2014

HIV AND OPIATES-MEDIATED NEUROTOXICITY: GSK3β IS A POTENTIAL THERAPEUTIC TARGET

Ruturaj Masvekar
Virginia Commonwealth University

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

Part of the Medicine and Health Sciences Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3496

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.
HIV AND OPIATES-MEDIATED NEUROTOXICITY: GSK3β IS A POTENTIAL THERAPEUTIC TARGET

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

By
Ruturaj R. Masvekar,
B.S. Shivaji University, 2006
M.S. Louisiana Tech University, 2009

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

Virginia Commonwealth University
Richmond, Virginia
August 2014
ACKNOWLEDGEMENT

I would like to express my gratitude towards all those who gave me a possibility to complete my PhD dissertation. Firstly, I would like to thank my advisor Dr. Pamela E. Knapp for providing me this project. Her constant encouragement, guidance and valuable advice throughout the process of my dissertation immensely contributed to my professional and personal advancement.

I would like to thank my committee members: Dr. Kurt F. Hauser, Dr. Nazira El-Hage, Dr. Jeff Dupree, and Dr. Michael Fox for their patience and willingness to be part of my advisory committee and helping me technically and intellectually throughout course of this project. I would like to thank each and every member of our research group for their help and cooperation throughout this work. Special thanks to Dr. Yun Kyung Hahn, Mr. Shiping (Patrick) Zou, Mr. Phu Vo, Dr. Sylvia Fitting, Mrs. Yun (Gigi) Ji and Ms. Joyce Balinang for their advice and support in my project.

I would like to thank the Department of Anatomy and Neurobiology, Neuroscience PhD program, and Virginia Commonwealth University for accepting me into the program and providing financial support during my tenure. Special thanks to Neuroscience program director Dr. John W. Bigbee for his intellectual support. He was always there to help me. I would like to extend my gratitude towards Global Education Office (GEO) for helping me through immigration issues.
I would like to express my wholehearted gratitude to my friends for their support and encouragement throughout my PhD. I would like to thank my parents for their support and blessings. Last but not least, I would like to thank my wife, Mrs. Poonam R. Masvekar for her love, attention, patience and faith in me.
# Table of Contents

List of Tables ........................................................................................................................................... v
List of Figures ............................................................................................................................................... vi
List of Abbreviations .................................................................................................................................. viii
Abstract ......................................................................................................................................................... xii

**Chapter 1** Introduction ............................................................................................................................. 1

**Chapter 2** Morphine Enhances HIV-1<sub>SF162</sub>-Mediated Neuron Death and Delays Recovery of Injured Neurites ................................................................................................................................. 13

**Chapter 3** Comparison of HIV-1<sub>LAI</sub> and HIV-1<sub>SF162</sub>-Mediated Neurotoxicity and Interactions with Opiates .................................................................................................................................................. 45

**Chapter 4** GSK3β-Activation is a Point of Convergence for HIV- and Opiate-Mediated Interactive Neurotoxicity .................................................................................................................................................. 66

**Chapter 5** Conclusions ............................................................................................................................... 87

List of References ......................................................................................................................................... 92

Vita .................................................................................................................................................................. 130
LIST of TABLES

Table 2.1  Treatment paradigm for neurite growth/regrowth assessments ..................................32
LIST of FIGURES

Figure 2.1 Concentration-dependent change in MTT reduction.........................................33

Figure 2.2 Concentration-dependent neuronal death in cultures treated with HIV⁺_sup ± morphine ........................................................................................................................................34

Figure 2.3 Neuronal apoptosis induced by HIV⁺_sup ± morphine........................................36

Figure 2.4 HIV⁺_sup ± morphine-mediated neuronal death................................................38

Figure 2.5 HIV⁺_sup ± morphine-mediated neurite damage..............................................39

Figure 2.6 Reversibility of HIV⁺_sup ± morphine-mediated neurite damage.......................40

Figure 2.7 HIV⁺_sup ± morphine-mediated effects on secretion of growth factors and cytokines by glia..................................................................................................................................................42

Figure 2.8 HIV⁺_sup ± morphine-mediated GSK3β activation.............................................44

Figure 3.1 HIV⁺_sup ± morphine-mediated neuronal death................................................58

Figure 3.2 Neuronal apoptosis induced by HIV⁺_sup ± morphine........................................60

Figure 3.3 HIV⁺_sup ± morphine-mediated neurite damage..............................................61

Figure 3.4 Cytokines/chemokines secretion from HIV-1-infected cells................................62

Figure 3.5 HIV_virion ± morphine-mediated neuronal death................................................64

Figure 4.1 HIV⁺_sup ± morphine-mediated GSK3β activation.............................................79

Figure 4.2 HIV⁺_sup- and morphine-mediated interactive effects on GSK3β-activation........80

Figure 4.3 Role of GSK3β in HIV⁺_sup ± morphine-mediated cell death............................81
Figure 4.4  Role of GSK3β in HIV$^{+}_{\text{sup}} \pm$ morphine-mediated changes in neuritic arborization, MAP-2 and PSD-95..........................................................83

Figure 4.5  Effects of small molecule GSK3β-inhibitors..........................................................85
**LIST of ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV-associated neurocognitive disorders</td>
</tr>
<tr>
<td>cART</td>
<td>Combination antiretroviral therapy</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor type 5</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>BMVECs</td>
<td>Brain microvascular endothelial cells</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>QA</td>
<td>Quinolinic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>ANI</td>
<td>Asymptomatic neurocognitive impairments</td>
</tr>
<tr>
<td>MND</td>
<td>Mild neurocognitive disorders</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV-associated dementia</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>MORs</td>
<td>µ-opioid receptors</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>hPBMCs</td>
<td>Human peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>AZT</td>
<td>Azidothymidine</td>
</tr>
<tr>
<td>HIVE</td>
<td>HIV encephalitis</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>p90&lt;sup&gt;rsk&lt;/sup&gt;</td>
<td>p90 ribosomal s6 kinase</td>
</tr>
<tr>
<td>FGF1</td>
<td>Fibroblast growth factor 1</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin growth factor 1 (IGF-1)</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element binding protein (CREB)</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>HSF-1</td>
<td>Heat shock factor-1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’5,5’-Tetramethylbenzidine</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>Li</td>
<td>Lithium</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>MAPK10</td>
<td>Mitogen-activated protein kinase 10</td>
</tr>
<tr>
<td>PKR</td>
<td>Double-stranded RNA-activated protein kinase</td>
</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>PSD95</td>
<td>Postsynaptic density protein 95</td>
</tr>
</tbody>
</table>
ABSTRACT

HIV AND OPIATES-MEDIATED NEUROTOXICITY: GSK3β IS A POTENTIAL THERAPEUTIC TARGET

By Ruturaj R. Masvekar

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

Virginia Commonwealth University, 2014

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

HIV-1 enters the CNS soon after initial systemic infection. HIV-1 can induce a wide range of neurological deficits, collectively known as HIV-1-associated neurocognitive disorders (HAND). Mature neurons are not infected by HIV-1; instead, infected and/or activated glial cells release various viral and cellular factors that induce direct and/or indirect neuronal toxicity, leading to HAND. Injection drug abuse is a significant risk factor for HIV-infection, and opiate drug abusers show increased HIV-neuropathology, even with anti-retroviral treatments. Our previous work has largely modeled HIV-neuropathology using the individual viral proteins Tat or gp120, with murine striatal neurons as targets. To model disease processes more closely, the current study uses supernatant from HIV-1-infected cells.

Supernatant from HIV-1_{SF162}(R5-tropic)-infected differentiated-U937 cells (HIV^{+\sup}) was collected and p24 level was measured by ELISA to assess the infection. We assessed HIV^{+\sup} effects on neuronal survival and neurite growth/pruning with or without concurrent exposure to
morphine, an opiate that preferentially acts through μ-opioid receptors. Effects of HIV$^{+}\text{sup}$ ± morphine were assessed on neuronal populations, and also by time-lapse imaging of individual cells. HIV$^{+}\text{sup}$ caused dose-dependent toxicity over a range of p24 levels (10-500 pg/ml). Significant interactions occurred with morphine at lower p24 levels (10 and 25 pg/ml). In the presence of glia, selective neurotoxic measures were significantly enhanced and interactions with morphine were also augmented. Importantly, the arrest of neurite growth that occurred with exposure to HIV$^{+}\text{sup}$ was reversible unless neurons were continuously exposed to morphine. Thus, while reducing HIV-infection levels may be protective, ongoing exposure to opiates may limit recovery.

During early stage of HIV-infection R5-tropic viruses are predominant, but during later stages of disease X4-tropic viruses are more predominant; co-receptor usage switch from CCR5 to CXCR4 is crucial in disease progression to AIDS. Some previous studies have shown that drugs of abuse interact with virus or viral proteins in strain/tropism-dependent manner. Therefore, we also assessed neurotoxic effects and interactions with opiates by supernatant from HIV-1$\text{LAI}$ (X4-tropic)-infected H9 cells. Neurotoxic effects and the interactions with opiates of HIV-1$\text{LAI}$-supernatant are quantitatively similar to that of HIV-1$\text{SF162}$. Surprisingly, the cytokine/chemokine release profile of HIV-1$\text{LAI}$-infected H9 cells is similar to that of HIV-1$\text{SF162}$-infected U937 cells. Only in the presence of glia, HIV-1$\text{LAI}$ virion induced neurotoxic effects, but no interactions with morphine were seen. Also our studies have shown that HIV-1$\text{LAI}$ virions are slightly more neurotoxic than HIV-1$\text{SF162}$. Altogether, largely our results suggest that HIV$^{+}\text{sup}$ mediated neurotoxicity and the interactions with opiates are majorly attributed to cytotoxic factors released from infected and activated cells instead of viral strain specific factors.
Although there is a correlation between opiate drug abuse and progression of HAND, the mechanisms that underlie interactions between HIV-1 and opiates remain obscure. Previous studies have shown that HIV-1 induces neurotoxic effects through abnormal activation of GSK3β. Interestingly, expression of GSK3β has shown to be elevated in the brains of young opiate abusers suggesting that GSK3β is also linked to neuropathology seen with opiate abusing patients. Thus, we hypothesized that GSK3β activation is a point of convergence for HIV- and opiate-mediated interactive neurotoxic effects. Cultures of striatal neurons were treated with HIVα suppressive (R5-tropic), in the presence or absence of morphine and GSK3β inhibitors. Our results show that multiple GSK3β inhibitors significantly reduce HIV-1-mediated neurotoxic outcomes, and also negate interactions with morphine that result in cell death. This suggests that GSK3β-activation is an important point of convergence and a potential therapeutic target for HIV- and opiate-mediated neurocognitive deficits.
CHAPTER 1

Introduction

HIV/AIDS

Epidemiology of HIV/AIDS

Human immunodeficiency virus (HIV) infects immune system cells, primarily CD4\(^{+}\) T cells and monocytes/macrophages. HIV infection dysregulates normal immune system functioning and leads to acquired immunodeficiency syndrome (AIDS) [1-3]. Worldwide about 35.3 million people are infected with HIV. In 2012, 2.3 million people were newly infected with HIV, and 1.6 million died due to AIDS [4]. Due to limited access to combination antiretroviral therapy (cART), AIDS pandemic continues to spread unchecked in many underdeveloped countries; sub-Saharan Africa is badly affected region, where around 25 million people are living with HIV infection. In 2012, in these countries nearly 1.6 million people were newly infected with HIV and 1.2 million died due to AIDS. AIDS epidemic has eroded the socioeconomic stability of these countries.

HIV genome and life cycle

HIV has been characterized into two types, HIV type 1 (HIV-1) and type 2 (HIV-2). HIV-1 is relatively more pathogenic and thus is spread worldwide, while HIV-2 is relatively less virulent and thus is restricted to limited parts of the world [5, 6]. HIV-1 has single stranded RNA genome
consisting of 9 genes. The \textit{gag} genomic region encodes for gag polyprotein, which is mainly processed to p17 (matrix protein, MA), p24 (capsid protein, CA), p7 (nucleocapsid protein, NC), and p6 proteins, by the viral protease. The \textit{pol} gene encodes for reverse transcriptase (RT), RNase H, integrase and protease. The \textit{env} gene encodes for precursor glycoprotein gp160, which is processed to surface glycoprotein gp120 and transmembrane glycoprotein gp41, by furin, a host cell protease. Remaining 6 genes (\textit{tat, rev, nef, vpr, vif, vpu}) encode for regulatory proteins, involved in regulation of viral replication and release [3, 7-9].

HIV-1 surface glycoprotein gp120 binds/interacts to CD4 and a co-receptor, usually either C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) on the surface of host cells. After binding/interaction, conformational changes in gp120 and gp41 enable virus to fuse with host cells and release its genetic material into host cells. RT with help of host cofactors transcribes DNA from RNA template by process called reverse transcription. Integrase integrates reverse-transcribed viral genome (DNA genome) into host chromosome. Following integration, the virus uses host cell machinery to transcribe and/or translate its genetic material and proteins. The Viral protease processes viral precursor proteins. Viral proteins and genetic materials assemble at the host cell membrane and bud out as new virions, ready for next cycle of infection. In some cases, following integration into the host genome, virus can remain latent for prolonged periods [3, 7-9].

\textbf{HIV-1 tropism}

The preference of HIV-1 gp120 for the co-receptors, either CCR5 or CXCR4, defines viral-tropism [10-13]. M-tropic (macrophage/monocyte tropic, R5-tropic) strains of HIV-1 use \(\beta\)-chemokine receptor, CCR5, to attach to host cells, and thus they mainly infect monocytes and/or
macrophages. T-tropic (T-cell tropic, X4-tropic) strains have preference for $\alpha$-chemokine receptor, CXCR4, and thus they mainly infect CD4$^+$ T cells. Some strains of HIV-1 do not have preference for either CCR5 or CXCR4, and are known as dual-tropic (X4R5-tropic) strains. Previous studies have shown that also some other chemokine receptors, such as CCR2, CCR3 and C-X3-C chemokine receptor 1 (CX3CR1, fractalkine receptor), can serve as co-receptors for viral attachment/binding at host cell surface [14-18].

**NeuroAIDS**

**Invasion of HIV-1 into CNS**

HIV-1 not only disrupts normal immune system functioning, but can also induce neurological complications, known as neuroAIDS. The blood brain barrier (BBB) is a tight, cohesive barrier between blood and brain. The BBB regulates influx of selective compounds from blood in capillaries to brain parenchyma, important for homeostasis and proper functioning of the brain [19-21]. Also the BBB protects central nervous system (CNS) from many infections and toxic insults in blood/plasma. Unlike a variety of blood-borne insults, HIV-1 overcomes this tight barrier and can invade the brain. HIV-1 can enter into the CNS soon after an initial systemic infection [22-24]. The exact route/mechanism for HIV-1 entry across BBB is not known. There are three hypotheses for route/mechanism of viral infiltration into CNS [22, 25]. The first hypothesis suggests that HIV-1 may invade into CNS as a passenger within infected leukocytes, which normally traffic across the BBB as a part of immune surveillance of the brain, known as ‘trojan horse’ hypothesis [26-28]. The second hypothesis suggests that activated and/or infected immune cells release various cytokines/chemokines and viral proteins, which can disrupt BBB integrity [29-32]. Thus, cell free virus particles may cross through compromised BBB and invade
into CNS. Alternatively, studies in cell cultures have shown that brain microvascular endothelial cells (BMVECs) can internalize virus particles into intracellular vacuoles, traffic these vacuoles via transcytosis, and release them at other side of the barrier [33-35]. Thus, it can be predicted that HIV-1 may traverse the BBB via transcytosis through BMVECs. The third hypothesis suggests that HIV-1 may invade into CNS via transmission by infected BMVECs because some in vitro studies have shown that these cells are capable of transmitting virus to other cells by direct cell-to-cell contact [36].

**HIV-1 infection of cells in the brain**

The Perivascular region of the brain is closely interfaces with the periphery, thus it comes in early and direct contact with HIV-1-infected leukocytes and/or free virus particles that cross through the BBB. Cells in this region of the brain, mainly perivascular macrophages, microglia and astrocytes, are the major site of viral infection. Unlike other resident cells within the brain, microglia are derived from mesoderm; microglia are also known as immune component of the brain [37-39]. Macrophages and microglia express CD4 and CCR5, the major receptor and co-receptor for HIV-1 entry [40-42]. Various studies in cell cultures have shown that primary macrophages and microglia are productively infected with HIV-1 [43-46].

Markers of viral infection, proteins and nucleic acids, have been detected in GFAP positive astrocytes [47-50], suggesting that these cells can be infected with HIV-1. Several in vitro studies with primary cells and tumor derived cell lines have confirmed that astrocytes can be infected with HIV-1 [51-53]. However, after initial acute productive infection their viral production is reduced to very low or undetectable levels [52, 54]. But virus production in persistently infected astrocytes can be transiently activated by treatment with pro-inflammatory
stimuli [52, 54, 55]. Also, it has been shown that persistently infected astrocytes initiate viral production in co-cultured monocytes/macrophages and T-cells [52, 54, 56]. Thus, it can be concluded that astrocytes act as a viral reservoirs.

Other resident cells of the CNS, oligodendrocytes and neurons, are rarely infected with HIV-1. Very few studies have shown that these cells get infected [49, 57], while various other studies did not detect any markers of HIV-1 infection in these cells [47, 58, 59]. Some in vitro studies have shown that these cells can be infected with HIV-1 [60, 61], but it is a nonproductive infection.

**HIV-1-mediated neurotoxicity**

Though HIV-1 induces profound changes in neuronal morphology, function and survival, the virus itself rarely infects neurons [22, 47, 49, 58]; instead, viral proteins directly cause neuronal damage or infected and/or activated glial cells produce cellular factors that drive secondary toxicity in neurons, known as bystander effects [22, 62-65].

