HIV-1 Envelope Glycoprotein 120 Induced Neuroinflammation Does Not Result in Neurocognitive Impairment in An *in vivo* Rat Model

by

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A thesis submitted in conformity with the requirements for the graduate degree of Masters of Science

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Masters of Science

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\textbf{ABSTRACT}

Human immunodeficiency virus-1 (HIV-1) infected patients are increasingly experiencing neurocognitive deficits due to low level viral replication and associated inflammation in the central nervous system which results in cellular apoptosis in the brain. The aim of this MSc project was to examine cognitive impairments in learning/memory in the Morris Water Maze using our recently implemented \textit{in vivo} rat model of HIV-1-induced neuroinflammation by intracerebroventricular administration of HIV-1 gp120\textsubscript{ADA}. Our findings illustrate that a single dose of 8\textmu g R5-tropic HIV-1 gp120\textsubscript{ADA} or 50\textmu g lipopolysaccharide elicited a robust proinflammatory cytokine (IL-1\textbeta, TNF\alpha) and oxidative stress (iNOS) response in the brain, but only lipopolysaccharide administration activated caspase-3 expression in hippocampal neurons, possibly, resulting in the observed spatial learning/memory deficits. Our gp120\textsubscript{ADA}-induced neuroinflammatory rat model suggests that acute administration of HIV-1 viral coat protein does not result in neurocognitive impairments, thus alternative gp120 dosing regimens should be considered to establish a model of neurocognitive deficits.
ACKNOWLEDGEMENTS

First and foremost, my sincerest thanks goes to my supervisor, Dr. Reina Bendayan, for her invaluable advice, knowledge, support and guidance throughout my graduate studies. Thank you for giving me the opportunity to pursue my Master degree under your supervision and for providing me with many chances to present our research at international, national and local research conferences. I have learnt a great deal from your unfailing enthusiasm and optimism, and I had a wonderful learning experience in your laboratory.

I would like to sincerely thank my committee members Dr. Peter Pennefather and Dr. Adrianna Carvalhal for their continuous support, constructive criticism and guidance throughout my MSc. studies. I am truly grateful to all of you.

I thank Dr. Jeffrey Henderson for collaborating with us and providing me with the opportunity to learn immunohistochemistry in his facility. I also thank Dr. Andrew Elia and Leanne Tworzyanski from the Advanced Optical Microscopy Facility (AOMF; Toronto General Hospital) for their patience and aid in microtome sectioning and immunofluorescence staining.

My sincere thanks to the Canadian Institute of Health Research Strategic Program in Biotherapeutics (CIHR BT) and the Graduate Department of Pharmaceutical Sciences for their generous financial support and travel funds.

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A special thanks to Dr. Tozammel Hoque for his calming nature and willingness to provide much needed assistance in various experiments. I will remember the kindness you have shown and the encouragements in the past two years. I sincerely thank Dr. Chiping Wu for all his help with the animal surgeries and behavioural experiments.

I would also like to honor the hundreds of little lives that contributed to this project. Without rodents, mice and other laboratory animals, many scientific breakthroughs would not have been possible, including findings reported in this thesis.

Thank you so much to my dearest friend, Natalie Cheng and Jeffrey Chang for your constant encouragements. Your humour, advice and emotional support made the difficult times easier to bear. Thank you for being there for me during the ups and downs. I wish you all the best in your future endeavors.

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Success is living a few years of your life like most people won't so that you can live the rest of your life like most people can't.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ARV</td>
<td>Antiretroviral</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BMECs</td>
<td>Brain microvessel endothelial cells</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CPE</td>
<td>CNS penetration effectiveness</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6′-diamidino-2-phenylindole hydrochloride</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-Diaminobenzidine</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>CAMs</td>
<td>Endothelial cell adhesion molecules</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSS</td>
<td>Genotypic Susceptibility Score</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV-associated dementia</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV-associated neurocognitive disorder</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>icv</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>JAMs</td>
<td>Junctional adhesion molecules</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris Water Maze</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MRPs</td>
<td>Multidrug resistance proteins</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NFL</td>
<td>Neurofilament protein</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NSI</td>
<td>Non-syncytia-inducing</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMEA</td>
<td>9-(2-phosphonomethoxyethyl)-adenine</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RNase H</td>
<td>Ribonuclease H</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ssrna (single-stranded RNA)</td>
</tr>
<tr>
<td>gp120</td>
<td>Surface glycoprotein 120</td>
</tr>
<tr>
<td>SI</td>
<td>Syncytia-inducing</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris Water Maze</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UNAIDS</td>
<td>United Nations Program on HIV/AIDS</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VPS</td>
<td>Vacuolar protein sorting</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occluden 1</td>
</tr>
</tbody>
</table>
CHAPTER 1: BACKGROUND

1.1 HIV-1 Epidemiology

The Human Immunodeficiency Virus (HIV) is an enveloped lentivirus that targets the CD4+ T lymphocytes and macrophage populations of the immune system and is the cause of human acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi, 2009). There are several modes of HIV acquisition and transmission, the predominant ones include sexual transmission, intravenous drug use (i.e. needle sharing) as well as vertical transmission from mother to child. According to the 2014 Gap Report by the Joint United Nations Program on HIV/AIDS (UNAIDS), about 35 million people worldwide were living with HIV infection in 2013 (Figure 1). In the same year, approximately 2.1 million new HIV infections were reported comprising of 1.9 million adults and 240,000 children. As for AIDS-related deaths, 1.5 million deaths were documented in 2013, including 190,000 children under the age of 15. Over the last 30 years of the AIDS epidemic, enormous efforts and resources have been dedicated to HIV pharmacotherapy, community-based interventions, trauma management, and sexual education. These efforts have resulted in a 38% decline in new infections since 2001 as well as a 19% reduction in AIDS-related deaths in the last 3 years. The latter representing the largest annual decline in the past decade (UNAIDS, 2014).
Global summary of the AIDS epidemic | 2013

<table>
<thead>
<tr>
<th>Number of people living with HIV</th>
<th>Total</th>
<th>35.0 million [33.2 million – 37.2 million]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adults</td>
<td>31.8 million [30.1 million – 33.7 million]</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>16.0 million [15.2 million – 16.9 million]</td>
</tr>
<tr>
<td>Children (&lt;15 years)</td>
<td>3.2 million [2.9 million – 3.5 million]</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>People newly infected with HIV in 2013</th>
<th>Total</th>
<th>2.1 million [1.9 million – 2.4 million]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Adults</td>
<td>1.9 million [1.7 million – 2.1 million]</td>
</tr>
<tr>
<td></td>
<td>Children (&lt;15 years)</td>
<td>240 000 [210 000 – 280 000]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AIDS deaths in 2013</th>
<th>Total</th>
<th>1.5 million [1.4 million – 1.7 million]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adults</td>
<td>1.3 million [1.2 million – 1.5 million]</td>
</tr>
<tr>
<td></td>
<td>Children (&lt;15 years)</td>
<td>190 000 [170 000 – 220 000]</td>
</tr>
</tbody>
</table>

Source: UNAIDS

Figure 1 Global Summary of the AIDS epidemic updated July 2014 by the Joint United Nations Programme on HIV/AIDS (UNAIDS). Based on these numbers, it is estimated that there was a 38% decline in new infections since 2001 and a 19% decrease in AIDS-related deaths in the last 3 years.

Efforts and resources have been particularly concentrated in Sub-Saharan Africa, an area that is currently the most affected by HIV with approximately 24.7 million individuals (i.e., one in every 20 adults) living with the virus. This region accounts for 74% of all mortalities from AIDS-related causes in 2013. The large impact of HIV in Sub-Saharan Africa could be the result of limited access to antiretroviral (ARV) therapy and prevention resources. Treatment inaccessibility for people infected with HIV remains high in South Africa (58%), India (64%), and Nigeria (80%). However, since 1995 ARV therapy has averted 7.6 million deaths globally, including 4.8 million deaths in Sub-Saharan Africa. Although by the end of 2013 there were 12.9 million people receiving ARV therapy globally, there remains 63% of people living with HIV who do not have access to this therapy (UNAIDS, 2014). UNAIDS has implemented various initiatives and goals to decrease the number of people who do not have access to ARV therapy and reduce medical costs. On the other hand, due to the higher availability of ARV therapy in western/central Europe and North America, approximately 2.3 million people were estimated to be living with HIV in 2013, 4% of whom reside in Canada (UNAIDS, 2013). In these regions, only minor changes in incidence rates were seen in the last decade. The ambitious
‘90-90-90’ target of ending the AIDS epidemic as a public threat by 2030 has been provisionally endorsed (Figure 2). UNAIDS is targeting a 90% reduction in new HIV infections (i.e., towards achieving zero new HIV infections per annum), a 90% reduction in stigma and discrimination faced by people living with HIV-1 (i.e., towards achieving zero discrimination) as well as a 90% reduction in AIDS-related deaths (i.e., towards achieving zero AIDS-related deaths). Optimism is growing that these targets can be achieved through global efforts in education, social work and increase accessibility of preventative measures against transmission.

1.1.1 HIV-1 Life Cycle

As a result of independent zoonotic transfer from viruses infecting African primates, two unique types of HIV have been identified (i.e., HIV-1 and HIV-2) and known to cause AIDS in humans (Hahn et al., 2000). Other immunodeficiency-related viruses found in primates are collectively termed the simian immunodeficiency virus (SIV) with suffixes denoting species of origin (e.g., SIV$_{cpz}$ from chimpanzees; SIV$_{sm}$ from sooty mangabeys). While HIV-1 is mainly responsible for the global HIV epidemic and suspected to originate from SIV$_{cpz}$, HIV-2 is less common, predominantly found in West Africa, and believed to evolve from SIV$_{sm}$ (Stebbing and Moyle, 2003). Both HIV-1 and HIV-2 are species of the lentivirus genus of the orthoretrovirinae subfamily in the retroviridae family. Similar to other retroviruses, HIV-1 is a lipid enveloped RNA virus approximately 100nm in diameter.
Within the lipid bilayer, an icosahedral or conical capsid encloses a linear, positive single-stranded RNA (ssRNA) genome that encodes nine viral proteins termed env, gag, pol, vif, vpu, vpr, tat, rev and nef; each of which play a vital role in viral maturation and proliferation.

The outer leaflet of the HIV-1 virion lipid envelope bilayer is studded with spike proteins that are anchored to matrix proteins found on the inner leaflet. The surface spike protein is a heterotrimer composed of three molecules of surface glycoprotein 120 (gp120) and three molecules of transmembrane glycoprotein 41 (gp41), both encoded by the viral env gene. Viral binding occurs through gp120 binding with the cluster of differentiation (CD) 4 receptor (Figure 3). CD4 is a surface transmembrane glycoprotein expressed by monocytes, macrophages, and subsets of T-cells and dendritic cells. The gp120-CD4 complex causes a conformational change to expose a secondary binding site (i.e., co-receptor) for chemokine receptors. Depending on viral tropism, the gp120-CD4 complex can bind either (or both) CCR5 or CXCR4 chemokine co-receptor(s) for cellular entry, thus resulting in three tropic classes of HIV-1 (Table 1). Non-syncytia-inducing (NSI) strains bind to the CCR5 chemokine receptor and are predominantly expressed by macrophages, therefore referred to as macrophage (M) tropic or R5 tropic. Syncytia-inducing (SI) strains bind to the CXCR4 chemokine receptor and are predominantly expressed by T-cell populations, therefore referred to T-cell (T) tropic or X4 tropic. Dual tropic viruses can use either CCR5 or CXCR4 co-receptors infecting both macrophage and T-cell lines. Within an infected patient, there may be a mixed population of viruses (R5-tropic and/or X4-tropic), however during early stages of infection, R5 tropic viruses appear more prevalent whereas in late stages of infection (i.e., progression into AIDS) the X4/dual tropic virus seems to be more prevalent (Goodenow and Collman, 2006). Viral tropism, as well as strain and clade differences of HIV, may be attributed to amino acid differences in the variable regions of envelope protein, gp120, resulting in changes in secondary structural conformation (Samikkannu et al., 2015).

<table>
<thead>
<tr>
<th>M-Tropic non-syncytia-inducing</th>
<th>R5 tropic</th>
<th>T-Tropic Syncytia-inducing</th>
<th>X4 tropic</th>
<th>Dual-Tropic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADA, Bal, 96ZM651, SF162, JRFL, CM, CN54, YU2</td>
<td>MN, LAI, IIIB</td>
<td>MN, SF2</td>
<td></td>
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</tr>
</tbody>
</table>

Susceptibility to HIV-1 infection is highly dependent on the expression of chemokine co-receptors. Genetic mutations that decrease protein expression or alter protein functionality, such as the CCR5
(CCRΔ32) deletion, are possibly associated with decreased HIV-1 susceptibility (Liu et al., 1996).

Other determinants that influence HIV-1 susceptibility include other genetic anomalies such as lower copy number of CCL3L1 (Gonzalez et al., 2005), disturbances in the vaginal flora (Taha et al., 1998), male circumcision (Donoval et al., 2006) as well as the presence of previous infections such as sexually transmitted diseases (Galvin and Cohen, 2004).

Once the gp120-gp41 complex is engaged with the chemokine co-receptor, the gp41 fusion peptide, specifically the hydrophobic N-terminus, is inserted into the target cell membrane. This signals the rearrangement of gp41 into its post-fusion state allowing the viral and host cell membranes to come into close proximity, fuse, and release of the viral capsid into the cellular cytoplasm (Pancera et al., 2014). In the cytoplasm, the capsid disassociates to release viral reverse transcriptase (RT), ribonuclease H (RNase H), integrase and the viral genomic ssRNA that work in concert to transcribe viral ssRNA genome into double stranded DNA, a molecule which is then translocated into the nucleus. Within the nucleoplasm, HIV-1 integrase cuts a strand of chromosomal DNA and attaches the viral genome to free 5′-phosphates. The single-stranded 3′-hydroxide breaks are attached by host DNA repair mechanisms establishing a stable provirus. New viral proteins can be produced through cellular protein synthesis resulting in virion assembly and budding as orchestrated by the viral HIV-1 gag protein. At this stage, the proline rich motif (i.e., late-domain) of gag interacts with cellular class E vacuolar protein sorting (VPS) proteins including the four varieties of cellular endosomal sorting complex required for transport (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III). Under physiological conditions, VPS and ESCRT proteins aid the formation of membrane-coated vesicles and abscission; however, HIV-1 gag protein exploits this cellular machinery to release a nascent viral vesicle containing viral precursor polypeptide (Gag-Pol). HIV-1 Vpu, a transmembrane protein, aids the release of the immature viral particle from the cell by interacting with host restriction factor, tetherin (Figure 3). Tetherin is a type II transmembrane protein on the cell surface, consisting of four domains (i.e., N-terminal cytoplasmic domain, a single transmembrane domain, an extracellular domain, and a putative C-terminal glycosyl phosphatidylinositol anchor). The mechanism of restricting progeny virion release has been extensively investigated as a potential avenue of therapeutic intervention (Simon et al., 2015). The final maturation of the HIV-1 virion occurs in parallel, or shortly after, budding. Proteolysis of Gag–Pol precursor peptide results in the expression of structural components (e.g., capsid, matrix, nucleocapsid) and viral enzymes (protease, reverse
transcriptase, integrase) that are typically found in the mature, and infectious, virion (Engelman and Cherepanov, 2012).

Figure 3 the HIV-1 Viral Life Cycle. The viral entry is initiated by the binding of viral envelope spike protein (gp120 and gp41 complex), to the CD4 receptor. This triggers a conformational change and allows the subsequent binding of gp120 to the HIV-1 co-receptor (i.e., CCR5 and CXCR4). Binding of gp120 to the co-receptor causes the fusion of viral and cell membrane allowing the release of viral capsid into the cell cytoplasm. The capsid proteins disassociate, releasing viral genome, integrase, RNase H and reverse transcriptase. Viral RNA is immediately transcribed into double-stranded DNA by viral reverse transcriptase and transported into the nucleus for integration. Subsequent transcription of the genome results in the production of new HIV-1 viral mRNA that is translated into proteins. The protein precursors are cleaved by viral protease into functional proteins that assemble at the plasma membrane of the host cell. Once released, the new virions undergo maturation before becoming infectious. The six different classes of ARVs that target different phases of the HIV-1 life cycle are shown and include fusion inhibitor, entry (CCR5) inhibitor, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitor (INSTIs) and protease inhibitors (PIs). Host restriction factors such as tetherin restrict progeny virions release from the cell membrane. (Diagram from Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. Nature reviews Microbiology. 2012;10(4):279-90. doi:10.1038/nrmicro2747).

1.2 HIV-1 Infection of the Central Nervous System

The central nervous system (CNS) has been identified as a viral sanctuary of HIV-1. Upon infection, the patient initially experiences a brief period of influenza-like symptoms that subsides and enters into an asymptomatic phase that can persist for an indefinite period of time. During this phase, the virus evades the immune system by hiding and populating in immunologically privileged sites (e.g. brain, eyes and testicles), creating major obstacles in HIV-1 eradication. Viral sanctuaries harbour a
replication-competent form of the virus in cellular compartments such as macrophage and microglial cells (Glass et al., 1995, Sinclair et al., 1992). These viral populations are then able to reseed the infection when the immune system is hampered and further the progression into AIDS. Additional sanctuaries can be found in peripheral tissues (e.g., eye, testes), or within particular cellular populations forming viral reservoirs (e.g., resting memory CD4 positive T-cells, microglia and astrocytes) (Borrow, 2011).

