2015

Effects of HIV-1 viral protein Tat on the viability and function of oligodendroglial cells

ShiPing Zou
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd

Part of the Neurosciences Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/4009

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
Effects of HIV-1 viral protein Tat on the viability and function of oligodendroglial cells

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate in Philosophy in Neuroscience at Virginia Commonwealth University.

By
ShiPing Zou
B.S. in Genetics and Genetic Engineering, Fudan University, 1999
M.S. in Computer Science, University of Kentucky, 2004
M.S. in Biological Science, University of Kentucky, 2004

Director: Pamela E. Knapp, Ph.D
Professor, Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, Virginia
November, 2015
Acknowledgement

First of all, I would like to thank my wife, Yun Ji, for her unconditional love and constant support during the past 14 years that we spent together. It is impossible for me to accomplish this without you being next to me all the time. Thanks to my daughter, Olivia YuTong Zou, for being a part of my life. Watching you grow up is both exciting and painful, and it also grows me up.

I would love to express my gratitude to my advisor, Dr. Pamela Knapp. You set a great example for me as a scientist with vision, passion and integrity. You showed me that it is possible to be a good parent and a good scientist at the same time. Your guidance on research, career and life are invaluable and will benefit me throughout my life.

I would also like to thank my committee members, Dr. Kurt Hauser, Dr. Babette Fuss, Dr. Michael Fox and Dr. William Maragos. Thank you all for encouraging my research and help me grow as a scientist. Your brilliant comments and suggestion during the past four years have been priceless to me.

Thanks to Dr. Jeff Dupree and Judy Williamson. You have been generous to me with your time in teaching me using the electron microscope and reading the images. Many
thanks to the director of the neuroscience program, Dr. John Bigbee, for encouraging me to return to the graduate school and always supporting my research career.

To my friends here at VCU, Dr. Marie Bonhomme, Joyce Balinang, Melissa Denton, Dr. Sylvia Fitting, Sarah Kim, Will Marks, Dr. Jason Paris and Natalie Wheeler. Thank you for your friendship and for making the most wonderful academic working environment. The past 4 years have been an important part of my life, and I will cherish all the time we spent together forever.

Last but not least, I would like to dedicate this thesis to my parents, Ronghua Zou and Qiuhua Shen. They raised me up and imparted to me lessons in honest, confidence, and fortitude. For every major achievement in my life, I thank them for their unconditional love, their support and their faith in me.
# Table of Contents

List of Figures ........................................................................................................... v
List of Abbreviations .................................................................................................... vii
Abstract ...................................................................................................................... 1

## Chapter 1
Introduction to HIV-1 and WM injury in the CNS ................................................. 3

## Chapter 2
Effects of HIV-1 Tat on OLs in vivo ......................................................................... 12

## Chapter 3
Effects of HIV-1 Tat on oligodendroglial survival and function in vitro .......... 29

## Chapter 4
Tat affects mature OL membrane change via CaMKIIβ activation .......... 65

## Chapter 5
Tat reduces immature OL viability through GSK3β activation ......................... 85

## Chapter 6
Using co-culture or human brain aggregate model to study OL injury by HIV-1/Tat .......................................................... 101

## Chapter 7
Conclusions, pitfalls and future studies ................................................................. 122

List of References ................................................................................................... 133
Vita ......................................................................................................................... 154
List of tables, figures, abbreviations and symbols

Figure 2.1  Tat induces aberrant OL morphology *in vivo* .................................. 24
Figure 2.2  Ultrastructural examination of caudate putamen in Tat* mice.............. 26
Figure 2.3  *In vivo* Tat expression leads to decreased MBP and MAG............. 28
Table 3.1  Percentage of cells expressing neuron/glial specific markers at various
times *in vitro* ........................................................................................................ 51
Figure 3.1  Survival of Tat-treated OLs................................................................. 52
Figure 3.2  Tat reduces myelin-like membranes of mature OLs......................... 55
Figure 3.3  Tat induces Ca$^{2+}$ influx in mature and immature OLs.................. 57
Figure 3.4  Tat-induced [Ca$^{2+}$]i increases in OLs are partially reversed by iGluR
antagonists.............................................................................................................. 59
Figure 3.5  Blocking alternative, non-iGluR targets has no effect on Tat-induced
increases in [Ca$^{2+}$]i................................................................................................. 61
Figure 3.6  Effects of iGluR antagonists on Tat-induced functional effects......... 63
Figure 4.1  Expression of Tat *in vivo* does not alter CaMKIIβ expression, but
promotes CaMKIIβ activation............................................................................ 80
Figure 4.2  Expression of CaMKIIβ on OLs *in vitro*.......................................... 81
Figure 4.3  Tat induced CaMKIIβ activation in both immature and mature OLs.... 82
Figure 4.4  Effects of blocking iGluRs on Tat-induced CaMKIIβ activation........ 83
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>$\text{Ca}^{2+}$/Calmodulin dependent kinase II</td>
</tr>
<tr>
<td>cART</td>
<td>Combined anti-retroviral therapy</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase 3β</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV-associated neurocognitive disorders</td>
</tr>
<tr>
<td>hBrnAgg</td>
<td>Human brain aggregate</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency virus</td>
</tr>
<tr>
<td>iGluRs</td>
<td>Ionotropic glutamate receptors</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>NCBI</td>
<td>The National Center for Biotechnology Information</td>
</tr>
<tr>
<td>OL</td>
<td>Oligodendrocyte</td>
</tr>
<tr>
<td>OPCs</td>
<td>Oligodendrocyte precursor cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>WM</td>
<td>White matter</td>
</tr>
</tbody>
</table>
Abstract

EFFECTS OF HIV-1 VIRAL PROTEIN TAT ON THE VIABILITY AND FUNCTION OF OLIGODENDROGLIAL CELLS

By ShiPing Zou, Ph.D.

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

Virginia Commonwealth University, 2015

Major Director: Pamela E. Knapp, Ph.D., Professor of Anatomy and Neurobiology

Myelin pallor is frequently reported in HIV patients, and can occur in the CNS prior to other evidence of disease process. Our exploratory studies showed that oligodendrocytes (OLs) are direct targets of HIV-1 Tat (transactivator of transcription). Tat induces a dose-dependent increase of intracellular Ca^{2+} level ([Ca^{2+}]_{i}) in cultured murine OLs, which can be attenuated by ionotropic glutamate receptor (iGluR) antagonists MK801 and CNQX. The Tat-induced [Ca^{2+}]_{i} increase leads to increased death in immature (O4^{+}, MBP^{-}), but not mature (O4^{+}, MBP^{+}) OLs, over 96 h. In addition, Tat-induced [Ca^{2+}]_{i} increase also reduced myelin-like membrane production by mature OLs. Calcium/Calmodulin dependent kinase II\beta (CaMKII\beta) and glycogen synthase kinase 3\beta (GSK3\beta) have been known to regulate differentiation, myelination,
and apoptosis in OLs. Since both CaMKIIβ and GSK3β are important downstream modulators of $[\text{Ca}^{2+}]_i$ change, we hypothesized that the detrimental effects of Tat on immature/mature OL viability and function are mediated via CaMKIIβ and GSK3β activation. Our results showed that Tat activates both CaMKIIβ and GSK3β in immature OLs, but only activates CaMKIIβ in mature OLs. MK801 completely blocks Tat-induced CaMKIIβ and GSK3β activation in both immature and mature OLs, while CNQX blocks GSK3β activation, but has only a partial effect on CaMKIIβ activity. Blocking iGluRs or inhibiting GSK3β both rescue Tat-induced immature OL death, but only MK801 reverses the membrane injury in mature OLs. Together, these data strongly suggest that 1) activity of CaMKIIβ and GSK3β in OLs can be regulated by Tat-induced iGluRs activation and 2) OLs at different developmental stages show different responses to Tat, possibly due to activation of different signaling pathways.
Chapter 1

Introduction to HIV-1 and WM injury in the Central Nervous System

HIV-1 and AIDS Overall

HIV is a member of the lentivirus family. There are two types of HIV: HIV-1 and HIV-2. Although both types infect cells in similar ways and appear to cause clinically indistinguishable AIDS, studies showed that HIV-1 is the predominant virus type. Infections of HIV-2 are: 1) rarely found outside of West Africa (de Silva et al., 2008), and 2) seldom progressed to AIDS or lead to HAND (Rowland-Jones and Whittle, 2007). On the other hand, more than 34 million individuals were infected by HIV-1 by the end of 2009 (UNAIDS, 2010). Currently, four strains of HIV-1 have been identified: the major group M, the outlier group O and two new groups N and P. At least 9 genetically distinct subtypes, designated as clades A, B, C, D, F, G, H, J and K, were found in group M, which accounts for >90% of HIV-1 infection. Recombinant forms of HIV-1 were also discovered from individuals infected with multiple subtypes of HIV-1. Some of the recombinant forms were reported to be transmitted between individuals, thus called circulating recombinant forms. Among all the HIV-1 clades, clade B is the most prevailing subtype that affects America, most of Europe and Australia (Gilbert et al., 2007).

The single-stranded RNA genome of HIV-1 encodes nine proteins consisting of envelope (Env), core (Gag), polymerase (Pol) and six accessory proteins including Tat
and Nef. The first step of HIV infection is the binding of the viral envelope protein gp120 to the CD4 receptors on the surface of the host cell (Sattentau et al., 1986). This binding induces a conformational change in gp120, which exposes its binding site to its cellular co-receptors. CCR5 and CXCR4, two receptors first identified as CC- and CXC-chemokine receptors, respectively, are the most reported co-receptors for HIV infection. Other chemokine receptors, including CCR2, CCR3, and CX3CR1 have also been reported to be utilized by HIV as co-receptors (He et al., 1997; Garin et al., 2003; Puissant et al., 2003). The interaction between gp120 and these co-receptors triggers further conformational change and the insertion of HIV-1 gp41 into the host cell membrane, which initiates the fusion of the membranes and eventually leads to the entry of the virus genome and proteins. Once the host cell is infected, the viral reverse transcriptase makes viral DNA using its RNA genome template. The newly synthesized viral DNA binds to the viral protein integrase and some host proteins to form the pre-integration complex, which then translocate to the nucleus, integrate into the host genome, and form the provirus. Infected cells can either be latent, or actively transcribe and produce virus and viral proteins such as Tat, gp120, Nef and Vpr.

The first case of HIV infection in the United States can be dated back to the 1970s, and AIDS was reported in New York and California in the 1980s. By 1983, approximately 3000 AIDS patients had been reported and 1000 patients had died (Berkson, 1990). Over 33 million individuals are currently infected by HIV (Power et al., 2012), and CDC estimates 50,000 Americans becoming infected with HIV each year. Since the first case of HIV infection, nearly 619,400 people had died with AIDS. The development of cART in 1996 has dramatically decreased the incidence of mortality and
initial progress of neurological pathogenesis, but it had little effects on HAND, which is estimated to affect 60% of the HIV infected population (Williams et al., 2009). Until today, AIDS is still the most common cause of dementia in non-aged individuals in the US. Thus, CNS neuropathology, such as HIV-associated dementia and moderate cognitive and motor diseases, remains a serious issue in HIV patients.

**CNS dysfunction and WM neuropathology in HIV**

Although the initial HIV infection occurs in the periphery and targets CD4⁺ lymphocytes, HIV enters the CNS at early, asymptomatic stages (Kramer-Hammerle et al., 2005). There are multiple ways by which HIV penetrates the BBB and enter the CNS. The major components of the BBB, microvascular endothelial cells, have been reported to be infected by HIV-1, which leads to trans-cellular migration of the virus into the CNS (Bagasra et al., 1996). Alternatively, infiltrated macrophages carrying HIV-1 as provirus may release HIV-1 in the CNS (Merrill and Chen, 1991). Further, infection-induced inflammation has also been shown to disrupt the integrity of the BBB, which may facilitate the CNS entry of virus and infected cells (Eugenin et al., 2011).

A large retrospective study in 2000 using 450 AIDS autopsy cases between 1984 and 1999 found that the brain was second only to lung in HIV-induced pathology, with 39% to 70% of all AIDS patients developing neurological disorders (Jellinger et al., 2000; Maschke et al., 2000). Clinically, HIV-associated dementia and other HIV-induced brain injuries have been demonstrated by CT scans and MRI images. A study of HIV-infected children by Roy et al., (1992) reported that all of the 49 patients participating in the research exhibited cerebral WM atrophy on their CT-scans. Jernigan et al., (1993)
later compared structural MRI images of HIV$^+$ patients with HIV$^-$ individuals. Their results suggested that HIV$^+$ patients, with or without clinical symptoms, showed preferential damage to cerebral WM and subcortical brain structures. This is in agreement with Stout et al., (1998), who also reported that HIV$^+$ males had a more rapid loss of the cortical tissues than HIV$^-$ males, and the reduction in WM volume was accelerated in patients with clinical symptoms than those asymptomatic patients.

Myelin pallor is an abnormal appearance of WM in histological sections that indicates myelin and oligodendroglial injury. Although it is clinically considered as non-specific WM pathology and has been attributed largely to edema resulting from inflammation-mediated changes in the blood brain barrier, it is among the most frequently reported change in HIV patients, and can occur in the CNS prior to other evidence of a disease involvement. In the brains of HIV$^+$ patients, microgliosis and astrogliosis are significantly correlated to myelin pallor (Esiri et al., 1991). HIV-induced microgliosis and astrogliosis have been observed in patients and reported together with elevated levels of TNF-$\alpha$ and p24 antigen repeatedly (Gyorkey et al., 1987; Eilbott et al., 1989; Esiri et al., 1991). Infected microglial cells and infiltrated macrophages secrete pro-inflammatory cytokines and chemokines such as TNF-$\alpha$, IL-1$\beta$, IL-6, IFN-$\gamma$, MCP-1, MIP-1$\alpha$ and CXCL10 (Tyor et al., 1992; Sui et al., 2004; Herbein et al., 2010), together with HIV viral proteins, including Tat, Nef and gp120, which not only show direct toxicity to myelin and oligodendroglia but also dysregulate receptor and cytokine/chemokine expression on CNS cells (Sui et al., 2004). Some of the secreted viral proteins are also chemoattractants (Albini et al., 1998a; Albini et al., 1998b), and can function in concert with chemokines to attract more reactive astroglia and microglia to the lesion site. Viral
proteins like Tat and gp120 can induce production of toxic factors (Lipton, 1998; Nath and Geiger, 1998; Hurley et al., 2003) and ROS (Chauhan et al., 2003; King et al., 2006) from infected glial cells, as well as stimulate further viral replication and production. Collectively, dysregulated cytokine and chemokine levels (El-Hage et al., 2008b), increased ROS production (Viviani et al., 2001), cluster of multinucleated giant cells (Esiri et al., 1991) and decreased glutamate uptake (Wang et al., 2003) in the CNS of HIV+ patients may lead to HIV-encephalitis, which happened in 10-50% of HIV patients (Bell, 1998) and is characterized by reactive gliosis, myelin pallor and infiltration of multinucleated giant cells (Wiley et al., 1991). HIV-encephalitis is frequently associated with WM damage ranging from inflammatory to degenerative pathology. Studies using multiple imaging, immunocytochemistry and biochemical techniques have shown that a number of WM diseases, such as progressive multifocal leukoencephalopathy (Zanin et al., 2012), cytomegalovirus encephalitis (Bell, 1998) and vacuolar myelopathy (Tan et al., 1995), are all linked to HIV infection.

Together, these findings suggest that HIV infection causes progressive atrophy within the WM in the brain, and injuries to the WM were most severe in the advanced stages of disease but were also evident even in asymptomatic HIV-positive persons.

**OLs and myelin injuries in HIV**

Cells of the immune system and CNS are known to be the main targets of HIV (Fauci, 1988; Price et al., 1988). Microglia and infiltrating macrophages are the major productively infected cells in the CNS (Jordan et al., 1991; Merrill and Chen, 1991). Astroglial infection by HIV via a CD4-independent mechanism has been reported (Liu et
al., 2004). However, infected astrocytes are rarely found in AIDS patients, and produce newly assembled viral particles at very low levels (Gorry et al., 2003; Kramer-Hammerle et al., 2005). The cells in the CNS that are most vulnerable to HIV infection are neurons and OLs. OLs are the CNS cells whose main function is to maintain the myelin that wraps around the axon to ensure saltatory propagation of action potentials. In addition, OLs have also been shown to regulate outgrowth and regeneration of central neurons (Bastmeyer et al., 1991; Schwab and Schnell, 1991). OLs do not express the CD4 receptor. However, several research groups have shown that galactosylceramide (GalCer), a glycolipid that is highly expressed on the surface of OLs, can serve as an alternative receptor that mediates HIV infection (Harouse et al., 1991; Yahi et al., 1992; Albright et al., 1996). Hence, whether OLs are infected by HIV or not remains elusive.

On the other hand, OL injuries in HIV+ CNS were frequently reported. Gyorkey et al., (1987) found hyperplasia of OLs followed by degeneration in brain biopsies of AIDS patients. This may be one of the first published pieces of data suggesting HIV-infection has pathological effects on oligodendroglial cells in the brain. Similar results were also reported by Esiri et al., (1991) who examined CNS tissues from spinal cords of 22 HIV-1 infected individuals and 11 HIV- controls and concluded that HIV-1 infection leads to an increased number of oligodendroglial lineage cells with or without severe myelin pallor, suggesting OL injuries may precede myelin damage. More recent studies reported that OLs from post-mortem HIV tissues show up-regulated expression of p53 and BAX (Jayadev et al., 2007), indicating activation of death pathways. Approximately one-fourth of the patients that have died of AIDS showed a characteristic vacuolar myelopathy at autopsy (Eilbott et al., 1989). MBP immunostaining using tissues from
these patients revealed signs of demyelination as well as presence of MBP fluorescence in the cytoplasm of the macrophage, suggesting damage of myelin, and possibly also OLs, leading to their subsequent phagocytosis by infiltrated macrophages (Eilbott et al., 1989). All these studies suggested that although proof of OL infection by HIV is limited, OL and myelin impairment in AIDS patients are common. Even if the infection itself is unlikely to be the major cause of OL injury, CNS infection by HIV has direct or indirect effects on OLs.

**HIV-1 Tat**

Infected microglia/macrophages express all nine viral proteins that are encoded by the HIV genome. For the purpose of this thesis project, I will focus on HIV-1 Tat. Tat is a virally encoded transactivator that is conserved in the genomes of primate lentivirus. It is synthesized at both early and late stages of viral replication, and is essential for efficient transcription of HIV viral genes and for HIV replication. Extracellular Tat has the strong tendency to adhere to the surface of nearby cells. In addition, it accumulates in the extracellular matrix via interaction between its basic domains and heparin sulfate proteoglycans (HSPGs) (Chang et al., 1997). Tat also has the CCF motif, which mimics the CC-chemokines that attracts CCR2, CCR3 and CXCR4 expressing glial cells (Cardona et al., 2006). The mRNA of the Tat protein consists of two coding exons. The first exon encodes amino acids 1–72, and the second exon encodes amino acids 73–101. The recombinant Tat used in most research laboratories was a truncated version of amino acids 1-86. The full-length Tat protein contains five functional domains: the N-terminal domain; the cysteine-rich domain that contains a CCF motif which competes
with cognate ligands to bind CXCR4, CCR2 and CCR3 chemokine receptors (King et al., 2006); the core region and the basic region that possesses the neurotoxicity of Tat as well as a nucleus localization sequence (NLS) (Albini et al., 1998b); and the C-terminal domain contains an RGD motif that mediates its interaction with the integrin receptor (Chang et al., 1997; Albini et al., 1998a). Although the demarcation of these five domains is somewhat arbitrary, it is generally accepted that the first four domains were included in the first exon and the fifth domain was encoded by the second exon.