‘Direct injury’ hypothesis suggests that HIV-1 proteins, mainly transactivator of transcription (Tat), gp120, negative regulatory factor (Nef) and viral protein R (Vpr), directly cause neuronal damage or death without requiring the intermediary functions of non-neuronal cells [22, 62, 63]. Various studies using primary neuron and neuroblastoma cell line cultures have shown that individual viral proteins induce toxicity including neurite degeneration and cell death [66-74]. However, in most of these studies the concentrations of viral proteins used were well above the levels thought to be present in brains of HIV-1-infected patients [62, 63, 75]. Thus, we need to be cautious when extrapolating results from these in vitro studies to actual human disease settings.
‘Indirect injury’ or ‘bystander effects’ hypothesis suggests that cellular factors including cytokines, chemokines, excitotoxic factors and free radicals, released from infected and/or activated non-neuronal cells cause neuronal toxicity [22, 62, 63]. Several studies have shown that infected and/or activated non-neuronal cells, mainly perivascular macrophages, microglia and astrocytes, release various pro-inflammatory and/or neurotoxic factors including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, transforming growth factor-β (TGF-β) interferon-γ (IFN-γ), fractalkine (CX3CL1), monocyte chemotactic protein-1 (MCP-1; CCL2), RANTES (CCL5), matrix metalloproteinases (MMPs), arachidonic acids, glutamate, cysteine, quinolinic acid (QA), nitric oxide (NO), free radicals etc. [22, 62, 63, 76-84]. Virus and/or viral factors are also known to disturb neuron supportive functions of glia [51, 85, 86], which may exacerbate neuronal damage.

As available data support the role for both, ‘direct’ and ‘indirect’ injury hypotheses, two hypotheses/mechanisms might coexist. But in in vivo settings where neurons are in continuous contact/communication with glia, the ‘indirect injury’ hypothesis seems to predominate [62, 63].

Synaptodendritic degenerative changes are thought to be the major substrate underlying HIV-1 related neurocognitive impairments. At least during the early stage of disease, synaptodendritic changes are more predominant than cell death; in HIV-1-infected individuals the degree of neurocognitive deficits is closely correlated to degeneration of synaptodendritic structures [87-91]. Initial damage at synapses and dendrites may culminate into cell death; postmortem analyses of brains of HIV-1-infected patients have shown evidence for neuronal death [92-95].

Though HIV-1 rarely, if ever, infects mature neurons, there is some evidence that neural progenitor cells may be permissive to infection [96]. HIV-1 and gp120 are known to decrease
proliferation and/or differentiation capacity of neural progenitor cells [97, 98]. It is also reported that number of proliferating cells in the hippocampus of HIV-1-infected patients is reduced [97]. Thus, it can be predicted that HIV-1 causes neurocognitive deficits not only by damaging existing neurons but also by impairing neurogenesis [99].

**HAND**

HIV-1 can induce a wide range of CNS deficits, including cognitive decline, personality changes, motor deficits and dementia, collectively known as HIV-1-associated neurocognitive disorders (HAND) [100-104]. Nearly 50% of HIV-infected patients experience HAND [88, 105-107]. Neurological impairments tend to be milder during early phase of HIV-1 infection, but severity of HAND upsurges with disease progression [108-110]. The occurrence and severity of HAND varies among HIV-infected patients; it may be because of differences in the individual’s genetic control of immune system [103]. There are three diagnostic terms commonly used to describe the severity of HAND [88, 104]: (a) asymptomatic neurocognitive impairments (ANI): refers to mild neurocognitive deficits, do not interfere with patients’ everyday functioning; (b) mild neurocognitive disorders (MND): refers to mild to moderate neurocognitive impairments, interfere modestly with everyday functioning of patients; (c) HIV-associated dementia (HAD): refers to moderate to severe neurocognitive impairments, markedly disturb patients’ day-to-day functioning, these patients are incapable of living independently.

cART has markedly controlled systemic HIV-infection, and has improved the health status of a large segment of patients [105, 111, 112]. Although cART has reduced the overall severity of neurocognitive disorders in HIV-1 patients, the prevalence of HAND remains same [88, 105-107, 111, 113]. The persistence of relatively high rates of CNS disease is likely due to a
combination of longer patient survival, the relatively poor CNS penetrance of most antiretroviral
drugs [88, 114], and their neurotoxic effects [115, 116]. Inhibiting viral replication in periphery
by cART can partially reverse neurocognitive deficits [117, 118]. However, some patients who
have responded positively to early cART with time have reverted back and are showing
continued neurocognitive decline [119, 120]. In some cART receiving patients, although viral
replication in the periphery is significantly controlled, viral loads in the CSF are unchecked, a
phenomenon called CNS escape [121-123]. In some patients, even if the CNS viral load is
extremely low or undetectable, neurodegeneration can still occur in response to viral proteins,
such as Tat, that are released from cells even when viral replication has been inhibited [124].

**HIV and Opiate Drugs of Abuse**

Illicit drug abuse is a significant risk factor for HIV-transmission due to sharing of contaminated
needles and unsafe sexual activities. Nearly 30% of HIV-infected patients have history of drug
abuse, which frequently include opiates [125-127]. Other than illicit drug abuse, a large subset of
HIV+ patients are exposed to opiates for treatment of AIDS-related chronic pain syndromes.

Opiates by themselves are known to affect normal immune system function, including antibody
production, monocyte and T-cell functioning and natural killer (NK)-cells cytotoxicity [126,
128-130]. Opiates enhance immune cell inflammation and also increase expression of cytokine
receptors including CCR5 and CXCR4 [131-134], co-receptors for HIV-1-entry. Morphine, an
opiate that preferentially acts through µ-opioid receptors (MORs), enhances HIV-1
replication/expression in phytohaemagglutinin (PHA)-activated cultured human peripheral blood
mononuclear cells (hPBMCs) [135]. Chronic morphine treatment of simian immunodeficiency
virus (SIV)-infected rhesus macaques has shown to cause increase in viral replication, increased
rate of viral mutation and tolerance to azidothymidine (AZT or Zidovudine), an antiretroviral drug, and decrease in life span of infected animals [131, 136-138]. Thus, it is likely that opiate abuse not only increases risk of HIV-infection but also accelerates disease progression to clinical AIDS.

Since opiate drugs of abuse themselves are known to induce neurotoxic/neuropathological effects, such as blood brain barrier (BBB) breakdown, immune and glial cell activation, neuronal damage etc. [139-144], it can be predicted that opiate drugs of abuse will exacerbate HIV-1-associated pathogenesis in CNS. Opiates can interact directly to aggravate HIV-1-mediated neurodegeneration, or they can act indirectly via exacerbating HIV-1-mediated glial cell inflammation [125, 145-156]. Opiate drug abusing HIV+ patients are more susceptible to develop HIV encephalitis (HIVE), which may lead to HAND [157-159]. The severity of neurological deficits in opiate drug abusing HAND patients have been shown to be elevated compared to non-drug abusing patients [160-162].

GSK3β

Glycogen synthase kinase 3 (GSK3) was first identified as a regulator of glycogen metabolism; inactivating phosphorylation of glycogen synthase by GSK3 is a central regulatory process in glycogen biosynthesis [163-166]. GSK3 is a multifunctional serine/threonine kinase which is ubiquitously expressed in eukaryotes. GSK3 exists in two highly homologous isoforms, α and β. Both isoforms are highly conserved within their kinase domain; GSK3β is smaller, consists of 482 amino acids, while GSK3α additionally contains glycine-rich N-terminus extension [163, 165, 167-169]. Both isoforms are widely expressed in all tissues; however, in the brain GSK3β is present in high abundance. Widespread expression of GSK3β in the brain suggests that it plays a
vital role in various neuronal signaling pathways. GSK3β is known to be an important player in regulation of CNS development; it plays a critical role in neurogenesis, neuronal migration, neuronal polarization, and axon growth and guidance [163, 166, 167, 169-171].

GSK3β is a constitutively active enzyme. Various kinases are known to phosphorylate GSK3β at Ser-9 and thereby reduce its enzyme activity, including Akt (also known as protein kinase B; PKB), p90 ribosomal s6 kinase (p90Rsk), protein kinase A (PKA; also known as cyclic AMP-dependent protein kinase) and protein kinase C (PKC). On the contrary, phosphorylation of GSK3β at Tyr-216 by ZAK1 or Fyn increases its enzyme activity [163, 165, 169, 172, 173]. Various growth factors induce their neurotrophic effects via reducing the activity of GSK3β by phosphorylation at Ser-9, including fibroblast growth factor 1 (FGF1), FGF2, insulin growth factor 1 (IGF-1), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF); while pro-apoptotic stimuli increase GSK3β activity via phosphorylation at Tyr-216 [163, 165, 173, 174].

GSK3β plays a crucial role in regulating functioning of various structural, metabolic and signaling proteins in neurons including tau, MAP2, β-Catenin, activator protein 1 (AP-1), cyclic AMP response element binding protein (CREB), nuclear factor-kappa B (NF-κB), heat shock factor-1 (HSF-1) and others. These signaling proteins regulate a variety of cellular functions including neuronal plasticity, gene expression and cell survival [163, 165, 166, 175]. Dysregulated GSK3β activity appears to be operant in various neuropathological conditions, including Alzheimer’s disease, Parkinson’s disease, schizophrenia, autism and bipolar mood disorder, and pharmacological inhibition of GSK3β ameliorate these diseases [163, 169, 173, 175-183].
Role of GSK3β in HIV-1-mediated neurotoxicity

Various studies with neurons in cultures as well as with animal models have shown that HIV-1 induces neurotoxic effects via increase in the GSK3β activity, and the GSK3β inhibitors ameliorate these toxic effects [174, 184-188]. The pathways through which HIV-1 induces GSK3β activation are unclear. But the mechanisms through which GSK3β-activation promotes neuronal damage mainly include β-Catenin and tau phosphorylation [184, 185]. β-Catenin is an immediate downstream target of GSK3β; active GSK3β phosphorylates and thereby initiates rapid ubiquitin-mediated degradation of β-Catenin, which otherwise positively regulate cell survival and neurite outgrowth [173, 175, 189-191]. Tau-hyperphosphorylation has been associated with neurodegenerative disorders, including Alzheimer’s disease [192-194]. GSK3β is a principal tau-kinase [195-197]. Tau-hyperphosphorylation causes microtubule cytoskeleton destabilization, leads to neurite degeneration. In addition to β-Catenin and tau, GSK3β regulates the functioning/stability of various structural and signaling molecules that appears to play roles in cell death and neurite damage [165, 175].

Recent studies have identified GSK3β as a central mediator of pro-inflammatory processes in immune cells, including microglia [169, 198-200]. Thus, it is likely that GSK3β-activation may play role in HIV-1mediated immune and glial cell activation, and the release of pro-inflammatory cytokines, leading to ‘bystander’ neuronal damage. A recent study by Kehn-Hall et al. has shown that 6BIOder, a novel GSK3β-inhibitor, inhibits HIV-1 replication [201], suggesting that GSK3β-activation may play a role in viral propagation.

Taken together, these studies support a central role of GSK3β-activation in HIV-1-mediated neurotoxicity. In accord with these studies, several pilot clinical trials have demonstrated
improvements in neuropsychological performance in HAND patients treated with GSK3β inhibitors as adjunct therapy with stable cART [202-204]. Thus, it can be concluded that GSK3β-mediated signaling pathways are potential therapeutic target in treatment of neuroAIDS.

**Role of GSK3β in opiate-mediated neurotoxicity**

Results from some previous studies have conflict regarding whether opiates induce GSK3β activation or inactivation; some studies have shown that opiates induce GSK3β activation [205-207], while some have shown that they inactivate it [208, 209]. Altogether, from these studies it can be inferred that opiates induce dose- and time-dependent effects on GSK3β activity; acute activation of MORs inactivates GSK3β by inducing phosphorylation at Ser-9 via phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, while chronic activation of MORs induces GSK3β activation by inducing phosphorylation at Tyr-216, may be through elevation in cytosolic Ca$^{2+}$ [205].

Studies from brains of opiate abusing patients suggest a role of GSK3β-mediated signaling pathways in opiate-associated neuropathology. The accelerated deposition of hyperphosphorylated tau that occurs in the CNS of young opiate abusers [141, 142] is likely related to elevated GSK3β expression [141] since GSK3β is a principal tau kinase [195-197].
CHAPTER 2

Morphine Enhances HIV-1\textsubscript{SF162}-Mediated Neuron Death and Delays Recovery of Injured Neurites

(This chapter has been published as an article; PLoS ONE 9(6): e100196. doi:10.1371/journal.pone.0100196)

Introduction

Human immunodeficiency virus-1 (HIV-1) disrupts normal immune system function and leads to acquired immunodeficiency syndrome (AIDS). HIV-1 can also induce a wide range of central nervous system (CNS) deficits, collectively known as HIV-1-associated neurocognitive disorders (HAND). HIV-1 enters the CNS soon after initial systemic infection \cite{22, 24}. It is widely believed that virus penetrates the CNS within infected monocytes and lymphocytes \cite{22, 210}, which normally traffic across the blood-brain barrier (BBB) as a part of immune surveillance of the brain. Mature neurons are not infected by HIV-1; instead, infected and/or activated glial cells release various viral and cellular factors that induce direct and/or indirect neuronal toxicity, leading to HAND \cite{22, 65, 88, 211, 212}. Combination antiretroviral therapy (cART), which controls systemic HIV-infection, has improved the health status of a large segment of patients \cite{105, 111, 112}. Although cART has reduced the overall severity of neurocognitive disorders in HIV-1 patients, the prevalence of HAND remains at approximately 50\% \cite{88, 105-107, 111}. The persistence of relatively high rates of CNS disease is likely due to a combination of longer
patient survival, the relatively poor CNS penetrance of most antiretroviral drugs [88, 114], and their neurotoxic effects [115]. Even if the CNS viral load is extremely low or undetectable, neurodegeneration can still occur in response to viral proteins, such as transactivator of transcription (Tat), that are released from cells even when viral replication has been inhibited [124].

Injection drug abusers are at high risk of acquiring HIV-infection due to sharing of contaminated needles and unsafe sexual behavior. Nearly 30% of HIV-infected patients have a history of injection drug abuse involving opiates [125, 127]. Additionally, a subset of HIV+ patients is exposed to opiates through their legitimate use for treatment of AIDS-related chronic pain syndromes. As opiates by themselves are known to induce immunomodulatory or immunosuppressive effects, both in the periphery and CNS [132, 140], it is hypothesized that they may enhance virus spread or otherwise exacerbate disease processes. Experimental evidence also suggests that opiates can interact with HIV-1 or HIV-1-proteins directly on CNS cells and tissues [125, 145, 147-151, 153]. Among patients with HIV-1 infection, those who also abuse opiate drugs show faster progression to AIDS and more severe neurocognitive deficits [160-162].

Many previous studies have modeled HIV-neuropathology using individual viral proteins, such as Tat, glycoprotein 120 (gp120), and others. However, the CNS of HIV-infected patients is not only exposed to individual viral proteins, but instead to all cytokines/chemokines and other cellular products, viral proteins and virus particles released from infected and/or activated cells. Thus, to more closely model HIV-1-mediated neurotoxicity, we have used supernatant from HIV-1SF162-infected differentiated-U937 cells (HIV+ sup). The R5-tropic HIV-1SF162 strain was
used since R5-tropic (monocyte/macrophage-tropic) viruses are predominant in cerebrospinal fluid (CSF) and CNS parenchyma [213, 214].

Multiple outcome measures were studied after HIV$^{+}_{\text{sup}} \pm$ morphine treatments, including both cell death and neurite degeneration. Studies in the presence of glia allowed us to distinguish between direct and indirect neurotoxicity. Our results indicate that morphine worsens selective neurotoxic effects of HIV, and that glycogen synthase kinase-3β (GSK3β) signaling may be a point of convergence. Importantly, morphine limits the ability of neurons to recover from sublethal damage.

**Materials and Methods**

**Ethics statement**

Experiments were conducted in accordance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (Protocol Number: AM10158).

**Mixed glial cultures**

Cells were cultured from the striatum, a region targeted by HIV where opioid receptor levels are relatively high. Mixed glial cultures (astrocytes and microglia) from mouse striatum were prepared as previously described [151, 153], with minor modifications. Striata from P0-P1 ICR (CD-1) mice (Charles River Laboratories International, Inc., Wilmington, MA) were dissected, minced and enzymatically dissociated with trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and deoxyribonuclease (DNase; 0.015 mg/ml; Sigma-Aldrich) in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY) for 30 min at 37°C. Tissue was resuspended in
DMEM supplemented with 10% fetal bovine serum (FBS; Gibco), tritured and filtered through 100 and 40 μm nylon mesh pore filters respectively. Cells were plated and maintained in supplemented DMEM containing 10% FBS.

**Neuronal cultures**

Mouse striatal neuron cultures were prepared as previously described [151, 153]. Briefly, striata from E15-E16 ICR mice were dissected, minced and enzymatically dissociated with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in neurobasal medium (Gibco) for 30 min at 37°C. Tissue was resuspended in neurobasal medium supplemented with B-27 additives (Gibco), L-glutamine (0.5 mM; Gibco) and glutamate (25 µM; Sigma-Aldrich), tritured and filtered twice through 70 μm nylon mesh pore filters. Neurons were plated and maintained in supplemented neurobasal medium. Culture purity was determined by immunocytochemistry using anti-MAP-2 antibody (Abcam, Cambridge, MA; ab32454) and found to be > 80% neurons.