The CNS is protected from the systemic circulation by the blood-brain barrier (BBB). In 1904 Paul Ehrlich proposed a physical barrier between the peripheral and CNS when he observed cationic aniline dyes injected to the systemic circulation could stain most organs with the exception of the brain and spinal cord. Corroborating Ehrlich’s report, his student Edwin E. Goldman in 1913 directly administered trypan dyes into the cerebrospinal fluid (CSF), and observed staining of the central, but not peripheral, tissues. Shortly after the observations of a physical barrier was established using coloured dyes, the development of the electron microscope further substantiated the existence of a structural barrier using horseradish peroxidase (HRP). HRP is often employed as a vascular tracer for protein leakage since it is the same size as many serum proteins and can be detected through electron microscopy or spectrophotometrically. When administered into the mouse vascular endothelium, the lack of HRP perfusion in the CNS was observed. This was found later due to the existence of tight junction proteins that sutured endothelial cells together and restricted paracellular trafficking of substrates and molecules (Reese and Karnovsky, 1967, Stewart et al., 1992). Our knowledge to date illustrates the BBB as a multi-cellular monolayer composed of non-fenestrated brain microvessel endothelial cells (BMECs) and brain parenchymal cells (e.g., surrounding pericytes, adjacent astrocytes and neurons) that functions in concert to create a physical and biochemical barrier.

The physical barrier results from the expression of tight junction proteins (e.g., occludin, claudin) and junctional adhesion molecules (JAMs). These proteins significantly reduce paracellular permeability of small and hydrophilic molecules as well as free flow of water and other solutes (Hawkins and Davis, 2005). Since open pores or pathways allow flow of water and aqueous solutes, measuring the electrical resistance can be used as an indicator of endothelial permeability wherein the higher number of open pores (i.e., permeability) results in lower electrical resistance. A high transendothelial electrical resistance was measured at approximately 800 Ωcm² in an in vitro BBB model using porcine microvessel endothelial cells (Patabendige et al., 2013). This resistance was
observed to be higher in an in vivo model measuring at approximately 1500-2000 Ωcm². In addition to water channels, several receptors, ion channels, and influx/efflux transport proteins are prominently expressed at the luminal and abluminal membranes to form a biochemical barrier (Figure 4). A secondary biochemical barrier also exists at the membranes of brain parenchymal cells further limiting the access of endogenous and exogenous substrates into cellular components of the CNS (Ashraf et al., 2014b). However, the BBB is not entirely restrictive as hormones, plasma proteins and various nutrients (i.e., amino acids, nucleosides, glucose, and electrolytes) are able to penetrate into the CNS through receptor-mediated endocytosis and/or membrane influx/efflux transporters. During disease states such as HIV-1 infection, the integrity of the BBB may be compromised due to HIV-associated decrease in tight junction proteins as well as induced apoptosis of endothelial cells (Toborek et al., 2005).

Figure 4 Structural and Biochemical Barriers at the Blood-Brain Barrier. Tight junction proteins (occludin, claudin) and junction adhesion molecules (JAMs) are expressed between brain microvessel endothelial cells (BMECs) lining the capillary wall to restrict paracellular entry of small molecule solutes. This forms the physical barrier between the systemic and CNS circulation. A biochemical barrier exists by the expression of efflux/influx transporters on the luminal and abluminal membranes (other ion channels and receptors also exist but are not shown). These membrane proteins regulate the transcellular traffic of solutes, nutrients, and numerous xenobiotics. The arrows indicate the direction of substrate transport. (Adapted and modified from Ashraf T, Kao A and Bendayan R, 2014).

It is widely accepted that HIV-1 can penetrate the CNS approximately two weeks, to a few months, after systemic infection (An et al., 1999, Davis et al., 1992, Spudich et al., 2011). Crossing the BBB can occur either through the paracellular route which involves migration in-between adjacent BMECs and/or the transcellular route which occurs directly through an individual endothelial cell. HIV-1 paracellular migration may occur as a cell-free virion or within an infected monocyte, known as the “Trojan Horse” hypothesis (Davis et al., 1992). In HIV-infected patients, significant increases in vascular permeability and BBB abnormalities have been reported in neuropathological studies (Andersson et al., 2001, Berger et al., 2000, Petito and Cash, 1992, Singer et al., 1994). In support,
rodent models have demonstrated that tight junction proteins and JAMs, which normally function to disallow foreign materials from entering the CNS, can be down regulated upon exposure to HIV-1 proteins. For example, tight junction proteins such as zona occluden-1 (ZO-1) and occludin were expressed at significantly lower levels by human BMECs upon HIV-1 gp120 treatment (Kanmogne et al., 2005, Nakamuta et al., 2008, Toborek et al., 2005). These findings have also been demonstrated in in vivo rodent models employing in-tissue administration of recombinant viral proteins such as gp120 or tat; significantly decreased expression of permeability proteins such as claudin-5, ZO-1, and laminin was observed 24h after administration (Louboutin et al., 2010a, Pu et al., 2005). The transcellular route can be used by cell free virions as well as infected monocytes. Cell free virion can utilise the mannose-6-receptor thereby entering the CNS via receptor-mediated transcytosis (Dohgu et al., 2012), whereas infected monocytes may utilise a pore or channel on the luminal surface of endothelial cells (Wittchen, 2009). Peripheral monocyte trafficking into the CNS is guided by the expression or activation of monocytic cognate ligands such as αLβ2 (LFA-1) and α4β1 (VLA-4) which bind to endothelial cell adhesion molecules (CAMs) expressed by BMECs such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1. Increased mRNA and protein expression of ICAM-1 and VCAM-1 by brain endothelial cells has been demonstrated in an in vivo mouse model employing intra-hippocampal injection of HIV-1 tat, suggesting increased peripheral monocyte infiltration (Pu et al., 2003). In addition to the production of chemokines and adhesion molecules, infiltration of HIV-1 infected monocyte into the CNS can be facilitated by matrix metalloproteinase (MMP). MMPs are extracellular proteases that aid monocyte trafficking into the CNS and significantly increased levels have been identified in the CSF of early HIV-1 infected patients (n = 52, mean infection duration less than one year) (Li et al., 2013). Together, these findings provide neuropathological evidence to support HIV-1 penetrates the CNS during infection.

Within the brain, HIV-1 readily infects mononuclear phagocytes that express both CD4 and the chemokine co-receptors required for cellular entry. Specifically, perivascular macrophages and brain resident microglial cells are the major targets of HIV-1 that significantly contribute to HAND pathogenesis (Dalgleish et al., 1984, Deng et al., 1996, Persidsky and Gendelman, 2003). Upon interacting with HIV-1, microglial cells, which are normally found in a resting quiescent state in healthy individuals, become activated and release pro-inflammatory cytokines, prostaglandins, reactive oxygen species (ROS) and nitric oxide (NO), arachidonic/quinolinic acid, and other molecules (Garden, 2002, Persidsky and Gendelman, 2003). The expression and secretion of these
soluble factors contribute to viral spread in the CNS by triggering the activation of neighbouring microglia and astrocytes as well as recruiting peripheral monocytes from the systemic circulation. Generally, microglial cells are responsible for a productive infection whereas astrocytes harbour the virus to allow continual maturation and propagation at a later time (i.e., non-productive latent infection). Activated astrocytes are also morphologically dissimilar to their quiescent healthy state and can release inflammatory cytokines and oxidative stress molecules. Direct HIV-1 infection of astrocytes remains controversial due to the lack of CD4 receptor expression at the cell surface, though, it is possible that astrocyte infection occurs in a CD4-independent mechanism requiring gp120 interactions with glycosphingolipid galactosylceramide (Boutet et al., 2001, Cook et al., 1994, Gorry et al., 2003). It does not appear likely that astrocytes are capable of productive HIV infection due to restrictive viral replication including decreased export of viral mRNA into the cytoplasm by viral protein Rev (Neumann et al., 2001). Latent infection in astrocytes may result in viral sequestration leading to the formation of a viral reservoir in these cells. Cytokines, which are soluble proteins or glycoproteins released for intracellular communication, can be classified into four families: chemokines, hematopoietins, interleukins (IL) and tumor necrosis factor (TNF) family. In particular, production of IL-1β and TNFα have been strongly implicated in neurodegenerative diseases and detected in high levels in post-mortem HIV-1 infected tissues (Persidsky et al., 1997, Tyor et al., 1992). IL-1β and TNFα are biologically produced as an inactive zymogen requiring proteolytic cleavage by caspase-1 for activation. Upon binding to specific receptors at the surface of target cells, cytokines can influence the expression levels of various proteins via various intracellular signaling pathways. In addition, a number of other cytokines such as interleukin-6 (IL-6), IL-10, IL-18, interferon (IFN) α and γ are also known to be elevated during brain HIV-1 infection (Hult et al., 2008, Perrella et al., 1992, von Giesen et al., 2004).

Inflammatory cytokines can also induce oxidative stress in glial cells (Won et al., 2013). Reactive oxygen species (ROS) from host metabolism (e.g. superoxide anions) can react with nitric oxide (NO) to form toxic reactive nitrogen species (RNS) such as peroxynitrite (Kovacic and Somanathan, 2012). ROS and RNS can oxidize vital biomolecules (i.e. lipid membranes, proteins, and deoxynucleic acids), resulting in cellular proliferation, hypertrophy and/or apoptosis. Nitric oxide synthase (NOS) regulates the production of NO and exists in three isoforms in the CNS differentiated by cellular origins: eNOS (i.e., endothelial), nNOS (i.e., neuronal), and iNOS (i.e., glial) (Fischer and Maier, 2015). High levels of iNOS expression have been reported in brain tissue isolated from HIV-1
patients with encephalitis, localized within microglial nodules and reactive astrocytes (Zhao et al., 2001). In the CSF of HIV-1 infected individuals, significantly increased oxidative stress markers have also been detected (Sacktor et al., 2004).

In addition to inflammation and oxidative stress, virus-infected cells and activated glial cells are also sources of various neurotoxic factors such as quinolinic acid, platelet activating factor (PAF), glutamate, prostaglandins and arachidonic acid (Wang et al., 2003). Significantly elevated levels of quinolinic acid have been documented in brain tissues and CSF of HIV-infected patients (Giulian et al., 1993, Heyes et al., 1991). Quinolinic acid is an L-tryptophan metabolite that acts as an excitotoxin in the CNS resulting in hindered neuronal function or apoptosis (Perez-De La Cruz et al., 2012). PAF which regulates the release of glutamate and inflammatory cytokines has been found in higher levels in HIV-infected patients, suggesting a role of uncontrolled PAF signaling (Gelbard et al., 1994). In addition, a cytoprotective enzyme (i.e., heme oxygenase 1) was found significantly decreased due to HIV-1 replication in cultured macrophages and HO-1 deficiency was found to be associated with increased release of neurotoxic levels of glutamate (Ambegaokar and Kolson, 2014). The collective effect of microglia and astrocyte communication, together with the release of neurotoxic compounds, leads to neuronal cellular stress, injury, and death.

Neurons do not appear to be susceptible to HIV-1 infection. However, chronic exposure to neurotoxic, inflammatory and oxidative stress markers during HIV-1 infection can cause irreversible neuronal damage (Kaul et al., 2001). In addition, viral proteins (i.e., gp120, Tat, vpr) can be shed from the virus or from infected cells resulting in neurotoxicity though the over stimulation of the N-methyl-D-aspartate (NMDA) receptor. Excessive calcium influx can result in the loss of mitochondrial membrane potential, cytochrome c release, excitotoxicity due to potassium or glutamate, caspase-3 activation, and ultimately DNA fragmentation and cellular apoptosis (Ashraf et al., 2013, Kanmogne et al., 2005, Kaul and Lipton, 2006, Kraft-Terry et al., 2010, Lindl et al., 2010, Ronaldson et al., 2008). Rodent models have shown caspase-3 dependent neuronal apoptosis due to gp120 (Singh et al., 2004), neurodeenerative effects of Vpr (Jones et al., 2007) as well as potassium/glutamate-mediated excitotoxicity due to Tat (Liu et al., 2013). Figure 5 below summarizes the different mechanisms involved in HIV-associated neuropathogenesis and the convergent effect on neurons.
1.2.1 HIV-1 Associated Neurocognitive Disorders (HAND)

HIV-associated neurocognitive disorder (HAND) is estimated to affect 18-50% of HIV-infected patients, ranging from asymptomatic neurocognitive impairment to fully manifested dementia (Zayyad and Spudich, 2015). Common clinical presentations include, but are not limited to, behavioural abnormalities (i.e., chronic fatigue, major depressive disorders, and anxiety), motor dysfunctions (i.e. tremor, loss of fine motor coordination) and cognitive impairments (i.e. learning, memory, attention) (Holland and Tross, 1985, Warriner et al., 2010). These cognitive symptoms can interfere with daily life functioning (i.e. employment, medication management, and driving) and
decrease quality of life in HIV-infected patients. Applying neuropsychological testing, HAND is diagnosed when patients perform one or more standard deviations below the mean of normative scores in at least two of seven cognitive domains (e.g., attention/working memory, executive functions, learning, memory, motor skills, information processing speed, and verbal fluency) (Antinori et al., 2007). However, these neuropsychometric screening tests are designed to detect severe forms of impairment (i.e., dementia) and are not sensitive to milder forms of deficits and are not specific to HAND (Bottiggi et al., 2007, Sacktor et al., 2005, Valcour, 2011). Additional challenges associated with screening tests include limited number of tests, requirement for specialized equipment/apparatuses, highly trained personnel to administer, score and interpretation of screening tests as well as difficulties of validating results in various languages and requirement for repeated measures render routine screening not feasible. Notably, an automated proof-of-concept smartphone-based screen test (i.e., NeuroScreen) has been developed by Robbins et al., as an easily implemented alternative method of screening neurocognitive deficits in HIV-1 infected individuals (Robbins et al., 2014). Currently in its early stages of development, NeuroScreen demonstrated a moderate-to-high correlation to traditional paper tests, assessing similar cognitive abilities, and will be optimised to detect milder cognitive impairments. Nonetheless, the lack of robust screening methods for early HAND diagnosis and monitoring is a key obstacle, highlighting the need for alternative measures of neurocognitive function such as neuroimaging techniques or levels of biochemical markers in tissue, serum, and/or CSF.

1.2.2 Neurocognitive Impairments and CNS Changes in Early/Acute Human Infection

Although neurocognitive impairments as well as motor dysfunctions have been robustly observed in patients chronically infected with HIV-1 (Doyle et al., 2015, Wilson et al., 2013), early onset of deficits are not well defined and under explored. Some studies have suggested the lack of neurocognitive impairment within the initial two years of infection (Vo et al., 2013), however, others highlight impairments seen in early infected individuals lie in the intermediate between chronically infected and non-infected controls (Moore et al., 2011). In other reports examining different cohorts, prominent deficits were observed in information processing speed, psychomotor speed, working memory and verbal learning of acutely HIV infected patients (Agrawal et al., 2010, Doyle et al., 2013, Moore et al., 2011, Ragin et al., 2012, Wang et al., 2011, Weber et al., 2013). Weber and colleagues (2013) reported in a sample of 46 ARV-naïve individuals with acute/early HIV infection (i.e., an estimated duration of infection at 75 days) that they were about four times more
likely to develop neurocognitive impairment than their seronegative controls, predominantly in information processing speed and verbal learning (Weber et al., 2013). Two studies investigating cognitive differences in an observational investigation (i.e., the Chicago Early HIV Infection Study) reported significantly impaired performance on the digit-symbol test used to evaluate psychomotor skills in a sample with estimated infection of 100 days (Ragin et al., 2015) and less than one year (Wang et al., 2011). Other domains that were significantly impaired involved executive functioning (i.e., letter-number sequencing task) and visual memory (i.e., Rey-Osterrieth Complex Figure Delayed Recall Test). Although patient population is often limited in these studies, it is possible that neurocognitive impairments may present shortly after seroconversion.

There is some evidence to suggest neuroimaging can evaluate the development of neurocognitive disorder prior to clinical presentations. Neuroimaging studies of acutely infected HIV-1 patients reveal remarkable changes in neuroanatomy and CSF composition that correlates with degree of neurocognitive impairment. In patients infected with HIV-1 for less than one year, significantly decreased resting-state cerebral blood flow (Ances et al., 2009) was reported using a relatively novel technique (i.e., arterial spin labeling magnetic resonance imaging). Both cortical and subcortical regions exhibited significantly diminished resting cerebral blood flow in HIV-infected participants compared to case-matched seronegative controls were observed, this suggests decreased metabolic activity and neuronal dysfunction. Further investigations in brain regions associated with various neurocognitive domains reveal significantly decreased resting cerebral blood flow in the lateral occipital cortex but not in other areas (e.g., executive control, motor, and auditory) (Wang et al., 2011). It is possible that disruptions in the occipital cortex may represent as a causal factor for impaired performance on neuropsychological tests requiring visual-motor coordination, though additional longitudinal investigations are required. These observations in decreased resting-state cerebral blood flow have also been made in patient populations infected with HIV-1 up to 100 days (Ragin et al., 2012, Ragin et al., 2015). In addition, Ragin et al., have also reported structural abnormalities in cortical and ventricular volume in these patients. Reduced cerebral cortex volume and swelling of the third ventricle may reflect general CNS specific disturbances or partially allude to various mechanisms of CNS penetration during HIV-1 pathogenesis. Changes in brain metabolite have also been noted within the first year of infection. Particularly in the frontal cortex, higher levels of choline and decreased levels of N-acetyl-aspartate, glutamate, and glutamine were observed (Lentz et al., 2011, Lentz et al., 2009). Since N-acetyl-
aspartate exists predominantly in neurons and correlates with the degree of neurocognitive dysfunction, it may indicate subclinical neurocognitive deficits. The severity of neurocognitive impairment may also be correlated with the degree of BBB breakdown, indicating downstream changes in brain physiology may be detected (Avison et al., 2004).

