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Interactions between HIV and opioids on antiretroviral accumulation, the blood brain barrier, and the inflammatory response in the brain.

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Acronyms

AAN	The American Academy of Neurology
ABC	Abacavir
ABCB	ATP-binding cassette class B
AIDS	Acquired immunodeficiency syndrome
ANI	Asymptomatic neurocognitive impairment
ANOVA	Analysis of variance
API	Atmospheric pressure ionization
ART	Antiretroviral therapy
ARV	Antiretroviral
BBB	Blood brain barrier
BCA	Bicinchoninic acid
BCRP	Breast cancer resistant protein
BLC	B lymphocyte chemoattractant
BMEC	Brain microvascular endothelial cell
BMVEC	Brain microvascular endothelial cell
CCL	C-C motif ligand
CCR	C-C motif receptor
CDC	Center for Disease Control
CLDN	Claudin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCL	C-X-C motif ligand
CXCR	C-X-C motif receptor
DNA	Deoxyribonucleic acid
DOX	Doxycycline chow
DTG	Dolutegravir
ELISA	Enzyme-linked immunoassay
ERK	Extracellular signal-regulated kinase
FDA	Federal Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
HAD	HIV associated dementia
HAND	HIV associated neurocognitive disorders
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
ICAM	Intracellular adhesion molecule
IFN	Interferon
IL	Interleukin
IMF	Illicitly-manufactured fentanyl
IV	Intravenous
JAMS	junction adhesion molecules
JNK	c-Jun N-terminal kinase
KC	keratinocyte-derived chemokine
LC-MS	Liquid chromatography mass-spectrometry

LIX	Lipopolysaccharide-induced CXC chemokine
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
MDC	Macrophage-derived chemokine
MDM	Monocyte-derived macrophage
MEK	Mitogen-activated protein kinase
MIG	Monokine induced by interferon-γ chemokine
MIP	Macrophage inflammatory protein
MOR	μ (mu) opioid receptor
NIDA	National Institute on Drug Abuse
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartate
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NVU	Neurovascular unit
OCT	Organic cation transporter
OUD	Opioid use disorder
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PFA	Paraformaldehyde
PI	Protease inhibitor
PLWH	People living with HIV
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcriptase
SA-PE	Streptavidin-phycoerythrin
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
STAT	Signal transducers and activators of transcription
TARC	Thymus and activation-regulated chemokine
TBST	Tris-buffered saline and polysorbate 20
3TC	Lamivudine
TEER	Transepithelial/transendothelial electrical resistance
TGX	Tris-Glycine eXtended
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRE	Tetracycline responsive element
UNAIDS	The Joint United Nations Program on HIV/AIDS
VCAM	Vascular cell adhesion molecule
ZO	Zonula occludens

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By Kara Rademeyer, PharmD
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Abstract

The complex mechanisms related to HIV infection, neurodegeneration, and chronic neuroinflammation collectively describe neuroHIV (Hauser et al. 2007; Chang et al. 2014; Smith et al. 2014). Specifically, opioid abuse, poor penetration of antiretroviral (ARV) drugs, chronic inflammation and neuronal injury/degeneration are all implicated in neuroHIV (Fantuzzi et al. 2003; Letendre et al. 2004; Verani et al. 2005; Duncan and Sattentau 2011; Hong and Banks 2015; Simoes and Justino 2015; Olivier et al. 2018; Murphy et al. 2019; Osborne et al. 2020). For the first time, we demonstrate that morphine, fentanyl, and methadone *in vivo* alter the brain accumulation of ARVs, impair the blood brain barrier (BBB), and dysregulate the immune response. The purpose of this body of work was to examine the interplay between HIV and opioids within the brain. Using two different animal model systems to mimic key aspects of the neuropathology of HIV, we examined three different opioids drugs and their effects on ARV accumulation in the striatum and hippocampus, transporters and tight junctions at the BBB, and chemokine concentrations. Morphine and fentanyl mediated BBB disruption through the modulation of tight junction and junction accessory proteins, claudin-5 and ZO-1, while methadone altered the expression of P-glycoprotein, an efflux transporter. All three opioids dysregulated chemokines in the presence of HIV-1 Tat and/or EcoHIV.

Chapter 1. Introduction

Globally, 38.4 million people were living with HIV in 2021, where 1.5 million accounted for new infections, and only 28.7 million had access to antiretroviral therapy (UNAIDS 2022). In the United States, 1.2 million people older than 13 were living with HIV in 2019 (HIV.gov 2021). The introduction of combination antiretroviral therapy (cART) in 1996 was the first major step in transforming the once deadly disease into a more manageable one (Paterson et al. 2000; Eggers et al. 2017; Javadi et al. 2018; Forsythe et al. 2019). Despite adequate viral suppression by combination antiretroviral therapy (cART), HIV-associated neurocognitive disorders (HAND) continue to persist and influence the quality of life for persons living with HIV-1 infection (Heaton et al. 2011; Eggers et al. 2017). The American Academy of Neurology (AAN) has established criteria for diagnosing HAND, and in the current post-cART era, there is clear evidence that milder forms of HAND persist and that people with mild HAND typically experience symptoms such as deficits in attention, learning and executive function (Heaton et al. 2011; Hong and Banks 2015; Saylor and Sacktor 2016; Sacktor 2018).

NeuroHIV describes the complex and overlapping mechanisms related to the pathogenesis of HAND; including drug use, persistent neuroinflammation, direct neurotoxicity in the brain (Hong and Banks 2015; Olivier et al. 2018; Murphy et al. 2019), poor efficacy of ARVs in the brain (Letendre et al. 2004; Simoes and Justino 2015; Osborne et al. 2020), and incomplete viral suppression in the brain (Hong and Banks 2015). Since the start of the HIV epidemic, opioid abuse and the subsequent opioid epidemic has remained interlinked (Leshner 1998). In 2017, approximately 1 in 10 new HIV infections were due to injection drug use (CDC 2022).

In the setting of HIV, opioid co-exposure exacerbates neuroHIV and substantially modulates the immune response. There is clear evidence that opioids act directly through the peripheral immune system, which is different from the effects of opioids in the brain (Hauser et al. 2007). Morphine, a prototype for studying drugs of abuse, has been demonstrated by various studies to increase the replication of HIV and enhance the migration of monocytes into the brain (Hauser et al. 2012; Murphy et al. 2019; Fitting et al. 2020)

HIV infection in the brain and the accompanying neuroinflammation associated with HAND is collectively known as neuroHIV (Hauser et al. 2007; Clifford and Ances 2013; Chang et al. 2014). NeuroHIV is greatly exacerbated by opioids, such as morphine and its metabolites. The exacerbation of neuroHIV by morphine has been demonstrated through increased expression of chemokine receptors necessary for viral cell entry, increased production of cytokines and chemokines and decreased antiretroviral drug concentrations in the brain (Horuk et al. 1997; Leibrand et al. 2019; Gabel et al. 2022). Once infected, cells within the brain can release direct neurotoxic viral proteins leading to the activation of bystander cells. The activation promotes the release of cytokines and chemokines, able to activate other cells and cascades (Meltzer et al. 1990; Fantuzzi et al. 2003). Opioids have immunomodulatory effects and increase reactive oxygen species (ROS) production by macrophages resulting in neurodegeneration and release of additional cytokines and chemokines (Hauser et al. 2007). Overall, the unbalanced

concentrations of cytokines, chemokines, ROS, other neurotoxic viral proteins within the brain, and the presence of opioids, promotes universal neuronal dysfunction and degeneration responsible for the neurocognitive manifestations of neuroHIV (Hauser et al. 2006, 2007, 2012; Fitting et al. 2010a).

1A: HIV

Human immunodeficiency virus (HIV) is a lentivirus originating from the *Retroviridae* family known to target the immune system (Levy 1993; Alam et al. 2016). The genome of the HIV retrovirus is composed of two identical RNA strands and structural genes *gag, pol,* and *env* (Fanales-Belasio et al. 2010). HIV isolates can be grouped into two types, HIV-1 and HIV-2, where HIV-1 is mostly responsible for the progression of HIV to acquired immunodeficiency syndrome (AIDS) world-wide and is the isolate referred to as HIV (Nyamweya et al. 2013; Bbosa et al. 2019). Between June and July of 1981, the first few cases of HIV were reported in the United States (US)(CDC 2021; KFF 2021). Upon discovery, it was not yet known how the virus was transmitted, but officials traced transmission to injection drug use as cases began to rise among people who inject drugs (CDC 2021). It would not be until 1982 that the U.S Food and Drug Administration licensed the first commercial blood kit that can detect HIV (CDC 2021). Since the first case, HIV and AIDS has been highly researched, however, a definite cure to the virus is yet to be developed (Wallet et al. 2019).

Since the discovery of the HIV virus, a considerable amount of literature describes the complex molecular and viral interactions that occurs when the lentivirus enters into a host (Levy 1993; Bagnarelli et al. 1996; Fanales-Belasio et al. 2010; Wilen et al. 2012; Bbosa et al. 2019). It is clear that HIV infection includes a multistep process requiring precise host and viral components (Bagnarelli et al. 1996; Wilen et al. 2012). During the first phase of the viral replication cycle, the HIV viral envelope consisting of subunits, gp41 and gp120, a viral membrane, and a variable loop 3, bind with the CD4 receptor and coreceptor on cells expressing the CD4 receptor resulting in a favorable conformation change (Haim et al. 2011; Grove and Marsh 2011; Wilen et al. 2012). Next, the conformational change allows for the fusion of subunit gp41 to the host membrane (Pan et al. 2010; Wilen et al. 2012). The third step of HIV infection is characterized by coreceptor binding, where HIV strains typically either bind to the chemokine coreceptor CCR5, termed R5 HIV, or to the chemokine coreceptor CXCR4, termed X4 HIV (Wilen et al. 2012).

Although most viral strains are either R5 HIV or X4 HIV, several viruses are able to use both coreceptors and are classified as R5X4 HIV (Berger et al. 1998). During coreceptor binding, a hydrophobic “fusion peptide” at the N-terminus of the gp41 subunit penetrates the cell membrane and anchors the virus to the host target cell (Bosch et al. 1989; Haim et al. 2011). Once anchored, the virus releases the viral core into the cytoplasm where it undergoes reverse transcription to produce double-stranded DNA containing long terminal repeats (LTRs) able to integrate into the host cell genome (Jonckheere et al. 2000; Ferguson et al. 2002; Hu and Hughes 2012). The complete double-stranded DNA with recognizable LTRs is then integrated by the viral integrase protein into the host chromosome, followed by the formation of the provirus (Ferguson et al. 2002). As the cell replicates host chromosomal

DNA, the viral genes are replicated as a consequence of integration (Ferguson et al. 2002). The LTR regions in the viral DNA signals host factors to synthesize, process, and translate viral mRNA that is later spliced resulting in the production of spliced mRNA encoding for HIV genes *tat, nef, rev, env, and gag* (Ferguson et al. 2002). Through complex interactions between the Gag protein and virus particles, virions assemble into infectious cylinder-shaped buds that are later released from the host cell and able to continue the infection and replication cycle in new cells expressing the CD4 receptor (Ferguson et al. 2002). Within the large group of CD4 expressing cells, several cells can become 'latent' viral reservoirs, in which, viral DNA is integrated in the genome of a long lived cell, the cell itself is in a resting stage allowing for viral DNA to persist undetected, or replication-competent viral components are present in the cell and able to undergo replication upon activation (Eisele and Siliciano 2012; Pedro et al. 2019).

1B. HIV in the Central Nervous System

Due to the biological nature of lentiviruses, HIV is able to infect and replicate in non-dividing cells such monocytes and macrophages (Meltzer et al. 1990; Verani et al. 2005; Eugenin et al. 2010). During the early stages of infection, HIV enters the CNS through the 'Trojan Horse' mechanism, whereby HIV-infected monocytes cross the blood-brain barrier (BBB), the physical and chemical gate keeper, and differentiate into perivascular macrophages (Eugenin et al. 2010; Lindl et al. 2010; Siliciano and Greene 2011). Macrophages are terminally-differentiated resident cells that play an important role in the innate immune system and act as an interface between the innate and adaptive immune system (Verani et al. 2005; Duncan and Sattentau 2011). Within HIV-infected monocytes, the virus is able to accumulate in the intracellular components of monocyte-derived macrophages unlike in CD_4^+ T lymphocytes (Fantuzzi et al. 2003). Under normal conditions, macrophages are highly involved in host defense, repairing tissues, and eliminating or controlling infections by destroying invasive pathogens or through the secretion of cytokines and chemokines (Verani et al. 2005; Galione and Davis 2018; Guerriero 2019; Dupont and Sattentau 2020).

Infected macrophages stray from their normal functions and promote neural inflammation as they enhance the secretion of cytokines, contribute in the production of neurotoxins, and display impaired immune-protective functions within the brain (Fantuzzi et al. 2003; Wong et al. 2019). A pivotal role in the pathogenesis of HIV infection in the CNS is the virus-induced recruitment of large numbers of monocytes and lymphocytes into the brain, that cross the BBB and release virus into the CNS creating a vicious cycle of viral replication and reservoir formation in the brain (Fantuzzi et al. 2003; Pedro et al. 2019). Macrophages constitutively express low levels of chemokine receptors that act as the HIV virus receptor sites, CD4, CCR5, and CXCR4. Macrophages, therefore, are subject to less efficient infection than typical infection of cells highly expressing the viral binding sites (Dupont and Sattentau 2020). The infection of macrophages is vastly different from that of a T cell and results in functional deficits and viral reservoir formation (Meltzer et al. 1990; Verani et al. 2005). Macrophage infection exists without obvious cytopathic effects in the brain, however, macrophages are sites where long-lived viral reservoirs are established and

where antiretroviral therapies are not able to reach (Verani et al. 2005; Nath and Clements 2011; Duncan and Sattentau 2011).

In addition to infection within macrophages, brain resident cells, such as microglia, are also susceptible to infection (Kitai et al. 2000; Wallet et al. 2019). Both infected macrophages and microglia are unfortunate victims for HIV reservoirs in the brain because of their long life span, in comparison to $CD4^+$ T cells (Castellano et al. 2019). Another hallmark of HIV infection in the CNS is related to the grave dysfunction of the immune response. As infected cells become activated by viral factors or host response factors, they stimulate the secretion of cytokines and chemokines further feeding into neuroinflammation (Dutta and Roy 2012; Fois and Brew 2015). The manifestations of HIV in the brain including overt neuroinflammation and neurotoxicity that impair neurotransmission and neuroimmune communication are collectively known as neuroHIV (Hauser et al. 2007; Smith et al. 2014; Gaskill et al. 2021).

To study HIV in the CNS, animal models are frequently utilized. The current research utilizes two types of animal models. The first model is the Tat-transgenic mouse model, which is commonly used in examining HIV-associated neurocognitive impairment and neurodegeneration (Kim et al. 2003; King et al. 2006; Leibrand et al. 2017, 2019; Joshi et al. 2020; Leibrand et al. 2022). The Tat-transgenic mouse model is created by inserting a tetracycline responsive element (TRE) and a glial fibrillary acidic protein (GFAP)-driven tetracycline promoter into the genome, which specifically results in Tat production in the brain (Bruce-Keller et al. 2008). Tat(+) experimental mice produce and express Tat in the brain once exposed to doxycycline in their chow, while Tat-control (Tat(–) mice do not have the TRE element and thus do not produce Tat in response to doxycycline exposure (Bruce-Keller et al. 2008). In experimental mice, Tat expression remains elevated in the brain for up to three weeks post doxycycline chow cessation (Guedia et al. 2016). This model has been used to study the various mechanisms related to the development of HAND with the understanding that Tat promotes neurotoxicity, neuroinflammation and dysregulation in cell signaling cascades in both infected and uninfected cells (King et al. 2006; Ajasin and Eugenin 2020; Nass et al. 2020; Leibrand et al. 2022).

Another model utilized in the current research is the EcoHIV model. Unlike the Tat-transgenic model, the EcoHIV mouse model is characterized by active infection, limited to rodents (Ceckova et al. 2018). To limit infection to rodents, constructing the EcoHIV model consists of replacing the HIV envelope glycoprotein, *gp120*, with *gp80* (Potash et al. 2005; Ceckova et al. 2018). Initial studies utilizing this model have revealed that infection is established after one inoculation and the virus can be detected in the spleen, macrophages, and the brain within two-to-twelve weeks after initial inoculation (Potash et al. 2005; Ceckova et al. 2018). Similar to HIV in humans, in EcoHIV infected mice, infected monocytes traffic into brain, the virus induces an immune response characterized by elevated cytokines and chemokines, produces neurotoxic insults, and results in the activation of brain resident cells (Kelschenbach et al. 2012; Olson et al. 2018; Dong et al. 2020). While both the Tat-transgenic and EcoHIV mouse models have been used to examine aspects of interacting mechanisms of opioids and HIV infection on the mechanisms of HIV-associated neurocognitive impairment and neurodegeneration, few studies have utilized these

models *in vivo* to examine the interactions between fentanyl and Tat/EcoHIV on the accumulation of ARVs within the brain, the regulation of the immune response, and any associated impairment or breakdown in the BBB.

1C. Neuroinflammation and HAND

Despite the development and widespread use of cART within the US, HIV-associated neurocognitive disorders continue to persist. HIV-neurocognitive disorders or HAND is collectively used to describe neurocognitive dysfunction, which is further characterized by asymptomatic neurocognitive impairment (ANI), mild-HAND to severe-HAND, or HIV-associated dementia (HAD) (Clifford and Ances 2013; Saylor et al. 2016). The prevalence of HAND among PLWH in the current era, is notably due to increased survival from the widespread use of cART (Nath et al. 2008; Alfahad and Nath 2013). Symptoms associated with HAND progress, but eventually include short term memory loss, mental slowing, difficulties in comprehending reading materials, gait disturbances, and apathy (Nath et al. 2008; Clifford and Ances 2013). During the initial phase of HIV infection, HAD is rare, even with comorbid opioid exposure (Nath et al. 2008). HAND has been associated with elevated chemokine, CC-motif ligand 2 (CCL2) levels, where the concentration of CCL2 increases over time of HIV infection and becomes predictive of the development of HAD (McArthur 2004). HAND is also accompanied by high concentrations of interleukin (IL)-8, CCL2/monocyte chemotactic protein (MCP)-1, CXCL10/induced protein (IP)-10, and granulocyte colony-stimulating factor (G-CSF), macrophage/monocyte derived tumor necrosis factor-alpha (TNF-α) (Wesselingh et al. 1993; Yuan et al. 2013).

Noticeable alterations in neuronal morphology and function, pronounced apoptosis of brain resident cells, chronic neuroinflammation, sustained immune activation, and existing viral reservoirs have been associated with HAND and are suggested to be responsible for the progression from ANI or mild-HAND to HAD (Kolson and González-Scarano 2000; Ellis et al. 2007; Duncan and Sattentau 2011; Clifford and Ances 2013; Hong and Banks 2015; Saylor et al. 2016). Upon activation by viral or immune factors or cell signaling cascades, HIV infected cells within the brain secrete neurotoxic HIV viral proteins such as HIV-1 Tat, Nef, Ref and gp120 (Chang et al. 1997; El-Hage et al. 2005; Hauser et al. 2006; King et al. 2006; Kovalevich and Langford 2012; Hategan et al. 2017; Ajasin and Eugenin 2020). The release of these toxins in the brain results in subsequent neuron and astrocyte dysfunction, both which are associated with HAND (Kaul et al. 2001; Wallace 2006). The viral proteins Tat and gp-120 are the most studied neurotoxic viral particles that contribute to the pathogenesis of HAND. Tat is found in the CSF of PLWH on cART with viral suppression (Johnson et al. 2013; Henderson et al. 2019) Both gp-120 and Tat have been implicated in modifying calcium homeostasis, increasing oxidative stress, and promoting neuronal excitotoxicity (Haughey and Mattson 2002; King et al. 2006; Hauser et al. 2007; Canonico et al. 2022). Microglia and astrocytes remain activated upon exposure to neurotoxins, resulting in increased production of inflammatory mediators including cytokines and chemokines (El-Hage et al. 2005; Pereyra et al. 2008; Liu et al. 2015; Walker et al. 2015; Chivero et al. 2017; Borrajo et al. 2021)

The direct microglial involvement in HAND pathogenesis is understood to be a major driver of HIV within the brain (Wallet et al. 2019; Castellano et al. 2019). Additionally, parallels between neurological disorders such as HAND, multiple sclerosis, and Alzheimer's disease reveal pronounced inflammatory processes caused by microglia including an overabundance of secreted cytokines, chemokines, and nitric oxide (Wallace 2006). Astrocyte involvement in the development of HAND and establishment of sustained neuroinflammation is observed by the exposure of Tat to astrocytes (Chauhan et al. 2003; El-Hage et al. 2005, 2006; Hauser et al. 2007; El-Hage et al. 2008; Hauser and Knapp 2014; Fitting et al. 2014; Joshi et al. 2020). It has been demonstrated that astrocytes transfected with HIV proviral DNA can support the expression of the Tat protein, and that the Tat expressed in astrocytes is not inherently toxic but makes the astrocyte resistant to oxidative stress, which in turn, promotes long-term survival of the viral genome within these cells (Chauhan et al. 2003; Zhou et al. 2004; Nookala and Kumar 2014). Astrocytes do not harbor actively dividing virus but remain viral reservoir sites where viral proteins such as Tat may be released (Pocernich et al. 2005; Li et al. 2020; Valdebenito et al. 2021). Intracellular Tat can be released by astrocytes and taken up by surrounding cells, such as macrophages/ monocytes, microglia, and additional astrocytes, where it produces neurotoxic effects and viral reactivation in viral cell reservoirs through transcellular transactivation of the HIV long-terminal repeat (LTR) (Chauhan et al. 2003; Ajasin and Eugenin 2020; Real et al. 2022). Astrocytes make up a majority of brain resident cells and act as immune cells through releasing cytokines and chemokines in response to injury or infection (Nookala et al. 2013). Astrocytes have been demonstrated to be more reactive to Tat than gp120, where extracellular Tat exposure results in the overproduction of cytokines and chemokines compared to basal levels produced in the absence of Tat (Fitting et al. 2010b; Podhaizer et al. 2012). Activated and infected microglia also release cytokines and chemokines which further increase the elevated circulating levels of these inflammatory mediators, ultimately leading to neurotoxicity (Garden 2002; Turchan-Cholewo et al. 2009). Infected and/or activated astrocytes and microglia contribute to the sustained secretion of cytokines and chemokines which recruits additional monocytes into the brain and maintain the activation of cells in the brain, creating a persistent loop of neuroinflammation and neurotoxic effects (Hauser et al. 2007). Macrophages in the brain also secrete cytokines and chemokines in response to several other factors, including gp120, Tat, HIV infection, viral transcription reactivation, or by constitutive functioning (Kaul et al. 2001; Herbein et al. 2010; Herbein and Varin 2010).

1D. Neuroinflammation and Chemokines

Transient neuroinflammation is a state of inflammation in the brain that is mediated by the production of cytokines, chemokines, reactive oxygen species, and secondary messengers in response to a foreign pathogen in the CNS (DiSabato et al. 2016). Because HIV infection causes severe dysfunctions in the immune response, neuroinflammation is chronic, and is characterized by an overabundance of the inflammatory, toxic, and secondary mediators that result in neurotoxicity and contribute to the pathogenesis of HAND (Kaul et al. 2001). Chronic inflammation in the brain, characterized by an

overabundance of cytokines and chemokines have been demonstrated not just in HIV and HAND, but also with other CNS inflammatory pathologies previously mentioned (Simpson et al. 1998; Cai et al. 2014). Chemokines represent a superfamily of small, highly conserved chemoattractant cytokines (Miller and Krangel 1992; Springer 1994; Zlotnik and Yoshie 2000). Chemokines are named based on the location of their cysteine residues and are then characterized by the “CC” or “CXC” prefixes (Miller and Krangel 1992; Springer 1994). Under normal conditions, chemokines function by stimulating the migration of white blood cells (leukocytes), regulating angiogenesis, exerting antimicrobial activity, and immunosurveillance (Hughes and Nibbs 2018). In the setting of HIV, chemokines display similar actions, with the exception that overexpression of chemokines alters the already dysfunctional cell-signaling cascades, which can result in neurotoxic insults in the brain.

The production or alteration of anti-inflammatory and proinflammatory cytokines/chemokines occurs rapidly during the first 1 to 2 weeks of primary HIV infection induced by widespread T cell activation (Teigler et al. 2018). Several studies reveal the involvement of chemokines in the neuropathogenesis of neuroHIV and subsequent development of HAND. Chemokine induced neurotoxicity is not a direct mechanism, but rather occurs indirectly as chemokines alter signaling cascades that are involved in carrying out direct neurotoxicity. Inflammatory cytokines and chemokines indirectly contribute to brain injury by activating additional infected cells in the brain and promoting the release of neurotoxic mediators (Kaul et al. 2001). Additionally, overexpression of cytokines and chemokines alters the physiological function of astrocytes, eventually resulting in neuronal toxicity and death (Rappaport and Volsky 2015). Specifically, CCL2 and CC-motif ligand 5 (CCL5) increase monocyte migration into the brain (He and Crews 2008; Li et al. 2011), potentially increasing viral entry into the brain through HIV infected monocytes (Schall et al. 1990; El-Hage et al. 2005; Stamatovic et al. 2005; Wong et al. 2019). CCL2 and CCL5 are also known to have additional effects. CCL2 decreases the BBB integrity by altering tight junction proteins and matrix metalloproteinases *in vitro* and *in vivo* (Stamatovic et al. 2005; Eugenin 2006; Dhillon et al. 2008). CCL5 also promotes a loss of BBB integrity through binding at the chemokine receptor, CCR1; this loss of barrier function allows unrestricted cell entry into the brain (Terao et al. 2008). Infected cells secrete cytokines, such as IL-1β, which signals for additional chemokine production and is responsible for inducing neuronal injury or death (El-Hage et al. 2008). There is a clear cycle of chemokine and cytokine induced signaling that maintains the persistent production of additional chemokines characteristic of chronic neuroinflammation (Kitai et al. 2000). While it is established that several chemokines are generally associated with worsened HIV outcomes and dysfunctional neuroinflammatory responses , other chemokines like, CCL3, CCL4, and CCL5 (Cheeran et al. 2001; Nakayama et al. 2006) produced by activated cells, have been demonstrated to have HIV suppressive activity (McManus et al. 1998; Cocchi et al. 2000; Cheeran et al. 2001; Nakayama et al. 2006; Flórez-Álvarez et al. 2018). Although CCL3, CCL4, and CCL5 have been demonstrated to have antiviral activity, other chemokines are known to further dysregulate HIV neurotoxicity by enhancing the production of inflammatory cytokines (Sindhu et al. 2019). The proinflammatory cytokines, TNF-α,

interleukin-1-beta (IL-1β), and interleukin-6 (IL-6) are well known to stimulate HIV replication in T cells and monocyte-derived macrophages, while IL-2 upregulates HIV replication and infection in T cells (Kedzierska et al. 2003). A subset of monocytes which are $CD16^+$ preferentially differentiate into macrophages and produce CC-motif ligand 22 (CCL22) and CC-motif ligand 17 (CCL17) (Ancuta et al. 2006). Both of these chemokines bind to the chemokine receptors, CCR3 and CCR4, present on T cells, resulting in T cell activation and enhanced susceptibility for productive HIV infection (Ancuta et al. 2006; Scheu et al. 2017).

While these are just a few examples, chemokines have remained a focus in the setting of HIV, because the virus utilizes chemokine receptors found on a variety of cells, to enter the cells and replicate. While chemokines play a role in the early stages of infection, they have also been demonstrated to have post-entry effects. In order for the virus to replicate within the cell, reverse transcription requires the polymerization of the actin component in the cytoskeletal network (Bukrinskaya et al. 1998). The exposure of cells with non-dividing virus to chemokines has been thought to activate these cytoskeletal processes through increasing intracellular calcium which in turn stimulates HIV replication (Kinter et al. 1998). Complicating this process is the exposure to HIV infected cells by opioids. Opioids have immunomodulatory effects, and can act in synergy with Tat to increase TNF-α, IL-1β and IL-6 and activate brain resident cells (Bokhari et al. 2011). Further, opioids enhance the expression of chemokine receptors, CCR5 and CXCR4, used by HIV thus promoting viral binding and HIV entry into cells(Steele et al. 2003; Mahajan et al. 2005; El-Hage et al. 2008). Additionally, opioids potentiate Tat effects through activating nuclear factor-kappa B (NF-κB) signaling which results in the production of chemokines in brain resident cells (El-Hage et al. 2008).

As inflammatory mediators, chemokines greatly contribute to the inflammatory response within the brain, and thus, are important markers to evaluate as it pertains to neuroHIV and the pathogenesis of HAND (Kinter et al. 2000; Hong and Banks 2015). The ability of certain chemokines to participate in chemokine-mediated regulation of enhanced viral replication demonstrates the importance of understanding chemokine concentrations in the brain with exposure to cART. Because opioids alone have immunomodulatory effects, it is also important to reveal opioid interactions on specific chemokines, especially those involved in maintaining homeostasis and viral suppression.

1E: Blood Brain Barrier

At its core physiological role, the blood brain barrier (BBB) exists as a diffusion barrier protecting the brain (Ballabh et al. 2004). The brain is shielded from toxic substances within the blood by the BBB, which also serves to filter substances going from the brain into the blood, and supplying nutrients to the brain tissues (Ballabh et al. 2004; Persidsky et al. 2006). Other barriers protecting the brain include the blood-cerebrospinal fluid (CSF) barrier, and the arachnoid barrier (Kadry et al. 2020). Endothelial cells, astrocyte end-feet, and pericytes make up the cellular component of the BBB, while the capillary basement membrane, microglial and neuronal cells complete the entire structure collectively called the

neurovascular unit (NVU) (Kadry et al. 2020). The brain microvascular endothelial cells (BMVEC) are essential for not just creating the basic barrier, but also for transporting nutrients, participating in receptor-mediated signaling, leukocyte trafficking and maintaining osmotic pressure (Persidsky et al. 2006). Structural components of the BMVEC are characterized by unique morphological, functional, and structural qualities that are involved in carrying out specific functions. Tight junctions are expressed between BMVEC, which serve to secure paracellular pathways between adjacent BMVEC (Persidsky et al. 2006). The tight junction proteins holding BMVEC together include claudin, occludin, and junction adhesion molecules (JAMS) which are accompanied by cytoplasmic accessory proteins such as Zonula occludens (ZO-1, ZO-2, ZO-3) and cingulin (Kadry et al. 2020). Cytoplasmic accessory proteins maintain the structural and functional integrity of the BBB endothelium formed by BMVEC (Kadry et al. 2020). Claudin-1 and claudin-5 make up the major components of tight junction proteins, where a primary seal is formed between tight junctions as claudin molecules on adjacent endothelial cells bind together and link with cytoplasmic proteins ZO-1, ZO-2, ZO-3, cingulin, and others (Furuse et al. 1999; Kadry et al. 2020). Zonula occludens proteins, including several other proteins, have the essential role for creating the cytoplasmic bridge that connects all tight junctions to the cytoskeleton of the endothelial barrier (Kadry et al. 2020). The cytoskeleton is further joined together by adherens junctions, which anchor tight junctions to the basement membrane by adhesive contacts between BMVEC (Kadry et al. 2020). Because adherens assemble through homophilic calcium-dependent interactions, any changes in either intracellular or extracellular calcium concentrations can affect the integrity and function of tight junctions (Abbott et al. 2006; Kadry et al. 2020). Tight junctions between BMVEC restrict the paracellular diffusion of polar molecules, while solute carriers and transport proteins regulate small molecule entry and efflux at the BBB (Laterra et al. 1999; Abbott and Friedman 2012). Several studies have revealed mechanisms related to HIV induced BBB dysfunction as well as alterations in the expression and function of drug transport proteins (Persidsky et al. 1999; Nottet 1999; Buckner et al. 2006; Konsman et al. 2006; Atluri et al. 2015).

HIV infection alone increases the expression of adhesion molecules, causes endothelial cell apoptosis, impairs astrocyte-end feet, and dysregulates calcium signaling all contributing to BBB impairment (Nottet et al. 1996; Eugenin et al. 2011). Further, it is believed that BBB dysfunction is a hallmark of HAND, which is represented by an increased expression of adhesion molecules responsible for creating dysfunction in the immune response and resulting in an overall loss of tight junction protein function and expression (Jones et al. 2016). A post-mortem analysis revealed that individuals with HAD displayed a fragmentation and an altered immune response by loss of ZO-1 and occludin (Dallasta et al. 1999; Toborek et al. 2005). Another study using primate lentivirus simian immunodeficiency virus (SIV), which is closely related to HIV, reported that loss of BBB integrity and function was associated with an accumulation of perivascular macrophages in the brain (Luabeya et al. 2000; Toborek et al. 2005). In a rodent HIV model, EcoHIV, it was reported that in a platelet activated-dependent manner, EcoHIV exposure resulted in a loss of tight junction protein, claudin-5, contributing to an alteration in BBB integrity

(Jones et al. 2016). HIV induced BBB alterations are further complicated by multiple intra- and intercellular mechanisms (Strazza et al. 2011). Cytokines and chemokines released from virally infected cells and uninfected cells acting in immunosurveillance have been implicated in altering the integrity of the BBB both directly and indirectly. The chemokine, CCL2, directly alters the integrity of the BBB by reducing the expression of tight junction proteins including ZO-1, claudin-1 and occludin (Eugenin 2006). Another method of examining the integrity and function of the BBB is the transendothelial electrical resistance (TEER) method. TEER is a measure of electrical resistance across an in vitro model of the BBB. TEER provides information about BBB integrity and function (Thanabalasundaram et al. 2011; Benson et al. 2013; Srinivasan et al. 2015). The cytokines, IL-6 and TNF-α, have been shown to decrease the TEER of BMVEC, indicating their role in dysregulation of the tight junction complex maintaining the BBB integrity (de Vries et al. 1996; Voirin et al. 2020). Indirectly, chemokines and cytokines also alter the BBB permeability by increased immune cell trafficking, activation of cells, release of neurotoxins which result in altering nearby cell functions and inducing cell death (Hong and Banks 2015). The HIV protein, gp120, induces BBB dysfunction through downregulating ZO-1, ZO-2, and occludin. The HIV-1 Tat protein alters the BBB integrity by decreasing the expression of ZO-1, occludin, and claudin-5 (Kanmogne et al. 2005; András et al. 2005; Chen et al. 2021). Through activating the signal transducers and activators of transcription (STAT) pathway, both chemokines and cytokines exacerbate gp120 and Tat induced BBB dysfunction (Dhillon et al. 2008; Yang et al. 2009).

Elevated levels of inflammatory mediators have also been associated with changes in BBB function. Increases in inflammatory mediators can induce alterations of the expression and function of drug transport proteins. At the BBB, drugs either cross by lipid-mediated diffusion, or are actively carried across the BBB by receptor-mediated transport systems (Pardridge 2003, 2012). While typically small lipophilic drugs cross the BBB through passive transcellular diffusion, hydrophilic drugs rely on cationic and anionic membrane transporters (Tamai and Tsuji 2000; Lin 2015). A major efflux transporter at the BBB is P-glycoprotein (P-gp/ABCB1), P-gp is located on the apical membrane of BMECs and effluxes drugs from the brain back into the blood thereby limiting the entry of many endogenous substances, drugs or toxic molecules into the brain (Miller et al. 2008; Chai et al. 2022). Modulation of this efflux transporter can impact the net flux of drugs in the brain. Inflammatory mediators can alter the permeability of the BBB by dysregulating the expression of P-gp (Tan et al. 2002; Fernandez et al. 2004; Seelbach et al. 2007). It has been suggested that chemokine activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, chemokines may indirectly increase the expression of P-gp because NF-κB activation upregulates the transcription for the gene encoding P-gp (Chai et al. 2022) (Yu et al. 2008; Liptrott et al. 2009; Chai et al. 2022). Studies further reveal P-gp expression was decreased by IL-6, and increased by IL-1β, IL-2, and TNF-α (Sukhai et al. 2001; Poller et al. 2010).

