p. injections of saline (SS). At 8:00 AM on Day 2, Group 1 received one i.p. injection with 5 mg/kg LPS (LL+L); Group 2 received one i.p. injection with saline (LL+S); Group 3 received one i.p. injection with 5 mg/kg LPS (SS+L); and Group 4 received one i.p. injection with saline (SS+S). The dosage of 5 mg/kg for the subsequent LPS injection was chosen based on previous studies using the HIV-1Tg rat model (Chen, Zhou et al. 2005). Two hours following the last injection, the brains, spleens, and blood were collected for RNA, protein, and serum preparation.

Protein extraction from the brain and spleen

Protein extracts were prepared from approximately 100 mg of brain and spleen tissue in a Tris lysis buffer containing 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1M NaF (all from Sigma Aldrich, St. Louis, MO), and 1 tablet of Protease Inhibitor (Roche, Mannheim, Germany). The tissues were disrupted using the Branson Sonifier 250 (VWR, Radnor, PA) with 15 sec bursts, a duty cycle setting of 40% (0.4 sec burst/0.6 sec

pause), and an output of 4. The concentration of protein from each of the tissue types was determined using the ProStain assay kit (Active Motif, Carlsbad, CA), by re-suspending the lyophilized dye in methanol and dilute this stock solution. This stock solution was added to each well of the microplate followed by adding the sample. Fluorescence intensity was measured against a BSA standard curve with the Spectra Max Gemini (Molecular Devices, Sunnyvale, CA).

Measurement of inflammatory cytokines

Protein levels of IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, and IL-13 were determined in undiluted serum and in extracts from 200 µg of brain and spleen using a 96-well inflammatory cytokine kit [MesoScale Discovery (MSD), Gaithersberg, MD]. The MSD plate is prepared per the kit instructions (http://www.mesoscale.com/CatalogSystemWeb/Documents/Rat_96_well_US.pdf) , the sample or calibrator are added, followed by the addition of detection antibody solution, followed by the read buffer. Measurement of electrochemiluminescent signal intensity was determined on the SECTOR 2400 instrument (MesoScale Discovery, Gaithersberg, MD). Calibrator solutions were diluted 4-fold over a concentration range of 40,000 pg/mL to 9.8 pg/mL.

RNA isolation and preparation of cDNA

Total RNA was extracted from brain and spleen homogenates using TRIZOL (Invitrogen, Carlsbad, CA). The extracts were then treated with Ambion® TURBO DNA-*free*™ (Ambion, Austin, TX) to remove contaminating DNA, and harvested using a RNeasy mini kit (Qiagen, Valencia, CA). The tissues were disrupted and the lysate was homogenized in the buffer, the lysates were centrifuged the supernantant was removed, 70% ethanol was added to the cleared lysate, sample was transfered to an RNeasy spin column and centrifuged, wash buffer was added followed by centrifugation (http://www.qiagen.com/products/rnastabilizationpurification/rneasysystem/rneasymini.aspx#Tabs=t2). RNA quality and quantity were assessed using a Nanodrop spectrophotometer. Equal amounts of RNA from each sample were then converted into first-strand cDNA using a RT2 First Strand Kit (SA Biosciences, Frederick, MD). The genomic DNA elmination mixture (with RNA sample and kit reagents) and the reverse transcription cocktail was prepared using the kit's instructions (http://www.sabiosciences.com/rt_pcr_product/HTML/330401.html). The 2 are mixed and incubated at 42°C for 15 min afterwhich the reaction is

stopped by heating at 95°C for 5 minutes and First Strand cDNA Synthesis Reaction is obtained.

Real-time PCR array

Detection and quantification of gene expression were performed using a Rat Inflammatory Cytokines and Receptors PCR Array and RT^2 SYBR Green Fluorescin qPCR Master (SA Biosciences, Frederick, MD) according to the manufacturer's instructions (http://www.sabiosciences.com/manuals/pcrarraywhitepaper.pdf). This kit was chosen because it includes diverse genes important in immune responses, including genes encoding CC chemokines (n = 16), CXC chemokines (n = 9), interleukin cytokines (n = 14), other cytokines (n = 11), chemokine receptors (n = 15), and cytokine receptors (n = 11), as well as other genes involved in the inflammatory response (n = 8).

Real-time PCR was performed using an ABI Prism 7900HT Fast Detection System (Applied Biosystems, Foster, CA). Each 10 µL reaction was performed in a 384-well format PCR array. The PCR mix was denatured at 95° C for 10 min before the first PCR cycle. The thermocycler parameters were 95° C for 10 min, followed by 40 cycles at 95° C for 15 s, and 60° C for 1 min.

PCR array data analysis

In order to be able to compare different PCR array results, the threshold and baseline values were set manually, according to the manufacturer's instructions, and the resulting threshold cycle value (CT) data were uploaded into the data analysis template on the manufacturer's website (http://www.sabiosciences.com/pcr/arrayanalysis.php). RNA expression of each gene was normalized using five housekeeping genes as controls. The relative expression of each gene, compared with the expression in the control group, was calculated on the website using the ΔΔCT method. A difference was considered significant at $p < 0.05$. In the expression studies, a gene was considered differentially regulated if the difference was >2-fold compared with the control, and markedly differentially regulated if the difference was >10-fold (DeRisi, Penland et al. 1996; DeRisi, Iyer et al. 1997; Linde, Sandhagen et al. 1999; Sudarsanam, Iyer et al. 2000; Wellmann, Thieblemont et al. 2000; Montgomery and Daum 2009) . Each reported value represents the mean increase or decrease of mRNA expression relative to the levels for the controls from three biological replicates.

Statistical analysis of Protein and Gene expression after LPS treatment

Inflammatory cytokine protein level data in this study are presented as the mean ± SD. Statistical analysis was done using Graphpad Prism 5.0. Differences among treatment groups were analyzed by a one-way ANOVA, followed by a Newman-Keuls *post hoc* test. The difference in the basal levels of cytokines/chemokines in the brain, spleen, and serum between F344 and HIV-1Tg rats was determined using the *Student's t*-test.

Animals used for CPP

Male HIV-1 transgenic (HIV -1Tg) rats and age-matched male Fisher /NHsd 344 (F344) control rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals were group housed 4-5 per cage in ventilated cages with a 12/12 hour day/night cycles (lights on at 7 AM) in a temperature controlled environment (21-22°C) with food and tap water available *ad libitum.* The animals were always tested in the light phase of the day/night cycle (9AM-2PM). Animals were acclimatized for approximately 2 months and handled for 3 consecutive days for 1-2 minutes each before beginning testing on them. The animals were approximately 3 months old (HIV-1Tg: 193-227g; F344: 228-277 g) when the experiment

was conducted on them. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

Drug

Morphine sulfate was obtained from (Sigma, St. Louis, MO). Morphine was administered intraperitoneally (i.p) as a 3.5 mg/kg dosage in the conditioning phase and as a 1mg/kg or a 3.5 mg/kg dosage in the reinstatement phase.

CPP apparatus

A commercially available CPP apparatus (MED Associates, East Fairfield, VT) was used. The apparatus consisted of an 18cm x 20cm white chamber with a grid floor and an18cm x 20cm black chamber with a rail floor connected by a side passage; this design ensures that the animal is always visible to the camera. This side passage in the middle of the two chambers had two doors on each chamber wall; the doors could be closed to isolate each chamber. The animal movement and time spent in each chamber was detected by an infrared camera (Stoelting ANY-maze, Wood Dale, IL). ANY-maze software (Stoelting ANY-maze, Wood Dale, IL) was

used to analyse the camera recordings to measure time spent in the each chambers.

Foot shock administration

A custom foot-shock administrating apparatus box 40 cm w x 40 cm d x 35 cm h, connected to an animal shocker, was purchased from Stoelting ANY-maze (Wood dale, IL). The apparatus was set to deliver foot shock to an animal in it intermittently, for 15 minutes, with an amplitude of 0.5 mA, and a 0.5 s duration, (off period 10-70 s, mean 40 s).

CPP, extinction and re-instatement procedure

There were five phases for this experiment. During the preconditioning phase (1 day), the animals were placed in the side passage with the door of the chambers open and allowed to explore the entire box for 15 minutes. In the conditioning phase (6 days), an approximately equal number of animals of each strain (HIV-1Tg and F344) were assigned to each type of chamber (white or black) as the morphine paired side. The animals were injected with 3.5 mg/kg morphine (dose based on pilot data) every other day and placed in the pre-assigned morphine-paired side and on the next day were injected with equivalent volume of saline and placed

in the saline-paired side, with the chamber door closed for 30 minutes. In the post-conditioning phase (1 day) the rats received no drug or saline and were placed in the side passage and allowed free access to the entire box for 15 minutes. For the first 8 days of the extinction procedure, the animals were put in the side passage and given free access to the box for 30 minutes. After the first 8 days of extinction (Extinction 1) testing there was no evidence of extinction so the extinction procedure was altered in the hope of accelerating extinction. Evidence suggests that handling and transportation cues contribute to contextual conditioning (Bevins, McPhee et al. 2000), thus during extinction day 9 through day 17 (Extinction 2) the extinction procedure was repeated twice daily for 15 minutes per session with an average interval of 2 hours and 17 minutes between the 2 sessions, to increase the number of exposures to the transportation cues without changing the duration of exposure to the context (30 minutes total). Dividing the extinction procedure into two separate sessions also permitted for the opportunity of spontaneous recovery (see Discussion). Moreover, the HIV-1Tg rats received 3 additional days of extinction (see Results). Following extinction the rats in each strain were distributed equally to either be assigned in the drug-induced reinstatement group or in the stress-induced reinstatement group. The rats in the drug-induced reinstatement group received 1mg/kg morphine on reinstatement day 1 and were given

free access to the entire box for 15 minutes; on re-instatement day 2 the rats were given 3.5 mg/kg morphine and were given free access to the entire box for 15 minutes. The rats in the stress-induced re-instatement group on day 2 of the re-instatement phase were administered foot-shock for 15 minutes and subsequently put in the CPP apparatus and given free access to the entire box for 15 minutes.

The movement of the rats in the CPP apparatus was tracked by the ANY-maze camera and software in all phases. The experiment was carried out daily.

Statistical Analysis

The means and standard deviations of the absolute time spent by animals (for each strain) in each chamber were calculated in all phases of the experiment. Inspection of the extinction data indicated that the chamber that was paired with morphine had a substantial effect on preference behaviour. To evaluate the effect of color/flooring of the chambers on the acquisition and extinction of CPP the data was analysed with color of the morphine-paired chamber as a factor. Careful inspection of the data also revealed systematic changes in preference within a test session suggesting that there was habituation of exploratory behaviour over time. To test this hypothesis the extinction sessions were separated into segments of five-

minutes. Analysis of variance (ANOVA) was used to study the interaction of strain (F344, HIV-1Tg), phase (pre-conditioning, post-conditioning, extinction 1 and2, and/or reinstatement), morphine-paired chamber, drug (morphine-paired, saline-paired). A value of $p \leq 0.05$ was considered statistically significant difference.

All analyses were done using the statistical software SPSS Statistics 17.0 IBM).

Results

Cytokine and chemokine protein expression in the serum, brain, and spleen of HIV-1Tg rats during endotoxin tolerance (ET)

The protein levels of IL-1β, KC/GRO, IL-4, IL-5, TNF-α, IFN-γ, and IL-13 were determined in the brain, spleen, and sera of HIV-1Tg and F344 rats rendered ET (LL), then challenged with either LPS (LL+L) or saline (LL+S), using an electrochemiluminescent (MSD) assay (Table 1-3). Saline-treated animals were used as controls (SS+S). There was no difference in the basal levels (SS+S group levels) of the cytokines/chemokines measured in the serum in the two strains of animals (Table 1). In the F344 serum, the levels of the pro-inflammatory cytokine, IFN-γ, and the anti-inflammatory cytokines, IL-4 and IL-13, in the single exposure group (SS+L) were significantly greater than in the control group (SS+S), and significantly less in the ET group (LL+L) than in the single exposure group (SS+L). In the HIV-1Tg serum, the levels of the pro-inflammatory cytokines, IL-1β, TNF-α, and IFN-γ, and the anti-inflammatory cytokines, IL-4 and IL-13, in the single exposure group (SS+L) were significantly higher than in the control group (SS+S), and significantly lower in the ET group (LL+L) than in the single exposure group (SS+L) [Table 1].

Table 1: Cytokine/Chemokine Profiles in F344 and HIV-1Tg Rat Serum

Cytokines and Chemokines in the Serum with LPS Treatment							
	Protein (pg/ml)(±SD)						
F344	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	IFN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - alpha	Interferon gamma	Interleukin 13
SS+S	83.0(±21.3)	1294.8(±589.7)	9.5(±0.6)	116.0(±23.7)	42.7(±6.1)	35.2(±7.2)	27.5(±1.1)
SS+L	272.1(±145.1)	7153.2(±2779.8)**	15.5(±1.7)***	348.7(±202.4)	7531.8(±7897.0)	138.6(±46.2)**	42.3(±9.5)**
LL+S	80.4(±42.4)	780.9(±846.6)	10.3(±51.2)	103.6(±32.6)	51.2(±12.4)	56.5(±19.6)	22.5(±1.1)
LL+L	125.1(±23.5)	3293.5(±680.6)*	11.1(±1.1)^^	210.0(±56.7)	88.2(±25.5)	61.8(±18.9)^^	26.5(±2.1)^
HIV-1Tg	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	IFN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - alpha	Interferon gamma	Interleukin 13
SS+S	85.2(±31.6)	1907.4(±978.9)	10.7(±2.0)	154.7(±83.6)	45.2(±11.4)	67.8(±32.2)	26.8(±0.5)
SS+L	307.4(±72.9)***	7002.2(±361.0)***	14.4(±2.5)	258.3(±37.3)	2366.7(±924.4)***	122.3(±13.4)*	36.2(±6.6)*
LL+S	47.9(±7.4)	327.6(±110.5)	8.0(±1.4)	29.7(±34.3)	42.8(±7.0)	18.3(±24.9)	22.3(±1.1)
LL+L	128.6(±3.0)^^^	3051.3(±1215.7)^^^	11.3(±0.8)^^	189.1(±121.1)	113.4(±51.8)^^^	71.1(±9.1)^	27.9(±4.2)^

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ greater than SS+S control; ^ $P<0.05$, ^^ $P<0.01$, ^^^ $P<0.001$ less than SS+L

F344 n=12, 3 in each group; HIV-1Tg n=12, 3 in each group

The basal levels of IL-4 and TNF-α in the F344 rat brain, and IL-4, IL-5, and TNF-α in the HIV1-Tg brain, were not detectable by electrochemiluminescent (MSD) assay. The basal levels of

cytokines/chemokines that were detectable in the brain were similar in both strains of animals (Table 2). An LPS challenge dose (SS+L and LL+L) did not significantly alter any of the cytokine/chemokine levels in the F344 rat brain (Table 2). However, in the HIV-1Tg rat brain, the levels of the pro-inflammatory cytokines, IL-1β and IFN-γ, were significantly higher in the SS+L group versus the control. The level of the pro-inflammatory cytokine, IFN-γ, was significantly lower in the LL+L group compared to the SS+L group (Table 2).