Neuroimaging studies are often invasive and include intravenous injection of tracers. Lumbar punctures may provide an alternative method of identifying markers of HAND progression. Compared to plasma, the CSF composition may provide additional information of cellular health within the CNS. An emerging CSF biomarker that demonstrates a high predictability of subclinical and clinical cognitive impairment is the light subunit of the neurofilament protein (NFL). NFL is a member of the intermediate filament protein family and is a major component the neuronal cytoskeleton. It is organised into thick filaments approximately 10nm in diameter and found predominantly in the axons and dendrites. By quantifying the level of NFL in the CSF in various neuropathologies, the extent of neuronal injury and monocyte activation could be estimated (McGuire et al., 2015, Norgren et al., 2005). Pertinent to HIV-1 infection, population-based studies have reported significantly increased CSF NFL levels that are reflective of cognitive status. For example, Jessen Krut (2014) recently reported in a cross-sectional study (n = 252), HIV-infected patients with dementia had significantly higher NFL concentrations compared to healthy controls (Jessen Krut et al., 2014). Other retrospective cross-sectional studies have also reported significantly higher levels of NFL in neurosymptomatic patients (Abdulle et al., 2007, Peterson et al., 2014). Furthermore, in a longitudinal Swedish study, significantly elevated CSF concentrations were found in HIV-infected patients who had developed dementia within two years (Gisslen et al., 2007). Notably, ARV treatment decreased NFL levels to baseline concentrations in approximately 50% of patients after 3 months, and almost 80% after 1 year of treatment (Mellgren et al., 2007). Levels of NFL may also correlate with extent of immune activation as in 33% of neuroasymptomatic patients enrolled, higher NFL was found predominately in individuals with low CD4+ cell counts (Abdulle et al., 2007, Jessen Krut et al., 2014). These studies highlight that evidence of neuronal injury continues to occur in patients despite the absence of clinical neurocognitive deficits. CSF concentrations of NFL may also become elevated early in infection, prior to the onset of neurological symptoms. Significantly elevated NFL was observed in a cross-sectional study involving treatment naïve HIV-infected patients with a mean duration of infection of 92 days (n = 82). These
studies suggest CSF levels of NFL may predict the development of neurocognitive deficits, however, clinical studies enrolling larger populations are required.

1.3 Pharmacotherapy of HIV-1 Infection in the Brain

In 1987, zidovudine became the first licensed ARV agent that marked the beginning of an intensive period of pharmacological research for the treatment of HIV. To date, there are currently six different classes of ARVs that target different phases of the HIV-1 life cycle including entry inhibitors (EIs), nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase inhibitor (INSTIs), protease inhibitors (PIs) and fusion inhibitors (FI) (Figure 3). In attempts to disrupt HIV-1 replication at multiple points in its life cycle, combinational therapy, otherwise known as highly active antiretroviral therapy (HAART) has become the standard form of care since 1996. Presently, an effective and specific treatment for HAND is not yet known as ARVs are ineffective in preventing patients from developing cognitive deficits (Sacktor et al., 2001). Nonetheless, the National Institute of Health published a set of guidelines that suggests preferred and alternative ARVs regimens for ARVs therapy-naïve patients (Table 2).

Viral load in the CSF is often used as a predictive measure of CNS infection and it has been documented at close to 900 copies/mL and 54,000 copies/mL in the CSF and plasma respectively in a sample of treatment naive patients (Marra et al., 2003). Fortunately, pharmacological adherence successfully suppresses systemic viral load to undetectable levels (i.e., < 50 viral RNA copies/ml of plasma, and CD4+ T-cells > 350 cells/mm³ in blood) (Thompson et al., 2010). As a result, decreases in opportunistic infections and mortality rates as well as increases in life expectancy of infected patients are observed (Johnson et al., 2013, Nakagawa et al., 2013). However, due to the presence of viral reservoirs established, in part, by blood-tissue barriers, viral eradication remains difficult.
Table 2 Recommended and Alternative Antiretroviral Regimen Options for antiretroviral Treatment-Naïve Patients. Adapted from Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents at http://aidsinfo.nih.gov/guidelines. PI based regiments are boosted with co-administration of Ritonavir

<table>
<thead>
<tr>
<th>Recommended Regimens</th>
<th>One of the following antiretroviral regimens</th>
<th>+ NRTI backbone</th>
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<tbody>
<tr>
<td>NNRTI based</td>
<td>efavirenz</td>
<td></td>
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<tr>
<td>PI based</td>
<td>atazanavir or darunavir</td>
<td>tenofovir disoproxil fumarate/emtricitabine</td>
</tr>
<tr>
<td>INSTI based</td>
<td>raltegravir</td>
<td></td>
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<tr>
<td></td>
<td>elvitegravir boosted with cobicistat</td>
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<tr>
<td></td>
<td>dolutegravir</td>
<td>abacavir/lamivudine</td>
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<tr>
<th>Alternative Regimens</th>
<th>One of the following antiretroviral regimens</th>
<th>+ NRTI backbone</th>
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</thead>
<tbody>
<tr>
<td>NNRTI based</td>
<td>rilpivirine</td>
<td>tenofovir disoproxil fumarate/emtricitabine or abacavir/lamivudine</td>
</tr>
<tr>
<td></td>
<td>efavirenz</td>
<td></td>
</tr>
<tr>
<td>PI based</td>
<td>atazanavir</td>
<td>abacavir/lamivudine</td>
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<td></td>
<td>darunavir</td>
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<tr>
<td></td>
<td>fosamprenavir</td>
<td>abacavir/lamivudine or tenofovir disoproxil fumarate/emtricitabine</td>
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<td></td>
<td>lopinavir</td>
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The prevalence of neurocognitive deficits have been increasing since HAART implementation. In recent reports, a paradigm shift that suggests subtle variations in cognitive impairments pre- and during HAART era has been suggested. Prior to the implementation of HAART, infected patients experienced more debilitating cognitive decline (e.g. motor dysfunctions, cognitive speed, and verbal fluency impairments) and was appropriately referred to as HIV-associated dementia. HAART appears to confer significant benefits in neuropathological features. In a recent retrospective study evaluating post-mortem brains, Bryant and colleagues report significantly less evidence of neurodegeneration in the mid-frontal cortex of patients receiving ARV compared to ARV-naïve (Bryant et al., 2015). Furthermore, HAART implementation may be delaying the progression into dementia as milder neurocognitive impairments (e.g. memory, learning and executive functioning deficits) are becoming more prevalent (Heaton et al., 2011). However, HAART administration does not appear capable of reversing or improving neurocognitive deficits. Some groups have reported
little to no improvements in these neurocognitive deficits in HAART-treated patients with undetectable peripheral viral loads (Heaton et al., 2010, Heaton et al., 2011, Schrier et al., 2015). This highlights the need for pharmacological interventions and additional monitoring mechanisms prior to neuronal apoptosis, which is an irreversible process. It was recently proposed that subclinical deficits may benefit from early ARV administration as improvements in learning were associated with increasing CD4 count (Ghate et al., 2015).

A major obstacle of HIV-1 pharmacotherapy in the CNS is achieving therapeutic ARV concentrations. Of the drugs known to date, lumbar puncture analyses in humans have revealed very low CSF drug concentrations compared to plasma indicating a low CNS permeability of ARVs (Table 3). In addition to the efflux transporters at the BBB and brain parenchymal cells, the CSF turn-over rate may also result in low CSF drug concentrations and contribute to low CNS penetration. This has been illustrated in an in vivo rodent model that injected the CSF with radiolabelled gp120 (l-gp120) (Banks and Kastin, 1998, Cashion et al., 1999). It was reported that only 27.8% of the administered dose remained in the CSF 120min after injection, indicating a half-life of 12.6min. Furthermore, the continuous production and flow of the CSF (i.e., approximately 350μl/min in humans representing a turnover rate of 0.4%/min), may prevent high concentrations of ARVs to establish in the CNS. Since a physiological gradient exists between the CSF and the brain (concentrations are usually higher in the tissue than CSF due to quick turn over), unrestricted diffusion results between the extracellular fluid of the brain into the CSF; molecules are then quickly eliminated due to CSF turnover causing a ‘sink effect’ as described by Hochwald et al., (Hochwald et al., 1976).
Table 3 Summary of ARV penetration into the cerebrospinal fluid in humans and brain tissue in animals compared to plasma levels. Adapted from Ashraf T et al., (2014) Curr Pharm Des.

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug Name</th>
<th>CSF : Plasma Ratio</th>
<th>Brain : Plasma % (animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRTIs</td>
<td>Zidovudine</td>
<td>0.02 (0-6.74)</td>
<td>16-25 (monkey)</td>
</tr>
<tr>
<td></td>
<td>Abacavir</td>
<td>0.35 (0.31-0.44)</td>
<td>21.6 ± 5.1 (guinea pig)</td>
</tr>
<tr>
<td></td>
<td>Lamivudine</td>
<td>0.23 (0-4.9)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Didanosine</td>
<td>0.16 (0.03-0.24)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Emtricitabine</td>
<td>0.26 (0.05-0.41)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Stavudine</td>
<td>0-0.20</td>
<td>34-62 ± 4-17 (rat)</td>
</tr>
<tr>
<td></td>
<td>Tenofovir</td>
<td>0.057 (0.004-0.84)</td>
<td>Negligible (rat)</td>
</tr>
<tr>
<td>NNRTIs</td>
<td>Nevirapine</td>
<td>0.63 (0.41-0.77)</td>
<td>45.6-59.4 ± 7.4-9.4 (guinea pig)</td>
</tr>
<tr>
<td></td>
<td>Etravirine</td>
<td>0.01 (0.005-0.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enfuvirtide</td>
<td></td>
<td>Negligible</td>
</tr>
<tr>
<td></td>
<td>Efavirenz</td>
<td>0.007 (0.003-0.011)</td>
<td>-</td>
</tr>
<tr>
<td>PIs</td>
<td>Indinavir</td>
<td>0.11 (0-0.47)</td>
<td>18 (rat)</td>
</tr>
<tr>
<td></td>
<td>Amprenavir</td>
<td>0.012 (0.008-0.018)</td>
<td>0.076 (rat)</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
<td>0.001-0.002</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>Below limit of detection</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Darunavir</td>
<td>0.009 (0.003-0.078)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>0.002 (0.001-0.005)</td>
<td>Not detectable (rat)</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>0.002 (0.001-0.008)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Atazanavir</td>
<td>0.008-0.02</td>
<td>-</td>
</tr>
<tr>
<td>EIs</td>
<td>Maraviroc</td>
<td>0.022 (0.004-0.17)</td>
<td>25 (rat)</td>
</tr>
<tr>
<td>INSTIs</td>
<td>Raltegravir</td>
<td>0.033-0.06 (0.01-0.54)</td>
<td>-</td>
</tr>
</tbody>
</table>

Since CSF concentrations are not indicative of whether the drug reached the active site, this gap in knowledge was addressed by a group at the University of California in San Diego led by Dr. Scott Letendre. He designed a more sophisticated system known as the CNS penetration effectiveness (CPE) scale (Letendre et al., 2008). This method was developed to quantify the relationship between ARV efficacy and CNS permeability (Table 4) by classifying various ARVs based on their ability to cross the BBB and control HIV replication (Letendre, 2011, Letendre et al., 2008). Unfortunately, conflicting reports have been published between the correlation of CPE scores and CSF viral load (Cross et al., 2013, Cusini et al., 2013, Smurzynski et al., 2011) or cognitive functioning (Garvey et al., 2011). This may result from investigational limitations such as degree of cognitive impairment of enrolled patients, medication adherence, clinical endpoint as well as racial background of the patient population. Genotypic differences have recently been shown in light of HIV-1 pharmacotherapy. Fabbiani and colleagues proposed a revised CPE score that integrates the genotypic susceptibility scores (GSS) of individual patients (Fabbiani et al., 2014). GSS is a numerical
value that is assigned based on the various polymorphisms that confer ARV drug resistance or decreases drug effectiveness (Anderson et al., 2008). Fabbiani and colleagues observed a higher correlation with neurocognitive performance in 215 patients using the CPE-GSS scale. However, additional efforts may be required to increase the robustness of the original CPE score. On the other hand, successful CNS penetration by antiretrovirals does not necessarily confer therapeutic benefit. High CPE scores have also shown adverse effects in patients, possibly due to toxic levels of antiretroviral compounds (Marra et al., 2009). For example, efavirenz, a benzoxamine NNRTI has been associated with psychiatric effects (e.g., mania, depression, suicidal thoughts, psychosis, and hallucinations) (Cavalcante et al., 2010) and other CNS toxicities (e.g., dizziness, irritability, headache, sleep disturbance) (Apostolova et al., 2015). Adverse side effects are not limited to efavirenz; mitochondrial toxicity has been commonly associated with NRTI’s (Schweinsburg et al., 2005). Therefore, safer adjuvant therapies are required for HIV-1 pharmacotherapy in the CNS.

Table 4 Central Nervous System Penetration Effectiveness Scale (CPE). Larger CPE scores reflect estimates of better penetration or effectiveness in the central nervous system (e.g., ranking of 4 indicates best penetration or effectiveness; /r indicates ritonavir-boosted). Adapted from Letendre S. (2011) Top Antivir Med.

<table>
<thead>
<tr>
<th>Drug Class</th>
<th>4</th>
<th>3</th>
<th>2</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoside Reverse Transcriptase Inhibitors</td>
<td>Zidovudine</td>
<td>Abacavir</td>
<td>Etravirine</td>
<td>Tenofovir</td>
</tr>
<tr>
<td></td>
<td>Emtricitabine</td>
<td>Didanosine</td>
<td>Zalcitabine</td>
<td></td>
</tr>
<tr>
<td>Nonnucleoside Reverse Transcriptase Inhibitors</td>
<td>Nevirapine</td>
<td>Delavirdine</td>
<td>Efavirenz</td>
<td></td>
</tr>
<tr>
<td>Protease Inhibitors</td>
<td>Indinavir/r</td>
<td>Darunavir/r</td>
<td>Atazanavir/r</td>
<td>Nelfinavir</td>
</tr>
<tr>
<td></td>
<td>Darunavir/r</td>
<td>Atazanavir/r</td>
<td>Atazanavir/r</td>
<td>Ritonavir</td>
</tr>
<tr>
<td></td>
<td>Fosamprenavir/r</td>
<td>Atazanavir/r</td>
<td>Saquinavir/r</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indinavir</td>
<td>Lopinavir/r</td>
<td>Lopinavir/r</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lopinavir/r</td>
<td>Fosamprenavir</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fosamprenavir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saquinavir/r</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saquinavir/r</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tipranavir/r</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maraviroc</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enfuvirtide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrase Strand Transfer Inhibitors</td>
<td>Raltegravir</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.1 Adjuvant Anti-Inflammatory Therapy

Most ARVs currently prescribed for HIV-1 therapy do not exhibit anti-inflammatory properties such as maraviroc and NRTIs (Fowler et al., 2014), however, a majority of them demonstrate poor brain permeability. Thus, a number of safer anti-inflammatoryities and/or anti-oxidant compounds have been explored as potential adjuvants that can be administered alongside accepted ARVs. These compounds exhibit higher brain permeability and are thought to exert therapeutic action by interacting with signaling cascades involved in the regulation of cytokine secretion. Downstream neurotoxic and cognitive dysfunctions may be prevented by limiting the expression of inflammatory mediators. There are several adjuvant compounds under investigation as reviewed by McGuire JD et al. (McGuire et al., 2014), we focus on minocycline and chloroquine.

1.3.1.1 Minocycline

Minocycline is a second generation tetracycline derivative known to elicit neuroprotective effects and its use in HIV cognitive-related diseases has been previously examined. Three publications have supported the potential of minocycline in attenuating neuroinflammation using an SIV model. A group at Baltimore, USA reported that 4mg/kg P.O. q.d x 2d of minocycline could significantly reduce encephalitis in SIV-infected pigtailed macaques (Zink et al., 2005). This study reported suppressed CNS viral load and decreased several CNS inflammatory markers (e.g. macrophage marker CD68, major histocompatibility complex class II, T-cell intracytoplasmic antigen 1, SIV glycoprotein 41, β-amyloid precursor protein, and activated p38) upon minocycline treatment. In a separate publication, this group further observed decreased low striatal dopamine levels that were corrected upon minocycline administration (Meulendyke et al., 2012). Another group in Boston evaluated neuroprotective potentials of minocycline by examining neuronal integrity in SIV-infected rhesus macaques. Using proton magnetic resonance spectroscopy in vivo and biomarkers in post-mortem tissues, it was reported that minocycline could attenuate a progressive decline in neuronal integrity, decrease glial activation and CSF and plasma viral loads (Ratai et al., 2010). This study was recently followed up with SIV-infected rhesus macaques showing decreased plasma virus and pro-inflammatory monocytes and reduced monocyte/macrophages recruitment to the CNS (Campbell et al., 2011). Unfortunately, despite the encouraging results in animal models, a recent clinical trial did not demonstrate significant effect in decreasing CSF viral load in seropositive patients (Ho et al., 2011) or improving neuropsychological test composite z-scores (Nakasujja et al., 2013). Sacktor and colleagues corroborated these findings in a sample of 107 HIV-1-infected individuals with cognitive
impairment, reporting the lack neurocognitive improvement and changes in CSF biomarkers for oxidative stress, neuronal injury, and inflammation (Sacktor et al., 2011, Sacktor et al., 2014). This discordance between animal models and humans may be time associated; the patients enrolled in the clinical trials were in late stages of infection. Therefore, it is possible that prophylactic treatment with anti-inflammatories may be more effective.