Studies utilizing brain capillary endothelial cells and primary brain micro-vessel endothelial cells reveal chronic cytokine exposure, by interferon-gamma (IFN-γ) and TNF-α resulted in a concentration-dependent decrease in the expression and function of P-gp thereby decreasing the efflux function of the

BBB (Théron et al. 2003; Poller et al. 2010; Lee et al. 2012). Adequate concentrations of ARVs in the brain are vital in order to eradicate viral reservoirs, however, certain ARVs tend to become more harmful over an extended period of time due to inherent neurotoxicity properties (Osborne et al. 2020). For example, efavirenz, a non-nucleoside reverse transcriptase inhibitor (NNRTI) has been demonstrated to disrupt the integrity of the BBB through altering claudin-5 expression and increasing the endothelium permeability (Bertrand et al. 2016). Stavudine and didanosine, two ARVs in the nucleoside reverse transcriptase inhibitors (NRTI) drug class, have been implicated in causing mitochondrial toxicity (Schweinsburg et al. 2005), although the clinical significance of this study is minimal because neither of these first generation NRTIs are still used clinically. Both *in vivo* and *in vitro* studies have demonstrated that more clinically used NRTI's including lamivudine, zidovudine, and abacavir have neurotoxic properties which are dependent upon the cumulative exposure of the drug (Lanman et al. 2021). These three ARVs may be associated with BBB impairment through inducing neuronal cell death, energy depletion in endothelial cells leading to cell death, endoplasmic reticulum stress in astrocytes, alterations in intracellular calcium, and increased production of ROS (Kakuda 2000; Bienstock and Copeland 2004; Nolan and Mallal 2004; Nooka and Ghorpade 2017; Lanman et al. 2021). A well-known non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz, mediates its toxic effects in a similar fashion to NRTIs causing BBB impairment in addition to dysregulating the immune response (O'Mahony et al. 2005; Ciavatta et al. 2017). Furthermore, the protease inhibitor (PI) drug class has been implicated in producing neurotoxic insults similar to those seen with certain NRTI's, where the insults include altering the integrity of the BBB, dysregulating cell signaling, and inducing neuronal damage(Robertson et al. 2012; Vivithanaporn et al. 2016; Gannon et al. 2017). The integrity of the BBB is essential for maintaining homeostasis and limiting neurotoxicity. In the presence of HIV, chronic neuroinflammation, certain ARVs, and drugs of abuse, the integrity of the BBB can become severely disrupted.

1F: Opioids and HIV

The use of opioids by PLWH is common; a major route of HIV transmission is via intravenous (IV) drug use (Mathers et al. 2008; Dutta and Roy 2012). Opioid drug use can intrinsically alter the course of HIV infection, contributing to neurological and behavioral deficits that occur with the development of HAND and neuroHIV (Hauser et al. 2006; Fitting et al. 2010a, 2020; Murphy et al. 2019). Opioids in HIV have also been shown to increase HIV replication in immune cells and suppress the immune system (Li et al. 2002), as well as acting in synergy with Tat to produce neurodegeneration (Gurwell et al. 2001). Opioid use in HIV perpetuates neuroinflammation because opioids activate corresponding opioid receptors on immune cells such as T-lymphocytes, monocytes, macrophages, astroglia, microglia and neurons (Gurwell et al. 2001). Our lab has demonstrated how co-exposure of morphine and Tat decreases ARV accumulation in a brain-region specific manner, upregulates P-gp expression, and dysregulates neuroinflammation (Leibrand et al. 2017, 2019, 2022). Direct acting opioids, such as heroin and morphine, are known to be immunomodulatory through their interference with cytokine and

chemokine secretion (Neri et al. 2005). Drugs used in the treatment of opioid use disorder (OUD), buprenorphine and methadone, may also have immunomodulatory effects within the brain, although the directionality of these effects are not well understood. While methadone has been shown to have mild immunosuppressive effects in mice with autoimmune disease, (Chavez-Valdez et al. 2013) methadone aggravates neuroinflammation by promotion of HIV replication in brain microglia and macrophages (Gabay 2006; Boland et al. 2014). HIV viral proteins and opioids exert immunomodulatory effects, however, the degree to which these effects hinder the accumulation and subsequent effectiveness of ARVs within the brain is not fully elucidated.

The effects of opioids such as morphine have been extensively studied in the setting of HIV, however, the interactions between fentanyl and HIV on outcomes related to neuroHIV and ARV drug distribution remain to be described. The current opioid epidemic in the United States is increasingly characterized by the alarming availability of fentanyl throughout the country. A report from 2018 provides that from 2017 - 2018, heroin-related deaths decreased, while deaths related to fentanyl ingestion increased by 10% (Hedegaard et al. 2020). To add to this, in 2020, the CDC reported that 57,834 opioid-related deaths were caused by fentanyl exposure, which increased by 15% in 2021, with 71,238 opioid-related deaths being attributed to fentanyl (National Center for Health Statistics). Pharmaceutical fentanyl is used as sedation for intubated patients and for the treatment of severe pain in patients with renal failure (Ramos-Matos et al. 2022). Illicitly-manufactured fentanyl (IMF) is sold as counterfeit pills, is mixed with other drugs to increase the potency, is sold as powder or nasal sprays and is camouflaged as other frequently abused opioids (Drug Enforcement Administration 2021). Fentanyl is a popular drug of abuse because of its faster onset as compared to heroin (Kong et al. 2022). Previous non-HIV studies examining the effects of fentanyl on cell signaling, cell survival, and immune regulation demonstrate that fentanyl exposure impairs mitochondrial function, induces apoptosis, alters cell signaling, and is immunosuppressive (Delogu et al. 2004; Kocak et al. 2017; Ma et al. 2017).

A recent study examining fentanyl in the setting of HIV revealed fentanyl enhances HIV replication and increases the expression of CCR5 and CXCR4 (Kong et al. 2022). Another *in vitro* study examined the effects of fentanyl on lipopolysaccharide (LPS)-induced inflammation, which is known to induce the expression of cytokines, IL-6, IL-10, and TNF-α. In the LPS inflammatory model, fentanyl decreased the expression of these cytokines, but failed to inhibit their release in the control group (no LPS) (Wu et al. 2009). Due to opioid abuse presenting as a major route of transmission for HIV and the rise of fentanyl in opioid drug abusers, it is crucial to elucidate how co-exposure of fentanyl and HIV may interact with HIV, neurotoxic viral proteins, ARV accumulation in the brain, and the immune response.

Methadone, which is also an opioid drug, is an FDA approved medication for treating OUD, and is frequently prescribed in the setting of HIV (Bruce 2010). Methadone treatment is associated with improved antiretroviral adherence in PLWH and comorbid OUD (Lucas et al. 2006). Methadone is used for OUD because it has a high oral bioavailability, does not produce euphoria through the dopaminergic system, has a longer half-life than other commonly abused opioids, and exhibits a higher relative intrinsic

efficacy at MOR (Barwatt et al. 2013; Cai et al. 2019). Methadone is able to passively diffuse across biological membranes, and has been shown to be a substrate and an inhibitor of P-gp at the BBB, although there is some debate regarding its inhibitory activity (Callaghan and Riordan 1993; Bouër et al. 1999; Eap et al. 2002; Kharasch 2003). Studies examining the gene encoding for P-gp, *ABCB1*, have revealed that polymorphisms or genetic differences in this gene, greatly affect the transport of methadone across membranes where the transporter is present (Dennis et al. 2014; Bart et al. 2021). Whether methadone exacerbates HIV infection and neuroinflammation remains unresolved and requires empirical testing (Wang et al. 2020; Fitting et al. 2020). *In vitro* data has revealed that methadone decreases the expression of inflammatory mediators responsible for neuroinflammation (Kafami et al. 2013). Another study demonstrated that methadone alone does not increase the production of chemokines or decrease neuronal survival, however, in conjunction with Tat, methadone increases neurotoxicity resulting in decreased neuronal survival and enhanced chemokine production compared to Tat alone (Fitting et al. 2015), which suggests that methadone may not be as safe in PWH as in people without HIV.
Methadone has a distinct mechanism of action because in addition to being an agonist at the mu-opioid receptor, it also possesses antagonistic behavior at NMDA receptors. Glutamate is one of the toxic byproducts produced by HIV infected cells that once released from infected cells, stimulates NMDA receptors in synergy with gp120 released from cells (Fontana et al. 1997). Several studies suggest stimulated NMDA receptors play a role in HIV neurotoxicity (Giulian et al. 1990; Jiang et al. 2001; O'Donnell 2006) through many downstream effects including increased influx of extracellular calcium (which can disrupt BBB integrity), increased nitric oxide production, enhanced neuronal cell death, and microglial activation (Choi 1994; Bagetta et al. 2004). Interestingly, NMDA antagonists protect against these deleterious effects (Savio and Levi 1993; Fontana et al. 1997; Epstein and Gelbard 1999; Wallace 2006; Barbour et al. 2020). While methadone may have the potential to protect against HIV induced neurotoxicity through NMDA receptor antagonism, it has a higher binding affinity for the (MOR). While most studies examine the mechanisms related to methadone drug-interactions in HIV, it remains unclear how methadone exposure is associated with the function of the BBB and subsequent cell-signaling cascades involved in carrying out neurotoxicities. Several studies reveal that the HIV protein Tat, and morphine, increases the expression of P-gp at the BBB, and therefore it is important to examine how Tat may alter the effects of methadone in the brain through dysregulating P-gp, and if methadone, an agonist at the same receptor as morphine, would affect P-gp (Haughey and Mattson 2002; Hayashi et al. 2005; Mahajan et al. 2008; Leibrand et al. 2017, 2022). In addition to potentially altering the integrity and function of the BBB, the higher binding affinity of MOR may result in activating more MOR and an enhanced dysregulation of the immune response as compared morphine (Morgan and Nicholson 2011; Reddy et al. 2012), further emphasizing the complexity of predicting the net pharmacodynamic effects of methadone treatment in PLWH.

Chapter 2: Aims

SPECIFIC AIMS. Despite adequate systemic viral suppression by combination antiretroviral (ARV) therapy (cART), HIV-associated neurocognitive disorders (HAND) remains prevalent in the United States (Saylor et al. 2016). Complex mechanisms of HAND are not fully understood(Murphy et al. 2019) and are further complicated by drug use, enhanced monocyte migration, and cART non-adherence(Ghosh et al. 2017; Rizzo et al. 2020; Osborne et al. 2020). Complicating HAND further is persistent neuroinflammation in comorbid HIV and drug abuse, as both HIV and drugs of abuse exert immunomodulatory effects in the brain promoting a spiraling inflammatory loop and cytotoxicity(Hauser and Knapp 2014; Fitting et al. 2020). Although cART successfully limits peripheral viral replication, eradicating the virus from the brain remains a challenge due to the central nervous system's (CNS) ability to create shelter for latent and actively dividing virus (Simoes and Justino 2015; Osborne et al. 2020) and the inability of ARVs to accumulate at sufficient concentrations in the CNS (Osborne et al. 2020). Opioid use amplifies the challenge of eradicating the virus from the brain. HIV infection and opioid use disorders (OUD) are interlinked epidemics, (Leshner 1998) as intravenous drug use is a major transmission route for many patients, some who have or may develop OUD(Mathers et al. 2008). In the current opioid epidemic, fentanyl, a μ-opioid receptor (MOR) agonist, use has risen dramatically within the last decade has taken the spot for the most popular abused opioid. While few studies examine the impact of fentanyl on outcomes related to neuroHIV, it is known that fentanyl has immunomodulatory effects and the potential to act in synergy with viral proteins to induce neurotoxicity (Delogu et al. 2004; Kocak et al. 2017; Ma et al. 2017). Methadone, is one of the FDA approved medications for treating OUD, and has demonstrated to have synergistic toxic effects with the HIV protein, Tat (Fitting et al. 2015). The understanding of how methadone interacts with HIV and ARV on neuroinflammation, and drug accumulation is complicated by the receptor binding properties of methadone. Methadone is an agonist at the MOR while an antagonist at NMDA receptors. The antagonism at NMDA receptors is thought to have protective effects in the setting of HIV, as NMDA receptor overstimulation in HIV has been associated with neurotoxicity (Savio and Levi 1993; Fontana et al. 1997; Epstein and Gelbard 1999; Jiang et al. 2001; Wallace 2006; O'Donnell 2006; Barbour et al. 2020).

Previously, our lab demonstrated that morphine alters the blood brain barrier (BBB) and decreases the accumulation of ARVs in a brain region specific manner (Leibrand et al. 2019). Additionally, our lab and others have demonstrated that both HIV-1 Tat and morphine decrease tight junction proteins, ZO-1, occludin, and JAM-2, while increasing P-gp expression (Hauser et al. 2012; Leibrand et al. 2017, 2019; Patel 2018). Our lab also has demonstrated the immunomodulatory effects of the HIV-1 viral protein, Tat; Tat exposed mice display significant increases (~5-fold) in the number of phagocytic macrophages in the striatum (Leibrand et al. 2017) and that Tat and morphine independently increase the recruitment of macrophages into striatum and hippocampus (Leibrand et al. 2022). Recently, we have also shown that in the presence of Tat, fentanyl alters chemokine concentrations in a region-specific manner (Chapter 3).

Methadone, morphine, and fentanyl are opioid agonists that bind similarly to opioid receptors as heroin, and therefore, may exert immunomodulatory effects and affect ARVs in the brain. However, it is *not yet known how* both fentanyl and methadone *interact with the immune response in HIV infection or in the presence of Tat, or how they impact ARV brain concentrations and whether the interactions are similar or different from morphine.* Achieving adequate concentrations of ARVs in the brain is critical for eliminating HIV brain reservoirs and limiting the loop of neuroinflammation (Osborne et al. 2020). Although morphine has been studied in the Tat-transgenic mouse model, and we have published data on the impact of morphine on the BBB, distribution of ARVs, and chemokine concentrations, we wanted to examine these outcomes with morphine in a model with infectious EcoHIV.

For this reason, these three separate studies seek to understand how fentanyl, morphine, and methadone affects the concentrations of antiretroviral drugs, the integrity of the blood brain barrier (BBB) and chemokines within the brain using the Tat-transgenic and EcoHIV mouse models. For each of the studies, concentrations of ARVs will be examined in the striatum and hippocampus in the presence of the respective opioid with a saline (control) control group. To assess the impact that these opioids may have on the integrity of the blood brain barrier, markers of the BBB integrity will be assessed in the presence of the opioids and saline (control). Furthermore, to advance our understanding of fentanyl, morphine, and methadone immunomodulatory effects, we will quantify inflammatory mediators known to contribute to HAND pathogenesis in the presence of all three opioids and ARVs in an infectious HIV-mouse model (EcoHIV).

To define and model the impact of opioids on brain concentrations of three ARV drugs (dolutegravir, abacavir, and lamivudine), BBB integrity and chemokine concentrations, we will use one of two models: infectious mouse ecotropic (pseudotyped HIV) viral model termed EcoHIV and Tat-transgenic mouse model.

Aim 1: Determine the impact of Tat and fentanyl on inflammatory mediators and BBB integrity

Aim 1a: Determine the impact of fentanyl and Tat on chemokine concentrations in the striatum and hippocampus using a 13-plex flow cytometry-based panel.

Aim 2b: Determine the integrity of the BBB by measuring BBB markers, VCAM, PDGFR-β and the BBB markers, claudin-5 and ZO-1. VCAM will be measured using enzyme-linked immunoassays (ELISA), PDGFR-β will be measured by immunoblotting, and both claudin-5 and ZO-1 will be measured by immunofluorescent staining and imaging.

Aim 2: Examine the effect of EcoHIV with fentanyl and morphine on inflammatory mediators, BBB integrity, and ARV concentrations.

Aim2a: Determine the effects of opioids ± EcoHIV on chemokine concentrations in the striatum and hippocampus using a 13-plex flow cytometry-based panel.

Aim2b: Determine the effects of opioids ± EcoHIV on blood brain barrier leakiness by measuring BBB tight junction proteins, claudin-5 and ZO-1, using enzyme-linked immunoassays (ELISA).

Aim2c: Determine the effects of opioids ± EcoHIV on ARV accumulation in the striatum and hippocampus. The concentrations of ARVs will be measured using LC-MS.

Aim 3: Determine the impact of Tat and methadone on inflammatory mediators, BBB integrity, and ARV concentrations.

Aim2a: Determine the effects of methadone ± Tat on ARV accumulation in the striatum and hippocampus. The concentrations of ARVs will be measured using LC-MS.

Aim 3b: Determine the effects of methadone ± Tat on BBB drug efflux transporter, P-gp, using immunoblotting.

Aim3c: Determine the effects of methadone ± Tat on chemokine concentrations in the striatum and hippocampus using a 13-plex flow cytometry-based panel.

Chapter 3. Fentanyl dysregulates neuroinflammation and disrupts blood-brain barrier integrity in HIV-1 Tat transgenic mice.

Introduction

HIV and drug abuse are interlinked epidemics (Leshner 1998). Opioid use disorder (OUD) in particular contributes to the spread of HIV through injection drug use and through the exchange of sex for drugs (Mathers et al. 2008; Blouin et al. 2016; Degenhardt et al. 2017). OUD also contributes to the pathogenesis of neuroHIV via direct actions in the central nervous system (CNS) (Hauser et al. 2012; Fitting et al. 2020) even with successful systemic suppression of the virus by cART (Anthony et al. 2005; Heaton et al. 2010; Harezlak et al. 2011). Morphine is the major bioactive metabolite of heroin in the CNS and a prototypic opioid whose impact has been widely examined in the context of neuroHIV pathogenesis and inflammation (Guo et al. 2002; Hall et al. 2006; Reddy et al. 2012; Dutta and Roy 2012; Tyagi et al. 2016; Al-Tayyib et al. 2017; Marsh et al. 2018). NeuroHIV encompasses the complex mechanisms of brain injury related to HIV infection and the subsequent chronic neuroinflammation (Hauser et al. 2007; Chang et al. 2014; Smith et al. 2014). These include, e.g., OUD, specific comorbid CNS diseases or injury, and poor CNS penetration of antiretroviral (ARV) drugs that may both enhance viral replication and promote the establishment and maintenance of latent viral reservoirs, chronic inflammation and neuronal injury/degeneration (Fantuzzi et al. 2003; Letendre et al. 2004; Verani et al. 2005; Duncan and Sattentau 2011; Hong and Banks 2015; Simoes and Justino 2015; Olivier et al. 2018; Murphy et al. 2019; Osborne et al. 2020).

The most commonly described theory of how HIV enters the brain is through the ‘Trojan Horse’ mechanism, whereby HIV-infected monocytes cross the blood brain barrier (BBB) and differentiate into perivascular macrophages (Eugenin et al. 2010; Lindl et al. 2010; Siliciano and Greene 2011). Infected cells in the brain contribute to neuroHIV by promoting neural inflammation, neurotoxin production, immune dysfunction, neurodegeneration and enhancing trafficking of peripherally infected cells into the brain (Weiss et al. 1999; Hauser et al. 2007; Eugenin et al. 2010; Wong et al. 2019). The BBB protects the brain against the entry of toxic substances or pathogens and the integrity and function of the BBB is compromised by HIV infection and opioids (Leibrand et al. 2017, 2019; Osborne et al. 2020). Morphine alters BBB integrity by reducing the expression of key tight junction proteins, decreasing brain concentrations of antiretroviral drugs (ARVs), increasing macrophage recruitment into the CNS, and dysregulating inflammatory responses in the brain (Leibrand et al. 2017, 2019, 2022). Typical opioids such as morphine synergize with several HIV proteins, including Tat and gp120, to enhance replication, promote neurodegeneration, destabilize calcium homeostasis and exacerbate neuroinflammation causing additional insults at the BBB (Gurwell et al. 2001; Haughey and Mattson 2002; Neri et al. 2005; Zou et al. 2011; Ellis et al. 2015; Fitting et al. 2020). Inflammatory cytokines and chemokines (Miller and Krangel 1992; Borish and Steinke 2003) can alter the function of the BBB by redistributing tight junction proteins such as claudin-5 and ZO-1 (Stamatovic et al. 2005; Chai et al. 2014; Wang et al. 2018b). HIV proteins

and opioids can trigger a variety of inflammatory processes that collectively contribute to BBB disruption (Buckner et al. 2006; Estevao et al. 2021). The loss of BBB integrity promotes the leakage of chemokines, the recruitment of additional infected/non-infected monocyte-derived macrophages (MDMs) and spiraling neuroinflammation potentially resulting in sustained neural injury and (El-Hage et al. 2011).

The opioid epidemic is increasingly characterized by the alarming availability of fentanyl throughout the United States. From 1999 to 2021, overdose deaths dramatically increased overall by 781% (National Safety Council 2023). Fentanyl-related overdose deaths increased from 730 deaths in 1999 to 70,601 deaths in 2021 (National Safety Council 2023). Despite the recent rise of fentanyl use, the interactive effects of fentanyl and HIV within the brain have not been widely studied. Fentanyl, a synthetic opioid, is 50 to 100 times more potent than morphine at μ opioid receptors (MORs) (Ramos-Matos et al. 2022). While the actions of fentanyl at MORs underlie most of its antinociceptive and addictive properties (Hammerslag et al. 2020; Vo et al. 2021; Sutcliffe et al. 2022; Kelly et al. 2023), many of fentanyl's deleterious effects (e.g., inflammatory) are opioid receptor independent (Torralva and Janowsky 2019; Torralva et al. 2020; Kelly et al. 2023) and mediated through alternative receptor types such as α_{1A}, α_{1B} and α_{1D} and α_2 adrenergic receptors (Torralva et al. 2020; Haouzi and Tubbs 2022; Kelly et al. 2023). Because fentanyl is highly potent at opioid receptors but also acts through non-opioid receptors, fentanyl's pharmacologic profile differs markedly from other "typical" opioids including morphine, heroin, methadone, and oxycodone. *In vitro*, fentanyl enhances HIV replication through upregulation of chemokine receptors involved in HIV pathogenesis and suppression of the host immune system (Kong et al. 2022; Yan et al. 2022). *In vivo* studies regarding the use of fentanyl on HIV outcomes are currently lacking. The purpose of this study was to examine the interplay between fentanyl and HIV-1 Tat on the BBB and on inflammatory signaling within the brain. Specifically, the effects of fentanyl and HIV-1 Tat on tight junction proteins, an adhesion molecule and chemokines were measured within two brain regions using the Tat-transgenic mouse model.

Materials and Methods

Animal Handling and Treatment

All studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and the experiments were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85–23).

Subjects and housing

Tat transgenic female mice (~4 months of age weighing ~25 g) were generated in the vivarium at Virginia Commonwealth University. All Tat transgenic mice (n = 30) were fed with doxycycline (Dox) containing chow (Dox Diet #2018; 6 g/kg; DOX-chow; purchased from Harlan Laboratories Madison, WI) for 7 days. DOX and fentanyl were administered concurrently. Alzet® osmotic pumps (2001, 1.0 μL/h, Cupertino, CA) containing fentanyl (0.05 mg/day) diluted in saline were subcutaneously implanted in the

subscapular area at the onset of DOX administration. DOX chow was provided *ad libitum* and fentanyl was delivered continuously for 7 days.

Surgical manipulation

Seven days after the initiation of Tat induction and the continuous delivery of fentanyl, mice were sacrificed by cervical dislocation under 4% isoflurane anesthesia and decapitation. After decapitation, brains were placed in a 15 mL conical tube filled with 10 mL cold normal saline and were gently swirled to facilitate removal of excess blood. The brain was then hemisected into the right and left hemispheres. The right hemisphere was further microdissected into the striatum and hippocampus, snap frozen, and stored at −80 °C until analysis.

Immersion Fixation and Staining

The left hemisphere was prepared for immersion fixation by gently rinsing the left hemisphere again in fresh cold normal saline to remove excess blood. After the second rinse, the left hemisphere was dropped in 4% paraformaldehyde (PFA) (enough to completely submerge brain; ~10 mL) using scintillation vials and were stored overnight at 4 °C. After submersion in the cold PFA fixative overnight (24 h), the brains were removed, lightly blotted with a paper towel, and were washed in fresh, cold PBS for 1 h on a shaker to remove additional PFA. Then the tissue was immersed in 10 % sucrose solution and stored overnight (24 h) at 4 °C. After the 10% sucrose solution immersion, the tissue was placed in 20% sucrose solution and stored overnight (24 h) at 4 °C. Hemisected brain samples were embedded in Tissue-Tek optimal cutting temperature (O.C.T) compound (Sakura Finetek, Torrence, CA), and stored at −80 °C until use. Frozen, O.C.T-embedded tissues were sectioned with a Leica CM cryostat (Leica Biosystems, Buffalo Grove, IL) at 20 µm thickness in the coronal plane. Striatal tissue sections were immersed in preboiled Tris-based antigen unmasking solution (H3301, Vector Laboratories, Newark, CA) for 3 min followed by a 1 × rinse in neutral pH phosphate buffered saline (PBS). Tissue sections were then blocked in Animal-Free Blocker® and Diluent solution (# SP-5035-100, Vector Laboratories) followed by overnight incubation in Alexa Fluor 488-conjugated-claudin-5 antibody (1:50, #352588, Thermo Fisher) and Zonula Occludens-1 (ZO-1) (1:100, #40-2200, Thermo Fisher). Following primary incubation, the tissue sections were incubated in species-specific secondary antibody for ZO-1.

Immunofluorescent imaging

Stitched photomicrographs of the striatum were taken at 63× magnification using a Zeiss confocal microscope (Zeiss Inc., Thornwood, NY) for intensity measurements. The photomicrographs for the striatum were obtained from bregma +1.10 mm to bregma +0.50 mm. Two sections were used for intensity measurements for each mouse. A 0.16 mm^2 field in the dorsal striatum was measured per section for intensity analysis. Mean fluorescent/pixel intensity values for Claudin-5 and ZO-1 corresponding to the level of immunostaining were measured in the dorsal striatum using Image J

software (NIH, Bethesda, MD) as previously described (Bertrand et al., 2016; Shihan et al., 2021; Wang et al., 2020; Xing et al., 2020). Background intensity was captured from unstained regions within each tissue section and used to obtain the corrected mean fluorescent intensity values.

Immunoblotting

Immunoblotting for platelet derived growth factor receptor-β (PDGFR-β) was performed on the striatal tissues obtained from the right hemisphere of mice exposed to fentanyl and Tat (7-day exposure) as described above. Fast-frozen striatal samples were homogenized in NP-40 buffer with cOmplete™, Mini Protease Inhibitor Cocktail (11836153001, Roche Diagnostics GmbH, Germany). The homogenate was centrifuged (16,000 × *g* at 4 °C for 10 min) and the supernatant was aliquoted and stored at −80 °C until use. Protein concentration was determined using the bicinchoninic acid protein assay method and 30 µg striatal samples were electrophoresed on 4-20% Tris-HCl gels. Separated proteins were transferred to PVDF membranes and non-specific binding sites were blocked using 5% nonfat dry milk in Tris-buffered saline containing 0.5% tween 20 (TBST) for 1 h at room temperature (RT). Membranes were incubated overnight at 4 °C with PDGFR-β (1:500, #13449-1-AP), Protein Tech group, Rosemont, IL). After washing in TBST, membranes were incubated in HRP-conjugated species-specific secondary antibody for 1 h at RT. GAPDH (1:2000, ab8245, Abcam) was used to assess loading. Membranes were scanned with a Bio-Rad ChemiDoc Imager (Bio-Rad, Hercules, CA). Optical densities of immunoreactive bands were determined using the Bio-Rad Image Lab software (V6.0.1, Bio-Rad).

Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was performed on striatal tissue lysates obtained from the right hemisphere to determine whether Tat and/or fentanyl exposure alters VCAM-1. Presence and relative amounts of VCAM-1 were detected in striatal samples using the mouse VCAM-1 Quantikine ELISA kit (#MVC00, R&D systems) per manufacturer's instructions and previous publications (Tanaka et al. 2001; Grabmaier et al. 2016). Briefly, 10 µg samples obtained from striatal lysates from the right hemisphere were incubated in microplate strips pre-coated with a monoclonal antibody specific for mouse VCAM-1. An enzyme-linked monoclonal antibody was used to selectively detect captured antigens (VCAM-1) via the conversion of an added substrate to a chromogenic reaction product which was read at an absorbance of 450 nm on a PHERAstar *FS* plate reader (BMG LabTech; Cary, NC). Sample values were then extrapolated from a generated standard curve.

Chemokine Assay

To measure chemokines in the hippocampus and the striatum from the right hemisphere, a Mouse 13-plex Proinflammatory Chemokine Panel (Catalog #740007; BioLegend) was used according to the manufacturer's instructions. The striatum and hippocampus were homogenized separately using the NP40 lysis buffer with cOmplete™, Mini Protease Inhibitor Cocktail (11836153001, Roche Diagnostics

GmbH, Germany). Brain homogenates were centrifuged (16,000 × *g* at 4 °C for 10 min) and supernatants were extracted and used to determine protein content using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 2020) and then stored in −80 ºC until chemokine analysis. The chemokine samples were prepared as instructed by the manufacturer (BioLegend) and the analysis was performed using the Cytek® Aurora flow cytometer (Cytek® Biosciences, Fremont, California). Briefly, sample lysates containing 12.5 µg of protein or standard were loaded in duplicates into wells of the filter plate. The sample and standard wells were incubated with chemokine antibody-conjugated beads (12.5 µL) for 2 h, after which biotinylated detection antibodies were added for 1 h. While the plate was incubated with both chemokine antibody-conjugated beads and biotinylated detection antibodies, the plate was placed on a shaker at 500 rpm. Following the first two incubation steps, streptavidin-PE (SA-PE) was added for signal amplification, and the plate was incubated for an additional 30 min on the shaker at 500 rpm. At the end of the 30 min, the filter plate was washed and sealed and placed in the refrigerator for 24 h until analysis. On the day of analysis, the samples were washed once more, resuspended for 5 min, and transferred to a U-bottom plate. The u-bottom plate was run on the Cytek® Aurora. Data was obtained using the LEGENDplex V8.0 software (BioLegend) which created a five-parameter logistic regression to fit the standard curves for each chemokine and duplicate plated samples.

Table 1. Chemokines assayed

Chemokine	Physiological Role
CCL2/MCP-1	Inflammatory (Deshmane et al. 2009; Singh et al. 2021)
CCL3/MIP-1α	Inflammatory (Maurer and von Stebut 2004; Bhavsar et al. 2015)
CCL4/MIP-1β	Inflammatory (Menten et al. 2002; Maurer and von Stebut 2004)
CCL5/RANTES	Inflammatory (Maurer and von Stebut 2004; Bhavsar et al. 2015)
CCL11/Eotaxin	Inflammatory (Parajuli et al. 2015; Ivanovska et al. 2020; Nazarinia et al. 2022)
CCL17/TARC	Dual-function* (Campbell et al. 1999; Gear and Camerini 2003; Weber et al. 2011)
CCL20/MIP-3α	Dual-function* (Zlotnik et al. 2011; Williams et al. 2014; Kwantwi et al.)
CCL22/MDC	Dual-function* (Greaves et al. 2001; Juremalm et al. 2005; Scheu et al. 2017)
CXCL1/KC	Inflammatory (Dong et al. 2013; Korbecki et al. 2022)
CXCL5/LIX	Dual-function* (Koltsova and Ley 2010; Chen et al. 2018)
CXCL9/MIG	Dual-function* (Metzemaekers et al. 2018; Tokunaga et al. 2018; Paparo 2019)
CXCL10/IP-10	Dual-function* (Strieter et al. 1995; Metzemaekers et al. 2018; Tokunaga et al. 2018)
CXCL13/BLC	Homeostatic (Widney et al. 2005; Williams et al. 2014; Gao et al. 2021)

*****Dual function: inflammatory and homeostatic

Chemokines included in the LEGENDplex 13-plex assay. Of the total chemokines measured, 10 were within the limit of detection (LOD), while 3 were below the LOD and were not statistically analyzed (CCL2, CCL5, and CXCL5).

Statistical analysis

Duplicate samples were analyzed and averaged for analysis by two-way analysis of variance (two-way ANOVA) to identify significant main effects or interactions of fentanyl and Tat exposure in the striatum and hippocampus. Two-way ANOVA testing was followed by Tukey HSD's multiple comparison test to make pairwise comparisons between group means.

For chemokine statistical analysis, CCL2, CCL5, and CXCL5 were all below the limit of detection, and therefore, statistical analysis was not performed on these chemokines. The data used for the analysis of claudin-5, ZO-1, PDGFR-β, and VCAM in the striatum, was each standardized as a percentage (%) of control values, i.e., relative to Tat (−) (control) mice that received vehicle-containing minipump implants. All data are presented as the mean with standard error of the mean (SEM) and results were considered statistically significant using a significance level of $\alpha = 0.05$. All statistical analyses were performed using GraphPad Prism Version 9.4.0 (GraphPad Software, LLC, La Jolla, CA).

Correlation Analysis

The Pearson correlation coefficient (r) method was used to measure linear correlations between Tat status and chemokines on claudin-5 and ZO-1, respectively. Results were considered statistically significant using a significance level of $\alpha = 0.05$. All statistical analyses were performed using GraphPad Prism Version 9.4.0 (GraphPad Software, LLC, La Jolla, CA).

Results

Blood Brain Barrier Measurements

To better understand the impact of Tat and fentanyl on blood-brain barrier integrity within the striatum, the areal intensity measurements of the tight junction and tight junction accessory proteins, ZO-1 and claudin-5 were measured via immunofluorescent imaging. Fentanyl decreased the expression of tight junction proteins, ZO-1 and claudin-5 in the striatum and Tat exposure also decreased the expression of claudin-5, but not ZO-1 [Table 2, **Fig. 1, Fig. 2a, 2b**], demonstrating that both fentanyl and Tat contribute to the disruption of the BBB.

Claudin-5 Areal Intensity Measurements in the Striatum

Irrespective of Tat status, fentanyl-treated mice had lower mean claudin-5 areal intensity measurements compared to vehicle-treated (saline)-mice. Additionally, Tat(+) mice exposed to saline had lower claudin-5 expression than Tat(−) mice exposed to saline. There were multiple interactions whereby Tat(−) mice exposed to saline had significantly higher claudin-5 expression compared all other

experimental groups [(Tat(+) saline-treated mice, Tat(−) fentanyl-treated mice, and Tat(+) fentanyl-treated mice) [$p = 0.001$, $p < 0.0001$, $p < 0.0001$, respectively]. Tat(+) mice exposed to saline had significantly higher claudin-5 expression compared to Tat(+) mice exposed to fentanyl ($p = 0.001$) [Table 2, **Fig. 2a**].

ZO-1 Areal Intensity Measurements in the Striatum

Fentanyl exposure resulted in a significant decrease in mean ZO-1 areal intensity measurements compared to mice receiving saline. Specifically, Tat(−) mice exposed to fentanyl had significantly lower ZO-1 expression compared to Tat(−) mice exposed to saline [$p = 0.02$] [Table 2, **Fig. 2b**].

PDGF receptor-β Western Blotting

Overall, Tat expression decreased PDGFR-β among experimental groups [Table 3]. Tat expression and fentanyl interacted, and Tat(+) and fentanyl co-exposed mice had significantly lower PDGFR-β expression compared to Tat(−) mice exposed to saline [$p = 0.02$] [Table 2]. Tat-induction by itself resulted in a significant decrease in PDGFR-β levels [$p = 0.02$] [Table 3, **Fig. 2c**].

VCAM ELISA

There were no significant differences in VCAM concentrations between the groups suggesting that Tat and fentanyl did not affect the expression of VCAM [Table 3, **Fig. 2d**].

Chemokine Concentrations in Striatum and Hippocampus

To determine the effects of Tat and fentanyl on chemokine concentrations within the brain, Tat induction and/or fentanyl exposure occurred for 7 days followed by measurement of 13 chemokine concentrations within the striatum and the hippocampus.

Inflammatory Chemokines

Post-hoc multiple comparisons

CCL3: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL3 in the striatum compared to Tat(−) and Tat(+) mice exposed to saline [$p = 0.0007$, $p = 0.0009$, $p = 0.0022$, $p = 0.0027$, respectively] [Table 4, **Fig. 3a**] and in the hippocampus respectively [$p < 0.0001$ for all hippocampus comparisons] [Table 4, **Fig. 3b**].

CCL4: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL4 in the striatum and hippocampus compared to Tat(−) and Tat(+) mice exposed to saline [$p < 0.0001$ for all] [Table 4, **Fig**. **3c, 3d**].

Dual function chemokines

Post-hoc multiple comparisons

CCL17: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL17 in the striatum compared to Tat(−) and Tat(+) mice exposed to saline [$p < 0.0001$ for all] [Table 4, **Fig. 4a**]. In the hippocampus, Tat(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL17 compared to Tat(+) mice exposed to saline [$p < 0.0001$] [Table 4, **Fig. 4b**].

CCL20: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL20 in the striatum compared to Tat(−) and Tat(+) mice exposed to saline [$p = 0.0087$, $p = 0.0321$, $p = 0.0087$, $p = 0.0321$, respectively] [Table 4, **Fig. 4c**]. In the hippocampus, Tat(−) and Tat(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL20 compared to Tat(−) and Tat(+) mice exposed to saline [$p < 0.0001$, $p < 0.0001$, $p = 0.0007$, $p = 0.0009$, respectively] [Table 4, **Fig. 4d**].

CXCL9: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *lower* CXCL9 concentrations in the striatum compared to Tat(−) and Tat(+) mice exposed to saline [$p = 0.0003$, $p = 0.0001$, $p = 0.0146$, $p = 0.0070$, respectively] [Table 4, **Fig. 4g**]. In the hippocampus, Tat(−) mice exposed to fentanyl had significantly *lower* concentrations of CXCL9 compared to Tat(−) mice exposed to saline [$p = 0.0052$] [Table 4, **Fig. 4h**].