Tat has been reported to induce a series of cellular responses in variable CNS cells. Tat induces expression of CCR3, CCR5 and CXCR4 in monocytes and macrophages in a concentration-dependent manner (Cardona et al., 2006). Since the CCF motif of Tat binds to β-chemokine receptors, up-regulation of these receptors facilitates a chemotactic response to Tat (Lafrenie et al., 1996). Tat has also been reported to cause increased [Ca\(^{2+}\)]\(_i\), either in a concentration-dependent manner through chemokine receptors (Albini et al., 1998b) or iGluRs (Song et al., 2003), or by activating the PLC, protein kinase C (PKC) and Gi/o protein pathway, which in turn opens IP3-gated intracellular Ca\(^{2+}\) stores (Power et al., 1998). The Tat-induced Ca\(^{2+}\) signaling in macrophages results in the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, TNF-β and TGF-β, as well as the cytokine receptor IL-4 receptor (IL-4R) (Puri and Aggarwal, 1992), which leads to inflammation and neuropathogenesis. Tat also increases production of free radicals in microglia. It has been shown that the Tat-induced expression of NO and iNOS is enhanced by IFN-γ, which is also elevated in the CNS of HIV/AIDS patients, suggesting a cooperative contribution between Tat and IFN-γ towards CNS injury (Polazzi et al., 1999). cAMP accumulation in microglia has
been known to induce expression of neuroprotective or immunosuppressive substances (Aloisi et al., 1999). Tat treatment decreases cAMP levels in microglial cultures in a dose- and time-dependent manner (Aloisi et al., 1999), indicating that Tat interferes with cAMP control of microglial activation. Tat also activates astrocyte production of pro-inflammatory cytokines and chemokines (El-Hage et al., 2008b; Zou et al., 2011b). Astrocytes exposed to Tat exhibit increased IκBα phosphorylation, followed by NFκB phosphorylation and nuclear translocation, and increased production of TNF-α, MIP-1 and IL-6 (El-Hage et al., 2008b), indicating activation of astroglial inflammatory responses. Tat has also been shown to cause increased production of free radicals and ROS in rat OLs, which leads to decreased glutathione (GSH) levels and increased lipid peroxidation (LPO), and eventually causes OL process retraction and apoptosis (Rathnasamy et al., 2011; Fernandez-Gamba et al., 2012).
Chapter 2 Effects of HIV-1 Tat on OLs in vivo

(This chapter, in part, was published as a paper in the Journal of Neuroscience in August of 2015, 35(32):11384-11398, entitled Oligodendrocytes are targets of HIV-1 Tat: NMDA and AMPA receptor-mediated effects on survival and development)

Introduction

Although combined anti-retroviral therapy (cART) dramatically decreases mortality and neurological deficits, 60% of the HIV-infected population still experience HIV-associated neurocognitive disorders (HAND) (Williams et al., 2009; Power et al., 2012). Despite viral suppression, infected brain cells, including microglia and astrocytes, continuously express and secrete viral proteins. Tat is a highly conserved HIV protein that can be detected in the blood (Xiao et al., 2000), extracellular matrix (Urbinati et al., 2005a), and cerebrospinal fluid (Wang et al., 2014) in HIV+ individuals, even with cART (Johnson et al., 2013). Besides its essential role in viral replication and efficient viral gene transcription, Tat has also been shown to be a toxic factor in the central nervous system (CNS) that leads to glial inflammatory responses (Nath et al., 2000; El-Hage et al., 2006; El-Hage et al., 2008a) and neuronal cell death (Zou et al., 2011b; Rumbaugh et al., 2013).
Oligodendrocytes (OLs) are myelinating CNS cells. OL dysfunction can lead to abnormal myelination, impaired neuronal signaling, and axon degeneration, resulting in serious neurological deficits. Tat-induced inflammatory response from astroglia or microglia may lead to OL injury. For example, TNF-α released from Tat-challenged astroglia has been shown to activate caspase-3 and induce apoptosis in both pre-myelinating and mature oligodendrocytes in rats (Rathnasamy et al., 2011). IL-1β, which is released by Tat-treated microglia, inhibits proliferation of rat oligodendroglial precursor cells (Kaur et al., 2012). Thus, WM injury in HIV+ patients may be attributed to Tat-induced glial inflammatory responses, which concurrently activates OL death signaling pathways and inhibits OL precursor cell proliferation.

Besides inflammation, Tat may also injure OLs via more direct mechanisms. OLs express variable surface molecules, including chemokine receptors CXCR4 (Patel et al., 2010), ionotropic (Salter and Fern, 2005) and metabotropic (Matute, 2011) glutamatergic receptors, integrin receptors (Laursen and Ffrench-Constant, 2007) and voltage-gated calcium channels (Kirischuk et al., 1995). These molecules have all been reported to interact with Tat in neurons (Diop et al., 1994; Albini et al., 1998b; Song et al., 2003; King et al., 2006; Li et al., 2008). The interactions between Tat and these molecules may underlie the mechanisms of direct effects of Tat towards OLs, which have not been extensively studied.

Early studies using Tat-transgenic mice showed that OLs exhibit caspase-3 activation and decreased process length when Tat expression was induced for 7 days (Hauser et al., 2009), suggesting that Tat may affect OLs morphology and function in vivo. The Tat-transgenic mouse was engineered to inducibly express HIV-1IIIb Tat1-86. In
these mice, Tat expression is controlled by a \textit{tet} responsive element, whose activation is controlled by a reverse tetracycline transactivator (\textit{rtTA}) transgene driven by a human glial fibrillary acidic protein (GFAP) promoter (Hauser et al., 2009; Hahn et al., 2012). Within the CNS, Tat expression is induced in astroglia by administration of DOX. Current studies use the same Tat-transgenic mice to systematically assess the effect of Tat on OLs. Using the Golgi–Kopsch procedure, we observed an increased occurrence of OLs with aberrant morphology in the corpus callosum and anterior commissure of transgenic mice expressing HIV-1 Tat for 7 days. Disrupted myelin structure was observed in the caudate putamen by electron microscopy after a longer Tat expression (12 wk). Western blot analysis of tissues from the same mice (12 wk Tat induction) show decreased expression of myelin protein MBP and MAG when compared with their Tat littermate fed with DOX-containing chow. Together, these observations indicating that expression of Tat \textit{in vivo} leads to OL injury at cellular, subcellular and protein levels. Further studies are needed to tell whether these OL injuries come from an inflammatory response or direct Tat toxicity.
Materials and Methods

All experiments involving animals were performed in compliance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

Tat transgenic mice and in vivo Tat expression

This study utilized a transgenic mouse engineered to inducibly express HIV-1_{IIIb} Tat_{1-86}. Tat expression is controlled by a *tet* responsive element, whose activation is controlled by a reverse tetracycline transactivator (*rtTA*) transgene driven by a human glial fibrillary acidic protein (GFAP) promoter (Hauser et al., 2009; Hahn et al., 2012). Within the CNS, Tat expression is induced in astroglia by administration of DOX. HIV transgenic (Tat⁺) and Tat⁻ control littermates were fed DOX-containing chow (6g/kg; Harlan Laboratories, Inc., Indianapolis, IN) *ad libitum* to induce expression of Tat. Both acute and chronic time periods were chosen for Tat induction to visualize early changes in OL processes (Golgi impregnation, 7 d) that might precede later myelin changes (EM, 12 wk) in Tat⁺ mice. The Tat⁻ mice express rtTA, but not Tat, and are a control for possible effects of the foreign transcription factor. The expression of the *rtTA* transgene in Tat⁻ mice and *rtTA* and *Tat* transgenes in Tat⁺ mice was confirmed by genotyping. The OL and myelin injury was observed in three different CNS regions (EM: caudate putamen; Golgi-impregnation: corpus callosum and anterior commissure), our studies were not designed to determine if defects were region specific.
Western Blotting

Striatal tissue from Tat transgenic mice was homogenized with 3 strokes (15 s/stroke) in a 2 ml ceramic bead tube (MO BIO Laboratories, Carlsbad, CA) using a Precellys 24 Homogenizer (Bertin Technologies, Rockville, MD) before protein extraction using RIPA buffer with protease and phosphatase inhibitors. The concentration of extracted proteins was assessed using the BCA assay (Thermo Scientific) before lysates were mixed at 1:1 ratio with 2x Laemmli buffer (BioRad, Hercules, CA). Equal amounts of total protein (5 - 10 µg) from each sample were loaded on Criterion 4–20% SDS-polyacrylamide gels (BioRad) and electrophoretically separated under constant voltage (130 V) for 1.5 h. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes (BioRad) with constant current (0.6 A, 2 h, 4°C). After transfer, membranes were blocked in 0.1% casein solution (BioRad, 1 h, room temperature) before probing with primary antibodies for MBP (1:5000, Abcam), MAG (1:3000, Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:3000, Abcam) at 4°C overnight. Membranes were incubated with appropriate IRDye secondary antibodies (1:3000, Li-COR, Lincoln, NE) for 1 h at room temperature, and imaged using an Odyssey Imager (Li-COR). Protein bands were quantified using Li-COR image studio software.

Electron Microscopy

Tissue processing and EM were performed as previously published (Hahn et al., 2012). In brief, Tat+ mice fed with DOX-containing chow for 3 months were perfused with phosphate buffer (pH=7.4, Sigma) containing 2% paraformaldehyde and 2%
glutaraldehyde before fixation/staining in 1% osmium tetroxide for 1 h. Fixed tissues were dehydrated through graded ethanols and infiltrated overnight in Embed 812 (EMS, Hatfield, PA), then polymerized at 60 °C for 2 d. Tissue blocks were sectioned at 600 – 700 Å thickness on a Leica EM UC6i ultramicrotome (Leica Microsystems), collected onto formvar-coated grids, and stained with uranyl acetate (5%) and Reynolds’s lead citrate (Reynolds, 1963). EM images were taken with a JEOL JEM-1230 Transmission Electron Microscope (JEOL USA, Inc. Peabody, MA) at 10,000—200,000x magnification using a Gatan Ultrascan 4000 digital camera with DigitalMicrograph™ software (Gatan Inc., Pleasanton, CA).

Golgi–Kopsch Impregnation

12-wk old Tat transgenic mice received normal (Tat−) or DOX-containing chow (Tat− and Tat+) for 7 d. A modified Golgi-Kopsch procedure as previously published (Hauser et al., 1989) was used to randomly impregnate neurons and glia. In brief, mice were anesthetized by isoflurane inhalation and euthanatized by intracardiac perfusion with 2% potassium dichromate and 5% glutaraldehyde. Whole forebrains were taken out, immersed in perfusion solution for 5 d before rinsed and re-placed in aqueous 0.75% silver nitrate solution for another 5 d. After that, tissues were infiltrated with graded sucrose solutions containing 0.75% silver nitrate for 24 h and frozen on dry ice before cutting into 120-µm thick serial coronals sections and thaw-mounting onto Superfrost-Plus slides (Fisher). Fluid to tissue volume ratios were ≥ 50:1, and non-metallic devices were used throughout. Images of impregnated OLs were taken randomly from corpus callosum and anterior commissure. Impregnated OLs were
identified using strict morphological criteria, including small (~10 µm diameter) cell body, myelin-forming processes aligned parallel to WM tracts, and appropriate localization. For each experimental condition (Tat⁺, Tat⁻ + DOX and Tat⁺ + DOX), 4 mice were used (N=4) and at least 40 cells were examined per mouse.
Results

Oligodendrocyte morphology in Tat transgenic mice

Fibrous astrocytes and OLs, the major cell types with cell bodies in the corpus callosum and anterior commissure, can be easily distinguished from each other in Golgi-Kopsch impregnated sections by their distinct morphologies. Significantly more OLs with abnormal morphology, including stunted or swollen processes, large (several microns in diameter), club-like cytoplasmic endings, and aberrant cell bodies were noted in Tat⁺ mice when compared with their Tat⁻ siblings with/without DOX (Fig. 2.1).

Ultrastructure of Tat⁺ mice

We next asked whether Tat induction by DOX caused any injury at the subcellular level in the transgenic mice. Electron microscopy revealed that many OLs in Tat⁺ mice had a seemingly normal morphology and subcellular structure after a 3-month DOX induction (Fig. 2.2A), yet a subpopulation showed very clear deficits. Enlarged periaxonal collars with dark cytoplasm were relatively common, potentially indicating accumulation of periaxonal fluid due to a deficit in exuding ion/fluid in OLs (Byun and Delpire, 2007) (Fig. 2.2B, C, E). Myelin splitting was also common, and associated with both normal-appearing axons (Fig. 2.2C, D, M-P) and axons exhibiting increased axoplasmic density (Fig. 1.2E). Abnormal membranous structures, and membrane inclusions with apparent periodicity were often observed (Fig. 2.2D-F, H), indicating potential deficits in OL membrane turnover. Hypomyelinated axons (1.D, N) and myelin membranes without an associated axon were also noted (Fig. 2.2J, L).
Tat Expression leads to down-regulated myelin proteins \textit{in vivo}

A deficiency in myelin associated glycoprotein (MAG), which mediates axon-glial contact, has been reported to cause enlarged periaxonal collars (Trapp et al., 1984; Montag et al., 1994); and decreased levels of myelin basic protein (MBP) are often found in transgenic mice with myelin defects (Popko et al., 1987; Imgrund et al., 2009). We thus examined the expression level of MAG and MBP in Tat transgenic mice. Western blots of lysates from striatum showed that Tat$^+$ mice exhibit significantly decreased levels of MAG and MBP when compared with their Tat$^-$ littermates after 3-month DOX-induction (Fig. 2.3).
Discussion

We found from these in vivo studies that transgenic mice with inducible Tat expression had higher numbers of OLs with an abnormal phenotype in Golgi-impregnated cells, disturbances in myelin ultrastructure, and decreased myelin protein (MBP and MAG) expression.

Tat transgenic mice were separated into three treatment groups. Group 1: Tat’ mice fed with normal diet without Dox; Group 2: Tat’ mice fed with DOX-containing chow; and Group 3: Tat’ mice fed with DOX-containing chow. While comparisons between group 2 and group 3 show the effects of in vivo Tat expression on OLs, group 1 serves as a control for non-specific effects of DOX intake. As Fig 2.1 showed, no differences were found between group 1 and group 2, suggesting the effects on OL morphology are unlikely to be caused by DOX.

We chose the anterior commissure and the corpus callosum as the two areas of interest, since Goigi-impregnation cannot be performed concurrently with other procedures (e.g. immunostaining) to discern different cell types. The anterior commissure and corpus callosum are the two prominent white matter tracts that can be unambiguously distinguished from other functional areas in brain sections. In addition, the majority of CNS cells with cell bodies residing in WM are fibrous astrocytes and OLs. A series of morphological criteria that was defined in previous studies (Hauser et al., 2009) were used to differentiate these two cell types from each other. In these two brain regions, OLs are easily discernable by their small soma size (~10 µm in diameter), outreaching processes that are parallel to myelinated axons, and appropriate location (associated with white matter bundles).
Our result suggested that inducing Tat expression for 7 days significantly increased the occurrence of OLs with abnormal morphology in the corpus callosum and anterior commissure. It is worth pointing out that the OLs we counted were mature, myelinating OLs, characterized by myelinating processes attached to the axon tracts. Migrating OPCs that rise from the subventricular zone can also be found in white matter tracts. Due to the restriction of this method, however, we are not able to unambiguously identify OPCs by its morphology.

We next investigated whether Tat expression also leads to injuries in myelin structure. Multiple abnormalities, including enlarged periaxonal collar, myelin splitting, hypomyelinated axons, abnormal membranous bodies, and the inclusion of myelin-like membranes, were found in the caudate putamen of Tat⁺ mice by EM (Fig. 2.2). Among these aberrant structures, enlarged periaxonal collars and split myelin have been the most frequently observed. In the peripheral nervous system, the formation and maintenance of the regular periaxonal collar requires the presence of MAG in the Schwann cell periaxonal membrane (Trapp et al., 1984). On the other hand, uncompact myelin lamellae have been found in the brain of myelin deficient mice (mld) that lack MBP expression (Matthieu, 1981). We thus tested the expression of MBP and MAG in the CNS of these mice. Consistent with our EM observation, western blot results showed significantly decreased expression of MAG and MBP in the brain of Tat⁺ mice compare to Tat⁻ mice (Fig. 2.3).

Multiple cellular abnormalities have been reported in Tat-transgenic mice, including reactive astrogliosis and microgliosis (Bruce-Keller et al., 2008), elevated caspase-3 activation in OLs (Hauser et al., 2009), and synaptodendritic injury (Kim et
al., 2003; Fitting et al., 2014), which coincide with deficits in behavior including learning and memory (Carey et al., 2012; Fitting et al., 2013) and cognitive/motor impairments (Hahn et al., 2015). Here, our in vivo studies showed that Tat also caused myelin damage and OL injury. Most previous studies suggest Tat-induced inflammation as the predominant cause of CNS injury. However, controlled trials of cART with adjunctive 'anti-inflammatory' agents do not show greater efficacy against HAND than cART alone (Tan and McArthur, 2012), suggesting neurotoxic effectors besides inflammation. We next used a highly purified culture system (>90% OLs) as our in vitro model to further elucidate whether there is a direct Tat toxicity to OLs.
**Figure 2.1.** Tat induces aberrant OL morphology in vivo. Golgi-Kopsch impregnation was used to visualize the structure of OLs in Tat⁺ mice receiving normal chow, and in Tat⁻ and Tat⁺ mice fed DOX-containing chow for 7 d. Sample brightfield images (A-H) are labeled with the genotype and treatment. (A-E) The majority of OLs from corpus callosum and anterior commissure of Tat⁻ mice fed normal or DOX-containing chow show normal morphology. These OLs have small (~10 µm diameter) cell bodies with smooth edges and relatively thick and continuous processes extending from their cell body to the white matter tracts. (F-H) Although most OLs in DOX-induced Tat⁺ mice appear normal, a significantly higher percentage had an aberrant phenotype. These OLs exhibited abnormal varicosities along or at the end of their processes (arrowheads), thin and disrupted processes, and irregular cell bodies (arrows). (I)
Quantification of OLs with abnormal morphology in the corpus callosum and the anterior commissure of Tat\(^-\) and Tat\(^+\) mice. In both regions, Tat induction resulted in significantly more OLs with pathological phenotype. (*\(p<0.05\); one-way ANOVA followed by post-hoc Bonferroni’s test; \(n=4\) individual experiments; >40 cells were counted for each \(n\); scale bar = 10 \(\mu\text{m}\) for all images).
Figure 2.2
Figure 2.2 Ultrastructural examination of caudate putamen in Tat\(^+\) mice. (A) Even after 3 months of continuous Tat expression, most OLs show a normal phenotype and subcellular structure (G: Golgi apparatus; RER: rough endoplasmic reticulum). (B-P) Despite this observation, multiple abnormal structures were commonly observed and documented in a subset of Tat exposed OLs. These include: i) Enlarged periaxonal collars containing dark cytoplasm (black arrows in B, C & E), possibly due to accumulation of periaxonal fluid; ii) Myelin splitting, suggesting a potential deficit of myelin compaction, is found both with normal-appearing axons (M, O, P; arrowheads in C, D, N) and axons exhibiting an abnormally dense cytoplasm (arrowhead in E); iii) Abnormal membranous structures (white arrows in D, E) and myelin-like membrane inclusions with obvious periodicity (F, H), indicating potential deficits in OL membrane turnover; iv) Hypomyelination, as indicated by axons of similar calibers in the same region invested with myelin sheaths of different thickness (N, compare axon groups a, b, c vs. a*, b*, c*, respectively). Also, in D, the axon denoted with # is unmyelinated, while neighboring axons with similar diameters have normal compact myelin; v) Axons with an abnormally dense cytoplasm, (Ax in E) and a myelin membrane in the absence of an axon, (J, L) were also noted.
Figure 2.3. *In vivo* Tat expression leads to decreased MBP and MAG. Western blot analysis of tissue lysate from transgenic mice after 3 month Tat induction showed decreased levels of both MBP and MAG. Protein expression was normalized to GAPDH. (n=4, Values are mean ± SEM; *p<0.05, t-test)
Chapter 3 Effects of HIV-1 Tat on oligodendroglial survival and function in vitro

(This chapter, in part, was published as a paper in the Journal of Neuroscience in August of 2015, 35(32):11384-11398, entitled Oligodendrocytes are targets of HIV-1 Tat: NMDA and AMPA receptor-mediated effects on survival and development)

Introduction

As mentioned in earlier chapters, adding adjunctive ‘anti-inflammatory’ agents to cART had no significant effect on HAND in HIV+ patients (Tan and McArthur, 2012). Since our in vivo studies showed that OLs and myelin were injured in Tat-transgenic mice, we hypothesized that Tat may be directly toxic to OLs. To exclude the impacts from other cell types, a highly purified OL culture was used to study the direct effects of Tat on OLs.

In vivo OL development is a complex process where migratory and mitotic OPCs arise from certain regions of the ventricular zones, move away to different brain regions and differentiate into myelinating OLs, which produce myelin to ensheath axons. During this process, a series of developmental markers are sequentially expressed, and can be used to divide the lineage into distinct phenotypic and biological stages. Early studies have shown that the capacity of OL progenitors to differentiate into OLs in vitro is identical to those in tissue (Temple and Raff, 1986). In this study, dissociated OL cultures were developed from A2B5+ OPCs harvested from 7-8 days old primary...
mixed glia cultures (Knapp et al., 1987; Knapp et al., 2009), and differentiated into OLs using specified differentiation medium containing T3 and CNTF. Immature and mature OLs were defined as OPCs cultured for 2 days and 7 days in the differentiation medium, respectively. At these time points, the majority of the cells are O4⁺, MBP⁻/⁺ in the immature OL population, and O4⁺, MBP⁺ in the mature OL population.

OL lineage cells express functional ionotropic glutamate receptors (iGluRs), including AMPA-Rs and NMDA-Rs, both in vivo and in vitro (Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006; Alix and Fern, 2009). The expression of these receptors on OLs is highly heterogeneous. Individual cells express different levels of iGluRs, and the distribution, subunit components, and downstream signaling pathways of different iGluRs may vary (Karadottir et al., 2005; Matute et al., 2006; Micu et al., 2006). Adding further complexity, iGluR expression in OLs is also developmentally regulated. AMPA-Rs are expressed at all developmental stages, while NMDA-Rs show later expression (Salter and Fern, 2005). In addition, the majority of NMDA-Rs expressed on OLs are clustered on processes and the myelin sheath while AMPA-Rs are evenly distributed on the cell body (Micu et al., 2006). Activation of iGluRs results in immature OL death (Deng et al., 2003; Follett et al., 2004) and myelin disruption (Micu et al., 2006). Importantly, since OL NMDA-Rs are less susceptible to Mg²⁺ blockade (Karadottir et al., 2005), and OL AMPA-Rs lack the Ca²⁺-impermeable GluR2 subunit (Hollmann and Heinemann, 1994; Matute et al., 2002), the level of extracellular glutamate necessary to injure OLs may be much lower than required for excitotoxic neuronal injury.
Since Tat-induced activation of iGluRs and resulting Ca\(^{2+}\) influx cause neuronal injury (King et al., 2006; Kim et al., 2008), we tested whether a similar mechanism exists in OLs. Increased [Ca\(^{2+}\)], occurred in immature and mature OLs with Tat treatment, and both were attenuated by NMDA-R and AMPA/KA-R antagonists. Additionally, Tat caused immature OL death and reduced myelin-like membrane production by mature OLs, both in a dose-dependent manner. Both effects were blocked by MK801, while CNQX only blocked immature OL death. Since these experiments were performed using a highly purified OL culture model, the effects likely reflect direct actions of Tat, rather than a response to Tat-induced inflammation.
Materials and Methods

Oligodendroglial cultures

Primary cultures were prepared from mice (CD-1, Charles River Laboratory, Wilmington, MA) of both sexes at postnatal day 0-1 as previously published (Zou et al., 2011b). In brief, whole brains were dissected and minced before being incubated (37°C, 5% CO₂) with 2.5 mg/ml trypsin (Sigma, St. Louis, MO) and 0.015 mg/ml DNase (Sigma) in Dulbecco's Modified Eagle’s Medium (DMEM) (Life Technologies, Carlsbad, CA) for 30 min. Tissue was triturated and re-suspended in DMEM supplemented with 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT), 6 g/l glucose (Sigma), sodium bicarbonate, and penicillin/streptomycin (Life Technologies), filtered through nylon mesh with pore sizes 100 µm and 40 µm, and then plated in T25 flasks (Corning Inc., Corning, NY) pre-coated with poly-L-lysine (1 mg/ml, Sigma) at a density of 2 brains/flask.