1.3.1.2 Chloroquine

Chloroquine is a widely used anti-malarial compound with anti-inflammatory properties that may confer therapeutic effects to HIV-infected individuals (Murray et al., 2010). Clinical trials involving chloroquine have reported mixed results. Anti-HIV effects of chloroquine were initially reported in an American population (n = 40) that showed significant decline of HIV RNA in plasma over an 8-week observational period (Sperber et al., 1995). This reduction in systemic immune activation was later replicated in thirteen chronically infected HIV individuals enrolled in a double-blind randomized placebo-controlled trial in the US (Murray et al., 2010) as well as in a European population (n = 20) of HIV-infected immunologic non-responders (Piconi et al., 2011). However, these encouraging results could not be replicated in a larger adult population (n = 83) from the United Kingdom (Paton et al., 2012), a paediatric population (n = 55) from Thailand (Engchanil et al., 2006) or in a Canadian adult population (n = 19) receiving ARVs (Routy et al., 2014). It is unclear whether chloroquine can confer therapeutic effects in the systemic circulation and its effect in the CNS in reversing cognitive deficits have not yet been explored. Similarly, in SIV-rhesus macaque disease model, it remains unclear whether chloroquine can confer anti-HIV effects and elicit a therapeutic response (Ma et al., 2012, Vaccari et al., 2014). In brief, chloroquine’s clinical application as an HIV adjuvant remains controversial, despite encouraging results in in vivo rodent models and cell systems (Hagihara et al., 2000, Hong et al., 2004, Naarding et al., 2007, Royle et al., 2013).

1.4 Gram Negative Bacterial Endotoxin Lipopolysaccharide in HIV-1 Infection

Lipopolysaccharide (LPS) is a ubiquitously expressed endotoxin composed of lipids and sugar side-chains found on the outer cell wall of gram-negative bacteria. It is composed of three major molecules including a lipid moiety (i.e., lipid A), covalently bonded to a polysaccharide core (i.e., inner and outer core), and a variable O-polysaccharide chain. Lipid A is anchored to the outer leaflet of the bacterial cell wall and is recognised as the key immunogenic component. Proximal to lipid A is
the inner core of the polysaccharide domain composed of various uncommon sugar molecules (i.e., 3-deoxy-D-manno-octulosonic acid and L-glycero-D-manno heptose) whereas the outer core contains common hexose and hexosamines. The O-polysaccharide chain varies according to the strain of gram negative bacteria and is composed of repeating carbohydrate subunits, typically hexose sugars. Gram negative bacteria (as well as gram positive bacteria) are commonly found in the human body, establishing a microbiota on the surface and deep layers of skin and mucosal tissues (e.g., gastrointestinal (GI) tract). In the gut of healthy individuals, commensal bacteria do not elicit an inflammatory response as they are restricted to the lumen by an intact lining of intestinal epithelium. However in disease states, circulating LPS (i.e., lipid A) is recognized by LPS binding protein that brings it to CD14, a membrane receptor that is in close proximity to an ‘activation cluster’ containing the toll-like receptor (TLR) 4 on the cell surface. Upon binding to TLR4, myeloid differentiation primary response protein 88 (MyD88) dependent or independent pathway ultimately activates nuclear factor κB (NFκB) through the degradation of its inhibitor, IκB, and results in the increased production of pro-inflammatory cytokines, chemokines and other factors associated with the adaptive immune response (Triantafilou and Triantafilou, 2002). Additional signaling pathways including the MAPK p38 kinases have been implicated. TLRs are a family of type I transmembrane signaling receptors that recognize pathogen-associated molecular patterns such as LPS and bacterial DNA (i.e., 16s rDNA). To date, ten TLRs (TLR1 – TLR10) have been identified in humans, and twelve in murine.

Since the intestinal immune system hosts a large population of CD4+CCR5+ T cells, it serves as a major target of HIV-1 and AIDS pathogenesis (Lackner et al., 2009). Upon HIV-1 infection, intestinal CD4+ T-cells are depleted more rapidly than peripheral blood and do not appear to recover despite adherence to ARV therapy (Mehandru et al., 2004). In HIV-1 infected individuals, bacteria are able to translocate from the GI lumen into the peripheral circulation and contribute to systemic immune activation. Studies have shown significantly elevated levels of LPS and bacterial 16S rDNA in plasma from HIV-1 infected patients (Jiang et al., 2009, Vassallo et al., 2012) as well as increased levels of soluble CD14, a marker of LPS-induced monocyte and macrophage activation, and pro-inflammatory cytokines such as TNF and IL-1 (Brenchley et al., 2006, Jiang et al., 2009). Commensal bacterial products may leak into circulation due to a compromised intestinal epithelium as well as decreased regulation of enteric microbes due to HIV-1 infection (Shan and Siliciano, 2014). Active viral replication in the intestines causes the production of cytokines that may disturb the ionic balance
within enterocytes as well as changes in the cellular scaffolding such as tubulin depolymerisation or increased pore-formation (Brenchley and Douek, 2008, Epple et al., 2009). In addition, a subset of helper T-cells known as Th17 cells which regulates enteric microbiota through the expression of cytokines are preferentially depleted during HIV-1 infection (Ancuta et al., 2010, Fevrier et al., 2011). These aberrant changes in cellular structure and function results in microbial translocation into the systemic circulation.

Systemic infection may exacerbate neuroinflammation during HIV-1 infection (Perry et al., 2007). It has been shown in vivo that LPS minimally penetrates the intact rodent BBB wherein approximately 0.025% of the intravenously administered dose of radiolabelled LPS with iodine (i.e., I-LPS) was taken up into the brain (Banks and Robinson, 2010). This suggests that receptors on the luminal membrane of BMECs may activate intracellular signaling mechanisms in order to release cytokines/chemokines and soluble factors at the abluminal surface (Dohgu and Banks, 2013, Verma et al., 2006, Wang et al., 2008). It has been shown that circulating LPS enhances monocyte trafficking into the CNS via IL-6 and granulocyte-macrophage colony-stimulating factor (Dohgu et al., 2011, Vassallo et al., 2013, Wang et al., 2008). In addition, brain pericytes may increase LPS transcytosis across the BBB, thus furthering neuroinflammation and aiding the progression of neurocognitive impairments (Dohgu and Banks, 2013). Microglial cells are highly susceptible to LPS activation due to the expression of TLR4 on the cell surface (Lehnardt et al., 2002). It has been shown in vitro that TLR4 activates the intracellular signaling pathway involving myeloid differentiation primary response protein 88 (MyD88) which induces microglial cells to secrete soluble factors (e.g., cytokines, chemokines, and chemotaxic molecules) that are directly neurotoxic to neurons (Dean et al., 2010). Circulating levels of LPS may also be correlated to the severity of neurological impairments as significantly higher levels of serum LPS (i.e., greater than 79pg/mL) have been documented in HIV-1 patients who developed dementia, compared to patients with milder neurocognitive deficits (Ancuta et al., 2008). This suggests that LPS plays a critical role in contributing to systemic immune activation as well as exacerbating neuroinflammation during HIV-1 infection.

1.5 Spatial and Contextual Learning in Small Rodents

There are various tasks that are designed to evaluate neurocognitive functioning in small rodents including motor coordination tasks (i.e., open field tests (Abraham et al., 2008, Bjugstad et al.,
spatial learning and memory in land or water-based mazes (i.e., radial maze (Li et al., 2004), T-maze foot shock (Farr et al., 2002), Morris water maze (Glowa et al., 1992)) as well as fear conditioning using auditory or contextual cues (i.e., contextual fear testing (Pugh et al., 2000)). Notably, neuropsychiatric conditions (e.g., social interactions, anxiety-like and depressive-like behaviors) can also be modeled using behavioural tasks listed above (Barak et al., 2002b, Sanchez-Alavez et al., 2000). Rodent models are cost-efficient and easily implementable methods of modeling human behaviour, however these behavioural tasks often require specific equipment, apparatuses, methodologies and measurements. Since the neurobiological basis of learning and memory is inherently complex due to the various inputs that contribute to memory formation (i.e., visual, auditory, olfactory, etc.), we will focus specifically on spatial memory in the Morris Water Maze (MWM) task as used in this study.

Prior to implementing a behavioural task, there are various parameters of experimental design that require careful consideration due to high inter-subject variation as well as high sensitivity to external conditions (i.e., room, temperature, presence of different experimenters). In a recent publication by Hanell and Marklund, they discuss some of the key aspects to consider in rodent behavioural tests (Table 4) such as the source of motivation and variability, impact of experimenter-rodents interactions, functionality of rodent sensory inputs required to complete the task as well as costs and practical logistics (Hanell and Marklund, 2014). It has been shown, and well accepted, that laboratory settings influence rodent behaviour resulting in variable findings depending on experimenter, equipment set-up, and animal housing (Chesler et al., 2002, Crabbe et al., 1999, Richter et al., 2010). Novel rodent behavioural tasks are under development to achieve robust and reproducible results by relying more on automated processes or computer-controlled measurements. Currently, selecting the most suitable behavioural task to date is highly project-dependent. In learning and memory projects, the ability of the rodent to complete the task is used as a measure of neurocognitive function. For example, in the MWM the accurate identification of a target over time is indicative of learning. However, a key aspect of completing a given task is motivation; this can appear in many forms including fear (i.e., fear of drowning, falling, or being shocked), hunger (i.e., positive reinforcement for food deprived animals) or inherent curiosity of rodents (i.e., exploration of novel environment) (Hanell and Marklund, 2014). Depending on the level of motivation, rodents may complete the task quicker than anticipated, or not at all, thereby
skewing the findings. This can be minimized by optimizing water temperature as in the MWM or increasing the shock voltage as in fear conditioning chambers.

It is also known that rodents are highly sensitive to olfactory inputs thus inconsistent environments represents a fundamental source of variation in baseline response. This may include the sex of experimenter (i.e., presence of male, but not females, may induce stress and analgesia in rats (Sorge et al., 2014)), level of comfort of the experimenter in working with rodents (van Driel and Talling, 2005), and potentially remaining trace odors from household pets. Accordingly, it is reasonable to allow one experimenter perform the entire behavioural experiment. While automated testing and computerised systems can potentially decrease variability associated with data collection/measurements, the choice of subject (i.e., mice or rats) and their breeding origins (i.e., in-bred or out-bred) is also an important aspect to consider. It is reasonable to attribute greater inter-animal variability in out-bred animals due to genetic diversity, however, in-bred animals may also exhibit micro-genetic differences such as polymorphic copy numbers, degree of methylation, and presence of mini-satellite regions (Lathe, 2004). Choice of species is often dependent on the choice of the behavioural task and will be discussed in the context of the MWM and fear conditioning experiments used in our studies. Validity of the experiment inherent to the behavioural task should be acknowledged in interpreting findings, such as those described in Table 5.
Table 5 Key points to consider when evaluating a behavioural test in rodents. Adapted and modified from Anders Hånell and Niklas Marklund (2014).

<table>
<thead>
<tr>
<th>Motivation</th>
<th>How are the animals motivated to perform the task? Is the level of motivation high and stable? Can the source of the motivation interfere with the disease model?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal-Experimenter Interaction</td>
<td>Are the experimenter and the animal in close contact during testing? Can handling be performed prior to testing to allow the animals to adjust to human contact?</td>
</tr>
<tr>
<td>Dynamic Range</td>
<td>Can the test accurately measure the ability of both naïve and severely impaired animals? Is there a risk for flooring or ceiling effects? Can the test difficulty be adjusted within or between trials?</td>
</tr>
<tr>
<td>Repeatability</td>
<td>Can the test be repeated to assess changes in ability over time?</td>
</tr>
<tr>
<td>Data Collection</td>
<td>How are the results collected? Is there a risk for subjective effects in the evaluation? Can the results be collected automatically?</td>
</tr>
<tr>
<td>Results Evaluation &amp; Interpretation</td>
<td>Which statistical tests can be used to evaluate the results? How are the results presented? Can the results be attributed to a single domain or can, for example, changes in general activity level interfere with the measurements?</td>
</tr>
<tr>
<td>Automation</td>
<td>How much of the test procedure is automated? Does the test have the potential to be fully automated?</td>
</tr>
<tr>
<td>Variability</td>
<td>Can the results be related to baseline performance to mitigate the effects of variability? Can the estrous cycle of female animals be measured to restrict testing to a single day in the cycle?</td>
</tr>
<tr>
<td>Experimental design</td>
<td>Can the test be performed in a blinded fashion? Can the test be used for both mice and rats of different strains to obtain more robust results?</td>
</tr>
<tr>
<td>Sensory modality</td>
<td>Which sensory modality does the animal use to solve the task? Is it possible to confirm intact sensory functions?</td>
</tr>
<tr>
<td>Validity (Ethological/face/intrinsic/extrinsic)</td>
<td>Does the test resemble natural rodent behavior? Is it immediately apparent what the test is intended to measure? Does the test give the same result when experiments are repeated? Does the test give the same result when performed in, for example, different strains, age group or species?</td>
</tr>
<tr>
<td>Throughput</td>
<td>How long does it take to run the test? Do the animals have to be trained before performing the test? Can several animals be tested in parallel? Is the collection and evaluation of the results time consuming?</td>
</tr>
<tr>
<td>Costs</td>
<td>How much does the equipment cost? How much lab space has to be devoted to the test? How much staff time does the test require?</td>
</tr>
<tr>
<td>Practical considerations</td>
<td>Can the equipment be stored away when not in use? Is extensive training of the experimenter required to carry out the test? Do the tests have to be performed on several consecutive days which may overlap with weekends, holidays and vacations? Can the test be run by a substitute in case of sick leave? Can the test equipment be easily cleaned and disinfected?</td>
</tr>
</tbody>
</table>

1.5.1 Spatial Learning in the Morris Water Maze

In 1981, British neuroscientist Richard G. Morris proposed the MWM as an alternative method of examining navigational strategies employed by rats. It offers several advantages over traditional dry-land maze tasks (e.g., radial arm maze, T-maze) to acutely discern memory-based navigation in
space. In dry-land mazes, non-spatial memory formation may occur through external olfactory cues (e.g., rat scent trails) and/or direction of exploration (e.g., maze arms form alleyway), both potential confounding factors that are absent in the MWM (Morris, 1984). Since then, the relative simplicity of the MWM has allowed it to become, perhaps, the most commonly employed protocol in behavioural neuroscience. In brief, this navigational task motivates rodents via fear of drowning to identify a platform located at or below the surface of an opaque pool of water given spatial cues, both locally and distally. A typical set up is shown in figure 6. The pool is often divided into various quadrants for analysis.

The water, rendered opaque via milk powder or non-toxic paint, should be kept at an optimized temperature for mild discomfort to encourage exploration, rather than floating. Notably, cognitive functioning is hampered by stress (Conrad, 2010), thus it is beneficial to further decrease hypothermia risk by increasing inter-trial resting time and retrieval trials (Livonen et al., 2003). Successful memory acquisition requires multiple retrieval trials over several consecutive days (e.g., four trials per day for 5 days). While there are a vast number of ways to design a training protocol, studies have shown learning at a slower pace for a longer time allows animals to learn more effectively (Commins et al., 2003, Rick et al., 1996, Sisti et al., 2007). At first, a search strategy is employed to contact the platform by chance wherein subsequent retrieval trials encourage successful acquisition and consolidation. This is evaluated by the subjects’ ability to identify the
platform, quickly and directly, from any point of the pool circumference. The final retrieval task is modified by removing the platform altogether to observe spatial bias as an indicator of memory retention. Several different quantifications can be used to interpret cognitive functioning including latency time (i.e., time required to locate the platform), swim distance and/or speed, swim path and thigmotaxis behaviour. Thigmotaxis is a behavioural tendency of rodents to remain close to the periphery/walls of the environment, which may complicate findings, and can be reduced depending on species and strain. Equal exploration during the probe test is often interpreted as impairments in memory consolidation or retrieval, but could also indicate differential learning patterns. Pertinent to spatial learning, two coding systems have been proposed: utilisation of distal (i.e., allocentric navigation) or internal cues (i.e., egocentric navigation) (Vorhees and Williams, 2014). Egocentric navigation employs the navigator as the point of reference acquiring and retaining all external cues relative to the navigator; whereas allocentric navigation requires only the spatial arrangement of external cues and is irrelevant of the navigators’ position. Cunningham and Sanderson suggested allocentric learning results in a preference for the target quadrant whereas egocentric learning leads to equal exploration in the pool (Cunningham and Sanderson, 2008). Thigmotaxic analysis can also indicate that rats are employing a stress-minimising strategy rather than failure to complete the maze (Simon et al., 1994, Treit and Fundytus, 1988). Using these navigational strategies, its proposed that a ‘concept map’ is constructed, thereby, allowing the rats to identify the target platform (O'Keefe and Nadel, 1978). The neurobiological basis of this concept map was significantly substantiated by the discovery of place cells in 1971 by J. O'Keefe and J. Dostrovsky (O'Keefe and Dostrovsky, 1971). Place cells are hippocampal pyramidal neurons that are specifically activated depending on the location of the animal in the environment. Recently, time cells provide a secondary dimension to which are hippocampal neurons that activate at specific moments of a conditioned response (Eichenbaum, 2014). The synergistic effect of space- and time-specific neuronal activation allows the animal to construct a ‘cognitive map’ of the environment and may provide the molecular basis of visual memory.

