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Morphine increases hippocampal viral load and suppresses frontal lobe CCL5 expression in the LP-BM5 AIDS model

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Abstract

Chronic opiate abuse accelerates the development of cognitive deficits in human immunodeficiency virus (HIV)-1 patients. To investigate morphine's effects on viral infection of the central nervous system, we applied chronic morphine treatment to the LP-BM5 murine acquired immunodeficiency syndrome (MAIDS) model. LP-BM5 infection induces proinflammatory cytokine/chemokine production, correlating to increased blood-brain barrier permeability. Morphine treatment significantly increased LP-BM5 viral load in the hippocampus, but not in the frontal lobe. Morphine reduced the chemokine CCL5 to non-infected levels in the frontal lobe, but not the hippocampus. These data indicate a region-specific mechanism for morphine's effects on virally-induced neurocognitive deficits.

Keywords

HIV encephalitis; opiate abuse; neuroinflammation; blood-brain barrier; cytokines; claudin-5

1. Introduction

Nearly one-third of the 35 million people living with human immunodeficiency virus (HIV) infection worldwide will develop symptoms of HIV-associated neurocognitive disorder (HAND) (McArthur, 2004; UNAIDS, 2013). While improved antiretroviral therapies have reduced dementia rates to ~1% of the total HIV-positive population, milder HAND deficits including motor and cognitive impairment have not declined (McArthur, 2004; Yadav and Collman, 2009). Heroin and its active metabolite, morphine, accelerate disease progression

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into acquired immunodeficiency syndrome (AIDS) and increase both the risk and severity of HAND (Bell et al., 1998; McArthur, 2004; Nath et al., 2002; Robertson et al., 2007). With up to one-third of HIV-positive patients reporting opiate abuse, the interaction between morphine and HAND represents a serious concern in long-term HIV care (Bell et al., 1998; UNAIDS, 2013). However, the mechanism for morphine's effects on central nervous system (CNS) infection and neurological damage in HIV-1 infected patients remains controversial.

The CNS defends against viral infection through i) the blood-brain barrier (BBB), which prevents viral entry; and ii) the glial innate immune response, which initiates inflammation and recruits peripheral immune cells to the site of infection (Strazza et al., 2011; Yadav and Collman, 2009; Yao et al., 2010). The BBB consists of brain endothelial cells bound by cell-cell tight junctions which restrict paracellular movement of compounds from the blood into the CNS parenchyma. The barrier allows facilitated transportation of essential nutrients and small ions while restricting entry of most serum macromolecules (Abbott et al., 2006). Barrier permeability undergoes rapid, transient changes to accommodate local metabolic demands, but prolonged increases in BBB permeability can lead to an influx of neurotoxic compounds, metabolic imbalances and cell death (Guo and Lo, 2009; Willis and Davis, 2008).

The importance of the BBB in restricting viral entry into the brain parenchyma has been shown in *in vitro* studies. Fiala showed that an intact BBB prevented direct entry of cell-free HIV-1 (Fiala et al., 1997). However, other *in vitro* studies have shown that the HIV-1 viral protein Tat reduced transendothelial electrical resistance and decreased expression of the tight junction protein occludin in human brain microvascular endothelial cells, indicative of reduced BBB integrity. Morphine exacerbates the effects of HIV-1 Tat on BBB integrity *in vitro*, but the mechanism is not known (Mahajan et al., 2008).

Disruption of the BBB is also a feature of HIV-1-associated encephalitis (HIVE) in humans (Strazza et al., 2011). HIV-1 can be detected in the cerebrospinal fluid of the CNS as early as 15 days post-infection (Tomlinson et al., 1999). Viral particle entry into the CNS may be due to either direct entry of viral particles or the migration of infected monocytes and macrophages into the brain parenchyma to supplement brain macrophage populations (Strazza et al., 2011). Once viral pathogens enter the brain parenchyma, glia form the second line of defense. Both astrocytes and microglia produce proinflammatory cytokines upon exposure to HIV-1 proteins such as Tat and gp120 (El-Hage et al., 2005). While essential for containment of the virus, the glial inflammatory response contributes to HIVE. This damaging encephalitis is characterized by reactive gliosis, myelin pallor, BBB dysfunction and neurodegeneration, particularly in the hippocampus, a region crucial to memory formation (Strazza et al., 2011; Yadav and Collman, 2009).