At day 8, primary glial cultures were put on an orbital shaker (100 rpm, 20 min) to dislodge microglia loosely attached to the culture surface. Medium was replaced and the flasks were hit sharply against the table 5-10 times to release as many O2A/glial progenitor cells as possible. The suspension was panned on a plastic, non-tissue culture dish for 2 h to remove adherent astroglia and microglia. Progenitors were plated in serum-free medium supplied with 10 ng/ml CNTF (Peprotech, Rocky Hill, NJ), 5 µg/ml NAC (Sigma) and 15 nM triiodothyronine (Sigma) on culture surfaces coated with poly-L-lysine as described above. Medium was changed every 3 d.
Viral protein and receptor antagonist treatments

OL cultures were treated with HIV-1 Tat\textsubscript{1-86} (clade B, ImmunoDX LLC, Woburn, MA) at 1 nM, 10 nM or 100 nM ± MK801 (20 µM, Tocris Bioscience, Bristol, UK) and CNQX (20 µM, Tocris), at day 2 or day 7 after plating the enriched cultures. In selected studies, heat-inactivated Tat\textsubscript{1-86} was used as a control. If the cells were treated concurrently with Tat and MK801 or CNQX, the receptor antagonists were added 30 min before Tat.

MK801 and CNQX concentrations were chosen to maximally block the NMDA-R and AMPA/KAR-R, respectively, as previously reported (Salter and Fern, 2005). Nimodipine (10 µM), dantrolene (10 µM), DL-TBOA (100 µM), RS102895 (10 µM), SB328437 (10 µM) and the RGDS peptide (10 µM) (all from Tocris Bioscience) were used when assessing the effects of different inhibitors on Tat-induced $[\text{Ca}^{2+}]_i$ change in OLs and were added 20 min before Tat treatment and Ca\textsuperscript{2+} imaging.

Time-lapse analysis of individual OLs

Enriched immature OLs were plated on a 12-well plate at a density of $1.5 \times 10^4$ cells/well. After Tat ± MK801/CNQX treatment, the plate was transferred to a heating insert M12 (Pecon, Erbach, Germany) and put on the scanning stage of a Zeiss Axio Observer Z1 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) equipped with an environmental incubator (Pecon), with a constant temperature (37°C) and CO\textsubscript{2} level (5%) during the experimental period. Both the heating insert and the environmental incubator were pre-warmed to 37°C at least 1 h before the start of the experiment. For each treatment, more than 25 OLs were systematically yet arbitrarily chosen, and
repeatedly imaged every hour using an automated stage controlled by the Zeiss Axiovision 4.8 software (Carl Zeiss). Viability of the cells was confirmed at the end of each experiment using calcein-AM (Life Technologies) staining. Time of cell death was determined by carefully assessing all pre-selected OLs in hourly digital images, using rigorous morphological criteria, including but not restricted to: (1) loss of phase brightness; (2) degenerating processes; and, (3) involution or fragmentation of cell soma. Data were analyzed at 4 h intervals and presented as mean percentage of survival ± standard error of the mean (SEM) from at least 4 individual experiments (n ≥ 4; at least 50 cells analyzed per N). Repeated measures ANOVA and post hoc Bonferroni’s test were used to compare whether differences between treatments were significant (p ≤ 0.05) or not (p > 0.05).

**Live-Dead Assay**

Cell viability after Tat treatment was confirmed using the LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies). Twenty microliters of 2 mM ethidium homodimer-1 (EthD-1) stock solution and 5 µl of 4 mM calcein-AM stock solution were added to 10 ml of sterile, tissue culture–grade D-PBS sequentially and vortexed for 20 seconds to make the working solution. Five hundred microliters working solution were added to each well of a sterile 24-well plate. OLs were plated on 12-mm diameter German glass cover slips (Chemglass Life Sciences, Vineland, NJ) and treated with vehicle or 100 nM Tat for 24, 48, and 72 h. After treatment, coverslips were quickly rinsed in Dulbecco’s Phosphate-Buffered Saline once and transferred to individual wells of a 24-well plate to incubate in working solution (30 min, room temperature). Coverslips
were then carefully inverted onto a clean Superfrost Plus Gold microscope slide (Fisher brand) with 10 µl fresh D-PBS and sealed with clear fingernail polish. Fluorescent (Ex/Em at ~495/~515 for calcein-AM and Ex/Em ~528/~617 for EthD-1) and phase images were taken using a Zeiss Axio Observer Z1 microscope with Zeiss Axiovision 4.8 software (Carl Zeiss). Live or dead cells were defined by green (calcein-AM) or red (EthD-1) fluorescence, respectively. For each n (individual experimental group), 20 images were systematically, yet arbitrarily taken and analyzed, and the relative ratio of live/dead cells was quantified by numbers of green/red fluorescent pixels using ImageJ software (National Institutes of Health).

**Intracellular calcium assessment**

After 2 or 7 d culture, OLs plated on 35 mm glass-bottom culture dishes (MatTek, Ashland, MA) were loaded with 2.5 µM fura-2-AM (Life Technologies) for 30 min at 37°C. Cells were washed twice and incubated in DMEM for another 30 min to ensure de-esterification of the acetoxy methylester (AM) group before transfer to the environmental incubator of the Zeiss Axio Observer microscope. Cytosolic [Ca^{2+}]_{i} was assessed using the physiology module of the AxioVision software. Fura-2-AM was excited at 340 and 380 nm, and emissions collected at 510-540 nm. Data were collected every second for the first 90 sec, then every 5 sec for the next minute, then every 30 sec for the rest of the 20 min experimental period. The F340/F380 ratio was converted to calcium concentration via a standard curve generated using a calcium calibration buffer kit (Life Technologies). Data were presented as mean [Ca^{2+}]_{i} ± SEM from at least 4 individual experiments (>25 cells analyzed per n).
**CM-Dil live Staining**

Cells from day 8 primary cultures were plated on 35 mm glass-bottom culture dishes (1.5 × 10^4 cells/dish) and incubated for 6 d in growth medium as described above. 2 µg/ml CM-Dil (Life Technologies) was added to the medium, and cells were incubated for 5 min before being transferred to 4°C for another 15 min. Cells were washed with pre-warmed culture medium, and placed back in the incubator. Treatments were applied the next day, prior to placement in the stage incubator. Individual OLs were selected as described in the repeated measure paradigm. Fluorescent (Ex at 560/40, Em at 630/75) and phase images were taken at 0, 24, and 48 h after treatment. The membrane area of individual cells, defined as the total red fluorescent area surrounding the cell body minus the area of the cell body (Dennis et al., 2008), was quantified using IP Lab imaging software (BD Biosciences, Franklin Lakes, NJ).

**Immunostaining**

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde and permeabilized with Triton-X 100. Neurons, astroglia, microglia, and OLs, were identified with antibodies specific to microtubule associated protein 2 (MAP2, 1:1000, Abcam, Cambridge, UK), glial fibrillary acidic protein (GFAP, 1:1000, Millipore, Billerica, MA), ionized calcium-binding adaptor molecule 1 (Iba-1, 1:500, Wako Chemicals, Richmond, VA), and myelin basic protein (MBP, 1:1000, Abcam), and the O4 monoclonal antibody (grown in our lab from hybridoma cells (Knapp and Hauser, 1996), 1:20), respectively. Antibodies specific to αv or β3 subunits (1:1000, Abcam) were used to identify integrins on OLs. Corresponding secondary antibodies were conjugated to Alexa 488 and 594.
(1:2000, Life Technologies). Cell nuclei were visualized with Hoechst 33342 dye (1:2000, Life Technologies); coverslips were mounted with Prolong Gold Antifade reagent (Life Technologies). Images were acquired using a Zeiss LSM 700 laser scanning confocal microscope and processed using the maximum projection module of Zen 2010 software (Carl Zeiss) to show cells in their entirety.
Results

Characterization of OL cultures

We used dissociated murine OL cultures to study whether the abnormalities of OLs and myelin \textit{in vivo} were a direct effect of Tat, or an indirect effect of Tat-induced inflammation. The percentage of cells expressing specific neural and glial cell markers was examined in cultures at 2 - 10 d after enrichment. Identification of OLs was made by immunostaining with the O4 antibody, which primarily detects the sulfated galactolipid sulfatide. In the CNS, sulfatide is expressed only by OLs; it is synthesized by immature, proliferating OLs and its expression is maintained in mature cells. As shown in Table 1, less than 5% of cells are positive for cell markers other than O4 in day 2 and day 5 OL cultures. This ratio increases to 7 - 10% at day 7 and day 10. Hence, even after 10 d culture, $\geq 90\%$ of cells in our culture are OLs. The majority of cells that are not O4$^+$ are GFAP$^+$ astrocytes (Table 3.1).

Dose-dependent effect of Tat on viability of immature, but not mature OLs \textit{in vitro}.

D2 and D7 OL cultures were treated with Tat or vehicle, and imaged hourly for 72 h. At D2, almost all OLs showed an immature phenotype, with two or more fine processes extending from relatively condensed, ovoid cell bodies. In addition, there are no myelin-like membranes extended from processes (Fig. 3.1A-B). Over time, vehicle treated cells showed signs of differentiation. The cell body became much larger with increased cytoplasmic area (Fig. 3.1A). The maturing OLs developed multiple, thick, primary processes from which emanate an extensive network of thinner processes with large, myelin-like membrane expansions that are also O4$^+$. Compared to controls, the
viability of immature OLs was significantly decreased in a dose-dependent manner after treatment with HIV-1 Tat (Fig. 3.1B, D-G). In addition, OLs that survived after 72 h lacked the extensive network of branching processes and myelin-like membrane extensions that are typical for healthy OLs at this age (Fig. 3.1A). A Live/Dead assay confirmed that OLs treated with Tat for 24-72 h exhibited significantly more EthD-1 staining and less calcein-AM staining than OLs in vehicle-treated groups (Fig. 3.1F-G). On the other hand, Tat treatment had no effect on the viability of mature OLs (D7) (Fig. 3.1C-D).

Myelin-like membranes are reduced by Tat exposure

Since Tat did not reduce the viability of mature OLs, we examined whether it might affect their ability to form myelin-like membranes. Differentiating OLs in vitro produce characteristic membranes that are thought to be analogous to myelin membranes in the brain, and which contain all major lipids and proteins found in myelin within the CNS (Dubois-Dalcq et al., 1986; Knapp et al., 1987; Waggener et al., 2013). OL cultures at D7 were labeled with CM-DiI before being treated with vehicle or Tat. Randomly selected cells, with the morphology of maturing OLs, were followed using a computer-controlled stage for 48 h; phase contrast and fluorescent images were taken at 0 h, 24 h and 48 h. Calcein-AM was added to the cultures at the end of the experiments to verify cell viability (Fig. 3.2A). Membrane area per OL, as indicated by CM-DiI labeling, was calculated using imageJ software as previously published (Dennis et al., 2008). In vehicle-treated cultures, individual OLs showed a ~30% increase of membrane area at 24 h, suggesting new membrane production in addition to cell
growth. This increased membrane size was maintained at 48 h (Fig. 3.2B). 1 nM Tat did not have a significant effect on the OL membrane area. However, adding 10 or 100 nM Tat to the medium significantly decreased OL membrane area at 24 h (~75% of 0 h), and this decreased membrane size was also maintained at 48 h (Fig. 3.2B).

Tat induces Ca\(^{2+}\) influx in both mature and immature OLs in vitro.

Since Tat has been reported to destabilize Ca\(^{2+}\) signaling in neurons (Kim et al., 2008; Perry et al., 2010), astroglia (El-Hage et al., 2005) and microglia (Sheng et al., 2000; Lokensgard et al., 2001), we hypothesized that the above-observed effects of Tat on OLs were mediated by changes in OL [Ca\(^{2+}\)]. To test this hypothesis, we first investigated whether Tat induces OL [Ca\(^{2+}\)] alterations. Fig. 3.3 shows that Tat caused an increase in [Ca\(^{2+}\)] in both D2 and D7 OLs in a dose-dependent manner. The Tat-induced Ca\(^{2+}\) influx seemed to start from distant regions of processes upon Tat addition to the medium (Fig. 3.3B, D). When challenged with 100 nM Tat, [Ca\(^{2+}\)] levels rose to ~150 nM in D2 OLs and ~230 nM in D7 OLs, and these increased [Ca\(^{2+}\)] levels were maintained during a 20 min experimental period (Fig. 3.3B, D-F). The Tat-induced [Ca\(^{2+}\)] increase was dose dependent; with a lower Tat (1 nM) exposure, [Ca\(^{2+}\)] returned to the control level after 5 min (Fig. 3.3E-F, cyan line). Furthermore, the Tat-induced [Ca\(^{2+}\)] increase was completely abolished in Ca\(^{2+}\)-free medium (Fig. 3.3E-F, blue line) and greatly attenuated when Tat was heat-inactivated (Fig. 3.3E-F, black line).
Blocking iGluRs attenuates the Tat-induced $[\text{Ca}^{2+}]_i$ increase

Interactions between Tat and iGluRs lead to iGluR activation and Ca$^{2+}$ influx, which have been reported extensively in neurons (Haughey et al., 2001; Kim et al., 2008). We therefore examined whether the Tat-induced Ca$^{2+}$ influx in OLs is also mediated by iGluRs. Since iGluR expression and subunit composition is developmentally regulated and highly heterogeneous (Gallo and Ghiani, 2000; Itoh et al., 2002; Cavaliere et al., 2012) in OLs, we first verified the expression of functional iGluRs on our cultured OLs. In immature, D2 OLs, NMDA at both 50 µM and 300 µM induced a minimum Ca$^{2+}$ response (Fig. 3.4A, cyan and blue lines), while either 50 µM or 300 µM AMPA induced a significant Ca$^{2+}$ influx lasting over 20 min (Fig. 3.4A, yellow and brown lines). These results are consistent with the fact that the majority of the iGluRs expressed on immature OL somas are AMPA/KA receptors (Gallo and Ghiani, 2000; Itoh et al., 2002). In mature OLs, 50 µM NMDA invoked a small Ca$^{2+}$ response (~60 nM) that was significantly above the control level (~40 nM) (Fig. 3.4B, cyan line vs. white line). A higher NMDA exposure (300 µM) elicited a sharp increase of $[\text{Ca}^{2+}]_i$ to a level similar to that induced by Tat (Fig. 3.4B blue line, vs. Fig. 3.3F red line). This high $[\text{Ca}^{2+}]_i$ level came down to ~60 nM within 10 min, but remained significantly higher than in vehicle-treated OLs. The AMPA responses of mature OLs were somewhat different. 50 µM AMPA caused a moderate $[\text{Ca}^{2+}]_i$ increase in mature OLs that recovered within 10 min (Fig. 3.4B, yellow line). 300 µM AMPA induced an initial $[\text{Ca}^{2+}]_i$ increase to ~110 nM, which then rose consistently over the 20 min experimental period (Fig. 3.4B, brown line).
We next examined whether blocking iGluRs affected Tat-induced \([\text{Ca}^{2+}]\), changes in OLs. In immature OLs, the \([\text{Ca}^{2+}]\), increase induced by 10 nM Tat was completely blocked by both MK801 and CNQX (Fig. 3.4C and E, yellow line), but \([\text{Ca}^{2+}]\), increases induced by 100 nM Tat were only reduced to ~100 nM (Fig. 3.4C and E, brown line). MK801 and CNQX additively inhibited the effect of 100 nM Tat, but could not completely block the \([\text{Ca}^{2+}]\), increase (Fig. 3.4G, brown line). The response in mature OLs was different. With 10 nM Tat, the initial \([\text{Ca}^{2+}]\), level was significantly attenuated by MK801 or CNQX, and the \([\text{Ca}^{2+}]\), level gradually returned to control over 5 min (Fig. 3.4D and F, yellow line). When MK801 and CNQX were added together, the effect of 10 nM Tat was completely blocked (Fig. 3.4H, yellow line). In contrast, only MK801 attenuated the initial \([\text{Ca}^{2+}]\), rise caused by 100 nM Tat, which was reduced to ~ 90 nM and maintained at this level over the 20 min experimental period. With CNQX, \([\text{Ca}^{2+}]\), levels were reduced only at later time points (Fig. 3.4D & F, brown line). MK801 and CNQX together had no additive effect on 100 nM Tat-induced \([\text{Ca}^{2+}]\), change in mature OLs (Fig. 3.4H, brown line). These results indicate that Tat-induced \([\text{Ca}^{2+}]\), changes in OLs were mediated by both NMDA-R and AMPA/KA-R activation. Further, since iGluR antagonists (20 µM) did not completely block \([\text{Ca}^{2+}]\), changes induced by the higher Tat concentration (100 nM), Tat may activate iGluRs in a way that cannot be completely inhibited by MK801/CNQX, or Tat may interact with other molecules on the surface of OLs that mediate \([\text{Ca}^{2+}]\), change.
Blocking alternative, non-iGluR targets has no effect on Tat-induced increases in [Ca\(^{2+}\)]

Since Tat-induced [Ca\(^{2+}\)]\(_i\) increases were not completely blocked by inhibiting iGluRs, we tested a series of alternative Ca\(^{2+}\) modulators. The Tat-induced OL [Ca\(^{2+}\)]\(_i\) increase was abolished in Ca\(^{2+}\)-free medium (Fig. 3.5 E-F). To test whether the initial Ca\(^{2+}\) influx triggers Ca\(^{2+}\)-induced-Ca\(^{2+}\) release from internal stores, immature/mature OLs were treated with 10 µM dantrolene for 20 min before addition of Tat, but this had no effect on Tat-induced [Ca\(^{2+}\)]\(_i\) change in OLs. OLs express abundant α\(_{v}\)β\(_3\) integrin (Fig. 3.5B), which has been reported to interact with Tat (Urbinati et al., 2005b; Urbinati et al., 2012). However, blocking integrin with the RGDS inhibitor peptide had no effect on Tat-induced [Ca\(^{2+}\)]\(_i\) increase in immature or mature OLs (Fig. 3.5A). Similarly, the L-type voltage-gated calcium channel blocker nimodipine (10 µM), CCR2 receptor inhibitor RS102895 (10 µM), CCR3 receptor inhibitor SB328437 (10 µM) and sodium-dependent Glutamate transporter inhibitor DL-TBOA (100 µM) all had no effect on Tat-induced [Ca\(^{2+}\)]\(_i\) changes in either immature or mature OLs (Fig. 3.5A).

**CNQX and MK801 effects on OL survival and membrane production**

Since Tat-induced [Ca\(^{2+}\)]\(_i\) changes were attenuated by MK801 and/or CNQX, we examined whether blocking iGluRs would also reverse Tat effects on immature OL survival. Both CNQX and MK801 (20 µM) completely reversed the effect of Tat on OL viability at 24, 48, and 72 h. CNQX by itself had no effect on OL viability, while cells treated with MK801 alone had a significantly higher survival rate than control (Fig. 3.6A). We next tested whether blocking iGluRs could also reverse the effect of Tat on the area of myelin-like membranes produced by mature OLs. Neither CNQX or MK801...
by themselves had any effect on the area of myelin-like membranes produced by mature OLs at either 24 or 48 h. Exposure to 100 nM Tat significantly decreased the size of the membrane area by >20% after 24 h. This effect was completely reversed by MK801, but not by CNQX. Results at 48 h were similar (Fig. 3.6B).
Discussion

Our findings through these studies establish that HIV-1 Tat, through activation of iGluRs, increases [Ca^{2+}]_i in OLs. In highly purified murine OL cultures, the outcome of Tat exposure varies with the stage of differentiation. At the level in which Tat reduced immature OL survival, mature OLs remain viable despite persistent elevations in [Ca^{2+}]_i, but have reduced myelin-like membrane areas. Blocking either NMDA-Rs or AMPA/KA-Rs by MK801 or CNQX, respectively, completely reversed Tat-induced immature OLs death, but only MK801 reverses Tat-induced membrane area reduction in mature OLs. These results suggest that both NMDA-Rs and AMPA/KA-Rs participates in Tat-induced immature OLs death, but only NMDA-Rs played a role in mature OLs membrane production.

Tat dosage

Tat is a highly conserved viral protein, essential for viral replication and efficient transcription of viral genes, which can be found in blood (Ensoli et al., 1993), extracellular matrix (Urbinati et al., 2005a), and cerebrospinal fluid (Tardieu et al., 1992). Importantly, Tat is produced by infected cells despite cART (Johnson et al., 2013). Levels of soluble Tat in the sera of HIV-1-infected individuals are within the range of 2 – 40 ng/ml (Xiao et al., 2000). Tat has a high affinity for cell surface proteins such as heparin sulfate proteoglycan (Liu et al., 2000) and extracellular cellular matrix components such as glycosaminoglycans (Xiao et al., 2000). Microdomains near productively-infected cells may have even higher levels. Thus, brain cells may be exposed to higher levels of Tat than what is measured in blood. It has also been
reported that the potency of recombinant Tat, as used in our experiments, is much less
than Tat secreted by infected cells (Li et al., 2008). Hence, the concentrations of Tat
used in our experiments (1-100 nM), are a reasonable approximation of titers in HIV-
infected brains.

