Human Neural Progenitor Cells are Productively Infected by R5-tropic HIV-1: Opiate Interactions on Infection and Function Involve Cdk5 Signaling

Joyce Magat Balinang
Human Neural Progenitor Cells are Productively Infected by R5-tropic HIV-1: Opiate Interactions on Infection and Function Involve Cdk5 Signaling

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

By
Joyce M. Balinang
Master of Science, Virginia Commonwealth University
Bachelor of Science, Virginia Commonwealth University

Virginia Commonwealth University
Richmond, Virginia
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# Table of Contents

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

Chapter 1  
Introduction to HIV-1 and Opiate Interaction in the Central Nervous  
System......................................................................................................... 1  

Chapter 2  
Interactive Effect of HIV-1 and Morphine on the Functions and Productive  
Infection of Primary Human Neural Progenitor Cells.............................. 28  

Chapter 3  
Central Role of CDK5 in Human Neural Progenitor Cell Responses to HIV-1  
and Opiate Exposure............................................................................... 55  

Chapter 4  
Regulation of MOR-1 and MOR-1K Splice Variants by HIV-1; Potential  
Function and Mechanism of MOR-1K in Human Neural Progenitor  
Cells........................................................................................................... 95  

Chapter 5  
Synopsis, Perspectives and Final Conclusion........................................114  

List of References.......................................................................................136  
Vita ...........................................................................................................xiv
### List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Classification of HIV strains</td>
<td>21</td>
</tr>
<tr>
<td>1.2</td>
<td>Prevalence of HAND subtypes in the pre-highly active antiretroviral therapy (HAART) era and in the current era of widespread HAART usage</td>
<td>22</td>
</tr>
<tr>
<td>1.3</td>
<td>Neuropathogenic mechanisms of HIV-1</td>
<td>23</td>
</tr>
<tr>
<td>1.4</td>
<td>Opiates exacerbate HIV-1 neuropathogenesis via the action of MOR expressed on CNS cells</td>
<td>24</td>
</tr>
<tr>
<td>1.5</td>
<td>Generation of human MOR splice variants from alternative splicing of the OPRM1 gene</td>
<td>25</td>
</tr>
<tr>
<td>1.6</td>
<td>HIV-1 and morphine co-exposure modulates expression of genes related to neurogenesis in immortalized hNPCs</td>
<td>26</td>
</tr>
<tr>
<td>2.1</td>
<td>Detailed characterization of the hNPC cultures</td>
<td>45</td>
</tr>
<tr>
<td>2.2</td>
<td>Morphine co-exposure prolongs the productive infection of hNPCs by R5-tropic HIV-1\textsubscript{BaL}</td>
<td>47</td>
</tr>
<tr>
<td>2.3</td>
<td>HIV-1 ± morphine co-exposure modulates DNA synthesis and doubling time of hNPCs</td>
<td>49</td>
</tr>
<tr>
<td>2.4</td>
<td>HIV-1 ± morphine co-exposure promotes neuronal and astrocytic differentiation of hNPCs</td>
<td>51</td>
</tr>
<tr>
<td>2.5</td>
<td>Infection of hNPCs is not required for the anti-proliferative effect of HIV-1</td>
<td>53</td>
</tr>
<tr>
<td>3.1</td>
<td>HIV\textsubscript{sup} and morphine-mediated changes in hNPC gene expression</td>
<td>79</td>
</tr>
<tr>
<td>3.2</td>
<td>RT-qPCR validation of PCR array data on the regulation of CDK5 related genes</td>
<td>81</td>
</tr>
</tbody>
</table>
3.3 HIV-1$_{\text{sup}}$ and morphine co-exposure increases protein level of Cdk5, p35 and calpain in hNPCs

3.4 Subcellular localization of Cdk5, p35 and calpain in hNPCs exposed to HIV-1$_{\text{sup}}$ and morphine

3.5 Design and optimization of siRNA Cdk5

3.6 Proliferative and protective role of Cdk5 in hNPCs exposed to HIV-1 and morphine

3.7 Involvement of Cdk5 in the interaction of HIV-1 and morphine on hNPC MAP2 differentiation

3.8 Roscovitine but not siCdk5 attenuates the effect of morphine on hNPCs GFAP differentiation

4.1 HIV-mediated regulation of exon 1-utilizing MOR (MOR-1$_{(\text{exon1-2})}$) and MOR-1k in immortalized hNPCs

4.2 HIV-mediated regulation of exon 1 utilizing MOR (MOR-1$_{(\text{exon1-2})}$) and MOR-1k in primary hNPCs

4.3 Regulation of FLNA by HIV-1 in immortalized and primary hNPCs

4.4 siRNA-mediated knockdown of MOR-1 and MOR-1K splice variants in primary hNPCs.

5.1 Potential cross-talk of Cdk5 with MOR in the mechanism of HIV-1 and morphine interaction on hNPCs
## List of Tables

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2.1</strong></td>
<td>Interactive effect of HIV-1 and morphine on hNPC doubling time</td>
<td>54</td>
</tr>
<tr>
<td><strong>2.2</strong></td>
<td>Primer sequences for all target genes used in Chapter 2 studies</td>
<td>54</td>
</tr>
<tr>
<td><strong>3.1</strong></td>
<td>mRNA expression fold change value of target genes in treated hNPCs</td>
<td>94</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>7TM</td>
<td>Seven transmembrane</td>
</tr>
<tr>
<td>ABCB7</td>
<td>ATP-binding cassette sub-family B member 7</td>
</tr>
<tr>
<td>ADORA2A</td>
<td>Adenosine A2a receptor</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALDH1L1</td>
<td>Aldehyde dehydrogenase 1 family member L1</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma receptor tyrosine kinase</td>
</tr>
<tr>
<td>ANI</td>
<td>Asymptomatic neurocognitive impairment</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BMECs</td>
<td>Brain microvascular endothelial cells</td>
</tr>
<tr>
<td>BMP4</td>
<td>Bone morphogenic protein 4</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cART</td>
<td>Combination antiretroviral therapy</td>
</tr>
<tr>
<td>CCR5</td>
<td>C-C chemokine receptor 5</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CD11b</td>
<td>Cluster of differentiation molecule 11B</td>
</tr>
<tr>
<td>CDK5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CDK5R1</td>
<td>Cyclin-dependent kinase 5 regulatory subunit 1</td>
</tr>
<tr>
<td>CDK5RAP2</td>
<td>CDK5 regulatory subunit associated protein 2</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>------------</td>
<td>----------------------------------------------------------------</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CXCR1</td>
<td>C-X-C motif chemokine receptor 1</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C chemokine receptor type 4</td>
</tr>
<tr>
<td>DAMGO</td>
<td>D-Ala², N-MePhe⁴, Gly-ol]-enkephalin</td>
</tr>
<tr>
<td>DOR</td>
<td>Delta opioid receptor</td>
</tr>
<tr>
<td>DRD2</td>
<td>Dopamine receptor 2</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulating kinase 1 and 2</td>
</tr>
<tr>
<td>FLNA</td>
<td>Filamin A</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Gi</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>gp41</td>
<td>Glycoprotein 41</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GRIN1</td>
<td>Glutamate ionotropic receptor NMDA type subunit 1</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV-associated dementia</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV-1-associated neurocognitive disorders</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HIVE</td>
<td>HIV-associated encephalitis</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus type 1</td>
</tr>
<tr>
<td>HIV-1sup</td>
<td>Supernatant from HIV-1-infected cells</td>
</tr>
<tr>
<td>HIV-2</td>
<td>Human immunodeficiency virus type 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HL-6</td>
<td>Human promyelocytic leukemia cell</td>
</tr>
<tr>
<td>hNPCs</td>
<td>Human neural progenitor cells</td>
</tr>
<tr>
<td>IDUs</td>
<td>Injection drug users</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>K\textsubscript{ring}3</td>
<td>Potassium inwardly-rectifying channel 3</td>
</tr>
<tr>
<td>KOR</td>
<td>Kappa opioid receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MND</td>
<td>Mild neurocognitive disorder</td>
</tr>
<tr>
<td>MOR</td>
<td>Mu-opioid receptor</td>
</tr>
<tr>
<td>M-tropic</td>
<td>Macrophage-tropic</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative regulatory factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural progenitor cells</td>
</tr>
<tr>
<td>NTN</td>
<td>Netrin 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PAX-3</td>
<td>Paired box-3</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PNCs</td>
<td>Perineurial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>RGC</td>
<td>Radial glial cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>Tumor necrosis factor (\alpha)</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex-determining region Y</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>UNF(_{sup})</td>
<td>Supernatant from un-infected cells</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein unique</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
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</tbody>
</table>
Human immunodeficiency virus type 1 (HIV-1) is known to cause a spectrum of neurological, behavioral and motor deficits collectively termed as HIV-1 associated neurocognitive impairments (HAND). Opiates augment HIV-related CNS complications through both direct and indirect mechanisms that disrupt glial and neuronal function. All CNS macroglia and neurons derive from neural progenitor cells (NPCs) during development, and NPCs in the adult brain contribute to repair processes. Since disruptions in NPC function are known to impact CNS populations and brain function in a number of disease/injury conditions, we determined whether HIV ± opiate exposure affected the maturation and fate of human NPCs (hNPCs). As hNPC infection by HIV has occasionally been reported, we also reexamined this question, and parsed between effects due directly to hNPC infection by HIV, or to hNPC dysfunction caused by the
infective milieu. Multiple approaches confirmed the infection of hNPCs by R5 tropic (CCR5 utilizing) HIV_{BaL}, and demonstrated that active infection could be sequentially transferred to naïve hNPCs. Exposure to supernatant from HIV_{BaL}-infected cells (HIV_{sup}) reduced hNPC proliferation and led to premature differentiation into astrocytes and neurons. Morphine co-exposure prolonged hNPC infection and exacerbated functional effects of HIV_{sup}. Neither purified virions nor UV-inactivated HIV_{sup} altered proliferation, indicating that this effect did not require infection. Gene array analysis and RT-qPCR with immunoblot validation suggested that Cdk5 signaling was involved in HIV-morphine interactions. siRNA-mediated knockdown of Cdk5 expression attenuated the effect of HIV-1 and morphine on hNPC proliferation and MAP2 differentiation, but also increased hNPC death. Furthermore, in an attempt to understand the role of mu-opioid receptor (MOR) splice variants on the interactive effect of HIV-1 and morphine on hNPCs, we found that both MOR-1 and MOR-1K are differentially regulated by HIV-1 in these cells. This suggests that these splice variants may have differential actions in the response of hNPCs to HIV-1 and morphine co-exposure. Given the overlap of Cdk5 and MOR signaling, it is likely that MOR-1K and/or MOR-1 converge with Cdk5 in the mechanism underlying HIV-1 and morphine interaction in hNPCs.

Overall, dysregulation of hNPC functions by the infectious environment may create cell population imbalances that contribute to CNS deficits in both adult and pediatric patients. Additionally, infected hNPCs may pass virus to their progeny, and serve as an unappreciated viral reservoir. The recent epidemic of opiate/heroin abuse highlights the clinical importance of HIV and opiate interactions.
Biology and Origin of HIV

Human immunodeficiency virus (HIV) is a retrovirus that specifically targets the immune system, causing a progressive loss of immune competence that can lead to Acquired Immunodeficiency Syndrome (AIDS). This disease is characterized by a profound decrease in the relative number and quantity of cluster differentiation 4 (CD4)-expressing T cells, accompanied by susceptibility to infection by microorganisms that are not normally pathogenic. HIV belongs to the Retroviridae family, a large and diverse family of enveloped retroviruses that replicate in host cells via the process of reverse transcription (Barre-Sinoussi, et al. 1983). The HIV virion is a spherical structure composed of two single ribonucleic acids (RNA) that encode for the virus’ nine genes (gag, pol, env, tat, rev, nef, vpr, vif, vpu), as well as viral proteins (e.g., reverse transcriptase, integrase and protease) essential for initial replication. All of the viral RNA and proteins are enclosed within the viral envelope consisting of a lipid bilayer taken from host cell membrane and viral proteins gp120 and gp41 (Gonda 1988; Watts, et al. 2009).

There are at least two types of HIV that have been identified, HIV Type 1 (HIV-1) and HIV Type 2 (HIV-2). HIV-1 has origins in Simian Immunodeficiency Virus (SIV) from infected chimpanzees (Gao, et al. 1999; Huet, et al. 1990), while HIV-2 originated from SIV strain that infects the sooty mangabey (Hirsch, et al. 1989). The HIV viruses are clinically indistinguishable and demonstrate similar pathological process. However, studies showed that HIV-1 is the predominant cause of HIV infection worldwide, and is
associated with higher virulence, faster rate of CD4+ T cell decline, and disease progression (Barre-Sinoussi, et al. 1983; Marlink, et al. 1994). HIV-2 on the other hand is mostly confined in West Africa where it is currently endemic (Clavel 1987; Romieu, et al. 1990).

HIV-1 consists of four distinct lineages and are designated as groups: M (main), O (outlier), N (non-M, non-O) and P (Figure 1.1). These groups have different geographic distribution but all produce similar clinical symptoms. Identified first, Group M is the most prevalent group and is responsible for the majority of the global HIV epidemic, while N, O and P are limited and restricted to specific locations (Sharp and Hahn 2011). Group O was discovered in 1990 and it represents about 1% of global HIV-1 infection. This strain is largely restricted to Cameroon, Gabon and neighboring countries (Mauclere, et al. 1997; Peeters, et al. 1997). Group N was identified in 1998, and is even less prevalent than Group O with only about 13 cases documented in Cameroon (Simon, et al. 1998). Group P was recently discovered in two cases, also in Cameroon (Vallari, et al. 2011). Group M is further classified into ten different subtypes or clades: A-D, F-H, J, K and circulating recombinant forms (CRFs) (Taylor and Hammer 2008). The clades have regional specificity, with B being the most prevalent affecting the Americas, much of Europe and Australia (Taylor and Hammer 2008), C affecting much of Africa and Asia and the others having smaller distributions (Gilbert, et al. 2007).

The extensive genetic diversity of HIV-1 is believed to stem from high replication rate, spontaneous mutation and recombination events that may occur during infection. These mechanisms, particularly spontaneous mutation play a central role in HIV-1 biology, enabling the virus to successfully evade the immune system, rapidly change
tropism, and evolve drug resistance (Smyth, et al. 2012). HIV-1 mutations arise either through the error prone viral reverse transcriptase (RT) that lacks proofreading activity, or through the action of mutagenic host cell factors such as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) (Ji and Loeb 1992; Mansky 1996). Initial measurements made in vitro revealed that HIV-1 RT had an unprecedented high mutation rate with an error rate in the order of $5 \times 10^{-4}$ – $6.7 \times 10^{-4}$ (Bebenek, et al. 1993; Roberts, et al. 1988). Subsequent studies looking at single cycles of viral replication indicated a mutation rate of HIV-1 of about $1.4 \times 10^{-5}$ errors per base, per replication cycle (Abram, et al. 2010). Recently, quantification of spontaneous mutation in peripheral blood mononuclear cell (PBMC) cDNA derived from HIV-1 infected subjects indicated a significantly higher rate of in vivo HIV-1 mutation, with about two orders of magnitude higher than that predicted by in vitro studies (Cuevas, et al. 2015).

**HIV Pathogenesis**

The precise mechanism(s) whereby HIV-1 induces pathogenic events leading to AIDS in vivo is incompletely understood. However, insight into this complex process has been explored from epidemiological studies, in vivo animal models, ex-vivo models and culture systems that aimed to understand the cellular behavior and pathogenicity of HIV (Evans and Levy 1989; Fauci 1993; Fauci, et al. 1996; Goldmann and Zuck 1989). It is well established that HIV infects various lymphocyte subsets including CD8+ T cells, B lymphocytes, monocytes, dendritic cells, and natural killer (NK) cells. However, the virus preferentially replicates and propagates in circulating CD4+ T cells and macrophages (Klatzmann, et al. 1984a; Klatzmann, et al. 1984b). The ability of HIV to establish infection
in these cells is dictated by the strong interaction between viral glycoproteins, gp120 and gp42 and the CD4 molecule on the target cells, along with co-receptors, primarily C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) (Dragic, et al. 1996; Feng, et al. 1996; Wu, et al. 1996). CCR5 is the main receptor for macrophage-tropic HIV-1 strains (M tropic), which comprise the majority of transmitted HIV-1. CXCR4 is the receptor for T-tropic isolates (X4 tropic) that generally infect CD4+ T cells and also macrophages (Berkowitz, et al. 1998). HIV-infection leads to dysfunction of CD4+ T cells and dysregulation of CD8+ T cells, B cells and NK cells (Fauci 1993). The long term consequences of HIV infection are progressive depletion of CD4+ T cells and generalized immunodeficiency, ultimately leading to opportunistic infections characteristic of AIDS (Barre-Sinoussi 1996).

**Epidemiology of HIV-1 and Outcome of cART**

According to a 2014 report by the World Health Organization (WHO), since the first outbreak in 1981, HIV has claimed more than 34 million lives worldwide, with 1.2 million reported in 2014 alone. A large percentage of HIV-related deaths are found in sub-Saharan Africa and Southeast Asia. Close to 60 million people have contracted the virus since it was first discovered and it is estimated that approximately 40 million people globally are living with HIV/AIDS today (http://www.who.int/hiv/en/). Although HIV infection remains a major health and socioeconomic issue around the world, viral burden has been dramatically controlled with the advent of combination antiretroviral therapy (cART).
The drug therapy consists of five classes of drugs that act on the different stages of the HIV life-cycle and include: nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists). The clinical benefits of cART are evident, with majority of patients showing significant reduction of plasma viral load and an increase in circulating CD4+ T cells (Daniel, et al. 1999). In addition, the partial restoration of a functional immune system by cART has also led to marked declines in the incidence and effects of opportunistic infection (French and Price 2001; Jacobson and French 1998). Overall, cART has dramatically reduced the morbidity and mortality associated with HIV-1 (Palella, et al. 1998). Today, HIV is considered a chronic disease and AIDS-related illnesses are no longer the major cause of death in the HIV-infected population in areas where cART is readily available.

Although cART has significantly improved the prognosis of HIV-1 infection in many patients, the increased life span of infected individuals has unmasked pre-existing complications related to chronic HIV-1 infection. Studies suggest that HIV-1 patients under cART are at higher risk for developing non-AIDS defining illnesses including cardiovascular disease, cancer, kidney disease, liver disease and neurocognitive disease. The emergence of these non-AIDS defining illnesses has raised new questions concerning the cause of non-AIDS defining illnesses and the limitations of cART for treating individuals (Deeks, et al. 2013; Wester, et al. 2011)

**HIV-Associated Neurocognitive Impairments**
HIV infection is known to cause neurological dysfunctions that can result in cognitive and behavioral impairments (Boisse, et al. 2008; Gabuzda and Hirsch 1987). Despite the success and widespread use of cART, central nervous system (CNS) impairments persist in a substantial proportion of individuals living with HIV-1. Currently, it is estimated that about 30-50% of HIV-1-infected patients suffer from various cognitive and motor deficits, collectively known as HIV-1 associated neurocognitive disorders (HAND). These conditions range from asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND), to profoundly disabling HIV-associated dementia (HAD) and HIV-associated encephalitis (HIVE) (Antinori, et al. 2007).

With the introduction of cART, the pattern of HAND has dramatically shifted (Figure 1.1). Cross-sectional observations comparing HIV-1 cases in the pre- and post-cART era indicated an overall decrease in the incidence of HAD and HIVE in the post-cART era, along with significant decline in opportunistic CNS infections. However, the prevalence of ANI and MND is increasing in the post-cART era, with a reported rate between 40-60% of HIV-infected individuals (Ellis, et al. 2007; Gray, et al. 2003; Sacktor, et al. 2002) (Figure 1.2). The clinical features of HAND have also changed, whereas deficits in motor skill, cognitive process, and verbal fluency were common in the pre-cART era, impairments in memory, learning, and executive functions are more frequent in the post-cART era (Heaton, et al. 2011). The causes of persistent high rates of HAND in the post-cART era are uncertain, but multiple possibilities have been suggested. These include irreversible brain injury prior to cART, incomplete viral suppression due to inadequate CNS penetration of most antiretroviral drugs, the presence of drug resistant viral strains,
chronic low levels of viral replication and prolonged inflammatory response, toxic effects of prolonged cART use, as well as age-related processes (Gray, et al. 2003).

**Mechanism of HIV-1 Neuropathogenesis in the CNS**

While it seems that the virus enters the CNS early after initial infection, the mechanism by which it does so remains poorly understood (Valcour, et al. 2012). Various mechanisms of HIV-1 neuroinvasion have been suggested from free virus to cellular-mediated entry. The consensus theory is that soon after systemic infection, HIV-1 crosses the blood brain barrier (BBB) and enters the CNS via infiltrating monocytes and T cells (An, et al. 1999; Davis, et al. 1992). *In situ* hybridization and immunohistochemical studies demonstrated that HIV accumulates in the perivascular region of the brain (Fischer-Smith, et al. 2004; Takahashi, et al. 1996; Wiley, et al. 1986). In the perivascular region, HIV-infected monocytes come in directed contract with several CNS cell types, mainly perivascular macrophages, microglia and astrocytes (Takahashi, et al. 1996; Wiley, et al. 1986). Among these, perivascular macrophages and microglia play a central role on the infection of the brain by HIV-1. Although some infection of astrocytes and endothelial cells has been reported, HIV is concentrated and replicates mainly in perivascular macrophages and microglia, which constitute a major cellular reservoir of HIV-1 infection in the CNS (Cosenza, et al. 2002; Persidsky and Gendelman 2003; Williams, et al. 2001). In addition to generating new virions and propagating CNS infection, infected microglia and perivascular macrophages also release toxic viral proteins (*e.g.*, gp120, Tat, vpr), inflammatory mediators (tumor necrosis factor alpha [TNF-α], interleukin-1 [IL-1], interferon-γ [IFN-γ]), potentially excitotoxic factors
(glutamate, arachidonic acid) and reactive oxygen species (ROS) (Persidsky and Gendelman 2003; Sippy, et al. 1995; Vitkovic, et al. 1994; Wesselingh, et al. 1993). A small number of astrocytes may also be infected, but they lack the ability to produce new virions (Brack-Werner 1999; Sabri, et al. 1999). However, similar to microglia, HIV-infected astrocytes may produce and release viral toxic proteins as well as pro-inflammatory cytokines and chemokines further exacerbating neuroinflammation. The essential homeostasis functions of astrocytes are also modulated by HIV-1 infection, such as the ability to buffer extracellular glutamate and provide trophic factors to neurons (Figure 1.3) (Wang, et al. 2003b)

It is generally accepted that neurons cannot be infected by HIV-1, although neuronal injury is a hallmark feature in the development of HAND. Different mechanisms of HIV-1 neuropathogenesis have been reported with the consensus suggesting that HIV-1 infection causes neuronal injury either by a direct mechanism (viral protein effects on neurons) or by an indirect or “bystander” effect (inflammatory/toxic conditions) (Gonzalez-Scarano and Martin-Garcia 2005; Kaul, et al. 2001). It is highly likely that both types of effects contribute to neuronal dysfunction over the course of the disease. The theory that HIV proteins can target neurons directly is supported by various studies using human ex-vivo paradigms, and cell culture systems showing that viral proteins directly cause neuronal injury and even cell death. For instance, in vitro studies reported the toxicity of viral protein gp120 on murine hippocampal neurons and human neuronal cells lines (Bardi, et al. 2006; Hesselgesser, et al. 1998; Lannuzel, et al. 1995). Relatively similar effects were observed using HIV-1 Tat. Treatment with Tat resulted in substantial
neurotoxicity, increased the level of intracellular calcium and altered neuronal excitability in cultured human neuronal cells (Haughey, et al. 1999; Nath, et al. 1996).