**Neuron-mixed glial co-cultures**

All cultures were prepared in 24 well plates pre-coated with poly-L-lysine (0.5 mg/ml; Sigma-Aldrich). Neurons were plated alone in 12 wells (neuron cultures); in the remaining 12 wells we established neuron-glia co-cultures as previously described [153]. Briefly, two deep midline grooves were made into the culture surface to restrict the movement of glial cells between sides. Glial cells (2×10^5 cells/well) were plated on one side of the grooves; when they became confluent (10 d), neurons (0.25×10^5 cells/well) were plated onto the entire culture surface. In these wells, all neurons are exposed to glial conditioned medium, but neurons on one side of the grooves contact the glial bedlayer, while neurons on the other side grow in isolation on the culture surface. It is difficult to visualize all neurite extensions when neurons are in contact with
the glial bedlayer. Thus, the co-culture studies used neurons that did not have direct contact with glia. All cultures were maintained in supplemented neurobasal medium; neurons were allowed to mature for 5-7 d prior to treatment.

**Supernatant from HIV-infected cells**

U937 cells (ATCC, Manassas, VA), a leukemic monocyte cell line originally derived from a histiocytic lymphoma, were plated at $0.5 \times 10^5$ cells/ml in RPMI-1640 media (Gibco) supplemented with 10% FBS, and activated/differentiated with interleukin-2 (IL-2, 100 ng/ml; Sigma-Aldrich), phytohaemagglutinin (PHA, 5 μg/ml; Sigma-Aldrich), and phorbol 12-myristate 13-acetate (PMA, 100 ng/ml; Sigma-Aldrich), for 48 h. Activated/differentiated cells were treated with Polybrene (2 μg/ml; Sigma-Aldrich) for 30 min at 37°C, resuspended in fresh medium and exposed to HIV-1SF162 (p24 = 50-100 pg/ml; from Dr. Jay Levy [215], through the NIH AIDS Research and Reference Reagent Program, Germantown, MD). After 7 d, supernatants were collected by filtering through a 0.20 μm filter. HIV infection was confirmed by quantification of p24 levels (HIV-1 p24 Antigen Capture Assay; Advanced Bioscience Laboratories, Rockville, MD) in culture supernatants; a 4-6 fold increase in p24 antigen levels was typical over 7 d. Supernatants from uninfected/untreated U937 cells (Control$_{sup}$) were used as a control. Cell culture supernatants were aliquoted and stored at -80°C.

**Treatments**

Morphine is the major metabolite of heroin in the CNS [216]; it preferentially targets μ-opioid receptors (MORs). Since opiates by themselves can affect HIV-1 infection and replication [217], it was important to assess the effects of opiate interactions using cell-free supernatants. $\text{HIV}^+_\text{sup}$
or Controlsup was added to neuronal cultures in the presence or absence of morphine sulfate (500 nM; Sigma-Aldrich) ± naloxone (1.5 µM; Sigma-Aldrich), a general opioid receptor antagonist.

**MTT assay**

At specific times after treatment, cells were rinsed and incubated with 1.2 mM 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldrazotium bromide (MTT; Molecular Probes, Grand Island, NY) in fresh, pre-warmed media at 37°C for 4 h. The medium was gently aspirated, and formazan crystals, the product of reduction of MTT by mitochondrial dehydrogenase in live cells, were dissolved in 100 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 37°C for 10 min. The amount of formazan was measured by absorbance at 540 nm using a PHERAstar microplate reader (BMG LABTECH Inc., Cary, NC).

**TUNEL assay**

At specific intervals after treatments, cells were fixed overnight at 2-8°C in 4% paraformaldehyde (Sigma-Aldrich), permeabilized at room temperature in 0.1% Triton-X 100 (Molecular Probes) and 0.1% BSA (Invitrogen, Grand Island, NY) for 15 min, and blocked in 0.1% BSA and 1% horse serum for 30 min. Fixed cells were stained for Hoechst 33342 (Sigma-Aldrich) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Roche Applied Sciences, Mannheim, Germany). Cells were visualized and digital images were acquired using an Axio Observer Z.1 microscope and Zen 2010 software (Zeiss Inc., Thornwood, NY). Neuronal apoptosis was assessed by manually counting the percentage of TUNEL(+) cells.
Assessment of neuronal viability

In each culture well, at least 50 healthy neurons were initially selected in 6-8 non-overlapping fields. After treatment, repeated images of pre-selected cells were captured at 1 h intervals, using a microscope with a computer-regulated stage (Axio Vision 4.6; Carl Zeiss Inc.) under controlled environmental conditions (37°C, 95% humidity and 5% CO₂) [151, 153]. At the end of each experiment, pre-selected neurons were assessed for viability at 6 h intervals in the digital images. Cell death was confirmed using rigorous morphological criteria including abnormal shrinking of the cell body and eventual cell body fragmentation, nuclear destruction, loss of phase-brightness, and excessive neurite loss [151, 153, 218]. In some experiments, live and dead cells were confirmed at the end of the experiment by staining respectively with calcein-AM and ethidium homodimer-1 (LIVE/DEAD Viability/Cytotoxicity Kit; Molecular Probes, Grand Island, NY). Findings were reported as the average percentage of neuron survival, with respect to pre-treatment neuron count ± standard error of the mean (SEM), and analyzed using a repeated measure analysis of variance (ANOVA) and Duncan’s post hoc test using Statistica 8.0 (StatSoft, Tulsa, OK).

Assessment of neurite length

At specific intervals after treatments, cells were fixed, permeabilized, blocked, and subsequently stained for TUNEL and MAP-2 (Abcam; ab32454); cells were visualized and digital images were acquired. In digital images, neuritic arborization was quantified only for live [TUNEL(-)] neurons, using modified Sholl analysis. A ‘Sholl score’ was measured by counting the number of intersections of MAP-2-positive neurites with equidistant concentric circles of increasing radius,
centered on the cell body [219]. The Sholl score was converted into neurite length in µm via micrometer calibration at the same magnification.

**Assessment of neurite growth/regrowth after treatment removal**

Prior to treatment, at least 15 healthy neurons were selected in 7-8 non-overlapping fields per well. Repeated images of pre-selected cells were captured at 1 h intervals after treatment onset. After 24 h, cells were gently rinsed with pre-warmed medium and returned either to a ‘control ± opiate environment’, which had Control_sup, or to a ‘HIV ± opiate environment’ which had HIV+sup (Table 1). We then continued to capture images of the same neurons at 1 h intervals for an additional 48 h (total 72 h). Thus, there were a total of 9 groups with varying exposure times to HIV+sup or Control_sup, in the presence or absence of morphine ± naloxone; these are outlined in Table 1.

Neurons that remained alive until the experiment end (72 h) were assessed for neuritic arborization in images taken at 0 h, 24 h, and 72 h using Sholl analysis. The findings were reported as average Sholl scores at each time, normalized to pre-treatment (0 h) scores ± SEM. Data were analyzed using a repeated measure ANOVA and Duncan’s post hoc test using Statistica 8.0 (StatSoft).

**ELISA**

Conditioned medium from mixed glial cultures were collected on the schedule described in Table 1 and assessed for brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), interleukin 6 (IL-6) and tumor necrosis factor α (TNFα) by ELISA according to the manufacturer’s instructions (BDNF and GDNF ELISAs: Abcam; IL-6 and TNFα ELISAs: R&D Systems, Minneapolis, MN). 3,3’,5,5’-tetramethylbenzidine (TMB)
substrate was added for color development and plates were read at 450 nm using a PHER Aster microplate reader immediately after terminating the reaction. Protein levels were determined based on a standard curve.

**Immunoblotting**

Whole cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (cOmplete - protease inhibitor cocktail tablets, and PhosSTOP - phosphatase inhibitor cocktail tablets; Roche), and total protein concentrations were determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL). Cell lysates containing equal amounts of total protein (~5-10 μg) were heated at 100º C for 5 minutes in laemmli buffer (Sigma-Aldrich), electrophoretically separated on a 10% SDS-polyacrylamide gels (Criterion Precast Gel; Bio-Rad, Hercules, CA), and transferred onto polyvinylidene difluoride (PVDF) membranes (Immun-Blot; Bio-Rad). Membranes were incubated with primary antibodies for phospho-GSK3β-Ser9 (p-GSK3β-S9; Cell Signaling Technology, Danvers, MA; 5558), GSK3β (t-GSK3β; Cell Signaling Technology; 9832) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam; ab8245). Appropriate horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) were used. Membranes were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and visualized using a Kodak Image Station 440CF.

**Statistical Analyses**

All data were expressed as average ± SEM. Unless otherwise indicated, data were analyzed statistically using a one-way ANOVA followed by Duncan’s post hoc test using Statistica 8.0 (StatSoft); an α level of $p < 0.05$ was considered significant.
**Results**

**Dose dependent neuron death and interactions with morphine**

To determine the HIV\(^{+}\)\(_{\text{sup}}\) concentration for subsequent experiments we assessed concentration-dependent toxicity in the presence or absence of 500 nM morphine, a titer chosen to maximally stimulate neuronal and glial MORs *in vitro*, and to result in dynamic Ca\(^{2+}\) changes and secretion of multiple cytokines and chemokines [145-147, 149, 151, 153, 220, 221]. HIV\(^{+}\)\(_{\text{sup}}\) at p24 ≥ 25 pg/ml and above showed significant toxicity even in the absence of morphine in an MTT assay. As expected, there was a concentration-dependent decrease in MTT reduction, indicating lower mitochondrial activity at higher HIV\(^{+}\)\(_{\text{sup}}\) levels. At p24 concentrations ≤ 10 pg/ml, HIV\(^{+}\)\(_{\text{sup}}\) did not affect the MTT assay unless cells were co-exposed to morphine, indicating a significant synergistic effect (Fig. 2.1). At p24 concentrations ≥ 25 pg/ml, there were no significant interactions of HIV\(^{+}\)\(_{\text{sup}}\) with morphine. The MTT assay is a measure of the activity of NADH and NADPH-dependent cellular oxidoreductase enzymes [222-224]. Although it is frequently used as an indicator of cell survival/toxicity or proliferation, it is only an indirect measure. Therefore, neuron survival was directly assayed using time-lapse imaging (Fig. 2.2). Like the MTT assay, time-lapse image analysis showed a p24 concentration-dependent decrease in neuron survival over a 48 h period (Fig. 2.2B). In contrast to the MTT assay, time-lapse image analysis also revealed interactive effects between HIV\(^{+}\)\(_{\text{sup}}\) and morphine on cell death at p24 concentrations of 10 and 25 pg/ml. As a p24 concentration of 25 pg/ml caused significant death/toxicity and also showed interactive effects with morphine, this titer was used in all further experiments. Importantly, 25 pg/ml falls within the range of p24 levels detected in the CSF of HAND patients on antiretroviral therapy (p24 = 43.2 ± 16.8 pg/ml) [225].
Toxic effects of HIV ± morphine in neuron cultures

Neuronal apoptosis was assessed using TUNEL staining (Fig. 2.3); all HIV$^+$$_{sup}$ treatment groups showed significantly enhanced neuronal apoptosis at all assessed time-points (Fig. 3B; Neuron panel). At all time-points, except 12 h, morphine significantly enhanced HIV$^+$$_{sup}$-mediated neuronal apoptosis and the interactive effects of morphine were blocked by naloxone. TUNEL staining is specific for death involving apoptotic pathways, and may not detect all dying neurons. Additionally, TUNEL does not distinguish and permit the exclusion of cells that were dead at the start of the treatment. Time-lapse imaging was used to more exactly follow cell survival/death (Fig. 2.4). Over the period of 72 h, HIV$^+$$_{sup}$ ± morphine treatments significantly reduced neuronal survival in cultures without glia (Fig. 4; Neuron panel). Morphine significantly enhanced neuronal death mediated by HIV$^+$$_{sup}$, and interactive effects of morphine were blocked by naloxone.

Sublethal synaptic losses and neuritic pruning are thought to be a major substrate of neurocognitive disorders [87-91]. Therefore, effects of HIV$^+$$_{sup}$ ± morphine treatment on neuritic arborization were assessed using MAP-2-immunostaining followed by modified Sholl analysis (Fig. 2.5); only those neurons determined to be alive by TUNEL assay were used in the analysis. At all assessed time-points, HIV$^+$$_{sup}$ ± morphine treatment groups showed significantly reduced neurite length (Fig. 2.5B; Neuron panel). Morphine did not show a significant interaction with HIV$^+$$_{sup}$ at any time.

Role of glia in HIV ± morphine-mediated neurotoxicity

HIV does not infect mature neurons; instead, virotoxins can cause indirect neuron damage via inducing an inflammatory response in activated and/or infected glia [22, 65, 88, 211, 212]. To
determine the role of glia in HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated neurotoxicity, treatments were carried out either in the presence or absence of glia. The presence of glia significantly increased the proportion of HIV\textsuperscript{+}\textsubscript{sup} ± morphine-induced TUNEL(+) neurons (Fig. 2.3B; compare panels). At the earliest time point examined (12 h), HIV\textsuperscript{+}\textsubscript{sup} and morphine displayed a significant interaction; however this only occurred in the presence of glia. Thus, glia appeared to accelerate the HIV\textsuperscript{+}\textsubscript{sup}-morphine interaction. At all time points except 72 h, the interactive effects of morphine were significantly attenuated by naloxone. Chronic exposure to naloxone is occasionally ineffective, even when acute blockade reverses morphine effects [226]. This may be due to blocking the cellular effects of the multiple opioids normally released by glia [227, 228]. The presence of glia significantly enhanced HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated neuron death over the entire 72 h experimental period (Fig. 2.4; compare panels).

In the subpopulation of neurons that survived, HIV\textsuperscript{+}\textsubscript{sup} induced significant neurite pruning or growth arrest. The presence of glia did not significantly affect HIV\textsuperscript{+}\textsubscript{sup}-induced neurite pruning, even when neurons were co-exposed to morphine. In fact, in the presence of glia, Control\textsubscript{sup}-treated groups had significantly longer neurites (Fig. 2.5B; compare panels).

**Reversibility of HIV ± morphine-mediated neurite damage**

Neurons appear to recover from certain types of sublethal damage caused by HIV-related insults [87, 88, 90]. Therefore, the reversibility of HIV- and morphine-mediated neurite damage was tested. HIV\textsuperscript{+}\textsubscript{sup} ± morphine treatments caused significant neurite growth arrest over the period of 24 h in cultures without glia (Fig. 2.6B; Neuron panel). With continuous exposure to HIV\textsuperscript{+}\textsubscript{sup} ± morphine for 72 h, neurite length remained significantly reduced. When HIV\textsuperscript{+}\textsubscript{sup} was removed at 24 h, neurites resumed their growth. Interestingly, sustained exposure to morphine by itself was
sufficient to reduce and/or delay neurite recovery/outgrowth despite the removal of HIV\textsuperscript{+}sup. This effect of morphine was blocked by naloxone.

Since glia support neurite outgrowth and synapse remodeling through multiple mechanisms [229-232] we tested whether glia play a role in the reversibility of HIV- and morphine-mediated neurite pruning/growth arrest. As in the neuron-only cultures, HIV\textsuperscript{+}sup ± morphine treatments caused significant neurite growth arrest over 24 h when glia were present (Fig. 2.6B; Neuron-glia panel). In the presence of glia, neurite outgrowth was significantly faster after removal of HIV\textsuperscript{+}sup than it was in neuron-only cultures (Fig. 2.6B; compare panels).

**HIV- and morphine-mediated effects on secretion of growth factors and cytokines by glia**

Our results show that glial effects on neuron injury and recovery are dependent on the context of HIV and morphine. Glia enhanced HIV-driven neuronal death (Fig. 2.3B and 2.4), but accelerated neurite recovery after removal of HIV (Fig. 2.6B). To determine how glia might direct these outcomes, we examined whether HIV and morphine affect glial production of secreted factors known to influence neuronal survival and outgrowth. ELISA was used to assay levels of the neurotrophic factors (BDNF and GDNF), as well as cytokines (IL-6 and TNFα) that indicate glial inflammatory activation (Fig. 2.7); they showed multiple response patterns. BDNF levels were significantly reduced by HIV\textsuperscript{+}sup ± morphine treatments. BDNF recovered to control levels after removal of HIV\textsuperscript{+}sup, even though morphine remained present. GDNF levels were unaffected by any treatment. IL-6 levels were increased by HIV\textsuperscript{+}sup or morphine treatment alone, and in addition, morphine significantly augmented the effect of HIV\textsuperscript{+}sup. Although IL-6 levels returned to control after removal of HIV\textsuperscript{+}sup, the elevated levels were maintained in the continued presence of morphine. TNFα release was significantly increased by HIV\textsuperscript{+}sup alone, but not by...
morphine alone, although morphine co-treatment augmented the effect of HIV$^+$sup. TNF$\alpha$ levels returned to control after removal of HIV$^+$sup, even in the continuous presence of morphine. Thus, among the secreted factors whose levels were influenced by HIV and morphine, BDNF, TNF$\alpha$ and IL-6 responded to HIV$^+$sup alone, while only IL-6 was affected by morphine itself. Both TNF$\alpha$ and IL-6 showed HIV-morphine interactive effects. Only IL-6 continued to respond to morphine exposure after HIV removal.

**GSK3$\beta$ as a point of convergence for HIV and morphine**

Previous studies have shown that HIV-1 induces neurotoxic effects by enhanced activation of GSK3$\beta$ [174, 184-188], and that GSK3$\beta$ is also linked to neuropathology seen with opiate-abusing patients [141, 142]. We therefore tested whether GSK3$\beta$ might be a site of HIV and morphine interactions. Neurons grown in isolation were lysed at 24 h after treatments with HIV$^+$sup ± morphine and immunoblotted for phospho-GSK3$\beta$-Ser9 (p-GSK3$\beta$-S9; an inactive form of GSK3$\beta$ [165, 173, 175, 233]), GSK3$\beta$ (total GSK3$\beta$; t-GSK3$\beta$) and GAPDH (Fig. 2.8). HIV$^+$sup and morphine by themselves induced significant reduction in p-GSK3$\beta$-S9 with respect to t- GSK3$\beta$. Morphine co-treatment significantly augmented HIV$^+$sup-mediated effects. All of the effects of morphine were blocked by naloxone.