Tat-induced [Ca\(^{2+}\)]\(_i\) increase

Tat disrupts Ca\(^{2+}\) homeostasis in astroglia (El-Hage et al., 2005; El-Hage et al.,
2008b), microglia (Sheng et al., 2000; Lokensgard et al., 2001), macrophages
(Contreras et al., 2005), and neurons (Haughey and Mattson, 2002; Zhu et al., 2009).
We found that Tat induced a concentration-dependent [Ca\(^{2+}\)]\(_i\) increase in both immature
and mature OLs. The primary source of Ca\(^{2+}\) was extracellular, since the [Ca\(^{2+}\)]\(_i\)
increases were abolished in Ca\(^{2+}\)-free medium. Transient [Ca\(^{2+}\)]\(_i\) increases occurred at 1
nM Tat with more sustained [Ca\(^{2+}\)]\(_i\) elevations at 10-100 nM Tat. Higher Tat
concentrations (\(\geq 10\) nM) may elicit an initial [Ca\(^{2+}\)]\(_i\) increase that depolarizes the
membrane, leading to secondary Ca\(^{2+}\) influx via voltage-gated calcium channels. Such
channels are expressed by OLs and activated by the cation influx via glycine receptors
and Na\(^{+}\)-dependent transporters (Kirischuk et al., 1995; Belachew et al., 2000).
Alternatively, the [Ca\(^{2+}\)]\(_i\) increase induced by 1 nM Tat may be below the threshold
required to activate Ca\(^{2+}\)-induced Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores. Neither the L-
type voltage-gated calcium channel blocker nimodipine nor the ryanodine receptor
inhibitor dantrolene affected Tat-induced [Ca\(^{2+}\)]\(_i\) increases (Fig. 3.5A), indicating the
involvement of alternative molecular targets (see below). Notably, 1 nM Tat does not
affect immature OL viability or mature OL membrane area. These results strongly
suggest the existence of a [Ca$^{2+}$]$_i$ threshold above which downstream signaling molecules involved in functional effects, such as CaMKIIβ, will be activated.

**iGluRs expression on OLs**

We found that stage of development was a significant determinant in the OL response to Tat, possibly due to developmentally-regulated expression of iGluRs on OLs (Deng et al., 2003). In OL cultures, AMPA elicited a dose-dependent [Ca$^{2+}$]$_i$ increase in immature OLs, while even high NMDA concentrations elevated [Ca$^{2+}$]$_i$, minimally (Fig. 3.4A). Mature OLs responded to both AMPA and NMDA with prominent [Ca$^{2+}$]$_i$ increases. These results are consistent with earlier studies showing that NMDA-evoked currents were detected in precursor, immature and mature OLs isolated from murine cerebellum and corpus callosum (Karadottir et al., 2005; Salter and Fern, 2005). A more recent study by Alix and Fern (2009) also demonstrated the co-localization of NMDA receptor NR1 subunit and CNPase$^+$ oligodendroglial processes in P10 rat optic nerve. At odds with these reports, De Biase et al., (2010) assessed gene expression in acutely purified/sorted CNS cells (Cahoy et al., 2008) and concluded that levels of NMDA-Rs and AMPA-Rs are high in NG2$^+$ OL progenitors, but decline markedly with maturation. Different receptor levels might reflect the effect of isolation procedures, although this has not been explored.

NMDA-Rs on myelinating OLs *in vivo* were found clustered in processes and on myelin (Salter and Fern, 2005; Micu et al., 2006). Consistent with this localization, the [Ca$^{2+}$]$_i$ increases mediated by Tat-NDMA-Rs interactions began at distal processes and traveled proximally (Fig. 3.3). De Biase et al., (2010) released caged glutamate by UV
photolysis in a 100-µm circle centered on the cell body, and only detected slight NMDA currents. Glutamate released in this way may be sequestered by neighboring cells prior to stimulating NMDA-Rs on more distal OL processes. It is noteworthy that Ca\(^{2+}\) responses to AMPA in immature OLs are more homogeneous than in mature OLs, suggesting a higher variability of AMPA-R expression or a greater diversity in the composition of AMPA-R subunits with maturation, as seen in vivo (Matute et al., 2002; Williams et al., 2009).

iGluRs mediate Tat-Induced [Ca\(^{2+}\)]\(_i\) increase

It is not surprising that Tat interacts with NMDA-Rs in OLs, since Tat-NMDA-R interactions occur in neurons (King et al., 2006; Kim et al., 2008; Rumbaugh et al., 2012; Capone et al., 2013). However, since NMDA minimally affected [Ca\(^{2+}\)]\(_i\) in immature OLs, it was intriguing that Tat-induced [Ca\(^{2+}\)]\(_i\) increases were attenuated by MK801. Tat and NMDA appear to interact differently with NMDA-Rs, since Tat-NMDA-R interactions cause persistent, rather than transient, [Ca\(^{2+}\)]\(_i\) increases (Fig. 3.4 and (Fitting et al., 2014)). CNQX also attenuated the effects of Tat on [Ca\(^{2+}\)]\(_i\), which was unexpected since direct Tat-AMPA/KA-Rs interactions have never been reported. In neurons, activation of cytoplasmic CaMKII recruits AMPA-Rs from intracellular vesicles to cytoplasmic membranes, and increases the likelihood of channel opening by phosphorylating Ser-831 of AMPA-R GluA1 subunits (Milstein and Nicoll, 2008; Derkach, 2011; Kristensen et al., 2011). We found that exposure to Tat leads to phosphorylation and activation of CaMKIIβ in OLs (see Chapter 5). Since the expression of the major cytoplasmic Ca\(^{2+}\) binding protein calbindin is extremely low
compared to other CNS cells, (Baimbridge et al., 1992; Zhang et al., 2014), we propose that Tat-NMDA-R interactions lead to an initial Ca\(^{2+}\) influx that activates CaMKIIβ, which then recruits and phosphorylates AMPA-Rs and raises [Ca\(^{2+}\)]\(i\) levels. Combined MK801 and CNQX had no additive effect, implicating additional, non-iGluR cellular components in Ca\(^{2+}\) responses to Tat. Blocking experiments preclude a role for integrins, chemokine receptors CCR3 and CCR2, sodium-dependent glutamate transporters, and ryanodine receptors in Tat-induced [Ca\(^{2+}\)]\(i\) increases (Fig. 3.5). Similar to neurons (Fitting et al., 2014), Tat-activated NMDA-Rs might cause Na\(^+\) influx, reversing Na\(^+\)/Ca\(^{2+}\) exchange and triggering mitochondrial Ca\(^{2+}\) release.

**Effects of Tat on OL viability and function**

Ca\(^{2+}\) influx via AMPA/KA-Rs can activate various death pathways in OLs (Matute, 2011). AMPA-R activation leads to caspase-8 recruitment and Bid truncation, while KA-R activation has been linked to caspase-3 and caspase-9 activation (Sanchez-Gomez et al., 2003; Matute et al., 2006). We found that CNQX completely reversed Tat-induced death of immature OLs, implicating activation of AMPA/KA-R-mediated death pathways. Surprisingly, MK801 had a similar effect, suggesting a requirement for both NMDA-Rs and AMPA/KA-Rs. This further implicates NMDA-R-mediated AMPA/KA-R activation. Interestingly, MK801 increased baseline survival of immature OLs, suggesting that some glutamate is released from OLs injured during the culture process.

Mature OLs remained viable when exposed to Tat, even when [Ca\(^{2+}\)]\(i\) was elevated to a level that resulted in immature OLs death. This is consistent with previous
findings that Ca\textsuperscript{2+}-dependent excitotoxicity decreases with OL maturation (McDonald et al., 1998; Kavanaugh et al., 2000; Itoh et al., 2002; Deng et al., 2003). Rumbaugh et al. (2013) have proposed that the peptide Tat\textsubscript{31-61} directly binds the NR1 subunit resulting in NMDA-R activation. \textit{In vivo}, NMDA-Rs are mainly clustered on cytoplasmic processes and the myelin sheath, and their activation causes process degeneration and myelin injury (Micu et al., 2006). Consistent with this subcellular distribution of NMDA-Rs, our experiments showed that Tat-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases in mature OLs originate at the distal processes and/or the peripheral edge of the myelin-like membranes (Fig. 3.3D). Importantly, the resultant reduction in myelin-like membranes can be reversed by MK801, but not CNQX (Fig. 3.6), suggesting that membrane retraction/OL injury is mediated by a common mechanism involving NMDA-R activation and Ca\textsuperscript{2+} influx. It is possible that Ca\textsuperscript{2+} influx due to Tat-NMDA-R interactions will generate a highly localized increase in [Ca\textsuperscript{2+}]\textsubscript{i} within distal processes/myelin membranes that leads to local CaMKII\textbeta activation (See Chapter 5). Waggener et al. (2013) showed that CaMKII\textbeta regulates OL maturation and CNS myelination via stabilizing the actin cytoskeleton. Our findings suggest that CaMKII\textbeta activation by Tat may also underlie some myelin damage with HIV infection.
Table 3.1. Percentage of cells expressing neuron/glial specific markers at various times *in vitro*

<table>
<thead>
<tr>
<th></th>
<th>O4+</th>
<th>GFAP+</th>
<th>MAP2+</th>
<th>Iba+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>98.76 ± 0.73%</td>
<td>1.00 ± 0.58%</td>
<td>0%</td>
<td>0.24 ± 0.24%</td>
</tr>
<tr>
<td>(n=4, 372 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>96.35 ± 0.49%</td>
<td>3.15 ± 0.62%</td>
<td>0%</td>
<td>0.50 ± 0.29%</td>
</tr>
<tr>
<td>(n=4, 413 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 7</td>
<td>93.18 ± 0.15%</td>
<td>5.35 ± 0.61%</td>
<td>0.25 ± 0.25%</td>
<td>1.22 ± 0.46%</td>
</tr>
<tr>
<td>(n=4, 396 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>90.60 ± 0.39%</td>
<td>7.31 ± 0.37%</td>
<td>0%</td>
<td>2.10 ± 0.44%</td>
</tr>
<tr>
<td>(n=4, 383 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n: number of individual cultures.
Figure 3.1. Survival of Tat-treated OLs. (A-C) Sample images of OL cultures treated with vehicle or Tat (100 nM) after 0, 24, 48 and 72 h. (A) Immature OLs treated with vehicle grow normally and differentiate over the 72 h period. At the end of 72 h, cells have complex process networks with myelin-like membranes. (Scale bar: 100 µm for A-C) (B) Immature OLs treated with 100 nM Tat. Both OLs in the field were healthy at 0 h. One OL was dead after 72 h (white arrow). Although the other OL survived, its process network was greatly reduced. An astrocyte at the lower right corner migrated out of the field by 24 h. (C) A mature OL treated with 100 nM Tat appeared healthy and still maintained a complex process network after 72 h, even though it underwent extensive process reorganization. The double arrow marks a major process that was retracted. (D) OL vulnerability to Tat was dependent on differentiation. Almost all mature OLs (>95%) survived for the 72 h experimental period, whether they were treated with vehicle or 100 nM Tat. In contrast, survival of immature OLs was significantly reduced by 100 nM Tat, even at 24 h. (*p<0.05 vs. immature OL controls; one-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; at least 25 cells were counted for each n) (E) The dose-dependent effect of Tat on immature OL survival. Immature OLs at day 2 were treated with 1, 10 and 100 nM Tat for 24, 48 and 72 h. 1 nM Tat shows no obvious effect on OL survival, while 10 nM Tat reduces survival by 10 – 15% when compared to controls at 24, 48 and 72h. The cytotoxic effect of 100 nM Tat is even more significant (22 – 35% reduction in survival). (*p<0.05 vs. control; #p<0.05 vs. 10 nM Tat group; two-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; >40 cells were counted for each n.) (F-G) The Live-Dead assay shows that 100 nM Tat significantly increased immature OL death at 24, 48 and 72 h.
(F) Corresponding phase and fluorescent images of calcein-AM (live cells, green) and EthD-1 (dead cells, red) labeled of D2 immature OLs treated with vehicle or 100 nM Tat for 72 h. A majority of cultured cells appeared to shift from green to red with Tat treatment. (G) At all time points, 100 nM Tat significantly increased the proportion of red pixels and reduced numbers of green pixels, indicating less OL survival with Tat and validating the visual observation in F. (*p<0.05 number of red pixels; Tat vs. control of same time point; #p<0.05, number of green pixels; Tat vs. control of same time point; one-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; 20 randomly selected images were analyzed for each n.)
Figure 3.2. Tat reduces myelin-like membranes of mature OLs. (A). Sample images showing changes in mature OL membrane area over 48 h. Cells were labeled with 2 μg/ml CM-Dil 1 d before Tat treatment. Phase contrast and fluorescent images of the same cell were taken at 0, 24, and 48 h. Phase and fluorescent images were merged to better visualize the myelin-like membrane structures. At the end of the experimental period, cells were labeled with calcein-AM to verify viability (green fluorescence). The yellow arrowhead indicates an area with an obvious reduction in the area of the cellular processes (loss of red fluorescence) over time. (B) Quantification of the change in membrane area in vehicle and Tat-treated OLs. At both 24 h and 48 h, vehicle-treated OLs showed ~25% growth of membrane area. 1 nM Tat did not have any significant effect on the membrane area when compared with controls. However, 10 or 100 nM Tat-treated OLs at 24 h and 48 h exhibited ~20% reductions in membrane area.
compared to 0 h (* p<0.05, two-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; at least 25 cells were counted for each n).
Figure 3.3. Tat induces Ca\textsuperscript{2+} influx in mature and immature OLs. (A-D) Sample images showing changes of intracellular Ca\textsuperscript{2+} concentration over a 20-min period in
immature (A-B) and mature (C-D) OLs treated with vehicle (A & C) or 100 nM Tat (B & D). [Ca\textsuperscript{2+}] was assessed by fura-2 AM 340/380 fluorescence ratio and was displayed as pseudocolored images generated by Zeiss AxioVision 4.8 software. (E-F) Tat induces dose-dependent [Ca\textsuperscript{2+}] increases in both immature (E) and mature (F) OLs. This effect is completely abolished in Ca\textsuperscript{2+}-free medium, and greatly attenuated by heat inactivation of Tat. (Vertical dashed line indicates the time point (30 s) when treatment was added *p<0.05 vs. control; #p<0.05, vs. 100 nM Tat; two-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; at least 7 cells were counted for each n. Scale Bar: 50 µm).
Figure 3.4

A) Immature OLs

B) Mature OLs

C)

D)

E)

F)

G)

H)
Figure 3.4. Tat-induced $[\text{Ca}^{2+}]_i$ increases in OLs are partially reversed by iGluR antagonists. (A-B): Expression of functional NMDA-R and AMPA-Rs on OLs. (A) In immature OLs, AMPA (at both 50 and 300 µM) causes $[\text{Ca}^{2+}]_i$ increases while NMDA responses are minimal. (B) In mature OLs, NMDA leads to a dose-dependent $[\text{Ca}^{2+}]_i$ increase. AMPA elicits a $[\text{Ca}^{2+}]_i$ increase only at the higher 300 µM concentration, and variability between cells is quite high. (C-H) iGluR antagonists attenuate Tat-induced $[\text{Ca}^{2+}]_i$ increases in OLs. (C, E, G) In immature OLs, MK801 and/or CNQX completely blocks the $[\text{Ca}^{2+}]_i$ increase induced by 10 nM Tat but only partially inhibits the $[\text{Ca}^{2+}]_i$ increase elicited by 100 nM Tat. (D, F, H). In mature OLs, the 10 nM Tat-induced $[\text{Ca}^{2+}]_i$ increase is similarly abolished by MK801 and/or CNQX. In contrast, the $[\text{Ca}^{2+}]_i$ increase induced by 100 nM Tat is only partially blocked by MK801 and/or CNQX (*$p<0.05$ vs. Control; #$p<0.05$ vs. 100 nM Tat; two-way ANOVA followed by post-hoc Bonferroni's test; n=4 individual experiments; at least 7 cells were counted for each n).
Figure 3.5. Blocking alternative, non-iGluR targets has no effect on Tat-induced increases in \([\text{Ca}^{2+}]_i\). A. Immature or mature OLs were exposed to nimodipine (L-type voltage-gated calcium channel inhibitor, 10 µM), dantrolene (ryanodine receptor inhibitor, 10 µM), DL-TBOA (sodium-dependent glutamate transporter inhibitor, 100 µM), RS102895 (CCR2 inhibitor, 10 µM), SB328437 (CCR3 inhibitor 10 µM), or the RGDS peptide (integrin inhibitor, 10 µM) for 20 min prior to Tat treatment. None of these inhibitors affected Tat-induced OL \([\text{Ca}^{2+}]_i\) increases. (*p<0.05 vs. control; two-way ANOVA followed by post-hoc Bonferroni’s test; n=3 individual experiments; at least 7 cells were counted for each n). B. Expression of αV and β3 integrin on O4+ and MBP+ OLs (Scale bar: 50 µm).
Figure 3.6. Effects of iGluR antagonists on Tat-induced functional effects. (A) Immature OLs treated with 100 nM Tat exhibit a significantly reduced survival rate at all time points, compared to control. Either 20 µM CNQX or 20 µM MK801 completely reversed the Tat-induced cell death at all time points. 20 µM CNQX by itself did not change the viability of immature OLs. On the other hand, at both 48 and 72 h, 20 µM MK801 significantly increased the viability of OLs (*p<0.05, less survival vs. control; #p<0.05, greater survival vs. control; one-way ANOVA followed by post-hoc Bonferroni’s test; n=4 individual experiments; at least 25 cells were counted for each n). (B) At both 24 h and 48 h, mature OLs treated with 100 nM Tat exhibit a significant, ~20% reduction in total membrane area when compared to 0 h. In contrast, the membrane area of vehicle-treated OLs increased significantly by ~25% at both time points. 20 µM MK801,
but not CNQX, reversed the reduction in membrane area caused by Tat. Neither MK801 nor CNQX by itself had any effect on OL membrane production (*p<0.05 vs. 0 h; one-way ANOVA followed by post-hoc Bonferroni’s test. n=4 individual experiments; at least 25 cells were counted for each n).
Chapter 4 Tat affects mature OL membrane change via CaMKIIβ activation

(This chapter, in part, was published as a paper in the Journal of Neuroscience in August of 2015, 35(32):11384-11398, entitled Oligodendrocytes are targets of HIV-1 Tat: NMDA and AMPA receptor-mediated effects on survival and development)

Introduction

It is interesting that mature OLs exposed to HIV-1 Tat exhibit no changes of viability, but local degeneration of processes and membranes, which leads to an overall reduction of myelin-like membrane area. We further investigated the pathways involved in mature OL membrane change, and focused on molecules involved in Ca\textsuperscript{2+} signaling and cytoskeleton changes.

CaM is the major intracellular receptor for Ca\textsuperscript{2+}. Studies by Faas et al., (2011) showed that Ca\textsuperscript{2+} binds more rapidly to CaM than to other Ca\textsuperscript{2+}-binding proteins. Besides, the expression of the major cytoplasmic Ca\textsuperscript{2+} binding protein calbindin is extremely low compared to other CNS cells, (Baimbridge et al., 1992; Zhang et al., 2014). Hence, CaM may be the major determinant of [Ca\textsuperscript{2+}]), after iGluRs-mediated Ca\textsuperscript{2+} influx. Ca\textsuperscript{2+}/CaM binds and activates Ca\textsuperscript{2+}/CaM-dependent protein Kinase II (CaMKII), which is central to the coordination of Ca\textsuperscript{2+} signal transduction. The predominant isoform of CaMKII in OLs, CaMKIIβ, has been repeatedly reported to be involved in
actin cytoskeleton re-organization (O'Leary et al., 2006; Okamoto et al., 2007; Lin and Redmond, 2008; Okamoto et al., 2009), and thus has the potential to be the mediator of Tat-induced membrane reduction in mature OLs.

The holoenzyme of CaMKII has been proposed to be a dodecameric structure composed of 12 monomers that form two stacked hexagonal rings (Kolodziej et al., 2000). Each CaMKII monomer contains four functional domains, a kinase catalytic domain, an autoinhibitory (regulatory) domain, an association (oligomerization) domain and a central variable domain that is subject to alternative splicing (Hudmon and Schulman, 2002a). The family of CaMKII isoforms is encoded by 4 closely related genes (α, β, γ, δ), among which CaMKIIβ is the major isoform expressed in murine OLs (Waggener et al., 2013) and has morphogenic functions not shared by other isoforms (O'Leary et al., 2006). The v1 variable domain of CaMKIIβ, which located C-term to the Ca²⁺/CaM binding domain, has been shown to bundle F-actin under resting state (Meyer et al., 1992; O'Leary et al., 2006; Okamoto et al., 2007) and stabilize cytoskeleton structure. Ca²⁺/CaM binding induces a conformational change in CaMKIIβ, which activates its kinase activity. More importantly, this conformational change also exposes a critical phosphorylation site (T287) on CaMKIIβ, which can be autophosphorylated by activated neighboring subunits in the same holoenzyme (Hudmon and Schulman, 2002a). T287 autophosphorylation not only increases the affinity between CaMKIIβ and Ca²⁺/CaM by ~1000 fold (Meyer et al., 1992), but also induces generation of autonomous activity in CaMKIIβ, which phosphorylates its neighboring CaMKIIβ subunits on multiple sites, including T306/307 in the variable domain. It has been reported that binding of Ca²⁺/CaM and autophosphorylation on
T306/307 result in dissociation of CaMKIIβ from F-actin, followed by cytoskeleton collapse in hippocampal neurons (Okamoto et al., 2007; Okamoto et al., 2009).

