2021

Effect of KCC2-enhancing prodrug CLP290 on Tat ± morphine treated female mice at the behavioral and molecular levels

Michael Marone
Virginia Commonwealth University

Effect of KCC2-enhancing prodrug CLP290 on Tat ± morphine treated female mice at the behavioral and molecular levels

A dissertation submitted in partial fulfillment of requirements for the degree of Master of Science at Virginia Commonwealth University

By

Michael Marone

Bachelor of Science, University of South Carolina

Director: Dr. Kurt Hauser

Professor, Department of Pharmacology and Toxicology

Virginia Commonwealth University

Richmond, Virginia

December 2021

Acknowledgements

First, I am grateful for the guidance, patience, and support of my advisor Dr. Kurt Hauser. You always supported my ideas, my goals, allowed me to pursue any question I had or technique I wanted to try, and always challenged me to be a better scientist.

I also would like to thank everyone who is and was in the Hauser-Knapp Lab during my time here. From day one I knew this was an extremely supportive lab environment. No matter how busy of you were, any time I needed help or guidance everyone was willing to drop what they were doing to help, and I am extremely grateful for all of you. I'd especially like to thank Dr. Yun Hahn, Dr. Ginny McLane, Dr. Sara Nass, Dr. Aaron Barbour, and Dr. Arianna Lark for their training, input, and support over the years and on this project.

To my Mom Dorothy, Dad John, and Sister Jacki: You have all been there for me, no matter what, in anything I have ever pursued in life. The pride you've all had in me during grad school pushed me to be better and work hard every day. Mom, thank you for being there day and night to stay on the phone with me to listen to me vent, complain, and go on rants constantly for the past 4.5 years. Dad, thank you for always having my back, giving me endless support, and trusting and being there for me in all my academic pursuits. Jacki, you have always been my source of inspiration and curiosity in science, and I truly appreciate you always being there to reach out and keep us close.

To my partner Cheryll, you have been my rock since the day I met you and I would not be graduating today without you. Your support, love, guidance, and inspiration has kept me going in my hardest times and continues to inspire me to be better person every day. No matter what I was going through, no matter what future and career I wanted to pursue, no matter how long I had to vent or talk, you were there to listen and support my dreams and aspirations. I cannot thank you enough, I love you.

List of Figures

List of Abbreviations

ANI	Asymptomatic neurocognitive impairment
ANO1	Anoctamin-1
ART	Anti-retroviral therapy
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CaCC	$Ca2^{+}$ - activated Cl- channel
cAMP	cyclic adenosine monophosphate
cART	combination anti-retroviral therapy
CCL11	C-C motif chemokine 11
CCR2	C-C chemokine receptor type 2
CCR3	C-C chemokine receptor type 3
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTZ	Cyclothiazide
CXCR4	C-X-C chemokine receptor type 4
D1R	Dopamine-1 receptor
D2R	Dopamine-2 receptor
DC	Dendritic cell
DOR	Δ-opioid receptor
DOX	Doxycycline
DMSO	Dimethyl sulfoxide
E_{cl}	Equilibrium potential
EGRF	Epidermal growth factor receptor
ER	Endoplasmic reticulum
Erk1/2	Extracellular signal-regulating kinase

FPKM	Fragments per kilobase exon per million mapped fragments
GABA	Gamma aminobutyric acid
GFAP	Glial-acidic fibrillary protein
gp	glycoprotein
GPCR	G-protein coupled receptor
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
HPCD	2-hydroxypropyl-beta-cyclodextrin
IFN-β	Interferon-β
IL-23	Interleukin 23
KCC2	K^+Cl^- cotransporter 2
KOR	κ-opioid receptor
MOR	μ-opioid receptor
MSN	Medium spiny neuron
Nac	Nucleus accumbens
NFkB	nuclear factor kappa-light-chain-enhancer of B-cells
NKCC1	Na-K-2Cl cotransporter 1
NMDAR	N-methyl-D-aspartate receptor
NPY	Neuropeptide Y
OUD	Opioid use disorder
PKC	Protein-kinase C
PLWH	People living with HIV
PNS	Peripheral nervous system
PP1	Protein phosphorylase 1
PV	Parvalbumin
s.c.	subcutaneous
SOM	Somatostatin
Tat	Transactivator of transcription
VTA	Ventral tegmental area

Table of Contents

Abstract

Effect of KCC2-enhancing prodrug CLP290 on Tat ± morphine treated female mice at the behavioral and molecular levels

A dissertation submitted in partial fulfillment of requirements for the degree of Master of Science at Virginia Commonwealth University

By
Michael Marone
Bachelor of Science, University of South Carolina

Major Director: Dr. Kurt Hauser
Professor, Department of Pharmacology and Toxicology

HIV-1 infection is highly comorbid with opioid use disorder. Prescription and illicit use of opioids are particularly risky for HIV-1 infected individuals due to potential synergistic neurotoxic effects mediated by viral proteins and opioids. Additionally, opiate drug abuse has been shown to exacerbate neuropathogenesis of HIV-1^{+} individuals. Development of combination antiretroviral therapies (cART) has drastically improved the life expectancy of these patients. Unfortunately, although the severity of neurological and neurocognitive complications has declined, the incidence of HIV-associated neurocognitive disorders (HAND) remains 30-50% in the post-cART era and is worsened by opioid use disorder. HIV-1 proteins, inflammatory mediators, and excitotoxins released and recruited by infected cells lead to sublethal neuronal pathologies, providing the neural basis of HAND. The striatum is a central interface for locomotor and addiction circuits that becomes dysregulated by opioids and is susceptible to harboring high viral loads leading to marked

neuropathology in HIV infected individuals. While neurons cannot be infected with HIV, glial cells, such as astrocytes, are preferentially targeted and are the source of many sublethal neuronal effects associated with HAND. While it has been demonstrated HIV viral protein, Tat, is synergistically excitotoxic with morphine through an NMDA receptor-mediated manner, little is known about how HIV-1 Tat interacts with opioids on GABAergic systems. The studies in this dissertation use a glial fibrillary acidic protein (GFAP)-driven doxycycline (DOX)-inducible Tat-transgenic model in female mice that accurately mimics neurological deficits in people living with HIV (PLWH).

Previous studies using this model in male mice showed diminished levels of K^+ Cl^- cotransporter 2 (KCC2) a neuron specific cotransporter that is essential for maintaining low levels of intracellular Cl^- required for proper $GABA_AR$-mediated hyperpolarization. Additionally, loss of overall KCC2 levels and KCC2 membrane localization via decreased phosphorylation of Serine 940-KCC2 were rescued by oral gavage of KCC2-enhancing prodrug CLP290. We performed similar studies in female DOX-inducible Tat-transgenic mice and administered a ramping dose of morphine to understand the interactive effects with Tat. We found that morphine administration increased anxiety-related behavior and decreased exploration in an open field test. Using western blot analysis to reveal potential changes in expression levels, we found CLP290 increased overall KCC2 levels in the striatum but had no effect on p940-KCC2 or on the immature neuron cotransporter Na-K-2Cl cotransporter 1 (NKCC1). Interestingly, we found that Tat and CLP290 independently diminished levels of calcium-activated chloride channel TMEM16a, suggesting an alternative mechanism where Tat may dysregulate intracellular Cl^-. Overall, these studies demonstrate a potential sex difference in the effects of this Tat-transgenic model and

CLP290 on KCC2 that should be further investigated in side-by-side studies to better understand KCC2 as a prospective treatment target for HAND and opiate use.

Chapter 1: Introduction

Human immunodeficiency virus (HIV)

In 2020 there were 37.7 million PLWH with 1.5 million new HIV infections during this year alone (UNAIDS 2021). HIV is in the lentivirus genus within the retrovirus subfamily and can be classified as HIV-1 or HIV-2 subtypes. HIV-1 is the canonical subtype associated as HIV and is divided into M, N, O, and P strains with M viruses being the responsible for a majority of global HIV-1 infections since the emergence of the HIV pandemic (Bbosa, Kaleebu, & Ssemwanga, 2019). HIV is diagnosed by quantifying the number of cluster of differentiation (CD) CD4+ T-cells in patient plasma samples which determines the level of immunodeficiency. Upon diagnosis, measurement scores of plasma viral load, CD4+ count, and clinical indicators are used as criteria for planning of anti-retroviral therapy (ART) (Simon, Ho, & Karim, 2006).

HIV viral entry and life cycle

There are three main steps during HIV entry sequence which consists of CD4 binding, co-receptor binding, and membrane fusion/cell entry. The HIV-1 lipid bilayer envelope contains the integral membrane glycoprotein (gp)160 that is comprised of gp120 and gp41, which are responsible for attachment to host cells, primarily CD4+ T-cells and macrophages. CD4 receptor binding leads to conformational changes in gp120 variable loops, particularly the V3 loop, which is critical to C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) co-receptor binding. Viruses that utilize a particular coreceptor are termed R5- or X4-tropic viruses, respectively (Wilen, Tilton, &

Doms, 2012). Upon co-receptor binding, a conformational changes in gp41 allows for viral-host membrane fusion and subsequent viral insertion into the host cell (Doms & Moore, 2000; Wilen et al., 2012). Understanding of the viral entry process and tropism of HIV-1 has led to the development of more effects ARTs. For example, ART drugs have been developed to block multiple stages of the viral entry process in attachment inhibitors that prevent HIV-CD4 interaction, CXCR4 and CCR5 coreceptor inhibitors, and fusion inhibitors which inhibit virus and cell membrane attachment (Lobritz, Ratcliff, & Arts, 2010). Once inside the host cell, HIV hijacks host cell machinery to produce low levels of viral transcript. One of the first viral proteins produced is trans-activator of transcription (Tat). Tat enhances viral replication by binding the TAR region of new transcripts as well as recruiting transcriptional proteins for more efficient replication (Bannwarth & Gatignol, 2005; Das, Harwig, & Berkhout, 2011).

HIV-associated neurocognitive disorders

The spectrum of neurocognitive impairments associated with HIV-1 infection are termed HIV-associated neurocognitive disorders (HAND). This spectrum ranges from the mildest form in asymptomatic neurocognitive impairment (ANI) to the most severe form in HIV-associated dementia (HAD). While PLWH need impairment in 2 or more neurocognitive domains to be diagnosed with HAND, people with HAD need to show marked impairment in daily life as opposed to people with ANI show no impairment in daily life. People affected by HAND classically present with memory impairment, executive dysfunction, lack of impulse control, emotional dysregulation, and motor impairments (Saylor et al., 2016; Antinori et al., 2007).

Viral entry into the brain and CNS can be detected early after infection. This was initially shown though synthesis of HIV antibodies within the blood-brain barrier (BBB) and isolation of the virus from cerebrospinal fluid (CSF) of asymptomatic HIV-patients (Gendelman et al., 1989). While still under debate, the predominant theory describing HIV entry into the brain is the "Trojan Horse" hypothesis in which dendritic cells (DCs) endocytose and harbor pathogens, including HIV, and *in vitro* evidence suggests DCs transmit virions to neighboring T-cells via exocytosis (Cavrois, Neidleman, & Greene, 2008). HIV is thought to enter the brain concealed within infected CD4+ monocytes and macrophages across the BBB (Bock, Zhai, Sharer, Mccomb, & Swindells, 2021; Jaeger & Nath, 2012). While neurons do not become infected with HIV, virus is detected in perivascular macrophages, parenchymal microglia, and astrocytes of the brain (Thompson, Cherry, Bell, & McLean, 2011). Infection of resident microglia and astrocytes leads to glial activation and cell specific effects to function and morphology.

Opiates, HIV, and the Striatum

The United States is in the midst of an opioid drug epidemic with over 1.6 million people having opioid use disorder (OUD) and 70,000 deaths from opioid overdoses ("About the Epidemic | HHS.gov," n.d.). Morphine, isolated from opium in 1806, is one of the most potent and readily prescribed analgesic drugs used today but has strong abuse potential (Brownstein, 1993; Darcq & Kieffer, 2018). Discovering that opioids such as morphine bind to endogenous opioid receptors in the brain unlocked an era of research to understanding the analgesic and addictive properties of opioids. Opioids mimic the endogenous opioid system by binding the μ (MOR), κ (KOR), and δ (DOR) opioid

receptors. These G-protein coupled receptors (GPCRs) are distributed throughout the CNS and mediate analgesia, addiction, emotion, and stress responses (Benarroch, 2012). The most extensive studied of these receptors is the MOR, which is highly expressed in the midbrain. Activation of MOR classically inhibits GABAergic neurons of the ventral tegmental area (VTA) via $G_{i/o}$ signaling. This leads to disinhibition of downstream dopaminergic neurons and subsequent increased dopamine release within the nucleus accumbens (NAc). Hijacking of this mesolimbic dopamine circuit has long been implicated behind the development of addictive behaviors (Koob & Volkow, 2010; Lobo & Nestler, 2011; Yager, Garcia, Wunsch, & Ferguson, 2015).

There are extensive findings describing that prescription or illicit use of opiate drugs can worsen the HIV neuropathology mediated by viral proteins and opioids. Opiates accelerate the pathogenesis of HIV-1 infection and worsen neurological outcomes through direct interactions in the central nervous system (CNS) (Chang & Connaghan, 2012; Hauser, Fitting, Dever, Podhaizer, & Knapp, 2005; Reddy, Pilakka-Kanthikeel, Saxena, Saiyed, & Nair, 2012). While development of combination antiretroviral therapies (cART) has drastically improved the life expectancy of patients, neurocognitive disorders still affect 30-50% of HIV+ individuals (Heaton et al., 2010; Saylor et al., 2016). Morphine can modulate various levels of HIV infection, viral entry, inflammation, and alter levels of HIV-1 coreceptors CCR5 and CXCR4 (Guo et al., 2002; Rogers, 2020; Sengupta et al., 2009). Morphine can potentiate HIV-1 replication and viral entry in neonatal monocyte-derived macrophages (Li et al., 2003). Morphine and HIV-1 Tat can work alone and in a synergistic fashion to decrease blood-brain barrier (BBB) integrity, decrease trans-endothelial electric resistance, decreasing expression of tight junction proteins, and

increase *trans*-migration of PBMCs across an *in vitro* BBB (Mahajan et al., 2008; Williams et al., 2014)

Particular brain regions are more vulnerable to the effects of HIV infection. The striatum is a central interface for motor, reward, and addiction circuits, which can become disrupted by drugs of abuse and is profoundly affected by HIV (Du Plessis et al., 2014; Zhou et al., 2009). Synergistic neurotoxic effects of HIV-1 and opioids on neurons are primarily mediated by mu-opioid receptor (MOR) expressing glia (S. Kim et al., 2018; Zou et al., 2011).Infected macrophages, microglia, and affected astrocytes can release viral proteins such as trans-activator of transcription (Tat) and envelope glycoprotein gp120. Tat and gp120 are contributors to HIV-1 induced synaptodendritic damage and cell death. Loss of synaptic connectivity and dendritic arborization are likely substrates of cognitive impairment in HAND (E, Masliah, N, Ge, M, Morey, R, DeTeresa, RD Terry, 1992; Eliezer Masliah et al., 1997b), as well as opiate-HIV interactive deficits in CNS structure and function (Anthony, Arango, Stephens, Simmonds, & Bell, 2008; E, Masliah, N, Ge, M, Morey, R, DeTeresa, RD Terry, 1992; Fitting et al., 2010; Sá et al., 2004).

Neuronal dysfunction in HAND

Behavioral and cognitive deficits in HAND are likely due to reduced dendritic arborization and synaptic degeneration (Eliezer Masliah et al., 1997a). While HIV does not directly infect neurons, HIV-viral proteins released by glial cells such as Tat and gp120 have direct effects on neurons. Striatal neurons exposed to Tat *in vitro* and *in* vivo show reduced dendritic length, reduced number of dendritic spines, total dendrite number, as well as increased dendritic swelling and varicosities (Fitting et al., 2014, 2010; Irollo,

Luchetta, Ho, Nash, & Meucci, 2021; Y. Liu et al., 2018). This is likely due to Tat-induced direct and indirect excitotoxic mechanisms. HIV-1 Tat has an affinity for the *N*-methyl-D-aspartate receptor (NMDAR) and has been shown increase NMDAR activation, expression, and glutamate release (Eugenin et al., 2011; Haughey, Nath, Mattson, Slevin, & Geiger, 2001; Hu, 2016). Tat-induced activation of NMDARs leads to prolonged and elevated levels of intracellular Ca^{2+} in striatal neurons in both the cytoplasm and dendrites. In addition to NMDAR-mediated Ca^{2+} influx, downstream signaling through ryanodine receptors causes release of intracellular Ca^{2+} from endoplasmic reticulum (ER) internal stores. Internal stores alone have been shown to be a large supply of excessive intracellular calcium in neurons *in vitro* where calcium levels and elevation of striatal neurons in calcium-free mediate in the presence of Tat are no different than with calcium-containing medium (Fitting et al., 2014). Tat-mediated excessive calcium influx and internal release both contribute to excitotoxic damage behind neuronal cell death and dendritic damage.

While overactivation of excitatory ions and signaling have been shown as a strong contributor to neuronal dysfunction in HAND, recent evidence is elucidating the importance of inhibitory signaling. Autopsy studies from HIV-infected individuals display highly significant alterations in *GAD1* and *GABRA1* (Buzhdygan et al., 2016; Gelman, Chen, et al., 2012) involved in GABAergic neurotransmission that rival alterations in genes involved in glutamate transmission (Gelman, Lisinicchia, et al., 2012). Tat-expressing transgenic mouse show a selective loss of reductions in somatostatin-immunopositive (SOM+)/neuropeptide Y-immuno-negative (NPY−), parvalbumin-immunopositive (PV+), and neuronal nitric oxide synthase-immunopositive (nNOS+)

GABAergic interneurons in hippocampal area CA1 (Marks et al., 2016) and a loss of inhibitory postsynaptic currents in *ex vivo* slices (Xu & Fitting, 2016). Interestingly, the loss of GABAergic markers does not appear to be accompanied by the loss of GABAergic neurons, suggesting alterations to GABAergic function at the molecular level (Buzhdygan et al., 2016; E. Masliah, Achim, Hansen, & Wiley, 1992). Thus, selective deficits in GABAergic function and neuronal markers suggests the preferential loss of inhibitory function may underlie the apparent increases in excitotoxicity in HIV-related neuronal dysfunction.

Neuronal chloride regulation and GABAergic function

Vulnerability of inhibitory signaling in HIV has been shown through reduction in the expression of GABAergic markers and frequency and amplitude of IPSCs. Additionally, studies investigating other neurodegenerative diseases are also finding disruptions to GABAergic machinery, showing the delicate nature of inhibitory signaling regulation (Akbarian et al., 1995; Lanoue, Dumitriu, Myers, & Soghomonian, 2010; Volk, Austin, Pierri, Sampson, & Lewis, 2000). GABAergic signaling is highly dependent upon intracellular ($[Cl^-]_i$) homeostasis to allow proper GABAergic inhibition. Minor changes in $[Cl^-]_i$ can dramatically affect the strength and polarity of inhibitory $GABA_A$ receptor function (De Koninck, 2007). Slight increases in [Cl⁻]i (as little as 5 mV) can shift the Cl^- equilibrium potential (E_{Cl}) (Prescott, Sejnowski, & De Koninck, 2006), and reverse the polarity of $GABA_A$ currents—such that an inhibitory transmitter signal can be excitatory (Chamma, Chevy, Poncer, & Lévi, 2012; De Koninck, 2007). Unlike glutamate in excitatory neurotransmission, GABA function changes over the course of development

as well as shifts in the $[Cl^-]_i$ gradient (Ben-Ari, Khalilov, Kahle, & Cherubini, 2012). To understand the role of Cl^- regulation in HIV-mediated disruptions to GABAergic transmission, we investigated into several candidate Cl^- regulating channels (KCC2, NKCC1, and TMEM16A) that will be discussed further.

NKCC1 and KCC2:

The hyperpolarizing nature of GABA relies on a gradient with low levels in intracellular Cl^- allowing for rapid Cl^- influx and hyperpolarization upon GABA receptor (GABAR) binding. The dichotomic nature of GABA signaling over the course of development is due to dynamic changes in the expression and function of neuronal neurotransmitter receptors, ion channels, and transporters (Ben-Ari et al., 2012; Chamma et al., 2012). The Cl^- gradient during development is primarily driven by the Na^+-K^+- $2Cl^-$ cotransporter NKCC1 and drives the inward transport of Cl^- to neurons leading to high levels of intracellular Cl^- (Mahadevan & Woodin, 2016). This allows for outward flow of Cl^- down its electrochemical gradient during GABAR binding, therefore having a depolarizing effect on neurons. While typically not associated with disease, recent evidence has pointed toward mutations and deletions in the NKCC1 encoding *SLC12A2* gene associated with several disease phenotypes (Koumangoye, Bastarache, & Delpire, 2020).

In contrast to immature neurons, mature neurons maintain high low levels of intracellular Cl^- which correlates with higher levels of K-Cl cotransporter isoform 2 (KCC2) (Payne, 1997). KCC2 is a neuron specific transmembrane cotransporter under the solute-carrier 12 (*SLC12*) family and is encoded by the *SLC12A5* gene (R. Liu, Wang, Liang,

Zhang, & Yang, 2020). $GABA_A$-mediated hyperpolarization is dependent on KCC2 function to allow for rapid Cl^- influx and synaptic inhibition. KCC2 is upregulated during development by brain-derived neurotrophic factor (BDNF) signaling via the tropomyosin receptor kinase-B (TrkB) leading to displacement of NKCC1 (Lee-Hotta, Uchiyama, & Kametaka, 2019). The developmental switch from NKCC1 to KCC2 ionic regulation is vital to postnatal survival as KCC2-deficient mice die immediately after birth due to severe motor defects and lack of respiration (CA Hübner, V Stein, I Hermans-Borgmeyer, T Meyer, K Ballanyi, 2001).

While hyperexcitability and neurotoxicity from excessive glutamate and NMDAR activation have long been at the forefront of many neuroinflammatory diseases, decreased expression of KCC2 is reported in several neurological disorders, the most prominent being epilepsy (Chen et al., 2017; Pisella et al., 2019; Rivera et al., 2002). Chen et al. showed that severity of epileptiform activity in cyclothiazide (CTZ)-induced seizure mice was correlated with changes to membrane levels of KCC2. The membrane stability and activity of KCC2 is highly regulated via phosphorylation of several tyrosine and serine residues along the C-terminal of membrane-bound KCC2 (Lee HH, R Jurd, 2010; Pisella et al., 2019; Silayeva L, TZ Deeb, RM Hines, MR Kelley, MB Munoz, HH Lee, NJ Brandon, J Dunlop, J Maguire, PA Davies, 2015). Of importance, Lee et al. (2015) showed that glutamate induces dephosphorylation of S940 via a Ca^{2+}-dependent mechanism through NMDARs and pS940 levels are correlated with membrane levels of KCC2. While loss of pS940-KCC2 does not affect its basal activity, there is a chloride extrusion deficit following glutamate exposure (Silayeva L, TZ Deeb, RM Hines, MR Kelley, MB Munoz, HH Lee, NJ Brandon, J Dunlop, J Maguire, PA Davies, 2015). These

studies show the importance of KCC2 regulation in neuroinflammatory diseases, especially during scenarios involving excitotoxicity.