CXCL10: Tat(−) and Tat(+) mice exposed to fentanyl had significantly *higher* concentrations of CXCL10 in the striatum compared to Tat(−) and Tat(+) mice exposed to saline [$p = 0.0228$, $p = 0.0132$, $p = 0.0006$, $p = 0.0004$, respectively] [Table 4, **Fig. 4i**].

Homeostatic chemokines

Within the striatum and hippocampus, fentanyl exposure resulted in a significant *increase* in CXCL13 concentrations (main effects only) [Table 4, **Fig. 5a**].

Correlations between Tat status and chemokines on claudin-5 and ZO-1

To understand the associations between Tat status and chemokines on blood brain barrier transport and accessory transport proteins, correlations were performed using the Pearson correlation coefficient (r) method.

Inflammatory chemokines

In Tat(−) mice, there was a positive correlation between the inflammatory chemokines CCL3 and CCL11 and claudin-5 [**Fig. 6a, 6d**] as well as ZO-1 [**Fig. 7a, 7d**]. In both Tat-groups, the inflammatory chemokine CCL4 was negatively correlated with claudin-5 expression [**Fig. 6b, 6c**], while ZO-1 was only negatively correlated with CCL4 in Tat(+) mice [**Fig. 7b**].

Dual-function chemokines

CXCL9 was positively correlated with claudin-5 and ZO-1 expression in Tat(−) mice [**Fig. 6g, 7g**, respectively], while CCL20 was negatively correlated with ZO-1 [**Fig. 7f**]. In both Tat-groups, CCL17 was positively correlated with claudin-5 and ZO-1 expression in the striatum [**Fig. 6e, 6f, 7d, 7e**]. In Tat(+) mice, CXCL10 was negatively correlated with claudin-5 and ZO-1 expression [**Fig. 6h, 7h**, respectively].

Discussion

The current study reveals that fentanyl, and to a lesser extent HIV-1 Tat, has profound effects on blood-brain barrier integrity and dysregulating chemokines. These studies also demonstrate unique regional differences in the production of chemokines in response to fentanyl and HIV-1 Tat.

The dysregulation of the CNS immune response by fentanyl in the context of HIV has not previously been studied. Quite unexpectedly, depending on the chemokine, fentanyl exposure by itself often caused as dramatic an increase in chemokine production as seen with exposure to Tat alone. The heightened chemokine production seen with fentanyl in the striatum and hippocampus is unlike that seen with morphine, which has little or no effect on chemokine release by itself (Fitting et al. 2010a; McLane et al. 2014; Hermes et al. 2020). In our study, HIV-1 Tat only dysregulated CXCL1 in the striatum and CCL11 in the hippocampus. Immune response dysregulation by HIV-1 Tat has been described previously (Nath et al. 1999; Dandekar et al. 2004; Hong and Banks 2015; Chivero et al. 2017; Roberto Pinto et al. 2022), as well as the interaction between Tat and morphine (D'Aversa et al. 2005; Hermes et al. 2020; Leibrand et al. 2022; Nass et al. 2023). Our current results demonstrate that fentanyl had a greater impact on the immune response in comparison to HIV-1 Tat.

Blood brain barrier impairment in the presence of fentanyl

In the present study, fentanyl decreased claudin-5 and ZO-1 in the striatum. Our lab has previously demonstrated that morphine alters BBB integrity *in vivo* resulting in leakage of high molecular weight tracers, 40 kDa-labeled fluorescein-conjugated dextran and 70kDa-labeled Texas Red®-conjugated dextran into the brain and that morphine also and alters the distribution of ZO-1 along the blood-brain barrier (Leibrand et al. 2019). Several *in vitro* studies have also demonstrated decreases in ZO-1 (Mahajan et al. 2008; Wen et al. 2011) and claudin-5 (Peyravian et al. 2022) expression after exposure to morphine, while other *in vivo* studies have shown that morphine decreases claudin-5 expression within the brain (Kosson et al. 2014; McLane et al. 2014). In the saline treatment groups, Tat(+) mice had significantly reduced claudin-5 as compared to the Tat(−) mice, which agrees with the findings of other investigators examining the impact of Tat on claudin-5 expression using both *in vitro* (András et al. 2005; Zhong et al. 2008; Liao et al. 2020) and *in vivo* models (András et al. 2003; Pu et al. 2007; Huang et al. 2014; Khan et al. 2022). Furthermore, the present study is in accordance with our previous *in vivo* data, demonstrating that Tat disrupted BBB integrity resulting in leakage of sodium-

fluorescein (0.376kDa) and horseradish peroxidase into the brain (Leibrand et al. 2017). In addition, we found that the co-exposure of Tat and fentanyl decreased claudin-5 in the striatum.

Although this study did not examine specific mechanisms related to the observed alterations in ZO-1 or claudin-5 , others have reported that Tat alters the distribution of ZO-1 and claudin-5 through mitogen-activated protein kinase 1 and 2 (MEK1/2) signaling (Wang et al. 2004; András et al. 2005; Pu et al. 2005; Toschi et al. 2006; Shin et al. 2015). Not unexpectedly, fentanyl also activates extracellular-signal regulated kinase (ERK1/2) (Macey et al. 2006), which is downstream of MEK1/2, suggesting that both fentanyl and Tat may use the same or similar mitogen-activated protein kinase (MAPK) cascade to disrupt BBB integrity. Furthermore, brain-region specific cytokine and chemokine secretion in the presence of both Tat and morphine has been associated with regional differences in BBB impairment (Fitting et al. 2010a; Nass et al. 2020).

To further understand the relationship between BBB integrity and inflammation, we performed correlation analyses between Tat status and chemokines on claudin-5 and ZO-1. The activation of nuclear factor-κB (NF-κB) by IL-2 or TNF-α results in the production of cytokines and chemokines such as IL-6 and CCL4 (Pahl 1999; Romio et al. 2011; Sindhu et al. 2019). Increased CCL4 concentrations have been demonstrated to alter the integrity of the blood brain barrier by decreasing ZO-1 through the activation of p38 mitogen-activated kinase (p38MAPK) (Li et al. 2015; Estevao et al. 2021). Activation of p38MAPK destabilizes the cytoskeleton, alters brain endothelial cell permeability, and disrupts the expression and distribution of ZO-1 and claudin-5 (González-Mariscal et al. 2008; Li et al. 2015; Jin et al. 2018; Estevao et al. 2021).

Of the dual function chemokines, CXCL10 activates the c-Jun N-terminal kinase (JNK) pathway, induces the production of TNF-α, leading to decreased claudin-5 and ZO-1 (Wang et al. 2018b) Among other cytokines, IL-6 is increased when TNF-α is overexpressed (Pahl 1999; Romio et al. 2011; Rochfort et al. 2016; Sindhu et al. 2019) and both IL-6 and TNF-α contribute to alterations in BBB integrity by increasing reactive oxygen species (ROS) that contribute to the loss of claudin-5 and ZO-1(Rochfort et al. 2014; Rochfort and Cummins 2015).

In addition to paracellular diapedesis, monocytes may enter the brain through transcellular diapedesis, which does not involve tight junctions, but is rather characterized by the concentration of adhesion molecules like ICAM and VCAM on BMECs (Ivey et al. 2009). Many studies reveal the mechanisms by which HIV, HIV proteins, and HIV-induced inflammatory mediators affect the BBB through upregulating ICAM-1/VCAM-1, inducing BMEC apoptosis, and altering the expression of tight junction proteins and accessory proteins (Toborek et al. 2005; András et al. 2005; Eugenin et al. 2006, 2010; Song et al. 2007; Zhong et al. 2008; Mahajan et al. 2008; Xu et al. 2012). In the present study, we did not find a change in VCAM in the presence of Tat and/or fentanyl, however, we did find Tat-induced alterations in PDGF receptor-β (PDGFR-β) expression. PDGFR-β and platelet-derived growth factor-BB (PDGF-BB) signaling mediates the recruitment and integrity of pericytes able to restore dysfunction at the BBB (Funa and Sasahara 2014; Montagne et al. 2015; Shen et al. 2019). Increased PDGF-BB by HIV-1

Tat leads to the autocrine activation of PDGFR-β and pericyte migration, overall resulting in the disruption of BBB integrity (Niu et al. 2014). Morphine induces PDGF-BB expression while at the same time, morphine decreases the expression of ZO-1 (Wen et al. 2011).

Fentanyl dysregulated the immune response in Tat-transgenic mice

The striatum and hippocampus are both key targets for HIV infection, and are abundantly rich in opioid receptors and affected by opioid exposure (Berger and Arendt 2000; Gurwell et al. 2001; Haughey and Mattson 2002; Nath 2015). Between the striatum and hippocampus, chemokine concentrations varied with exposure to fentanyl, consistent with previous reports of regional differences in cytokines and chemokines in the presence of morphine (Fitting et al. 2010b; Hermes et al. 2020; Nass et al. 2021). Cytokines and chemokines are proteins involved in the innate and adaptive immune responses that have roles in regulating cell differentiation and activation (Miller and Krangel 1992; Borish and Steinke 2003). In general, *homeostatic chemokines* are constitutively expressed, *inflammatory chemokines* are inducible, and *dual function chemokines* are both constitutively expressed and inducible [Table 1] (Moser and Loetscher 2001). CXCL13 is the only *homeostatic* chemokine included in the present study and is involved in B-cell, T-cell, and macrophage chemotaxis (Bekele Feyissa et al. 2021; Gao et al. 2021). Increased CXCL13 concentrations in human plasma have been suggested to be positively correlated with HIV disease progression and mortality risk (Wada et al. 2016; Mehraj et al. 2019). Most chemokines involved in the immune response and protection against pathogens are under the control of NF-κB transcription factors (Richmond 2002; Oeckinghaus and Ghosh 2009; Fiume et al. 2015). Cytokines and stimuli like ROS, activate the NF-κB pathway which activates gene expression responsible for the production of chemokines (Richmond 2002; Collino et al. 2006). In the brain, chemokines are involved in regulating neuroinflammation and neurodegeneration as they interact with their receptors, cells, and cell signaling cascades (Le Thuc et al. 2015). In our study, we have demonstrated that fentanyl dysregulates neuroinflammation by increasing CCL4, CCL20, and CXCL10.

Fentanyl increased CCL4 in the striatum and hippocampus. Increased CCL4 has been previously associated with neurological conditions such as Alzheimer's and Parkinson's disease that share similar mechanisms of neuronal degeneration as seen in HAND (Zhu et al. 2014; Canet et al. 2018; Calvani et al. 2020; Estevao et al. 2021; Priyanka and Seth 2022). In addition, CCL4 activates p38MAPK (Estevao et al. 2021) which stimulates the NF-κB pathway (Vanden Berghe et al. 1998; Collino et al. 2006). Interestingly, *in vitro* studies reveal NF-κB suppression by fentanyl, suggesting that fentanyl may specifically interfere with the immune response through this pathway (Liu et al. 2006; Ma et al. 2017). Indeed, inflammatory responses are attenuated when NF-κB activation is inhibited (Collino et al. 2006). In addition to CCL4, cytokines such as TNFα, and the HIV viral proteins, Tat and gp120, are known to activate NF-κB (Chen et al. 1999; Santoro et al. 2003; Ben Haij et al. 2015; Planès et al. 2016; Mussbacher et al. 2019). It is suggested that concurrent activation at MOR by morphine and the

activation at CCR5 by HIV-1 Tat upregulates CCL4 (Hahn et al. 2010) promotes neuroinflammation and neurotoxicity (Malik et al. 2011; Zou et al. 2011; Fitting et al. 2014; Gonek et al. 2018). Therefore, the elevated CCL4 concentrations induced by fentanyl in the current study is consistent with previous reports demonstrating interactive effects of Tat and an opioid agonist (Zou et al. 2011; Gonek et al. 2018).

CCL20, also known as macrophage inflammatory protein-3α (MIP-3α), is a major chemokine involved in the immune response that binds selectively to the CCR6 receptor (Terao et al. 2009; Zlotnik et al. 2011; Williams et al. 2014; Das et al. 2019). In studies examining traumatic brain injury (Leonardo et al. 2012) and cerebral ischemia (Terao et al. 2009), CCL20 was demonstrated to participate in neurodegeneration through inducing neuronal cell death (Leonardo et al. 2012), inducing CCR6 gene expression (Terao et al. 2009), and stimulating chemotaxis of CD_4^+ T cells by activating CCR6 (Leonardo et al. 2012). In addition, proinflammatory cytokines like IL-1β and TNF-α, which can be increased in a temporally-dependent manner with Tat-induction in our model (Nass et al., 2020; Hermes et al., 2021), stimulate the production of CCL20, which maintains the inflammatory cascade (Terao et al. 2009). Fentanyl increased CCL20 in the striatum, while reducing CCL20 in the hippocampus. These regional specific alterations are in agreement with previous work demonstrating spatial differences in cytokines that are suggested to be related to regional specific BBB disruption and/or glial cell activation (Arima et al. 2012; Drabek et al. 2015; Leibrand et al. 2017, 2019; Nass et al. 2020).

Similar to CCL20, CXCL10, is an important mediator of the immune response and binds with high specificity to CXCR3 (Bajetto et al. 2002; Cho et al. 2009). CXCL10 is interesting especially in the setting of neuroHIV because elevated CXCL10 is associated with altered neurological function and both morphine and fentanyl have shown to inhibit TNF-α induced CXCL10 production (Davis et al. 2007; Cho et al. 2009; Michlmayr and McKimmie 2014). CXCL10 can dysregulate Ca^{2+} homeostasis and promote excitotoxicity and synaptodendritic degeneration (Nelson and Gruol 2004; Sui et al. 2006; Williams et al. 2009a, b). Tat can disrupt Ca^{2+} homeostasis and promote excitotoxicity in neurons ultimately causing neuronal cell death (Haughey and Mattson 2002; Fitting et al. 2014). Similarly, opioid activation of MOR regulates ionic currents and calcium homeostasis in neurons (Lemos et al. 2012; Seseña et al. 2014). Fentanyl dysregulates calcium homeostasis, promoting overall hyperexcitability in neurons, (Khomula et al. 2019) and activates Toll-like receptor 4 (TLR4) (Stevens et al. 2013). Taken together, it may be suggested that through upregulating CXCL10, fentanyl contributes to neurodegeneration by dysregulating calcium homeostasis and neuronal dysfunction.

Fentanyl appears to severely dysregulate the CNS immune response causing discordant increases and decreases in related chemokines. For example, fentanyl increased CCL4, CCL20 and CXCL10, while reducing CCL3, CCL17, and CXCL9 levels. The discrepancy between CCL3 and CCL4 (in the striatum and hippocampus) is interesting because both are inducible by IL-1β (Zhu et al. 2014; Guo et

al. 2014), and activate CCR5, although CCL3 also binds to CCR1 (Takahashi et al. 2015). As an inducible chemokine, astroglial CCL3 recruits MDMs and promotes neuroinflammation (Pelisch et al. 2020).

An *in vitro* study examined the immunologic effects of fentanyl in the setting of sepsis, a condition characterized by inflammation and deficits in motor and sensory functions (Bolton et al. 1984; Gotts and Matthay 2016; van der Poll et al. 2017; Hirotsu et al. 2022; Bissell et al. 2023). Similar to the present study, fentanyl exposure significantly decreased the expression CCL3 and IL-1β in mice with sepsis compared with saline-treated mice (Bissell et al. 2023).

CCL17 is a regulatory chemokine involved in T_{reg} responses and immune homeostasis (Scheu 2017). A study utilizing an intracerebral hemorrhage mouse model revealed that CCL17 has neuroprotective properties; CCL17 decreased the activation of microglia and macrophages and inhibited neuronal cell death (Deng et al. 2021). The decrease in CCL17 also might be related to TNF-α, which has previously been demonstrated to decrease in response to fentanyl (Wen et al. 2015; Wang et al. 2018a).

To conclude, the blood brain barrier and the immune response were both significantly dysregulated in the presence of HIV-1 Tat and fentanyl [Fig. 8]. The integrity and function of the blood-brain barrier was altered as evidenced by dysregulated claudin-5, ZO-1, and PDGFR-β. Inflammatory chemokines were severely altered in the striatum and hippocampus, with the exception that CCL11 was only altered in the hippocampus and CXCL1 was only altered in the striatum. Dual-function chemokines were also dysregulated in both brain regions with the exception that CXCL10 was only altered in the striatum and CCL22 was not altered at all. To elucidate on the mechanisms responsible for the dysregulation of BBB integrity and chemokine responsiveness, future experiments should examine the events signaling intracellular signaling pathways, such as those involving NK-κB and p38MAPK (Liu et al. 2006; Ma et al. 2017), in addition to cytokines involved in BBB regulation and production.

Table 2. Effects of fentanyl and HIV-1 Tat exposure on claudin-5 and ZO-1 levels in the striatum (areal intensity).

	Fentanyl (Main effect)		**HIV-1 Tat (Main effect)**		**Interaction**	
	$F_{(1,10)}$	*p*	$F_{(1,10)}$	*p*	$F_{(1,10)}$	*p*
CLAUDIN-5	59.06	**< 0.0001**✧	20.08	0.0012	8.8	**0.0142**≒
ZO-1	8.93	**0.0136**✧	0.038	0.85	3.237	0.102

Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline-treated], significant main effect of Tat status (ⓣ) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (≒).

Table 3. Effects of fentanyl and HIV-1 Tat exposure on VCAM and PDGFR-β levels in the striatum.

	Fentanyl (Main effect)		**HIV-1 Tat (Main effect)**		**Interaction**	
	$F_{(1,25)}$	*p*	F	*p*	$F_{(2,26)}$	*p*
VCAM	1.91	0.18	1.11	0.30	1.53	0.23
PDGFR-β	2.61	0.12	7.16	**0.013♏**	4.55	**0.04≒**

Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline-treated], significant main effect of Tat status (♏) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (≒).

Table 4. Effects of fentanyl and HIV-1 Tat exposure on chemokine levels in the striatum and hippocampus.

Brain Region		Fentanyl (Main effect)		Tat (Main effect)		Interaction	
Striatum							
	Chemokine	$F_{(1,26)}$	*p*	$F_{(1,26)}$	*p*	$F_{(1,,26)}$	*p*
	CCL3	35.69	**< 0.0001**✧	0.06	0.81	0.133	0.72
	CCL4	67.93	**< 0.0001**✧	0.26	0.61	0.04	0.84
	CCL11	0.05	0.83	1.18	0.29	1.01	0.32
	CCL17	2973	**< 0.0001**✧	0.21	0.65	0.21	0.65
	CCL20	21.74	**< 0.0001**✧	0.14	0.71	0.14	0.71
	CCL22	9.13	**0.005**✧	2.57	0.12	0.44	0.51
	CXCL1	0.49	0.49	7.17	**0.01**ⓗ	0.0005	0.98
	CXCL9	36.31	**< 0.0001**✧	1.64	0.21	0.76	0.39
	CXCL10	31.70	**< 0.0001**✧	0.69	0.41	1.29	0.27
	CXCL13	4.65	**0.04**✧	0.006	0.94	1.32	0.26
Hippocampus							
	Chemokine	$F_{(1,26)}$	*p*	$F_{(1,26)}$	*p*	$F_{(1,,26)}$	*p*
	CCL3	205.0	**< 0.0001**✧	1.80	0.33	2.57	0.12
	CCL4	76.74	**< 0.0001** ✧	0.1345	0.69	0.3441	0.54
	CCL11	1.41	0.25	4.96	**0.03**ⓗ	1.23	0.28
	CCL17	3190	**< 0.0001**✧	1.17	0.29	2.40	0.13
	CCL20	51.90	**< 0.0001**✧	0.72	0.40	1.03	0.32
	CCL22	2.78	0.11	1.87	0.18	1.62	0.21
	CXCL1	0.13	0.72	2.88	0.10	2.36	0.14
	CXCL9	17.13	**0.0003**✧	1.33	0.26	1.22	0.28
	CXCL10	3.86	0.06	1.22	0.28	1.30	0.26
	CXCL13	3.80	0.06	0.01	0.90	0.59	0.45

Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline-treated], significant main effect of Tat status (ⓗ) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (⍇).

Chapter 3 Figures.

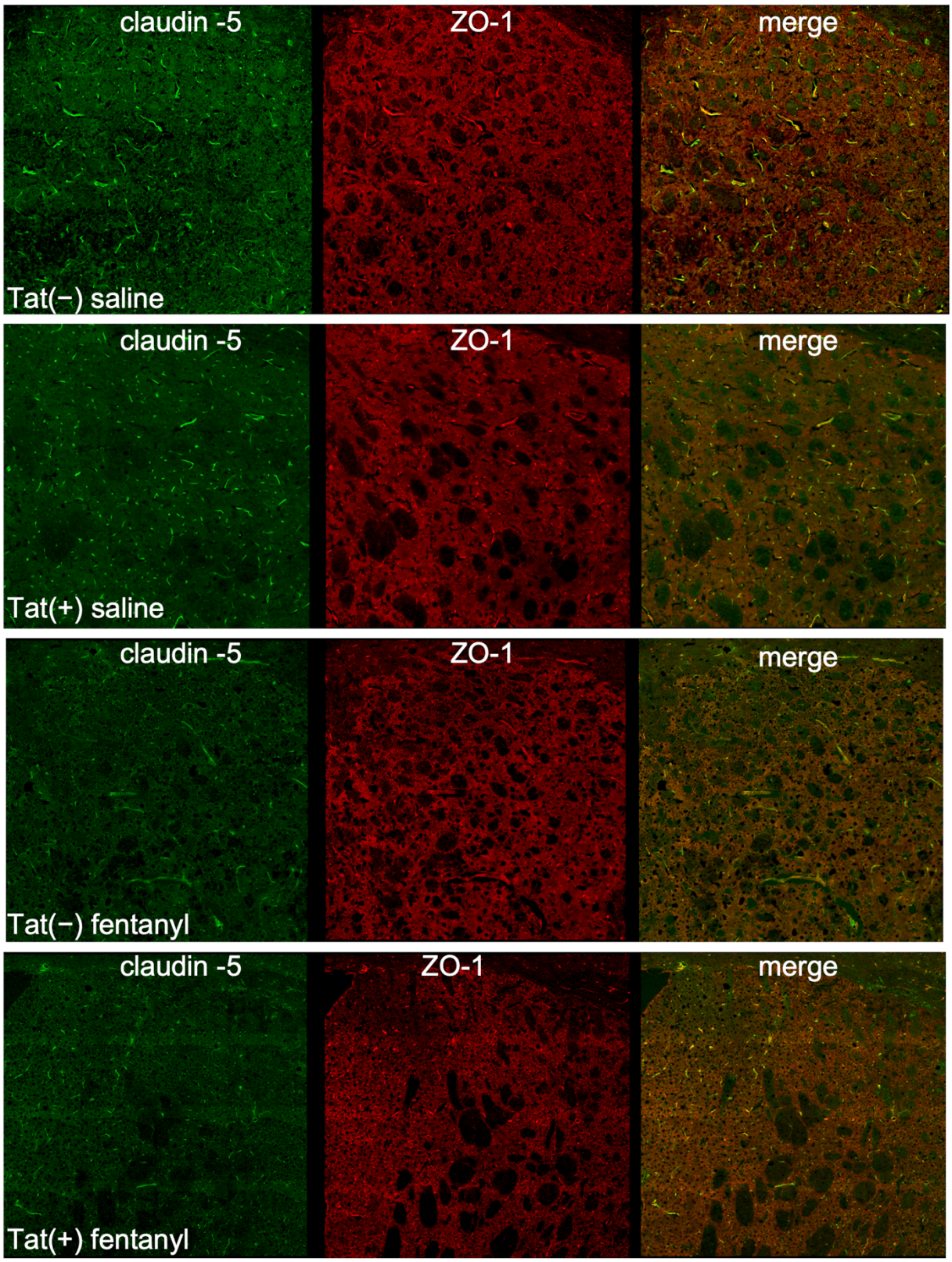

Fig. 1 Photomicrographs of claudin-5 and ZO-1 immunofluorescence in the striatum.

Areal measurements of mean fluorescent pixels (intensity above a specific threshold) immunoreactive for ZO-1 and claudin-5 were assessed within optical z-plane sections using confocal microscopy and Image J software (NIH, Bethesda, MD) as described before (Bertrand et al. 2016; Logsdon et al. 2018; Wang et al. 2020a; Xing et al. 2020; Shihan et al. 2021).

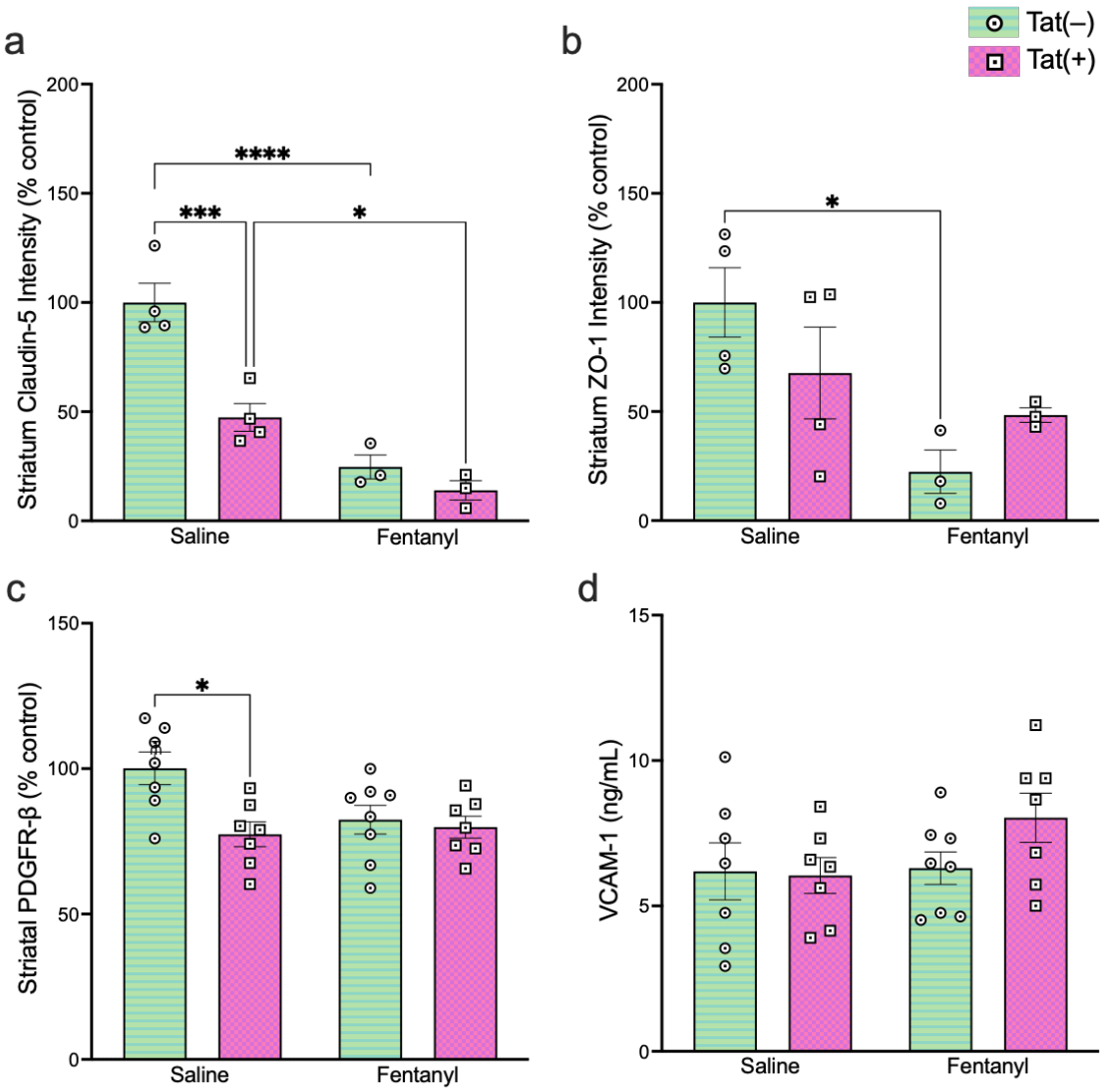

Fig. 2 Areal intensity measurements of claudin-5 (a) and ZO-1 (b) and enzyme-linked immunosorbent assay (c) and immunoblot (d) results. Tat(−) mice are depicted on the bar graph by green with horizontal lines or white circles with a black dot where Tat(+) mice are depicted by pink and purple squares and white squares with black a dot. Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline], significant main effect of Tat status (⋒) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (⩲) and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$ for post-hoc testing.

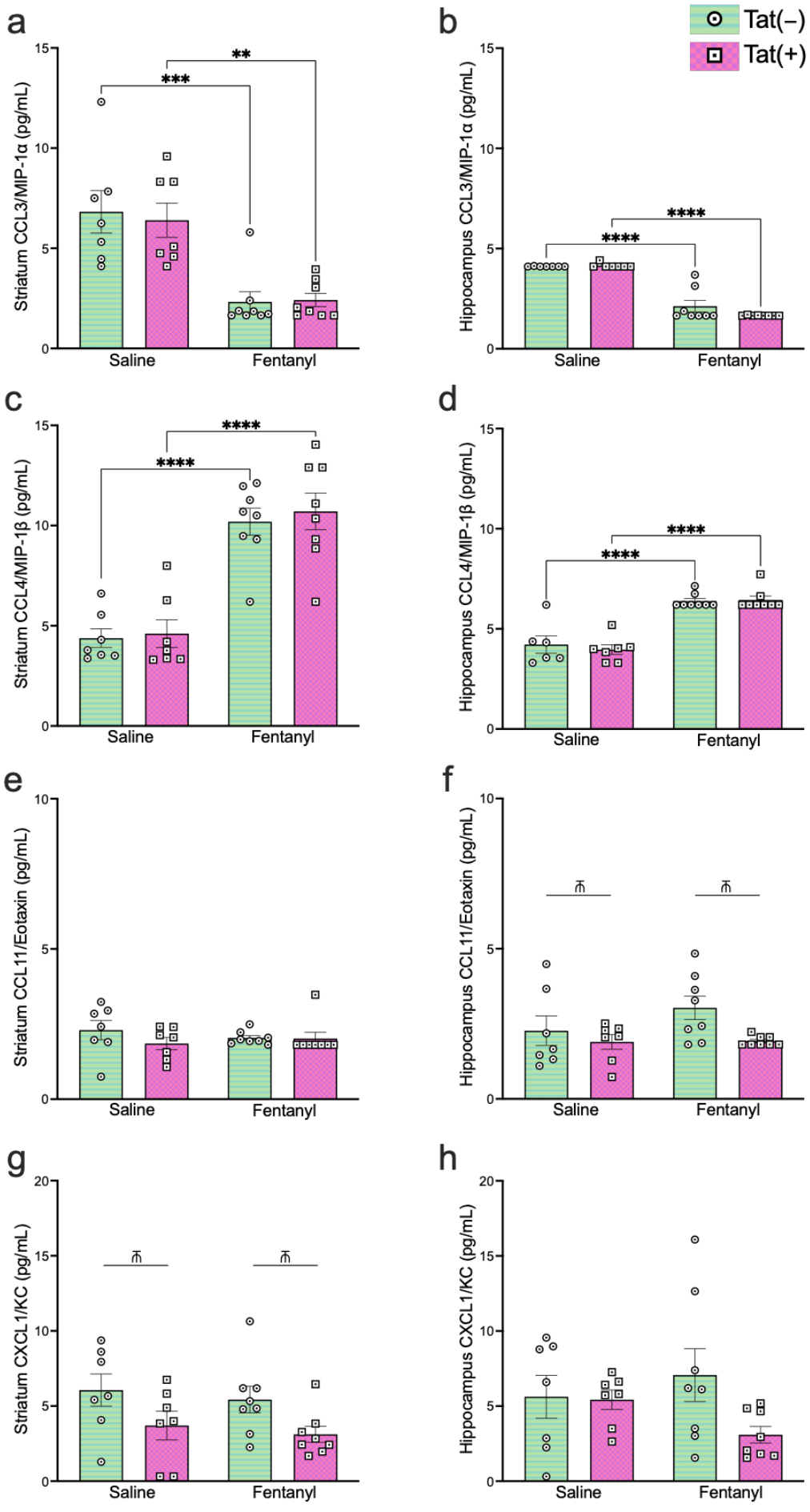
a
b
c
d
e
f
g
h
Tat(−)
Tat(+)
Striatum CCL3/MIP-1α (pg/mL)
Hippocampus CCL3/MIP-1α (pg/mL)
Striatum CCL4/MIP-1β (pg/mL)
Hippocampus CCL4/MIP-1β (pg/mL)
Striatum CCL11/Eotaxin (pg/mL)
Hippocampus CCL11/Eotaxin (pg/mL)
Striatum CXCL1/KC (pg/mL)
Hippocampus CXCL1/KC (pg/mL)
Saline
Fentanyl
**
***
****
ns

Fig. 3 *Inflammatory chemokines* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(−) mice are depicted on the bar graph by green with horizontal lines or white circles with a black dot where Tat(+) mice are depicted by pink and purple squares and white squares with black a dot Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline], significant main effect of Tat status (⋔) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (⩲) and significant differences at $\alpha < 0.05$ are denoted by $*p < 0.0032$, $**p < 0.0021$, $***p < 0.0002$, and $****p < 0.0001$ for post-hoc testing.

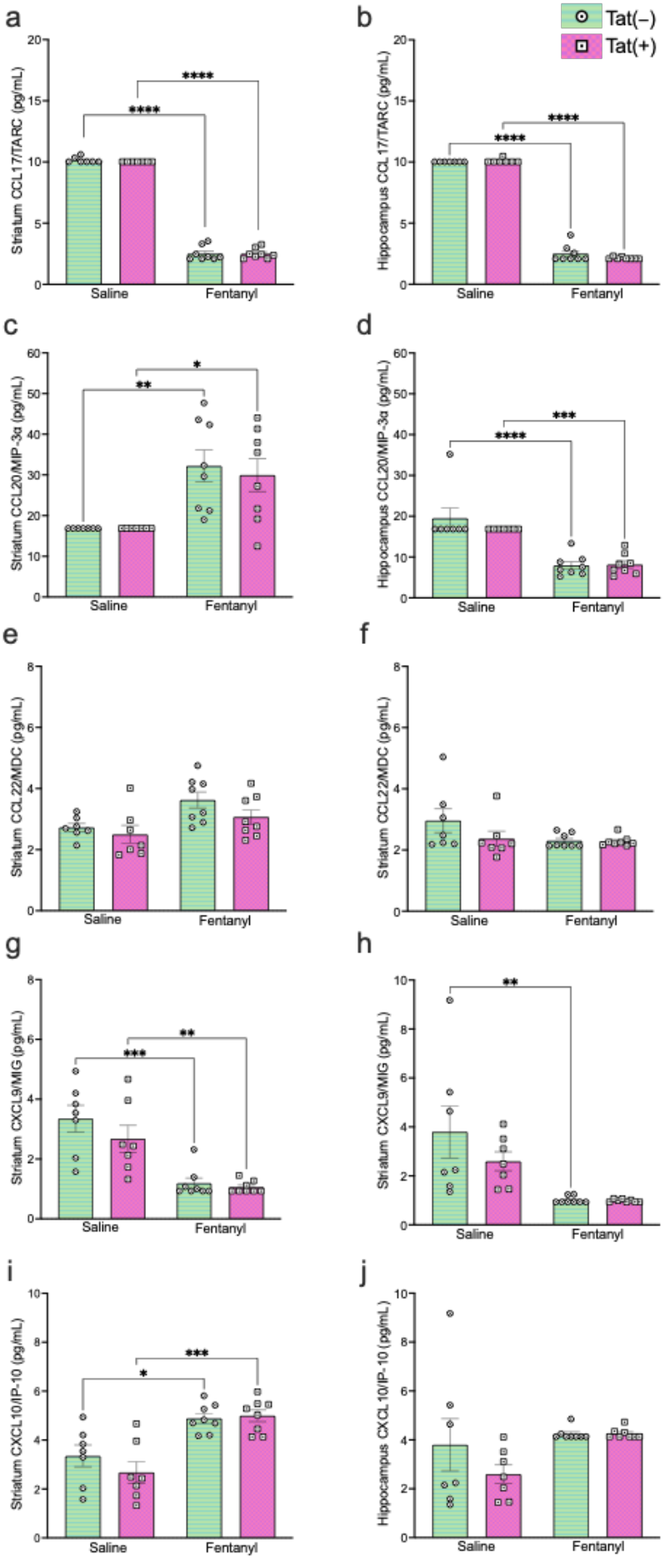
a
b
Tat(−)
Tat(+)
Striatum CCL17/TARC (pg/mL)
Hippocampus CCL17/TARC (pg/mL)
Striatum CCL20/MIP-3α (pg/mL)
Hippocampus CCL20/MIP-3α (pg/mL)
Striatum CCL22/MDC (pg/mL)
Striatum CXCL9/MIG (pg/mL)
Striatum CXCL10/IP-10 (pg/mL)
Hippocampus CXCL10/IP-10 (pg/mL)
Saline
Fentanyl

Fig. 4 *Dual-function chemokines* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(−) mice are depicted on the bar graph by green with horizontal lines or white circles with a black dot where Tat(+) mice are depicted by pink and purple squares and white squares with black a dot. Main effects are represented by significant main effect of treatment (✧) [fentanyl vs. saline], significant main effect of Tat status (⋔) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (⩲) and significant differences at $\alpha < 0.05$ are denoted by $*p < 0.0032$, $**p < 0.0021$, $***p < 0.0002$, and $****p < 0.0001$ for *post-hoc* testing.