**Discussion**

Our studies conclusively show that opiates can directly exacerbate the deleterious effects of HIV-1 on neurons in an infective model in vitro, although past studies have demonstrated that morphine interacts with the HIV-1 proteins Tat [149, 153] and gp120 [151]. The present studies also confirm and extend prior findings of glial involvement in interactions between opiates and
HIV proteins, demonstrating that combined morphine and HIV-1_{SF162} neurotoxicity can be amplified in the presence of glia. Lastly, we found that continuous morphine exposure significantly restricted the ability of neurons to recover from exposure to HIV^{+}_{sup}. This suggests that HIV-opiate co-exposure may trigger maladaptive cellular responses that persist in the presence of opiates alone, even after HIV infection is mitigated. Importantly, this situation is relevant to opiate-exposed patients whose HIV infection is controlled with cART.

**Experimental models for HIV ± opiate-mediated neurotoxicity**

Since HIV is a human-specific disease, models in other species have deficiencies as well as strengths. For example, non-human primates have been an invaluable model to assess interactive effects of the HIV-like simian immunodeficiency virus (SIV) and opiates [234, 235]. However, limited availability and the lack of established simian culture models make mechanistic studies difficult. There are rodent in vivo models that closely mimic viral infection, including an HIV-1 transgenic rat that expresses a majority of HIV-1 proteins without viral replication [236], and “humanized” SCID mice in which establishment of a human immune system in mice permits HIV infection [237]. However, in both cases the peripheral and central target cells are those of the rodent host. Our past *in vivo* studies have used a Tat transgenic mouse in which Tat production is largely restricted to the CNS [145, 148], and we have also examined effects of HIV-1 proteins on murine cells *in vitro* [149, 151-153, 218, 238]. In general, the findings in culture have paralleled outcomes *in vivo*; all have closely modeled key aspects of neurodegeneration and inflammatory biomarker production seen clinically in the CNS. We are specifically interested in effects on striatal neurons, since the striatum is a major target of HIV-1, and since levels of opioid receptors in the striatum are relatively high [149, 239, 240]. Although primary human cells may be preferable as an *in vitro* model, we have used murine targets since
human neurons/glia from specific brain regions are not consistently available, and outcomes frequently show regional specificity [241, 242]. Additionally, murine cultures (a) eliminate human genetic variability in terms of MOR [243], CCR5 [244, 245], and other factors that influence infective and neurodegenerative processes; and, (b) are free from any confounding effects of morphine on HIV replication in human microglia [217]. Still, the issue of species mixing must be considered when interpreting results in this model.

Neurotoxicity induced by HIV ± morphine

The extent to which opiates contribute to the progression of HAND in the era of cART remains controversial, although some large clinical studies now support moderate interactive effects [160, 161]. Opiate drugs of abuse have been shown to enhance particular damaging effects of HIV-1 proteins in vitro [149-153]. However, the CNS of HIV-1-infected patients is exposed to a great many other cellular and viral factors released from infected and/or activated cells. Current studies therefore used supernatant from HIV-infected cells to more fully represent the variety of those toxic and protective elements. HIV\textsuperscript{+}	extsuperscript{sup} caused neuronal death in a concentration-dependent manner over a range of p24 levels (10-500 pg/ml, Fig. 2.1 and 2.2), but significant morphine interactions were observed only at lower p24 levels (10 and 25 pg/ml). Very high levels of neuronal death at p24 ≥ 100 pg/ml may have masked interactive effects. If, as our data suggest, HIV-1-opiate interactions are partly governed by the level of infection, HIV-1 patients receiving cART may be especially vulnerable to opiate interactions since cART has greatly reduced the viral load [105, 111, 112]. The sensitivity of HIV-opiate interactions to levels of infection may also explain some controversy concerning the role of opiates in severity of HAND.
Since synaptic losses and neuritic pruning/degeneration are thought to be the principal substrate underlying HAND [87-91], we also examined length of neurites in cells that survived treatments. Our results show that HIV\textsuperscript{+} reduced the length of neurites, but unlike the cell death results, there were no significant morphine interactions (Fig. 2.5). Since HIV\textsuperscript{+} and morphine can induce multiple pathways, it is easily envisioned that interactions may differ between outcome measures. In some instances, cumulative reductions in synapses and dendritic simplification may culminate in cell death. Alternatively, neurite pruning may result in significant loss of cellular functions, but neurons may remain alive [238, 246]. Control treated groups actually showed an increase in the length of neurites over the same timeframe. This suggests that neurite length changes mainly reflected neurite growth arrest/inhibition. Results from repeated neurite length assessments of individual cells (Fig. 2.6) support this hypothesis. These conclusions are in conflict with some previous studies [152, 238, 247], where reduction in neurite length was mainly attributed to pruning of existing neurites. Disparate findings may reflect different types of neurons, their age and relative maturity, the response of neurons to individual viral proteins versus the multiple stimuli in HIV\textsuperscript{+}, and the selection criteria for neurons; we specifically evaluated sub-lethal neurite length changes by assessing only [TUNEL(-)] cells instead of the entire population.

Although many experimental and epidemiological studies have indicated a link between opiate drug exposure and HAND severity, the mechanisms underlying interactions between HIV-1 and opiates remain largely obscure. HIV-1 is known to induce neurotoxic effects through abnormal activation of GSK3β, and the GSK3β inhibitors, lithium (Li) and valproic acid (VPA), ameliorate HIV-1-mediated neurotoxicity [174, 184-188]. GSK3β signaling is also implicated in neuropathologic responses to opiates. For example, the accelerated deposition of
hyperphosphorylated tau that occurs in the CNS of young opiate abusers [141, 142] may be related to elevated GSK3β expression seen in opiate abusers [141] since GSK3β is a principal tau kinase [195-197]. GSK3β plays a crucial role in regulating the levels and function of various structural and signaling proteins in neurons including tau, MAP2, β-catenin, activator protein 1 (AP-1), cyclic AMP response element binding protein (CREB), heat shock factor-1 (HSF-1), and among others, all of which regulate neuronal plasticity, gene expression and survival [165, 166, 175]. GSK3β is thus well-positioned to be a potential convergence point for interactions between HIV-1 and opiates that regulate neuronal damage. Our results show that morphine co-exposure significantly augments HIV⁺SUP-mediated GSK3β-activation (Fig. 2.8), supporting this hypothesis.

**Role of glia in HIV ± morphine-mediated neurotoxicity**

Opiates exacerbate the release of numerous factors with neurotoxic potential from glia exposed to HIV [125, 147, 155], and alone or in concert with HIV can disrupt certain neuron-supportive functions of glia, including glutamate buffering, free radical scavenging, phagocytosis and release of neurotrophic factors [153, 155, 248, 249]. It is easily appreciated that glia might play a crucial role in HIV-opiate interactions; in our previous studies glia were actually required for interactive neurotoxicity between morphine and HIV-1 Tat [153]. In the present study, morphine significantly enhanced HIV⁺SUP-mediated striatal neuron death even in the absence of glia. One obvious interpretation is that morphine interacts with factors in addition to HIV-1 Tat in the HIV⁺SUP. Even among R5 strains, unique gp120 sequences may result in a different degree of interaction between opiates and HIV [151]. While glia are clearly important determinants of neurotoxic HIV-opiate interactions, some interactions, perhaps those involving factors other than Tat, seem to occur directly upon neurons.
Glia also modified neurite recovery, enhancing outgrowth when HIV was removed. The effect of glia on neurons is never entirely positive or negative but instead reflects the net input of various effectors that either promote or damage neurite/neuron structure and function [229-232]. In this context, our finding that glial production of BDNF is suppressed by HIV\textsuperscript{+\_sup} but then rebounds to control levels after removal of HIV\textsuperscript{+\_sup} shows a return towards a more trophic glial function. The normalization of proinflammatory cytokines TNF\textalpha{} and IL-6 after HIV removal indicates a similar trend, although note that continued exposure to morphine partly abrogates the effect of removing HIV (Fig. 2.7).

Overall, our results show that cellular and viral products released from HIV\textsubscript{1}\textsubscript{SF\textsubscript{162}}-infected leukemic monocytes have significant negative consequences on striatal neurons. Coincident exposure to morphine worsens neuronal outcomes in a concentration- and time-dependent manner. This is especially true when glia are present, although the net effects of glial exposure depend upon the local levels of virus and opiates. At lower viral titers, HIV\textsuperscript{+\_sup} has sublethal effects on growth of neurite arbors, indicating that neurons may undergo functional changes long before they die. This may be quite relevant to the situation in HIV-infected patients where dendritic/synaptic plasticity, not neuron death, is the presumed substrate of HAND. Diminished infection levels in the CNS are probably critical in reversing HIV-driven neurite damage, although our results caution that chronic exposure to opiates may inflict damage even in the absence of HIV.
### Tables

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Treatment from 0 to 24 h</th>
<th>Treatment from 24 to 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>72h (C)</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>72h (C + M)</td>
<td>Control + Mor</td>
<td>Control + Mor</td>
</tr>
<tr>
<td>72h (C + M + N)</td>
<td>Control + Mor + Nal</td>
<td>Control + Mor + Nal</td>
</tr>
<tr>
<td>72h (H)</td>
<td>HIV</td>
<td>HIV</td>
</tr>
<tr>
<td>72h (H + M)</td>
<td>HIV + Mor</td>
<td>HIV + Mor</td>
</tr>
<tr>
<td>72h (H + M + N)</td>
<td>HIV + Mor + Nal</td>
<td>HIV + Mor + Nal</td>
</tr>
<tr>
<td>24h (H) then 48h (C)</td>
<td>HIV</td>
<td>Control</td>
</tr>
<tr>
<td>24h (H + M) them 48h (C + M)</td>
<td>HIV + Mor</td>
<td>Control + Mor</td>
</tr>
<tr>
<td>24h (H + M + N) then 48h (C + M + N)</td>
<td>HIV + Mor + Nal</td>
<td>Control + Mor + Nal</td>
</tr>
</tbody>
</table>

Table 2.1: Treatment paradigm for neurite growth/regrowth assessments. Control = Control\(_{sup}\); HIV = HIV\(_{sup}\) (p24 = 25 pg/ml); Mor = morphine sulfate (500 nM); Nal = naloxone (1.5 μM).
**Figure 2.1: Concentration-dependent change in MTT reduction.** Cell toxicity/proliferation was analyzed in neuron cultures at 48 h after treatment using an MTT assay. The findings were reported as percent of control absorbance at 540 nm ($A_{540}$) ± SEM. Significance was analyzed using a one-way ANOVA and Duncan’s post hoc test, from $n = 3$ separate experiments. All treatment groups, except morphine alone [Control + Mor] and p24 = 10 pg/ml of HIV$^+$ sup [HIV(10)], showed significantly decreased absorbance at 540 nm ($^*p < 0.05$ vs. Control), likely reflecting neurotoxicity. HIV$^+$ sup caused a concentration-dependent reduction in $A_{540}$ ($^s p < 0.05$). Morphine did not show a significant interaction with HIV$^+$ sup at any p24 level. Control = Control$^+$ sup; HIV = HIV$^+$ sup (concentration of p24 in pg/ml is specified in parentheses); Mor = morphine sulfate (500 nM).
Figure 2.2: Concentration-dependent neuronal death in cultures treated with HIV^{+}_{sup} \pm \text{morphine}. Individual striatal neurons were selected prior to treatment and repeatedly imaged for 48 h after treatment. (A) Digital images of the same cells/fields, at 0 h, 24 h and 48 h after treatment (white arrowheads indicate cells that have died over the previous 24 h period). Live
and dead cells were confirmed at the end of the experiment by staining respectively with calcein-AM (green) and ethidium homodimer-1 (red); scale bar = 40 µm. (B) Cells were assessed for viability at 6 h intervals in digital images. Findings were reported as the average neuronal survival as a percent of pre-treatment neuron count ± SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test, from n = 3 separate experiments (at least 150 neurons per treatment group). Over the period of 48 h, all treatment groups except morphine alone [Control + Mor] and p24 = 10 pg/ml of HIV\textsuperscript{+} sup alone [HIV(10)], showed significantly reduced neuronal survival (*p < 0.05 vs. Control). Neuronal survival declined in a concentration dependent manner with HIV\textsuperscript{+} sup treatment (\$p < 0.05). Morphine showed significant interaction with HIV\textsuperscript{+} sup, but only at lower levels of exposure (p24 = 10 and 25 pg/ml) (#p < 0.05 vs. HIV\textsuperscript{+} sup alone at corresponding titer). Control = Control\textsubscript{sup}; HIV = HIV\textsuperscript{+} sup (concentration of p24 in pg/ml is specified in parentheses); Mor = morphine sulfate (500 nM).
**Figure 2.3: Neuronal apoptosis induced by HIV\(^+\)sup ± morphine.** Cells were fixed at specific intervals after treatment and labeled for Hoechst 33342 (blue) and TUNEL (red). (A) Digital images of neuronal cultures at 72 h after treatment; scale bar = 40 µm. (B) Apoptosis was assessed by manually counting the percentage of TUNEL(+) cells. Findings were reported as the average percentage of TUNEL(+) cells ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test, from \(n = 4\) separate experiments. At all assessed time points, in both culture systems, all groups exposed to HIV\(^+\)sup showed significantly enhanced neuronal apoptosis (*\(p < 0.05\) vs. respective C group). In all cases, except at 12 h in cultures with neurons alone, morphine significantly augmented HIV\(^+\)sup-mediated neuronal apoptosis (#\(p < 0.05\) vs. respective H group). In all cases, except for 72 h in neuron-glia cultures, the interactive effects of morphine were significantly attenuated by naloxone. In most cases, the presence of glia significantly enhanced HIV\(^+\)sup ± morphine-mediated neuron apoptosis (\(\gamma p < 0.05\) vs. corresponding treatment...
in neuron cultures; compare panels). C = Control_{sup}; H = HIV^{+}_{sup} (p24 = 25 pg/ml); M = morphine sulfate (500 nM); N = naloxone (1.5 \mu M).
Figure 2.4: HIV$^+$ sup ± morphine-mediated neuronal death. Neurons were repeatedly imaged for 72 h after treatment. Cells were assessed for viability at 6 h intervals in digital images. The findings were reported as the average percentage of neuron survival with respect to pre-treatment neuron count ± SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test, from n = 6 separate experiments. Over the period of 72 h, in both culture systems, all groups exposed to HIV$^+$ sup showed significantly reduced neuronal survival (*p < 0.05 vs. C). Morphine significantly enhanced HIV$^+$ sup-mediated neuronal death (#p < 0.05 vs. H), and the interactive effects of morphine were blocked by naloxone. In the presence of glia, HIV$^+$ sup ± morphine-mediated neuronal death was significantly enhanced (§p < 0.05 vs. corresponding treatment in neuronal cultures; compare panels). C = Control sup; H = HIV$^+$ sup (p24 = 25 pg/ml); M = morphine sulfate (500 nM); N = naloxone (1.5 µM).
**Figure 2.5: HIV\textsuperscript{+} sup ± morphine-mediated neurite damage.** Cells were fixed at specific intervals after treatment and labeled for MAP-2 (green) and TUNEL (red). (A) Digital images of neuronal cultures at 72 h after treatment; scale bar = 40 µm. (B) The ‘Sholl score’ was assessed only for TUNEL(-) neurons in the digital images and converted into neurite length in µm via a micrometer-scale calibration. The findings were reported as average total neurite length per neuron (µm) ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test from $n = 4$ separate experiments. At all time-points and in both culture systems, all groups exposed to HIV\textsuperscript{+} sup showed significantly reduced neurite length (*$p < 0.05$ vs. C). Morphine did not show a significant interaction with HIV\textsuperscript{+} sup treatment. The presence of glia did not have a significant effect on HIV\textsuperscript{+} sup ± morphine-mediated neurite damage, but in the presence of glia, Control\textsubscript{sup}-treated groups showed significantly longer neurite length ($^{\$}p < 0.05$ vs. corresponding treatment in neuron cultures; compare panels). C = Control\textsubscript{sup}; H = HIV\textsuperscript{+} sup (p24 = 25 pg/ml); M = morphine sulfate (500 nM); N = naloxone (1.5 µM).
Figure 2.6: Reversibility of HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated neurite damage. Images of pre-selected neurons were captured for 24 h after initial treatments, and for an additional 48 h after treatments were changed as described in Table 1. (A) Digital images of neuronal cultures after specified time and treatments (white arrowheads indicate area of neurite outgrowth since previous image); scale bar = 40 µm. (B) Neurons that remained alive until the experiment end
(72 h) were assessed for their arborization in images taken at 0, 24 and 72 h, using Sholl analysis. The findings were reported as average Sholl scores at each time, normalized to pretreatment (0 h) scores ± SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test, from \( n = 45-60 \) neurons per treatment group (sampled from 3 separate experiments; at least 15 neurons per group per experiment). Over the period of 24 h, and in both culture systems, HIV\(^+\)\(_{\text{sup}}\) ± morphine treatments induced neurite growth arrest; in neuron-glia cocultures, HIV\(^+\)\(_{\text{sup}}\) + morphine treatment appeared to cause neurite pruning (*\( p < 0.05 \) vs. 0 h, for corresponding treatment). After removing HIV\(^+\)\(_{\text{sup}}\) at 24 h, neurite growth arrest was reversible (\( \$ p < 0.05 \) vs. 24 h, for corresponding treatment); however, if HIV\(^+\)\(_{\text{sup}}\) ± morphine treatments were continued for 72 h, then neurite growth arrest was persisted. If morphine treatment continued after the removal of HIV\(^+\)\(_{\text{sup}}\), neurite outgrowth was significantly reduced/delayed compared to neurons returned to Control\(_{\text{sup}}\) (*\( p < 0.05 \) vs. ‘24 h (H) then 48 h (C)’). This effect of morphine was blocked by naloxone. In the presence of glia, neurite outgrowth after removal of HIV\(^+\)\(_{\text{sup}}\) was significantly enhanced, even in the continued presence of morphine (\( \$ p < 0.05 \) vs. corresponding treatment and time point in neuronal cultures; compare panels). C = Control\(_{\text{sup}}\); H = HIV\(^+\)\(_{\text{sup}}\) (p24 = 25 pg/ml); M = morphine sulfate (500 nM); N = naloxone (1.5 µM).
**Figure 2.7: HIV$^{+}_{\text{sup}} \pm$ morphine-mediated effects on secretion of growth factors and cytokines by glia.** After specified times and treatments, conditioned medium from mixed glial cultures was collected and assessed for levels of BDNF, GDNF, IL-6 and TNF$\alpha$ by ELISA; Growth factor / cytokine levels were determined based on a standard curve. The findings were reported as average concentrations (pg/ml) ± SEM. Significance was analyzed using a one-way ANOVA and Duncan’s post hoc test, from $n = 3$ separate experiments. **BDNF:** HIV$^{+}_{\text{sup}} \pm$ morphine treatments significantly reduced levels of BDNF (*$p < 0.05$ vs. ‘72 h (C)’); after removal of HIV$^{+}_{\text{sup}},$ BDNF returned to control levels ($^{\delta}p < 0.05$). **GDNF:** HIV$^{+}_{\text{sup}} \pm$ morphine
treatments did not have significant effects on GDNF levels. **IL-6**: HIV\textsuperscript{+\_sup} treatment significantly enhanced levels of IL-6; morphine treatment alone also significantly elevated IL-6 levels (*p < 0.05 vs. ‘72 h (C)’), and morphine co-treatment significantly augmented HIV\textsuperscript{+\_sup}-mediated effects (#p < 0.05). After removal of HIV\textsuperscript{+\_sup}, IL-6 returned to control levels (#p < 0.05); in the continuous presence of morphine, IL-6 remained at a significantly higher level than control (*p < 0.05) and [24h (H) then 48h (C)]-treatment group (#p < 0.05). **TNFα**: HIV\textsuperscript{+\_sup} treatment significantly enhanced levels of TNFα (*p < 0.05 vs. ‘72 h (C)’); morphine co-treatment significantly enhanced the HIV\textsuperscript{+\_sup}-mediated effect (#p < 0.05). After removal of HIV\textsuperscript{+\_sup}, TNFα levels returned to control values (#p < 0.05). C = Control\textsubscript{sup}; H = HIV\textsuperscript{+\_sup} (p24 = 25 pg/ml); M = morphine sulfate (500 nM).
Figure 2.8: HIV⁺SUP ± morphine-mediated GSK3β activation. Cells were lysed and immunoblotted for p-GSKβ-S9 (an inactive form of GSKβ), t- GSKβ (total GSKβ) and GAPDH in neuronal cultures at 24 h after treatment. Findings were reported as a percent of control values of p-GSKβ-S9 levels normalized with t-GSKβ (p-GSKβ-S9/t-GSKβ) ± SEM. Significance was analyzed using a one-way ANOVA and Duncan’s post hoc test, from n = 3 separate experiments. HIV⁺SUP caused significant loss of p-GSKβ-S9 (*p < 0.05 vs. C). Treatment with morphine alone also caused significant loss of p-GSKβ-S9, and morphine co-treatment significantly augmented the HIV⁺SUP-mediated effect (#p < 0.05). The effects of morphine were blocked by naloxone. C = ControlSUP; H = HIV⁺SUP (p24 = 25 pg/ml); M = morphine sulfate (500 nM); N = naloxone (1.5 μM).
CHAPTER 3