As the importance of post-translational modification and membrane stability of KCC2 in CNS disorders became more documented, Gagnon et al. (Gagnon, M, MJ Bergeron, G Lavertu, A Castonguay, S Tripathy, RP Bonin, J Perez-Sanchez, D Boudreau, B Wang, L Dumas, I Valade, K Bachand, M Jacob-Wagner, C Tardif, I Kianicka, P Isenring, G Attardo, JA Coull, 2013) developed the compound CLP257 to combat loss of KCC2 activity. This group used a high throughput drug screen to determine the best compounds the enhance Cl^- extrusion in cells expressing low levels of KCC2, mimicking pathological conditions. Through this they discovered CLP257 which was able to restore Cl^- efflux in mature spinal cord neurons as well as increase cell surface expression of KCC2. To increase efficacy and pharmacokinetics of CLP257, Gagnon et al. (2017) designed the CLP257 prodrug CLP290 which improved the overall pharmacokinetic profile compared to CLP257.

TMEM16a:

While KCC2 is the main transporter for maintaining Cl^- equilibrium potential, ionic regulation a highly regulated process performed by many transmembrane proteins and intracellular signals. Following NMDAR- and Ca^{2+}-mediated KCC2 downregulation, it is possible other channels or transporters compensate for the lack of KCC2 Cl^- extrusion. Following Tat ± morphine exposure, neurons overload with Ca^{2+} via both NMDAR activation and release from internal stores. Ca^{2+} is a strong downstream signaling molecule that regulates many cellular processes. To investigate other possible KCC2-

related mechanisms, we sought to investigate other Cl^- regulating channels involving Ca^{2+}.

TMEM16a or Anoctamin-1 (ANO1) is a Ca^{2+}-activated Cl^- channel (CaCC) involved in cellular processes such as cell survival, proliferation, and neuronal excitation (Dulin, 2020). CaCCs have primarily been identified in small and large sensory neurons that are sensitive to elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in both the CNS and peripheral nervous system (PNS) (Ji et al., 2019), although recent evidence has shown TMEM16a is expressed in cholinergic neurons of the medial habenula via *in situ* hybridization (Cho et al., 2020). TMEM16a is classically studied in the context of cancer and lung function. TMEM16a is overexpressed in many tumors which can lead to overactivation of several cancer cell survival and signaling pathways such as extracellular signal-regulating kinase (Erk1/2), nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), and epidermal growth factor receptor (EGFR) (H. Wang et al., 2017). Additionally, TMEM16a has recently been reported to improve airway function in cystic fibrosis as an alternative mechanism for Cl^- excretion. A tissue specific knockout of TMEM16a in ciliated airway epithelial cells (by crossing of TMEM16A$^{flox/flox}$ mice with *FoxJ1-Cre* mice) leads to mucus accumulation during inflammatory lung disease by disrupting Cl^- conductance and subsequent fluid and mucus secretion (Benedetto, Cabrita, Schreiber, & Kunzelmann, 2019; Benedetto et al., 2017). While there have been no reports of TMEM16a being directly involved in HIV neuropathogenesis, alterations in TMEM16a are known to contribute to HIV-induced diarrhea, which is most likely results from excess Cl^- and water retention in the colon (Chaudhury, 2015). Crofelemer, an FDA approved, antiretroviral drug blocks TMEM16a mediated Cl^- and fluid secretion which

would lead to HIV-induced diarrhea (Crutchley, Miller, & Garey, 2010). These findings suggest overactivation or expression of TMEM16a at least in secretory cells of the intestines during HIV and potentially other regions highly affected by HIV such as the brain.

Chapter 2: *In vivo* morphine administration leads to anxiety-like behavior and decreased exploration

Abstract

While cART has improved the lifespan and quality of life of PLWH, there is still approximately 50% of patients who present with HAND which is reflective of the pre-cART era. This is likely due, in part, to the expression, harboring, and transfer of the HIV-1 viral protein Tat even in people on cART. Tat triggers the release of proinflammatory cytokines and a neurotoxic environment leading to sublethal neuronal damage and GABAergic dysfunction, which can be exacerbated with opioid use. In these studies, we used the GFAP-driven DOX-inducible Tat-transgenic mouse model in combination with 2-weeks of escalating morphine administration ± the KCC2 enhancing prodrug CLP290 to understand the role of KCC2 function in Tat- and morphine-induced neurodegeneration in female mice. Here, we demonstrate that 1 hour after the final morphine administration, morphine by itself increased anxiety-like behavior and decreased exploration regardless of genotype or CLP290 administration, while Tat had minimal effect on these outcomes. Enhancement of KCC2 with CLP290 has no effect on morphine-induced behavioral changes. These data provide evidence that 1 hour after morphine administration morphine is anxiogenic, as well as a sex-dependent divergence in behavioral responses to Tat expression when compared to previous studies.

Introduction

Behavioral tests are a strong method to examine overarching phenotypes of CNS and other disease models that allow for more targeted investigations at the molecular level. To understand *in vivo* contributions of intrinsically neurotoxic HIV-1 proteins,

transgenic models for both Tat and gp120 have been generated. We use a Tat-transgenic model as Tat levels still persist in the CNS and continue to be transcribed in infected macrophages even in patients with successful cART treatment, making it a good model to study many features of HAND (Carvallo et al., 2017). The doxycycline (DOX)-inducible driven *tet*-on HIV-1_{IIIB} Tat_{1-86} transgenic mouse model has been extensively used to study the effects of HIV-1 Tat expression alone and in combination with opioids (Barbour, Nass, Hahn, Hauser, & Knapp, 2021; Bruce-Keller et al., 2008; Fitting et al., 2012; Hauser et al., 2009). *In vivo* exposure to Tat causes increased anxiety (Yun K Hahn et al., 2016; Schier et al., 2017b), locomotor changes (Barbour et al., 2021; Fitting et al., 2012), and learning and memory deficits (Carey, Sypek, Singh, Kaufman, & McLaughlin, 2012; Marks et al., 2021, 2016).

The striatum is the main output center of the basal ganglia and is involved in functions such as reward, anxiety, locomotion, and addiction and is particularly vulnerable to HIV. Within the striatum, the cellular composition consists primarily of GABAergic medium spiny neurons (MSNs) which make up 95% of all neurons. MSNs can be divided into two classes, the dopamine-1 receptor (D1R)-expressing and the dopamine-2 receptor (D2R)-expressing neurons. Exposure to HIV-1 Tat ± morphine leads to decreased dendritic spine density, spine plasticity, GABAergic function, and importantly loss of KCC2 on MSNs and within the striatum (Barbour, Hauser, McQuiston, & Knapp, 2020; Hauser et al., 2005; Schier et al., 2017a). Interestingly, in addition to overall lower KCC2 protein levels within the striatum, Barbour et al. discovered selective loss of membrane localized KCC2 on D2R-MSNs specifically. This region-specific loss of KCC2 was found in Tat-transgenic mice that displayed hyperactivity in an open field test(Barbour

et al., 2021). Interestingly, administration of KCC2-enhancing pro-drug CLP290 rescued the hyperactive phenotype.

To further understand the role of KCC2 function in HIV-1 Tat striatal neurodegeneration and behavioral phenotypes, we sought to understand (i) how HIV-1 Tat exposure effects female mice, (ii) whether there is any independent or interactive effect of morphine, and (iii) how CLP290 may influence any of these outcomes. We found that a 2-week ramping dose of morphine lead to increased anxiety-like behavior and decreased exploration in both mice lacking (Tat-) and expressing (Tat+) the *tat*-transgene. Interestingly, we did not see any effects of Tat or CLP290 in any behavioral outcomes.

Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were in accordance with ethical guidelines defined by the National Institutes of Health. For all studies, 4–6-month-old female doxycycline (DOX)-inducible HIV-1 Tat transgenic mice were used. Mice expressing the rtTA ± the *tat* transgene were fed a standard chow supplemented with DOX (6 mg/g, Harlan Indianapolis, IN) for two weeks prior to experimentation. Mice were housed 4-5 per cage with *ad libitum* access to food and water in a temperature- and humidity-controlled facility on a 12:12h light-dark cycle.

Drug administration

A subset of mice was administered a ramping dose (10, 20, 30, and 40 mg/kg) of morphine sulfate (s.c.) twice per day (b.i.d.) with the dose increasing 10 mg/kg at 2-day intervals until a 40 mg/kg, b.i.d., dose is reached on day 7. A 40 mg/kg morphine b.i.d. dose is given thereafter (days 7-14). As a control, another group of mice received saline injections (s.c.) twice per day in equivalent volumes. Additionally, select mice received 30 mg/kg (200 µl/mouse) of KCC2 enhancing pro-drug CLP290 via oral gavage once per day prior to morphine or saline injection. Control mice for CLP290 were administered the drug vehicle solution (20% hydroxypropyl-beta-cyclodextrin (HPCD) in dimethyl sulfoxide (DMSO)) via oral gavage (200 µl /mouse).

Behavioral Assays

Prior to behavioral assays, all mice were acclimated to the testing room overnight prior to testing day. On the test day, treatment timing was staggered so each mouse was tested at the same time interval following treatment.

Open Field: Spontaneous locomotor activity was assessed using an open field chamber 1 hour-post morphine (or saline) injections at 9 am. Mice were placed in the center of a square Plexiglas box (40 × 40 × 35 cm; ANY-maze, Stoelting Co., Wood Dale, IL) and allowed to explore freely for 20 minutes. ANY-maze software was used for three-point video tracking of all mice while in the open-field. The area within the open field was arbitrarily divided into a 3 × 3 grid consisting of 9 ‘zones’. Computer-aided video tracking using ANY-maze software was used to determine the number of zone entries and time spent within each zone during each 20-minute trial. Average speed, distance traveled, time spent mobile, time in center zone, number of center zone crossings, and number of

rears were tracked and used as indices of motor and exploratory, and anxiety-like behaviors.

Rotarod: Evoked locomotion was assessed using a single trial of a rotarod test. Mice were placed on a 3 cm diameter immobile rod suspended at a height of 44.5 cm (Columbus Instruments) facing the back of the rotarod chamber (Rotamex-5, Columbus Instruments, Columbus, OH). The rotarod apparatus consists of a rotating spindle (3 cm diameter) separated by dividers into four sectors (9.5 cm wide) allowing for the simultaneous testing of four mice. Mice were tested on a single accelerating speed trial (starting at 0 rpm, acceleration step of 1 rpm, 7.5 s intervals) with no performance ceiling. Evoked locomotor behavior was recorded using Rotamex-5 software and assessed by measuring latency to fall.

Statistics

For all experiments, a 3-way analysis of variance (ANOVA) was used to examine main effects and interactions between Tat ± morphine and ± CLP290 treatment groups. To look at multiple comparisons, a post-hoc analysis was performed using a Fishers-LSD test. Data are shown as the mean ± the standard error of mean (SEM) and the findings are considered significant if $p < 0.05$. All data was analyzed using Prism 9 (GraphPad) statistical software.

Results

Morphine-treated mice show an increase in anxiety like behavior

Exposure to a ramping dose of morphine increased behavior that is indicative of anxiety in mice and reduced exploratory behavior in an open field test. Both Tat(−) and Tat(+) (*n* = *8*) morphine-treated mice had fewer center entries, time spent in center of the box, and less rears compared to saline treated mice over the 20-minute test ($p < 0.001$) **(Fig. 1)**. These effects are not due to motor impairment or lethargy as mice in all groups did not differ in their distance traveled or performance on a rotarod test. Mice also did not differ in mean speed or time spent mobile during the open-field test **(Table 1)**.

Discussion

The studies described in this chapter outline the effects of 2-week Tat ± morphine exposure and how enhancement of KCC2 function via CLP290 administration influenced behavioral outcomes in female mice. We show that a 2-week ramping dose of morphine leads to anxiety-like behavior through decreased center entries, time spent in center, and decreased exploratory behavior through decreased rearing. These data suggest that morphine is disrupting specific CNS circuitry but not locomotor activity as all mice performed similarly in an evoked locomotor task and time mobile, distance traveled, and speed in the open-field test.

Depending on the duration, method of exposure, and dosage, HIV-1 Tat can have differential effects. For example, the DOX-inducible Tat-transgenic model used in these studies displays a milder pathology compared to other Tat expression models (B. O. Kim et al., 2003; W Zou, BO Kim, BY Zhou, Y Liu, A Messing, 2007). We chose our model on the basis that this system has a more prolonged onset that is more representative of that seen in HIV patients. As we are looking at how Tat ± morphine mediated effects on KCC2, we wanted to study a timeframe in which electrophysiological, synaptodendritic

manifestations, and mild behavioral abnormalities appear prior to overt cell death and extreme behavioral effects. Previous work at the same 2-week exposure duration demonstrated that Tat-TG mice display decreased dendritic spine complexity and density, increased dendritic damage, anxiety-like behavior, and hyperlocomotion along with loss of KCC2 (Barbour et al., 2020, 2021; Schier et al., 2017b). While we saw an anxiety-like behavior phenotype, it was solely due to morphine administration with no interactions with Tat-expression or KCC2 modulation. Further testing of other anxiety-like behavior using a light-dark box, Although duration of Tat exposure in the present experiment was identical to our previous studies, one difference was the use of female mice. In most models of HIV-1 Tat exposure *in vivo*, experiments are conducted using only male mice. Interestingly, Hahn et al. showed that there are sex differences in the phenotypes observed using the same HIV-1 Tat-transgenic mouse model following 3-months of Tat induction as these studies presented with males being more vulnerable than females (Yun Kyung Hahn et al., 2015). Compared to the studies performed by Hahn et al. our paradigm had the added stressor of daily s.c. injections. Morphological and physiological differences in microglia between sexes could be another way in which Tat exposure could lead to divergent responses. Under basal conditions, male microglia show higher expression of the antigen presenting molecules major histocompatibility complex II, motility regulator P2Y12, as well as larger K^+ current responses to ATP-stimulation, suggestive of heighten reactivity compared to female microglia (Guneykaya et al., 2018; Yanguas-Casás, 2020). Additionally, in a comparative study using a NFκB-*luc2* mouse model to investigate inflammatory status of cells *in vivo*, RNA sequencing analysis of isolated microglia showed 79% of 95 inflammatory genes were upregulated in males

compared to females (Villa et al., 2018). These studies could explain a lack of a Tat response due to weakened immune and inflammatory response to Tat expression compared to male mice. Further, side-by-side investigations into male versus female responses to Tat at the behavioral level as well as molecular studies looking into sex differences in glial inflammatory responses are needed. Lastly, careful examination of the video recordings in morphine-treated mice revealed considerable fine stereotypic motor movements (e.g., pivoting back-and-forth on hindpaws that are stationary) that the ANY-maze software often interpreted as inactivity. Future studies should consider examining additional fine motor behaviors, such as wet-dog shakes or paw flutters, which can accompany opioid withdrawal, when assessing the interactive effects of opioids and HIV in mice.

In addition to our use of female mice, our studies differed from similar studies by Barbour et al. these mice received s.c. injections twice a day for the duration of the study. Studies have shown that repeated injections lead to a generalized vital stress response in mice with an increase in heart rate and body temperature lasting around 30 minutes (Meijer, Spruijt, Van Zutphen, & Baumans, 2006) and Paris et al. have found increases in corticosteroids in male mice injected with morphine or saline regardless of whether they express Tat (Paris et al., 2020 Neurobiology of Stress). This body response is likely related to increased levels of serum proinflammatory cytokines and increased corticosterone following saline injection (Du Preez et al., 2020). Although our behavioral tests were done 1 hour following saline or morphine injection, it is still possible a stress response from the injection could have induced behavioral changes in the open field test when combined with HIV-1 Tat exposure that differ from non-injected Tat-transgenic

mice. Addtionally, males and females can have differing responses to stress and related disorders. For example, corticotropin-releasing factor (CRF) levels are higher in females compared to males such as the paraventricular nucleus and amygdala (Iwasaki-Sekino, Mano-Otagiri, Ohata, Yamauchi, & Shibasaki, 2009). Excessive release of CRF is implicated in depression, stress, and anxiety disorders which are more common in females compared to males (Wiersielis et al., 2016). This suggests that females could be more sensitive to the stressors in our studies and another reason why the anxiety-like behaviors of less center entries and time spent in center may not be seen in previous studies with males (Barbour et al., 2021). In future studies, evaluation of the estrous cycle in female mice should be considered as proestrus females have higher neuronal activation within the infralimbic region as well as a positive correlation with grooming behavior that are not seen in diestrus females or males (Wiersielis et al., 2016). While we performed behavioral tests and treatment at the same time every day, differences in estrous cycle between mice could be masking or biasing results seen.

We wanted to test the direct response to morphine administration on behavioral outcomes, but most studies involving anxiety and opioids involves looking at the withdrawal response. While the withdrawal response to opioids, such as morphine, results in anxiolytic behavior, the direct response following administration has been shown to be anxiogenic in rodents (Chaoliang, Gu, Li Peng, Hu Bi, Ouyang Xinping, Fu Juan, Gao Jun, Song Zeng, Han Li, Ma Yuanye, Tian Shaowen, 2008; Kudryavtseva, Gerrits, Avgustinovich, Tenditnik, & Van Ree, 2004). Compared to the κ- (kappa) and δ-(delta) opioid receptors, morphine has the strongest affinity for the mu opioid receptor. While any morphine effects is most likely through the μ-receptor, both the μ-opioid and κ-opioid

receptor-specific agonists can produce anxiogenic behaviors in rodents (Kudryavtseva et al., 2004; Y. J. Wang et al., 2016). The results of opioid receptor activation are dependent on the route of administration, dosage, and timing. For example, Intraperitoneal (i.p.) administration of the kappa-opioid receptor agonist U50,488H produces an anxiolytic effect at low doses but an anxiogenic effect at higher doses which correlate with respective changes to ERK1/2 phosphorylation (Y. J. Wang et al., 2016). Additionally, we pursued the acute response to morphine due to its interactions with KCC2 regulation. Downregulation of KCC2 is a key component to the development of morphine-induced hyperalgesia (Tang, D, AH Qian, DD Song & WY, Yao, J Sun, WG Li, TL Xu, 2015). Weakening of GABAergic inhibition of spinal dorsal horn neurons from KCC2 downregulation as well as morphine-induced hyperalgesia are restored with concurrent CLP290 administration (F Ferrini, T Trang, TA Mattioli, S Laffray, T Del'Guidice, LE Lorenzo, A Castonguay, N Doyon, W Zhang, AG Godin, D Mohr, S Beggs, K Vandal, JM Beaulieu, CM Cahill, MW Salter, 2013; Ferrini, Lorenzo, Godin, Quang, & De Koninck, 2017). We performed our open field test 60 minutes following morphine injections and we could have potentially missed the window for morphine-induced locomotion. Several studies have seen that immediately post-morphine administration and then approximately 2-hours post-administration there are increases in locomotion (Murphy, Lam, & Maidment, 2001; Vanderschuren, Schoffelmeer, Mulder, & De Vries, 1999). Additionally, the half-life of morphine in plasma is under 60 minutes in mice therefore reduced levels of morphine at our experimental timepoint could result in lack of locomotion (Zelcer et al., 2005). Our results highlight and partially recapitulate the effect of morphine on anxiogenic behavior. To understand the role of sex differences in Tat-induced behavioral

phenotypes, direct side-by-side comparisons of males and females need to be explored in further studies.

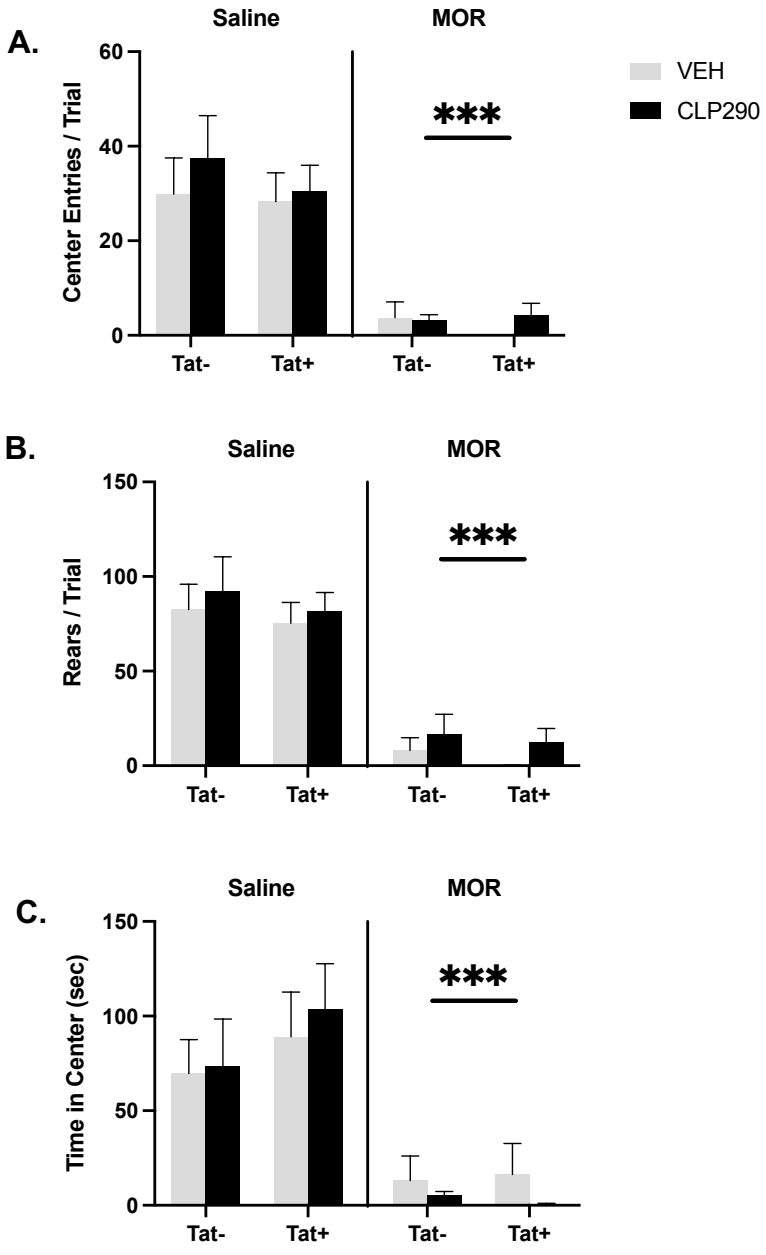

Fig 1. Morphine-treated mice showed anxiety-like behavior and decreased exploratory behavior. Morphine-treated mice showed anxiety-like behavior with less center entries compared to the saline group (A) as well as fewer entries into the center zone of the open-field (B) (***, $p < 0.001$, $n=8$). Additionally, morphine-treated mice showed less exploratory behavior measured by number of rears during the trial (C) (***, $p < 0.001$, $n = 8$).