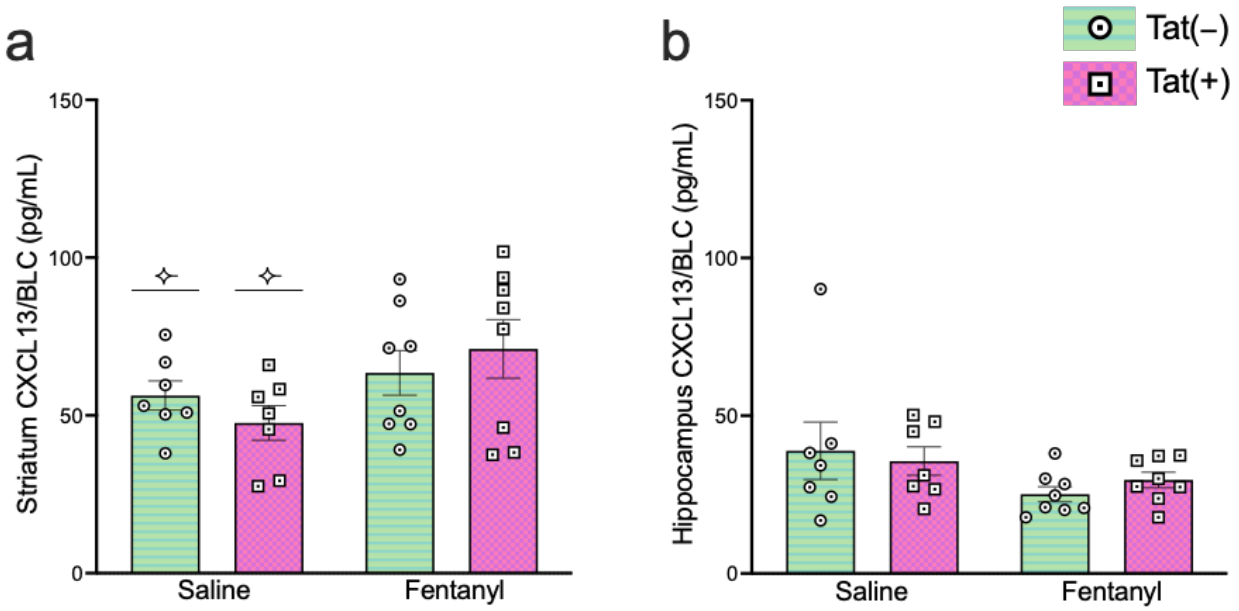

Fig. 5 *Homeostatic chemokine* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(−) mice are depicted on the bar graph by green with horizontal lines or white circles with a black dot where Tat(+) mice are depicted by pink and purple squares and white squares with black a dot. Significant main effects of fentanyl (✧) [fentanyl vs. saline], Tat exposure (m̄) [Tat(−) vs. Tat(+)], and interactions between fentanyl and Tat (≒) were noted.

Correlation analyses

To further understand the impact of chemokines on BBB integrity, Pearson r correlation analyses were performed between Tat status and chemokines on claudin-5 and ZO-1, respectively.

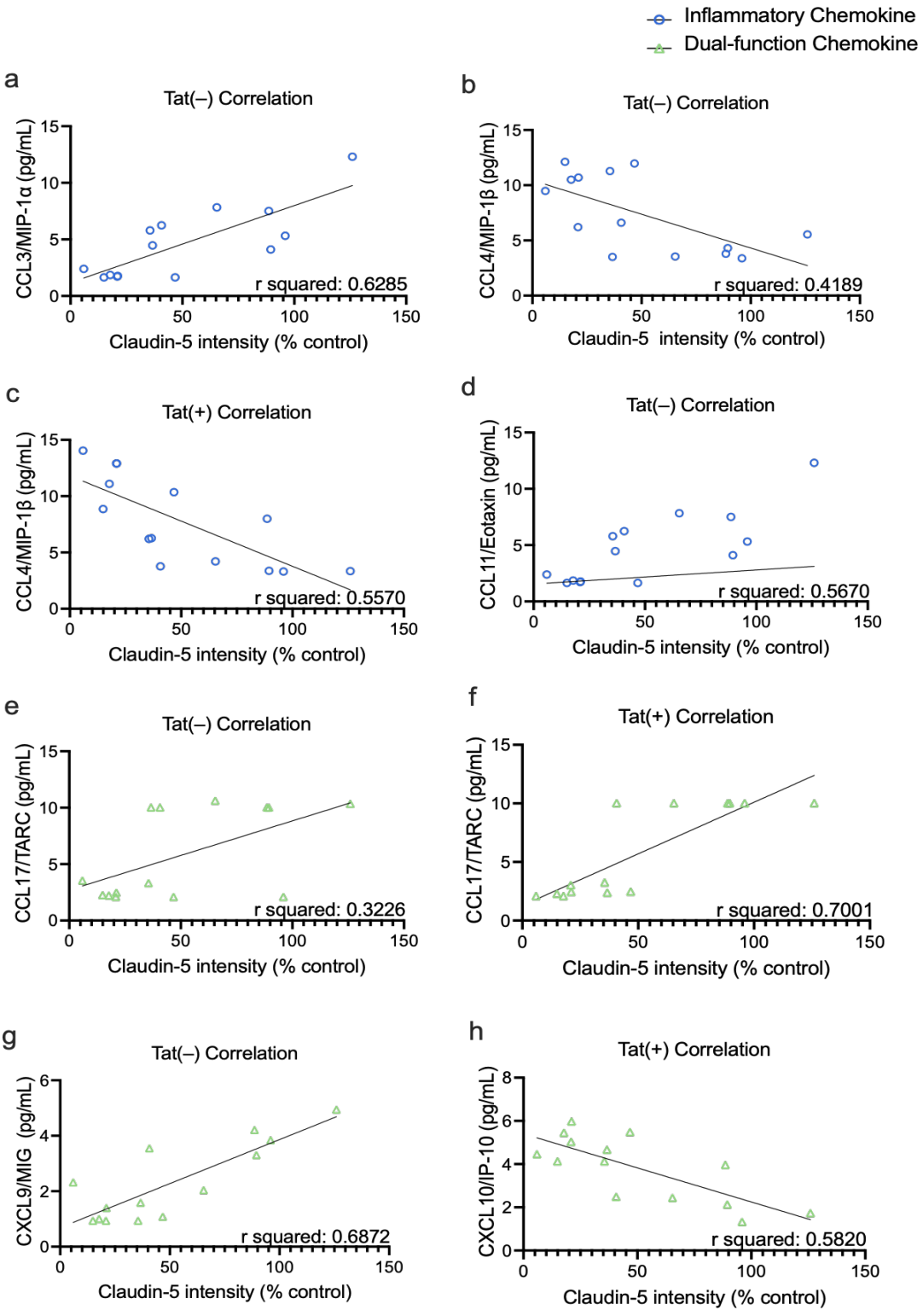

Fig. 6 Correlation analyses in the striatum between chemokines and claudin-5. Inflammatory chemokines are represented by open-blue circles. Dual-function chemokines are represented by open-green triangles.

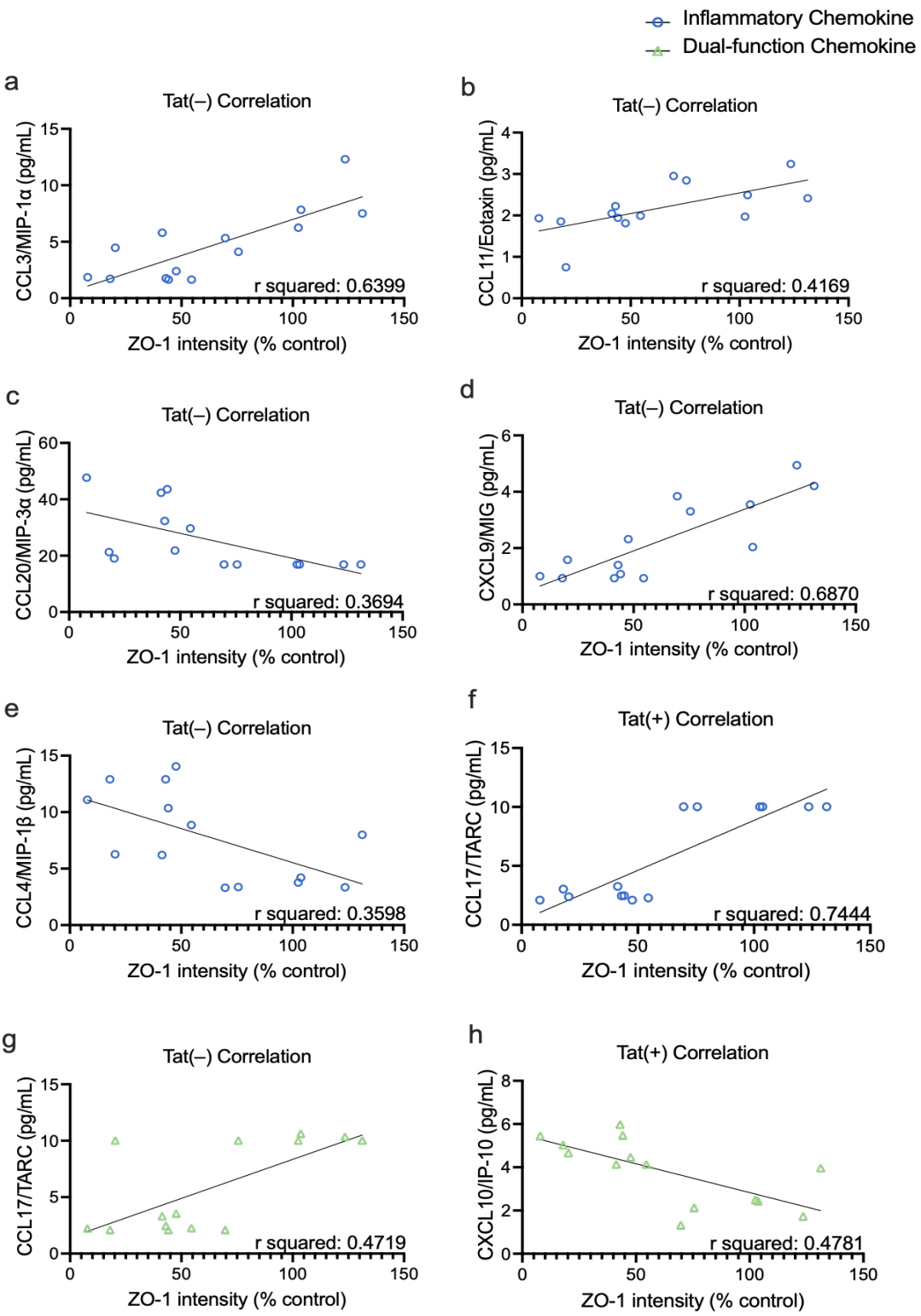

Fig. 7 Correlation analyses in the striatum between chemokines and ZO-1. Inflammatory chemokines are represented by open-blue circles. Dual-function chemokines are represented by open-green triangles.

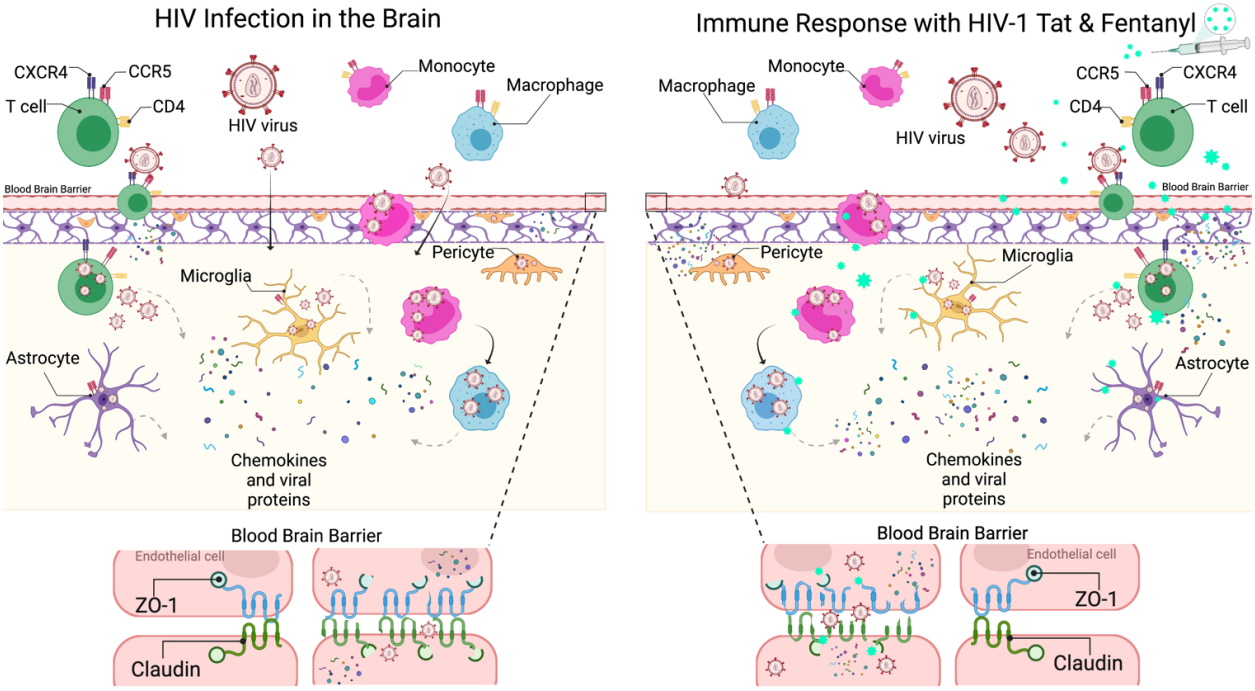

Fig. 8 Immune response depicted with chemokines in the brain in the presence of Tat and fentanyl.

Chapter 4. Morphine and fentanyl dysregulate neuroinflammation and disrupts blood-brain barrier integrity in EcoHIV infected mice.

Introduction

Intravenous (IV) drug use is suggested to be the highest risk factor for new HIV diagnoses; a recent study demonstrates that 62% of people living with HIV (PLWH) reported daily injection drug use and that 1 in 4 PLWH share needles (Alpren et al. 2020). It is also estimated that one third of acquired HIV infections are attributed to injecting drugs such as opioids (Banerjee et al. 2011). In addition to being a risk factor for HIV, opioid use in the setting of HIV increases neuroHIV, which describes mechanisms related to HIV-associated neurocognitive disorders (HAND) (Hauser et al. 2012; Fitting et al. 2020). Opioid abuse in the setting of HIV contributes to cognitive impairment, poor antiretroviral accumulation in the brain, and chronic inflammation and neuronal injury (Fantuzzi et al. 2003; Letendre et al. 2004; Verani et al. 2005; El-Hage et al. 2005; Martin et al. 2018; Olivier et al. 2018; Osborne et al. 2020).

The 'Trojan Horse' hypothesis describes one of the mechanisms of how HIV enters the brain via infected monocytes crossing the blood brain barrier (BBB) (Eugenin et al. 2010; Lindl et al. 2010; Siliciano and Greene 2011). Once in the brain, infected monocytes differentiate into long-lived macrophages, where the virus replicates and establishes viral reservoirs (Fantuzzi et al. 2003; Verani et al. 2005; Duncan and Sattentau 2011). Viral reservoirs greatly contribute to neuroHIV (Crowe et al. 2003; Fois and Brew 2015) based on the understanding that many first-line antiretrovirals therapies (ARVs) do not accumulate in the brain at concentrations sufficient for limiting viral replication (He et al. 2018; Wong et al. 2019; Osborne et al. 2020). Complicating this further is the presence of opioids, such as morphine,, which further limits the penetration of ARVs into the brain and also damages the BBB (Leibrand et al. 2019). The BBB serves to protect the brain from harmful substances or pathogens (Abbott et al. 2010). Neurotoxic and neuroinflammatory signals have been implicated in mediating cell death at the BBB (Yang et al. 2009; Eugenin et al. 2011; Kadry et al. 2020). The HIV viral protein Tat (transactivator of transcription) alters the integrity of the BBB by decreasing the expression and/or altering the distribution of claudin-1 and claudin-5, ZO, and adhesion molecules (Pu et al. 2003; András et al. 2003, 2005; Leibrand et al. 2017). The integrity of the BBB is also disrupted by toxic insults from opioids (Wen et al. 2011; Kosson et al. 2014) and inflammatory mediators (Stamatovic et al. 2005; Terao et al. 2008). Activation of cells by either opioids or HIV viral proteins results in excessive calcium mobilization, production of cytokines/chemokines, and increased reactive oxygen species (ROS) production that destabilizes the BBB (El-Hage et al. 2005; Bruce-Keller et al. 2008; Turchan-Cholewo et al. 2009; Zou et al. 2011; Kim et al. 2018).

Recently, the opioid epidemic has largely consisted of fentanyl abuse as evidenced by a decrease in heroin-related deaths and an increase in fentanyl-related deaths (Hedegaard et al. 2020). Fentanyl is a synthetic opioid agonist that binds with the highest affinity at the μ-opioid receptor (Comer and Cahill 2019). However, most of fentanyl's inflammatory effects are not mediated through opioid

receptors, and are thought to occur through alternative receptors such as adrenergic receptors (Torralva and Janowsky 2019; Torralva et al. 2020; Kelly et al. 2023). Fentanyl is 400 times more potent than morphine and 30-50 times more potent than heroin intravenously (Ciccarone et al. 2017; Comer and Cahill 2019; Moss and Carlo 2019) and it displays a vastly different pharmacological profile in comparison to morphine and heroin (Torralva and Janowsky 2019; Torralva et al. 2020; Kelly et al. 2023). Despite the increased abuse of fentanyl and the interlinked opioid and HIV epidemics (Leshner 1998), the effects of fentanyl on the neuropathology of HIV has yet to be fully examined. To date, the interaction between HIV fentanyl has only been examined *in vitro,* where fentanyl increases chemokine co-receptors necessary for HIV viral replication and suppresses the immune response (Kong et al. 2022; Yan et al. 2022).

The current study utilizes the EcoHIV model, which involves infection of immunocompetent mice with an actively replicating mouse-tropic chimeric virus that can establish latent reservoirs similar to HIV (Gu et al. 2018). EcoHIV is constructed by replacing the *gp120* viral envelope protein gene with *gp80* limiting infection to rodents (Ceckova et al. 2018). EcoHIV mimics active viral infection, virus can be detected in the brain, spleen, and peritoneal macrophages within 2-12 weeks after a single inoculation (Potash et al. 2005; Ceckova et al. 2018). EcoHIV infection elicits similar responses as HIV, such as increased chemokine secretion, neurotoxicity, and impaired cognition (Kelschenbach et al. 2012; Olson et al. 2018; Dong et al. 2020). Few studies have used this model to examine the effects of drugs of abuse, such as fentanyl and morphine, on the inflammatory state and antiretroviral drug distribution in the brain. The present study sought to compare the effects of morphine and fentanyl on the BBB integrity, inflammatory mediators, and ARV accumulation within the brain using the infectious mouse model of HIV, EcoHIV.

Materials and Methods

Animal Handling and Treatment

All studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and the experiments were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85–23).

EcoHIV

EcoHIV viral stocks were prepared through the transfection of 293T cells by plasmid EcoHIV DNA (Potash et al. 2005). The resultant EcoHIV was titered for core antigen, p24 content (Gu et al. 2018).

Subjects and housing

Male and female C57BL/6 mice (Envigo, ~4 months of age weighing ~25 g) were anesthetized (3-4% isoflurane) and injected intraperitoneally with 2 million picograms of p24 EcoHIV [EcoHIV(+)] or with normal sterile saline [EcoHIV(−), uninfected mice]. Mice were housed 4 per cage and were separated by EcoHIV(+) and uninfected mice. Food and water was provided *ad libitum* for the duration of the study.

Treatment

Triumeq® tablets, purchased from the VCU Health Systems Pharmacy, were used to prepare a drug solution containing abacavir 2.5 mg/day, lamivudine 1.2 mg/day, and dolutegravir 0.2 mg/day. Doses were prepared using allometric scaling (25 g mouse) as previously described (Nair and Jacob 2016)). Fentanyl (NIDA Drug Supply Program, Bethesda, MD), morphine (NIDA Drug Supply Program, Bethesda, MD) or sterile saline (control) was added to the antiretroviral solution and loaded into the ALZET® pump at a concentration sufficient to deliver 1.92 mg/day morphine, 0.05mg/day fentanyl, or saline. Antiretroviral drugs were co-administered with each treatment (morphine, fentanyl, or saline) for 5 days. Drug preparations were made in batches to minimize dosing variability.

Surgical manipulation

Fifteen days post inoculation, Alzet® osmotic pumps (2001, 1.0 µL/h, Cupertino, CA) containing ARVs drugs ± morphine ± fentanyl ± saline were subcutaneously implanted in the mice. After 5 days of continuous delivery of ARVs ± opioids, mice were anesthetized (4% isoflurane) and sacrificed by exsanguination to collect blood. Brain and spleen were harvested from each mouse and ½ of fresh spleen was used for DNA isolation. The brain was hemisected into left and right hemispheres, and further microdissected into striatum and hippocampus. Tissues were snap frozen and stored at −80 °C until analysis. Plasma was separated from whole blood by centrifugation (5,000 × *g* at 4 °C for 10 min) and stored at −80 °C until analysis.

Antiretroviral Quantification Analysis

Antiretroviral concentrations were measured in the plasma and tissues using LC-MS/MS, as described previously (Leibrand et al. 2019). Briefly, the right striatum and hippocampus frozen samples were weighed and homogenized using Precellys® hard tissue grinding kit tubes (Cayman Chemical, MI, USA) containing 1 mL of 70:30 acetonitrile:1mM Ammonium phosphate (pH 7.4). Lamivudine and abacavir plasma and tissue homogenates were extracted by protein precipitation with the isotopically labeled internal standards, abacavir-d 4 and lamivudine-15 N-d 2. Chromatographic separation was achieved by reverse phase chromatography on a Waters Atlantis T3 (50 x 2.1 mm, 3 µm) analytical column. Dolutegravir plasma and tissue homogenates were extracted by protein precipitation with the isotopically labeled internal standard, dolutegravir- 13 C-d 5. Chromatographic separation was achieved by reverse phase chromatography on a Waters XTERRA MS C18 (50 × 2.1 mm, 3.5 µm) analytical column. An AB Sciex API-5000 triple quadrupole mass spectrometer was used to detect all the analytes and internal standards under positive ion electrospray conditions. Precision and accuracy of the calibration standards and quality control samples were within 15% (lamivudine, abacavir in plasma) or 20% (lamivudine, abacavir in tissues and dolutegravir in plasma and tissues) for the following dynamic ranges: 50.0-4,000 ng/mL of lamivudine in plasma; 5.00 - 4,000 ng/mL of abacavir in plasma; 20.0 - 4,000

ng/mL of dolutegravir in plasma; 0.100 - 50.0 ng/mL of lamivudine, abacavir in tissue; and 0.025 - 50.0 ng/mL of dolutegravir in tissue. Final concentrations were normalized to tissue mass and reported in ng/g units.

Enzyme-linked Immunosorbent Assay

Enzyme-linked Immunosorbent Assay (ELISA) was performed on left striatal and hippocampus tissue lysates to determine whether EcoHIV and morphine or fentanyl exposure alters the concentrations of claudin-5 and ZO-1 in the striatum and hippocampus. Presence and relative amounts of tight junction and tight junction accessory protein in striatal and hippocampal samples were detected using the mouse tight junction protein 1 (ZO1) ELISA KIT (#MBS2604345, MyBioSource, San Diego, CA) and mouse claudin 5 (CLDN5) ELISA kit per manufacturer's instructions (#MBS456204, MyBioSource, San Diego, CA). Briefly, 5 µg samples of striatal and hippocampus lysates from the left hemisphere were used. For ZO-1, samples were incubated in pre-coated microplate wells, followed by washing steps to remove unbound antibodies and impurities. Biotinylated antibodies that are specific for ZO-1 or claudin-5 were added to each well, then horseradish + avidin, and finally substrates for the horseradish peroxidase (HRP) reaction. The microplate was washed thoroughly before each step. The microplates were then read on a BioTek plate reader using an absorbance of 450 nm (Synergy H1, 269978, BioTek). During the plate reading process, samples were normalized to the blank-control wells that contained only the standard diluent for ZO-1 or diluent buffer for claudin-5. Samples were then extrapolated from a generated standard curve. Samples were run in duplicate and the averages were used for statistical analysis.

Chemokine Assay

Chemokines were measured in the left striatum and left hippocampus using a Mouse 13-plex Proinflammatory Chemokine Panel, (Catalog #740007; BioLegend) according to the manufacturer's instructions. The striatal and hippocampal tissues were homogenized separately using NP40 lysis buffer containing cOmplete™ Mini Protease Inhibitor Cocktail (11836153001, Roche Diagnostics GmbH, Germany). The homogenates were centrifuged (16,000 x *g* at 4 °C for 10 min) and the extracted supernatants were used to determine protein content using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 2020) and then stored in −80 °C until chemokine analysis. Samples were prepared according to the manufacturer (BioLegend) and were analyzed by the Cytek® Aurora flow cytometer (Cytek® Biosciences, Fremont, California). Sample lysates containing 12.5 µg of protein or standard were loaded in duplicate and both sample and standard wells were incubated with chemokine antibody-conjugated beads (12.5 µL) for 2 h, followed by incubation with biotinylated detection antibodies for 1 h. During both incubation periods, the filter plate was placed on a shaker at 500 rpm. After both incubations, streptavidin-PE (SA-PE) was added for signal amplification and one final incubation period of 30 min was conducted. The filter plate was washed and sealed and stored at 4 °C for 24 h until analysis.

Immediately before analysis on the Cytek® Aurora, samples were washed and resuspended for 5 min, and then transferred to a u-bottom plate. The u-bottom plate was run on the Cytek® Aurora. Data was obtained using the LEGENDplex V8.0 software (BioLegend). A five-parameter logistic regression was performed by the LEGENDplex™ Data Analysis Software Suite to fit the standard curve of each chemokine with the duplicated samples.

Statistical Analysis

A two-way analysis of variance (two-way ANOVA) was performed on averaged duplicate samples to determine main effects or interactions between EcoHIV status and treatment (fentanyl, morphine, or saline) for the striatum and hippocampus. The Tukey HSD's multiple comparison test was used for pairwise comparisons between group means.

For the ARV quantification in tissues, plasma-normalized values that were below the limit of quantification were set to zero [Plasma LLOQ: abacavir 5 ng/mL, lamivudine 50 ng/mL, dolutegravir 20 ng/mL; Tissue LLOQ dependent on sample].

For chemokine analysis, sex was collapsed, and chemokine results were presented by their respective functions: inflammatory, dual, and homeostatic. The chemokines, CCL20, CXCL1, CXCL5, and CXCL9 were below the limit of detection and were therefore not included in the statistical analysis.

Statistical analyses were performed, and results graphed using GraphPad Prism 9.0, version 9.4.2 (GraphPad Software, LLC, La Jolla, CA). A significance level of $\alpha = 0.05$ was used and if the calculated *p*-value was less than $p < 0.05$ results were considered statistically significant. Uninfected mice are represented as EcoHIV(–) or Eco(–).

Claudin-5 and ZO-1 Statistical Analysis

Sample lysates used for ELISA analysis for claudin-5 and ZO-1 were performed in duplicate. Duplicates were averaged and fit to a standard curve to determine claudin-5 and ZO-1 concentrations for the females and males in the striatum and hippocampus. The resultant concentrations of claudin-5 and ZO-1 in females and males within the striatum and hippocampus were used to conduct a two-way analysis of variance (two-way ANOVA) to determine significant main effects and interactions between infection status and treatment (fentanyl, morphine, or placebo) in the striatum and hippocampus. The Tukey HSD's multiple comparison test was used for pairwise comparisons between group means.

Results

Antiretroviral Concentrations

To better understand the impact of EcoHIV infection and opioids on antiretroviral therapies, plasma and plasma-normalized tissue concentrations for the striatum and hippocampus were quantified by LC-MS/MS as previously described (Leibrand et al. 2019). Morphine decreased abacavir tissue concentrations in the striatum and hippocampus of infected mice, and fentanyl appeared to reverse the decrease in striatal

abacavir concentrations. In uninfected mice, lamivudine and dolutegravir concentrations were significantly lower in the striatum compared to fentanyl (uninfected mice). In the hippocampus, infected mice treated with fentanyl had significantly lower dolutegravir than infected mice treated with saline, and uninfected mice treated with fentanyl.

Plasma-normalized Antiretroviral Concentrations in the Striatum

In the striatum, plasma-normalized abacavir was significantly *lower* in EcoHIV(+) mice exposed to morphine compared to both EcoHIV(+) mice exposed to saline and EcoHIV(+) mice exposed to fentanyl [$p = 0.02$, $p = 0.01$, respectively] [Table 1, **Fig. 2a**]. Plasma-normalized lamivudine and plasma-normalized dolutegravir were significantly *lower* in EcoHIV(–) mice exposed to morphine compared to EcoHIV(–) mice exposed to fentanyl [$p = 0.02$ for both] [Table 1, **Fig. 2b, 2c**].

Plasma-normalized Antiretroviral Concentrations in the Hippocampus

In the hippocampus, plasma-normalized abacavir was significantly *lower* in EcoHIV(+) mice exposed to morphine compared to EcoHIV(+) mice exposed to saline [$p = 0.01$] [Table 1, **Fig. 2d**]. Plasma-normalized dolutegravir was significantly *higher* in EcoHIV(+) mice exposed to saline and EcoHIV(–) mice exposed to fentanyl compared to EcoHIV(+) mice exposed to fentanyl [$p = 0.03$, $p = 0.006$, respectively] [Table 1, **Fig. 2f**].

Blood Brain Barrier Measurements

To better understand the impact of EcoHIV and fentanyl or morphine on blood-brain barrier integrity within the striatum and hippocampus, the concentrations of the tight junction protein and tight junction accessory proteins, ZO-1 and claudin-5, were determined in male and female mice. Morphine and fentanyl dysregulated both claudin-5 and ZO-1, however, the alterations were not always consistent between female and male mice.

Claudin-5 ELISA in Female Striatum and Hippocampus

EcoHIV(–) mice exposed to morphine and EcoHIV(–) mice exposed to fentanyl had significantly *lower* claudin-5 in the striatum compared to EcoHIV(–) mice exposed to saline ($p = 0.01$, $p = 0.0009$, respectively) [Table 2, **Fig. 3a**]. EcoHIV(–) mice exposed to morphine and EcoHIV(+) mice exposed to saline had significantly *lower* claudin-5 in the hippocampus compared to EcoHIV(–) mice exposed to saline [$p = 0.005$, $p = 0.003$, respectively] [Table 2, **Fig. 3b]**.

Claudin-5 ELISA in Male Striatum and Hippocampus

EcoHIV(–) mice exposed to morphine and EcoHIV(–) mice exposed to fentanyl had significantly lower claudin-5 in the striatum compared to EcoHIV(–) mice exposed to saline [$p = 0.003$, $p = 0.007$]. EcoHIV(+) mice exposed to saline had significantly *lower* claudin-5 in the striatum compared to EcoHIV(–) mice

exposed to saline [p = 0.002] [Table 2, **Fig. 3e**]. EcoHIV(+) mice exposed to saline had significantly *lower* claudin-5 in the hippocampus compared to EcoHIV(–) mice exposed to saline [p = 0.01]. EcoHIV(–) mice exposed to morphine had significantly *higher* claudin-5 in the hippocampus compared to EcoHIV(+) mice exposed to morphine [p = 0.01]. EcoHIV(–) mice exposed to fentanyl had significantly *higher* claudin-5 in the hippocampus compared to EcoHIV(+) mice exposed to fentanyl [p = 0.001] [Table 2, **Fig. 3f**].

ZO-1 ELISA in Female Striatum and Hippocampus

EcoHIV(–) mice exposed to morphine and EcoHIV(–) mice exposed to fentanyl had significantly *lower* ZO-1 in the striatum compared to EcoHIV(–) mice exposed to saline [p = 0.01, p = 0.02, respectively]. EcoHIV(+) mice exposed to morphine had significantly *lower* ZO-1 in the striatum compared to EcoHIV(+) mice exposed to fentanyl [p = 0.01] [Table 2, **Fig. 3c**]. EcoHIV(–) mice exposed to morphine, EcoHIV(–) mice exposed to fentanyl, and EcoHIV(+) mice exposed to saline had significantly *lower* ZO-1 in the hippocampus compared to EcoHIV(–) mice exposed to saline [p < 0.0001 for all] [Table 2, **Fig. 3d**].

ZO-1 ELISA in Male Striatum and Hippocampus

EcoHIV(+) mice exposed to morphine and EcoHIV(–) mice exposed to saline had significantly *lower* ZO-1 in the striatum compared to EcoHIV(+) mice exposed to saline [p = 0.001, p = 0.01, respectively] [Table 2, **Fig. 3g**]. In the hippocampus, ZO-1 was significantly *lower* in EcoHIV(+) mice exposed to saline compared to EcoHIV(–) mice exposed to saline [p =0.0003]; EcoHIV(+) mice exposed to morphine mice compared to EcoHIV(–) mice exposed to morphine mice [p < 0.0001]; and EcoHIV(+) mice exposed to fentanyl mice compared to EcoHIV(–) mice exposed to fentanyl [p = 0.0006] [Table 2, **Fig. 3h**].

Chemokine Concentrations in Striatum and Hippocampus

Inflammatory Chemokine Concentrations

With the sex variable collapsed to include females and males, we determined the effect of EcoHIV and fentanyl or morphine on five inflammatory chemokine concentrations in the brain. Chemokines were analyzed from striatum and hippocampus 21 days post EcoHIV infection and after 5 days of continuous delivery of either drug (morphine or fentanyl) or vehicle (saline).

Inflammatory Chemokines

Post-hoc multiple comparisons

CCL2: In the striatum, EcoHIV(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL2 compared to EcoHIV(–) mice exposed to fentanyl and EcoHIV(+) mice exposed to saline [p = 0.03, p < 0.0001, respectively] [**Fig. 4a**]. In the hippocampus, EcoHIV(+) mice exposed to morphine and EcoHIV(+) mice exposed to fentanyl both had significantly *higher* concentrations of CCL2 compared to EcoHIV(+) mice exposed to saline [p = 0.0004, p = 0.002, respectively]. EcoHIV(–) mice exposed to

morphine mice had significantly *lower* concentrations of CCL2 compared to EcoHIV(+) mice exposed to morphine [$p = 0.007$] [**Fig. 4b**].

CCL3: In the striatum, EcoHIV(–) mice exposed to morphine had significantly *higher* concentrations of CCL3 compared to EcoHIV(–) mice exposed to saline [$p = 0.009$]. EcoHIV(–) mice exposed to morphine and EcoHIV(+) mice exposed to morphine, respectively, had significantly *higher* concentrations of CCL3 compared to EcoHIV(–) mice exposed to fentanyl and EcoHIV(+) mice exposed to fentanyl [$p < 0.0001$ for both]. EcoHIV(–) mice exposed to fentanyl and EcoHIV(+) mice exposed to fentanyl, respectively, had significantly *lower* concentrations of CCL3 compared to EcoHIV(–) mice exposed to saline and EcoHIV(+) mice exposed to saline [$p = 0.04$, $p < 0.0001$, respectively]. EcoHIV(+) mice exposed to fentanyl had significantly *lower* concentrations of CCL3 in the striatum compared to EcoHIV(–) mice exposed to fentanyl [$p = 0.04$] [**Fig. 4c**]. In the hippocampus, EcoHIV(+) mice exposed to fentanyl had significantly *lower* CCL3 concentrations compared to EcoHIV(–) mice exposed to fentanyl, Eco(+) mice exposed to morphine, and EcoHIV(+) mice exposed to saline [$p = 0.0005$, $p < 0.0001$, $p < 0.0001$, respectively]. Eco(+) mice exposed to morphine had significantly *higher* CCL3 concentrations compared to Eco(–) mice exposed to morphine and EcoHIV(+) mice exposed to fentanyl mice [$p = 0.001$] [**Fig. 4d**].

CCL4: In the striatum, EcoHIV(–) mice exposed to fentanyl and EcoHIV(+) mice exposed to fentanyl, respectively, had significantly *higher* concentrations of CCL4 compared to EcoHIV(–) mice exposed to saline and EcoHIV(+) mice exposed to saline [$p = 0.001$, $p < 0.0001$, respectively]. EcoHIV(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL4 compared to EcoHIV(–) mice exposed to fentanyl [$p < 0.0001$]. EcoHIV(+) mice exposed to morphine had significantly *higher* concentrations of CCL4 compared to EcoHIV(–) mice exposed to morphine and EcoHIV(+) mice exposed to saline [$p = 0.002$, $p < 0.0001$] [**Fig. 4e**]. In the hippocampus, EcoHIV(+) mice exposed to saline, EcoHIV(–) mice exposed to morphine, and EcoHIV(–) mice exposed to fentanyl mice had significantly *higher* concentrations of CCL4 compared to EcoHIV(–) mice exposed to saline [$p < 0.0001$ for all]. EcoHIV(+) mice exposed to morphine and Eco(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL4 compared to EcoHIV(+) mice exposed to saline [$p < 0.0001$ for all]. EcoHIV(+) mice exposed to morphine and EcoHIV(–) mice exposed to fentanyl had significantly *higher* concentrations of CCL4 concentrations compared to EcoHIV(–) mice exposed to morphine [$p < 0.0001$ for all] [**Fig. 4f**].

CCL5: In the striatum, EcoHIV(–) mice exposed to morphine and EcoHIV(+) mice exposed to morphine had significantly *higher* concentrations of CCL5 compared to EcoHIV(–) mice exposed to saline and EcoHIV(+) mice exposed to saline [$p = 0.001$, 0.008, 0.001, 0.008, respectively]. EcoHIV(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL5 compared to Eco(+) mice exposed to saline [$p = 0.002$] [**Fig. 4g**]. In the hippocampus, EcoHIV(+) mice exposed to morphine had significantly *higher*

concentrations of CCL5 compared to EcoHIV(–) mice exposed to morphine and EcoHIV(+) mice exposed to saline [$p = 0.001$, $p = 0.01$, respectively] [**Fig. 4h**].

CCL11: In the striatum, EcoHIV(–) mice exposed to fentanyl and EcoHIV(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL11 concentrations compared to EcoHIV(–) mice exposed to saline and EcoHIV(+) mice exposed to saline [$p = 0.0006$, $p = 0.002$, $p < 0.0001$, $p < 0.0001$, respectively]. EcoHIV(–) mice exposed to morphine had significantly *lower* concentrations of CCL11 compared to EcoHIV(–) mice exposed to fentanyl [$p = 0.01$] [**Fig. 4i**]. In the hippocampus, EcoHIV(–) mice exposed to fentanyl had significantly *higher* concentrations of CCL11 concentrations compared to EcoHIV(–) mice exposed to saline [$p = 0.02$] [**Fig. 4j**].

Dual-function and Homeostatic Chemokine Concentrations
Post-hoc multiple comparisons
CCL17: In the striatum, EcoHIV(+) mice exposed to saline and EcoHIV(–) mice exposed to fentanyl had significantly *higher* concentrations of CCL17 concentrations compared to EcoHIV(–) mice exposed to saline [$p = 0.01$, $p = 0.0001$, respectively]. EcoHIV(–) mice exposed to morphine had significantly *lower* concentrations of CCL17 concentrations compared to EcoHIV(–) mice exposed to fentanyl [$p = 0.01$] [**Fig. 5a**]. In the hippocampus, EcoHIV(+) mice exposed to morphine had significantly *higher* concentrations of CCL17 compared to EcoHIV(+) mice exposed to saline and EcoHIV(–) mice exposed to morphine [$p = 0.0001$, $p = 0.0004$, respectively]. Eco(+) mice exposed to fentanyl had significantly *higher* concentrations of CCL17 concentrations compared to Eco(–) mice exposed to fentanyl [$p = 0.01$] [**Fig. 5b**].

CCL22: In the striatum, EcoHIV(+) mice exposed to saline had significantly *lower* concentrations of CCL22 compared to EcoHIV(–) mice exposed to saline [$p = 0.03$] [**Fig. 5c**]. In the hippocampus, Eco(–) mice exposed to fentanyl had significantly *higher* concentrations of CCL22 compared to Eco(–) mice exposed to saline [$p = 0.004$] [**Fig. 5d**].

CXCL10: In the striatum, EcoHIV(–) mice exposed to saline, EcoHIV(+) mice exposed to morphine, and EcoHIV(+) mice exposed to fentanyl had significantly *lower* concentrations of CXCL10 compared to EcoHIV(+) mice exposed to saline [$p = 0.01$, $p = 0.0001$, $p < 0.0001$, respectively] [**Fig. 5e**].
In the hippocampus, EcoHIV(–) mice exposed to fentanyl had significantly *lower* concentrations of CXCL10 compared to EcoHIV(–) mice exposed to saline [$p = 0.0009$]. EcoHIV(–) mice exposed to saline, Eco(+) mice exposed to morphine, and EcoHIV(+) mice exposed to fentanyl mice had significantly *lower* concentrations of CXCL10 compared to EcoHIV(+) mice exposed to [$p = 0.01$, $p = 0.002$, $p = 0.0001$, respectively] [**Fig. 5f**].

CXCL13: In the striatum, Eco(–) mice exposed to saline, EcoHIV(–) mice exposed to morphine, and EcoHIV(+) mice exposed to morphine had significantly *higher* concentrations of CXCL13 compared to EcoHIV(+) mice exposed to saline [$p = 0.001$, $p = 0.006$, $p = 0.01$, respectively] [**Fig. 5g**].

Discussion

Our results reveal novel interactions between infectious HIV, morphine, and fentanyl, on the integrity of the BBB and the regulation of the immune response in two brain regions. Opioid drug use in HIV modulates the integrity and function of the BBB, alters the immune response, and worsens neuroHIV (Hauser et al. 2007, 2012; Chang et al. 2014; Smith et al. 2014; Fitting et al. 2020; Leibrand et al. 2022). In the presence of EcoHIV, morphine and fentanyl, the integrity of the BBB was altered and chemokines were dysregulated. The alteration in BBB integrity and dysregulation of the immune response are well described mechanisms involved in neuroHIV (Resnick et al. 1988; Ho et al. 1994; Toborek et al. 2005; Falasca et al. 2017; Williams et al. 2020). In our study, all experimental groups received combination antiretroviral therapy (cART) consisting of abacavir, lamivudine, and dolutegravir.

Alterations in antiretroviral concentrations

Inflammatory mediators and opioids have been demonstrated to alter the integrity of the BBB (Huber et al. 2001; Seelbach et al. 2007; Mahajan et al. 2008; Yousif et al. 2008). Alterations in BBB integrity have implications for successful antiretroviral drug delivery to the brain. Similar to our previous study (Leibrand et al. 2019), in the present study, both morphine and fentanyl altered the distributions of antiretroviral drugs in a regional manner, dependent on infection status. Abacavir and dolutegravir alterations may be related to the regulation of the drug efflux transporter, P-glycoprotein, as both antiretrovirals are substrates for this transporter (Shaik et al. 2007; Giri et al. 2008; ViiV Healthcare 2015, 2017, 2020). Alterations in lamivudine would not have been affected by opioid interactions at P-gp, because lamivudine is excreted by the kidney (van Leeuwen et al. 1992; Johnson et al. 1999; Minuesa et al. 2009; Müller et al. 2013; Ceckova et al. 2016; Liu et al. 2018). Our results suggest that morphine and fentanyl may affect drug transporters and/or renal function, in a drug-specific manner, and future studies are warranted to examine these mechanisms related to antiretroviral pharmacokinetics in the setting of HIV and OUD.

EcoHIV and opioids altered proteins involved in maintaining blood brain barrier integrity

While the BBB serves as the main protector of the brain against toxic or foreign molecules and pathogens, HIV and neuroinflammation compromise the integrity and function of the BBB (Leibrand et al. 2017, 2019; Osborne et al. 2020) potentially making the brain even more susceptible to the infiltration of, and damage by, circulating toxins. In the present study, claudin-5 and ZO-1 in the striatum and hippocampus were altered in the presence of EcoHIV and opioid, although regional differences, sex specific differences and opioid specific differences were observed.

Opioids, like morphine, can disrupt the integrity of the BBB by decreasing the expression of claudin-5 and ZO-1 (Mahajan et al. 2008; András and Toborek 2011; Peyravian et al. 2022), leading to increased permeability of the barrier. In this study, fentanyl or morphine alone disrupted claudin-5 expression primarily in the striatum. Interestingly, when the mice were infected with EcoHIV, co-exposure with fentanyl resulted in a significant increase in claudin-5 expression in females but not males. There were also sex and brain region differences observed in claudin-5 in response to EcoHIV infection. Within the striatum, EcoHIV infection appeared to reverse the opioid-mediated decreases (fentanyl effects in female mice and morphine effects in male mice) in claudin-5 to bring the expression level back to baseline (saline treated, uninfected mice). Surprisingly, in the hippocampus, the effects of EcoHIV infection appeared to be opposite of that observed in the striatum. In males, claudin-5 expression decreased in all experimental groups (saline, morphine and fentanyl-exposed) upon infection with EcoHIV whereas in females, the only significant effect of infection on claudin-5 was in the saline treated group.

Brain region and sex differences were also observed for ZO-1 expression in response to EcoHIV infection and/or opioids. Morphine exposure resulted in significant decreases in ZO-1 in the striatum and hippocampus of female mice; where co-exposure to morphine and EcoHIV decreases ZO-1 in the male striatum. Fentanyl also decreased ZO-1 expression in females in both brain regions, but had no effect in the males. EcoHIV infection significantly decreased ZO-1 expression in the hippocampus of saline treated female mice and in all experimental groups in the male hippocampus. In the striatum of male mice, EcoHIV infection increased ZO-1 expression. Overall tight junction proteins within the hippocampus appear to be more vulnerable to EcoHIV infection than the striatum or than females. In contrast, within the striatum, female and male mice were both susceptible to opioids with male mice less reactive to tight junction changes to opioid exposure in the hippocampus.

It has been reported elsewhere that estrogen and testosterone are both involved in region-specific regulation of claudin-5 and ZO-1 within the prefrontal cortex, striatum, and hippocampus (Burek and Förster 2009; Oh et al. 2016; Atallah et al. 2017; Yoest et al. 2018; Solarz et al. 2021; Dion-Albert et al. 2022). In addition, activated microglia *in vivo* produce cytokines that are associated with the loss of tight junction proteins (Bolton et al. 1998; Haruwaka et al. 2019). Region-dependent sex-differences have also been shown in rats where the concentrations of basal- and inflammation-induced inflammatory mediators (cytokines/chemokines) are different in the striatum and hippocampus between females and males (Nonoguchi et al. 2022). Further, sex-dependent responses of microglia activation by morphine have been demonstrated, where female rats experience higher microglial activation in the periaqueductal gray compared to males in the presence of morphine (Doyle et al. 2017). In general, women experience increased HIV immune activation, faster disease progression, and more severe cognitive dysfunction than men with HIV (Meier et al. 2009; Griesbeck et al. 2016; Martin et al. 2018), although, at least with respect to tight junction changes, in this study, male mice were more susceptible to infection.

EcoHIV dysregulates the immune response

The activation of brain cells by HIV or HIV proteins and the resulting cytokine secretion, and subsequent neuronal toxicity has been previously studied *in vitro* (Johnston et al. 2001; Fitting et al. 2010b) and *in vivo* (Schier et al. 2017; Nass et al. 2020). HIV and the mouse tropic EcoHIV modulate immune responses through regulation of several cytokines including IL-6, IL-1β, and TNF-α, which are all three involved in regulating chemokine production (Ellegård et al. 2015; Omeragic et al. 2020; Li et al. 2020). In this study, EcoHIV infection resulted in perturbations in chemokine concentrations for selected chemokines, although the directionality of the effect was not always the same. Of the inflammatory chemokines examined herein, only CCL4 concentrations were altered by EcoHIV such that EcoHIV infection resulted in a significant increase in CCL4 within the hippocampus. Another study found that EcoHIV infection significantly increases CCL2 and CXCL11 15 days post infection, with normalization of these chemokines by day 30 (Kelschenbach et al. 2012). They did not examine CCL4, an HIV-suppressive and neuroprotective chemokine produced by T cells during immune activation (Cocchi et al. 1995; Hudspeth et al. 2012; Kelschenbach et al. 2012).

Among the chemokines examined herein, EcoHIV infection dysregulated homeostatic and dual function chemokines and in a brain region-dependent manner. Compared to the hippocampus, the striatum exhibits higher vulnerability to HIV infection and Tat-associated neurotoxicity (El-Hage et al. 2005; Nath 2015; Nass et al. 2020). EcoHIV infection has been shown to produce Tat (Potash 2005). Tat induces regional- and duration-dependent neurotoxicities (Fitting et al. 2013; Marks et al. 2016; Schier et al. 2017; Barbour et al. 2021; Nass et al. 2021) and these neurotoxicities are exacerbated by opioids (Gurwell et al. 2001; Zou et al. 2011; Fitting et al. 2014).

Alterations in chemokine concentrations by interactive effects between EcoHIV and opioids

Several studies reveal interactive effects between HIV-1 Tat and opioids on mechanisms related to neuroinflammation (Hauser et al. 2005; El-Hage et al. 2008; Turchan-Cholewo et al. 2009; Fitting et al. 2010a), while only one other study has examined the effects of opioids and EcoHIV on three cytokine concentrations in the brain (Sindberg et al. 2015). In the present study, there were interactive effects between EcoHIV and fentanyl or morphine exposure for several of the chemokines studied. Fentanyl and EcoHIV co-exposure increased CCL2, CCL4, CCL17 in the striatum but decreased CCL3 in both the striatum and hippocampus. Likewise there were brain region differences in the interactive effects between EcoHIV and morphine with increases in only CCL4 and CCL17 in the striatum but increases in CCL2, CCL3, CCL4, CCL5 and CCL17 within the hippocampus. The perturbations observed in chemokine concentrations in the current study were more severe in response to opioids or the interaction between EcoHIV and opioids compared to EcoHIV alone. In the present study, all mice were exposed to antiretroviral drugs regardless of EcoHIV infection. Another study demonstrates that total viral DNA and integrated viral DNA is reduced in EcoHIV mice exposed to antiretroviral drugs (Gu et al. 2018),

suggesting that in the present study, the immune response was more susceptible to opioid dysregulation compared to EcoHIV in the presence of antiretroviral therapy.

Morphine and fentanyl independently dysregulate the immune response

The striatum and hippocampus are abundantly rich in opioid receptors (Banghart et al. 2015; Hu 2016; Shi et al. 2020). Cells expressing opioid receptors become activated upon opioid binding which results in altered immune responses and the disruption of cell signaling cascades (Bokhari et al. 2011; Reddy et al. 2012; Murphy et al. 2019). Independent *in vitro* and *in vivo* studies reveal that fentanyl and morphine participate in stimulating cytokine production (Wang et al. 2012; Liang et al. 2016; Chang et al. 2018; Hu et al. 2021).

In the present study, morphine and fentanyl, independently, resulted in perturbations in chemokine concentrations, although the directionality of the effects were not universal. Compared to infected mice treated with saline, morphine dysregulated inflammatory and dual-function chemokines in the striatum and hippocampus. Morphine was the only opioid that dysregulated the homeostatic chemokine, CXCL13. In comparison, chemokine dysregulation by fentanyl (compared to infected mice treated with saline) appeared to be regional and chemokine dependent. Dysregulation of inflammatory chemokines by fentanyl appear to occur in the same direction, while dual-function chemokines are either increased or decreased depending on the region and chemokine.

The induction of inflammatory cytokines and several chemokines is partially regulated by transcription factors such as NF-κB and STAT3 (Tato et al. 2006; Iyer and Cheng 2012; Nookala et al. 2013; Taniguchi and Karin 2018). Furthermore, opioid activation of the toll-like receptor 4 (TLR4) signaling pathway promotes NF-κB expression which signals for additional cytokine production (Wang et al. 2012; Stevens et al. 2013; Xie et al. 2017). While fentanyl results in minor activation of TLR4, it has been suggested that fentanyl alters the inflammatory response through suppressing NF-κB (Liu et al. 2006; Stevens et al. 2013; Ma et al. 2017). Although both morphine and fentanyl are opioid drugs, there is evidence that morphine and fentanyl act differently with respect to ligand binding, intracellular signaling events and receptor activation (Lipiński et al. 2019b, a; Ricarte et al. 2021), which may contribute to the differences observed within our study.

Dysregulation of inflammatory chemokines by morphine and fentanyl

In this study, inflammatory chemokines within the striatum and hippocampus were dysregulated by fentanyl and morphine in the presence of infectious HIV and antiretroviral therapy. All five of the inflammatory chemokines, CCL2, CCL3, CCL4, CCL5, and CCL11 are induced and not constitutively expressed (Maurer and von Stebut 2004; Bhavsar et al. 2015). These inflammatory chemokines are induced by various inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (Jedrzkiewicz et al. 2000; Lebovic et al. 2001; Zhang et al. 2003; Boekhoudt et al. 2003; Lee et al. 2006; Wang and Knaut 2014; Zegeye et al. 2018; Hyvärinen et al. 2019). In addition,

both CCL5 and CCL11 are inducible by IL-4 and IL-10 in the presence of TNF-α (Kumagai et al. 2000; Rokudai et al. 2006; Zheng et al. 2009; Kim and Kim 2014; Kindstedt et al. 2017; Ivanovska et al. 2020).

Inflammatory chemokines were dysregulated in both the striatum and hippocampus while CCL11 changes were mostly seen in the striatum with only one difference in the hippocampus. Elevated CCL11, which induces chemotaxis of eosinophils, is typically involved in allergic reactions or asthma (Chandra et al. 2016). Although most studies examine peripheral CCL11, elevated CCL11 has been found in post-mortem brain tissue from patients with Parkinson's disease, and has been associated with cognitive decline and aging (Villeda et al. 2011; Chandra et al. 2016; Sirivichayakul et al. 2019). HIV-1 Tat increases CCL11 in the striatum and is associated with depressive-like behavior (Nass et al. 2020, 2023). The differences between morphine and fentanyl effects on CCL11 might be related to morphine and fentanyl cytokine modulation; fentanyl exposure results in a greater production of IL-4 in T lymphocytes, than does morphine exposure (Börner et al. 2013) and fentanyl increases IL-10 (Yadav and Bhattacharya 2017) while morphine decreases IL-10 (Franchi et al. 2019). These findings may explain the observation herein of increased CCL11 in the presence of fentanyl, but not morphine, although we did not measure the aforementioned cytokines. Compared to CCL11, effects of morphine and/or fentanyl on CCL2, CCL3, CCL4, and CCL5 were observed in both the striatum and hippocampus, although the directionality was not always consistent. These four chemokines are mostly regulated by IL-6, IL-1β, and TNF-α, which engage in the NF-κB signaling cascade (Cao et al. 2006; Wang et al. 2013; Ahmad et al. 2019). Both fentanyl and morphine have been shown to modulate NF-κB signaling and cytokine production (Roy et al. 1998; Houghtling et al. 2000; Liu et al. 2006; Ma et al. 2017; Haghjooy-Javanmard et al. 2018; Liu and Xu 2023). In light of these findings, our results warrant additional studies to examine how morphine and fentanyl dysregulate the NF-κB-cytokine signaling pathway involved in the production of CCL2, CCL3, and CCL5 in HIV.

Dysregulation of dual-function and homeostatic chemokines by morphine and fentanyl

In addition to inflammatory chemokines, morphine and fentanyl both dysregulated dual-function chemokines and the homeostatic chemokine, CXCL13, in the striatum and hippocampus in the presence of infectious HIV. Dual-function chemokines, CCL17, CCL22, and CXCL10, are constitutively expressed and can be induced by various factors (Greaves et al. 2001; Gear and Camerini 2003; Weber et al. 2011; Scheu et al. 2017; Metzemaekers et al. 2018; Tokunaga et al. 2018). The cytokines IL-4, IL-13, and IL-22 induce both CCL17 and CCL22, however, the induction of CCL17 by IL-4 or IL-3 requires TNF-α (Faffe et al. 2003; Yasuoka et al. 2011; Villarreal et al. 2014; Gougeon 2017). Unlike CCL17 and CCL22, the induction of CXCL10 is mainly dependent upon signaling synergy between interferon-gamma (IFNγ) and TNF-α (Williams et al. 2009a, b).

In the present study, CCL17 and CXCL10 were dysregulated by EcoHIV and both morphine and fentanyl in the striatum and hippocampus. The alterations of morphine and fentanyl treated mice suggest EcoHIV and opioids are involved in dysregulating cytokine signaling between IL-3, IL-4, and TNF-α

(Wang et al. 2012; Ellegård et al. 2015; Chang et al. 2018; Omeragic et al. 2020), however, additional studies are warranted to examine these mechanisms. EcoHIV decreased CCL22 concentrations in saline-treated mice in the striatum and fentanyl increased CCL22 in uninfected mice compared to uninfected saline-treated mice in the hippocampus. Our findings put together with previous findings suggest that both EcoHIV and fentanyl modulate cytokines and IL-33/IFNγ signaling cascades (Martinez et al. 2009; Scheu et al. 2017) involved in CCL22 induction in a region-specific manner.

CXCL10 is upregulated by both HIV and EcoHIV and reduced by fentanyl and morphine (Davis et al. 2007; Ellegård et al. 2015; Omeragic et al. 2020). Specifically, morphine reduces the concentration of IFNγ, which is one of the main cytokines involved in the production of CXCL10 (Peterson et al. 1987; Asadikaram et al. 2015; Chen et al. 2019). In agreement with previous studies, we reveal significant increases in CXCL10 by EcoHIV in saline-treated mice, while at the same time, in EcoHIV infected mice, CXCL10 concentrations were significantly reduced by both morphine and fentanyl in the striatum and hippocampus.

To conclude, our study examined the regional distribution of antiretrovirals in the brain, proteins involved in maintaining BBB integrity, and chemokines which are involved in the immune response in the presence of infectious HIV and opioids. Our results suggest that morphine and fentanyl both interfere with the activation of cells and transcriptional signaling pathways involved in maintaining the integrity of the BBB and producing chemokines. It is clear that the opioids exert distinct effects as perturbations vary by region and chemokine, which is supported by inherent pharmacological differences between the two opioids. The alterations in BBB integrity and the sex differences we observed in the present study are supported by previous studies demonstrating sex differences in the pathogenesis of HIV, sex-specific vulnerabilities to neurotoxic insults (Griesbeck et al. 2016; Scully 2018; Bagdas et al. 2020), and the effects of sex-hormones on the BBB (Burek and Förster 2009; Oh et al. 2016; Atallah et al. 2017; Yoest et al. 2018; Solarz et al. 2021; Dion-Albert et al. 2022). Future studies should investigate sex-differences in antiretroviral distribution in the brain and the regulation of the immune response in the setting of opioids and HIV.

Table 1. Effects of fentanyl or morphine and EcoHIV infection on antiretroviral concentrations in plasma and tissue.

Antiretroviral Drug Concentrations	**Opioid /Treatment (Main effect)**		**EcoHIV (Main Effect)**		**Interaction**	
Plasma	$F_{(2,39)}$	*p*	$F_{(1,39)}$	*p*	$F_{(2,39)}$	*p*
Plasma ABC	3.509	**0.03**✧	0.8078	0.37	4.116	**0.02**≒
Plasma 3TC	0.2303	0.79	0.3826	0.53	2.768	0.07
Plasma DTG	0.2729	0.76	1.121	0.29	0.3755	0.68
Striatum	$F_{(2,32)}$	*p*	$F_{(1,32)}$	*p*	$F_{(2,32)}$	*p*
T:P* ABC	3.947	**0.02**✧	0.3627	0.55	4.829	**0.01**≒
T:P* DTG	3.942	**0.02**✧	0.4678	0.49	1.493	0.24
T:P* 3TC	3.919	**0.03**✧	0.7103	0.40	2.291	0.11
Hippocampus	$F_{(2,32)}$	*p*	$F_{(1,32)}$	*p*	$F_{(2,32)}$	*p*
T:P* ABC	4.912	**0.01**✧	2.114	0.15	2.931	0.06
T:P* DTG	4.719	**0.01**✧	13.57	**0.0008**₥	1.786	0.18
T:P* 3TC	1.396	0.26	5.905	**0.02**₥	1.178	0.32

*****T:P: plasma-normalized tissue concentrations
ABC = abacavir, DTG = dolutegravir, 3TC = lamivudine

Main effects are represented by significant main effect of treatment (✧) [morphine or fentanyl vs. saline-treated], significant main effect of EcoHIV status (₥) [EcoHIV(−) vs. EcoHIV(+)], and the interaction between Treatment and EcoHIV status (≒).

Table 2. Effects of fentanyl or morphine and EcoHIV infection on claudin-5 and ZO-1 concentrations in the striatum and hippocampus.

Antiretroviral Drug Concentrations	**Opioid / Treatment (Main effect)**		**EcoHIV (Main Effect)**		**Interaction**	
Females	$F_{(2,16)}$	*p*	$F_{(1,16)}$	*p*	$F_{(2,16)}$	*p*
Claudin-5 Striatum	15.52	**0.0002**✧	2.471	0.13	18.14	**< 0.0001**≒
Claudin-5 Hippocampus*	5.767	**0.01**✧	13.28	**0.003**₥	7.391	**0.008**≒
ZO-1 Striatum	7.945	**0.004**✧	0.099	0.75	9.236	**0.002**≒
ZO-1 Hippocampus*	26.24	**< 0.0001**✧	10.74	**0.006**₥	31.15	**< 0.0001**≒
Males	$F_{(2,13)}$	*p*	$F_{(1,13)}$	*p*	$F_{(2,13)}$	*p*
Claudin-5 Striatum	1.231	0.32	0.003	0.95	20.77	**< 0.0001**≒
Claudin-5 Hippocampus	3.99	**0.04**✧	58.59	**< 0.0001**₥	0.6082	0.55
ZO-1 Striatum	9.493	**0.002**✧	8.324	**0.01**₥	6.301	**0.01**≒
ZO-1 Hippocampus	2.226	0.14	126.6	**< 0.0001**₥	0.59	0.56

*Female hippocampus treatment main effect and interaction [$F_{(2,12)}$]; main effect for EcoHIV [$F_{(1,12)}$]

Main effects are represented by significant main effect of treatment (✧) [morphine or fentanyl vs. saline-treated], significant main effect of EcoHIV status (₥) [EcoHIV(−) vs. EcoHIV(+)], and the interaction between Treatment and EcoHIV status (≒).

Table 3. Effects of fentanyl and HIV-1 Tat exposure on chemokine levels in the striatum and hippocampus.

Brain Region		Opioid / Treatment (Main effect)		EcoHIV (Main Effect)		Interaction	
Striatum							
	Chemokine	$F_{(2,37)}$	*p*	$F_{(1,37)}$	*p*	$F_{(2,37)}$	*p*
	CCL2	24.36	**< 0.0001**✧	7.01	**0.01**₥	4.794	**0.01**≒
	CCL3	68.71	**< 0.0001**✧	1.025	0.31	5.117	**0.01**≒
	CCL4	46.34	**< 0.0001**✧	38.30	**< 0.0001**₥	5.517	**0.008**≒
	CCL5	19.49	**< 0.0001**✧	2.054	0.16	3.510	**0.04**≒
	CCL11	25.66	**< 0.0001**✧	0.3268	0.57	0.9692	0.38
	CCL17	9.741	**0.0004**✧	5.260	**0.02**₥	4.398	**0.01**≒
	CCL22	1.416	0.255	1.009	0.32	4.417	**0.01**≒
	CXCL10	19.33	**< 0.0001**✧	0.7878	0.38	6.327	**0.004**≒
	CXCL13	2.512	0.09	10.92	**0.002**₥	4.381	**0.01**≒
Hippocampus							
	Chemokine	$F_{(2,34)}$	*p*	$F_{(1,34)}$	*p*	$F_{(2,34)}$	*p*
	CCL2	12.05	**0.0001**✧	7.889	**0.008**₥	4.247	**0.02**≒
	CCL3	19.42	**< 0.0001**✧	1.153	0.29	21.28	**< 0.0001**≒
	CCL4	249.5	**< 0.0001**✧	80.70	**< 0.0001**₥	18.95	**< 0.0001**≒
	CCL5	5.176	**0.01**✧	24.82	**< 0.0001**₥	1.811	0.1789
	CCL11	6.558	**0.003**✧	2.089	0.15	0.9634	0.39
	CCL17	3.773	**0.03**✧	11.27	**0.002**₥	12.59	**< 0.0001**≒
	CCL22	5.675	**0.007**✧	0.2387	0.62	3.243	0.05
	CXCL10	22.90	**< 0.0001**✧	13.46	**0.0008**₥	0.8908	0.41
	CXCL13	1.347	0.27	4.422	**0.04**₥	2.729	0.07

Main effects are represented by significant main effect of treatment (✧) [morphine or fentanyl vs. saline-treated], significant main effect of EcoHIV status (₥) [EcoHIV(−) vs. EcoHIV(+)], and the interaction between Treatment and EcoHIV status (≒).

Table 4. Post-hoc effects of saline and morphine and EcoHIV infection on chemokine levels in the striatum and hippocampus.

Brain Region	Chemokine	Saline vs. Morphine (Post-hoc effect)	*p-value*
Striatum			
	CCL2	Eco(+) < Eco(+) morphine	0.006
	CCL3	Eco(–) < Eco(–) & Eco(+) morphine	0.009, 0.003
	CCL4	Eco(–) & Eco(+) < Eco(+) morphine	< 0.0001, < 0.0001
	CCL5	Eco(–) < Eco(–) & Eco(+) morphine Eco(–) & Eco(+) morphine	0.001, 0.008 0.001, 0.008
	CXCL10	Eco(+) > Eco(–) & Eco(+) morphine	0.0003, 0.0001
	CXCL13	Eco(+) < Eco(–) & Eco(+) morphine	0.006, 0.01
Hippocampus			
	CCL2	Eco(–) & Eco(+) < Eco(+) morphine	0.0006, 0.0004
	CCL4	Eco(–) < Eco(–) & Eco(+) morphine Eco(+) < Eco(–) & Eco(+) morphine	< 0.0001, < 0.0001 0.0001, < 0.0001
	CCL5	Eco(–) & Eco(+) < Eco(+) morphine	0.0002, 0.01
	CCL17	Eco(+) < Eco(+) morphine	0.0003
	CXCL10	Eco(+) > Eco(–) & Eco(+) morphine	< 0.0001, 0.0002

*Eco(–): uninfected saline-treated group

Results from Tukey-HSD post-hoc analysis in the striatum and hippocampus between treatment [morphine and saline-treated] including EcoHIV infection status.

Table 5. Post-hoc effects of saline and fentanyl and EcoHIV infection on chemokine levels in the striatum and hippocampus.

Brain Region	Chemokine	Saline vs. Fentanyl (Post-hoc effect)	*p-value*
Striatum			
	CCL2	Eco(–) & Eco(+) < Eco(+) fentanyl Eco(+) < Eco(–) fentanyl	< 0.0001, <0.0001 0.006
	CCL3	Eco(–) > Eco(–) & Eco(+) fentanyl Eco(+) > Eco(–) & Eco(+) fentanyl	0.04, < 0.0001 0.001, < 0.0001
	CCL4	Eco(–) < Eco(–) & Eco(+) fentanyl Eco(+) < Eco(+) fentanyl	0.001, < 0.0001 < 0.0001
	CCL5	Eco(–) & Eco(+) < Eco(+) fentanyl	0.0002, 0.0002
	CCL11	Eco(–) < Eco(–) & Eco(+) fentanyl Eco(+) < Eco(–) & Eco(+) fentanyl	0.0006, 0.002 < 0.0001, < 0.0001
	CCL17	Eco(–) < Eco(–) & Eco(+) fentanyl	0.0001, 0.001
	CXCL10	Eco(–) > Eco(+) fentanyl Eco(+) > Eco(–) & Eco(+) fentanyl	0.03 0.0003, < 0.0001
Hippocampus			
	CCL2	Eco(–) & Eco(+) < Eco(+) fentanyl	0.004, 0.002
	CCL3	Eco(–) & Eco(+) > Eco(+) fentanyl	< 0.0001, < 0.0001
	CCL4	Eco(–) < Eco(–) & Eco(+) fentanyl Eco(+) < Eco(–) & Eco(+) fentanyl	< 0.0001, < 0.0001 < 0.0001, < 0.0001
	CCL5	Eco(–) < Eco(+) fentanyl	0.006
	CCL11	Eco(–) < Eco(–) & Eco(+) fentanyl	0.02, 0.009
	CCL22	Eco(–) < Eco(–) fentanyl	0.004
	CXCL10	Eco(–) > Eco(–) fentanyl Eco(+) > Eco(–) & Eco(+) fentanyl	0.009 < 0.0001, 0.0001

*Eco(–): uninfected saline-treated group

Results from Tukey-HSD post-hoc analysis in the striatum and hippocampus between treatment [fentanyl and saline-treated] including EcoHIV infection status.

Table 6. Post-hoc effects of morphine and fentanyl and EcoHIV infection on chemokine levels in the striatum and hippocampus.

Brain Region	Chemokine	Morphine vs. Fentanyl (Post-hoc effect)	*p-value*
Striatum			
	CCL2	Eco(–) morphine < Eco(+) fentanyl	< 0.0001
	CCL3	Eco(–) morphine > Eco(–) & Eco(+) fentanyl Eco(+) morphine > Eco(–) & Eco(+) fentanyl	< 0.0001, < 0.0001 <0.0001, < 0.0001
	CCL4	Eco(–) morphine < Eco(+) fentanyl	< 0.0001
	CCL11	Eco(–) morphine < Eco(–) & Eco(+) fentanyl	0.01, 0.03
	CCL17	Eco(–) morphine < Eco(–) fentanyl	0.01
Hippocampus			
	CCL2	Eco(–) morphine < Eco(+) fentanyl	0.04
	CCL3	Eco(–) morphine > Eco(+) fentanyl Eco(+) morphine > Eco(–) & Eco(+) fentanyl	0.0005 0.01, < 0.0001
	CCL4	Eco(–) morphine < Eco(–) & Eco(+) fentanyl	< 0.0001, < 0.0001
	CCL5	Eco(–) morphine < Eco(+) fentanyl Eco(+) morphine > Eco(–) fentanyl	0.04 0.004
	CCL17	Eco(+) morphine > Eco(–) fentanyl	0.0001

*Eco(–): uninfected group

Results from Tukey-HSD post-hoc analysis in the striatum and hippocampus between treatment [morphine and fentanyl] including EcoHIV infection status.

Chapter 4 Figures.

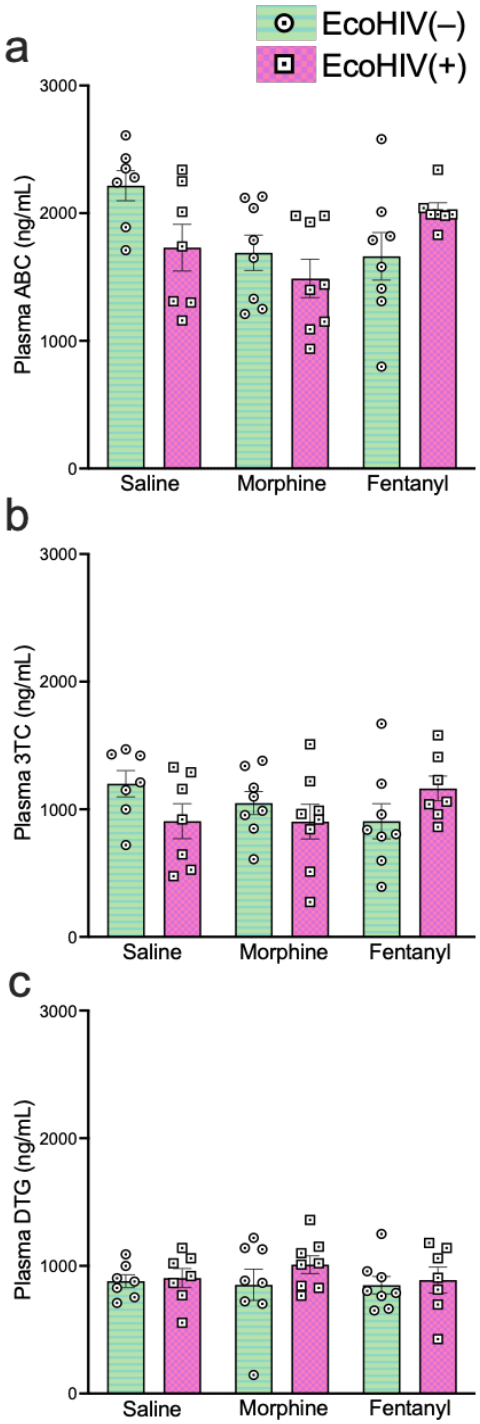

Fig. 1 *Concentrations of abacavir, lamivudine, and dolutegravir* results from Tukey-HSD post-hoc analysis in the plasma. EcoHIV(–) mice are depicted by horizontal green lines and white circles with a black dot. EcoHIV(+) mice are depicted by pink squares and white squares with a black dot. Data represents the

mean plasma concentration for each ARV ± SEM; n = 7 EcoHIV(–)/saline, n = 7 EcoHIV(+)/saline, n = 8 EcoHIV(–)/morphine, and n = 8 EcoHIV(+)/morphine, n = 8 EcoHIV(–)/fentanyl, and n = 7 EcoHIV(+)/fentanyl and significant differences at $\alpha < 0.05$ are denoted by *$p < 0.0032$, **$p < 0.0021$, ***$p < 0.0002$, and ****$p < 0.0001$ for post-hoc testing.

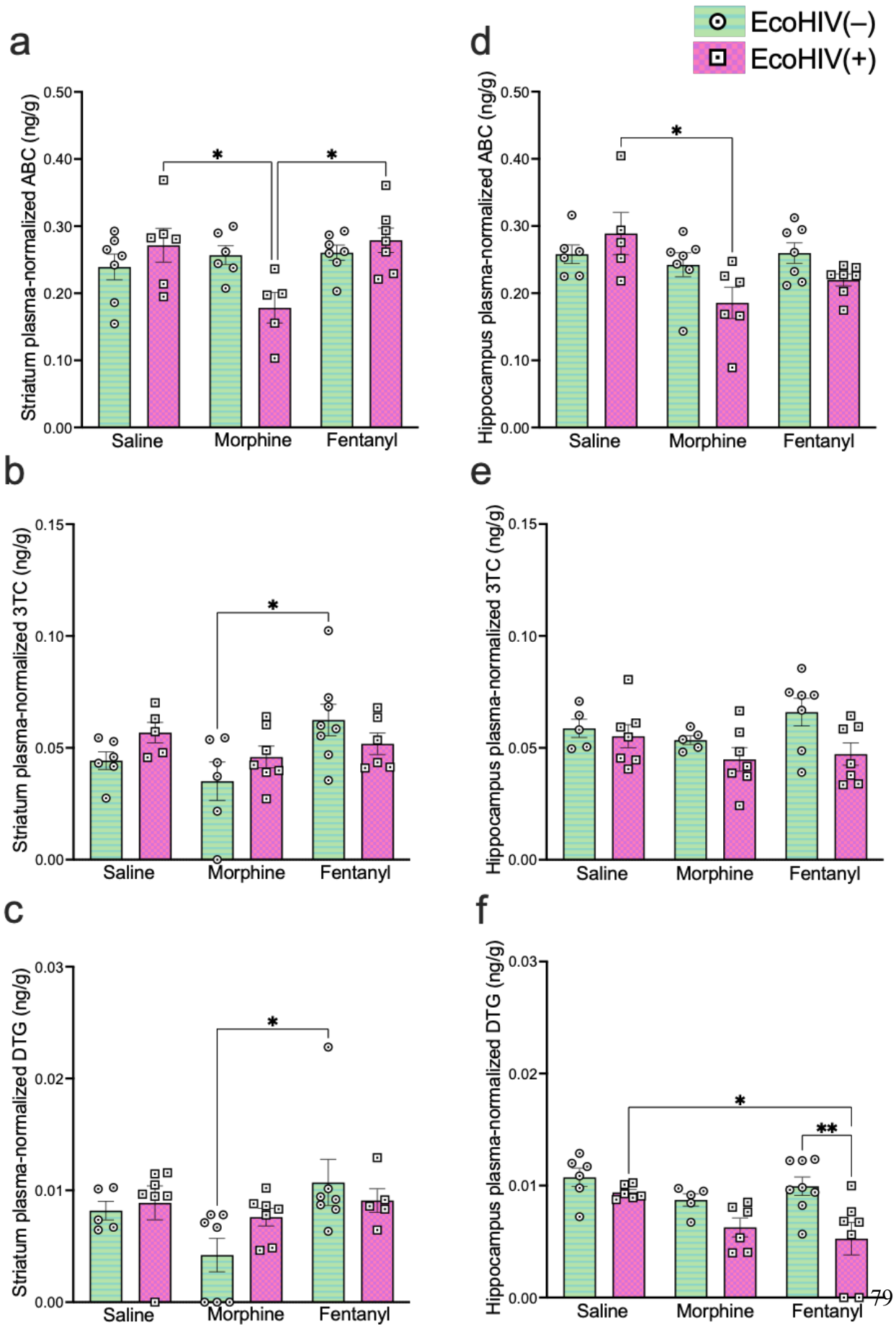
a
Striatum plasma-normalized ABC (ng/g)
0.50
0.40
0.30
0.20
0.10
0.00
Saline
Morphine
Fentanyl
*
*
d
EcoHIV(–)
EcoHIV(+)
Hippocampus plasma-normalized ABC (ng/g)
*
b
Striatum plasma-normalized 3TC (ng/g)
0.15
0.10
0.05
0.00
*
e
Hippocampus plasma-normalized 3TC (ng/g)
c
Striatum plasma-normalized DTG (ng/g)
0.03
0.02
0.01
f
Hippocampus plasma-normalized DTG (ng/g)
**

Fig. 2 *Plasma-normalized tissue concentration* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. EcoHIV(–) mice are depicted by horizontal green lines and white circles with a black dot. EcoHIV(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean plasma-normalized tissue concentration for each ARV ± SEM; striatum: n = 5-7 EcoHIV(–)/saline, n = 6-7 EcoHIV(+)/saline, n = 6-7 EcoHIV(–)/morphine, and n = 5-7 EcoHIV(+)/morphine, n = 7 EcoHIV(–)/fentanyl, and n = 5-7 EcoHIV(+)/fentanyl; hippocampus: n = 5-6 EcoHIV(–)/saline, n = 5-7 EcoHIV(+)/saline, n = 5-7 EcoHIV(–)/morphine, and n = 6-7 EcoHIV(+)/morphine, n = 7-8 EcoHIV(–)/fentanyl, and n = 7 EcoHIV(+)/fentanyl; and significant differences at $\alpha < 0.05$ are denoted by *$p < 0.0032$, **$p < 0.0021$, ***$p < 0.0002$, and ****$p < 0.0001$ for post-hoc testing.

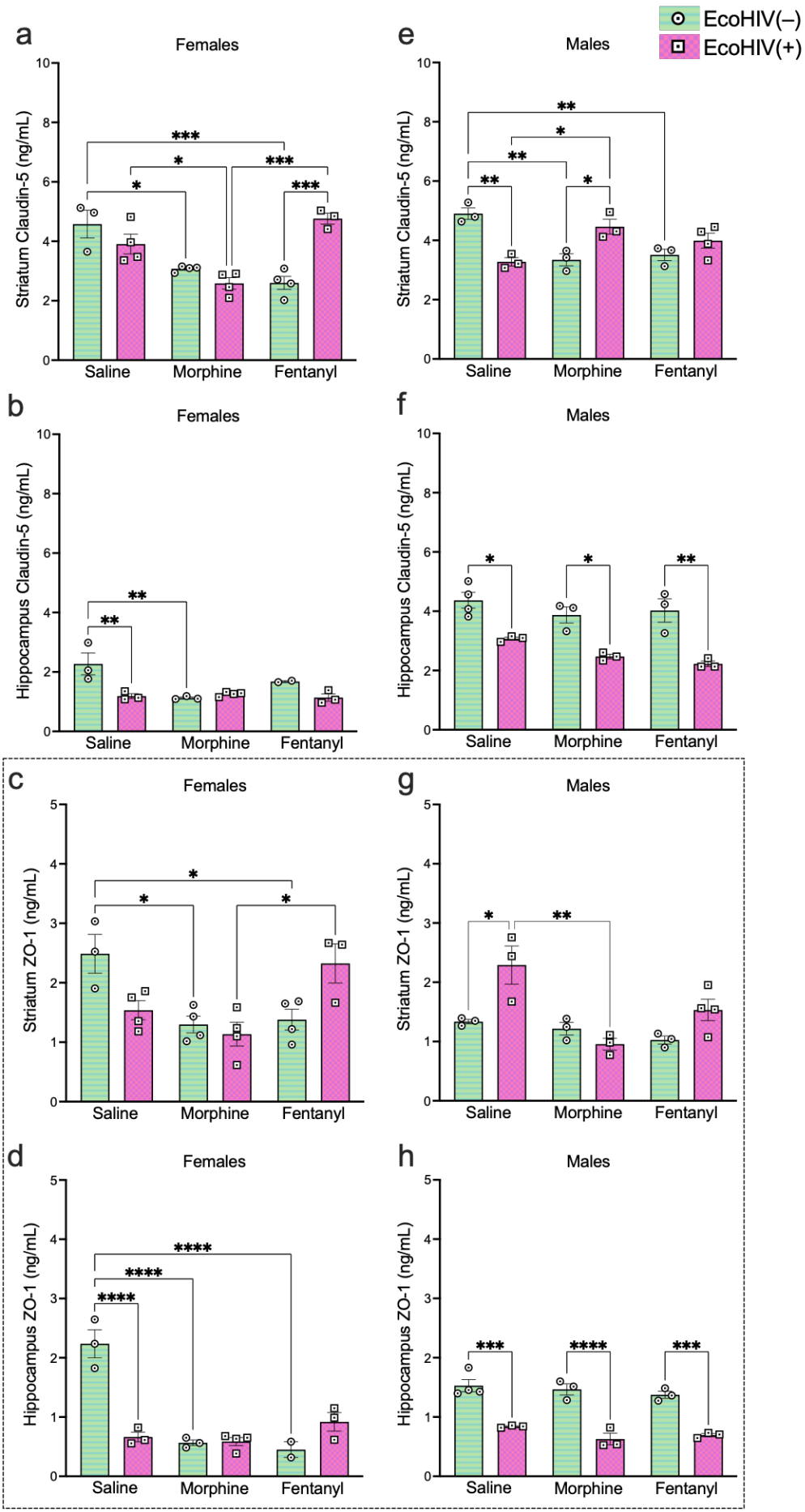
EcoHIV(–)
EcoHIV(+)
a
Females
Striatum Claudin-5 (ng/mL)
Saline
Morphine
Fentanyl
e
Males
b
Hippocampus Claudin-5 (ng/mL)
f
c
Striatum ZO-1 (ng/mL)
g
d
Hippocampus ZO-1 (ng/mL)
h

Fig. 3 *Female and male claudin-5 and ZO-1* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. EcoHIV(–) mice are depicted by horizontal green lines and white circles with a black dot. EcoHIV(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean claudin-5 and ZO-1 concentrations for females and males ± SEM; females n = 3 EcoHIV(–)/saline, n = 3-4 EcoHIV(+)/saline, n = 3-4 EcoHIV(–)/morphine, and n = 4 EcoHIV(+)/morphine, n = 2-4 EcoHIV(–)/fentanyl, and n = 3 EcoHIV(+)/fentanyl; males n = 3-4 EcoHIV(–)/saline, n = 3 EcoHIV(+)/saline, n = 3 EcoHIV(–)/morphine, and n = 3 EcoHIV(+)/morphine, n = 3 EcoHIV(–)/fentanyl, and n = 3-4 EcoHIV(+)/fentanyl and significant differences at $\alpha < 0.05$ are denoted by *$p < 0.0032$, **$p < 0.0021$, ***$p < 0.0002$, and ****$p < 0.0001$ for post-hoc testing.

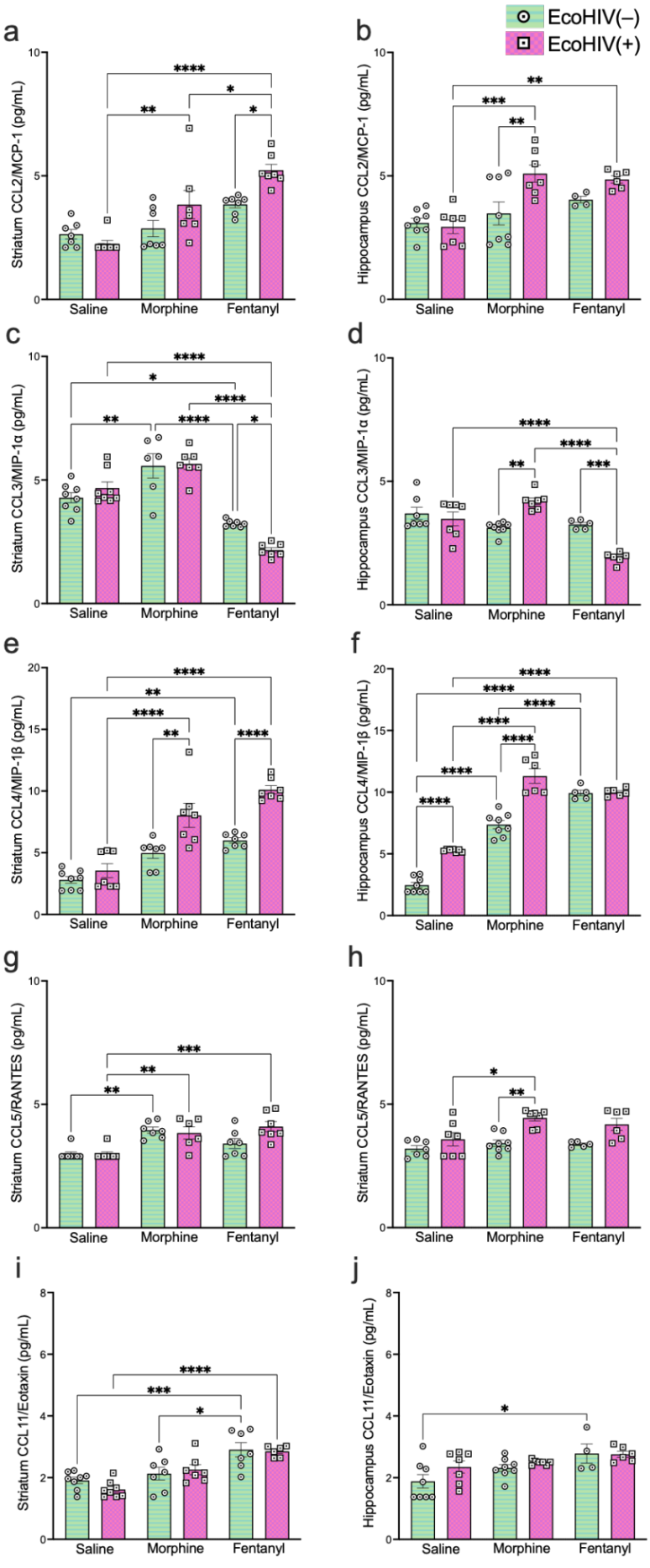
EcoHIV(–)
EcoHIV(+)
a
Striatum CCL2/MCP-1 (pg/mL)
b
Hippocampus CCL2/MCP-1 (pg/mL)
c
Striatum CCL3/MIP-1α (pg/mL)
d
Hippocampus CCL3/MIP-1α (pg/mL)
e
Striatum CCL4/MIP-1β (pg/mL)
f
Hippocampus CCL4/MIP-1β (pg/mL)
g
Striatum CCL5/RANTES (pg/mL)
h
Striatum CCL5/RANTES (pg/mL)
i
Striatum CCL11/Eotaxin (pg/mL)
j
Hippocampus CCL11/Eotaxin (pg/mL)
Saline
Morphine
Fentanyl

Fig. 4 *Inflammatory chemokine* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. EcoHIV(–) mice are depicted by horizontal green lines and white circles with a black dot. EcoHIV(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean concentration for each chemokine ± SEM; striatum n = 7-8 EcoHIV(–)/saline, n = 7-8 EcoHIV(+)/saline, n = 6-7 EcoHIV(–)/morphine, and n = 6-7 EcoHIV(+)/morphine, n = 7 EcoHIV(–)/fentanyl, and n = 6-7 EcoHIV(+)/fentanyl; hippocampus n = 7-8 EcoHIV(–)/saline, n = 7 EcoHIV(+)/saline, n = 8 EcoHIV(–)/morphine, and n = 6-7 EcoHIV(+)/morphine, n = 4-5 EcoHIV(–)/fentanyl, and n = 6 EcoHIV(+)/fentanyl and significant differences at $\alpha < 0.05$ are denoted by *$p < 0.0032$, **$p < 0.0021$, ***$p < 0.0002$, and ****$p < 0.0001$ for post-hoc testing.

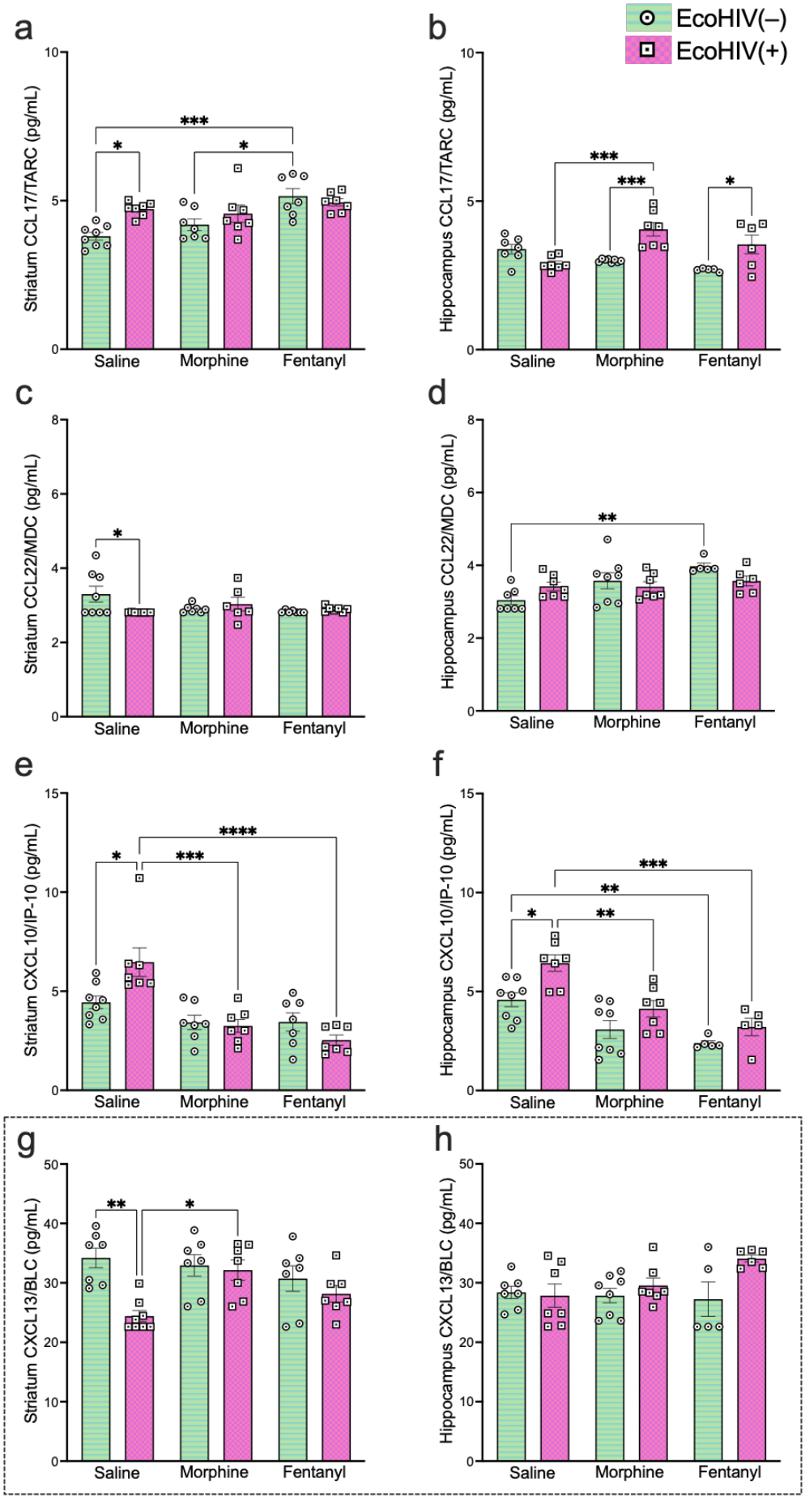
a
b
c
d
e
f
g
h
EcoHIV(–)
EcoHIV(+)
Striatum CCL17/TARC (pg/mL)
Hippocampus CCL17/TARC (pg/mL)
Striatum CCL22/MDC (pg/mL)
Hippocampus CCL22/MDC (pg/mL)
Striatum CXCL10/IP-10 (pg/mL)
Hippocampus CXCL10/IP-10 (pg/mL)
Striatum CXCL13/BLC (pg/mL)
Hippocampus CXCL13/BLC (pg/mL)
Saline
Morphine
Fentanyl

Fig. 5 *Dual-function and homeostatic chemokine* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. EcoHIV(–) mice are depicted by horizontal green lines and white circles with a black dot. EcoHIV(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean concentration for each chemokine ± SEM; striatum n = 8 EcoHIV(–)/saline, n = 7-8 EcoHIV(+)/saline, n = 7-8 EcoHIV(–)/morphine, and n = 6-7 EcoHIV(+)/morphine, n = 7 EcoHIV(–)/fentanyl, and n = 7 EcoHIV(+)/fentanyl; hippocampus n = 7-8 EcoHIV(–)/saline, n = 7 EcoHIV(+)/saline, n = 8 EcoHIV(–)/morphine, and n = 7 EcoHIV(+)/morphine, n = 5 EcoHIV(–)/fentanyl, and n = 5-6 EcoHIV(+)/fentanyl and significant differences at $\alpha < 0.05$ are denoted by *$p < 0.0032$, **$p < 0.0021$, ***$p < 0.0002$, and ****$p < 0.0001$ for post-hoc testing.

Chapter 5. Methadone alters antiretroviral concentrations, dysregulates neuroinflammation, and disrupts P-glycoprotein expression in the brains of HIV-1 Tat transgenic mice.

Introduction

In 2019, 1.2 million people in the United States were living with HIV, where 34,800 people accounted for newly diagnosed infections (Centers for Disease Control and Prevention 2021). Closely associated with the ongoing HIV epidemic is the opioid epidemic which was characterized by 10.1 million people aged 12 or older who misused opioids in 2019 (Substance Abuse Mental Health Services Administration 2020). The two epidemics, HIV and opioid abuse, are interlinked because intravenous drug use is a major transmission route of HIV (Leshner 1998; Mathers et al. 2008). NeuroHIV describes mechanisms related to neuronal injury and neuroinflammation involved in the pathogenesis of HIV-associated neurocognitive disorders (Hauser et al. 2007; Chang et al. 2014; Smith et al. 2014). Opioids exacerbate neuroHIV through accelerating HIV replication, increasing glial activation, enhancing monocyte migration, and exerting neurotoxic effects (Bell 1998; Bell et al. 2002; Anthony et al. 2005; Anthony 2008; Purohit et al. 2011; Devlin et al. 2012; F. Hauser et al. 2012; Byrd et al. 2012; Fitting et al. 2020).

In order to replicate in host cells, the HIV virus requires the presence of the trans-activator of transcription (Tat) protein. HIV-1 Tat is one of the first expressed proteins after initial infection, and is able to support productive replication, contribute to the persistence of viral reservoirs in macrophages, and induce cytotoxic insults (Manganaro et al. 2010; Bagashev and Sawaya 2013; Meltzer et al. 2018) Tat also contributes to neuroHIV due to its ability to induce alterations in the blood brain barrier (BBB), the immune response, and cell signaling cascades (Gurwell et al. 2001; Haughey and Mattson 2002; Neri et al. 2005; Zou et al. 2011; Ellis et al. 2015; Fitting et al. 2020). The Tat-transgenic mouse model has been commonly used to examine neuroinflammation (Hauser et al. 2007; Clifford and Ances 2013; Chang et al. 2014) and the interactive effects between HIV, drug abuse, inflammation, and antiretroviral accumulation (Kim et al. 2003; Langford et al. 2018; Leibrand et al. 2019, 2022). This model is useful for studying the neuroHIV because Tat is primarily localized to the CNS in GFAP expressing cells, compared to other models where Tat is constitutively expressed in all tissues (Kim et al. 2003) Furthermore, in virally suppressed patients, Tat remains elevated in the cerebrospinal fluid (CSF) and upregulates cytokine production (Johnson et al. 2013; Henderson et al. 2019). Studies using the Tat-transgenic mouse model further demonstrate that the Tat protein induces neuronal injury and produces learning and memory impairments (Wood et al. 2005; Fitting et al. 2013; Nass et al. 2020).

Previously, our lab has demonstrated that Tat exposure increases the recruitment of phagocytic macrophages, decreases antiretroviral drug concentrations in a brain-region manner, and dysregulates the immune response (Leibrand et al. 2017, 2019, 2022). During initial infection, HIV enters the brain by three proposed mechanisms, paracellularly through gaps in the blood brain barrier, transcellularly through

endothelial cells and through infected monocytes. Specific to HIV infected monocytes is that once in the brain, monocytes differentiate into long-lived macrophages where viral reservoirs are established (Eugenin et al. 2010; Siliciano and Greene 2011; Jaureguiberry-Bravo et al. 2016). Viral reservoirs are essentially hidden from the immune response and are difficult to target with antiretroviral therapies (Duncan and Sattentau 2011; Wong et al. 2019; Astorga-Gamaza et al. 2022). Actively dividing virus or viral proteins, including HIV-1 Tat, are released from infected/activated brain cells resulting in the infection or activation of nearby brain resident cells that sustains neuroinflammation (Rayne et al. 2010; Meltzer et al. 2018; Wallet et al. 2019; Wong et al. 2019; Ajasin and Eugenin 2020). Extensive research described the immunomodulatory effects of opioids (endogenous and exogenous) through their associations with opioid receptors on immune cells (Li et al. 2009; Reddy et al. 2012; Liang et al. 2016). Our previous studies have demonstrated that HIV-1 Tat disrupts the integrity of the BBB through altering the expression of a tight junction protein, ZO-1, and, together with morphine, decreases the penetration of select antiretroviral drugs and increases the expression of a drug efflux protein P-glycoprotein (P-gp) (Leibrand et al. 2019). P-glycoprotein is a major drug transporter at the BBB which limits the entry of many endogenous substances, drugs, and toxic molecules into the brain (Kharasch 2003; Miller et al. 2008). Inflammatory mediators can similarly increase the permeability of the BBB and alter the expression of P-glycoprotein and therefore impact the net flux of drugs into the brain (Tan et al. 2002; Fernandez et al. 2004; Seelbach et al. 2007).

Methadone is one of the FDA approved medications for the treatment of opioid use disorder (OUD) and is often used to treat OUD in PLWH (Huang et al. 1996; Roncero et al. 2017; Bart et al. 2021; Zamani et al. 2022), despite numerous potential drug interactions with HIV therapies (Gourevitch and Friedland 2000; Bart et al. 2021). Methadone is of interest to the current study due its pharmacologic profile (Hewitt 2000; Murphy et al. 2019). Methadone has been shown to dysregulate the immune response and activate both microglia and astrocytes (Wang et al. 2020; Navaei et al. 2022). *In vitro*, methadone exacerbated HIV-1 Tat induced neurotoxicity by decreasing neuronal survival and interacted with HIV-1 Tat resulting in the enhanced production of inflammatory mediators (Fitting et al. 2014). In addition, *in vitro* methadone enhanced HIV infection in monocyte-derived macrophages and activated latently infected peripheral blood mononuclear cells (Li et al. 2002). While the effects of methadone on HIV replication and neuroinflammation have been studied, it is less clear how methadone impacts antiretroviral therapies, the BBB, and neuroinflammation concurrently. Studies have examined drug-drug interactions between methadone and antiretrovirals, however, how these drug-drug interactions may impact antiretroviral brain concentrations remains unknown. The goals of this study are to investigate the impact of HIV-1 Tat and methadone on antiretroviral drug accumulation, and the immune response within the two brain regions highly affected by HIV, the striatum and hippocampus.

Materials and Methods

Animal Handling and Treatment

All studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University, and the experiments were conducted in accordance with ethical guidelines defined by the National Institutes of Health (NIH Publication No. 85–23).

Subjects and housing

Tat-transgenic female mice (~6 months old weighing ~25 g) were generated in the vivarium at Virginia Commonwealth University. All Tat transgenic mice (n = 37) were fed with doxycycline (Dox) containing chow (Dox Diet #2018; 6 g/kg; DOX-chow; purchased from Harlan Laboratories Madison, WI) for 14 days. After the Tat-induction period, mice were switched to regular chow and Alzet® osmotic pumps (2001, 1.0 µL/h, Cupertino, CA) containing methadone or sterile saline (control) were implanted subcutaneously. Antiretroviral (ARV) drugs were administered concurrently with methadone and control. Chow (Dox and regular) was provided *ad-libitum* and ARVs ± methadone were delivered continuously for 5 days.

Treatment

Triumeq®, the commercially available tablet containing abacavir (ABC), lamivudine (3TC), and dolutegravir (DTG) was purchased from the VCU Health Systems Pharmacy. Antiretroviral solutions were prepared in sterile saline, as described previously (Leibrand et al. 2019b). Allometric scaling (25g mouse) was used as previously described (Nair and Jacob 2016), to administer abacavir 2.5 mg/day, lamivudine 1.2 mg/day, and dolutegravir 0.2 mg/day. Methadone (NIDA Drug Supply Program, Bethesda, MD), was prepared in the solution containing the antiretroviral drugs at a concentration sufficient to deliver 1.92 mg/day. Drug preparations were made in batches to minimize dosing variability.

Surgical Manipulation

Five days after the continuous delivery of antiretroviral drugs ± methadone mice were anesthetized (isoflurane 4% induction, 2% maintenance) and sacrificed by exsanguination to collect blood. After collecting blood, a brief perfusion with phosphate buffered saline (PBS) was performed to clear the vasculature of blood content. The brain was collected, hemisected into right and left hemispheres. Each hemisphere was further microdissected into the striatum and hippocampus; tissues were weighed and snap-frozen (stored at −80 °C) until analysis. Plasma was separated by centrifugation (5,000 x *g* at 4 °C for 10 min) and stored at −80 °C until analysis.

Antiretroviral Quantification Analysis

Antiretroviral concentrations were measured in the plasma and tissues using LC-MS/MS, as described previously (Leibrand et al. 2019b). Briefly, the right striatum and hippocampus frozen samples were weighed and homogenized using Precellys® hard tissue grinding kit tubes (Cayman Chemical, MI,

USA) containing 1 mL of 70:30 acetonitrile:1mM Ammonium phosphate (pH 7.4). Lamivudine and abacavir plasma and tissue homogenates were extracted by protein precipitation with the isotopically labeled internal standards, abacavir-d 4 and lamivudine- 15 N-d 2. Chromatographic separation was achieved by reverse phase chromatography on a Waters Atlantis T3 (50 x 2.1 mm, 3 µm) analytical column. Dolutegravir plasma and tissue homogenates were extracted by protein precipitation with the isotopically labeled internal standard, dolutegravir- 13 C-d 5 . Chromatographic separation was achieved by reverse phase chromatography on a Waters XTERRA MS C18 (50 × 2.1 mm, 3.5 µm) analytical column. An AB Sciex API-5000 triple quadrupole mass spectrometer was used to detect all the analytes and internal standards under positive ion electrospray conditions. Precision and accuracy of the calibration standards and quality control samples were within 15% (lamivudine, abacavir, dolutegravir in plasma and dolutegravir in tissues) or 20% (lamivudine, abacavir in tissues) for the following dynamic ranges: 50.0 - 4,000 ng/mL of lamivudine, abacavir, dolutegravir in plasma; 0.100 - 50.0 ng/mL of 3TC in tissue; 0.200 - 50.0 ng/mL of abacavir in tissue; and 0.025 - 50.0 ng/mL of dolutegravir in tissue. Final concentrations were normalized to tissue mass and reported in ng/g units.

Immunoblotting

Immunoblotting for P-glycoprotein was performed on the left striatum and hippocampus from mice exposed to ARVs ± methadone as described above. Tissue samples were homogenized in NP-40 buffer prepared with a cOmplete™, Mini Protease Inhibitor tablet (11836153001, Roche Diagnostics GmbH, Germany). Homogenized tissue samples were centrifuged (16,000 × *g* at 4 °C for 10 min) and supernatant was removed, aliquoted, and stored at −80 °C until use. The bicinchoninic acid protein assay was performed to determine protein concentrations and 20 µg of striatal and hippocampal tissues were electrophoresed on 4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (#4568093, Bio-Rad Laboratories, Inc.) following the General Stain-Free Western Blotting Protocol (Bulletin 6390, Bio-Rad Laboratories, Inc.). The separated proteins were transferred to PVDF membranes using the Trans-Blot® Turbo™ Transfer System (1704150, Bio-Rad Laboratories, Inc.) after which transfer verification was performed by ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). Membranes were blocked in EveryBlot Blocking Buffer (12010020, Bio-Rad Laboratories, Inc.) and incubated for 1 h with primary antibody diluted (1:200 dilution) in EveryBlot Blocking Buffer (D14EF01114, COVANCE, Dedham MA, USA). The resultant chemiluminescent immunoblots were imaged using the ChemoDoc MP Imaging System. The Bio-Rad Image Lab software (V6.0.1, Bio-Rad) was used to normalize the chemiluminescent immunoblot with the Stain-Free transfer blot. Bands and optical densities for the immunoreactive P-gp band (170kDa) were determined using the Bio-Rad Image Lab software (V6.0.1, Bio-Rad) and the P-gp band was identified using the Precision Plus Protein Unstained Standard (1610363, Bio-Rad). Data obtained from the Image Lab Software was then analyzed for both striatal and hippocampal immunoblots using GraphPad Prism 9.0, version 9.4.2 (GraphPad Software, LLC, La Jolla, CA).

Chemokine Assay

The left striatum and left hippocampus were used for chemokine analysis. Chemokines were measured with a Mouse 13-plex Proinflammatory Chemokine Panel (Catalog #740007; BioLegend) and samples were prepared according to the manufacturer's instructions. In brief, tissue samples were homogenized in a cocktail composed of NP-40 lysis buffer with a tablet of cOmplete™ Mini Protease Inhibitor (11836153001, Roche Diagnostics GmbH, Germany). After homogenization, tissue samples were centrifuged (16,000 × *g* at 4 °C for 10 min) followed by the removal of the supernatant. Protein content was determined using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 2020), and samples were stored in −80 ºC until analysis. Sample lysates from the striatum and hippocampus containing 12 ug of protein per sample were prepared in duplicate along with standards and were incubated in chemokine antibody-conjugated beads (2 h, agitated at 500 rpm), followed by biotinylated detection antibodies (1 h, agitated at 500 rpm). Lastly, samples and standards were incubated with streptavidin-PE (SA-PE) for 30 min (agitated at 500 rpm). The filter plate was sealed and stored for 24 h at 4 °C. After the 24 h incubation period, samples and standards were resuspended (5 min, agitated at 500 rpm) and transferred to U-bottom plates. The Cytek® Aurora flow cytometer (Cytek® Biosciences, Fremont, California) was used to run the U-bottom plate according to the manufacturer's instructions. The LEGENDplex V8.0 software (BioLegend) was used to create a five-parameter logistic regression to fit the standard curve of each chemokine with the duplicated samples to be used for analysis.

Statistical analysis

Duplicate samples were averaged and analyzed by two-way variance (2-way ANOVA) to determine main effects of Tat status and methadone exposure (treatment) and an interaction between Tat status and treatment. For pairwise comparisons between group means, the 2-way ANOVA was followed by Tukey-HSD's multiple comparisons test.

For plasma-normalized antiretroviral values in the striatum or hippocampus, values that were below the limit of quantification were set to zero [Plasma LLOQ: abacavir 5 ng/mL, lamivudine 50 ng/mL, dolutegravir 20 ng/mL; Tissue LLOQ dependent on sample].

For immunoblotting analysis, data was normalized to the protein content in the Tat(–) group (control) from the Stain-Free transfer blot as instructed by the manufacturer (Bulletin 6390, Bio-Rad Laboratories, Inc.). P-glycoprotein in the Tat(–) group (control) was then set to 1, and the fold change from control was calculated for the Tat(+) group (control) and Tat(–) and Tat(+) methadone groups.

For the chemokine analysis, CCL2 and CXCL9 were below the LOD and were not included in statistical analyses.

Results were statistically significant using a significance level of $\alpha = 0.05$. Data is presented as the mean with the standard error of the mean (S.E.M.). GraphPad Prism 9.0, version 9.4.2 (GraphPad Software, LLC, La Jolla, CA) software was used for the statistical testing and to graph the results.

Results

Antiretroviral Concentrations

To better understand the impact of Tat and methadone on antiretroviral therapies, plasma and plasma-normalized tissue concentrations for the striatum and hippocampus were quantified by LC-MS/MS as previously described (Leibrand et al. 2019).

Antiretroviral Concentrations in the Plasma

Plasma concentrations were only different for lamivudine, where concentrations were significantly lower in Tat(+) placebo mice compared to Tat(+) methadone mice ($p = 0.001$, respectively) [Table 1, **Fig. 1b**].

Plasma-normalized Antiretroviral Concentrations in the Striatum

In the striatum, plasma-normalized abacavir was significantly *lower* in Tat(–) mice treated with saline and Tat(+) mice treated with methadone compared to Tat(+) mice treated with saline [$p = 0.003$, $p = 0.001$, respectively] [Table 1, **Fig. 2a**].

Blood Brain Barrier Measurements

To investigate the impact of HIV-1 Tat and methadone on P-glycoprotein expression, immunoblotting was performed to examine P-glycoprotein expression the striatum and hippocampus [Table 2].

In the striatum, Tat(–) mice treated with saline and Tat(+) mice treated with methadone, P-gp expression was significantly *lower* compared to Tat(+) mice treated with saline [$p < 0.0001$, $p = 0.0007$, respectively] [Table 2, **Fig. 3a**]. In the hippocampus, Tat(–) mice treated with methadone and Tat(+) mice treated with saline had significantly *higher* P-gp expression compared to Tat(–) mice treated with saline [$p < 0.0001$, $p = 0.02$, respectively] [Table 2, **Fig. 3b**].

Chemokine Concentrations in Striatum and Hippocampus

Inflammatory Chemokine Concentrations

To determine the effect of HIV-1 Tat and methadone on chemokine concentrations, 13 chemokines were analyzed from striatum and hippocampus after 5 days of continuous delivery of either methadone or placebo [Table 3].

Inflammatory Chemokines

Post-hoc multiple comparisons

CCL3: In the striatum, Tat(–) mice treated with saline had significantly *lower* concentrations of CCL3 compared to Tat(+) mice treated with saline [$p = 0.0003$] [Table 3, **Fig. 4a**]. In the hippocampus, Tat(–) mice treated with saline and Tat(+) mice treated with methadone had significantly *lower* concentrations of CCL3 compared to Tat(+) mice treated with saline [$p = 0.01$, $p = 0.02$, respectively] [Table 3, **Fig. 4b**].

CCL4: In the striatum, Tat(–) mice treated with saline had significantly *lower* concentrations of CCL4 compared to Tat(+) mice treated with saline [$p = 0.02$] [Table 2, **Fig. 4c**]. In the hippocampus, Tat(–) mice treated with methadone had significantly *higher* concentrations of CCL4 compared to Tat(–) mice treated with saline and Tat(+) mice treated with methadone [$p = 0.0001$, $p < 0.0001$, respectively]. Tat(+) mice treated with saline had significantly *higher* concentrations of CCL4 compared to Tat(–) mice treated with saline and Tat(+) mice treated with methadone [$p = 0.01$, $p = 0.006$, respectively] [Table 2, **Fig. 4d**].

CCL5: In the striatum, Tat(–) mice treated with saline had significantly *lower* concentrations of CCL5 compared to Tat(+) mice treated with saline [$p = 0.01$]. Tat(–) mice treated with methadone had significantly *lower* concentrations of CCL5 compared to Tat(+) mice treated with methadone [$p = 0.03$] [Table 3, **Fig. 4e**]. In the hippocampus, Tat(–) mice treated with saline and Tat(+) mice treated with methadone had significantly *lower* concentrations of CCL5 compared to Tat(+) mice treated with saline [$p = 0.0005$, $p = 0.007$, respectively] [Table 3, **Fig. 4f**].

CCL11: In the striatum, Tat(+) mice treated with methadone had significantly *higher* concentrations of CCL11 compared to Tat(+) mice treated with saline and Tat(–) mice treated with methadone mice [$p < 0.0001$ for all] [Table 3, **Fig. 4g**]. In the hippocampus, Tat(–) mice treated with saline had significantly *lower* concentrations of CCL11 compared to Tat(+) mice treated with saline [$p = 0.04$] [Table 3, **Fig. 4h**].

CXCL1: In the striatum, Tat(+) mice treated with saline and Tat(–) mice treated with methadone had significantly *higher* concentrations of CXCL1 compared to Tat(–) placebo mice [$p < 0.0001$ for all]. Tat(+) methadone mice had significantly *higher* concentrations of CXCL1 compared to Tat(–) methadone mice [$p = 0.01$] [Table 3, **Fig. 6a**]. In the hippocampus, Tat(+) methadone mice had significantly *higher* concentrations of CXCL1 compared to Tat(+) placebo and Tat(–) methadone mice [$p = 0.001$, $p = 0.003$, respectively] [Table 3, **Fig. 6b**].

Dual-function and Homeostatic Chemokine Concentrations

Post-hoc multiple comparisons

CCL17: In the striatum, Tat(+) methadone mice had significantly *higher* concentrations of CCL17 compared to Tat(+) placebo and Tat(–) methadone mice [$p < 0.0001$ for all] [Table 3, **Fig. 5a**]. In the hippocampus, Tat(+) methadone mice had significantly *higher* concentrations of CCL17 compared to Tat(+) placebo and Tat(–) methadone mice [$p = 0.001$, $p = 0.001$, respectively] [Table 3, **Fig. 5b**].

CCL20: In the striatum, Tat(–) placebo mice had significantly *lower* concentrations of CCL20 compared to Tat(+) placebo mice [$p = 0.03$] [Table 3, **Fig. 5c**].

CCL22: In the striatum, Tat(–) methadone mice and Tat(+) methadone mice, respectively, had significantly *higher* CCL22 concentrations compared to Tat(–) placebo and Tat(+) placebo mice [p = 0.0005, p = 0.001, respectively] [Table 3, **Fig. 5e]**.

CXCL5: In the striatum, Tat(–) mice treated with saline had significantly *higher* concentrations of CXCL5 compared to Tat(+) mice treated with saline (p = 0.004). Tat(–) mice treated with methadone mice had significantly *higher* concentrations of CXCL5 compared to Tat(–) mice treated with saline and Tat(+) mice treated with methadone [p = 0.04, p = 0.004, respectively] [Table 3, **Fig. 6c**].

CXCL10: In the striatum, Tat(–) mice treated with saline had significantly *lower* concentrations of CXCL10 concentrations compared to Tat(+) mice treated with saline [p = 0.04] [Table 3, **Fig. 6e**]. In the hippocampus, Tat(–) mice treated with saline had significantly *lower* concentrations of CXCL10 compared to Tat(+) mice treated with saline [p = 0.007] [Table 3, **Fig. 6f**].

CXCL13: In the striatum, Tat(–) mice treated with methadone had significantly *lower* concentrations of CXCL13 compared to Tat(+) mice treated with methadone [p = 0.03] [Table 3, **Fig. 6g**]. In the hippocampus, Tat(–) mice treated with methadone had significantly *lower* concentrations of CXCL13 concentrations compared to Tat(–) mice treated with saline and Tat(+) mice treated with methadone [p < 0.0001, p = 0.02, respectively] [Table 3, **Fig. 6h**].

Discussion

The current study examined the effects of HIV-1 Tat and methadone on antiretroviral accumulation within the brain, on drug transport proteins, and on neuroinflammation. HIV-1 Tat and opioids are known to contribute to neuroinflammation by dysregulation of cytokines and chemokines (Nath et al. 1999; Toborek et al. 2003; Gabay 2006; Boland et al. 2014), activation of viral replication (Li et al. 2002; Das et al. 2011; Azzoni et al. 2022) and alterations in drug transporters at the BBB (Miller et al. 2008; Leibrand et al. 2019). In the current study, HIV-1 Tat and methadone influence the expression of P-glycoprotein, antiretroviral plasma and tissue concentrations, and the regulation of chemokine concentrations in the striatum and hippocampus.

Alterations in antiretroviral concentrations and P-glycoprotein expression

The antiretroviral drugs examined in this study are substrates for several drug efflux transporters at the BBB. Abacavir and dolutegravir are known substrates for P-gp (Shaik et al. 2007; Giri et al. 2008; ViiV Healthcare 2015, 2017, 2020). In the striatum, methadone treatment reversed the Tat-induced increases in P-gp whereas, interestingly, in the hippocampus, methadone treatment caused an increase in P-gp expression in Tat(–) mice. Methadone is a substrate and inhibitor of P-gp and an inducer of an additional efflux transporter, breast cancer resistant protein (BCRP) (Störmer et al. 2001; Hassan et al.

2009; Tournier et al. 2010; Hung et al. 2013; Neradugomma et al. 2017). The alterations observed in abacavir concentrations within the striatum may have been associated with mechanisms not examined herein, such as BCRP at the BBB (Hernández-Lozano et al. 2021).

Although no drug-drug interactions were expected between methadone and lamivudine (Bruce et al. 2013), μ-opioid receptor-binding opioids reduce renal blood flow (Mercatello 1990; Mallappallil et al. 2017), and methadone has shown to potently inhibit renal OCT2 *in vitro* (Hacker et al. 2015). Lamivudine is not significantly metabolized by the liver and is primarily excreted unchanged by the kidney. Lamivudine is excreted by active secretion and is a substrate for renal drug transporters, including organic cationic transporter 2 (OCT2) (van Leeuwen et al. 1992; Johnson et al. 1999; Minuesa et al. 2009; Müller et al. 2013; Ceckova et al. 2016; Liu et al. 2018). In the presence of an OCT2 inhibitor, the renal clearance of lamivudine decreased while plasma concentrations increased (Moore et al. 1996; Müller et al. 2013). It is possible that methadone may interfere with renal blood flow or the renal uptake of lamivudine by OCT2 resulting in lamivudine accumulation in the plasma, although additional studies are warranted to fully examine this potential mechanism.

In the present study, there was an increase in P-gp expression in Tat(+) mice. The signaling pathways responsible for regulating P-gp expression and function are complex and require several elements (Hartz et al. 2006; Miller et al. 2008; Rigor et al. 2010; Chen et al. 2015). In both *in vitro* and *in vivo* studies, Tat induces the production of cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Nath et al. 1999; Yang et al. 2010; Nass et al. 2020), which may simultaneously increase the expression and function of P-gp in the brain (Ronaldson and Bendayan 2006; Bauer et al. 2007; Lee et al. 2012; Wang et al. 2018). Tat also activates the nuclear factor-κB (NF-κB) pathway which is involved in regulating P-gp gene expression; inhibition of NF-κB results in decreased P-gp expression (Demarchi et al. 1999; Toborek et al. 2003; Bentires-Alj et al. 2003; Hayashi et al. 2005). These findings are consistent with the present study, where HIV-1 Tat increased P-gp in the striatum and hippocampus.

Our findings also demonstrated that, in Tat(+) mice, methadone significantly decreased P-gp expression within the striatum. Methadone is an agonist at μ-opioid receptors and an antagonist at NMDA receptors(Hewitt 2000; Murphy et al. 2019). Both μ-opioid receptors and NMDA receptors have been shown *in vivo* to interact with P-gp expression (Zong and Pollack 2003; Yousif et al. 2012). Chronic morphine exposure significantly enhanced the expression of P-gp *in vitro*, however, antagonizing NMDA receptors inhibited morphine-induced alterations of P-gp *in vivo* (Yousif et al. 2012). NMDA receptors can affect P-gp expression; activation results in glutamate-mediated increases in P-gp whereas inhibition at NMDA receptors blocks glutamate-mediated expression of P-gp (Zhu and Liu 2004; Miller et al. 2008). Therefore, in the present study, the antagonism of the NMDA receptor by methadone may have masked the effects of methadone binding at the μ-opioid receptor (Rodríguez-Muñoz et al. 2012), suggesting that the NMDA receptor was largely involved in the regional regulation of P-gp expression.

Previously, we have examined the impact of Tat and morphine, another opioid agonist, on antiretroviral concentrations and P-gp expression in Tat transgenic mice (Leibrand et al. 2019). The

results between the two studies are partially inconsistent, where previously the data did not find a significant influence of Tat on antiretroviral concentrations or on P-gp expression. In the present study, however, Tat expression resulted in a significant increase in P-gp expression in saline-treated mice in both the striatum and the hippocampus. Although the reason for the discrepancy is not known, it could be due to differences in study design. In the prior study, after 14 days of doxycycline administration, there was a 5-day washout period between doxycycline discontinuation and the initiation of the antiretroviral and morphine treatment. Although it is thought that Tat expression remains elevated after discontinuation of doxycycline (Qrareya et al. 2022), as others have demonstrated long-lasting effects of Tat-induction on learning and memory up to 1 month after DOX induction was completed (Carey et al. 2012), there may be changes in overall concentrations of Tat that resulted in the discrepant findings herein.

Immune system dysfunction by HIV-1 Tat and methadone

Tat secreted from HIV-infected cells acts in a neurotoxic manner by inducing astrocytosis and neuronal apoptosis, increasing the infiltration of immune cells, and recruiting proinflammatory mediators involved in the pathogenesis of HAND (Liu et al. 2002; Kim et al. 2003; Gonek et al. 2018). NeuroHIV describes the mechanisms associated with HIV brain injury and chronic neuroinflammation, which are further dysregulated in the presence of opioids (El-Hage et al. 2006; Hauser et al. 2006, 2007; Bruce-Keller et al. 2008; Byrd et al. 2012). Neuroinflammatory conditions such as Alzheimer's, Parkinson's disease (Garcia-Marin et al. 2009; Sama and Norris 2013; Zaichick et al. 2017; Andersen et al. 2022), and HIV-associated neurocognitive disorders (Haughey and Mattson 2002; Andhavarapu et al. 2020; Khodr et al. 2022), share similar pathologies that include perturbations in cell signaling and dysfunction of the immune response in the brain.

Dysregulation of inflammatory chemokines by HIV-1 Tat and methadone

Independent studies reveal both Tat and methadone are able to activate neurons, astrocytes, and microglia (Haughey et al. 1999; Zhou et al. 2004; Norman et al. 2007; Lee et al. 2021; Zamani et al. 2022). In the presence of HIV, activated brain cells release neurotoxins involved in the dysregulation of inflammatory mediators (Liu et al. 2002; Eugenin et al. 2005; El-Hage et al. 2006; Turchan-Cholewo et al. 2009). Chemokine production can be stimulated by cytokines and by signaling mechanisms such as the activation of NF-κB and toll-like receptors (Stevens et al. 2013; Xie et al. 2017). The cytokines TNF-α and IL-1β are produced by various cells and stimulate the production of the inflammatory chemokines examined herein (Jedrzkiewicz et al. 2000; Lebovic et al. 2001; Zhang et al. 2003; Lee et al. 2006; Wang et al. 2013; Hyvärinen et al. 2019).

Overall, the effects of Tat on inflammatory chemokines resulted in several notable observations. HIV-1 Tat significantly increased CCL3, CCL4, and CCL5 in both brain regions examined, while CCL11 was significantly increased only in the hippocampus. Increases in these chemokines would contribute to neuroinflammation through their known effects of increasing immune cell recruitment into the brain. Both

CCL3 and CCL4 may be induced by the cytokine IL-1β *in vitro* (Guo et al. 2003; Zhang et al. 2003). Furthermore, an *in vitro* assay examining Tat and methadone demonstrated Tat-dependent increases in CCL4 and CCL5. Therefore it is plausible that the changes in CCL3 and CCL4 observed herein may have been mediated by Tat induction of IL-1β, although this was not tested experimentally. Methadone treatment also altered the levels of these inflammatory chemokines, although fewer changes were seen than with Tat exposure. Mice treated with methadone had significantly increased CCL11 in the striatum and decreased CCL5 in the hippocampus. The production of CCL5 and CCL11 are partially mediated by IL-1β (Jedrzkiewicz et al. 2000; Lebovic et al. 2001). Additionally, antagonism of NMDA receptors *in vitro* has shown to inhibit the NMDA-activated production of IL-1β (Gérard and Hansson 2012). In addition to IL-1β, an *in vitro* study shows that IL-6 can stimulate the production of CCL5 (Weber et al. 2020), and methadone has been shown to decrease IL-6 concentrations in the blood (Chavez-Valdez et al. 2013). In the hippocampus, methadone significantly decreased CCL5. Although not examined herein, it is reasonable that methadone altered CCL5 and CCL11 independent of IL-1β or by interacting with IL-6 in a regional-dependent manner.

Dysregulation of dual-function chemokines by HIV-1 Tat and methadone

In the present study, dual-function chemokines were dysregulated by HIV-1 Tat and methadone. Chemokines that are both constitutively expressed in the thymic and lymphoid tissues and inducible by several factors or activated cells are considered dual-function chemokines (Williams et al. 2014).

The binding of CCL20 to its receptor, CCR6, has been suggested to play a role in HIV latency (Cameron et al. 2010; Lee and Körner 2017). An *in vitro* study using resting $CD4^+$ cells treated with two HIV viral strains, demonstrated increased viral integration with minimal reverse transcriptase production in the presence of the CCL20/CCR6 axis (Cameron et al. 2010). Our study reveals that HIV-1 Tat expression significantly increased CCL20 in the striatum. CCL20 has been associated with *in vitro* neurodegeneration in traumatic brain injury (Das et al. 2011), spinal cord injury (Hu et al. 2016), and HIV infection (Sperk et al. 2021). *Ex vivo*, elevated CCL20 was associated with psychiatric conditions; compared to healthy controls, monocytes from bipolar patients display significantly higher levels of CCL20 (Padmos et al. 2008).

HIV-1 Tat expression also significantly increased CXCL1 in the striatum and in the hippocampus. CXCL5 concentrations were decreased by Tat exposure in the striatum but were unchanged in the hippocampus. Although the effect of Tat on CXCL1 has not been directly measured, behavioral deficits have been associated with elevated CXCL1 in mice chronically exposed to Tat (Nass et al. 2023). Additionally, *in vitro* studies demonstrate that monocytes and macrophages exposed to either TNF-α or IL-1β are able to produce CXCL1 (Lane et al. 2001; Issa et al. 2006). In a similar manner to HIV-1 Tat (Haughey et al. 1999), *in vitro* studies demonstrate that CXCL10 mediates neuronal cell death by dysregulating Ca^{2+} homeostasis and promoting excitotoxicity (Nelson and Gruol 2004; Sui et al. 2006; van Weering et al. 2011). Our findings are consistent with these studies, as HIV-1 Tat expression increased CXCL10 concentrations in both the striatum and hippocampus.

Increases in CCL17 and CCL22, as were seen with methadone and Tat exposure, are often associated with neurologic injury and immune response and the recruitment of T cells (Banisor et al. 2005; Pulliam et al. 2007; Osborn et al. 2011; Burman et al. 2014), which may dampen the immune response and promote viral persistence. The production of CCL17 and CCL22 involves the cytokine induction by IL-3 and/or IL-4 (Faffe et al. 2003; Yasuoka et al. 2011; Villarreal et al. 2014; Gougeon 2017) and an *in vivo* study shows that methadone increases IL-4 concentrations (Franchi et al. 2019). Based on prior studies, the increased concentrations of CCL17 and CCL22 by methadone are not surprising, however, further analysis is warranted to examine the mechanism involved.

Tat and methadone increased the inflammatory chemokine, CXCL1, and decreased the dual-function, CXCL5 chemokine, which are both involved in stimulating neutrophil recruitment and infiltration into the brain (Bozic et al. 1995; Tessier et al. 1997; Chintakuntlawar and Chodosh 2009). The influx of neutrophils may contribute to the pathogenesis of HIV through contributing to the neuroinflammatory response. Although other opioids, such as morphine, have been shown to dysregulate CXCL1 (Lin et al. 2015) and CXCL5 (Jalodia et al.), very little is currently known about the impact of methadone on these chemokines. One *in vitro* study using peripheral blood mononuclear cells isolated from rats, reported significant increases in TNF-α and CXCL1 in response to methadone exposure (Newville et al. 2020) which is consistent with our findings. This study demonstrates that methadone treatment can dysregulate CXCL1 and CXCL5 in a brain region specific manner, although to a lesser extent that Tat exposure.

Of the chemokines examined herein, the only homeostatic chemokine included was CXCL13. As a homeostatic chemokine, CXCL13 mediates B-cell, T-cell, and macrophage chemotaxis (Bekele Feyissa et al. 2021; Gao et al. 2021). In HIV, CXCL13 is increased (Widney et al. 2005; Cagigi et al. 2008) and an *in vitro* analysis using B-cells demonstrates that the cytokines, IL-2 and IL-10, are involved in the activation of B-cells which results in the production of CXCL13 B-cells (Cagigi et al. 2008). In the striatum and hippocampus, Tat increased CXCL13 in the presence of methadone. In the hippocampus, methadone decreased CXCL13. During neuroinflammation, CXCL13 recruits B-cells (Kowarik et al. 2012) and the accumulation of B-cells in the hippocampus has been associated with cognitive dysfunction in mice (Doyle et al. 2015; Shen et al. 2020).

In conclusion, HIV-1 Tat and methadone have multiple effects on cells and cell signaling cascades in the CNS resulting in the alteration of BBB integrity and the dysregulation of immune responses (Badou et al. 2000; Kim et al. 2003; King et al. 2006; Abbas and Herbein 2013; Azzoni et al. 2022). As an opioid, it is not surprising that methadone dysregulated chemokines in the striatum and hippocampus (Bokhari et al. 2011; Reddy et al. 2012; Murphy et al. 2019). Our study provides novel findings regarding the interactions between HIV-1 Tat and methadone on the accumulation of antiretrovirals, the impairment of the BBB, and the dysregulation of the immune response. Despite established drug-drug interactions between methadone and several antiretroviral therapies (Gourevitch and Friedland 2000; Bart et al. 2021), at least in this study, methadone and ARV co-exposure did not significantly impact ARV accumulation in the brain. The successful delivery- and accumulation of ARVs

into the brain is necessary for the treatment of HIV in the brain (Rao et al. 2009). Although additional studies are necessary to reproduce the current findings, clinically, our results suggest that treatment with abacavir, lamivudine, and dolutegravir in the setting of methadone, for opioid-use disorder, would not significantly impact the brain distribution of these antiretrovirals. Our results warrant additional studies to explore the interactions of methadone on antiretroviral drugs and the regulation of cytokines involved in chemokine production.

Table 1. Plasma and plasma-normalized antiretroviral concentrations in the striatum and hippocampus.

Antiretroviral Drug Concentrations	**Methadone (Main effect)**		**HIV-1 Tat (Main effect)**		**Interaction**	
Plasma	*F*	*p*	*F*	*p*	*F*	*p*
Plasma ABC [$F_{(1,32}$]	2.115	0.15	1.106	0.30	4.910	**0.03**≒
Plasma 3TC [$F_{(1,31)}$]	14.47	**0.0006**✧	0.0286	0.86	4.129	0.05
Plasma DTG [$F_{(1,31)}$]	2.053	0.1622	0.7373	0.39	2.018	0.16
T:P Striatum						
T:P* ABC [$F_{(1,32}$]	7.054	**0.01**✧	12.30	**0.001**₥	9.215	**0.004**≒
T:P* 3TC [$F_{(1,31)}$]	4.795	**0.03**✧	0.1850	0.67	1.061	0.31
T:P* DTG [$F_{(1,33)}$]	0.114	0.73	1.624	0.21	0.03	0.86
T:P Hippocampus						
T:P* ABC [$F_{(1,32}$]	2.945	0.09	4.864	**0.03**₥	1.945	0.17
T:P* 3TC [$F_{(1,31)}$]	11.23	**0.002**✧	4.444	**0.04**₥	0.1784	0.67
T:P* DTG [$F_{(1,33)}$]	0.7339	0.39	6.839	**0.01**₥	0.0002	0.98

*****T:P: plasma-normalized tissue concentrations
ABC = abacavir, DTG = dolutegravir, 3TC = lamivudine

Main effects are represented by significant main effect of treatment (✧) [methadone vs. saline-treated], significant main effect of Tat status (₥) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (≒).

Table 2. Effects of methadone and HIV-1 Tat exposure on P-glycoprotein levels in the striatum and hippocampus.

Brain Region	Methadone (Main effect)		HIV-1 Tat (Main effect)		Interaction	
Striatum*	$F_{(1,31)}$	p	$F_{(1,31)}$	p	$F_{(1,31)}$	p
	8.77	**0.005**✧	37.22	**< 0.0001**♒	10.78	**0.002**≒
Hippocampus*	$F_{(1,33)}$	p	$F_{(1,33)}$	p	$F_{(1,33)}$	p
	0.0961	0.75	62.15	**< 0.0001**♒	15.86	**0.0004**≒

***** Data presented as fold change from control Tat(–) placebo

Main effects are represented by significant main effect of treatment (✧) [methadone vs. saline-treated], significant main effect of Tat status (♒) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (≒).

Table 3. Effects of methadone and HIV-1 Tat exposure on chemokine concentrations in the striatum and hippocampus.

Brain Region		Methadone (Main effect)		HIV-1 Tat (Main effect)		Interaction	
Striatum							
	Chemokine	$F_{(1,28)}$	*p*	$F_{(1,28)}$	*p*	$F_{(1,28)}$	*p*
	CCL3	1.832	0.18	15.35	**0.0005**♏	2.915	0.09
	CCL4	6.425	0.10	29.94	**0.001**♏	0.9728	0.51
	CCL5	0.0737	0.78	18.83	**0.0002**♏	0.1104	0.74
	CCL11	52.47	**< 0.0001**✧	51.28	**< 0.0001**♏	30.99	**< 0.0001**♒
	CCL17	56.19	**< 0.0001**✧	61.18	**< 0.0001**♏	42.45	**< 0.0001**♒
	CCL20	0.1743	0.67	5.965	**0.02**♏	2.147	0.15
	CCL22	38.35	**< 0.0001**✧	10.15	**0.003**♏	0.0382	0.84
	CXCL1	28.61	**< 0.0001**✧	55.04	**< 0.0001**♏	8.694	**0.006**♒
	CXCL5	14.44	**0.0007**✧	28.33	**< 0.0001**♏	0.0006	0.97
	CXCL10	0.0972	0.75	10.16	**0.003**♏	0.5636	0.45
	CXCL13	0.0016	0.96	5.11	**0.03**♏	2.665	0.11
Hippocampus							
	Chemokine	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*	$F_{(1,30)}$	*p*
	CCL3	4.430	**0.04**✧	4.43	**0.04**♏	5.419	**0.02**♒
	CCL4	0.6462	0.42	2.227	0.14	36.39	**< 0.0001**♒
	CCL5	1.979	0.16	8.417	**0.006**♏	13.53	**0.0009**♒
	CCL11	0.0067	0.93	5.732	**0.02**♏	2.560	0.12
	CCL17 [$F_{(1,31}$]	5.970	**0.02**✧	5.97	**0.02**♏	14.82	**0.0006**♒
	CCL20	0.2063	0.65	2.669	0.11	0.0741	0.78
	CCL22	15.69	**0.0004**✧	6.293	**0.01**♏	2.622	0.11
	CXCL1	19.42	**0.0001**✧	12.78	**0.0012**♏	2.574	0.11
	CXCL5	0.1893	0.66	2.784	0.10	1.348	0.25
	CXCL10	0.2032	0.65	6.365	**0.01**♏	5.965	**0.02**
	CXCL13	24.31	**< 0.0001**✧	5.052	**0.03**♏	3.187	0.06

Main effects of methadone and Tat on chemokine levels in the striatum (*n* = 32) and hippocampus (*n* = 34). Main effects are represented by significant main effect of treatment (✧) [methadone vs. saline-treated], significant main effect of Tat status (⋒) [Tat(−) vs. Tat(+)], and the interaction between Treatment and Tat status (≒).

Chapter 5 Figures.

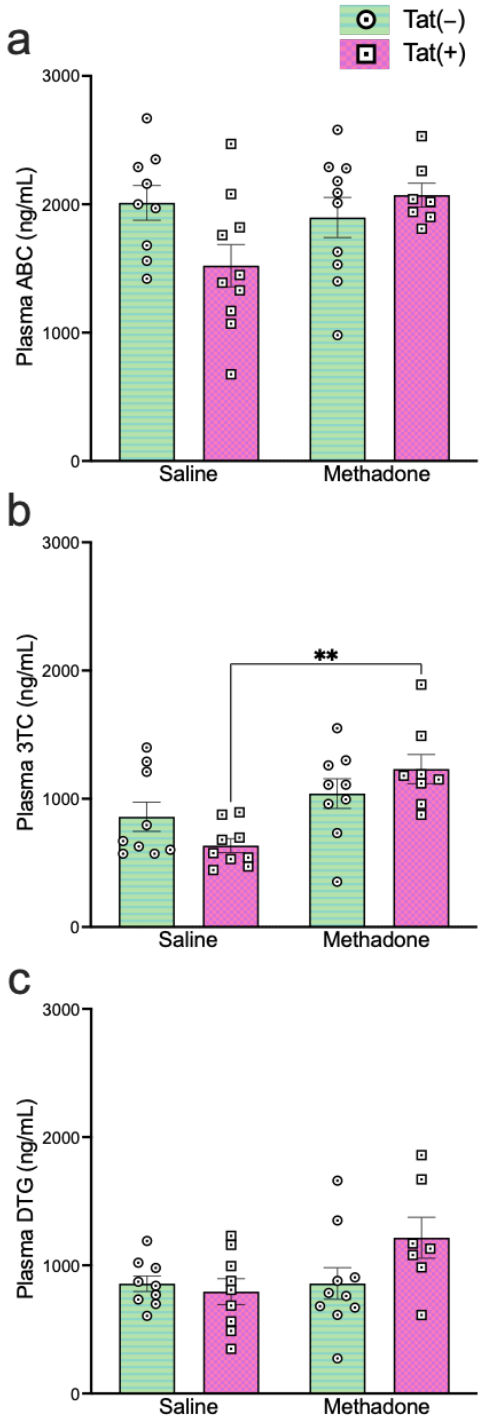

Fig. 1 *Plasma concentrations* of abacavir, lamivudine, and dolutegravir results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Data represents the mean ± SEM plasma concentrations of each antiretroviral; n = 9 Tat(–)/saline, n = 10 Tat(+)/saline, n = 10 Tat(–)/methadone, and n = 8 Tat(+)/methadone and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$.

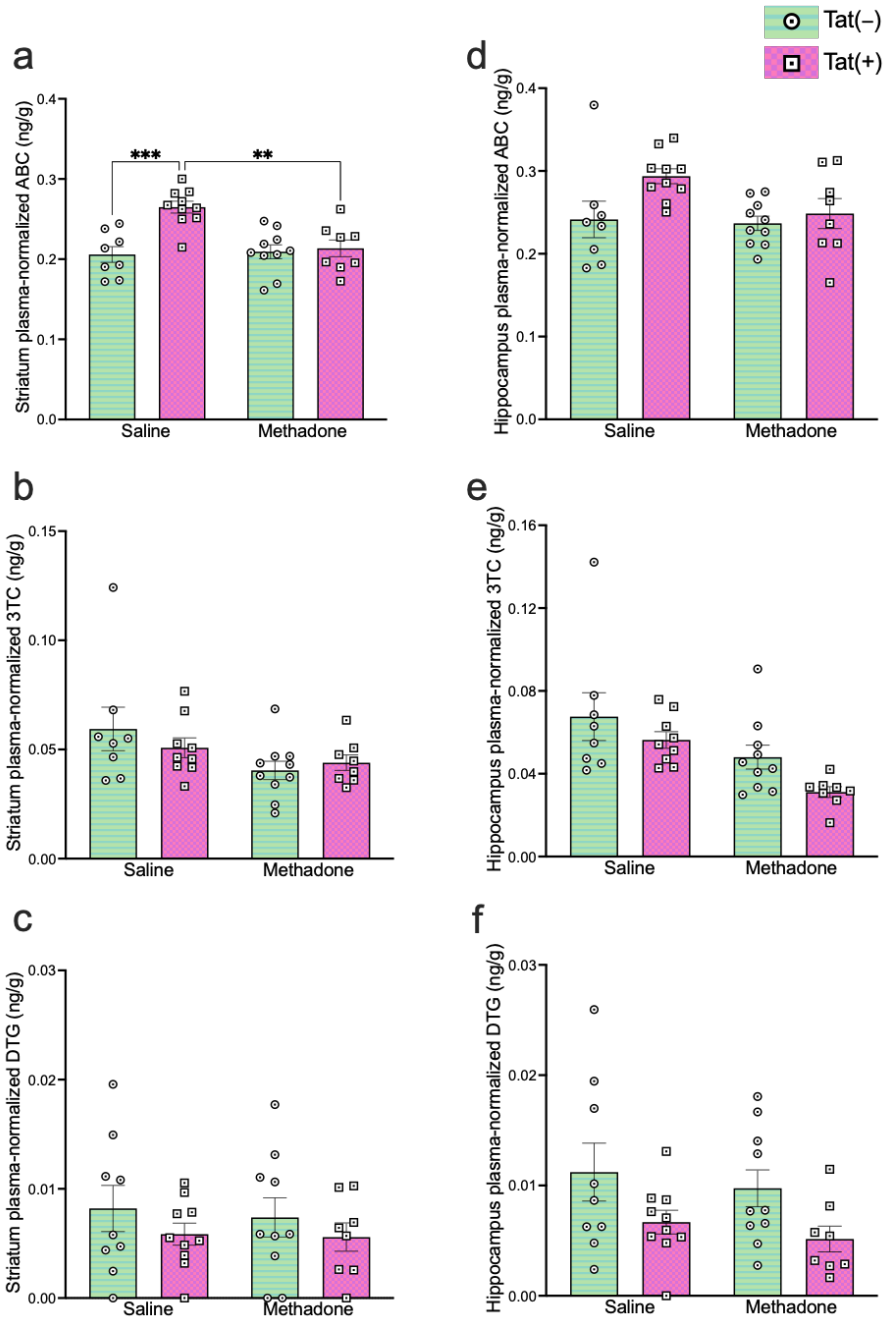

Fig. 2 *Plasma-normalized* tissue concentrations of abacavir, lamivudine, and dolutegravir from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(–) mice are depicted by horizontal green lines and white circles with a black dot. Tat(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean ± SEM tissue-normalized plasma concentrations of each antiretroviral; n = 9 Tat(–)/saline, n = 9-10 Tat(+)/saline, n = 9-10 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$.

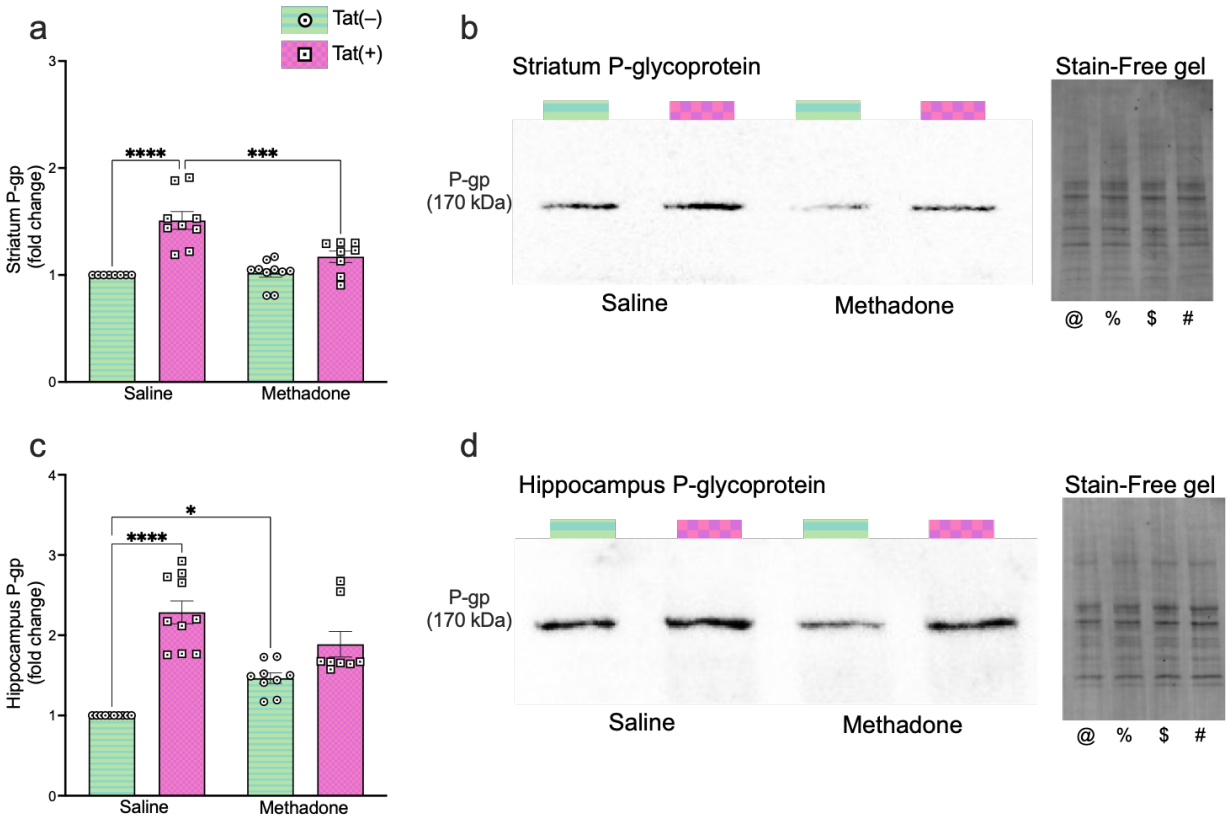

Fig. 3 P-glycoprotein expression in the striatum (a-b) and hippocampus (c-d) **i**mmunoblot results. Stain-Free transfer blot was used for normalization calculation of the fold change from control (Tat(–) saline-treated; P-gp = 1) in the striatum and hippocampus where lanes are designated for Tat(–) saline-treated (@), Tat(+) saline-treated (%), Tat(–) methadone-treated ($) and Tat(+) methadone-treated (#), respectively. Data represents the fold-change in mean fluorescence intensity ± SEM; striatum: *n* = 8 Tat(–)/saline, *n* = 9 Tat(+)/saline, *n* = 10 Tat(–)/methadone, and *n* = 8 Tat(+)/methadone; hippocampus: (*n* = 10 Tat(–)/saline, *n* = 10 Tat(+)/saline, *n* = 9 Tat(–)/methadone, and *n* = 8 Tat(+)/methadone.

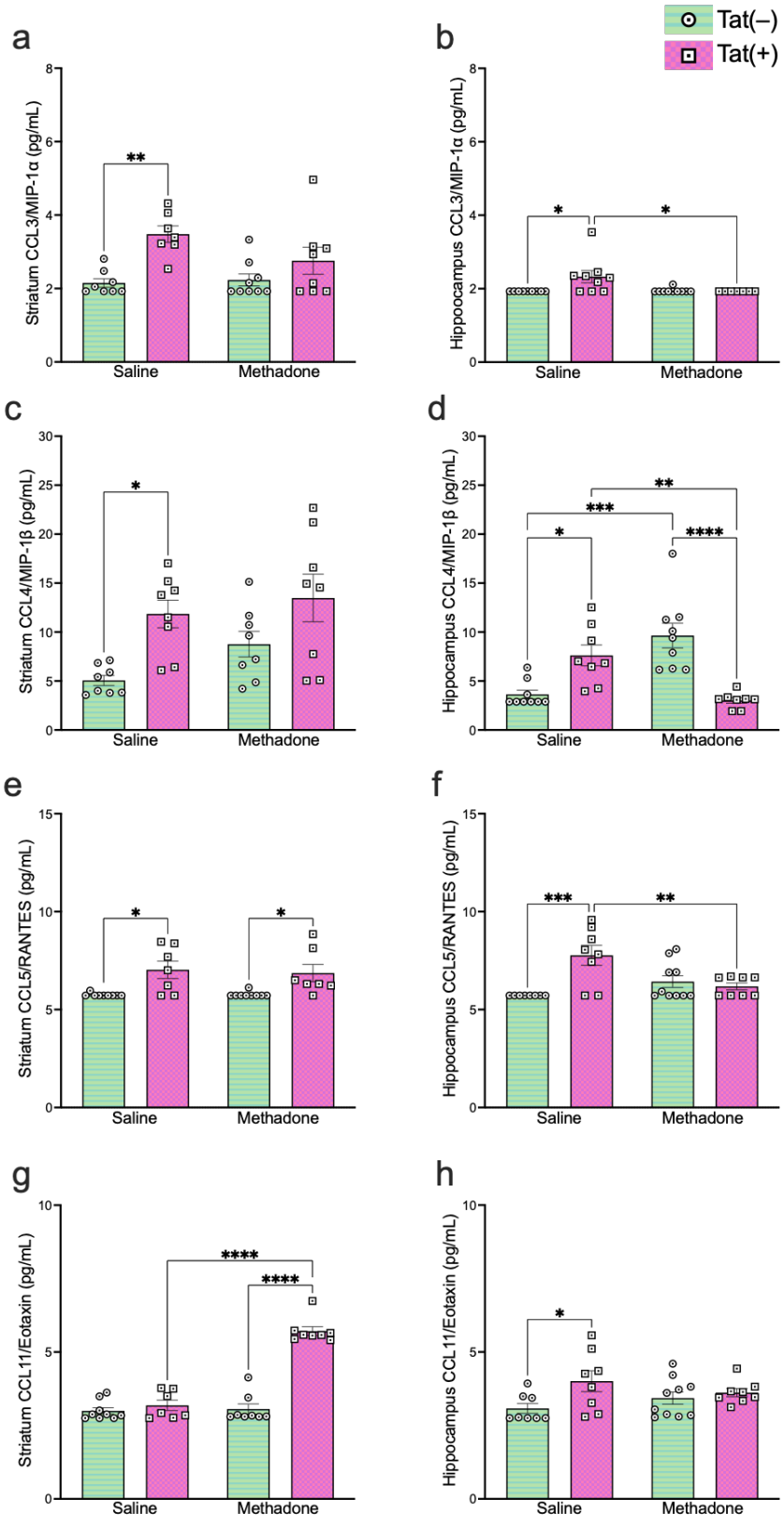
a
b
c
d
e
f
g
h
Tat(–)
Tat(+)
Striatum CCL3/MIP-1α (pg/mL)
Hippocampus CCL3/MIP-1α (pg/mL)
Striatum CCL4/MIP-1β (pg/mL)
Hippocampus CCL4/MIP-1β (pg/mL)
Striatum CCL5/RANTES (pg/mL)
Hippocampus CCL5/RANTES (pg/mL)
Striatum CCL11/Eotaxin (pg/mL)
Hippocampus CCL11/Eotaxin (pg/mL)
Saline
Methadone

Fig. 4 *Inflammatory chemokine* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(–) mice are depicted by horizontal green lines and white circles with a black dot. Tat(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean concentration ± SEM; striatum: n = 8-9 Tat(–)/saline, n = 7-8 Tat(+)/saline, n = 8-9 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone; hippocampus: n = 8-9 Tat(–)/saline, n = 8-9 Tat(+)/saline, n = 9-10 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$.

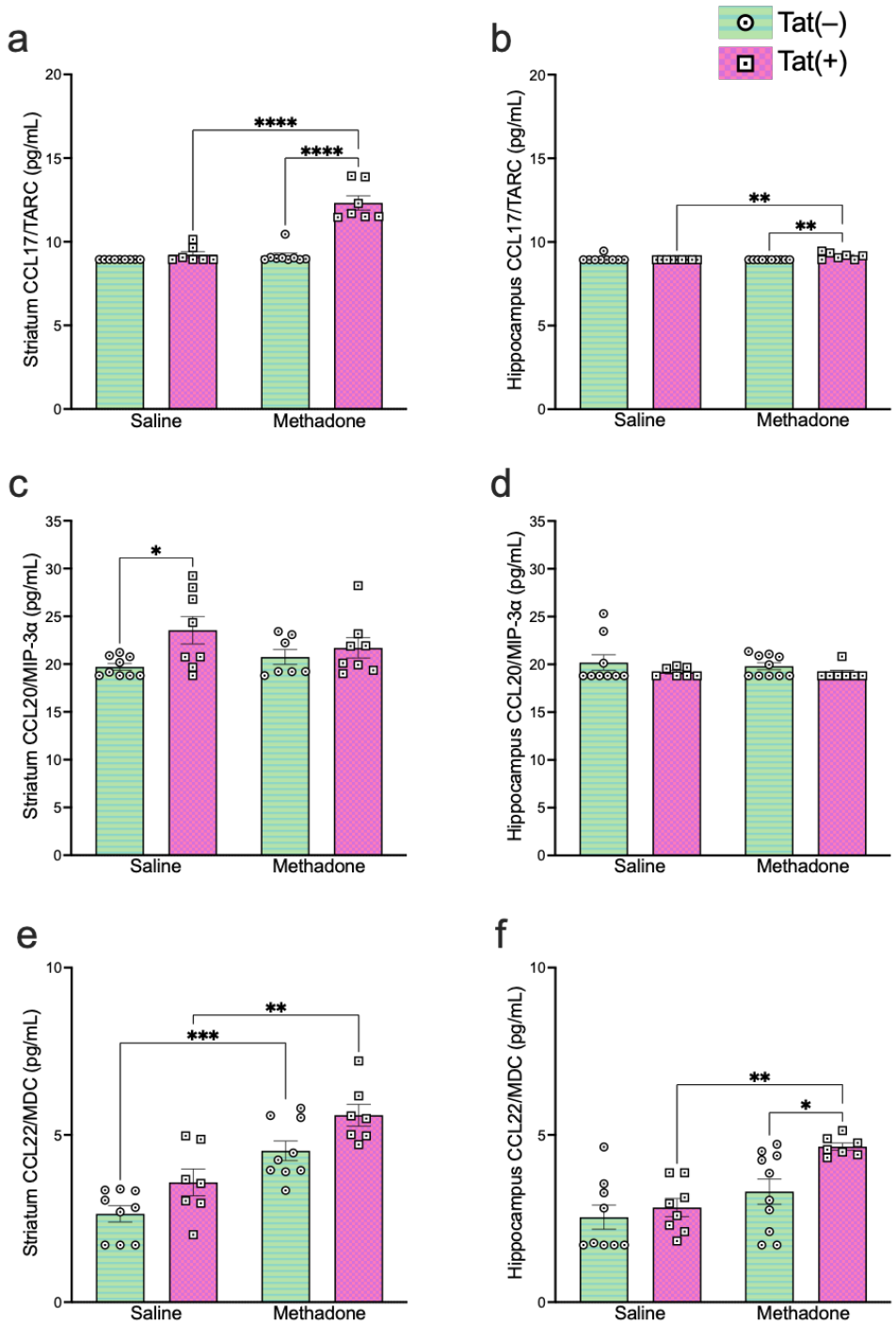

Fig. 5 *Dual function 'CCL-X' chemokines* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(–) mice are depicted by horizontal green lines and white circles with a black dot. Tat(+) mice are depicted by pink squares and white squares with a black dot. Data represents the mean concentration ± SEM; striatum: n = 9 Tat(–)/saline, n = 7-8 Tat(+)/saline, n = 7-9 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone; hippocampus: n = 9 Tat(–)/saline, n = 7-9 Tat(+)/saline, n = 10 Tat(–)/methadone, and n = 7 Tat(+)/methadone and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$.

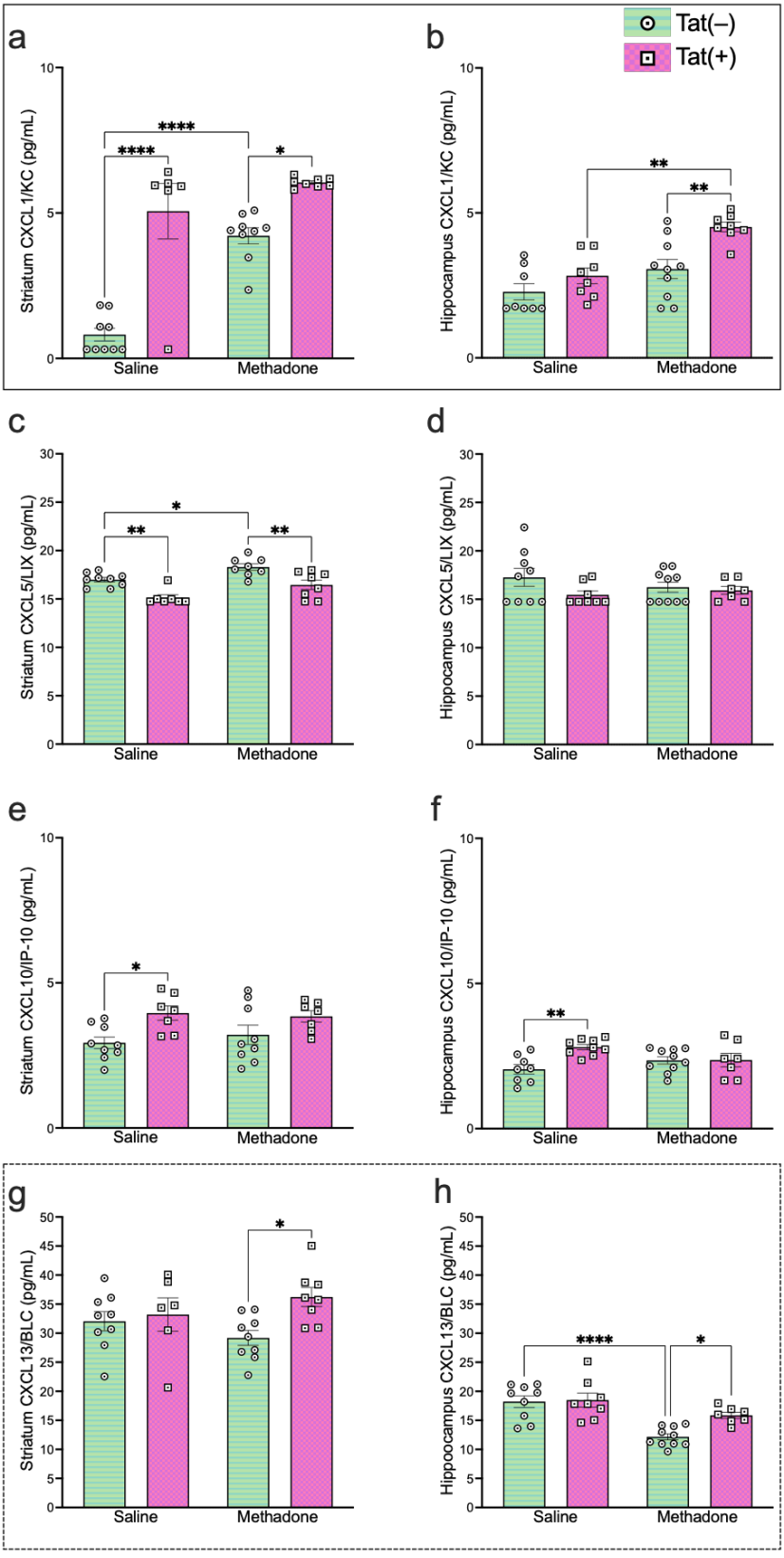
a
b
Tat(–)
Tat(+)
Striatum CXCL1/KC (pg/mL)
Hippocampus CXCL1/KC (pg/mL)
c
d
Striatum CXCL5/LIX (pg/mL)
Hippocampus CXCL5/LIX (pg/mL)
e
f
Striatum CXCL10/IP-10 (pg/mL)
Hippocampus CXCL10/IP-10 (pg/mL)
g
h
Striatum CXCL13/BLC (pg/mL)
Hippoocampus CXCL13/BLC (pg/mL)
Saline
Methadone

Fig. 6 *Inflammatory (a-b) and dual function (c-h) 'CXCL-X' chemokines* results from Tukey-HSD post-hoc analysis in the striatum and hippocampus. Tat(–) mice are depicted by horizontal green lines and white circles with a black dot. Tat(+) mice are depicted by pink squares and white squares with a black dot. The inflammatory chemokine CXCL1 is surrounded by a solid line box, and the homeostatic chemokine CXCL13 is surrounded by the dashed box. Data represents the mean concentration ± SEM; striatum: n = 9 Tat(–)/saline, n = 6-8 Tat(+)/saline, n = 6-9 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone; hippocampus: n = 8-9 Tat(–)/saline, n = 7-9 Tat(+)/saline, n = 10 Tat(–)/methadone, and n = 7-8 Tat(+)/methadone and significant differences at $\alpha < 0.05$ are denoted by $^{*}p < 0.0032$, $^{**}p < 0.0021$, $^{***}p < 0.0002$, and $^{****}p < 0.0001$.

Chapter 6. Overall Conclusions and Future Directions.

HIV in the brain and neuroHIV

The entry of HIV into the brain has been reviewed previously and is thought to occur by three potential mechanisms; through HIV-infected monocytes, endothelial transcytosis, or paracellularly through a 'leaky' blood brain barrier (Banks et al. 1999; Mattson et al. 2005; McRae 2016). The 'leaky' blood brain barrier (BBB) mechanism of HIV brain entry involves endothelial cells held together by tight junctions, which are structurally reinforced by tight junction proteins including claudins (Ivey et al. 2009; Kadry et al. 2020) and by tight junction accessory proteins such as Zonula occludens (ZO) (Kadry et al. 2020). HIV alters the integrity of the BBB by mediating cell signaling cascades that temporarily open tight junctions (Kadry et al 2020) and decrease claudin-5 and ZO expression (Dallasta et al. 1999; Kanmogne et al. 2005; András et al. 2005; Yang et al. 2009; McRae 2016). In a similar manner, opioids have demonstrated to alter the integrity of the BBB (Leibrand et al. 2017, 2019; Osborne et al. 2020) and reduce the accumulation of antiretrovirals in the brain, promote macrophage migration into the brain, and dysregulate the immune response (Leibrand et al. 2017, 2019, 2022; Osborne et al. 2020).

NeuroHIV collectively describes the mechanisms by which HIV injures brain cells and sustains neuroinflammation which are involved in the development of HIV-associated neurocognitive disorders (Hauser et al. 2007; Clifford and Ances 2013; Chang et al. 2014). The HIV-1 viral protein, Tat, is a major contributing component to neuroHIV (Hauser et al. 2012; Fitting et al. 2020) as HIV-1 Tat is neurotoxic and dysregulates cell signaling cascades involved in regulating the immune response (Badou et al. 2000; King et al. 2006; Herbein et al. 2010; Abbas and Herbein 2013; Fields et al. 2015). The exacerbation of neuroHIV by opioid abuse in the setting of HIV have been examined (Reddy et al. 2012; Hauser et al. 2012; Fitting et al. 2020). Figure 1 provides an overview of the mechanisms by which HIV in the brain and opioids maintain the cycle that contributes to neuroHIV.

Differences in the pharmacological profiles between the opioids examined

The three opioids examined within this large body of work included morphine, fentanyl, and methadone, which are all agonists at the μ-opioid receptor. Opioid receptor activation has been examined in HIV as cells expressing opioid receptors are activated upon the binding of an opioid resulting in altered immune responses and the disruption of cell signaling cascades (Bokhari et al. 2011; Wang et al. 2012; Murphy et al. 2019). In addition to the μ-opioid receptor, morphine, fentanyl, and methadone exhibit binding at κ-opioid receptors and δ-opioid receptors, although with different affinities for each receptor with methadone having very low affinity for κ-opioid receptors and δ-opioid receptor (Kristensen et al. 1995; Torralva et al. 2020).

The pharmacological profile for these opioids display some key differences. First, unlike morphine and fentanyl, methadone is an antagonist at NMDA receptors. The pharmacological profiles between morphine and fentanyl further diverges with fentanyl also binding to non-opioid receptors such as

adrenergic receptors (α_{1A}, α_{1B} and α_{1D} and α_2), dopamine D4.4 and D1 receptor subtypes, and the vesicular monoamine transporter type 2 (VMAT2) among others (Torralva et al. 2020; Haouzi and Tubbs 2022; Kelly et al. 2023).

Disruption of the blood brain barrier and drug efflux transporter, P-glycoprotein

As we have demonstrated within our three separate studies, fentanyl, morphine, and methadone each can impact the molecular characteristics of the brain environment differently. Fentanyl and morphine altered claudin-5 and ZO-1, although not always in the same direction or to the same extent [Fig. 2]. Methadone was also involved in alterations of the efflux transporter P-glycoprotein in the striatum and hippocampus. In Chapter 4, morphine increased claudin-5 in male striatum and fentanyl increased claudin-5 in female striatum [Fig. 2]. Compared to infected fentanyl-treated female mice, infected morphine-treated female mice had lower claudin-5 in the striatum of female infected mice. In the hippocampus, males appeared to be more vulnerable to claudin-5 alterations. Infected morphine- and fentanyl-treated male mice had significantly lower claudin-5 compared to their uninfected counterparts. In Chapter 5, we did not measure markers of blood brain barrier integrity, but instead examined the efflux drug transporter, P-glycoprotein as many antiretroviral drugs are substrates for the transporter (Chan et al. 2013; Agrawal et al. 2020). Methadone decreased P-glycoprotein in the striatum and increased P-glycoprotein in the hippocampus.

Alterations in the distribution of antiretroviral therapies

Although the treatment of HIV infection by combination antiretroviral therapies effectively limits HIV within the periphery (Wong et al. 1997), the complete eradication of HIV in the brain remains a challenge. Poor antiretroviral accumulation within the brain is associated with the persistence of viral reservoirs (Crowe et al. 2003; Fois and Brew 2015). Resting CD_4^+ T cells contain the majority of replication competent proviruses (reservoirs) and in order to replicate, the virus requires host cell activation which causes the host cell to express transcription factors (Kimata et al. 2016; Anderson and Maldarelli 2018). The non-transcriptionally active viral reservoirs in quiescent CD_4^+ T cells are not targeted by many first-line antiretrovirals therapies (ARVs), and many ARVs do not accumulate in the brain at concentrations sufficient for limiting viral replication especially once viral reservoirs become transcriptionally active (Fois and Brew 2015; Kimata et al. 2016; Anderson and Maldarelli 2018; Wong et al. 2019; Osborne et al. 2020)(He et al. 2018; Wong et al. 2019; Osborne et al. 2020).

In Chapter 4, morphine-treated infected mice had lower abacavir concentrations in the striatum and hippocampus compared to saline-treated infected mice. Morphine-treated infected mice also had lower abacavir concentrations in the striatum compared to fentanyl-treated infected mice. Lamivudine and dolutegravir concentrations within the striatum were lower in uninfected morphine-treated mice compared to uninfected fentanyl-treated mice. Lastly, in the hippocampus, infected fentanyl-treated mice had significantly lower concentrations of dolutegravir in the hippocampus compared to infected saline-treated

mice and uninfected fentanyl-treated mice. In Chapter 5, methadone increased plasma concentrations of lamivudine in Tat(+) mice. Abacavir concentrations in the striatum were higher in Tat(+) saline-treated mice compared to Tat(+) methadone-treated mice.

Abacavir and dolutegravir are both substrates for the the drug efflux transporter, P-glycoprotein, suggesting that alterations in their concentrations may be related to changes in their overall flux into the brain (Shaik et al. 2007; Giri et al. 2008; ViiV Healthcare 2015, 2017, 2020). Lamivudine is not a substrate for P-glycoprotein because it is primarily excreted by the kidneys (van Leeuwen et al. 1992; Minuesa et al. 2009; Müller et al. 2013; Ceckova et al. 2016). For renal excretion, lamivudine has to be actively secreted at the kidney by the uptake transporter, OCT2, suggesting that opioids may affect the functionality of this transporter (Hacker et al. 2015), possibly preventing the uptake of lamivudine into the kidney and causes lamivudine accumulation in the plasma.

Immune Response Dysregulation

In all of the studies, we examined 13 different chemokines, however, we would like to conclude with the findings of three inflammatory chemokines, CCL3, CCL4, and CCL5. These chemokines are thought to have very similar roles in regulating the immune system by recruiting and activating immune cells to a site of injury or inflammation (Maurer and von Stebut 2004).

With regards to the immune response, morphine, fentanyl, and methadone dysregulated chemokines, and three of them will be reviewed herein. In Chapter 4, morphine increased CCL3 in the striatum, fentanyl decreased CCL3 in the striatum and hippocampus. Chapter 5 demonstrated that methadone had no impact on CCL3. The second chemokine, CCL4, which is thought to have a similar function to CCL3 (Maurer and von Stebut 2004), was dysregulated by all three of the opioids examined herein. Morphine significantly increased CCL4 in the striatum and hippocampus, fentanyl increased CCL4 in the striatum, and methadone decreased CCL4 in the hippocampus. Lastly, similar to CCL3 and CCL4, the chemokine CCL5 is also involved in regulating the immune response through the recruitment of immune cells (Schall 1991). Morphine increased CCL5 in the hippocampus while fentanyl and methadone did not appear to have any significant effect on CCL5 in either brain region.

Although it might be assumed that a decrease in CCL3 would be beneficial compared to an increase in CCL3, both directions of dysregulation are potentially harmful. Increased CCL3 contributes to viral replication and subsequent neuroinflammation as CCL3 recruits monocyte-derived macrophages (Pelisch 2020). Because CCL3 is also involved in B lymphocyte function, decreased CCL3 contributes to the loss of antibody responses against HIV (Moir and Fauci 2009), therefore, also contributing to viral replication and subsequent neuroinflammation. The upregulation of CCL4 and CCL5 have been implicated in enhancing viral replication (Kinter et al. 1998), and elevated CCL5 has been demonstrated to play a role in Alzheimer's disease and Parkinson's disease (Fiala et al. 2005; Reale et al. 2009). Morphine, fentanyl, and methadone dysregulated chemokines, although not in the same direction or to

the same extent. With this being said, the opioids have demonstrated to dysregulate the immune response in a manner that would sustain the neuroHIV cycle.

Sex Differences in Tight Junctions

In Chapter 4, we revealed sex differences in claudin-5 and ZO-1 within the striatum and hippocampus in response to both EcoHIV and opioid exposure. No sex differences were observed in Chapter 3 or Chapter 4 because only female mice were used for those studies. In Chapter 4, tight junction proteins within the male hippocampus appeared to be more vulnerable to EcoHIV infection than the striatum or than females. In contrast, within the striatum, female and male mice were both susceptible to opioids. In general, male tight junctions were less reactive to changes to opioid exposure in the hippocampus.

Implications and Future Directions

Throughout these studies we have seen three μ-opioid receptor agonists affect the brain in three different ways. This indicates the complexity of opioid-HIV interactions within the brain and that more than the mu-opioid receptor is important in opioid effects.

Both opioid and NMDA receptors are involved in neuroHIV (Giulian et al. 1990; Jiang et al. 2001; O'Donnell 2006). as their activation results in perturbations of [Ca^{2+}] homeostasis, increased nitric oxide production, immune and glial cell activation and neuronal degeneration (Choi 1994; Bagetta et al. 2004). HIV infected cells release glutamate, a toxic byproduct, that stimulates NMDA receptors (Fontana et al. 1997) and glutamate-activation of NMDA receptors has been demonstrated to alter the expression of the efflux transporter, P-glycoprotein (Zhu and Liu 2004; Miller et al. 2008). Due to methadone binding at NMDA receptors, there is a potential that NMDA-glutamate independent mechanisms were involved in regulation of P-glycoprotein as inhibition of NMDA receptors has been shown to block the glutamate-mediated upregulation of P-glycoprotein (Zhu and Liu 2004; Miller et al. 2008). With respect to the inflammatory response, methadone did not appear to dysregulate chemokine production to the same extent as morphine and fentanyl. Although we hypothesize that NMDA receptor inhibition was not solely responsible for alterations in P-glycoprotein, there is a possibility that NMDA receptor inhibition was involved in the immune response, although this needs to be tested experimentally.

It would be expected that morphine and fentanyl produce different results from methadone in regards to the regulation of the blood brain barrier and the immune response, based on the NMDA activity of methadone. Interestingly, the blood brain barrier and immune responses were also different when examining morphine vs. fentanyl. Compared to fentanyl, morphine increased CCL3 in the striatum and hippocampus and decreased CCL4 in the hippocampus. The opioid-independent activity of fentanyl has implications for understanding opioid and HIV interactions as it relates to neuroHIV, although we did not examine these specifically. For example, α_{1A} adrenergic receptors and VMAT2 are expressed on astrocytes (Hertz et al. 2010; Wahis and Holt 2021). Fentanyl inhibits the uptake of neurotransmitters at

VMAT2 (Torralva et al. 2020), and inhibition of VMAT2 on astrocytes disrupts dopamine homeostasis and is involved in neurocognitive dysfunction (Petrelli et al. 2020). In addition, the activation of α_{1A} adrenergic receptors is involved in mediating intracellular $[Ca^{2+}]_i$ release from astrocytes (Ding et al. 2013) while activation of α_2 receptors enhances glutamate uptake (Wallace 2006; Hertz et al. 2010). Alterations in $[Ca^{2+}]_i$ and glutamate homeostasis are associated with excitotoxicity, microglial activation and neuron degeneration, which are mechanisms that contribute to neuroHIV (Haughey and Mattson 2002; El-Hage et al. 2005; Wallace 2006; Hauser et al. 2007; Fitting et al. 2014).

Overall, our findings reveal that opioids contribute to mechanisms related to neuroHIV. Compared to morphine, we hypothesize that fentanyl's opioid-independent activity contributes to the alterations we observed in the blood brain barrier integrity and immune response. Our findings of morphine-mediated alterations are mostly consistent with prior studies. However, to the best of our knowledge, this is the first study to examine and compare the effects of morphine and fentanyl in an infectious HIV animal model. Future studies should examine these opioid-independent mechanisms ons on the integrity of the blood brain barrier and the immune response in HIV. Even though methadone is used to treat opioid use disorder, it is clear that methadone use may come with some potentially negative effects within the system. The inhibition of the NMDA receptor blocks the disruption of calcium homeostasis in astrocytes and neurons, and alterations in calcium homeostasis are involved in astrocyte and neuron activation which stimulates the release of inflammatory mediators (Haughey et al. 2001; Nath 2002; El-Hage et al. 2008). Future studies should further examine mechanisms related to the potential negative effects for the drugs that are used to treat opioid use disorder because there may be unintended negative consequences that ultimately further worsen NeuroHIV.

Lastly, we did not find significant differences in the chemokines between male and female mice in either the EcoHIV mice or Tat-transgenic mice; these studies were not powered to find sex differences. Future studies should include larger sample sizes to better understand how HIV and opioid responses might be different in males versus females. Prior research from others strongly suggests that the cytokines, TNF-α and IL-1β, are highly involved in regulating the chemokines examined within our studies. We did not study the mechanisms of chemokine induction, however, our studies warrant further investigation to better understand the regulation of chemokine production in the setting of HIV and co-exposure of opioids. As mentioned, the pharmacological profile of opioids should be considered in future studies to further expand on our novel findings of the interactions between various opioids and HIV on mechanisms related to neuroHIV.

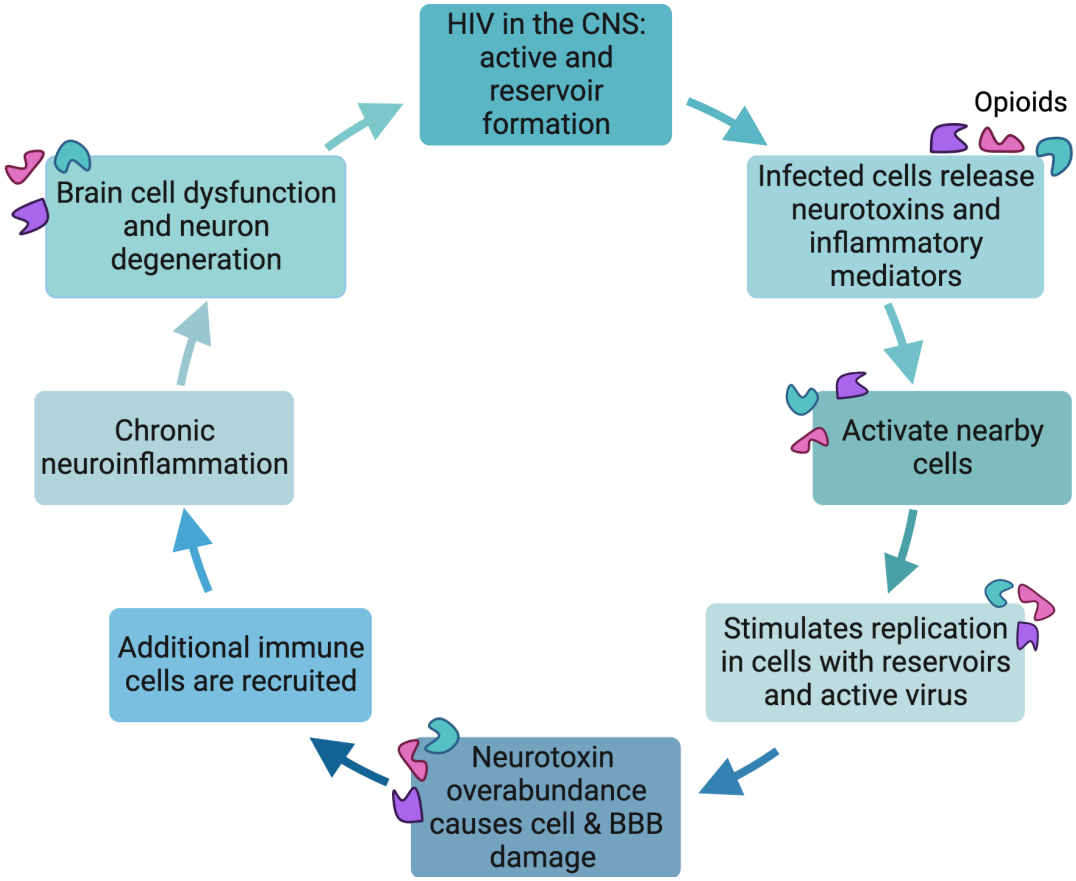

Fig. 1 The cycle of NeuroHIV is exacerbated by opioids.

In the brain, the HIV virus can actively replicate and establish viral reservoirs, where integrated viral DNA is non-transcriptionally active and remains undetected by the immune response and antiretroviral therapies. Infected cells in the central nervous system release neurotoxins and inflammatory mediators, which leads to the activation of additional bystander cells. Bystander cells that are either actively infected or which harbor viral DNA become activated, resulting in the stimulation of viral replication and additional release of neurotoxins and inflammatory mediators. Over time, there is an overabundance of neurotoxins within the brain that is involved in damaging glial cells and the blood brain barrier. The damage of glial cells and the blood brain barrier stimulates active recruitment of immune cells to the site of injury, which sustains the cycle of chronic neuroinflammation. Chronic neuroinflammation eventually leads to brain cell dysfunction and neutron degeneration. Opioids activate cells, dysregulate cell signaling cascades, impair the integrity of the blood brain barrier, and promote glial cell dysfunction and neuron degeneration.

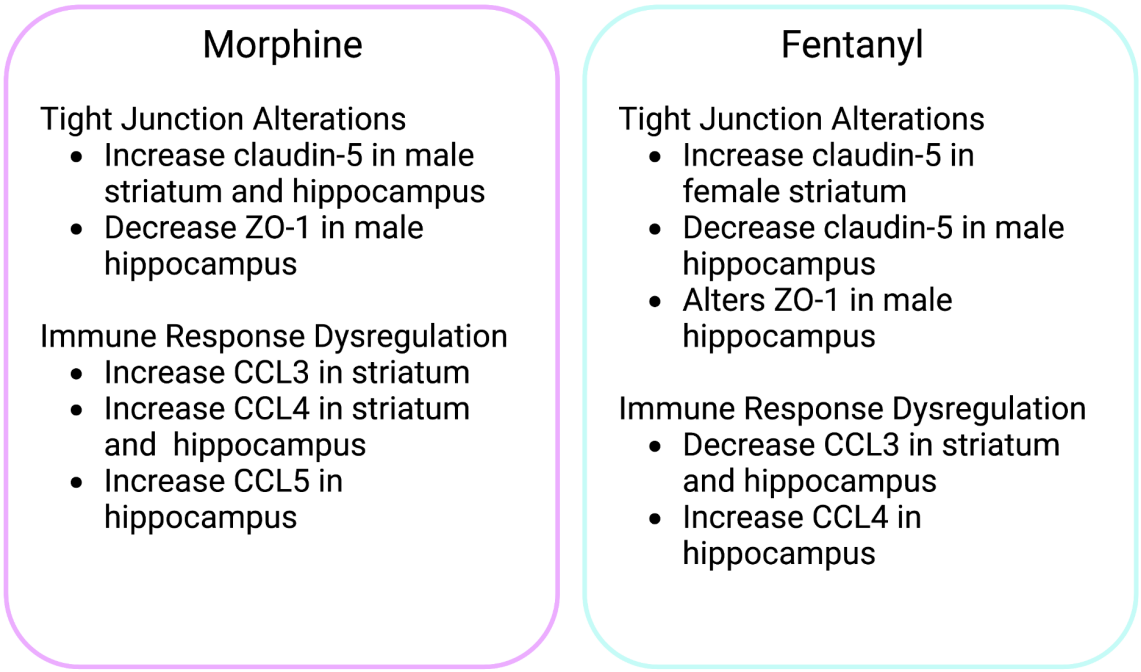

Fig. 2 A summary of the effects of morphine and fentanyl on the blood brain barrier and immune response in EcoHIV infected mice.

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