In addition to the direct actions of viral proteins, it is also postulated that the inflammatory and toxic conditions created by infected and/or activated non-neuronal cells can have deleterious consequences on neighboring neurons. Marked neuronal abnormalities resembling those in brains of HIV-1-infected humans were observed in HIV gp1200 transgenic mice, with the severity correlated with the brain level of gp120 expression (Toggas, et al. 1994). In addition, various investigations showed that HIV-1 and HIV gp120-induced neuronal injury and cell death appears to be mediated primarily via the release of soluble factors from microglia and macrophages. This includes reactive oxygen species, numerous inflammatory cytokines such TNF-α, IL-1, IFN-γ, IL-6, and chemokines (Bruce-Keller 1999; Chao, et al. 1992; Chen, et al. 2002; Iskander, et al. 2004; Kaul and Lipton 1999; Persidsky, et al. 1997; Seilhean, et al. 1997; Xiong, et al. 2000). Infected astrocytes also contribute to the neurotoxic process by releasing pro-inflammatory cytokines and toxic cellular products such as glutamate and ROS (Genis, et al. 1992; Kaul, et al. 2001; Nath, et al. 1999; Persidsky and Gendelman 2003). The inflammatory and toxic conditions induced by these infected cells ultimately promotes the activation of uninfected microglia and astrocytes, creating a pathological feedback loop in the CNS. Overall, the cumulative response of infected and/or activated macrophages, microglia, and astrocytes creates a pathophysiological condition that is detrimental to neighboring neurons, ultimately resulting in substantial neuronal dysfunctions and even death (Figure 1.3). Sub-lethal damage to the integrity of dendrites and synapses is
thought to underlie the development of neurological deficits characteristic of HAND in the post-cART era (Everall, et al. 1999; Masliah, et al. 1997)

**Embryonic and Adult Neural Progenitor Cells and Their Role in Brain Development and Plasticity**

Accumulating evidence suggests that, in addition to microglia, macrophages and astrocytes, neural progenitor cell (NPCs) may also be targets of the deleterious effect of HIV-1. NPCs are self-renewing, multipotent cells that give rise to all neuronal and glial populations in the CNS and are present in both the developing and adult system (Gage 2002). In the developing brain there are various types of progenitor cells, each generating different population of neurons, astrocyte and oligodendrocytes under a well-coordinated and regulated process. These progenitor cells all arise from a single layer of neuroepithelial cells. During early CNS development, neuroepithelial cells undergo several rounds of symmetrical division and form the germinal zones, namely the ventricle zone and subventricular zone. Neuroepithelial cells at these sites serve as neural stem cells, generating new neurons and glia via asymmetric division. After the early phases of neurogenesis, neuroepithelial cells begin to transform into Radial glial cells (RGCs) by the down-regulation of epithelial features such as tight junctions followed by up-regulation of glial specific markers such as glutamate aspartate transporter (GLAST) and brain lipid-binding protein (BLBP) (Barry, et al. 2014; Morest and Silver 2003). During the late stage of neurogenesis RGCs divide asymmetrically at the VZ to generate a new RGC and a postmitotic neurons or intermediate progenitor cell. Intermediate progenitor cells can directly and indirectly form neurons through symmetric and asymmetric division.
respectively (Gotz and Huttner 2005). At the end of cortical development, most RGCs lose their ventricular attachment and migrate toward the surface of the cortex, where they transform into astrocytes (Misson, et al. 1988). In addition, RGCs can also generate oligodendrocytes during the late stage of embryogenesis and in the early postnatal period (Casper and McCarthy 2006; Kessaris, et al. 2006).

Different from embryonic NPCs, adult NPCs are restricted to the dentate gyrus (DG) of the hippocampal formation and the subventricular zone (SVZ) of the lateral ventricle. They are normally relatively quiescent but can be activated and drive ongoing gliogenesis and neurogenesis, especially in response to insult or injury. Activated NPCs undergo a series of proliferation, differentiation and migration leading to the generation of new neurons and glial cells in surrounding tissues (Kernie and Parent 2010). This is a tightly regulated process dependent on both intrinsic and extrinsic factors (Corbin, et al. 2008). Though NPC activation in the adult brain can be advantageous in terms of learning and memory as well as replacing injured cells after acute injury, prolonged NPCs activation can perturb the balance of CNS cell populations, likely contributing to pathological processes. In fact, the consequences of NPC dysfunction have been implicated in various psychiatric (Kim, et al. 2012; Toro and Deakin 2007) and neurodegenerative diseases (Winner and Winkler 2015), as well as in injuries caused by inflammation (Ekdahl, et al. 2003; Monje, et al. 2003), stroke/ischemia (Kernie and Parent 2010), or epilepsy (Jessberger and Parent 2015).

Involvement of NPCs in HIV Neuropathogenesis
Given the fundamental role of NPCs on CNS cell generation that can ultimately dictate brain architecture and plasticity, NPC dysfunction may contribute to the cognitive and motor impairments observed in both children and adults living with HIV-1. In fact, various groups in the past have questioned the potential involvement of NPCs in the mechanism of HIV neuropathogenesis, and have explored both their ability to be infected by HIV and whether exposure to HIV alters their normal function. Limited HIV infection of human NPCs (hNPCs) was demonstrated by HIV p24 production from cultured, fetal brain-derived hNPCs treated with X4 tropic viruses HIV_{IIIB} and HIV_{NL4-3}. P24 production was measured for up to 10 days in culture after which the infection could be stimulated with TNF-α. Differentiation of infected hNPCs to astrocytes increased p24 production suggesting a phenotype difference in viral infection. (Lawrence, et al. 2004). Sustained p24 production was also detected, as well as proviral and HIV transcripts in v-myc immortalized human neural stem cell line (HNSC.100) treated with HIV_{IIIB}. In this study, viral activity was validated in HNSC.100 using a HIV Rev fluorescent-based reporter assay (Rothenaigner, et al. 2007). Subsequently, the likelihood for in vivo infection of hNPCs was reported by Schwartz et al. showing the presence of viral DNA in nestin⁺ NPC rich region in hippocampal slices from pediatric AIDS brain samples (Schwartz, et al. 2007). Despite this, the infection of hNPCs by HIV-1 continues to be a controversial topic, primarily because of the limitations of these earlier investigations. For instance, both in vitro studies assessed HIV-1 infection in hNPCs by measuring p24 production. Although this a classic method to determine infectivity in culture, the lack of subsequent experimental validation such as measuring production of other viral proteins makes it difficult to infer the productive infection of these cells. Although Rothenaiger et al. (2007)
demonstrated the presence of proviral DNA along with evidence of p24 production in HNSC.100, these cells are immortalized and may not represent the true behavior of hNPCs in the actual brain including their ability to be infected.

Several groups have examined the functional vulnerability of NPCs to HIV-1. Krathwohl et al. provided the initial evidence on the effect of HIV coat proteins on NPC proliferation using cultured human NPCs (hNPCs) and hippocampal slices. These findings were supported with in vivo data showing significant decrease in the number of hippocampal NPC obtained at autopsy from HIV-infected subjects with dementia compared to non-dementia counterparts (Krathwohl and Kaiser 2004). Subsequent investigations also indicated reduced proliferation of adult rodent NPCs exposed to gp120 (Okamoto, et al. 2007), and of fetal hNPCs to Tat (Malik, et al. 2014; Mishra, et al. 2010). Furthermore, our laboratory previously reported that HIV Tat and HIV-infected supernatant dramatically altered the proliferation and population of both murine NPCs and immortalized hNPCs, further supporting the notion of the anti-proliferative effect of HIV-1 on NPCs. In contrast, Peng et al. found that exposure to HIV-1-infected macrophage increased the proliferation of fetal hNPCs, as well as induced gliogenesis (Peng, et al. 2008), which was later revealed to be mediated by signal transducer and activator of transcription 3 (STAT3) activation (Peng, et al. 2011). In this study, the presence of lipopolysaccharide (LPS) in the HIV-1 supernatant, which was used to stimulate the macrophages prior to infection, may have confounding effects that likely influence the outcome. Although these early findings may be contradictory in part, they all highlight the vulnerability of NPCs to HIV-1, and imply that these cell types are likely additional targets of HIV-1 in the CNS.
Opiates Exacerbate CNS HIV-1-Infection and HAND

Injection drug abuse is a major vector for HIV-1 transmission. Nearly 30-50% of the HIV-1 infected population are injection drug users (IDUs), making injection drug abuse the fastest growing vector for the spread of HIV-1. Thus, IDUs, many of whom are reported to preferentially abuse opiates such as heroin, are at higher risk for contracting HIV-1 and developing systemic and neurological complications (Curran, et al. 1988). A growing body of evidence from experimental findings and epidemiology studies are starting to establish the link between opiate drug abuse and HIV neuropathogenesis. Early studies comparing opiate abusers to non-drug abusers within a population of HIV-infected subjects reported severe neuropathology and increased evidence of neuroinflammation in HIV-1-infected opiate abusers (Anthony, et al. 2008; Bell, et al. 2002; Bell, et al. 1998), suggesting that opiates heightened CNS disease progression mediated by HIV-1. The exact molecular mechanisms by which opiates augment HIV-1 neuropathogenesis remain a question and area of research that needs to be better developed. However, it is postulated that opiates promote HIV-1 replication in immune cells while suppressing immune functions. Opiates alone adversely affect the immune system by suppressing the functions of immune cells including NK cells (Carr and France 1993), phagocytic macrophages (Singhal, et al. 1998), neutrophils (Liu, et al. 1992), and monocytes (McCarthy, et al. 2001b). The use of morphine, the major metabolite of heroin, has been shown to hasten HIV viral replication in human peripheral blood mononuclear cells (Peterson, et al. 1990) and SIV replication in rhesus macaques (Bokhari, et al. 2011;
Chuang, et al. 1993; Kumar, et al. 2006). In addition, chronic morphine exposure accelerated the onset of AIDS in these primates infected with SIV (Kumar, et al. 2006).

In the CNS, morphine significantly exacerbates lymphocyte infiltration into the CNS following a neuroinflammatory stimulus (Olin, et al. 2012). In the context of HIV-1, morphine was found to potentiate HIV-1 mediated effects on cytokine and chemokine regulation of glial cells. This subsequently enhances neurodegenerative effects of HIV-1 by reverberating inflammatory/cytotoxic positive feedback between infected and/or activated CNS cells (El-Hage, et al. 2005; Gurwell, et al. 2001; Hauser, et al. 2006; Zou, et al. 2011). Similar to peripheral immune cells, morphine has been shown to enhance viral replication in cultured human brain cells, suggesting that morphine likely increases HIV infection in the CNS (Peterson, et al. 1994). Various groups including ours postulate that opiates modulate HIV neuropathogenesis through actions on opioid receptors, primarily the \( \mu \)-opioid receptor subtype (MOR). Although the MOR is expressed by both neurons and glia, evidence indicates that the neuropathological effects of opiates are largely mediated by glia (Zou, et al. 2011) (Figure 1.4).

The \( \mu \)-opioid Receptor (MOR) System

MORs are part of the opioid receptor family which is comprised of four major subtypes; MOR, kappa- (KOR), delta- (DOR), and nociception- (NOP) opioid receptor. All four are G-protein coupled receptors (GPCRs) sharing a similar, seven transmembrane (7TM) protein structure, and are activated by various opioids. Opioid receptors have no direct link with effector proteins, instead they relay their action via coupling with G-proteins. Generally, all three subtypes couple to inhibitory G-protein (G\(_{i/o}\)) to relay their
messages downstream upon activation by opioid ligand binding. Following receptor activation by agonists, either by endogenous opioids such as endorphin or exogenous opioids including morphine or fentanyl, the Gα subunit dissociates from the receptor and acts on various intracellular pathways. One of the classical and important aspects of opioid receptor signaling relates to their ability to regulate calcium and potassium channels. After opioid receptor activation, Gα dissociates and directly interacts with G-protein gated inwardly rectifying potassium channel (KIR3), leading to channel inhibition. Gα-mediated inhibition of KIR3 causes cellular hyperpolarization and inhibition of tonic neural activity (Torrecilla, et al. 2002). Like other GPCR that utilize G\textsubscript{i/o} for signal transduction, opioid receptors are known to inhibit adenylyl cyclase activity (Carter and Medzihradsky 1993; McKenzie and Milligan 1990; Sharma, et al. 1977), which have been implicated in suppressing the activity of pronociceptive sensory neurons (Crain and Shen 2000).

Morphine, the major metabolite of heroin and one of the most commonly used opiates for pain management, binds to all three opioid receptor subtypes. Early studies looking at its pharmacological properties showed that morphine displays the highest affinity for the MOR subtype (Ki = 1.2 – 14 nM) (Chen, et al. 1991; Lutz and Pfister 1992; Pert, et al. 1973), compared to KOR (538 nM-range) and DOR (>1000 nM) (Lutz and Pfister 1992). MORs are expressed widely in the CNS (Mansour, et al. 1995; Mansour, et al. 1994), and are critical for many physiological processes involving analgesia, immunological response, respiration, gastrointestinal (GI) tract functions, and hormone regulation (Pasternak and Pan 2011; Pattinson 2008; Roy, et al. 1998; Roy and Loh 1996). Initial cloning of MOR in SH-SY5Y cells showed that the receptor couples to G\textsubscript{i/o}
G_{o} subunits upon stimulation with morphine and D-Ala^{2}, N-MePhe^{4}, Gly-ol-[enkephalin (DAMGO) (Carter and Medzihradsky 1993). The subsequent downstream effects were later reported to include inhibition of adenylyl cyclase, decrease of cyclic adenosine monophosphate (cAMP) and intracellular calcium, as well as the induction of phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPK) pathways (Carter and Medzihradsky 1993; Chakrabarti, et al. 1995; Chen, et al. 1993).

In recent years, MORs have been implicated as cofactors in HIV-1 disease progression and neuropathogenesis. In addition to CNS tissues, MOR is also highly expressed in immune cells where it has been shown to modulate immune activities (Roy, et al. 2006). Activation of MOR was demonstrated to decrease lymphocyte proliferation, antibody production and macrophage mediated immunity (Bussiere, et al. 1993; McCarthy, et al. 2001a; Roy, et al. 2006). Additionally, MOR expression was found to be up-regulated primarily in macrophages from the HIV gp120 transgenic rat (Chang, et al. 2007). Related studies also reported gp120-induced up-regulation of MOR in human promyelocytic leukemia cell (HL-6) and vascular endothelium cells (Beltran, et al. 2006; Cadet, et al. 2001). MOR have also been shown to undergo heterologous cross-sensitization with major HIV-1 co-receptors CCR5 and CXCR4, indicating their potential role on HIV-cellular binding and entry (Chen, et al. 2004; Rogers, et al. 2000).

Early pharmacological studies examining receptor binding sites and actions, suggest the existence of more than one class of MOR (Ling, et al. 1985; Ling, et al. 1983). This has now been supported at the molecular level with the identification of numerous MOR splice variants through the alternative splicing of both murine and human OPRM1 (Pan, et al. 2005; Pan 2002). To this date, there are at least 21 human MOR splice
variants that have been characterized, many displaying distinct responses to opioids including morphine (Figure 1.5) (Pan 2005; Pasternak and Pan 2013). Despite the identification of these splice variants, the majority of studies on opiate interaction, including in HIV-mediated neurocognitive impairments, have concentrated on the canonical MOR-1. Our group initially profiled the expression MOR splice variants in various CNS cell types and in brain tissues from HIV-1 subjects to address the potential involvement of MOR splice variants in HIV-1 CNS related effects. We reported that selected C-terminal (MOR-1, MOR-1A, MOR-1X) and N-terminal (MOR-1K) truncated MOR splice variants were differentially expressed in primary human astrocytes, microglia and neurons, as well as in cell types associated to the BBB, brain microvascular endothelial cells (BMECs) and perineurial cells (PNCs). Furthermore, evidence of MOR splice variant regulation was observed in brain tissues obtained from HIV-1 infected subjects (Dever, et al. 2014; Dever, et al. 2012). Interestingly, among the splice variants that were profiled, MOR-1K was found to be specifically up-regulated in HIV-1 subjects and its expression correlated with the level of cognitive impairments (Dever, et al. 2014). Overall these studies suggest that the canonical MOR-1 may not be the predominant MOR expressed in the CNS, and that other splice variants such as MOR-1K, are likely differentially regulated in the brains of individuals with HIV-1/AIDS.

**Interactive Effect of HIV-1 and Opiates on Neural Progenitor Cells**

In addition to the independent effect of HIV-1 on NPCs, studies suggest that they are also vulnerable to the deleterious effects of HIV-1 and opiates. We have previously shown that immortalized human NPCs do express MOR, along with HIV-1 co-receptor
CCR5 and CXCR4 (Hahn, et al. 2012). Opiates alone perturb the proper functions of NPC including proliferation and survival (Arguello, et al. 2008; Eisch, et al. 2000; Kahn, et al. 2005). Given these findings, various groups including ours have recently examined the interaction of HIV-1 and morphine on NPCs to obtain insight as to how these cells contribute to the neuropathological events in the infected brain. We reported that morphine co-exposure augments the effect of HIV-1 supernatant and HIV-1 Tat on the proliferation and population of murine NPCs and human immortalized cells (Hahn, et al. 2012). This was subsequently supported by Malik et al., as they showed interaction of HIV-1 Tat and morphine on the proliferation of human fetal brain-derived NPCs, which was determined to be regulated by the extracellular signal-regulating kinase 1 and 2 (ERK1/2) signaling (Malik, et al. 2014). Despite these key studies, our understanding of the precise mechanism(s) underlying the interaction of HIV-1 and opiates on NPCs is not clear.

In an attempt to parse out the molecular events that may be involved in the interaction of HIV-1 infection and opiate exposure that we previously reported on immortalized hNPCs (Hahn, et al. 2012), we screened for changes in gene expression in these cells following exposure to HIV-1 infected supernatant (HIV-1_{sup}) and morphine. Preliminary data indicated specific regulation of several genes associated in the different aspect of neurogenesis by HIV-1 and morphine co-exposure. Out of the 82 genes examined, 20 were specifically up-regulated by HIV-1 and morphine. Many of these genes are associated with cell differentiation, neuronal migration, apoptosis, cell cycle and signaling (Figure 1.6). We further validated the regulation of these genes by performing independent qPCR using primers different from the PCR array. The results
showed increased expression dopamine receptor 2 (DRD2), adenosine A2a receptor (ADORA2A), pleiotrophin (PTN), bone morphogenic protein 4 (BMP4), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), glial cell derived neurotrophic factor (GDNF) in hNPC exposed to a combination of HIV-1sup and morphine compare to control, un-infected control and HIV-1 (Figure 1.6). This initial experiment evidence of an interactive effect of HIV-1 and morphine at the molecular level, in different aspects of hNPCs development such as migration, differentiation, apoptosis and growth. Moreover, the PCR array studies identified a group of potential candidates that may be involved in HIV-1 and morphine interactions in hNPCs. The findings from these PCR array experiments provided the groundwork for objectives in Chapter 3.

Chapter Summary

In summary, NPCs are likely targets of the deleterious combination of HIV-1 and opiates and represent an unappreciated source of viral-mediated dysfunctions and infection that contributes to the development of neuropathology. The work presented in the following chapters focused on investigating the functional effects of HIV-1 and morphine interaction in primary hNPCs and determining the molecular mechanism underlying this interaction. It is hypothesized that HIV-1 and morphine co-exposure modulates behaviors that are essential to hNPC function, including proliferation, differentiation, and survival. It is further hypothesized that interaction may be differentially mediated by the actions of MOR splice variants expressed by these cells. Overall, the findings from these novel questions will provide valuable insight into the potential role of
NPCs in the development and progression of CNS dysfunctions, especially in the context of opiate drug abuse.

**Figure 1.1 Classification of HIV strains.** There are two major strains of HIV, HIV Type 1 (HIV-1) and a relatively less virulent HIV Type 2 (HIV-2). HIV-1 is further classified into four groups: M (the major group), O (the outlier group), N (non-M and non-O) and P. Group M consists of ten major subtypes: A, B, C, D, F, G, H, J, K and CRFs (circulating recombinant forms).
Figure 1.2 Prevalence of HAND subtypes in the pre-highly active antiretroviral therapy (HAART) era and in the current era of widespread HAART usage. Pre-HAART or cART (before 1996) and HAART (after 1996). Adapted from McArthur et al., *Ann of Neurol*, 2010.
**Figure 1.3 Neuropathogenic mechanisms of HIV-1.** HIV carried by infiltrating monocytes enters the CNS by crossing BBB, and infects perivascular macrophages, microglia and astrocytes. (a). Infected macrophages and microglia are the main producers of new virions, as well as viral toxic proteins, inflammatory modulators and metabolic toxic factors. (b) Released factors in turn activates un-infected microglia and astrocytes, leading to release of chemokines and cytokines. (c) Activated and/or HIV-infected astrocytes alters BBB permeability, resulting in further recruitment of HIV-infected monocytes to the CNS. (e) Soluble factors released by infected and/or activated macrophages, microglia and astrocytes directly impacts neighboring neurons, promoting injury and even cell death. Adapted from Gonzales-Scarano and Martin-Garcia, *Nat Rev Immunol*, 2005.
Figure 1.4 Opiates exacerbate HIV-1 neuropathogenesis via the activation of MOR expressed on CNS cells. Opiates directly interacts with infected and/or activated microglia and astrocytes that express MOR, leading to enhanced viral replication, production of viral toxic proteins, secretion of proinflammatory cytokines and chemokines as well as increased release of ROS and toxic metabolic factors. In addition, opiates augment the inflammatory/cytotoxic positive feedback signaling between HIV-infected microglia and astrocytes. The response of HIV-infected and/or activated microglia and astrocyte, which is enhanced by opiates, ultimately promote lethal and sublethal damages on neurons. Adapted from Hauser et al. Curr HIV Res, 2012.
Figure 1.5 Generation of human MOR splice variants from the alternative splicing of the OPRM1 gene. Schematic diagram of OPRM1 gene containing exon 11, 1, 13, 2, 3, X, Y, 5, 4 and O with four identified promoters (E11, E1, E13 and E2). There are two major MOR splice variants, N-terminal truncated (spliced exon 11) and C-terminal truncated (spliced exon 4) MOR splice variants. Compare to the canonical MOR-1, MOR-
1K utilize promoter E13 upstream of novel exon 13 generating a mature transcript containing exon 13, 2,3 and 4. Adapted from Andersen et al., *PLOS One*, 2013.

**Figure 1.6** HIV-1 and morphine co-exposure modulates gene expression related to neurogenesis in immortalized hNPCs. (A) Gene expression profile was obtained from cultured hNPCs treated with HIV$_{sup}$ (500 pg/ml HIV p24) (HIV) ± morphine (500 nM) (H&M) for 48 using RT-PCR gene array. The array contained multiples primers against gene specifically regulated during neurogenesis. Several genes related to neuronal migration, apoptosis, cell cycle and cell differentiation were regulated by HIV$_{sup}$ and a
combination of HIV\textsuperscript{sup} and morphine compare to media only, and UNF\textsuperscript{sup} (UNF) control. (B) Independent RT-qPCR analysis of target genes; ADORA2A, BMP4, DRD2, GRIN1, GDNF in treated hNPCs. Error bars show mean ± SEM from n=3 biological repeats. Data is presented as fold change in expression relative to control. Fold change was derived using the ΔΔCT method.
Chapter 2

Interactive Effect of HIV-1 and Morphine on the Functions and Productive Infection of Primary Human Neural Progenitor Cells

(This chapter, in part, is under editorial review as a research article in AIDS, entitled Productive Infection of Human Neural Progenitor Cells by R5 tropic HIV-1: Opiate Co-Exposure Heightens Infectivity and Functional vulnerability)

Introduction

CNS complications occur in 40-50% of people living with HIV/AIDS (40-50%), even those receiving combination antiretroviral therapy (cART) (Heaton, et al. 2010; Mothobi and Brew 2012). Collectively known as HIV-1-associated neurocognitive disorders (HAND), these impairments manifest largely as asymptomatic or mild disease. More severe CNS disease, including HIV-associated dementia, has been diminished by cART, but remains problematic in more resource-limited settings (Gonzalez-Scarano and Martin-Garcia 2005). HIV-1 invades the CNS soon after systemic infection, predominantly infecting perivascular macrophages, microglia, and less frequently astrocytes, within the brain parenchyma (An, et al. 1999; Wiley, et al. 1986). It has been occasionally reported that hNPCs, which generate all CNS neurons and macroglia, can harbor infection (Lawrence, et al. 2004; Rothenaigner, et al. 2007; Schwartz, et al. 2007), although this remains controversial. The cumulative response of infected and/or activated CNS cells promotes inflammatory and toxic conditions, the combination of which leads to neuronal injury and
synaptic dysfunction (Epstein and Gelbard 1999; Kaul 2008). Sublethal damage to the integrity of dendrites and synapses is thought to be the basis of neurological impairments associated with HAND (McArthur, et al. 2005).


CCR5-tropic (R5) HIV-1 clone BaL.26 (HIV_{BaL}) and a primary hNPC culture system derived from 8-10-week old fetal brain tissues, and systematically confirmed that R5 HIV-1BaL infects hNPCs. A serial dilution and infection approach demonstrated that hNPCs propagate de novo viral infection in vitro; morphine interacted to sustain productive infection. In addition, we explored the effects of HIV-1BaL on hNPC function. Exposure to HIV_{sup} reduced proliferation and induced premature differentiation of hNPCs into both astrocytes and neurons; effects were significantly enhanced by morphine co-exposure. UV-inactivation showed that infection was not required for HIV/morphine-dependent alterations in proliferation, which were likely due to factors in the infectious supernatant.