Table 1. Effects of morphine and Tat and CLP290 on open-field behavior.

Behavioral Measure	Main Effect	Saline Mean ± SEM (CLP290)	Morphine Mean ± SEM (CLP290)	Significance (*p-value*)
Center Entries	Morphine	29.875 ± 7.645 (37.429 ± 0.055)	3.714 ± 3.386 (3.125 ± 4.250)	*** ; $p < 0.0001$
Time in Center	Morphine	69.625 ± 17.945 (73.286 ± 25.112)	13.114 ± 12.915 (5.013 ± 2.304)	*** ; $p < 0.0001$
Number of Rears	Morphine	82.500 ± 13.399 (92.286 ± 17.992)	8.000 ± 6.849 (16.625 ± 10.502)	*** ; $p < 0.0001$
Distance Traveled	None	29.542 ± 3.258 (30.594 ± 3.822)	24.310 ± 9.791 (43.236 ± 16.552)	*ns*
Time Mobile	None	591.038 ± 53.232 (597.129 ± 59.231)	546.686 ± 82.155 (626.875 ± 113.136)	*ns*
Mean Speed	None	0.025 ± 0.003 (0.026 ± 0.004)	0.020 ± 0.008 (0.036 ± 0.014)	*ns*
Rotarod	None	62.925 ± 9.485 (67.286 ± 11.744)	68.114 ± 11.474 (79.863 ± 7.890)	*ns*

Statistical outcome for all behavioral measures recorded. Main effects of the 3-way ANOVA are shown alongside mean values for each behavioral outcome ± the standard error of the mean (SEM) for saline and morphine treated mice in both the vehicle and CLP290 groups.

Chapter 3: CLP290 administration increases expression levels of KCC2 but decreases Cl^- channel TMEM16a in the striatum

Abstract

The striatum is highly affected by HIV and susceptible to neuronal damage by viral proteins such as Tat. HIV-1 proteins, inflammatory mediators, and excitotoxic molecules released and recruited by infected cells lead to sublethal neuronal damage, providing the neural basis of HAND. While it has been demonstrated that the HIV viral protein, Tat, is synergistically excitotoxic with morphine through mechanisms involving NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation (Fitting et la., 2014), little is known about how HIV-1 Tat interacts with opioids on GABAergic systems. Recent work has shown that following exposure to HIV-1 Tat downregulates KCC2 *in vitro* and *in vivo,* reduced KCC2 phosphorylation at S940 *in vivo*, and disrupts $GABA_AR$ mediated hyperpolarization *in vitro* (Barbour et al., 2020, 2021). We used DOX-inducible, GFAP driven Tat-transgenic mice and used western blotting analysis to assess protein level changes in the striatum. We found that CLP290 increased levels of KCC2 but did not increase phosphorylation at S940, but no treatment had any effects on NKCC1. Lastly, we found that Tat and CLP290 decreased levels of the chloride channel TMEM16a. These results show that in female mice, Tat and morphine have minimal effects on KCC2 protein levels and phosphorylation. Additionally, our data suggests TMEM16a is another way in which Tat can dysregulate GABAergic function which could be linked to KCC2 as CLP290 decreased TMEM16a immunoreactivity.

Introduction

Recent evidence in post-mortem tissue samples from HIV-infected individuals and in Tat transgenic mice *in vivo* suggests GABAergic neurons are selectively vulnerable to (Buzhdygan et al., 2016; Marks et al., 2016; Xu & Fitting, 2016). The cellular composition of the striatum is unique compared to other brain regions as a vast majority of the principal, projection neuronal populations, and some interneuronal subpopulations, are GABAergic. Within the striatum, about 90-95% of neurons in adult rodents are GABAergic MSNs and 4-5% being various types of GABAergic interneurons (Gagnon et al., 2017; Gerfen & Surmeier, 2011). GABAergic neurons rely on a strong inward driving force for Cl^- through maintenance of low levels of intracellular Cl^-. This ionic gradient allows for the rapid hyperpolarization and proper inhibition of neurons upon $GABA_A$ receptor ($GABA_AR$) binding. In mature neurons the Cl^- gradient is primarily controlled by KCC2. KCC2 is highly regulated at both the transcriptional and post-translational level (Kahle et al., 2013; Lee-Hotta et al., 2019). Previous work has shown that KCC2 is downregulated in the striatum of Tat-transgenic mice (Barbour et al., 2020). While administration of CLP290 does not increase total KCC2 levels, CLP290 reverses Tat-dependent decreases in KCC2 phosphorylation at S940—resulting in increased KCC2 phosphorylation, trafficking to the cell membrane, and activation. Moreover, The Tat-dependent reductions in KCC2 p-S940 and localization at the cell membrane KCC2 was only evident in D2R (but not D1R) medium spiny neurons (MSNs) (Barbour et al., 2021), although deficits in KCC2 may occur in D1R MSNs with more prolonged (> 1 month) Tat exposure since D1 MSNs display a more delayed onset, less severe pathology than D2R MSNs following 2 months of Tat induction (Lark et al., submitted for publication). Our work plays off these studies

and looks to advance this line of research by investigating morphine by itself and in combination with Tat .

Ionic homeostasis is controlled by a series of checks and balances through the actions of transmembrane channels and transporters. While channels allow for the flow of ions based on the driving force determined by their respective electrochemical gradients, transporters use ATP-driven energy to move ions against their electrochemical gradients (Dubyak, 2004). In Tat-transgenic mice, striatal neurons are overloaded with Ca^{2+}, which results in dendritic spine swelling and varicosities. While KCC2 is downregulated via a Ca^{2+}-dependent mechanism, it is logical that other channels or transporters attempt to compensate for deficits in Cl^- extrusion accompanied by shortfalls in KCC2 function. The CaCC, TMEM16a is activated by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) resulting in increased Cl^- conductance. TMEM16a dysfunction is implicated in some neoplasias including gastrointestinal, squamous cell carcinomas, breast, pancreatic, and prostate cancers (Le, Correspondence, Yang, & Yang, 2020). While there is not yet a link to HIV-mediated neurodegeneration, blockage of TMEM16a by ART drugs leads to HIV-induced diarrhea (Crutchley et al., 2010). TMEM16a is functionally active in cholinergic neurons of the medial habenula and plays an important role in regulating anxiety-like behavior (Cho et al., 2020). Additionally, there is evidence that the related CaCC, TMEM16b is active in GABAergic neurons of the amygdala and that deleting the TMEM16b encoding gene *ano2* leads to impaired context-independent fear memory (Li Ke-Xin, He Mu, Ye Wenlei, Simms Jeffrey, Gill Michael, Xiang Xuaner, Jan Yuh Nung, 2019). This finding led to us investigate whether the expression of TMEM16a was affected by Tat ± morphine-dependent deficits in KCC2 and the extent to

which KCC2 enhancement with CLP290 might reverse putative alterations in TMEM16a. Although we found there was no influence of Tat and or morphine on KCC2 or pS940-KCC2 levels, Tat expression did lower levels of TMEM16a. Although CLP290 did not rescue KCC2 and TMEM16a levels of both KCC2 and TMEM16a tended to increase in mice administered CLP290.

Methods

Western Blotting

Whole striata (single hemisphere) from 4-6 month old female mice used in the behavioral assessments were harvested at 1pm each testing day. Striata were homogenized in RIPA buffer (Sigma) containing 100× HALT™ Protease and Phosphatase inhibitor cocktail (Thermo Fisher) to prevent protein degradation. Protein lysate supernatants were aliquoted and stored at -80º C until use. Protein concentration was measured using a BCA protein assay (Pierce, Rockford, IL). Samples of 40 µl lysates were loaded into 4-20% gradient Criterion Pre-Cast Gels (Bio-Rad Laboratories, Hercules, CA) and ran at 100V. Gels were transferred onto PVDF membranes (Bio-Rad) and probed with anti-KCC2 (1:2000; Protein Tech, Rosemont, IL), anti-pS940 KCC2 (1:1000, Abcam), anti-NKCC1 (1:1000; Protein Tech, Rosemont, IL), anti-TMEM16a (1:500; Bioss), and anti-GAPDH (1:2000, Abcam) primary antibodies. KCC2 and GAPDH were detected by measuring fluorescent signal from Alexa Fluor 488 and 647-conjugated secondary antibodies (1:1000). TMEM16a, NKCC1, and pS940-KCC2 were detected using a horseradish peroxidase (HRP) anti-rabbit secondary antibody and Super Signal West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher).

Results

CLP290 administration increased striatal KCC2 levels but had no effect on pS940-KCC2 levels

We have seen reduced striatal KCC2 expression in Tat transgenic male mice following 2 weeks of DOX-administration. To see if these findings translate to female mice, we performed western blot analysis of harvested striatal tissue. In female mice, overall mice administered showed higher levels of KCC2 regardless of genotype or morphine treatment (Fig. 2A). While KCC2 levels in Tat+ mice were not significantly different from control, Tat+ mice ± morphine displayed significantly higher KCC2 levels compared to non-CLP treated mice (Fig. 2A, D, E).

As CLP290 enhanced KCC2 activation via phosphorylation, we investigated levels of pS940-KCC2. Interestingly, while CLP increased overall KCC2 levels, CLP had no effect on pS940-KCC2 levels (Fig. 2B, D, E) and there were no effects of either morphine or Tat exposure. These data potentially suggest an alternative mechanism by which CLP is increasing KCC2 levels such as increased protein stability or lack of degradation. Additionally, to investigate a potential reversal of the developmental shift between NKCC1 and KCC2 expression, we probed for levels of NKCC1. We saw no significant changes in striatal NKCC1 levels due to Tat, morphine, or CLP290 treatments (Fig 3A-C).

HIV-1 Tat and CLP290 independently decreased TMEM16a immunoreactivity

To investigate potential connected or compensatory changes to related Cl^- regulators, we probed for TMEM16a from striatal tissue lysates. Western blot analysis showed a main effect of CLP290 decreasing TMEM16a immunoreactivity (Fig. 3; $p <$

0.05) with no interaction with genotype or morphine treatment. Post-hoc analysis revealed Tat expressing mice have decreased TMEM16a levels compared to saline-vehicle controls regardless of CLP290 administration (Fisher's LSD, $p < 0.05$). These data suggest that Tat exposure either directly or indirectly decreases TMEM16a levels but this appears to be unrelated to KCC2 stability.

Discussion

The studies in this chapter describe the effects of Tat expression and morphine administration on Cl^- transporter and channel levels in striatal tissue lysates and the role of KCC2 via enhancement with CLP290. We show that CLP290 increases striatal KCC2 immunoreactivity but does not influence pS940 levels. Also, there were no changes to the ratio of pS940-KCC2:KCC2 or NKCC1 levels. These data suggest that TMEM16a is an alternative target for HIV-1 Tat to disrupt Cl^- regulation and GABAergic function. Additionally, these data show a potential sex-difference in KCC2 regulation by Tat and CLP290 as our results differ from previous literature which uses male mice (Barbour et al., 2020, 2021). Tat induction has been shown to decrease striatal KCC2 levels through a decrease in phosphorylation at S940, with CLP290 administration rescuing pS940-KCC2 levels and KCC2 membrane stability (Barbour et al., 2020). While CLP290 did in fact increase KCC2 levels, we did not see a rescue as there was no Tat induced KCC2 loss nor did CLP290 increase pS940 levels. This suggests that in our cohort of female mice, CLP290 increased basal KCC2 protein expression through a mechanism other than stabilizing S940 phosphorylation. KCC2 is highly regulated at the post-transcriptional level and its membrane stability is regulated by phosphorylation at S940 via protein-

kinase C (PKC) (Lee et al., 2007). Conversely, dephosphorylation of KCC2 at S940 is mediated through protein phosphorylase 1 (PP1) through and NMDA dependent mechanism (Lee, Deeb, Walker, Davies, & Moss, 2011). There is no conclusive evidence to whether CLP290 acts directly at KCC2, the PKC, or PP1 levels. Our data shows that there is no increase in phosphorylation but KCC2 levels are increased with CLP290 administration. This suggests that in our studies, CLP290 is not enhancing phosphorylation of KCC2 but potentially blocking dephosphorylation through PP1 to increase stability. Whether PP1 activity or expression is influenced by CLP290 needs to be explored in further studies. Additionally, a cohort of both male and female mice should be used in future studies to explore sex differences in Tat and morphine induced KCC2 dysregulation as well as differential CLP290 effects.

Our studies are the first investigating TMEM16a in the brain in regard to HIV-1 Tat expression. While the cell type expression of TMEM16a in the brain is not fully understood, the Brain Seq database (https://www.brainrnaseq.org) from the Barres Laboratory shows that TMEM16a encoding gene *ano1* is detected at low levels in neurons, oligodendrocytes, and microglia with highest expression in astrocytes (Zhang et al., 2014). More specifically in the lateral habenula, the loss of TMEM16a expression in cholinergic neurons has been implicated in mediating anxiety-like behaviors in mice (Cho et al., 2020). To further understand TMEM16a function in the striatum, fluorescent *in situ* hybridization and immunohistochemistry studies should be performed to investigate TMEM16a expression at the gene and protein level in specific cell types. Performing these studies in Tat-transgenic mice would confirm our immunoblotting results, as well as determine if loss of TMEM16a immunoreactivity is specific to a particular cell type.

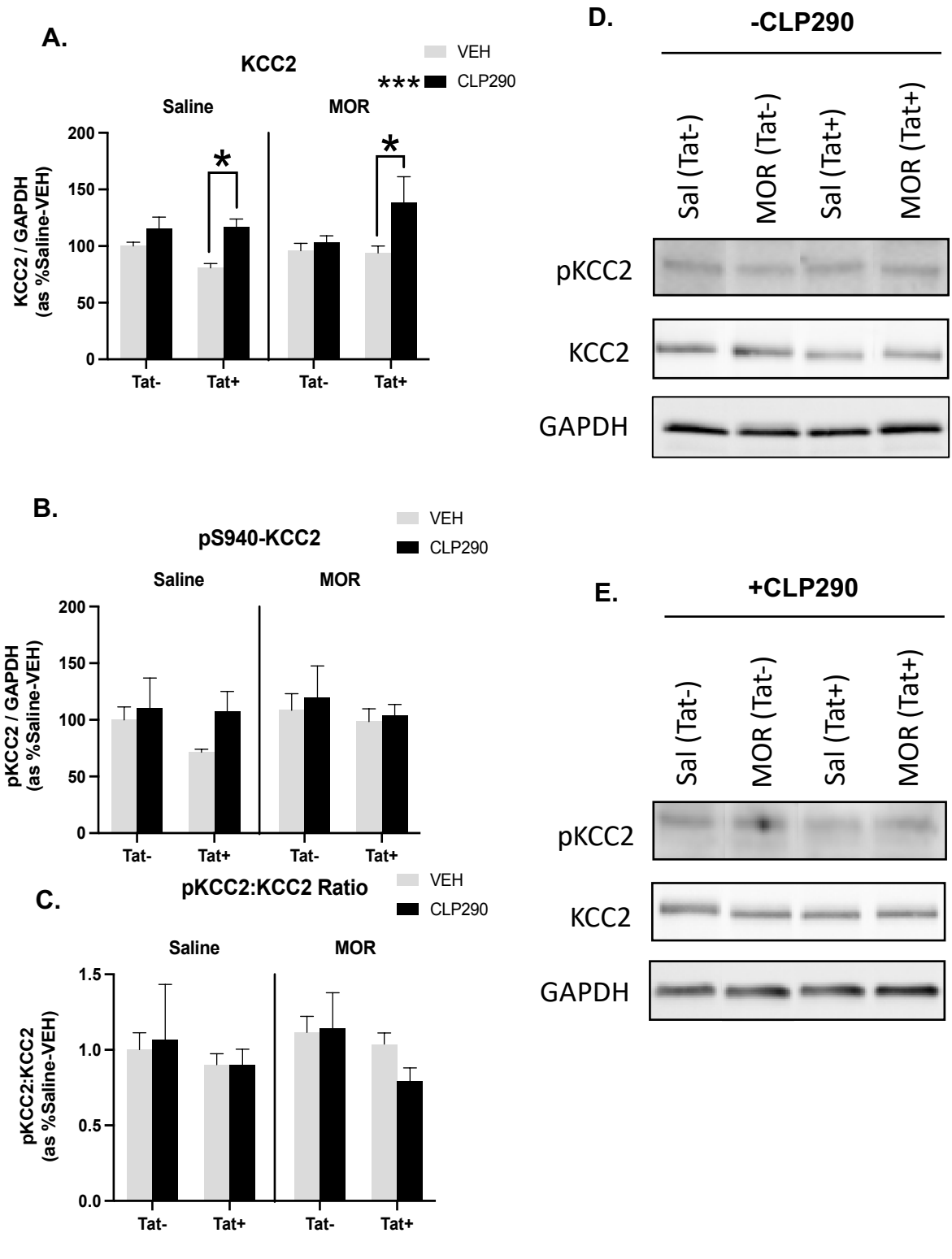
A.
KCC2
VEH
***
CLP290
Saline
MOR
KCC2 / GAPDH
(as %Saline-VEH)
200
150
100
50
0
*
*
Tat-
Tat+
Tat-
Tat+
B.
pS940-KCC2
VEH
CLP290
Saline
MOR
pKCC2 / GAPDH
(as %Saline-VEH)
200
150
100
50
0
Tat-
Tat+
Tat-
Tat+
C.
pKCC2:KCC2 Ratio
VEH
CLP290
Saline
MOR
pKCC2:KCC2
(as %Saline-VEH)
1.5
1.0
0.5
0.0
Tat-
Tat+
Tat-
Tat+
D.
-CLP290
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
pKCC2
KCC2
GAPDH
E.
+CLP290
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
pKCC2
KCC2
GAPDH

Fig 2. CLP increases striatal KCC2 immunoreactivity but has no effect on S940 phosphorylation. Representative western blot bands from striatal protein lysates from all treatment groups either administered drug vehicle (D) or CLP290 (E). Mice administered CLP290 show increased levels of KCC2 immunoreactivity (A) with no difference in levels of pS490-KCC2 (B), the ratio of pS940: unphosphorylated KCC2 (C), (*$p < 0.05$; ***$p < 0.001$; $n = 6$).

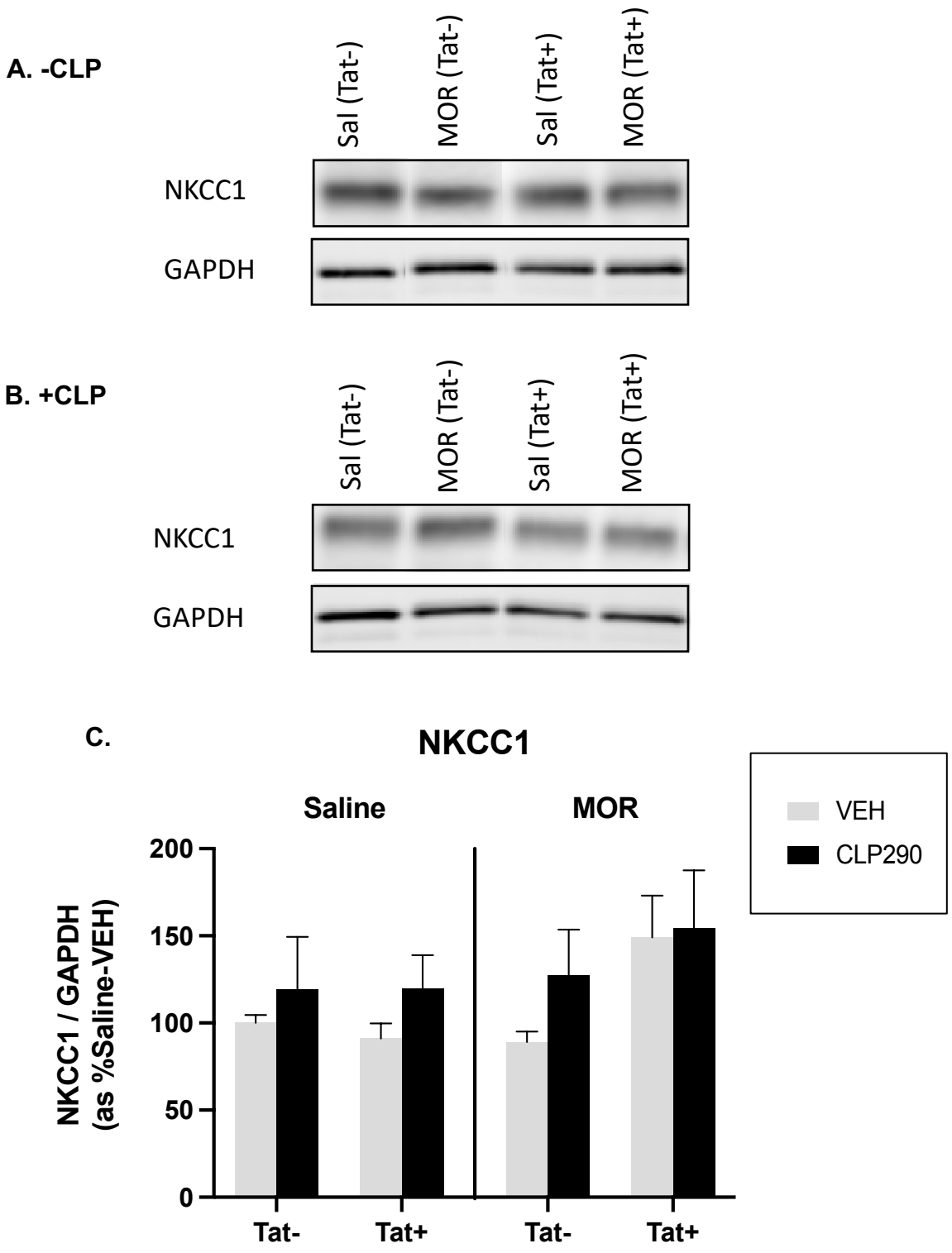
A. -CLP
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
NKCC1
GAPDH
B. +CLP
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
NKCC1
GAPDH
C.
NKCC1
Saline
MOR
VEH
CLP290
NKCC1 / GAPDH
(as %Saline-VEH)
200
150
100
50
0
Tat-
Tat+
Tat-
Tat+

Fig 3. NKCC1 protein levels are unaltered by Tat or morphine exposure nor by modulation of KCC2. Representative western blots with each band representing an individual animal. Bands from striatal protein lysates from all treatment groups either administered drug vehicle (A) or CLP290 (B).