Comparison of HIV-1\textsubscript{LAI} and HIV-1\textsubscript{SF162}-Mediated Neurotoxicity and Interactions with Opiates

(This chapter is in preparation to submit for publication)

Introduction

Nearly half of the human immunodeficiency virus-1 (HIV-1)-infected individuals suffer from a wide range of neurological disorders, including motor disturbances, dementia and behavioral changes, collectively known as HIV-1-associated neurocognitive disorders (HAND) [100-103]. HIV-1 enters the brain parenchyma soon after initial systemic infection [22-24, 250]. HIV-1 itself rarely infects neurons [22, 47, 49, 58]; instead, infected and/or activated glial cells produce pro-inflammatory cytokines/chemokines and other cellular factors that, in conjunction with secreted viral proteins, induce ‘bystander’ damage to neurons and other cells in the CNS [22, 62-65, 251-253]. Though combination antiretroviral therapy (cART) has significantly improved the health status of a large number of AIDS patients, it has not been proved equally effective for HAND [88, 105-107, 111, 113, 254]. This high incidence of HAND despite cART may be due to the longer survival of AIDS patients with milder neurocognitive disorders or result from limited penetration of available anti-retroviral drugs across blood-brain barrier (BBB) [88, 112, 114], or may be because of both.
Nearly one third of HIV-infected patients have a history of drug abuse, commonly involving opiates [125-127]. Because of sharing of contaminated needles and unsafe sexual activities, drug abuse is a major risk factor for HIV-transmission. Since opiate drugs of abuse induce immunosuppressive/immunomodulatory effects [132, 140, 255], they not only increase the risk of HIV-infection but also exacerbate viral spread and disease progression. As opiate drugs of abuse themselves induce neurotoxic effects, such as BBB breakdown, glial cell activation, and neuronal injury [139, 140, 154, 158], it can be predicted that they may exacerbate HIV-1 pathogenesis in the CNS and the severity of HAND. Many previous studies have shown that opiates interact with and exacerbate HIV-1-mediated neurotoxic effects [125, 145, 148-153, 256]. This mimics more severe neurocognitive deficits observed in opiate drug abusing HIV+ patients [160-162].

Although many studies have indicated the link between opiate drug abuse and HAND progression, the extent to which opiates exacerbate neurocognitive deficits is still controversial [257-260]. In current studies, we tried to determine whether morphine, the major active narcotic metabolite of injected heroin [216], exacerbate HIV-1-mediated striatal neuron toxicity; we tested our hypothesis using striatal cell cultures because the striatum is a principal target of HIV-1 in the CNS and expresses high levels of opioid receptors [239, 240, 261, 262].

During initial stages of HIV-infection, R5-tropic strains predominate. However, during course of infection, the virus goes under evolution, in later stages of disease X4-tropic strains are more predominant. The co-receptor usage switch from CCR5 to CXCR4 is crucial in disease progression to AIDS [263-267]. Some previous studies have shown that drugs of abuse interact with virus or viral proteins in a strain/tropism-dependent manner [151, 268]. Our previous studies have shown that certain neurotoxic effects induced by HIV-1_SFL62, a R5-tropic strain, are
significantly enhanced by co-exposure to morphine [256]. The main aim of current work is to determine whether HIV-1$_{LAI}$, a X4-tropic strain induces similar neurotoxic effects and has similar interactions with opiates, or whether induces differential effects in strain-dependent manner.

Neurotoxic outcome measures were studied after treatments either with supernatant from HIV-1-infected cells (HIV$^+$$_{sup}$) or with purified virions (HIV$_{virion}$), in the presence or absence of morphine. Surprisingly, we found very little differences in X4 versus R5-tropic HIV$^+$$_{sup}$ mediated neurotoxicity and interactions with opiates. Interestingly, without intermediate effects of glia, purified virions did not induce significant neurotoxic effects and also did not cause significant interactions with morphine. Suggesting that HIV$^+$$_{sup}$ mediated neurotoxic effects and interactions with opiates are mainly due to cytotoxic/inflammatory milieu instead of virion itself.

**Materials and Methods**

Experiments were conducted in accordance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

**Mixed glia cultures**

Mouse striatal mixed glia (astrocytes and microglia) cultures were prepared as previously described [151, 153, 256]. Briefly, striata from P0–P1 ICR (CD-1) mice (Charles River Laboratories International, Inc., Wilmington, MA) were dissected, minced, and incubated with trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and deoxyribonuclease (DNase; 0.015 mg/ml; Sigma-Aldrich) in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY) for 30 min at 37°C. Tissue was resuspended in DMEM supplemented with 10% fetal bovine
serum (FBS; Gibco), triturated, and filtered through 100 and 40 μm pore nylon mesh filters. Cells were plated and maintained in DMEM supplemented with 10% FBS.

Neuron cultures

Neuron cultures from E15-E16 ICR (CD-1) mouse striatum were prepared as previously described [151, 153, 256]. Striata were dissected, minced, and enzymatically dissociated with trypsin (2.5 mg/ml) and DNase (0.015 mg/ml) in neurobasal medium (Gibco) for 30 min at 37°C. Tissue was resuspended in neurobasal medium supplemented with B-27 additives (Gibco), L-glutamine (0.5 mM; Gibco) and glutamate (25 μM; Sigma-Aldrich), triturated and filtered twice through 70 μm pore nylon mesh filters. Neurons were plated and maintained in supplemented neurobasal medium.

Neuron-mixed glia co-cultures

All cultures were prepared in 24 well plates coated with poly-L-lysine lysine (0.5 mg/ml; Sigma-Aldrich). In half of the wells neurons were cultured alone (indicated as neuron or neuron-alone cultures). In the remaining wells, we established neuron-mixed glia co-cultures (indicated as neuron-glia cultures) as described previously [153, 256]. Two deep midline grooves were made into the culture surface with a hot scalpel to inhibit the movement of glial cells between sides. Glial cells (2×10^5 cells/well) were plated on one side of grooves by tilting the plate at a sufficient angle. After glia on one side of the grooves were confluent (10 d), neurons (0.25×10^5 cells/well) were plated over the entire surface of the well. Neurons on one side of the grooves contact the glial bedlayer, while neurons on other side grow on the culture surface, without direct glial contact. However, all neurons are exposed to glial conditioned medium. It is hard to visualize the neurite extensions when neurons are in contact with glia. Thus, for co-culture studies we only
examined neurons growing on culture surface. Neuron and neuron-glia co-cultures were similarly maintained in supplemented neurobasal medium. Neurons were allowed to mature for 6-7 d prior to treatment.

**Supernatant from HIV-infected cells**

H9 and U937 cells (ATCC, Manassas, VA; 0.5 × 10⁵ cells/ml) were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS, and stimulated with interleukin-2 (IL-2, 100 ng/ml; Sigma-Aldrich), phytohaemagglutinin (PHA, 5 µg/ml; Sigma-Aldrich), and phorbol 12-myristate 13-acetate (PMA, 100 ng/ml; only for U937 cells to induce differentiation; Sigma-Aldrich), for 48 h. Stimulated cells were treated with polybrene (2 µg/ml; Sigma-Aldrich) for 30 min at 37°C, resuspended in fresh medium and exposed respectively to HIV-1\textsubscript{LAI} and HIV-1\textsubscript{SF162} (p24 = 25-50 pg/ml; X4-tropic, HIV-1\textsubscript{LAI}, from Dr. Jean-Marie Bechet and Dr. Luc Montagnier [269, 270], and R5-tropic, HIV-1\textsubscript{SF162}, from Dr. Jay Levy [215] through NIH AIDS Research and Reference Reagent Program, Germantown, MD). After 5-7 d, supernatants (HIV\textsuperscript{+}\textsubscript{sup}) were collected by filtering through a 0.20-µm filter, and viral productive infection was confirmed by quantifying HIV-p24 levels using ELISA (Advanced Bioscience Laboratories, Rockville, MD; typically a 3-5 fold increase in 5-7 d). Supernatants from uninfected/untreated H9 and U937 cells (Control\textsubscript{sup}) were used as a control. Cell culture supernatants were aliquoted and stored at -80°C.

**Treatments**

Our previous studies have shown that R5-tropic HIV\textsuperscript{+}\textsubscript{sup} induces neurotoxic effects in a concentration-dependent manner over a range of p24 levels (10-500 pg/ml). However, the interactions with morphine were significant only at lower HIV-exposure levels (p24 = 10 and 25 pg/ml) [256]. Accordingly, the present studies used a HIV\textsuperscript{+}\textsubscript{sup} exposure level of p24 = 25 pg/ml.
In addition, the neurotoxic effects mediated by purified virus (virus particles or virion; HIV\textsubscript{virion}) at the same p24 exposure level were tested. HIV\textsuperscript{+}/Control\textsuperscript{+} or HIV\textsubscript{virion} were added to neuronal cultures in the presence or absence of morphine sulfate (morphine; 500 nM; Sigma-Aldrich) ± naloxone (1.5 µM; Sigma-Aldrich), a general opioid receptor antagonist.

**Neuron viability assessments**

After treatment, digital images of pre-selected neurons were recorded at 1 h interval for 72 h using a microscope with computer-regulated stage (Axio Vision 4.6; Zeiss Inc., Thornwood, NY) and controlled environment (37°C, 95% humidity and 5% CO\textsubscript{2}). Pre-selected neurons were visually assessed for viability in digital images at 6 h intervals. Cell death was confirmed using rigorous morphological criteria, including excessive neurite disintegration, cell body fragmentation, nuclear destruction and loss of phase brightness [151, 153, 218, 256]. Findings were reported as average percentage of neuron survival as a proportion of pre-treatment neuron count ± standard error of the mean (SEM). The effect of each treatment on neuron survival was analyzed using a repeated measures analysis of variance (ANOVA) followed by Duncan’s post hoc test (Statistica 8.0; StatSoft, Tulsa, OK).

**TUNEL assay**

At specified intervals after treatment, cells were rinsed with PBS (Gibco) and fixed overnight at 4°C in 4% paraformaldehyde (Sigma-Aldrich). Cells were permeabilized at room temperature with 0.1% Triton-X 100 (Molecular Probes) and 0.1% BSA for 15 min, and subsequently blocked in 0.1% BSA (Invitrogen, Grand Island, NY) and 1% horse serum (Invitrogen) for 30 min. Cells were labeled with Hoechst 33342 (Sigma-Aldrich) and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling; Roche Applied Sciences, Mannheim,
Germany). Cells were visualized and digital images were acquired using an Axio Observer Z.1 microscope and Zen 2010 software (Zeiss Inc.). Neuronal apoptosis was assessed by manually counting the percentage of TUNEL(+) cells in digital images.

**Neurite length assessments**

At specified intervals after treatment cells were fixed, permeabilized, blocked and subsequently labeled with TUNEL and MAP-2 (Abcam, Cambridge, MA; ab32454). Cells were visualized and digital images were acquired. Neurite length was assessed in digital images using modified Sholl analysis. To specifically evaluate sub-lethal neurite length changes, only TUNEL(-) cells were examined. Sholl score was calculated by evaluating the cumulative intersections of MAP-2-positive neurites with equidistant concentric circles of increasing radius, centered on the cell body [219]. Sholl score was converted into neurite length in µm using micrometer-scale calibration.

**ELISA**

Conditioned medium from HIV-1\textsubscript{LAI}-infected H9 cells or HIV-1\textsubscript{SF162}-infected U937 cells (HIV\textsuperscript{+}\textsubscript{sup}) and uninfected H9 or U937 cells (Control\textsubscript{sup}) were collected at day 5 and assessed for levels of interleukin 6 (IL-6), tumor necrosis factor α (TNFα), RANTES (also known as CCL5), and monocyte chemotactic protein-1 (MCP-1, also known as CCL2), using ELISA (R&D Systems, Minneapolis, MN). 3,3’,5,5’-tetramethylbenzidine (TMB) substrate was added for color development and plates were read at 450 nm using a PHERAstar microplate reader immediately after terminating the reaction. Protein levels were determined based on a standard curve.
Statistical Analyses

All data were expressed as average ± SEM. Unless otherwise indicated, data were statistically analyzed using a one-way ANOVA and Duncan’s post hoc test (Statistica 8.0; StatSoft). An α level of $p < 0.05$ was considered significant.

Results

Toxic effects of HIV$^+$ sup ± morphine in neuron cultures

Cell survival was assessed using time-lapse imaging analysis (Fig. 3.1). In neuron-alone cultures, over the period of 72 h, HIV$^+$ sup significantly reduced neuron survival (Fig. 3.1B; Neuron panel). HIV$^+$ sup-mediated neuron death was significantly enhanced by morphine co-treatment, and the interactive effect of morphine was blocked by naloxone. Cell death was additionally confirmed using TUNEL-staining (Fig. 3.2). At all assessed time-points, HIV$^+$ sup significantly increased the percentage of TUNEL(+) neurons, which was augmented by morphine co-treatment (Fig. 3.2; Neuron panel). Interactive effects of morphine were blocked by naloxone.

Since sublethal synaptic losses and neurite damage are considered to be a principal substrate for HAND [87-91], changes in neurite lengths were assessed using MAP-2 immunostaining followed by Sholl analysis (Fig. 3.3). To specifically evaluate sublethal neurite length changes, only TUNEL(-) cells were assessed instead of whole cell population, where inclusion of dead cells may distort the findings. In neuron-alone cultures, HIV$^+$ sup significantly reduced neurite lengths at all assessed time-points. No significant morphine interactions were noted (Fig. 3.3B; Neuron panel).
Role of glia in HIV$^{+}_{\text{sup}} \pm$ morphine-mediated neurotoxic effects

HIV-1 itself rarely infects neurons [22, 47, 49, 58]; instead it induces indirect neurotoxic effects via release of cellular and viral toxic products from infected and activated glial cells [22, 62-65, 251-253]. To assess the role of glia in HIV $\pm$ morphine-mediated neurotoxicity all treatments were carried out either in presence or absence of glia. In the presence of glia, HIV$^{+}_{\text{sup}} \pm$ morphine-mediated neuron death was significantly enhanced (Fig. 3.1B and 3.2; compare panels). The presence of glia did not cause any significant effect on HIV$^{+}_{\text{sup}} \pm$ morphine-mediated neurite pruning (Fig. 3.3B; compare panels). In contrast, Control$_{\text{sup}}$-treated groups had significantly longer neurites.