We speculate that HIV and opiate-mediated disruptions in the genesis of new CNS cells may create an imbalance in overall neuronal and glial populations. This may be critical in the developing brain of children and adolescents, as well as for maintaining neuroplasticity in the adult brain. Importantly, infection of hNPCs and perhaps their progeny may create an additional viral reservoir in the CNS compartment. The recent escalation in opioid dependence and misuse, which includes adolescents and women of child-bearing age, underscores the potential clinical importance of our findings.

**Materials and Methods**

**Ethics Statement**

All human materials used in this study were purchased from biological supply vendors, were anonymized, and not classified as human subject research. Human blood Leuko Paks were purchased from ZenBio, a FDA licensed facility (Research Triangle Park, NC).
HIV-1 (BaL strain) directed pelleted virus was purchased from Advanced Biotechnologies INC, Eldersburg, MD. Human fetal brain tissue samples were obtained from Advanced Bioscience Resources, Alameda CA.

**Primary hNPCs culture.** hNPCs were derived from 8-10 week-old fetal brain tissues (Advanced Bioscience Resources, Alameda CA), a developmental stage when mature, ramified microglia are generally not present (80). The tissues were homogenized in 0.1% Trypsin, and cultured in NPC media (0.6% glucose, 5 mM HEPES, 10% B27 minus Vitamin A, 1% Pen/Strep, 20 ng/mL FGF, 20 ng/mL EGF, 10 ng/mL LIF). To induce differentiation, hNPCs were maintained in NPC media without FGF, EGF, and LIF.

HIV-1 propagation in human PBMCs. PBMCs were isolated from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) via ficoll gradient centrifugation. For HIV-1 infection, PBMCs activated with phytohemagglutinin (PHA) were infected with HIV-1BaL (Advanced Biotechnology) (1 ng/mL p24) for 72 h. The level of viral infection was quantified by assaying using Alliance p24 Antigen ELISA kit (Advanced Bioscience).

HIV-1 treatments for proliferation and differentiation assays. hNPCs were treated with supernatant from HIV-infected PBMCs (HIVsup) at level of 0.5, 5.0, 50 or 500 pg/mL p24. Controls included treatment with media only (control), and equal volume of supernatant from un-infected, PHA-activated PBMCs (UNFsup) at the same initial cell density. Morphine (500 nM) treatment was performed along with supernatant treatment. For UV-inactivation of HIV, a sample of HIVsup was UV-crosslinked at a setting of 1200 j/sm2 for 5 min on ice.
HIV-1 infection of hNPCs. hNPCs were initially exposed to purified HIV-1BaL at various concentrations (1.0 – 100 ng/mL p24) for 24 h. The cells were then washed thoroughly to remove residual virus and incubated in NPC media for a specific duration of time, depending on the experimental outcome.

**Immunostaining.** Treated hNPCs were fixed using 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, then immunostained with primary antibody for nestin (Abcam), SOX2 (Abcam), GFAP (Chemicon) or MAP2 (Millipore), BrdU (Dako), HIV Nef (Abcam), Iba-1 (Abcam), then conjugated with Alexa Fluor 488 (Oregon green) or Alexa Fluor 594 (Thermofisher) secondary antibody. Nuclei were stained with Hoechst 33342. Samples were imaged using a Zeiss LSM 700 laser scanning confocal microscope under 20x or 63x objective. The images were collected using ZEN 2009 Light Edition software (Carl Zeiss, NY).

**RT-PCR.** Total RNA purified from cultured hNPCs using RNeasy Mini Kit (Qiagen, Inc.) was used to generate cDNA templates via reverse transcription using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Target genes were amplified from cDNA clones via PCR using MangoMixTM (end point PCR) or FastStart DNA Master SYBR Green (qPCR) and gene specific primer sets. See Table 2.2 for sequence of the primers used for this study.
**BrdU incorporation and analysis.** Cultured hNPCs treated for 12, 24, and 48 h, and pulsed with 1 mM of BrdU at the final 6 h of treatment. Fixed cells were immunostained using anti-mouse BrdU antibody (Dako, CA).

**Cell growth and doubling time.** Cultured hNPCs were treated for 5 d, and cell samples were harvested every 24 h using Accutase cell detachment solution (Millipore). The cell density was determined using Cellometer cell counting chambers and Cellometer Auto T4 Counter (Nexcelom, MA). Doubling time was calculated using the equation $D(t) = T \ln 2 / \ln(X_e/X_b)$ (ATCC Animal Cell Culture Guide).

**Live/Dead staining.** Changes in hNPCs’ survival in response to treatment were monitored using LIVE/DEAD Reduced Biohazard Viability kit (Molecular Probes, Inc) according to the manufacturer’s instructions.

**Statistics.** Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc). Unless otherwise indicated, data were analyzed by one-way ANOVA followed by Bonferroni post-hoc test. A value of $p<0.05$ was considered significant.

**Results**

1. **Characterization of primary hNPC cultures**

   Human cells may more accurately reproduce disease features involving species-dependent interactions. To minimize variability, samples were carefully matched for age,
sex, and passage number, and cell composition was characterized at each passage. Cellular composition was examined via immunostaining and RT-PCR. Immunostaining results showed that more than 90% expressed NPC markers nestin and/or SOX2, with smaller percentages expressing the astrocytic marker GFAP (<20%) or the neuronal marker MAP2 (<5-10%). Within populations expressing GFAP and MAP2, 80-90% also expressed SOX2, indicating maintenance of an immature phenotype (Figure 2.1a-2.1b). RT-PCR findings demonstrated that cells expressed the NPC genes PAX3 and NOTCH1, the astrocyte ALDH1L1 and the neuronal gene dlg4a that encodes human postsynaptic density protein 95 (PSD-95) (Figure 2.1c). The sex of each culture was determined by the presence of Y- and X-chromosome related genes (Figure 2.1d). Male cultures were utilized to avoid any sex-specific effects.

2. Sustained and productive infection of hNPCs by R5 HIV-1BaL

To determine the contribution of hNPCs to HIV-1 and opiate neuropathogenesis, we first reassessed prior reports that hNPCs could be infected by HIV (Rothenaigner, et al. 2007; Schwartz, et al. 2007; Schwartz and Major 2006). De novo infection was confirmed by monitoring production of HIV transcripts and proteins using multiple methods. PCR analysis indicated expression of HIV Tat in hNPCs 72 h post-infection at all HIV-1BaL concentrations (Figure 2.2a). Robust expression of HIV Nef immunoreactivity was also observed in HIV-1BaL treated hNPCs (Figure 2.2b). Nef expression was detected in 5-8% of cultured hNPCs exposed to either 10 or 100 ng/ml. The presence of microglia, macrophages and T-cells was examined to rule out potential contributions to infectivity results. The microglia/macrophage specific Iba-1 protein and
transcript were not detected (Figure 2.2b, 2.2c). The mRNAs of other human microglia/macrophage genes including CX3CR1 and cd11b, as well as T cell genes CD4 and CD8 were not detected in three independent cultures (Figure 2.2c, 2.2d). Furthermore, earlier reports (Lawrence, et al. 2004; Rothensaigner, et al. 2007) of p24 production in the culture supernatant of infected hNPCs were confirmed by ELISA (Figure 2.2g).

To test whether the infection observed in hNPCs was productive, we adapted a serial dilution and passaging approach. Initial infections were performed by treating cultured hNPCs with purified HIV-1BaL; infection was confirmed 3 d post-wash by ELISA for p24 production. Supernatant was removed and diluted (1:100), then placed on naïve hNPCs for 3 d. Three serial, 1:100 dilutions were performed and p24 was measured at the end of each infection period (Figure 2.2e). In the absence of new infection, the p24 level is expected to decline at each passage (overall 1x106 dilution). Instead, the p24 level was sustained over time, suggesting that the infection was productive and transferable (Figure 2.2f). Morphine did not augment the infectivity of hNPCs during the initial infection or at dilutions, even when the infection period was extended to 5 d (not shown). Collectively, the findings from these multiple experimental approaches show that R5 HIV-1 can infect primary hNPCs, and also that hNPCs are capable of propagating de novo infection in vitro.

3. Morphine sustains productive R5 infection of hNPCs

The effect of morphine on the infection process was further analyzed at later stages of infection. Cultured hNPCs were initially infected with purified HIV-1BaL at 1.0,
10 and 100 ng/mL p24. p24 production was monitored in cultures ± morphine for up to 15 d post-infection. A gradual increase in p24 production was measured at all infection levels, was sustained for up to 12 d, and eventually plateaued after 15 d. Morphine did not enhance the early infection, but p24 production was significantly increased by morphine at 12 and 15 d post-infection in hNPCs initially infected at 100 ng/ml p24 (Figure 2.2g). Morphine also increased HIV Tat mRNA levels at 9 d post-infection, a period preceding the morphine interaction on p24 (Figure 2.2h). We confirmed that the difference in p24 production and Tat expression was not due to death of hNPCs exposed to HIV-1BaL ± morphine. A significant difference in DNA content between groups was not observed (Figure 2.2i).

4. HIV-1 and Morphine Affect DNA Synthesis and Doubling Time

Separate from the question of hNPC infection, we tested whether HIV-1 and morphine co-exposure affected hNPCs proliferation, their DNA synthesis, and doubling time. To model inflammatory conditions associated with HIV infection, we treated hNPCs with HIVsup at multiple p24 levels. Bromodeoxyuridine (BrdU) was used to label cells entering the S-phase of DNA synthesis. BrdU immunoreactivity was significantly decreased versus media control at 24 h in hNPCs treated with higher HIVsup concentrations (50 and 500 pg/ml p24), and in all tested HIVsup concentrations at 48 h. Significant differences between the HIVsup and the uninfected supernatant (UNF) control at 24 h (500 pg/ml p24) and 48 h (50-500 pg/ml p24) indicated a specific response to the infected condition (Figure 2.3a). Morphine enhanced this effect, as evidenced by further
reduction of BrdU labeling in hNPCs treated with combined HIVsup and morphine (Figure 2.3b, 2.3c).

Changes in DNA synthesis were reflected in hNPC replication. hNPCs were treated for 5 d with HIVsup at concentrations where interactive effects on BrdU incorporation were observed (50 and 500 pg/ml p24). Cell density was measured every 24 h to calculate the population doubling time. Co-exposure to morphine at higher HIVsup concentration significantly prolonged doubling time (Table 2.1). Effects on proliferation and cell division were not attributable to changes in cell death (Figure 2.3d).

5. HIV-1 and Morphine Affect hNPC Differentiation

hNPCs were treated with HIVsup (50 and 500 pg/ml p24) ± morphine for 12 d, and immunolabeled with antibodies to nestin, SOX2, GFAP, and MAP2. Compared to controls, cultures treated with both HIVsup concentrations had significantly higher percentages of both GFAP+ and MAP2+ cells. HIV-1 and morphine interactions were observed on neuronal and astroglial differentiation, evidenced by increased percentages of MAP2+ cells in response to morphine and HIVsup at 50 and 500 pg/ml p24, and GFAP+ cells in response to morphine and HIVsup at 500 pg/ml p24. The ratio of neurons to astroglia was unchanged by any treatment (data not shown). It is notable that despite the increased expression of MAP2 and GFAP upon HIVsup ± morphine treatment, the percentage of nestin- and SOX2-expressing cells was unchanged (Figure 2.4a, 2.4b).

6. HIV-1 Infection is not Required for Effects on Proliferation
As hNPCs were productively infected by HIV-1, we tested whether HIV-1 infection underlies the functional responses of hNPCs by comparing the effects of UV-inactivated HIVsup and concentrated HIV-1 virions. UV irradiation inactivates virions in the infective supernatant without eliminating inflammatory or other deleterious factors. Inactivation was confirmed by lack of HIV p24 production by PBMCs after exposure to UV-irradiated HIV (Figure 2.5a). BrdU analysis showed that 48 h exposure to concentrated, infectious, HIV-1BaL did not change the percentage of BrdU+ hNPCs relative to control. However, a significant decrease of BrdU incorporation was observed in hNPCs treated with UV-inactivated supernatant (Figure 2.5b). Thus, changes in hNPC proliferation are not due to infection per se, but rather mediated by the combined effect of inactive virions, HIV proteins, and inflammatory conditions created by infected cells.

**Discussion**

Our findings demonstrate that hNPCs are targets for HIV-1 infection at two levels. Conditions created by HIV-1 infection significantly restrict hNPC proliferation and alter the dynamics of their differentiation. Also, hNPCs can be infected and can propagate active infection, at least under certain conditions in vitro. Many outcomes were exacerbated by opiate co-exposure. Injection drug abuse is a major vector for HIV transmission, and opiate abuse is re-emerging as a major public health crisis, underscoring the likely clinical importance of HIV and opiate interactions.

NPCs are present throughout the developing brain, and persist in the dentate gyrus and subventricular zone of the adult brain (Gage 2002). NPCs are highly mitotic and migratory in the developing brain; adult NPCs are more quiescent but can be activated to
proliferate, particularly in response to insult/injury (Kernie and Parent 2010). Aberrant NPC activation can perturb the balance of CNS populations, and the consequences of hNPC dysfunction have been implicated in various psychiatric (Kim, et al. 2012; Toro and Deakin 2007) and neurodegenerative diseases (Winner and Winkler 2015), as well as in injuries caused by inflammation (Ekdahl, et al. 2003; Monje, et al. 2003), stroke/ischemia (Kernie and Parent 2010), or epilepsy (Jessberger and Parent 2015).

The concept that hNPCs may be infected by HIV remains controversial despite culture studies which showed limited p24 production by fetal-derived hNPCs exposed to X4-tropic HIV\textsubscript{IIIB} and viral isolates HIV\textsubscript{NL4-3} (Lawrence, et al. 2004), and the presence of pro-viral DNA in an immortalized human neural stem cell line treated with HIV\textsubscript{IIIB} (Rothenaigner, et al. 2007). The presence of viral DNA was also noted in nestin+ hNPCs microdissected from the hippocampus of pediatric AIDS patients (Schwartz, et al. 2007). We hoped to clarify this controversy by systematically examining the capacity of primary hNPCs from multiple independent samples to be infected and to propagate infection. De novo infection resulted in detection of HIV-1 Tat, Nef, and p24. PCR confirmed that microglia, monocytes/macrophages, and both CD4+ and CD8+ cells were absent from the cultures and could not contribute to infection. Confident that a small percentage of hNPCs were infected, we tested whether infection could be transferred to naïve cells. The p24 titer was sustained even after a series of three dilution/infection cycles, providing compelling evidence that hNPCs can actively propagate HIV-1 infection. A mechanism for HIV entry into hNPCs remains to be determined, although we found no evidence of CD4 expression, suggesting that infection is CD4 independent. CCR5 and CXCR4 are
expressed in immortalized hNPCs (Peng, et al. 2011); these HIV co-receptors may be involved in hNPC infection.

hNPCs were only infected at relatively high viral titers, suggesting that NPCs in close proximity to HIV-infected microglia/macrophages in the brain parenchyma may be most vulnerable. This likely occurs in NPC-rich brain regions such as the hippocampus, where a high density of infected microglia and macrophages has been demonstrated (Wiley, et al. 1998). Viral tropism may also influence the capacity for hNPC infection. Studies here used an R5-preferring strain, but hNPCs might also be susceptible to infection by other variants, perhaps requiring a lower viral titer to achieve infection. In fact, two studies reported evidence of p24 production in progenitor cultures exposed to the X4-tropic HIV-1IIIB strain (Lawrence, et al. 2004; Rothenaigner, et al. 2007). While R5 viruses represent the predominant virus population early after infection, and persist throughout disease, X4 viruses are generally associated with heightened virulence, rapid CD4+ T cell-decline, CNS injury, and accelerated AIDS progression (Connor, et al. 1997; Kaul, et al. 2007; Meucci, et al. 1998; Scarlatti, et al. 1997; Schramm, et al. 2000). The likelihood of hNPC infection is increased if they are vulnerable to X4 as well as R5 strains.

Exposure to HIV-1 at p24 levels measured in the infected brain (Achim, et al. 1993; Brew, et al. 1994) affected critical hNPC functions. HIVsup dramatically decreased BrdU incorporation, and prolonged the doubling-time of hNPCs. Reduced proliferation was observed with HIV proteins (Hahn, et al. 2012; Krathwohl and Kaiser 2004; Mishra, et al. 2010; Okamoto, et al. 2007); we showed a similar effect of HIVsup on immortalized hNPCs (Hahn, et al. 2012). Hippocampal tissue from autopsied HIV-1 dementia patients showed reduced Ki-67+ cells (Krathwohl and Kaiser 2004), which would be consistent
with reduced NPC proliferation. In contrast, supernatant from LPS-stimulated, HIV-infected macrophages increased hNPC proliferation (Peng, et al. 2011), although the proliferative effects of acute LPS on NPCs raised a confound (Go, et al. 2009). Morphine co-exposure accentuated the effect of HIVsup on both hNPC DNA synthesis and cell numbers, paralleling interactions previously reported between morphine and HIV-1 Tat on fetal hNPCs (Malik, et al. 2014), and morphine and HIV-1 Tat or HIVsup on immortalized hNPCs (Hahn, et al. 2012). Effects were unrelated to hNPC survival, which was unchanged by any treatment. Morphine by itself did not significantly affect hNPC proliferation, although this was previously reported in rodent NPCs (Arguello, et al. 2008; Eisch, et al. 2000), and hNPCs (Malik, et al. 2014), perhaps reflecting species or timing differences.

We also explored hNPC differentiation and found that HIVsup exposure induced the expression of differentiation markers, MAP2 and GFAP suggesting that these cells are likely in the early phases of premature differentiation into both neurons and astrocytes. Morphine co-exposure significantly enhanced this effect. The effects of HIVsup ± morphine on proliferation and differentiation suggest that extended exposure will alter the balance/size of MAP2+ and GFAP+ cell populations. A meaningful, in vivo assessment of this will require stereological assessment of potentially affected brain regions.

Are these in vitro results relevant to human disease? NPCs are present in both developing and adult systems, albeit in different regions. Adult and immature NPCs also have unique characteristics that may influence their response to HIV infection and to opiate exposure. An accurate balance between NPC proliferation and differentiation is
necessary for establishing and maintaining cell populations, and perturbations to these regulated processes can influence overall CNS architecture and function (Caviness, et al. 1995; Kempermann, et al. 2004; Ladran, et al. 2013). Cortical atrophy is a common feature of pediatric neuroAIDS, especially in cART-naïve children (DeCarli, et al. 1993; George, et al. 2009). The reduced NPC production and early differentiation described here may contribute to cortical atrophy and other neurological manifestations in children and adolescents with HIV-1 (Epstein, et al. 1986; Mintz and Epstein 1992; Schwartz and Major 2006). Although cART has greatly reduced the incidence of HAND in children, currently 2.6 million children worldwide are infected with HIV-1, and children represent 1-2% of cases in the United States (http://www.who.int/hiv/en/).

Unlike NPCs in the developing brain, adult NPCs are restricted to the subventricular zone and the subgranular zone of the hippocampal dentate gyrus (Eriksson, et al. 1998; Gage 2002). There is a strong correlation between hippocampal atrophy/dysfunction and reduced spatial recognition and learning/memory ability, both in HIV experimental models (Fitting, et al. 2006; Fitting, et al. 2013; Harricharan, et al. 2015; Marks, et al. 2016; Torres and Noel 2014) and in HIV patients (Ortega, et al. 2013; Pfefferbaum, et al. 2014; Wang, et al. 2015). The hippocampus of HIV-1 seropositive adults exhibits high viral load (Wiley, et al. 1998) and often shows a significant atrophy (Archibald, et al. 2004; Wang, et al. 2015), suggesting that NPCs may have limited expansion. Krathwohl et al. demonstrated that proliferating hippocampal NPCs from adult subjects with HIV-1 dementia were significantly reduced when compared to infected subjects without dementia, also suggesting that hippocampal impairments might involve failure to generate normal numbers of adult NPCs (Krathwohl and Kaiser 2004).
There could be multiple clinical consequences of productive hNPC infection. First, HIV-1 infected hNPCs might augment overall CNS disease by increasing the levels of virus, viral proteins, and inflammation in the brain. The ability of HIV-1 infection to disrupt hNPC proliferation without affecting viability might promote HIV neurovirulence, similar to other cells with active viral replication. The virus may be able to maintain persistent infection by switching host DNA synthesis to viral DNA synthesis in infected cells (Das and Basu 2011), as has been shown for hNPCs upon infection with human cytomegalovirus (HCMV) (Svant, et al. 1998). Secondly, infected hNPCs may pass the infection to long-lived progeny, generating new viral reservoirs and new barriers for eradication. In support of this concept, inflammatory mediators such as TNF-α (Lawrence, et al. 2004) and IL-1β (Sabri, et al. 1999) were found to reactivate viral production in cultured NPC-derived astrocytes. The ability of NPCs to pass infection to progeny might also explain reports of infected, immature neurons (Canto-Nogues, et al. 2005; Torres-Munoz, et al. 2001).

Morphine exacerbated the effect of HIV-1 on the proliferation and differentiation and sustained de novo viral production in vitro. If these results can be extrapolated to the human brain, it suggests that the extent of dysfunction and infection in both adult and young NPCs will be greater with opiate co-exposure. These findings are especially concerning given the current opiate drug crisis. Markedly increased heroin use and overdose-related hospitalizations were documented over the past 10 years (Cicero, et al. 2014; Jones 2013); the number of heroin users nearly tripled between 2005-2012 (Bruneau, et al. 2012). A major, coincident, consequence of increased opiate abuse has been the rising outbreak of HIV infection outside urban communities, where new
infections occur frequently in young adults, almost half of whom are women (Conrad, et al. 2015; Strathdee and Beyrer 2015). Although perinatal HIV transmission has been significantly decreased by cART, the CDC reported 174 new cases of HIV in children in the US in 2014, as well as 1,999 children living with perinatal HIV. Furthermore, an estimated 9,131 adults and adolescents were living with HIV acquired perinatally (http://www.cdc.gov/hiv/group/gender/pregnantwomen/). Importantly, maternal drug use is strongly associated with increased risk of perinatal transmission (Magder, et al. 2005; Van Dyke, et al. 1999). A high percentage of children and young adults with HIV/AIDS also receive opiates for pain intervention, as pain prevalence among this group is reportedly as high as 60% (Hirschfeld, et al. 1996).