Chronic morphine abuse increases macrophage populations, CD8⁺ T cell infiltration, and viral load in the hippocampus and striatum of HIV-1 infected patients (Bell et al., 1998; Reddy et al., 2012). Murine astrocytes co-treated with morphine and HIV-1 Tat protein increased their production of cytokines and chemokines including tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and CCL5/RANTES (regulated upon activation, normal T-cell expressed and secreted) in both *in vitro* and *in vivo* mouse models (El-Hage et al. 2008a; El-

Hage et al. 2005). These studies have led to the proposal that morphine acts in synergism with HIV-1 – particularly the inflammatory HIV-1 proteins, Tat and gp120 - to tip the immunological balance towards chronic inflammation that contributes to the development of encephalitis in the CNS (El-Hage et al. 2008b; Nath et al. 2002; Reddy et al. 2012). Prolonged inflammation can lead to oxidative stress, neurodegeneration, and further alterations in BBB permeability, all of which can increase access of peripheral virions to the CNS (Kraft-Terry et al., 2009). However, other *in vitro* studies showed that morphine decreased Tat-induced production of interleukin (IL)-8 by astrocytes (Reddy et al., 2012) and the production of TNF- α , IL-6, and CCL2/MCP-1 (monocyte chemoattractant protein-1) by microglia (Jadwiga Turchan-Cholewo et al., 2009). Morphine also disrupts type 1 interferon signaling, leading to a dysregulated cellular antiviral response and facilitating increased viral replication (Cheung et al., 1991; Wang et al., 2011). These conflicting results underline the need for further investigation into the effects of morphine in the HIV-1-infected CNS.

Taking these results together, morphine appears to influence HIV-1 CNS infection and inflammation through its effects on interactions between microglia, astrocytes, and the BBB in the context of peripheral immunodeficiency. We therefore hypothesized that chronic morphine potentiates the virally-induced increase in BBB permeability and CNS cytokine production. This could accelerate the influx of blood-borne viral particles and infected cells, exacerbating infection-induced encephalitis.

To test this hypothesis *in vivo*, we applied chronic morphine treatment to the murine acquired immunodeficiency syndrome (MAIDS) model. LP-BM5 retroviral infection induces severe immunodeficiency (Morse et al., 1992). Like HIV-1, LP-BM5 infiltrates the CNS rapidly, generating encephalitis, BBB dysfunction and spatial memory deficits within 6-8 weeks of infection (Cao et al., 2012; Kustova et al., 1999; Sei et al., 2006). This model provides a unique advantage over many available HIVE models, which often lack the viral infection and virally-induced immunodeficiency that is central to HIV-1 infection (Jaeger and Nath, 2012).

Using quantitative real-time PCR and immunohistochemistry, we investigated morphine's influence on viral load, BBB integrity and the CNS cytokine/chemokine response following LP-BM5 infection. We focused our efforts on the hippocampus, which exhibits high viral loads and inflammation in HIV-1 patients, and the frontal lobe, which shows significantly less viral infection in HIV-1 patients (Wiley et al., 1998; Yadav and Collman, 2009). In the present study, we show that in the MAIDS model, chronic morphine exerted an unexpected immunosuppressive effect on CNS cytokine/chemokine production, reducing frontal lobe CCL5 expression while increasing hippocampal CNS viral load. These studies will help elucidate the effects of chronic morphine abuse on the development and progression of HIV-associated neurocognitive disorders.

2. Materials and Methods

2.1. Animals and treatment

Seven-wk-old male C57BL/6 mice (National Cancer Institute, Frederick, MD, USA) were maintained on a 12-hour light/dark cycle and provided food and water *ad libitum*. After a week of habituation to the housing environment, mice were injected intraperitoneally with 4×10^5 plaque-forming units of LP-BM5 retroviral mixture. At 7 wks post-infection, morphine (25 mg) or placebo pellets (NIDA, Bethesda, MD, USA) were implanted subcutaneously. Previous studies have shown that morphine serum levels from these pellets begin to decline after 72 hours (Kitano and Takemori, 1979). To maintain high serum levels, a second subcutaneous morphine or placebo pellet was implanted 72 hours later. At 8 wks post-infection, animals were sacrificed by CO₂ inhalation. All experimental procedures were approved by the Institutional Animal Care and Use Committee at University of New England (Biddeford, ME, USA) and in compliance with the Animal Welfare Act and NIH Guide for the Care and Use of Laboratory Animals.

2.2. LP-BM5 virus stock

LP-BM5 retroviral stock was derived from a complete LP-BM5 isolate provided by Dr. Green of Dartmouth College (Hanover, NH, USA). Viral stock was maintained in our laboratory as previously described (Cao et al., 2012). Viral titer was determined by XC plaque assay (Cao et al., 2012).