Secretion of cytokines/chemokines from HIV-1-infected T-cells

The ‘indirect injury’ or ‘bystander damage’ hypothesis suggests that cytotoxic factors released from infected and activated cells cause neuronal injury [22, 62, 63]. Secretion of pro-inflammatory factors was analyzed by assessing levels of cytokines/chemokines in conditioned medium from HIV-1$_{\text{LAI}}$-infected H9 cells and HIV-1$_{\text{SF162}}$-infected U937 cells using ELISA (Fig. 3.4). After HIV-1-infection/exposure, with both viral strains, levels of IL-6, TNF$\alpha$ and RANTES were significantly increased. Infection of U937 cells with HIV-1$_{\text{SF162}}$ caused significant increase in levels of MCP-1, but no significant change in MCP-1 levels was seen after infection of H9 cells with HIV-1$_{\text{LAI}}$.

HIV$_{\text{virion}} \pm$ morphine-mediated neuronal death

Many previous studies have shown that HIV-1 proteins as well as cytotoxic factors released from infected and activated cells induce neuronal damage [22, 62, 63, 67, 68, 71, 72]. But the neurotoxic effects produced by the virion itself and their interactions with opiates are not known.
HIV-1-virion (HIV-1LAI and HIV-1SF162) and morphine-mediated neuronal death was assessed using time-lapse imaging analysis (Fig. 3.5). In neuron-alone cultures, over the period of 72 h, no significant change in cell survival was seen after any treatments (Fig. 3.5; Neuron panel). In neuron-glia co-cultures, only HIV-1LAI alone significantly reduced neuron survival (Fig. 3.5; Neuron-glia panel). Both viral strains significantly induced cell death when co-treated with morphine. Presence of glia significantly increased HIV-1LAI + morphine-mediated effects.

**Discussion**

The neurotoxic effects and interactions with opiates of HIV-1LAI-supernatant (a X4 tropic strain) are quantitatively similar to that of HIV-1SF162 (a R5-tropic strain). This suggests that the toxic effects are largely attributable to cellular factors released from infected and activated cells instead of viral strain-specific factors. Secretion of certain cytokines/chemokines by H9 cells was elevated after infection/exposure to HIV-1LAI. Surprisingly, the cytokine/chemokine release profile of HIV-1LAI-infected H9 cells is almost similar to that of HIV-1SF162-infected U937 cells. This is the first demonstration that HIV-1LAI-virion alone induces deleterious effects on neurons; however, this occurs only in the presence of glia. These results extend prior findings regarding the role of glia in HIV-1 mediated neurotoxicity.

Our results show that opiates interact with and exacerbate HIV$^+$sup$^{-}$mediated effects that result in neuronal death. But unlike cell death, opiates did not cause significant effects on HIV$^+$sup$^{-}$mediated neurite pruning. From these results it can be interpreted that HIV-1 induces multiple pathways in neurons that result in different outcomes, and opiates interact with only certain pathways. HIV-1 alone or in concert with opiates induces release of various neurotoxic factors from glia [125, 147, 155], they also disturb neuron-supportive functions of glia, such as
glutamate buffering, free radical scavenging, release of neurotrophic factors and others [153, 155, 248, 249]. Accordingly, our results also showed that HIV ± opiate mediated certain neurotoxic outcomes were exacerbated in presence of glia. Also severity of neurotoxic interactions between HIV-1 and opiates were enhanced in presence of glia. But HIV-1 and opiates showed some significant interactions in absence of glia too. Therefore, it can be concluded that though glia are important in HIV-1-opiate interactions, some interactions occur directly upon neurons.

The deleterious effects of HIV-1LAI-supernatant on neurons are quantitatively similar to that of HIV-1SF162, as previously observed [256]. These results are in conflict with some previous studies [271, 272], where strain/tropism specific differences in neuropathological profiles have been seen. Also some previous studies have shown that drugs of abuse interact with HIV-1-proteins in strain dependent manner [151, 268]. But in our studies we did not find quantitative differences between interactions of X4 and R5-tropic viral strain supernatants with opiates. Disparate findings may reflect the differences in response of neurons to individual viral proteins versus the multiple stimuli in HIV+ sup. Our results suggest that neurotoxic effects mediated by HIV+ sup are mainly attributed to common cytotoxic factors released from infected and activated cells instead of virion (viral strain) specific factors. These results support the theory that ‘indirect injury’ hypothesis is predominate over ‘direct injury’ hypothesis [62, 63].

Many previous studies have modeled HIV-1-neuropathology using individual viral proteins. But CNS of HIV-1-infected patients is not only exposed to individual viral proteins instead it is exposed to all cellular and viral factors and virions released from infected and/or activated cells. To model disease process more closely we used supernatant from HIV-1-infected cells. ‘Indirect injury’ or ‘bystander damage’ hypothesis suggest that HIV-1-mediated neurotoxic effects are
mainly attributed to cellular factors including cytokines, chemokines, excitotoxic factors and free radicals, released from infected and/or activated cells [22, 62-65, 251-253]. Our results show that after infection/exposure of H9 cells to HIV-1<sub>LAI</sub> secretion of certain cytokines including IL-6, TNFα and RANTES was enhanced. Surprisingly, cytokine/chemokine release profile, for assessed molecules, of HIV-1<sub>LAI</sub>-infected H9 cells is almost similar to that of HIV-1<sub>SF162</sub>-infected U937 cells; suggests reason behind the similarity between the neurotoxicity profiles of different viral strain-supernatants. Certain cellular factors are known to play role in HIV-1-mediated neurotoxicity [82, 273-276]. Although in current studies we have assessed only four cytokines/chemokines there must be other cellular factors released from infected and activated cells playing role in HIV-1-mediated neurotoxicity.

Our studies demonstrated that HIV<sub>virion</sub> induces neurotoxic effects but only in the presence of glia. Since we are using murine cell culture model, HIV<sub>virion</sub> cannot infect the glia in our cultures. Thus, it can be predicted that HIV<sub>virion</sub> may interact with glia and may induce release of cytotoxic factors and may also disturbs neuronal supportive functions of glia. These studies support and extend prior findings regarding the role of glia in HIV-1 mediated neurotoxicity [145, 153, 155, 277]. Though we have used HIV<sup>+</sup><sub>sup</sub> and HIV<sub>virion</sub> at same p24 concentration our results show that HIV<sup>+</sup><sub>sup</sub> is highly neurotoxic than HIV<sub>virion</sub>. One of the obvious interpretations for these findings is that HIV<sup>+</sup><sub>sup</sub> includes HIV<sub>virion</sub> as well as cellular and viral factors released from infected and activated cells. Greater neurotoxic outcomes than seen with HIV<sup>+</sup><sub>sup</sub> can be attributed to cellular and viral factors secreted from activated and infected cells. Also, the opiate-interactions seen with HIV<sup>+</sup><sub>sup</sub> are not evident with HIV<sub>virion</sub>. The neurotoxic interactions between HIV-1 and opiates are mainly caused by secreted factors instead of by HIV<sub>virion</sub>. 
Though we did not observe quantitative strain/tropism-specific differences in HIV$^{+}_{\text{sup}}$-mediated neurotoxic outcomes; surprisingly, HIV$^{+}_{\text{virion}}$ displayed strain-specific differences. It can be concluded that in case of HIV$^{+}_{\text{sup}}$, strain specific-effects by HIV$^{+}_{\text{virion}}$ are overshadowed by common secreted factors. Our results indicate that HIV-1$^{\text{LAI}}$ is slightly more neurotoxic than HIV-1$^{\text{SF162}}$. Our results are in accord with some previous studies which also have showed that X4-tropic viral strains are more neurotoxic than R5-tropic strains [271, 272].

Overall our results show that opiates interact with and exacerbate HIV$^{+}_{\text{sup}}$-mediated effects that result in cell death in striatal neuron cultures. HIV-1 and opiate mediated selective neurotoxic outcomes were enhanced in the presence of glia, suggesting role of glia in these processes. Surprisingly neurotoxic outcomes profile by X4 and R5-tropic HIV-1-supernatants were quantitatively similar. Since assessed cytokine/chemokine release profile by X4-tropic virus infected T-cells and R5-tropic virus infected monocytes were almost similar HIV$^{+}_{\text{sup}}$-mediated neurotoxic outcomes are mainly attributed to cytotoxic factors released from infected and activated cells. Our results show that X4-tropic virion is slightly more neurotoxic than R5-tropic virion, may explain why co-receptor usage switch from CCR5 to CXCR4 is crucial in disease progression [264, 267].
Figure 3.1. HIV$^+$ sup ± morphine-mediated neuronal death. (A) Cells were repeatedly imaged for 72 h after treatments; digital images of the same cells/fields at 0, 24 and 72 h (white arrowheads indicate dead cells that were alive in previous image). (B) Cells were assessed for viability at 6 h intervals in digital images. The findings were reported as the average percentage of neuron survival as a proportion of pre-treatment neuron count ± SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test; n = 4 separate experiments.
(at least 150 neurons per treatment group). In both culture systems, HIV$^+$ sup significantly reduced neuronal survival (*$p < 0.05$ vs. C), which was significantly augmented by morphine co-treatment (#$p < 0.05$ vs. H). Interactive effects of morphine were blocked by naloxone. In the presence of glia, HIV$^+$ sup $\pm$ morphine-mediated neuronal death was significantly enhanced ($^5p < 0.05$ vs. corresponding treatment in neuron-alone cultures; compare panels). C = Control$^{sup}$; H = HIV$^+$ sup; M = morphine sulfate; N = naloxone (1.5 $\mu$M).
Figure 3.2. Neuronal apoptosis induced by HIV$^{sup}$$\pm$ morphine. Cells were fixed at specific intervals after treatment and labeled for Hoechst 33342 (blue) and TUNEL (red). The rate of neuron apoptosis was assessed by counting the percentage of TUNEL(+) cells, and reported as average % of TUNEL(+) cells ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test; $n = 3$ separate experiments. At all assessed time points, in both culture systems, HIV$^{sup}$ significantly increased % of TUNEL(+) cells (*$p < 0.05$ vs. respective C group). Morphine significantly augmented HIV$^{sup}$-mediated neuronal apoptosis (#$p < 0.05$ vs. respective H group), interactive effects of morphine were blocked by naloxone. In the presence of glia, HIV$^{sup}$$\pm$ morphine-mediated neuronal apoptosis was significantly augmented ($^\$$p < 0.05 vs. corresponding treatment in neuron cultures; compare panels). C = Control$^{sup}$; H = HIV$^{sup}$; M = morphine sulfate; N = naloxone.
**Figure 3.3. HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated neurite damage.** Cells were fixed at specific intervals after treatment and labeled for MAP-2 (green) and TUNEL (red). (A) Digital images of neuronal cultures at 72 h after treatment. (B) The ‘Sholl score’ was assessed only for TUNEL(-) neurons in the digital images and converted into neurite length in \( \mu \text{m} \) via a micrometer-scale calibration. The findings were reported as average neurite length per neuron (\( \mu \text{m} \)) ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test from \( n = 3 \) separate experiments. At all time-points, in both culture systems, HIV\textsuperscript{+}\textsubscript{sup} significantly reduced neurite length (\( ^*p < 0.05 \) vs. C), without any significant morphine interaction. In the presence of glia, Control\textsubscript{sup}-treated groups showed significantly longer neurite length (\( ^S_p < 0.05 \) vs. corresponding treatment in neuron cultures; compare panels). C = Control\textsubscript{sup}; H = HIV\textsuperscript{+}\textsubscript{sup}; M = morphine sulfate; N = naloxone.
Figure 3.4. Cytokines/chemokines secretion from HIV-1-infected cells. Conditioned medium from HIV-1<sub>LAI</sub> (X4)-infected H9 cells or HIV-1<sub>SF162</sub> (R5)-infected U937 cells (HIV<sup>+</sup><sub>sup</sub>) and uninfected H9 or U937 cells (Control<sub>sup</sub>) at day 5 were collected and assessed for levels of IL-6, TNFα, RANTES and MCP-1, using ELISA. The findings were reported as average concentrations (pg/ml) ± SEM. Significance was analyzed using a one-way ANOVA and Duncan’s post hoc test, from \( n = 3 \) separate experiments. HIV-1-infection, with both viral strains,
elevated IL-6, TNFα and RANTES levels (*p < 0.05 vs. Control_{sup}). Infection of U937 cells with HIV-1_{SF162} caused significant increase levels of MCP-1, but no significant change in MCP-1 levels was seen after infection of H9 cells with HIV-1_{LAI}. 
Figure 3.5. HIV\textsubscript{virion} \pm morphine-mediated neuronal death. Neurons were repeatedly imaged for 72 h after treatment. Cells were assessed for viability at 6 h intervals in digital images. The findings were reported as the average percentage of neuron survival with respect to pre-treatment neuron count \pm SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test, from $n = 4$ separate experiments. In neuron-alone cultures, no significant change in cell survival was seen after any treatments. In the presence of glia, only HIV-1\textsubscript{LAI} alone significantly reduced neuron survival (*$p < 0.05$ vs. C), while both viral strains significantly increased cell death when co-treated with morphine. Presence of glia significantly increased
HIV-1_{LAI} + morphine-mediated neuronal death ($p < 0.05$ vs. corresponding treatment in neuronal cultures; compare respective panels). C = Control (cell culture medium); H = HIV_{virion} (pure virus; HIV-1_{LAI} and HIV-1_{SF162}); M = morphine sulfate.
CHAPTER 4

GSK3β-Activation is a Point of Convergence for HIV- and Opiate-Mediated Interactive Neurotoxicity.

(This chapter is in preparation to submit for publication)

Introduction

Infection of the central nervous system (CNS) with human immunodeficiency virus-1 (HIV-1) occurs rapidly after primary peripheral infection [22-24, 278]. HIV-1 can induce a wide range of CNS deficits, collectively known as HIV-1-associated neurocognitive disorders (HAND); nearly 50% of the HIV-1-infected individuals suffer from HAND [100-103]. Infected and/or activated glial and immune cells in the CNS release various viral and cellular factors that drive direct and indirect neuronal toxicity, leading to HAND [22, 62-65]. The advent of combination antiretroviral therapy (cART) has reduced the severity of HAND, but the prevalence of disease remains the same [88, 105-107, 111, 113, 254], likely due to longer patient survival [112], the relatively poor CNS penetrance of available antiretroviral drugs [88, 114], their neurotoxic effects [115], or a combination of all.

Drug abuse is a major risk factor for HIV infection; nearly 30% of HIV+ patients have a history of drug abuse involving opiates [125, 127]. A large fraction of HIV+ patients are also exposed to opiates for treatment of AIDS-related chronic pain syndromes. Opiate drugs of abuse modulate immune function [132, 140, 255], and studies also have shown that opiates promote HIV-1
replication in vitro [135, 217]. Thus, opiate abuse not only increases the risk of HIV-infection but may also exacerbate disease progression. Since opiates by themselves have outcomes involved in neuronal dysfunction, such as blood brain barrier breakdown, immune and glial cell activation, and neuronal damage [139, 140, 154, 158], it can be predicted that opiate drug abuse might exacerbate HIV-1 pathogenesis in the CNS. Our previous work has shown that certain neurotoxic effects induced by the individual HIV-1 proteins, trans-activator of transcription (Tat) and glycoprotein 120 (gp120) [148, 149, 151-153], and by HIV+ supernatant (HIV+ sup) [256], are enhanced by co-exposure to morphine, the major metabolite of heroin in the CNS [216]. This mimics co-morbid neurological effects observed in opiate-abusing HIV+ patients [160, 161]. Although there is a correlation between opiate drug abuse and HAND progression, the mechanisms that underlie interactions between HIV-1 and opiates remain obscure; the main aim of this work was to identify point(s) of convergence for HIV-1 and morphine signaling in neurons.

Identified originally as a regulator of glycogen metabolism, glycogen synthase kinase-3β (GSK3β) is a central component of various signaling pathways in neurons including those affecting neuronal plasticity, gene expression, and cell survival [163, 165, 173]. Dysregulated GSK3β activity appears to be operant in various neuropathological conditions, including Alzheimer’s disease, Parkinson’s disease, schizophrenia, autism, and bipolar mood disorder, and pharmacological inhibition of GSK3β can ameliorate these diseases [163, 169, 173, 175, 176, 180-183]. Previous studies have shown that HIV-1 induces neurotoxic effects through abnormal activation of GSK3β [174, 184-188], and that GSK3β is also linked to neuropathology seen with opiate-abusing patients [141, 142]. We therefore tested GSK3β-activation as a point of convergent signaling for interaction between HIV-1 and morphine.
Both lethal and sublethal effects of HIV$^{+}$ sup $\pm$ morphine treatments on neurons were assessed in populations, and also by time-lapse imaging of individual cells over 72 h. Multiple GSK3β inhibitors significantly reduced HIV-1-mediated neurotoxic outcomes, and also negated interactions with morphine that resulted in cell death.

**Materials and Methods**

All experimental procedures were reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

**Striatal neuron cultures**

Our culture model system uses striatal neurons because the striatum is a principal target of HIV-1 and levels of opioid receptors in the striatum are relatively high [239, 240, 261]. Mouse striatal neurons were cultured as previously described [151, 153, 256, 279]. In brief, striata from E15-E16 ICR (CD-1) mice (Charles River Laboratories International, Inc., Wilmington, MA) were dissected, minced and incubated with trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and deoxyribonuclease (DNase; 0.015 mg/ml; Sigma-Aldrich) in neurobasal medium (Gibco, Grand Island, NY) for 30 min at 37°C. Tissue was resuspended in neurobasal medium supplemented with B-27 additives (Gibco), L-glutamine (0.5 mM; Gibco), glutamate (25 $\mu$M; Sigma-Aldrich) and Antibiotic-Antimycotic (Gibco), triturated, and filtered twice through 70 $\mu$m nylon mesh pore filters (BD Biosciences, San Jose, CA). Cells were seeded into culture plates (Corning Inc., Corning, NY) pre-coated with poly-L-lysine (0.5 mg/ml; Sigma-Aldrich), in supplemented neurobasal medium. Purity was determined by immunostaining for microtubule-associated
protein 2 (MAP-2, a dendrite marker; Abcam, Cambridge, MA; ab32454), and cultures were found to be > 80% neurons.