In conclusion, our study shows that cultured hNPCs are capable of sustaining and propagating HIV-infection. Even in the absence of infection, hNPC function is compromised by exposure to HIV. Dysregulation of these proliferative, multipotent cells by HIV-mediated inflammatory/toxic insults may intensify neuropathology, leading to greater and longer-lasting deficits in HIV-infected children and adolescents. Infected hNPCs may also serve as an additional CNS viral reservoir. The significant interactions between morphine and HIV suggest that HIV patients with a history of opiate abuse or exposure may be particularly vulnerable to HIV-related cognitive and motor deficits.
Figure 2.1 Detailed characterization of the hNPC cultures. (a) Representative fluorescent images of hNPCs double-immunostained for nestin (green) and glial fibrillary acidic protein (GFAP) (green), microtubule associated protein 2 (MAP2) (green) and sex-determining region Y-box2 (SOX2) (red). Hoechst 33342 (blue) staining indicates cell nuclei. Image was obtained using 20x magnification. Scale bar represents 50 μM. (b) The percentage of hNPCs expressing nestin, SOX2, GFAP, and MAP2 at passage 2, 4, and 6 (P2, P4, P6) were calculated from a population of >200 Hoechst+ cells. Error bars show mean ± SEM from four studies (n=4), each using hNPCs derived from independent tissue samples. (c) 1% agarose gel showing PCR products for NPC genes; paired box 3 (PAX3), Notch homology 1 (NOTCH1), glial genes; astrocyte gene aldehyde dehydrogenase 1 family, member L1 (ALDH1L), myelin basic protein (MBP), and neuronal gene (Psd95),
amplified from purified RNA of cultured hNPCs. (d) Sex of individual hNPC cultures was determined via RT-PCR and primers specific for the Y-chromosome sex determining region Y (SRY) and X-chromosome ATP-binding cassette sub-family B member 7 (ABCB7) and Filamin A (FLNA).
**Figure 2.2** Morphine co-exposure prolonged the productive infection of hNPCs by R5-tropic HIV-1BaL. (a) Agarose gel containing PCR product for HIV trans-activator of transcription (Tat) amplified from hNPCs infected with purified HIV-1BaL at 1.0, (HIV 1.0), 10 (HIV 10), and 100 ng/ml p24 (HIV 100) for 72 h. Control represents cultured hNPCs not exposed to HIV-1BaL. (b) Representative confocal images of HIV negative regulatory factor (Nef) (green) and nestin (red), IgG isotype control (green), and ionized calcium-binding adapter molecule-1 (Iba-1) (red) immunostaining of uninfected (control) and HIV-
1_{BaL} -infected (HIV-infected) hNPCs. (c) mRNA expression analysis of Iba-1, fractalkine receptor 1 (CX3CR1), and integrin alpha M (CD11b) from hNPC and PBMC cultures. (d) mRNA expression analysis of T-cell surface glycoproteins, cluster of differentiation 8 (CD8) and cluster of differentiation 4 (CD4) in cultured hNPCs and PBMCs. The PCR results from (a), (c) and (d) are representative of n=3 independent cultures. (e) Schematic diagram of the method for serial dilution and infection of hNPCs with HIV-1. (f) p24 measurements from cultured hNPCs with or without morphine co-exposure after each 3 d incubation with diluted culture supernatant. Initial p24 level before dilution in hNPC supernatants ranged between 263.50 ± 78.51 and 412.90 ± 71.11 pg/mL. Data are presented as log10 of p24 concentration (pg/mL) measured at each dilution. Dotted blue line represents the expected p24 concentration after each dilution if there is no new virus production. (g) De novo infection was monitored for up to 15 d via p24 ELISA in cultured hNPCs initially infected with 100 (HIV 100) ng/mL HIV-1_{BaL} alone or with morphine. Data are from n=4 cultures derived from independent tissue samples. (*) P <0.05 vs. HIV 100 alone. (h) Quantification of Tat mRNA expression in hNPCs exposed to HIV-1_{BaL} at concentrations of 1.0 ng/mL (H1.0), 10 ng/mL (H10), or 100 ng/mL (H100) p24 alone or with morphine (M) for 9 d. (i) Spectrophotometer measurement of total DNA content from hNPC cultures exposed to morphine, purified HIV-1_{BaL} at 10 ng/ml p24 (H10) or 100 ng/ml p24 (H100) alone or with morphine (M) for 15 d. Error bars show mean ± SEM from n=3 hNPC cultures derived from independent tissue samples. * P <0.05.
Figure 2.3 HIV-1 ± morphine co-exposure modulates DNA synthesis of hNPCs.  (a) BrdU immunostaining analysis on hNPCs treated with HIV<sub>sup</sub> at 0.5 - 500 pg/ml HIV p24 (HIV 0.5, HIV 5.0, HIV 50, HIV 500) for 12, 24 and 48 h. Cells were pulsed with BrdU (1mM) 6 h prior to the end of treatments. Controls included NPC media only (control), and equal volumes of uninfected supernatant (UNF 0.5 – UNF 500) for each HIV<sub>sup</sub> level. (b) BrdU analysis on hNPCs treated with HIV<sub>sup</sub> at 5.0 – 500 pg/ml HIV p24 ± morphine (500 nM), equal volumes of uninfected supernatant (UNF<sub>sup</sub>), and media only (control) for 48 h. Controls included NPC media only (control) and equal volumes of uninfected supernatant (UNF<sub>sup</sub>) for each HIV<sub>sup</sub> level. (c) The LIVE/DEAD viability assay (Thermo Fisher Scientific) was used to analyze cell death rates in hNPCs treated with morphine,
HIV sup at 5.0-500 pg/ml HIV p24 (HIV 5.0, HIV 50, HIV 500) ± morphine (M) for 48 h. Data were derived from >200 hNPCs, and presented as percentage of DEAD Red (an ethidium homodimer derivative that identifies apoptotic cells) positive cells relative to the total cells. (d) Representative images showing BrdU (green) immunostaining of hNPCs treated with 500 pg/ml p24 HIV sup (HIV 500) alone or with morphine (HIV 500 + M). Data for (a) and (b) was derived from >200 Hoechst+ hNPCs, and presented as percentage of BrdU+ cells relative to the total cells. For (a), (b) and (c), error bars show mean ± SEM from n=4 studies, each using hNPCs derived from independent tissue samples. Significance in (b) was determined by two-way ANOVA with Bonferroni post-hoc testing. ($) $P <0.05$ vs. control, (*) $P <0.05$. 
Figure 2.4 HIV-1 ± morphine co-exposure promotes MAP2 and GFAP expression of hNPCs. (a) Representative immunostaining of SOX2 (red), GFAP (green), and MAP2 (green) immunostaining of hNPCs treated with morphine (500 nM), HIV_{sup} at 500 pg/ml p24 alone (HIV 500) or with morphine (HIV 500 + M) in differentiation medium for 12 d. (b) Immunostaining analysis of hNPCs treated with morphine, HIV_{sup} at 50 pg/ml p24 (HIV 50), 500 pg/ml p24 (HIV 500) alone or with morphine (M), and equal dilutions of supernatant from uninfected PBMCs (UNF50 or UNF500) alone or with morphine (M). The percent of hNPCs expressing nestin, SOX2, MAP2, and GFAP from each treatment group was calculated from >200 Hoechst\(^*\) cells. Error bars show mean ± SEM from \(n=5\)
studies each using hNPCs derived from independent tissue samples. ($) $P < 0.05$ vs. control, (*) $P < 0.05$. 
Figure 2.5 Infection of hNPCs is not required for the anti-proliferative effect of HIV-1. (a) p24 measurement in cultured PBMCs treated with HIVsup (1.0 ng/mL p24) or an equal volume of UV-inactivated HIVsup for 3, 6 and 9 d. (b) BrdU immunostaining analysis in hNPCs treated with purified HIV-1Bal at concentrations of 1.0 (HIVBal 1.0), 10 (HIVBal 10), and 100 (HIVBal 100) ng/mL p24 or UV-inactivated HIVsup from infected PBMCs used in (a) at concentrations of 5 (UV H5.0), 50 (UV H50), or 500 (UV H500) pg/mL p24 for 48 h. Error bars show mean ± SEM from n=3 hNPC cultures derived from independent tissue samples. (#) P < 0.05 vs. control, and all purified HIVBal exposure levels.
Table 2.1 Interactive effect of HIV-1 and morphine on hNPC doubling time.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Doubling Time (h)</th>
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<th>Doubling Time (h)</th>
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<tbody>
<tr>
<td>Control</td>
<td>43.58 ± 1.27</td>
<td>HIV50 + M</td>
<td>63.44 ± 4.62 *</td>
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<tr>
<td>Morphine</td>
<td>49.04 ± 1.68</td>
<td>UNF500</td>
<td>47.98 ± 4.10</td>
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<tr>
<td>UNF50</td>
<td>47.90 ± 1.51</td>
<td>UNF500 + M</td>
<td>46.71 ± 1.12</td>
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<tr>
<td>UNF50 + M</td>
<td>47.20 ± 1.84</td>
<td>HIV500</td>
<td>55.98 ± 3.09</td>
</tr>
<tr>
<td>HIV50</td>
<td>55.03 ± 6.16</td>
<td>HIV500 + M</td>
<td>68.65 ± 6.20 *</td>
</tr>
</tbody>
</table>

* p < 0.05 vs. Control

Table 2.2 Primer sequences for all target genes used in Chapter 2 studies

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer Sequence</th>
</tr>
</thead>
</table>
| PAX3        | for 5'-GTGCCGTCAGTGAGTTCCAT-3'  
              | rev 5'-GAAGGGACCTGATCCGAGC-3'   |
| NOTCH1      | for 5'-GCAGAGGCGTGCAAGCAGCTAT-3'  
              | rev 5'-GGGCCACGATTTCCTCGA-3'    |
| ALDH1L1     | for 5'-ATCTTTGTGACTGATGCACCT-3'  
              | rev 5'-GCACCTCTTTGCTACCTCTC-3'  |
| MBP         | for 5'-GCTGTCACACATGCATACAGTAC-3'  
              | rev 5'-CCCCAGCTAAATCTGCTCA-3'    |
| Psd95       | for 5'-CCCCCAACATGCGATCTGTCCT-3'  
              | rev 5'-CCCCGTTCACATCCTGCTGAGG-3' |
| SRY         | for 5'-CACCAGTGACTGCACTTACC-3'   
              | rev 5'-GCCAATGTTACCCGATTGTC-3'  |
| ABCB7       | for 5'-CTGGCTCCAGTGCGATCTGTCCT-3'  
              | rev 5'-GTGGCATCTACCTGCGTAC-3'   |
| FLNA        | for 5'-TAGACTGCAACAGTGCGT-3'     
              | rev 5'-TCTAGAGAAACTGAAAGCCACCG-3' |
| IBA-1       | for 5'-ACCAGGGATTTCAGGGGGA-3'    
              | rev 5'-TCCAGCATTGTTTCCAGGGA-3'  |
| CXCR1       | for 5'-AGGTGGCAACACACTGAGAC-3'   
              | rev 5'-ATGGTGAAGGCTCTTAGTGCG-3' |
| Cd11b       | for 5'-CATGACATAAGGCTAAGGCTGT-3'  
              | rev 5'-GCTTTGGTGCTTCCCTGTG-3'   |
| Tat         | for 5'-GGGATTCATGAGCCAGTCATGCCT-3'  
              | rev 5'-TTATCATTGGTGAAGAAACTTG-3' |
| GAPDH       | for 5'-CATGGCACCAGTCAAGGGCTGAGAA-3' 
              | rev 5'-CAGTGGCATCCACACGACTCA-3' |
Central Role of CDK5 in Human Neural Progenitor Cell Responses to HIV-1 and Opiate Exposure

(This chapter, in part, will be prepared as a research article in Journal of Neurochemistry, entitled Central Role of CDK5 in Human Neural Progenitor Cell Responses to HIV-1 and Opiate Exposure)

Introduction

Infection with human Immunodeficiency virus type-1 (HIV-1) can lead to neurological and motor impairments for which there are currently no effective treatments. About 30-50% of individuals living with HIV/AIDS suffer from these complications, collectively known as HIV-1 Associated Neurocognitive Disorders (HAND) (Anthony and Bell 2008; Gonzalez-Scarano and Martin-Garcia 2005). The persistence of neurological impairments in these patients, despite the use of combination antiretroviral therapy (cART), is thought to be related to recurrent viral replication, viral resistance, dysregulated immune response, and poor CNS penetration of most antiretroviral drugs (Anthony and Bell 2008). Worldwide, HAND remains a common cause of cognitive impairments, and is becoming a major health and socioeconomic issue in industrialized countries, particularly in the United States (Heaton, et al. 2010).
HIV-1 enters the CNS immediately after systemic infection with the infiltration of infected monocytes, and creates a unique, long-lasting viral compartment (An, et al. 1999; Wiley, et al. 1986). Perivascular macrophages and microglia are the main source of productive viral replications in the CNS (Cosenza, et al. 2002; Takahashi, et al. 1996; Williams, et al. 2001). A small number of astrocytes can also harbor virus but lack the ability to replicate active infection in the brain (Brack-Werner 1999; Sabri, et al. 1999). HIV-infected astrocytes and microglia can become activated by HIV, leading to the production of viral toxic proteins, inflammatory mediators, excitotoxic factors, and reactive oxygen/nitrosative species (Gonzalez-Scarano and Martin-Garcia 2005). The cumulative response of infected and/or activated CNS cells promotes inflammatory and excitotoxic conditions, the combination of which leads to neuronal injury and synaptic dysfunction (Epstein and Gelbard 1999; Kaul 2008). It is postulated that the sub-lethal damage to the integrity of dendrites and synapses of affected neurons is the basis of most neurological impairments associated with HAND (McArthur, et al. 2005).

Opiate drug abuse has consistently been shown in animal and human systems, to exacerbate CNS deficits caused by HIV-1 infection (Fitting, et al. 2014; Gupta, et al. 2010; Hu, et al. 2005; Kumar, et al. 2006). Opiate drugs such as heroin and morphine, modulate CNS immunity and subsequently enhancing disease progression by directly interacting with infected and/or activated glial cells. Opiates are thought to modulate HIV neuropathogenesis through the action of opioid receptors, particularly the mu opioid receptor (MOR), expressed on neurons and glial cells (Zou, et al. 2011). Despite this, the mechanism(s) by which opiates exacerbate HIV-1 neuropathogenesis remain largely
unknown. Understanding the distinct targets of opiate/HIV interaction is a critical step in developing therapeutic strategies for HAND in the opiate-abusing, HIV-infected population.

In recent years, several studies have started to establish the role of neural progenitor cells (NPCs) as additional targets of the interaction of HIV-1 and opiates. NPCs are the precursors of all neurons and glial cells in the CNS. They are found throughout the developing brain and persist in the dentate gyrus and the subventricular zone (SVZ) of the adult brain, where they are critical for ongoing neurogenesis and gliogenesis. In addition, NPCs have been shown to play a central role in cognitive functions such as learning and memory (Eriksson, et al. 1998; Gage 2000; Gage 2002). The consequences of NPC dysfunction have been documented in various neurodegenerative (Desplats, et al. 2012; Mu and Gage 2011; Winner and Winkler 2015) and psychiatric (Jessberger and Parent 2015) diseases, as well as in central injuries caused by inflammation (Ekdahl, et al. 2003). The independent effects of HIV-1 and opiates on murine and human NPCs have also been extensively reported (Hahn, et al. 2012; Krathwohl and Kaiser 2004; Malik, et al. 2014; Okamoto, et al. 2007; Peng, et al. 2008), indicating their potential vulnerability to HIV-1 and morphine interaction. We and others have initially questioned the interactive effect of HIV-1 and morphine on the functions of NPCs, and reported that morphine co-exposure exacerbates the anti-proliferative and differentiation effect of HIV Tat and HIV-infected supernatant on NPCs both in vitro and in vivo (Hahn, et al. 2012; Malik, et al. 2014). Malik et al. provided initial mechanistic details into the interaction of HIV-1 and morphine on the proliferation of human NPCs (hNPCs), suggesting the involvement of the extracellular signal-regulated kinase (ERK1/2)-p53-p21 axis (Malik, et
al. 2014). Despite this, the precise mechanism(s) on how morphine augments the functional consequences of HIV-1 on hNPCs remain to be fully identified, and therefore warrant further investigation.

In this study, we attempted to elucidate the downstream molecular mechanism underlying the interaction of HIV-1 and morphine in primary hNPCs. Gene array techniques were used to screen downstream molecular targets involved in HIV-1 and morphine effects on primary hNPCs. We hypothesized that exposure to HIV-infected supernatant (HIV\textsuperscript{sup}) and morphine would regulate the expression of genes relating to neurogenesis, thus alternating signaling pathways that mediate proliferation and differentiation of hNPCs. HIV and morphine independently and interactively caused extensive modulation of gene expression in hNPCs, including genes associated with Cdk5 signaling. Cdk5 is a serine/threonine cyclin-dependent kinase, largely found in the CNS where it regulates various essential neuronal processes. Substantial studies have shown the prominent role of Cdk5 in the normal CNS development (Ko, et al. 2001; Paglini and Caceres 2001), neuronal migration during corticogenesis (Ohshima, et al. 1996), neurite outgrowth (Nikolic, et al. 1996), synaptic plasticity (Cheng and Ip 2003), neurotransmission (Cicero and Herrup 2005), and cognitive function (Angelo, et al. 2006). Recently, Cdk5 has emerged as a regulator of neuronal survival, neuroprotection, and neuronal death pathways (Ohshima, et al. 1996; Vartiainen, et al. 2002; Wang, et al. 2003a). Subsequent validation via independent RT-qPCR and immunoblotting indicated the specific up-regulation of Cdk5 and its regulators p35 by HIV\textsuperscript{sup} and morphine co-exposure. Calpain, the calcium-dependent protease that cleaves p35 to the constitutively active isoform p25, was also elevated by HIV\textsuperscript{sup} and morphine co-exposure. Using siRNA
and a pharmacological inhibitor, we demonstrated that Cdk5 plays a central role on HIV-morphine-induced alterations of hNPC survival, proliferation, and differentiation.

**Materials and Methods**

**Primary hNPC culture.** Primary hNPCs were obtained from 8-10 week-old fetal brain tissues (Advanced Bioscience Resources, Alameda CA). Brain tissues were homogenized in 0.1% trypsin, and cultured in NPC media (0.6% glucose, 5 mM HEPES, 10% B27 minus vitamin A, 1% Penicillin-Streptomycin, 20 ng/mL FGF, 20 ng/mL EGF, 10 ng/mL LIF). Cultured hNPCs were differentiated in NPC media without FGF, EGF, and LIF.

**Generation of HIV-infected supernatant (HIV\textsubscript{sup}).** Isolated PBMCs from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) purified via ficoll gradient centrifugation were activated with phytohemagglutinin (PHA) for 3 d, then infected with R5 tropic HIV-1\textsubscript{Bal} (Advanced Biotechnology) (1 ng/mL p24) for 72 h. The culture supernatant was harvested and passed through 0.2 \(\mu\)m filter. The level of viral infection in the infected supernatant was quantified by assaying using Alliance p24 Antigen ELISA kit (Advanced Bioscience).

**HIV\textsubscript{sup} and morphine treatment for PCR array, immunoblot, proliferation and differentiation studies.** Cultured hNPCs were treated with HIV\textsubscript{sup} at a level 50 or 500 pg/mL p24 for 48 h (PCR array, immunoblot, proliferation studies), 72 h (immunoblot
studies) or 12 d (differentiation studies). Controls included treatment with NPC media only (media), and equal volume of supernatant from un-infected, PHA-activated PBMCs (UNF<sub>sup</sub>) at the same initial cell density. Morphine (500 nM) treatment was performed along with supernatant treatment. For each independent hNPC culture, treatments with roscovitine, vehicle control, siRNA and siRNA transfection control were performed in the same 24 well plate.

**PCR Array and RT-qPCR.** Total RNA was purified from treated hNPCs using RNeasy Mini Kit (Qiagen, Inc.). For PCR array studies, cDNA templates were generated from purified hNPC RNA using RT² First Strand Kit (Qiagen, Inc.) according to the manufacturer's instructions. One microgram of cDNA and SYBR® Green Master Mix (Qiagen, Inc) were used to amplify target genes contained in the RT² PCR Array Human Neurogenesis (Qiagen, Inc). For validation studies, cDNA templates derived from treated hNPCs were used to amplify target genes via qPCR using FastStart DNA Master SYBR Green.

**Immunostaining.** Cultured hNPCs were fixed in 4% paraformaldehyde (PFA), permeabilized with 0.1% Triton X-100, then incubated in blocking solution (5% BSA 0.1% Tween in 1X TBS) for 1 h at room temp. The samples were immunostained with primary antibody for nestin (Abcam), SOX2 (Abcam), GFAP (Chemicon) or MAP2 (Millipore), BrdU (Dako) overnight at 4°C then conjugated with Oregon Green® 488 or Alexa 594 secondary antibody (both Thermo Fisher Scientific). Nuclei were stained with Hoechst 33342. Samples were imaged using a Zeiss LSM 700 laser scanning confocal microscope.
under 20x or 63x objective. The images were collected using ZEN 2009 Light Edition software (Carl Zeiss, NY).

Subcellular fractionation. For whole cell lysate isolation, treated hNPCs were harvested using RIPA Lysis Buffer (Sigma) supplemented with protease inhibitor cocktail tablets (Roche). For nuclear and cytoplasmic fractionation, treated hNPCs were initially harvested in lysis buffer (10 mM HEPES pH 7.9 (Thermo Fisher Scientific), 50 mM sodium chloride (NaCl), 0.5 M sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% Triton X-100) supplemented with 1 mM dithiothreitol (DTT) and 1 tablet of cOmplete EDTA-free Protease Inhibitor Cocktail (Roche), then centrifuged to separate the cytoplasmic (supernatant) and nuclear fraction (pellet). The initial pellet was washed twice with Buffer A (0 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 M EGTA) then centrifuged at 14,000 rpm for 15 min at 4°C to pellet the nuclear fraction. The nuclear pellet was resuspended in Buffer C (10 mM HEPES pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis (β-aminoethyl ether)-N, N’, N’’-tetraacetic acid (EGTA)). The protein concentration of whole cell, cytoplasmic, and nuclear samples were determined using BCA™ Protein Assay (Thermo Fischer).

Immunoblotting. Protein lysates prepared in 4x Laemmli sample buffer (BioRad) were boiled for 5 min. Whole cell lysates, cytoplasmic fractions, and nuclear fractions were loaded, separated on a 4-20% Tris-HCl Criterion pre-cast gel (BioRad), electrophoresed in 1X Running Buffer (BioRad), and transferred onto PVDF membranes (BioRad). The membrane blots were blocked in 5% BSA and 0.1% Tween in 1X TBS for 1 h. The
membranes were incubated overnight at 4ºC with primary antibodies for Cdk5 (Cell Signaling), p35/p25 (Santa Cruz), calpain (Cell Signaling), histone 3 (Cell Signaling), GAPDH (Millipore), and β-actin (Millipore). After primary incubation, the membranes were washed in 0.1% Tween in 1X TBS, then incubated with secondary, species-specific antibodies for 1 h at room temperature (1: 10,000 anti-rabbit IRDye 680CW, 1: 10,000 anti-mouse IRDye 800CW) The images were obtained with Odyssey CLx Imaging System (LICOR Bioscience).

Cdk5 inhibition via siRNA transfection and roscovitine treatment. Cultured hNPCs were pre-transfected with Cdk5 siRNA or pre-treated with roscovitine. The cells were transfected with 250 nM Cdk5 siRNA (Mission siRNA Sigma) using Viromer® Black siRNA transfection reagent (Lipocalyx, Germany) for 4 h, then washed prior to HIV-1 ± morphine treatment. For transfection control (siRNA Veh), we treated hNPCs with equal volume of Viromer Lipocalyx transfection reagent without siRNA for 4 h. For siRNA control (siRNA Neg), we transfected hNPCs with MISSION® siRNA universal negative control (200 nM) for 4 h. hNPCs were pre-treated with 10 μM roscovitine (Sigma) for 1 h, then washed prior to HIV-1 ± morphine treatment.

BrdU incorporation and analysis. Cultured hNPCs treated for 12, 24, and 48 h, and pulsed with 1 mM of BrdU during the final 6 h of treatment. Fixed cells were immunostained using anti-mouse BrdU antibody (1:200, Dako, CA).
Live/Dead staining. Changes in hNPCs’ survival were monitored using LIVE/DEAD Reduced Biohazard Viability kit (Molecular Probes, Inc) according to the manufacturer's instructions.

Statistics. Statistical analyses were derived using one-way (GraphPad Prism 5) and three-way ANOVE (SPSS) followed by Bonferroni post-hoc testing. A value of p<0.05 was considered significant. Details are provided in each figure legend.

Results
1. HIV<sub>sup</sub> and Morphine Interact to Alter Signaling Pathways Related to Neurogenesis

Changes in hNPC gene expression were evaluated using a PCR gene array relevant to neurogenesis. Comparative heat map analysis showed that 75 of the 84 neurogenesis-related genes were expressed at varying levels among the treatment groups. The group receiving only morphine showed the least divergence in expression pattern from control, with the majority of genes showing no change (black) or down-regulation (green) on the heat map (Figure 3.1A). Up-regulation (red) of genes involved in neurogenesis was observed in all other groups versus control, suggesting that treatments containing supernatant in general had more of an effect on neurogenesis-related processes in hNPCs. Scatter plot analysis of the PCR array data clearly illustrated that compared to control, a combination of HIV<sub>sup</sub> and morphine treatment showed the highest degree of genetic changes, as majority of the target genes shifted to the left relative to the center line (control) upon treatment. (Figure 3.1B). Based on the calculated
CT values of the target genes, we determined that 26 of the 84 targets were specifically upregulated by HIV$_{\text{sup}}$ and morphine co-exposure (Table 3.1). We selected five candidate genes related to Cdk5 signaling pathway to further investigate: netrin 1 (NTN1), anaplastic lymphoma receptor tyrosine kinase (ALK), pleiotrophin (PTN), cyclin-dependent kinase 5 regulatory subunit 1 (CDK5R1) and CDK5 regulatory subunit associated protein 2 (CDK5RAP2). The protein products of NTN1, ALK and PTN are known downstream substrates of Cdk5 (Del Rio, et al. 2004; Horiuchi, et al. 2006). CDK5R1 and CDK5RAP2 each encodes for Cdk5 regulatory proteins p35 and Cdk5rap2 respectively. We validated the regulation of NTN1, ALK, PTN, CDK5R1 and CDK5RAP2 in treated hNPCs via RT-qPCR experiments using different primers and buffers than those used in the array study. The primers were designed against an exon-exon junction for each genes using NCBI Primer-Blast program. The result was consistent with the array data. Even though the magnitude fold changes are different between the array and independent qPCR, which is likely due to the different primer efficiency, the pattern of changes are similar in the two system. We demonstrated a significant upregulation of NTN, ALK, PTN, CDK5R1 and CDK5RAP2 expression in hNPCs treated with a combination of HIV$_{\text{sup}}$ and morphine compared to controls. We also measured the expression of CDK5, which was not included in the gene panel used in this study. Cdk5 gene (CDK5) expression also increased in HIV$_{\text{sup}} \pm$ morphine treated hNPCs (Figure 3.2). Out of the 6 target genes, the expression of 3 genes (ALK, CDK5R1 and CDK5) was found to be significantly different between the HIV$_{\text{sup}}$ and HIV$_{\text{sup}} +$ morphine groups, demonstrating a HIV-morphine interaction on the regulation of these genes. Based on the
gene array findings, we more closely examined the role of Cdk5 in the HIV/morphine effects on hNPC functions.