2.3. Quantification of MAIDS development post-LP-BM5 infection

Development of MAIDS was quantified by measuring serum immunoglobulin (Ig) loads and spleen weight. Blood was drawn by cardiac puncture. Serum was collected and stored at -20°C . Serum Ig was quantified through established enzyme-linked immunosorbent assays (ELISAs) for IgG2a and IgM (Cao et al., 2012; Li and Green, 2006). Spleens were weighed immediately upon dissection and stored at -80°C for further analysis.

2.4. RNA isolation and quantitative real time polymerase chain reaction (qRT-PCR)

Tissue for PCR was frozen on dry ice immediately after dissection and stored at -80°C . Brain tissue RNA was isolated and purified with the RNeasy Lipid Tissue kit (QIAGEN, Germantown, MD, USA) per the manufacturer's instructions. Splenic tissue RNA was isolated and purified with the RNeasy Plus kit (QIAGEN). RNA (1 μg) was reverse-transcribed to 20 μl complimentary DNA (cDNA) via the iScript kit (Bio-Rad, Hercules, CA, USA).

For qRT-PCR, 0.5 μl of cDNA was amplified with Sybr Green Supermix (Bio-Rad) and quantified on a Bio-Rad iCycler system. To measure viral RNA, primers for BM5eco and BM5def *gag* genes and β -actin were used (Cook et al., 2003). The PCR protocol was as follows: 95°C for 8 min, followed by 80 cycles of 94°C for 15 sec, 63°C for 45 sec, and 72°C for 15 sec. Viral data was normalized to β -actin and relative expression levels were calculated with the C_t method, as described previously (Cao et al., 2012; Cook et al., 2003). To measure cytokines, chemokines, and GAPDH, the PCR protocol was as follows: 95°C for 15 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. Cytokine

expression was normalized to GAPDH using the Ct method. Sequences of cytokine primers were described previously (Christophi et al., 2009; Zhao et al., 2009). Sequences are summarized in Table 1. All primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

2.5. Immunohistochemistry

Coronal hippocampal sections were excised immediately after sacrifice and flash-frozen in 2-methylbutane at -30°C . Sections were stored at -80°C until they were mounted in Tissue-tek (Sakura Finetek, Torrance, CA, USA) and cut to 15 μm sections on a cryostat (Leica CM1950, Leica Microsystems, Buffalo Grove, IL, USA). Slides were stored at -80°C until stained. Immunohistochemistry was performed as described previously, at room temperature (Willis et al., 2004). In brief, tissue sections were air-dried for 10-15 min and fixed in 100% ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Slides were then washed with phosphate buffered saline (PBS), followed by PBS/bovine serum albumin (BSA)/Tween (P/BS/T: 1.0% BSA, 0.05% Tween 20 in PBS; Sigma-Aldrich). Sections were blocked with normal goat serum (1.9 mg/mL in P/BS/T, Dako, Carpinteria, CA, USA) for 30 min. All primary antibodies were diluted in P/BS/T and included claudin-5 (rabbit anti-claudin-5, 0.5 $\mu\text{g/mL}$, Life Technologies, Carlsbad, CA, USA), platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) (rat anti-mouse CD31, 0.5 $\mu\text{g/mL}$, clone ER-MP12, AbD Serotec, Raleigh, NC, USA); or laminin (rabbit anti-laminin, 0.95 $\mu\text{g/mL}$, Dako). Tissue sections were incubated with selected primary antibodies for 90 min. Secondary antibodies included goat anti-rat Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 (Life Technologies). Secondary antibodies were diluted to 4 $\mu\text{g/mL}$ in P/BS/T and incubated with tissue sections for 60 min in the dark. For identification of mouse IgG, goat anti-mouse Alexa Fluor 488 (4 $\mu\text{g/mL}$, Life Technologies) was incubated with tissue sections for 90 min. Following incubation with secondary antibodies, slides were washed with P/BS/T followed by PBS, and coverslipped with Fluoromount G (Southern Biotech, Birmingham, AL, USA) containing 1.5 $\mu\text{g/mL}$ DAPI (Sigma-Aldrich) to visualize nuclei.