Activation of CaMKII by NMDA-Rs is an important step in long-term potentiation of synaptic transmission in hippocampal neurons (Malenka and Nicoll, 1999). NMDA-R-mediated Ca\(^{2+}\) influx activates CaMKII, which promotes synaptic insertion and single channel conduction of AMPA-Rs (Rongo and Kaplan, 1999; Hayashi et al., 2000). Studies by Bayer et al., (2001) further shown that interactions between NR2B subunit of NMDA-Rs can induce synaptic translocation of CaMKII, which facilitates its activation by NMDA-Rs-mediated Ca\(^{2+}\) influx. It is also noteworthy that the interaction between NR2B and CaMKII generates a Ca\(^{2+}\)/CaM-independent autonomous activity that is similar to CaMKII T287 autophosphorylation, and thus possessing the potential of T306/T307 phosphorylation. In addition, a recent study also showed that pharmacologically inhibiting CaMKIIβ activation in vitro restrains the morphological maturation of differentiating rat OLs, while systemic knock-out of CaMKIIβ in vivo leads to an increase in the g-ratio (decrease in thickness) of the myelin sheath (Waggener et al., 2013), suggesting that CaMKIIβ plays an important role in OL differentiation and function. Results from our previous work indicated that Tat induces a persistent [Ca\(^{2+}\)]\(_i\) increase via NMDA-Rs and AMPA/KA-Rs activation. We thus hypothesized that the [Ca\(^{2+}\)]\(_i\) increase in mature OLs activates CaMKIIβ and leads to membrane collapse. Our results showed that CaMKIIβ activation, which is assessed by the level of T287 phosphorylation, was significantly increased in Tat-treated OLs. The Tat-induced CaMKIIβ activation can be completely blocked by MK801, coincident with our previous experimental results that MK801 completely reversed the Tat-induced myelin-like
membrane reduction in mature OLs (chapter 3). CNQX only partially reverse the Tat-induced CaMKIIβ activation, suggesting that Ca^{2+} influx via AMPA/KA-Rs also activates CaMKIIβ, but to a less extent.
**Materials and Methods:**

**Western blotting of CaMKIIβ and T-287 phosphorylated CaMKIIβ**

Western blotting was performed as described previously (Chapter 3). In short, proteins were extracted from cultured OLs or brain tissues using RIPA buffer with protease and phosphatase inhibitors. The concentration of extracted proteins was assessed using the BCA assay. Target proteins were probed with primary antibodies for CaMKIIβ (1:1000, Life Technologies) and phosphor-CaMKIIβ (T287) (1:1000, Abcam) at 4°C overnight. Membranes were incubated with appropriate IRDye secondary antibodies (1:3000, Li-COR, Lincoln, NE) for 1 h at room temperature, and imaged using an Odyssey Imager (Li-COR). Protein bands were quantified using Li-COR image studio software.

**RT-PCR analysis of CaMKII transcription**

RNAs were extracted from OLs or cerebral cortex of P2 ICR mice using the miRNAeasy Mini Kit (QIAGEN Inc. Valencia, CA). Concentration of RNAs of each sample was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Equal amounts of RNAs were then used as templates to transcribe cDNA using a high capacity cDNA reverse transcription kit (Life Technologies).

Gene-specific primer pairs were designed using the NCBI Gene database and primer-design web tool ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The web tool generates an array of primer sets that fit the parameters defined by users. These parameters include size of the final product and the primers, whether primers span the junctions between exons, GC contents of the primer etc. For each isoform of CaMKII, two primer sets with the
least GC contents at the 3'-terminus were picked and tested using SensiMi SYBR Hi-ROX Kit (Bioline USA Corp, Taunton MA) on a Roche Lightcycler 96 real-time PCR system (Roche Diagnostics Corporation, Indianapolis, IN). First, a test run using a range of T\textsubscript{m} (56 – 66°C) were performed to determine the optimal T\textsubscript{m} for all the primer sets. Efficiency of all primer sets was then decided by running RT-PCR with T\textsubscript{m} set at the optimal temperature (60°C) and a series of dilution (1:2.5 – 1:20) of transcribed cDNA. For each isoform of CaMKII, the primer set with better efficiency was chosen to be used in determining the expression of CaMKII in cultured OLs. The sequences of these primer sets are listed below:

**CaMKII\textalpha:**

Primer pair 2 (Efficiency = 1.98)

Forward Primer: 5'-AGACACCAAAGTGCGCAAAC-3'

Reverse primer: 5'-TTCCAGGGTCGCACATCTTC-3'

**CaMKII\textbeta**

Primer Pair 3 (Efficiency = 1.92)

Forward primer 5'-GGTTTGGATTTGCGGGAACG-3'

Reverse primer 5'-ACAGGATCACCCCACATGCC-3'

**CaMKII\textgamma**

Primer pair 2 (Efficiency = 1.98)

Forward Primer: 5'-CACCGACGACTACCAGCTTT-3'

Reverse primer: 5'-TTTCTGATGATCTCGGGCGG-3'

**CaMKII\textdelta**

Primer pair 1 (Efficiency = 2.00)
Forward Primer: 5’-TCGGAGGAGGGGCTTCCATTA-3’
Reverse primer: 5’-TCTCAGGCTTTAGGTCCCGA-3’

siRNA and antisense oligonucleotide sequence that targets mRNA of CaMKIIβ

ON-TARGETplus SMARTpool siRNA against mice Camk2b were commercially available from Thermo Fisher Scientific/Dharmacon Inc., Lafayette, CO. The SMARTpool siRNA contains siRNAs specific to 4 different sequences in the Camk2b gene. These sequences are listed as below:

siRNA 1 (J-062002-05): target sequence: 5’-GCACGUCAUUGCGAGGAU-3’
siRNA 2 (J-062002-06): target sequence: 5’-GAAGGAGGCCUACGGCAAA-3’
siRNA 3 (J-062002-07): target sequence: 5’-CCAAGAUCAUUAAUACCAA-3’
siRNA 4 (J-062002-08): target sequence: 5’-CCAUGGGUCUGCCAACGUU-3’

Mice CaMKIIβ mRNA sequence was acquired from the NCBI Gene Database (NCBI Reference Sequence#: NM_001174053.1). The antisense oligonucleotide sequence was designed by Integrated DNA Technologies (IDT) AntiSense Design tool (http://www.idtdna.com/Scitools/Applications/AntiSense/Antisense.aspx?source=menu). The tool generates a maximum of 20 antisense nucleotides sequences at one time, among which three antisense oligonucleotide sequences (Sequence 1, 14 and 15) targeting different, non-overlapping regions were picked and tested. Their sequences are listed as below:

Sequence 1: 5’-ACCTCCATACCTCACTCCC-3’
Sequence 14: 5’-GCCACTCAGATGTCTTACCT-3’
Sequence 15: 5’-GCTTGTGTTGGTCTCATCCC-3’
All sequences ordered are phosphorothioate-modified to be nuclease-resistant.

**Antisense oligonucleotide sequence delivery**

To inhibit expression of CaMKIIβ, several methods have been used to deliver the antisense oligonucleotide sequence or ON-TARGETplus SMARTpool siRNA to mature OLs.

1. **Direct feed:** 5 µM antisense oligonucleotides were added to the culture medium and replenished every 48 h.

2. **Lipofectamine transfection:** Lipofectamine working solution was prepared by adding 1 µl lipofectamine 2000 (Life Technologies) to 50 µl culture medium. Oligonucleotide/siRNA working solution was prepared by adding 1 µg oligonucleotides/siRNA and 1 nM siGLO green (Life Technologies) to 50 µl culture medium. Both working solutions were left at room temperature for 5 min before being mixed together gently. The 100 µl mixed solution was then left at room temperature for another 15 min before being added to a mature OL culture, and incubated in 37°C for 2 h. Transfection medium containing oligonucleotides/siRNA and lipofectamine were replaced with normal culture medium after 3 hrs. Mature OLs were kept in normal culture medium for another 48-72 h before transfection efficiencies were determined by fluorescent microscopy.

3. **Viromer transfection:** Viromer Blue (Lipocalyx, Halle, Germany) and oligonucleotides/siRNA were mixed with transfection buffer to a final concentration of 50 µM and 25 nM, respectively, and left at room temperature for 10 min before being added to the mature OL culture. Transfection medium containing viromer-oligonucleotides/siRNA
was replaced after 4 h with fresh culture medium.

4. RVG9R (Rabies Virus Glycoprotein 9R) procedure: RVG-9R (Peptide sequence: YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRR) was purchased from Bachem, Torrance, CA. Oligonucleotide/siRNA and RVG9R were mixed in a ratio of 1:10. One nanomolar siGLO were then added to this mixture. The solution was left at room temperature for 30 min before being added to the culture medium. Delivery of oligonucleotide/siRNA to the mature OLs was tested 48 h after the procedure with fluorescent microscopy and RT-PCR.
Results

Expression of Tat in vivo does not alter CaMKIIβ expression, but increase CaMKIIβ activation

We first examined whether expression of Tat altered CaMKIIβ levels in Tat transgenic mice. To rule out the possibility that the level of CaMKIIβ may change with development, Tat expression was not induced until the mice were 3-month old. Western blot results showed that there was no significant difference in CaMKIIβ expression between Tat+ and Tat− mice in cortex, cerebellum, striatum or hippocampus (Fig. 4.1A). The same samples were also used to test whether CaMKIIβ activation was changed by Tat expression. A significantly increased level of CaMKIIβ-T287 phosphorylation was detected in all four regions that assessed, indicating increased CaMKIIβ activation by Tat expression in these areas (Fig. 4.1B).

Tat induced CaMKIIβ activation in both immature and mature OLs

Immunostaining results showed that CaMKIIβ was expressed on both immature and mature OLs in vitro. In immature OLs, most of the CaMKIIβ was observed in processes. In mature OLs, CaMKIIβ was localized in both cytoplasm and major processes (Fig. 4.2). We next investigated whether CaMKIIβ was activated as a consequence of the Tat-induced [Ca2+]i increase. Both immature and mature OLs exposed to 100 nM Tat for 30 min showed significantly increased levels of CaMKIIβ Thr-287 autophosphorylation, suggesting increased CaMKIIβ activation (Fig. 4.3B). In either immature or mature OLs, the expression level of total CaMKIIβ was not affected by Tat
treatment (Fig. 4.3A). Further, CaMKIIβ expression level in mature OLs is significantly higher than in immature OLs (Fig. 4.3A).

**Effects of blocking iGluRs on Tat-induced CaMKIIβ activation**

We next assessed whether blocking iGluRs had any effect on Tat-induced CaMKIIβ activation. Immature and mature OLs were treated with MK801 and CNQX with or without 100 nM Tat. Western blots showed that CaMKIIβ phosphor-T287 level was significantly elevated (~2×) by 30-min Tat treatment. This Tat-induced CaMKIIβ T-287 phosphorylation was completely reversed by MK801, but only partially inhibited by CNQX (Figure 4.4).

**Efficiency of CaMKII primer sets**

Existing inhibitors to CaMKIIβ are either non-specific or toxic to OLs in vitro. Hence, we tried to silence CaMKIIβ by interrupting its expression in OLs. To be able to detect the change of CaMKIIβ transcription in OLs, we first designed primer sets to all 4 CaMKII isoforms and tested the efficiency of these primer sets. It is known that expression levels of different isoforms of the CaMKII varied greatly in murine OLs (Waggener et al., 2013). Hence, we used RNAs extracted from cerebral cortex of P2 ICR mice to test the efficiency of CaMKII primer sets. As Table 5.1 shows, efficiency of designed primer sets of CaMKIIα, β, γ, ξ were determined to be 1.98, 1.92, 1.98 and 2.00, respectively, indicating that RT-PCRs using these primer sets can faithfully reflect the mRNA contents in tissues. Same primer sets were then used to determine the expression level of CaMKII in cultured OLs.
Attempt to delivery antisense oligonucleotide to mature OLs

Several methods have been tried to deliver the antisense oligonucleotide or SMARTpool siRNAs specific to Camk2b gene to D7, mature OLs. We first tried a direct feed methodology, where the phosphorothioate-modified oligonucleotide was added to the culture medium with a final concentration at 5 µM, in expecting that endocytosed oligonucleotide will escape to the cytoplasm and bind to the mRNA of CaMKIIβ, which leads to RNase-H mediated degradation of CaMKIIβ mRNA. RT-PCR showed that expression level of CaMKIIβ was not changed in antisense oligonucleotide-treated culture between 0 - 72 h. We next tried several transfection reagents including lipofectamine®, viromer® and the rabies virus glycoprotein 9R (RVG9R). These transfection reagent in theory will form a complex with oligonucleotides/siRNA and carry them through the cellular membrane. A small fluorescent molecule, siGLO, was co-transfected with the antisense oligonucleotides/siRNAs, so that the transfection efficiency can be evaluated using fluorescent microscopy. Both RT-PCR and fluorescent microscopy experiments suggested that none of these methods successfully down-regulate expression of CaMKIIβ in mature OLs.
Discussion

Our early studies showed that in mature OLs, Tat-induced \([\text{Ca}^{2+}]_i\) increases had no effect on cell survival, but instead causes a 20 - 25\% decrease of the total membrane area. The Tat-induced membrane loss can be completely blocked by MK801, suggesting that it is mainly mediated by NMDA-Rs. This is consistent with \textit{in vivo} studies from other groups, which reported that \text{Ca}^{2+} influx via ischaemia-mediated NMDA-R activation causes OL process degeneration and demyelination (Karadottir et al., 2005; Micu et al., 2006). We hypothesized that CaMKII\(\beta\) activation may be the downstream signaling events that links \([\text{Ca}^{2+}]_i\) increase and membrane loss in mature OLs. Our results showed that 30 min Tat treatment leads to CaMKII\(\beta\) activation in mature OLs, which can be completely blocked by NMDA-Rs, but only partially blocked by CNQX.

We found that expression of Tat did not change the total amount of CaMKII\(\beta\) \textit{in vivo} (Fig. 4.1A), but leads to its activation in all of the four brain regions (cortex, cerebellum, striatum and hippocampus) that we sampled (Fig. 4.1B). In the murine CNS, CaMKII\(\beta\) expression was not restricted to oligodendroglial cells (Zhang et al., 2014). Hence, we used cell culture to test whether Tat activates CaMKII\(\beta\) in OLs. Our results showed that Tat induced a \(\sim2\)-fold increase of CaMKII\(\beta\)-T287 phosphorylation in both immature and mature OLs. Since >90\% of the cells in our culture are from oligodendroglial lineage (Table 3.1), and the protein samples were harvested 30 min after Tat treatment, it is highly possible that CaMKII\(\beta\) activation is a direct result of Tat challenge, rather than a secondary effect mediated by other cell types.
Several lines of evidences indicate CaMKIIβ as the signaling molecule that mediates the Tat effects on OL membrane change. First, Ca$^{2+}$/CaM activates CaMKIIβ. Since the expression levels of other intracellular Ca$^{2+}$ binding molecule, such as Calbindin, are relatively low in OLs (Zhang et al., 2014), Tat-induced Ca$^{2+}$ influx most likely will result in increased levels of Ca$^{2+}$/CaM complex, which leads to CaMKIIβ activation. This is consistent with our Western blot results, which found that CaMKIIβ was indeed activated by Tat in both immature and mature OLs (Fig. 4.3B). Second, it has been reported that activation of NMDA-Rs promotes an interaction between its NR2B subunit and CaMKIIβ, which facilitates CaMKIIβ activation (Bayer et al., 2001; Bayer et al., 2006). Coincidently, we found that CaMKIIβ activation by Tat is completely abolished by MK801 (Fig. 4.4), indicating the major activator of CaMKIIβ is NMDA-R-mediated Ca$^{2+}$ influx. Third, CaMKIIβ has been known to have a structural role besides its kinase activity (Hudmon and Schulman, 2002b). At resting state, CaMKIIβ binds F-actin and stabilizes cytoskeleton structures. Activation of CaMKIIβ by Ca$^{2+}$/CaM leads to a conformational change of its structure and disrupts this interaction, which destabilizes actin cytoskeleton (Okamoto et al., 2007; Okamoto et al., 2009). This interaction is relevant to OL maturation and myelination because knockout CaMKIIβ leads to hypomyelination, while a CaMKIIβ mutant (CaMKIIβ$^{A303R}$) lacks its kinase activity, but preserves its actin-binding capacity showed normal myelination (Waggener et al., 2013). Further, recent studies have suggested that constitutive elevation of [Ca$^{2+}$], may inactivate the actin binding properties of CaMKIIβ via phosphorylation of S371, which leads to collapse of the actin cytoskeleton and affects OL maturation (Martinez-Lozada et al., 2014).
Based on the results from our experiments and the evidences reported by other groups, we proposed the following model for the role of CaMKIIβ in Tat-induced mature OL membrane reduction. At resting state, CaMKIIβ binds F-actin and stabilizes the cytoskeleton. Activation of NMDA-Rs due to physiological activities leads to a transient CaMKIIβ activation. This allows CaMKIIβ to dissociate from F-actin for a short period of time and facilitates cytoskeleton growth. Under pathological conditions when Tat was secreted by infected cells, interaction between Tat and NMDA-Rs leads to a persistent [Ca^{2+}]_i increase and prolonged CaMKIIβ activation in OLs, which dissociates CaMKIIβ from actin for a long-period of time and destabilizes the cytoskeleton, eventually leading to reduction of membrane structure.

Unfortunately, we are still lacking data that directly links CaMKIIβ to OL membrane change. Currently there are no inhibitory drugs that are both specific to CaMKIIβ and non-toxic to cells. Multiple attempts have been made to deliver siRNAs specific to CaMKIIβ to mature OLs to stop its expression. However, mature OLs are very resistant to transfection reactions, and the efficiency of transfection is too low to be meaningful to any further experiments (Data not shown). In addition, preventing CaMKIIβ expression is not equivalent to inhibiting CaMKIIβ activation. OLs cultured from CaMKIIβ^{A303R} mutant mice (O'Leary et al., 2006; Lin and Redmond, 2008; Waggener et al., 2013) will be a better model to verify our proposed mechanism. In these mice, the mutated CaMKIIβ^{A303R} preserves the ability of binding and stabilizing actin filaments, but was unable to bind Ca^{2+}/CaM, and thus would be predicted to not be activated by Tat-induced [Ca^{2+}]_i increase.
Figure 4.1 Expression of Tat in vivo does not alter CaMKIIβ expression, but promotes CaMKIIβ activation. (A) Western blot using proteins extracted from 4 different brain regions (cortex, cerebellum, striatum and hippocampus) of the Tat transgenic mice showed that in vivo expression of CaMKIIβ in the brain was not significantly affected by Tat. (B) Ratio of p-CaMKIIβ/t-CaMKIIβ was significantly increased in cortex, cerebellum, striatum and hippocampus in Tat+ mice, suggesting that Tat expression leads to increased CaMKIIβ activation in these areas. (*: p<0.05 vs. Tat- mice; N=3 individual experiments, t-test)
Figure 4.2 Expression of CaMKIIβ on OLs in vitro. Immunostaining showed that CaMKIIβ was expressed on both immature and mature OLs, and mainly localized in cytoplasm and processes (Scale bar = 10 µm)
Figure 4.3 Tat induced CaMKIIβ activation in both immature and mature OLs. (A)
Western blot results indicated that expression of CaMKIIβ in immature OLs is significantly lower than in mature OLs in vitro (*: p<0.05 vs. immature OL), and is not affected by Tat treatment. (B) CaMKIIβ activity was significantly upregulated in both immature and mature OLs after 1 h Tat treatment (*: p<0.05 vs. vehicle treatment)
Figure 4.4 Effects of blocking iGluRs on Tat-induced CaMKIIβ activation. Immature and mature OLs were treated with MK801 or CNQX with or without Tat for 30 min. 100 nM Tat induced a 2-fold increase of CaMKIIβ T287 phosphorylation when compared with vehicle treated OLs. MK801 completely reversed the CaMKIIβ activation in both immature and mature OLs, while CNQX only showed a partial effect. (N=4, values are presented as mean ± SEM; *p<0.05 vs. control; #p<0.05 vs. Tat treated group; one-way ANOVA)
### Table 4.1 Efficiency of CaMKII primer sets.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>CaMKIIα</th>
<th>CaMKIIβ</th>
<th>CaMKIIγ</th>
<th>CaMKIIδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency</td>
<td>1.98</td>
<td>1.92</td>
<td>1.98</td>
<td>2.00</td>
</tr>
<tr>
<td>Y-intercept</td>
<td>19.6</td>
<td>17.14</td>
<td>17.19</td>
<td>22.26</td>
</tr>
</tbody>
</table>
Chapter 5 Tat reduces immature OL viability through GSK3β activation

(This chapter, in part, was in preparation as a manuscript, entitled HIV-1 Tat affects oligodendrocyte survival via GSK3β activation, to be submitted to the Journal of Neuroscience)

Introduction

Our previous studies showed that OLs are direct targets of HIV-1 Tat. Interactions between Tat and NMDA-Rs and AMPA/KA-Rs elicit a dose-dependent increase of [Ca$^{2+}$]i in OLs, which leads to increased CaMKIIβ activation and reduced cell survival in the immature OL population (chapter 3; (Zou et al., 2015)). We thus investigated other signaling pathways that are activated by increased OL [Ca$^{2+}$]i and involved in cell death. Since HIV-1/Tat toxicity in neurons has been shown to be mediated by abnormal GSK3β activation (Maggirwar et al., 1999; Masvekar et al., 2015), we hypothesized the existence of similar mechanisms in OLs.