The MWM task was initially designed for use with rats who are naturally strong swimmers. Comparisons with mice revealed that rats displayed more stable trial-to-trial performance, exhibit less floating time in the water and show stronger exploratory abilities by exhibiting less thigmotaxis (D’Hooge et al., 1999). Furthermore, profound strain-related differences in cognitive performance have been documented in a study involving four unique rat strains in four separate behavioural
domains (van der Staay and Blokland, 1996) as well as sex-related differences in performance wherein male rats, who are more territorial (i.e., implying superior spatial retention abilities), appear to benefit from higher levels of testosterone over female rats; this advantage was reversed by testosterone treatment in female rats (Roof, 1993, Terry, 2009). Thus, in efforts to minimize experimental variations when employing the MWM, several subject characteristics (e.g., strain, age and stress) and equipment-related parameters (e.g., environment, training protocol, and quantification) require thorough consideration and optimisation.

1.6 Role of the Hippocampus and Cortical Regions in Visual Learning and Memory

Franz Joseph Gall’s doctrine of localization was among the earlier studies of neuropsychology. He described that specific regions of the brain supported specific cognitive functioning which, when used more extensively, would exert significant pressure against the skull causing abnormalities in the shape and size of the head (Zola-Morgan, 1995). In contrast, Karl Lashley later discovered that memories were not localised to a specific region of the brain, but were distributed throughout the cortex suggesting that memory loss is a function of the amount, not location, of tissue damage (Bruce, 2001). A Canadian student of Lashley conceptualised that formation of long-term memory occurs from repeated activation of the same synapses which results in structural changes whereas short-term memory is mediated by temporary synaptic activity (Brown and Milner, 2003). To date, neuroscientists view memory formation as a complicated neural network that integrates several regions of the brain. However, the hippocampus is believed to play a crucial role due to a case study of an epileptic patient who received surgical resection of the medial temporal lobe containing the hippocampus and could no longer form new memories nor remember events that occurred shortly before surgery (Scoville and Milner, 2000). Since this report, animal models have provided strong evidence indicating the hippocampus is a major region for memory formation, particularly in spatial and contextual learning. In order to evaluate the role of the hippocampus, lesions using electrolytic or excitotoxic effects before (anterograde damage) or after (retrograde damage) behavioural conditioning have been employed in rodents. Retrograde (and to a lesser extent anterograde) damage have been shown to impair contextual fear conditioning, highlighting the hippocampus as a vital region (Anagnostaras et al., 1999, Frankland et al., 1998, Kim and Fanselow, 1992, Maren et al., 1997). Furthermore, in fear conditioning studies, it appears that hippocampal lesions could independently impair contextual fear conditioning, without affecting auditory cued fear (Blanchard and Fial, 1968, Kim and Fanselow, 1992, Phillips and LeDoux, 1992). In the MWM, rats with lesions
of the hippocampus were unable to acquire the hidden platform, but, could accurately identify a visible platform (i.e., platform above the surface of the water) possibly due to visual acuity and instinctive behaviours (Broadbent et al., 2004, Leggio et al., 2006). In addition performance in other navigation-based tasks were severely impaired including dry-land mazes (Ramos, 2013), visual recognition tasks (Clark et al., 2000) and other spatial assessments (Dudchenko et al., 2000).

Notably, the hippocampus is not solely responsible for successful visual conditioning. Parahippocampal regions such as the perirhinal and postrhinal cortices appear to play a significant role in contextual and spatial learning. It was reported that individual and combined damage to the parahippocampal cortices significantly and robustly impaired contextual conditioning but not spatial learning in the MWM (Burwell et al., 2004). Furthermore, the striatum appears to play a role in selecting which spatial navigation strategies is employed. Rats with caudate-putamen lesions exhibited a significantly different pattern of behaviour when exposed to the MWM including higher thigmotaxis-like behaviour (i.e., swimming in large circles), decreased motor functioning and failure of identifying the removed platform (Block et al., 1993, Whishaw et al., 1987). In order for spatial memory formation, both the hippocampus and striatum require reciprocal communications with the cortex, an area known for planning and processing information. Severe impairments of the MWM were observed with lesions to the prefrontal cortex, but not the parietal cortex (Mogensen et al., 1995) and damage to the anterior cingulate cortex produced only a mild acquisition deficit whereas no behavioural impairments were observed with lesions to the posterior cingulate cortex (Warburton et al., 1998). Other regions including the basal forebrain and cerebellum have also been implicated in poor MWM performance, highlighting the synergistic effect of the brain.

1.7 Neuroinflammation and Neurocognitive Impairments

Inflammation-induced neurotoxicity has been shown in vitro as well as in vivo. In mice, spatial memory impairments were observed upon IL-1β administration, regardless of administration route or origins of IL-1b (Gibertini et al., 1995, Oitzl et al., 1993). For example, in one study, C57BL/6 mice were intraperitoneally injected with 100ng of exogenous IL-1β or forced to produce endogenous IL-1b via exposure to gram-negative bacterium. Both methods of IL-1β exposure rendered the mouse incapable of acquisition or retention (Gibertini et al., 1995). Furthermore, direct injection of IL-1b into the rat CNS (i.e., intracerebroventricular injection) disturbed memory processes of acquisition and retention as they required significantly more time to locate the platform (Oitzl et al., 1993).
However, behavioural neuroscience is often faced with low reproducibility owing to the sensitivity of the experiment and variability of the subjects. It is not surprising to see that some groups have reported daily administration of IL-1β results in no effect in spatial learning in mice (Lacosta et al., 1999) and, in other cases, even enhances spatial acquisition and retention (Gibertini, 1998, Goshen et al., 2007, Song et al., 2003). On the other hand, not only is IL-1β potentially associated with inducing cognitive impairments, daily TNFα administration to rats (Bjugstad et al., 1998) or overexpression of TNFα in the CNS of transgenic mice (Aloe et al., 1999, Fiore et al., 1996) have also been reported to impair spatial learning and memory. Studies have also shown direct LPS injection into the CNS can activate immune-related pathways (e.g., MAPK, NFκB, and COX-2) and increase the expression of inflammatory cytokines (e.g., TNFα, IL-1β); in these studies, acute intracerebroventricular (icv) of 50μg LPS impaired spatial navigation 10-13 days after injection in rats, possibly through unsuccessful memory consolidation (Guo et al., 2010, Li et al., 2011). At the cellular level, icv administration of up to 50μg LPS, significantly activated caspase-3 immunoreactive neurons in the cortex 24-72 hour post injection in Sprague–Dawley rats (Song et al., 2014).

In models of inflammation, adult rodents are favoured over juvenile rodents for several reasons including hormonal stability and ease of handling. Rats may also be a more favourable model as it has also been shown that inflammatory conditions induced by exogenous agents could not be established in adult mice. In this publication that compared the effects of age it was reported that in-bred male BALB/c adult mice (3-6 months; 13-26 week) did not show significant up-regulation of pro-inflammatory cytokine mRNA (IL-1β, IL-6) 24 hours after chronic HIV-1<sub>SF162</sub> gp120 icv injections compared to saline-treated mice; however, in aged mice (22-24 months) significant fold-changes (4-700%) in transcripts were observed (Abraham et al., 2008). Notably, mouse models of other neuroinflammatory disorders have been implemented and established such as the transgenic mice models of Alzheimer’s Disease and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced mice model of Parkinson’s Disease (Heneka et al., 2014).

1.7.1 HIV-1 gp120-Induced Spatial Deficits

HIV-1 gp120 has been demonstrated to induce inflammation and cause behaviour abnormalities in rodents. We have extensively characterised a robust inflammatory response generated by gp120 in primary cultures of astrocytes (Ronaldson and Bendayan, 2006) and in an in vivo rat model using icv administration of HIV-1 gp120 (Ashraf et al., 2014a). In our in vivo rat model, we observed the
highest expression of IL-1β and TNFα in the hippocampus when compared to the frontal cortex and striatum of control rats (Ashraf et al., 2014a, Pu et al., 2003). In addition to our findings, direct and indirect neurotoxicity have also been demonstrated by exposure to viral protein, gp120. For example, in cell systems co-culturing cortical brain neurons with HIV-1 infected macrophages resulted in the release of numerous toxins detrimental to neuronal health (Giulian et al., 1993). In addition, in primary mice cortical neuronal cultures, gp120-induced neurotoxicity occurred through RNA-activated protein kinase, a stress kinase (Alirezaei et al., 2007). In another study using a similar model, repeated icv administration of gp120 at 100ng for 3 or 7 days resulted in significant caspase-3 activation and neuronal apoptosis in the cerebral cortex (Acquas et al., 2004).

Furthermore, an SV-40 derived vector expressing gp120 injected into the rat caudate-putamen led to chronic apoptosis of microglia and neurons, secretion of MIP-1α and an increase in oxidative stress (Louboutin et al., 2010b). These in vitro and in vivo studies highlight viral coat glycoprotein, gp120, as a key mediator of neuroinflammation and caspase-3 dependent neurotoxicity. In addition, HIV-1 proteins appear to induce robust inflammatory response in regions highly involved in learning and memory paradigms (i.e., frontal cortex, striatum and hippocampus). Using the MWM task, a highly hippocampus-dependent test, several groups have documented significant memory impairments due to central administration of HIV-1 in rodents (Harezlak et al., 2011, Masliah et al., 2004, Pu et al., 2003). Following icv administration of gp120, significant changes in cognition and behaviour such as spatial memory (Glowa et al., 1992, Tang et al., 2009), locomotion (Abraham et al., 2008, Barak et al., 2002a, Barak et al., 2002b, Bjugstad et al., 2004), contextual fear (Farr et al., 2002) and other behavioural assessments were observed. Furthermore, significant neuronal loss in the brain were detected in gp120-treated rats peaking at 24 hours post injection (Louboutin et al., 2007), particularly in the cerebral cortex (Acquas et al., 2004, Bagetta et al., 1995). These studies highlight gp120 as a key mediator of the inflammatory response, associated cognitive changes and, potentially, neuronal death.

1.8 Overall Goal

The goal of this project was to implement an in vivo rodent model of HIV-associated brain inflammation and neurocognitive deficits using a single icv administration of a high dose of HIV-1 gp120 in both lateral ventricles in rats.
1.9 Rationale

HIV-infection is commonly found in association with chronic and uncontrolled inflammation in the CNS leading to neurocognitive deficits as well as motor dysfunctions (Kaul and Lipton, 2006). Irreversible neuronal injury and loss can be caused by the collective insult of inflammatory mediators and oxidative stress present in the systemic circulation that cross the BBB and/or mediators secreted by brain glial cells. Our laboratory has investigated the efficacy of anti-inflammatory agents (minocycline, chloroquine, simvastatin) in reversing this inflammatory response through the MAPK/NFkB pathway, but their effects on reversing or delaying cognitive impairments (learning/memory) is not yet known. Continuing with our recently implemented in vivo model of HIV-1-induced neuroinflammation by icv administration of HIV-1 gp120 in rats, we would like to further this research to examine behavioural changes in spatial learning and memory. Using the rat model developed and optimised herein, future work could aim to evaluate the efficacy of anti-inflammatory agents in reversing memory and learning impairments post gp120-induced brain inflammation.

1.10 Hypothesis

We hypothesize that HIV-1 gp120 associated inflammation and oxidative stress in rodent CNS result in neuronal death which ultimately leads to neurocognitive deficits.

1.11 Specific Objectives

i. To characterize HIV-1 gp120ADA and LPS associated neurocognitive deficits (learning/memory) in rats by examining the latency to escape and enter the target quadrant in the MWM

ii. To characterize the brain inflammatory response following icv administration of HIV-1 gp120 and LPS by measuring levels of pro-inflammatory cytokines (Il-1β, TNFα) and oxidative stress markers (iNOS) in several brain regions in rodents

iii. To characterise neuronal death due to HIV-1 gp120ADA and LPS administration in the hippocampus of rodents by quantifying and localizing activated caspase-3 expression
CHAPTER 2: HIV-1 ENVELOPE GLYCOPROTEIN 120 INDUCED INFLAMMATION DOES NOT RESULT IN NEUROCOGNITIVE IMPAIRMENT IN A RAT MODEL

This work is in preparation for Journal Submission.

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Conducted Experiments and Data Analyses:

Kao ACC (behavioural work, qPCR, immunoblotting), Wu Chiping (animal surgeries and dissections), Hoque MT (western blotting), Bendayan R (overall design of experimental plan and overview of data analysis and interpretation)

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Kao ACC (draft, submissions), Hoque MT and Henderson JT (editorial review of the manuscript), Bendayan R (overall guidance for the preparation of the manuscript, editorial review of the many drafts of the manuscript)
2.1 Abstract

Background: The prevalence of neurological complications in Human Immunodeficiency Virus-1 (HIV-1) infected patients is increasing dramatically with 18-50% of patients developing HIV-associated neurocognitive disorders (HAND). Symptomatically, these patients appear to be aging prematurely, possibly due to persistent low-level viral replication and associated brain inflammation leading to tissue damage and neuronal loss. The goal of this project was to develop an in vivo rat model of brain inflammation manifesting neurocognitive deficits in spatial acquisition and retention by intracerebroventricular (icv) administration of HIV-1 viral coat glycoprotein (gp120) or lipopolysaccharide (LPS) in adult male Wistar rats. Methods: Anesthetized rats were administered a single high dose of HIV-1 gp120_{ADA} in the lateral ventricles (4μg/ventricle) or LPS (25μg/ventricle). Real-time qPCR was used to assess gene expression of inflammatory/oxidative stress markers in the hippocampus, frontal cortex, and striatum. Cognitive deficits in spatial learning and memory were characterised using the Morris Water Maze (MWM) test. Expression of activated caspase-3 was determined through immunoblotting and immunofluorescence. Results: Our data demonstrated that administration of LPS significantly impaired spatial learning/memory compared to saline injected controls (p < 0.001; n = 12-16), while gp120_{ADA} treatment did not result in significant cognitive delay. Consistent with our behavioural observations, high levels of cytokines (IL-1β and TNFα) and oxidative stress (iNOS) transcripts were detected in the brain regions at 6hr, 24hr, and 6 days after administration of gp120 or LPS. In addition, significant activation of caspase-3 was observed in rats treated with LPS, but not with gp120_{ADA}, in both the frontal cortex (p < 0.01; n = 6) and hippocampus (p < 0.001; n = 6). In these animals both neuronal and glial cells demonstrated the presence of activated caspase-3 particularly within the dentate gyrus of the hippocampus. Conclusions: Our findings illustrate that both HIV-1 gp120_{ADA} and LPS elicit a robust inflammatory response in the brain, but only LPS induced caspase-3 activation in hippocampal neurons, which may be related to the observed learning and memory deficits.

Key Words: HIV Associated Neurocognitive Disorder, Morris Water Maze, gp120, LPS, neuroinflammation
2.2 Background

Although highly active antiretroviral therapy can suppress human immunodeficiency virus (HIV) viral loads to undetectable levels in the periphery, a significant number of HIV-infected patients develop cognitive deficits, particularly during advanced stages of infection (Zayyad and Spudich, 2015). HIV-associated neurocognitive disorder (HAND) is the clinical diagnosis for patients whom experience cognitive deficits which can interfere with daily life functioning. Shortly after seroconversion, neuropsychological testing reveals that HIV-infected patients appear to learn at a markedly reduced rate compared to healthy age-matched controls (Doyle et al., 2013). Such observations are made using a comprehensive battery of neuropsychological test designed to assess seven neurocognitive domains relevant to HAND: attention/working memory, executive functions, learning, memory, motor skills, information processing speed, and verbal fluency (Gisslen et al., 2011). A possible mechanism for cognitive impairments (i.e., memory acquisition and retention) is sustained inflammation due to persistent low-level viral replication (Harezlak et al., 2011). Early in infection, HIV-1 enters the brain predominantly through infected monocytes (Davis et al., 1992). Once inside the central nervous system (CNS), the major cellular targets of HIV are macrophage and microglial cells. Upon interacting with infected monocytes, microglia and astrocytes become activated exhibiting various morphological and biochemical changes (Nash et al., 2011), including the secretion of cytokines, enhanced generation of reactive oxygen species, enhanced glutamate release, and other neurotoxins. Interaction of shed viral proteins (e.g., gp120, Tat) with neuronal chemokine receptors can result in excitotoxicity, loss of mitochondrial membrane potential, caspase activation, and DNA fragmentation, which promote neuronal injury (Kaul et al., 2001, Lindl et al., 2010). These interactions can result in irreversible apoptotic and immune mediated injury ultimately resulting in cognitive deficits. In particular, HIV-1 viral coat glycoprotein 120 (gp120), is a key mediator of HIV-1 cellular entry and pathogenesis (Kaul and Lipton, 2006, Kaul et al., 2005). Our laboratory has extensively investigated gp120-mediated release of pro-inflammatory cytokines (e.g., TNFα, IL-1β) and oxidative stress (inducible nitric oxide synthase (iNOS)) in primary cultures of rodent astrocytes and recently in an in vivo rat model applying daily icv gp120ADA administration for 7 days (Ashraf et al., 2014a, Ronaldson and Bendayan, 2006, 2008). In addition, behavioral abnormalities have also been demonstrated using icv administration of gp120 in rodents including impairments in spatial memory (Glowa et al., 1992, Tang et al., 2009), locomotion (Abraham et al., 2008, Barak et al., 2002a, Barak et al., 2002b, Bjugstad et al., 2004) and auditory and contextual cued fear (Farr et al., 2002).
High plasma levels of lipopolysaccharide (LPS), a bacterial endotoxin, have been detected in HIV-infected patients (Vassallo et al., 2012). It has been suggested that this activity contributes to the chronic immune dysregulation seen in HIV-1 patients. In addition to being a potent stimulator of the immune function, LPS has been shown to interact with cell surface receptors such as Toll-like receptor 4 on neural cells (Triantafilou and Triantafilou, 2002). The source of LPS, as well as bacterial DNA, has been suggested to arise from HIV-mediated disruption of mucosal-associated lymphoid tissues within the gastrointestinal tract. This intestinal mucosal barrier is disrupted in untreated HIV-1 patients due to changes in mucosal cytokines which alter tight junction properties and promote epithelial apoptosis (Eppe et al., 2009). At the blood-brain barrier, released LPS may enhance monocyte trafficking into the CNS by acting at the luminal surface of BMECs to induce release of cytokines from its abluminal surface, thus furthering the inflammatory cascade (Dohgu and Banks, 2013, Vassallo et al., 2013, Wang et al., 2008). Moreover, high plasma level of LPS have been reported as a contributory factor to HAND in the presence of co-infections such as hepatitis C virus (Vassallo et al., 2013). Previous rodent studies, central injection of LPS have been shown to increase the expression of inflammatory cytokine tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) and activates mitogen-activated protein kinases and nuclear factor κB signalling (Guo et al., 2010, Li et al., 2011). In these studies, acute intracerebroventricular (icv) injection of 50μg LPS into rats impaired spatial navigation 10-13 days after administration. At the cellular level, icv administration of 50μg LPS, significantly enhanced caspase-3 activation in neurons of the cerebral cortex 24-72 hour post injection in Sprague–Dawley rats (Song et al., 2014). Accordingly, due to its relevance to HIV-1 and its established neurotoxicity profile (e.g., behavioural deficits and caspase-3 activation), we examined the effect of icv administration of LPS in the context of the cognitive dysfunction observed following HIV-1 infection in our studies. The goal of this project was to implement an in vivo rat model of neurocognitive delay in spatial acquisition and retention by icv administration of a single high dose of HIV-1 viral coat glycoprotein, gp120ADA or LPS, in adult male Wistar rats. We used an R5 tropic strain (i.e., gp120ADA), as opposed to a dual or X4 tropic, since R5-tropic viruses appear to be the most prevalent in the brain (Gabuzda and Wang, 2000). Molecular characterization of neuroinflammatory response and neurotoxicity due to gp120 or LPS administration was also performed.
2.3 Materials and Methods