2. Elevated Cdk5, p35 and p25 Protein Expression in hNPCs Exposed to HIV-1<sub>sup</sub> and Morphine

We tested whether the observed change in CDK5 mRNA expression was reflected by increased protein (Cdk5) expression. Cdk5 levels were compared among hNPCs treated with media (control), morphine, UNF<sub>sup</sub> ± morphine, or HIV<sub>sup</sub> ± morphine for 48 and 72 h. We also examined the levels of p35 a known regulator of Cdk5 activity under normal conditions. We also probed for the level of p25, an aberrant activator of Cdk5, which is generated by calpain-dependent cleavage of p35 under pathological conditions. Compared to control, morphine and UNF<sub>sup</sub> ± morphine, treatment with HIV<sub>sup</sub> and HIV<sub>sup</sub> + morphine significantly increased the production of Cdk5 in hNPCs after 48 h (Figure 3.3A, 3.3B). We did not detect a difference between the HIV<sub>sup</sub> and HIV<sub>sup</sub> + morphine groups. Elevated p35 production was measured in both HIV<sub>sup</sub> and HIV<sub>sup</sub> + morphine treatments. A faint signal for the p25 band was observed only with HIV<sub>sup</sub> + morphine treatment. Calpain expression was present at the same level in all treatment groups. After a longer treatment time of 72 h, p25 expression was detected in all treatment groups except control and morphine, but there is no difference between treatments (Figure 3.3C, 3.3D). However, there was a robust upregulation of calpain expression in hNPCs exposed to HIV<sub>sup</sub> ± morphine after 72 h, with a significantly higher level in the HIV<sub>sup</sub> + morphine group, demonstrating an interactive effect.
3. Subcellular Localization of Cdk5 and p35 in hNPCs Exposed to HIV-1\textsubscript{sup} and Morphine

Under normal conditions, neuronal Cdk5 localizes to the cytoplasm; nuclear translocation is associated with activation of cell death pathways (Zhang, et al. 2008a). Thus, we next examined whether up-regulation of Cdk5 by HIV-1 and morphine occurs in a specific cellular compartment, using subcellular fractionation to separate the nuclear and the cytoplasmic after 48 and 72 h treatment. Immunoblot analysis indicated the presence of Cdk5, p35 and calpain in the cytoplasmic compartment at 48 h and 72 h (Figure 3.4A, 3.4C). At both times, Cdk5 and p35 production were both elevated by HIV\textsubscript{sup} and HIV\textsubscript{sup} + morphine treatment (Figure 3.4B, 3.4D). We detected a significant difference in Cdk5 level between HIV\textsubscript{sup} and HIV\textsubscript{sup} + morphine groups at 72 h, indicating an interaction. Calpain level remained unchanged at 48 h in all treatments, but greatly increased after 72 h treatment with HIV\textsubscript{sup} ± morphine.

We also observed cytoplasmic p25 at 72 h, but at a very low level compared to p35, and its level remained constant in all treatments (Figure 3.4C). Interestingly, Cdk5 was found in the nuclear compartment in all treatment groups. Analysis showed a significant difference in nuclear Cdk5 level in hNPCs treated with HIV\textsubscript{sup} + morphine compared to control, morphine, UNF\textsubscript{sup} ± morphine as well as HIV\textsubscript{sup} demonstrating an interactive effect. In all, the results showed that HIV-1 and morphine co-exposure enhances both cytoplasmic and nuclear Cdk5 expression, and also may regulate cytoplasmic Cdk5 activity with the increased expression of activator p35 in hNPCs.
4. Cdk5 is Necessary for the Interactive Effect of HIV-1 and Morphine on hNPC Proliferation.

HIV-1 and morphine have been shown to alter the proliferation of murine and human NPCs (Hahn, et al. 2012; Malik, et al. 2014) (Balinang 2016). Considering this, and the findings from this study, we next examined the role of Cdk5 signaling on HIV-1 and morphine interaction in hNPC proliferation. To do this, we directly targeted Cdk5 expression using siRNA methods. Pre-designed and pre-validated siRNAs against the human CDK5 gene were obtained from Mission® Sigma siRNA. The siRNAs targeted exon 7, 8, 9 of human CDK5 (Figure 3.5A). The specificity of Cdk5 knockdown was confirmed by immunoblot analysis following siRNA transfection with various concentrations of Cdk5 siRNA (50-500 nM). Among the tested concentrations, 200 nM siRNA resulted in the greatest Cdk5 protein knockdown (70-80%) in transfected hNPCs (Figure 3.5B). The inhibition of Cdk5 protein expression was maintained for 48 h after transfection. Based on this, we transfected hNPCs with 200 nM Cdk5 siRNA (siCdk5) for 4 h, then treated the cells with varying concentrations of HIV_sup (50-500 pg/ml p24) ± morphine for 48 h. The treated cells were pulsed with 10 µM of BrdU during the last 6 h of treatment. For transfection control (siRNA Veh), we treated hNPCs with equal volume of Viromer Lipocalyx transfection reagent without siRNA for 4 h. For siRNA control (siRNA Neg), we transfected hNPCs with MISSION® siRNA universal negative control (200 nM) for 4 h. Exposure to HIV_sup at both 50 and 500 pg/ml levels significantly reduced the percentage of BrdU^+ hNPCs and this effect was exacerbated with morphine co-exposure (Figure 3.6A, 3.6B). Similar findings were observed in a previous report (Balinang 2016). The result showed that siCdk5 attenuated the anti-proliferative effect of HIV_sup and
morphine co-exposure at the 50 pg/ml HIV level, indicating the functional role of Cdk5 in
the interaction of HIV-1 and morphine on the proliferation of hNPCs.

5. Protective Role of Cdk5 in the Response of hNPCs to HIV$_{sup}$ and Morphine Co-
exposure

Earlier reports demonstrated a role of Cdk5 on pro-survival signaling pathways in
neurons (O'Hare, et al. 2005; Zhang, et al. 2008a). Here we questioned whether Cdk5
mediates to the observed resistance of primary hNPCs to the deleterious effect of HIV-1
and morphine. We again transfected cultured hNPCs with siCdk5 prior to HIV$_{sup}$ (50-500
pg/mL p24) ± morphine exposure for 48 h. LIVE/DEAD analysis confirmed that treatment
with HIV$_{sup}$ ± morphine at either level did not induce significant cell death in the hNPCs
culture. siRNA-mediated knockdown of Cdk5 resulted in about 2-4-fold increase in the
percentage of cell death in the HIV$_{sup}$ and HIV$_{sup}$ + morphine groups (Figure 3.6C, 3.6D).
These results suggest in hNPCs Cdk5 may be involved in the prosurvival mechanism
induced in these cells against HIV-1 and morphine co-exposure.

We next tested whether the effect siCdk5 on the viability of hNPCs exposed HIV$_{sup}$
± morphine was due specifically to the knockdown of Cdk5 and not due to indirect effect
of siRNA transfection. Here, we transfected hNPCs with equal concentration of Mission$^\text{®}$
Sigma siRNA universal negative controls (siNeg) for 4 h then treated with HIV$_{sup}$ ±
morphine. Compared to siCdk5, siNeg transfection did not induce significant cell death
suggesting that the potential toxic effect of siRNA transfection was not mediating the
observed effect on hNPC viability (Figure 3.6E).
6. Cdk5 is Necessary for the Interactive Effect of HIV-1 and Morphine on hNPC Neuronal Differentiation

Several studies of CDK family members identify Cdk5 as the essential cyclin-dependent kinase in the process of neuronal development and maturation (Cicero and Herrup 2005; Jessberger, et al. 2009; Nikolic, et al. 1996; Paglini and Caceres 2001). Based on previous reports that HIV$_{sup}$ and morphine exposure induced premature MAP2 differentiation of hNPCs (Balinang 2016) we tested the role of Cdk5 on this process. Following Cdk5 inhibition via siRNA transfection, cultured hNPCs were exposed to HIV$_{sup}$ (50-500 pg/ml p24) ± morphine for 12 d in differentiation media. Neuronal, as well as astrocyte differentiation, was assessed via immunostaining using antibodies against the neuronal marker, MAP2, and the astrocyte marker, GFAP (Figure 3.7A). Immunostaining analysis showed that MAP2 and GFAP immunoreactivity were both increased after HIV$_{sup}$ ± morphine treatment (Figure 3.7B). Cdk5 siRNA blocked the effect of HIV$_{sup}$ on MAP2 immunoreactivity, particularly at the highest HIV. Furthermore, the interaction of HIV-1 and morphine on neuronal differentiation was suppressed with siRNA-mediated knockdown of CDK5. Interestingly, when we blocked Cdk5 using pharmacological Cdk5 inhibitor roscovitine the effect of HIV$_{sup}$, as well as the combinatory effect of HIV$_{sup}$ + morphine on neuronal differentiation was also blocked. The result suggests that both Cdk5 expression and activity are essential in the interaction between HIV-1 and morphine that facilitate differentiation of NPCs into MAP2$^+$ neurons.

Interestingly, while siCdk5 did not influence the effect of HIV-1 and/or morphine on GFAP differentiation of hNPCs, we did however observe the attenuation of morphine interaction with roscovitine treatment. roscovitine alone increased GFAP-
immunoreactivity in morphine treated hNPCs. While roscovitine did not alter HIV-mediated increases in GFAP, it significantly blocked morphine potentiation of this response in cells treated with both levels of HIVsup (Figure 3.8A, 3.8B)

Discussion

Neural progenitor cells are critical determinants of the overall cell population in children, adolescents and adults. Emerging evidence suggests the vulnerability of these cells to the interaction of HIV-1 and opiates, which would affect both neuronal and macroglial populations throughout the CNS. Given this, NPCs dysfunctions may underlie the neurological impairments observed in children and adults living with HIV-1, especially those with a history of opiate use either through illicit drug use or pain management. Our findings may be of great clinical significance considering the emergent opiate drug crisis among young adults and women. The present findings begin to elucidate the molecular events that underlie the HIV-1 and morphine effects and interactions on hNPCs. Initial gene array data suggested that HIVsup and morphine interaction altered the expression of a network of genes associated with Cdk5 signaling.

* * * 

*Cdk5 in neurodegenerative disorders, opiate drug abuse, and HAND*

Cdk5 belongs to the family of serine/threonine cyclin-dependent kinases (CDK) best known for their role in cell cycle regulation and proliferation. Despite having ~60% genomic sequence homology to that of Cdk4 and cell division cycle kinase 2 (Cdc2), Cdk5 is not believed to influence normal cell cycle (Hellmich, et al. 1992; Lew, et al. 1994).
Cdk5 is widely expressed in various tissues, but its highest expression and associated kinase activity have been detected within the CNS (Hellmich, et al. 1992; Tsai, et al. 1993). It is largely found in post-mitotic neurons and to a lesser extent in astrocytes, throughout the developing brain, and in neurogenic regions of the adult brain (Hellmich, et al. 1992; Tsai, et al. 1993). Unlike mitotic CDKs, Cdk5 does not require cyclin for activation. Rather it depends on association with the regulatory proteins, p35 and p39 (Lew, et al. 1994). When Cdk5 is activated by p35, it regulates a wide range of CNS cellular events via phosphorylation of protein substrates involved in cytoskeletal organization, motility, and cell signaling (Dhavan and Tsai 2001; Grant, et al. 2001; Smith 2003; Tanaka, et al. 2001). As such, it plays a significant role in developmental processes including neuronal differentiation (Ko, et al. 2001; Paglini and Caceres 2001), neurite outgrowth (Nikolic, et al. 1996), and cortical migration (Ohshima, et al. 1996), and also influences synaptic plasticity (Cheng and Ip 2003), neurotransmission (Cicero and Herrup 2005), and cognitive function (Angelo, et al. 2006). Cdk5 has also recently been implicated as a regulator of pathways associated with neuronal survival and cell death (Ohshima, et al. 1996; Vartiainen, et al. 2002; Wang, et al. 2003a). Aberrations in Cdk5 activity have been implicated in the etiology of several neurodegenerative disorders including Alzheimer’s disease (AD), Parkinson’s disease (PD) (Qu, et al. 2007), and amyotrophic lateral sclerosis (ALS) (Nguyen, et al. 2001). The proteolytic cleavage of Cdk5 activator p35 to an aberrant and constitutively active p25 by the calcium-dependent protease calpain has been strongly linked to neurodegeneration (Patrick, et al. 1999). These mechanisms are not fully understood, but may involve aberrant Cdk5 phosphorylation of Tau protein, microtubule-associated proteins (MAPs), and proteins

Cdk5 and its activators p35 and p25 have been implicated in the neuroplasticity and neuropathology induced by opiate drugs and HIV-1 infection independently. Neuronal Cdk5/p35 was down-regulated in opioid-addicted individuals and rodents (Ferrer-Alcon, et al. 2003), and reduced Cdk5 activity has been linked to morphine-induced tolerance (Wang, et al. 2004), psychological dependence (Narita, et al. 2005), and opioid receptor regulation (Beaudry, et al. 2015), suggesting that Cdk5 may play an important role in both cellular and behavioral adaptations to opiate drug abuse. In the context of HIV-1, early gene array studies in brain tissues from HIV-positive individuals revealed abnormal expression of Cdk5 signaling-related genes specifically in patients with HIVE (Masliah, et al. 2004), suggesting a role for Cdk5 signaling in the progression of HIV-related neuropathology in vivo. In support of that concept, Cdk5 and p35 levels were increased in the brains of both HIVE patients and gp120 transgenic mice, and were associated with abnormal tau phosphorylation and dendritic degeneration (Patrick, et al. 2011). Furthermore, exposure to HIV-infected supernatant increased NMDAR-mediated calpain activation, and cleavage of p35 to p25 in rat hippocampal (O'Donnell, et al. 2006), and cortical neurons (Wang, et al. 2007), resulting in substantial neuronal cell death. Although the mechanism(s) of HIV neurotoxicity via Cdk5 is not well understood, it was recently suggested that increased Cdk5 translocation from the nucleus to the cytoplasm mediated by HIV, facilitates aberrant phosphorylation of Tau, doublecortin and myocyte enhancer factor-2 (Mef2), resulting in abnormal neuronal functions (Fields, et al. 2015).
HIV-1 and morphine regulation of Cdk5 in hNPCs.

Treatment with HIV\textsubscript{sup} and morphine increased both Cdk5 and p35 mRNA and protein levels. These findings are consistent with earlier reports showing increased protein expression of Cdk5 and p35 in brain tissues from HIV\textsubscript{E} patients, HIV gp120 transgenic mice (Patrick, et al. 2011), and in cultured rodent cortical neurons exposed to HIV-infected supernatant (O'Donnell, et al. 2006; Wang, et al. 2007). Morphine by itself did not change Cdk5 or p35 levels in cultured hNPCs, although an earlier report indicated negative regulation of Cdk5/p35 after more chronic opiate-exposure \textit{in vivo} (Ferrer-Alcon, et al. 2003). Although calpain production significantly increased with HIV\textsubscript{sup} + morphine for 72 h, the level of p25 remained very low in all groups except for control, and its level did not change with treatments. This was unexpected as we speculated based on earlier studies correlating elevated p25 with neuropathogenesis in HIV-1 individuals and animals (Patrick, et al. 2011), that exposure to HIV\textsubscript{sup} ± morphine would induce p25 production. The discrepancy in the results may be due to the timing of p25 production in hNPCs. As Patrick et al. showed increased p25 production in the brain of HIV-infected subjects with HIV\textsubscript{E} and not those without cognitive impairments. It is likely that p25 production is a consequence of chronic HIV-1 exposure, and that longer HIV\textsubscript{sup} and morphine exposure in hNPCs may be required to detect the induction of p25. It is also possible that the activity of calpain is affected by HIV\textsubscript{sup} ± morphine exposure, preventing the cleavage of p35 to p25. Although we detected a strong up-regulation of calpain protein production, we did not measure whether its activity is also elevated.

We detected an increase of Cdk5 expression primarily in the cytoplasmic compartment of hNPCs upon HIV\textsubscript{sup} ± morphine treatment. Studies suggest that the
Localization of Cdk5 and its activators dictates the downstream outcomes of Cdk5 signaling (Ino and Chiba 1996; Zhang, et al. 2008a). Cdk5 is normally found in both the nuclear and cytoplasmic compartments of neurons, but at relatively higher levels in the latter, where p35 expression is also predominantly located (Ino and Chiba 1996). In the cytoplasm, Cdk5/p35 phosphorylates multiple proteins including MAP1B, PAK1 and β-APP, leading to regulation of neuronal differentiation, migration, neurite outgrowth, and axonal transport (Dhavan and Tsai 2001). Recently, cytoplasmic localization of Cdk5 was demonstrated to promote the survival of post-mitotic neurons upon exposure to oxidative stress and glutamate toxicity (O'Hare, et al. 2005; Zhang, et al. 2010). It was later suggested that Cdk5 attenuates neuronal cell death by inhibiting the translocation of forkhead box protein O1 (FOXO1) to the nucleus and preventing the transcription of pro-apoptotic genes such as BIM (Zhou, et al. 2015). Furthermore, Zhang et al. demonstrated the role of nuclear Cdk5 along with p35, cyclin p27 and E2F1 on cell cycle arrest and maintenance of the post-mitotic status of neurons (Zhang, et al. 2008a; Zhang, et al. 2010). In terms of HIV-1 neuropathogenesis, earlier studies in individuals with HIVE, and in HIV gp120 expressing mice, showed accumulation of cytoplasmic Cdk5 leading to aberrant phosphorylation of Tau, DCX and CRPM2, all associated with neuronal degeneration and neurocognitive impairments (Fields, et al. 2015).

*Cdk5 modulates effects of HIV-1 and morphine co-exposure on hNPC differentiation*

We previously found that HIV$_{sup}$ and morphine co-exposure caused the premature differentiation of cultured hNPCs into both neurons and astrocytes (Balinang 2016). Here we reduced Cdk5 expression and activity using siRNA and roscovitine, respectively, and
found that loss of Cdk5 partially reversed the effect of HIV\textsubscript{sup} and morphine to aberrantly accelerate hNPC differentiation into young, MAP2\textsuperscript{+} neurons. The critical function of Cdk5 on neuronal differentiation under normal condition had been established by various groups (Cicero and Herrup 2005; Nikolic, et al. 1996; Zheng, et al. 2010), however whether or how Cdk5 impacts this process under pathological conditions has not been explored, until now. Although the exact mechanism as to how Cdk5 mediates hNPC neuronal differentiation in the context of HIV-1 and morphine interaction is not known, we could speculate that aberrant Cdk5 activation promotes hyper-phosphorylation of MAP2, DCX and p27. The phosphorylation of these proteins by Cdk5 had been shown to be crucial to neuronal differentiation both \textit{in vivo} and \textit{in vitro} (Cicero and Herrup 2005; Zheng, et al. 2010).

Interestingly, while roscovitine reversed premature differentiation of HIV\textsubscript{sup} + morphine treated hNPCs into GFAP\textsuperscript{+} astroglia, siCdk5 did not. This discrepancy is likely related to the fact that roscovitine also inhibits other CDKs, including Cdc2, and Cdk2/Cyclin A, Cdk2/Cyclin B (Meijer, et al. 1997). Although roscovitine has a relatively stronger effect on Cdk5 compared to the other CDKs (Meijer, et al. 1997), it is still possible that the observed effect on GFAP differentiation may be due to the inhibition of either Cdc2 or Cdk2. Indeed, Cdk2 is expressed by NPCs, and has been demonstrated to be important in the proliferation and self-renewal processes (Caillava, et al. 2011; Jablonska, et al. 2007).

\textit{Cdk5 modulates the anti-proliferative effect of HIV-1 and morphine co-exposure.}
In addition to altering hNPC differentiation, HIV\textsubscript{sup} treatment reduced the proliferation of hNPCs; proliferation was further reduced by morphine. We found that siCdk5 blocked the anti-proliferative effect of HIV\textsubscript{sup} and morphine co-exposure in hNPCs, and increased the percentage of BrdU\textsuperscript{+} cells to control levels. This suggest that under pathological conditions, such as those observed in the HIV-infected brain, Cdk5 may function as a cell cycle regulator. Earlier investigation also reported the ability of Cdk5 to regulate cell cycle in neurons of Cdk5 deficient mice (Cicero and Herrup 2005; Zhang, et al. 2008a). It is postulated that Cdk5 promote cell cycle arrest by forming a complex with p35 and transcription factor E2F1, and preventing E2F1 from binding to promoters of various cell cycle regulators (Zhang, et al. 2010). It is interesting that we see a similar pattern of cell cycle arrest in hNPCs culture system when Cdk5 is upregulated by HIV-1 and morphine. This suggest that Cdk5 under pathological conditions such as that created by HIV-1 and morphine interaction, may act as a cell cycle suppressor in hNPCs.

**Cdk5 role in hNPC survival**

While HIV\textsubscript{sup} causes a dose-dependent loss of significant numbers of primary neurons (Masvekar, et al. 2014), hNPCs are resistant to such toxicity, even at much higher exposure levels, and even in the presence of morphine (Balinang 2016). Considering the emerging role of Cdk5 as a regulator of neuronal survival (Tanaka, et al. 2001; Zhou, et al. 2015), we tested whether Cdk5 was involved in the ability of hNPCs to survive HIV-1 ± morphine exposure. siRNA-mediated knockdown of Cdk5 significantly increased the percentage of hNPC death in cultures exposed to HIV\textsubscript{sup}, as well as to a combination of HIV\textsubscript{sup} and morphine. Cdk5 signaling is thus implicated in the relative
ability of hNPCs to survive conditions induced by HIV$_{sup} \pm$ morphine exposure. The exact mechanism(s) that underlie Cdk5-mediated survival of hNPCs remains to be determined. However, Cdk5 is known to phosphorylate certain apoptotic regulators whose genes were identified in our array data (e.g. BCL-2, APOE, NTN and ALK) (Del Rio, et al. 2004; Zhou, et al. 2016) suggesting that there are multiple Cdk5 targets that might influence survival. For example, Cdk5 phosphorylates BCL-2, which results in the suppression of cell apoptosis (Brinkkoetter, et al. 2009; Cheung, et al. 2008; Wang, et al. 2006a). Cdk5 may also regulate activity of upstream transcription factors, preventing the expression of apoptotic genes, as previously reported by Zhou et al. (2015) who showed that cytoplasmic Cdk5 altered the localization of transcription factor FOX01, ultimately leading to suppression of cell death in post-mitotic neurons (Zhou, et al. 2015).