GSK3 was first identified as a phosphorylating and an inactivating agent of glycogen synthase. Two isoforms of GSK3, encoded by two separated genes, GSK3α and GSK3β, were originally identified in rat (Woodgett, 1990). Later studies by Mukai et al., (2002) found that in the CNS there exists an alternative splice variant of GSK3β, GSK3β2. The expression of GSK3β2 is neuron-specific and developmentally regulated. Although the exact function of GSK3β2 remains to be determined, silencing GSK3β2 using siRNA inhibits axon growth in rat cortical neurons, suggesting that it may play a
role in neuron morphogenesis (Castano et al., 2010). In contrast, the ubiquitously expressed isoform GSK3β1 has been shown to mediate multiple signaling pathways, including the conserved Wnt/β-Catenin pathway that regulates cell fate decisions during development.

Different from most kinases, basal activity of GSK3 is relatively high in resting cells. Multiple signaling molecules have been shown to regulate activity of GSK3. For example, the PI3K/Akt signaling pathway has been reported to phosphorylate the N-term serine residue of GSK3 (Ser9 in GSK3β and Ser21 in GSK3α) and inhibit GSK3β activity (Cross et al., 1995). The p38 mitogen-activated protein kinase (p38MAPK) has been shown to phosphorylate the C-term serine residue (Ser 389) of GSK3β, which also leads to its inactivation (Thornton et al., 2008). Besides inhibitory phosphorylation, activity of GSK3 is also down-regulated when it is bound to other proteins such as disrupted in schizophrenia 1 (DISC1) (Mao et al., 2009) and the partitioning defective homologue (PAR) complex (Schlessinger et al., 2007). On the other hand, PYK2 phosphorylates the tyrosine residue of GSK3 (Tyr279 in GSK3α and Tyr216 in GSK3β), which increases its substrate accessibility and promotes its activity (Hartigan et al., 2001). Importantly, studies by Hartigan et al., (1999) reported that increased [Ca^{2+}]_i results in up-regulated GSK3 activity, implicating that Tat-induced [Ca^{2+}]_i increase in OLs may lead to GSK3 activation.

Activity of GSK3 has been shown to mediate physiological and pathological changes in oligodendroglial lineage cells. For example, decreased GSK3 activity leads to β-catenin nuclear translocation, which affects OL differentiation and maturation (Fancy et al., 2009; Ye et al., 2009). \textit{In vivo} studies using a range of GSK3β inhibitors
also showed that blocking GSK3β activity increased the proliferation and survival of OPCs (Azim and Butt, 2011). The same study further found that besides the pro-survival effects on OPCs, GSK3β inhibition increased CREB activity, which up-regulates OL differentiation and myelination. Thus, GSK3β appear to be a good candidate for mediating Tat-induced OL injuries.

Our studies showed that Tat expression in vivo leads to increased activity of GSK3β in multiple brain regions. In vitro, Tat upregulates GSK3β activity and decreases cell survival in immature, but not mature OLs, which can be rescued by GSK3β inhibitors VPA or SB415286. In addition, we found that expression of GSK3β and CaMKIIβ in OLs was developmentally regulated; immature OLs expressing more GSK3β but less CaMKIIβ than mature OLs. The Tat-induced elevation of GSK3β and CaMKIIβ activities in OLs can be blocked by iGluRs antagonists MK801 and CNQX. Further, pharmacologically inhibiting CaMKIIβ activity increases GSK3β activity in both immature and mature OLs. Together, our results suggest that the effects of Tat on OL viability were mediated through GSK3β activity, and point to CaMKIIβ-GSK3β signaling as a potential therapeutic target for OL injuries in HAND patients.
Materials and Methods

Oligodendroglial cultures

Immature and mature OL cultures were performed as described in chapter 3. For Western blot detection of GSK3β and phosphor-GSK3β-S9, O2A/glial progenitor cells harvested from 8-day primary mixed culture were plated in a density of approximated 500,000 cells/dish on poly-L-lysine coated 35-mm dishes. Progenitors were fed with differentiation medium [(DMEM supplied with CNTF (10 ng/ml), NAC (5 µg/ml) and triiodothyronine (15 nM)] for 2 days or 7 days to reach immature and mature stages, respectively. Medium was changed every other day. For repeated measure experiments, plating density of progenitors was the same as described in chapter 3.

Viral protein and drug treatments

OLs were treated with HIV-1 Tat1-86 (1—100 nM), VPA (0.01–1 mM, Sigma), SB415286 (0.01–0.05 mM, Sigma), KN-92 (10 µM, Tocris), KN-93 (10 µM, Tocris), MK801 (20 µM) and CNQX (20 µM), after 2 days in enriched culture. All inhibitors were added 30 min before Tat if treated concurrently. Concentrations of drugs were chosen as previously reported ((Salter and Fern, 2005; Waggener et al., 2013; Masvekar et al., 2015)).

Induction of Tat expression in vivo

The same Tat-transgenic mice as described in previous chapters (Chapter 2) were used here. Three-month old mice (4 Tat⁻ and 4 Tat⁺) were fed with Dox-containing chow ad libitum for 10 days to induce Tat expression. Different regions of the brain,
including the frontal cortex, cerebellum, striatum, hippocampus, and midbrain, were dissected on ice and stored in -80°C before proteins were extracted for western blotting.

**Time-lapse analysis**

Time-lapse analysis was performed as described in chapter 3. In brief, mature and immature OLs were cultured in 12-well plates and treated with Tat and/or GSK3β inhibitors. Plates were then transferred to the Zeiss Axio Observer Z1 system, and placed in the environmental controlled chamber that maintains 37°C temperature and supplied with 5% CO2. For each treatment group, ≥50 OLs were randomly picked and imaged hourly for 96 h. Data were presented as mean percentage of survival ± standard error of the mean (SEM) from at least 4 individual experiments. Two-way ANOVA and post hoc Bonferroni's test was used to compare whether differences between treatments were significant (p<0.05).

**Live-Dead Assay**

Survival/death of OLs was confirmed using LIVE/DEAD Viability/Cytotoxicity Kit (Life Technologies) as previously described (chapter 3). In short, 96 h Tat- or vehicle-treated OL cultures were washed once with D-PBS and immersed in a working solution containing 4 µM EthD-1 and 2 µM Calcein-AM for 30 min at room temperature, before mounted on a clean, Fisherbrand Superfrost Plus Gold microscope slide (Fisher), and sealed with clear fingernail polish. For each individual treated group, 20 images were taken randomly using a Zeiss Axio Observer Z1 microscope with Zen 2012 software (Carl Zeiss). Live/dead cells were defined by green (Calcein-AM, Em ~515 nm) or red
(EthD-1, Em ~617 nm) fluorescence, respectively, and quantified by the number of green/red fluorescent pixels using ImageJ software (National Institutes of Health).

**Western Blot**

Western blot procedures are performed as previously described (Chapter 2). Primary antibodies specific to phospho-GSK-3β-Ser9 or total GSK3β (Cell Signaling Technology), phosphor-CaMKIIβ (Abcam, 1:1000) and total CaMKIIβ (Life Technologies, 1:1000) were used at 1:1000 as suggested by manufacturer. Bound antibodies were detected by appropriate IRDye secondary antibodies (1:3000, Li-COR, Lincoln, NE), and imaged using an Odyssey Imager (Li-COR). Protein bands were selected and quantified using Li-COR image studio software. The anti-phospho-GSK3β antibody also has weak affinity to phospho-GSK3α (Ser21). However, phospho-GSK3β-S9 (46kDa) can be easily distinguished from phospho-GSK3α-S21 (51kDa) due to size differences.
Results

Effects of Tat on OL survival and GSK3β expression.

We first examined whether adding HIV-1 Tat to culture medium affects the OL viability. The viability of D7, mature OLs was not affected by Tat treatment (Fig. 5.1A). In comparison, the viability of D2, immature OLs exposed to Tat decreased in a dose-dependent manner. Approximately 65% OLs treated with vehicle or 1 nM Tat survived after 96 h. This rate dropped to ~55% with 10 nM Tat and further decreased to ~40% with 100 nM Tat. The effects of 100 nM Tat on immature OL survival reach statistical significance by 24 h, while 10 nM Tat effects become significant at ~ around 60 h (Fig. 5.1A). A live/dead assay was performed on cells after these experiments (at 96 h) to verify the counting results. The Tat (100 nM) treated group exhibited significantly more red fluorescent (EthD-1+) and less green fluorescent (Calcein-AM+) cells when compared to the control group, indicating less cell survival when exposed to Tat (Fig. 5.1B). GSK3β signaling pathways have been recently shown to play an important role in Tat-mediated neurotoxicity and HIV replication (Kehn-Hall et al., 2011; Zhou et al., 2013; Guendel et al., 2014). We thus examined whether Tat affects GSK3β expression. We did not detect any difference in GSK3β expression between Tat- and Tat+ mice in cortex, cerebellum, striatum, or hippocampus (Fig. 5.1C). In vitro experiments using pure OL culture showed that expression of total GSK3β in immature OLs was significantly higher than in mature OLs (Fig. 5.1D), and these levels were not affected by Tat treatment (Fig. 5.1E).

Inhibiting GSK3β activation rescues Tat-induced immature OLs death in vitro.
Since Tat did not affect GSK3β expression, we next investigated whether Tat affects GSK3β activity, by examining the level of GSK3β-S9 inhibitory phosphorylation (Hur and Zhou, 2010). In vivo, expression of Tat leads to significantly decreased ratio of phosphorylated GSK3β-S9 over total GSK3β (p-GSK3β/t-GSK3β) in cortex, striatum and hippocampus, indicating upregulated GSK3β activity (Fig. 5.2A). The GSK3β activity in the cerebellum was not affected. To further elucidate whether Tat regulates OL GSK3β activity, cultured OLs were treated with Tat for 24 h. In immature OLs, 10 or 100 nM Tat leads to significantly increased GSK3β activity when compared with vehicle or 1nM Tat treated groups (Fig. 5.2B). The upregulation of GSK3β activity by Tat (100 nM) can be detected as early as 2h, and persists over 96 h (Fig. 5.2C). In comparison, GSK3β activity was not affected by 1 – 100 nM Tat in mature OLs (Fig. 5.2B).

We further investigated whether blocking GSK3β can reverse the effect of Tat on immature OL survival. VPA, a drug that inhibits GSK3β through activation of its upstream WNT signaling pathway (Hall et al., 2002), and SB415286, a small molecule that competes with ATP on binding GSK3β (Liang and Chuang, 2007) were picked to block GSK3β activities in OLs. Efficacy studies showed that 0.1 - 1 mM VPA or 0.01 - 0.05 mM SB415286 are enough to block GSK3β activities in immature OLs (Fig. 5.2D). When VPA (100 µM) or SB415286 (10 µM) were added to immature OLs together with Tat (100 nM), both drugs completely reversed the increased GSK3β activity (Fig. 5.2D), and immature OL death induced by Tat (Fig. 5.2E).
Tat-induced CaMKIIβ activation inhibits GSK3β activity and cell death in mature OLs

Activities of CaMKIIβ and GSK3β have been known to be regulated by iGluRs-mediated increase of intracellular calcium (Bayer et al., 2001; Hudmon and Schulman, 2002; De Montigny et al., 2013). Since the Tat-induced [Ca2+]i change and cell death in immature OL can be blocked by N-methyl-D-aspartate receptor (NMDA-R) antagonist MK801 or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor (AMPA/KA-R) antagonist CNQX (Zou et al., 2015), we assessed the effects of MK801/CNQX on CaMKIIβ and GSK3β activities in Tat-treated OLs. In both immature and mature OLs, MK801 completely reversed Tat-induced increase of CaMKIIβ activity, while CNQX only partially blocks the upregulated CaMKIIβ activity (Fig. 5.3A). The upregulated GSK3β activities in immature OLs were also completely abolished when MK801 or CNQX were added concurrently with Tat (Fig. 5.3B). Further, CaMKIIβ inhibitor KN93 and its inactive derivative KN92 were added together with Tat to test the effects of CaMKIIβ inhibition on Tat-induced GSK3β activation. At both 2 h and 6 h, adding KN93 and Tat together significantly increased GSK3β activity in mature OLs. In immature OLs, the Tat-induced upregulation of GSK3β activity was further enhanced by KN93 (Fig. 5.3C).
Discussion

Previously, we reported that Tat induces increased \([\text{Ca}^{2+}]_i\) and Ca\(^{2+}\)/CaM dependent kinase II\(\beta\) (CaMKII\(\beta\)) activity in both immature and mature OLs, but only decreases the cell viability in immature OL population. It is feasible that there may exist a causal relationship between the Tat-induced \([\text{Ca}^{2+}]_i\) increase and immature OL death because 1) both effects are Tat dose-dependent, with increased cell death only occurring when a significant \([\text{Ca}^{2+}]_i\) increase was induced by high Tat concentration; and 2) Tat-induced cell death can be rescued by attenuating \([\text{Ca}^{2+}]_i\) increase (Zou et al., 2015). It has been shown that the neurotoxicity of Tat can be rescued by inhibiting GSK3\(\beta\) activity (Chao et al., 2014; Masvekar et al., 2015). Since the activities of GSK3\(\beta\) are upregulated by iGluR-mediated \([\text{Ca}^{2+}]_i\) increase (Hartigan and Johnson, 1999), and function as a negative regulator of OL proliferation and myelination (Azim and Butt, 2011), we hypothesized that GSK3\(\beta\) is the downstream signaling molecule that mediates the different effects of Tat on immature and mature OL viability.

Dysregulation of GSK3\(\beta\) activity has been reported to be involved in multiple neurodegenerative processes, including HAND (Schifitto et al., 2006; Crews et al., 2009; Jacobs et al., 2012). Interestingly, our results showed that GSK3\(\beta\) activity was upregulated in cortex, hippocampus and striatum, but not cerebellum, when Tat expression was induced \textit{in vivo} for 10 days (Fig. 5.2A). Cerebellar astrocytes exposed to Tat secrets significantly less cytokines/chemokines than astrocytes from cortex or spinal cord (Fitting et al., 2010), in accordance with the fact that HIV-related neuropathology in cerebellum was rarely reported. Since GSK3\(\beta\) activation was regulated by an array of extracellular signals, Tat-induced inflammatory responses in
cerebellum may be less potent than other brain regions to stimulate GSK3β activation. Alternatively, cerebellum may have a specific mechanism that inhibits GSK3β activation. An example of such mechanisms has been described in cerebellar granule neurons (CGNs), where depolarization-coupled CaMKII activation inhibits the GSK3β activity and promotes neuronal survival (Song et al., 2010). In support of this idea, our experiments also found that the activity of CaMKIIβ, the predominant isoform of CaMKII in both OLs and CGNs (Burgin et al., 1990; Waggener et al., 2013), was significantly upregulated by Tat expression in the cerebellum (Fig. 4.1).

GSK3β is ubiquitously expressed in most CNS cell types, we thus used highly purified cell cultures to assess whether Tat regulates GSK3β activities in OLs. Our results showed that Tat causes a dose-dependent GSK3β activation in immature, but not mature, OLs, parallel with the findings that the viability of mature OLs was not affected by Tat (Fig. 5.1A). MK801 or CNQX, which attenuates Tat-induced $[\text{Ca}^{2+}]_i$ increase and rescues Tat-induced immature OL death (Zou et al., 2015), reversed OL GSK3β activation by Tat (Fig. 5.3B), consistent with the idea that activation of GSK3β can be caused by iGluR-mediated $[\text{Ca}^{2+}]_i$ increase (Hartigan and Johnson, 1999). Further, VPA and SB415286, two GSK3β inhibitors that block GSK3β activity with different mechanisms, both reversed Tat-induced immature OL death (Fig. 5.2E). Together, these data strongly suggest a Tat-induced death signaling mechanism where interactions between Tat and iGluRs lead to $[\text{Ca}^{2+}]_i$ increase and GSK3β activation in immature OLs, which causes cell death. Studies by other groups have reported that GSK3β inhibition prevents mitochondrial *cytochrome c* release and apoptosis (Maurer et
al., 2006; Ngok-Ngam et al., 2013), implying mitochondria dysfunction as the next downstream death signaling after OL GSK3β activation. Theoretically, VPA and SB415286 also inhibit GSK3α. However, our experiments showed that Tat, VPA or SB415286 had no effect GSK3α activity in OLs (Data not shown).

It is intriguing that GSK3β was not activated in mature OLs, since Tat also elevates [Ca^{2+}] in mature OLs (Zou et al., 2015). Cellular calcium signaling is affected by many factors such as amplitude and frequency of [Ca^{2+}] change, source of Ca^{2+}, and availability of different Ca^{2+} binding proteins and downstream components (Mattson et al., 1988; Quinlan and Halpain, 1996). For example, mature OLs express higher levels of NMDA-Rs than immature OLs (Salter and Fern, 2005; Micu et al., 2006; De Biase et al., 2010). Tat-induced NMDA-R activation (Haughey et al., 2001; Deng et al., 2003; Kim et al., 2008) may lead to downregulated GSK3β activity in mature OLs, since NMDA-R-mediated GSK3β inhibition has been reported (De Montigny et al., 2013). Several studies revealed that CaMKII is efficiently activated following NMDA-R-activation, possibly due to its interaction with the NR2B subunit, which brings it close to the NMDA-R channel (Bayer et al., 2006; Lee et al., 2009). Our previous study also showed that Tat activates CaMKIIβ, the predominant isoform of CaMKII, in OLs (Zou et al., 2015), and inhibition of GSK3β by CaMKIIβ has also been reported (Song et al., 2010). The results from this study revealed that mature OLs express significantly more CaMKIIβ and less GSK3β than immature OLs (Fig. 4.3A), and inhibiting CaMKIIβ promotes Tat-induced GSK3β activation (Fig. 5.4C). Collectively, these data strongly suggest that the different level of GSK3β activity in immature and mature OLs may
attribute to their distinct level of inhibition by CaMKIIβ.

In vivo, elevated CaMKIIβ activity was also observed in the cortex, striatum and hippocampus (Fig. 4.1B), hence, it is also intriguing that GSK3β activity was still upregulated in these regions (Fig. 5.2A). Compared to CaMKIIβ, expression levels of GSK3β are significantly higher in astrocytes (~20×), immature OLs (~6×) and endothelial cells (~15×) (Zhang et al., 2014). Our results also showed that Tat did not alter the total expression levels of GSK3β or CaMKIIβ in these regions (Fig. 5.1C, 4.1A). Activated GSK3β in those cell types may not be inhibited by CaMKIIβ, which could result in increased overall GSK3β activity.

WM injuries, such as decreased WM volume (Sarma et al., 2014), increased occurrence of abnormal axonal tracts (Gongvatana et al., 2009), and degenerated OLs (Gyorkey et al., 1987; Jayadev et al., 2007), contribute to HAND pathogenesis in HIV+ patients. Our current findings suggest that upregulated GSK3β activity is essential to Tat-induced immature OL death. Hence, interfering with GSK3β activity, by modulating NMDA-R and CaMKIIβ activation, might be a potential adjunctive therapy to relieve HAND in HIV patients.
**Figure 5.1.** Effects of Tat on OL survival and GSK3β expression. (A) Tat treatment leads to a dose-dependent decrease of immature, but not mature, OL survival (*,##: p<0.05 vs. control or 100 nM Tat, respectively, at the same time point.). (B) Results from (A) were confirmed by Live Dead assay using Calcein-AM (Live cell, green) and EthD-1 (Dead cell, red). Sample images were chosen from vehicle and 100 nM Tat treated immature OL groups and stained at 96 h (Scale bar: 10 µm). (C) In vivo, expression of Tat has no effect on total GSK3β level in any of the regions examined. (D-E) In vitro, expression of total GSK3β in immature OLs were significantly (~3 folds) higher than in mature OLs (D), and were not affected by Tat treatment within 96 h (E).
Figure 5.2 Inhibiting GSK3β activity rescues Tat-induced immature OLs death. (A) In vivo, Tat expression leads to upregulated activity of GSK3β (decreased ratio of p-GSK3β-S9/t-GSK3β) in cortex, striatum and hippocampus, but not cerebellum (*: p<0.05 vs. corresponding Tat- control). (B-C) Western blots showed that Tat induces a dose-dependent upregulation of GSK3β activity in immature, but not mature OLs (B), and the upregulated GSK3β activity by 100 nM Tat in immature OLs persists over 96 h (C) (*: p<0.05 vs. corresponding control; #: p<0.05 vs. 10 nM Tat). (D) VPA (0.1 – 10 mM) or SB415286 (0.01 - 0.05 mM) downregulates GSK3β activity in immature OLs, and reverses upregulated GSK3β activity by Tat (*,#: p<0.05 vs. Control). (E) Either VPA (100 µM) or SB415286 (10 µM) rescues increased immature OL death induced by 100 nM Tat, while neither VPA nor SB415286 alone affects immature OL viability (*: p<0.05 vs. all other groups at the same time point).
Figure 5.3

**Figure 5.3 CaMKIIβ inhibits GSK3β activation in Tat-treated OLs.** (A) Tat-induced upregulation of CaMKIIβ activity can be completely inhibited by MK801, and partially reversed by CNQX (*: p<0.05 vs. Control; #: p<0.05 vs. 100 nM Tat). (B) Either MK801 or CNQX fully reverses Tat-induced upregulation of GSK3β activity (*: p<0.05 vs. Control). (C) In vitro, inhibiting CaMKIIβ by KN93 for 2 h or 6 h significantly promotes Tat-induced GSK3β activation (*: p<0.05, vs. Vehicle; #: p<0.05 vs. Tat).
Chapter 6 Using co-culture or human brain aggregate model to study OL injury by HIV-1/Tat

Introduction

In vivo, OLs do not exist as an isolated population. Rather, they forms a complex network with all the other major cell types in the CNS. Signaling between OLs and neurons, astrocytes and microglia have been frequently reported (Bastmeyer et al., 1991; Schwab and Schnell, 1991). To further assess whether injury mechanisms we observed in vitro faithfully reflect HIV pathogenesis in vivo, we used two models: a co-culture model where OLs are cultured on top of a mixed glial layer and a brain aggregate model where dissociated progenitors acquired from mice or human fetal tissues were supplied with serum and allowed to aggregate spontaneously.