Table 2: Cytokine/Chemokine Profiles in the F344 and HIV-1Tg Rat Brain

Cytokines and Chemokines in the Brain with LPS Treatment							
	Protein (pg/ml)(±SD)						
F344	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	IFN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - alpha	Interferon gamma	Interleukin 13
SS+S	42.2(±8.8)	74.4(±23.3)	ND	56.5(±)	ND	15.3(±7.5)	4.4(±1.8)
SS+L	178.4(±162.1)	1325.3(±1431.3)	13.5()	102.8(±61.1)	30.9	20.5(±11.9)	4.8(±3.1)
LL+S	59.1(±26.3)	112.6(±35.6)	3.4	74.7	ND	14.7(±7.0)	6.9(±1.4)
LL+L	207.0(±133.0)	1036.2(±685.6)	2.9(±1.3)	141.9(±98.3)	ND	21.9(±6.5)	5.3(±2.3)
HIV-1Tg	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	IFN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - alpha	Interferon gamma	Interleukin 13
SS+S	37.3(±17.1)	125.3(±87.8)	ND	ND	ND	11.7(±5.2)	5.1(±0.4)
SS+L	361.7(±104.6)*	2991.3(±491.5)**	9.2(±3.1)	164.2(±35.3)	109.5	47.1(±14.5)*	9.3(±4.2)
LL+S	56.5(±15.1)	128.1(±61.6)	ND	ND	ND	8.9(±5.9)	4.6(±1.4)
LL+L	208.6(±167.4)	1312.9(±1039.2)^	8.1(±1.6)	181.2(±50.0)	27.3	24.3(±14.5)^	7.3(±3.7)

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ greater SS+S control; ^ $P<0.05$, ^^ $P<0.01$, ^^^ $P<0.001$ less than SS+L

F344 n=12, 3 in each group; HIV-1Tg n=12, 3 in each group

The basal level of TNF-α was not detectable in the spleen of either the F344 or HIV1-Tg rats (Table 3). There was no significant difference in the basal levels of any of the cytokines/chemokines that were detectable in the spleen of the F344 and HIV-1Tg rats, with the exception of IFN-γ, which

was significantly greater in the F344 rat spleen compared to that in the HIV-1Tg rat spleen (Table 3). In the F344 spleen, the levels of the pro-inflammatory cytokines, IL-1β and IFN-γ, and the anti-inflammatory cytokines, IL-4 and IL-13, in the single exposure group (SS+L) were significantly higher than in the control. In the F344 rats, the levels of the pro-inflammatory cytokines, IL-1β and IFN-γ, and the anti-inflammatory cytokine, IL-4, were significantly lower in the spleen of the LL+L group compared to the single exposure group (SS+L). In the HIV-1Tg spleen, the levels of the pro-inflammatory cytokines, IL-1β and IFN-γ, and the anti-inflammatory cytokine, IL-4, were significantly higher in the single exposure group (SS+L) and in the endotoxin tolerant group (LL+L) compared to the control. In the LL+L group of HIV-1Tg rats, the pro-inflammatory cytokines, IL-1β and IFN-γ, and the anti-inflammatory cytokine, IL-4, were significantly lower than in the single exposure (SS+L) group (Table 3).

Table 3: Cytokine/Chemokine Profiles in the F344 and HIV-1Tg Rat Spleen

Cytokines and Chemokines in the Spleen with LPS Treatment							
	Protein (pg/ml)(±SD)						
F344	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	FN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - alpha	Interferon gamma	Interleukin 13
SS+S	1802.9(±635.7)	264.7(±122.7)	6.9(±3.3)	119.5(±20.0)	ND	34.5(±13.6)	3.7(±2.3)
SS+L	19030.7(±11365.4)*	20065.6(±11858.1)	50.7(±16.0)**	344.4(±83.9)**	2943.6(±2752.3)	203.3(±71.5)**	22.1(±6.0)**
LL+S	3325.3(±440.6)	506.6(±205.0)	6.8(±5.7)	120.0(±28.8)	ND	32.5(±28.1)	3.8(±3.0)
LL+L	7898(±1996.9)^	11818.7(±9648.2)	21.6(±10.2)^^	246.6(±79.4)	228.7(±19.6)	105.3(±45.0)*^	14.2(±6.1)
HIV-1Tg	IL-1β	KC/GRO	IL-4	IL-5	TNF-α	FN-γ	IL-13
	Interleukin 1 beta		Interleukin 4	Interleukin 5	Tumor necrosis factor - α	Interferon gamma	Interleukin 13
SS+S	1274.5(±122.3)	361.1(±187.7)	3.8(±1.4)	107.7(±33.0)	ND	10.8(±4.8)	0.8(±0.7)
SS+L	19951.8(±4023.8)**	25812.5(±3014.5)**	41.8(±5.7)**	395.4(±93.3)**	2755.4(±1596.0)	296.9(±83.4)**	32.0(±4.9)*
LL+S	5087.1(±1675.4)	961.8(±89.1)	7.3(±2.0)	178.4(±57.2)	ND	19.8(±2.6)	6.6(±6.4)
LL+L	11443.1(±7400)*^	12754.4(±11613.3)^	27.3(±12.6)*^	239.4(±77.1)^	356.7(±149.4)	151.5(±77.6)*^	19.8(±11.4)

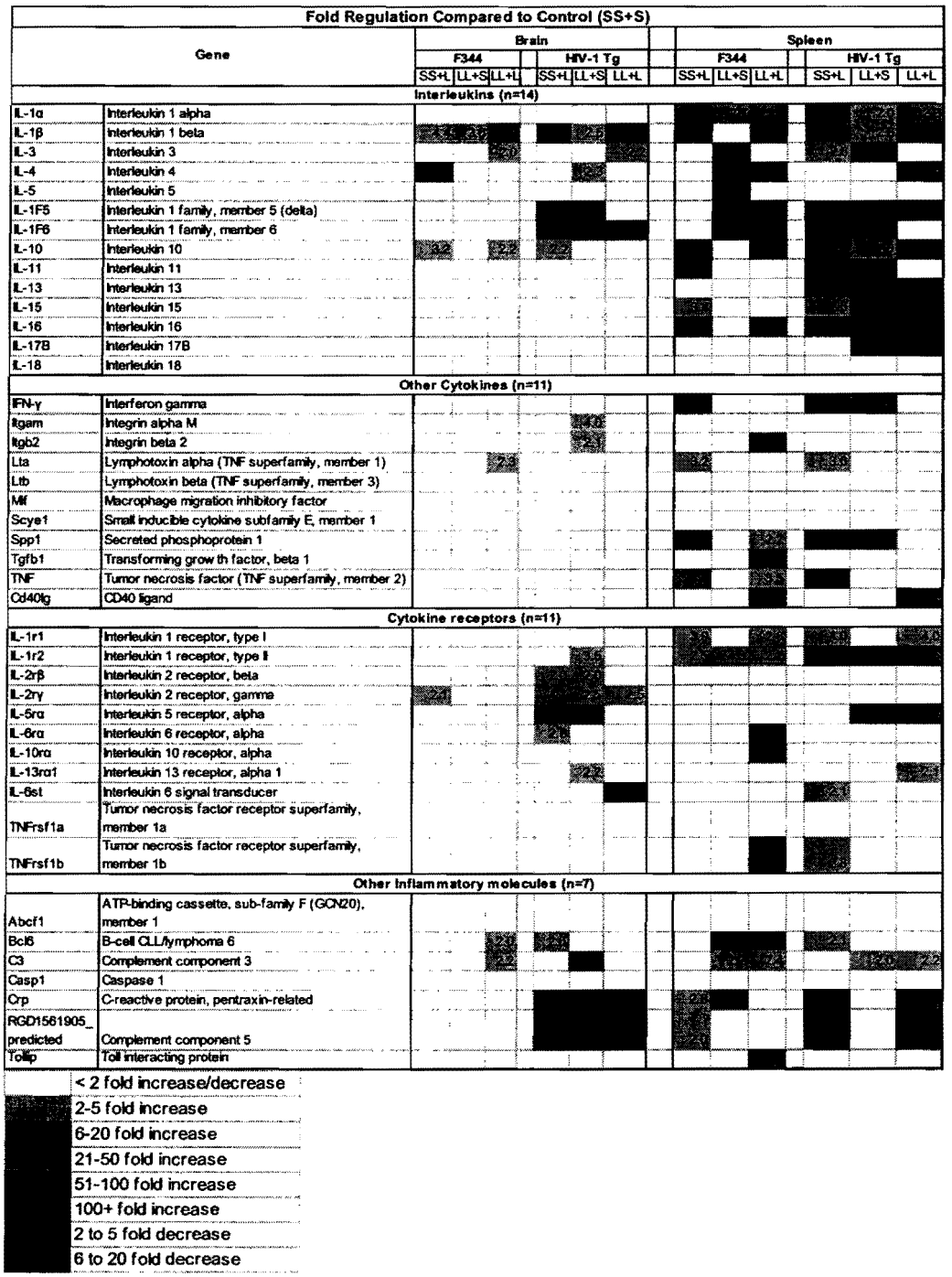
* $P<0.05$, ** $P<0.01$, *** $P<0.001$ greater than SS+S control; ^ $P<0.05$, ^^ $P<0.01$, ^^^ $P<0.001$ less than SS+L

F344 n=12, 3 in each group; HIV-1Tg n=12, 3 in each group

Expression profiles of cytokines, cytokine-receptors, and other inflammatory molecules in the brain and spleen of the HIV-1Tg rat following LPS treatment

The gene expression profiles of an array of cytokines, cytokine receptors, and other inflammatory molecules were determined in the brain and spleen of the three LPS treatment groups (SS+L, LL+S, and LL+L) and compared to the control group (SS+S) of both the HIV-1Tg and F344 rats (Figure-1). In the F344 and HIV-1Tg rat brain, the level of the pro-inflammatory cytokine, IL-1β, was elevated in the LL+L (>9 fold) and SS+L (>4 fold) groups. The level of the anti-inflammatory cytokine, IL-10, was elevated in the brain of both the SS+L (>3 fold) and LL+L (>2 fold) groups in the F344 rats, but only in the brain of the SS+L (>2 fold) group in the HIV-1Tg rat brain (Figure-1). In the spleen of the F344 and HIV-1Tg rats, the levels of the pro-inflammatory cytokines, IL-1α, IL-1β, were elevated in both the SS+L (>10 fold) and LL+L (>5 fold) groups. The levels of the pro-inflammatory cytokines, IFN-γ and TNF-α, were increased in the SS+L group (>6 fold), and the anti-inflammatory cytokine, IL-10, was higher in the SS+L (> 5 fold) and LL+L (>7 fold) groups (Table-4).

Table 4: Cytokine Expression in the Brain and Spleen of F344 and HIV-1Tg Rats



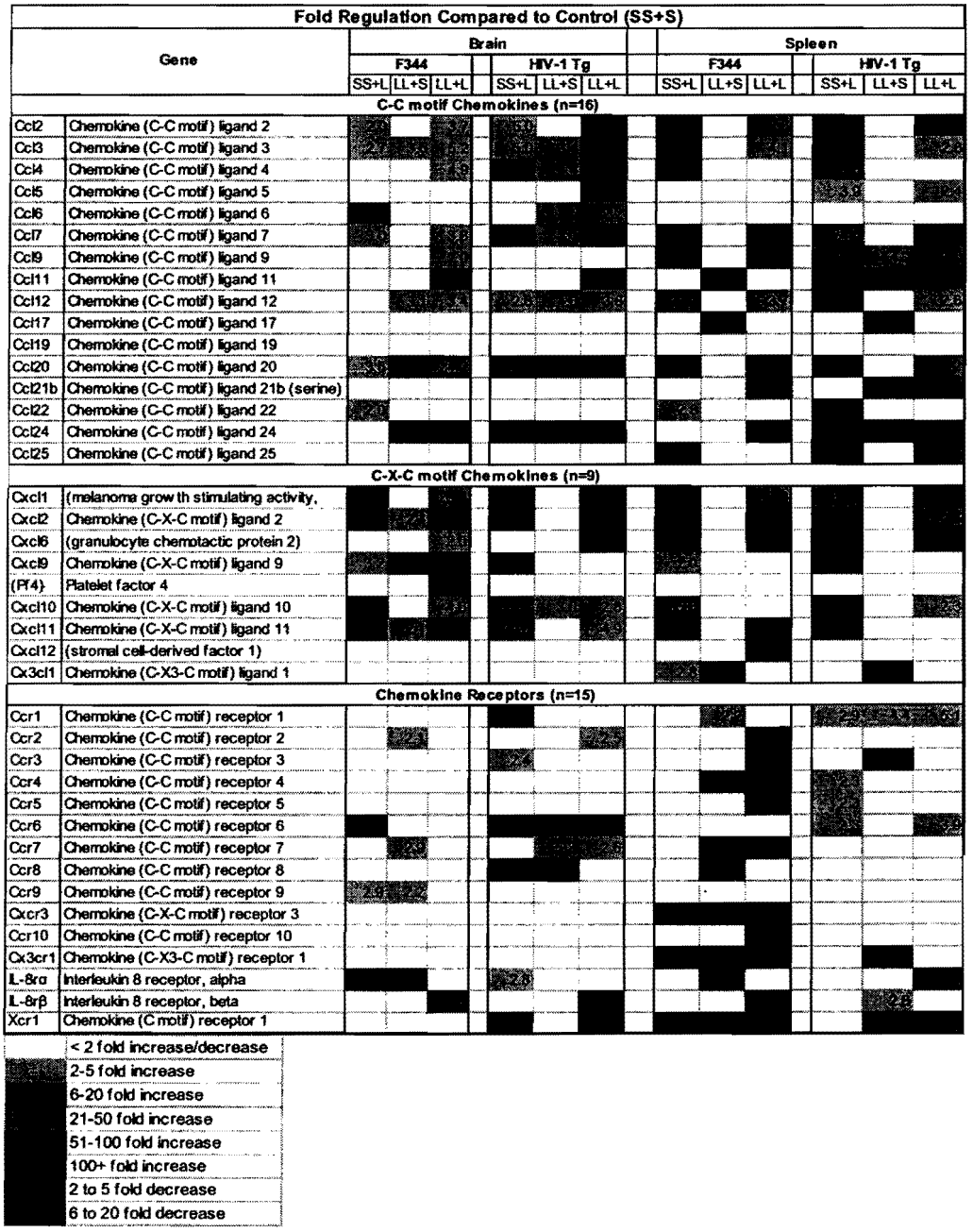
F344 n=12, 3 in each group; HIV-1Tg n=12, 3 in each group

Expression profiles of chemokine and chemokine receptors in the brain and spleen of the HIV-1Tg rat following LPS treatment

The gene expression profiles of various chemokines and chemokine receptors were examined in the three LPS treatment groups and compared to the control group (SS+S) in both the HIV-1Tg and F344 rats (Figure-2). The gene expression of the inflammatory cc chemokines, Ccl2, Ccl3, Ccl7, and Ccl20, and inflammatory Cxc chemokines, Cxcl1, Cxcl2, and Cxcl10, were up-regulated in the brain and spleen in the SS+L and LL+L groups of both the F344 and HIV-1Tg rats. The gene expression of the inflammatory cc chemokines, Ccl11 and Ccl24, were down-regulated in the brain of the LL+L group of both the F344 and HIV-1Tg rats, and in the SS+L and LL+L groups in the spleen of the HIV-1Tg rats.

Ten of the 15 (10/15) chemokine receptors studied were down-regulated in the LL+L group of the spleen of the F344 rats. One of 15 (1/15) and three of 15 (3/15) chemokine receptors were down-regulated in the LL+L group of the brain of the F344 and HIV-1Tg rats, respectively; ten of 15 (10/15) and two of 15 (2/15) chemokine receptors were diminished in the LL+L group of the spleen of the F344 and HIV-1Tg rats (Figure-2).