Overall, the current study provides novel insights into the molecular events underlying the interactive effects of HIV-1 and morphine on primary hNPCs. NPCs play a critical role the generation of all neuronal and macroglia population in both the developing and adult brain, and alteration of these cells at both stages can greatly influence overall CNS cell populations and their function (Kempermann, et al. 2004; Ladran, et al. 2013). NPC dysfunction has been implicated in various psychiatric (Braun and Jessberger 2014) and neurodegenerative disease (Desplats, et al. 2012; Mu and Gage 2011), as well as in injuries caused by inflammation (Ekdahl, et al. 2003), stroke/ischemia (Kernie and Parent 2010) or epilepsy (Jessberger and Parent 2015). In the developing brain, perturbation of NPC functions during corticogenesis profoundly impacts overall brain development (Caviness, et al. 1995). Thus, NPC dysfunction may underlie many of the neurological
impairments observed in individuals living with HIV-1/AIDS. Furthermore, given the crucial role of NPC on brain development, their functional impairment may in some part explain the greater frequency and severity of neurological complications observed in children and adolescent with HIV-1 (Epstein, et al. 1986; Mintz and Epstein 1992). Our demonstration that Cdk5 influences the functional response of hNPCs to HIV and morphine suggests its potential role as a point of convergence in the development of CNS complications due to HIV-1 infection. Thus, identifying key molecular factors that may be altering the essential functions of NPCs due to HIV-1 and opiate comorbidity can be critical for the development of therapeutic strategies for a majority of the HIV population.
Figure 3.1 HIV$_{\text{sup}}$ and morphine-mediated changes in hNPC gene expression. (A) Heat map analysis showing changes of gene expression in hNPCs treated with morphine (500 nM), HIV$_{\text{sup}}$ at a level of 500 pg/ml HIV p24 [HIV] + morphine [HIV + M] and equal volume of UNF$_{\text{sup}}$ [UNF] + morphine [UNF + M] compare to media only control. Shades of red correspond to increase in gene expression, whereas shades of green corresponds to decreased in gene expression. (B) Scatter plot analysis comparing normalized expression of every gene on the array between the control group and morphine, UNF, UNF + M, HIV and HIV + M groups. Data are presented as log 10 of $\Delta\Delta$CT values of
treated groups (Y-axis) versus log 10 of ΔΔCT values of control group. Center line region contains unchanged genes. Red dot indicates increased gene expression and green dot indicates decreased gene expression compared to control.
Figure 3.2 RT-qPCR validation of PCR array data on the regulation of CDK5 related genes. RT-qPCR validation of array data using primers different from those use in the array studies. Changes in mRNA expression of *NTN*, *ALK*, *PTN*, *CDK5RAP2*, *CDK5R1* and *CDK5* in treated hNPCs were calculated using $2^{\Delta\Delta Ct}$ method and presented as fold change relative to control. Error bars show mean ± SEM from $n=3$ studies each using hNPCs derived from independent tissue samples. Significance was determined by one-way ANOVA with Bonferroni post-hoc testing. (#) $P < 0.05$ vs. control and morphine, (*) $P < 0.05$. 


Figure 3.3 HIV-1$_{sup}$ and morphine co-exposure increases protein level of Cdk5, p35 and calpain in hNPCs. (A, C) Representative immunoblot of whole cell lysates (20-30 µg) obtained from hNPCs treated with 500 nM morphine [M], HIV$_{sup}$ at 500 pg/ml HIV p24 [H] ± morphine [HM], equal volume UNF$_{sup}$ [U] ± morphine [UM], and NPC media [C] for 48 h ad 72 h probed with antibodies against Cdk5, p35, p25, calpain, Gapdh for 48 h and 72 h treatment. (B, D) Immunoblot analysis of whole cell lysates from hNPCs treated with
morphine, HIV$_{\text{sup}}$ [HIV] + morphine [HIV+M], and equal volume of UNF$_{\text{sup}}$ [UNF] + morphine [UNF + M] for 48 and 72 h. Data expressed as ratio relative to Gapdh level. Error bars show mean ± SEM from $n=3$ studies each using hNPCs derived from independent tissue samples. Significance was determined by one-way ANOVA with Bonferroni post-hoc testing. (*) $P < 0.05$. 
Figure 3.4 Subcellular localization of Cdk5, p35 and calpain in hNPCs exposed to HIV-1<sub>sup</sub> and morphine. (A, C) Representative immunoblots probed with antibodies against Cdk5, p35, p25, calpain, β-actin and Histone 3 (H3) using cytoplasmic (20-30 μg) and nuclear (10-20 μg) fractions isolated from treated hNPCs with NPC media [C], morphine [M], HIV<sub>sup</sub> at 500 pg/ml HIV p24 [H] ± morphine [HM], equal volume of UNF<sub>sup</sub> [U] ± morphine [UM] for 48 and 72 h. (B, D) Densitometry analysis showing significant up-regulation of cytoplasmic p35 by HIV<sub>sup</sub> and HIV<sub>sup</sub> + morphine treatment, as well as calpain in all treatments compare to control. Marked increase of cytoplasmic Cdk5, p35 and calpain was measured in hNPCs treated with HIV<sub>sup</sub> ± morphine for 72 h. (E) HIV<sub>sup</sub> and morphine treatment for 72 h significantly increased Cdk5 level in the nuclear
compartment compare to control, morphine, UNF ± morphine and HIV sup. The purity of
the isolated cytoplasmic and nuclear fraction was verified by the presence of cytoplasmic
\( \beta \)-actin and nuclear Histone 3. Error bars show mean ± SEM from \( n=3 \) studies each using
hNPCs derived from independent tissue samples. Significance was determined by one-
way ANOVA with Bonferroni post-hoc testing. (#) \( P <0.05 \) vs. control and morphine, (*) \( P <0.05 \).
Figure 3.5 Design and optimization of siRNA Cdk5. (A) Schematic diagram of the human CDK5 mRNA containing 12 exons (E1-E12). Red lines indicate the three sites of siRNA target for CDK5. Brief description of each siRNA used to inhibit CDK5 including the base count and the site of target. (B) Representative Immunoblot probed against Cdk5 and Gapdh using whole cell lysates from cells transfected with 50, 100, and 200 nM siRNA Cdk5 and 100 nM Gapdh siRNA [G]. Veh represent siRNA transfection control.
where the cells were exposed to equal volume of Viromer Lipocalyx siRNA transfection reagent. Control group [C] represents un-transfected hNPCs. Densitometry analysis of siRNA immunoblot showing relative level of Cdk5 protein level. Gapdh served as loading control.
Figure 3.6 Proliferative and protective role of Cdk5 in hNPCs exposed to HIV-1 and morphine. (A) BrdU immunostaining showing siRNA-Ck5 transfected (siCdk5) exposed to NPC media [Media], morphine, HIV_sup at 500 pg/ml HIV p24 [H500] ± morphine [HM500] for 48 h. siRNA transfection control (siRNA Veh) represents hNPCs treated with equal Viromer Lipocalyx transfection reagent without siRNA. (B) BrdU analysis on siCdk5 transfected hNPCs exposed to NPC media [Media], HIV_sup at 50-500 pg/ml HIV p24 [H50, H500] ± morphine [HM50, HM500], equal volume of UNF_sup [U50, U500] + morphine [UM50, UM500] for 48 h. Control groups represent un-transfected hNPCs exposed to the appropriate treatment. Data was derived from >200 Hoechst^+ hNPCs, and presented as percentage of BrdU^+ cells relative to the total cells. (C) Representative Dead RED live staining of treated hNPCs. (D) LIVE/DEAD assay analysis of siCdk5 transfected hNPCs.
exposed to morphine, HIV_{sup} at 50-500 pg/ml HIV p24 [H50, H500] ± morphine [M] for 48 h. Control groups represent un-transfected hNPCs exposed to the appropriate treatment. Data were derived from >200 hNPCs, and presented as percentage of DEAD Red positive cells relative to the total cells. (E) LIVE/DEAD assay analysis of siCdk5 transfected hNPCs, as well as hNPCs transfected with equal concentration of Sigma siRNA universal negative control (siNeg). For (B, D, E) error bars show mean ± SEM from n=3 studies, each using hNPCs derived from independent tissue samples. Significance was determined by three-way ANOVA with Bonferroni post-hoc testing. (#) $P<0.05$ vs. control, (%) $P<0.05$ vs. HIV and (*) $P<0.05$. 
Figure 3.7 Involvement of Cdk5 in the interaction of HIV-1 and morphine on hNPC MAP2 differentiation. (A) Representative immunostaining of SOX2 (red), MAP2 (green) of siRNA-Cdk5 transfected (siCdk5), roscovitine pre-treated (Roscovitine) exposed to NPC differentiation media only [Media], HIV\textsubscript{sup} at 500 pg/ml p24 alone (HIV 500) and HIV\textsubscript{sup} with morphine [HIV 500 + M]. siRNA transfection control (siRNA Veh) represents hNPCs treated with equal Viromer Lipocalyx transfection reagent without siRNA. (B) Immunostaining analysis of siCdk5 transfected and roscovitine pre-treated hNPCs treated with morphine, HIV\textsubscript{sup} at 50 pg/ml p24 [HIV 50], 500 pg/ml p24 [HIV 500] alone or with morphine [M]. Control groups indicate un-transfected and roscovitine naïve hNPCs. Error bars show mean ± SEM from \textit{n}=4 studies each using hNPCs derived from
independent tissue samples. Significance was determined by three-way ANOVA with Bonferroni post-hoc testing. (#) $P < 0.05$ vs. control, (%) $P < 0.05$ vs. HIV and (*) $P < 0.05$. 
Figure 3.8 Roscovitine but not siCdk5 attenuates the effect of morphine on hNPC GFAP differentiation. (A) Representative immunostaining of GFAP (green) and SOX2 (red) on siCdk5 transfected and roscovitine pre-treated hNPCs exposed to HIV$_{sup}$ at 500 pg/ml + morphine (HM500). Hoechst staining (blue) indicate cell nuclei. (B) Immunostaining analysis of siCdk5 transfected and roscovitine pre-treated hNPCs treated with morphine, HIV$_{sup}$ at 50 pg/ml p24 [HIV 50], 500 pg/ml p24 [HIV 500] alone or with morphine [M]. Control groups represent un-transfected and roscovitine naïve hNPCs. Error bars show mean ± SEM from $n=4$ studies each using hNPCs derived from...
independent tissue samples. Significance was determined by three-way ANOVA with Bonferroni post-hoc testing. (#) \( P < 0.05 \) vs. control, (\%) \( P < 0.05 \) vs. HIV and (*) \( P < 0.05 \).
Table 3.1 mRNA expression fold change of target genes in treated hNPCs.

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<th>UNF&lt;sub&gt;sup&lt;/sub&gt; + M</th>
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Chapter 4

Regulation of MOR-1 and MOR-1K Splice Variants by HIV-1; Potential Function and Mechanism of MOR-1K in Human Neural Progenitor Cells

(This chapter, in part, will be prepared as a communication article in AIDS, entitled
Regulation of MOR-1 and MOR-1K Splice Variants by HIV-1; Potential Function and
Mechanism of MOR-1K in hNPCs)

Introduction

There is substantial evidence suggesting that opiates may have a direct role in
HIV-1 infection and disease progression, including in the central nervous system (CNS)
ilicit drug users, who account for about 30-50% of HIV-1 infected population (Donahoe
and Vlahov 1998), have higher prevalence of cognitive disorders (Chiesi, et al. 1996;
Nath, et al. 2001) and display more severe neuropathological features (Anthony, et al.
2008; Bell, et al. 2002). Many of these individuals reported to preferentially abuse opiate
drugs including heroin. We and other have hypothesized that opiates might affect the
course of HIV-1 neuropathogenesis and enhance CNS disease progression through the
direct actions of opiate drugs on opioid receptors, specifically the \( \mu \)-opioid receptor (MOR)
subtype expressed on neurons and glial within the brain. This is supported by key studies
demonstrating up-regulation of MOR expression by HIV gp120 in various human brain
cells (Beltran, et al. 2006; Cadet, et al. 2001) and evidence of heterologous cross-sensitization of MOR with HIV-1 co-receptors CCR5 and CCR4 (Chen, et al. 2004; Rogers, et al. 2000). Recently we reported using neuron-glia co-culture model that HIV Tat mediated neurotoxicity is exacerbated by the presence of MOR on glial cells (Zou, et al. 2011).

MOR are one of the three subtypes of opioids receptors that display the strongest affinity for clinically used and abused opiates. Alternative splicing of the OPRM1 gene generates at least 21 human MOR splice variants with many displaying differences in the C-terminus region of the receptor (Pan 2005). Despite the identification of these splice variants, the majority of the studies examining opiate interactions including in HIV-1 neuropathogenesis, have been focused on the canonical splice variant MOR-1. Given that alternative splicing of MOR was found to underlie the different nociceptive effects of morphine (Liu, et al. 2011), it is likely that opiates may differentially mediate HIV-1-related CNS effects via the actions of multiple MOR splice variants. This could be of critical importance to understanding how opiates augment HIV-1 CNS disease progression. In fact, we previously showed that selected C-terminal and N-terminal truncated MOR splice variants are indeed expressed in human CNS cell types and also in brain tissues of HIV-infected subjects, suggesting that that the canonical MOR-1 may not be the predominant MOR expressed in the CNS, and that these MOR splice variants may be differentially regulated in the brains of HIV-infected individuals with varying level of cognitive impairment (Dever, et al. 2014; Dever, et al. 2012). Among the splice variants that we profiled, MOR-1K is of great interest in our group due to its distinct protein characteristic and functions which are suggested to uniquely activate neuroinflammation associated
with opioid-dependent hyperalgesia, tolerance and dependence (Gris, et al. 2010). We have shown that MOR-1K mRNA expression is specifically elevated in brain tissues of HIV-1 infected subjects with HIV encephalitis (HIVE), and also that its expression is correlated with the level of neurocognitive impairments in non-HIVE subjects. In addition, increased MOR-1k expression in HIVE subjects occurs simultaneously with increased mRNA of known inflammatory mediators (Dever, et al. 2014). We speculate that in the brain of HIV-infected drug users, opiates facilitate neuroinflammatory events that ultimately leads to CNS dysfunctions through in part the action of MOR-1K. The involvement of MOR splice variants, especially MOR-1K on the mechanism(s) of HIV-1 and opiate interaction that leads to CNS dysfunctions is yet to be determined. Moreover, the question remains whether expression of specific MOR splice variants may make particular cell types more vulnerable to the deleterious effect of HIV-1 and opiates in the CNS. Understanding these key questions can provide insight on how opiates at the cellular level augments HIV neuropathogenesis which can be valuable to the development of therapeutic targets for a subpopulation of HIV-1 infected individuals that abuse opiates.

Neural progenitor cells (NPCs), the precursor cells of all neurons and glial in the CNS have been shown to also express MOR, along with HIV-1 co-receptors, CCR5 and CXCR4 (Hahn, et al. 2012). Moreover, these cells are suggested to be additional targets of HIV-1 and opiate interaction, as the independent effect of HIV (Hahn, et al. 2012; Krathwohl and Kaiser 2004; Malik, et al. 2014; Okamoto, et al. 2007; Peng, et al. 2011; Peng, et al. 2008) and morphine (Arguello, et al. 2008; Eisch, et al. 2000; Kahn, et al. 2005; Mandyam, et al. 2004) on murine and human NPCs have been extensively studied.
We and others have provided initial evidence on the interactive effect of HIV-1 exposure / HIV proteins and morphine on the proliferation and population dynamics of murine and human NPCs (Hahn, et al. 2012; Malik, et al. 2014). Subsequently, our group expanded these early studies and demonstrated that morphine exacerbates the functional consequences of the toxic/inflammatory conditions of HIV-1 infection in fetal brain-derived hNPCs. We also reported that morphine sustains the productive infection of hNPCs by R5 tropic HIV-1 \textit{in vitro} (Balinang 2016). These recent findings further reinforced the notion that these highly mitotic, multipotent cells are likely important targets for the deleterious effect of HIV-1 and opiates co-exposure. The precise mechanism(s) underlying HIV-1 and morphine interaction on the function and infectivity of hNPCs, and whether the presence of MOR splice variants on these cells impact the outcome of this interaction are not completely known. In this short communication, we investigated the expression of MOR splice variants, MOR-1 and MOR-1K in cultured hNPCs, and determined whether their expression is regulated by HIV-1. Furthermore, we briefly tested potential mechanism and function of MOR-1K and MOR-1 in the response of hNPCs to HIV-1.

**Material and Methods**

**Primary hNPC culture.** Primary hNPCs were derived from fetal brain tissues (8-10 week old). Cells were maintained in NPCs media (0.6% glucose, 5 mM HEPES, 10% B27 minus Vitamin A, 1% Pen/Strep, 20 ng/mL FGF, 20 ng/mL EGF, 10 ng/mL LIF) or differentiation media (NPC media without FGF, EGF and LIF)
Immortalized hNPC culture: ReNcell VM human neural progenitor cell line was purchased from Millipore and maintained in ReNcell NSC Maintenance Medium.

Generation of HIV-infected supernatant (HIV_{sup}). HIV_{sup} were collected from cultured PBMCs infected with 1 ng/mL R5 tropic HIV-1_{BaL} (Advanced Biotechnology). Briefly, PBMCs were isolated from peripheral blood Leuko Paks (ZenBio, Research Triangle Park, NC) via ficoll centrifugation, activated with phytohemagglutinin (PHA) for 3 d, then infected with HIV-1_{BaL} for 3-5 d. The culture supernatant was harvested and passed through 0.2 μm filter. The level of viral infection in the infected supernatant was quantified by assaying using Alliance p24 Antigen ELISA kit (Advanced Bioscience).

RT-qPCR. Treated hNPCs were harvested and RNA was purified using RNeasy Mini Kit (Qiagen, Inc.). cDNA templates were generated using RT² First Strand Kit (Qiagen, Inc.) according to the manufacturer's instructions. One microgram of cDNA and SYBR® Green Master Mix (Qiagen, Inc) were used to amplify target genes. MOR products were generated using the following primers: for MOR-1K forward 5'-CGGGAAATGAGTGGTTCCCA-3' and reverse 5'-TCCAAATGGCCATGTTCCCA-3'; for MOR-1K forward 5'-TTGTCAGATACACCAAGATGAAGA-3' and reverse 5'-GGGTGGCAGACTGCAATGTA-3'.

Immunoblotting. Protein lysates prepared in 4x Laemmli sample buffer (BioRad) were boiled for 5 min, and loaded on a 4-20% Tris-HCl Criterion pre-cast gel (BioRad), electrophoresed in 1X Running Buffer (BioRad), and transferred onto PVDF membranes
Blots were blocked in 5% BSA and 0.1% Tween in 1X TBS, incubated with primary antibodies for MOR (Abcam), and GAPDH (Millipore) overnight, then incubated with secondary, species-specific antibodies for 1 h at room temperature (Oregon Green® 488 or Alexa 594 secondary antibody both Thermo Fisher Scientific). The images were obtained with ChemiDoc™ MP System (Biorad).

MOR-1 and MOR-1K inhibition via siRNA transfection. Cultured hNPCs were transfected with 25-200 nM MOR-1 or MOR-1K siRNA (Mission siRNA Sigma) using Viromer® Black siRNA transfection reagent (Lipocalyx, Germany) for 4 h. The cells were washed and harvested after 48 h in culture.

Statistics. Statistical analyses including one-way ANOVA followed by Bonferroni post-hoc testing, were performed using GraphPad Prism 5 software (GraphPad Software, Inc). A value of p<0.05 was considered significant.

Results
1. HIV-1 Mediated Regulation of MOR-1 and MOR-1K in Immortalized and Primary Human NPCs.

We previously showed the protein expression of MOR in immortalized hNPCs, along with evidence of their functional vulnerability to the interactive effect of HIV-1 and morphine in vitro (Hahn, et al. 2012). To determine whether MOR-1 and/or MOR-1K splice variants are specifically involved in the mechanism of HIV-1 and morphine interaction in these cells, we tested the presence of these variants via RT-qPCR. Due to
the high sequence similarities of the canonical MOR-1 with other variants, the MOR-1 primers used in this study detect other exon 1-utilizing MOR splice variants, in addition to MOR-1. Thus this pool of MORs are defined as MOR-1_{(exon \ 1-2)} (Figure 4.1A). On the other hand, MOR-1K primers only detect MOR-1k, as they are designed to target the novel exon 13 (MOR-1K_{(exon \ 13-2)}) (Dever, et al. 2014). We confirmed the expression of both MOR-1_{(exon \ 1-2)} and MOR-1K_{(exon \ 13-2)} in immortalized hNPCs (Figure 4.1B), and found the relative level of MOR-1_{(exon \ 1-2)} is about 100-200 fold higher versus MOR-1K_{(exon \ 13-2)}. The larger level of MOR-1_{(exon \ 1-2)} detected may represent the total pool of exon 1-utilizing MORs including MOR-1 expressed in hNPCs.

We next tested whether the expression of MOR-1K_{(exon \ 13-2)} and MOR-1K is regulated by HIV-1. To model the physiological condition found in the HIV-infected brain, we treated immortalized hNPCs with supernatant from human peripheral blood mononuclear cells (PBMCs) infected with R5 tropic HIV-1, HIV_{Bal} (HIV_{sup}). The cells were treated with 50 \( \mu g/ml \) HIV p24, a level that we previously reported to have an effect on human neurons and astrocytes (El-Hage, et al. 2013; Masvekar, et al. 2014), for 12, 24, 48 and 72 h. RT-qPCR analysis revealed significant up-regulation of MOR-1K at 48h and 72 h compare to control and un-infected supernatant (UNF_{sup}) control (Figure 4.1C). Interestingly, there was no difference in MOR-1_{(exon \ 1-2)} expression amongst all the treatment groups. The result indicates splice variant-specific regulation of HIV-1, where MOR-1K expression in particular is modulated by HIV-1 exposure in immortalized hNPCs.

The previous findings were validated in a primary hNPC culture system. Primary hNPCs were derived from 10-12 weeks old human fetal brain tissues. The cellular composition of the primary hNPC culture was previously characterized, showing that 90-
95% of the cells expressed NPC markers, nestin and Sex determining region Y – box 2 (SOX2) (Figure 2.1). We expanded the initial study in immortalized cells and treated primary hNPCs with various concentrations of HIV$_{sup}$ (0.5 – 500 $\mu$g/ml HIV p24) at 12, 24 and 48 h. The cells were harvested and analyzed via RT-qPCR. Similar to immortalized hNPCs, both splice variants were detected in the primary hNPCs, with the relative level MOR-1$_{(exon\ 1-2)}$ being significantly higher that MOR-1K$_{(exon\ 13-2)}$ (data not shown). In addition, we found with 12 h treatment the expression of MOR-1$_{(exon\ 1-2)}$ and not MOR-1K$_{(exon\ 13-2)}$ was specifically up-regulated in primary hNPCs treated with higher HIV$_{sup}$ levels (50-500 $\mu$g/ml HIV p24) compared to control and UNF$_{sup}$ controls (Figure 4.2). At 24 h, we continued to measure an up-regulation of MOR-1$_{(exon\ 1-2)}$ at 5.0 – 500 HIV$_{sup}$ levels. Also at this time point, MOR-1K$_{(exon\ 13-2)}$ expression increased with 50 – 500 HIV$_{sup}$ levels compared to UNF$_{sup}$ controls. Interestingly at 48 h we observed a specific up-regulation of MOR-1K$_{(exon\ 13-2)}$ only in hNPCs exposed to the highest HIV$_{sup}$ level of 500 $\mu$g/ml HIV p24. A similar observation was observed in immortalized hNPCs (Figure 4.1C).

2. Potential Mechanism of HIV-1 mediated Regulation and Intrinsic Function of MOR-1K in hNPCs.

In an earlier study using network analysis, we revealed in brain tissues of HIV-infected subjects with HIV encephalitis (HIVE), filamin A (FLNA) as a possible interaction partner with MOR-1K. In addition, over-expression of FLNA promoted the trafficking of MOR-1K to the cell surface *in vitro*. We speculate that filamin A is an important chaperone of MOR-1K, facilitating ligand-binding activation and/or cross-sensitization with CXCR4 and CCR5 at the cell surface (Dever, et al. 2014). In an attempt to understand the
mechanism of MOR-1K in hNPCs, we sought to determine whether FLNA is also regulated in hNPCs treated with HIV sup, similar to what we observed in HIV+ brain tissues. Using RT-qPCR analysis, we found that FLNA expression in immortalized hNPCs remained unchanged with HIV sup (500 pg/ml HIV p24) treatment for 48 h (Figure 4.3). However, it is interesting to see that FLNA expression significantly increased upon 48 h treatment with un-infected and HIV-infected supernatant treatment in primary hNPCs compared to control. More importantly, a significant difference was measured between the UNF and HIV groups, indicating the specific HIV-1 effect on FLNA expression. The result suggests that in primary hNPCs, HIV-1 exposure promotes the expression of FLNA leading to increase trafficking of MOR-1K and/or MOR-1 variants to the cell surface for activation.

In addition to elucidating a potential mechanism of MOR-1K activation in hNPCs exposed to HIV-1, we also examined the possible functional role of MOR-1K in these cells compared to the canonical MOR-1. To do this, we employed siRNA to target exon 13 of MOR-1K and exon 1 of MOR-1 (Figure 4.4A). The efficiency of siRNA transfection in primary hNPCs was determined using GAPDH siRNA (100 nM) and measured about 50-70% protein knock down at 48 h post transfection (4 h) (Figure 4.4B). Using the same approach, we transfected our cells with MOR-1K or MOR-1 siRNA with varying concentrations (25 – 200 nM), then analyzed protein level of MOR-1 and MOR-1K via immunoblotting. The result showed that MOR-1 siRNA, especially at the lower siRNA concentration decreased MOR-1 protein level to up to 60% (Figure 4.4C, 4.4D). Unfortunately, as common with commercially available antibodies for MOR, we were unable to efficiently detect the MOR-1K species at 25 kDA and evaluate its protein
knockdown. However, looking closely at the immunoblot data, we discovered that targeting exon 13 of MOR-1K dramatically decreased MOR-1 protein expression, at a much larger extent compare to MOR-1 siRNA (80-90% knockdown). This unexpected result indicates that potential regulatory function of MOR-1K on the canonical MOR-1.