**HIV supernatants**

Cells of the acute monocytic leukemia cell line, THP1 (ATCC, Manassas, VA) were cultured at 0.5 × 10⁵ cells/ml in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/ml Penicillin-Streptomycin (Gibco), and stimulated with interleukin-2 (IL-2; 100 ng/ml; Sigma-Aldrich) and phytohaemagglutinin (PHA; 5 μg/ml; Sigma-Aldrich), for 48 h. Stimulated cells were treated with polybrene (2 μg/ml; Sigma-Aldrich) for 30 min at 37°C, resuspended in fresh medium and exposed to HIV-1₁₆₂ (p24 = 25-50 pg/ml; a R5-tropic HIV-1 strain; from Dr. Jay Levy [215], through NIH AIDS Research and Reference Reagent Program, Germantown, MD). After 5 d, supernatants (HIV⁺ sup) were collected by filtering through a 0.20-μm filter, and viral infection was confirmed by quantifying HIV-p24 levels using ELISA (Advanced Bioscience Laboratories, Rockville, MD; typically a 3-5 fold increase in 5 d). Supernatants from uninfected/untreated THP1 cells (Control sup) were used as a control. Cell culture supernatants were aliquoted and stored at -80°C.

**Treatments**

As opiates can enhance HIV replication [135, 217, 280], we assessed the interactive effects of morphine with cell-free, viral supernatant. Our previous studies showed that supernatants from HIV-infected cells induce neurotoxic effects in a concentration-dependent manner over a range of p24 levels (10-500 pg/ml), but the interactions with morphine were significant only at lower HIV-exposure levels (p24 = 10 and 25 pg/ml) [256]. Current studies used HIV⁺ sup exposure level of p24 = 25 pg/ml. HIV⁺ sup and Control sup were added to neuronal cultures in the presence or
absence of morphine (morphine sulfate; Sigma-Aldrich), at a dose that maximally stimulate
neuronal and glial μ-opioid receptors (MORs) in vitro (500 nM) [145, 147, 149, 151, 153, 220,
221, 256]. To determine the role of GSK3β in HIV- and opiate-mediated neurotoxicity, HIV^+sup
± morphine treatments were carried out either in the presence or absence of a standard GSK3β
inhibitor, sodium valproate (valproic acid, VPA; 1 mM; Sigma-Aldrich), or one of two small
molecule inhibitors, SB415286 (10 µM; Sigma-Aldrich) and GSK3β inhibitor XXVI (XXVI; 1
µM; EMD Millipore, Billerica, MA).

**TUNEL assay**

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C, permeabilized in
0.1% Triton-X 100 (Molecular Probes) and 0.1% BSA (Invitrogen, Grand Island, NY) for 15
min, blocked in 0.1% BSA (Invitrogen, Grand Island, NY) and 1% horse serum (Invitrogen) for
30 min, and subsequently labeled with Hoechst 33342 (Sigma-Aldrich) and TUNEL (terminal
deoxynucleotidyl transferase dUTP nick end labeling; Roche Applied Sciences, Mannheim,
Germany). Cells were visualized and digital images were acquired using the Axio Observer Z.1
microscope and Zen 2010 software (Zeiss Inc., Thornwood, NY). Neuronal apoptosis was
assessed by manually counting the percentage of TUNEL(+) cells.

**Assessment of neuron viability**

Time-lapse digital images of pre-selected neurons were captured at 1 h interval for 72 h after
treatments using a microscope with computer-regulated stage (Axio Vision 4.6; Carl Zeiss Inc.)
and controlled environment (37°C, 95% humidity, 5% CO₂). In digital images, neurons were
assessed for viability at 6 h intervals using rigorous morphological criteria confirming cell death,
including cytoplasmic swelling, nuclear destruction, cell body fragmentation, excessive neurite
degeneration, and loss of phase brightness [151, 153, 218, 256, 279]. Findings were reported as the average percentage of neuronal survival, with respect to pre-treatment neuron count ± standard error of the mean (SEM). The effect of each treatment on neuronal survival was analyzed using a repeated measures analysis of variance (ANOVA) followed by Duncan’s post hoc test (Statistica 8.0; StatSoft, Tulsa, OK) from n = 3-5 experiments (at least 50 neurons per treatment group per experiment; total, 150-250 neurons per treatment group).

Assessment of neuritic arborization

Cells were fixed, permeabilized, blocked, and subsequently labeled for MAP-2 (Abcam), TUNEL (Roche Applied Sciences), and Hoechst 33342 (Sigma-Aldrich). Cells were visualized and digital images were acquired. Neuritic arborization was evaluated using Sholl analysis; a ‘Sholl score’ was measured by counting the number of intersections of MAP-2-positive neurites with equidistant concentric circles of increasing radius, centered on the cell body [219]. To avoid the inclusion of dead cells, we specifically evaluated neuritic arborization changes by including only TUNEL(-) cells in our assessments.

Immunoblotting

Whole cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) with protease and phosphatase inhibitors (Roche Applied Sciences), and total protein concentrations were determined by bicinechonic acid (BCA) assay (Thermo Fisher Scientific, Rockford, IL). Cell lysates containing equal amounts of total protein (~5-10 μg) were heated at 100º C for 5 minutes in laemml buffer (Sigma-Aldrich), electrophoretically separated on a 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were incubated with primary antibodies to

71
phospho-GSK3β-Ser9 (Cell Signaling Technology, Danvers, MA; 5558), GSK3β (Cell Signaling Technology; 9832), β-Catenin (Cell Signaling Technology; 9562), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam; ab8245), MAP-2 (Abcam; ab32454) and postsynaptic density protein 95 (PSD-95; UC Davis/NIH NeuroMab Facility, Davis, CA; 73-028). Appropriate horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech, Birmingham, AL) were used. Membranes were detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), and visualized using a Kodak Image Station 440CF.

**Statistical Analyses**

All data were expressed as average ± SEM. Unless otherwise indicated, data were analyzed statistically using a one-way ANOVA followed by Duncan’s post hoc test using Statistica 8.0 (StatSoft); an α level of $p < 0.05$ was considered significant.

**Results**

**HIV- and morphine-mediated GSK3β activation**

GSK3β activation/inactivation was assessed at 4 h after treatments. Cell lysates were immunoblotted for phospho-GSK3β-Ser9 (p-GSK3β-S9; an inactive form of GSK3β [165, 173, 175, 233]), GSK3β (total GSK3β; t-GSK3β), β-Catenin, and GAPDH (Fig. 4.1). $\text{HIV}^+_{\text{sup}}$, with or without morphine, significantly decreased p-GSK3β-S9 (normalized with t-GSK3β; p-GSK3β-S9/t-GSK3β; Fig. 4.1A), suggesting an increase in GSK3β activation. VPA significantly abrogated $\text{HIV}^+_{\text{sup}} \pm$ morphine-mediated effects. β-Catenin is a downstream target of GSK3β; active GSK3β phosphorylates β-Catenin and initiates rapid ubiquitin-mediated degradation by the proteasome [173, 175, 189]. $\text{HIV}^+_{\text{sup}}$, with or without morphine, significantly reduced β-
Catenin levels (Fig. 4.1B), suggesting an enhancement in GSK3β activity. VPA completely inhibited the effect of HIV\textsubscript{sup} + morphine and partially inhibited the effect of HIV\textsubscript{sup} alone, returning both to levels that were not different from control levels.

Opiates are known to induce time-dependent effects on GSK3β activation [205, 206]. Since significant individual or interactive effects of morphine were not detected at 4 h, HIV\textsubscript{sup} and morphine-mediated effects were assessed at longer time intervals (Fig. 4.2). At all assessed time points (12, 24, and 72 h), HIV\textsubscript{sup} significantly reduced p-GSK3β-S9. At 24 and 72 h, morphine alone also induced significant loss of p-GSK3β-S9, but a significant interaction with HIV\textsubscript{sup} was seen only at 24 h.

**Role of GSK3β in HIV ± morphine-mediated cell death**

Neurons exposed to morphine and/or VPA had survival values equivalent to control, while all groups treated with HIV\textsubscript{sup} showed significantly reduced neuronal survival (time-lapse imaging analysis; Fig. 4.3B). Morphine significantly enhanced HIV\textsubscript{sup}-mediated neuronal death. HIV\textsubscript{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment. The survival of neurons treated with ‘HIV\textsubscript{sup} + VPA’ and ‘HIV\textsubscript{sup} + morphine + VPA’ was not significantly different, showing that VPA effectively negated any HIV\textsubscript{sup}-morphine interactions. Cell death was additionally confirmed using TUNEL staining (Fig. 4.3C). HIV\textsubscript{sup} induced a significant increase in TUNEL(+) neurons, which was augmented by morphine co-treatment. HIV\textsubscript{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment. As seen for the time-lapse analyses, VPA also nullified the interactions between HIV\textsubscript{sup} and morphine.
Role of GSK3β in HIV ± morphine-mediated changes in neuritic arborization, MAP-2 and PSD-95

Since neurite degeneration and synapse losses are the major substrate for HAND [87-91] we assessed changes in neuritic arborization using MAP-2 immunostaining followed by Sholl analysis (Fig. 4.4A and B). At 72 h, HIV$^+$ sup induced a significant reduction in neuritic arborization (Fig. 4.4B). There was no morphine interaction, and the HIV effect was partially, but significantly, reversed by VPA co-treatment. Neuritic/synaptic degeneration was additionally confirmed by immunoblotting for MAP-2 and PSD-95 (Fig. 4.4C and D). At 72 h, HIV$^+$ sup significantly decreased MAP-2. Similar to the Sholl analysis, there was no morphine interaction (Fig. 4.4C), and VPA significantly abrogated HIV$^+$ sup-mediated loss of MAP-2. HIV$^+$ sup also induced a significant loss in PSD-95, but in this case there was an increased loss with morphine co-treatment (Fig. 4.4D). VPA partially, but significantly, reversed HIV$^+$ sup ± morphine effects on PSD-95. The HIV-morphine interaction on PSD-95 levels was maintained in the presence of VPA.

Effects of small molecule GSK3β-inhibitors

The role of GSK3β activation in HIV$^+$ sup ± morphine-mediated neurotoxicity was confirmed by the additional use of the small molecule GSK3β-inhibitors, SB415286 or XXVI (Fig. 4.5). Both small molecule inhibitors partially, but significantly, reduced HIV$^+$ sup ± morphine-mediated neuronal death, and also abrogated HIV$^+$ sup-morphine interactions (Fig. 4.5A and B). Both inhibitors reversed the effect of HIV$^+$ sup on neurite loss, although SB415286 only resulted in partial recovery (Fig. 4.5C).
Discussion

The current studies show that GSK3β inhibitors reduce many neurotoxic effects of HIV+sup ± morphine, including cell death, neurite pruning, and loss of structural and functional proteins, indicating that GSK3β plays an active role in multiple, degenerative processes. Interactions between HIV+sup and morphine enhanced neuronal death and increased the loss of PSD95, similar to interactions that we have previously reported for HIV proteins and morphine [148, 149, 151-153]. Importantly, GSK3β-inhibitors entirely negated the HIV-morphine interactive effect on neuron death, demonstrating for the first time that GSK3β-activation is an important point of convergence for neurotoxic interactions between HIV and opiates. GSK3β-inhibitors only partially reversed certain effects of HIV+sup, and did not disrupt HIV-morphine interactive effects on PSD95, indicating the likely involvement of other signaling pathways in various aspects of HIV± morphine-related neurotoxicity.

Though HIV-1 induces profound changes in neuronal morphology, function, and survival, the virus itself rarely infects neurons [22, 47, 49, 58]; instead, infected and activated glial cells produce cellular and viral products that drive secondary toxicity in neurons, known as ‘bystander damage’ [22, 62-65]. Our previous studies have shown that glia play a crucial role in determining the extent of neurotoxic interactions between the HIV-1 proteins, Tat or gp120 and opiates [151, 153] as well as those between supernatant from HIV-1 infected cells and opiates [256]. As the present studies primarily focus on understanding the pathway(s) in neurons that underlie HIV-1 and opiate interactions, we excluded glia from our culture model system. Still, it must be remembered that glial mechanisms play important roles in HIV- and opiate-mediated neurotoxicity.
In accord with many previous studies [174, 184-188], our results show that HIV$^+$sup induces GSK3β-activation, and that treatments to inhibit GSK3β activation ameliorate HIV$^+$sup-mediated neurotoxic effects. GSK3β-activation clearly plays a central role in HIV-1-associated neuronal damage. Abnormally active GSK3β can promote neuronal damage via dysregulating function/stability of various structural, metabolic, and signaling proteins, including tau, β-catenin, MAP-2, activator protein 1 (AP-1), cyclic AMP response element binding protein (CREB), nuclear factor-kappa B (NF-κB), heat shock factor-1 (HSF-1), and others. [163, 165, 166, 175]. In our study, certain neurotoxic outcomes due to HIV$^+$sup were only partially reversed by co-treatment with GSK3β inhibitors, suggesting the involvement of other factors such as mitogen-activated protein kinase 10 (MAPK10), double-stranded RNA-activated protein kinase (PKR), and cyclin-dependent kinase 5 (CDK5) [174, 281, 282].

Studies from the brains of opiate-abusing patients have indirectly implicated GSK3β-activation in opiate-associated neuropathology [141, 142]. However, previous studies have disagreed on whether opiates induce GSK3β activation [205, 206] or cause inactivation [208, 209]. Opiates induce effects on GSK3β-activity in a dose- and time-dependent manner [206]. Acute activation of μ-opioid receptors (MORs) inhibits GSK3β activity via phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway [209], while chronic activation of MORs elevates GSK3β activity [205, 206], may be through transient increase in intracellular calcium [205, 283, 284]. In accord with these studies, our results also show that morphine exerts time-dependent effects on GSK3β activation (Fig. 4.1 and 4.2). At earlier time points (4 and 12 h) morphine effects were not observed, but at later time points (24 and 72 h) morphine significantly induced GSK3β activation.
At 24 h, morphine significantly augmented HIV\textsuperscript{+}\textsubscript{sup}-mediated GSK3\textbeta activation (Fig. 4.2), but this transient additive/interactive effect of morphine was not seen at 72 h. By 72 h, HIV\textsuperscript{+}\textsubscript{sup} + morphine treatment induced nearly 50\% cell death (Fig. 4.3 and 4.5). Therefore, may be due to excessive cell death or selective loss of MOR-expressing neurons interactive effect of morphine is vanished. Though at 72 h, no significant interactive effect of morphine was seen, overall tendency of morphine is to induce GSK3\textbeta activation.

Although there was no additive/interactive effect of morphine on p-GSK3\textbeta at 72 h, morphine did exacerbate some neurotoxic effects of HIV\textsuperscript{+}\textsubscript{sup} at that time (cell death and loss of PSD-95). This discordance may be due to a difference in the time-frame of the ‘cause’ and the ‘effect’. The morphine-mediated interactive elevation in GSK3\textbeta activity seen at 24 h is the cause, resulting in an ‘effect’, aggravation of neurotoxic outcomes that is seen at a somewhat later time.

Certain neurotoxic outcomes of HIV\textsuperscript{+}\textsubscript{sup} were completely reversed by VPA co-treatment (loss of MAP-2; Fig. 4.4C), while some were only partially reversed (cell death and loss of PSD-95; Fig. 4.3 and 4.4D). In terms of HIV\textsuperscript{+}\textsubscript{sup}-morphine interactions, VPA co-treatment negated the interaction that augmented neuronal death (Fig. 4.3), but did not reduce the interaction that augmented PSD-95 loss (Fig. 4.4D). These discrepancies likely reflect the role(s) of other molecules or pathways, which, given the multiple downstream pathways and events triggered by opiates, HIV virions, and factors secreted from HIV-infected cells, is predictable. For example, CDK5, like GSK3\textbeta, is known to be involved in both HIV- and opiate-associated neuropathology [141, 174, 207, 281, 285], and might be a likely candidate in this regard.

In previous studies, VPA has significantly ameliorated HIV-mediated neurotoxic outcomes both in experimental models [174, 184, 204] and in patients [203]. Though VPA is a potent GSK3\textbeta
inhibitor, it is not a specific inhibitor; VPA targets additional molecules/pathways, including HDAC, Na⁺ channels, Ca²⁺ channels, voltage-gated K⁺ channels, GSK3α, and others [286]. Therefore, we also tested effects of the small molecule GSK3β-inhibitors SB415286 and XXVI which have much greater target specificity. Since both small molecule inhibitors similarly inhibited HIV⁺ sup ± morphine-mediated neurotoxic outcomes (Fig. 4.5), the role of GSK3β in these processes is confirmed.