Image analysis focused on the dentate gyrus region of the hippocampus. Confocal z-stack images were obtained on a Leica SP5 laser scanning confocal microscope (Leica Microsystems) utilizing 405 nm diode/argon/DPSS 561 nm lasers. Epifluorescent images were obtained on an Olympus BX53 upright microscope with an Olympus DP72 digital camera (Olympus, Center Valley, PA, USA). Images were analyzed in ImageJ (NIH, Bethesda, MD, USA). For IgG epifluorescent images, pixels above background threshold of intensity were selected to determine the area of 'leak' around vessels. Blood vessels were selected by laminin immunoreactivity and excluded from IgG analysis. For claudin-5 confocal image analysis, vessels were selected by CD31 immunoreactivity within a maximum projection image, and claudin-5 immunoreactivity measured within vessels. Contrast and brightness of the raw images were not adjusted for analysis.

2.6. Statistics

Statistical analysis was performed using Sigmapstat 3.5 (Systat Software, Inc. San Jose, CA, USA). Two-way ANOVA was used with "infection" and "morphine" as the factors. Pairwise comparisons were performed with a Student-Newman-Keuls (SNK) *post hoc* test.

Values of $p < 0.05$ were considered statistically significant. All data were presented as mean $\pm$ SEM.

3. Results

3.1. MAIDS development was unaffected by chronic morphine

LP-BM5 infection induces an immunodeficiency syndrome characterized by splenomegaly, lymphadenopathy and hypergammaglobulinemia (Morse et al., 1992). To quantify this, we measured spleen weight and serum IgG2a and IgM concentrations. Spleen weight (fig. 1A) was normalized to the individual mouse body weight to account for variation in size between animals. Normalized spleen weight increased significantly with LP-BM5 infection ($p < 0.001$), but decreased significantly in morphine-treated infected mice ($p = 0.038$). However, morphine-treated infected spleens were still significantly larger than those of non-infected morphine-treated mice ($p < 0.001$). The observed morphine effects on spleen weight may be due to morphine-potentiated splenocyte apoptosis (McCarthy et al., 2001) or efflux of splenocytes into the vasculature (Olin et al., 2012). LP-BM5 infection increased serum IgG2a ($p = 0.003$) and IgM ($p = 0.043$), as measured by ELISA, but morphine had no effect on serum Ig levels (fig. 1B, C). Beyond the decrease in spleen size, morphine did not appear to significantly inhibit the development of LP-BM5-induced MAIDS.

3.2. Chronic morphine induced regional changes in CNS LP-BM5 viral loads

The LP-BM5 retroviral mixture includes the non-pathological ecotropic (BM5eco) helper virus and the pathological defective (BM5def) virus. While BM5def induces the disease, it requires BM5eco to replicate. To investigate the effects of chronic morphine on LP-BM5 viral loads within the CNS, viral *gag* RNA relative expression was quantified with qRT-PCR in the hippocampus (fig. 2B, E) and frontal lobe (fig. 2C, F) in comparison to the spleen (fig. 2A, D). LP-BM5 infected mice showed significant increases in both BM5def and BM5eco relative expression in the spleen, hippocampus, and frontal lobe ($p < 0.001$). Further, morphine significantly increased BM5 def viral *gag* RNA expression in the hippocampus alone (fig. 2E, $p = 0.003$). No morphine effects were detected in frontal lobe or spleen. These data suggest a region-specific morphine effect in the CNS.

3.3. LP-BM5 decreased blood-brain barrier integrity in the hippocampus

To investigate the effects of chronic morphine and LP-BM5 infection on BBB integrity, we probed for serum proteins (IgG) and endothelial cell tight junctions (claudin-5) in the dentate gyrus of the hippocampus. Large serum proteins such as IgG do not cross the intact BBB, as observed in non-infected animals (fig. 3A, C). In LP-BM5 infected tissue, IgG immunoreactivity was observed surrounding blood vessels in the parenchyma (arrows, fig. 3B, D). Preliminary data indicated that IgG was detectable in the parenchyma as early as wk 4 post-infection, and peaks by wk 8 (data not shown). To avoid confounds with the hypergammaglobulinemia symptom in infected mice, BBB leak was quantified in ImageJ as the area of IgG immunoreactivity outside of blood vessels (fig. 3E). LP-BM5 infection alone significantly increased the area of IgG immunoreactivity in the parenchyma ($p = 0.004$). Chronic morphine did not affect IgG immunoreactivity within either non-infected ($p = 0.980$) or LP-BM5 infected ($p = 0.601$) animals. To investigate the functional basis of IgG leak,

hippocampus tissue was stained for the endothelial cell tight junction protein claudin-5. In non-treated control animals, claudin-5 appeared as continuous, bright immunoreactivity along blood vessels (fig. 4A, C). LP-BM5-infected animals showed regions of low claudin-5 immunoreactivity with or without chronic morphine treatment (arrows, fig. 4B, D). Previous studies have correlated this loss of tight junction expression with focal compromise of BBB integrity (Willis et al., 2004). To quantify changes in claudin-5 localization, blood vessels were selected by PECAM-1 immunoreactivity, and the average claudin-5 intensity within those vessels was measured (fig. 4E). LP-BM5 infection significantly reduced claudin-5 intensity ($p < 0.001$), which was not affected by chronic morphine treatment ($p = 0.563$).