2.3.1 Materials

Purified, full length recombinant HIV-1\textsubscript{ADA} gp120 (R5 tropic) protein was obtained from Immunodiagnostics Inc. (Woburn, Massachusetts, United States). Live camera SMART Video tracking system (PanLab S.L.U, Harvard Apparatus) was used to record latency and swim parameters (i.e., path, speed, distance, etc.). TRIzol™ was obtained from Invitrogen™ Life Technologies (Carlsbad, California, United States). High capacity reverse transcriptase cDNA synthesis kit and TaqMan FastMix were obtained from Applied Biosystems (Foster City, California, United States) and Quanta Biosciences Inc. (Gaithersburg, Maryland, United States), respectively. Protein assay and chemiluminescent (ECL) reagent were purchased from Bio-Rad Laboratories Inc. (Hercules, California, United States) and Thermo Fischer Scientific Inc. (Waltham, Massachusetts, United States), respectively. Activated anti-caspase-3 (Asp175) antibody was purchased from Cell Signaling Technology Inc. (Danvers, Massachusetts, United States). Fluorescent cyanine 2 and 3 (Cy2/Cy3) secondary antibodies were obtained from Jackson Laboratory. Anti-microtubule-associated protein-2 (MAP-2) antibody (product M4403 clone Hm-2), 3,3'-Diaminobenzidine (DAB), and 4',6-diamidino-2-phenylindole (DAPI) were all purchased from Sigma-Aldrich Co. LLC. (Oakville, Ontario, Canada). Entellan rapid mounting medium was purchased from EMD Millipore Corp. (Etobicoke, Ontario, Canada).

2.3.2 Animals and Stereotaxic Intracerebroventricular (icv) Administration

Adult Wistar male rats, 250-300g, were purchased from Charles River Laboratories (St. Constant, QC, Canada) and were housed with rodent chow and water \textit{ad libitum} on a 12hr light-dark cycle. All procedures were carried out in accordance with the approval of the University of Toronto Animal Care Committee and University Health Network Research Ethics Board. Rodents were randomly assigned to different treatment groups: wildtype, saline, gp120\textsubscript{ADA} or LPS. The same number of LPS and gp120 treated animals were included in each experimental replicate. Experimenters were blinded during stereotaxic injections and throughout the behavioural work. Sterile stereotaxic techniques was performed for all rat brain injections as previously described by our group (Ashraf \textit{et al.}, 2014a) and as outlined by Pritchett-Corning et al. (Pritchett-Corning \textit{et al.}, 2011). In brief, 2-3% isoflurane was used to induce surgical anesthesia. Bilateral icv injection into the lateral ventricles was performed at the following coordinates according to Paxinos and Watson (1986): 0.9mm posterior to bregma, 1.6mm lateral from midline and 3.4mm ventral from the surface of the brain. A
gas tight 10μl Hamilton syringe (maximum volume 8μl; rate 1μl/minute) was utilised for injections. A single dose of 8μg R5-tropic gp120ADA or 50μg LPS was suspended in 8μl cold sterile normal saline and 4μl was administered into each ventricle. Vehicle control animals received a similar volume of saline, while wildtype animals did not undergo stereotaxic surgery.

2.3.3 Morris Water Maze

The Morris Water Maze (MWM) was used to evaluate cognitive changes in spatial memory acquisition and retention in rats. Behavioural assessments were carried out in a circular dark blue plastic pool (1.8m diameter, 0.6m height), filled with 0.5m of opaque (non-toxic dark blue tempera paint) water maintained at 21±1°C and divided into nine zones within four quadrants. The pool was situated with numerous extra-maze cues which remained consistent throughout the experiment. A transparent Plexiglas platform (5 × 8.5 × 0.5 cm) was fixed in the pool and served as an egress target. At the start of each trial, rats were placed gently in the water, facing the wall, at a pseudorandom location with respect to the platform: center, left, or right. A SMART Video tracking system was fixed directly above the pool, which recorded animal performance (i.e., latency to find the platform, speed, distance, path etc.) for up to 60sec. Subjects who did not find the platform within the specified time were guided to the hidden platform and were allowed to remain on the platform for 10sec. Animals were then removed from the maze, towel dried, and allowed to rest for 20min under a heating lamp before proceeding with successive trials. Animals received four-trials-per day for five consecutive days. The first day was used to acclimatised animals to the environment wherein remaining trials on days 2-5 were performed with the platform submerged 2cm below the surface in opaque water (i.e., hidden). Memory retention was assessed in a probe test 24hr following the final training trial. The platform was removed during the probe test where animals were scored during one 60sec trial. The time spent in each quadrant, in particular the target quadrant, was recorded and compared between experimental groups.

2.3.4 Tissue Collection

At the designated time points after injection (6hr, 24hr, and 6 days), animals were anaesthetized and perfused through the left ventricle of the heart with 120ml of chilled phosphate-buffered saline without Ca^{2+} and Mg^{2+} pH 7.2 (PBS). For biochemical analyses, whole brains were removed directly following perfusion, dissected on wet ice, and stored at -80°C until analyzed. Whole rat brains were dissected to isolate frontal cortex, hippocampus and striatum. These regions were selected based
on previous studies that demonstrated HIV-1 proteins induced robust inflammatory response in rodents (Ashraf et al., 2014a, Harezlak et al., 2011, Pu et al., 2003). For imaging studies, cardiac perfusion was employed with 60ml of PBS-/ followed by perfusion of 60ml of cold 4% paraformaldehyde (PFA) in PBS-/ solution. Whole rat brains were then extracted, post-fixed at 4°C in 4% PFA with light agitation for 24hr, and stored at 4°C for paraffin embedding.

2.3.5 Quantitative real-time PCR

Real-Time Quantitative Polymerase Chain Reaction (qPCR) was used to determine transcript levels of inflammatory and oxidative stress markers, as previously described in our laboratory (Ashraf et al., 2014a). Total RNA was extracted from brain regions using TRIzol™ using standard methods and the concentration of RNA was quantified spectrophotometrically by measuring absorbance at 260nm. Extracted RNA was treated with amplification grade DNase I (Invitrogen) to remove contaminating genomic DNA. The High Capacity cDNA Reverse Transcriptase Kit was used to synthesize first-strand cDNA. Rat primer pairs for amplification were purchased from Life Technologies for the following genes and were examined using TaqMan: IL-1β (Rn00580432_m1), TNFα (Rn99999017_m1), iNOS (Rn99999069_m1) and cyclophilin B gene (utilized as housekeeping gene control; Rn00835638_m1). Expression levels were normalized to cyclophilin B, and compared to saline-treated vehicle control group using the comparative Ct (ΔΔCt) method.

2.3.6 Immunoblot Analysis

Immunoblotting was performed as previously described (Ronaldson and Bendayan, 2006). In brief, tissue homogenates were prepared using a lysis buffer [1% (v/v) NP-40 in 20mM Tris, 150mM NaCl, 5mM EDTA at pH 7.5 containing 1mM phenylmethane-sulfonylfluoride, and 0.1% (v/v) protease inhibitor cocktail]. Tissues were sonicated for 10sec, vortexed every 20min for 30sec over 1hr on ice, and centrifuged at 40 000g for 20min at 4°C to remove cellular debris. Protein concentrations of tissue homogenates were determined using Bradford’s protein assay. Total proteins (5μg to 50μg) were separated on 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking with 5% skim milk for 1.5hr, the membrane was probed with primary antibody (e.g., cleaved caspase-3, 1:200). β-actin (1:1000) was used as loading control. HRP-conjugated secondary antibody was added after 3 washes in Tris-buffered saline with Tween. Bands were detected using enhanced chemiluminescent reagent (Thermo Fischer Scientific). Densitometric analysis was performed using AlphaDigiDoc RT2 software to quantify relative protein expression. The graphs
represent relative density of the bands of interest, normalized to corresponding β-actin, and reported as fold-changes from saline.

2.3.7 Immunohistochemistry and Imaging

Immunohistochemical staining and imaging results were performed at the Princess Margaret Hospital’s Advanced Optical Microscopy Facility (AOMF). Primary anti-cleaved Caspase-3 antibody (1:000) or anti-MAP-2 antibody (1:000 – Sigma) and Cy2/Cy3 secondary antibody (1:500) were used for staining. Four percent PFA fixed brain tissues were paraffin embedded and sectioned at 4.5µm as coronal serial sets. Slides were dewaxed in 100% xylene and rehydrated through a series of ethanol baths before rinsing three times in PBS. Thionin staining was performed on brain sections to assess cellular anatomy and morphology. Immunofluorescence for MAP-2 and cleaved caspase-3 was carried out following antigen retrieval using sodium citrate as previously described (Shi et al., 1993), then incubated in blocking buffer (5% goat serum and 0.2% Triton X-100 in 0.1M PBS) for 1hr followed by overnight incubation with anti-caspase-3 antibody at 4°C. Sections were then washed thoroughly in PBS and incubated for 35min in secondary antibody (1:500). Following the incubation, the slides were again thoroughly washed in PBS and treated with DAPI for 10min. The slides were then washed and cover slipped using Entellan. Images were collected using Volocity (Perkin Elmer) on a Leica DM6000 microscope equipped with a Hamamatsu ORCA-ER C4742-95 camera for fluorescence microscopy. For immunoperoxidase staining, after dewaxing and rehydration, the slides were treated with 0.3% hydrogen peroxide in PBS for 15min to eliminate endogenous peroxidase activity and were incubated overnight at 4°C with anti-caspase-3 antibody. Following washes, slides were incubated with the appropriate secondary antibody (1:400) for 35min at room temperature and washed in PBS. All slides were scanned using Nanozoomer 2.0 HT (Hamamatsu Photonics) and the images were viewed using NDP.view2 software (Hamamatsu Photonics). All bright field and fluorescent images were taken using Velocity (Perkin Elmer) on a Leica DM6000 microscope equipped with a Hamamatsu ORCA-ER C4742-95 camera for fluorescence microscopy and a Q-Imaging MicroPublisher 5.0 RTV colour camera for bright-field analysis. All images were processed using Adobe Photoshop CS2.

2.3.8 Data Analysis

Data were analyzed using GraphPad Prism 5 software (San Diego, CA, USA). Student’s t-test was used to determine statistical significance between two groups. Multiple comparisons were
performed using ANOVA and Bonferroni’s post-hoc analysis and behavioural assessments were analyzed using two-way ANOVA. A p-value less than 0.05 was considered statistically significant.

2.4 Results

2.4.1 Behavioural Effects due to HIV-1 gp120 or LPS Administration
Stereotaxic administration of saline to the lateral ventricles of adult Wistar rats resulted in no significant differences between wildtype and saline groups in the MWM (four-trials-per-day with 20min inter-trial time) with respect to learned platform acquisition over the period of training (Figure 7a; p = 0.39). In wildtype rats, mean escape latency decreased from 49±3.0sec on day 1 to 18±3.9sec on day 5. A significant reduction in mean escape latency between day 5 and days 1 (p<0.001) and 2 (p<0.05; figure 7a) was verified by t-tests suggesting animals acquired the location of the platform over 5 days. Similarly, saline-administered rats showed decrease in mean escape latency from 43±4.1sec on day 1 to 17±5.6sec on day 5. Spatial retention was evaluated 24hr post-acquisition (Figure 7b). In the absence of the platform, the percentage of time spent searching for the quadrant that initially contained the platform (i.e., target) was evaluated over 60sec. Wildtype rats spent 18±1.7% of the time in the target quadrant, whereas saline-treated animals spent 16±2.9%. Student t-tests confirmed no significant difference between the two groups (Figure 7b, p = 0.55), hence, only saline vehicle controls were examined in successive experiments.
In our model, administration of 50μg LPS in 8μl sterile saline significantly impaired spatial acquisition (Figure 8a; p = 0.001). Compared to saline controls, a delay of 7±1.0sec in mean escape latency was observed in 50μg LPS-treated rats whereas only a 0.6±1.5sec delay was seen in 8μg gp120ADA-treated rats. Spatial memory was evaluated 24hr after acquisition where the percentage of time spent in each quadrant was measured. LPS-treated rats spent approximately 9±1.9% less time in the target quadrant compared to saline and gp120ADA-treated rats (Figure 8b; p < 0.01). Furthermore, examining the time spent in the opposite, left, and right quadrants (with respect to the target quadrant) revealed that LPS-treated rats explored all quadrants equally, with no significant preference for the target quadrant (figures 8e). In gp120ADA-treated rats, no significant difference was observed compared to saline controls in the percentage of time spent in the target quadrant (Figure 8b; p > 0.05). Furthermore, gp120ADA-treated rats displayed a significant preference for the target quadrant, which was similar to saline-treated animals (Figure 8c, d; p < 0.001). These results suggest that spatial acquisition and retention were impaired, or delayed, upon LPS-treatment, but not with saline or gp120ADA administration.
Figure 8 Effect of icv administration of 8μg HIV-1 gp120ADA or 50μg LPS on MWM escape latency. (a) Depiction of learning curve over 5 days of training, 4 trials per day. (b) Performance during probe test examining spatial memory as percentage of time spent in target quadrant. (c-e) Amount of time spent in all quadrants during 60sec probe test of (c) saline, (d) gp120ADA and (e) LPS treated animals. Results are expressed as mean±SEM. All groups are representative of n = 12-16. Asterisk represents data point significantly different from saline-treated animals using ANOVA with bonferroni post-hoc (* p<0.05; ** p<0.01; ***p<0.001).
2.4.2 Molecular Characterization of gp120 and LPS Stress Response in the CNS in vivo

Similar to other groups, we have previously demonstrated the presence of gp120 elicits an inflammatory response in neural cells both in vitro (e.g., primary cultures of rat astrocytes (Ronaldson and Bendayan, 2006)) and in vivo (i.e., daily icv administration of 500ng HIV-1 gp120ADA for 7 days in rats (Ashraf et al., 2014a)). Our current model employs acute icv administration of a single dose of either 50μg LPS or 8μg HIV-1ADA gp120 in adult male Wistar rats. We observed expression of high levels of inflammatory cytokines (TNFα, IL-1β) and oxidative stress (iNOS) marker in the hippocampus, frontal cortex and striatum at 6hr, 24hr and 6 days after icv injection of either gp120 or LPS. Substantially elevated levels of IL-1β mRNA were seen in LPS-treated animals 6 and 24hrs following injection compared to saline controls in all the brain regions (figures 9a-c; p < 0.001). This response remained elevated up to 6 days post injection (Figure 9). In gp120ADA-treated rats, significant elevation of IL-1β mRNA was observed at 6hr post injection, though not as pronounced as LPS-treated animals. This effect subsided by 24hr post injection in the frontal cortex (Figure 9b), but remained modestly elevated in the hippocampus (Figure 9a), and striatum (Figure 9c). Levels of IL-1β transcript returned to baseline in gp120ADA-treated rats by 6 days post injection, but not in LPS-treated rats for all brain regions (Figure 9).
Effect of icv administration of gp120ADA or LPS on the mRNA expression of IL-1β.