HIV⁺ patients who abuse opiates show more serious neuropathologies and behavioral and cognitive deficits [160, 161]. The current work implies that GSK3β-mediated signaling pathway(s) are mechanistic in HIV-opiate interactions. Our recent studies have shown that the continuous presence of morphine limits the ability of neurons to recover from an HIV insults, even after HIV is removed [256]. In the post-cART era those studies are quite critical because they suggest that continual exposure to opiate drugs of abuse, and perhaps also opiates prescribed for pain, can limit the reversibility of HIV insults in the CNS. If true, this will impact the effectiveness of neurotherapeutic efforts. Based on the current findings it is reasonable to predict that opiates might limit neurite recovery after HIV insults via a GSK3β-mediated signaling mechanism. A recent study by Kehn-Hall et al. has shown that 6BIOder, a novel GSK3β-inhibitor, inhibits HIV-1 replication [201]. Also GSK3β is identified as a central mediator of pro-inflammatory processes in CNS [169, 198-200]. Thus, therapeutically targeting GSK3β along with cART may further diminish viral replication, neuroinflammation and neuronal damage, and may improve recovery.
Figure 4.1. HIV$^+$sup ± morphine-mediated GSK3β activation. At 4 h after treatment, cells were lysed and protein levels were detected using immunoblot analysis. Findings were reported as average normalized protein levels (% control) ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test; $n = 4$ separate experiments. (A) Immunoblotting for phospho-GSK3β-Ser9 (p-GSKβ-S9) and total GSK3β (t-GSK3β); levels of p-GSKβ-S9 were normalized with t-GSK3β (p-GSKβ-S9/t-GSK3β). HIV$^+$sup significantly reduced p-GSK3β-S9 (*$p < 0.05$ vs. Control), without any significant morphine interaction. VPA significantly abrogated HIV$^+$sup ± morphine-mediated effects (#$p < 0.05$). (B) Immunoblotting for β-catenin and GAPDH; levels of β-catenin were normalized with GAPDH (β-catenin/GAPDH). HIV$^+$sup significantly decreased β-catenin (*$p < 0.05$ vs. Control) without any morphine interaction. VPA restored β-catenin levels to control values (#$p < 0.05$), although in the case of neurons treated with HIV$^+$sup alone the effect was somewhat less significant (HIV + VPA was not different from either HIV or control). Control = control$^+$sup; HIV = HIV$^+$sup; Mor = morphine sulfate; VPA = sodium valproate.
Figure 4.2. HIV$^+$sup and morphine-mediated interactive effects on GSK3β-activation. At 12, 24 and 72 h after treatment, cells were lysed and immunoblotted for p-GSKβ-S9 and t-GSK3β. Findings were reported as average levels of p-GSKβ-S9 normalized with t-GSK3β (p-GSKβ-S9/t-GSK3β) as a percentage of control values ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test; $n = 3$ separate experiments. At all times, HIV$^+$sup significantly reduced p-GSK3β-S9 (*$p < 0.05$ vs. Control). At 24 and 72 h, morphine alone also induced significant loss of p-GSK3β-S9. A significant interaction with HIV$^+$sup was seen at 24 h (§$p < 0.05$). Control = control$^+$sup; HIV = HIV$^+$sup; Mor = morphine sulfate.
Figure 4.3. Role of GSK3β in HIV^{+}sup ± morphine-mediated cell death. (A) Cells were repeatedly imaged for 72 h after treatments. Digital images show the same cells/fields at 0, 24 and 72 h (white arrowheads indicate dead cells that were alive in previous image). (B) Cells were
assessed for viability at 6 h intervals in digital images. Findings were reported as the average percentage of neuronal survival as a proportion of pre-treatment neuron count ± SEM. Significance was analyzed by repeated measures ANOVA and Duncan’s post hoc test; $n = 3$ separate experiments (at least 150 neurons per treatment group). HIV$^+$ sup significantly reduced neuronal survival ($^*p < 0.05$ vs. Control), which was significantly augmented by morphine co-treatment ($^\delta p < 0.05$). HIV$^+$ sup ± morphine-mediated effects were partially, but significantly, reversed by VPA ($^# p < 0.05$). VPA also effectively negated HIV$^+$ sup-morphine interactions. (C) At 72 h after treatment, cells were fixed, permeabilized, and labeled for TUNEL and Hoechst 33342. The rate of neuronal apoptosis was reported as the average percentage of TUNEL(+) cells ± SEM. Significance was analyzed by one-way ANOVA and Duncan’s post hoc test; $n = 3$ separate experiments. HIV$^+$ sup significantly increased the percentage of TUNEL(+) cells ($^*p < 0.05$ vs. Control), and morphine augmented the effect ($^\delta p < 0.05$). HIV$^+$ sup ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment ($^# p < 0.05$), and VPA negated interactions between HIV$^+$ sup and morphine. Control = control$^+$ sup; HIV = HIV$^+$ sup; Mor = morphine sulfate; VPA = sodium valproate.
Figure 4.4. Role of GSK3β in HIV\(^{+}\) sup ± morphine-mediated changes in neuritic arborization, MAP-2 and PSD-95. (A) At 72 h after treatment, cells were fixed, permeabilized, and labeled for MAP-2 (green), TUNEL (red) and Hoechst 33342 (blue) (B) Neurite arborization was measured by Sholl analysis in digital images of TUNEL(-) neurons. The findings were reported as average Sholl score ± SEM. HIV\(^{+}\) sup significantly reduced the Sholl score (*p < 0.05 vs. Control), without any significant morphine interaction. HIV\(^{+}\) sup ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment (#p < 0.05). (C) At 72 h after treatments, cells were lysed and immunoblotted for MAP-2 and GAPDH. Findings were reported
as average normalized MAP-2 (% control) ± SEM. HIV\textsuperscript{+}\textsubscript{sup}, with or without morphine, significantly reduced MAP-2 (*p < 0.05 vs. Control). VPA significantly inhibited HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated effects (#p < 0.05). (D) Cell lysates were immunoblotted for PSD-95 and GAPDH. Findings were reported as average normalized PSD-95 (% control) ± SEM. HIV\textsuperscript{+}\textsubscript{sup} induced a significant loss of PSD-95 (*p < 0.05 vs. Control), which was augmented by morphine co-treatment ($p < 0.05$). HIV\textsuperscript{+}\textsubscript{sup} ± morphine-mediated effects were partially, but significantly, reversed by VPA co-treatment (#p < 0.05). Even in the presence of VPA, morphine significantly augmented the effects of HIV\textsuperscript{+}\textsubscript{sup}-mediated effects ($p < 0.05$). In all studies, significance was analyzed by one-way ANOVA and Duncan’s post hoc test, from $n = 3$ separate experiments. Control = control\textsubscript{sup}; HIV = HIV\textsuperscript{+}\textsubscript{sup}; Mor = morphine sulfate; VPA = sodium valproate.
Figure 4.5. Effects of small molecule GSK3β-inhibitors. (A) Cell viability was assessed by time-lapse imaging analysis. Neurons exposed to ‘Control + SB’, ‘Control + XXVI’, ‘Mor’, ‘Mor + SB’ and ‘Mor + XXVI’ treatments had survival equivalent to ‘Control’ but are not shown in the figure to highlight other groups. HIV\textsuperscript{+}sup significantly reduced neuronal survival (*p < 0.05 vs. Control), with a significant morphine interaction (**p < 0.05). The effects of HIV\textsuperscript{+}sup were partially, but significantly, reversed by both small molecule inhibitors (#p < 0.05 vs. respective HIV or HIV + Mor). Both SB415286 and XXVI also effectively negated interactions between HIV\textsuperscript{+}sup and morphine. (B) Cell death was examined using TUNEL-staining. HIV\textsuperscript{+}sup significantly increased the percentage of TUNEL(+) cells (*p < 0.05 vs. Control), with a significant morphine interaction (**p < 0.05). Both small molecule inhibitors partially reversed HIV\textsuperscript{+}sup ± morphine-mediated effects (#p < 0.05) and also eradicated the HIV\textsuperscript{+}sup-morphine interaction. (C) Neuritic arborization was evaluated using MAP-2 immunostaining and Sholl
analysis. HIV$_{sup}^+$ significantly reduced the Sholl score ($^*p < 0.05$ vs. Control) without a morphine interaction. All losses in arborization were partially, but significantly, reversed by both small molecule inhibitors ($^#p < 0.05$), except that XXVI completely reversed the effect of HIV$_{sup}^+$. Control = control$_{sup}$; HIV = HIV$_{sup}^+$; Mor = morphine sulfate; SB = SB415286; XXVI = GSK3β inhibitor XXVI.
HIV-1 disturbs normal immune system functioning and leads to AIDS. Soon after initial systemic infection HIV-1 can crossover BBB and enter into CNS [22-24, 278]. HIV-1 can induce a wide range of neurocognitive deficits, collectively known as HAND. Nearly 50% of HIV-1+ patients suffer from HAND [100-103]. After the advent of cART, the survival rates and health status of AIDS patients has been tremendously improved [105, 111, 112]. Though cART has reduced the severity of HIV-associated CNS deficits, the overall prevalence of the disease remains the same [88, 105-107, 111].

Injection drug abuse is a major cause of acquiring HIV-infection. Nearly 50% of HIV+ patients have a history of drug abuse, commonly involving opiates [125, 127]. Opiate abusing HIV+ patients have high risk for faster progression of AIDS [132, 135, 140, 217, 255]. Many experimental evidences have shown that opiates exacerbate HIV-1-mediated neurotoxic outcomes on CNS cells and tissues [125, 145, 147-151, 153], mimics co-morbid neurological deficits observed in opiate abusing HIV+ patients [160, 161]. Though many experimental and epidemiological findings have indicated a link between opiate drug abuse and HAND progression, the extent to which opiates exacerbate neurological deficits is still controversial.

In our current studies, we have tried to determine: (a) whether opiates have any effect HIV-1-mediated neurotoxic outcomes; (b) strain/tropism-specific differences in HIV-1 mediated
neurotoxicity and interactions with opiates; (c) point(s) of convergence for HIV-1 and opiate mediated signaling.

Many previous studies have modeled HIV-1-mediated neuropathology using individual viral proteins. But the CNS of HIV-1-infected patients is not only exposed to individual viral proteins instead it is exposed to all cytotoxins, virotoxins and virus particles released from infected and/or activated cells. To model disease process more closely, we have used supernatant from HIV-1-infected cells. R5-tropic HIV-1 strains are predominant in CNS [213, 214]. Therefore, in chapter 2 we used a R5-tropic strain, HIV-1_{SF162}. We assessed different neurotoxic outcomes mediated by HIV^{+}\text{sup}, in the presence or absence of morphine.

Our results showed that HIV^{+}\text{sup} induce neuronal death in concentration-dependent manner. But interestingly, significant interactive effects of morphine were seen only at lower HIV-1 exposure levels. Interactive effects of morphine may get masked due to excessive cell death at higher HIV-1 exposure levels. These results may explain controversy concerning the role of opiates in severity of HAND. In addition, our results caution that patients receiving cART may be highly vulnerable to opiate interactions since cART has greatly reduced the viral load [105, 111, 112].

Our results showed that opiates interact with and exacerbate only selective neurotoxic outcomes by HIV-1 (compare neuronal death and neurite pruning results). Suggesting that HIV- and opiate-mediated neurotoxicity is induced via multiple pathways that may lead to different outcomes, and interactions occur for selective pathways only. Importantly, our results showed that morphine limits neurite recovery after HIV-1-insults. Altogether, our results suggest that continual exposure to opiate drugs of abuse may adversely impact the neurotherapeutic efforts of cART.
Glia played dual role in HIV-1-mediated neurotoxicity. On one hand, HIV-1 ± morphine-mediated neuronal death was exacerbated in presence of glia; by contrast, glia enhanced neurite outgrowth when HIV was removed. Damaging effects by glia in presence of HIV-1 are largely attributable to the enhanced release of pro-inflammatory cytokines, and neurotrophic effects after removal of HIV may due to the rebound in release of growth factors.

We propagated HIV-1 in human cell lines, but due to inconsistency in availability of human brain cells from specific regions, we used murine cell culture model system. Therefore, we need to keep in mind the issue of species mixing while interpreting results from our model system. Cell culture model systems are easy to maintain and manipulate and provide a general idea about actual in vivo system. However, we need to be cautious when extrapolating these results because there are various additional factors present in actual in vivo system which may interfere with these processes.

Though during early stage of infection R5-tropic HIV-1 strains are predominant during late stage of disease X4-tropic strains are predominant; co-receptor usage switch from CCR5 to CXCR4 is crucial in disease progression to AIDS [263-267]. Some previous studies have shown that HIV-1 induces neurotoxic effects and interacts with drugs of abuse in strain/tropism-specific manner [151, 268, 271, 272]. Therefore, in chapter 3 we tried to compare neurotoxic effects of HIV-1_{LAI} (X4-tropic) and HIV-1_{SF162} (R5-tropic) and their interactions with opiates.

Surprisingly, our results showed that the neurotoxic effects and interactions with opiates by X4 and R5-tropic HIV-1-supernatants are quantitatively similar. Also cytokine/chemokine release profile of X4 and R5-tropic HIV-1-infected cells is almost similar. Altogether our results suggest that HIV-1-supernatant mediated neurotoxicity is mainly attributed to common cytotoxic factors
released from infected and/or activated cells instead of viral strain specific factors. Our results support ‘indirect injury’ or ‘bystander damage’ hypothesis.

Interestingly, studies with HIV-1 virion showed strain-specific differences. May be in case of HIV-1-superntant these strain-specific differences are overshadowed by other cytotoxic factors. But in case of HIV-1 virion neuronal death was observed in the presence of glia only, confirms role of glia/glia-activation in HIV-1 mediated neurotoxicity. In accord with some previous studies [271, 272] our results showed that HIV-1_{LAI} (X4-tropic) is slightly more neurotoxic than HIV-1_{SF162} (R5-tropic); these results may explain why co-receptor usage switch is crucial in disease progression [264, 267].

Though many previous studies have indicated a link between opiate drug abuse and HAND progression [125, 145, 147-151, 153, 160, 161], the mechanisms that underlie interactions between HIV-1 and opiates remain largely obscure. Previous studies have shown that HIV-1 induces neurotoxic effects via abnormal activation of GSK3\(\beta\) [174, 184-188], GSK3\(\beta\) is also linked to neuropathology seen in opiate abusing patients [141, 142]. Therefore, in chapter 4, we tested role of GSK3\(\beta\) as a point of convergence for interactions between HIV-1 and morphine.

Our results showed that HIV-1 induces GSK3\(\beta\) activation; morphine alone and in concert with HIV-1 induced GSK3\(\beta\) activation in time-dependent manner. Multiple GSK3\(\beta\)-inhibitors significantly reduced HIV-1 \pm morphine-mediated neurotoxic outcomes, suggesting role of GSK3\(\beta\) in these processes. Importantly, GSK3\(\beta\)-inhibitors negated selective neurotoxic interactions between HIV-1 and morphine. Since certain neurotoxic outcomes and interactive effects were only partially reversed also the role of other factors, such as CDK5, is suggested.
Altogether our results suggest that GSK3β is a potential therapeutic target for HIV-1- and opiate-mediated neurocognitive deficits.
LIST of REFERENCES


75. Klasse PJ, Moore JP. Is there enough gp120 in the body fluids of HIV-1-infected individuals to have biologically significant effects? *Virology* 2004,323:1-8.


90. Kim HJ, Martemyanov KA, Thayer SA. Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death. *J Neurosci* 2008, **28**:12604-12613.


126. Donahoe RM, Vlahov D. Opiates as potential cofactors in progression of HIV-1 infections to AIDS. *J Neuroimmunol* 1998, **83**:77-87.


163. Frame S, Cohen P. GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 2001, **359**:1-16.


221. Miyatake M, Rubinstein TJ, McLennan GP, Belcheva MM, Coscia CJ. Inhibition of EGF-induced ERK/MAP kinase-mediated astrocyte proliferation by mu opioids:
integration of G protein and beta-arrestin 2-dependent pathways. *J Neurochem*
2009, **110**:662-674.

222. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization,
substrate dependence, and involvement of mitochondrial electron transport in MTT
reduction. *Arch Biochem Biophys* 1993, **303**:474-482.

223. Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-
dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J Neurochem*
1997, **69**:581-593.

Biol* 2011, **731**:237-245.

225. Royal W, 3rd, Selnes OA, Concha M, Nance-Sproson TE, McArthur JC. Cerebrospinal
fluid human immunodeficiency virus type 1 (HIV-1) p24 antigen levels in HIV-1-related

226. Hauser KF, Hahn YK, Adjan VV, Zou S, Buch SK, Nath A, *et al.* HIV-1 Tat and
morphine have interactive effects on oligodendrocyte survival and morphology. *Glia*
2009, **57**:194-206.

227. McMillian MK, Hong JS. Regulation of preproenkephalin expression in astrocytes: is
there a role for glia-derived opioid peptides in reactive gliosis? *Crit Rev Neurobiol*
1994, **9**:91-103.


VITA

Ruturaj R. Masvekar was born on August 4, 1984, in Kolhapur, India. He graduated from Jawahar Navodaya Vidyalaya, Kagal, India in 2002. He received his Bachelor of Engineering in Biotechnology from Shivaji University, Kolhapur, India in 2006. From August 2006 to January 2007 he worked as a lecturer at Kolhapur Institute of Technology’s College of Engineering, India, where he taught Protein Engineering and Biopharmaceuticals courses to senior undergraduate students. In Spring 2007, Ruturaj joined University of Houston-Clear Lake for Master of Science in Biotechnology, subsequent Fall he transferred to Louisiana Tech University, Ruston, LA, and earned Master of Science in Molecular Science and Nanotechnology, in August 2009. In Fall 2009, Ruturaj joined Virginia Commonwealth University, Richmond, VA for PhD in Neuroscience. In Fall 2010 he joined Dr. Pamela E. Knapp’s laboratory for his PhD dissertation. As a graduate student, Ruturaj presented his research at Society for Neuroscience meeting, International Society for Neurochemistry meeting, International Symposium on NeuroVirology, and many other institutional presentations. Ruturaj earned various awards, such as best poster award at Central Virginia Chapter of Society for Neuroscience Fall 2013 Poster Session, presentation award at John C. Forbes Graduate Student Honors Colloquium 2014, Charles C. Clayton fellowship award 2013. Because of his good academic credentials Ruturaj received membership of The Honor Society of Phi Kappa Phi. Manuscript resulting from Ruturaj R. Masvekar’s work at Virginia Commonwealth University: