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KCC2: a novel therapeutic target to rescue GABAergic dysfunction and behavioral deficits induced by HIV and opiate use

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

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Abbreviations

Aβ	Amyloid β
AIDS	Acquired immunodeficiency syndrome
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANI	Asymptomatic neurocognitive impairment
ATP	Adenosine triphosphate
ARV	Antiretroviral
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BrAgg	Brain aggregate
$[Ca^{2+}]_i$	Intracellular Ca^{2+}
cART	Combined antiretroviral therapy
CCL2	C-C motif chemokine ligand 2
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
$[Cl^-]_i$	Intracellular Cl^-
CNPase	2’3’-cyclic-nucleotide 3’-phosphodiesterase
CPP	Conditioned place preference
CSF	Cerebrospinal fluid
CTD	C-terminal domain
CXCR4	C-X-C motif chemokine receptor type 4
D1R	Dopamine receptor D1
D2R	Dopamine receptor D2
DA	Dopamine
DAT	Dopamine transporter
dNTP	Deoxynucleoside triphosphate

DOR	δ-opioid receptor
DOX	Doxycycline
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GAD67	Glutamic acid decarboxylase 67
GABA	γ-amino butyric acid
$GABA_AR$	GABA type A receptor
$GABA_BR$	GABA type B receptor
GECI	Genetically encoded Ca^{2+} indicator
GEVI	Genetically encoded voltage indicator
GFAP	Glial fibrillary acidic protein
gp120	glycoprotein 120
gp41	glycoprotein 41
GSK3β	Glycogen synthase kinase 3β
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
hIPSC	Human induced pluripotent stem cells
HIV	Human immunodeficiency virus
HIV_{sup}	HIV-infected PBMC supernatant
hSyn	Human synapsin promoter
IDU	Injection drug use
IFNγ	Interferon γ
IL	Interleukin
IN	Integrase
INI	Integration inhibitors
IPSC	Inhibitory postsynaptic current
KCC2	K^+- Cl^- cotransporter 2
KOR	κ-opioid receptor
LIF	Leukemia inhibitory factor
LTR	Long terminal repeat

MAP2	Microtubule-associated protein 2
MAPK	Mitogen activated protein kinase
MNI	Mild neurocognitive impairment
MOR	μ-opioid receptor
MSN	Medium spiny neuron
MVC	Maraviroc
NAcc	Nucleus accumbens
neuroHIV	HIV-associated neuropathology
NFκB	Nuclear factor κ-light-chain enhancer of activated B cells
NKCC1	Na^+- K^+- $2Cl^-$ cotransporter
NMDAR	N-methyl-D-aspartate receptor
NNRTI	Non-nucleoside/tide reverse transcriptase inhibitor
NPC	Neural progenitor cell
NRTI	Nucleoside/tide reverse transcriptase inhibitor
NTD	N-terminal domain
p-TEFb	Protein transcription elongation factor-b
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PKC	Protein kinase C
PI	Protease inhibitor
PP1	Protein phosphatase 1
PWH	People infected with HIV
pS940	Phospho-serine 940
REST	RE1-silencing transcription factor
ROS	Reactive oxygen species
RT	Reverse transcriptase
SUD	Substance use disorder
TAR	Transactivation response element
Tat	Transactivator of transcription
TBI	Traumatic brain injury

TNF	Tumor necrosis factor
TrkB	Tropomyosin receptor kinase B
UNF	Uninfected, but activated PBMC supernatant
VTA	Ventral tegmental area

Abstract

KCC2: a novel therapeutic target to rescue GABAergic dysfunction and behavioral deficits induced by HIV and opiate use

By Aaron J. Barbour

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

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Department of Anatomy & Neurobiology

With adherence to combined antiretroviral therapy (cART), HIV infection can be considered a controllable chronic condition, but quality of life issues remain. The preeminent of which, HIV-associated neurocognitive disorders (HAND), encompasses an array of neurological complications and has persisted despite cART implementation. The symptoms of HAND can be exacerbated by opiate use, a common comorbidity for people infected with HIV (PWH). While neurons are not infected by HIV, they incur sublethal damage, with γ-amino butyric acid- (GABA)ergic function being particularly vulnerable to viral and inflammatory factors released by infected/affected glia. This dissertation presents studies on novel organoid and dissociated primary human CNS models of HAND, the latter of which showed diminished levels of K^+ - Cl^- cotransporter 2 (KCC2), a neuronal transporter that maintains low intracellular Cl^-, after exposure to HIV-1 and HIV proteins ± morphine. $GABA_AR$-mediated hyperpolarization is predicated upon activity of KCC2 and functional examination of these neurons revealed decreased hyperpolarization

and disinhibition in response to $GABA_AR$ activation due KCC2 loss. Additionally, we identified the mechanisms through which HIV-1 mediates KCC2 reduction: the HIV protein, transactivator of transcription (Tat), through activation of N-methyl-D-aspartate receptor (NMDAR), and the HIV protein, glycoprotein 120 (gp120), through a novel mechanism involving CCR5 activation. We also found that morphine acts through the μ opioid receptor (MOR) to dysregulate KCC2. Pharmacological maintenance of KCC2 with the KCC2 enhancer, CLP257, rescued HIV, Tat, and morphine effects on KCC2 and $GABA_AR$ activity.

Common neurological deficits in PWH include memory and motor dysfunction which are likely the manifestations of HIV-induced hippocampal and striatal degeneration. Thus, we expanded our *in vitro* results to the glial fibrillary acidic protein (GFAP)-driven, doxycycline(DOX)-inducible Tat-transgenic mouse model of HAND. No changes in KCC2 in the hippocampus were seen, but we did find significant Tat-induced loss of KCC2 in the striatum which was associated with locomotor abnormalities in these mice. We also rescued phosphorylation of serine 940-KCC2 leading to increased KCC2 membrane localization and restoration of baseline motor activity with oral gavage of the prodrug of CLP257, CLP290. Overall, our *in vitro* and *in vivo* results demonstrate KCC2 as a promising, novel therapeutic target to alleviate the symptoms associated with HAND ± opiate use.

Chapter 1: Introduction

The human immunodeficiency virus (HIV)

Worldwide, 37.9 million people are infected with HIV with 1.7 million new infections in 2018 (UNAIDS, 2019). HIV infects cells of the immune system and can lead to nadir T-cell counts below 200, a point at which someone is classified as having acquired immunodeficiency syndrome (AIDS). Both HIV subtypes (HIV-1, -2) are members of the lentivirus genus within the retrovirus family. HIV-1 is more transmissible and more prevalent worldwide than HIV-2 and is the focus of this dissertation. Four strains of HIV-1 have been identified: M, O, N, and P. The most prevalent is strain M, which consists of 10 clades (A, B, C, D, F, G, H, J, K), and circulating recombinant forms with clade B being the most prevalent subtype in America, Europe, and Australia (Gilbert et al., 2007).

HIV was first found to be the virus that causes AIDS in 1983 (Gallo et al., 1983) and later found to be derived from simian immunodeficiency virus (Gao et al., 1999, Bailes et al., 2003, Chen et al., 1997). Initial cases were found in vulnerable communities including homosexual men (Brennan and Durack, 1981), injection drug users (CDC, 1982b), and hemophiliacs (CDC, 1982a), reflecting the modes of HIV transmission. HIV can be passed through venous injection or exposure of mucous membrane or damaged tissue to certain bodily fluids (blood, semen, rectal fluids, vaginal fluids, and breast milk) of someone infected with HIV. Thus, HIV is most commonly transmitted by sexual intercourse and needle sharing. Initial treatments for HIV-infection primarily consisted of prophylaxis against opportunistic infections that arose due to reduced immune competence (CDC, 1997, Moore and Chaisson, 1996). Combined antiretroviral therapy

(cART), became the recommended treatment for HIV/AIDS patients in 1997 and dramatically shifted the progression of HIV (Gulick et al., 1997, Hammer et al., 1997, Moore and Chaisson, 1999). The World Health Organization estimates that 62% of PWH now have access to cART, which involves the administration of three or more antiretrovirals (ARVs) simultaneously. ARVs are designed to interfere with each stage in the life cycle of HIV. Until 2015, it was recommended to begin HIV+ patients on a cART regimen after their cluster of differentiation (CD) 4 count dropped below 350 cells/mL, but this was revised to begin cART as early as possible (UNAIDS, 2016). Implementation of cART dramatically reduced viral replication and the viral load in many patients' plasma to undetectable levels (<50 RNA copies/mL) and reduced the mortality rate of HIV by 50 – 80% in the first ten years of use (Delaney, 2006).

HIV structure and life cycle

The HIV membrane is comprised of a lipid bilayer and proteins crucial for binding and fusion with host cells, namely, glycoprotein 120 (gp120) and glycoprotein 41 (gp41). The core of the HIV virion houses the viral genome, two single stranded RNA molecules, and the viral proteins reverse transcriptase (RT) and integrase (IN) (Briggs et al., 2006). Following mucosal invasion via interactions with dendritic cells (Turville et al., 2005, Turville et al., 2004), HIV-1 has six major steps in its life cycle and, thus, there are six major classes of ARVs: (1) binding/fusion (entry inhibitors), (2) reverse transcription (Nucleoside/tide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs)), (3) integration (integration inhibitors (INIs)), (4) transcription/translation, (5) budding, and (6) proteolysis/maturation (protease inhibitors (PIs)). Viral entry is initiated with binding of the viral coat protein, gp120, to CD4, found

primarily on helper T cells and macrophages/monocytes. Bound gp120 induces a conformational change, exposing a binding site for either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) co-receptors, introducing the different tropisms of HIV (Alkhatib et al., 1996, Deng et al., 1996, Feng et al., 1996, Trkola et al., 1996). CCR5-tropic strains (R5-tropic) are the predominant HIV-1 strains for active transmission and replication and preferentially bind CCR5 receptors found on macrophages, monocytes and T-cells (Roos et al., 1992, Zhu et al., 1993, Schweighardt et al., 2004) and are the most common type detected in the brain (Strizki et al., 1996, Cunningham et al., 1997, Gorry et al., 2001). Some variants may shift co-receptor preference to CXCR4 (X4-tropic variants) found on T-cells at later stages of infection (Ho et al., 2007, Tasca et al., 2008, Ribeiro et al., 2006). Engagement of the co-receptor leads to a conformational change in gp41, fusing the viral and host membranes to allow for the insertion of the viral core. Entry inhibitors (maraviroc (MVC) and efurvitide) block the actions of gp120/gp41 to prevent CD4/co-receptor binding and viral and host membrane fusion. MVC is a CCR5 antagonist; efuvirtide binds gp41 preventing membrane fusion. After membrane fusion, partial uncoating of the capsid proteins of the core occurs, providing RT access to host deoxyribonucleoside triphosphates (dNTPs) to initiate reverse transcription of viral RNA to cDNA, while forming the pre-integration complex which can pass through the intact nuclear envelope (Forshey et al., 2002). NRTIs and NNRTIs target reverse transcription to halt the virus life cycle. NRTIs are analogs of dNTPs that can enter the catalytic site of RT; NNRTIs are allosteric modulators of RT (Sarafianos et al., 1999, Huang et al., 1998). Both inhibit RT activity, preventing conversion of viral RNA to cDNA. Host enzymes complete integration by repairing the

single strand gaps resulting in the HIV provirus, which can remain latent indefinitely (Folks et al., 1986), which is a feature common to lentiviruses. Activation of immune cells by the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) or nuclear factor of activated T-cells induce low level transcription of the integrated viral DNA from the U3 promoter located in the upstream long terminal repeat (LTR) (Nabel and Baltimore, 1987, Siekevitz et al., 1987, Duh et al., 1989, Kinoshita et al., 1998). Transactivator of transcription (Tat) and Rev are the first proteins transcribed and translated. Tat is necessary for efficient transcription of other viral proteins. Tat recruits the host cellular protein transcription elongation factor-b (p-TEFb) to viral transactivation response element (TAR) to enhance the activity of host RNA polymerase II (Tahirov et al., 2010, Barboric et al., 2001). Viral mRNAs are produced as numerous alternate splice forms. Smaller viral mRNAs can be transported directly out of the nucleus, while larger species require Rev binding of Rev response element on the env protein to create a nuclear export sequence (Daugherty et al., 2010). Once in the cytoplasm, viral mRNAs are translated into immature polypeptides by host machinery. Viral proteins congregate on the cellular membrane and begin to bud off of the host cell (Briggs et al., 2009, Carlson et al., 2010). Concurrent with or directly after budding, HIV-1 protease cleaves the immature polypeptides into mature proteins resulting in a mature infective virion (Pettit et al., 1994, de Marco et al., 2010). PIs do not affect the ability of HIV to replicate and escape host cells it has already infected, but do prevent affected virion from infecting new cells (Kohl et al., 1988).

HIV-associated neurocognitive disorders

Snider et al. (Snider et al., 1983) found that neurological impairments accompany AIDS. Early investigations of this phenomenon discovered encephalopathy in many HIV patients, demonstrating that HIV can harm the CNS (Masliah et al., 1992, Shaw et al., 1985). Neurological impairments can range from asymptomatic (ANI) to HIV-associated dementia (HAD), collectively termed HIV-associated neurocognitive disorders (HAND). Affected neurological domains from HIV damage can include verbal/language ability, attention/working memory, executive function, memory, speed of information processing, sensory perception, and motor skills (Antinori et al., 2007). ANI and mild neurocognitive impairment (MNI) are diagnosed as impairment in two cognitive domains with and without impairment to daily life activities, respectively. HAD requires marked impairment in two or more domains with significant daily functional impairment (Antinori et al., 2007, Saylor et al., 2016). While the introduction of cART has reduced the prevalence of HAD, ANI and MNI have increased with an overall prevalence of HAND remaining stable (Heaton et al., 2010). While effective cART can reduce HIV to nondetectable levels in the periphery (Autran et al., 1997, Komanduri et al., 1998, Lederman et al., 1998), the brain can act as a reservoir of HIV, which may be implicated in the persistent neurological effects of HIV infection (Gelman et al., 2013). The introduction of cART has also shifted domains of neurocognitive impairment. Pre-cART era patients had a significantly higher percent of impairment in tasks related to motor skills, cognitive speed, and verbal fluency, whereas, cART era patients had a higher percent impairment in processing speed, memory, and executive functioning (Heaton et al., 2010, Maki et al., 2015, Rubin et al., 2017). These changes reflect a shift from subcortical and white matter disruption to cortical impairment from pre-cART to cART eras (Cysique et al., 2004). In a 2008 review, Anthony and Bell

(Anthony and Bell, 2008) posit that a shift in pathology has occurred from the basal ganglia to the hippocampus, entorhinal cortex, and temporal cortex, reflecting the neurocognitive findings of the clinical data. cART has had subtle/variable effects on HAND partially due to variable blood brain barrier (BBB) penetrance of the ARVs used. More effective penetrance may help dampen some aspects underlying HAND, but some studies have found that those on cART with higher BBB penetrance have worsened neurological impairment (Marra et al., 2009). This may be a result of direct ARV neurotoxicity, potentially contributing to the continuation of HAND and the shift in affected brain areas and symptomology noted. *In vitro* studies on ARVs have found that some induce dendritic beading at physiologically relevant concentrations (Robertson et al., 2012) and can prevent microglial phagocytosis of amyloid β (Aβ) (Giunta et al., 2011) offering mechanisms by which certain ARVs may contribute to CNS pathology. Even with effective cART, the neurotoxic HIV protein Tat can be found in the cerebrospinal fluid (CSF) of PWH on cART and is correlated with worse performance in motor speed and information processing (Henderson et al., 2019).

The predominant theory underlying HIV entry into the CNS, dubbed the Trojan Horse Theory, posits that HIV crosses the BBB early after infection via infiltrating monocytes/macrophages and, possibly T cells. Specifically, infected CD14+/CD16+ monocytes are highly vulnerable to infection and critical for viral seeding of the CNS (Fischer-Smith et al., 2001, Ellery et al., 2007). Additionally, they have increased transmigration across the BBB due to HIV-induced C-C chemokine receptor type 2 (CCR2) elevation and heightened sensitivity to chemokine C-C motif ligand 2 (CCL2) (Williams et al., 2013). After entry, monocytes establish residence and produce virus

within the brain, which then infects susceptible CNS cells. Productive infection occurs in perivascular macrophages and microglia. There is also strong evidence showing infection of astrocytes (with unproductive viral replication) (Eugenin et al., 2011, Wiley et al., 1986, Deiva et al., 2006), and neural progenitor cells (NPCs) (Balinang et al., 2017, Lawrence et al., 2004, Schwartz et al., 2007, Skowronska et al., 2018). While microglia and macrophages are widely accepted to actively replicate and release infective HIV, both infected astrocytes and microglia also release neurotoxic HIV proteins. Further, even uninfected microglia and astrocytes respond to viral insult by shifting towards a proinflammatory phenotype. While neurons themselves are not infected, they incur damage through viral and proinflammatory factors released by infected/activated glia resulting in reduced axodendritic complexity, hyperexcitability, and possibly excitotoxicity. This neuronal damage resulting in disrupted circuitry, is thought to underlie the symptoms associated with HAND (Masliah et al., 1997).

The role of microglia and resident macrophages in HAND

Microglia are the resident immune cells of the CNS and play an integral role in synaptic and dendritic maintenance and, thus, facilitate neuronal circuitry through phagocytosis of cellular debris and synapses. Expression of CD4 and CCR5 begets microglial susceptibility to R5-tropic strains of HIV-1 infection (Gelman et al., 1997, Epstein and Gendelman, 1993). Along with perivascular macrophages and monocyte derived macrophages microglia are the primary sites of active replication in the brain. Viral DNA can be found in all three cell types (Wiley et al., 1986, Koenig et al., 1986) even with effective cART (Ko et al., 2019) and are thought to be sites of viral reservoir in the CNS (Wallet et al., 2019). At basal conditions, microglia have ramified morphology,

surveying synapses and extracellular matrix for debris, but microglia of the HIV-infected brain have a reactive, ameboid morphology (Everall et al., 2009). Pre-cART era, postmortem staining of human brain sections found increased interleukin-1 β (IL-1β) and tumor necrosis factor (TNF) in the cerebral cortex and white matter of HIV patients compared to controls. Microglial activation can be found even in patients receiving effective cART (Garvey et al., 2014). In addition to increased markers of reactive microglia/inflammation, HIV Tat-exposed microglia have reduced phagocytosis *in vitro* (Giunta et al., 2008) which may contribute to the accumulation of Aβ in the brains of cART era HIV patients, a hallmark of neurodegeneration and Alzheimer's Disease (Green et al., 2005).

Microglia are exposed to HIV proteins *in vivo* including, Tat, gp120, Vpr, and nef. *In vitro* studies have examined how these factors affect microglia and their cytokine/chemokine profile after exposure. Microglia respond to HIV-1 infection, and Tat or gp120 exposure with a proinflammatory phenotype releasing CCL2 , CCL4, CXCL10, interferon γ (IFNγ), C-X-C motif chemokine ligand type 8, IL-1β, TNF, CCL5, IL-6, and IL-8 *in vitro* which contributes to neuroinflammation and neurotoxicity in the HIV-infected brain (D'Aversa et al., 2004, Chivero et al., 2017, Jin et al., 2012, Sheng et al., 2000, El-Hage et al., 2015). Several of these factors are also upregulated in *in vivo* models of HAND including TNF and IL-1β (Chivero et al., 2017, Jin et al., 2012). Tat exposure can also induce glutamate release by microglia, directly contributing to hyperexcitability (Gupta et al., 2010). Overall, microglia are susceptible to HIV infection, serving as a reservoir of virus in the brain. They exhibit decreased phagocytic capacity, and release neurotoxic inflammatory mediators that are either directly neurotoxic, or that result in

secondary neurotoxic outcomes via the activation of astrocytes and other resident CNS cells. They are critical drivers of the neuronal dysfunction that underlies the symptoms associated with HAND.

The role of astrocytes in HAND

Astrocytes perform a myriad of functions including phagocytosis, cytokine/chemokine release, metabolic/trophic support to neurons, and synaptic cleft clearance. These functions become dysregulated as a result of viral and inflammatory insult. Some studies provided evidence that astrocytes can be infected by HIV, albeit, unproductively (Churchill et al., 2006). While astrocytes express no detectable CD4, evidence indicates that they can be infected by HIV *in vivo* (Tornatore et al., 1994, Ranki et al., 1995, Takahashi et al., 1996, An et al., 1999, Anderson et al., 2003, Churchill et al., 2009) and *in vitro* under specific conditions (Li et al., 2020). Lacking CD4, astrocytic infection requires an alternate route of viral integration or internalization. The generally accepted mechanism involves CD4-independent endocytosis (Chauhan et al., 2014, Hao and Lyman, 1999). A recently published study described in detail the mechanism underlying productive HIV infection in human fetal astrocytes (Li et al., 2020). Their proposed mechanism involves CD4-indpendent, CXCR4-dependent endocytosis of immature virion and escape of endolysosomal degradation to establish productive infection. Immature virions are vital to this proposed mechanism. Maturation of the HIV viral particle occurs soon after budding from the host cell, thus, an infected lymphocyte must be in close proximity to the astrocyte, which corresponds with earlier proposed mechanisms that require cell-to-cell contact (Nath et al., 1995, Li et al., 2015, Luo and He, 2015) and post mortem studies showing that infected astrocytes are primarily found

in close proximity to vasculature (whereby astrocytic end feet would be in close proximity to circulating, infected lymphocytes). While difficult to quantify, evidence from studies that combined immunohistochemistry for astrocytic and microglial markers, laser microdissection, and PCR demonstrated that as much as 19% of astrocytes contain HIV DNA and the frequency of astrocytic infection was correlated with severity of neurological complications (Churchill et al., 2009). There is still some dispute regarding the presence of astrocytic infection *in vivo* (Ko et al., 2019), but the small sample size in this study may suggest that astrocytic infection varies between PWH, and may be dependent on viral strains present and lymphocyte infiltration of the CNS. These variables exemplify some components of cellular and viral heterogeneity of HIV infection and disease progression which likely underlie the heterogeneity of HAND.

Regardless of how much infection occurs *in vivo*, astrogliosis has been well documented in postmortem tissue of HIV-1-infected subjects (Vitkovic and da Cunha, 1995, Desplats et al., 2013, Xing et al., 2009) and astrocytes show elevated release of proinflammatory cytokines/chemokines in response to HIV proteins *in vitro*. HIV-1 Tat and gp120 stimulate an inflammatory response and the release of IL1β, CCL2, IL6, TNFα, CCL5, and CCL2 (Wesselingh et al., 1997, Nottet et al., 1995, El-Hage et al., 2009, El-Hage et al., 2005, Weiss et al., 1999). Astrocytes also release stromal cell-derived factor 1 in response to IL1β released by HIV infected macrophages (Churchill et al., 2009). Astrocytes take up extracellular Tat (Ma and Nath, 1997) and infected astrocytes can release Tat and gp120 (Fan and He, 2016, Rahimian and He, 2016, Chauhan et al., 2003, Toggas et al., 1994). These factors, along with those released by microglia, contribute to neuroinflammation of the HIV-infected brain.

Beyond their immune roles, astrocytes participate in the tripartite synapse, clearing excess neurotransmitters and, in some cases, releasing neurotransmitters themselves to modulate neuronal plasticity (Nedergaard, 1994, Parpura et al., 1994, Araque et al., 1998, Wang et al., 2012a). Astrocytic excitatory amino acid transporter (EAAT) 1 and EAAT2 are the primary sources of glutamate clearance in the brain (Bergles and Jahr, 1997, Schousboe et al., 1977, Gundersen et al., 1995). EAAT2 is decreased after exposure to HIV proteins *in vitro* (Wang et al., 2003, Pappas et al., 1998) as well as in postmortem brain tissue of HIV-1 infected subjects (Xing et al., 2009). Resultant excess glutamate is then available to bind Ca^{2+} permeable channels such as N-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) contributing to aberrant neuronal activation and, thus, neuronal hyperexcitability.

The extreme metabolic demands of neuronal activity beget their reliance on astrocytic support in the form of lactate via the astrocyte-neuronal lactate shuttle. Lactate derived from astrocytes is released into the extracellular matrix for neurons to uptake to allow neuronal adenosine triphosphate (ATP) generation needed for synaptic transmission while bypassing the glycolytic pathway (Suzuki et al., 2011, Vilchez et al., 2007). Tat induced rises in Ca^{2+} were found to increase mitochondrial Ca^{2+} uniporter Ca^{2+} uptake in astrocytes, resulting in decreased glycolysis and lactate transport to neurons (Natarajaseenivasan et al., 2018). Thus, Tat-induced astrocytic misallocation of energy substrates may deprive neurons of the metabolic support needed for proper neuronal activity.

HIV-induced neuronal dysfunction

In addition to HIV/HIV protein-induced, glial-mediated neuroinflammation and neural homeostasis disruption, Tat and gp120 have direct neuronal targets. Tat has affinity for NMDAR (Haughey et al., 2001, Prendergast et al., 2002, Chandra et al., 2005, Self et al., 2004), and can activate AMPAR (Longordo et al., 2006, Fitting et al., 2014), voltage-gated L-type Ca^{2+} channels (Napier et al., 2014), and the transient receptor potential canonical channel (Peng et al., 2012), all of which lead to increased neuronal $[Ca^{2+}]_i$. In addition to dysregulating cell membrane Ca^{2+} channels, Tat induces release of Ca^{2+} through internal sources. Tat induction of inositol 1,4,5-triphosphate receptor and ryanodine receptor signaling pathways leads to increased $[Ca^{2+}]_i$ from endoplasmic reticulum stores (Haughey et al., 1999, Perry et al., 2010). External influx and internal release disrupts Ca^{2+} homeostasis leading to excessive activation, metabolic disruption, and neuronal death *in vitro* (Kim et al., 2018, Nath et al., 1996, Brailou et al., 2008, Cheng et al., 1998, Norman et al., 2007, Zou et al., 2011). gp120 targets vary depending on tropism (CXCR4, CCR5, or dual (CXCR4- and CCR5-tropic)) and CXCR4 and CCR5 can both be found on subpopulations of neurons. Thus, CXCR4, CCR5, and dual-tropic gp120 can also induce enhanced $[Ca^{2+}]_i$ and neuronal death *in vitro* (Podhaizer et al., 2012, Kaul et al., 2007, Xu et al., 2011). While neuronal death is not often seen *in vivo*, the pathways initiated by Tat and gp120 can lead to loss of synaptodendritic complexity, hyperexcitability, and circuitry disruption found in these models of HAND and regulate, along with glial-mediated inflammation and metabolic disruption, neuropathology seen in PWH.

The prevailing viewpoint on HIV-induced neuronal excitotoxicity and/or hyperexcitability has centered around excessive glutamate and neuronal $[Ca^{2+}]_i$, which

can result in a positive feedback loop, through increased $[Ca^{2+}]_i$ stimulating glutamate release, which, in turn, activates $[Ca^{2+}]_i$ permeable channels; a process that is further exacerbated by decreased astrocytic glutamate transport and increased microglial glutamate release. While increased excitation certainly tips the scale of neuronal excitatory-inhibitory balance, this only takes into account one side of the story. Increasing evidence of vulnerability of GABAergic markers and function has underscored the importance of loss of inhibition in HIV-induced excitatory-inhibitory imbalance. Human post-mortem brain gene expression array studies found downregulation of mRNA associated with GABAergic transmission in the neocortex and striatum of patients with HAND correlating with neurocognitive impairment (Gelman et al., 2012a, Buzhdygan et al., 2016). Corollaries to the changes in human gene expression have been found in DOX-inducible, GFAP-driven Tat transgenic mice. Mice expressing the Tat transgene (Tat+) showed decreased inhibitory synaptic markers in the hippocampus associated with deficits in spatial memory compared to those lacking the Tat transgene (Tat-) mice (Fitting et al., 2013). These results were expanded upon to demonstrate selective loss of parvalbumin+, nitric oxide synthase+, neuropeptide Y- interneurons (Marks et al., 2016). Additionally, Tat+ striatal GABAergic neurons, particularly dopamine receptor D2 (D2R)-expressing medium spiny neurons (MSNs) displayed reduced spine density and increased dendritic varicosities compared to their control counterparts (Fitting et al., 2010, Schier et al., 2017). Studies have also found disrupted GABA release and reduced amplitude and frequency of miniature induced post synaptic currents (mIPSC) from Tat exposure (Musante et al., 2010, Xu et al., 2016). These studies provide evidence of disinhibition through loss of GABAergic synapses and transmission after exposure to HIV

or HIV proteins and impetus to further study loss of inhibition in relation to the HIV-induced excitatory-inhibitory imbalance.

Opiate use and its impact on HAND progression

The United States is in the midst of an opioid epidemic with approximately 11 million people reported to have misused opioids in 2017 (SAMHSA, 2017). Opiates act by mimicking endogenous opioid ligands, activating the μ-opioid receptor (MOR), and δ-opioid receptor (DOR) and κ-opioid receptor (KOR) to a lesser extent. Due to their strong analgesic effects, opioids have been the primary source of prescription pain medication (National Academies of Sciences and Medicine, 2017). Activation of the classic reward pathway by opiates occurs through MOR activation and subsequent inactivation of GABAergic neurons resulting in disinhibition of ventral tegmental area (VTA) dopamine (DA)-ergic neurons and increased DA release in the nucleus accumbens (NAcc) (Johnson and North, 1992) which underlies the abuse liability of opiates.

HIV infection and substance use disorders (SUD) are interlinked epidemics. Injection drug use (IDU) contributed to 20% of recorded HIV cases in 2016 (NIDA, 2019) and people who inject drugs are 22 times more likely to acquire HIV (UNAIDS, 2019). Illicit drug use is not the only route through which PWH are exposed to opiates. Recent studies found that 36% of PWH had chronic pain diagnoses and up to 50% of PWH were prescribed opiates (Chilunda et al., 2019, Denis et al., 2019). Drug use is associated with poor cART adherence and increased viral load in the CSF (Denis et al., 2019, Canan et al., 2018, King et al., 2012). Clinical data has been inconsistent in establishing a clear link between opiate use and the progression of HAND. One consistent confound being that most drug abusers use more than one substance, limiting conclusions that can be drawn

about the effects of opiates themselves. Despite this, some evidence has linked opiate use to worsened neurocognitive progression and higher rates of dementia (Chiesi et al., 1996, Bell et al., 1998, Bell et al., 2002, Lucas et al., 2006, Byrd et al., 2011). Byrd and colleagues (Byrd et al., 2011) found an association between lifetime heroin use and poor recall association among PWH. Post mortem studies have also found that HIV encephalopathy indicators and markers of neuroinflammation were more prevalent in IDU than non-IDU HIV patients (Smith et al., 2014, Bell et al., 1998). Despite confounds, much of the literature associates higher prevalence and more rapid progression of neurocognitive impairment in HIV+ IDU than non-IDU which is in accordance with studies involving models of HAND and opiate exposure. Mechanisms underlying these changes involve convergence of cellular and molecular pathways that induce activation of viral reservoirs, enhanced cytokine and chemokine release, enhanced reductions of synaptodendritic complexity, increased glutamate release/decreased uptake, resulting in circuitry disruption and subsequent behavioral and cognitive deficits.

Accumulating evidence showing reward deficits in PWH (Anderson et al., 2016, Plessis et al., 2015) suggests that the HIV-exposed CNS may have altered responses to drugs of abuse, possibly increasing the likelihood of drug seeking behavior and relapse in PWH. Imaging and postmortem studies have shown disrupted DAergic systems, such as reduced DA, dopamine transporters (DAT), D2R, and DA reuptake in the striatum (Chang et al., 2008, Wang et al., 2004, Kumar et al., 2009, Ferris et al., 2010), suggesting that HIV targeting of DAergic systems may underlie changes in reward processing. Several studies utilizing the DOX-inducible, GFAP-driven Tat-transgenic (Tat-transgenic) mice have identified potential neuronal and molecular targets underlying these clinical

effects. Tat exposure decreased DA levels (Kesby et al., 2016), impaired DAT function (Ferris et al., 2009a), decreased stimulated DA release (Ferris et al., 2009b), and decreased DA receptor expression in these mice (Kesby et al., 2016). Acute Tat exposure also increased the rewarding effects of drugs of abuse measured by behavioral assessment (Paris et al., 2014a, McLaughlin et al., 2014). Together, these studies suggest HIV infection of the CNS may increase the risk of substance abuse by altering the rewarding properties of drugs of abuse through impairment of DAergic circuitry.

Opiates primarily exert their effects through activation of MOR, which can be found heterogeneously on neurons and glia. Opiates can increase HIV replication by decreasing IFN expression (Wang et al., 2012b) and susceptibility to infection by increasing CCR5 expression (Guo et al., 2002, Li et al., 2003). Morphine, the primary metabolite of heroin, can reduce EAAT1 and 2 *in vivo*, directly contributing to excess glutamate, and has an additive effect with Tat to decrease glutamate buffering and increase microglial glutamate release, *in vitro* and exacerbate reactive oxygen species (ROS) release in a MOR dependent manner (Zou et al., 2011, Gupta et al., 2010, Turchan-Cholewo et al., 2009). Morphine enhanced Tat effects can result in exacerbated neuronal death *in vitro* through glial MOR activation (Zou et al., 2011) and may be dependent on the presence of glial CCR5 (Kim et al., 2018). Elevated neurotoxicity due to gp120-morphine co-exposure is dependent on gp120 tropism. Morphine significantly enhanced dual CXCR4-CCR5 tropic (MN) gp120-induced neurotoxicity while having no effects on CCR5 (ADA) or CXCR4 (IIIB) toxicity (Podhaizer et al., 2012). Viral factors and morphine neurotoxic interactions may be mediated by glycogen synthase kinase 3β (GSK3β) signaling (Masvekar et al., 2014, Masvekar et al., 2015) and may be glial-mediated (Zou et al., 2011).

Neuroinflammation in the HIV-opiate co-exposed brain may be sustained by astrocytic-microglial crosstalk. Further, Tat and gp120-induced release of cytokines and chemokines are synergistically increased with morphine co-exposure (El-Hage et al., 2005, El-Hage et al., 2006, El-Hage et al., 2008). Combined inflammatory effects of morphine and Tat emerge from convergence in elevated [Ca^{2+}] and NFκB activation (El-Hage et al., 2008). Thus, morphine and HIV-induced inflammatory astrocytic response activates microglia to release proinflammatory factors resulting in a positive feedback loop creating a cycle of sustained neuroinflammation and hyperexcitability (Hauser et al., 2012).

KCC2

Preferential vulnerability of inhibitory neurotransmission to HIV-induced degeneration has been studied in regard to reduction of GABAergic machinery and synaptic markers (mRNA and protein) and frequency and amplitude of IPSCs. Other neurological disorders are increasingly finding GABAergic disruption through collapse of postsynaptic ionic gradients important for fast synaptic inhibition. Importantly, therapeutic efficacy has been demonstrated in targeting the transporters responsible for this ionic balance, such as K^+- Cl^- cotransporter-2 (KCC2).

Slc12a encodes 9 cation-Cl^- transporters, including the central neuron specific, KCC2 (Hebert et al., 2004). KCC2 is a 12 membrane-spanning protein that can exist as monomers, dimers, trimers, or tetramers (Uvarov et al., 2009). Alternative splicing of the KCC2 gene can give rise to two isoforms that differ by 40 N-terminal amino acid residues, KCC2a and KCC2b (Uvarov et al., 2007). The longer isoform, KCC2a, is found more prominently in the neonatal brain stem and spinal cord while KCC2b is strongly

upregulated during development and is the predominant isoform in the adult brain (~90% of KCC2 expression in mature cortex) (Uvarov et al., 2007, Uvarov et al., 2009). Studies have not noted a difference in function between the isoforms. Both are central neuron-specific (Uvarov et al., 2009) due to activity of the RE1-silencing transcription factor (REST) on the neuron restrictive silencer element upstream of the KCC2 promoter (Yeo et al., 2009).

KCC2 is an electroneutral secondary active transporter that utilizes the K^+ gradient established by Na^+-K^+ adenosine 5'-triphosphate-(ATP)ase to extrude Cl^-. This activity maintains low intracellular Cl^- ($[Cl^-]_i$) driving the influx of this anion via the ligand gated, Cl^- permeable $GABA_A$ and glycine receptors. Upon ligand binding, the inward rush of Cl^- leads to hyperpolarization and inhibition of the postsynaptic neuron. KCC2 maintains low (<10 mM) $[Cl^-]_i$ in mature mammalian neurons resulting in the reversal potential of GABAergic currents near resting membrane potential (Delpire and Staley, 2014). While setting the stage for postsynaptic hyperpolarization and inhibition through Cl^- regulation, KCC2 has also been implicated in excitatory transmission. It has somatodendritic localization and clusters around dendritic spines (Gulyas et al., 2001, Gauvain et al., 2011, Chamma et al., 2013) and through its interaction with cytoskeleton-associated protein 4.1 N KCC2 participates in stabilizing actin (Li et al., 2007) and is integral to dendritic spine formation and stability (Fiumelli et al., 2013), and, thus, is vital for glutamatergic plasticity. Taking into consideration its Cl^- extrusion capacity to increase GABAergic inhibition and its localization to spines, KCC2 is fundamental to the excitatory-inhibitory balance of neurons.

KCC2 is upregulated during development, due to brain derived neurotrophic factor (BDNF) activation of tropomyosin receptor kinase B (TrkB) on immature neurons, while a transporter with opposing Cl^- displacement, Na^+-K^+-2Cl^- cotransporter (NKCC1), is downregulated. This developmental shift was thought to result in the change of GABA from an excitatory to an inhibitory neurotransmitter (Rivera et al., 1999, Ben-Ari et al., 1989, Chudotvorova et al., 2005), although some data suggested that conditions of these studies produced confounds that resulted in GABAergic excitation (Bregestovski and Bernard, 2012). In particular, the metabolic changes that occur to neurons during the slicing process for *ex vivo* slice electrophysiology may result in the accumulation of neuronal Cl^- (Dzhala et al., 2012), thus, confounding experiments performed in these conditions. *In vivo* studies also demonstrated that application of $GABA_AR$ antagonists (gabazine and bicuculine) increased neuronal activity in rat pups (P3 - P5), suggesting inhibitory effects of $GABA_AR$ activation in the immature brain (Baram and Snead, 1990, Bernard and Axelrad, 1993). Some evidence from these studies has been refuted (Ben-Ari et al., 2012). In direct contradiction to the studies above, experiments demonstrated that in P1 – 5 rats GABA does, in fact, excite immature neurons of the intact hippocampus *in vitro* (Valeeva et al., 2013) and that, in immature slice preparations, physiological concentrations of lactate or pyruvate do not alter the depolarizing effects of GABA, suggesting that depolarizing GABA is not an artifact of inadequate energy supplies (Tyzio et al., 2011). Further, blockade of NKCC1 with bumetanide *in vivo* during the early post-natal period suppressed sharp waves in the hippocampus (Sipila et al., 2006), and attenuated electrographic seizures (Dzhala et al., 2005), suggesting depolarizing actions of GABA. These inconsistencies may be resolved through a suggested dual excitatory

and inhibitory role for GABA in the developing brain (Ben-Ari, 2014). This paradox may be due to shunting inhibition, whereby depolarizing GABA initially increases synchronous neuronal activation followed by reduced network activity by inhibiting depolarization above the GABA reversal potential, decreasing glutamate receptor currents (Khalilov et al., 1999). An attempt to finally resolve the role of GABA during development was made by Kirmse and colleagues (Kirmse et al., 2015), in which *in vivo* whole-cell voltage-clamp and 2-photon Ca^{2+} recordings of P3 - 5 mouse V1 neurons were made during puff application of GABA. Their voltage-clamp findings demonstrated that GABA is depolarizing in the majority of cells, but did not induce action potentials and, thus, lacked direct excitatory effects. *In vivo* Ca^{2+} recording revealed that application of GABA did not induce Ca^{2+} transients and that application of GABA agonist (benzodiazepine) and antagonist (gabazine) strongly inhibited and promoted spontaneous networks, respectively. Taken together, these data show that GABA is depolarizing, but inhibitory at the network level (likely through shunting inhibition) in the immature CNS (Kirmse et al., 2015). While there has been debate as to the exact postsynaptic effects of GABA in the developing CNS, it should be noted that a shift in neuronal expression pattern from NKCC1 dominant to KCC2 dominant has not been questioned, but rather the physiological consequences of this phenomenon.

Membrane localization of KCC2 is largely post-translationally regulated in mature neurons. With an average membrane turnover rate of 20 – 30 minutes (Lee et al., 2010, Rivera et al., 2004), its trafficking, membrane stability, and degradation are tightly regulated by several phosphorylation sites (Come et al., 2019). The KCC2 N-terminal domain (NTD) was demonstrated to be required for membrane trafficking through a study

involving NTD deletion and the C-terminal domain (CTD) is involved in membrane stability and contains the majority of phosphorylation sites (Friedel et al., 2017) . Phosphorylation of S932, T934, S937, S940 increase membrane stability while phosphorylation of T1009, T1007, T906, Y903, Y1087, decrease its stability and activity. Proteomic studies identified several other phosphorylation sites on KCC2, but whether they play a role in transportation and/or stability remains unknown (Cordshagen et al., 2018, Weber et al., 2014). Phosphorylation and consequential changes in KCC2 activity are mediated by several upstream pathways. One well-studied pathway involves protein kinase C (PKC) and protein phosphatase 1 (PP1)-dependent phosphorylation and dephosphorylation of serine 940 (S940), respectively. NMDAR activation and subsequent $[Ca^{2+}]_i$ increase leads to a molecular pathway involving PP1 dephosphorylation of S940 and its internalization, evidenced by KCC2 rescue via AP5 antagonism of NMDAR (Lee et al., 2011). In contrast, PKC phosphorylation of S940 increases its membrane stability and decreases clathrin-induced internalization (Lee et al., 2007). Conversely, phosphorylation of Y903 and Y1087 result in loss of KCC2 activity via clathrin dependent internalization and lysosomal degradation (Lee et al., 2010). Interestingly, both excitatory and inhibitory neurotransmission differentially regulate KCC2 membrane stability and activity. Postsynaptic excitation by glutamate decreases KCC2 membrane stability through NMDAR-associated mechanisms discussed above. GABA was recently shown to be an upstream regulator of KCC2 as well. Post synaptic increase in Cl^- reduced with-no-lysine 1 (WNK1) activity and subsequent phosphorylation of T1007, increasing its stability (Heubl et al., 2017). Thus, NMDAR-mediated Ca^{2+}-induced decrease in activity and $GABA_AR$-mediated Cl^--induced increase provides another mechanism by which neurons

can fine tune the balance of inhibition/excitation through KCC2 membrane stability within localized dendritic compartments.

The link between KCC2 and neurological disorders has grown steadily over the last two decades. Loss of KCC2 activity was first linked to models of epilepsy (Rivera et al., 2002) and mutations in Slca12 were later found to underlie febrile seizures and generalized epilepsy in small cohorts of humans (Puskarjov et al., 2014, Kahle et al., 2014). KCC2 loss precedes cyclothiazide-induced epileptiform activity, suggesting that it is the trigger setting the stage for seizures rather than a consequence of excessive NMDAR activation in these settings (Chen et al., 2017a). Hyperexcitability is a hallmark of many neuroinflammatory disorders. Evidence continues to accumulate linking KCC2 loss and subsequent Cl^- dysregulation as a mediator of hyperexcitability in several models of these diseases including Alzheimer's disease (Chen et al., 2017b), traumatic brain injury (TBI) (Lizhnyak et al., 2019), Huntington's (Dargaei et al., 2018), Rett syndrome (Tang et al., 2016), and neuropathic pain (Mapplebeck et al., 2019, Coull et al., 2005). A recent publication (Pisella et al., 2019) found that mice carrying a heterozygous KCC2 mutation to reduce its Cl^- extrusion capacity had 'Autism-like' phenotypes. Behaviors included abnormal ultrasonic vocalizations and social deficits which correlated with delayed GABAergic maturation.

Opiate use and dependence are well known to dysregulate both spinal and striatal circuitry. Loss of KCC2 activity was found in lamina 1 neurons of the spinal dorsal horn disinhibiting spinal nociceptive output, after chronic morphine exposure inducing hyperalgesia (Ferrini et al., 2013, Ferrini et al., 2017). In contrast to immature neurons, TrkB activation downregulates KCC2 in mature neurons, which was shown to be the

mechanism here. Pharmacological maintenance of KCC2 was demonstrated to restore morphine's analgesic effects in rats (Ferrini et al., 2017). During the development of morphine dependency, GABA switched from an inhibitory to excitatory signal in rats (Laviolette et al., 2004, Vargas-Perez et al., 2009). While not explicitly linked to KCC2, Cl^- dysregulation is a likely mechanism underlying this switch and, thus, KCC2 is also likely dysregulated.

Due to the increasing reports of KCC2 dysregulation in neurological diseases, Gagnon et al. (Gagnon et al., 2013) sought to design a compound that could maintain KCC2 activity. Through development of a novel KCC2 activity assay, they isolated CLP257, an arylmethylidine compound that restored Cl^- extrusion in neurons treated with BDNF to reduce KCC2 activity and rescued KCC2 membrane expression. To enable *in vivo* study of pharmacological reintroduction of Cl^- extrusion, a prodrug of CLP257, CLP290, was designed. CLP290 has a half-life of 5 h and is non-toxic with a maximum daily dose of 2,000 mg in rats (Gagnon et al., 2013). It was administered twice a day at 100 mg/kg to rats to reverse morphine-induced hyperalgesia (Ferrini et al., 2017) and at 50 mg/kg once a day to restore membrane expression and rescue rotarod performance after TBI (Lizhnyak et al., 2019). In mice, daily 35 mg/kg intraperitoneal injections of CLP290 restored stepping ability in spinal cord injured mice by restoring spinal cord inhibitory interneuron circuitry (Chen et al., 2018) and daily injections of 100 mg/kg to amyloid precursor protein knockouts rescued hippocampal inhibitory post synaptic current amplitude (Chen et al., 2017b).

KCC2 activity and turnover is tightly regulated through the activity of REST, TrkB, kinase, and phosphatase activity mediated by numerous upstream pathways that play

important physiological roles in healthy CNS development and activity. These events include a developmental GABAergic switch, maintaining low $[Cl^-]_i$ for postsynaptic hyperpolarization/inhibition, and maintenance of excitatory synapses and glutamatergic plasticity. These events can become dysregulated and contribute to hyperexcitability and circuit dysregulation under pathological conditions.

Chapter 2

In vitro primary human CNS models

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Introduction

In vitro models allow for the study of precise cellular and molecular pathways with control that may not be attainable using *in vivo* models. Rodent *in vitro* models have been instrumental in unravelling the mechanistic pathophysiology of many CNS disorders. Although, inherent drawbacks arise in the attempt to model human-specific diseases such as Alzheimer's Disease, Parkinson's Disease, and HIV-associated neurocognitive disorders (HAND) in nonhuman cells. Human induced pluripotent stem cells (hiPSCs) were first generated from reprogrammed human somatic skin cells (Takahashi et al., 2007, Yu et al., 2007). hiPSCs have since advanced to the point of induction and differentiation of physiologically active human neurons with the capability of driving neuronal subset differentiation. These induced neurons (iNeurons) are critical to model human-specific CNS diseases. As with any model, there are inherent weaknesses found in iNeurons, including lost cytoarchitecture, reduced intercellular interactions, and artificial differentiation/development among others. Thus, for every study one must determine which model(s) can provide the most effective route towards answering the question at-hand and must take into consideration the inherent strengths and weaknesses of each.

To attempt to restore aspects of cytoarchitecture and cellular diversity found *in vivo* while retaining the control provided by *in vitro* models, 3-dimensional (3D) organoid models have grown in favor. One early CNS organoid system, human brain aggregates (BrAgg), described by Pulliam and colleagues contain the major cellular subsets of the CNS in proportions similar to the human brain (40% neurons, 40% astrocytes, 15% oligodendrocytes, 5% microglia) (Pulliam et al., 1988, Pulliam et al., 1991, Pulliam et al., 1998). Recent advances have shown the promise of such brain organoid development. Long-term culturing of hiPSC-derived organoids show major characteristics of the developing cortex including apical-basal polarity, interkinetic nuclear migration, neuronal migration and six cortical layers and brain region-specific organoids (Qian et al., 2018, Qian et al., 2016).

Here, we sought to develop and characterize translationally relevant primary human CNS models. We developed a differentiation protocol for a novel primary mixed astrocyte-neuron culture model (hNeuron) and further characterized BrAgg, initially described by Pulliam et al. (Pulliam et al., 1988) and demonstrate their utility as a model for HAND. The use of primary cells derived from human fetal CNS tissue, maintains natural cellular development until the point of pregnancy termination and BrAgg maintains critical intercellular interactions and provides stability for long-term culture experiments.

Materials and Methods

Primary hNPC dissociated culture.

Human neural progenitor cells (hNPCs) were derived from human CNS tissue at 15 - 17 weeks gestation (Advanced Biosciences Resources; Alameda, CA). Tissue was

passed through 120 μM mesh, centrifuged and washed with rinse media (DMEM supplemented with 1.5% glucose, 1% amphotericin B (Thermo Fisher), 50 ng/mL gentamycin, and 1% Pen/Strep) twice, and dissociated cells were plated on poly-L-lysine (10 μg/mL) and laminin (2 μg/mL) coated flasks. hNPCs were maintained in NPC media: DMEM F12 supplemented with 0.6% glucose, 10% B27 (without Vit A; Thermo Fisher Scientific), 20 ng/mL fibroblast growth factor (FGF; R&D Systems, Minneapolis MN), 20 ng/mL Epidermal growth factor (EGF; EMD Millipore, Billerica, MA), 10 ng/mL leukemia inhibitory factor (LIF; EMD Millipore, Billerica, MA), 1% Pen/Strep, 5 mM HEPES (N-2-hydroxyethylpiperazine-N-3-ethane sulfonic acid; Thermo Fisher Scientific) with media changed every 3 or 4 days.

Dissociated neuronal differentiation.

Upon 80 - 90% confluency, hNPCs were detached using Accutase cell detachment solution (Millipore) and plated on poly-L-lysine (50 μg/mL) and laminin (100 μg/mL) coated 12-mm-diameter coverslips (immunostaining) or glass bottomed MatTek dishes (functional imaging) at 12,000 - 15,000 cells/coverslip in DMEM-F12 medium supplemented with 0.6% glucose, 10% B27 (without vitamin A; Thermo Fisher Scientific), 1% Pen/Strep, 5 mM HEPES (Thermo Fisher Scientific), and 10 ng/mL brain derived neurotrophic factor (BDNF; Sigma). After 7 DIV, medium was exchanged gradually (50% on days 7 and 9) and then fully (100% every 72 - 96 h) to BrainPhys™ (STEMCELL Technologies Inc, Vancouver, BC, CAN) supplemented with SM1 (STEMCELL Technologies Inc).

BrAgg culture.

BrAgg cultures were initiated and maintained using protocols established by Pulliam et al (Pulliam et al., 1988). Briefly, 15 – 17 wk human fetal CNS tissue (Advanced Biosciences Resources; Alameda CA) was passed through 120 μM mesh, centrifuged, and washed twice with rinse media. Cells were then transferred to Sigmacote^{TM} (Sigma) coated 25 mL DeLong flasks at 30 million cells per flask containing 5 mL growth media (DMEM supplemented with 0.75% glucose, 50 ng/mL gentamycin (Thermo Fisher), 10% fetal bovine serum (FBS), and 1% Pen/Strep). After 48 h, BrAgg were transferred to sigmacote-coated 50 mL DeLong flasks containing 10 mL exchange media (DMEM with 0.75% glucose, 50ng/mL gentamycin, 15% FBS, and 1% Pen/Strep). BrAgg were maintained rotating at 80 RPM in 10% CO2 and 37° C with 50% media change (exchange media) every 48 h.

HIV-1 propagation and HIV$_{sup}$ preparation.

HIV-1$_{BaL}$ was propagated using methods previously established (Balinang et al., 2017). Briefly, isolated peripheral blood mononuclear cells (PBMCs) from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) were activated with 1 mg/mL phytohemagglutinin (PHA) for 48 h, then infected with HIV-1$_{BaL}$ (NIH AIDS Reagent Program) at 1 ng/mL [p24]. After 72 h, infection was assayed using p24 antigen ELISA kit (Advanced Bioscience, Rockville, MD) and supernatant harvested and frozen at −80° C for HIV$_{sup}$ experiments. HIV$_{sup}$ used in all BrAgg experiments was from the same source sample to reduce variability.

Immunocytochemistry.

hNeuron were fixed with 4% paraformaldehyde for 15 min after 21 DIV. Coverslips were then permeablilized with 0.1% Triton-X 100 in 0.1% BSA and blocked for 1 h at room

temperature in 0.1% BSA and 1% goat serum. Cellular markers were detected by incubating for 1 h with antibodies specific for microtubule-associated protein 2 (MAP2) (1:500; Millipore, Burlington, MA), glial fibrillary acidic protein (GFAP; 1:1000; Millipore), GAD67 (1:500; Abcam), glutaminase (1:500, Abcam), tyrosine hydroxylase (TH; 1:1000; Abcam), MOR (1:500; Antibodies Incorporated; Davis, CA), KCC2 (1:1000, Protein Tech), or NKCC1 (1:100; Abcam). AlexaFluor 488 and 594-conjugated secondary antibodies (1:1000, Thermo Fisher) were used and Hoechst 33342 (1:10000; Invitrogen) detected nuclei.

BrAgg were fixed with 4% paraformaldehyde for 30 min, permeabilized for 30 min, and blocked for 1 h. BrAgg were incubated overnight with antibodies specific for MAP2, GFAP, Iba-1 (1:1000; Wako), GAD67, and 2'3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase; 1:500; Abcam) to determine cellular subsets present. AlexaFluor 488 and 594-conjugatged secondary antibodies and Hoeschst33342 were used.

Both hNeuron and BrAgg were visualized and images obtained with Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 and Zen 2010 software (Zeiss Inc.).

BrAgg infection with HIV-1$_{Ba-L}$.

To determine infectivity of BrAgg, we incubated cultures with varying concentrations of purified HIV-1$_{Ba-L}$ or HIV-1$_{Ba-L}$-infected PBMC supernatant (HIV$_{sup}$) during culture initiation. BrAgg supernatants were collected and frozen with HALT^{TM} Protease & Phosphatase inhibitor Cocktail (Thermo Fisher) to prevent protein

degradation at -80º C every 48 h. HIV-1 concentrations were then estimated using p24 antigen ELISA kit (Advanced Bioscience, Rockville, MD).

HIV_{sup} Preparation.

HIV-1_{BaL} was propagated using methods previously established (Balinang et al., 2017). Briefly, isolated PBMCs from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) were activated with 1 mg/mL PHA for 48 h, then infected with HIV-1_{BaL} (NIH AIDS Reagent Program) at 1 ng/mL p24. After 72 h, infection was assayed using p24 antigen ELISA kit (Advanced Bioscience, Rockville, MD) and supernatant harvested and frozen at −80° C for HIV_{sup} experiments. HIV_{sup} used in all experiments was from the same source sample to reduce variability.

Statistics.

Changes in BrAgg [p24] within subjects (across days) were assessed by repeated-measures analysis of variance (ANOVA) in R. Bonferroni post hoc analysis corrected for multiple comparisons after comparisons of simple main effects. Effects were considered significant when $p < 0.05$.

Results

hNPC differentiation and hNeuron characterization.

We developed a protocol to differentiate hNPCs into mature hNeurons. EGF, FGF, and LIF initially maintain hNPC pluripotency. These factors were replaced with BDNF to promote neuronal survival and differentiation for seven days. Next, cells were gradually transitioned to BrainPhys medium, which more accurately mimics *in vivo* physiological conditions, increases spontaneous and evoked action potentials, and increases

frequency and amplitude of spontaneous and induced inhibitory post synaptic potentials (IPSCs) compared to classic basal media (Bardy et al., 2015). After a total of 3 weeks *in vitro*, 53 ± 3.3% of cells are MAP2+/ GFAP- neurons and 44 ± 3.5% GFAP+ astrocytes (Fig. 2.1). Neurons were found to be primarily glutaminase+ (~75%), and glutamate decarboxylase+ (~25%), with few tyrosine hydroxylase+ (~1%) neurons (Fig. 2.2). Both neurons and glia present in these cultures were found to express MOR (Fig. 2.2). NKCC1 is expressed on immature neurons and opposes KCC2 activity by increasing neuronal $[Cl^-]_i$. Its expression decreased to undetectable levels by 21 days *in vitro* (DIV), while KCC2 expression increased, as expected from studies on neuronal maturation (Schulte et al., 2018) (Fig. 2.1). These characteristics suggested that hNeurons had matured enough to hyperpolarize in response to $GABA_AR$ activation, as evident in experiments performed in Chapter 3.

BrAgg characterization.

BrAgg were fixed and stained for cellular markers from five to 29 DIV. We detected the presence of astrocytes (GFAP+), neurons (MAP2+/GFAP-), oligodendrocytes (CNPase+), and microglia (Iba1+) (Fig. 2.2). Neurons and astrocytes were present by 5 DIV and make up a majority of the cellular subsets (~40% each) (Fig. 2.2). Oligodendrocytes and microglia make up the majority of the remaining cells present. Oligodendrocytes were detected at 21 DIV. Microglia were detected at 5 DIV (the earliest time point at which BrAgg were fixed and immunostained), but have distinct lineage from NPC derived neurons, astrocytes, and oligodendrocytes, appear in the human CNS by 5 gestational weeks and, thus, are present in BrAgg from culture initiation (Bertrand et al., 2005, Rezaie and Male, 1999). All microglia imaged have amoeboid morphology

suggesting a proinflammatory phenotype. The neurons present appear to be GABAergic, evidenced by detection of GAD67 on the majority of neurons visualized (Fig 2.2).

HIV-1_{Ba-L} infection of BrAgg.

We sought to develop a model of HAND that more accurately mimics the cytodiversity and chronic infective and inflammatory environment of the HIV-infected brain while retaining the high levels of control and manipulation afforded by *in vitro* models. HIV is capable of infecting microglia and astrocytes, thus, we wanted to create a model reflecting this aspect of the disease. In order to achieve this goal, we determined if we could gain active HIV-1_{Ba-L} infection of BrAgg. Challenges inherent to this process include determining the ideal paradigm (pure HIV-1_{Ba-L} vs. HIV_{sup}, concentration) to infect BrAgg and washing out potentially new infective virus with each media change. We exposed BrAgg to 0.5 – 2.5 ng of purified HIV-1_{BA-L} or HIV_{sup} (added to achieve a final concentration of 1 ng/mL [p24]). To tackle the issue of 'lost' virus at each media change (50% change every 48 h), we calculated the levels at which [p24] would be expected given day 1 concentrations and simply divided by two for each consecutive media change ([$p24_{exp}$]) and subsequently subtracted that from the observed [p24] of each sample ([$p24_{obs}$]) . For example, if [p24] on Day 1 was 10 pg/mL, then by Day 9 (after media changes on days 1, 3, 5, and 7) [$p24_{exp}$] = 0.625 pg/mL. Thus, the adjusted [p24] ([$p24_{adj}$]) = [$p24_{obs}$] – [$p24_{exp}$]. The resultant figure (Fig 2.4) displays this [$p24_{adj}$] value. This is not an ideal model of infectivity, given the activity of proteases etc. that would further decrease p24 concentrations, but quantifying these data in this way does allow for the quantification of 'new' [p24], and, therefore, better represents the infectivity of BrAgg. Analysis with repeated measures ANOVA revealed a significant increase in [$p24_{adj}$] within groups

exposed to 1.25 ng HIV-$1_{Ba\text{-}L}$ on days 9 and 21 compared to day 1 ($p < 0.05$; n = 4; Fig 2.4), suggesting active infection of BrAgg by HIV-$1_{Ba\text{-}L}$.

Discussion

The studies outlined here describe primary human CNS models involving the differentiation of human gestational CNS derived hNPCs. We demonstrated that hNeuron increase immunoreactivity for KCC2 while decreasing NKCC1 expression throughout our novel differentiation protocol. These features allow for the mechanistic study of human neuronal and GABAergic maturation and should be explored in future studies. hNeurons also show promise for the study of human specific disorders in which either of these proteins may become dysregulated resulting in disrupted GABAergic inhibition. We also characterized BrAgg and found that all major cellular subsets of the CNS are present and demonstrate its utility as a novel model for HAND in which there was active HIV-1 infection of CNS cells.

Drawbacks are inherent to all models of neurodegenerative disorders and the models described here are not immune to certain pitfalls. Tissue samples acquired for these studies are tested for HIV infection prior to shipment, but not all potential confounds can be controlled for. We cannot determine reasons for pregnancy termination (whether medical necessity or otherwise), control for exogenous substance exposure (drugs of abuse and others), or obtain maternal or paternal genetic information. We also did not control for potential genetic abnormalities, nor did we determine if the tissue had been exposed to other viruses. Early gestational tissue may also be difficult to acquire depending on the everchanging legal landscape surrounding legal access to legitimate centers for termination procedures. Once acquired, this is less of a concern for the

dissociated cultures, for which, tissue from one source can be proliferated as hNPCs, frozen, and used for several passages, allowing for each sample to be used in several experiments. Due to the sheer number of cells necessary for one flask of BrAgg (30 million cells), it is not feasible to expand and freeze hNPC for use as BrAgg. Rather, for each BrAgg culture initiation, a separate sample source is required. The BrAgg studies here are limited by sample size. This particularly affects studies with higher variability, such as our assessment of infectivity, limiting conclusions from these results. A higher sample size may have resulted in significant changes in [$p24_{adj}$] at levels other than 1.25 ng HIV-$1_{Ba\text{-}L}$.

In this chapter, we described a novel primary human mixed astrocyte-neuron culture model containing both GABAergic and glutamatergic neuronal subsets with matured Cl^- transporter expression. This dissociated model is particularly useful to elucidate mechanisms underlying neuronal disruption for human specific disorders. We also characterized BrAgg organoids which maintain components of the cyto-diversity and proportionality of cell types of the intact human CNS. Both show promise as translationally relevant *in vitro* models of human-specific neurological disorders.

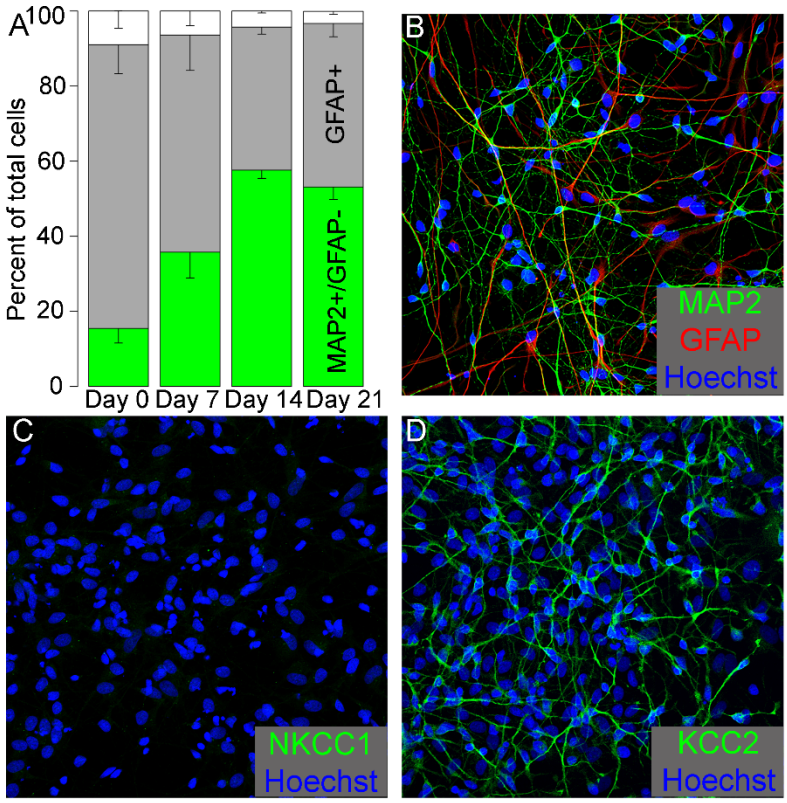

Figure 2.1. Differentiation and characterization of hNeuron. hNPCs differentiate to ~55% MAP2^{+}/GFAP^{-} (green) and 40% GFAP (red)$^{+}$ by 14 DIV (*A*). Representative images from hNeuron cultures showing immunofluorescence of MAP2 and GFAP after 21 DIV (*B*). hNeurons lose NKCC1 (*C*) and gain KCC2 (*D*) immunoreactivity by 21 DIV.

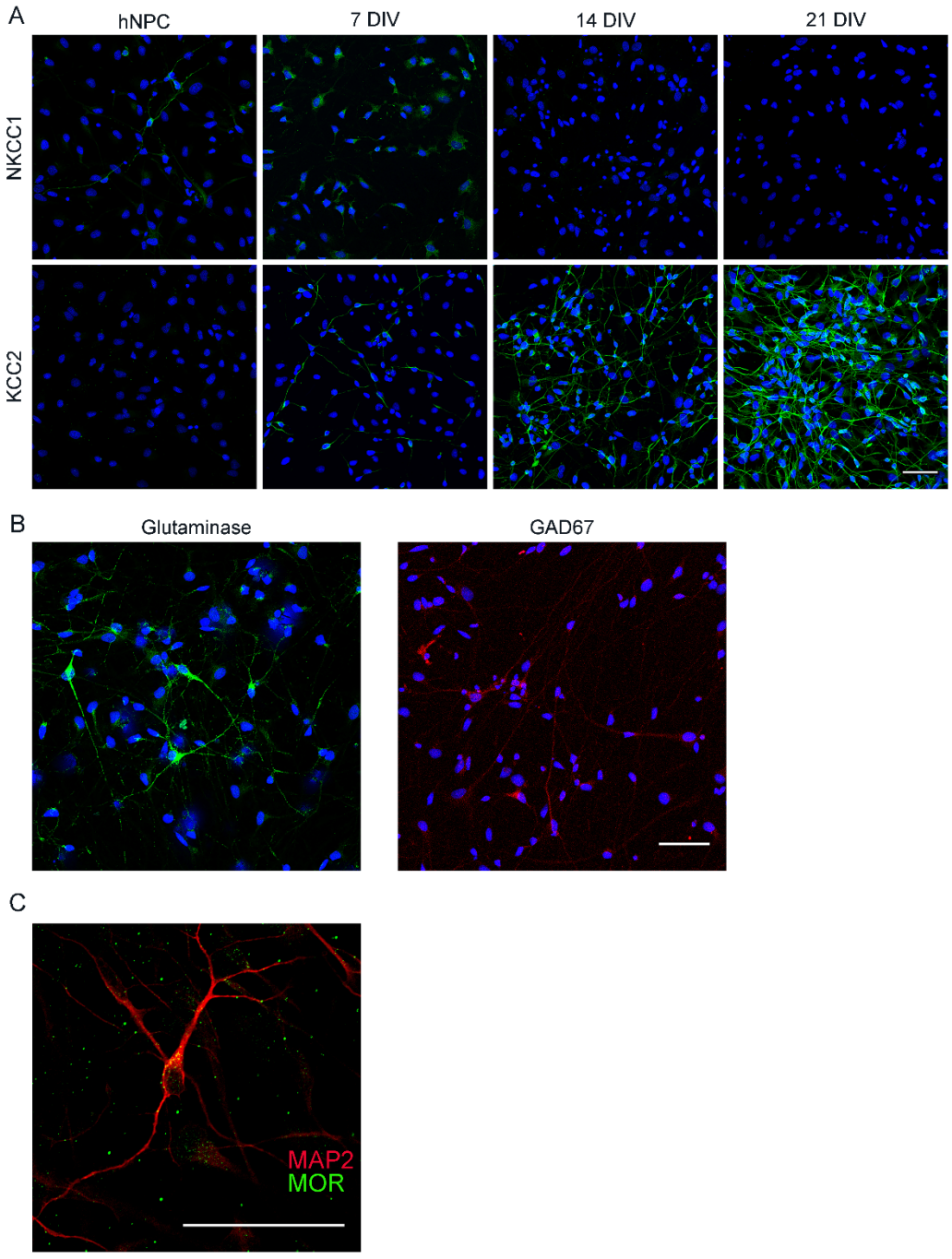
A
hNPC
7 DIV
14 DIV
21 DIV
NKCC1
KCC2
B
Glutaminase
GAD67
C
MAP2
MOR

Figure 2.2. Extended hNPC Differentiation. Following our neuronal differentiation protocol, cells lose NKCC1 expression and gain KCC2 expression by 21 DIV (*A*). After 21 DIV, immunoreactivity for glutaminase and GAD67 demonstrate both excitatory and inhibitory neuronal subsets were present, respectively (*B*). hNeuron express MOR (*C*). Scale bars = 50 μM.

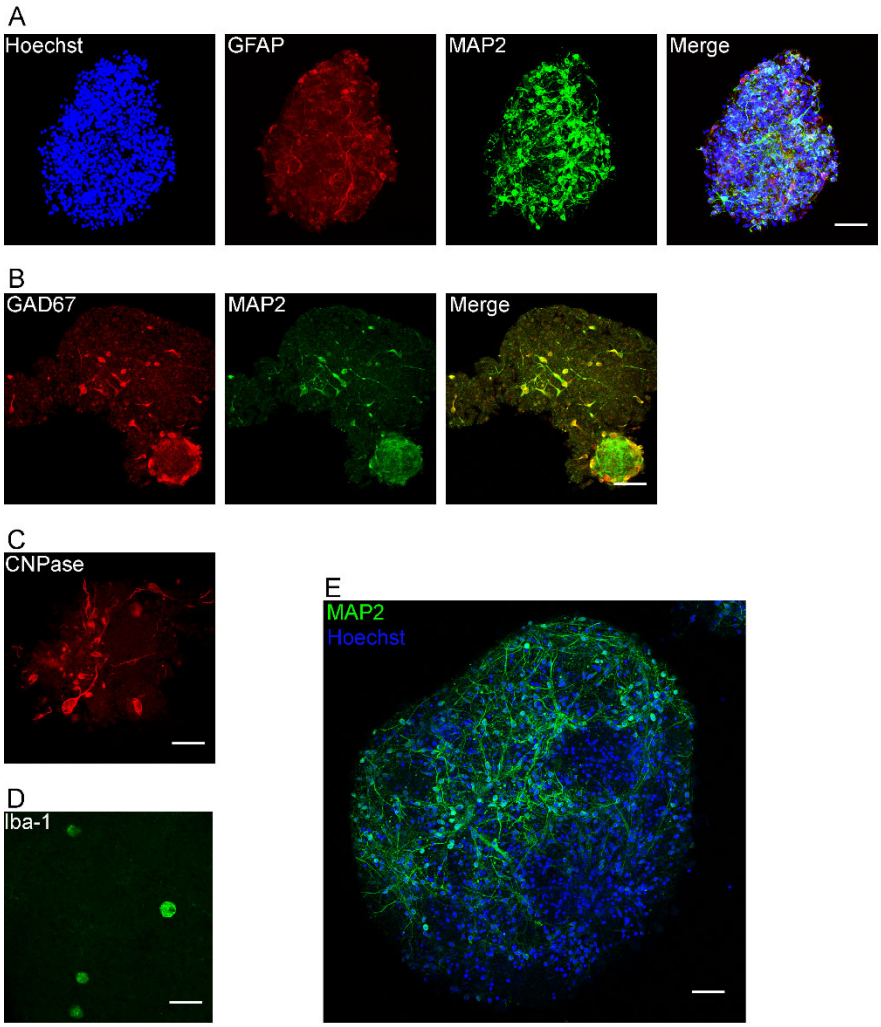

Figure 2.3. BrAgg characterization. BrAgg contain neurons and astrocytes by 5 DIV, indicated by positive staining for MAP2 and GFAP, respectively (*A*). Most neurons present appear to be GABAergic due to high colocalization between MAP2+ and GAD67+ cells (*B*). A minority of cells are also CNPase+ or Iba1+ indicating the presence of oligodendrocytes and microglia, respectively (*C*, *D*). BrAgg can vary in size from ~150 – 400 μM in diameter (*A*, *E*). Scale bars = 50 μM.

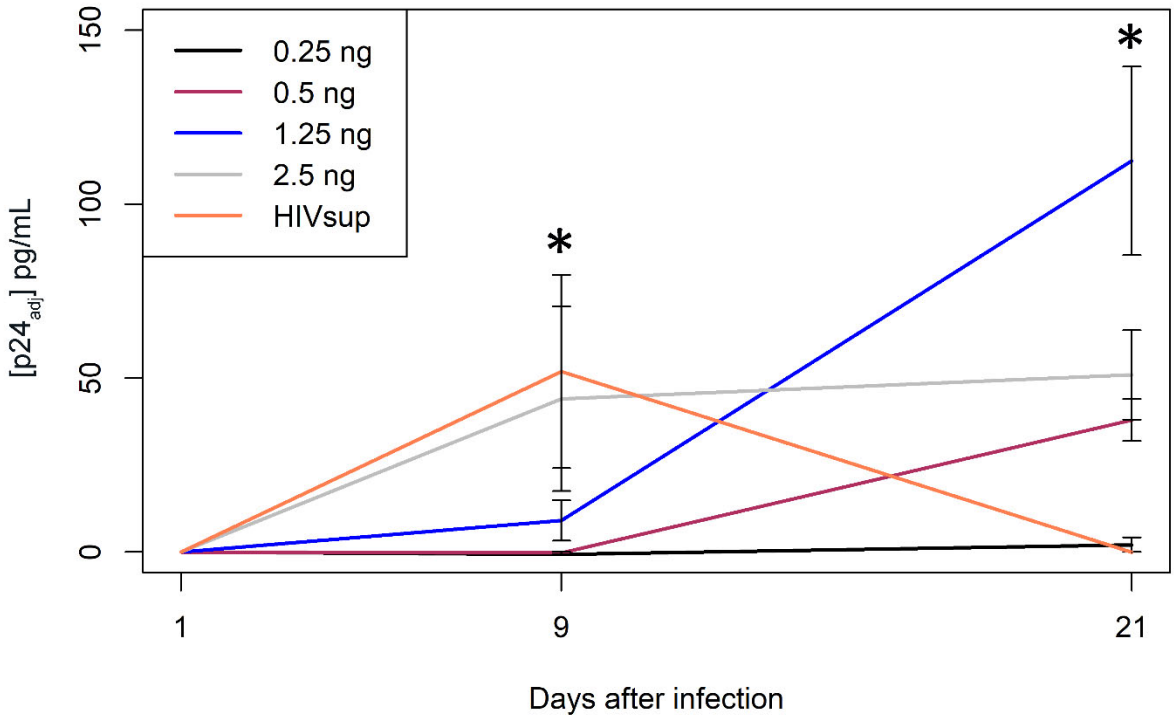

Figure 2.4 Infection of BrAgg with HIV-1$_{Ba-L}$. Exposure to 1.25 ng/mL [p24] purified HIV-1$_{Ba-L}$ (blue) results in a significant increase in [p24] adjusted for initial [p24] and media changes on days 9 and 21 compared to day 1 (*; $p < 0.05$; n = 4) ([p24$_{adj}$]). No significant differences were found at other exposure concentrations nor exposure to HIV$_{sup}$.

Chapter 3

HIV and opiates dysregulate K^+-Cl^- cotransporter 2 (KCC2) to cause GABAergic dysfunction in primary human neurons and Tat-transgenic mice

(This chapter was accepted for publication in *Neurobiology of Disease, article in press*)

Abstract

Approximately half of people infected with HIV (PWH) exhibit HIV-associated neuropathology (neuroHIV), even when receiving combined antiretroviral therapy. Opiate use is widespread in PWH and exacerbates neuroHIV. While neurons themselves are not infected, they incur sublethal damage and GABAergic disruption is selectively vulnerable to viral and inflammatory factors released by infected/affected glia. Here, we demonstrate diminished K^+-Cl^- cotransporter 2 (KCC2) levels in primary human neurons after exposure to HIV-1 or HIV-1 proteins ± morphine, resulting in disruption of $GABA_AR$-mediated hyperpolarization/inhibition. We found that the HIV proteins Tat (acting through NMDA receptors), and R5-tropic gp120 (acting via CCR5), and morphine (acting through μ-opioid receptors) induce KCC2 loss. We demonstrate that modifying KCC2 levels or function, or antagonizing NMDAR, CCR5 or MOR rescues KCC2 and $GABA_AR$-mediated hyperpolarization/inhibition in HIV, Tat, or gp120 ± morphine-exposed neurons. Using an inducible, Tat-transgenic mouse neuroHIV model, we found that chronic exposure to Tat also reduces KCC2. Our results identify KCC2 as a novel therapeutic target for ameliorating the pathobiology of neuroHIV, especially PWH exposed to opiates.

Introduction

Close to 38 million people worldwide are infected with HIV, with 50,000 new diagnoses of HIV-1 infection per year in the US alone (UNAIDS, 2019). Up to 50% of those infected with HIV-1 have CNS complications including HIV-associated neurocognitive disorders (HAND) (Antinori et al., 2007, Heaton et al., 2010). It is widely accepted that neurons are not infected by HIV-1. Neurological deficits in patients receiving combined antiretroviral therapy (cART) are likely due to sublethal neuronal stress and injury induced by viral proteins released from infected cells and persistent neuroinflammation, resulting in hyperexcitability (Anthony et al., 2005, Everall et al., 2009, Nath and Steiner, 2014, Neuenburg et al., 2002). Exposure to HIV-1 transactivator of transcription (Tat) depolarizes neurons and leads to electrophysiological dysfunction and hyperexcitability, partly through interactions with *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Philippon et al., 1994, Krogh et al., 2014). Another HIV-1 protein, gp120, binds co-receptors C-C chemokine receptor type 5 (CCR5) and/or C-X-C chemokine receptor type 4 (CXCR4) (Wang et al., 2003) (Kaul et al., 2007), leading to neurotoxic glial and inflammatory effects, as well as direct neurotoxicity. Opiate use is often comorbid with HIV-1 infection and has been implicated in increased severity of HAND (Byrd et al., 2011, Carrico, 2011, Bell et al., 2006, Smith et al., 2014). This comorbidity is highlighted by recent HIV-1 outbreaks in communities experiencing a surge in opiate abuse (Conrad C, 2015). *In vivo* and *in vitro* models of HAND demonstrate that morphine, the primary bioactive metabolite of heroin in the CNS, exacerbates HIV-1-induced neuropathogenesis primarily through the activation of the μ-opioid receptor (MOR) on glia, and subsequent modulation of

neuroinflammation and reduced glutamate buffering (Rodriguez et al., 2017, Bokhari et al., 2009, Zou et al., 2011).

GABAergic neurons seem to be selectively vulnerable to damage by Tat and HIV-1 infection in mouse models and post-mortem brain tissue from HIV-infected patients, highlighting disinhibition as a potential mechanism underlying hyperexcitability in the HIV-infected brain (Marks et al., 2016, Fitting et al., 2013, Buzhdygan et al., 2016, Gelman et al., 2012a). The deficits appear to result from a loss of GABAergic markers (including *GAD1*, *GAD2*, and *GJD2*) rather than the death of GABAergic interneurons in autopsy samples from PWH (Buzhdygan et al., 2016). Importantly, deficits in GABAergic markers differed significantly among brain regions and linked to worse cognitive performance, but these markers were unaffected by a history of drug abuse and differed minimally when comparing pre- and post-cART autopsy samples (Buzhdygan et al., 2016).

KCC2 extrudes Cl^- to maintain a low intracellular Cl^- concentration ($[Cl^-]_i$) in mature neurons (Blaesse et al., 2009). $GABA_A$ and glycine receptor-induced fast synaptic inhibition is predicated on the maintenance of low neuronal $[Cl^-]_i$ provided by KCC2 activity. While upregulation and subsequent increases in KCC2 activity have been extensively studied in development, loss of KCC2 activity and/or expression have more recently been noted in a number of neurological disorders including epilepsy (Kahle et al., 2014, Puskarjov et al., 2014, Silayeva et al., 2015), traumatic brain injury (Lizhnyak et al., 2019), schizophrenia (Hyde et al., 2011, Tao et al., 2012, Arion and Lewis, 2011), Rett syndrome (Tang et al., 2016), Huntington's disease (Dargaei et al., 2018), and morphine-induced hyperalgesia (Ferrini et al., 2013, Ferrini et al., 2017). The KCC2 activity enhancer, CLP257, and its prodrug, CLP290, have allowed manipulation of KCC2

activity and rescued deficits in KCC2-deficient states (Gagnon et al., 2013). While models of HAND have been shown to disrupt GABAergic functioning, loss of KCC2 activity has not yet been implicated in these deficits.

In addition to working with individual HIV-1 proteins, we exposed primary human neurons (hNeurons), derived and matured from neural progenitors (hNPC), to supernatant from HIV-1 infected monocytes (HIV_{sup}). This supernatant reflects the complexity of an infective milieu in that it contains multiple inflammatory/reactive factors and virions, as well as viral proteins. The genetically encoded voltage (GEVI) and Ca^{2+} indicators (GECI), Archon1 (Piatkevich et al., 2018) and GCaMP6f (Chen et al., 2013), respectively, were used to examine the functional outcomes of interactions between an HIV+ environment and morphine on hNeurons. Specifically, we used Archon1 and GCaMP6f to examine changes in $GABA_AR$-mediated hyperpolarization and inhibition, respectively. Importantly, this optical approach permitted us to manipulate and test functional outcomes absent the biohazards inherent with invasive electrophysiological measurements in the presence of infective HIV. Assessment of electrophysiological activity by Archon1 is equivalent to patch-clamp recording of neuronal spiking and subthreshold millivolt scale activity (Piatkevich et al., 2018). We demonstrate that HIV_{sup} and morphine target KCC2 in hNeurons, resulting in loss of $GABA_AR$ mediated hyperpolarization (measured by Archon1) and inhibition (measured by GCaMP6f). This suggests that dysregulation of KCC2 may play a role in circuit hyperexcitability in the HIV-infected brain. Further, we identify Tat, acting via an NMDA receptor (NMDAR)-mediated mechanism, and $gp120_{ADA}$, acting via a novel, CCR5-mediated mechanism, as viral proteins that can dysregulate KCC2 and $GABA_AR$-mediated inhibition. We also examined

the effects of Tat exposure on KCC2 in an *in vivo* model of HAND and found significantly diminished KCC2 in the striatum of these animals. Our results suggest KCC2 and its upstream regulators as promising therapeutic targets to alleviate the symptoms of HAND ± comorbid opiate use.

Materials and Methods

Primary hNPC culture.

Human neural progenitor cells (hNPCs) were derived from human CNS tissue at 15 - 17 weeks gestation (Advanced Biosciences Resources; Alameda, CA). Tissue was passed through 120 μM mesh, centrifuged and washed with medium twice, and dissociated cells were plated on poly-L-lysine (10 μg/mL) and laminin (2 μg/mL) coated flasks. hNPCs were maintained in NPC medium: DMEM F12 supplemented with 0.6% glucose, 10% B27 (without vitamin A; Thermo Fisher Scientific), 20 ng/mL fibroblast growth factor (FGF; R&D Systems, Minneapolis, MN), 20 ng/mL epidermal growth factor (EGF; EMD Millipore, Billerica, MA), 10 ng/mL leukemia inhibitory factor (LIF; EMD Millipore, Billerica, MA), 1% Pen/Strep, 5 mM HEPES (N-2-hydroxyethylpiperazine-N-3-ethane sulfonic acid; Thermo Fisher Scientific) with medium changed every 3 - 4 days.

Neuronal Differentiation.

Upon 80 - 90% confluency, hNPCs were detached using Accutase cell detachment solution (Millipore) and plated on poly-L-lysine (50 μg/mL) and laminin (100 μg/mL) coated 12-mm-diameter coverslips (immunostaining) or glass bottomed MatTek dishes (functional imaging) at 12,000 - 15,000 cells/coverslip in DMEM-F12 medium supplemented with 0.6% glucose, 10% B27 (without vitamin A; Thermo Fisher Scientific),

1% Pen/Strep, 5 mM HEPES (Thermo Fisher Scientific), and 10 ng/mL brain derived neurotrophic factor (BDNF; Sigma). After 7 DIV, medium was exchanged gradually (50% on days 7 and 9) and then fully (100% every 72 - 96 h) to BrainPhys™ (STEMCELL Technologies Inc, Vancouver, BC, CAN) supplemented with SM1 (STEMCELL).

HIV-1 propagation and HIV_{sup} preparation.

$HIV\text{-}1_{BaL}$ was propagated using methods previously established (Balinang et al., 2017). Briefly, isolated PBMCs from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) were activated with 1 mg/mL phytohemagglutinin (PHA) for 48 h, then infected with $HIV\text{-}1_{BaL}$ (NIH AIDS Reagent Program) at 1 ng/mL p24. After 72 h, infection was assayed using p24 antigen ELISA kit (Advanced Bioscience, Rockville, MD) and supernatant harvested and frozen at −80° C for HIV_{sup} experiments. HIV_{sup} used in all experiments was from the same source sample to reduce variability.

Treatments.

hNeuron cultures were treated with HIV_{sup} at 125 - 500 pg/mL [p24] or HIV protein (10 - 100 nM HIV-1 $Tat_{1\text{-}86}$ IIIB (clade B) or 250 pM - 1 nM R5-tropic $gp120_{ADA}$, X4-tropic $gp120_{IIIB}$, or dual-tropic gp120 MN; ImmunoDx, Woburn MA) ± 500 nM morphine sulfate for 6 or 24 h. Drug treatments: 10 μM CLP257 (Sigma), 50 μM AP5 (Alomone Labs, Jerusalem IL), 50 nM maraviroc (MVC; BOC Sciences, Shirley, NY) were applied 30 min prior to HIV_{sup}, HIV protein, and morphine for 24 h experiments. 50 μM VU02440551 (Tocris, Bristol UK) was applied 2 h prior to GEVI/GECI imaging experiments. Supernatant from uninfected but activated monocytes (UNF) was used as a control for HIV_{sup}. Vehicle controls were used for all other treatments.

Immunocytochemistry and Cell Counting.

Neuronal cultures were fixed for 15 min (4% paraformaldehyde), permeabilized for 15 min (0.1% Triton-X 100, 0.1% BSA) and blocked for 1 h at room temperature (0.1% BSA, 1% goat serum in PBS). Coverslips were then immunostained with rabbit anti-KCC2 (1:1000; Protein Tech, Rosemont, IL) and mouse anti-microtubule-associated protein (MAP2) (1:500; Millipore, Burlington, MA) for KCC2 immunoreactivity studies Alexa Fluor 488 and 594-conjugated secondary antibodies (1:1000; Thermo Fisher, Waltham, MA) were used for all studies as well as Hoechst 33342 (1:10000; Invitrogen, Carlsbad, CA) to detect nuclei. Cells were visualized and images obtained using a Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 and Zen 2010 software (Zeiss Inc., Thornwood, NY). Cells were manually counted using the CellCounter plugin for Image J. At least 200 Hoechst+ cells were counted per sample.

GEVI/GECI Expression.

AAV serotype 1 containing GCaMP6f and Archon1 were acquired from AddGene (plasmid # 100837; Watertown, MA) and Vector Biolabs (Malvern, PA), respectively. hNeurons were treated with AAV1.hSyn.GCaMP6f.WPRE.SV40 or AAV1.hSyn.Archon1.WPRE.SV40 for 5 - 7 days prior to experiments. Human synapsin promoter (hSyn) was used to ensure neuronal specificity.

GEVI/GECI Imaging and Analysis.

Archon1 and GCaMP6f recordings were acquired at 2 or 1 KHz, respectively, with a Neuro-CCD camera (RedShirt Imaging, Decatur, GA) mounted on a Zeiss Axio Observer with 40× oil immersion objective. Archon1 and GCaMP6f illumination was

achieved with an LED (UHP-T-LED; Prizmatix, Holon, IL) 640 nm excitation, 660LP emission for Archon1 (custom-made filter cube; Omega Optical, Brattleboro, VT) and 470/40 nm excitation, 495 dichroic, 525/50 nm emission for GCaMP6f (filter set 38; Zeiss). All recordings were performed in BrainPhys solution (STEMCELL) with 2 μM CGP55845 ($GABA_BR$ antagonist; Abcam) or 2 μM CGP55845 and 100 μM picrotoxin (PTX) ($GABA_A$ and glycine receptor antagonist; Tocris) to validate Archon1 results (Fig. S3).

Soma ROI were used for all experiments. Archon1 and GCaMP6f traces were analyzed with a custom code in R. Both Archon1 and GCaMP6f traces were 5-point Savitzky-Golay smoothed and GCaMP6f traces were Loess subtracted, to remove slower transients. Fluorescence changes were calculated as $\Delta F/F = (F_A - F_{bl})/F_{bl}$, where F_A is the fluorescence level during perfusion and F_{bl} is the average baseline fluorescence intensity 2.5 s prior to perfusion. Significant Ca^{2+} transients were counted when the trace increased by more than four-fold of the standard deviation (SD) of background activity (measured 2.5 s prior to perfusion) and returned to baseline levels (defined as within 0.5 × SD of mean activity).

Animals.

Male, doxycycline (DOX)-inducible, GFAP driven HIV-1 Tat-transgenic mice (10 – 12 weeks old) were used to explore the effects of Tat expression on KCC2. Mice expressing the *tat* and *rtTA* transgenes (Tat+) or mice lacking the *tat* transgene, but expressing the *rtTA* transgene (Tat−), were fed doxycycline-containing chow (6 mg/g, Harlan Indianapolis, IN) for 2 weeks prior to sacrifice. Animal procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee

and are in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Immunoblot.

KCC2 presence was examined by immunoblotting striatal tissue from Tat+ and Tat- mice. Striata were freshly harvested and homogenized in RIPA lysis buffer (Sigma) and Halt™ Protease & Phosphatase Inhibitor Cocktail (Thermo Fisher). Lysates were then centrifuged and stored at -80º C. Protein concentration was measured using BCA protein assay (Pierce, Rockford, IL). 40 µg of lysates were loaded per well onto 4 – 20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to PVDF membranes (Bio-Rad) and probed with antibodies against KCC2 (1:2000; Protein Tech) and GAPDH (1:2000; Abcam). Alexa Fluor 647 and 488-conjugated secondary antibodies (1:2000; Thermo Fisher) were used to detect proteins. A Bio-Rad ChemiDoc™ MP Imaging System and Image Lab were used to measure and analyze protein levels, respectively.

Statistics.

KCC2 immunocytochemistry studies were assessed using three or two-way analysis of variance (ANOVA; R) to determine the effects of Tat_{1-86}/gp120/HIV_{sup}, CLP257/AP5/MVC, and morphine on KCC2 expression at 24 h following viral/drug exposure. The 6 h studies were assessed using two-way ANOVA with HIV (i.e., Tat_{1-86}, gp120, or HIV_{sup}) and morphine treatments as factors. Interactions and main effects for both were examined via simple main effects using a Bonferroni *post hoc* test to determine individual group differences. Functional imaging studies were analyzed as above except the assessment of VU0240551 was determined separately using a two-tailed Student's *t*

test. A two-tailed Student's *t* test was also used to examine differences between Tat+ and Tat− mice in western blots. Data were expressed as mean value ± standard error of the mean. Analyses were considered significant if $p < 0.05$. Cook's distance was used to determine and remove outliers.

Results

HIV-1-infected PBMC supernatant reduces KCC2 immunoreactivity.

Previous studies have shown selective vulnerability of GABAergic neurons to HIV-1 and Tat (Marks et al., 2016, Gelman et al., 2012a). Here, we examined KCC2 immunoreactivity to determine if $[Cl^-]_i$ dysregulation might relate to GABAergic deficits. To model the HIV-infected human brain, we exposed hNeurons to HIV_{sup}. Experiments show ~55 % of Hoechst+ cells in control groups are immunoreactive for KCC2 (Fig. 3.1A), with significant reductions in co-expression when hNeurons were exposed for 6 or 24 h to HIV_{sup} at 250 - 500 pg/mL [p24] (but not 125 pg/ml [p24]) or 500 nM morphine ($p < 0.01$; n = 6) (Fig. 3.1A,B). Loss of KCC2 was not due to cell death in the exposure groups examined as evidenced by continued MAP2 antigenicity and the LIVE/DEAD™ viability/cytotoxicity assay (Molecular Probes, Eugene, OR) (Fig. 3.8). Pretreatment (30 min prior to HIV_{sup} ± morphine) with the KCC2 enhancer, CLP257 (10 μM), restored KCC2 immunoreactivity ($p < 0.05$; n = 6) (Fig 3.1B). These results suggest that Cl^- regulation and, therefore, GABA function may be altered in neurons of the HIV-1-exposed brain. HIV_{sup} and morphine interactions were examined at 125 pg/mL p24, but not found). This interaction was not examined at 250 - 500 pg/mL p24 since cell death can occur with morphine co-exposure at the higher p24 levels (Masvekar et al., 2015) and which might be confounding for KCC2 immunoreactivity.

The HIV proteins Tat and gp120 are both well-known to influence neuronal hyperexcitability and progression of HAND (Nath and Steiner, 2014), so we tested their contribution to the loss in KCC2 immunoreactivity induced by HIV_{sup}. Since NMDAR and CCR5 are respective major targets of Tat and gp120, we pretreated cultures with the NMDAR antagonist AP5 (50 μM) and/or the CCR5 antagonist, MVC (50 nM). Independent exposures showed that both significantly increase KCC2 immunoreactivity at 500 pg/mL HIV_{sup} and AP5 at 250 pg/mL HIV_{sup} ($p < 0.05$, n = 5 – 6) with a trend towards rescue with MVC at 250 pg/mL HIV_{sup} ($p = 0.061$, n = 5 – 6) (Fig 3.1D). When combined, MVC and AP5 are significantly different than either individually and completely restored KCC2 immunoreactivity ($p < 0.01$; n = 5 - 6) (Fig 3.1D), suggesting NMDAR and CCR5 as the pathways mediating HIV_{sup}-induced KCC2 reduction. Further examination revealed a significantly increased KCC2 after AP5 exposure compared to MVC exposure suggesting that while both CCR5 and NMDAR activation play a role, NMDAR is the dominant pathway towards diminished KCC2 in this context ($p < 0.01$; n = 5). Both the pan-opioid receptor antagonist, naloxone, and the selective MOR antagonist, CTAP, reversed KCC2 losses induced by morphine, suggesting MOR activation can suppress KCC2 activity ($p < 0.01$; n = 6) (Fig 3.1C).

HIV_{sup} ± morphine diminishes $GABA_AR$ mediated hyperpolarization.

The GEVI, Archon1, is a far-red-shifted, membrane-bound, voltage-sensitive, opsin-based fluorescent protein that changes fluorescence intensity with membrane potential. This high speed, noninvasive measurement of electrophysiological activity was shown to be identical to patch-clamp recording of neuronal spiking (100 Hz) and subthreshold millivolt scale activity (Piatkevich et al., 2018). To determine HIV_{sup} ±

morphine effects on $GABA_AR$ activation, we measured Archon1 activity in cultures perfused with 100 μM GABA and $GABA_BR$ antagonist, CGP55845 (2 μM) after 24 h exposure to 125 - 500 pg/mL [p24] HIV_{sup} ± morphine or to vehicle/UNF controls. Archon1-expressing hNeuron control groups showed robust hyperpolarization with peak ΔF/F deflection of −8.7 ± 0.5% during 115 s perfusion of 100 μM GABA, CGP55845 (Fig. 3.2). hNeurons exposed for 24 h to varying concentrations of HIV_{sup} (125 - 500 pg/mL p24) or morphine (500 nM) showed 16 - 34% reductions of peak ΔF/F deflection during perfusion ($p < 0.01$; n = 15 - 24), suggesting reduced hyperpolarization in response to $GABA_AR$ activation (Fig. 3.2). Analysis revealed a main effect of CLP257 treatment in restoring $GABA_AR$-mediated hyperpolarization. A comparison of individual groups revealed that CLP257 reversed the effects of 500 pg/mL [p24] HIV_{sup} and morphine ($p < 0.05$; n = 15 - 24) (Fig. 3.2). Groups exposed to the KCC2 antagonist, VU0240551 (50 μM), recapitulated results of HIV_{sup} and morphine groups (28% ΔF/F reduction; $p < 0.05$; n = 15) (Fig. 3.2). We confirmed that ΔF/F changes above were, in fact, due to $GABA_AR$ activation by perfusing GABA, CGP55845, and the noncompetitive Cl^- channel blocker, PTX (100 μM), and found no fluctuations in ΔF/F (Fig. 3.3). These results suggest that loss of KCC2 activity due to HIV_{sup} ± morphine exposure results in dysregulated $[Cl^-]_i$ and subsequent deficits in $GABA_AR$ activity, and that these deficits can be rescued by maintaining KCC2 expression with CLP257.

HIV-1 proteins reduce KCC2 immunoreactivity.

Since HIV_{sup} contains multiple inflammatory and viral factors found in the HIV-infected CNS, it served as a more translational model of neuronal injury. To determine the contribution of individual viral proteins to effects seen with HIV_{sup}, we exposed

hNeurons to Tat_{1-86} and gp120 (R5-tropic, X4-tropic, and dual-tropic), both of which are thought to be primary factors in neurodegenerative outcomes and HAND symptoms. We first examined the effects of Tat_{1-86} exposure in isolation because NMDAR is a primary target of Tat and its activation with regard to induction of KCC2 degradation is well-known (Medina et al., 2014). Both morphine (500 nM) and Tat_{1-86} (50 – 100 nM) alone significantly reduced percentages of Hoechst+ cells displaying KCC2 immunoreactivity, after both 6 and 24 h exposure, compared to vehicle groups ($p < 0.01$; n = 6) (Fig. 3.4B, C). Further, there was a significant interaction between Tat_{1-86} (50 nM) and morphine with 24 h co-exposed groups displaying exacerbated KCC2 loss ($p < 0.05$; n = 5 – 6), suggesting signaling convergence. Again, cell death was not responsible for Tat_{1-86} effects since the number of MAP2+ cells was stable (Fig. 3.4A) and no increases in dying/dead cells were evident using a cytotoxicity assay (Fig. 3.8). CLP257 significantly rescued KCC2 immunoreactivity in Tat_{1-86}, morphine, and co-exposed groups ($p < 0.01$; n = 6) (Fig. 3.4C). Pharmacological blockade of NMDAR with AP5 rescued KCC2 immunoreactivity (Fig. 3.4C). These data suggest that Tat_{1-86} activation of NMDAR may suppress KCC2 levels and may play a role in reducing the number of HIV_{sup}-exposed KCC2 immunoreactive hNeurons without causing their death.

Effects on KCC2 vary with gp120 tropism.

We tested concentration -dependent effects of gp120 (250 pM-1 nM) from three different tropic strains of HIV. Exposure to $gp120_{ADA}$ (R5-tropic; $p < 0.01$; n = 6), but not $gp120_{IIIB}$ (X4-tropic) ($p = 0.23$; n = 5) or $gp120_{MN}$ (dual-tropic) ($p = 0.078$; n = 5) strains, significantly reduced KCC2 expression at both 6 h and 24 h compared to controls (Fig. 3.5 A, B). Further, there was a trend towards interaction with morphine and $gp120_{ADA}$ at

the 500 pM level (p = 0.068; n = 6). The reduction in KCC2 immunoreactivity was not due to cell death, although exposure to 1 nM $gp120_{MN}$ trended towards significant cell loss (p = 0.06; Fig. 3.8). While KCC2 is a well-studied target of NMDAR activation in other neuropathologic diseases, CCR5-mediated KCC2 regulation has not been described by others. To validate that CCR5 activation triggered KCC2 loss, we pretreated cultures with the CCR5 antagonist, MVC (50 nM), which maintained KCC2 expression (49.19 ± 1.2%; p < 0.01; n = 5). Interestingly, pretreatment with CLP257 did not rescue KCC2 immunoreactivity in these experiments. These results describe a novel mechanism of KCC2 regulation involving CCR5 and suggest that the activation of CCR5 by $gp120_{ADA}$ may contribute to KCC2 reductions after HIV_{sup} exposure.

HIV_{sup}, Tat, and gp120 ± morphine did not alter NKCC1 immunoreactivity.

NKCC1 acts in opposition to KCC2 and is the primary neuronal Cl^- transporter in the immature brain. To examine another potential route of neuronal Cl^- dysregulation, we immunostained hNeurons for NKCC1 after 24 h exposure to 125 – 500 pg/mL [p24] HIV_{sup}, 10 – 100 nM Tat, and 250 – 1000 pM gp120 ± morphine. NKCC1 was not detectable with exposure any of the viral factors or morphine at any of the concentrations examined (n = 4) (data not shown).

HIV-1 proteins reduce inhibitory potential of $GABA_AR$ activation.

We next examined the functional effects of Tat_{1-86}, $gp120_{ADA}$, and morphine on $GABA_AR$-induced inhibition by expressing the intracellular Ca^{2+} reporter GCaMP6f in hNeurons using AAV1.hSyn.GCaMP6f.WPRE. $GABA_AR$-mediated inhibition was examined in these cells by recording neuronal Ca^{2+} activity during perfusion of BrainPhys solution containing 100 µM GABA, 25 µM glutamate, and 2 µM CGP55845 for 55 s and

quantifying resultant neuronal spiking (the total number of significant Ca^{2+} transients during 55 s recording period). Analysis revealed a significant increase in 50 nM Tat_{1-86} and morphine groups, without interaction comparedto vehicle ($p < 0.01$; n = 15 – 21) (Fig. 3.6B). Thus, Tat_{1-86} and morphine independently reduced the inhibitory potential of $GABA_AR$ activation. Pretreatment with CLP257 ameliorated Tat_{1-86} and morphine effects ($p < 0.01$; n = 15 - 21), suggesting rescue of inhibitory activity in these groups, consistent with immunoreactivity results showing restored levels of KCC2. We also found disinhibition of hNeurons, as determined by significantly increased Ca^{2+} spiking in groups exposed to 500 pM $gp120_{ADA}$ (Fig. 3.6B). Three-way ANOVA revealed a main effect of $gp120_{ADA}$ regardless of CLP257 or morphine application ($p < 0.05$; n = 15 - 18). Pretreatment with MVC showed a strong tendency to prevent gp120-induced excitation presumably by retaining/restoring KCC2 function ($p = 0.059$). Consistent with immunoreactivity results, these results suggest a failure of CLP257 to maintain KCC2 expression following $gp120_{ADA}$ exposure and, thus a failure to restore the inhibitory effects of $GABA_AR$ activation. Selectively inhibiting KCC2 with VU0240551 recapitulated the effects of Tat_{1-86} or $gp120_{ADA}$ ± morphine effects (n = 15, $p < 0.01$) independently confirming the role of KCC2 in the overexcitation. These results suggest that hNeurons become disinhibited after Tat_{1-86} or $gp120_{ADA}$ ± morphine exposure due to a loss of KCC2 activity and that pharmacological maintenance of KCC2 can prevent or rescue the deleterious effects of Tat and/or morphine.

Tat expression reduces KCC2 in the striatum of Tat-transgenic mice.

HIV-1 Tat transgenic mice show neuropathology and behavioral deficits similar to PWH (Schier et al., 2017, Fitting et al., 2013). To examine if KCC2 abnormalities found

in culture could be seen in Tat-transgenic mice, we performed western blot analysis of striatal tissue after two weeks of Tat induction by DOX. Tat+ mice showed a significant decrease in total KCC2 levels compared to Tat− control mice (Fig. 3.7) ($p < 0.05$; n = 12). These results confirm that chronic Tat exposure induces KCC2 loss *in vivo* as well as in cultured hNeurons.

Discussion

Previous studies including human post-mortem (Buzhdygan et al., 2016, Gelman et al., 2012a) and *in vivo* rodent studies (Marks et al., 2016, Xu et al., 2016, Fitting et al., 2013) have shown selective changes in GABAergic markers and function, driving an emerging concept that disrupted GABAergic transmission may be central to the development of HIV-related CNS dysfunction. Our data strengthen this perspective by demonstrating that KCC2 levels and subsequent GABAergic activity are diminished by an infective, HIV+ environment and by exposure to $gp120_{ADA}$ *in vitro* or HIV-1 Tat, both *in vitro* and *in vivo*. Importantly, HIV-infected individuals demonstrate a loss of GABAergic markers without the loss of GABAergic neurons (Buzhdygan et al., 2016) as seen in the present study. Taken together, our data along with findings from other investigators suggest that there may be an overall loss of inhibition via reduced inhibitory synapses and/or GABAergic function, and as we demonstrate here, diminished hyperpolarization at remaining inhibitory synapses caused by deficits in KCC2.

Endogenous levels of chemokine (C-C motif) ligand 5 (CCL5) are almost undetectable in the healthy CNS, but dramatic increases are found in several neuroinflammatory disorders (Louboutin and Strayer, 2013) including HAND (El-Hage et al., 2005, Letendre et al., 1999). In multiple sclerosis, CCL5 is elevated during early

stages of the disease and is correlated with hyperexcitability (Sorensen et al., 1999, Mori et al., 2016). Our results demonstrate KCC2 as a target of CCR5 activation that may underlie this hyperexcitability. Targeting CCR5, perhaps with MVC, should be further investigated for therapeutic potential in multiple sclerosis and in other disorders in which elevated CCL5-CCR5 signaling may be operative, such as in neuroHIV (Kim et al., 2018).

Additionally, our results using the MOR-selective antagonist, CTAP (Fig. 3.1C) demonstrate that neuronal KCC2 expression is a target of MOR activation. It is important to note that we have used a mixed neuron-astrocyte culture model. Outcomes may thus be due to direct activation of neuronal MOR, or a secondary effect of MOR activation on astroglia. Our results only demonstrated an interaction between morphine and viral proteins at the 24 h timepoint. Our previous studies have shown neurotoxic interactions between morphine and HIV/viral proteins at 24 – 72h and also that neurotoxicity was largely if not completely driven by glia (Zou et al., 2011) [or similarly by CCR5 deletion from glia (Kim et al., 2018)]. Thus, astrocytes may be acting as the intermediary through which MOR activation leads to neuronal KCC2 loss in the data presented here. Overall, our implication of MOR in KCC2 loss is in line with previous findings that $GABA_AR$ activation on neurons in the ventral tegmental area of rats switches from an inhibitory to excitatory signal during the development of opiate dependency (Laviolette et al., 2004, Vargas-Perez et al., 2009), and that KCC2 loss contributes to states of opiate withdrawal and morphine-induced hyperalgesia via mechanisms involving microglia-mediated BDNF release and subsequent TrkB activation (Ferrini et al., 2013, Ferrini et al., 2017, Taylor et al., 2016). Further, we found an exacerbation of KCC2 losses at 50 nM Tat with co-exposure to morphine, suggesting that opiates and HIV protein interactions may similarly

occur in the CNS and underlie the increased incidence/severity of HAND that has been noted in PWH who abuse opiates (Byrd et al., 2011, Bell et al., 2006, Carrico, 2011, Smith et al., 2014).

Interestingly, CLP257 was able to maintain KCC2 expression and functional response of hNeurons to Tat, but had no effect on gp120-exposed groups. While many factors regulate KCC2 activity/degradation, the mechanism by which CLP257 preserves KCC2 activity remains elusive. KCC2 stability is regulated by phosphorylation at multiple sites. For example, phosphorylation of S940 or T1007 by protein kinase C and WNK increases and decreases KCC2 membrane stability, respectively (Lee et al., 2011, Inoue et al., 2012). NMDAR activation can lead to protein phosphatase 1 activation and S940 dephosphorylation leading to decreased membrane localization and subsequent calpain-induced KCC2 degradation in the cytoplasm (Lee et al., 2011). Thus, there are several mechanisms that may underlie the effects of CLP257. gp120 and Tat may have differential effects on one or more of these pathways, resulting in these inconsistencies. MVC, an FDA-approved CCR5 antagonist commonly prescribed to HIV-infected individuals to prevent HIV from binding to this co-receptor, was able to rescue both KCC2 expression and functional responses to $GABA_AR$ activation in $gp120_{ADA}$-exposed hNeurons. Thus, MVC treatment might prevent $gp120_{ADA}$-induced KCC2 loss upstream of CLP257 and could be considered more widely for PWH with HAND symptomology.

Neuronal electrophysiological properties govern CNS function, and elucidating deficits at the cellular level can identify circuitry imbalances that underlie neurological disorders. To study electrophysiological responses in HIV-1 exposed neurons, we used the GEVI, Archon1. This noninvasive interrogation has the advantages of allowing

ensemble activity to be studied both *in vitro* and *in vivo*, with the capability of tracking specific neurons long-term. This is a particularly useful alternative to traditional electrophysiology when biosafety is a concern. Most of the experiments here utilized acute exposure paradigms (6 and 24 h). While this approach allowed us to uncover novel targets and subsequent functional responses in primary human neurons, PWH are exposed to these factors over a much longer timeframe. Thus, we began to validate the observed changes using a chronic *in vivo* model and found reduced KCC2 in the striatum of Tat+ transgenic mice after 2 weeks of Tat exposure. Further studies are underway to test the effectiveness of a CLP257 prodrug (CLP290) to reverse KCC2 loss, the resultant electrophysiological and behavioral changes observed with Tat and/or CLP290 exposures, and any preferential vulnerability between the prominent neuronal subtypes in the striatum (dopamine D1 receptor-expressing vs. dopamine D2 receptor-expressing) to KCC2 loss. Interestingly, the loss of the expression of GABAergic markers is inversely correlated with increases in dopamine D2 receptor (*DRD2L*) in PWH and increases in *DRD2L* in the prefrontal cortex positively correlated with the development of HAND (Gelman et al., 2012b, Buzhdygan et al., 2016). HIV-induced neurocognitive detriments vary among individuals, and length of exposure is likely a contributing factor along with age, genetics, comorbidities (including drug use), and the particular strains of HIV present. We used the HIV_{BaL} strain to generate HIV_{sup} as R5 tropism is prominent in the CNS (Schnell et al., 2011). We also began to address viral heterogeneity by comparing effects of three different gp120 strains. Results here suggest that R5-tropic strains may be more likely to disrupt GABAergic circuitry. CSF viral titers and levels of HIV-1 Tat have been shown to vary among infected individuals whose blood titers are well-controlled

(Johnson et al., 2013, Henderson et al., 2019). Thus, we expect that levels of viral proteins and inflammation within the brain parenchyma must also vary among PWH. As these levels are not easily measured, our experimental paradigm may over- or underestimate CNS exposure levels in HIV-infected individuals.

Similar situations of disinhibition through dysregulated $[Cl^-]_i$ appear to contribute significantly to hyperexcitability in other neurological disorders, and restoration of KCC2 with CLP257 or its prodrug, CLP290, has shown promise in reversing the hyperexcitability in models of these disorders (Chen et al., 2018, Ferrini et al., 2017). Overall, our studies further implicate KCC2 in novel ways that could have broad impact for human health. We established KCC2 and upstream pathways as promising therapeutic targets to restore GABAergic function and to treat the symptoms of HAND, and this approach may be particularly relevant for PWH that use opiates. We also identified KCC2 as a novel link underlying hyperexcitability in conditions that involve elevated CCL5 and/or CCR5 activation in the CNS. Our results also highlight that opiate drugs of abuse may independently dysregulate KCC2 levels to cause GABAergic dysfunction. This finding has significance for the large population of opiate abusers who are not infected with HIV.

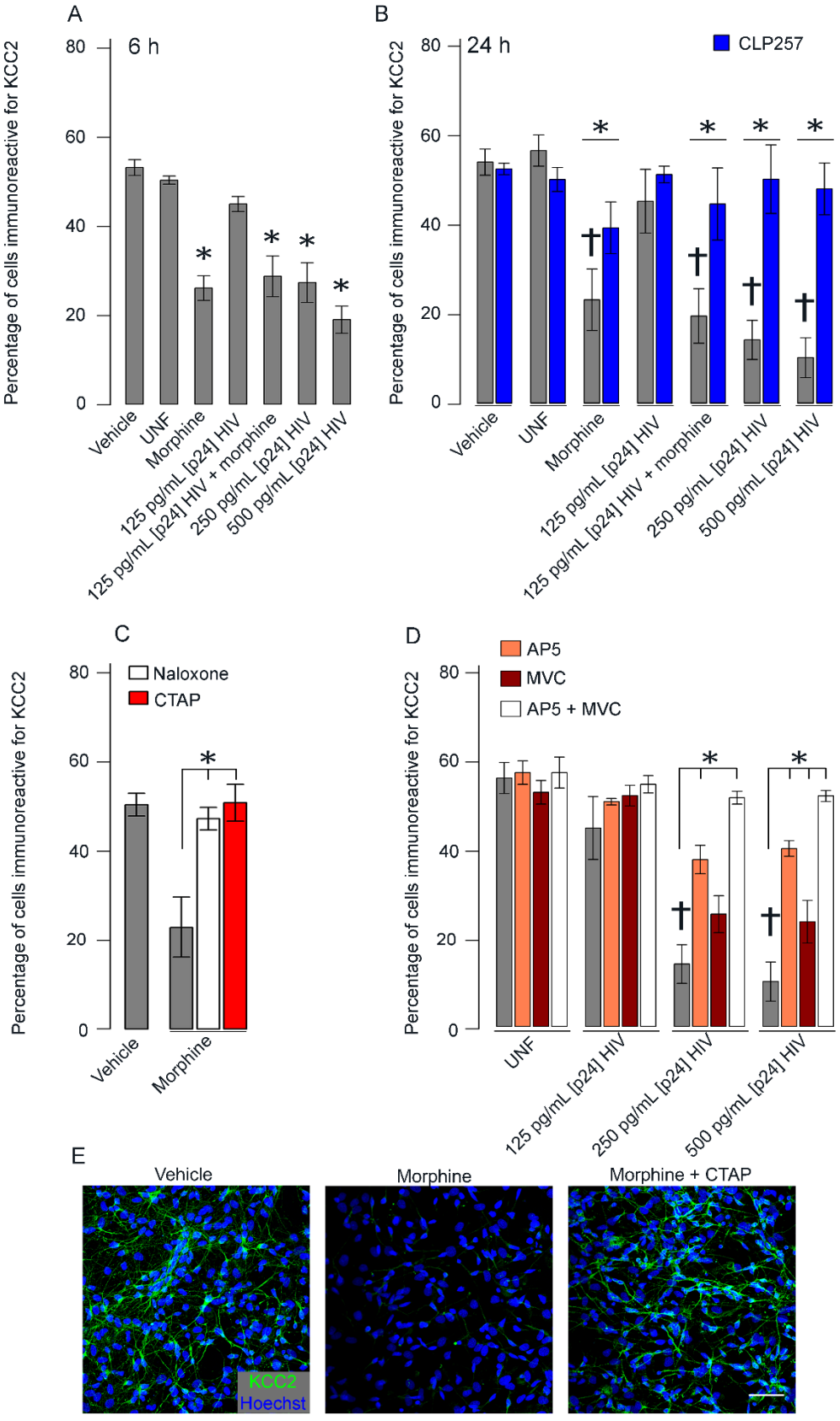
A
6 h
Percentage of cells immunoreactive for KCC2
Vehicle
UNF
Morphine
125 pg/mL [p24] HIV
125 pg/mL [p24] HIV + morphine
250 pg/mL [p24] HIV
500 pg/mL [p24] HIV
B
24 h
CLP257
C
Naloxone
CTAP
D
AP5
MVC
AP5 + MVC
E
Vehicle
Morphine
Morphine + CTAP
KCC2
Hoechst

Figure 3.1. HIV_{sup} and morphine significantly reduce the percentage of cells immunoreactive for KCC2. Treatment for 6 h (*A*) or 24 h (*B*) with 250 – 500 pg/mL [p24] HIV_{sup} ± morphine significantly reduces the number of KCC2 immunoreactive cells ($^{\dagger}p < 0.01$ for respective controls; n = 6). CLP257 (blue) rescued this effect across all groups ($^{*}p < 0.05$, n = 6). Naloxone and CTAP both antagonized the effects of morphine (*C*), suggesting the involvement of MOR ($^{*}p < 0.01$; n = 6). AP5 (orange) restored KCC2 immunoreactivity after 250 – 500 pg/mL HIV_{sup} exposure while MVC (maroon) significantly increased KCC2 in 500 pg/mL ($^{*}p > 0.05$, n = 5 – 6). A trend towards MVC rescue of KCC2 immunoreactivity was found at 250 pg/mL HIV_{sup} ($p = 0.061$, n = 5 – 6). Co-exposure of HIV_{sup} with both AP5 and MVC fully restored KCC2 levels, suggesting NMDAR and CCR5 as the primary pathways of HIV_{sup}-mediated KCC2 reduction. Representative images for KCC2 immunofluorescence of vehicle, morphine, and morphine + CTAP-treated hNeurons. Scale bar = 50 μm (E).

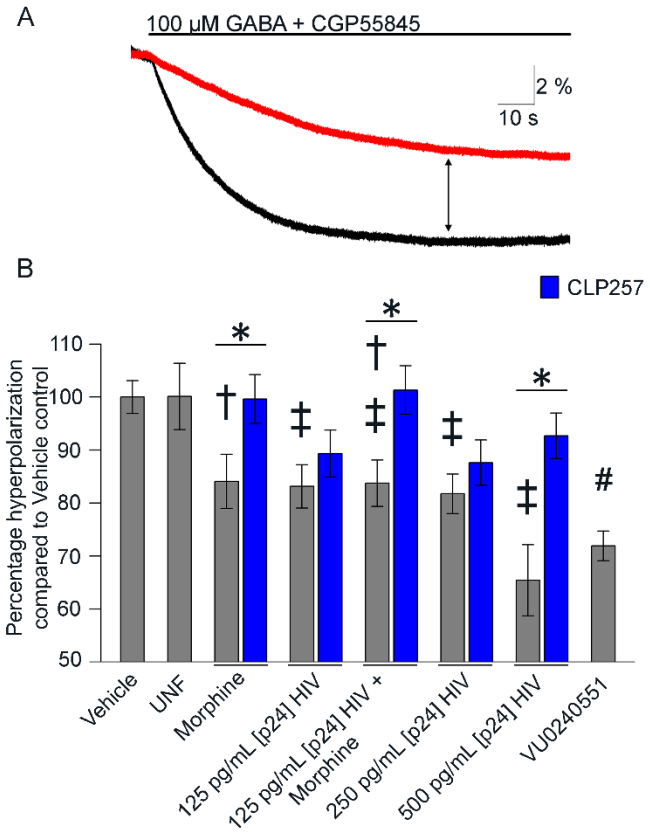
A
100 µM GABA + CGP55845
2 %
10 s
B
CLP257
Percentage hyperpolarization compared to Vehicle control
110
100
90
80
70
60
50
*
*
*
†
†
‡
‡
‡
‡
#
Vehicle
UNF
Morphine
125 pg/mL [p24] HIV
125 pg/mL [p24] HIV + Morphine
250 pg/mL [p24] HIV
500 pg/mL [p24] HIV
VU0240551

Figure 3.2. HIV_{sup} and morphine decrease $GABA_AR$-mediated hyperpolarization. (*A*) Representative traces of 500 pg/mL [p24] HIV_{sup} treated (red) and vehicle control (black) during GABA and CGP55845 perfusion (115 s). Arrow is representative of the diminished hyperpolarization quantified in the bar graph. (*B*) Bar graph shows peak ΔF/F deflection from each treatment groups normalized as a percentage of vehicle control corresponding to that sample during 115 s perfusion of 100 μM GABA and CGP55845. hNeurons expressing Archon1 exposed to 125 - 500 pg/mL [p24] HIV ± morphine shows reduced hyperpolarization ($^{\dagger}p < 0.01$, n = 15 - 21) and/or UNF ($^{\ddagger}p < 0.01$, n = 15 - 21). Analysis revealed a main effect of CLP257 and group comparisons showed that CLP257 significantly reversed the effects of 500 pg/mL HIV_{sup} and morphine groups (blue) ($^{*}p < 0.05$, n = 15 - 21). Further, 2 h treatment with VU02440551 showed similar results as HIV_{sup} ± morphine groups, significantly reducing percentage of cells immunoreactive for KCC2 ($^{\#}p < 0.05$, n = 15 - 21), suggesting that effects of HIV_{sup} and morphine are due to loss of KCC2 activity. Data were analyzed from 15 - 21 cells from 5 – 6 separate tissue samples per group.

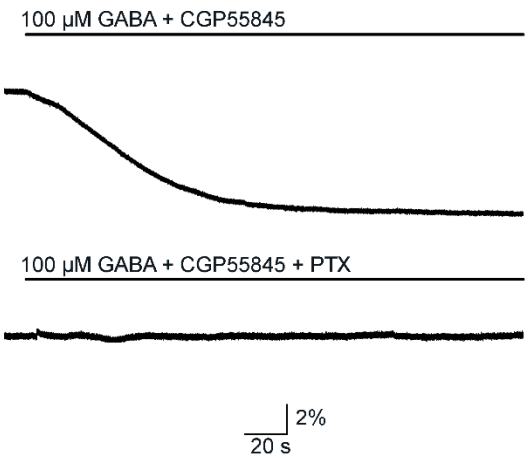

Fig. 3.3. Validation of Archon1. Representative trace of Archon1 activity in hNeuron during perfusion of 100 µM GABA and CGP55845 (Top) and 100 µM GABA and CGP55845 and PTX (bottom). Antagonizing $GABA_AR$ and $GABA_BR$ prevented ΔF/F changes, suggesting that fluorescence shifts seen in the top trace and Fig. 3.2 are due to $GABA_AR$ activation (n = 9).

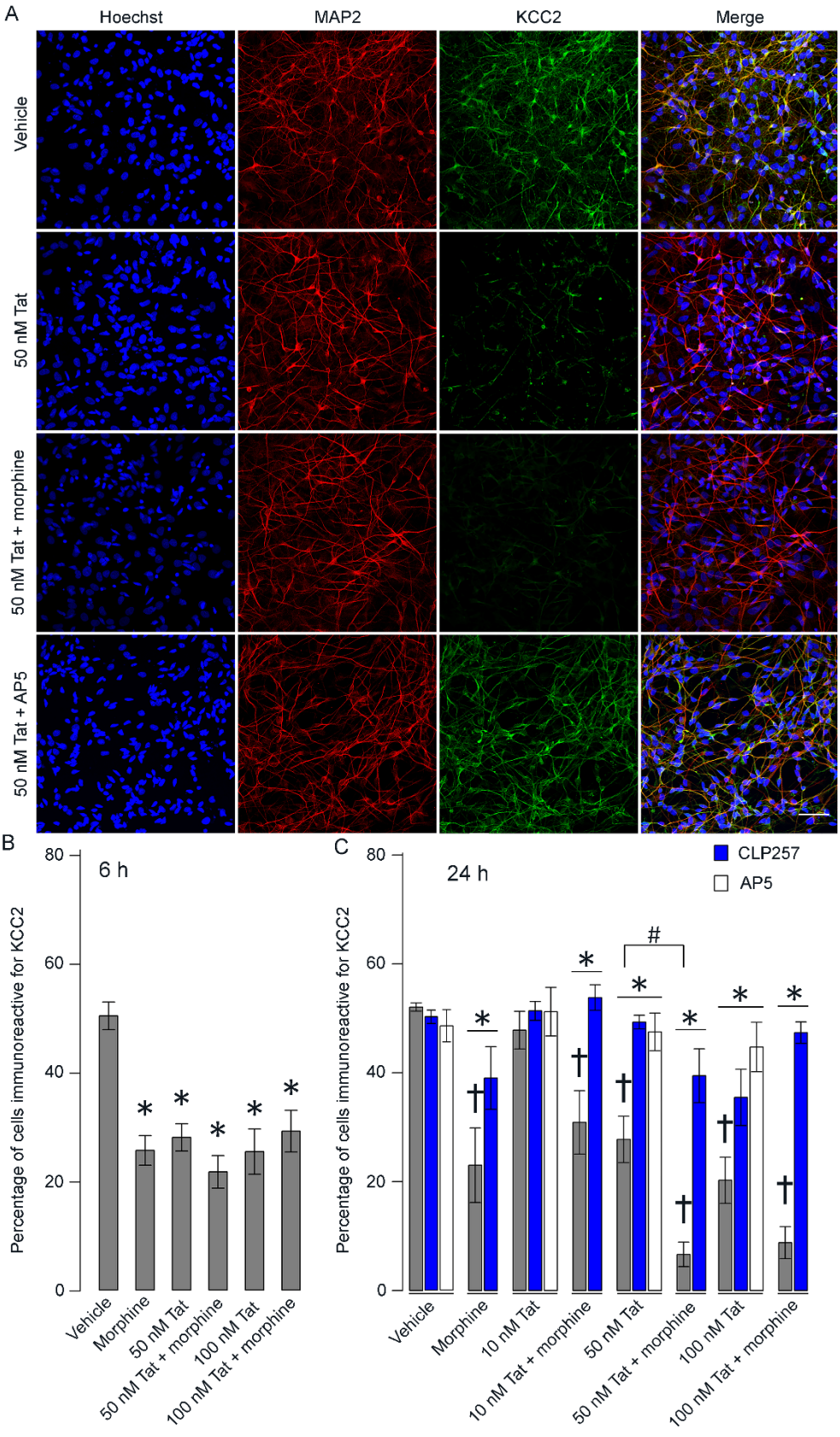
A
Hoechst
MAP2
KCC2
Merge
Vehicle
50 nM Tat
50 nM Tat + morphine
50 nM Tat + AP5
B
6 h
Percentage of cells immunoreactive for KCC2
Vehicle
Morphine
50 nM Tat
50 nM Tat + morphine
100 nM Tat
100 nM Tat + morphine
C
24 h
CLP257
AP5
Percentage of cells immunoreactive for KCC2
Vehicle
Morphine
10 nM Tat
10 nM Tat + morphine
50 nM Tat
50 nM Tat + morphine
100 nM Tat
100 nM Tat + morphine

Figure 3.4. hNeurons lose KCC2 immunoreactivity after exposure to HIV-Tat ± morphine. (*A*) Representative images of hNeuron cultures immunolabeled for MAP2 and KCC2. Loss of KCC2 immunoreactivity can be noted in cells treated with both 50 nM $Tat_{1\text{-}86}$ and 50 nM $Tat_{1\text{-}86}$ + morphine. Rescue is seen in 50 nM $Tat_{1\text{-}86}$ + AP5 (scale bar = 50 µm). (*B, C*) The percentage of cells immunoreactive for KCC2. Both 6 h (*B*) and 24 h (*C*) exposure to 50 - 100 nM $Tat_{1\text{-}86}$ ± morphine results in significant decrease in KCC2 immunoreactivity compared to their respective controls ($^{\dagger}p < 0.01$; n = 6 – 7). Further, a significant interaction between morphine and $Tat_{1\text{-}86}$ was found at the 50 nM $Tat_{1\text{-}86}$ level after 24 h exposure ($^{\#}p < 0.05$). Application of CLP257 (blue) and AP5 (white) showed significant restoration of KCC2 immunoreactivity across all groups and $Tat_{1\text{-}86}$ exposed groups, respectively ($^{*}p < 0.01$).

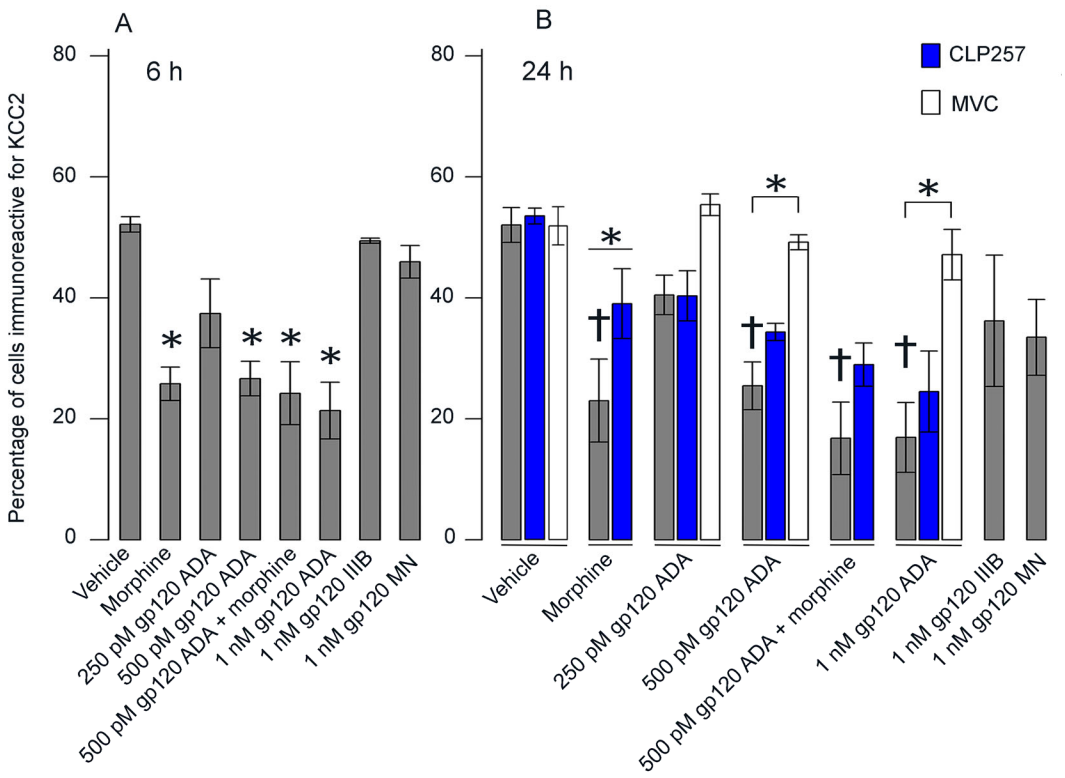

Figure 3.5. gp120 ADA reduces KCC2 immunoreactivity. (*A, B*) Bar graphs quantifying percentage of cells immunoreactive to KCC2. Both 6 h (*A*) and 24 h (*B*) exposures to 500 pM - 1 nM gp120 ADA significantly reduced the percentage of KCC2 immunoreactive cells compared to their respective controls ($^{\dagger}p < 0.01$; n = 6), while gp120 IIIB or gp120 MN had no significant effect on KCC2 expression. Interestingly, CLP257 co-exposure failed to maintain KCC2 immunoreactivity in gp120 ADA exposed groups, but MVC showed significant rescue ($^{*}p < 0.05$; n = 5 - 6), suggesting that CCR5 activation is responsible for gp120 loss.

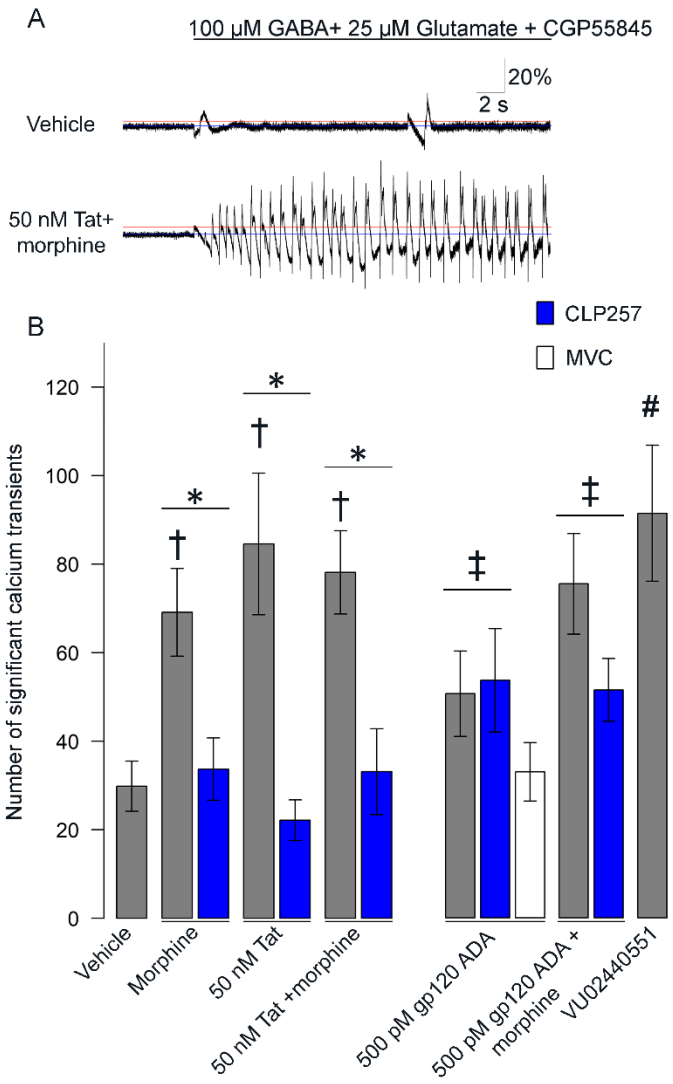
A
100 µM GABA+ 25 µM Glutamate + CGP55845
20%
2 s
Vehicle
50 nM Tat+
morphine
B
CLP257
MVC
Number of significant calcium transients
0
20
40
60
80
100
120
Vehicle
Morphine
50 nM Tat
50 nM Tat +morphine
500 pM gp120 ADA
500 pM gp120 ADA +
morphine
VU0240551

Figure 3.6. Tat_{1-86}, gp120 ± morphine exposure decreased $GABA_AR$ mediated inhibition. (*A*) Representative 30 s sample GCaMP6f traces of vehicle (top) and 50 nM Tat_{1-86} + morphine (bottom) exposed hNeurons during 25 μM glutamate, 100 μM GABA, and CGP55845 perfusion. (*B*) Exposure to 50 nM Tat_{1-86} ± morphine (500 nM) resulted in significantly increased Ca^{2+} transients measured by GCaMP6f activity during 55 s perfusion of 25 μM glutamate, 100 μM GABA, and CGP55845 compared to control (†; $p < 0.05$; n = 15 - 21). CLP257 (blue) rescued these effects (*; $p < 0.01$; n = 15 - 21). Exposure to 500 pM gp120 ADA showed significant increase in neuronal activity, regardless of CLP257 or morphine cotreatment (†; n = 15 - 21; $p < 0.05$). MVC exposure trended towards reversing gp120 effects ($p = 0.059$, n = 18). KCC2 antagonist, VU02440551 recapitulated results of Tat_{1-86}, gp120, and morphine, suggesting that these effects are due to loss of KCC2 activity ($^{\#}p < 0.01$, n = 15). Ca^{2+} transients were considered significant and counted when the spike amplitude was > 4 SD above background activity (determined 2.5 s prior to perfusion; red line) and returned to within 0.5 x standard deviation of background activity (blue line).

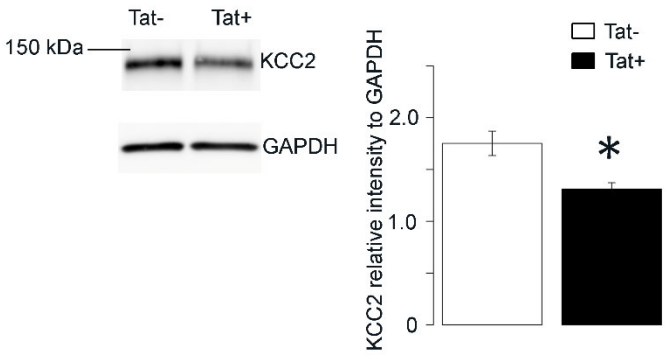

Figure 3.7. Tat+ mice show reduced striatal KCC2 compared to control Tat- mice. Two weeks of Tat expression significantly reduced the amount of KCC2 detected by western blot (n = 12; $p < 0.05$). Data are displayed as KCC2 intensity normalized to GAPDH.

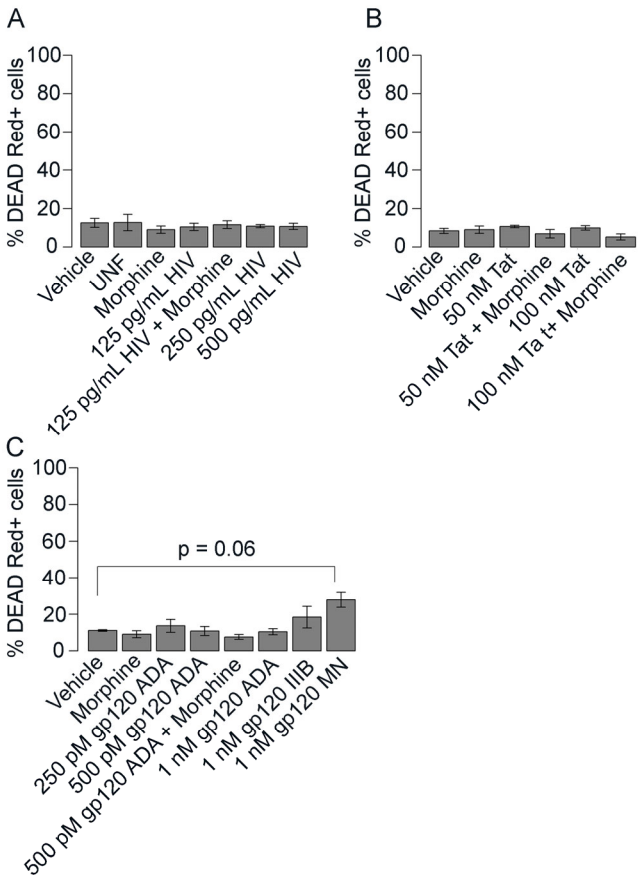

Fig. 3.8. HIV_{sup}, Tat, gp120 ± morphine effects on cellular viability. Treatments for 24 h of 125 – 500 pg/mL [p24] HIV_{sup}, 50-100 nM Tat ± 500 nM morphine had no significant effect on cellular viability (*A*, *B*). Treatments for 24 h of 250 – 1000 pM gp120 ADA and 1 nM gp120 IIIB do not affect cellular viability while 1 nM gp120 trended towards significantly increasing cellular death (*C*). n = 4.

Chapter 4

Restoration of KCC2 membrane localization in striatal D2R-expressing medium spiny neurons rescues behavioral deficits in HIV Tat-transgenic mice

(This chapter is in preparation for publication)

Abstract

People infected with HIV (PWH) are highly susceptible to hippocampal and striatal damage from the neurotoxic HIV protein, transactivator of transcription (Tat). Memory and motor impairment are common among these patients, likely as behavioral manifestations of damage to these brain regions. GABAergic dysfunction from HIV infection and Tat exposure has been well documented. We recently demonstrated that the neuron specific Cl^- extruder, K^+ Cl^- cotransporter (KCC2), is diminished after exposure to HIV proteins resulting in disrupted $GABA_AR$-mediated hyperpolarization and inhibition. Here, we utilized doxycycline (DOX)-inducible, GFAP-driven Tat transgenic mice to further explore this phenomenon. We found no changes in hippocampal KCC2, but a significant decrease in the striatum associated with hyperlocomotion in the open field assay in mice expressing the Tat transgene with two wks of DOX treatment. We were able to restore KCC2 activity and baseline locomotion with the KCC2 enhancer, CLP290. Additionally, we found that CLP290, whose mechanism of action had yet to be described, acts to restore phosphorylation of serine 940 resulting in increased KCC2 membrane localization. We also examined neuronal subpopulation contributions to the noted effects and found that dopamine receptor D2-expressing MSNs are selectively vulnerable to Tat-induced KCC2

loss with no changes seen in dopamine receptor D1-expressing MSNs. These results provide a mechanism underlying motor impairment in PWH and suggest that targeting KCC2, perhaps with CLP290, is a viable treatment for those with HIV-associated neurocognitive disorders.

Introduction

While the advent of combined antiretroviral therapy (cART) has greatly improved the prognosis of HIV-1 infection, quality of life issues remain. One of the most prevalent being HIV-associated neurocognitive disorders (HAND), with about half of people infected with HIV (PWH) experiencing detriment in varying neurocognitive domains including memory recall and sensorimotor function (Heaton et al., 2010, Antinori et al., 2007). These cognitive and behavioral alterations are likely due to synaptodendritic damage and circuitry disruption caused by direct and secondary damage from HIV proteins and persistent neuroinflammation (Masliah et al., 1997, Brailou et al., 2008, Tavazzi et al., 2014, Alakkas et al., 2019). While neurons are not infected by HIV, both microglia and astrocytes can be infected and release viral proteins and inflammatory factors damaging neurons as bystanders. Both the hippocampus and striatum seem to be particularly vulnerable to these factors in humans and *in vivo* models of HAND (Fitting et al., 2010, Marks et al., 2016, Fitting et al., 2013, Gelman et al., 2006, Chang et al., 2008, Maki et al., 2009, Alakkas et al., 2019).

The HIV-1 protein, transactivator of transcription (Tat), is a primary mediator of HIV-induced CNS degeneration, is secreted by infected cells, and its expression is seen in PWH even when receiving effective cART (Henderson et al., 2019). Use of the doxycycline (DOX)-inducible, glial fibrillary acidic protein (GFAP)-driven Tat transgenic

mouse has been used to study the neuropathological features of Tat exposure *in vivo* and recapitulates many of the behavioral abnormalities seen in HAND patients (Paris et al., 2014b, Hahn et al., 2016, Fitting et al., 2012, Marks et al., 2016, Fitting et al., 2013, Carey et al., 2012, Kim et al., 2003). Tat is capable of inducing proinflammatory phenotypes in both astrocytes and microglia, promoting neuroinflammation and has direct neuronal targets. Through activation of NMDAR, AMPAR, L-type voltage gated Ca^{2+} channels, Tat can induce focal disruptions in Ca^{2+} homeostasis resulting in loss of synaptodendritic complexity (Fitting et al., 2014, Schier et al., 2017, Fitting et al., 2010, Chandra et al., 2005, Napier et al., 2014).

Hyperexcitability is a hallmark of the HIV-exposed CNS and excessive glutamate release and reduced clearance have been well studied (Longordo et al., 2006, Pappas et al., 1998, Wang et al., 2003, Musante et al., 2010). Accumulating evidence has also revealed the importance of GABAergic disruption in hyperexcitability. Human postmortem tissue from HAND patients as well as *in vivo* models of HAND display reduced GABAergic markers (Fitting et al., 2013, Marks et al., 2016, Buzhdygan et al., 2016, Gelman et al., 2012a) and functionality (Xu et al., 2016) as we also demonstrated in Chapter 3. K^+ -Cl^- cotransporter 2 (KCC2) is neuron-specific and functions to extrude Cl^- to maintain low the intracellular levels upon which $GABA_AR$ mediated hyperpolarization is predicated. Membrane localization and, therefore, functionality of KCC2 is mediated by several phosphorylation sites (Cordshagen et al., 2018). One well-studied residue, serine 940 (S940), can be phosphorylated by protein kinase C (PKC) to increase membrane stability and localization and can be dephosphorylated by protein phosphatase 1 (PP1) resulting in internalization (Lee et al., 2007, Lee et al., 2011). A loss of KCC2 and/or its membrane

localization results in diminished $GABA_AR$-mediated hyperpolarization/inhibition and has been found to be a mediator of neuronal disinhibition in several neurological disorders (Lizhnyak et al., 2019, Arion and Lewis, 2011, Boulenguez et al., 2010, Chen et al., 2017a, Dargaei et al., 2018, Chen et al., 2017b). Importantly, these deficits have been rescued in many cases with the KCC2 activity enhancer, CLP257, and its prodrug, CLP290, although the mechanism underlying their mechanism of action remains elusive. We have recently shown that KCC2 is a target of the HIV proteins Tat and gp120 in primary human neurons *in vitro* and in an *in vivo* model of neuroHIV and, thus, may be implicated in neuronal dysfunction underlying HAND.

The striatum is composed primarily of dopamine receptor D1 (D1R)-expressing medium spiny neurons (MSNs) and dopamine receptor D2 (D2R)-expressing MSNs. This area is particularly vulnerable to HIV-induced damage in PWH (Gelman et al., 2006, Alakkas et al., 2019), and *in vivo* models of neuroHIV (Fitting et al., 2014, Schier et al., 2017, Fitting et al., 2010). Striatal MSNs, particularly those expressing D2R (Schier et al., 2017) are vulnerable to Tat-induced spine reductions due to dendritic Ca^{2+} influx (Fitting et al., 2014). Given striatal vulnerability to Tat-induced KCC2 loss, and clinical evidence demonstrating motor deficits in PWH, we sought to further explore this phenomenon by examining potential motor dysfunction caused by diminished striatal KCC2, whether these effects were reversible using CLP290, and if there was preferential vulnerability between D1R-expressing or D2R-expressing MSNs. We found a loss of KCC2 selectively in D2R-expressing MSNs of the striatum of mice expressing the Tat transgene (Tat+) vs those without the transgene (Tat-), correlating with motor hyperactivity after two wks DOX

administration. Additionally, we were able to rescue phosphorylation of S940-KCC2, membrane localization of KCC2, and motor activity with CLP290 oral gavage.

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.

Mice used for behavioral and western blot experiments were male, doxycycline (DOX)-inducible, GFAP driven HIV-1 Tat-transgenic mice aged 10 – 12 weeks. Mice that expressed the rtTA and Tat transgenes (Tat+) or mice only expressed rtTA without Tat (Tat-), were fed DOX-containing chow (6 mg/g, Harlan indianapolis, IN) for two weeks prior to experimentation.

Tat+ and Tat- mice were crossed with B6.Cg-Tg (Drd1a-tdTomato)6Calak/J line 6 mice (#016204; The Jackson Laboratory) or Drd2-eGFP (#036931 – UCD; Mutant Mouse Resource and Research Centers) mice (Ade et al., 2011) to detect D1R-expressing and D2R-expressing neuronal subpopulations, respectively, and were used for immunohistochemistry (IHC)/colocalization and electrophysiological experiments.

All mice were housed two to five per cage with *ad libitum* access to food and water in a temperature- and humidity-controlled facility on a 12:12 h light – dark cycle.

Drug administration

Mice were treated with either the KCC2 enhancer, CLP290 (50 mg/kg; Aobious), freshly suspended in dimethyl sulfoxide (DMSO) and 20% 2-hydroxypropyl-β-cyclodextrin (HPCD; Tocris) or vehicle once per day (morning) by oral gavage (200 µL total volume) for seven days during the second week of DOX administration.

Behavior

After two weeks DOX administration, mice were assayed for locomotor effects with the open field assay by placing mice in the top left corner of a 40 x 40 x 35 cm Plexiglas box and recording their activity for 20 min. Rearing responses were recorded when the animal breaks the array of photo-beams and total distance and time spent in center was recorded and encoded using the ANY-maze behavioral tracking system (Stoelting).

Western Blot

KCC2 was quantified by western blot of striatum from Tat+ and Tat- mice. Freshly harvested whole striata were homogenized in RIPA buffer (Sigma) with HALT^{TM} Protease & Phosphatase inhibitor Cocktail (Thermo Fisher) to prevent protein degradation. Lysates were separated and stored at -80º C. Protein concentration was measured using BCA protein assay (Pierce, Rockford, IL). 40 µg lysate per sample were loaded into 4 – 20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to PVDF membranes (Bio-Rad) and probed with anti-KCC2 (1:2000; Protein Tech, Rosemont, IL) or anti-pS940 KCC2 and GAPDH (1:2000; Abcam) antibodies. KCC2 and GAPDH proteins were detected measuring fluorescent signal from Alexa Fluor 647 and 488-conjugated secondary antibodies (1:1000; Thermo Fisher), respectively. pS940-KCC2 proteins were detected using pS940-KCC2 specific antibody (1:2000; Novus Bio),

horseradish peroxidase (HRP; Southern Biotech; Birmingham AL) and SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher).

Membrane bound KCC2 was examined by western blot by first performing membrane fractionation on striata from Tat transgenic mice. Briefly, tissue was homogenized in lysis buffer (4 mM HEPES (N-2-hydroxyethylpiperazine-N-3-ethane sulfonic acid; Thermo Fisher Scientific), 320 mM sucrose, 5 mM EDTA (ethylenediaminetetraacetic acid; Thermo Fisher Scientific), and HALT™ Protease & Phosphatase inhibitor cocktail (Thermo Fisher Scientific)), centrifuged at 100,000 x g, and the pellet containing membrane fraction was resuspended in lysis buffer and stored at -80º C. Protein concentration measurement and western blot for these samples were performed as above, except that a 20-µg lysate load was used per sample lane. KCC2 was normalized to total lane protein detected by Revert™ 700 Total Protein Stain (LI-COR, Lincoln, NE), and Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher) were used to detect KCC2.

IHC

After two wks DOX administration, Drd1a-tdTomato/Drd2-eGFP X Tat+/Tat- mice were fixed by cardiac perfusion with 4% paraformaldehyde (PFA). Brains were then dissected, submerged in 4% PFA for 24 h, washed in PBS, and sequentially placed in 10%, 20%, and 30% sucrose for 24 h each before being embedded in OCT. Serial 20 µM sections were cut on a cryostat, thaw mounted, dried, and stored frozen at -80º C. IHC was then performed by permeabilizing for 30 min (0.01% Triton-X100), blocking for 1 h, and overnight incubation with primary antibody for KCC2 (1:500; Protein Tech) at 4º C. The following day, Drd1a-tdTomato and Drd2-eGFP sections were washed and incubated for

1 h with 488 and 594 Alexa-Fluor conjugated secondary antibodies (1:1000; Invitrogen, Carlsbad, CA), respectively, for 1 h. Hoechst 33342 (1:10000, Thermo Fisher) was used to identify nuclei and weighted coverslips were adhered with ProLong™ Gold Antifade Mountant (Thermo Fisher).

Colocalization

To visualize KCC2 immunofluorescence and tdTomato or eGFP, Z-stack images were obtained with a Zeiss LSM 700 confocal module configured to an Axio Observer Z.1 with 20 x objective and Zen 2010 software (Zeiss Inc., Thornwood, NY). 3D reconstruction and colocalization between KCC2 and tdTomato (D1R-expressing MSNs) or eGFP (D2R-expressing MSNs) was performed with Imaris software (Bitplane, South Windsor, CT). Colocalization thresholding was determined using secondary control (without primary antibody incubation) sections from the same mice. Thresholds were equivalent across all mice and set to virtually no colocalization (< 1%) between tdTomato/eGFP and KCC2 immunofluorescence for control slices. Colocalization results are displayed as the percentage of voxels with tdTomato or eGFP fluorescence colocalized with voxels containing KCC2 immunofluorescence.

Statistics

A two-tailed Student's *t* test was used to examine potential differences in KCC2 western blots between Tat+ and Tat- mice and for colocalization experiments. Two-way analysis of variance (ANOVA) was used to examine behavioral assays and western blots with drug (CLP290/Vehicle) and genotype (Tat+/Tat-) as factors. Interactions and main effects were examined via simple main effects using Bonferroni *post hoc* test to determine group

differences. Data are displayed as mean value ± standard error of the mean. Results were considered significant when $p < 0.05$. All statistical analyses were performed in R.

Results

Two weeks of DOX-induced Tat expression reduces total KCC2 in the striatum of Tat-transgenic mice.

Clinical evidence and results from Tat-transgenic mice have demonstrated vulnerability of the striatum and hippocampus to HIV- and Tat-induced neuropathology. Thus, we examined total KCC2 by western blot in hippocampal and striatal tissue from Tat-transgenic mice with DOX administration for two and four weeks (striatum) or two, four, and eight weeks (hippocampus). There was no difference found in hippocampus at any time points (two wk DOX: $p = 0.82$, n = 6; four wk DOX: $p = 0.51$, n = 6; eight wk DOX: $p = 0.65$, n = 7) (Fig 4.1A). Nor were significant differences found in the striatum after four wks of DOX-induced Tat exposure ($p = 0.28$, n = 6) (Fig 4.1B). However, we found a significant decrease in total KCC2 in the striatum of Tat+ mice compared to Tat- mice on DOX for two wk ($p = 0.001$, n = 11 - 12) (Fig 4.1B; data also shown in Fig 3.8, included here for clarity). These results suggest that the striatum is vulnerable to Tat-induced KCC2 loss in a biphasic, or possibly, more complex manner. This suggests that striatal circuitry may be disrupted due to deficits in $GABA_AR$-mediated inhibition after two wks of Tat exposure.

Tat expression reduced total KCC2 in the striatum and CLP290 rescued pS940-KCC2 and KCC2 membrane localization.

To determine if striatal KCC2 loss could be reversed, we treated Tat+ and Tat- mice with 50 mg/kg CLP290 or vehicle by oral gavage once per day during the second week of DOX administration. Western blot analysis revealed a main effect of genotype to reduce total KCC2 in the striatum of Tat+ compared to Tat- mice without interaction (main effect: $p = 0.002$, n = 12 – 18), suggesting that Tat induction diminished the presence of KCC2 and that CLP290 failed to rescue total KCC2 levels (Fig 4.2A). Membrane localization of KCC2 and, thus, Cl^- extrusion activity is promoted by phosphorylation of S940. We sought to determine if CLP290 treatment was capable of rescuing phosphorylation of S940 (pS940). Analysis of western blot of striata harvested from Tat+ and Tat- mice treated with either CLP290 or vehicle for pS940-KCC2 revealed a drug by genotype interaction ($p < 0.05$, n = 8 – 12). Investigation of simple main effects determined that Tat expression reduced pS940-KCC2 and administration of CLP290 rescued this effect ($p < 0.05$; n = 8 - 12) (Fig 4.2B) and, thus, likely restores membrane localization and Cl^- extrusion capacity of KCC2. To confirm this, we performed a membrane fraction separation and western blot for KCC2 and found that membrane localization of KCC2 was, again, significantly reduced by two wks Tat exposure and was, in fact, restored by CLP290 administration ($p < 0.05$, n = 8 – 9) (Fig 4.2C). These data suggest that CLP290 is able to rescue the functional state of KCC2 and provide insight into a mechanism involving phosphorylation of S940 that may underlie the effects of CLP290.

Tat+ mice displayed hyperactive locomotion with rescue seen via CLP290 administration.

To determine potential behavioral manifestations of Tat effects on disrupted striatal activity, we assayed locomotor activity of Tat+ and Tat- mice treated with either CLP290

or vehicle with the open field test. Significant increases were found in the distance travelled and rearing number in the Tat+/Veh groups with rescue in the Tat+/CLP290 group ($p < 0.05$, n = 8 – 9) (Fig 4.3 A, B). These results suggest motor hyperactivity in Tat+ animals with CLP290 restoration of KCC2 membrane localization sufficient to restore baseline activity. We also examined the time spent in center zone as one measure of anxiety-like behavior and found no significant differences (n = 8 – 9) (Fig 4.3C). Overall, these results suggest that diminished KCC2 activity in the striatum, likely resulting in neuronal disinhibition, leads to excessive locomotion.

Previous work in our lab demonstrated a loss in dendritic spines selectively in D2R-expressing MSNs of Tat+ mice (Schier et al., 2017). Given the biochemical and behavioral results presented here and the role KCC2 plays in dendritic spine maintenance (Li et al., 2007, Fiumelli et al., 2013), we hypothesized that D2R-expressing MSNs are selectively vulnerable to Tat-induced KCC2 loss, contributing towards disrupted striatal circuitry manifesting as hyper locomotor activity.

D2R-expressing MSNs show enhanced KCC2 loss in response to Tat induction compared to their D1R-expressing counterparts.

We crossed Tat+ and Tat- mice with Drd1a-tdTomato and Drd2-eGFP mice to allow for independent examination of D1R-expressing and D2R-expressing MSNs to determine if there is a preferential vulnerability of D1R or D2R-expressing MSNs to Tat-induced KCC2 loss. To visualize KCC2 colocalization with tdTomato (D1R-expressing MSNs) or EGFP (D2R-expressing MSNs) we performed IHC with antibodies specific for KCC2 and either 488 or 594 secondary antibodies, respectively. Z stacks of striata were obtained and 3D reconstruction and subsequent colocalization analysis was performed

with Imaris. We found a loss of KCC2 colocalization with eGFP in Tat+ x Drd2-eGFP mice compared to Tat- x Drd2-eGFP mice ($p < 0.05$, n = 8) and no significant changes in KCC2 colocalization between Tat+/Tat- x Drd1a-tdTomato mice (n = 3 – 12) (Fig 4.4). It does not appear that there is overt, total loss of KCC2 in some D2R-expressing MSNs, thus, there is likely a more subtle decrease across a large proportion. Particularly noticeable is that somatic KCC2 staining appears primarily around the membrane in D1R-expressing MSNs and Tat- D2R-expressing MSNs (yellow arrows, Fig 4.4), this localization is lost in Tat+ D2R-expressing MSNs (white arrows, Fig 4.4), suggesting shifted reversal potential of Cl^- and diminished hyperpolarization in this population of neurons. Overall, data from these experiments suggest a preferential vulnerability D2R-expressing MSNs to Tat-induced KCC2 loss and provide some insight to the circuit disruption underlying the behavioral abnormalities found above.

Discussion

These studies highlight a novel route of Tat-mediated neuronal damage to induce motor dysfunction. Loss of KCC2 detection was associated with motor hyperactivity after two wks of DOX-induced Tat exposure and we found preferential vulnerability of D2R-expressing MSNs. Importantly, we were able to rescue membrane-localized KCC2 and behavioral abnormalities with the KCC2 activity enhancer, CLP290, demonstrating the potential efficacy of KCC2 as a therapeutic target for the treatment of HAND.

The classic striatal model of motor initiation involves a balance between activation of the D1R-expressing MSN-mediated ‘Go-pathway’ and D2R-expressing MSN-mediated ‘No Go-pathway’ (Gerfen and Young, 1988, Kravitz et al., 2010). Based on this model, we would expect to have seen a preferential vulnerability of D1R-expressing MSNs to

Tat-induced KCC2 loss resulting in disinhibition of the D1R-mediated 'Go pathway' of motor initiation. Our data do not support this hypothesis of motor initiation and lend credence to the growing body of evidence refuting this model. Recent prevailing theories suggest more subtle circuitry underlying striatal motor initiation and have demonstrated increased D1R- and D2R-expressing MSN activity during movement (Parker et al., 2018, Cui et al., 2013, Klaus et al., 2017, Barbera et al., 2016, Kupchik et al., 2015). Thus, disinhibition of either D1R- or D2R-expressing MSNs may lead to abnormal locomotion and as our data suggest, D2R-expressing MSN disinhibition induces motor hyperactivity in the open field assay.

Motor impairment in PWH was initially described in the first publication outlining neurocognitive decline associated with HIV (Navia et al., 1986) and has persisted into the era of cART treatment (Valcour et al., 2008, Heaton et al., 2010, Robinson-Papp et al., 2008). Typically, HIV-induced motor deficits are associated with hypokinetic symptoms such as bradykinesia (Mirsattari et al., 1998, Bhidayasiri and Tarsy, 2012, Sullivan et al., 2011, Valcour et al., 2008), but hyperkinetic states like tremor which can be caused by disinhibition of the striatum (Oran and Bar-Gad, 2018) can be seen as well and may occur with or without other signs of Parkinsonism (Mirsattari et al., 1998, Cardoso, 2002, Nath et al., 1987). Our results demonstrate motor hyperactivity after two wks of DOX-induced Tat exposure (Fig 4.3 A, B) associated with reduced membrane localization of KCC2 (Fig 4.2 C) and may serve as a mechanism underlying heightened striatal output of HIV-induced tremor. Studies utilizing positron emission tomography to measure basal ganglia metabolism found that early stages of HIV-infection/HAND were associated with hyperactivity of the basal ganglia, followed by basal ganglia hypometabolism and

bradykinesia as the disease progresses (von Giesen et al., 2000). Our results with two wks DOX treatment may represent early disease stages, resulting in excessive striatal activity caused by hyperactivity in D2R-expressing MSNs of the striatum and increased locomotion. In fact, we have found that Tat transgenic mice also display a biphasic change in locomotor activity, whereby, as we have demonstrated here, hyperactivity after two wks of Tat exposure (Fig 4.3 A, B) and previous studies from our lab found decreased locomotion after four wks DOX-induced Tat expression (Hahn et al., 2016), a time point at which we found no differences in KCC2 (Fig 4.1), mirroring the clinical results showing increased then decreased striatal activation (von Giesen et al., 2000). Importantly, we also found that we could mitigate Tat-induced motor deficits with pharmacological maintenance of membrane-bound KCC2 exemplifying the utility of targeting this pathway as a potential therapy for PWH with motor impairment. Based on the involvement of KCC2 in motor behavior, it is reasonable to hypothesize that KCC2 dysregulation may contribute to other behavioral or behavioral and cognitive deficits in Tat-transgenic mice and PWH, respectively, and should be explored going forward. While we found no overt changes in total KCC2 in the hippocampus, there may be more subtle alterations in specific neuronal subsets that may not be detectable by western blot of the entire hippocampus and changes to KCC2 localization and/or function may be present and operative in memory deficits seen in Tat-transgenic mice and PWH.

Our results should be validated and expanded upon by examining the functional responses of D1R- and D2R-expressing MSNs by either gramicidin perforated patch clamp or tight-seal cell-attached current-clamp to measure Cl^- reversal and GABA-mediated postsynaptic potentials (Perkins, 2006), respectively. We hypothesize that

D2R-expressing MSNs would preferentially show functional deficits based on our colocalization results demonstrating selective vulnerability of D2R-expressing MSNs to Tat-induced KCC2 loss. We previously demonstrated D2R-expressing MSN vulnerability to Tat-induced decrease in dendritic spine density (Schier et al., 2017). Given the importance of KCC2 in dendritic spine stability (Fiumelli et al., 2013, Li et al., 2007), and our results demonstrating D2R-expressing MSN vulnerability to both KCC2 and dendritic spine loss, future studies should examine whether maintenance of KCC2 with CLP290 is sufficient to restore dendritic spines. This would further increase the therapeutic scope of CLP290 for utility in restoration of GABAergic function as well as excitatory circuitry. Interestingly, we had previously not found significant changes in locomotion in the open field assay after two wks DOX treatment (Schier et al., 2017). It is important to note that in our previous studies, we had used Tat+ and Tat- mice crossed with D1-tdTomato and D2-eGFP mice for all experiments reported in that paper, while the behavioral assays here utilized Tat+ and Tat- mice (not crossed), potentially suggesting strain differences in the effects of Tat on locomotion. Previous examinations of D1-tdTomato and D2-eGFP mice showed no difference and elevated motor activity, respectively, compared to C57Bl/6 control mice (Ade et al., 2011). Motor hyperactivity found in the D2-eGFP mice may have confounded data from the open field assay in our previous study, resulting in the disparate outcomes between the data presented here and those from our earlier study (Schier et al., 2017). Other subtle differences in experimental paradigm may explain these differences as well. The behavioral battery in the prior study more intensively explored anxiety-like behaviors measured with the elevated plus maze on the same day as the open field assay and differences in assay length: 20 min versus 30 min for the present

study and previous examinations, respectively. Whether these or other potential confounds (such as experimenter or prior handling) produced the discrepancies between these studies should be examined going forward.

HIV infection has many comorbidities including substance use disorder (SUD) and drug use can affect the progression of HAND (NIDA, 2019, Canan et al., 2018, Denis et al., 2019, Bell et al., 2002, Byrd et al., 2011). PWH have also been shown to have disrupted reward processing, placing them at higher risk for adverse outcomes associated with SUD (Anderson et al., 2016, Plessis et al., 2015). Drugs of abuse have profound impact on striatal circuitry and can result in long term circuit changes resulting in dependency (Koob and Volkow, 2010). Our previous work demonstrated that activation of μ-opioid receptor by morphine can reduce KCC2 in human neurons *in vitro* (Chapter 3) and previous studies have shown that opiate dependency can induce a GABA switch from inhibitory to excitatory (Laviolette et al., 2004, Vargas-Perez et al., 2009). While not directly explored, changes in KCC2 activity may underlie this shift. Fitting et al. (2010) also demonstrated that opiate exposure can exacerbate Tat-induced striatal dendritic pathology (Fitting et al., 2010). Given striatal vulnerability to disruption from both HIV and drugs of abuse, the experiments performed here should be expanded to examine how drugs of abuse may interact with Tat to affect striatal KCC2, GABAergic function, and associated behavioral outputs. Further, Tat exposure has increased cocaine induced motor hyperactivity (Harrod et al., 2008, Paris et al., 2014a). Thus, these factors may converge to dysregulate KCC2, exacerbating motor hyperactivity.

CLP290 administration had previously been utilized to restore KCC2 Cl^- extrusion and walking in spinal cord injured mice (Chen et al., 2018), morphine-induced

hyperalgesia in rats (Ferrini et al., 2017), and somatosensory-related behavior after traumatic brain injury (Lizhnyak et al., 2019). Here, we increased membrane-localized KCC2 and restored motor activity to baseline levels after two wks of CNS exposure to Tat. Membrane-localization and degradation of KCC2 is tightly regulated by several different phosphorylation events. While its efficacy has been well documented, the mechanism by which CLP290 reestablishes KCC2 activity remains elusive. Our results suggest that CLP290 rescues KCC2 activity by restoring phosphorylation of S940 to increase membrane stabilization of KCC2 and, therefore, Cl^- extrusion. Whether CLP290 directly interacts with KCC2 to maintain S940 phosphorylation, or if it enhances PKC or inhibits PP1 to increase or decrease S940 phosphorylation, respectively, is unknown and should be explored in future studies.

These experiments demonstrate the role of KCC2 in behavioral deficits induced by CNS exposure to Tat which add to our previous *in vitro* studies in which we first identified dysregulation of KCC2 by HIV-1 and the Tat and $gp120_{ADA}$ HIV proteins. KCC2 is emerging as a key regulator of hyperexcitability in several neurological disorders and the use of CLP290 has shown promise for pharmacological reintroduction of KCC2 and rescue of physiological and behavioral deficits. We provided evidence that begins to uncover the mechanism by which CLP290 reinstates KCC2 activity which had previously not been described. Overall, we add *in vivo* biochemical and behavioral evidence to the utility of targeting KCC2, perhaps with CLP290, as a potential therapy for PWH with HAND, particularly those with motor impairment.

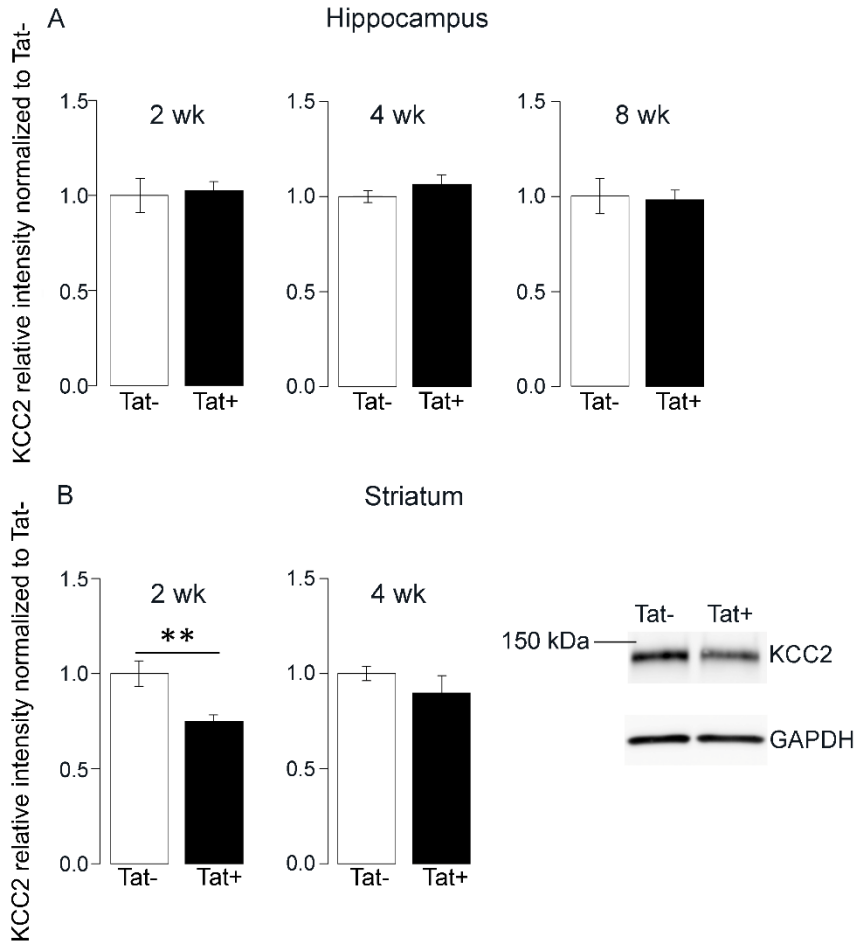

Figure 4.1. Two wk DOX-induced Tat expression in Tat+ mice reduced total striatal KCC2. Analysis of western blot from hippocampus animals administered DOX for two (p = 0.82, n = 6), four (p = 0.51, n = 6), and eight wks (p = 0.65, n = 7) show no significant differences between Tat+ and Tat- mice (*A*). Two wk DOX administration significantly reduced total KCC2 in the striatum of Tat+ mice compared to Tat- mice (** p = 0.001, n = 11 – 12) and four wk DOX groups showed no significant differences (p = 0.28, n = 6). Representative blots showing decreased KCC2 in Tat+ mice compared to Tat- after two wk DOX treatment (*B*; right). All KCC2 western blots are represented as relative intensity to GAPDH normalized to Tat- groups.

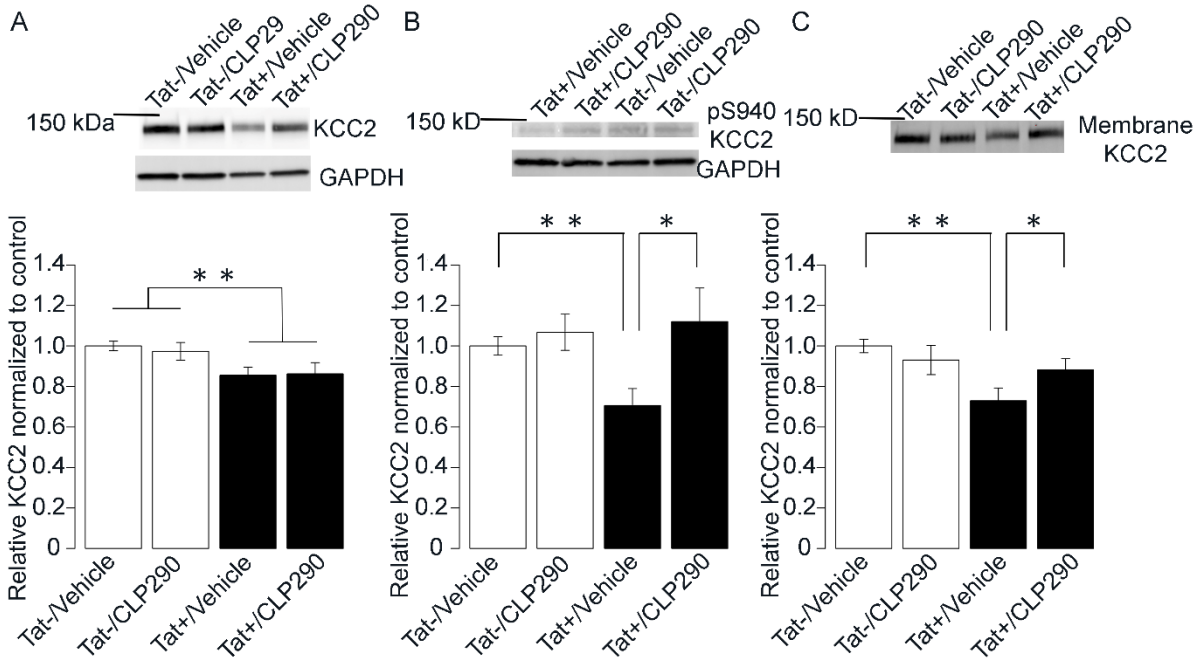

Figure 4.2. CLP290 administration rescues phosphorylation of S940 and membrane localization of KCC2. Two-way analysis revealed a main effect of Tat expression to reduce total KCC2 (**, $p < 0.01$, n = 12 – 18) suggesting that CLP290 failed to rescue total KCC2 levels (*A*). Examination of phosphorylation of S940 revealed a significant decrease in the Tat+/Vehicle group compared to control (** $p < 0.01$, n = 8 – 12) and rescue with CLP290 (* $p < 0.05$, n = 8 – 12) (*B*) suggesting that membrane bound KCC2 may be altered, which was confirmed by western blot on from membrane fractionation (** $p < 0.01$; * $p < 0.05$, n = 8 – 9) (*C*). Blots for total and pS940-KCC2 are represented as intensity relative to GAPDH normalized to Tat-/Vehicle controls (*A*, *B*). Blots for membrane bound KCC2 are relative to total lane protein normalized to controls (*C*).

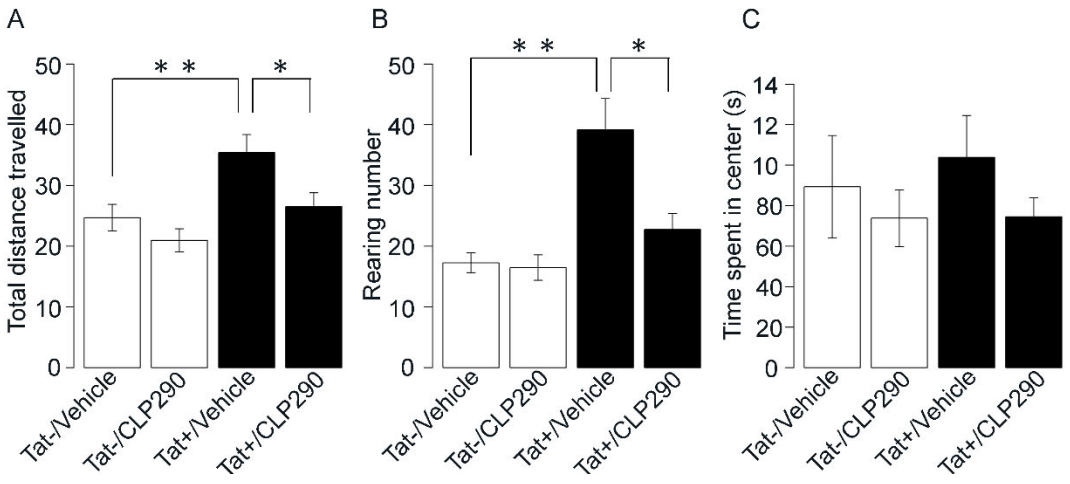

Figure 4.3. Tat+ mice show hyperactive locomotion in the open field assay with CLP290 able to rescue this effect. Tat+ mice given vehicle showed increased motor activity compared to controls in the open field assay measured by both total distance travelled (*A*) and rearing number (*B*) (**, $p < 0.01$, n = 8 – 9). CLP290 administration significantly decreased total distance travelled and rearing number in Tat+ mice (*, $p > 0.05$, n = 8 – 9), suggesting that restoration of membrane-localized KCC2 was sufficient to rescue abnormal locomotor activity in these animals. We also examined time spent in center zone as a measure of anxiety-like behavior and found no significant differences (n = 8 – 9) (*C*).

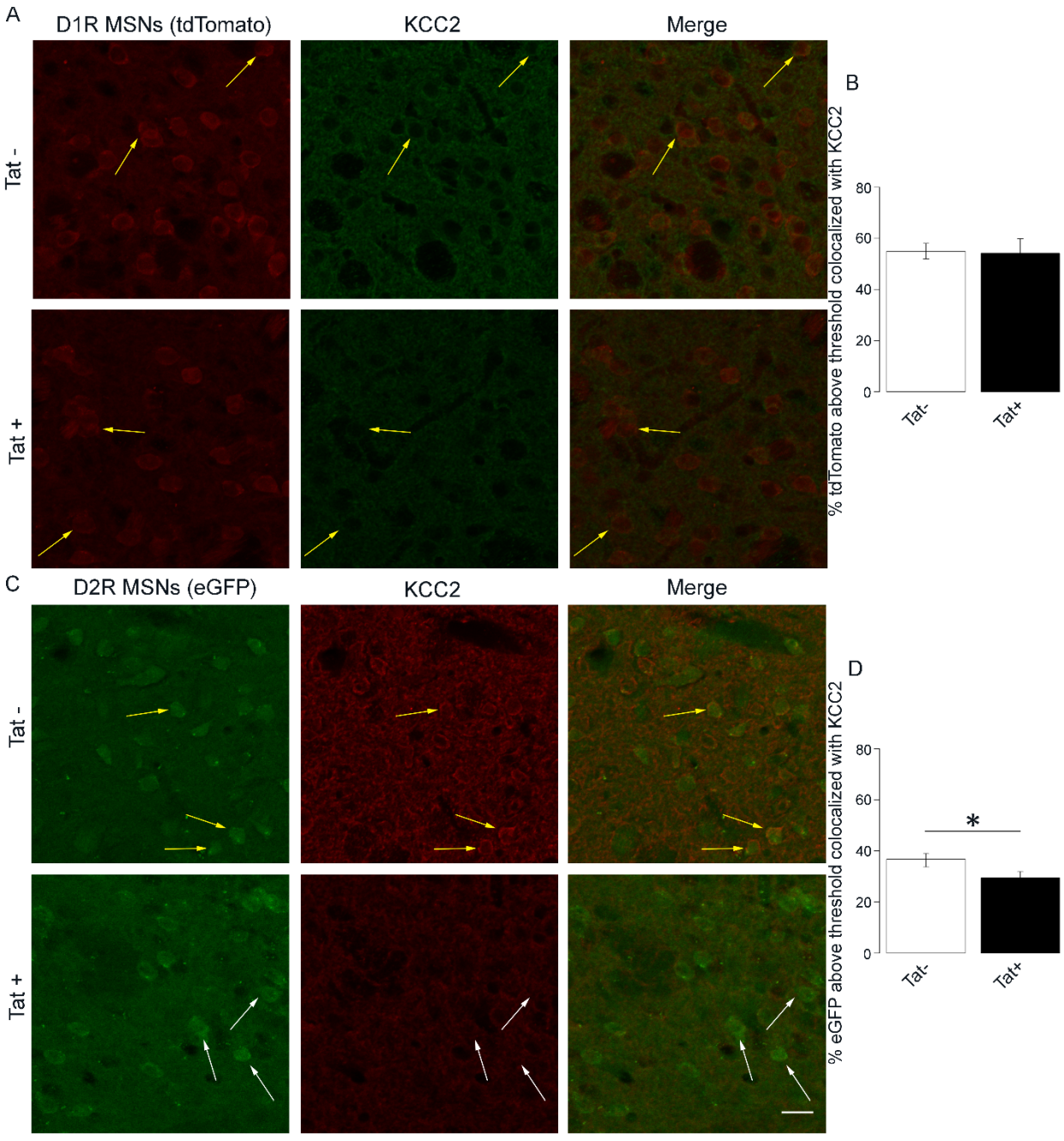
A
D1R MSNs (tdTomato)
KCC2
Merge
Tat -
Tat +
B
% tdTomato above threshold colocalized with KCC2
80
60
40
20
0
Tat-
Tat+
C
D2R MSNs (eGFP)
KCC2
Merge
Tat -
Tat +
D
% eGFP above threshold colocalized with KCC2
80
60
40
20
0
*
Tat-
Tat+

Figure 4.4. D2R-expressing MSNs display Tat-induced KCC2 loss while D1R-expressing MSNs do not. Colocalization analyses revealed no change in KCC2 immunofluorescence with tdTomato between Tat+ x Drd1a-tdTomato and Tat- x Drd1a-tdTomato mice (n = 3 (Tat-), 12 (Tat+)) (*A*, *B*). Representative images showed no change in KCC2 immunofluorescence in D1R-expressing MSNs (tdTomato). Yellow arrows exemplify somatic KCC2 staining, localized around the cell membrane, in D1R-expressing MSNs (*A*). Tat+ x Drd2-eGFP mice showed decreased colocalization with KCC2 immunofluorescence compared to Tat- x Drd2-eGFP mice (*$p < 0.05$; n = 8) (*C*, *D*). Representative images showed strong colocalization in D2R-expressing MSNs of Tat- mice, while Tat+ mice showed decreased immunofluorescence colocalized with D2R-expressing MSNs (eGFP) (*C*). Yellow arrows exemplify somatic membrane KCC2 colocalization with D2R-expressing MSNs (top *C*). White arrows exemplify D2R-expressing neurons with reduced KCC2 cell membrane localization and decreased colocalization with eGFP (bottom *C*). These results suggest a preferential vulnerability of D2R-expressing MSNs to Tat-induced KCC2 loss. Scale bar = 20 μM.

Chapter 5

Conclusions and Future Directions

The introduction of cART has changed the prognosis of an HIV-1 infection from a virtual death sentence to a manageable chronic condition. Thus, the focus of treatments for these patients has shifted from prophylaxis against opportunistic infection to viral suppression and improvement of quality of life. Even with cART adherence, HAND is a preeminent complication for up to 50% of PWH (Heaton et al., 2010). Neuronal hyperexcitability along with reduced synaptodendritic complexity underlies disrupted circuitry that manifests as cognitive and behavioral alterations associated with HAND (Masliah et al., 1997). Heightened glutamatergic signaling plays a significant role and has classically been the center of focus when studying hyperexcitability. Mounting evidence also demonstrates disrupted GABAergic signaling as a vital component to this process (Gelman et al., 2012a, Buzhdygan et al., 2016). The evidence presented in this dissertation demonstrates that KCC2 plays an integral role in disrupting the postsynaptic response in GABAergic signaling, contributing to hyperexcitability of the HIV-exposed CNS. Through experiments involving a novel human *in vitro* model and DOX-inducible, GFAP-driven Tat-transgenic mice we determined that HIV-1, and viral proteins diminish KCC2 resulting in $GABA_AR$ signaling deficits and behavioral abnormalities. Most of these deficits were ameliorated pharmacologically using the KCC2 enhancer, CLP257, and its prodrug, CLP290, demonstrating the efficacy of targeting KCC2 as a novel therapy for HAND.

SUD is a common comorbidity of HIV-infection. IDU was responsible for ~20% of new HIV-1 cases in 2016 and up to 50% of PWH are prescribed opiates (NIDA, 2019, Denis et al., 2019, Chilunda et al., 2019). Regardless of whether use is illicit or prescribed, opiate exposure results in faster progression of AIDS (Peterson et al., 1990, Peterson et al., 2004), and possibly, exacerbated HAND symptomology (Bell et al., 2002, Bell et al., 1998, Byrd et al., 2011, Chiesi et al., 1996, Lucas et al., 2006). These enhanced symptoms are likely the result of cellular signaling convergence from HIV proteins and opiate activation of MOR in areas of the brain vulnerable to insult from both factors, such as the hippocampus and striatum. Previously identified targets of morphine-HIV neuronal signaling convergence include GSK3β (Masvekar et al., 2015), and p38 mitogen activated protein kinases (MAPK) (Mukerjee et al., 2008, Hu et al., 2005) which can enhance neuronal death *in vitro*. Our results demonstrate that morphine activation of MOR can independently reduce KCC2 and disrupt $GABA_AR$-mediated hyperpolarization and inhibition and has a significant or trend toward a combinatorial effect with HIV-Tat and gp120, respectively, to enhance KCC2 loss under certain conditions (Chapter 3; Fig 3.1, 3.2, 3.4, 3.5, 3.6). Thus, our *in vitro* results provide a cellular/molecular substrate upon which opiates disrupt GABA signaling and can converge with HIV protein-mediated pathways to enhance these effects.

Opiate administration activates MOR on GABAergic interneurons of the VTA, inhibiting GABA release from this neuronal subpopulation, thus, disinhibiting DAergic neurons, increasing DA release on the NAcc inducing feelings of euphoria and promoting opiate dependence. Previous studies have demonstrated that opiate dependence and withdrawal cause a switch of GABA on VTA GABAergic terminals from inhibitory to

excitatory, although they did not provide a mechanistic cause of this physiological reversal (Laviolette et al., 2004, Vargas-Perez et al., 2009). Our results provide the first direct link between MOR activation and diminished KCC2 and neuronal disinhibition (Chapter 3; Fig 3.1, 3.6). Thus, it is feasible that MOR activation after repeated exposures to opiates *in vivo* results in decreased KCC2 on GABAergic interneurons of the VTA. This, in turn, necessitates continual MOR activation to maintain their inhibition (through neuronal $G_{i/o}$ signaling) thereby maintaining VTA DAergic activity, which would promote opiate dependency. Removal of MOR activation disinhibits these GABAergic interneurons, which is now exacerbated by reduced KCC2 and $GABA_AR$-mediated excitation and thus, strongly inhibits VTA DAergic neuronal activity on the NAcc inducing a withdrawal state. This proposed mechanism of KCC2-driven opiate dependency and withdrawal should be investigated *in vivo* to determine if opiate dependency and withdrawal reduce KCC2 in GABAergic interneurons of the VTA and if modulation (perhaps with CLP290) affects outcomes associated with excitatory GABA and opiate dependency and withdrawal.

Signaling from HIV and drugs of abuse converge on the striatum and studying their interactive effects may give insight to the vulnerability of PWH to reward deficits. These deficits have been reflected in studies in which Tat+ mice showed a three-fold increase in both ethanol and cocaine conditioned place preference (CPP) compared to controls (Paris et al., 2014a, McLaughlin et al., 2014). These behavioral changes are accompanied by significant changes in DAergic markers (Kesby et al., 2016, Ferris et al., 2009a, Ferris et al., 2009b) and drug of abuse enhancement of striatal MSN damage (Fitting et al., 2010, Bruce-Keller et al., 2008). Classic theories on reward circuitry, typically studied in regard to drugs of abuse, proposed a diametrically opposed role of

D1R and D2R-expressing MSNs. D1R-expressing MSNs theoretically encode reward and are activated by drugs of abuse and whose stimulation can induce/potentiate CPP, whereas D2R-MSNs encode aversion and inhibit drugs of abuse-induced CPP (Lobo et al., 2010, Hikida et al., 2010, Tai et al., 2012, Kravitz et al., 2012). This controversial theory of reward circuitry has come into question in more recent studies (Soares-Cunha et al., 2016, Natsubori et al., 2017, Cole et al., 2018) exemplified by a 2019 publication that demonstrated that both D1R and D2R-expressing MSNs can encode reward, dependent on neuronal stimulation frequency, determined by selective optogenetic activation of D1R and D2R-expressing MSNs (Soares-Cunha et al., 2019). Our data suggests GABAergic disruption in the striatum of Tat+ mice, specifically, disinhibition of D2R-expressing MSNs is mediated through reduced KCC2 (Chapter 4, Fig 4.3, 4.4). These may be involved in the sequelae of events resulting in Tat-potentiated cocaine and ethanol reward salience and reward deficits in PWH. Future studies should examine if modulation/restoration of GABAergic inhibition of D2R-expressing MSN could ameliorate Tat-potentiated CPP.

It is important to note the hNeuron *in vitro* model described in Chapter 2 and utilized in Chapter 3 is a mixed neuron-glia (astrocyte) co-culture. Thus, it may be direct neuronal MOR activation or activation of MOR on glia, which through an unknown mediator, acts on neurons to diminish KCC2. Ferrini et al (Ferrini et al., 2013) demonstrated that morphine-induced KCC2 reductions in the spinal cord induce hyperalgesia and were mediated by microglial purinergic receptor activation. Additionally, previous studies in our lab have rarely found interactions between HIV proteins and opiates *in vitro* with less than 24 h exposures and combinatorial effects from these factors at later time points are

consistently mediated by glia (Zou et al., 2011, Kim et al., 2018). When taken together with our results showing morphine-Tat interactions at 24 h, but not at 6 h, it may be that morphine-induced KCC2 loss is glial-mediated (Fig 5.1).

The role of KCC2 dysregulation has been investigated in several neurological disorders (outlined in Chapter 1) and we are the first to demonstrate its role in neuroHIV (Chapters 3, 4). While these neurodegenerative disorders have different etiologies, there are several common hallmarks between them such as neuroinflammation and elevated glutamatergic signaling. Another emerging commonality is neuronal Cl^- gradient collapse via reduced KCC2 activity, resulting in neuronal disinhibition through diminished $GABA_AR$- mediated hyperpolarization and inhibition, and in some cases, $GABA_AR$-mediated depolarization and excitation. While there are common pathways, the cognitive and behavioral consequences are often vast. These differences arise in age of onset, brain regions and neuronal subsets affected, white matter involvement, and differences in immune recruitment, among others. For example, Rett Syndrome has large-scale behavioral and cognitive abnormalities compared to those typically seen with HAND. These differences arise in their separate etiologies: Rett is a genetic disorder and, thus, these KCC2 deficits result in improper GABAergic tuning during a critical period of CNS development (as well as other cellular and molecular sequelae differences), resulting in large-scale cognitive deficits. HIV is typically acquired after these critical periods of development, and, thus, the deficits of HAND are comparatively, subtle. Loss of KCC2 function has a well-established link to epilepsy via neuronal disinhibition, but seizures are not always seen in states of lowered KCC2 and while the rates of epilepsy due to HIV infection (~4%) are higher than the general population (1.2%), they are still low

(Kellinghaus et al., 2008). The involvement of KCC2 in epileptogenesis is likely due to extent of KCC2 activity loss and brain regions and neuronal subsets affected. Extreme loss of KCC2 activity may result in accumulation of $[Cl^-]_i$ to the point of Cl^- efflux upon $GABA_AR$ activation resulting in depolarization, and possibly neuronal excitation. The subsets of neurons affected have drastic effects on neuronal micro- and macro-circuitry. For example, ablation of KCC2 in pyramidal neurons of the hippocampus resulted in $GABA_AR$-mediated depolarization and was sufficient to induce temporal lobe epilepsy (Kelley et al., 2018). In contrast, our results suggest that HIV_{sup} ± morphine-induced KCC2 activity loss are not as profound, as there is still hyperpolarization due to $GABA_AR$ activation (Fig 3.2). Our *in vivo* data showed KCC2 loss in D2R-expressing MSNs of the striatum, but we found no overt loss of KCC2 in the hippocampus (Fig. 4.1, 4.4) and while Tat+ mice have displayed lower kainite-evoked seizure thresholds (Zucchini et al., 2013), epilepsy has not been noted in Tat-transgenic mice. Thus, HIV exposure may be associated with KCC2 loss and hyperexcitability, but the development of epilepsy depends on differences in extent of $[Cl^-]_i$ accumulation and neuronal subsets and brain regions with reduced KCC2 activity. Despite differing etiologies, neuronal subpopulation and brain region involvement, and cognitive/behavioral consequences, the commonalities underlying the neuropathology of these disorders suggest that there may be common therapies between them.

CLP257 and its prodrug, CLP290, are capable of maintaining membrane expression of KCC2, and thus maintain neuronal Cl^- extrusion, but the mechanism underlying these effects has yet to be elucidated. We demonstrated its utility *in vitro* in maintaining KCC2 immunoreactivity and $GABA_AR$-mediated hyperpolarization and

inhibition after exposure to HIV_{sup}, Tat, and morphine exposure (Chapter 3, Fig 3.1, 3.2, 3.4, 3.5, 3.6). *In vivo* studies found that CLP290 could restore KCC2 and GABAergic activity to reactivate neuronal circuitry after spinal cord injury reinstating walking (Chen et al., 2018), restore somatosensation after TBI (Lizhnyak et al., 2019), and reverse morphine induced hyperalgesia (Ferrini et al., 2017). We found that daily oral gavage of CLP290 for seven days during the second week of DOX treatment was capable of ameliorating abnormal motor activity in Tat+ mice (Chapter 4, Fig 4.4). While unable to rescue total KCC2 levels, CLP290 administration did rescue pS940-KCC2, and membrane localized KCC2, suggesting rescued neuronal Cl^- gradient and proper GABAergic transmission (Chapter 4, Fig 4.3). These results also provide insight to a possible mechanism that CLP290 acts. Our results demonstrate that CLP290 rescues membrane localization of KCC2 through maintenance of pS940. Although whether CLP290 interacts directly with KCC2 to prevent PP1 dephosphorylation of KCC2, inhibits PP1 itself, enhances PKC phosphorylation of S940, or modulates activity upstream of PP1 or PKC is unknown. It is also possible that CLP290 maintains other phosphorylation states important for membrane localization/degradation and these phosphorylation specific residues should be examined going forward. Our results demonstrated a failure of CLP257 to restore KCC2 and $GABA_AR$-mediated inhibition after exposure to $gp120_{ADA}$ (Chapter 3, Fig 3.5, 3.6). Taken together with our data implicating involvement of pS940 in CLP290s mechanism of action, suggests that $gp120_{ADA}$ may dysregulate pathways involved in the mechanism of action of CLP290.

The primary therapy for HAND is viral suppression via cART. Since its introduction, there has been a reduction of the most severe form of HAND, HAD, but there has been

an increase in the two milder forms with an overall prevalence remaining the same (Heaton et al., 2010). While able to control viral loads in the periphery, the CNS can act as a reservoir for HIV which may play a role in the persistence of HAND. Other factors that may be implicated in continuing neurocognitive detriment include an aging HIV population and, potentially, neurotoxic effects from some ARVs. Regardless of the cause, this preeminent quality of life issue for PWH underscores the necessity for adjuvant therapies to ameliorate the symptoms of HAND. As the preeminent theories for neuronal dysfunction in HAND centered around glutamatergic and Ca^{2+} signaling, so did trials for adjuvant therapies. Trials for NMDAR antagonist, memantine, and voltage gated L-Type Ca^{2+} channel, nimodipine had little to no success at restoring or decelerating deficits of HAND (Schifitto et al., 2007, Navia et al., 1998). Other adjuvant therapies to reach clinical trial have been a monoamine oxidase B inhibitor, selegiline, (Sacktor et al., 2009), TNF antagonist, CPI-1189 (Clifford et al., 2002) an acetylcholinesterase inhibitor, rivastigmine (Sacktor et al., 2011), and valproic acid (Schifitto et al., 2006), none of which significantly improved symptoms of HAND. Going forward, targeting KCC2, perhaps with CLP290 may prove to be beneficial to restore GABAergic inhibition and ameliorate the excitatory-inhibitory imbalance of the HIV-exposed CNS as discussed thoroughly in chapters 3, 4, and 5. At this time, CLP290 has not been tested in clinical trials. Bumetanide, a diuretic that is approved for clinical use, has been shown to restore neuronal Cl^- gradient via antagonism of NKCC1. Acting in opposition to KCC2, NKCC1 increases neuronal $[Cl^-]_i$, is the dominant neuronal Cl^- transporter in the immature brain and is aberrantly increased in some neurological disorders. Clinically, bumetanide significantly reduced seizure frequency in temporal lobe epilepsy (Eftekhari et al., 2013) and benefited neurocognitive

and behavioral outcomes for those suffering from drug-resistant epilepsy (Gharaylou et al., 2019), autism (Lemonnier and Ben-Ari, 2010, Lemonnier et al., 2012, Lemonnier et al., 2017), and schizophrenia (Lemonnier et al., 2016) through restoration of inhibitory GABAergic signaling. While we saw no changes in NKCC1 in our *in vitro* experiments, future studies should examine NKCC1 changes in Tat transgenic mice and human tissue to determine if this could be a potential adjuvant therapy for PWH experiencing neurocognitive decline.

Our discovery that CCR5 mediates KCC2 immunoreactivity and inhibitory effects of GABA (Chapter 3; Fig 3.5, 3.6) is a novel mechanism of neuro-immune interaction and may have implications for PWH as well as other neuroinflammatory disorders. MVC, an FDA approved CCR5 antagonist, is a commonly prescribed ARV to prevent viral entry with high CNS penetrance (Yilmaz et al., 2009). A clinical study found that when MVC use was intensified, PWH with neurocognitive impairment had significant improvements in global functioning, learning and memory, and executive function (Ndhlovu et al., 2014). Thus, restored KCC2 and GABAergic hyperpolarization may be a molecular and physiological mechanism underlying improvements in these subjects. To our knowledge, this is the first time a chemokine receptor has been linked to changes in KCC2. This route of neuro-immune crosstalk demonstrates a novel pathway by which immune activation can fine-tune neuronal circuitry and induce hyperexcitability and, therefore, may be involved in other disorders that show operative CCR5-CCL5 signaling. For example, multiple sclerosis increases CCL5 (Sorensen et al., 1999). Of particular relevance, one study with multiple sclerosis patients used a paired pulse transcranial magnetic stimulation (TMS) protocol to induce intracortical inhibition (inter stimulus interval of 2

ms), which is $GABA_AR$ mediated (Di Lazzaro et al., 2006), and found decreased paired pulse-induced cortical inhibition was significantly correlated with subjects CSF CCL5 levels (Mori et al., 2016). Thus, these results may be explained by decreased $GABA_AR$ inhibition via increased CCR5 activation and subsequent reduction in KCC2 activity. Given this novel CCR5-KCC2 signaling cascade, MVC should be considered more heavily for PWH experiencing neurocognitive decline and should be examined for use in other disorders in which elevated CCL5-CCR5 signaling is a component of neuroinflammation.

D2R-expressing MSNs seem to be particularly vulnerable to hyperexcitability (Cepeda et al., 2008) and excitotoxicity through glutamate receptor activation (Mesco et al., 1992). Previous studies from our lab have demonstrated vulnerability of striatal neurons, particularly D2R-expressing MSNs, of Tat+ mice to dendritic spine loss induced by elevated $[Ca^{2+}]_i$ (Fitting et al., 2014, Fitting et al., 2010, Schier et al., 2017). KCC2 interactions with cytoskeletal elements to maintain dendritic spines and our results showing Tat-induced, NMDAR-dependent KCC2 loss (Fig 3.1, 3.4), suggest that the Tat-induced neuropathology (dendritic spine and KCC2 loss) noted between these studies may be part of the same signaling cascade. We propose that Tat-mediated NMDAR-dependent $[Ca^{2+}]_i$ elevation induces dephosphorylation of S940 and KCC2 internalization and degradation, preferentially in D2R-expressing MSNs, destabilizing and ultimately reducing dendritic spine density. Thus, Tat induction induces striatal inhibitory and excitatory dysfunction through KCC2 reduction and subsequent dendritic spine loss, respectively. These interactions should be examined by determining if KCC2 maintenance (with CLP290) is sufficient to restore dendritic spines on D2R-expressing

MSNs which may also broaden the therapeutic potential of targeting KCC2 with drugs like CLP290.

While motor disorders associated with HAND typically involve hypokinetic states, such as bradykinesia, our results demonstrate hyper locomotion in the open field assay after two wks DOX treatment. These seemingly paradoxical results may actually reflect the biphasic nature of striatal activation and motor impairment seen in PWH (von Giesen et al., 2000). Early in disease progression, subjects showed hypermetabolism of the striatum measured by positron emission tomography, while in more advanced stages of HIV infection subjects showed striatal hypometabolism and motor slowing. When taken together, the behavioral results presented in this dissertation showing increased motor activity after two wks of DOX-induced Tat expression (Fig 4.3) and previous work form our lab demonstrating decreased locomotion after 4 wk DOX treatment, reflect the progression of motor changes in PWH. Further, these locomotor changes also follow the biphasic response of KCC2 loss examined in Chapter 4 (Fig 4.1, 4.2). In accordance with decreased KCC2 in D2R-expressing MSNs (and, therefore, likely disinhibition of these cells), there is an increase in motor activity and as we see a recovery of KCC2 to baseline levels at four wk DOX-induced Tat exposure, there is a reversal in motor effects to hypolocomotion in the open field assay (Hahn et al., 2016). These results stand in contradiction to the classic ‘Go/ No Go’ theory of striatal motor activation and support emerging evidence showing that either D1R- or D2R-expressing MSN activation are implicated in motor initiation which is covered in the discussion of Chapter 4. Overall, we propose that the increased striatal output seen early in disease progression in PWH may be a result of decreased striatal KCC2 (preferentially in D2R-expressing MSNs), resulting

in neuronal disinhibition, thus increasing striatal activity, and as KCC2 returns to baseline, giving way to more prolonged circuit changes and DA depletion, there is a reversal of striatal activity manifesting as hypokinesia, resulting in the striatal hypometabolism and Parkinsonian-like states described clinically.

Final Conclusion

The work performed for this dissertation described two *in vitro* models with particular utility for HAND, one of which we used to identify a novel target of HIV and opiates to disrupt GABAergic signaling. Pharmacological manipulation of this novel target, KCC2, with CLP257 restored $GABA_AR$-mediated hyperpolarization and inhibition. *In vitro* experiments performed in Chapter 3 have implications beyond the neuroHIV and drug abuse fields. We identified CCR5-KCC2 as a novel intermediary of neuro-immune interaction by which chemokine receptor signaling can disrupt $GABA_AR$ activity. Additionally, these studies demonstrated that morphine activation of MOR decreases KCC2, and may provide insight to the molecular substrate implicated in the synchrony of events resulting in GABAergic reversals in opiate-dependent animals. We expanded our *in vitro* KCC2 results to the Tat-transgenic mouse and found reduced striatal KCC2 after two wks of Tat expression. Additionally, D2R-expressing MSNs of the striatum seem to be preferentially vulnerable to KCC2 loss and these changes were associated with hyper locomotion. We were able to restore basal motor activity and membrane localized KCC2 through rescue of phosphorylation of S940-KCC2 with CLP290 oral gavage, providing insight to a potential mechanism by which this drug works. In total, the studies presented describe a promising therapeutic target for HAND and implicate a common mechanism

between HIV proteins and morphine to dysregulate GABAergic activity, which may have additional relevance in opiate abusing populations.

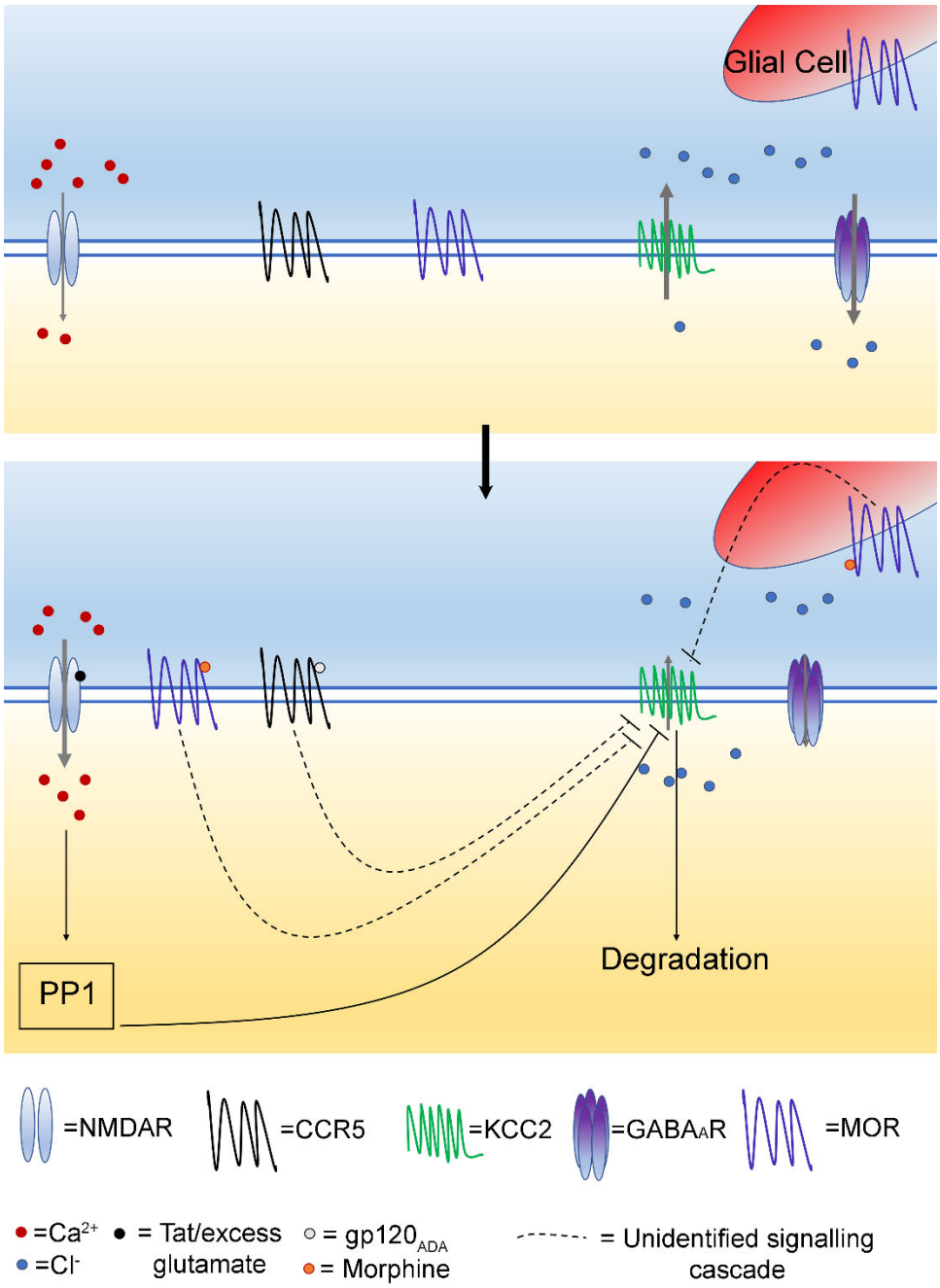

Figure 5.1. Mechanisms underlying Tat, gp120$_{ADA}$, and morphine-induced KCC2 loss and GABA$_A$R dysfunction.

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Vita

Education

Virginia Commonwealth University

August 2014- Present (Expected Graduation Spring 2020)

Neuroscience PhD Candidate

GPA: 3.9

Virginia Polytechnic Institute and State University

Graduation Date: May, 2012

B.S., Biological Sciences, Psychology

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August 2014 - Present

KCC2: a novel therapeutic target to rescue GABAergic dysfunction and behavioral deficits induced by HIV and opiate use

· Genetically encoded voltage (FlicR1, Archon1) and calcium (GCaMP6f) indicators

·Rig construction

·Transfection/infection (chemical and AAV)

·Recording

·Custom analysis in R

· Human cell line differentiation and culture techniques

· Development of primary human neuron differentiation protocol

· Human brain organoid culture techniques and characterization

· Mouse handling and surgical techniques

· Mouse behavioral assays and analysis (ANY-maze™)

· Mouse neuron and mixed glia culture techniques

· Fluorescent immunocytochemistry and immunohistochemistry

· Confocal laser scanning microscopy

· 3-D neuron reconstruction and morphological analysis (Imaris)

· Polymerase chain reaction (PCR)

· Enzyme-linked immunosorbent assay (ELISA)

· Western Blot

Research Technician, *Virginia Tech Carilion Research Institute*, *Advisor: Dr. William. J. Tyler*

August 2012- March 2014

· Collected data using EEG, fMRI, ECG, MEPs, fNIRS, GSR, neuronavigation

· Administered noninvasive neurostimulation protocols using TMS, tDCS, tACS, tFUS

· Analyzed EEG, ECG, MEP, fMRI, and behavioral data using Matlab, SPM8, BrainVision™, Spike 2™

Teaching Experience

Private Chemistry Tutor, Blacksburg, VA

August 2011- August 2012

Publications

Barbour AJ, Nass SR, Hahn YK, Hauser KF, Knapp PE. Restoration of KCC2 membrane localization in striatal D2R-expressing medium spiny neurons rescues behavioral deficits in HIV Tat-transgenic mice. In preparation.

Marks WD, Paris JJ, **Barbour AJ**, Moon J, McClane VD, Lark A, Nass SR, McQuiston AR, Knapp PE, Hauser KF. The effects of Tat and morphine on the structure and function of CA1. In preparation.

Barbour AJ, Hauser KF, McQuiston AR, Knapp PE (2020). HIV and opiates dysregulate KCC2 to cause GABAergic dysfunction in primary human neurons. Article accepted, in press. *Neurobiology of Disease*.

Schier CJ, Marks WD, Paris JJ, **Barbour AJ**, McQuiston AR, Knapp PE, Hauser KF (2017). HIV-1 Tat causes selective vulnerability of D2-expressing medium spiny neurons. *Journal of Neuroscience*, 37 (23):5758-69.

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Invited Talks

Local

HIV and HIV proteins ± morphine dysregulate KCC2 inducing GABAergic deficits in primary human neurons and Tat-transgenic mice. ***VCU Department of Anatomy & Neurobiology Seminar Series.*** 2019.

National Meetings

HIV and morphine induce GABAergic deficits through dysregulation of KCC2. ***Nanosymposium - HIV-associated neurocognitive disorders***. Society for Neuroscience, Chicago, IL. 2019.

Optical electrophysiology of primary human neurons: role of KCC2 in hyperexcitability induced by HIV ± morphine exposure. ***Presentation of selected abstracts.*** American Society for Neurochemistry, Riverside, CA. 2018.

Abstracts

Barbour AJ, McQuiston AR, Hauser KF, Knapp PE (2019). HIV and morphine dysregulate KCC2 and induce GABAergic deficits in neurons via NMDAR, CCR5, and MOR. International Society for Neurochemistry – American Society for Neurochemistry, Montreal, QC, Canada.

Barbour AJ, Balinang J, McQuiston AR, Hauser KF, Knapp PE (2018). HIV-1 & HIV proteins interact with morphine to induce loss of KCC2 & GABAergic dysfunction in primary human neurons in a NMDAR & CCR5 dependent manner. Society for Neuroscience, San Diego, CA.

Barbour AJ, Balinang J, McQuiston AR, Hauser KF, Knapp PE (2018). Optical electrophysiology of primary human neurons: role of KCC2 in hyperexcitability induced by HIV ± morphine exposure. International Society of Neurovirology/Society for NeuroImmune Pharmacology, Chicago, Il.

Barbour AJ, Balinang J, McQuiston AR, Hauser KF, Knapp PE (2017). Optical electrophysiology of human primary neurons: role of KCC2 in excitatory-inhibitory imbalance induced by HIV ± morphine exposure. Society for Neuroscience, Washington, DC.

Moon J, Marks WD, **Barbour AJ**, Knapp PE, Hauser KF (2017). HIV-1 Tat causes selective reductions in dendritic spines in the stratum oriens and the stratum radiatum, but not the stratum lacunosum moleculare of hippocampal CA1 pyramidal cells. Society for Neuroscience Washington, DC.

Marks WD, **Barbour AJ**, Paris JJ, Schier CJ, Denton MD, Fitting S, McQuiston AR, Knapp PE, Hauser KF (2016). HIV-1 Tat causes structural abnormalities in CA1 regional microcircuitry, and disturbances in CA1 function and memory formation. Platform talk (Marks). Society for Neuroscience, San Diego, CA.

Marks WD, **Barbour AJ**, Paris JJ, Schier CJ, Denton MD, Fitting S, McQuiston AR, Knapp PE, Hauser KF (2016). Interaction between HIV-1 Tat and morphine causes disruption of GABAergic systems within CA1. International Symposium on Neurovirology, Toronto, Ontario, Canada.

Schier CJ, Marks WD, Paris JJ, **Barbour AJ**, McQuiston AR, Knapp PE, Hauser KF (2016). CNS effects of opiate exposure in a mouse model of NeuroAIDS: selective vulnerability of D1 and D2-expressing medium spiny neurons. American Society for Neurochemistry, Denver, CO.

Opitz A, Legon W, Mueller J, **Barbour AJ**, Bickel W, Paulus W, Tyler WJ (2014). Weak electric field effects from sham transcranial magnetic stimulation over the dorsolateral prefrontal cortex on EEG dynamics and working memory. Biomedical Engineering Society. San Antonio, TX.

Lofti M, Legon W, **Barbour AJ**, Williams A, Tyler WJ (2013). Neurophysiological responses to short duration transcranial direct current stimulation of human prefrontal cortex. Society for Neuroscience. San Diego, CA.

Legon W, **Barbour AJ**, Tyler W, Williams A, Opitz A, Tyler WJ (2013). Transcranial ultrasound modulates human brain activity. Human Brain Mapping. Seattle, WA.

Opitz A, Legon W, Mueller J, **Barbour AJ**, Williams A, Paulus W, Tyler WJ (2013). Effects of the residual electric field induced by sham coils should not be neglected in TMS studies. Human Brain Mapping. Seattle, WA.

Professional Memberships

Society for Neuroscience

International Society for Neurochemistry - American Society for Neurochemistry

Awards

MVC Alumni Association Scholarship.
2020

Ruth L. Kirchstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31). National Institute of Drug Abuse (NIDA). F31DA047195.
2018-2019

Phi Kappa Phi Scholar Award (for outstanding academic performance). School of Medicine, Virginia Commonwealth University.
2018

C.C. Clayton Award (for outstanding academic and research progress in the biomedical sciences). School of Medicine, Virginia Commonwealth University.
2017