Adult Wistar rats were administered, bilateral icv, a total of 8μl of saline containing 8μg HIV-1 gp120ADA or 50μg LPS. Wildtype (WT) and saline injected animals were analysed as surgical and vehicle controls, respectively. IL-1β expression was analyzed in the (a) hippocampus (n = 8-13), (b) frontal cortex (n = 6-7), and (c) striatum (n = 3-7) at 6hr, 24hr, and 6 days post icv injection. Results are expressed as mean±SEM at 6hr, 24hr and 6 days post icv injection relative to saline. Asterisk represent data point significantly different from saline administered animals at respective time points using student t-tests (* p <0.05; ** p<0.01; ***p<0.001).

Similarly, levels of TNFα transcripts were significantly elevated in both LPS- and gp120ADA-treated animals at 6hr and 24hr post injection in all the brain regions compared to saline-administered animals (Figure 10). Although a significant elevation in TNFα mRNA was detected in the hippocampus, frontal cortex, and striatum upon gp120ADA injection (i.e., 6hr post injection; figures 10a-c), this response quickly subsided by 24hr post injection and remained at baseline thereafter (i.e., after behavioural studies). Levels of LPS-induced TNFα transcripts were most pronounced in the hippocampus at 6hr post injection and remained modestly elevated by 6 days post injection (Figure 10a). In the frontal cortex and striatum, LPS induced significant levels at 6hr post injection which dropped to baseline at 6 days post injection (figures 10b-c). It appears that persistently high
levels of inflammation (i.e., cytokine levels) in the brain during the course of the MWM could only be achieved by LPS injections, but not gp120\textsubscript{ADA}.

**Figure 10** Effect of icv administration of gp120\textsubscript{ADA} or LPS on the mRNA expression of TNFα. Adult Wistar rats were administered, bilateral icv, a total of 8μl of saline containing 8μg HIV-1 gp120\textsubscript{ADA} or 50μg LPS. Wildtype (WT) and saline injected animals were analysed as surgical and vehicle controls, respectively. TNFα expression was analyzed in the (a) hippocampus (n = 8-13), (b) frontal cortex (n = 6-7), and (c) striatum (n = 3-7) at 6hr, 24hr and 6 days post icv injection. Results are expressed as mean±SEM at 6hr, 24hr and 6 days post icv injection relative to saline. Asterisk represent data point significantly different from saline administered animals at respective time points using student t-tests (* p <0.05; ** p<0.01; ***p<0.001).

Compared to saline-controls, LPS-treated animals had significantly elevated iNOS levels in the brain at both 6hr and 24hr post injection, most pronounced in the frontal cortex (Figure 11b). As for gp120\textsubscript{ADA}-treated rats, a significant increase in iNOS transcript was observed at 6hr post injection, and remained elevated only in the frontal cortex at 24hr post injection (Figure 11b). Baseline levels of iNOS expression were observed at 6 days post administration of gp120\textsubscript{ADA} or LPS (Figure 11).
Figure 11 Effect of icv administration of gp120ADA or LPS on the mRNA expression of iNOS. Adult Wistar rats were administered, bilateral icv, a total of 8μl of saline containing 8μg HIV-1 gp120ADA or 50μg LPS. Wildtype (WT) and saline injected animals were analysed as surgical and vehicle controls, respectively. iNOS expression was analyzed in the (a) hippocampus (n = 8-13), (b) frontal cortex (n = 6-7), and (c) striatum (n = 3-7) at 6hr, 24hr and 6 days post icv injection. Results are expressed as mean±SEM at 6hr, 24hr and 6 days post icv injection relative to saline. Asterisk represent data point significantly different from saline administered animals at respective time points using student t-tests (* p <0.05; ** p<0.01; ***p<0.001).

2.4.3 Caspase-3 Activation and Localization in HIV-1 gp120 or LPS Treated Rats

Given that we observed significant and persistent upregulation of cytokine and oxidative stress marker following LPS administration, and more modestly in gp120ADA-treated animals, we next examined the extent of apoptotic programmed cell death in these regions. Caspase-3 has been widely utilized as a downstream apoptotic marker of programmed cell death in numerous tissues in vivo. For example, in a previous study that employed in-tissue injection of M-tropic gp120BAL into the caudate-putamen, significant neuronal loss peaking, substantially, at 24hr post injection was observed (Louboutin et al., 2007). Similarly, daily icv administration of up to 100ng T-tropic gp120IIIIB for 7 days in rats significantly up-regulated activated caspase-3 expression, particularly in the somatosensory cortex 24hr after the last injection (Acquas et al., 2004, Bagetta et al., 1995). We chose to examine caspase-3 activation at 24hr post injection. Compared to saline-treated rats, we
observed caspase-3 activation is significantly elevated in the frontal cortex (Figure 12a; p < 0.01; n = 6) and hippocampus (Figure 12b; p < 0.001; n = 6) of rats treated with LPS, but not with gp120ADA. Quantitative immunoblotting analyses demonstrate that LPS administration enhanced activated caspase-3 expression by an average of 3.1-fold in the frontal cortex (Figure 12c) and a 3.5-fold in the hippocampus (Figure 12d). For gp120ADA, these data did not reach statistical significance (figures 12b-c).

![Immunoblot analysis of caspase-3](image)

**Figure 12** Effect of icv administration of gp120ADA or LPS on the protein expression of activated caspase-3. Adult Wistar rats were administered, bilateral icv, a total of 8μl of saline containing 8μg gp120ADA or 50μg LPS. A representative immunoblot of activated caspase-3 from the (a) frontal cortex (n = 6) and (b) hippocampus (n = 6) at 24hr post icv injection is shown along with corresponding densitometric analysis. A total of 100μg of protein was loaded in each sample. All results are expressed as mean±SEM relative to saline-administered controls. Asterisk represent data point significantly different from saline administered animals at respective time points using one-way ANOVA with bonferroni post-hoc (*p <0.05; **p<0.01; ***p<0.001).

To better localise the distribution of cells undergoing programmed cell death in the CNS following LPS treatment, immunofluorescence and immunohistochemistry were utilized to trace caspase-3 cleavage in the brain. There is strong evidence implicating the hippocampus as the major region in spatial mapping, therefore, we examined 4.5μm coronal hippocampal slices from wildtype rats as well as those treated with saline, 8μg gp120ADA or 50μg LPS at 24hr post injection. We employed DAB-peroxidase staining to visualise activated caspase-3 and immunolabelled neuron-specific marker, MAP-2, with Cy3 secondary antibody. The hippocampus dentate gyrus (DG) appeared most sensitive to our bilateral icv treatment thus a representative figure of the DG is shown in figure 13. Wildtype animals did not present with prominent activated caspase-3 immunostaining in the hippocampus and surrounding cortex (Figure 13a, DAB-peroxidase panel). As expected neuronal
morphology appeared normal (Figure 13a, Cy3-MAP-2 panel). Similarly, treatment with saline (Figure 13b) and gp120ADA (Figure 13c) resulted in modest levels of activated caspase-3 staining in the DG, suggesting minor stress due to stereotaxic surgery. Caspase-3 positive signals obtained in these sections were sporadic, possibly due to transient glial cellular turn over in the brain (figures 13b-c, overlay). However, LPS treatment resulted in numerous caspase-3 positive cells, clustering predominantly in the hippocampal DG (Figure 13d). A majority of caspase-3 immunostaining was found in areas lacking MAP-2 immunoreactivity, suggesting neuronal cell bodies were the main source of caspase-3 expression. Neuronal morphology appeared condensed and shrunken compared to wildtype controls which is consistent with apoptotic features of cellular death (Bonde et al., 2002). These results qualitatively support our immunoblot observations. In summary, LPS-induced caspase-3 activation, particularly in the neurons, may have resulted in the significant impairments in a spatial navigation task; whereas the lack of caspase-3 activation in our gp120ADA model along with to the modest inflammatory response compared to LPS, could explain the absence (or mildness) of neurocognitive deficits as measured by the MWM.
Figure 13 Cellular localization of activated caspase-3 in the hippocampus 24hr post icv administration. (a) Wildtype adult Wistar rats were administered, bilateral icv, a total of (b) 8μl of saline containing (c) 8μg HIV-1 gp120ADA or (d) 50μg LPS. Bright field images were used to observe immunoperoxidase staining of caspase-3 while fluorescence cy3 images were used to observe neuronal marker MAP-2. Majority of caspase-3 immunoreactive cells were found in the hippocampus DG thus images were focused in this area. Representative images were selected from one biological replicate from each experimental group: wildtype (n = 4), saline (n = 4), gp120ADA (n = 4), and LPS (n = 10). Both brightfield and fluorescence images are photographed at 20x; scale bar indicate 50μm.
2.5 Discussion

Ample evidence suggests an active role for inflammation and oxidative stress in the development and progression of neurocognitive disorders in patients that are chronically infected with HIV-1 (Kaul et al., 2001, Zayyad and Spudich, 2015). Herein, we aimed to implement an in vivo rat model of neuroinflammation, neurotoxicity, and neurocognitive impairments in spatial acquisition and retention by icv administration of a single, high dose of HIV-1 viral coat glycoprotein, gp120\textsubscript{ADA}, or LPS, in adult male Wistar rats. In brief, we observed significant deficits in spatial learning and memory as a result of 50μg LPS administration, but not 8μg gp120\textsubscript{ADA}. Significantly elevated levels of TNFα, IL-1β, and iNOS transcripts were observed in whole brain regions up to 6 days post LPS injection, but only up to 24hr post gp120\textsubscript{ADA} injection. Furthermore, administration of LPS, but not gp120\textsubscript{ADA}, increased expression of activated caspase-3 in neuronal cell bodies, particularly in the hippocampal DG, possibly resulting in the observed delayed spatial acquisition and retention.

To the best of our knowledge, acute (i.e., single injection) gp120 administration in adult rats has not been previously investigated using the MWM, a highly hippocampus-dependent task. Pugh and colleagues reported a lack of impairment in auditory-cued fear, but not contextual fear, after administering up to 8μg gp120\textsubscript{MN} icv in mice (Pugh et al., 2000), suggesting gp120-induced neurocognitive impairments occurs through hippocampal-dependent mechanisms. Although we detected significantly elevated levels of inflammatory cytokines/oxidative stress marker in the hippocampus after gp120 administration, the lack of caspase-3 activation was observed. This suggests that the mild latency delay (i.e., 0.60sec delay; Figure 8a) in learning due to acute gp120\textsubscript{ADA} administration is likely attributed to inter-rat variability. In other studies that examined gp120-induced spatial impairments using the MWM, multiple icv injections were employed (Bjugstad et al., 2004, Glowa et al., 1992, Tang et al., 2009). For example, in the initial report of gp120-induced spatial deficits, Glowa et al. injected 12ng gp120\textsubscript{SF} or gp120\textsubscript{IIIb} for 7 days prior to MWM training and continued injections throughout the remainder of the experiment (Glowa et al., 1992). Rats were trained one-trial-per-day for 15 days (i.e., total of 15 trials) where the greatest effect of gp120-induced learning delays occurred over the first 9 days of training and remained significantly slower throughout the remainder of the experiment. This is in comparison to significant deficits reported by Tang et al. who examined the effect of administering 150ng of only the V3 loop peptide from the dual tropic gp120\textsubscript{MN} on spatial memory (Tang et al., 2009). The V3 loop of gp120 is a conserved region located on the outer domain and is considered essential for viral infectivity (Ivanoff et al.,}
1992). Rats received daily administration of 150ng of the V3 loop peptide in artificial cerebrospinal spinal fluid per day, for 3 days, and their MWM performance were recorded at four-trials-per-day for 5 days (i.e., 20 trials) (Tang et al., 2009). V3 injected rats displayed significant spatial memory impairments as measured by time spent and frequency of entering the target quadrant. On the other hand, multiple dosing did not result in robust neurocognitive deficits as a lack of impairments were reported in rats injected icv with 50ng gp120IIIB per day for 7 days, prior to MWM training (Bjugstad et al., 2004).

Since our acute gp120ADA model mimics biochemical changes during early period of infection, and neurocognitive deficits do not typically present until several years after seroconversion, it may be reasonable that we observed a lack of spatial impairments. Nonetheless, clinical studies have reported the presence of subclinical impairments intermediate to chronically infected and non-infected controls; these untreated subclinical impairments may progress into HAND (Moore et al., 2011). Although some studies have suggested the lack of neurocognitive impairment within the initial two years of infection (Vo et al., 2013). Other studies of acute infection viewed neurocognitive disorder not as a collection of neuropsychometric tasks, but as individual cognitive domains, and as a result, prominent deficits in information processing speed, psychomotor speed, working memory and verbal learning have been observed in acutely HIV-1 infected patients (Doyle et al., 2013, Moore et al., 2011, Ragin et al., 2012). Thus similarly to mild/transient deficits present in various neurocognitive domains, it is likely that rodent models of gp120-induced neurocognitive deficits may require the evaluation in a spectrum of behavioural tasks assessing several neurocognitive domains such as locomotor activity, contextual/auditory-fear, motivation, speed of movement, and stress in addition to spatial memory. In regards to LPS, it is not surprising that icv administration of 50μg LPS (i.e., our positive control) induced significant deficits in spatial learning and memory as our observations are consistent with previous reports that examined the effect of 50μg LPS icv in rats using the MWM (Guo et al., 2010, Li et al., 2011).

Cytokines production may induce neurocognitive impairments, in part, through neuronal death (Kaul and Lipton, 2006), thus we documented pro-inflammatory cytokines and oxidative stress levels as well as activated caspase-3. We observed robust mRNA levels of TNFα, IL-1β, and iNOS in the frontal cortex, hippocampus, and striatum in rats treated with LPS, and to a lesser extent gp120ADA. This is consistent with our previous findings in an in vivo rodent model employing daily administration of 500ng HIV-1 gp120ADA for 7 days where we documented significantly elevated
levels of TNFα, IL-1β, and iNOS in the frontal cortex, but only IL-1β and iNOS were elevated in the hippocampus and striatum, 24hr after the last injection (Ashraf et al., 2014a). These markers are known to be upregulated in HIV-1 infection and have been observed in post-mortem brains of HIV-1 infected patients (Bagetta et al., 1999, McGuire et al., 2015). The release of pro-inflammatory cytokines, particularly TNFα, contributes to neurodegeneration (Carvey et al., 2005, Fischer and Maier, 2015). For example, significant dopaminergic neuronal loss was reported as a result of 200ng TNFα and 10ng IL-1β administered, alone and in combination, into the right medial forebrain bundle of rats (Carvey et al., 2005). Neuronal loss was more pronounced when TNFα and IL-1β were administered together, implicating an additive effect of cytokines. Furthermore, in rat studies, daily icv administration of 50ng TNFα for 7 days (Bjugstad et al., 1998) and icv administration of 100ng IL-1β (Oitzl et al., 1993) resulted in significant delays in MWM escape latencies. In our study, significant levels of IL-1β and TNFα up to 6 days post injection in the hippocampus of LPS-treated rats may have had an additive effect that resulted in hippocampal-dependent impairments (i.e., spatial learning/memory), whereas gp120-induced cytokine production subsided by 24hr post injection and did not result in spatial impairments. Accordingly, gp120ADA administration did not result in significant activated caspase-3 expression. This lack of effect may be due to dose, duration and/or strain differences of the gp120 molecule we used as well as the mode of administration (i.e., icv, intrahippocampal, intrastriatal, etc.). For example, in a study that administered 400ng gp120IIIIB directly into the rat striatum a significant number of caspase-3-positive cells were observed and identified as neurons (Nosheny et al., 2004). Notably, repeated icv administration of 100ng gp120IIIIB for 3 or 7 days in rats could also result in significant neuronal caspase-3 activation (Acquas et al., 2004). This is in agreement with studies in humans; brain tissues from pediatric patients with HIV-1 encephalitis revealed marked increase in pro-caspase-3 immunostaining, particularly in neurons, macrophages, and microglia, but not in astrocytes (James et al., 1999). Significant expression of activated caspase-3 was also reported in cultured cerebromotoral neurons from patients with HIV-1 associated dementia/encephalitis as well as cultured rat cerebromotorial cells treated with gp120SF (Garden et al., 2002). It is possible that neurons are particularly sensitive to inflammation as central LPS administration resulted in significant monocyte activation and loss of pyramidal neurons in the CA3-CA4 region as well as the DG of the hippocampus (Hauss-Wegrzyniak et al., 2000). To further highlight the role of the hippocampus in neurocognitive processes, in studies employing the MWM, rats with lesions of the hippocampus were unable to identify the hidden platform in both recent and remote memory recall evaluations (Broadbent et al., 2004). Notably, rats with such hippocampus
lesion were able to accurately identify a visible platform (i.e., platform above the surface of the water), possibly due to visual acuity and instinctive/survival behaviours. Hippocampal lesions severely impaired performance in other navigation tasks including dry-land mazes (Ramos, 2013), visual recognition tasks (Clark et al., 2000) and other spatial assessments (Dudchenko et al., 2000). Furthermore, hippocampal substructures such as the DG has been implicated in disrupting contextual encoding, a necessary component in spatial navigation tasks (Lee and Kesner, 2004). These studies corroborate our findings wherein LPS administration resulted in significant caspase-3 activation in neuronal cells of the DG. It is possible that the marked increase in activated caspase-3 expression, suggesting DNA fragmentation and apoptosis, was sufficient for the appearance of spatial memory deficits.