Co-cultures of neurons and mixed glia have been used to study the synergistic toxic effects of HIV-1 Tat and morphine on neurons (Zou et al., 2011b). In this model, a confluent layer of mixed primary glial cells, consisting of mainly astroglia with a small percentage of microglia, were cultured initially. Neuronal progenitor cells were then plated on the bed layer of glial cells and induced to differentiate into mature neurons in defined medium. Treatments of drugs or viral proteins were added in the medium, and affected both neurons and the glial bed layer. It has been shown that glial cells treated with Tat produce inflammatory cytokines and reactive oxygen species, both of which are
toxic factors to OLs (El-Hage et al., 2006; Eugenin et al., 2007; Zou et al., 2011b). On the
other hand, the glutamate transporter on astrocytes may take up excessive extracellular glutamate released by injured cells, thereby preventing the spread of excitotoxic injury (Danbolt, 2001; Beart and O'Shea, 2007). Similarly, microglia may be protective to OLs by endocytosing cellular components, fragmented nucleotides and degraded peptides released from dying/dead cells. In the OL-Glia culture, instead of neuronal progenitor cells, we seeded OPCs on top of the glial bed layer and cultured the cells in OL differentiation medium. We used this model to answer the question of whether other glial cells played a role in Tat-induced OL injury.

HIV is a human disease, thus, we also used a hBrnAgg model (Pulliam et al., 1988) with live HIV-1 virus to assess whether the OL injury we observed in mice may happen in human. Different from dissociated culture, which usually advances the enrichment of certain cell types by a defined set of growth factors or metabolic inhibitors, hBrnAggs are formed by the spontaneous aggregation of primary CNS cells acquired from dissociated fetal brain tissues. Early studies have shown that the hBrnAggs are viable for 60 days in culture and contains approximately 10% OLs, ≥40% neurons, and ≥40% astrocytes after 10 days in culture. Importantly, myelin sheaths were observed in the hBrnAggs using EM (Pulliam et al., 1988), suggesting that it preserves the histological diversity of the brain. Hence we surmised that the hBrnAggs could be used to study the effects of HIV on human CNS neuropathologies.
Methods and materials

All the experiments involving live HIV-1 were performed in BSL-2+ viral facility.

OL-Glia co-culture

To establish OL-glia cultures, mixed glial cultures were prepared first as previously described in Chapter 3. In brief, dissociated primary glial cells acquired from P0-P2 ICR pups are plated in poly-L-lysine coated, 12-well plate at a density of 200,000 cells per well and allowed to grow in DMEM supplied with 10% fetal bovine serum for 6 days. After 6 days, OPCs harvested from 8-day primary cultures were seeded on the bed layer of glial cells with a density of 20,000 cells/well and the mixed culture was incubated in OL differentiation medium containing T3, NAC and insulin for another 3 days to allow OPCs to differentiate into immature OLs.

hBrnAgg culture

Brain tissue (17-wk in age) received from Advanced Bioscience Resources, Inc (ABR, Alameda, CA) were mechanically dissociated and gently pushed through two nylon mesh pockets with pore sizes of 250 µm and 135 µm, respectively. Cells were then washed twice with Rinse Medium (DMEM + 12 g/L glucose, 2.5 mg/L fungizone and 50 mg/L gentamicin). A 4 ml cell suspension with a density of $1 \times 10^7$ cells/ml was added to a 25-ml DeLong flask (Corning) and placed on an orbital shaker with a constant rotation speed of 78 rpm in a culture incubator ($37^\circ$C, 10% CO$_2$). The next day, 1 ml Exchange Medium (Rinse Medium + 15% FBS) was added to the flask. After 3 d, the BrnAggs were transferred to 50-ml DeLong flasks; 5 ml of Exchange Medium was
added for a total of 10 ml. Medium was exchanged every 3 d for as long as the culture was maintained.

**PBMCs isolation and infection**

PBMCs were isolated from Leukopak (HemaCare Corporation, Van Nuys, CA) using Lymphocyte separation medium (LSM) (MP biomedicals, LLC, Santa Ana, CA). In short, leukopak were diluted with PBS-EDTA in a 1:1 ratio before loaded on top of the LSM layer (30 ml diluted blood per 12.5 ml LSM) in a 50 ml tube and centrifuged at 2000 rpm for 30 min at room temperature. Cells were then collected and re-suspended in PBS-EDTA (Miltenyi Biotec Inc. San Diego, CA), and treated with ACK lysis buffer (Life technologies) for 5 min at room temperature before culture in a T150 flask (Corning) in 20 ml HMDM medium [RPMI1640 medium supplied with L-Glutamine (1%), fetal bovine serum (10%) and penicillin/streptomycin (1%)] overnight. On the next day, cells were collected and re-plated in T75 flasks at a density of 40 million cells per 20 ml HMDM per flask. Phytohemagglutinin (PHA-M) was added in the medium to a final concentration of 5 µg/mL to activate the PBMCs. After activation, 1 ng HIV-1 p24/ml HIV-1Ba-L virus, measured by HIV-1 p24 ELISA (ABL Inc., Rockville, MD), was added to the culture medium to infect the PBMCs for 3 days.

**Treat hBrnAgg with HIV-1 (R5) or supernatant from HIV-infected PBMCs**

Human BrnAgg were transferred to a 24-well plate and suspended in DMEM supplemented with 15% fetal bovine serum overnight. The next day, pure HIV-1Ba-L was added to the cells at a final concentration of 1 ng/ml, 10 ng/ml and 100 ng/ml p24. Equal
amount of UV-inactivated HIV-1$_{\text{Ba-L}}$ were used as a negative control. UV-inactivation of HIV-1 was performed by exposing the virus to UV light (3 ml/10 cm$^2$ dish) for 4 min at 999 mJ/cm$^2$ using a Spectroline UV Crosslinker XL-1000 (Spectronics).

Three days after PBMCs were infected by HIV-1$_{\text{Ba-L}}$, supernatant was collected and passed through a 0.2-µm filter and stored at -80°C. To treat hBrnAggs, an aliquot of the supernatant containing 6.77 ng/ml p24 was used. Supernatant collected from non-infected PBMCs were used as negative controls.
Results

Characterization of hBrnAgg

After 10 days in culture, healthy hBrnAggs appeared as ~1 mm diameter spheres with smooth edges. The majority of the inner cells of the hBrnAggs are not stained by trypan blue, indicating that they are viable (Fig. 6.1A). To further confirm the cell viability, 10-day old hBrnAggs were transferred to a laminin-coated tissue culture dish and supplied with DMEM + 15% serum. The aggregates adhered to the bottom of the dish within 2 hrs. After 2 days, cells already grew out of the edge of the adherent hBrnAggs and can be easily observed using light microscope with a 10x objective (Fig. 6.1B). Immunostaining using antibodies specific to MAP2, MBP or GFAP showed that neurons, OLs and astrocytes were all present in the outgrowing cultures, with GFAP+ astrocytes dominating the population (Fig. 6.2). EM examination of hBrnAggs at 30 days reveals the subcellular structures inside these cell aggregates. Normal-looking mitochondria, and large amount of ribosomes are frequently found (Fig. 6.3 A), indicating active cellular activities and protein translation in these cells. Synaptic structures consisting of vesicle-clustering pre-synaptic terminals and post-synaptic densities were also found in the aggregates (Fig. 6.3 B). Although myelin structures in this model have been described by other investigators (Pulliam et al., 1988), we did not find typical, compact myelin structures with periodicity of major dense lines and intraperiod lines in our cultures. Western blot did not detect the existence of MBP in the hBrnAggs either (Data not shown). However, loosely wrapped membrane structures were observed (Fig. 6.3 D&E). We also noticed groups of dark-pigmented structures in
these aggregates that possibly originate from endocytosed dead cells or cell debris (Fig. 6.3 C).

**hBrnAgg was not infected by HIV$_{Ba-L}$**

In an effort to investigate whether HIV infection leads to human oligodendroglial lineage cell injury, we first tried to infect hBrnAggs with HIV$_{Ba-L}$. One-month old hBrnAggs were cultured in regular DMEM supplemented with 15% FBS and treated with HIV stock containing 1, 10 or 100 ng/ml p24, measured by ELISA. Culture medium was changed once at 3rd day and HIV stock was replenished. After 7 days, supernatant of the culture was collected and p24 level was measured with ELISA. We did not detect any p24 in the supernatant of control- or HIV$_{Ba-L}$-treated hBrnAggs (Data not shown).

**High level of HIV$_{Ba-L}$ activates GSK3β, but not CaMKIIβ in hBrnAgg**

We next examined whether GSK3β activity was affected when hBrnAggs were exposed to HIV$_{Ba-L}$. As Fig. 6.4 shows, hBrnAggs treated with HIV$_{Ba-L}$ stock containing 100 ng/ml p24 showed a significantly increased GSK3β activity when compared with control. When hBrnAggs were exposed to HIV$_{Ba-L}$ with lower p24 levels (1 or 10 ng/ml), GSK3β activity was not significantly affected. On the other hand, CaMKIIβ activity was not affected by HIV$_{Ba-L}$ (Data not shown).

**Repeated measure using OL-Glia co-culture**

Early studies from the lab showed that glia exacerbate the toxic effect of Tat on neurons in vitro. We thus used an OL-glia co-culture system to study whether glial cells also played a role in OL injuries by Tat. In the co-culture model, OPCs were seeded on
top of a confluent primary glial bed layer and fed with OL differentiation medium. Similar to the pure OL culture, in ~2 days, most of the OPCs differentiate into immature OLs with multiple processes and minimal of production of myelin-like membranes. However, it seems that culturing on top of the glia bed layer accelerated the maturation process of OLs. At day 5, most of the OLs showed mature morphology with plenty of membrane productions (Fig. 6.4). To characterize the percentage of different cell types in the culture, immunostaining using antibodies specific to major CNS cell surface markers was performed. We found that the majority of cells in the co-culture are O4+ oligodendroglial cells (9 - 11%) or GFAP+ astrocytes (85 – 90%). Only 1-2% of all cells are MAP2+ neurons or Iba+ positive microglia (Table 6.1).

We next investigated whether HIV-1 Tat had similar effects on OL viability with glial cells present. Co-cultures at Day 2 (immature OLs) or Day 5 (mature OLs) were treated with vehicle or Tat and individual cells were followed for 96 h. Presence of glial cells greatly reduced the toxicity of HIV-1 Tat on immature OL viability. Although 100 nM Tat still leads to significantly more OL death between 72 and 96 h, 1 – 10 nM Tat had no impact on immature OL survival when compared to control. Viability of mature OLs is not affected by Tat treatment (Fig. 6.5). As a matter of fact, all the mature OLs we tracked survived the 96 h experiment, vehicle-treated or Tat-treated.
Discussion

Our works using high-purity, dissociated mice OL culture had shed some lights on how HIV-1 Tat injures OLs. However, there are two limitations that restrict the direct link between our findings and the CNS pathogenesis of HIV infection in human. First, HIV is a human disease, thus, it is important to understand that cellular responses to HIV/viral proteins may diverge significantly between species. For example, mice are resistant to HIV infection because the mice CD4 receptor does not bind to HIV envelope protein gp120, even though its sequence and structure are similar to human CD4 receptors (Littman, 1987; Littman and Gettner, 1987; Landau et al., 1988). Studies by Seok et al., (2013) also demonstrated that there exist significant differences between mice and human in genomic responses to human inflammatory diseases. Second, cell cultures used in our experiments are highly purified and lacking the complex cellular composition and architecture of human CNS. Although the purpose of the study was to investigate whether Tat possesses direct toxicities to OLs, isolating OLs from other CNS cells may have an impact on its gene expression and physiological properties.

Ideally, the hBrnAgg model would address both issues: it consists of primary human cells and contains all major CNS cell types. The original publication reported that there are ≥40% GFAP⁺, ≥40% NSE⁺, ~10% β-gal⁺ and ~10% MBP⁺ cells in the hBrnAggs after 10 days in culturing medium (Pulliam et al., 1988). We adopted the same procedures and used the same reagents as Pulliam et al., (1988) used to reproduce the hBrnAggs culture. The majority of the cells in our hBrnAggs are not permeable to trypan blue after 10 days (Fig. 6.1), suggesting that the hBrnAggs are formed mostly by live cells. Immunostaining and EM were performed on these
aggregates to verify the existence of different cell types and subcellular structures (Fig. 6.2 & 6.3). At odds with Pulliam et al., (1988), although MBP\(^+\) cells were found in the culture, we did not observe normal-looking, compact myelin structure in the hBrnAggs. Instead, loosely wrapped membrane structures were seen under EM. There are several explanations for this. First, most of the primary human cells were differentiated at the time of tissue acquisition, and differentiated OLs have been shown to be more vulnerable to excitotoxicity than other cell types (Oka et al., 1993; Matute et al., 2007). While Pulliam et al., (1988) received their cells within 3 h of tissue procurement, it took averagely ~18 h for the cells to be delivered to us after tissue procurement. Hence, there may be much reduced amount of OLs at the time we started the culture. Second, it has also been reported that factors in culture may facilitate progenitor cells to take the astroglial fate (Raff et al., 1983). Consistent with this, when hBrnAgg were allowed to attach to the bottom of a laminin-coated dish and grow in the same medium as they were cultured in suspension for 2 days, ≥80% of cells were found to be GFAP\(^+\). Thus, there may be much less OLs and neurons in our hBrnAggs, which greatly decreased the probability of OLs locate close enough to an axon and receive the proper signal to initiate myelination.

Although normal myelin was not observed in our hBrnAggs, plenty of loosely wrapped membrane structures that mimic un-compact myelin sheath were found. In addition, positive MBP immunostaining on adherent hBrnAgg also indicates the existence of functional OLs that producing myelin protein. Thus, we tried to infect the hBrnAgg with HIV\(_{Ba-L}\) and investigate whether viral infection had any effect on OLs. We did not detect any p24 in the supernatant after 7 days of infection, indicating that the
hBrnAggs are not infected by HIV_{Ba-L}. This is not surprising because immunostaining using antibodies specific to ionized calcium binding adaptor-1 (IBA-1) did not detect microglia in the hBrnAggs. Although we cannot completely rule out the possibility that there may be a small amount of microglia existing in the aggregates, they may be buried in the aggregates and not accessible to the virus. Intriguingly, GSK3β activation was still observed in hBrnAggs treated with high concentrations of HIV_{Ba-L} (100 ng/ml p24). Taking into account that the hBrnAggs are not infected by HIV, GSK3β activation may be a result of interactions between cell surface molecule and the HIV envelope protein gp120, which has been shown to be a toxic factor to neurons (Podhaizer et al., 2012), and triggers astroglial inflammatory responses (Pulliam et al., 1993; Benos et al., 1994; Nath et al., 2000; Zou et al., 2011a). The gp120 effect has also been shown to be independent of the major HIV receptor CD4, suggesting that it could affect both astrocytes and neurons in the absence of microglia (Kaiser et al., 1990). Since GSK3β is universally expressed in all CNS cell types, and its activation by viral protein Tat has been reported in neurons and OLs (Maggirwar et al., 1999) we cannot pin down the activation of GSK3β to OLs. Future experiments using purified human OLs may answer the question whether HIV activates GSK3β in OLs.

As we mentioned earlier, using hBrnAgg culture as a model to study CNS diseases has several advantages. It preserves histological diversity of human CNS and is easy to be maintained for long experiment period (up to 60 days). Drug treatment or viral infection can be easily realized by addition to the culture medium. The reason why our experiments did not meet our expectation is likely the imbalance of cell components in our aggregates. One possible improvement to this model is to add human OPCs.
purchased from ScienCell (ScienCell, Carlsbad, CA) to dissociated brain cells before hBrnAggs forms. This may help increase the percentage of oligodendroglial lineage cells and facilitate myelination of axons in the aggregates. To further manipulate the system, human microglia, even HIV-infected microglia or PBMCs can also be added at the time of aggregation. Another way to improve this technique is to use fluorescence-activated cell sorting (FACS) to separate dissociated cells based on specific cell markers, and then use a fixed ratio of different cell types to form the hBrnAggs. In theory, hBrnAggs formed this way should be more reproducible.

We also used an OL-glia co-culture to answer the question whether the existence of glial cells have impact on Tat-induced OL injury. As Table 6.1 showed, the co-culture contains ~10% OLs, >85% astrocytes and 1-2% microglia. Since glial cells enhanced the toxic effects of Tat on neurons, we hypothesized that OL injury by Tat may also be exacerbated in OL-glia co-culture. Surprisingly, the existence of glial cells actually increased the viability of Tat-treated OLs. All the mature OLs treated with 100 nM Tat survived over 96 h experimental periods. For immature OLs, 1-10 nM Tat had no effect on cell viability, while 100 nM Tat significantly reduced OL survival only after 72 h. OL development and survival in vitro has been known to be dependent on various extracellular cues including cytokines/chemokines and neurotropic factors secreted from surrounding cells. It has been long known that astrocytes play an important role in maintaining environmental homeostasis and provide structural, metabolic and trophic support for other cells (Markiewicz and Lukomska, 2006). Recent studies by Pang et al., (2013) has shown that astrocyte-conditioned medium protects OPCs from apoptosis and significantly increases OL long-term survival. Similar protective role of astrocyte-
conditioned medium has also been reported to attenuate glutamate-induced neuron apoptosis (Lu et al., 2015). Mechanistic studies revealed that the metabotropic glutamate receptor 4 (mGlu4) expressed in astrocytes enhances OL viability by increasing TGFβ1 expression (Spampinato et al., 2014). Similar OL protection may also happen in Tat-induced excitotoxicity in OLs, where the presence of astrocyte leads to increased TGFβ1 level in the medium and enhanced OL survival. Since mGlu4s are activated under excitotoxic conditions, we did not find significant changes in OL viability when co-culture was treated with vehicle (Fig 4.1 vs. Fig 6.5).
Figure 6.1 Cultured hBrnAggs. (A) After 10 days in culture, cells are tightly packed and organized into a true sphere of ~1-mm diameter. Trypan blue (dark cytoplasm) staining showed that most cells in 10-day aggregates are viable. (B) 10-day old hBrnAgg was transferred into a laminin-coated dish and cultured for 2 days. The aggregate attached to the bottom of the dish and started to extend its colony. Outgrowth of cells around its edge are clearly visible. (Scale bar = 100 nm)
Figure 6.2 Immunostaining of adherent hBrnAggs. Sample images are taken at areas ~ 700 nm away from the center of the adherent aggregate. MAP2+ neurons, MBP+ OLs and GFAP+ astrocytes have all been found to be present in the culture, although GFAP+ astrocytes seem to be the dominant cell type. (Scale bar = 10 µm)
Figure 6.3 EM examination of 30-day old hBrnAgg. (A) Healthy looking cells were found everywhere, with typical healthy-looking mitochondria, and plenty of ribosomes indicating active cellular activities and protein translations in process. (B) Synaptic structures consist of both the vesicle-clustering pre-synaptic terminals and the post-synaptic density were also observed. (C) Groups of dark-pigmented structures, possibly formed by endocytosed cell debris or dead cells, were also observed. (D-E) Membrane structures resembling un-compact myelin are often found.
Table 6.1. Percentage of cells expressing neuron/glial specific markers at various times in OL-Glia co-culture

<table>
<thead>
<tr>
<th></th>
<th>O4+</th>
<th>GFAP+</th>
<th>MAP2+</th>
<th>Iba+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>11.64 ± 0.92%</td>
<td>85.69 ± 1.50%</td>
<td>0.99 ± 0.32%</td>
<td>1.68 ± 0.38%</td>
</tr>
<tr>
<td>(n=4, 1618 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 5</td>
<td>10.69 ± 0.71%</td>
<td>86.67 ± 0.87%</td>
<td>0.48 ± 0.20%</td>
<td>2.17 ± 0.23%</td>
</tr>
<tr>
<td>(n=4, 1891 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 8</td>
<td>8.98 ± 0.51%</td>
<td>89.53 ± 0.53%</td>
<td>0.30 ± 0.13%</td>
<td>1.20 ± 0.21%</td>
</tr>
<tr>
<td>(n=4, 1988 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n: number of individual cultures.
Figure 6.4 HIV<sub>Ba-L</sub> activates GSK3β in hBrnAggs. Thirty-day old hBrnAggs were treated with control or HIV<sub>Ba-L</sub> stock with different levels of p24 (1, 10 or 100 ng/ml). Western blot results showed that the highest titer of HIV<sub>Ba-L</sub> (100 ng/ml p24) leads to a significant increase of GSK3β activity when compared with control. Lower titers of HIV<sub>Ba-L</sub> had no effect on GSK3β activity in hBrnAggs (* p<0.05 vs. control; One-Way ANOVA with post-hoc bonferroni’s test, n=3).
Figure 6.5

<table>
<thead>
<tr>
<th>Phase</th>
<th>2 Day</th>
<th>5 Day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>O4</td>
<td><img src="image3.jpg" alt="Image" /></td>
<td><img src="image4.jpg" alt="Image" /></td>
</tr>
<tr>
<td>GFAP</td>
<td><img src="image5.jpg" alt="Image" /></td>
<td><img src="image6.jpg" alt="Image" /></td>
</tr>
<tr>
<td>Hoechst</td>
<td><img src="image7.jpg" alt="Image" /></td>
<td><img src="image8.jpg" alt="Image" /></td>
</tr>
<tr>
<td>Merge</td>
<td><img src="image9.jpg" alt="Image" /></td>
<td><img src="image10.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 6.5 OL-Glia co-culture. Immunostaining using antibodies specific to O4 and GFAP showed that OLs can be co-cultured on top of glial cell bed layer. Different from pure OL culture, OPCs grow on top of glial cells differentiate faster and show plenty of myelin-like membrane production after 5 days.
Figure 6.6 Effects of Tat on OL viability in OL-Glia co-culture. Day 2 or Day 5 OL-Glia co-culture were treated with vehicle or different concentration of HIV-1 Tat and OLs were tracked for 96 h. Mature OL viability were not affected by Tat treatment. For immature OLs, 1 nM or 10 nM Tat had no effect when compare to vehicle treatment. 100 nM Tat leads to significantly less cell survival, but only between 72 to 96 h. (*: p<0.05 vs. control, two-way ANOVA followed by post-hoc Bonferroni’s test, N=3)
Chapter 7 Conclusions, pitfalls and future studies