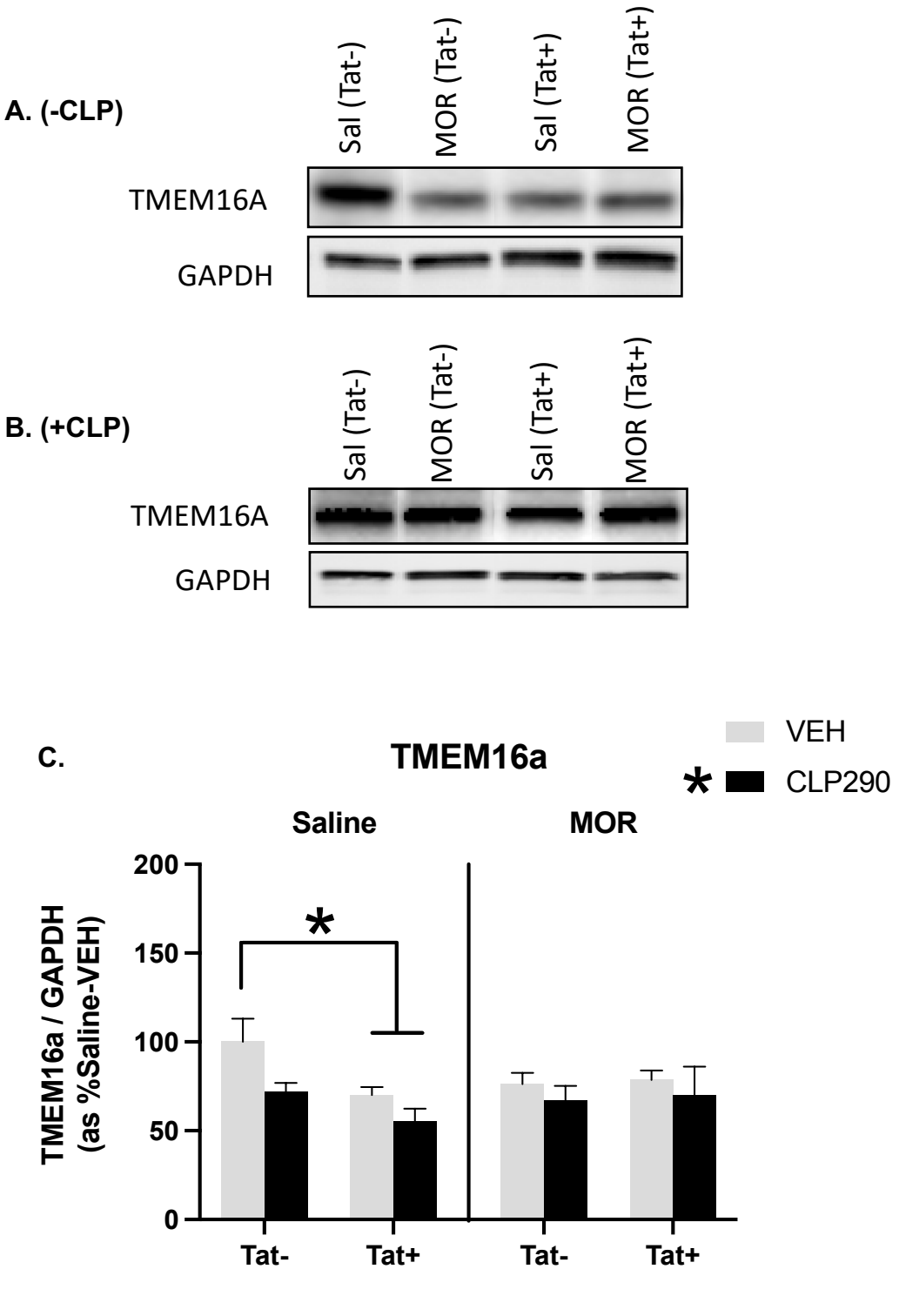
A. (-CLP)
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
TMEM16A
GAPDH
B. (+CLP)
Sal (Tat-)
MOR (Tat-)
Sal (Tat+)
MOR (Tat+)
TMEM16A
GAPDH
C.
TMEM16a
VEH
CLP290
Saline
MOR
TMEM16a / GAPDH
(as %Saline-VEH)
200
150
100
50
0
Tat-
Tat+
Tat-
Tat+

Fig 4. CLP290 reduces striatal TMEM16a levels. Representative western blots from striatal protein lysates from all treatment groups either administered drug vehicle (*n = 4-6*) (A) or CLP290 (B). Mice administered CLP290 show decreased TMEM16a immunoreactivity (B,C) compared to drug vehicle (* $p < 0.05$). Post-hoc analysis shows a significant decrease of TMEM16a in Tat+ mice ± CLP compared to Tat- vehicle controls (* $p < 0.05$) (C).

Chapter 4: Investigating other mechanisms of Cl^- dysregulation and inflammation

Abstract

The striatum is a central interface for addiction circuits that becomes dysregulated by opiates and harbors high viral loads making it susceptible to neuropathology in HIV infected individuals. While neurons do not become infected with HIV, glial cells, such as microglia and astrocytes, are preferentially targeted and are the source of many sublethal neuronal effects associated with HAND. Exposure to HIV or viral proteins leads to release of proinflammatory cytokines and chemokines from glial cells which is exacerbated by opioids such as morphine. We used the LEGENDplex™ bead-based immunoassay on the striatum of DOX-inducible, GFAP-driven Tat-transgenic mice to analyze cytokine and chemokine levels. We found that morphine alters levels of proinflammatory cytokines IL-23 and IFN-β and that CLP290 treated Tat+ mice show significantly higher levels of IFN-β compared to Tat+ mice administered drug vehicle. When total mean cytokine concentrations were averaged, we found that cytokine levels increase with age in Tat- mice with increasing age, while they decreased from 4-6 months in Tat+ mice. Additionally, chemokine levels were relatively unchanged, but morphine did alter concentrations of Eotaxin (CCL11) depending on Tat-transgene expression. Contrary to cytokines, we found that total mean chemokine levels decreased with age in both Tat- and Tat+ mice. Lastly, we mined existing Next-Gen RNA seq data and found no detectable gene expression changes following acute Tat and morphine exposure in genes encoding Cl^- channels, transporters, or GABA receptors.

Introduction

Even with effective cART, PLWH still display high levels on inflammation compared to non-infected individuals (Neuhaus et al., 2010). Sustained inflammation without ongoing viral replication is likely due to cellular reservoirs of HIV-1 in the brain. Microglia are the primary reservoir harboring latent HIV-1 and while a low percentage of astrocytes integrate HIV-1 DNA, they are capable of transferring virus and viral protein to other cells (Valdebenito, Castellano, Ajasin, & Eugenin, 2021; Wallet et al., 2019). HIV-1 and its viral proteins lead to activation of both microglia and astrocytes which results in expression and release of proinflammatory cytokines and chemokines. PLWH show an increased level of proinflammatory markers such as IFN-γ, TNFα, IL-6, IL-1α, IL-2, and IL-12, some of which can still be elevated following well beyond the initiation of cART treatment (Abassi et al., 2017; Nolting et al., 2012; Osuji, Onyenekwe, Ahaneku, & Ukibe, 2018).

Prescription and illicit use of opioids are highly comorbid with HIV-1 infection. Drugs of abuse, including opioids, have been shown to worsen HIV neuropathology (Bell, Brettle, Chiswick, & Simmonds, 1998; Steele, Henderson, & Rogers, 2003) and neurological outcomes (Byrd et al., 2011; Cernasev et al., 2020) in HAND in both the pre-cART and cART eras.

Methods

LEGENDplex Flow Cytometry Cytokine and Chemokine Panels

All reagents used were from LEGENDplex™ Mouse Inflammation Panel (BioLegend, San Diego, CA) and LEGENDplex™ Mouse Proinflammatory Chemokine Panel (BioLegend, San Diego, CA) kits. Using the same harvested tissue from the 4-6 month female mice for western blot analysis, the opposite hemisphere for each mouse

was homogenized in LEGENDplex Assay Buffer. Protein lysate supernatants were aliquoted and stored at −80 ºC until use. Reconstituted standard cocktail were prepared in a 1:4 serial dilution starting at 10,000 pg/mL. Diluted standards and samples were added to specified wells of a 96-well plate along with mixed beads and assay buffer. Filled plate was mixed at room temperature (RT) for 2 hours at 800 rpm. Plate was centrifuged at 350 g for 5 minutes until pellet formation, supernatant was decanted, and pellet was washed with 1X wash buffer (this step is repeated). Pellet was mixed with detection antibody and shaken at 800 rpm for 1 hour at RT. Streptavidin-PE was added to each well to enhance biotin antibody signal and plate was shaken at 800 rpm for 30 additional minutes. Plate was spun at 350 g for 5 minutes and pellets were washed as described above. Final pellets were resuspended in 150 µL of wash buffer and plate was stored at 4 ºC until use.

Plate was read on a Cytek Aurora flow cytometer with a yellow-green (YG) laser by the VCU Flow Cytometry Core. Beads were gated automatically by LEGENDplex software and manually checked and or readjusted as needed.

Next Gen RNAseq datamining

Existing RNAseq data from male mice acutely exposed to Tat and morphine was mined for differences in gene expression. ENSEMBLE IDs for each gene were translated into their respective gene names using https://www.biotools.fr/mouse/ensembl_symbol_converter and gene expression levels were presented as fragments per kilobase exon per million mapped fragments (FPKM).

FPKM values for *n* = 3 mice per treatment group were averaged and presented on a logarithmic scale using Prism 9 software.

Statistics

For all experiments, a 3-way analysis of variance (ANOVA) was used to examine main effects and interactions between Tat ± morphine and ± CLP290 treatment groups. Data is shown as the mean ± SEM and these data are considered significant if $p < 0.05$. Gene expression data was analyzed using Prism 9 statistical software and LEGENDplex data was analyzed using Prism 9 and JMP software. To look at multiple comparisons, post-hoc analyses were performed using a Fishers-LSD test.

Results

Morphine administration affects IL-23 and INF-β concentrations and total cytokine levels alter with age depending on Tat expression in the striatum

We first validated our data by confirming proper standard curve generation, bead gating, and analyte detection (**Fig 5A-D**). Animals treated with a 2-week ramping dose of morphine showed an increased concentration of proinflammatory cytokine IL-23 (3-way ANOVA, $p < 0.05$) and a decrease in IFN-β (3-way ANOVA, $p < 0.05$) (**Fig. 6**). Though IL-23 only showed a main effect of morphine, Tat+ mice showed a trending decrease in IL-23 levels compared to Tat− controls ($p = 0.062$). INF-β showed a significant increase in Tat+ mice when treated with CLP290 compared to Tat+ vehicle treated mice that brings IFN-β around Tat− control levels (**Fig. 6B**). The mean difference between saline and morphine groups for IFN-β is minimal (0.11), there is a trending interaction with genotype

suggesting that the morphine main effect is mainly driven by Tat genotype. While only individual 2 of 13 analytes showed, we wanted to investigate all cytokines combined and examine the role of aging. Over the course of a lifetime, physiological deterioration from tissue atrophy, metabolism, reduced cell counts, and tissue proteins are a result of aging (Park & Yeo, 2013). One of the most common phenomena seen in the aging process is chronic low levels of inflammation (Chung et al., 2019; Loeser, 2010). When we average individual analytes across treatment groups, generating one mean cytokine value per animal, and split the cohort by age, we see that Tat− mice show a significant increase in the mean concentration of cytokine markers from 4 to 5 months in age ($p< 0.05$), 4 to 6 months ($p < 0.05$), and 5 to 6 months ($p < 0.05$) (**Fig. 7A**). Although Tat+ mice start at a higher baseline value of mean cytokine concentration ($p < 0.01$) compared to Tat- mice, Tat+ mice show a decrease in mean cytokine concentration from 4 to 6 months in age ($p < 0.05$). Interestingly, these effect trends hold when we split the mice into their respective treatment groups and when cytokines are split into pro- (solid line) and anti-inflammatory (dotted line) markers (**Fig. 8**). These data suggest that Tat expression can alter age induced cytokine alterations in females and further studies into aging markers and phenotypes could bring more clarity to our findings.

Chemokine concentrations are relatively unaffected Tat expression and morphine

Identical to the cytokine panel, we validated our data for each cytokine by confirming standard curve generation, bead gating, and analyte detection (**Fig 9A-D**). Unfortunately, only 5 of 13 chemokines were within the limits of detection, therefore our analysis only focuses on these. Eotaxin (CCL11) was the only chemokine to show any

significant effect with a genotype x morphine interaction (3-way ANOVA, $p < 0.05$) (**Fig 10A)**. Multiple comparisons showed that Tat- morphine treated animals that were administered CLP show an increased concentration of eotaxin compared to vehicle administered mice (Fisher's LSD, $p < 0.05$). We also investigated the extent to which mean chemokine levels were altered during aging and found a main effect of age in which overall chemokine concentrations increased with age in control. Tat− mouse striatum (2-way ANOVA, $p < 0.05$). Post-hoc analysis showed that compared to the 4-month-old mice, 6-month-old mice had a lower mean chemokine concentration (Fisher's LSD, $p < 0.05$) (**Fig 11A-C)**. Interestingly, chemokines show an opposing trend compared to the cytokines in our studies which increased with age.

Genes encoding Cl^- channels, transporters, or GABA receptors were unaffected by morphine and/or Tat

To investigate whether Tat and morphine exposure altered the expression of genes relevant to KCC2 and chloride regulation, RNA sequencing data was mined from the striatum of mice acutely exposed to Tat ± morphine. We found no changes in FPKM values between groups in any of the genes analyzed (**Fig. 12**). Looking at more prolonged exposures to Tat and morphine could provide more insight into genes involved in chloride regulation.

Discussion

The studies in this chapter investigate inflammatory changes in the striatum and the extent that Tat ± morphine induced KCC2 deficits or KCC2 enhancement by CLP290

might underlie Tat ± morphine dependent inflammation. We show that 2-week morphine administration altered striatal concentrations of IL-23 and IFN-β and that while mean cytokine concentrations increase with age in Tat- mice, Tat+ mice slightly decrease concentrations. Additionally, we found that there is little alteration to individual chemokines, but mean chemokine concentrations decrease with age, as opposed to the cytokine results. Lastly, we mined existing RNA seq data from a separate cohort of male mice acutely exposed to Tat with or without morphine to investigate gene expression changes to genes encoding Cl^- channels, transporters, and GABA receptors. We saw no significant differences but increasing sample size and or duration of Tat and morphine treatment may uncover more differences.

While acute inflammation is vital to rapid responses to infection and injury, chronic, low level inflammation can lead to tissue degeneration (Franceschi & Campisi, 2014). Immunosenescence is a hallmark of aging and is characterized by decreased T-cell function and altered cytokine responses (Michaud et al., 2013). In particular, TNF-α and IL-6 are linked to cognitive decline with age and are important factors in other inflammatory diseases (Frolich, Hofman, Jolles, Breteler, & Westendorp, 2007; Holmes et al., 2009). While we did not see significant differences in concentrations of TNF-a or IL-6 in the striatum, it is possible we missed the window for elevated levels of these early cytokines, but we did see morphine effects in both IL-23 and IFN-β. IFN-β is an antiviral cytokine and Tat-dependent increases in IFN-β were not surprising. Unlike in the periphery, however, IFN-β can act in both a pro- and anti-inflammatory manner in the CNS and is a well-studied therapy for people with relapsing-remitting multiple sclerosis

(Filipi & Jack, 2020). Interestingly, morphine can suppress both IL-23 production and IFN signaling, and both have implications in HIV infection.

Regulatory T lymphocytes (T_{regs}) are critical for maintaining immune tolerance to self and preventing autoimmunity. IL-23 increases T_{reg} plasticity by increasing the recuritment/differentiation of T helper 17 (Th17) cells that have positive of negative consequences depending on timing and context (Kannan et al., 2019). In the periphery, IL-23 is a clinical indicator and target for inflammatory diseases such as rheumatoid arthritis and irritable bowel disease (Cayatte et al., 2012; Zaky & El-Nahrery, 2016). In the CNS, IL-23 may contribute to the accumulation of β-amyloid and neurodegeneration in Alzheimer's disease (Nitsch, Schneider, Zimmermann, & Müller, 2021). Morphine inhibits IL-23 signaling via Toll-like receptor 2-dependent disruption of host defenses against infection (J. Wang, Ma, Charboneau, Barke, & Roy, 2011). Additionally, *in vitro* infection of T-cells with HIV blocks IL-23 signaling, even in cells isolated from patients on highly activate anti-retroviral therapy (Fernandes, Berthoud, Kumar, & Angel, 2017). Fernandes et al. suggest that anti-retroviral therapy is incapable to reactivate IL-23 signaling in T-cells *in* vitro could influence immune activation in PLWH despite minimal viral replication from cART treatment.

Persistent HIV-mediated inflammation both systemically (Deeks, Tracy, & Douek, 2013) and in the brain (McArthur & Johnson, 2020), in addition to aging-induced inflammation, prompted us to investigate cytokine and chemokine levels by age. While we saw an expected increase in mean cytokine levels in the striatum of Tat− mice, we surprisingly saw that Tat+ mice showed a slight decrease in cytokines from 4 to 6 months in age. We hypothesize that chronic low levels of inflammatory signaling in Tat-transgenic

mice could be preventing any age-related vamping of inflammation levels as the basal level of mean cytokine concentrations is higher in Tat+ compared to Tat- mice. Contrary to cytokines, we saw a decrease in mean chemokine concentrations from 4 to 6 months in age. Additionally, we saw that Eotaxin (CCL11) was the only chemokine individually altered where concentrations were increased in morphine treated mice that were Tat- but decreased in morphine treated mice that were Tat+. Eotaxin released from activated astrocytes promotes neuronal death through its receptor CCR3 (can also bind CCR2 and CCR5) on microglia, in turn leading to oxidative stress and glutamate-mediated neurotoxicity (Parajuli, Horiuchi, Mizuno, Takeuchi, & Suzumura, 2015). Further investigating microglial activation and inflammatory signaling could uncover more about the role of cytokines and chemokines Tat ± morphine mediated neurodegeneration as well as the connection between inflammation and KCC2 signaling.

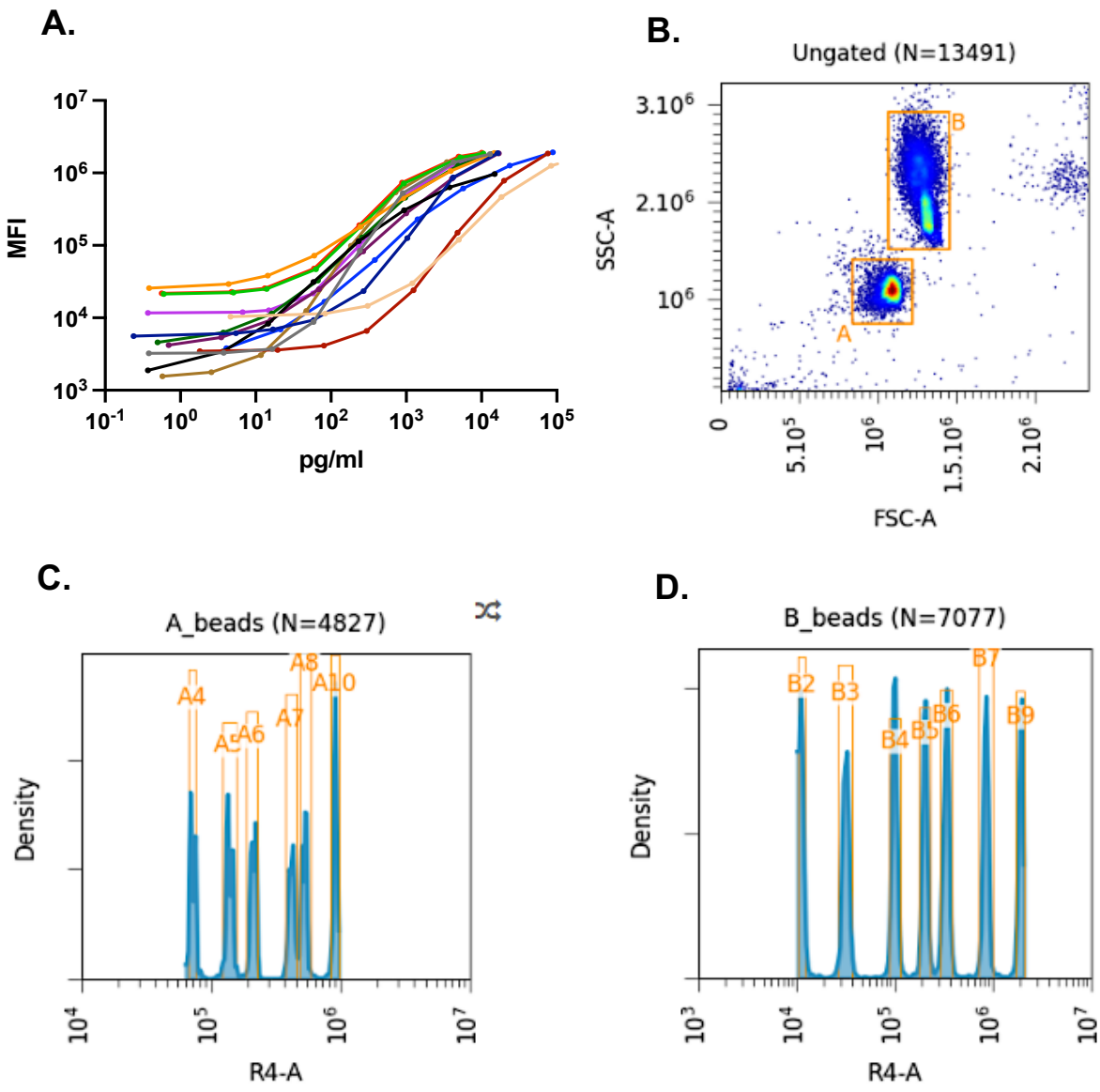

Figure 5. Standard curves and bead gating for LEGENDplex Inflammation Panel. Standard curves were plotted by measured standard concentration and mean fluorescence intensity (MFI) for each analyte (**A**). Representative bead scatter plot showing gating of A and B bead populations (**B**). Histogram of analyte density detected by A and B beads showing 13 populations represented by individual peaks for each cytokine (**C,D**).

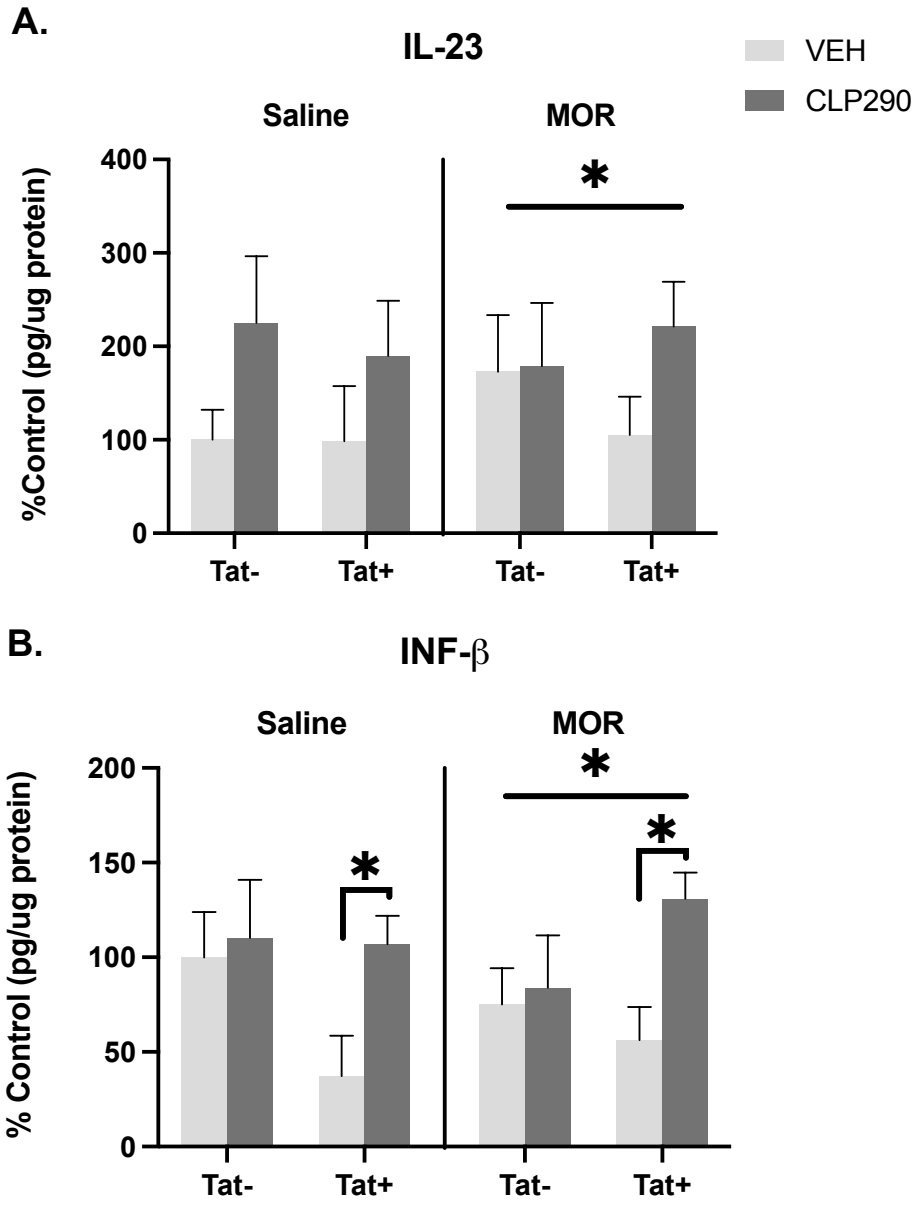

Figure 6. Morphine administration increases levels of pro-inflammatory cytokines IL-23 and IFN-β. A main effect of morphine was detected for both IL-23 (**A**) and IFN-β (**B**) cytokine concentrations (3-way ANOVA, *$p < 0.05$, $n = 5$). Fisher's LSD Post-hoc analysis show that in Tat+ mice administered CLP290, IFN-β concentrations were higher compared to Tat+ mice administered vehicle ($p < 0.05$) (**B**).

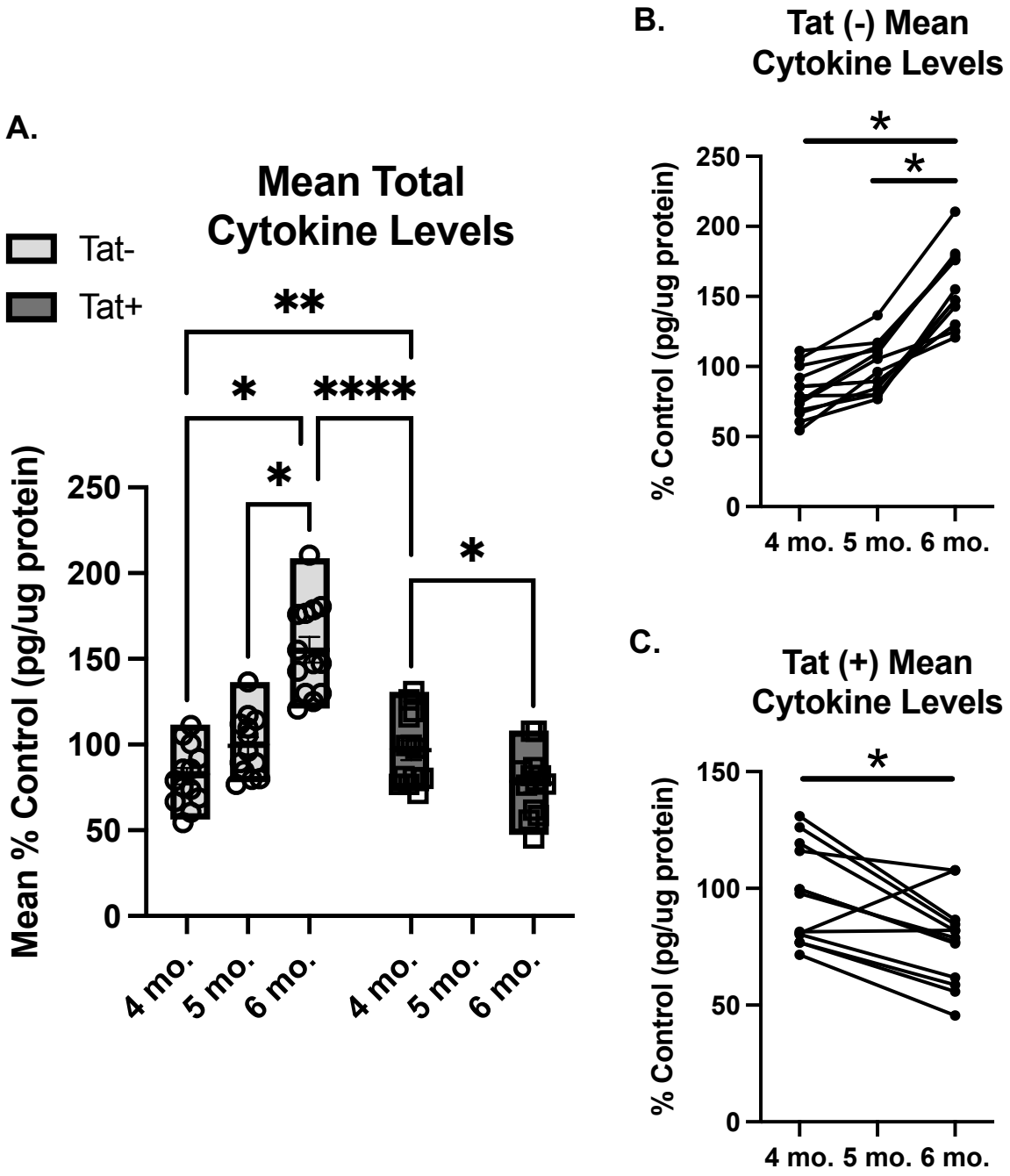
A.
Mean Total Cytokine Levels
Tat-
Tat+
**
*
****
*
*
Mean % Control (pg/ug protein)
250
200
150
100
50
0
4 mo.
5 mo.
6 mo.
4 mo.
5 mo.
6 mo.
B.
Tat (-) Mean Cytokine Levels
*
*
% Control (pg/ug protein)
250
200
150
100
50
0
4 mo. 5 mo. 6 mo.
C.
Tat (+) Mean Cytokine Levels
*
% Control (pg/ug protein)
150
100
50
0
4 mo. 5 mo. 6 mo.

Figure 7. Age related alterations to cytokine levels are altered by Tat expression. 2-way ANOVA shows main effects of age and genotype (2-way ANOVA, age, $^{**}p < 0.01$; genotype, $^{***}p < 0.001$, $n = 13/group$). Post-hoc analysis reveals mean cytokine concentrations increase with age in Tat- mice, while showing a decrease with age in Tat+ mice. Tat+ mice show an elevated mean concentration of cytokines at 4 months compared to Tat- mice (** $p < 0.01$) (**A**). Mean levels of cytokines increase stepwise from 4 to 6 months in age in Tat- mice (* $p < 0.05$) (**B**) while decrease from 4 to 6 months in Tat+ mice (* $p < 0.05$). (**C**).

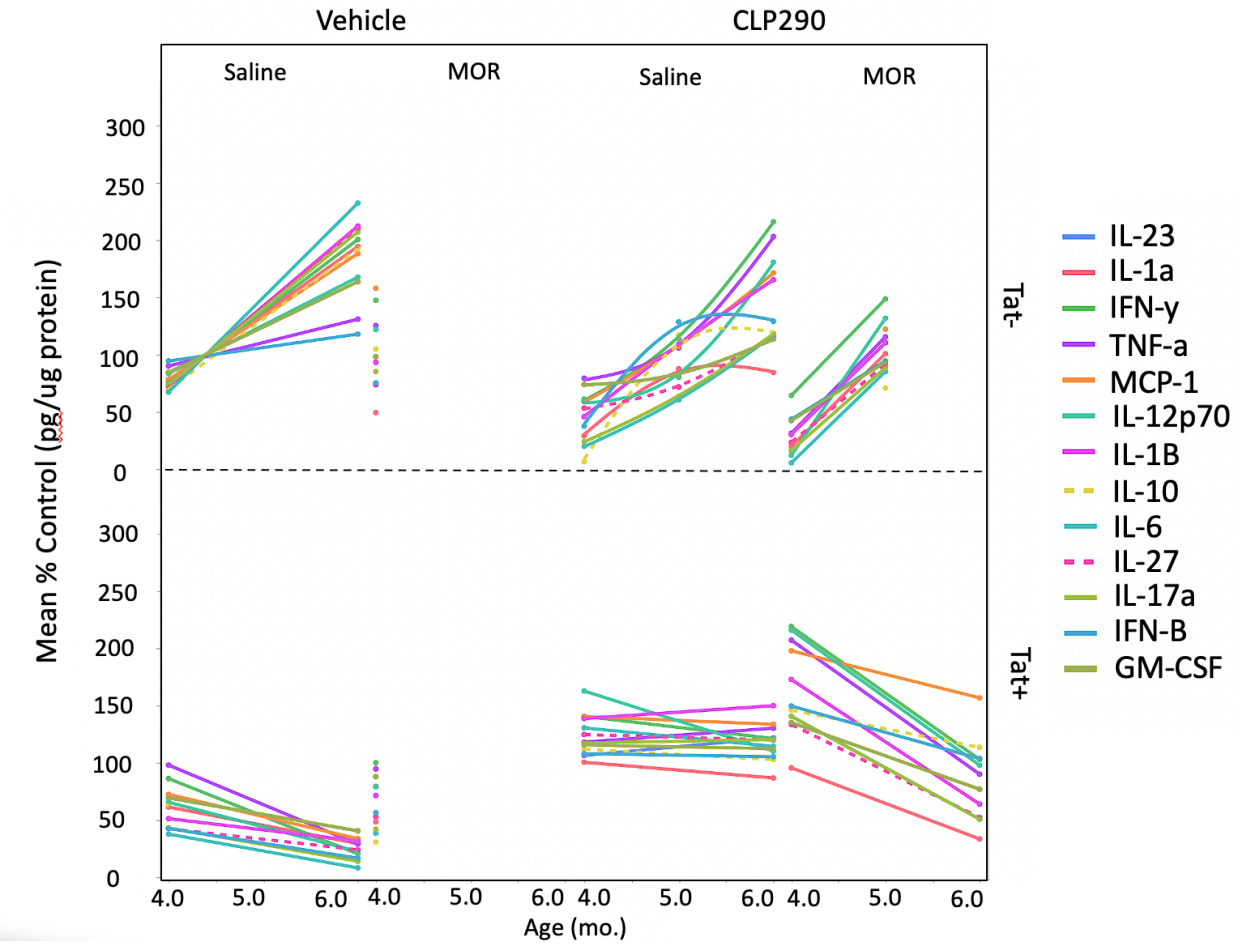

Figure 8. Age and genotype related alteration to cytokine levels show same trends when split into CLP290 and morphine treatment groups. Mean cytokine concentrations were visualized by genotype and per treatment group. When split between morphine and CLP290 groups, cytokine values show similar trends when visualized (increase in Tat-, decrease in Tat+), suggesting so influence of morphine or KCC2 on this observation. Smoothened trend lines are solid for pro-inflammatory cytokines and dotted for anti-inflammatory cytokines.

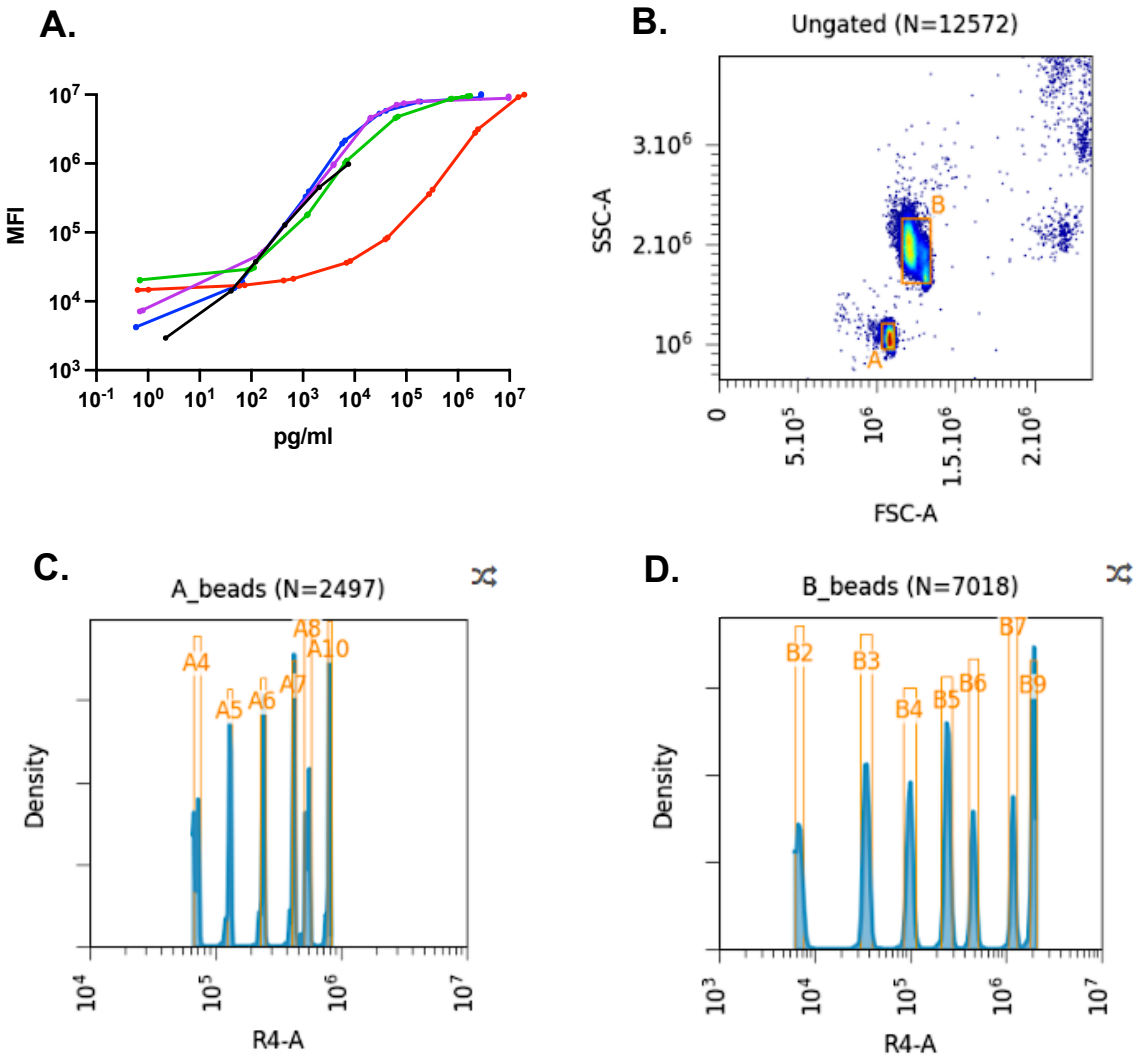

Figure 9. Standard curves and bead gating for LEGENDplex Proinflammatory Chemokine Panel. Standard curves were plotted by measured standard concentration and mean fluorescence intensity (MFI) for each analyte (**A**). Representative bead scatter plot showing gating of A and B bead populations (**B**). Histogram of analyte density detected by A and B beads showing 13 populations represented by individual peaks for each chemokine (**C,D**).

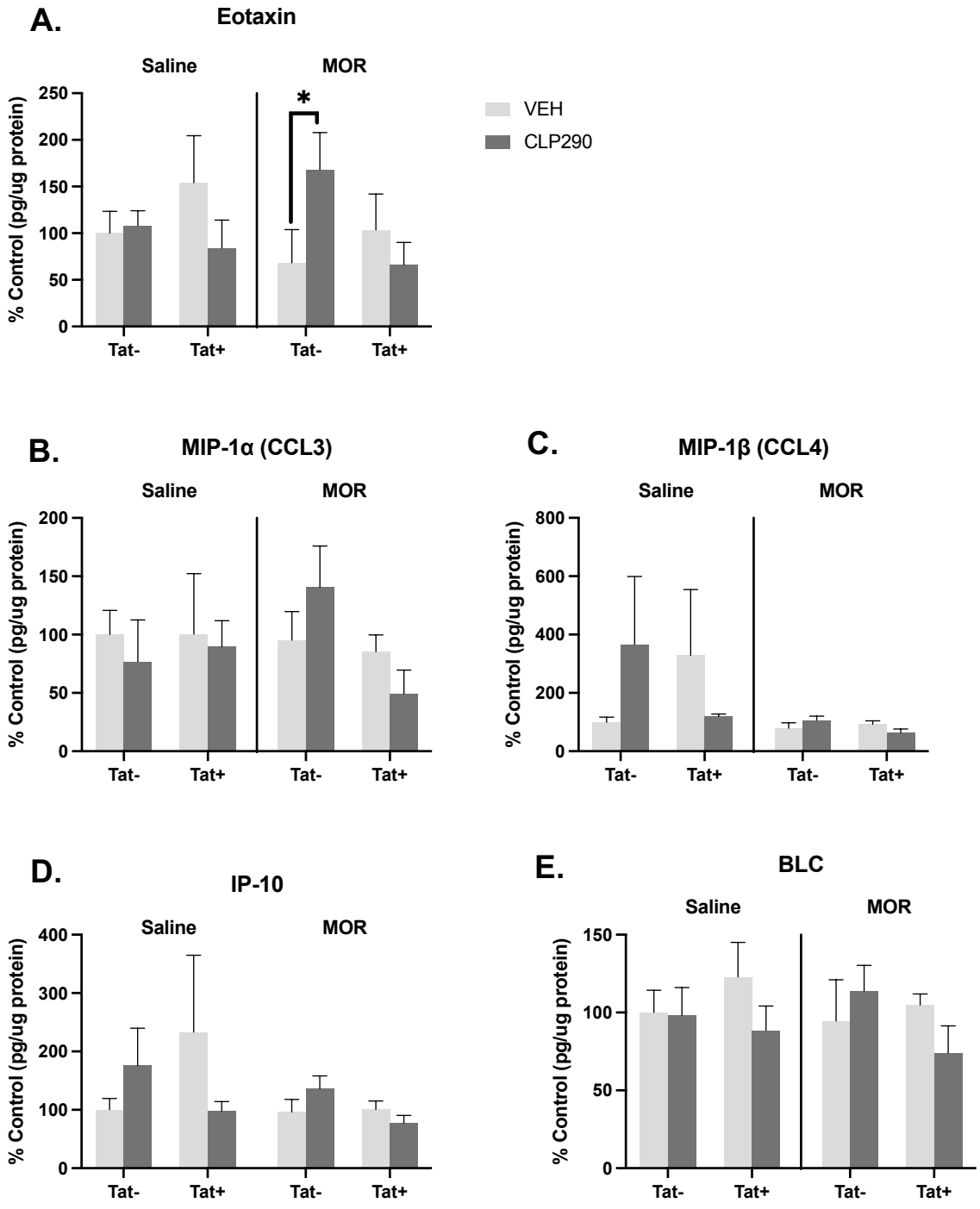
A.
Eotaxin
Saline
MOR
VEH
CLP290
% Control (pg/ug protein)
250
200
150
100
50
0
*
Tat-
Tat+
Tat-
Tat+
B.
MIP-1α (CCL3)
Saline
MOR
% Control (pg/ug protein)
200
150
100
50
0
Tat-
Tat+
Tat-
Tat+
C.
MIP-1β (CCL4)
Saline
MOR
% Control (pg/ug protein)
800
600
400
200
0
Tat-
Tat+
Tat-
Tat+
D.
IP-10
Saline
MOR
% Control (pg/ug protein)
400
300
200
100
0
Tat-
Tat+
Tat-
Tat+
E.
BLC
Saline
MOR
% Control (pg/ug protein)
150
100
50
0
Tat-
Tat+
Tat-
Tat+

Figure 10. Only Eotaxin (CCL11) is altered by morphine but depends on Tat expression. Eotaxin concentrations were increased in morphine treated animals that were Tat-, but were decreased in animals that were Tat+ (Genotype x Morphine interaction, $p < 0.05$, $n = 5$) (**A**). No significant differences were detected for the chemokines CCL3, CCL4, IP-10, or BLC (**B-E**).

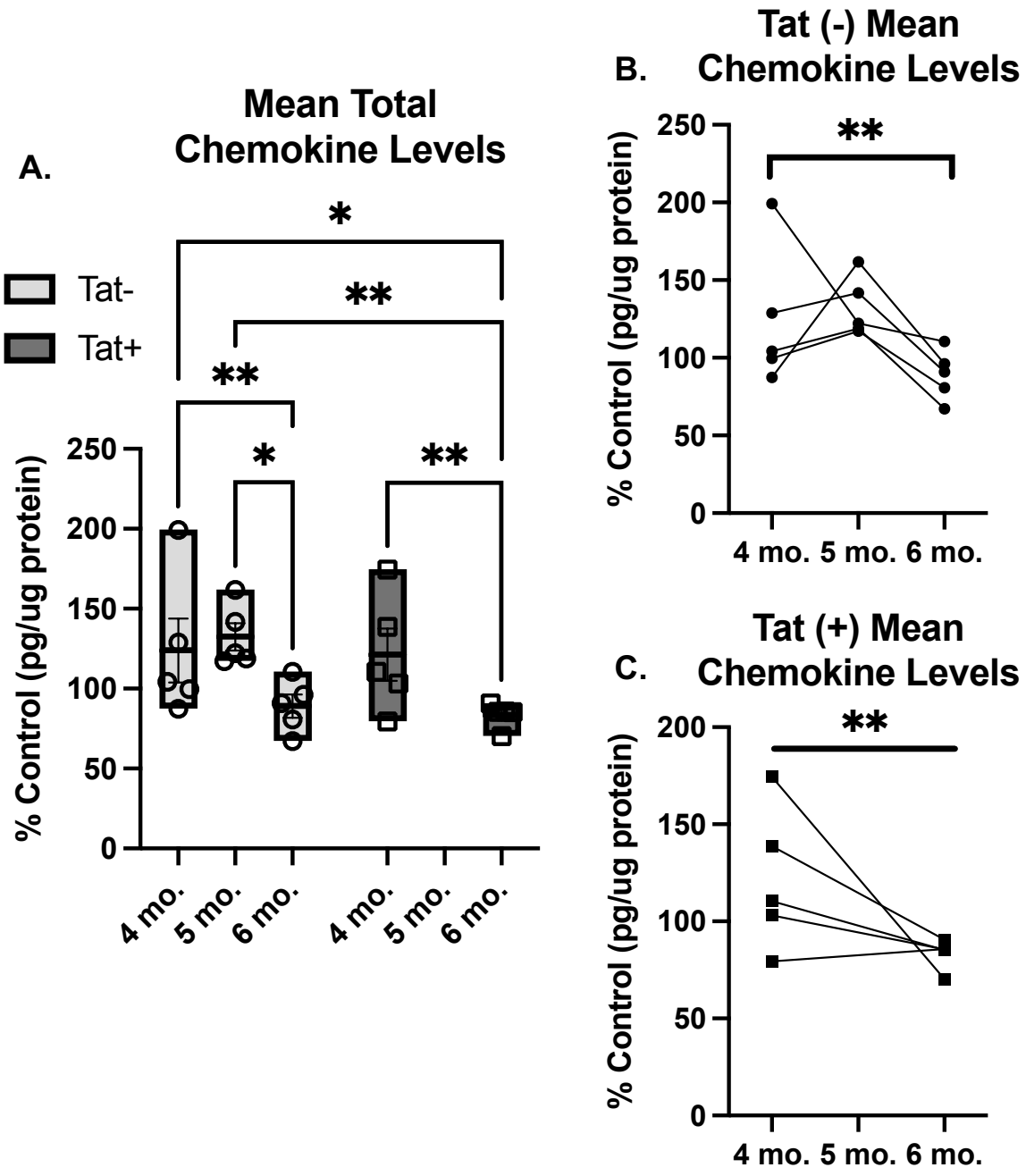

Figure 11. Mean chemokine concentrations lower with age. 2-way ANOVA shows a main effect of age (2-way ANOVA, age, $p < 0.05$; genotype, $n = 5/group$). Post-hoc analysis reveals mean chemo concentrations decrease with age from 4 to 6 months in age in both Tat- and Tat+ mice (** $p < 0.01$) (**A**). Mean levels of chemokines decrease from 4 months compared to 6 months in age in Tat- and Tat+ mice (* $p < 0.05$) (**B, C**).

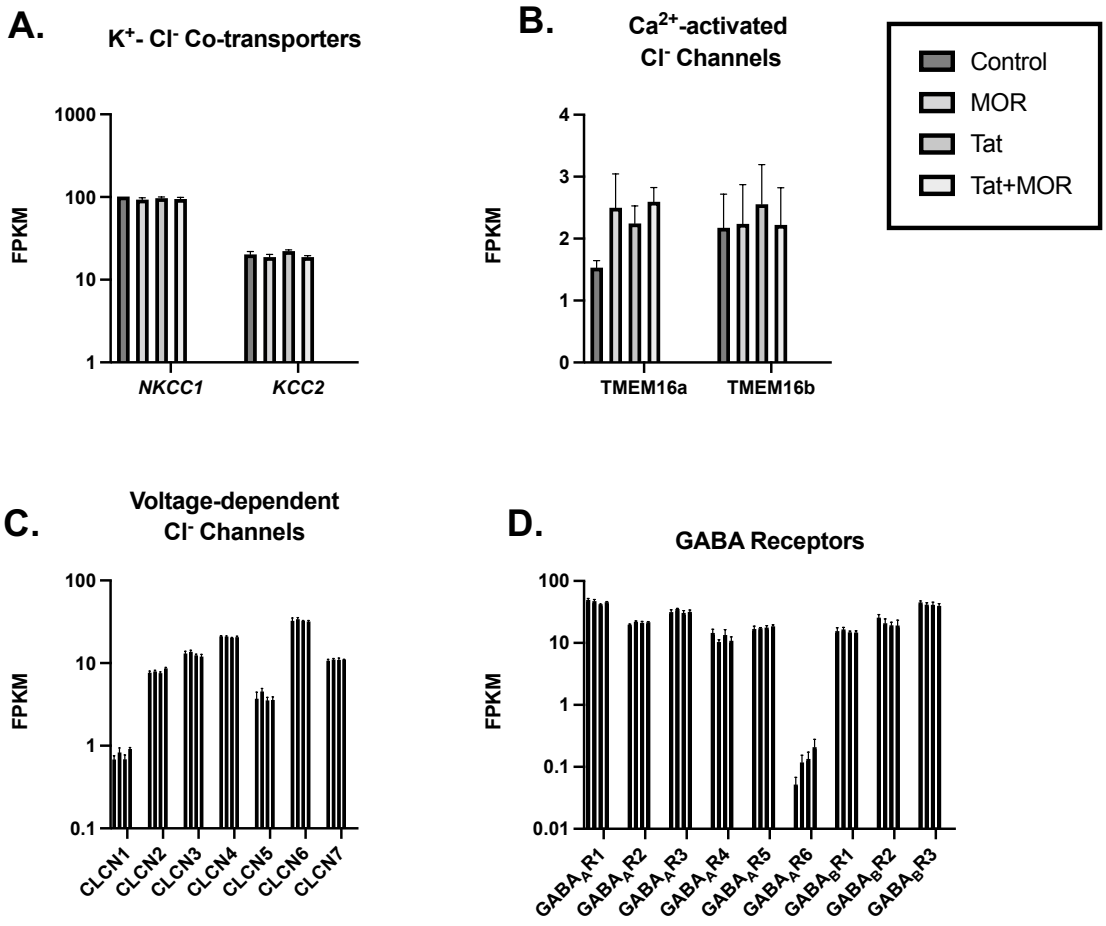

Figure 12. No detected gene expression changes following acute Tat and morphine exposure in genes encoding Cl^- regulators. FPKM values for *n* = *3* animals per group were averaged and grouped based on encoded protein function. There were no detected differences between any groups within a gene for K^+Cl^- co-transporters (**A**), CaCCs (**B**), voltage-dependent Cl^- channels (**C**), or $GABA_A$ receptors (**D**).

Chapter 5: Conclusions and Future Directions

The Tat-transgenic mouse model enabled us to study the *in vivo* role of KCC2 in Tat ± morphine induced neurodegeneration. *In vivo* Tat-expression has been shown to induce anxiety-like behavior and hyperactivity in male mice. We sought to see if these results were the same in female mice and enhance these studies with morphine co-treatment along with CLP290 administration to understand the role of KCC2. Interestingly, we found no effect of Tat expression on behavioral outcomes in an open field task but did see a major effect morphine leading to decreased exploratory behavior and increased anxiety like behavior. These results were not due to locomotor impairment as all groups had similar distance traveled in the open field and performed similarly on a rotarod task.

Following behavioral testing, we wanted to investigate molecular changes in the striatum that could be tied to our behavioral results by western blot analysis. While the striatum is classically known for its role in locomotion via the direct and indirect pathways (Fieblinger, 2021), striatal circuitry is also implicated in anxiety and depression (Macpherson & Hikida, 2019; Quarmley, Nelson, Clarkson, White, & Jarcho, 2019). We found CLP290 increased overall levels of KCC2 but not pS940-KCC2 or NKCC1. Although the behavioral results did not coincide with Tat and/or morphine-dependent changes in levels of pS940-KCC2 or total KCC2 and we unaffected by CLP290 in female mice, we found Tat-expressing mice showed lower levels of TMEM16a and CLP290 lowered TMEM16a levels overall. We focused on the striatum specifically as previous studies in male mice showed region specific loss of KCC2 within the striatum (Barbour et al., 2021). Further investigation into KCC2 within more specific anxiety related brain

regions such as the amygdala and prefrontal cortex may lead to a better understanding of molecular changes connected to our behavioral results.

We designed our behavioral studies to attempt to observe the effects of morphine and any potential interaction with Tat and CLP290. Accordingly, tests were conducted 1-hour following a 40 mg/kg morphine injection. To examine the acute effects of morphine, while avoiding the effects of physical withdrawal, we chose our 1-hour timepoint. Most studies examining spontaneous morphine withdrawal wait at least 5 hours after the final injection (Ayoub et al., 2021; Papaleo & Contarino, 2006; Quock & Brewer, 2019), since the half-life of morphine in wildtype mice is typically less than 60 minutes (Zelcer et al., 2005). While we assessed behavior close to the final injection time, it is still possible that the mice are undergoing signs of somatic withdrawal or withdrawal events at the molecular level which could be having an impact on behavior. Thus, a better understanding of the temporal window for morphine effects and morphine withdrawal in our mice is needed and how Tat may interfere with pharmacodynamics of morphine. For example, Fitting et al. found that 2-week Tat induction reduces the antinociceptive efficacy of 8 mg/kg morphine administration in a tail flick assay 20 minutes post-final injection (Fitting et al., 2016). A more acute time course of withdrawal effects from morphine such as wet dog shakes, jumping, and paw tremors at 20, 40, 60, and 120 minutes in both Tat- and Tat+ mice would be beneficial to ensure behavioral testing is done before any somatic withdrawal signs appear. Additionally, molecular changes in the brain such as increased phosphorylation of cAMP-response element binding protein and *c-fos* expression could be used as markers in these further studies or on the animals in these studies to assess

morphine withdrawal (Chan & Lutfy, 2016; Georges, Stinus, & Le Moine, 2000; Martín, Laorden, & Milanés, 2009).

Previous studies in Tat-transgenic mice have found that KCC2 levels as well as phosphorylation at S940 are decreased following two weeks of Tat expression and these changes are reversed with CLP290 (Barbour et al., 2020, 2021). While our results support CLP290 increasing KCC2 immunoreactivity, we see no changes in KCC2 levels in Tat-expressing mice nor in phosphorylation at S940. The main difference between our results and previous findings is our studies were performed in female rather than male mice. Perrot-Sinal et al. have shown that there are sex differences in the expression of both NKCC1 and KCC2 in the developing hypothalamus (Perrot-Sinal, Sinal, Reader, Speert, & McCarthy, 2007). Additionally, *in situ* hybridization studies have shown KCC2 mRNA expression is higher in females than males irrespective of age in the substantia nigra pars reticulata (Galanopoulou, Kyrozis, Claudio, Stanton, & Moshé, 2003). Higher expression at the mRNA level in females could compensate for any potential *slc12a* gene expression downregulation by Tat. These findings remain true at the protein level as KCC2 immunoreactivity in the mediobasal hypothalamus of female rats is significantly higher than in males (Perrot-Sinal, Sinal, Reader, Speert, & Mccarthy, 2007). Unfortunately, all studies are performed during the perinatal period and no insights into potential sex differences during adulthood have been found. Further investigation with both males and females within the same experimental cohort and identical treatment paradigm is necessary to definitively determine if there is a sex difference in KCC2 regulation and effects from Tat ± morphine.

Surprisingly, LEGENDplex analysis of cytokines and chemokines in the striatum did not reveal major changes to individual analytes following Tat ± morphine exposure. Both Tat ± morphine have been shown to induce alterations in cytokine and chemokine levels both *in vitro* (El-Hage et al., 2008, 2005) and *in vivo* (Gonek et al., 2018; Hermes et al., 2020; Nass et al., 2020). Additionally, cytokine and chemokine release is extremely dynamic and can depend on the type of insult, duration of treatment, or even time of day (Nakao, 2014; Tweedie et al., 2020). We collected brain samples following 2-weeks of Tat induction and morphine administration, it is possible that cytokine and chemokine levels were elevated initially but declined due to tolerance of from chronically activated signaling (Gillen et al., 2021). Possible ways we could combat this in the future is to collect serum from the mice and analyze systemic inflammation versus a specific brain region or assess changes to inflammatory markers at the gene level. Cytokine and chemokines have very short half-lives (Aziz et al., 2016; Lotze et al., 1985) and due to their instability, then can be difficult to detect. Collecting mRNA and running quantitative PCR could give a more accurate insight into the inflammatory markers and signaling pathways being activated.

Final Conclusions: This work sought out to understand the role of KCC2 on Tat ± morphine induced neurodegeneration in female mice through administration of the KCC2 enhancing pro-drug CLP290. We identified that a 2-week ramping dose of morphine led to anxiety-like behavior and decreased exploration. Using striatal protein lysates from these mice, we saw CLP290 increased levels of KCC2 but had no effect on pS940-KCC2, suggesting a potential alternative mechanism through which CLP290 can regulate KCC2.

Additionally, we saw a Tat mediated decrease in immunoreactivity of the CaCC TMEM16a and a main effect of CLP290 on TMEM16a. As both TMEM16a and KCC2 regulate Cl^-, modulation of KCC2 could be impacting function and expression of TMEM16a. Lastly, the studies in Chapter 4 described how cytokine and chemokine levels in the striatum of these mice showed differential trends depending on age and genotype. Additionally, we saw alteration to concentrations of IL-23, IFN-β, and Eotaxin (CCL-11) providing potential insight into activated signaling pathways. Overall, these studies give insight into the need to perform identical studies using males and females to investigate potential sex differences of Tat and or morphine on KCC2 regulation, spontaneous locomotion, and anxiety as well as sex-specific changes to inflammatory signaling.

References

Abassi, M., Morawski, B. M., Nakigozi, G., Nakasujja, N., Kong, X., Meya, D. B., … Boulware, D. R. (2017). Cerebrospinal fluid biomarkers and HIV-associated neurocognitive disorders in HIV-infected individuals in Rakai, Uganda. *Journal of NeuroVirology*, *23*(3), 369–375. https://doi.org/10.1007/S13365-016-0505-9/TABLES/3

About the Epidemic | HHS.gov. (n.d.). Retrieved October 17, 2021, from https://www.hhs.gov/opioids/about-the-epidemic/index.html

Akbarian, S., Kim, J. J., Potkin, S. G., Hagman, J. O., Tafazzoli, A., Bunney, W. E., & Jones, E. G. (1995). Gene expression for glutamic acid decarboxylase is reduced without loss of neurons in prefrontal cortex of schizophrenics. *Archives of General Psychiatry*, *52*(4), 258–266. https://doi.org/10.1001/ARCHPSYC.1995.03950160008002

Anthony, I. C., Arango, J. C., Stephens, B., Simmonds, P., & Bell, J. E. (2008). The effects of illicit drugs on the HIV infected brain. *Frontiers in Bioscience*. Front Biosci. https://doi.org/10.2741/2762

Antinori, A., Arendt, G., Becker, J. T., Brew, B. J., Byrd, D. A., Cherner, M., … Wojna, V. E. (2007). Updated research nosology for HIV-associated neurocognitive disorders. *Neurology*, *69*(18), 1789. https://doi.org/10.1212/01.WNL.0000287431.88658.8B

Ayoub, S. M., Piscitelli, F., Silvestri, C., Limebeer, C. L., Rock, E. M., Smoum, R., … Parker, L. A. (2021). Spontaneous and Naloxone-Precipitated Withdrawal Behaviors From Chronic Opiates are Accompanied by Changes in N-Oleoylglycine and N-Oleoylalanine Levels in the Brain and Ameliorated by Treatment With These

Mediators. *Frontiers in Pharmacology*, *12*, 2371. https://doi.org/10.3389/FPHAR.2021.706703/BIBTEX

Aziz, N., Detels, R., Quint, J. J., Li, Q., Gjertson, D., & Butch, A. W. (2016). Stability of cytokines, chemokines and soluble activation markers in unprocessed blood stored under different conditions. *Cytokine*, *84*, 17. https://doi.org/10.1016/J.CYTO.2016.05.010

Bannwarth, S., & Gatignol, A. (2005). HIV-1 TAR RNA: The Target of Molecular Interactions Between the Virus and its Host. *Current HIV Research*, *3*(1), 61–71. https://doi.org/10.2174/1570162052772924

Barbour, A. J., Hauser, K. F., McQuiston, A. R., & Knapp, P. E. (2020). HIV and opiates dysregulate K+-Cl- cotransporter 2 (KCC2) to cause GABAergic dysfunction in primary human neurons and Tat-transgenic mice. *Neurobiology of Disease*, *141*, 104878. https://doi.org/10.1016/j.nbd.2020.104878

Barbour, A. J., Nass, S. R., Hahn, Y. K., Hauser, K. F., & Knapp, P. E. (2021). Restoration of KCC2 Membrane Localization in Striatal Dopamine D2 Receptor-Expressing Medium Spiny Neurons Rescues Locomotor Deficits in HIV Tat-Transgenic Mice: *Https://Doi.Org/10.1177/17590914211022089*, *13*. https://doi.org/10.1177/17590914211022089

Bbosa, N., Kaleebu, P., & Ssemwanga, D. (2019). HIV subtype diversity worldwide. *Current Opinion in HIV and AIDS*, *14*(3), 153–160. https://doi.org/10.1097/COH.0000000000000534

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

involvement. *Brain*, *121*, 2043–2052.

Ben-Ari, Y., Khalilov, I., Kahle, K. T., & Cherubini, E. (2012). The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. *Neuroscientist*, *18*(5), 467–486. https://doi.org/10.1177/1073858412438697

Benarroch, E. E. (2012). Endogenous opioid systems. *Neurology*, *79*(8), 807–814. https://doi.org/10.1212/WNL.0B013E3182662098

Benedetto, R., Cabrita, I., Schreiber, R., & Kunzelmann, K. (2019). TMEM16A is indispensable for basal mucus secretion in airways and intestine. *The FASEB Journal*, *33*(3), 4502–4512. https://doi.org/10.1096/FJ.201801333RRR

Benedetto, R., Ousingsawat, J., Wanitchakool, P., Zhang, Y., Holtzman, M. J., Amaral, M., … Kunzelmann, K. (2017). Epithelial Chloride Transport by CFTR Requires TMEM16A. *Scientific Reports 2017 7:1*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-10910-0

Bock, G., Zhai, Q. H., Sharer, L. R., Mccomb, R. D., & Swindells, S. (2021). Mechanisms for the transendothelial migration of HIV-1-infected monocytes into the brain. *The Journal of Immunology*, 1284–1295. Retrieved from http://www.jimmunol.org/

Brownstein, M. J. (1993). A brief history of opiates, opioid peptides, and opioid receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(12), 5391. https://doi.org/10.1073/PNAS.90.12.5391

Bruce-Keller, A. J., Turchan-Cholewo, J., Smart, E. J., Geurin, T., Chauhan, A., Reid, R., … Hauser, K. F. (2008). Morphine causes rapid increases in glial activation and neuronal injury in the striatum of inducible HIV-1 tat transgenic mice. *Glia*, *56*(13),

1414–1427. https://doi.org/10.1002/GLIA.20708

Buzhdygan, T., Lisinicchia, J., Patel, V., Johnson, K., Neugebauer, V., Paessler, S., … Gelman, B. (2016). Neuropsychological, Neurovirological and Neuroimmune Aspects of Abnormal GABAergic Transmission in HIV Infection. *Journal of Neuroimmune Pharmacology 2016 11:2*, *11*(2), 279–293. https://doi.org/10.1007/S11481-016-9652-2

Byrd, D. A., Fellows, R. P., Morgello, S., Franklin, D., Heaton, R. K., Deutsch, R., … Grant, I. (2011). Neurocognitive impact of substance use in HIV infection. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *58*(2), 154–162. https://doi.org/10.1097/QAI.0B013E318229BA41

CA Hübner, V Stein, I Hermans-Borgmeyer, T Meyer, K Ballanyi, T. J. (2001). Disruption of KCC2 reveals an essential role of K-Cl cotransport already in early synaptic inhibition. *Neuron*, *30*(2), 515–524. https://doi.org/10.1016/S0896-6273(01)00297-5

Carey, A. N., Sypek, E. I., Singh, H. D., Kaufman, M. J., & McLaughlin, J. P. (2012). Expression of HIV-Tat protein is associated with learning and memory deficits in the mouse. *Behavioural Brain Research*, *229*(1), 48–56. https://doi.org/10.1016/J.BBR.2011.12.019

Carvallo, L., Lopez, L., Fajardo, J. E., Jaureguiberry-Bravo, M., Fiser, A., & Berman, J. W. (2017). HIV-Tat regulates macrophage gene expression in the context of neuroAIDS. *PLOS ONE*, *12*(6), e0179882. https://doi.org/10.1371/JOURNAL.PONE.0179882

Cavrois, M., Neidleman, J., & Greene, W. C. (2008). The Achilles Heel of the Trojan

Horse Model of HIV-1 trans-Infection. *PLOS Pathogens*, *4*(6), e1000051. https://doi.org/10.1371/JOURNAL.PPAT.1000051

Cayatte, C., Joyce-Shaikh, B., Vega, F., Boniface, K., Grein, J., Murphy, E., … Beaumont, M. (2012). Biomarkers of Therapeutic Response in the IL-23 Pathway in Inflammatory Bowel Disease. *Clinical and Translational Gastroenterology*, *3*(2), e10. https://doi.org/10.1038/CTG.2012.2

Cernasev, A., Veve, M. P., Cory, T. J., Summers, N. A., Miller, M., Kodidela, S., & Kumar, S. (2020). Opioid Use Disorders in People Living with HIV/AIDS: A Review of Implications for Patient Outcomes, Drug Interactions, and Neurocognitive Disorders. *Pharmacy: Journal of Pharmacy Education and Practice*, *8*(3), 168. https://doi.org/10.3390/PHARMACY8030168

Chamma, I., Chevy, Q., Poncer, J. C., & Lévi, S. (2012). Role of the neuronal K-Cl co-transporter KCC2 in inhibitory and excitatory neurotransmission. *Frontiers in Cellular Neuroscience*, *6*(JANUARY). https://doi.org/10.3389/FNCEL.2012.00005

Chan, P., & Lutfy, K. (2016). Molecular Changes in Opioid Addiction: The Role of Adenylyl Cyclase and cAMP/PKA System. https://doi.org/10.1016/bs.pmbts.2015.10.005

Chang, S. L., & Connaghan, K. P. (2012). Behavioral and molecular evidence for a feedback interaction between morphine and HIV-1 viral proteins. *Journal of Neuroimmune Pharmacology*, *7*(2), 332–340. https://doi.org/10.1007/S11481-011-9324-1/FIGURES/5

Chaoliang, Gu, Li Peng, Hu Bi, Ouyang Xinping, Fu Juan, Gao Jun, Song Zeng, Han Li, Ma Yuanye, Tian Shaowen, H. X. (2008). Chronic morphine selectively impairs

cued fear extinction in rats: Implications for anxiety disorders associated with opiate use. *Neuropsychopharmacology*, *33*(3), 666–673. https://doi.org/10.1038/sj.npp.1301441

Chaudhury, A. (2015). VIP in HIV diarrhea: Finding links for the “slim disease.” *Frontiers in Physiology*, *6*(DEC), 402. https://doi.org/10.3389/FPHYS.2015.00402/BIBTEX

Chen, L., Wan, L., Wu, Z., Ren, W., Huang, Y., Qian, B., & Wang, Y. (2017). KCC2 downregulation facilitates epileptic seizures. *Scientific Reports 2017 7:1*, *7*(1), 1–13. https://doi.org/10.1038/s41598-017-00196-7

Cho, C.-H., Lee, S., Kim, A., Yarishkin, O., Ryoo, K., Lee, Y.-S., … Park, J.-Y. (2020). TMEM16A expression in cholinergic neurons of the medial habenula mediates anxiety-related behaviors. *EMBO Reports*, *21*(2), e48097. https://doi.org/10.15252/EMBR.201948097

Chung, H. Y., Kim, D. H., Lee, E. K., Chung, K. W., Chung, S., Lee, B., … Yu, B. P. (2019). Redefining Chronic Inflammation in Aging and Age-Related Diseases: Proposal of the Senoinflammation Concept. *Aging and Disease*, *10*(2), 367. https://doi.org/10.14336/AD.2018.0324

Crutchley, R. D., Miller, J., & Garey, K. W. (2010). Crofelemer, a Novel Agent for Treatment of Secretory Diarrhea: *Http://Dx.Doi.Org/10.1345/Aph.1M658*, *44*(5), 878–884. https://doi.org/10.1345/APH.1M658

Darcq, E., & Kieffer, B. L. (2018). Opioid receptors: drivers to addiction? *Nature Reviews Neuroscience 2018 19:8*, *19*(8), 499–514. https://doi.org/10.1038/s41583-018-0028-x

Das, A. T., Harwig, A., & Berkhout, B. (2011). The HIV-1 Tat Protein Has a Versatile

Role in Activating Viral Transcription. *Journal of Virology*, *85*(18), 9506. https://doi.org/10.1128/JVI.00650-11

De Koninck, Y. (2007). Altered chloride homeostasis in neurological disorders: a new target. *Current Opinion in Pharmacology*, *7*(1), 93–99. https://doi.org/10.1016/J.COPH.2006.11.005

Deeks, S. G., Tracy, R., & Douek, D. C. (2013). Systemic Effects of Inflammation on Health during Chronic HIV Infection. *Immunity*, *39*(4), 633. https://doi.org/10.1016/J.IMMUNI.2013.10.001

Doms, R. W., & Moore, J. P. (2000). HIV-1 Membrane Fusion: Targets of Opportunity. *The Journal of Cell Biology*, *151*(2), f9. https://doi.org/10.1083/JCB.151.2.F9

Du Plessis, S., Vink, M., Joska, J. A., Koutsilieri, E., Stein, D. J., & Emsley, R. (2014). HIV infection and the fronto-striatal system: A systematic review and meta-analysis of fMRI studies. *AIDS*, *28*(6), 803–811. https://doi.org/10.1097/QAD.0000000000000151

Du Preez, A., Law, T., Onorato, D., Lim, Y. M., Eiben, P., Musaelyan, K., … Fernandes, C. (2020). The type of stress matters: repeated injection and permanent social isolation stress in male mice have a differential effect on anxiety- and depressive-like behaviours, and associated biological alterations. *Translational Psychiatry 2020 10:1*, *10*(1), 1–17. https://doi.org/10.1038/s41398-020-01000-3

Dubyak, G. R. (2004). Ion homeostasis, channels, and transporters: an update on cellular mechanisms. *Advances in Physiology Education*, *28*(1–4), 143–154. https://doi.org/10.1152/ADVAN.00046.2004

Dulin, N. O. (2020). Calcium-Activated Chloride Channel ANO1/TMEM16A: Regulation

of Expression and Signaling. *Frontiers in Physiology*, *0*, 1428. https://doi.org/10.3389/FPHYS.2020.590262

E, Masliah, N, Ge, M, Morey, R, DeTeresa, RD Terry, C. W. (1992). Cortical dendritic pathology in human immunodeficiency virus encephalitis. *Laboratory Investigation*, *66*(3), 285–291.

El-Hage, N., Bruce-Keller, A. J., Yakovleva, T., Bazov, I., Bakalkin, G., Knapp, P. E., & Hauser, K. F. (2008). Morphine Exacerbates HIV-1 Tat-Induced Cytokine Production in Astrocytes through Convergent Effects on [Ca2+]i, NF-κB Trafficking and Transcription. *PLoS ONE*, *3*(12). https://doi.org/10.1371/JOURNAL.PONE.0004093

El-Hage, N., Gurwell, J. A., Singh, I. N., Knapp, P. E., Nath, A., & Hauser, K. F. (2005). Synergistic increases in intracellular Ca 2+ , and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat ‡, *50*(2), 91–106. https://doi.org/10.1002/glia.20148

Eugenin, E. A., King, J. E., Hazleton, J. E., Major, E. O., Bennett, M. V. L., Zukin, R. S., & Berman, J. W. (2011). Differences in NMDA receptor expression during human development determine the response of neurons to HIV-Tat-mediated neurotoxicity. *Neurotoxicity Research*, *19*(1), 138–148. https://doi.org/10.1007/s12640-010-9150-x

F Ferrini, T Trang, TA Mattioli, S Laffray, T Del'Guidice, LE Lorenzo, A Castonguay, N Doyon, W Zhang, AG Godin, D Mohr, S Beggs, K Vandal, JM Beaulieu, CM Cahill, MW Salter, Y. D. K. (2013). Morphine hyperalgesia gated through microglia-mediated disruption of neuronal Cl^- homeostasis. *Nature Neuroscience*, *16*(2),

183–192. https://doi.org/10.1038/NN.3295

Fernandes, J. R., Berthoud, T. K., Kumar, A., & Angel, J. B. (2017). IL-23 signaling in Th17 cells is inhibited by HIV infection and is not restored by HAART: Implications for persistent immune activation. *PLoS ONE*, *12*(11). https://doi.org/10.1371/JOURNAL.PONE.0186823

Ferrini, F., Lorenzo, L.-E., Godin, A. G., Quang, M. Le, & De Koninck, Y. (2017). Enhancing KCC2 function counteracts morphine-induced hyperalgesia. *Scientific Reports 2017 7:1*, *7*(1), 1–8. https://doi.org/10.1038/s41598-017-04209-3

Fieblinger, T. (2021). Striatal Control of Movement: A Role for New Neuronal (Sub-) Populations? *Frontiers in Human Neuroscience*, *15*, 419. https://doi.org/10.3389/FNHUM.2021.697284/BIBTEX

Filipi, M., & Jack, S. (2020). Interferons in the Treatment of Multiple Sclerosis: A Clinical Efficacy, Safety, and Tolerability Update. *International Journal of MS Care*, *22*(4), 165. https://doi.org/10.7224/1537-2073.2018-063

Fitting, S., Knapp, P. E., Zou, S., Marks, W. D., Scott Bowers, M., Akbarali, H. I., & Hauser1, X. F. (2014). Interactive HIV-1 tat and morphine-induced synaptodendritic injury is triggered through focal disruptions in Na^+ influx, Mitochondrial instability, and Ca^{2+} overload. *Journal of Neuroscience*, *34*(38), 12850–12864. https://doi.org/10.1523/JNEUROSCI.5351-13.2014

Fitting, S., Scoggins, K. L., Xu, R., Dever, S. M., Knapp, P. E., Dewey, W. L., & Hauser, K. F. (2012). Morphine efficacy is altered in conditional HIV-1 Tat transgenic mice. *European Journal of Pharmacology*, *689*(1–3), 96. https://doi.org/10.1016/J.EJPHAR.2012.05.029

Fitting, S., Stevens, D. L., Khan, F. A., Scoggins, K. L., Enga, R. M., Beardsley, P. M., … Hauser, K. F. (2016). Morphine Tolerance and Physical Dependence Are Altered in Conditional HIV-1 Tat Transgenic Mice. *The Journal of Pharmacology and Experimental Therapeutics*, *356*(1), 96–105. https://doi.org/10.1124/jpet.115.226407

Fitting, S., Xu, R., Bull, C., Buch, S. K., El-Hage, N., Nath, A., … Hauser, K. F. (2010). Interactive comorbidity between opioid drug abuse and HIV-1 Tat: Chronic exposure augments spine loss and sublethal dendritic pathology in striatal neurons. *American Journal of Pathology*, *177*(3), 1397–1410. https://doi.org/10.2353/ajpath.2010.090945

Franceschi, C., & Campisi, J. (2014). Chronic Inflammation (Inflammaging) and Its Potential Contribution to Age-Associated Diseases. *The Journals of Gerontology: Series A*, *69*(Suppl_1), S4–S9. https://doi.org/10.1093/GERONA/GLU057

Frolich, M., Hofman, A., Jolles, J., Breteler, M. M., & Westendorp, R. G. J. (2007). Systemic markers of inflammation and cognitive decline in old age. *Journal of the American Geriatrics Society*, *55*(5), 708–724. https://doi.org/10.1111/j.1532

Gagnon, M, MJ Bergeron, G Lavertu, A Castonguay, S Tripathy, RP Bonin, J Perez-Sanchez, D Boudreau, B Wang, L Dumas, I Valade, K Bachand, M Jacob-Wagner, C Tardif, I Kianicka, P Isenring, G Attardo, JA Coull, Y. D. K. (2013). Chloride extrusion enhancers as novel therapeutics for neurological diseases. *Nature Medicine*, *19*(11), 1524–1528. https://doi.org/10.1038/NM.3356

Gagnon, D., Petryszyn, S., Sanchez, M. G., Bories, C., Beaulieu, J. M., De Koninck, Y., … Parent, M. (2017). Striatal Neurons Expressing D1 and D2 Receptors are

Morphologically Distinct and Differently Affected by Dopamine Denervation in Mice. *Scientific Reports 2017 7:1*, *7*(1), 1–16. https://doi.org/10.1038/srep41432

Galanopoulou, A. S., Kyrozis, A., Claudio, O. I., Stanton, P. K., & Moshé, S. L. (2003). Sex-specific KCC2 expression and GABAA receptor function in rat substantia nigra. *Experimental Neurology*, *183*(2), 628–637. https://doi.org/10.1016/S0014-4886(03)00213-9

Gelman, B. B., Chen, T., Lisinicchia, J. G., Soukup, V. M., Carmical, J. R., Starkey, J. M., … Morgello, S. (2012). The National NeuroAIDS Tissue Consortium Brain Gene Array: Two Types of HIV-Associated Neurocognitive Impairment. *PLOS ONE*, *7*(9), e46178. https://doi.org/10.1371/JOURNAL.PONE.0046178

Gelman, B. B., Lisinicchia, J. G., Chen, T., Johnson, K. M., Jennings, K., Freeman, D. H., & Soukup, V. M. (2012). Prefrontal Dopaminergic and Enkephalinergic Synaptic Accommodation in HIV-associated Neurocognitive Disorders and Encephalitis. *Journal of Neuroimmune Pharmacology 2012 7:3*, *7*(3), 686–700. https://doi.org/10.1007/S11481-012-9345-4

Gendelman, H. E., Orenstein, J. M., Baca, L. M., Weiser, B., Burger, H., Kalter, D. C., & Meltzer, M. S. (1989). The macrophage in the persistence and pathogenesis of HIV infection. *AIDS*. https://doi.org/10.1097/00002030-198908000-00001

Georges, F., Stinus, L., & Le Moine, C. (2000). Mapping of c-fos gene expression in the brain during morphine dependence and precipitated withdrawal, and phenotypic identification of the striatal neurons involved. *The European Journal of Neuroscience*, *12*(12), 4475–4486. https://doi.org/10.1046/J.0953-816X.2000.01334.X

Gerfen, C. R., & Surmeier, D. J. (2011). Modulation of striatal projection systems by dopamine. *Annual Review of Neuroscience*, *34*, 441. https://doi.org/10.1146/ANNUREV-NEURO-061010-113641

Gillen, J., Ondee, T., Gurusamy, D., Issara-Amphorn, J., Manes, N. P., Yoon, S. H., … Nita-Lazar, A. (2021). LPS tolerance inhibits cellular respiration and induces global changes in the macrophage secretome. *Biomolecules*, *11*(2), 1–19. https://doi.org/10.3390/biom11020164

Gonek, M., Mclane, V. D., Stevens, D. L., Lippold, K., Akbarali, H. I., Knapp, P. E., … Paris, J. J. (2018). CCR5 mediates HIV-1 Tat-induced neuroinflammation and influences morphine tolerance, dependence, and reward. *Brain Behav Immun*, *69*, 124–138. https://doi.org/10.1016/j.bbi.2017.11.006

Guneykaya, D., Ivanov, A., Hernandez, D. P., Haage, V., Wojtas, B., Meyer, N., … Wolf, S. A. (2018). Transcriptional and Translational Differences of Microglia from Male and Female Brains. *Cell Reports*, *24*(10), 2773-2783.e6. https://doi.org/10.1016/J.CELREP.2018.08.001/ATTACHMENT/A9C31F9C-687C-4A82-B502-8DCB5DF30B6B/MMC5.XLS

Guo, C. J., Li, Y., Tian, S., Wang, X., Douglas, S. D., & Ho, W. Z. (2002). Morphine Enhances HIV Infection of Human Blood Mononuclear Phagocytes through Modulation of β-Chemokines and CCR5 Receptor. *Journal of Investigative Medicine : The Official Publication of the American Federation for Clinical Research*, *50*(6), 435. https://doi.org/10.1136/JIM-50-06-03

Hahn, Yun K, Paris, J. J., Lichtman, A. H., Hauser, K. F., Sim-Selley, L. J., Selley, D. E., & Knapp, P. E. (2016). Central HIV-1 Tat exposure elevates anxiety and fear

conditioned responses of male mice concurrent with altered mu-opioid receptor-mediated G-protein activation and β-arrestin 2 activity in the forebrain HHS Public Access. https://doi.org/10.1016/j.nbd.2016.01.014

Hahn, Yun Kyung, Podhaizer, E. M., Farris, S. P., Miles, M. F., Hauser, K. F., & Knapp, P. E. (2015). Effects of chronic HIV-1 Tat exposure in the CNS: heightened vulnerability of males versus females to changes in cell numbers, synaptic integrity, and behavior. *Brain Structure & Function*, *220*(2), 605. https://doi.org/10.1007/S00429-013-0676-6

Haughey, N. J., Nath, A., Mattson, M. P., Slevin, J. T., & Geiger, J. D. (2001). HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *Journal of Neurochemistry*, *78*(3), 457–467. https://doi.org/10.1046/J.1471-4159.2001.00396.X

Hauser, K. F., Fitting, S., Dever, S. M., Podhaizer, E. M., & Knapp, P. E. (2005). *Opiate Drug Use and the Pathophysiology of NeuroAIDS*. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3431547/pdf/CHIVR-10-435.pdf

Hauser, K. F., Hahn, Y. K., Adjan, V. V., Zou, S., Buch, S. K., Nath, A., … Knapp, P. E. (2009). HIV-1 Tat and morphine have interactive effects on oligodendrocyte survival and morphology. *Glia*, *57*(2), 194–206. https://doi.org/10.1002/GLIA.20746

Heaton, R. K., Clifford, D. B., Franklin, D. R., Woods, S. P., Ake, C., Vaida, F., … Grant, I. (2010). HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology*, *75*(23), 2087. https://doi.org/10.1212/WNL.0B013E318200D727

Hermes, D. J., Jacobs, I. R., Key, M. C., League, A. F., Yadav-Samudrala, B. J., Xu, C.,

… Fitting, S. (2020). Escalating morphine dosing in HIV-1 Tat transgenic mice with sustained Tat exposure reveals an allostatic shift in neuroinflammatory regulation accompanied by increased neuroprotective non-endocannabinoid lipid signaling molecules and amino acids. *Journal of Neuroinflammation*, *17*(1). https://doi.org/10.1186/S12974-020-01971-6

Holmes, C., Cunningham, C., Zotova, E., Woolford, J., Dean, C., Kerr, S., … Perry, V. H. (2009). Systemic inflammation and disease progression in Alzheimer disease. *Neurology*, *73*(10), 768. https://doi.org/10.1212/WNL.0B013E3181B6BB95

Hu, X.-T. (2016). HIV-1 Tat-Mediated Calcium Dysregulation and Neuronal Dysfunction in Vulnerable Brain Regions. *Current Drug Targets*, *17*(1), 4. Retrieved from /pmc/articles/PMC4772427/

Irollo, E., Luchetta, J., Ho, C., Nash, B., & Meucci, O. (2021). Mechanisms of neuronal dysfunction in HIV-associated neurocognitive disorders. *Cellular and Molecular Life Sciences 2021 78:9*, *78*(9), 4283–4303. https://doi.org/10.1007/S00018-021-03785-Y

Iwasaki-Sekino, A., Mano-Otagiri, A., Ohata, H., Yamauchi, N., & Shibasaki, T. (2009). Gender differences in corticotropin and corticosterone secretion and corticotropin-releasing factor mRNA expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala in response to footshock stress or psychological stress in rats. *Psychoneuroendocrinology*, *34*(2), 226–237. https://doi.org/10.1016/J.PSYNEUEN.2008.09.003

Jaeger, L. B., & Nath, A. (2012, May). Modeling HIV-associated neurocognitive disorders in mice: New approaches in the changing face of HIV neuropathogenesis.

DMM Disease Models and Mechanisms. https://doi.org/10.1242/dmm.008763

Ji, Q., Guo, S., Wang, X., Pang, C., Zhan, Y., Chen, Y., & An, H. (2019). Recent advances in TMEM16A: Structure, function, and disease. *Journal of Cellular Physiology*, *234*(6), 7856–7873. https://doi.org/10.1002/JCP.27865

Kahle, K. T., Deeb, T. Z., Puskarjov, M., Silayeva, L., Liang, B., Kaila, K., & Moss, S. J. (2013). Modulation of neuronal activity by phosphorylation of the K–Cl cotransporter KCC2. *Trends in Neurosciences*, *36*(12), 726. https://doi.org/10.1016/J.TINS.2013.08.006

Kannan, A. K., Su, Z., Gauvin, D. M., Paulsboe, S. E., Duggan, R., Lasko, L. M., … Gauld, S. B. (2019). IL-23 induces regulatory T cell plasticity with implications for inflammatory skin diseases. *Scientific Reports 2019 9:1*, *9*(1), 1–8. https://doi.org/10.1038/s41598-019-53240-z

Kim, B. O., Liu, Y., Ruan, Y., Xu, Z. C., Schantz, L., & He, J. J. (2003). Neuropathologies in Transgenic Mice Expressing Human Immunodeficiency Virus Type 1 Tat Protein under the Regulation of the Astrocyte-Specific Glial Fibrillary Acidic Protein Promoter and Doxycycline. *The American Journal of Pathology*, *162*(5), 1693. https://doi.org/10.1016/S0002-9440(10)64304-0

Kim, S., Hahn, Y. K., Podhaizer, E. M., McLane, V. D., Zou, S., Hauser, K. F., & Knapp, P. E. (2018). A central role for glial CCR5 in directing the neuropathological interactions of HIV-1 Tat and opiates. *Journal of Neuroinflammation*, *15*(1), 285. https://doi.org/10.1186/s12974-018-1320-4

Koob, G. F., & Volkow, N. D. (2010). Neurocircuitry of Addiction. *Neuropsychopharmacology*, *35*(1), 217. https://doi.org/10.1038/NPP.2009.110

Koumangoye, R., Bastarache, L., & Delpire, E. (2020). NKCC1: Newly Found as a Human Disease-Causing Ion Transporter. *Function*, *2*(1), 28. https://doi.org/10.1093/FUNCTION/ZQAA028

Kudryavtseva, N. N., Gerrits, M. A. F. M., Avgustinovich, D. F., Tenditnik, M. V., & Van Ree, J. M. (2004). Modulation of anxiety-related behaviors by μ- And κ-opioid receptor agonists depends on the social status of mice. *Peptides*, *25*(8), 1355–1363. https://doi.org/10.1016/j.peptides.2004.05.005

Lanoue, A. C., Dumitriu, A., Myers, R. H., & Soghomonian, J. J. (2010). Decreased glutamic acid decarboxylase mRNA expression in prefrontal cortex in Parkinson's disease. *Experimental Neurology*, *226*(1), 207. https://doi.org/10.1016/J.EXPNEUROL.2010.09.001

Le, S. C., Correspondence, Y., Yang, L. &, & Yang, H. (2020). An Additional Ca 2+ Binding Site Allosterically Controls TMEM16A Activation . *CellReports*, *33*, 108570. https://doi.org/10.1016/j.celrep.2020.108570

Lee-Hotta, S., Uchiyama, Y., & Kametaka, S. (2019). Role of the BDNF-TrkB pathway in KCC2 regulation and rehabilitation following neuronal injury: A mini review. https://doi.org/10.1016/j.neuint.2019.04.003

Lee, H. H. C., Deeb, T. Z., Walker, J. A., Davies, P. A., & Moss, S. J. (2011). NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor mediated currents. *Nature Neuroscience*, *14*(6), 736. https://doi.org/10.1038/NN.2806

Lee, H. H. C., Walker, J. A., Williams, J. R., Goodier, R. J., Payne, J. A., & Moss, S. J. (2007). Direct protein kinase C-dependent phosphorylation regulates the cell

surface stability and activity of the potassium chloride cotransporter KCC2. *Journal of Biological Chemistry*, *282*(41), 29777–29784. https://doi.org/10.1074/jbc.M705053200

Lee HH, R Jurd, S. M. (2010). Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2. *Molecular and Cellular Neurosciences*, *45*(2), 173–179. https://doi.org/10.1016/J.MCN.2010.06.008

Leibrand, C. R., Paris, J. J., Jones, A. M., Masuda, Q. N., Halquist, M. S., Kim, W.-K., … McRae, M. (2019). HIV-1 Tat and opioids act independently to limit antiretroviral brain concentrations and reduce blood-brain barrier integrity. *Journal of Neurovirology*, *25*(4), 560. https://doi.org/10.1007/S13365-019-00757-8

Li Ke-Xin, He Mu, Ye Wenlei, Simms Jeffrey, Gill Michael, Xiang Xuaner, Jan Yuh Nung, J. L. Y. (2019). TMEM16B regulates anxiety-related behavior and GABAergic neuronal signaling in the central lateral amygdala. *ELife*, *8*. https://doi.org/10.7554/ELIFE.47106

Li, Y., Merrill, J. D., Mooney, K., Song, L. I., Wang, X. U., Guo, C.-J., … Ho, W.-Z. (2003). Morphine Enhances HIV Infection of Neonatal Macrophages. https://doi.org/10.1203/01.PDR.0000074973.83826.4C

Liu, R., Wang, J., Liang, S., Zhang, G., & Yang, X. (2020). Role of NKCC1 and KCC2 in Epilepsy: From Expression to Function. *Frontiers in Neurology*, *0*, 1407. https://doi.org/10.3389/FNEUR.2019.01407

Liu, Y., Zhou, D., Feng, J., Liu, Z., Hu, Y., Liu, C., & Kong, X. (2018). HIV-1 Protein Tat1–72 Impairs Neuronal Dendrites via Activation of PP1 and Regulation of the CREB/BDNF Pathway. *Virologica Sinica*, *33*(3), 261.

https://doi.org/10.1007/S12250-018-0031-4

Lobo, M. K., & Nestler, E. J. (2011). The striatal balancing act in drug addiction: Distinct roles of direct and indirect pathway medium spiny neurons. *Frontiers in Neuroanatomy*, *0*(JULY), 41. https://doi.org/10.3389/FNANA.2011.00041/BIBTEX

Lobritz, M. A., Ratcliff, A. N., & Arts, E. J. (2010). HIV-1 Entry, Inhibitors, and Resistance. *Viruses*, *2*(5), 1069. https://doi.org/10.3390/V2051069

Loeser, R. F. (2010). Age-Related Changes in the Musculoskeletal System and the Development of Osteoarthritis. *Clinics in Geriatric Medicine*, *26*(3), 371. https://doi.org/10.1016/J.CGER.2010.03.002

Lotze, M. T., Matory, Y. L., Ettinghausen, S. E., Rayner, A. A., Sharrow, S. O., Seipp, C. A., … Rosenberg, S. A. (1985). In vivo administration of purified human interleukin 2. II. Half life, immunologic effects, and expansion of peripheral lymphoid cells in vivo with recombinant IL 2. *The Journal of Immunology*, *135*(4).

Macpherson, T., & Hikida, T. (2019). Role of basal ganglia neurocircuitry in the pathology of psychiatric disorders. *Psychiatry and Clinical Neurosciences*, *73*(6), 289–301. https://doi.org/10.1111/PCN.12830

Mahadevan, V., & Woodin, M. A. (2016). Regulation of neuronal chloride homeostasis by neuromodulators. *The Journal of Physiology*, *594*(10), 2593. https://doi.org/10.1113/JP271593

Mahajan, S. D., Aalinkeel, R., Sykes, D. E., Reynolds, J. L., Bindukumar, B., Fernandez, S. F., … Schwartz, S. A. (2008). Tight junction regulation by morphine and HIV-1 tat modulates blood-brain barrier permeability. *Journal of Clinical Immunology*, *28*(5), 528–541. https://doi.org/10.1007/S10875-008-9208-1

Marks, W. D., Paris, J. J., Barbour, A. J., Moon, J., Carpenter, V. J., McLane, V. D., … Hauser, K. F. (2021). HIV-1 Tat and Morphine Differentially Disrupt Pyramidal Cell Structure and Function and Spatial Learning in Hippocampal Area CA1: Continuous versus Interrupted Morphine Exposure. *ENeuro*, *8*(3). https://doi.org/10.1523/ENEURO.0547-20.2021

Marks, W. D., Paris, J. J., Schier, C. J., Denton, M. D., Fitting, S., Mcquiston, A. R., … Org, K. H. (2016). HIV-1 Tat causes cognitive deficits and selective loss of parvalbumin, somatostatin, and neuronal nitric oxide synthase expressing hippocampal CA1 interneuron subpopulations HHS Public Access. *J Neurovirol*, *22*(6), 747–762. https://doi.org/10.1007/s13365-016-0447-2

Martín, F., Laorden, M. L., & Milanés, M. V. (2009). Morphine withdrawal regulates phosphorylation of cAMP response element binding protein (CREB) through PKC in the nucleus tractus solitarius-A2 catecholaminergic neurons. *Journal of Neurochemistry*, *110*(5), 1422–1432. https://doi.org/10.1111/J.1471-4159.2009.06234.X

Masliah, E., Achim, C. L., Hansen, L. A., & Wiley, C. A. (1992). Selective Neuronal Vulnerability in HIV Encephalitis. *Journal of Neuropathology & Experimental Neurology*, *51*(6), 585–593. https://doi.org/10.1097/00005072-199211000-00003

Masliah, Eliezer, Heaton, R. K., Marcotte, T. D., Ellis, R. J., Wiley, C. A., Mallory, M., … Grant, I. (1997a). Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. HNRC Group. The HIV Neurobehavioral Research Center. *Annals of Neurology*, *42*(6), 963–972. https://doi.org/10.1002/ANA.410420618

Masliah, Eliezer, Heaton, R. K., Marcotte, T. D., Ellis, R. J., Wiley, C. A., Mallory, M., … Grant, I. (1997b). Dendritic injury is a pathological substrate for human immunodeficiency virus-related cognitive disorders. *Annals of Neurology*, *42*(6), 963–972. https://doi.org/10.1002/ana.410420618

McArthur, J. C., & Johnson, T. P. (2020). Chronic inflammation mediates brain injury in HIV infection: relevance for cure strategies. *Current Opinion in Neurology*, *33*(3), 397–404. https://doi.org/10.1097/WCO.0000000000000807

Meijer, M. K., Spruijt, B. M., Van Zutphen, L. F. M., & Baumans, V. (2006). Effect of restraint and injection methods on heart rate and body temperature in mice. *Laboratory Animals*.

Michaud, M., Balardy, L., Moulis, G., Gaudin, C., Peyrot, C., Vellas, B., … Nourhashemi, F. (2013). Proinflammatory Cytokines, Aging, and Age-Related Diseases. *Journal of the American Medical Directors Association*, *14*(12), 877–882. https://doi.org/10.1016/J.JAMDA.2013.05.009

Murphy, N. P., Lam, H. A., & Maidment, N. T. (2001). A comparison of morphine-induced locomotor activity and mesolimbic dopamine release in C57BL6, 129Sv and DBA2 mice. *Journal of Neurochemistry*, *79*(3), 626–635. https://doi.org/10.1046/J.1471-4159.2001.00599.X

Nakao, A. (2014). Temporal regulation of cytokines by the circadian clock. *Journal of Immunology Research*, *2014*. https://doi.org/10.1155/2014/614529

Nass, S. R., Hahn, Y. K., McLane, V. D., Varshneya, N. B., Damaj, M. I., Knapp, P. E., & Hauser, K. F. (2020). Chronic HIV-1 Tat exposure alters anterior cingulate cortico-basal ganglia-thalamocortical synaptic circuitry, associated behavioral

control, and immune regulation in male mice. *Brain, Behavior, & Immunity - Health*, *5*, 100077. https://doi.org/10.1016/J.BBIH.2020.100077

Neuhaus, J., Jacobs, D. R., Baker, J. V., Calmy, A., Duprez, D., Rosa, A. La, … Neaton, J. D. (2010). Markers of Inflammation, Coagulation and Renal Function Are Elevated in Adults with HIV Infection. *The Journal of Infectious Diseases*, *201*(12), 1788. https://doi.org/10.1086/652749

Nitsch, L., Schneider, L., Zimmermann, J., & Müller, M. (2021). Microglia-Derived Interleukin 23: A Crucial Cytokine in Alzheimer's Disease? *Frontiers in Neurology*, *12*, 503. https://doi.org/10.3389/FNEUR.2021.639353/BIBTEX

Nolting, T., Lindecke, A., Hartung, H. P., Koutsilieri, E., Maschke, M., Husstedt, I. W., … Arendt, G. (2012). Cytokine levels in CSF and neuropsychological performance in HIV patients. *Journal of NeuroVirology*, *18*(3), 157–161. https://doi.org/10.1007/S13365-012-0091-4/TABLES/2

Osuji, F. N., Onyenekwe, C. C., Ahaneku, J. E., & Ukibe, N. R. (2018). The effects of highly active antiretroviral therapy on the serum levels of pro-inflammatory and anti-inflammatory cytokines in HIV infected subjects 11 Medical and Health Sciences 1103 Clinical Sciences 11 Medical and Health Sciences 1107 Immunology. *Journal of Biomedical Science*, *25*(1), 1–8. https://doi.org/10.1186/S12929-018-0490-9/TABLES/5

Papaleo, F., & Contarino, A. (2006). Gender- and morphine dose-linked expression of spontaneous somatic opiate withdrawal in mice. *Behavioural Brain Research*, *170*(1), 110–118. https://doi.org/10.1016/J.BBR.2006.02.009

Parajuli, B., Horiuchi, H., Mizuno, T., Takeuchi, H., & Suzumura, A. (2015). CCL11

enhances excitotoxic neuronal death by producing reactive oxygen species in microglia. *Glia*, *63*(12), 2274–2284. https://doi.org/10.1002/GLIA.22892

Park, D. C., & Yeo, S. G. (2013). Aging. *Korean Journal of Audiology*, *17*(2), 39. https://doi.org/10.7874/KJA.2013.17.2.39

Payne, J. A. (1997). Functional characterization of the neuronal-specific K-Cl cotransporter: implications for [K+]oregulation. *Https://Doi.Org/10.1152/Ajpcell.1997.273.5.C1516*, *273*(5 42-5). https://doi.org/10.1152/AJPCELL.1997.273.5.C1516

Perrot-Sinal, T. S., Sinal, C. J., Reader, J. C., Speert, D. B., & Mccarthy, M. M. (2007). Sex differences in the chloride cotransporters, NKCC1 and KCC2, in the developing hypothalamus. *Journal of Neuroendocrinology*, *19*(4), 302–308. https://doi.org/10.1111/J.1365-2826.2007.01530.X

Perrot-Sinal, T. S., Sinal, C. J., Reader, J. C., Speert, D. B., & McCarthy, M. M. (2007). Sex Differences in the Chloride Cotransporters, NKCC1 and KCC2, in the Developing Hypothalamus. *Journal of Neuroendocrinology*, *19*(4), 302–308. https://doi.org/10.1111/J.1365-2826.2007.01530.X

Pisella, L. I., Gaiarsa, J. L., Diabira, D., Zhang, J., Khalilov, I., Duan, J. J., … Medina, I. (2019). Impaired regulation of KCC2 phosphorylation leads to neuronal network dysfunction and neurodevelopmental pathology. *Science Signaling*, *12*(603), 300. https://doi.org/10.1126/SCISIGNAL.AAY0300

Prescott, S. A., Sejnowski, T. J., & De Koninck, Y. (2006). Reduction of anion reversal potential subverts the inhibitory control of firing rate in spinal lamina I neurons: towards a biophysical basis for neuropathic pain. *Molecular Pain*, *2*, 32.

https://doi.org/10.1186/1744-8069-2-32

Quarmley, M. E., Nelson, B. D., Clarkson, T., White, L. K., & Jarcho, J. M. (2019). I Knew You Weren't Going to Like Me! Neural Response to Accurately Predicting Rejection Is Associated With Anxiety and Depression. *Frontiers in Behavioral Neuroscience*, *13*, 219. https://doi.org/10.3389/FNBEH.2019.00219/BIBTEX

Quock, R. M., & Brewer, A. L. (2019). Establishing a time course for spontaneous opioid withdrawal in male and female outbred mice using an abbreviated dependence paradigm. *The FASEB Journal*, *33*(S1), 663.6-663.6. https://doi.org/10.1096/FASEBJ.2019.33.1_SUPPLEMENT.663.6

Reddy, P. V. B., Pilakka-Kanthikeel, S., Saxena, S. K., Saiyed, Z., & Nair, M. P. N. (2012). Interactive effects of morphine on HIV infection: Role in HIV-associated neurocognitive disorder. *AIDS Research and Treatment*, *2012*. https://doi.org/10.1155/2012/953678

Rivera, C., Li, H., Thomas-Crusells, J., Lahtinen, H., Viitanen, T., Nanobashvili, A., … Saarma, M. (2002). BDNF-induced TrkB activation down-regulates the K+–Cl− cotransporter KCC2 and impairs neuronal Cl− extrusion. *Journal of Cell Biology*, *159*(5), 747–752. https://doi.org/10.1083/JCB.200209011

Rogers, T. J. (2020). Bidirectional Regulation of Opioid and Chemokine Function. *Frontiers in Immunology*, *11*, 94. https://doi.org/10.3389/FIMMU.2020.00094/BIBTEX

Sá, M. J., Madeira, M. D., Ruela, C., Volk, B., Mota-Miranda, A., & Paula-Barbosa, M. M. (2004). Dendritic changes in the hippocampal formation of AIDS patients: A quantitative Golgi study. *Acta Neuropathologica*, *107*(2), 97–110.

https://doi.org/10.1007/s00401-003-0781-3

Saylor, D., Dickens, A. M., Sacktor, N., Haughey, N., Slusher, B., Pletnikov, M., … McArthur, J. C. (2016). HIV-associated neurocognitive disorder — pathogenesis and prospects for treatment. *Nature Reviews Neurology*, *12*(4), 234–248. https://doi.org/10.1038/nrneurol.2016.27

Schier, C. J., Marks, W. D., Paris, J. J., Barbour, A. J., McLane, V. D., Maragos, W. F., … Hauser, K. F. (2017a). Selective vulnerability of striatal D2 versus D1 dopamine receptor-expressing medium spiny neurons in HIV-1 tat transgenic male mice. *Journal of Neuroscience*, *37*(23), 5758–5769. https://doi.org/10.1523/JNEUROSCI.0622-17.2017

Schier, C. J., Marks, W. D., Paris, J. J., Barbour, A. J., McLane, V. D., Maragos, W. F., … Hauser, K. F. (2017b). Selective Vulnerability of Striatal D2 versus D1 Dopamine Receptor-Expressing Medium Spiny Neurons in HIV-1 Tat Transgenic Male Mice. *The Journal of Neuroscience*, *37*(23), 5758–5769. https://doi.org/10.1523/JNEUROSCI.0622-17.2017

Sengupta, R., Burbassi, S., Shimizu, S., Cappello, S., Vallee, R. B., Rubin, J. B., & Meucci, O. (2009). Morphine Increases Brain Levels of Ferritin Heavy Chain Leading to Inhibition of CXCR4-Mediated Survival Signaling in Neurons. *Journal of Neuroscience*, *29*(8), 2534–2544. https://doi.org/10.1523/JNEUROSCI.5865-08.2009

Silayeva L, TZ Deeb, RM Hines, MR Kelley, MB Munoz, HH Lee, NJ Brandon, J Dunlop, J Maguire, PA Davies, S. M. (2015). KCC2 activity is critical in limiting the onset and severity of status epilepticus. *Proceedings of the National Academy of*

Sciences of the United States of America, *112*(11), 3523–3528. https://doi.org/10.1073/PNAS.1415126112

Simon, V., Ho, D. D., & Karim, Q. A. (2006). HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. *Lancet*, *368*(9534), 489. https://doi.org/10.1016/S0140-6736(06)69157-5

Steele, A. D., Henderson, E. E., & Rogers, T. J. (2003). Mu-opioid modulation of HIV-1 coreceptor expression and HIV-1 replication. *Virology*, *309*(1), 99–107. https://doi.org/10.1016/S0042-6822(03)00015-1

Tang, D, AH Qian, DD Song, Q. Ben, & WY, Yao, J Sun, WG Li, TL Xu, Y. Y. (2015). Role of the potassium chloride cotransporter isoform 2-mediated spinal chloride homeostasis in a rat model of visceral hypersensitivity. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *308*(9), G767–G778. https://doi.org/10.1152/AJPGI.00313.2014

Thompson, K. A., Cherry, C. L., Bell, J. E., & McLean, C. A. (2011). Brain Cell Reservoirs of Latent Virus in Presymptomatic HIV-Infected Individuals. *The American Journal of Pathology*, *179*(4), 1623. https://doi.org/10.1016/J.AJPATH.2011.06.039

Tweedie, D., Karnati, H. K., Mullins, R., Pick, C. G., Hoffer, B. J., Goetzl, E. J., … Greig, N. H. (2020). Time-dependent cytokine and chemokine changes in mouse cerebral cortex following a mild traumatic brain injury. *ELife*, *9*, 1–29. https://doi.org/10.7554/ELIFE.55827

Update, G. A. (2021). UNAIDS 2021.

Valdebenito, S., Castellano, P., Ajasin, D., & Eugenin, E. A. (2021). Astrocytes are HIV

reservoirs in the brain: A cell type with poor HIV infectivity and replication but efficient cell-to-cell viral transfer. *Journal of Neurochemistry*, *158*(2), 429–443. https://doi.org/10.1111/JNC.15336

Vanderschuren, L. J. M. J., Schoffelmeer, A. N. M., Mulder, A. H., & De Vries, T. J. (1999). Lack of Cross-Sensitization of the Locomotor Effects of Morphine in Amphetamine-Treated Rats. *Neuropsychopharmacology 1999 21:4*, *21*(4), 550–559. https://doi.org/10.1016/s0893-133x(99)00051-2

Villa, A., Gelosa, P., Castiglioni, L., Cimino, M., Rizzi, N., Pepe, G., … Maggi, A. (2018). Sex-Specific Features of Microglia from Adult Mice. *Cell Reports*, *23*(12), 3501. https://doi.org/10.1016/J.CELREP.2018.05.048

Volk, D. W., Austin, M. C., Pierri, J. N., Sampson, A. R., & Lewis, D. A. (2000). Decreased Glutamic Acid Decarboxylase67 Messenger RNA Expression in a Subset of Prefrontal Cortical γ-Aminobutyric Acid Neurons in Subjects With Schizophrenia. *Archives of General Psychiatry*, *57*(3), 237–245. https://doi.org/10.1001/ARCHPSYC.57.3.237

W Zou, BO Kim, BY Zhou, Y Liu, A Messing, J. H. (2007). Protection against human immunodeficiency virus type 1 Tat neurotoxicity by Ginkgo biloba extract EGb 761 involving glial fibrillary acidic protein. *The American Journal of Pathology*, *171*(6), 1923–1935. https://doi.org/10.2353/AJPATH.2007.070333

Wallet, C., Rovere, M. De, Assche, J. Van, Daouad, F., Wit, S. De, Gautier, V., … Schwartz, C. (2019). Microglial Cells: The Main HIV-1 Reservoir in the Brain. *Frontiers in Cellular and Infection Microbiology*, *9*, 362. https://doi.org/10.3389/FCIMB.2019.00362

Wang, H., Zou, L., Ma, K., Yu, J., Wu, H., Wei, M., & Xiao, Q. (2017). Cell-specific mechanisms of TMEM16A Ca2+-activated chloride channel in cancer. *Molecular Cancer 2017 16:1*, *16*(1), 1–17. https://doi.org/10.1186/S12943-017-0720-X

Wang, J., Ma, J., Charboneau, R., Barke, R., & Roy, S. (2011). Morphine inhibits murine dendritic cell IL-23 production by modulating Toll-like receptor 2 and Nod2 signaling. *The Journal of Biological Chemistry*, *286*(12), 10225–10232. https://doi.org/10.1074/JBC.M110.188680

Wang, Y. J., Hang, A., Lu, Y. C., Long, Y., Zan, G. Y., Li, X. P., … Liu, J. G. (2016). κ Opioid receptor activation in different brain regions differentially modulates anxiety-related behaviors in mice. *Neuropharmacology*, *110*, 92–101. https://doi.org/10.1016/j.neuropharm.2016.04.022

Wiersielis, K. R., Wicks, B., Simko, H., Cohen, S. R., Khantsis, S., Baksh, N., … Bangasser, D. A. (2016). Sex differences in corticotropin releasing factor-evoked behavior and activated networks. *Psychoneuroendocrinology*, *73*, 204. https://doi.org/10.1016/J.PSYNEUEN.2016.07.007

Wilen, C. B., Tilton, J. C., & Doms, R. W. (2012). HIV: Cell Binding and Entry. *Cold Spring Harbor Perspectives in Medicine*, *2*(8). https://doi.org/10.1101/CSHPERSPECT.A006866

Williams, D. W., Veenstra, M., Gaskill, P. J., Morgello, S., Calderon, T. M., & Berman, J. W. (2014). Monocytes Mediate HIV Neuropathogenesis: Mechanisms that Contribute to HIV Associated Neurocognitive Disorders. *Current HIV Research*, *12*(2), 85. https://doi.org/10.2174/1570162x12666140526114526

Xu, C., & Fitting, S. (2016). Inhibition of GABAergic Neurotransmission by HIV-1 Tat

and Opioid Treatment in the Striatum Involves μ-Opioid Receptors. *Frontiers in Neuroscience*, *10*, 497. https://doi.org/10.3389/fnins.2016.00497

Yager, L. M., Garcia, A. F., Wunsch, A. M., & Ferguson, S. M. (2015). The ins and outs of the striatum: role in drug addiction. *Neuroscience*, *301*, 529–541. https://doi.org/10.1016/j.neuroscience.2015.06.033

Yanguas-Casás, N. (2020). Physiological sex differences in microglia and their relevance in neurological disorders. *Neuroimmunology and Neuroinflammation*, *7*(1), 13–22. https://doi.org/10.20517/2347-8659.2019.31

Zaky, D. S. E., & El-Nahrery, E. M. A. (2016). Role of interleukin-23 as a biomarker in rheumatoid arthritis patients and its correlation with disease activity. *International Immunopharmacology*, *31*, 105–108. https://doi.org/10.1016/j.intimp.2015.12.011

Zelcer, N., Van De Wetering, K., Hillebrand, M., Sarton, E., Kuil, A., Wielinga, P. R., … Borst, P. (2005). Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(20), 7274–7279. https://doi.org/10.1073/PNAS.0502530102

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., … Wu, J. Q. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *Journal of Neuroscience*, *34*(36), 11929–11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014

Zhou, L., Rua, R., Ng, T., Vongrad, V., Ho, Y. S., Geczy, C., … Saksena, N. K. (2009). Evidence for predilection of macrophage infiltration patterns in the deeper midline and mesial temporal structures of the brain uniquely in patients with HIV-associated

dementia. *BMC Infectious Diseases*, *9*, 192. https://doi.org/10.1186/1471-2334-9-192

Zou, S., Fitting, S., Hahn, Y.-K., Welch, S. P., El-Hage, N., Hauser, K. F., & Knapp, P. E. (2011). Morphine potentiates neurodegenerative effects of HIV-1 Tat through actions at m-opioid receptor-expressing glia. *A JOURNAL OF NEUROLOGY*. https://doi.org/10.1093/brain/awr281