Discussion

In this brief study, we provided the first report of the expression of exon 1 utilizing MOR splice variants, including canonical MOR-1 and newly identified MOR-1K in hNPCs. We also demonstrated that MOR-1_{(exon1-2)} and MOR-1K are selectively regulated by HIV-1, suggesting that MOR-1K and MOR-1_{(exon12)} may have differential role in the response of hNPCs to the infected environment. Moreover, FLNA which encodes for filamin A, a known MOR chaperone was also up-regulated by HIV-1 in primary hNPCs, indicating a potential mechanism for MOR-1K and/or MOR-1 activation during infection. Targeting exon 13 of MOR-1K via siRNA greatly reduced MOR-1 protein expression, demonstrating that this N-terminal truncated splice variant may function as an intrinsic regulator of MOR-1 expression in hNPCs. Overall, our study highlights the possible role of MOR-1_{(exon1-2)} and MOR-1K in the response of hNPCs to HIV-1 and reinforces a potentially critical mechanism of opiate interaction with HIV-1 via the involvement of MOR splice variants in the CNS.

Although the precise mechanism of MOR-1K in hNPCs have not been identified, we speculate based on previous investigations that MOR-1K activation will modulate downstream molecular pathways related to NPC functions. The canonical MOR-1 follows the classical MOR signaling(Pasternak and Pan 2013), while MOR-1K stimulates the
opposite effects including activating adenylyl cyclase (AC) and increasing cAMP by coupling with G\(\alpha_s\) (Gris, et al. 2010). Accordingly, MOR have been shown to regulate rat NPC proliferation via the activation of AC leading to increased cAMP production and induction of the mitogen-activated protein kinases (MAPK) signaling cascade (Persson, et al. 2003). cAMP has also been reported to play a critical role in the proper proliferation and differentiation of adult rat hippocampal NPCs via the involvement of a phosphatidylinositol 3-kinase (PI3k)/Akt (PI3k/Akt) cascade (Peltier, et al. 2007). It is evident that the distinct signaling of MOR-1k overlaps with those associated with NPC functions. Thereby it is likely that these molecular targets participate in the mechanism of MOR-1K in the response of hNPCs to HIV-1 infection. Additional biochemical studies such as immunoblotting and activity based-assays measuring the induction of these downstream targets especially AC and cAMP in hNPCs exposed to HIV-1 will enable one to confirm the involvement of MOR-1k. Equally important, an experiment examining the coupling of MOR-1k with G\(\alpha_s\) subunit will provide direct evidence of MOR-1K activation in these cells.

Filamin A is a cytoskeleton protein that crosslinks actin filaments into orthogonal network and maintains cell cytoskeleton integrity. Filamin A also participates in the anchoring of transmembrane proteins to the plasma membrane (van der Flier and Sonnenberg 2001). It’s been previously shown that filamin A interacts with the C-terminal tail of the conical MOR-1 and this interaction specifically altered receptor trafficking (Onoprishvili, et al. 2003; Simon and Onoprishvili 2010). Recently, we discovered that Filamin A also interacts with the same C-terminal sequence on MOR-1K resulting in the trafficking of MOR-1K from the intracellular compartment to the plasma membrane.
(Dever, et al. 2014) This mechanism can be critical given that MOR-1K is normally found intracellularly and would need to be expressed on the cell surface in order to bind to the appropriate ligand for activation. In addition to promoting ligand binding, filamin A can also impact the ability of MOR-1K to affect HIV viral entry. Opioid receptors, including MOR, have been previously demonstrated to form complexes with both CXCR4 and CCR5 to affect cellular signaling and possibly viral entry via bidirectional heterologous interactions (Chen, et al. 2004; Patel, et al. 2006; Rogers and Peterson 2003; Rogers, et al. 2000). Accordingly, we showed that the mRNA that encodes for filamin A is greatly up-regulated in primary hNPCs exposed to HIV-1. This coincides with the increased expression of both MOR-1_{(exon1-2)} and MOR-1K, suggesting a potential interaction between filamin A and these splice variants in hNPCs upon HIV-1 treatment. This suggests that MOR-1k may be more highly expressed on the surface of these cells, where it can be activated as well as interact with HIV co-receptors, ultimately altering hNPC response to HIV-1.

In this study, we also showed that targeting exon 13 of MOR-1K resulted in reduced protein expression of the canonical MOR-1 in hNPCs, at much greater extent than targeting MOR-1 itself. The result suggests that MOR-1K may function as a potent regulator of MOR-1, perhaps stabilizing the receptor and preventing its turn-over. This regulatory mechanism has been described previously by other MOR splice variants. Using a Tet-OFF cell system, Xu et. al. demonstrated that single transmembrane (TM) domain MOR variants dimerize with full length MOR-1 in the endoplasmic reticulum (ER) and increase MOR-1 protein expression via a chaperone like function that prevent ER-associated degradation of MOR-1 (Xu, et al. 2013). Given that MOR-1K is normally found
in the intracellular compartment, it is possible that this variant may function as a chaperone for other MORs. This is not limited to the MOR subtype, as truncated δ-opioid receptor-1 (DOR-1) variants have been identified to function similarly as the single TM MOR-1 variants (Gaveriaux-Ruff, et al. 1997). Given that MOR-1K is normally found in the cytoplasmic compartment, it is possible that it also functions as a regulatory chaperone, interacting with internalized MOR-1 at the ER and increasing MOR-1 protein level preventing its degradation. Along with the FLNA data, we can speculate that MOR-1/MOR-1K complex is trafficked back to the cell surface via filamin A and prolong MOR-1 activation. Further experiments examining this cellular interaction such as co-immunoprecipitation and fluorescence immunostaining is necessary to verify whether MOR-1K functions as a regulatory chaperone for the canonical MOR-1.
Figure 4.1 HIV-mediated regulation of exon 1-utilizing MOR (MOR-1\textsubscript{(exon1-2)}) and MOR-1K in immortalized hNPCs. (A) Schematic representation of the spliced mRNA sequence of MOR-1 containing exon 1, 2, 3 and 4 and MOR-1K containing exon 13, 2, 3 and 4. Red arrows denote the location of PCR primers used to detect MOR-1\textsubscript{(exon 1-2)} and MOR-1K. Primers used for MOR-1\textsubscript{(exon 1-2)} detect the canonical MOR-1 as well as a pool of exon 1 utilizing MORs. (B) Quantified relative mRNA level of MOR-1\textsubscript{(exon1-2)} and MOR-1K in immortalized hNPCs treated with HIV\textsubscript{sup} (50 pg/ml HIV p24) (HIV) or equal volume of UNF\textsubscript{sup} (UNF) for 12, 24, 48 and 72 h. (C) RT-qPCR analysis of MOR-1\textsubscript{(exon 1-2)} and MOR-1K expression in treated immortalized hNPCs. Dotted line indicates the control.
expression level for each variant and data presented as fold change relative to internal control GAPDH. Fold change values were derived using the ∆∆CT method. Error bars show mean ± SEM from n=3 independent experiments. Significance was determined by one-way ANOVA with Bonferroni post-hoc testing. (#) $P < 0.05$ vs. control, (*) $P < 0.05$. 
Figure 4.2 HIV-mediated regulation of exon 1-utilizing MOR (MOR-1\textsubscript{(exon1-2)}) and MOR-1K in primary hNPCs. Quantitation of MOR-1\textsubscript{(exon 1-2)} and MOR-1K expression in primary hNPCs treated with varying concentration of HIV\textsubscript{sup} (0.5-500 pg/ml HIV p24) for 12, 24 and 48 h. Dotted line indicates the control expression level for each variant and data presented as fold change relative to internal control GAPDH. Fold change values were derived using the $\Delta\Delta$CT method. Error bars show mean ± SEM from $n=3$ hNPC culture derived from independent tissue samples. Significance was determined by one-way ANOVA with Bonferonni post-hoc testing. (♯) $P<0.05$ vs. control, (*) $P<0.05$. 
Figure 4.3 Regulation of FLNA by HIV-1 in immortalized and primary hNPCs. RT-qPCR quantitation of FLNA mRNA expression in immortalized and primary hNPCs treated with HIV\textsubscript{sup} at a 500 pg/ml level (HIV), equal volume of UNF\textsubscript{sup} (UNF), and NPC media (Control) for 48 h. Error bars show mean ± SEM. Data on immortalized hNPCs were derived from \(n=3\) biological repeats. Data on primary hNPCs were obtained from \(n=3\) hNPC culture derived from independent tissue samples. Significance was determined by one-way ANOVA with Bonferroni post-hoc testing. (#) \(P < 0.05\) vs. control, (*) \(P < 0.05\).
Figure 4.4 siRNA-mediated knockdown of MOR-1 and MOR-1K splice variants in primary hNPCs. (A) Schematic diagram of MOR-1 and MOR-1K splice variant mRNA. Blue and red lines denote the location of designed siRNA for MOR-1 and MOR-1K respectively. (B) Representative immunoblot image probed with antibody for GAPDH using whole cell protein lysates (30 μg) from un-transfected hNPC (UT), vehicle treated hNPCs (control) and transfected hNPC with GAPDH siRNA (siRNA). (C) Immunoblot analysis of MOR-1 and GAPDH protein expression in whole cell lysates (30 μg) obtained
from cultured hNPCs transfected with various concentrations of MOR-1 or MOR-1K siRNA (25-200 nM). (D) Densitometry analysis of the relative protein expression of MOR-1 in hNPCs transfected MOR-1 and MOR-1K siRNA (25-200 nM). Control group represent un-transfected hNPCs. Error bars show mean ± SEM. Data were obtained from n=3 hNPC culture derived from independent tissue samples. Significance was determined by one-way ANOVA with Bonferonni post-hoc testing. (#) P <0.05 vs. control, (*) P < 0.05.
Chapter 5
Synopsis, Perspective and Final Conclusion

Synopsis and perspectives

Human NPCs, which give rise to all the neurons and glial cells in the CNS, are potential targets of the deleterious effect of HIV-1 and opiates and represent an unappreciated source of viral-mediated dysfunctions and infection that contributes to the development of neuropathology. The overall goals of this study were to investigate the functional consequence(s) of HIV-1 exposure on primary hNPCs in vitro, determine whether morphine interacts with HIV-1 to augment these changes and to identify the molecular mechanism(s) underlying such interaction. It was hypothesized that HIV-1 and morphine co-exposure modulates behaviors that are essential to hNPC function, including proliferation, differentiation and survival. Moreover, we hypothesized that interaction may be differentially mediated by the actions of MOR splice variants expressed by these cells. The major findings and implications of these studies are outlined below.

1. Productive infection of primary hNPCs by R5 tropic HIV-1 BaL (HIV$_{BaL}$).

The infection of hNPCs by HIV-1 has been questioned previously. Production of HIV p24 and the presence of proviral DNA were detected in both cultured fetal brain-derived (Lawrence, et al. 2004) and immortalized hNPCs (Rothenaigner, et al. 2007). These studies were further supported by in vivo evidence of HIV-1 infection of nestin-expressing NPCs in archival pediatric brain tissues (Schwartz, et al. 2007). Although these studies have been critical in highlighting the potential infection of hNPCs, the
findings are limited in different aspects, which is likely the very reason why the notion of HIV-infected hNPCs remains controversial. For example, the in vitro studies demonstrated evidence of productive infection via the production of supernatant p24 only. Although this is a classic method of determining HIV infectivity in culture, subsequent experimental methods examining the production of other viral proteins will make the case stronger. In addition, even though Rothenaiger et al. (2007) demonstrated the presence of proviral DNA along with evidence of p24 production in HNSC.100, these cells are immortalized and may not represent the true behavior of hNPCs in the actual brain including their ability to be infected. In our study, not only did we confirmed the productive infection of hNPCs using various experimental approaches, we also provided the first evidence that these cells can propagate active infection in vitro. Treatment with purified R5 tropic HIV, HIV_{BaL} resulted in the de novo expression of HIV p24, Nef and Tat in cultured hNPCs. Using a serial dilution approach, it was demonstrated that hNPCs can transfer active infection to naïve cells. The clinical implications of productive HIV-1 infection of hNPCs could be profound. Infected hNPCs may exacerbate overall disease progression by elevating both viral replication and production of toxic viral proteins including Tat and gp120. In addition, these cells can propagate infection to their progeny, many of which are long-lived in the brain, thereby creating new viral reservoirs of latent infection. Overall, the presence of HIV-infected hNPCs and perhaps their cellular derivatives can contribute to the current barrier to the eradication of HIV-1 in the CNS.

While we have shown that the infection of hNPCs is CD4 independent, the exact mechanism of viral infection in these cells is not known. Our laboratory previously reported the expression of HIV-1 co-receptors CXCR4 and CCR5 on immortalized
hNPCs, suggesting that viral entry and subsequent infection may be mediated by these receptors (Hahn, et al. 2012). Additional characterization and functional studies will be needed to confirm this hypothesis. First, one may need to verify that primary hNPCs indeed express CXCR4 and CCR5 in order to infer that viral entry is facilitated by these receptors. This can be assessed by measuring the transcript level of CXCR4 and CCR5 via RT-PCR or performing immunostaining/immunoblotting to detect the receptors. Second, studies directly targeting these receptors such as gene editing or the use of CCR5 antagonist maraviroc can validate the involvement of CXCR4 and CCR5 on the initiation of viral infection in primary hNPCs.

2. Morphine sustained productive infection of hNPCs.

Morphine has in the past been shown to enhance HIV viral replication of cultured immune and brain cells (Peterson, et al. 1994; Peterson, et al. 1993; Peterson, et al. 1990). In addition, chronic morphine exposure promotes CNS viral replication and accelerates the onset of AIDS in SIV-infected macaques (Kumar, et al. 2006). In our study, we demonstrated that co-treatment with morphine sustained HIV p24 production and increased the HIV Tat expression in HIV\text{Ba}_L treated hNPCs compared to control, indicating an interaction of morphine on the infection of hNPCs. These results suggest that in an environment where morphine present in high titers, such as in the brain of HIV-infected opiate drug users, the productive infection of hNPCs and perhaps of their progeny is heightened. However, it remains unknown how morphine exacerbates the productive infection of cultured hNPCs. Subsequent studies using pharmacological inhibitors of opioid receptors to block the action of morphine on HIV-infected hNPCs may
provide insight on the underlying mechanism of morphine-mediated enhanced infection. Although we speculate that the interaction is mediated primarily through the MOR subtypes, it is also possible that other opioid receptors are involved. As such the use of naloxone, a competitive antagonist for the MOR, DOR and KOR, and a selective MOR antagonist, CTAP will enable one to differentiate the action of all subtypes versus MOR.

3.Interaction of HIV-1 and morphine on hNPC proliferation and cell growth.

HIV\textsubscript{sup} treatment significantly reduced the percentage of hNPC entering S-phase of DNA synthesis, which was exacerbated by morphine co-exposure. In addition, HIV\textsubscript{sup} and morphine co-treatment greatly reduced hNPC cell growth leading to prolonged doubling time. There was no evidence of significant cell death in the hNPC culture exposed to HIV\textsubscript{sup} ± morphine indicating that the findings were not in part due to changes in cell viability. In sum, the results demonstrate an interactive effect of HIV-1 and morphine on the proliferation and cell growth of primary hNPCs. Similar changes in DNA synthesis was observed by other groups including us, using HIV-1 Tat and morphine on primary hNPCs and HIV-1 supernatant/ HIV-1 Tat on rodent and immortalized human NPCs (Hahn, et al. 2012; Malik, et al. 2014). Similarly, the resistance of NPC to HIV-1/ HIV-1 Tat and a combination of HIV-1/HIV-1 Tat and morphine exposure had been previously observed both in the rodent and human system (Hahn, et al. 2012; Malik, et al. 2014; Okamoto, et al. 2007; Rothenaigner, et al. 2007). HIV-1 reduction of hNPC proliferation without affecting viability may potentially be a mechanism of neurovirulence in these cells, similar to has been observed in cells with active viral replication. The virus may be able to maintain persistent infection by switching host DNA synthesis to viral DNA synthesis in
infected cells (Das and Basu 2011), as has been shown for hNPCs upon infection with human cytomegalovirus (HCMV) (Salvant, et al. 1998).

In our attempt to understand whether the proliferative effect of HIV-1 involved the infection of hNPCs, we treated the cells with purified virions and UV-inactivated HIV$_{sup}$. UV-crosslinking inactivated any virions present in the supernatant, and enabled us to distinguish the effect of the inflammatory/toxic condition found in the supernatant versus the effect of HIV-infection. The data showed that exposure of UV-inactivated HIV$_{sup}$ but not treatment with pure virion reduces BrdU incorporation of hNPCs, indicating that soluble factors/viral proteins in the infectious supernatant, rather than infection per se, were the cause. These findings reinforced the idea proposed by many that the CNS dysfunction is not due to the virus, but largely due to the neuroinflammatory/toxic conditions created as a consequence of HIV-1 infection.

4. Interaction of HIV-1 and morphine on hNPC differentiation.

HIV$_{sup}$ treatment induced premature differentiation of hNPC toward both the neuronal and astrocyte lineage. Morphine co-treatment enhanced the effect of HIV$_{sup}$, further increasing the percentage of MAP2 and GFAP expressing hNPCs. To our knowledge this is the first evidence on the interactive effect of HIV-1 and morphine on hNPC differentiation. These results, particularly on the independent effect of HIV-1 on hNPC differentiation stood apart from earlier studies. Exposure to HIV-infected-LPS-activated macrophages promoted astrocyte (GFAP) but not neuronal (β-tubulin) differentiation of primary hNPCs (Peng, et al. 2008), however the presence of LPS in the HIV-infected supernatant may have confounding effect on the differentiation results. The
action of LPS independent of HIV-1 on NPC neuronal differentiation has been previously reported by various group (2008) (Butovsky, et al. 2006; Liu, et al. 2005). Although the response prolife of LPS versus PHA-M is not known, we hypothesized that each compound will induce relatively different response, leading to distinct supernatant prolife. The difference in soluble factors found in each supernatant may underlie the contrasting differentiation results observed in hNPCs. Reduced neuronal differentiation (Tuj-1 [β-tubulin] and doublecortin [DCX]) was also measured in primary hNPCs treated with HIV Tat (Okamoto, et al. 2007). The use of Tat versus HIV-infected supernatant, which contains Tat and other soluble viral/host factors, may also cause the discrepancy in the result. Despite the contradicting findings, all of these studies including ours, indicate the dynamic response of these cells to HIV-1 and suggest that in the HIV-infected brain, the ability of NPC to differentiate normally is severely impaired. This can have profound consequence on the overall CNS cell population and function. Too many or too little neurons, astrocytes and oligodendrocytes can perturb neuronal processes, leading to neurological dysfunction. Furthermore, these NPC-derived neurons or glial cells can be inherently impaired, contributing more to the development of pathological functions.

In the future, it will be worthwhile to examine whether these newly GFAP and MAP2-expressing hNPCs are functional or inherently impaired. This can be assessed by performing electrophysiology studies measuring the excitability of these cells. We suspect that these premature MAP2⁺ and GFAP⁺ hNPCs are dysfunctional and will display abnormal electrical activity. If true, then we can speculate that these NPC-derived astrocyte and neurons may contribute to CNS dysfunctions and cognitive impairments observed in HIV animals and HIV-infected individuals.
One limitation of our study is that we did not evaluate the effect of HIV-1 and morphine on the overall hNPC population. The effects of HIV$_{\text{sup}}$ ± morphine on proliferation and differentiation suggest that exposure over time will alter the balance of MAP2$^+$ and GFAP$^+$ cell populations. A meaningful assessment of this will require stereological assessment of potentially affected brain regions.

5. Modulation of gene expression related to neurogenesis in hNPCs exposed to HIV$_{\text{sup}}$ ± morphine.

Preliminary PCR array data demonstrated the consequence of HIV-1 and morphine co-exposure on neurogenesis related gene expression in immortalized hNPCs. Here we employed a similar array technology to question whether aspects of neurogenesis are also regulated by HIV$_{\text{sup}}$ and morphine co-exposure in primary hNPCs, with a goal of identifying downstream molecular targets that contribute in the response of hNPCs to the interaction of HIV-1 and morphine. Treatment with HIV$_{\text{sup}}$ and HIV$_{\text{sup}}$ ± morphine altered the expression of several genes (75 out of 84) associated with neurogenesis processes (neural migration, differentiation, cell cycle, etc.) in primary hNPCs. Among the genes that were specifically up-regulated with treatment, we selected five candidates linked to the Cdk5 signaling pathway to further investigate: CDK5RAP2, CDK5R1, NTN, ALK and PTN. Independent RT-qPCR analysis validated the array data showing significant up-regulation of the five targets, as well as CDK5 upon treatment with HIV$_{\text{sup}}$ ± morphine.

Earlier array study demonstrated dysregulation of gene expression connected to neuronal processes in the brains of HIVE AIDS patients. However, where our result
showed a robust up-regulation of $CDR5R1$, which encodes for Cdk5 activator p35, in
HIV$_{\text{sup}}$ treated hNPCs, Masliah et al. (2004) reported down-regulation of this gene in the
frontal cortex of HIVE subjects (Masliah, et al. 2004). In this study, they concluded based
on $CDR5R1$ expression alone, that the components of Cdk5 signaling are altered in these
patients with HIVE. However, the level of $CDK5$ and other factors of Cdk5 signaling were
not examined, leading us to question whether their conclusion really reflects what is
occurring in vivo. The difference in $CDR5R1$ expression between our study and Maslial
et al. (2004) however may be due to several factors including; in vivo versus in vitro
model, chronic vs. acute HIV-1 exposure, single cell type vs. a population of cells. More
importantly, the discrepancy in $CDR5R1$ expression may be due to the fact that the
expression analysis in this earlier study was performed in the frontal cortex of adult
individuals. As mentioned before, adult NPCs are predominantly found in two discrete
brain regions, the DG of the hippocampal formation and SVZ of the lateral ventricle.
Although adult NPCs may be found in other brain regions such as the frontal cortex, there
are currently no strong evidence to support this. Thus Masliah (2004) findings may
represent the response of non-NPCs in the frontal cortex. Equally important, the age of
the HIV brain tissue versus our culture may also underlie the difference in the results, as
our cell are derived from fetal brain tissues.

5. HIV-1 and morphine mediated up-regulation of protein Cdk5 and its activator p35
in primary hNPCs.

Subsequent immunoblot studies showed increased level of protein Cdk5 as well
as p35, a known Cdk5 regulator encoded by the $CDK5R1$ gene in hNPCs following HIV$_{\text{sup}}$
± morphine treatment. Protein level of calpain, a calcium-dependent protease that cleaves p35 to p25, was also elevated in HIV\textsubscript{sup} and HIV\textsubscript{sup} + morphine groups. Although p25 was detected in all treated hNPCs, its level remained unchanged despite the presence of HIV-1 and/or morphine. These results along with the RT-qPCR data underscore the potential involvement of Cdk5/p35 in the mechanism of HIV-1 and morphine interaction in primary hNPCs. This is of clinical relevance as previous studies have shown similar up-regulation of Cdk5 and p35 in brain tissue of HIVE subjects and HIV gp120 transgenic mice (Lee, et al. 2013; Patrick, et al. 2011).

\textit{In vivo} studies suggest that the cellular localization of Cdk5 and its regulators may be play an important role on the mechanism neurodegeneration and disease progression mediated by HIV-1 (Fields, et al. 2015). Cdk5 is normally found in the cytoplasmic compartment of post-mitotic neurons, but under stress conditions, Cdk5/p25 accumulates in the nucleus where it has been shown to phosphorylate pro-survival transcription factors and cell cycle regulators leading to cell-cycle re-entry and ultimately cell death (Zhang, et al. 2008a). In addition, aberrant cytoplasmic Cdk5 activity has also been reported to promote accumulation of phosphorylated Tau leading to neuronal cytoskeleton alteration and neurodegeneration (Tsai, et al. 1993). Interestingly, our results showed specific up-regulation of Cdk5, p35 and calpain by HIV\textsubscript{sup} and morphine primarily in the cytoplasmic compartment, and to a lesser extent in the nucleus at later exposure. The result may suggest the effect of HIV-1 and morphine interaction in hNPCs promote enhance Cdk5-mediated phosphorylation of substrates that may be involved in differentiation. The presence of nuclear Cdk5 expression potentially influence cell cycle progression, leading to altered proliferation. The specific function(s) of cytoplasmic versus nuclear Cdk5 in
HIV_{sup} and morphine treated hNPCs remain to be identified. Additional immunoblot and immunostaining experiments examining the phosphorylation states of downstream targets both in the cytoplasmic and nuclear compartments will enable one to elucidate the molecular mechanism of Ckd5 in hNPCs, especially in the context of HIV-1 and morphine. Details on these future experiments are describe on the next point.