WM injury in HIV patients was first discovered almost at the same time when HIV was reported. However, the effects of HIV infection on the WM, as well as the mechanisms that underlie the injury of OL/myelin, have not yet been systematically assessed (Masters and Ances, 2014). Most of the existing studies on WM injuries in HIV are descriptive, using modern techniques such as functional magnetic resonance imaging (fMRI), diffusion tensor imaging (DTI) and magnetic resonance imaging (MRI). Other studies simply attribute these injuries to infection-related inflammatory responses. As a recent clinical study showed, various adjunctive anti-inflammatory drugs combined with cART does not improve neurocognitive scores of HIV patients when compared with cART alone (Tan and McArthur, 2012), suggesting that there must exist factors other than inflammation that leads to WM injury. Such factor(s) must have appeared in the CNS during an early stage of infection, and continued to exist even when the peripheral viral load is reduced to below the clinical detection limit after cART. One candidate of such factor(s) is the HIV viral protein Tat, which is expressed across the HIV lifespan, and has been reported to be a toxic factor to neurons in the CNS. We thus hypothesized that HIV-1 Tat possesses direct toxicity to OLs/myelin. Based on the results of all the experiments reported in this thesis, and existing publications (Oka et al., 1993; Maggirwar et al., 1999; Salter and Fern, 2005; Okamoto et al., 2007; Alix and Fern, 2009; Song et al., 2010; Rumbaugh et al., 2013; Waggener et al., 2013; Fitting et
al. 2014; Masvekar et al., 2015), we proposed the following model for Tat-mediated injuries in immature and mature OLs. In immature OLs, interaction between Tat and NMDA-Rs activates NMDA-Rs and leads to Ca\(^{2+}\) influx. Since CaMKII\(\beta\) binds NR2B and co-localizes with NMDA-Rs, the NMDA-R-mediated Ca\(^{2+}\) influx efficiently activates CaMKII\(\beta\), which recruits and activates AMPA-Rs, and leads to more Ca\(^{2+}\) influx and persistent [Ca\(^{2+}\)]\(_i\) increase. This elevated [Ca\(^{2+}\)]\(_i\) then activates GSK3\(\beta\), triggers downstream death signaling pathways, and eventually results in cell death. In mature OLs, Tat also activates NMDA-Rs and leads to Ca\(^{2+}\) influx and CaMKII\(\beta\) activation. Since NMDA-Rs are mostly located at processes and myelin-like membranes, activated CaMKII\(\beta\) dissociates from actins and destabilizes the cytoskeleton, resulting in membrane disintegration. In addition, activated CaMKII\(\beta\) also migrates to the cell soma, where it recruits and activates AMPA/KA-Rs, which leads to more Ca\(^{2+}\) influx. Meanwhile activated CaMKII\(\beta\) also phosphorylates GSK3\(\beta\)-S9 and inhibits its activation, thus protects mature OLs from death (Fig 7.1).

We used the Tat-transgenic mice to examine whether in vivo expression of Tat in the CNS had any effects on WM. One benefit of the Tat-transgenic mice that we used is the temporal control of Tat expression by DOX. Since active myelination during mouse development happened in the first month after birth, we did not feed the mice with DOX until they were 3-months old. Thus, the abnormality of OLs and myelin we observed, as well as the reduced expression of myelin protein MBP and MAG, are likely to be caused by Tat-induced injury. Restricted by the length of experiments and amount of mice, all the subcellular structure change observed by EM are descriptive. Adding quantitative EM studies such as investigating whether the g-ratio of WM tracts is
affected by Tat expression can strengthen this study, as we have observed groups of hypomyelinated axons in Tat+ mice.

To answer the question whether Tat had direct toxicity on OLs, we used a highly purified murine OL culture. Based on our immunostaining experiments, this culture contains >96% O4+ OLs within the first 5 days, and >90% O4+ OLs between day 6 and day 10. Although this model can greatly reduce the impact of other CNS cell types, we cannot completely rule out the possibility that other cell types, mainly astrocytes, may still affect the results we observed, especially at later time points. Using this model, we found that viability of mature OLs was not affected by 100 nM Tat. Consistently, early studies by Hauser et al., (2009) using the same transgenic mice did not find an increase of TUNEL staining in APC+ mature OLs after Tat induction, suggesting that the Tat expression in vivo does not affect mature OL viability. However several recent studies reported that astrocytes protect OLs from iGluR-mediated excitotoxicity (Pang et al., 2013; Lu et al., 2015). Our repeated measure experiments using OL-glia co-culture also showed that glial cells attenuated the effect of Tat-induced OL death. Thus, the conclusion that Tat does not affect viability of mature OLs is still debatable. Using fluorescence-activated cell sorting (FACS) technique to isolate olig2+ OPCs from primary mixed glial culture may be a way to acquire close to 100% pure mature OL population, which in theory can give a definitive answer whether Tat directly affects mature OL viability.

We observed that Tat leads to persistent [Ca2+]i increase in both immature and mature OLs, which can be attenuated by either MK801 or CNQX. Interaction between Tat and the NR1 subunit of NMDA-Rs has been documented (Rumbaugh et al., 2013).
It is thus proposed that this interaction causes a conformational change of the NMDA receptor, which leads to its activation. On the other hand, physical binding between Tat and AMPA/KA-Rs has never been reported. Although other studies also reported that Tat activates AMPA/KA-Rs (New et al., 1998), the mechanism remains unclear. AMPA/KA-Rs-mediated Ca\(^{2+}\) influx has been shown to usually lead to activation of various death pathways in OLs (Stys, 2004; Matute, 2010; Domercq et al., 2011; Matute, 2011), while to our best knowledge, the OL death pathways triggered by NMDA-R activation have not been seriously studied. Thus, understanding how Tat activates AMPA/KA-R may have important therapeutic significance. As mentioned in chapter 3, studies by other investigators have shown that CaMKII recruits AMPA-Rs from intracellular vesicles to cytoplasmic membranes, and increases the likelihood of channel opening by phosphorylating Ser-831 of AMPA-R GluA1 subunits (Milstein and Nicoll, 2008; Derkach, 2011; Kristensen et al., 2011). Since NMDA-R-mediated CaMKII\(\beta\) activation has been observed in OLs treated with 100 nM Tat, it is possible that AMPA/KA-R activation is a downstream event following Tat-induced NMDA-R and CaMKII activation. Measuring AMPA/KA-R current in OLs while inhibiting NMDA-R and/or CaMKII can test this hypothesis. Since the duration of these experiments should not go beyond 1 h, existing CaMKII\(\beta\) inhibitors such as KN-93 can be used for this purpose.

It is really interesting that inhibiting both NMDA-Rs and AMPA/KA-Rs can not completely block Tat-induced [Ca\(^{2+}\)]i increases. Since our Ca\(^{2+}\) imaging experiments found that the primary source of [Ca\(^{2+}\)]i increase is extracellular Ca\(^{2+}\), most of the inhibitors we tested target OL surface molecule or channels. Internal Ca\(^{2+}\) stores such
as mitochondria and endoplasmic/sarcoplasmic reticulum also play important role in maintaining intracellular Ca^{2+} homeostasis. Fitting et al., (2014) had reported that the interaction between Tat and NMDA-Rs leads to Na^{+} influx, which reverses Na^{+}/Ca^{2+} exchange and triggers mitochondrial Ca^{2+} release. The same mechanism may also exist in OLs. We found that using dantrolene to block ryanodine receptors (RYRs) had no effect on Tat-induced [Ca^{2+}]_{i} increase. However, it has been reported that dantrolene had no effect on the RYR2 isoform (Zhao et al., 2001). Thus, using inhibitors such as ryanodine that blocks all 3 isoforms of RYRs can tell whether Ca^{2+}-induced-Ca^{2+} release from internal stores contributes to Tat-induced [Ca^{2+}]_{i} increases. It is well established that Tat can be taken up by cells via macropinocytosis (Wadia et al., 2004; Kaplan et al., 2005). Using biotin-conjugated Tat_{1-86}, we found that Tat enters the OL cytoplasm within an hour (Fig 7.2). Hence, it is also plausible that Tat may interact with surface molecules or channels on the internal Ca^{2+} store and results in Ca^{2+} release. Adding cyclopiazonic acid (CPA) to deplete internal Ca^{2+} stores right before assessing Tat induced OL [Ca^{2+}]_{i} change can be a future experiment to tell whether Ca^{2+} release from internal stores plays a role in Tat-induced [Ca^{2+}]_{i} increases in OLs.

It is generally accepted that resting CaMKIIβ bundles and stabilizes the actin cytoskeleton. Transient activation of CaMKIIβ relaxes the cytoskeleton, promotes actin polymerization and CaMKIIβ recruitment, and favors cytoskeletal growth. This model has been shown in both synaptogenesis (Okamoto et al., 2007) and OL maturation (Waggener et al., 2013). Based on this theory, we proposed that prolonged CaMKIIβ activation by NMDA-R-mediated Ca^{2+} influx leads to destabilization of the actin cytoskeleton and membrane disruption in mature OLs. Although our experiments
unambiguously showed that NMDA-R activation results in CaMKIIβ activation, direct proof that links CaMKIIβ activation and membrane reduction are lacking. All the existing CaMKIIβ inhibitors are either non-specific or highly toxic to OLs (usually kills cells within 10 h). Since the membrane reduction in OLs is a long-term phenomenon (in our experiments paradigm, it is observed at 24 and 48 h), pharmacologically blocking CaMKIIβ activation is unrealistic. Although an ON-TARGETplus SMARTpool siRNA specifically against mice Camk2b was commercially available, we failed to find an effective way to deliver the siRNA into mature, adherent OLs. Plus, hypomyelination in ventral spinal cord has been reported in CaMKIIβ KO mice, suggesting that silencing the CaMK2b gene itself already had an impact on the actin cytoskeleton. Thus, inhibiting CaMKIIβ expression may also not be an ideal way to assess whether mature OL membrane reduction is the result of prolonged CaMKIIβ activation. As mentioned in chapter 4, a repeated measure experiment using OLs from CaMKIIβ^{A303R} mice will be the best way to investigate this question. The CaMKIIβ^{A303R} mutant retains its ability to bundle F-actin, but loses its Ca\(^{2+}\)/CaM binding capacity. Based on our proposed model, Tat-induced [Ca\(^{2+}\)]\(_i\) increase should have no effect on OL membrane area since Ca\(^{2+}\)/CaM cannot bind and activate CaMKIIβ^{A303R}, and promotes its release from actin cytoskeleton.

Most of our experiments are done in mouse cells. Since HIV is a human disease, we used an hBrnAgg model to try to verify our findings in human cells. We speculated that the hBrnAgg model would serve our experiments best because 1) it mimics the \textit{in vivo} cell composition with the presence of all major CNS cell types; 2) myelination has been observed in the model by other investigators, thus fulfilling the need of studying
the effect of HIV/Tat on both OLs and myelin; and 3) the hBrnAgg model can be maintained for a long period of time (up to 60 days), making it a good model to study the long-term effects of HIV/Tat.

Our immunostaining experiments suggested that there are far less OLs in the aggregates than we expected. Under EM, plenty of synaptic structure, both symmetric and asymmetric, were observed in the hBrnAgg. However, cells with typical mature OL characteristics, including dark cytoplasm and nucleus, elongated, thin strands of endoplasmic reticulum; numerous mitochondria, and stacks of Golgi apparatus, were rarely found. Although we detected expression of myelin protein MBP in the outgrowth of aggregate cultures, we did not detect MBP directly in the hBrnAggs, and compact myelin was also never observed in these aggregates under EM. As we mentioned in chapter 6, a possible explanation is that too much time (>18 h) has passed between tissue procurement and the initiation of culture. OL lineage cells are known to be vulnerable to excessive extracellular glutamate, inflammatory cytokines and reactive species (Oka et al., 1993), all of which were produced during the procurement procedure. Hence, the brain tissue that we received may only have a small amount of surviving OLs, which results in a relatively rare probability that OLs are located in proximity to axons. It has been shown that in vitro OLs will ensheath anything within its reach that feels like an axon (Rosenberg et al., 2008). However, to a great extent myelination is determined by axonal activity-dependent release of glutamate, adenosine, neuregulin and BDNF, which promotes OL NMDA-R expression and activation, and facilitates myelin membrane compaction (Demerens et al., 1996; Stevens et al., 2002; Lundgaard et al., 2013). Consistently, we found plenty of loosely-
wrapped myelin-like membrane in the hBrnAgg under EM, indicating that membranes produced by OLs lacking signaling for myelin compaction. Hence, future studies using this model should pay attention to the composition of different cell types in the hBrnAgg. As proposed in chapter 6, using FACS technique to sort dissociated brain cells, and use fixed ratio of different cell type to form the hBrnAgg may be a good strategy. Not only it may lead to better reproducibility of experiments, but also by this way investigators can further adjust the system to meet requirements of different studies.
Figure 7.1

Immature OL

Mature OL
**Figure 7.1 Proposed model for the direct effects of HIV-1 Tat on OLs.** In immature OLs, interactions between Tat and NMDA-Rs (1) leads to Ca$^{2+}$ influx (2), which binds to CaM (3) and activates CaMKIIβ (4). Activated CaMKIIβ recruits AMPA-Rs to the cell surface (5) and phosphorylates AMPA-Rs at S831 (6), which increases the conductance of AMPA-Rs and leads to more Ca$^{2+}$ influx (7), and results in an overall [Ca$^{2+}$] elevation. This increased [Ca$^{2+}$] dephosphorylates and activates GSK3β (8), and activated GSK3β may lead to increased DNA damage, ER stress and hypoxia and inhibition of prosurvival transcription factors such as CREB and heat shock factor-1, which eventually resulted in cell death. Similarly, in mature OLs, Tat-NMDA-R interaction (1) leads to Ca$^{2+}$ influx (2), and CaMKIIβ activation by Ca$^{2+}$/CaM complex (3,4). Activated CaMKIIβ relaxed its interaction with F-actins and dissociated from actin cytoskeleton (4), which caused the cytoskeleton destabilization and the reduction of the area of myelin-like membrane (5). Dissociated CaMKIIβ may move to cell body, where they recruits and phosphorylates AMPA-Rs, which results in increased [Ca$^{2+}$], just as what happened in immature OLs (6,7,8). In the cell soma of mature OLs, incoming activated CaMKIIβ also phosphorylates GSK3β at Ser-9, which inhibits GSK3β activation and prevents activation of death signaling pathways in mature OLs.
Figure 7.2 Immunostaining of 1h Biotin-conjugated Tat treated OLs. Immunostaining using antibodies specific to O4 and biotin showed that Biotin-conjugated Tat presents in the cytoplasm of both immature and mature OLs after being added to the culture medium for 1 h.
List of References


Vita

ShiPing Zou was born in Shanghai, China on April 14th, 1978, and is a Chinese citizen. He received his Bachelor of Science in Genetics and Genetic Engineering from Fudan University in Shanghai. He subsequently received two Masters of Science in Computer Science and Biological Science from University of Kentucky, Lexington, Kentucky in 2004. He then worked for 7 years as research associate before entering the neuroscience Ph.D program at Virginia Commonwealth University in 2011.

Academic Background

1995-1999  **B.S. Genetics and Genetic Engineering**

Fudan University, Shanghai, China

1999-2004  **M.S. Biological Science**

**M.S. Computer Science**

University of Kentucky, Lexington, KY

2011-present  **Ph.D Candidate Neuroscience**

Virginia Commonwealth University, Richmond, VA

Research & Work Experience

Graduate Student Researcher

2011-present  Virginia Commonwealth University

- Advisor: Pamela Knapp, Ph.D
- Dissertation Project: Effects of HIV-1 viral protein Tat on the viability and
phenotype/function of oligodendroglial cells

**Grant Review Specialist**

2014 Virginia Center on Aging

- Reviewing research grants for the Geriatric Training and Education (GTE) Award

**Grant Review Specialist**

2013-2014 Virginia Center on Aging

- Reviewing research grants for Alzheimer's and Related Disease Research Award Fund (ARDRAF)

**Lab and Research Specialist**

2007-2011 Virginia Commonwealth University

- Project: Toxic interactions between opiates and HIV viral proteins in Striatal Neurons
- Project: Role of the phosphatase and tensin homologue on chromosome 10 (PTEN) in mediating envelope glycoprotein 120 (gp120)-induced neurotoxicity in the striatum

**Laboratory Technician**

2004-2007 University of Kentucky

- Project: Effects of HIV-1 Tat and opiates on transcription factor activity in astrocytes

**Graduate Student Researcher**

1999-2004 University of Kentucky

Biological Science:

- Advisor: Chuck Staben, Ph.D
Project: A computation algorithm for protein secondary structure prediction

Computer Science:

- Advisor: Fuhua Cheng, Ph.D
- Project: Constrained scaling of Catmull-Clark subdivision surface

Professional/ Academic Honors and Awards

02/2015 American Society for Neurochemistry (ASN) Young Investigator’s Educational Enhancement (YIEE) Travel Awards
01/2015 VCU Neuroscience Student Travel Award
05/2014 C. C. Clayton Award
03/2014 Central Virginia Chapter of the Society for Neuroscience (CVCSN) 2014 Graduate Student/Postdoc Poster award
11/2013 Honor Society of Phi Kappa Phi
04/2013 International Society for Neurochemistry (ISN) travel award for the joint ISN-ASN meeting
1997 Fudan University People’s Scholarship
1996 Fudan University People’s Scholarship

Publications


identification by the combination of in situ polymerase chain reaction and immunohistochemistry. AIDS 10:573-585.


microglia through the expression of MCP-1 and alternative chemokines. Glia 53:132-146.


4.

Invited Talks

1. HIV-1 Tat disrupts oligodendrocyte viability and functions: Ca\(^{2+}\) mediated glutamatergic mechanisms (University of North Dakota, Grand Folks, ND. July. 2015)

2. Effects of HIV-1 Tat on oligodendrocyte viability: iGluR-mediated Ca\(^{2+}\) dysregulation and GSK3\(\beta\) activation (Central Virginia Chapter of the Society for Neuroscience, Richmond, VA. 2015)

3. Oligodendrocytes are targets of HIV-1 Tat: NMDA and AMPA-receptor-mediated effects on survival and development. (The 43\(^{rd}\) Annual John C. Forbes Research Colloquium, Richmond, VA. 2015)

4. Stage-specific effects of HIV-1 Tat on the viability and phenotype of oligodendroglia: interactions with glutamatergic receptors activate CaMKII\(\beta\) and GSK3\(\beta\). (The Virginia Symposium on Brain Immunology and Glia, Charlottesville, VA. 2014)

5. Glutamate receptors and calcium signaling through CaMKII\(\beta\) mediate effects of HIV-1 Tat on the viability and phenotype of oligodendroglia. (Virginia Universities AIDS Research Consortium, Richmond, VA. 2014)

Conference Poster Presentations

1. ShiPing Zou, Babette Fuss, Kurt F. Hauser, and Pamela E. Knapp. HIV-1 Tat affects the viability of immature and mature oligodendrocytes via Ca2+ dysregulation and
2. ShiPing Zou, Babette Fuss, Kurt F. Hauser, and Pamela E. Knapp. Stage-specific effects of HIV-1 Tat on the viability and phenotype of oligodendroglia: interactions with glutamatergic receptors activate CaMKIIβ and GSK3β. (Oral Presentation and Poster, The Virginia Symposium on Brain Immunology and Glia, Charlottesville, VA. 2014)


4. ShiPing Zou, Babette Fuss, Kurt F. Hauser, and Pamela E. Knapp. Glutamate receptors and calcium signaling through CaMKIIβ mediate effects of HIV-1 Tat on the viability and phenotype of oligodendroglia. (Poster, 45th ASN annual meeting, Long Beach, CA. 2014)

5. ShiPing Zou, Kurt Hauser and Pamela Knapp. Glutamate receptors mediate stage-specific effects of HIV-1 Tat on the viability and phenotype of oligodendroglia. (Poster, 12th ISNV abstracts, Washington, D.C. 2013)

6. ShiPing Zou, Kurt Hauser and Pamela Knapp. Glutamate receptors mediate stage-specific effects of HIV-1 Tat on the viability and phenotype of oligodendroglia. (Poster, 9th Great Glia Meeting abstracts, Traverse City, MI. 2013)

7. S. Zou, K.F.Hauser, Brandon Layne and P.E.Knapp. Stage-specific effects of HIV-1 Tat on the viability and phenotype of oligodendroglia: interactions with glutamatergic receptors alter Ca$^{2+}$ dynamics. (Poster, 24th ISN-ASN Biennial Joint Meeting
abstracts, Cancun, Mexico 2013)

8. **S. Zou**, K.F.Hauser, Brandon Layne and P.E.Knapp. HIV-1 Tat and gp120 differentially affect the viability and phenotype of maturing oligodendroglia: potential interactions with opiates alter $\text{Ca}^{2+}$ dynamics. (Poster, Society For Neuroscience abstracts, New Orleans, LA. 2012)


15. **S. Zou**, S. Fitting, K.F. Hauser, & P.E. Knapp. HIV-1 Tat affects the viability and differentiation of oligodendroglia through glutamatergic interactions. (Poster, 42nd Annual ASN Meeting abstracts, St. Louis, MO 2011)