Table 5: Chemokine Expression in the Brain and Spleen of F344 and HIV-1Tg Rats

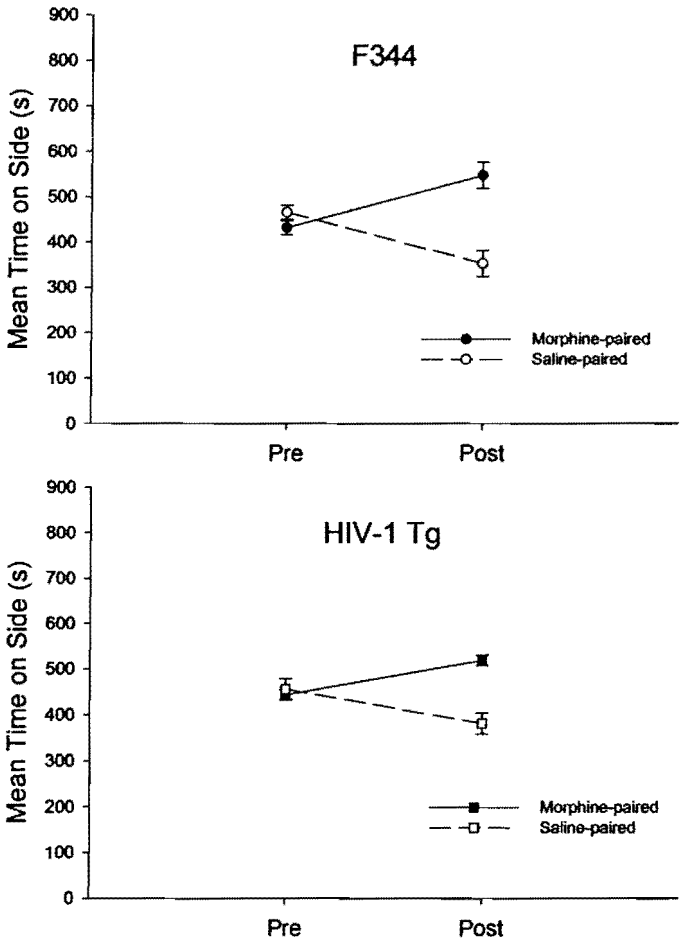


F344 n=12, 3 in each group; HIV-1Tg n=12, 3 in each group

Morphine-induced place preference

Fourteen HIV-1Tg and 14 F344 rats were placed in the CPP apparatus and given free access for 15 minutes (pre-conditioning) to determine if either strain showed a preference for one of the chambers. As shown in Figure 1 neither the HIV-1Tg rats nor the F344 rats showed a preference for either the black or white chambers in the pre-conditioning phase. After 6 sessions of conditioning with 3.5 mg/kg morphine, both HIV-1Tg and F344 rats showed a CPP (Figure 1). A Strain (F344, HIV-1 Tg) x Phase (Pre, Post) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA yielded a significant Phase x Side interaction $F(1,26) = 31.03$, $p = .000$, such that more time was spent in the morphine-paired side than in the saline-paired side post-conditioning but not pre-conditioning.

Figure 1: Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain in pre-conditioning and post-conditioning phase is shown. On post-conditioning day animals (F344 n=14; HIV-1Tg n=14) were conditioned daily for 15 minutes following a 3.5mg/kg morphine (i.p.) dose every other day for 6 days. The following day after 6 conditioning sessions, animals were given free access to entire apparatus and tested for CPP.

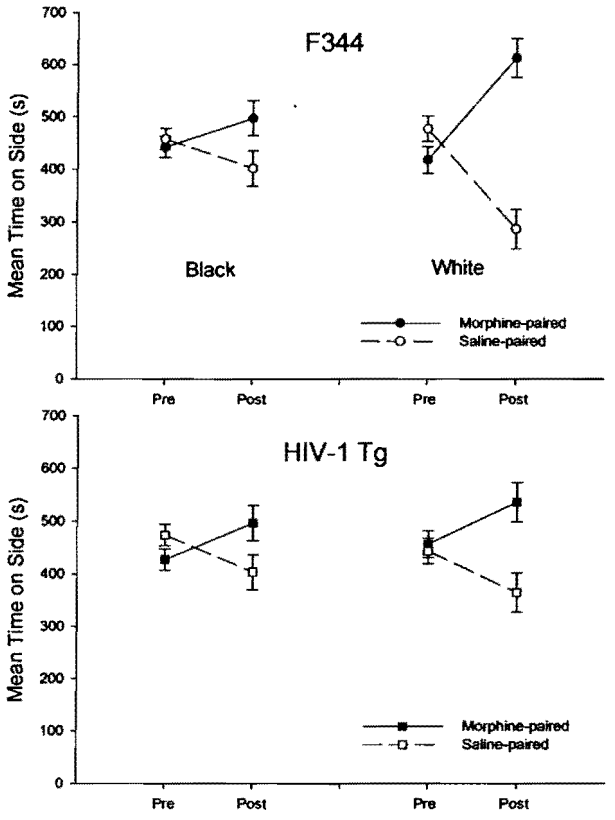


To counterbalance for the morphine/side pairings, the strains were divided so that approximately half received morphine paired with the black side and

the remainder received morphine paired with the white side. In addition to the color difference, the two sides also had different floors (grid and rail). Since the rats were tested under dim red-light illumination the floor was a much more salient cue than the color, eliminating a natural bias for the black chambers (Roma and Riley 2005). Nevertheless, for convenience the sides are referred to as black or white. The absence of a side preference during the 15 min pre-conditioning session suggested that the CPP apparatus used in this study was an unbiased apparatus. However after introducing the extinction procedure it was observed that there was a very high resistance to extinction in many animals. Inspection of the data indicated a stronger preference during extinction in the white-morphine paired animals than the black-morphine paired animals. Thus, to confirm that a CPP occurred in the white-paired and black-paired animals the data was analyzed with the counterbalancing factor (color of morphine-paired chamber) included as an independent variable (Figure 2). A Strain (F344, HIV-1Tg) x Phase (Pre, Post) x Morphine Color (Black, White) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA yielded a significant four way interaction, $F(1,24) = 4.59$, $p = .043$, that was due to a greater CPP when morphine was paired with the white side in the F344 animals [Phase x Drug x Morphine Color: $F(1,12) = 7.54$, $p = .018$] but not the HIV-1Tg rats. In the HIV-1Tg rats the significant CPP (Phase x Drug: $F(1,12) = 20.83$, $p =$

.001) was not affected by the color of the morphine-paired chamber [Phase x Drug x Morphine Color: $F(1,12) < 1$, $p = .784$] (Figure 2).

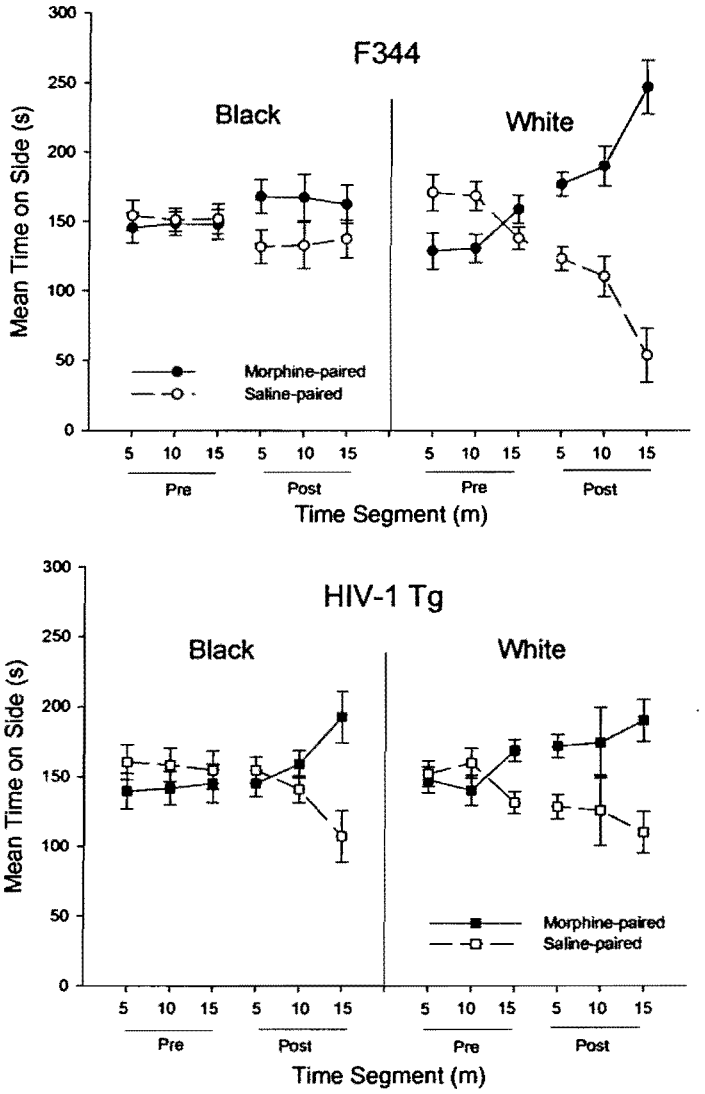
Figure 2: Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain and by color of morphine-paired chamber, in pre-conditioning and post-conditioning phase is shown. (F344 n=14; HIV-1Tg n=14).



To investigate further the effect of time on the development of CPP we analysed the data in segments of 5-minutes. Figure 3 shows the pre-conditioning and post-conditioning results separated into three 5-minute

time segments. The rats were again divided in terms of the side that was paired with the drug (color of morphine-paired chamber). When the pre-conditioning data were analysed, no statistically significant effects were observed. However, the post-conditioning data shows clear changes in preference over the three, 5-minute periods. A Strain (F344, HIV-1Tg) x Phase (Pre, Post) x Morphine Color (Black, White) x Drug (Morphine-Paired, Saline-Paired) mixed ANOVA confirmed a 4- way interaction $F(2,48) = 4.27$, $p = .02$. Generally, the rats engaged in considerable exploratory behaviour often crossing between chambers, but as time passed rats explored less and spent more time on the morphine-paired side. This can be seen in the black-paired and white-paired HIV-1Tg groups (bottom graph). Although this pattern of increasing preference over time was not seen in the black-paired F344 group it was very pronounced in the white-paired F344 rats (top graph). When the animals were first placed in the CPP apparatus they explored and might have spent more time exploring in the preferred sides, but as the exploratory behaviour habituates the time spent in the preferred side increases even more. The data suggests that the F344 rats that experienced morphine paired with the white side had the strongest preference for the morphine-paired side (Figure 3).

Figure 3: Time spent in the morphine-paired and saline-paired side of the CPP apparatus by strain and by color of morphine-paired chamber separated into three 5-minute time segments, in pre-conditioning and post-conditioning phase is shown. (F344 n=14; HIV-1Tg n=14)



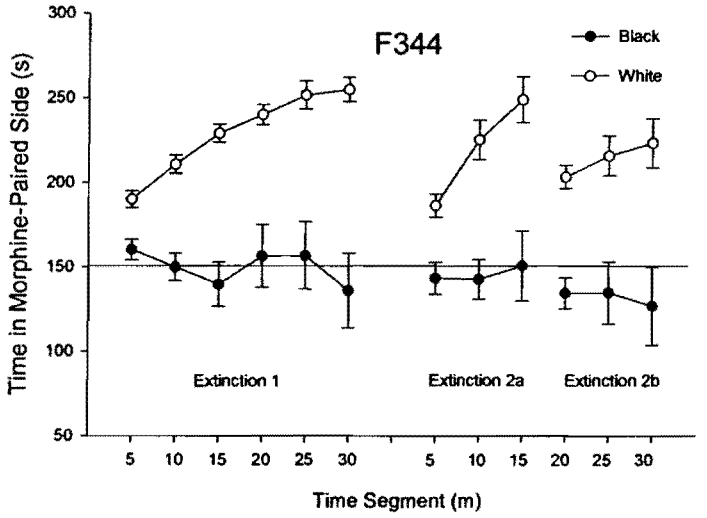
Extinction

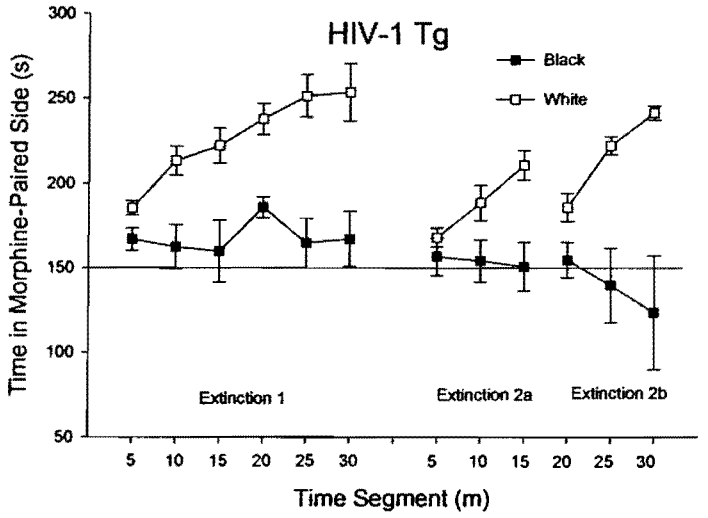
To test for extinction (no difference in time spent in the morphine-paired side and saline-paired side) animals were given free access to the both chambers for 30 minutes per day for 8 days (Extinction 1) and for 2 sessions of 15 minutes per day thereafter (Extinction 2a and 2b).

The time spent in the morphine-paired side in 5-minute time segments for two phases of extinction is shown in Figure 4. A score of 150 indicates equal time spent in the black and white chambers per time segment (no preference); a score greater than 150 indicates a preference for the morphine-paired side. The average across all days for each extinction phase is plotted in order to show the high resistance to extinction in the rats that experienced morphine paired with the white chamber and to demonstrate the observed within-session changes in preference. We hypothesize that the within-session increase in preference is due to habituation of exploratory behaviour in the presence of a highly preferred white chamber. Evidence consistent with this habituation interpretation is provided in Extinction 2 where spontaneous recovery of exploratory behaviour was observed as a result of the delay between Extinction 2a and 2b (Figure 4). These results suggest that the rats had a natural preference

for the white side that was not evident in the preconditioning phase, but when paired with morphine it became a much stronger preference that was highly resistant to extinction.

Figure 4: Extinction phase: To test for extinction, animals (F344 (n=14), HIV-1Tg (n=14)) were given free access to the entire apparatus for 30 mins (F344: 8 days, HIV-1Tg: 8 days) everyday (Extinction 1) and then for 2 fifteen min sessions/day (F344:9 days, HIV-1Tg: 13 days) (Extinction 2a and 2b) until all animals reach extinction. Average time spent in the morphine-paired side by strain and by color of morphine-paired chamber separated into three 5-minute time segments (group means) in the 2 phases of extinction (Extinction 1 and Extinction 2 (a and b)) is shown.



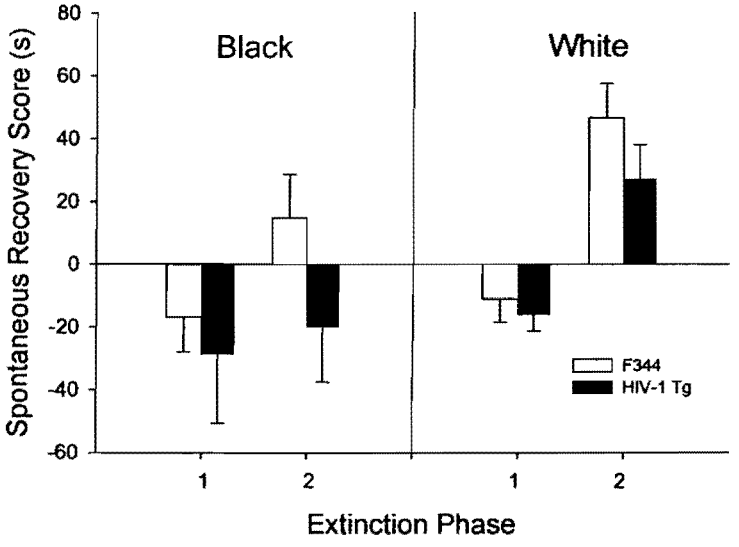


If habituation of exploratory behaviour contributes to the increasing preference over time in the CPP test, as hypothesized here, then the preference at the beginning of Extinction 2b (minutes 16-20) should be less than the preference observed at the end of Extinction 2a (minutes 10-15) because the delay causes the spontaneous recovery of exploratory

behaviour. This is the pattern seen in the Extinction 2 data of the white-morphine paired groups that are plotted in Figure 4. Note that this drop in preference between minutes 10-15 and minutes 16-20 is not seen in Extinction 1 since there was no delay imposed between these time segments to allow for spontaneous recovery of exploratory behaviour. To confirm these observations a spontaneous recovery score was calculated for all rats by subtracting the time spent on the morphine-paired side during the fourth time segment (minutes 16-20) from the time spent on the morphine-paired side during third time segment (minutes 11-15). A positive score indicates spontaneous recovery, whereas a negative score indicates continued habituation. To facilitate statistical analysis of Extinction 1 (which had 8 days) and Extinction 2 (which had 9 days), the last day of extinction 2 was omitted. The data was then analyzed with a Strain (2) x Morphine Color (2) x Extinction Phase (2) by Days (8) mixed ANOVA. The white-morphine paired and black-morphine paired HIV-1Tg and F344 groups averaged across days for the two extinctions sessions are shown in Figure 5. Spontaneous recovery was observed in the white-morphine paired groups (right panel) in Extinction 2, when a delay was present, but not in Extinction 1 when there was no delay. These observations were supported by a significant main effect of Extinction Phase, $F(1,24) = 17.3$, $p = .000$. A significant main effect of color of morphine-paired chamber, $F(1,24) = 6,14$,

$p = .021$, supports the impression that spontaneous recovery was greater in the white-morphine paired groups than the black-morphine paired groups. There was no significant effect of days. The results also suggest that spontaneous recovery was greater in the F344 rats than the HIV-1Tg rats, but the main effect of strain fell short of significance, $F(1, 24) = 3.29$, $p = .08$ and strain did not interact with any other factor.

Figure 5: Spontaneous Recovery Score- The time spent on the morphine-paired side during the 4th time segment (minutes 16-20) was subtracted from the time spent on the morphine-paired side during third time segment (minutes 11-15). A positive score indicates spontaneous recovery, whereas a negative score indicates continued habituation. The results for the white-morphine paired and black-morphine paired HIV-1 and F344 groups averaged across days for the two extinctions sessions are shown below. To facilitate statistical analysis of Extinction 1 (which had 8 days) and Extinction 2 (which had 9 days), the last day of extinction 2 was omitted. (F344 n=14; HIV-1Tg n=14)

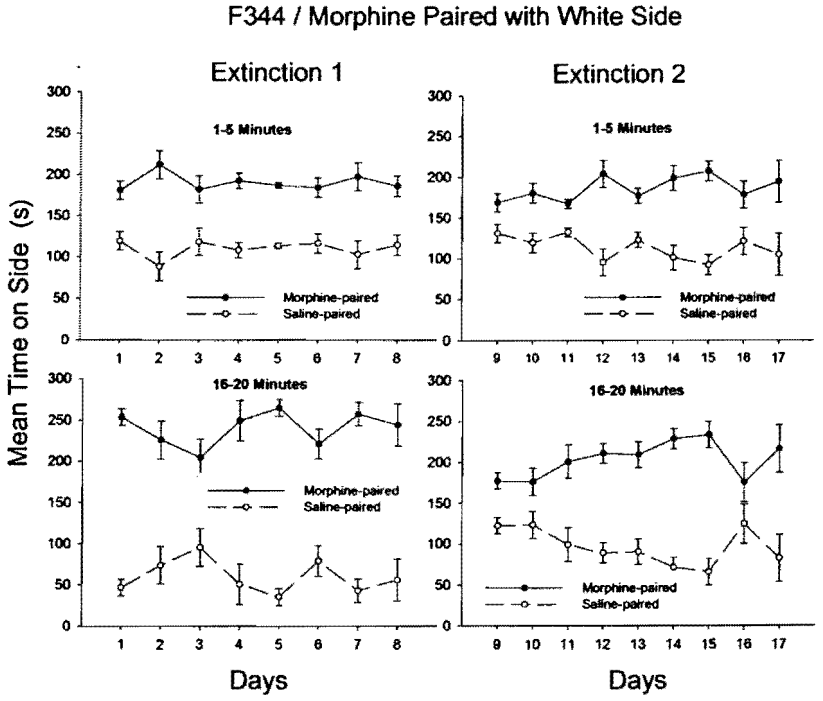


These pattern of data suggest that during a CPP test there are several factors that influence overall behaviour: a conditioned place preference, a natural preference (when present), and the elicitation of exploratory

behaviour that interacts with the conditioned and natural preferences. The impact of the two preferences on performance differs across a session as exploratory behaviour undergoes habituation, with the stronger preference dominating control of behaviour later in a session as the rats begin to "settle into" one chamber. Whereas a strong natural preference for one side may obscure a more modest conditioned place preference later in a session, the conditioned place preference may have a greater contributing role in overall performance earlier in a session when exploratory behaviour is at its maximum. We suggest that the strong preference for the white chamber in the rats that received the white side paired with morphine is a result of an enhanced natural preference for the white side rather than a result of the summation of a conditioned place preference with a natural preference. If the latter was true, then preference in the first 5 minutes of an extinction session (when a modest CPP is having its greatest impact on performance) should decline over days as the CPP undergoes extinction, whereas the preference later in a session (which reflects the stronger natural preference) should persist over days. However, if morphine induced the enhancement of a natural preference that is resistant to extinction, then the observed preference should be seen at all time intervals across all days with no evidence of extinction. The time in the morphine- and saline-paired sides of the F344 animals that received morphine paired with the white side is

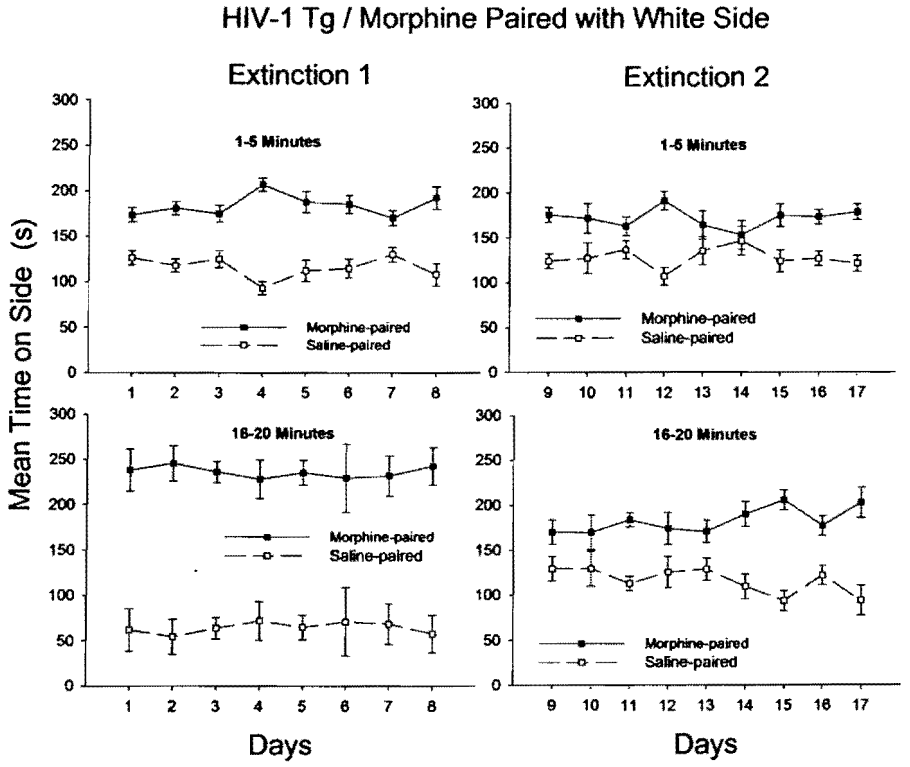
shown over days for two of the six 5-minute time segments in Figure 6. A preference for the morphine-paired side in the first 5 minutes (top panels) is observed that does not change over days in Extinction 1, [Effect of Drug: $F(1,5) = 63.62$, $p = .000$; Drug x Days: $F(7, 35) < 1$, n.s.], or in Extinction 2 [Effect of Drug: $F(1,5) = 28.27$, $p = .003$; Drug x Days: $F(8, 40) = 1.34$, $p = .251$]. Thus, there is no evidence of extinction over days, even when just the first 5 minutes, when a CPP is likely to have its greatest impact, are examined. By the fourth time segment (minutes 16-20) of Extinction 1 the preference for the morphine-paired time is even greater (bottom, left panel) as exploratory behaviour begins to habituate. However, during Extinction 2, when the 16 – 20 minute time segment was preceded by a break, the preference is again reduced as exploratory behaviour spontaneously recovers. Yet there is still no indication of a decreased preference for the morphine-paired side over days, [Drug x Days: $F(8, 40) = 1.73$, $p = .121$].

Figure 6: The time spent in the morphine- and saline-paired sides over days of the F344 animals that received morphine paired with the white side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown The top graphs show the first time segment (minutes 1 – 5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behavior. (F344 rats with morphine paired with white side n=7).



The same pattern is also seen in the HIV-1Tg rats (Figure 7).

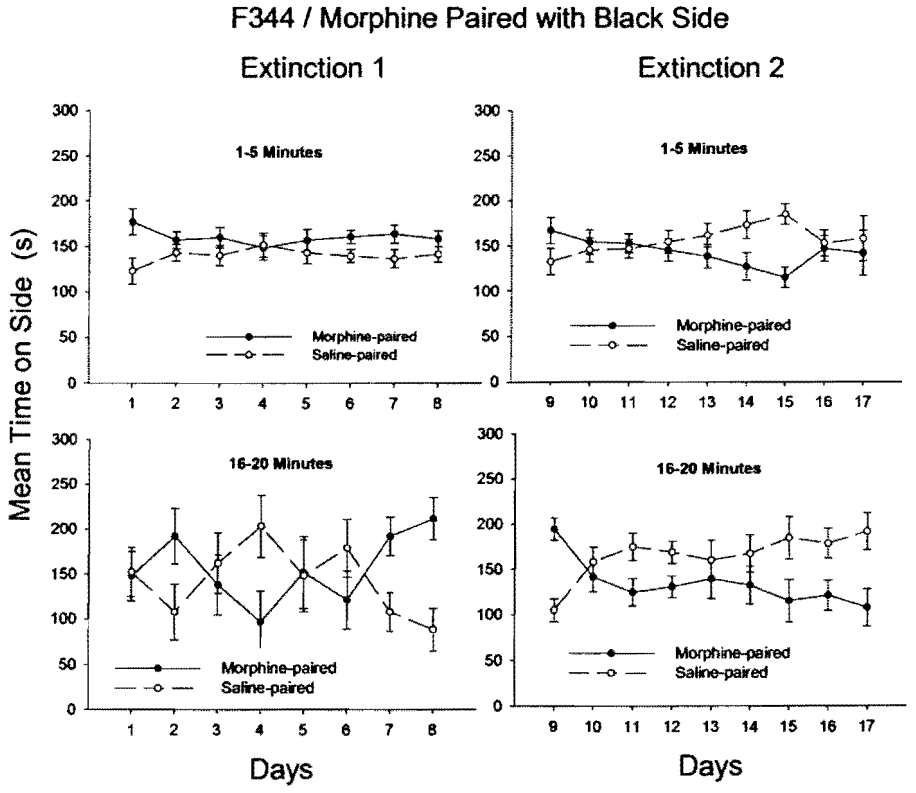
Figure 7: The time spent in the morphine- and saline-paired sides over days of the HIV-1Tg animals that received morphine paired with the white side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1 – 5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behavior. (HIV-1Tg rats with morphine paired with white side n=7).



The pattern of results for the rats that received morphine paired with the black side was very different. Figures 8 and 9 show the data for the F344

and the HIV-1Tg rats respectively. These were plotted in the same manner as the previous graphs. The Extinction 1 and 2 data were analysed separately with a Strain (2) x Drug (2) x Days (8 or 9) mixed ANOVA. During the first 5 minutes of Extinction 1 there was a significant preference for the morphine-paired side that persisted across days of Extinction 1 in both strains [Main Effect of Drug: $F(1,12) = 6.32$, $p = .027$; Drug x Days: $F(7,84) < 1$, n.s.]. There were no significant effects of strain. By Extinction 2 the main effect of Drug was no longer significant [$F(1,12) < 1$, n.s.] indicating the extinction of a CPP. Once again there were no significant effects of strain.

Figure 8: The time spent in the morphine- and saline-paired sides over days of the F344 animals that received morphine paired with the black side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1 – 5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behavior. (F344 rats with morphine-paired with black side n=7)



During the fourth time segment (minutes 16-20) a reversal in preference from the black morphine-paired side to the white saline-paired side took place during Extinction 2 in the F344 rats (Figure 8) [$F(7, 49) = 2.41$, $p =$

.034]. The absence of a significant preference during the 16-20 minute time segment of Extinction 1 may reflect the persistence of a conditioned preference for the black chamber that competed with the natural preference for the white chamber. By extinction 2 the extinguished CPP no longer competed with the natural preference, reversing the side preference. This reversal during minutes 16 – 20 in Extinction 2 was not observed in the HIV-1Tg rats [$F(7,35) < 1$, n.s.] (Figure 9), however, suggesting that CPP was not fully extinguished in the HIV-1Tg rats. Thus, the HIV-1Tg rats were tested for 3 additional days for extinction.

Figure 9: The time spent in the morphine- and saline-paired sides over days of the HIV-1Tg animals that received morphine paired with the black side. The Extinction 1 graphs are shown on the left side and Extinction 2 are shown on the right. Data from only 2 of the 6 time segments are shown. The top graphs show the first time segment (minutes 1 – 5) and the lower graphs show the fourth time segment (minutes 16-20). During Extinction 2 the 16-20 minute time segment was preceded by a break from the previous session to allow for spontaneous recovery of exploratory behavior. (HIV-1Tg rats with morphine paired with black side n=7).

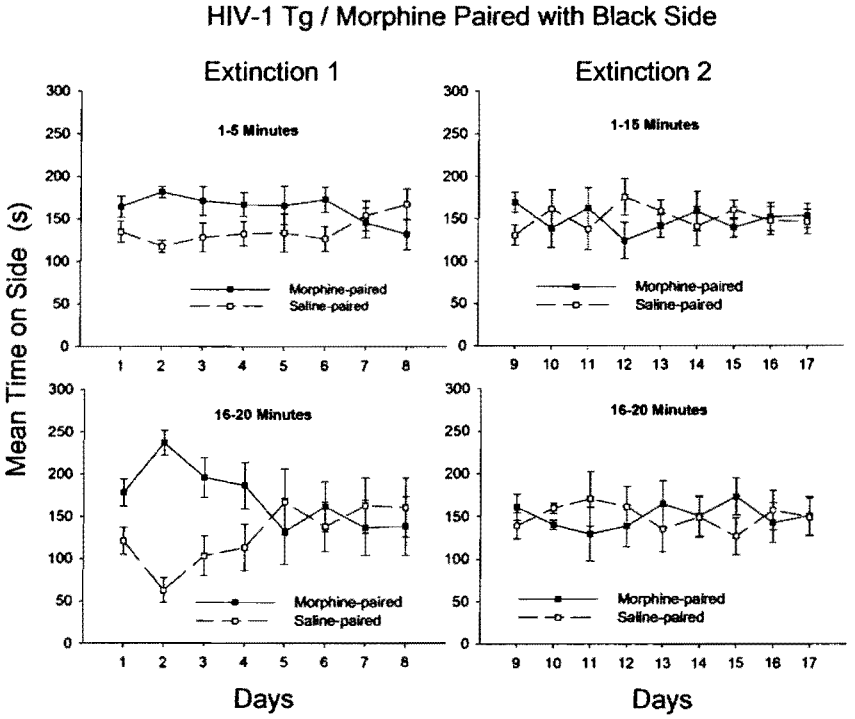
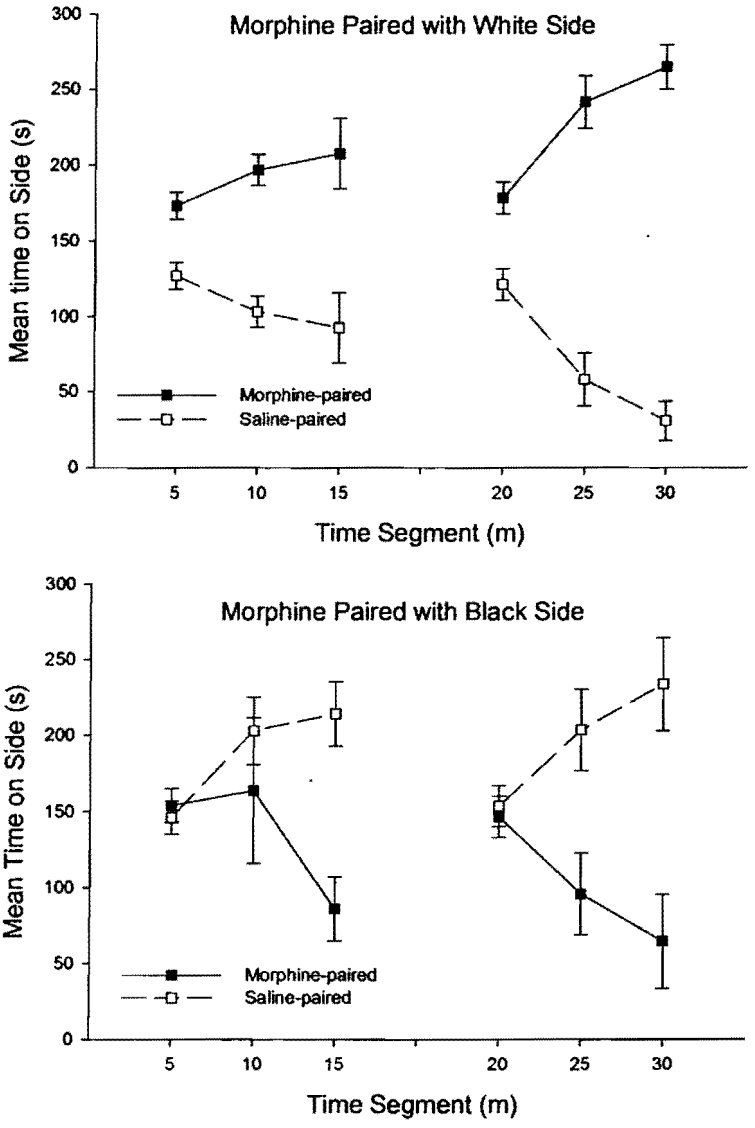


Figure 10 shows the results of the last three extinction days for the HIV-1Tg rats. There was no effect of days; therefore the means across all three days were presented. A significant four-way interaction was found $F(4,48) = 3.39$, $p = .016$. The rats for whom morphine was paired with the

white side (top graph) continued to show a significant preference for the morphine-paired side at all time segments and the preference increased across the first session as well as across the second session after spontaneous recovery of exploratory behaviour. The rats for whom morphine was paired with the black side (lower graph) did not show a preference during the first time segments of both extinction sessions (time segments 1-5 and 16-20) but showed a reverse preference in the subsequent sessions. Thus, although a reverse preference was not observed in the first five minutes of extinction like in the F344 rats, the strong preference for the saline-paired side later in the sessions is consistent with the suggestion that the white chamber is a highly preferred chamber that was not evident in preconditioning test.

Figure 10 – Time in Morphine-paired chamber over time segments for HIV-1Tg rats given three additional days of extinction during the second extinction phase (Mean of Days 18-20). (HIV-1Tg rats with morphine paired with white side n=7; (HIV-1Tg rats with morphine paired with black side n=7).).



Reinstatement with re-introduction of morphine

F344 (n=7) and HIV-1Tg (n=7) after reaching the extinction state were given 1mg/kg morphine and tested for CPP by giving them free access to the entire apparatus for 15 minutes. On the next day the same animals were given a higher dose of morphine (3.5 mg/kg, i.p.) and then free access to the entire apparatus for 15 minutes. The HIV-1Tg and F344 animals that had morphine paired with the black side showed a preference for the saline-paired side during the reinstatement test. This supports the argument that the rats had a natural preference for the white side that was counteracted by the CPP, but when the CPP is extinguished the animals chose to spend more time on the white side during all three time segments despite the reinstatement attempt. The data from the HIV-1Tg and F344 rats that had the morphine paired with the white side simply confirms that they had a preference for the white side. It cannot be assumed that this preference is a reinstatement effect because they never extinguished this preference during the extinction phase.

Reinstatement with a foot shock stressor.

After reaching extinction the F344 (n=7) and HIV-1Tg (n=7) were administered a foot-shock for 15 min. intermittently and the tested for CPP by giving them free access to the entire apparatus for 15 min. The F344 and HIV-1Tg rats that had the morphine paired with the white side showed a preference for the white chamber that increases across the time segments (Tim Seg x Drug: F344, $F(2,4) = 29.245$, = .004; HIV-1Tg, $F(2, 6) = 7.773$, $p = .022$). These groups, of course, cannot be used to assess reinstatement because their preference for white never extinguished, but it does confirm that the white preference in the white-morphine paired groups observed throughout extinction is still present. However, no statistically significant effects were observed in the F344 or HIV-1Tg rats that had morphine paired with the black side.

Discussion

Endotoxin tolerance (ET) is a phenomenon in which previous exposure of cells or organisms to microbial products, such as the endotoxin, LPS, induces a transient period of hypo-responsiveness to a subsequent endotoxin challenge. Exposure to an endotoxin initiates the production of pro-inflammatory cytokines and a subsequent production of anti-inflammatory cytokines by immune cells (Foster, Hargreaves et al. 2007; Mages, Dietrich et al. 2007; del Fresno, Garcia-Rio et al. 2009; Draisma, Pickkers et al. 2009). ET is characterized by diminished release of pro-inflammatory cytokines, such as IL-1β, IL-1α, and TNF-α, and increased expression of anti-inflammatory cytokines, such as IL-10 (Biswas, Bist et al. 2007) . This negative feedback mechanism is important for protecting the host from tissue damage and death caused by excessive inflammation. The differential expression of cytokines/chemokines in different tissues and at different times has been examined in Sprague Dawley rats to investigate the modulation of immune responses (Chen, Zhou et al. 2005).

The occurrence of ET has been shown to increase the incidence of several diseases, including sepsis, pancreatitis, trauma and surgery (Biswas and Lopez-Collazo 2009). The CDC has identified the increase of HIV-1 positive population as a major factor in the increased incidence of sepsis(Angus, Linde-Zwirble et al. 2001). Studies have shown that the

presence of HIV viral proteins causes a compromised immune response in HIV-1 patients (Capobianchi 1996; Schols and De Clercq 1996; Wilson, Habeshaw et al. 1997; Herbeuval and Shearer 2006; Boasso, Shearer et al. 2009). The phenomenon of ET during HIV-1 infection has not been studied through animal models. Reid and colleagues (Reid, Sadowska et al. 2001) established a non-infectious HIV-1 transgenic (HIV-1Tg) rat model that expresses an HIV-1 provirus regulated by the viral promoter, but with a functional deletion of *gag* and *pol*. The characteristics of the HIV-1Tg rat include immunologic dysfunction, nephropathy, muscle wasting, skin lesions, and cataracts. We studied the systemic effect of the concurrent presence HIV-1 viral proteins and ET on the inflammatory response to bacterial endotoxin using the HIV-1Tg rat model. We examined the LPS-induced gene expression of 84 cytokines, chemokines, and their receptors in the blood, brain, and spleen of the endotoxin tolerant HIV-1Tg rat. Based on previous studies, we used two injections of a low dose of LPS (250 μg/kg) administered 9 h apart to induce ET, and a challenge injection with a high dose of LPS (5 mg/kg) 12 h later. A single exposure to a high dose of endotoxin should cause a significant increase in the levels of pro-inflammatory and anti-inflammatory cytokines compared to control (Chen, Zhou et al. 2005). In the ET state, when animals are exposed to repeated low doses of endotoxin, upon a subsequent challenge with a high dose of

the endotoxin, one would expect that the increase in the pro-inflammatory cytokine levels would be lower compared to that in the single high dose exposure group (Biswas, Bist et al. 2007). To confirm that the animals were in the ET state, we measured the protein levels of inflammatory cytokines after the LPS challenge dose. Although the basal levels in the brain, spleen, and serum of the control groups (SS+S) of F344 and HIV-1Tg rats were similar, the changes in the cytokine and chemokine profiles in response to LPS were different in the HIV-1Tg and F344 rats. We found that, during ET, there was a significantly diminished expression of pro-inflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α, in response to LPS (LL+L) compared to a single exposure of LPS (SS+L).

Of particular interest was the finding that the basal level of IFN-γ in the spleen was lower in the SS+S group of HIV-1Tg rats than of the F344 rats, but was significantly increased in the SS+L and LL+L groups of the HIV-1Tg rats compared to the F344 rats (Table 3). A recent study showed that IFN-γ countered ET by facilitating Toll-like receptor (TLR)-induced chromatin remodeling (Chen and Ivashkiv 2010). ET was prevented in IFN-γ pre-treated primary human monocytes, and production of pro-inflammatory cytokines, such as TNF-α and IL-6, was restored by facilitating TLR-induced chromatin remodeling (Chen and Ivashkiv 2010). It would be interesting to examine what role IFN-γ plays in the restoration of the production of pro-

inflammatory cytokines at the transcriptional level in the HIV-1Tg rat compared to control.

We found that, in both the ET state and after a single exposure to LPS, there was an altered response to LPS in terms of pro-inflammatory cytokine production in the spleen of the HIV-1Tg rats compared to the F344 rats. The expression level of the pro-inflammatory cytokines, IL-1α, IL-1β, and IFN-γ, was 4- to 82-fold greater in the spleen of the HIV-1Tg rats compared to the F344 rats in both the single LPS exposure (SS+L) and ET (LL+L) groups, indicating that the presence of viral proteins may have an effect on innate immune responses. There was also a difference in the expression of cytokine receptors in the brain of the HIV-1Tg rats compared to the F344 rats in response to LPS in both the ET state and after a single exposure to LPS, suggesting that HIV-1 viral proteins may interact with or work through cytokine receptors in the brain. These data warrant further investigation into the neuroimmune effects of HIV-1 viral proteins.

Studies show that there is an alteration of cytokine receptors in the brain during HIV infection(Masliah, Ge et al. 1994) and that there is an association of IL2Ralpha, IL4Ralpha, IL10Ralpha, and IFN (gamma) R1 cytokine receptor genes with AIDS progression(Do, Vasilescu et al. 2006) and that cytokine receptor deficiencies may contribute to immune deficiency in HIV-infected patients (Pahwa 2007). Sirskyj D and colleagues

comprehensively review the effects of the disruption of the cytokine/cytokine receptor network in T-cells during HIV infection(Sirskyj, Theze et al. 2008). However, the molecular mechanisms causing this are largely unknown, due to the due to the incredibly complex interplay between viral factors and host cytokine signaling pathways (Sirskyj, Theze et al. 2008).

During ET, the production of pro-inflammatory cytokines, such as IL-1β, IL-4, and IL-5, in response to LPS (LL+L) was diminished in contrast to that elicited by a single exposure to the endotoxin (SS+L). This altered response was seen in the spleen and serum in both the HIV-1Tg and F344 ET rats.

The spleen is an immune system organ, and, as such, one would expect that a greater number of cytokine and chemokine genes would be changed in the spleen in response to an immune challenge compared to the brain, and that is what we found in this study. In both the HIV-1Tg and F344 rats, there was a greater response to LPS in the spleen than in the brain.

Chemokines and their receptors have been implicated in the neuropathogenesis of HIV-1 infection (Mahajan, Schwartz et al. 2002). There were significant differences in chemokine expression in response to LPS in the HIV-1Tg rats compared to the F344 rats. Enhanced levels of Ccl2 in HIV-1 patients have been associated with HIV-1-associated

dementia (Ansari, Heiken et al. 2007). Our results indicate that Ccl2 levels were increased to a greater extent (3- to 5-fold) in the brain of the SS+L and LL+L groups of HIV-1Tg rats versus the F344 rats, corroborating the role of upregulation of Ccl2 and its implications on HIV encephalopathy. The levels of the inflammatory CXC chemokines, Cxcl1, Cxcl2, Cxcl10, and Cxcl11, were elevated to a greater extent (0.1- to 40-fold) in the brain of the SS+L and LL+L groups of the F344 rats compared to the HIV-1Tg rats. Ccl2, Ccl7, and Ccl9,levels were increased to a greater extent in the SS+L and LL+L groups of the spleen of the HIV-1Tg rats in comparison to the F344 rats. Cxcl1 and Cxcl2 levels were elevated to a lesser extent in the spleen of the SS+L and LL+L groups of the F344 rats compared to the HIV-1Tg rats.

Chemokines and chemokine receptors define a network throughout the body, playing critical roles in immune and inflammatory responses as well as in many pathological processes, in diseases such as multiple sclerosis, Alzheimer's disease, and HIV/AIDS (Martin-Garcia, Kolson et al. 2002). Cxcr4 and Ccr5 are reported to be co-receptors that mediate HIV-1 entry (Bachis, Cruz et al. 2010). In our study, the gene expression of the chemokine receptors, Ccr2, Ccr3, Ccr4, Ccr5, Ccr7, Cxcr3, Ccr10, Ccr3, Cx3cr1, IL-8rβ, and Xcr1, in the spleen of the ET group (LL+L) of F344 rats were down-regulated, whereas those in the HIV-1Tg spleen were not

significantly different compared to the control group (SS+S). All these receptors have been shown to function as co-receptors for HIV-1infection *in vitro* (Starr-Spires and Collman 2002), which suggests that HIV-1 viral proteins may interact with these chemokine receptors *in vivo*. There is also evidence that chemokines and chemokine receptors play an important part in the signaling of neuroprotective effects in the brain (Martin-Garcia, Kolson et al. 2002).

In this study, we noted a distinct pattern of cytokine/chemokine expression in the brain, spleen, and serum of the HIV-1Tg and F344 rats in response to LPS, both with and without ET. Identifying these distinct cytokine/chemokine profiles may potentially be useful as indicators of the onset and/or progression of certain disease processes, such as sepsis. Further studies will be done to determine the relationship of viral protein expression to the production of cytokines and chemokines during ET.

In our other study of CPP in the HIV-1Tg and control F344 animals, we explored the hypothesis that the HIV-1Tg rat is more prone to the addictive properties of morphine than the control animal F344 because expression of MOR is up-regulated in the HIV-1Tg rat (Chang, Beltran et al. 2007). We used the CPP test to measure the motivational salience of morphine paired cues of the HIV-1Tg rats versus control, F344 rats. A dose of 3.5mg/kg morphine successfully established a CPP in both the HIV-1Tg and F344

rats. However, an unanticipated finding was that rats that had morphine paired with the white chamber failed to show any evidence of extinction, whereas the rats that had morphine paired with the black chamber showed successful extinction and the emergence of a reversed preference. When the color of the morphine-paired chamber was not taken into consideration in the statistical analysis, group averages suggested that extinction occurred after 17 and 20 days for F344 and HIV-1Tg rats, respectively. However, when the morphine-paired chamber was included as a factor in the analysis, it was revealed that extinction occurred earlier in the rats for whom morphine was paired with the black chamber, but not in the rats for whom morphine was paired with the white chamber.

During a CPP test rats freely explore both sides of the apparatus, spending more time overall on the morphine-paired side if a CPP is established. The behavior of the rats in a CPP apparatus, however, is affected by factors other than a CPP that are not typically assessed systematically in CPP studies. When first placed in the apparatus the relative novelty of the environment activates exploratory behavior that declines or habituates over time. As exploratory behavior undergoes habituation the rats move less and choose to settle in a preferred chamber if a preference is present (Reid, Marglin et al. 1989). Rats may settle in one chamber because that chamber accrued rewarding properties when it was paired with morphine;

this is basic assumption of the CPP test. However, our present studies suggest that following habituation of exploratory behavior rats may also settle to one chamber when a natural preference for that chamber exists that may not have been apparent in the preconditioning phase when the novelty of the apparatus was activating high levels of exploratory behavior. The presence or absence of natural preferences is a characteristic of the CPP apparatus that has been considered in the literature. When morphine is paired with the naturally preferred chamber, the observed CPP is assumed to result from the sum of the natural preference and the conditioned preference. When morphine is paired with the non-preferred chamber, however, the conditioned rewarding properties of the previously less preferred chamber will compete with the naturally preferred chamber. If the newly acquired rewarding properties are sufficiently strong to compete successfully with the natural preference a CPP will be observed, although it is likely to be weaker than in subjects that had morphine paired with the naturally preferred side. This is the reason that the drug is paired with the non-preferred side when a biased CPP apparatus is used (Roma and Riley 2005). A successful CPP in a biased apparatus indicates that the conditioned rewarding properties were sufficiently strong to overcome the natural preference. With an unbiased apparatus the drug is not paired with a preferred side although the drug-paired chamber is typically

counterbalanced within groups. But the results of the present study suggest that natural chamber preferences may not be apparent when a single brief preconditioning test that is a common procedure as suggested in the CPP literature is used. This is because that there is not sufficient opportunity for the habituation of intense exploratory behavior to reveal the natural preference When we analyzed the pre-conditioning and post-conditioning results separated into three 5-minute time segments no statistically significant side preference was observed during preconditioning in the two-chamber CPP apparatus used in the present experiment. However, the post-conditioning data showed clear changes in preference over the three 5-minute periods. As time passed rats generally explored less and spent more time in the morphine-paired chamber. This pattern was most pronounced in the rats that experienced morphine paired with the white chamber.

There were animals that did not extinguish the morphine-induced CPP and there were other animals that did show extinction. For clarity these are discussed separately below.

Failed Extinction of a CPP

The extinction data indicated that the preference for the white morphine-paired side failed to weaken despite 17 (F344 rats) and 20 (HIV-1Tg rats) days of exposure to the extinction procedure. Moreover, within-session

increases in preference for the morphine-paired side was consistently observed in the white-morphine paired rats, but not in the black-morphine paired rats. We argue that the within-session increase in preference is due to habituation of exploratory behavior. Evidence consistent with this habituation interpretation is provided in Extinction 2 where spontaneous recovery of exploratory behavior was observed as a result of the delay between Extinction 2a and 2b. Our studies also show that the rats had a natural preference for the white chamber that was not evident in the preconditioning phase, but when paired with morphine this preference became more evident. The natural place preference plus the conditioned place preference may have synergized to augment the preference for the white side. An alternative explanation is that morphine enhanced the natural preference without inducing a CPP as it is typically characterized. If the increased preference for the white chamber was due to the summing of a natural preference and a CPP, then repeated exposure to the chamber cues in the absence of morphine treatment should decrease the place preference as the CPP undergoes extinction, but there was no evidence of extinction in the white-morphine paired groups. It cannot be ruled out that extinction would have been observed if additional extinction days were given. However, the expectation of an increased likelihood of extinction given sufficient extinction trials is based on the prevailing theoretical view

that explains CPP as a case of classical conditioning that involves predictive learning. Alternatively, the CPP procedure with morphine may result in another form of behavioral plasticity that does not conform to a predictive learning account of classical conditioning.

In the predictive learning model of classical conditioning the acquisition of CPP is seen as involving an association between the discriminative chamber cues and the rewarding properties of morphine, such that the chamber cues predict the availability of morphine. In the extinction procedure a new association is presumed to occur (i.e., the chamber cues no longer predict the availability of morphine) that supersedes the original association, causing CPP to extinguish. When the rats are "reminded" of the earlier predictive relationship in a reinstatement procedure the CPP reemerges; that is, the morphine effect is once again anticipated. However, there are several well documented cases of classical conditioning procedures that result in a conditioned preference that do not extinguish. When the consumption of a flavored saccharin solution is paired with a carbohydrate solution that is pumped directly into the stomach, for example, rats develop a strong preference for the flavored saccharin solution that is highly resistant to extinction(Elizalde and Sclafani 1990; Drucker, Ackroff et al. 1994). This suggests that some preferences that are established with classical conditioning procedures may not be easily eliminated by

extinction. A body of research has found similar effects in animals and humans and is referred to as evaluative conditioning (Baeyens and De Houwer 1995; De Houwer, Thomas et al. 2001). In evaluative conditioning the positive value of the unconditioned stimilus is presumed to transfer to the conditioned stimulus, increasing the liking of the CS. Therefore, the positive value of the morphine US in the present study may have been transferred to preferential discriminative chamber cues (white chamber) to produce an enhanced preference that is highly resistant to extinction. Indeed a persistent preference for cues previously paired with a psychoactive drug would help explain an individual's life-time struggle with drug addiction – the acquired preference for drug-paired cues never really go away. Interestingly, numerous studies show that conditioned responses to drug-paired cues can undergo extinction in animals, but the evidence of similar extinction effects in humans is lacking (Myers and Carlezon 2010). It is unclear what factors may be involved in whether a CPP undergoes extinction or whether it persists indefinitely. One possible factor that may influence resistance to extinction may be the type of morphine-paired cue. A CPP that resists extinction may be more likely if there is a natural preference for the paired cue before conditioning, even if the natural preference is not immediately apparent early in testing because other behaviors (e. g., exploratory behavior) mask the initial preference. Clearly

not all preferred cues paired with a drug lead to CPP that fails to extinguish since extinction is often observed in biased CPP procedures where the drug is paired with an initially preferred side. An enhanced natural preference may be most prominant when morphine is paired with contact cues (that is, floor cues) because once rats make physical contact with the floor cues they may prefer to maintain contact with these cues and reduce their approach to other distal cues (Vezina and Stewart 1987).

The prevalence of CPP that is highly resistant to extinct is not well known. Investigators often do not test for extinction of CPP because it is not germane to the hypothesis being investigated. When extinction is a desired outcome in an experiment (e.g., to test for reinstatement, for example) any procedure that results in CPP that resists extinction may be interpreted as a failed procedure and abandoned in favor of procedures that yield CPP results more consistent with a predictive memory model interpretation. Nevertheless there are several examples of high resistance to extinction of morphine-induced CPP in the literature. Most recently, for example, Rutten and colleagues reported that it took 18 fifteen-minute-sessions to see extinction of a 3.0 mg/kg dose and up to 45 fifteen-minute-sessions for a 10 mg/kg dose of morphine (Rutten K 2011). In another recent study, Narp knockout mice failed to extinguish a morphine CPP. The authors conclude that the Narp deletion disrupts the ability to update the predictive

association between the CPP context cues and the drug reward (Crombag, Dickson et al. 2009) . However, this conclusion assumes that morphine CPP procedures result in associative predictive learning. If CPP can also result in the establishment of long lasting preference that is not subsequently altered through extinction, then it is difficult to determine if Narp deletion failed the updating of predictive learning (failed extinction) or if it resulted in an enhanced behavioural plasticity that is better described as evaluative conditioning. It is also possible that a preference for floor cues may have been altered in the transgenic mice, rather than their propensity to learn, and morphine induced an enhancement of this altered, natural preference. We suggest here that the interpretation of extinction performance of morphine CPP may be complicated by the behavioural plasticity noted in the present CPP experiment and a more detailed analysis of changing preferences across CPP sessions can help evaluate the factors that contribute to the acquisition and maintenance of a place preference. It is still plausible that a CPP was established along with an enhanced natural preference in the white morphine paired animals, but the natural preference dominated over the CPP. Thus, we examined place preference early in an extinction session (minutes 1 -5), when the animals are exploring at their maximum because habituation has not yet occurred. The exploratory behavior early in the session may be guided primarily by the

morphine predicitve cues (i.e., CPP) independent of a natural preference. The same can be said for minutes 16 -20 in Extinction 2b, because the delay allowed the spontaneous recovery of exploratory behavior in this time segment.

A preference for the morphine-paired side in the first 5 minutes was observed that did not decrease over days in Extinction 1 or Extinction 2. Thus, there was no evidence of extinction of morphine-pridictive cues over days, even when just the first 5 minutes are examined. By the fourth time segment (minutes 16-20) of Extinction 1 the preference for the morphine-paired time was even greater as exploratory behavior was habituating, but during Extinction 2 the preference is again reduced as exploratory behavior spontaneously recovers. Yet there is still no indication of a decreased preference for the morphine-paired side over days. The same pattern was also seen in the HIV-1Tg rats. The evidence suggests, therefore, that morphine induced enhanced natural preference when it was paired with the white chamber rather than a CPP. Perhaps this enhanced natural preference is best described as a type of evaluative conditioning (Baeyens and De Houwer 1995; De Houwer, Thomas et al. 2001). A limitation of the evaluation conditioning hypothesis is that a control group that received no morphine treatment but similar number of "extinction" exposures to the chamber was not available to assess the strength of the natural preference

in the absence of morphine. Nevertheless, the evaluative conditioning hypothesis is worth exploring given the many cases in the literature of preferences that are highly resistant to extinction.

Extinction of a CPP

The same analysis of the data over time segments from the black-morphine paired groups yielded evidence of the extinction of a CPP. The evidence suggests that these groups established a CPP that overcame the natural preference for the white chamber and that this CPP extinguished. Although the black-morphine paired groups did not experience an enhancement of the natural preference for the white chamber (because it was not paired with morphine) the hypothesized natural preference for the white side should still be present. As extinction of the CPP occurs the natural preference for the white side should emerge. This is what was observed. During the first 5 minutes of Extinction 1 there was a significant preference for the morphine-paired side that persisted across days of Extinction 1 in both the F344 and HIV-1Tg groups. By Extinction 2 the main effect of drug was no longer evident, indicating that the CPP had extinguished.

During the fourth time segment (minutes 16-20) of Extinction 2a significant reversal in preference from the black morphine-paired chamber to the white saline-paired chamber was observed in the F344 rats. This preference reversal from the black morphine-paired chamber to the white saline-paired

chamber during minutes 16 – 20 in Extinction 2 was not observed in the HIV-1Tg rats, however, suggesting that CPP was not fully extinguished in the HIV-1Tg rats. An additional 3 days of extinction for the HIV-1Tg rats still failed to result in a reverse preference during the first 5 minutes of the extinction sessions, but the reverse preference was clearly evident in the last 10 minutes of the extinction sessions.

The lack of preference reversal in the HIV-1Tg rats during the first 5 minutes of extinction suggests that the HIV-1Tg rats showed greater resistance to extinction of a CPP than the F344 rats. A more substantial resistance to extinction was observed in the HIV-1Tg rats in a previous study (Chang and Connaghan 2011), nevertheless both studies support the hypothesis that morphine has a greater rewarding efficacy in the HIV-1Tg rats (Rutten K 2011). The difference in the magnitude of the resistance to extinction that was observed between the two studies is most likely due to the apparatus used. In the previous study a custom-made three-compartment apparatus was used that resulted in a very strong natural preference for the smaller middle compartment. Many rats also showed a slight preference for one of the two larger compartments. Morphine was paired with the less preferred of the two larger chambers while saline was paired with the other large chamber. The middle chamber was not paired with any injection. To assess CPP the time in the two larger chambers

were compared while the time in the middle chamber was not taken into consideration. The rats spent most of their time in the naturally preferred chamber, but when the times in the two larger chambers were compared a significant CPP was observed that took significantly longer to extinguish in the HIV-1Tg rats. Thus, in the previous study with a three-compartment chamber the strong natural preference for the smaller chamber did not compete directly with the CPP that was established in the larger chambers, whereas in the current study with the two-compartment apparatus the natural preference competed with the CPP. When morphine was paired directly with the naturally preferred white chamber in the present study, the preference was enhanced to be greater than rats that did not experience morphine paired with their naturally preferred chamber.

Reinstatement of CPP

A salient feature of drug addiction is relapse (Koob and Nestler 1997; Kalivas and Volkow 2005). Even after long drug-free periods the subject can relapse to drug seeking and use if exposed to small quantities of the drug, to a drug-paired cue or to stress (O'Brien 1997; Kalivas and Volkow 2005). CPP has been used to measure reinstatement for extinguished place preference by reintroduction of the drug (drug-priming) or to stressors (Wang, Luo et al. 2000; Shaham, Shalev et al. 2003; Aguilar, Rodriguez-Arias et al. 2009; Chang 2010; Chang 2011).

In the present experiment, we used morphine primes of 1mg/kg and 3.5 mg/kg in the HIV-1Tg and F344 rats for whom the morphine-induced CPP was extinguished. The HIV-1Tg and F344 rats paired with the white and black chamber did not show re-instatement of CPP at either dose of morphine. The HIV-1Tg and F344 rats for whom morphine was paired with the black chamber, preferred the saline-paired side during the reinstatement test. This supports the argument that the rats had a natural preference for the white side that was counteracted by the CPP, but now that the CPP is extinguished they are choosing to spend more time on the white side during all three time periods despite the reinstatement attempt. It may be that this white side preference was overcome by the initial CPP, but the reinstatement effect was just not strong enough to overcome this natural preference.

There is considerable research indicating that there are major differences in the neurocircuitry and neurobiology of reinstatement by drug-priming, by cues and by stressors (Shaham, Shalev et al. 2003; Bossert, Ghitza et al. 2005). Stewart and colleagues show that the corticotrophin-releasing factor and noradrenergic dependent pathways were primarily involved in foot-shock stress induced relapse but had little effect on drug-induced relapse (Shaham, Erb et al. 2000; Stewart 2000; Erb S 2001; Stewart 2003). There are studies which indicate that stress is a co-risk-factor in the drug-addiction

relapse in the HIV-1 positive population (Demas, Schoenbaum et al. 1995; Rotheram-Borus, Rosario et al. 1995; Back, Dansky et al. 2001; Tull, Trotman et al. 2009). Under foot shock stress the F344 and HIV-1Tg rats that had the morphine paired with the white side continued to show a preference for the white side. However, no statistically significant effects were observed in the F344 or HIV-1Tg rats that had morphine paired with the black side. This result may suggest that the stress reinstated a CPP sufficient enough to counteract the preference for white, but not enough to reverse the preference for the morphine-paired side. An alternative explanation is that the black-morphine paired animals were showing a smaller preference for the white side by the end of extinction (the natural white side preference was not enhanced for them because it was never paired with morphine) and in these reinstatement tests the smaller preference is no longer evident because of the reduced statistical power as a result of reducing the number of subjects by about half.

The failure to observe reinstatement effects in this experiment precluded the test of the hypothesis that HIV-1Tg and F344 rats differ in their sensitivity to morphine. There was some evidence, however, that the HIV-1Tg rats display a greater resistance to extinction of a morphine CPP; a result that is consistent with a previous experiment using a three-compartment biased CPP apparatus (Chang 2011). These differences in

resistance to extinction of a morphine CPP supports the hypothesis that viral proteins alters the effects of morphine on behaviour, but more studies and a better understanding of the behavioural plasticity that occurs in the morphine CPP procedure is needed before these effects of viral proteins can be elucidated.

Conclusions and Future Direction

The data from our study endotoxin –induced cytokine chemokine-profile in the HIV-1Tg and control F344 animals provides a comprehensive picture of the neuroimmune responses to infection during ET, and strongly suggest that the presence of HIV-1 viral proteins may exacerbate those responses. These findings also suggest the potential use of experimentally defined cytokine/chemokine expression profiles as indicators of altered immune function in various disease states.

Our results of the CPP study in HIV-1Tg rats failed to show reinstatement however the HIV-1Tg rats did take a longer time to extinguish the morphine-induced CPP, which supports our hypothesis that the persistent presence of viral proteins may lead to an increased propensity for morphine induced motivational salience. Moreover, this data suggested that the CPP procedure itself may result in a behavioural plasticity that does not conform to the Pavlovian/memory model.

Future studies

Our study of endotoxin–induced cytokine- and chemokine-profiles in the HIV-1Tg, provided valuable information of the specific inflammatory cytokine, chemokine and their receptors' genes that were modulated during ET in the HIV-1Tg rat, it would be interesting to study the protein levels of these modulated genes:

- IL-1α, IL-1β, and IFN-γ in the spleen as these genes were 4- to 82-fold greater in the spleen of the HIV-1Tg rats compared to the F344 rats in both the single LPS exposure (SS+L) and ET (LL+L)
- Cytokine receptors – IL-1r2, IL-2rβ, IL-2rγ, IL-5rα, IL-6rα, IL-6st, in the brain as these were differently modulated in the brains of the HIV-1Tg rats compared to the F344 rats in response to LPS in both the ET state and after a single exposure to LPS
- In the brain, Ccl2, Cxcl1, Cxcl2, Cxcl10, and Cxcl11, which were levels, which differently modulated in the brains of the HIV-1Tg rats compared to the F344 rats in response to LPS in both the ET state and after a single exposure to LPS
- In the spleen, Ccl2, Ccl7, Ccl9, Cxcl1 and Cxcl2 levels
- Ccr2, Ccr3, Ccr4, Ccr5, Ccr7, Cxcr3, Ccr10, Ccr3, Cx3cr1, IL-8rβ, and Xcr1, in the spleen

MOR is upregulated in the HIV-1Tg rat and we hypothesize that this may have lead to the differences in the endotoxin-induced cytokine/chemokine profiles between the HIV-1Tg and control animals as MOR is known to modulate cytokine/chemokine networks. To explore the role of MOR in modulation of cytokine- chemokine networks during ET, it would be reasonable to study these cytokine/chemokine genes modulated in this study during ET in MOR knock out rats to compare with the results obtained with F344 rats in ET state in our study.

Our morphine CPP study in HIV-1Tg rats has characterized this important HIV-1 model and provides novel and valuable information on morphine-induced CPP, which may result in a behavioural plasticity that does not conform to the Pavlovian/memory model.

In our morphine-induced CPP study we noted that for all animals their preference for the white chamber was enhanced by morphine, however, this preference was not initially noted in the pre-conditioning phase. A future study could explore if there is an initial preference of the animals for a particular chamber by way of a longer pre-conditioning phase (3-days). If a preference for the animals is observed in the pre-conditioning phase then a biased design (versus the balanced design) for establishing a CPP could be used where morphine is paired with the non-preferred chamber during the

conditioning phase. It would be interesting to explore if the "enhanced" preference for one of the chambers still develops in the animals after morphine-treatment using a biased design. Further if a morphine-induced CPP is successfully produced using the biased design it would be interesting to study the difference in the rate of extinction and in the reinstatement of a drug-induced and/or stress-induced CPP between the HIV-1Tg and control F344 rats. This will investigate our hypothesis that HIV-1Tg may be more prone to morphine addiction due to the upregulation of MOR in HIV-1Tg rats.

It would be of interest to know if extinction would eventually happen the animals who had morphine paired with the white side and did not show extinction. But rather than give many more extinction trials, which may require weeks of extinction exposure, a more practical approach would be to reverse the conditions and retrain the animals but this time pair morphine with the black side chamber. This reverse pairing should change the preference as they learn that black is now associated with morphine (reversal and extinction are similar in that they both involve a new learning, but reversal learning should be faster because of the actual presence of morphine). Also, if it would let us explore if the HIV-1Tg rats do indeed learn more slowly, if any observed reversal of the preference is slower in the HIV-1Tg rats compared to the F344 rats.

References

Adib-Conquy M, Cavaillon JM (2009) Compensatory anti-inflammatory response syndrome. Thrombosis and Haemostasis 101:36-47.

Aguilar MA, Rodriguez-Arias M, Minarro J (2009) Neurobiological mechanisms of the reinstatement of drug-conditioned place preference. Brain Research Reviews 59:253-277.

Angus D, Linde-Zwirble W, Lidicker J, Clermont G, Carcillo J, Pinsky M (2001) Epidemiology of severe sepsis in the United States: Analysis of incidence, outcome, and associated costs of care. Critical Care Medicine 29:1303-1310.

Ansari AW, Heiken H, Moenkemeyer M, Schmidt RE (2007) Dichotomous effects of C-C chemokines in HIV-1 pathogenesis. Immunology Letters 110:1-5.

Bachis A, Cruz MI, Mocchetti I (2010) M-tropic HIV envelope protein gp120 exhibits a different neuropathological profile than T-tropic gp120 in rat striatum. European Journal of Neuroscience 32:570-578.

Back SE, Dansky BS, Carroll KM, Foa EB, Brady KT (2001) Exposure therapy in the treatment of PTSD among cocaine-dependent individuals: description of procedures. Journal of Substance Abuse Treatment 21:35-45.

Baeyens F, De Houwer J (1995) Evaluative conditioning is a qualitatively distinct form of classical conditioning: a reply to Davey (1994). Behaviour Research and Therapy 33:825-831.

Banerjee A, Strazza M, Wigdahl B, Pirrone V, Meucci O, Nonnemacher MR (2011) Role of mu-opioids as cofactors in human immunodeficiency virus type 1 disease progression and neuropathogenesis. Journal of Neurovirology 17:291-302.

Basselin M, Ramadan E, Igarashi M, Chang L, Chen M, Kraft AD, Harry GJ, Rapoport SI (2011) Imaging upregulated brain arachidonic acid metabolism in HIV-1 transgenic rats. Journal of Cerebral Blood Flow and Metabolism 31:486-493.

Bevins RA, McPhee JE, Ranhut AS, Ayres JJB (2000) One-trial context fear conditioning with immediate shock: The role of transport and contextual cues. Animal Learning and Behavior 28:162-171.

Beyrer C (2007) HIV epidemiology update and transmission factors: risks and risk contexts--16th International AIDS Conference epidemiology plenary. Clinical Infectious Diseases 44:981-987.

Biswas SK, Bist P, Dhillon MK, Kajiji T, Del Fresno C, Yamamoto M, Lopez-Collazo E, Akira S, Tergaonkar V (2007) Role for MyD88-independent, TRIF pathway in lipid A/TLR4-induced endotoxin tolerance. Journal of Immunology 179:4083-4092.

Biswas SK, Lopez-Collazo E (2009) Endotoxin tolerance: new mechanisms, molecules and clinical significance. Trends in Immunology 30:475-487.

Boasso A, Shearer GM, Chougnet C (2009) Immune dysregulation in human immunodeficiency virus infection: know it, fix it, prevent it? Journal of Internal Medicine 265:78-96.

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.

Borner C, Kraus J, Schroder H, Ammer H, Hollt V (2004) Transcriptional regulation of the human mu-opioid receptor gene by interleukin-6. Molecular Pharmacology 66:1719-1726.

Bossert JM, Ghitza UE, Lu L, Epstein DH, Shaham Y (2005) Neurobiology of relapse to heroin and cocaine seeking: an update and clinical implications. European Journal of Pharmacology 526:36-50.

Breen EC (2002) Pro- and anti-inflammatory cytokines in human immunodeficiency virus infection and acquired immunodeficiency syndrome. Pharmacology and Therapeutics 95:295-304.

Bruggeman LA, Dikman S, Meng C, Quaggin SE, Coffman TM, Klotman PE (1997) Nephropathy in human immunodeficiency virus-1 transgenic mice is due to renal transgene expression. Journal of Clinical Investigation 100:84-92.

Burbassi S, Aloyo VJ, Simansky KJ, Meucci O (2008) GTPgammaS incorporation in the rat brain: a study on mu-opioid receptors and CXCR4. Journal of Neuroimmune Pharmacology 3:26-34.

Cabral GA (2006) Drugs of abuse, immune modulation, and AIDS. J Neuroimmune Pharmacology 1:280-295.

Capobianchi MR (1996) Induction of lymphomonocyte activation by HIV-1 glycoprotein gp120. Possible role in AIDS pathogenesis. Journal of Biological Regulators and Homeostatic Agents 10:83-91.

Cavaillon JM, Adrie C, Fitting C, Adib-Conquy M (2005) Reprogramming of circulatory cells in sepsis and SIRS. Journal of Endotoxin Research 11:311-320.

Chang S, Connaghan K (2011) Behavioral and molecular evidence for a feedback interaction between morphine and HIV-1 viral proteins. Journal of Neuroimmune Pharmacology doi 10.1007/s11481-011-9324-1.

Chang SL, Beltran JA, Swarup S (2007) Expression of the mu opioid receptor in the human immunodeficiency virus type 1 transgenic rat model. Journal of Virology 81:8406-8411.

Chang SL, Frank Ocasio and Jose Beltran (2007) Immunodeficient parameters in the HIV-1 transgenic rat model. American Journal of Infectious Diseases 3:6.

Chang SL, Wu GD, Patel NA, Vidal EL, Fiala M (1998) The effects of interaction between morphine and interleukin-1 on the immune response. Advances in Experimental Medicine and Biology 437:67-72.

Chang SL, Connaghan KP (2011) Behavioral and Molecular Evidence for a Feedback Interaction Between Morphine and HIV-1 Viral Proteins. Journal of Neuroimmune Pharmacology (revision).

Chang SL, Kass MD (2010) Transgenic rodent models. In: The Neurology of AIDS(Howard E. Gendelman, I. G., Ian Paul Everall, Stuart A. Lipton and Susan Swindells (Eds.) ed) New York.: Oxford University Press Inc.

Chen J, Ivashkiv LB (2010) IFN-gamma abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodeling. Proceedings of the National Academy of Sciences of the United States of America 107:19438-19443.

Chen R, Zhou H, Beltran J, Malellari L, Chang S (2005) Differential expression of cytokines in the brain and serum during endotoxin tolerance. Journal of Neuroimmunology 163:53-72.

Cicala C, Arthos J, Selig SM, Dennis G, Jr., Hosack DA, Van Ryk D, Spangler ML, Steenbeke TD, Khazanie P, Gupta N, Yang J, Daucher M, Lempicki RA, Fauci AS (2002) HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication. Proceedings of the National Academy of Sciences of the United States of America 99:9380-9385.

Clapham PR, McKnight A (2001) HIV-1 receptors and cell tropism. British Medical Bulletin 58:43-59.

Crombag HS, Dickson M, Dinenna M, Johnson AW, Perin MS, Holland PC, Baraban JM, Reti IM (2009) Narp deletion blocks extinction of morphine place preference conditioning. Neuropsychopharmacology 34:857-866.

Davis CM, Roma PG, Dominguez JM, Riley AL (2007) Morphine-induced place conditioning in Fischer and Lewis rats: acquisition and dose-response in a fully biased procedure. Pharmacology, Biochemistry and Behavior 86:516-523.

De Houwer J, Thomas S, Baeyens F (2001) Associative learning of likes and dislikes: a review of 25 years of research on human evaluative conditioning. Psychological Bulletin 127:853-869.

del Fresno C, Garcia-Rio F, Gomez-Pina V, Soares-Schanoski A, Fernandez-Ruiz I, Jurado T, Kajiji T, Shu C, Marin E, Gutierrez del Arroyo A, Prados C, Arnalich F, Fuentes-Prior P, Biswas SK, Lopez-Collazo E (2009) Potent Phagocytic Activity with Impaired Antigen Presentation Identifying Lipopolysaccharide-Tolerant Human Monocytes: Demonstration in Isolated Monocytes from Cystic Fibrosis Patients. The Journal of Immunology 182:6494-6507.

Demas P, Schoenbaum EE, Wills TA, Doll LS, Klein RS (1995) Stress, coping, and attitudes toward HIV treatment in injecting drug users: a qualitative study. AIDS Education and Prevention 7:429-442.

DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, Chen Y, Su YA, Trent JM (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nature Genetics 14:457-460.

DeRisi JL, Iyer VR, Brown PO (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680-686.

Dewart T, Frank B, Schmeidler J (2006) The impact of 9/11 on patients in New York City's substance abuse treatment programs. American Journal of Drug and Alcohol Abuse 32:665-672.

Dinda A, Gitman M, Singhal PC (2005) Immunomodulatory effect of morphine: therapeutic implications. Expert Opinion Drug Safety 4:669-675.

Do H, Vasilescu A, Diop G, Hirtzig T, Coulonges C, Labib T, Heath SC, Spadoni JL, Therwath A, Lathrop M, Matsuda F, Zagury JF (2006) Associations of the IL2Ralpha, IL4Ralpha, IL10Ralpha, and IFN (gamma) R1 cytokine receptor genes with AIDS progression in a French AIDS cohort. Immunogenetics 58:89-98.

Donovan B, Padin-Rivera E, Kowaliw S (2001) "Transcend": initial outcomes from a posttraumatic stress disorder/substance abuse treatment program. Journal of Traumatic Stress 14:757-772.

Draisma A, Pickkers P, Bouw MP, van der Hoeven JG (2009) Development of endotoxin tolerance in humans in vivo. Critical Care Medicine 37:1261-1267.

Drucker DB, Ackroff K, Sclafani A (1994) Nutrient-conditioned flavor preference and acceptance in rats: effects of deprivation state and nonreinforcement. Physiology and Behavior 56:701-707.

El-Hage N, Dever SM, Fitting S, Ahmed T, Hauser KF (2011) HIV-1 coinfection and morphine coexposure severely dysregulate hepatitis C virus-induced hepatic proinflammatory cytokine release and free radical production: increased pathogenesis coincides with uncoordinated host defenses. Journal of Virology 85:11601-11614.

Elizalde G, Sclafani A (1990) Flavor preferences conditioned by intragastric polycose infusions: a detailed analysis using an electronic esophagus preparation. Physiology and Behavior 47:63-77.

Ellis RJ, Calero P, Stockin MD (2009) HIV infection and the central nervous system: a primer. Neuropsychology Review 19:144-151.

Erb S SY, Stewart J (2001) Stress-induced relapse to drug seeking in the rat: role of the bed nucleus of the stria terminalis and anygdala. Stress 4:289-303.

Fauci AS (1996) Host factors and the pathogenesis of HIV-induced disease. Nature 384:529-534.

Foster SL, Hargreaves DC, Medzhitov R (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. Nature; 447(7147):972-8.

Fowler CJ, Fraser GL (1994) Mu-, delta-, kappa-opioid receptors and their subtypes. A critical review with emphasis on radioligand binding experiments. Neurochemistry International 24:401-426.

Friedman H, Eisenstein TK (2004) Neurological basis of drug dependence and its effects on the immune system. Journal of Neuroimmunology 147:106-108.

Friedman SR, Ompad DC, Maslow C, Young R, Case P, Hudson SM, Diaz T, Morse E, Bailey S, Des Jarlais DC, Perlis T, Hollibaugh A, Garfein RS (2003) HIV prevalence, risk behaviors, and high-risk sexual and injection networks among young women injectors who have sex with women. American Journal of Public Health 93:902-906.

Gagin R, Kook N, Cohen E, Shavit Y (1997) Prenatal morphine enhances morphine-conditioned place preference in adult rats. Pharmacology, Biochemistry and Behavior 58:525-528.

Garza HH, Jr., Prakash O, Carr DJ (1996) Aberrant regulation of cytokines in HIV-1 TAT72-transgenic mice. Journal of Immunology 156:3631-3637.

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. New England Journal of Medicine 305:1425-1431.

Grakalic I, Schindler CW, Baumann MH, Rice KC, Riley AL (2006) Effects of stress modulation on morphine-induced conditioned place preferences and plasma corticosterone levels in Fischer, Lewis, and Sprague-Dawley rat strains. Psychopharmacology 189:277-286.

Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Resau JH, Wang JM, Ali H, Richardson R, Snyderman R, Oppenheim JJ (1998a) Opiates transdeactivate chemokine receptors: delta and mu opiate receptor-mediated heterologous desensitization. Journal of Experimental Medicine 188:317-325.

Grimm MC, Ben-Baruch A, Taub DD, Howard OM, Wang JM, Oppenheim JJ (1998b) Opiate inhibition of chemokine-induced chemotaxis. Annals of the New York Academy of Sciences 840:9-20.

Guo CJ, Li Y, Tian S, Wang X, Douglas SD, Ho WZ (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.

Haase AT (2010) Targeting early infection to prevent HIV-1 mucosal transmission. Nature 464:217-223.

Hahn JW, Jagwani S, Kim E, Rendell VR, He J, Ezerskiy LA, Wesselschmidt R, Coscia CJ, Belcheva MM (2010) Mu and kappa opioids modulate mouse embryonic stem cell-derived neural progenitor differentiation via MAP kinases. Journal of Neurochemistry 112:1431-1441.

Happel C, Steele AD, Finley MJ, Kutzler MA, Rogers TJ (2008) DAMGO-induced expression of chemokines and chemokine receptors: the role of TGF-beta1. Journal of Leukocyte Biology 83:956-963.

Herbeuval JP, Shearer GM (2006) Are blockers of gp120/CD4 interaction effective inhibitors of HIV-1 immunopathogenesis? AIDS Reviews 8:3-8.

Holguin A, O'Connor KA, Biedenkapp J, Campisi J, Wieseler-Frank J, Milligan ED, Hansen MK, Spataro L, Maksimova E, Bravmann C, Martin D, Fleshner M, Maier SF, Watkins LR (2004) HIV-1 gp120 stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). Pain 110:517-530.

House RV, Thomas PT, Bhargava HN (1996) A comparative study of immunomodulation produced by in vitro exposure to delta opioid receptor agonist peptides. Peptides 17:75-81.

Hu S, Chao CC, Hegg CC, Thayer S, Peterson PK (2000) Morphine inhibits human microglial cell production of, and migration towards, RANTES. Journal of Psychopharmacology 14:238-243.

Jiang J, Aiken C (2006) Maturation of the viral core enhances the fusion of HIV-1 particles with primary human T cells and monocyte-derived macrophages. Virology 346:460-468.

Jones LE, Perelson AS (2005) Opportunistic infection as a cause of transient viremia in chronically infected HIV patients under treatment with HAART. Bulletin of Mathematical Biology 67:1227-1251.

Joshi PC, Raynor R, Fan X, Guidot DM (2008) HIV-1-transgene expression in rats decreases alveolar macrophage zinc levels and phagocytosis. American Journal of Respiratory Cell and Molecular Biology 39:218-226.

Kalivas PW (2003) Predisposition to addiction: pharmacokinetics, pharmacodynamics, and brain circuitry. American Journal of Psychiatry 160:1-2.

Kalivas PW, Volkow ND (2005) The neural basis of addiction: a pathology of motivation and choice. American Journal of Psychiatry 162:1403-1413.

Klotman PE, Notkins AL (1996) Transgenic models of human immunodeficiency virus type-1. Current Topics in Microbiology and Immunology 206:197-222.

Koob GF, Nestler EJ (1997) The neurobiology of drug addiction. Journal of Neuropsychiatry and Clinical Neurosciences 9:482-497.

Koulierakis G, Gnardellis C, Agrafiotis D, Power KG (2000) HIV risk behaviour correlates among injecting drug users in Greek prisons. Addiction 95:1207-1216.

Kraus J, Borner C, Giannini E, Hickfang K, Braun H, Mayer P, Hoehe MR, Ambrosch A, Konig W, Hollt V (2001) Regulation of mu-opioid receptor gene transcription by interleukin-4 and influence of an allelic variation within a STAT6 transcription factor binding site. Journal of Biological Chemistry 276:43901-43908.

Kraus J, Borner C, Giannini E, Hollt V (2003) The role of nuclear factor kappaB in tumor necrosis factor-regulated transcription of the human mu-opioid receptor gene. Molecular Pharmacology 64:876-884.

Lane HC, Masur H, Edgar LC, Whalen G, Rook AH, Fauci AS (1983) Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. New England Journal of Medicine 309:453-458.

Lashomb AL, Vigorito M, Chang SL (2009) Further characterization of the spatial learning deficit in the human immunodeficiency virus-1 transgenic rat. Journal of Neurovirology 15:14-24.

Lester R, Yao X, Ball T, McKinnon L, Kaul R (2008) Toll-like-receptor expression and responsiveness are increased in viraemic HIV-1 infection. AIDS 22:685-694.

Levy JA (1998) HIV and the pathogenesis of AIDS. Washington, D.C.: ASM Press.

Leyton M, Stewart J (1990) Preexposure to foot-shock sensitizes the locomotor response to subsequent systemic morphine and intra-nucleus accumbens amphetamine. Pharmacology, Biochemistry and Behavior 37:303-310.

Li PF, Hao YS, Zhang FX, Liu XH, Liu SL, Li G (2004) Signaling pathway involved in methionine enkephalin-promoted survival of lymphocytes infected by simian immunodeficiency virus in the early stage in vitro. International Immunopharmacology 4:79-90.

Linde T, Sandhagen B, Backman U, Fellstrom B (1999) Altered flow properties of blood and increased plasma fibrinogen in cyclosporin-treated renal allograft recipients. Nephrology, Dialysis, Transplantation 14:1525-1529.

Lund AK, Lucero J, Herbert L, Liu Y, Naik JS (2011) Human immunodeficiency virus- transgenic rats exhibit pulmonary hypertension. American Journal of Physiology of Lung Cell Molecular Physiology.

Mages J, Dietrich H, Lang R (2007) A genome-wide analysis of LPS tolerance in macrophages. Immunobiology 212:723-737.

Mahajan SD, Aalinkeel R, Reynolds JL, Nair BB, Fernandez SF, Schwartz SA, Nair MP (2005a) Morphine exacerbates HIV-1 viral protein gp120 induced modulation of chemokine gene expression in U373 astrocytoma cells. Current HIV Research 3:277-288.

Mahajan SD, Schwartz SA, Aalinkeel R, Chawda RP, Sykes DE, Nair MP (2005b) Morphine modulates chemokine gene regulation in normal human astrocytes. Clinical Immunology 115:323-332.

Mahajan SD, Schwartz SA, Shanahan TC, Chawda RP, Nair MP (2002) Morphine regulates gene expression of alpha- and beta-chemokines and their receptors on astroglial cells via the opioid mu receptor. Journal of Immunology 169:3589-3599.

Martin-Garcia J, Kolson DL, Gonzalez-Scarano F (2002) Chemokine receptors in the brain: their role in HIV infection and pathogenesis. AIDS 16:1709-1730.

Masliah E, Ge N, Achim CL, Wiley CA (1994) Cytokine receptor alterations during HIV infection in the human central nervous system. Brain Research 663:1-6.

McCarthy L, Wetzel M, Sliker JK, Eisenstein TK, Rogers TJ (2001) Opioids, opioid receptors, and the immune response. Drug and Alcohol Dependence 62:111-123.

Midde NM, Gomez AM, Harrod SB, Zhu J (2011) Genetically expressed HIV-1 viral proteins attenuate nicotine-induced behavioral sensitization and alter mesocorticolimbic ERK and CREB signaling in rats. Pharmacology, Biochemistry and Behavior 98:587-597.

Mirani M El, Volpi S, Hiroi N, Chrousos GP, Kino T (2002) HIV-1 protein Vpr suppresses IL-12 production from human monocytes by enhancing glucocorticoid action: potential implications of Vpr coactivator activity for the innate and cellular immunity deficits observed in HIV-1 infection. Journal of Immunology 169(11):6361-6368.

Miyagi T, Chuang LF, Doi RH, Carlos MP, Torres JV, Chuang RY (2000) Morphine induces gene expression of CCR5 in human CEMx174 lymphocytes. Journal of Biological Chemistry 275:31305-31310.

Monneret G, Venet F, Pachot A, Lepape A (2008) Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony. Molecular Medicine 14:64-78.

Montgomery CP, Daum RS (2009) Transcription of inflammatory genes in the lung after infection with community-associated methicillin-resistant Staphylococcus aureus: a role for panton-valentine leukocidin? Infection and Immunity 77:2159-2167.

Munoz C, Carlet J, Fitting C, Misset B, Bleriot JP, Cavaillon JM (1991) Dysregulation of in vitro cytokine production by monocytes during sepsis. Journal of Clinical Investigation 88:1747-1754.

Myers KM, Carlezon WA, Jr. (2010) Extinction of drug- and withdrawal-paired cues in animal models: relevance to the treatment of addiction. Neuroscience and Biobehavioral Reviews 35:285-302.

Nyland SB, Specter S, Im-Sin J, Ugen KE (1998) Opiate effects on in vitro human retroviral infection. Advances in Experimental Medicine and Biology 437:91-100.

O'Brien CP (1997) A range of research-based pharmacotherapies for addiction. Science 278:66-70.

Ouimette P, Coolhart D, Funderburk JS, Wade M, Brown PJ (2007) Precipitants of first substance use in recently abstinent substance use disorder patients with PTSD. Addictive Behaviors 32:1719-1727.

Pahwa S (2007) Role of common gamma chain utilizing cytokines for immune reconstitution in HIV infection. Immunologic Research 38:373-386.

Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. New England Journal of Medicine 338:853-860.

Peng J, Vigorito M, Liu X, Zhou D, Wu X, Chang SL (2010) The HIV-1 transgenic rat as a model for HIV-1 infected individuals on HAART. Journal of Neuroimmunology 218:94-101.

Peterson PK, Molitor TW, Chao CC (1998) The opioid-cytokine connection. Journal of Neuroimmunology 83:63-69.

Prejean J, Song R, Hernandez A, Ziebell R, Green T, Walker F, Lin LS, An Q, Mermin J, Lansky A, Hall HI (2011) Estimated HIV incidence in the United States, 2006-2009. PLoS ONE 6:e17502.

Reid LD, Marglin SH, Mattie ME, Hubbell CL (1989) Measuring morphine's capacity to establish a place preference. Pharmacology, Biochemistry and Behavior 33:765-775.

Reid W, Sadowska M, Denaro F, Rao S, Foulke J, Jr., Hayes N, Jones O, Doodnauth D, Davis H, Sill A, O'Driscoll P, Huso D, Fouts T, Lewis G, Hill M, Kamin-Lewis R, Wei C, Ray P, Gallo RC, Reitz M, Bryant J (2001) An HIV-1 transgenic rat that develops HIV-related pathology and immunologic dysfunction. Proceedings of the National Academy of Sciences of the United States of America 98:9271-9276.

Risdahl JM, Khanna KV, Peterson PK, Molitor TW (1998) Opiates and infection. Journal of Neuroimmunology 83:4-18.

Roma PG, Riley AL (2005) Apparatus bias and the use of light and texture in place conditioning. Pharmacology, Biochemistry and Behavior 82:163-169.

Rotheram-Borus MJ, Rosario M, Reid H, Koopman C (1995) Predicting patterns of sexual acts among homosexual and bisexual youths. American Journal of Psychiatry 152:588-595.

Roy S, Charboneau RG, Barke RA, Loh HH (2001) Role of mu-opioid receptor in immune function. Advances in Experimental Medicine and Biology 493:117-126.

Roy S, Nirıkovic J, Banerjee S, Charboneau RG, Das S, Dutta R, Kirchner VA, Koodie L, Ma J, Meng J, Barke RA (2011) Opioid Drug Abuse and Modulation of Immune Function: Consequences in the Susceptibility to Opportunistic Infections. Journal of Neuroimmune Pharmacology.

Roy S, Wang J, Kelschenbach J, Koodie L, Martin J (2006) Modulation of immune function by morphine: implications for susceptibility to infection. Journal of Neuroimmune Pharmacology 1:77-89.

Rutten K vdKE, De Vry J, Tzchentke TM (2011) Critical evaluation of the use of extinction paradigms for the assessment of opiod-induced conditioned place preference in rats. Pharmacology 87:286-296.

Ruzicka BB, Thompson RC, Watson SJ, Akil H (1996) Interleukin-1 beta-mediated regulation of mu-opioid receptor mRNA in primary astrocyte-enriched cultures. Journal of Neurochemistry 66:425-428.

Schletter J, Heine H, Ulmer AJ, Rietschel ET (1995) Molecular mechanisms of endotoxin activity. Archives of Microbiology 164:383-389.

Schols D, De Clercq E (1996) Human immunodeficiency virus type 1 gp120 induces anergy in human peripheral blood lymphocytes by inducing interleukin-10 production. Journal of Virology 70:4953-4960.

Shaham Y, Erb S, Stewart J (2000) Stress-induced relapse to heroin and cocaine seeking in rats: a review. Brain Research Brain Research Reviews 33:13-33.

Shaham Y, Shalev U, Lu L, De Wit H, Stewart J (2003) The reinstatement model of drug relapse: history, methodology and major findings. Psychopharmacology 168:3-20.

Shalev U, Grimm JW, Shaham Y (2002) Neurobiology of relapse to heroin and cocaine seeking: a review. Pharmacological Reviews 54:1-42.

Shetty P (2011) Nora Volkow--challenging the myths about drug addiction. Lancet 378:477.

Simpkins JW, Taylor ST, Gabriel SM, Katovich MJ, Millard WJ (1984) Evidence that chronic hyperprolactinemia effects skin temperature regulation through an opioid mechanism. Neuroendocrinology 39:321-329.

Sirskyj D, Theze J, Kumar A, Kryworuchko M (2008) Disruption of the gamma c cytokine network in T cells during HIV infection. Cytokine 43:1-14.

Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J, Lebedeva M, DeCamp A, Li D, Grove D, Self SG, Borrow P (2009) Induction of a striking systemic cytokine cascade prior to peak viremia in acute human immunodeficiency virus type 1 infection, in contrast to more modest and delayed responses in acute hepatitis B and C virus infections. Journal of Virology 83:3719-3733.

Starr-Spires LD, Collman RG (2002) HIV-1 entry and entry inhibitors as therapeutic agents. Clinics in Laboratory Medicine 22:681-701.

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

Steffen T, Blattler R, Gutzwiller F, Zwahlen M (2001) HIV and hepatitis virus infections among injecting drug users in a medically controlled heroin prescription programme. European Journal of Public Health 11:425-430.

Stewart J (2000) Pathways to relapse: the neurobiology of drug- and stress-induced relapse to drug-taking. Journal of Psychiatry and Neuroscience 25:125-136.

Stewart J (2003) Stress and relapse to drug seeking: studies in laboratory animals shed light on mechanisms and sources of long-term vulnerability. American Journal on Addictions 12:1-17.

Sudarsanam P, Iyer VR, Brown PO, Winston F (2000) Whole-genome expression analysis of snf/swi mutants of Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 97:3364-3369.

Tull MT, Trotman A, Duplinsky MS, Reynolds EK, Daughters SB, Potenza MN, Lejuez CW (2009) The effect of posttraumatic stress disorder on risk-taking propensity among crack/cocaine users in residential substance abuse treatment. Depression and Anxiety 26:1158-1164.

Turnbull AV, Rivier CL (1999) Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiological Reviews 79:1-71.

Tzschentke TM (1998) Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. Progress in Neurobiology 56:613-672.

Tzschentke TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addiction Biology 12:227-462.

van Maanen M, Sutton RE (2003) Rodent models for HIV-1 infection and disease. Current HIV Research 1:121-130.

Vezina P, Stewart J (1987) Conditioned locomotion and place preference elicited by tactile cues paired exclusively with morphine in an open field. Psychopharmacology 91:375-380.

Vidal EL, Patel NA, Wu G, Fiala M, Chang SL (1998) Interleukin-1 induces the expression of mu opioid receptors in endothelial cells. Immunopharmacology 38:261-266.

Vigano A, Trabattoni D, Schneider L, Ottaviani F, Aliffi A, Longhi E, Rusconi S, Clerici M (2006) Failure to eradicate HIV despite fully successful HAART initiated in the first days of life. Journal of Pediatrics 148:389-391.

Wang B, Luo F, Zhang WT, Han JS (2000) Stress or drug priming induces reinstatement of extinguished conditioned place preference. Neuroreport 11:2781-2784.

Wang J, Barke RA, Charboneau R, Roy S (2005) Morphine impairs host innate immune response and increases susceptibility to Streptococcus pneumoniae lung infection. Journal of Immunology 174:426-434.

Wang J, Ma J, Charboneau R, Barke R, Roy S (2011a) Morphine inhibits murine dendritic cell IL-23 production by modulating Toll-like receptor 2 and Nod2 signaling. Journal of Biological Chemistry 286:10225-10232.

Wang X, Ye L, Zhou Y, Liu MQ, Zhou DJ, Ho WZ (2011b) Inhibition of anti-HIV microRNA expression: a mechanism for opioid-mediated enhancement of HIV infection of monocytes. American Journal of Pathology 178:41-47.

Wellmann A, Thieblemont C, Pittaluga S, Sakai A, Jaffe ES, Siebert P, Raffeld M (2000) Detection of differentially expressed genes in lymphomas using cDNA arrays: identification of clusterin as a new diagnostic marker for anaplastic large-cell lymphomas. Blood 96:398-404.

Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V (1995) HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. European Molecular Biology Organization Journal 14:546-554.

Wilson SE, Habeshaw JA, Addawe MA, Hounsell EF, Oxford JS (1997) HIV type 1 envelope glycoprotein 120 carboxy-terminal peptide-induced human T cell lines selectively suppress heterogeneous proliferative T cell responses to soluble antigens. AIDS Research and Human Retroviruses 13:1313-1324.

Yim HC LJ, Lau JS, Lau AS (2009) HIV-1 Tat dysregulation of lipopolysaccharide-induced cytokine responses: microbial interactions in HIV infection. AIDS 23:1473-1484.