One particular limitation of this study was determining whether HIV-1 and morphine directly induce Cdk5 kinase activity, which is a more direct indication of Cdk5 activation versus evaluating protein level of Cdk5 and p35/25. This involves measuring the accumulation of gamma labeled phosphate γ-[^{32}P] in Cdk5 specific substrate such as histone H1 and MAP2. Unfortunately, our laboratory lacked the capability and the resources to perform such radiolabeled assay. Verifying the aberrant activity of Cdk5 due to HIV-1 and morphine will certainly bolster the notion that Cdk5 dysregulation contributes to the development of hNPC impairments.

6. Involvement of Cdk5 in the interactive effect of HIV-1 and morphine on the proliferation, differentiation and survival of hNPCs.

Blocking Cdk5 expression and activity via siRNA transfection and roscovitine treatment respectively, attenuated the combinatory effect of HIV_{sup} and morphine on neuronal differentiation and proliferation of hNPCs. Furthermore, where we initially showed the persistence of hNPCs to HIV_{sup} ± morphine treatment, knocking down Cdk5 dramatically hindered their viability upon exposure to HIV_{sup} ± morphine. These results represent the first set of evidence on the functional role of Cdk5 on the interactive effect of HIV-1 and morphine in hNPCs.
Despite these novel findings, the precise mechanism of Cdk5 on the proliferation, differentiation and survival on primary hNPCs remains to be identified. Given what is known about Cdk5 in neurons we can speculate some pathways that may overlap in NPCs. Recently, Cdk5 has been implicated in cell cycle re-entry and cell death pathways in post mitotic neurons (Cicero and Herrup 2005; O'Hare, et al. 2005; Zhang, et al. 2010), and its localization with p35 and p25 in the nucleus is a critical determinant for its action on these processes (O'Hare, et al. 2005; Zhang, et al. 2008a). Considering these studies, we hypothesized that the elevated presence of nuclear Cdk5 in hNPCs may in part alter the proliferation dynamic and survival of hNPCs after HIV \text{sup} and morphine treatment. Nuclear Cdk5 was shown to be important in cell cycle arrest and maintaining the post-mitotic status of neurons. It is postulated that this mechanism prevents neurons from cell-cycle entry-mediated cells death. Nuclear Cdk5 may also function to promote cell cycle arrest in hNPCs, leading to reduced proliferation and enhancing survival in these cells following HIV-1 and morphine exposure. To verify this experiments examining the interaction of nuclear Cdk5 with known substrates involved in cell cycle and anti-apoptotic pathways such as myocyte enhancer factor -2 (MEF2), E2F transcription factor 1 (E2F1) and foxhead box O1 (FOXO1) (Zhang, et al. 2008a; Zhang, et al. 2010; Zhou, et al. 2015) will need to be performed.

The role of Cdk5 in neuronal differentiation have been extensively studied in Cdk5 deficient mice (Cicero and Herrup 2005; Nikolic, et al. 1996; Zheng, et al. 2010). In these animals, deficient neural differentiation was associated with diminished cortical MAP2 and \(\beta\)-tubulin expression. Interestingly in our system where Cdk5 is up-regulated by HIV-1 and morphine, MAP2 differentiation of NPCs is significantly increased. Being that MAP2
is a known downstream substrate of Cdk5, it is likely that HIV-1 and morphine interaction facilitates aberrant phosphorylation of MAP2 by Cdk5, resulting in an alteration of the neuronal cytoskeleton and its differentiation. We speculate that Cdk5-MAP2 interaction occurs in the cytoplasm, as previously described (Dhavan and Tsai 2001). Immunoblot experiments measuring the phosphorylation state of cytosolic MAP2 in HIV-1 and morphine treated hNPCs can confirm whether this mechanism indeed occurs in these cells.

One drawback of this particular study is the use of roscovitine to inhibit Cdk5 in hNPCs. Although this drug has been extensively used to examine the specific function of Cdk5 in cellular processes, including studies on HIV-1 neuropathogenesis and opiate drug use (Contet, et al. 2008; Lee, et al. 2013; Patrick, et al. 2011; Wang, et al. 2004; Wang, et al. 2007), it also targets other CDKS, including Cdc2, Cdk2/Cyclin A and Cdk2/Cylin B. Based on the IC50 values of roscovitine for each targets (0.65, 0.7, 0.7, and 0.16 μM for Cdc2, Cdk2/Cyclin A, Cdk2/Cylin B and Cdk5), the inhibitor should display higher efficiency with Cdk5. However, if Cdc2 or Cdk2 are expressed on hNPCs, especially at a higher level compared to Cdk5, then it is highly possible that the outcome of roscovitine treatment may be due to the inhibitions of these other CDKs. In fact, previous studies have shown the presence of Cdk2 in NPCs and demonstrated its role on NPC proliferation and differentiation (Caillava, et al. 2011; Jablonska, et al. 2007). Future experiments measuring the expression of Cdc2 and Cdk2 in hNPCs will be necessary to address the issue of roscovitine action independent of Cdk5. This can be performed using RT-qPCR to measure transcript level or immunoblot to measure protein level of Cdc2 and Cdk2.
If Cdk2 and Cdc2 are indeed expressed in hNPCs, their potential role on the mechanism of HIV-1 and morphine interaction in these cells may be of functional importance. Although the general thought is that Cdk5 is the predominant form of CDK in the CNS (Lew, et al. 1994), it is likely these less prevalent CDK members may also have important functions, especially in cycling cells including NPCs. To address whether Cdk2 and/or Cdc2 mediate(s) the observed HIV-1 and morphine effect on the proliferation and differentiation of hNPCs, subsequent siRNA experiments targeting these two CDKs can be employed. Given the results from roscovitine treatment, it is possible that all three CDKs have differential functions in hNPCs upon HIV-1 and morphine exposure. The findings from these studies can provide novel and substantial information on the role of CDKs in HIV-1 and morphine neuropathogenesis.

7. Potential role of MOR-1 and MOR-1K splice variants in the interactive effect of HIV-1 and morphine on primary hNPCs. RT-qPCR analysis showed that MOR-1 and MOR-1K splice variants are differentially regulated by HIV-1 in both immortalized and primary hNPCs. Furthermore, we showed that the expression of FLNA, which encodes for a known MOR chaperone filamin A, was also modulated by HIV-1 and its expression correlated with MOR-1 and MOR-1K. This suggest two things: first, that MOR-1 and MOR-1K may have differential outcomes on the interaction of morphine with HIV-1 in hNPCs and second, the mechanism of MOR-1 and/or MOR-1K activation and downstream functions likely involves filamin A. Moreover, siRNA studies demonstrated that targeting MOR-1K greatly reduced MOR-1 expression, at a significantly higher degree versus MOR-1 siRNA. This unexpected result implies that MOR-1K acts as an
intrinsic regulator for MOR-1, perhaps by stabilizing MOR-1 and preventing its degradation.

Although our studies provided novel insights on the mechanism and function of MOR splice variants, the results were largely descriptive. This is mainly due to experimental limitations that impeded our ability to further investigate the functional significance of these splice variants in hNPCs. As a result, the direction of my study regarding the mechanism of HIV-1 and morphine interaction in hNPCs was modified to investigate the involvement of other molecular targets. One major setback is the availability of effective antibodies recognizing individual MOR splice variants including MOR-1K. The majority of commercially available antibodies target the C-terminal amino acid sequence, a region shared by other MOR variants including MOR-1K. N-terminal MOR antibodies, which would detect MOR-1 and not MOR-1K, were found to be problematic and highly nonspecific. Without an effective antibody, we were unable to test the precise mechanism of MOR-1 and/or MOR-1K in hNPCs. For instance, the siRNA result indicates the potential regulatory function of MOR-1K on MOR-1 expression. This could occur either at the transcriptional or translational level. To test whether MOR-1k functions as a transcriptional regulator, binding on the promoter of MOR-1 and altering its transcription, chromatin immunoprecipitation (ChIP) can be performed. To assess if MOR-1k acts at the protein level and stabilizing MOR-1, experiments measuring the direct interaction of MOR-1 and MOR-1K intracellularly are necessary. Such methods may include immunofluorescence (IF) and immunoprecipitation (IP). In both cases, the use of a specific MOR-1K and MOR-1 antibody is necessary to pull down chromatin-bound MOR-1K for ChIP analysis, or detect MOR-1K protein expression for IF and IP. The
drawback of not having a specific MOR-1K and MOR-1 antibody can be circumvented by using FLAG-tagged MOR-1K and MOR-1 constructs that have been employed in earlier studies (Dever, et al. 2014; Gris, et al. 2010). Although this approach has been invaluable to understanding the fundamental actions of MOR-1K relative to MOR-1, it is an over-expression system measuring the action of exogenous MOR-1 and MOR-1K, which may reflect artificial interactions that may not normally occur in primary hNPCs upon exposure to HIV-1 and morphine.

For future reference, an alternative approach to circumvent the antibody challenge is performing *in situ* hybridization. This technique can be extremely helpful in detecting the precise expression and localization of mRNA of MOR splice variants in hNPCs. A specific MOR-1K probe can be design against exon 13, and MOR-1 against exon 1, 3 or 4. Fluorescent probes can be used on fixed cells to visualize the expression of MOR-1K relative to MOR-1 under a microscope. With this method, one can determine whether MOR-1K interacts with MOR-1 via examining their cellular localization relative to each other in hNPCs upon exposure to HIV-1 ± morphine. If in the context of HIV-1 and morphine, MOR-1K functions as regulator/chaperone stabilizing MOR-1, the *in situ* hybridization result will show co-localization of the two variants at the cell surface.

**Potential cross-talk between MOR-1 and/or MOR-1K with Cdk5 in the mechanism of HIV-1 and morphine interaction**

Taken together, the findings suggest that both Cdk5 and MOR-1 and/or MOR-1K may in part participate on the mechanism underlying HIV-1 and morphine interaction in hNPCs. This notion is supported by recent data demonstrating the interaction of Cdk5...
and MOR reported by various groups. In challenged human neuroblastoma cells (SH-SY5Y) and primary rodent hippocampal neurons, Cdk5 was found to be necessary and sufficient to mediate neuroprotection by MOR-agonist DAMGO (Wang, et al. 2006b). Moreover, Cdk5 has been shown to modulate MOR receptor-mediated anti-nociceptive and anti-hyperalgesia in morphine and complete Freund’s Adjuvant (CFA)-treated (inflammatory model) mice (Beaudry, et al. 2015). At the molecular level, many known substrates and proteins that interact with Cdk5 have been implicated in MOR-related signaling pathways. For example, Cdk5/p35 is involved in altering the MAPK/ERK pathway, a known downstream target of MOR activation (Belcheva, et al. 2003; Macey, et al. 2006), via the phosphorylation of MAP kinase kinase-1 (MEK1) and extracellular signal-regulated kinase (ERK) 1/2 (Ramos-Miguel and Garcia-Sevilla 2012; Sharma, et al. 2002). In addition, Cdk5 has also been shown to regulate other MOR associated targets including Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CAMKII) (Bruggemann, et al. 2000), NMDA (Li, et al. 2001) and voltage-dependent calcium channel (VDCC) (Tomizawa, et al. 2002).

Based on these studies, it is possible that a cross-talk between Cdk5 and MOR splice variants may exist in hNPCs upon exposure to HIV-1 and morphine (Figure 5.1). While the mechanism of Cdk5 and MOR splice variants interaction has not been identified, we speculate that Cdk5 may act at various levels in MOR-1 and/or MOR-1K mediated signaling in hNPCs. First, Cdk5 may phosphorylate MOR-1 and/or MOR-1K leading to either altered activity or localization. The ability of Cdk5 to regulate other receptors have been demonstrated previously, including the DOR subtypes (Jeong, et al. 2013; Zhang, et al. 2008b). Phosphorylation of a consensus Cdk5 phosphorylation motif
in the second intracellular loop of DOR by Cdk5 was found to regulate internalization and desensitization (Xie, et al. 2009). Second, it is possible that Cdk5 upregulation may be downstream of MOR-1 and/or MOR-1K activation. In an earlier study, MOR activation promotes the expression of delta c-fos (Shoda, et al. 2001), a transcription factor that is known to mediate the transcription of Cdk5 and p35 (Cheng and Scadden 2002). Thus in our paradigm, HIV-1 and morphine mediated activation of MOR-1 and/or MOR-1K may enhance the expression of delta c-fos ultimately leading to the up-regulation of Cdk5 and p35. MOR-1 and/or MOR-1K signaling may also converge with Cdk5-related pathways and augments the functional consequences of HIV-1 and morphine co-exposure in hNPCs. For instance, the classic downstream target of MOR activation is the induction of the ERK 1/2 pathway, leading to cellular outcomes associated with cell differentiation and proliferation. The different components of the ERK 1/2 signaling cascades are also known substrates of Cdk5. It was previously reported that Cdk5 interacts with p27, a downstream target of the ERK 1/2 cascade. This results in the nuclear translocation of Cdk5/p35 and promotes cell cycle arrest in post-mitotic neurons (Zhang, et al. 2008a). Interestingly the anti-proliferative effect of HIV-1 Tat and morphine co-exposure was shown to be mediated by the induction of ERK 1/2 and p27, inhibiting the action of cell cycle regulators Cyclin D, Cdk4 and Cdk6 in hNPCs (Malik, et al. 2014). Thus we speculate that a cross-talk may occur between Cdk5 and MOR signaling at the ERK 1/2 level, promoting cell cycle arrest in hNPCs by the action of nuclear Cdk5/p35/p27 interaction. In addition, the ERK 1/2 signaling cascade has also been implicated in astrocyte differentiation in the developing brain (Cheng, et al. 2013). Thus it is possible that Cdk5 and MOR converge at this point to regulate astrocyte differentiation of hNPCs.
exposed to HIV-1 and morphine. Cdk5 and MOR cross-talk may also exist in the mechanism of HIV-1 and morphine mediated neuronal differentiation via the induction of the p38/MAPK pathway.

MOR-1K in particular can potentially regulate the aberrant activity of Cdk5 leading to the development of pathological processes in hNPCs exposed to HIV-1 and morphine (Figure 5.1). Distinct to MOR-1, MOR-1K is coupled to Gs and can activate stimulatory events including increase intracellular calcium via the stimulation of adenylyl cyclase and cAMP (Gris, et al. 2010). Elevated intracellular calcium can activate calcium-dependent protease calpain, which we showed to be elevated in hNPCs exposed to HIV-1 and HIV-1 + morphine. Activation of calpain can lead to the cleavage of p35 to constitutively active isoform p25. As mentioned prior, aberrant activity of Cdk5 is mediated by the binding of p25 to Cdk5. This interaction is implicated with the development of pathological events in both in vitro and in vivo models of HAND (Fields, et al. 2015; Patrick, et al. 2011; Wang, et al. 2007)

**Final conclusion**

In this study, we have carefully investigated the functional consequence of HIV-1 and morphine co-exposure on primary hNPCs and determined potential molecular targets that may be involved on the interaction of HIV-1 and morphine. The interaction of morphine on HIV-1-mediated proliferation and differentiation changes in hNPCs is likely to have important consequences for adult patients, where NPCs are actively responding to the disease processes. This can also be significant in the children and adolescents with HIV-1, given that critical developmental processes are ongoing in the brains of these
individuals. HIV/opiate interactive effects may result in even greater and longer-lasting neurological deficits. Our findings are especially concerning given the existing opiate drug crisis, where the number of heroin users nearly tripled between 2005-2012 and overdose-related hospitalizations greatly increased over the past 10 years (source). The re-emergence of a heroin epidemic was largely driven by the relative availability and abuse of prescription opioids, such as oxymorphone (Senate Caucus on International Narcotics Control, 2014). The subsequent crackdown on prescription medication abuse fueled a transition to heroin, which is cheaper and more accessible. A major public health consequence of this drug epidemic is the rising outbreak of HIV infection in rural communities, where new infections occur most often in young adults, almost half of whom are women (Conrad, et al. 2015).
Figure 5.1 Potential cross-talk of Cdk5 with MOR in the mechanism of HIV-1 and morphine interaction on hNPCs. (A) HIV-1 exposure increases the protein level of Cdk5 and its activator p35, which likely leads to enhanced Cdk5 activity in primary hNPCs. Elevated Cdk5/p35 in the cytoplasm may facilitate HIV-mediated neuronal differentiation of hNPCs by phosphorylating substrates such as p38 and Map2, which have been shown to induce neuronal differentiation in vitro and in vivo. In addition, elevated cytoplasmic Cdk5/p35 may also promote astrocyte differentiation of hNPCs by activating the ERK1/2 signaling cascade. Cytoplasmic Cdk5/p35 could also play a protective role in hNPCs upon exposure to HIV-1 by preventing the nuclear translocation and activation of pro-apoptotic factor FOXO1. This action prevents the transcription of genes associated in the death signaling pathways including BIM. In addition, cytoplasmic Cdk5/p35 has also been shown phosphorylate anti-apoptotic factor Bcl-2, blocking its degradation and ultimately
maintain pro-survival signaling. In addition, cytoplasmic Cdk5/p35 may interact with p27, which is necessary to carry Cdk5/p35 to the nucleus. Once in the nucleus, p27 have been shown to promote cell cycle arrest in hNPCs by inhibiting the actions of cell cycle regulators such as Cyclin D, Cdk4 and Cdk6. It is likely that the nuclear Cdk5/p35/27 axis may have the same action in hNPC exposed to HIV-1 leading to reduced proliferation of these cells. Morphine exacerbates the effect of HIV-1 by activating MOR-1 and/or MOR-1K expressed on hNPCs. Potential crosstalk may occur between MOR and Cdk5 at several levels, which may ultimately augment the functional outcome of HIV-1. MOR activation leads to the release of inhibitory G protein, G\textsubscript{i}, which have been demonstrated to induce both the ERK1/2 and p38/MAPK signaling cascade. These events may enhance the effect of HIV-1 on the differentiation and proliferation of hNPCs. In addition, MOR can directly regulate the level of Cdk5 and p35 in hNPCs via the induction of delta c-fos, a transcription factor that promotes the expression of CDK5 and CDK5RAP1 (p35). (B) We speculate based on preliminary data from this study and previous findings by our group, that under a more pathological condition, MOR-1K actions can promote the aberrant activation of Cdk5. MOR-1K chaperoned by filamin A is activated at the cell surface, leading to the induction of stimulatory G protein, G\textsubscript{s}-mediated signaling. This include the activation of adenylyl cyclase and eventually increasing intracellular calcium level. Elevated intracellular calcium induces protease calpain and enhances the proteolytic cleavage of p35 to an aberrant and constitutively active p25. Aberrant activation of Cdk5 by Cdk5 is likely to play a role on the development of further dysfunctions and even cell death in hNPCs exposed to HIV-1 and co-exposed to HIV-1 and morphine at a longer
period. Dotted lines indicate protein localization, while dash lines represent process of proteolytic cleavage.
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Joyce M. Balinang, MS, PhD

Education

- Doctorate of Philosophy candidate: Neuroscience, School of Medicine, Virginia Commonwealth University (2012-2016)
- Master of Science: Microbiology and Immunology, School of Medicine, Virginia Commonwealth University (2010-2012)
- Bachelor of Science: Biology and Chemistry double major, Virginia Commonwealth University (2004-2008)

Research Experience

Ph.D. Graduate Research Associates August 2012-October 2016
Virginia Commonwealth University, Richmond, Virginia
Advisor: Pamela E. Knapp, Ph.D.

Projects:
1. Investigating the role of CDK5 signaling on the interactive effect of HIV-1 and opiates on the behavior and infection of human fetal brain-derived neural progenitor cells
2. HIV-1 mediated regulation of µ-opioid receptor (MOR) splice variants, MOR-1 and MOR-1K in human neural progenitor cells.

M.S. Graduate Research Associates August 2010-May 2012
Virginia Commonwealth University, Richmond, Virginia
Advisor: Shirley M. Taylor, Ph.D.

Projects: Understanding the transcription regulation of novel mitochondrial DNA Methyltransferase-1 (mtDMNT1) on the response to oxidative stress in human colon carcinoma cells (HCT116).

Research Techniques & Skills

Human and rodent cell Culture
- Primary and immortalized human cell culture (neural progenitor cells, neurons, astrocytes, macrophages, brain aggregates, HCT116, SH-SY5Y, HEK293)
- Primary murine mixed neuron-glia culture (E14-16, P0-P3)
- Human peripheral blood mononuclear cells (PBMCs) isolation and culture
- Culture protocol development (isolation protocol, media and substrate formulation)

Animal model
- HIV Tat transgenic mice

Molecular and cell biology
- RNA and DNA extraction and purification, RT-qPCR, PCR, PCR array, primer design
- siRNA gene knockdown, cell transfection, molecular cloning, plasmid purification
- Genetic sequencing and analysis, bisulfite sequencing and analysis
- ELISA, p24 antigen capture assay, cytokine arrays
- Chromatin immunoprecipitation (ChIP), methylated DNA immunoprecipitation (MeDIP), immunoprecipitation (IP), co-immunoprecipitation (CoIP)
- Immunoblotting, immunofluorescence staining, flow cytometry
- BrdU incorporation, cell growth assay, live/dead assay, Ficoll gradient, subcellular fractionation

Microscopy
- Confocal microscopy, fluorescence microscopy, time-lapse microscopy

Data Analysis
- EndNote X7, PRISM, SPSS, JMP, SigmaPlot

Other Skills and Experience
- Languages: English (fluent), Spanish (proficient writing and conversational), Tagalog (fluently), Kapampangan (fluently)
- Software: Microsoft Office programs, Adobe Illustrator, Adobe Photoshop, GraphPad, Prism, AxioVision.
- Hobbies/interest: outdoors sports, hiking, basketball, animal training, community volunteer, travelling, cooking.

Fellowship/Scholarships
- NRSA Pre-doctoral Training Fellowship, NIDA (2016)
- VCU Dean’s Scholarship (2004-2008)

Peer-Reviewed Publications, Abstracts, Awards

Peer-Reviewed Publication

• **Shipping Z.,** **Balinang, J.,** Paris, J., Hauser, KF., Knapp, PE. 2016. Effects of HIV-1 Tat on oligodendrocyte viability are mediated by CaMKIIβ-GSK3β interaction. *J. Neurosci.* submitted


Abstracts


• **Balinang, J.,** Hauser, KF., Knapp, PE. Morphine enhances the effect of HIV-1 on proliferation of primary human neural progenitor cells; role of µ-opioid receptor (MOR) splice variants. (Poster, 46th ASN Annual Meeting abstract, Atlanta, GA. 2015)

• **Balinang, J.,** Hauser, KF., Knapp, PE. Morphine enhances the effect of HIV-1 on proliferation of primary human neural progenitor cells; role of µ-opioid receptor (MOR) splice variants. (Poster, 6th CVCSN Annual Meeting abstract, Atlanta, GA. 2015)

• **Balinang, J.,** Hauser, KF., Knapp, PE. Interactive effects of HIV-1 and morphine on the proliferation, but not on the survival of primary hNPCs; role of µ-opioid receptor (MOR) splice variants. (Poster, 13th ISNV Annual Meeting abstract, San Diego, CA. 2015)


• **Balinang, J.,** Hauser, KF., Knapp, PE. Functional consequences and infection of human fetal brain-derived NPCs; interaction with morphine. (Poster, 47th ASN Annual Meeting abstract, Denver, CO. 2015).

• **Balinang, J.,** Hauser, KF., Knapp, PE. Human neural progenitor cells (hNPCs) are productively infected by R5-tropic HIV-1: Morphine interactions on infection and function involve Cdk5 signaling. (Poster, 14th ISNV Annual Meeting abstract, Toronto, Canada. 2016).

• Paris, J., Kim, S., **Balinang, J.,** Zhou, S., Knapp, PE., Hauser, KF. Allopregnanolone regulation of mitochondrial function may attenuate HIV-1 Tat and morphine-mediated neurotoxicity. (Nanosymposium, 46th SFN Annual Meeting abstract, San Diego, 2016)

Awards

• Multi-cultural Academic Award, VCU (2005-2006)

• Dean’s List Award, VC (2004-2008)

• Neuroscience Travel Award, VCU (2015)

• YIEE Travel Award, ASN (2015)

• Induction into the Honor Society of Phi Kappa Phi (2014)