3.4. Chronic morphine induced regional changes in CNS cytokine expression

Cytokine levels were assessed in the frontal lobe and hippocampus through qRT-PCR. Selected cytokines included IL-6, IL-12 p40, IFN- γ and the chemokine CCL5; measured but not shown were IL-1 β , TNF- α , CCL2, CCL3 and CXCL10. Previous studies have shown that LP-BM5 infection significantly increased expression of proinflammatory cytokines and chemokines (Cao et al., 2012; Cheung et al., 1991). Similar effects were observed in the present study in the frontal lobe and hippocampus of infected mice, with LP-BM5 treatment inducing significant increased CCL5 expression (fig. 5A, hippocampus, $p = 0.064$; fig. 5B, frontal lobe, $p = 0.050$). Chronic morphine treatment decreased CCL5 production in frontal lobe (fig. 5B, $p = 0.022$), returning expression to baseline levels. Similar trends were observed in frontal lobe IL-12 p40 (fig. 5F, $p = 0.521$) and frontal lobe IFN- γ (fig. 5D, $p = 0.128$) expression, but did not reach statistical significance. Unlike in the frontal lobe, chronic morphine did not reduce CCL5 expression in the hippocampus of LP-BM5 infected mice (fig. 5A, $p = 0.241$). Neither LP-BM5 nor chronic morphine had significant effects on IFN- γ or IL-12 p40 in the hippocampus (fig. 5C, E). No clear morphine effect was observed with other cytokines (IL-6 (fig. 5G-H; hippocampus, $p = 0.317$; frontal lobe, $p = 0.488$), TNF- α (data not shown)) or chemokines (CCL2, CCL3 (data not shown)). Altogether, these data suggest a CNS region-specific effect for chronic morphine on cytokine/chemokine production in LP-BM5 infected mice.

4. Discussion

In this study, we examined the effect of systemic chronic morphine administration on retroviral infection in the CNS. Development of MAIDS progressed uninhibited in chronic morphine-treated mice but had no effect on viral loads within the spleen and frontal lobe. Morphine significantly increased relative expression of BM5def viral *gag* RNA within the hippocampus, but did not increase BBB permeability. While LP-BM5 infection significantly increases expression of the proinflammatory chemokine CCL5, morphine reduced CCL5 expression to baseline levels in the frontal lobe, but not in the hippocampus. Similar trends were observed in frontal lobe IFN- γ and IL-12 p40 expression, while neither morphine nor LP-BM5 had significant effects on these cytokines in the hippocampus. Altogether, chronic morphine exerted a region-specific effect on CNS viral load and cytokine/chemokine expression in LP-BM5/MAIDS.

This study is one of the first to apply chronic morphine administration to the LP-BM5/MAIDS model, which provides distinct advantages over many other available *in vivo* murine HIV models. HIV-1 protein studies rely on intracerebral injection or genetic knock-in expression of HIV-1 Tat or gp120 in the absence of viral infection or virally-induced immunodeficiency. Humanized HIV-1 infection models offer a live viral alternative to HIV-1 Tat studies by implanting immunosuppressed animals with HIV-1-infected human-derived macrophages and lymphocytes. However, these human cells may not form functional interactions with the HIV-resistant murine glia of the CNS, and show donor-to-donor inconsistencies (Jaeger and Nath, 2012). LP-BM5 enters the CNS early in infection, where it induces BBB dysfunction, encephalitis and spatial memory deficits while productively infecting glia, similar to HIV-1 (Cao et al., 2012; Kustova et al., 1999; Sei et al., 2006; Yadav and Collman, 2009). Peripherally, LP-BM5 is marked by splenomegaly, lymphadenopathy, and hypergammaglobulinemia similar to HIV-1. Suppressed B- and T-cell responses paired with a loss of CD4⁺ T-cell function induce the HIV-1-like state termed murine AIDS (Li and Green, 2006); recently, Mutnal et al. observed a profound loss of circulating CD4⁺ and CD8⁺ T-cells in LP-BM5 animals, similar to HIV-1 patients (Mutnal et al., 2013). Altogether, LP-BM5/MAIDS offers the opportunity to explore morphine's effects within the context of an immunodeficiency-inducing virus which infiltrates the CNS, infects glial cells, and produces established effects on cognitive function.

As a major component of memory formation, the hippocampus represents a unique crossroads in this study. This region (i) expresses a high density of mu-opioid receptors (El-Hage et al., 2008b); (ii) undergoes rapid changes in BBB integrity, as seen in ischemia-reperfusion insults (Cavaglia et al., 2001); and (iii) shows increased HIV-1 viral loads and encephalitis in post mortem human studies (Wiley et al., 1998). In this study, we observed a regional increase in BM5def *gag* RNA in the hippocampus (fig. 2E) compared to frontal lobe (fig. 2F). These results are not attributable to changes in the peripheral replication of the virus, as relative expression of viral RNA remained the same in the spleen (fig. 2A, D). The stark differences in BM5def expression between hippocampus and frontal lobe suggest an intriguing region-specific mechanism for chronic morphine-potentiated viral infection in the CNS.

The chronic morphine-potentiated increase in hippocampal viral load may be due to accelerated replication of the virus, or entry of peripheral infected immune cells and/or blood borne viral particles. Viral replication is curtailed within cells by the type 1 interferon response. Mediated largely by IFN- α and IFN- β , type 1 interferon signaling increases cellular resistance to viral infection, facilitates interaction of infected cells with immune cells via increased MHC I expression, and impairs viral replication (Paul et al., 2007). Disruption of IFN- α , β signaling has been observed in morphine-treated human monocytes, leading to increased HIV-1 replication (Wang et al., 2011). Similarly, LP-BM5 infection itself was shown to disrupt the type 1 interferon response in splenocytes and other peripheral organs (Cheung et al., 1991; Pitha and Biegel, 1988). In our model, morphine could be acting synergistically with LP-BM5 to disrupt the type 1 IFN-mediated viral response, allowing for increased viral replication and facilitating infection of adjacent cells.

Alternatively, viral load could be increased due to a dysfunctional BBB, allowing increased access of infected cells and viral particles to the CNS. LP-BM5 increased the leak of serum proteins such as IgG and decreased tight junction immunoreactivity in the dentate gyrus of the hippocampus, indicating impaired BBB function (fig. 3, 4). This correlates to the reduced tight junction expression observed in HIV-1 patients (Dallasta et al., 1999). These alterations in BBB permeability can be driven by the cytokines themselves, which can reduce transendothelial electrical resistance and increase permeability of human endothelial cell monolayers to HIV-1, as observed with TNF- α in an *in vitro* BBB system (Fiala et al., 1997).

Microglia and astrocytes initiate and maintain the CNS immune response through their robust cytokine/chemokine production, yet previous studies have yielded conflicting results on morphine's influence on glial activation. In astrocytes, co-treatment with morphine and HIV-1 Tat increased the production of proinflammatory cytokines (IL-6, TNF- α) and chemokines (CCL2) through an NF- κ B-mediated pathway (El-Hage et al., 2005, 2008b). In contrast, microglia, perivascular macrophages, and peripheral blood mononuclear cells reduce production of TNF- α , CCL5 and other proinflammatory cytokines following morphine treatment (Friedman et al., 2003; Hu et al., 2000; J Turchan-Cholewo et al., 2009). In addition to its effects on astrocytic and microglial cytokine/chemokine production, morphine suppresses macrophage phagocytic activity (Friedman et al., 2003; Roy et al., 2011) and impairs the ability of human microglia to follow chemotactic signals such as CCL5 *in vitro* (Hu et al., 2000). We propose that morphine's suppressive influence on microglia and infiltrating macrophages in combination with the reduced antiviral type 1 IFN response described above could allow for accelerated HIV replication, prolonging CNS infection. The regional differences in morphine's influence on cytokine/chemokine production between the hippocampus and frontal lobe may be a reflection of regional differences in the proportion of astrocytes to microglia. The hippocampus shows increased density of microglia (Lawson et al., 1990) and astrocytes (Martin and O'Callaghan, 1995) compared to the frontal lobe. The known regional heterogeneity in astrocyte physiology could also influence these outcomes (Matyash and Kettenmann, 2010). These regional differences in glial proportion and physiology could impact both local viral infection and BBB function. While there are no known structural differences in BBB between the frontal lobe and the hippocampus, the hippocampus shows increased susceptibility to increased BBB permeability following ischemic insult (Cavaglia et al., 2001; Willis et al., 2010). In our future work, we aim to identify the effect of chronic morphine on the inflammatory responses of astrocytes and microglia in these regions in the LP-BM5/MAIDS model.

In this study, we have focused our efforts on two regions: the hippocampus and the frontal lobe. Human HIV-1 studies have shown that the hippocampus and caudate nucleus of the striatum express ten-fold higher concentrations of viral RNA than other regions, with viral load lowest in frontal cerebral cortex and cerebellar cortex (Wiley et al., 1998). The hippocampus and frontal lobe have served as representative regions of high and low HIV-1 infection susceptibility in this study. In future studies, we will expand our work to include the striatum, which is likewise sensitive to chronic morphine abuse in HIV-1 patients and is a crucial component of procedural memory formation (Reddy et al., 2012). This will enable

us to build a picture of the regional changes in vulnerability to HIV-1 infection and how these correlate to changes in cytokine/chemokine expression.

At the behavioral level, LP-BM5/MAIDS manifests as impaired performance on the Morris water maze, a spatial memory test (Sei et al., 2006). Chronic morphine injection has also been shown to induce Morris water maze deficits in mice (Li et al., 2001), but there are no studies, to our knowledge, looking at the influence of chronic morphine on LP-BM5/MAIDS-induced spatial memory deficits. As we begin to identify potential mechanisms for the changes in viral load and cytokine/chemokine production observed here, we will look towards improving these deficits in the chronic morphine LP-BM5/MAIDS model.

In the HIV-1 patient, morphine influences HIV through its combined effects on the glia and infiltrating immune cells of the CNS. Furthering our understanding of morphine's cumulative effects on viral infection and the CNS immune response is crucial to preventing the development of neurological deficits in HIV-1 patients. In this study, we observed an increase in hippocampal retroviral infection and a decline in cytokine expression with chronic morphine treatment in the CNS of MAIDS mice. Our LP-BM5 model suggests that morphine can exert CNS region-specific effects on cytokine/chemokine production and viral load. We propose that these observations stem from the conflicting effects of morphine on the glia that initiate the CNS innate immune response, facilitating BM5def viral replication within the hippocampus while impairing cytokine/chemokine production in the frontal lobe. LP-BM5/MAIDS infection provides an underexplored perspective for investigating these regional differences in the effects of morphine on astrocyte and microglial activation, BBB function, and peripheral immune cell infiltration in CNS viral infection. While other studies have examined the effect of chronic morphine on peripheral cytokine production and morphine metabolism in MAIDS (Chen and Watson, 1991), our study is the first, to our knowledge, to examine the effect of morphine on the CNS glial immune response in the MAIDS model. Based on the results presented in this study, we can begin to pursue mechanisms for the regional differences in cytokine/chemokine production and viral load observed here. The influence of chronic morphine on antiviral responses, infiltration of potentially infected peripheral immune cells, and the balance between pro- and anti-inflammatory responses of glial cells all represent potential pathways for chronic morphine-potentiated HIV/HAND. Identification of a potential pathway for the increased hippocampal viral load will open the gateway to pharmacological intervention, with the ultimate goal of improving spatial memory deficits in the MAIDS model. Through this work, we aim to unravel the contribution of opioid abuse to HIV-1-induced neurocognitive disorders and move towards improving the quality of life in HIV-1-positive patients.

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Highlights

- We applied chronic morphine treatment to the LP-BM5 MAIDS model.
- Morphine increased viral load in the hippocampus, but not the frontal lobe.
- Morphine reduced frontal lobe chemokine and cytokine production.
- We observed region-specific effects for chronic morphine in the MAIDS brain.

Figure 1.

Effects of chronic morphine treatment on peripheral LP-BM5 infection. Male C57BL/6 mice were selected for LP-BM5 infection or no infection. Mice received morphine (25 mg) or placebo pellets 7 wks post-infection. Spleens and serum were collected 8 wks post-infection. **A.** Spleen weights expressed as the ratio of spleen weight to body weight. **B, C.** ELISA was used to detect serum IgG2a (**B**) and IgM (**C**) levels. Data presented as mean $\pm$ SEM (n=11). Axis labels: BM5, LP-BM5 infected; NI, non-infected; Plac, placebo; Mor, morphine. Groups were compared using two-way ANOVA followed by a pairwise SNK *post hoc* test. *p < 0.05 between bracketed groups.

Figure 2.

Regional effects of chronic morphine on LP-BM5 viral load in spleen, hippocampus and frontal lobe. C57BL/6 mice were treated as described in fig. 1. q-RTPCR was used to quantify BM5-eco (A-C) and BM5-def (D-F) viral *gag* RNA in the spleen (A, D), hippocampus (B, E), and frontal lobe (C, F). Results were normalized to β -actin. Data presented as mean $\pm$ SEM (n=11). ND = non-detected. Axis labels: BM5, LP-BM5 infected; NI, non-infected; Plac, placebo; Mor, morphine. Groups were compared using two-way ANOVA followed by a pairwise SNK *post hoc* test. *p < 0.05 between bracketed groups.

Figure 3.

Effects of chronic morphine and LP-BM5 infection on blood-brain barrier integrity in hippocampus. C57BL/6 mice were treated as described in fig. 1. **A-D.** Representative widefield images of hippocampal dentate gyrus labeled for IgG (green) and laminin (red). Large serum proteins such as IgG are restricted to blood vessels (laminin, red) in the brain in control animals. LP-BM5 infected mice showed a significant increase in IgG immunoreactivity in the parenchyma (arrows, B, D). Images were taken via epifluorescence at 20 $\times$, scale bar = 150 μ m. **E.** To quantify the leak of IgG, ImageJ was used to quantify the

total area in pixels of immunoreactivity above background fluorescence. Blood vessels were selected by laminin immunoreactivity (not shown) and excluded from analysis. Data presented in **E** as mean $\pm$ SEM (n=11). Axis labels: BM5, LP-BM5 infected; NI, non-infected; Plac, placebo; Mor, morphine. Groups were compared using two-way ANOVA followed by a pairwise SNK *post hoc* test. *p < 0.05 between bracketed groups.

Figure 4.

Effects of chronic morphine and LP-BM5 infection on claudin-5 localization in the hippocampus. C57BL/6 mice were treated as described in fig. 1. **A-D**. Representative confocal images of claudin-5 in the dentate gyrus region of the hippocampus. In control mice (**A**, **B**) claudin-5 shows contiguous immunoreactivity along with PECAM-1-positive blood vessels (inset). Compromised vessels showed reduced claudin-5 immunoreactivity (arrows, **C**, **D**). **E**. Claudin-5 intensity was quantified in ImageJ. Blood vessels were selected by PECAM-1 immunoreactivity, and the average intensity of claudin-5 within these vessels was measured. Images were captured at 40×, scale bar = 100 μm. Data presented in **E** as mean ± SEM (n=11). Axis labels: BM5, LP-BM5 infected; NI, non-infected; Plac, placebo; Mor, morphine. Groups were compared using two-way ANOVA followed by a pairwise SNK *post hoc* test. * $p < 0.05$ between bracketed groups.

Figure 5.

Effects of chronic morphine on cytokine expression in the hippocampus and the frontal lobe. C57BL/6 mice were treated as described in fig. 1. Relative expression of CCL5 (A, B), IFN- γ (C, D), IL-12 p40 (E, F), and IL-6 (G, H) were analyzed in hippocampus and frontal lobe via qRT-PCR. Results were normalized to GAPDH. Data presented as mean $\pm$ SEM (n=11). Axis labels: BM5, LP-BM5 infected; NI, non-infected; Plac, placebo; Mor, morphine.

Groups were compared using two-way ANOVA followed by a pairwise SNK *post hoc* test.

* $p < 0.05$ between bracketed groups; † $p < 0.06$ between bracketed groups.

Table 1
Cytokine qRT-PCR Primers

Product of Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>CCL2</i> ¹	GTATGTCTGGACCCATTCC TTC	GCTGTAGTTTTTGTCAACCAAG C
<i>CCL3</i> ¹	CAGCCAGGTGTCATTTTCC T	AGGCATTCAAGTTCCAGGTCA
<i>CCL5</i> ¹	AGCTGCCCTCACCATCATC	CTCTGGGTGGCACACACTT
<i>CXCL10</i> ¹	TTTCTGCCTCATCCTGCTG	CTCATCATCTTTTTCATCGT G
<i>GAPDH</i> ¹	ACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA
<i>IFN-γ</i> ¹	GCCATCAGCAACAACATAA GC	CAGCAGCGACTCCTTTTCC
<i>IL-1β</i> ²	TCCAGGATGAGGACATGA GCAC	GAACGTCACACACCAGCAGG TTA
<i>IL-6</i> ¹	CAGAGGATACCACTACCA CAG	TCTCATTTCCACCACGATTTC CC
<i>IL-12 p40</i> ¹	CCATTGAACTGGCGTTGGA AG	CGGGTCTGGTTTGATGATGT CC
<i>TNF-α</i> ¹	TGAACTTCGGGGTGATCG GTC	AGCCTTGTCCTTGAAGAGA AC

¹ Christophi et al. 2009;

² Zhao et al. 2009