Since administering a single dose of R5 tropic gp120\textsubscript{ADA} did not result in significant caspase-3 activation or spatial impairments in the MWM, as shown in our study, we suggest the implementation of alternative regimens such as higher dosing, repeated daily administration, or chronic infusion using an osmotic pump in order to develop a robust model of HIV-1 gp120-induced neurocognitive impairment. Notably, it is difficult to establish a rodent model that accurately reflects HAND pathogenesis. We acknowledge the limitations of our current model which excludes clinical aspects such as recurrent viral entry and replication in the CNS and chronic immunosuppression in HIV infected patients. Nonetheless, short of employing a macaque model of HIV encephalitis, the use of gp120-induced neuroinflammation in rodents is a convenient, and relatively easily implemented, method to model HAND. Other studies have previously illustrated various neurobehavioral changes due to gp120 administration into the CNS (Barak et al., 2002a, Fitting et al., 2008, Pugh et al., 2000). Furthermore, the efficacy of various potential therapeutic adjuvants, including the delivery of anti-oxidant enzymes into the CNS (Louboutin et al., 2010b) and administering anti-inflammatory compounds early in infection (Ashraf et al., 2014a) have also been reported using a gp120 induced inflammatory model. These studies highlight the potential of employing a gp120 rodent model in studying pathogenesis and therapeutic options of HAND.

2.6 Conclusion

In summary, using an \textit{in vivo} gp120 or LPS induced neuroinflammation model, we have demonstrated significant levels of pro-inflammatory cytokines and oxidative stress marker in the brain. Since administering a single dose of R5 tropic gp120\textsubscript{ADA} did not result in significant caspase-3
activation as evaluated by immunoblotting and immunoperoxidase staining, and no significant spatial impairments were observed as indicated by escape latencies in the MWM, alternative gp120 regimens should be explored. In this model applying acute icv administration, we observed that neurons of the hippocampal DG were highly susceptible to LPS-induced caspase-3 activation, compared to gp120_{ADA} administration. Significant levels of caspase-3 activation upon LPS-administration, but not gp120, likely resulted in the observed delays in spatial learning and memory.

2.7 Conflicting Interests
The authors report no competing interests.

2.8 Authors Contributions
ACCK, MTH and RB designed the study, performed the literature searches/analyses and prepared the animal protocol. ACCK and RB contributed to the manuscript preparation. CW performed the animal stereotaxic surgery and brain tissue isolation. JH provided guidance with immunofluorescence studies.

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CHAPTER 3: OVERALL DISCUSSION AND CONCLUSION

3.1 Overall Discussion

HIV-associated neurocognitive disorder is increasing in prevalence as antiretroviral therapy allows patients to live an almost normal life span (Vivithanaporn et al., 2010). Depending on the context of investigation, between 18-50% of HIV-1 infected patients develop neurocognitive deficits that present as learning/memory loss, poor concentration, chronic pain, depression, fatigue, and motor abnormalities (Zayyad and Spudich, 2015). These neurocognitive symptoms significantly decrease quality of life and present a major public health issue. The development of cognitive impairments may be the result of low level viral replication in the CNS that induces the secretion of pro-inflammatory cytokines and oxidative stress markers (Glass et al., 1995, Persidsky et al., 1997, Tyor et al., 1992). Shed viral proteins (such as gp120, Tat and vpr) can also generate inflammatory response and oxidative stress in brain macrophages, microglia and astrocytes (Pu et al., 2003, Ronaldson and Bendayan, 2006, Torres and Noel, 2014). In addition, circulating enteric bacterial products further exacerbate the inflammatory response in the CNS by acting at the abluminal membrane or cellular transcytosis though an impaired blood-brain barrier (Verma et al., 2006). Persistent inflammation and oxidative stress induce neuronal apoptosis, wherein widespread neuronal apoptosis may provide the neural basis of neurocognitive impairments in HAND patients (Kaul, 2008, Kaul and Lipton, 2006). In the present study, we employed an in vivo model of neuroinflammation using an acute dosing of gp120 administered icv. In adult male Wistar rats, we demonstrated that using a single dose of R5 tropic gp120$_{ADA}$ would lead to significantly elevated levels of pro-inflammatory cytokines and oxidative stress markers in the brain up to 24hr post injection, but does not result in significant caspase-3 activation or spatial learning/memory impairments in the MWM. As for our positive control, administration of 50μg LPS resulted in significant deficits in spatial learning and memory as well as significantly high levels of TNFα, IL-1β and iNOS in whole brain regions up to 6 days post injection. Accordingly, significant caspase-3 activation in neuronal bodies, particularly in the hippocampal DG, were observed, which likely led to the observed delayed spatial acquisition and retention.

3.1.1 Effect of Acute HIV-1 gp120 Administration in Rats in the Morris Water Maze

Previous reports employing acute (i.e., single injection) gp120 administration in adult rats have shown significant deficits in other behavioural domains. For example, Barak and colleagues
administered 2μg gp120\textsubscript{IIIB} icv in rats and reported a significant decrease in locomotor activity (i.e., motivational movements) as well as other sickness behaviours (i.e., decreased exploratory behavior, suppressed consumption of food/ saccharin solution and reduced body weight) (Barak \textit{et al.}, 2002a, Barak \textit{et al.}, 2002b). However, these behavioural abnormalities reflect depression-like behaviour rather than neurocognitive deficits such as learning/memory. Interestingly, Pugh and colleagues observed a lack of impairment in auditory-cued fear, but not contextual fear, after administering up to 8μg gp120\textsubscript{MN} icv rats (Pugh \textit{et al.}, 2000). Contextual-fear is an aspect of spatial memory processing that requires the hippocampus to integrate various sensory inputs (i.e., visual, auditory, olfactory, etc.) into a conjunctive episode for memory consolidation, a theory known as cognitive mapping theory (O'Keefe and Nadel, 1978, Rudy \textit{et al.}, 2004). This suggests that the contextual-fear disruptions reported by Pugh and colleagues may be gp120-induced neurocognitive deficits occurring through hippocampal-dependent, rather than hippocampal-independent, memory consolidation mechanisms. Since the MWM is a highly hippocampus-dependent task, and although we observed significantly elevated levels of inflammatory cytokines/oxidative stress marker in the hippocampus, due to the lack of caspase-3 activation, it is reasonable to suggest that mild disruptions in spatial memory consolidation did not occur upon acute gp120\textsubscript{ADA} administration. In theory, prolonged exposure to gp120, or concurrent gp120 administration with behavioural testing may result in robust memory impairments in the MWM. However, several studies have reported discordant results (Bjugstad \textit{et al.}, 2004, Glowa \textit{et al.}, 1992, Tang \textit{et al.}, 2009). Due to the various experimental settings employed (i.e., gp120 regimen and strain, number of training days and number of trials per day in the MWM), it is difficult to directly compare our observations. It is likely that rodent models of gp120-induced neurocognitive deficits may require the evaluation of a spectrum of behavioural tasks assessing several neurocognitive domains such as locomotor activity, contextual/auditory-fear, motivation, speed of movement, and stress in addition to spatial memory.

Since our current model reflects an acute infection, it is interesting to note that mild or transient cognitive impairments and abnormalities have been documented during the very early period of HIV-1 infection. For example, although neurocognitive deficits do not typically present until several years after infection, remarkable changes in neuroanatomy and functioning have been documented early in HIV-1 infection such as decreased resting-state cerebral blood flow (Ances \textit{et al.}, 2009), reduced cerebral cortex volume and swelling of the third ventricle (Ragin \textit{et al.}, 2012, Ragin \textit{et al.}, 2015). Additionally, in a sample of early HIV-1 infected patients (n = 52, mean infection duration less
than one year) increased matrix metalloproteinase (MMP) was detected in the CSF (Li et al., 2013). These parameters may reflect general CNS-specific disturbances or HIV-1 induced alterations in vascular physiology (i.e., BBB integrity). Furthermore, deficits in specific neurocognitive domains have been widely reported and replicated in various cohorts. For example, Weber and colleagues (2013) reported in a sample of 46 ARV-naïve individuals with acute/early HIV-1 infection (i.e., an estimated duration of infection at 75 days) that they were four-times more likely to develop neurocognitive impairment than their seronegative controls, predominantly in information processing speed and verbal learning (Weber et al., 2013). Two groups investigated cognitive abilities in a subset of an ongoing, observational study (i.e., the Chicago Early HIV-1 Infection Study) and both reported significantly impaired performance in psychomotor skills (i.e., digit-symbol test) in patients infected for less than one year (Wang et al., 2011) or within 100 days (Ragin et al., 2015). Other domains that were significantly impaired involved executive functioning (i.e., letter-number sequencing task) (Ragin et al., 2012) and visual memory (i.e., Rey-Osterrieth Complex Figure Delayed Recall Test) (Wang et al., 2011). Although the patient population is often limited in these acute infection studies, there is evidence suggesting CNS alternations in both structure (i.e., anatomy) and function (i.e., psychological) occur shortly after HIV-1 infection.

3.1.2 LPS Induced Neurocognitive Impairments in Rats and Relevance in HIV-1 Infection

In regards to LPS, our observations are consistent with previous reports that examined the effect of administering 50μg LPS icv in rats using the MWM. In these studies, although spatial learning latencies were not reported, significant delays in spatial memory were observed in time (i.e., latency to find the platform) as well as distance (i.e., amount of distance travelled) (Guo et al., 2010, Li et al., 2011); in our study, LPS-treated rats spent approximately 9±1.9% less time in the target quadrant (i.e., approximately 5.5sec of the 60sec observational window of the MWM probe test) whereas a 35sec and 20sec delay was reported by Guo et al., and Li et al., respectively. With regards to spatial learning, we reported a 7±1.0sec delay in mean escape latency, while others who administered LPS icv have reported an average of 11.25sec (Gong et al., 2010), 12.75sec delay (Rosi et al., 2006) and 17sec (Cui et al., 2008). Notably, the length of acquisition differed among studies.

Recovery from neurocognitive impairments may occur slowly without pharmacological intervention. In a study that administered LPS into the fourth lateral ventricle using osmotic mini-pumps set to deliver approximately 0.25μg/h for 37 or 74 days, significant spatial learning delay was reported in
rats treated for 37 days, an effect that did not appear to be exacerbated by doubling the infusion time (i.e., 74 days) (Hauss-Wegrzyniak et al., 2000). In HIV-1 infected patients, gastrointestinal microbes and associated molecules (i.e., LPS or 16S rDNA) may enter the systemic circulation. It has been reported that significantly high levels of bacterial 16S rDNA was detected in plasma of HIV-1 infected patients (approximately 132.5 copies/μL) compared to case-controlled non-infected patients (0-5 copies/μl) (Jiang et al., 2009). Although it has been shown in vivo that LPS minimally penetrates the intact rodent BBB (Banks and Robinson, 2010), LPS may signal at the luminal side of BMECs to exacerbate HIV-associated neuroinflammation through mechanisms including increasing monocyte trafficking, decreasing expression of tight junction proteins, or enhancing the potential of HIV-1 transcytosis (Dohgu and Banks, 2013, Verma et al., 2006, Wang et al., 2008). Peripheral inflammatory conditions, caused by HIV-associated microbial translocation or co-infections, may also contribute to neuronal apoptosis and neurocognitive decline (Hernandez-Romero et al., 2012). For example, significantly higher levels of serum LPS (i.e., greater than 79pg/mL) were documented in HIV patients who developed dementia, compared to patients with milder neurocognitive deficits (i.e., HAND) (Ancuta et al., 2008). It is may be possible that microbial-induced systemic inflammation or presence of bacterial proteins (e.g., LPS or 16 rDNA), exacerbates HIV-associated neuroinflammation, which may provide some degree of insight on the neurocognitive status of patients.

3.1.3 HIV-1 gp120 and LPS Induced Neuroinflammation and Activated Caspase-3 Expression

During HIV-1 infection, the viral coat glycoprotein gp120 is often shed from the virus which contributes to the production of a number of pro-inflammatory cytokines (i.e., TNFα, IL-1β, IL-6, and IFN-γ) and oxidative stress markers (i.e., iNOS) (Naif, 2013). We observed robust mRNA levels of pro-inflammatory cytokines (i.e., TNFα, IL-1β) and oxidative stress markers (i.e., iNOS) in the frontal cortex, hippocampus, and striatum in rats. The production of IL-1β mRNA appeared most sensitive as dramatic increases were observed in all three regions of the brain 6hr post LPS administration, and gp120ADA to a lesser extent. In LPS-treated rats, levels of IL-1β mRNA remained significantly high and did not return to baseline up to 6 days post injection, whereas, gp120-induced IL-1β transcripts decreased to baseline levels by 24hr post injection. Levels of TNFα transcript were more pronounced in the hippocampus at 6hr post injection of LPS-treated rats, and remained significantly elevated up to 6 days post injection. As for gp120ADA administration, TNFα levels were elevated up to 6hr post injection and returned to baseline within 24hr post injection. Levels of iNOS appeared
most responsive in the frontal cortex after LPS and gp120\textsubscript{ADA} administration and remained elevated up to 24hr post injection, but returned to baseline by the end of the MWM in both LPS and gp120\textsubscript{ADA} treated rats.

Due to the nature of the compounds that were administered (i.e., bacterial vs. viral) dramatic differences in the cytokine profile were observed in our study. It is possible that the lack of gp120-induced impairments in the MWM is the result of the modest increase in inflammatory cytokines, compared to the LPS positive control. LPS is recognised by the TLR4 receptor and elicits an inflammatory response via multiple signaling pathways including p38 MAPK, ERK1/2, JNK and NFκB (Kawai \textit{et al.}, 1999). Furthermore, the physiological role of IL-1β and TNFα are slightly different although both are well known inflammatory cytokines that are elevated upon LPS administration. IL-1β signals through the type I IL-1 receptor/IL-1 accessory protein complex, leading to NFκB-dependent transcription of pro-inflammatory cytokines and chemokines (Cullinan \textit{et al.}, 1998, Ikejima \textit{et al.}, 1990). This allows it to act as an early inflammatory mediator to recruit other pro- or anti-inflammatory molecules as part of the innate immune response. On the other hand, TNFα signals through the TNF-receptor 1 or TNF-receptor 2 resulting in the recruitment of several signaling proteins such as TRADD, RIP1 and FADD (Brenner \textit{et al.}, 2015). TNF-induced apoptosis may be mediated by interaction of adapter proteins TRADD and FADD leading to the activation of caspase-8 (Bender \textit{et al.}, 2005, Ermolaeva \textit{et al.}, 2008). Caspase-8 is an apoptotic initiator protein that cleaves pro-caspase-3 into its physiologically active state (Stennicke \textit{et al.}, 1998). In our rat model, LPS treatment elicited a higher inflammatory response that persisted longer than gp120-administration. It is likely that the dramatically different inflammatory cytokine production profile observed in our study contributed to the results obtained in the MWM studies.

Notably, other markers of persistent inflammation have also been documented in late stage HAND patients including widespread reactive astrocytosis, activated resident microglia, monocyteid and blood derived macrophage infiltration (Kaul, 2008). In both HIV-1 gp120 and LPS infected states, activated monocytes elicit an immune response commonly characterised by the presence of pro-inflammatory cytokines (e.g., TNFα, IL-1β, IL-6, IL-8), other neurotoxins (e.g. arachidonic/quinolinic acid and metabolites, platelet activating factor, neurotoxic amines, and glutamate), and the activation of neighbouring astrocytes that further contributes to inflammation (Garden, 2002). Since neuronal cells express TNF-receptors on the surface membrane, it is likely that they are more sensitive to TNFα-associated neurodegeneration (Kaul, 2008). Significant neuronal loss have been
reported in in vivo studies as a result of central TNFα and/or IL-1β administration (Bjugstad et al., 1998, Carvey et al., 2005, Oitzl et al., 1993). In addition to neurotoxicity, TNFα can exacerbate inflammation by promoting the release of various reactive oxygen or nitrogen species (i.e., ROS or RNS) in activated glial cells and astrocytes (Mir et al., 2008). High levels of iNOS expression were reported in brain tissue isolated from HIV patients with encephalitis and localized particularly in microglial nodules and reactive astrocytes (Zhao et al., 2001). In addition to neurotoxicity, TNFα can exacerbate inflammation by promoting the release of various reactive oxygen or nitrogen species (i.e., ROS or RNS) in activated glial cells and astrocytes (Mir et al., 2008). Molecules such as superoxide anion radical (O2^-), hydroxyl radical (OH), and hydrogen peroxide (H2O2), nitric oxide (NO), inducible nitric oxide synthase (iNOS), can oxidise vital biomolecules such as lipid membranes, proteins, and nucleic acids, resulting in subsequent neuronal injury and cell death (Fischer and Maier, 2015, Kaul et al., 2001). High levels of iNOS expression were reported in brain tissue isolated from HIV patients with encephalitis and localized particularly in microglial nodules and reactive astrocytes (Zhao et al., 2001). We observed most pronounced levels of iNOS in the frontal cortex up to 24hr post injection of LPS, and to a lesser extent gp120. These studies are consistent with our observations in the MWM where the degree of inflammation and oxidative stress in the brain may reflect the extent of neuronal injury; thus, we sought to quantify and localize caspase-3 expression.

Many groups have evaluated neuronal death using a well-known apoptotic marker, caspase-3. Upon proteolytic cleavage at aspartic acid position 177, activated caspase-3 represents an early-stage biomarker that irreversibly commits the cell to apoptosis (Li and Yuan, 2008, Shalini et al., 2014). In our model, gp120ADA treatment did not result in a significant increase in caspase-3; this lack of effect may be due to dose, duration and/or strain differences of the gp120 treatment we used as well as the route of administration (i.e., icv, intrahippocampal, intrastratal, etc.) as such illustrated by Nosheny et al. and Acquas et al., (Acquas et al., 2004, Nosheny et al., 2004). As for LPS treatment in our model, we observed significantly high expression of activated caspase-3, predominantly in the neuronal cell bodies of the hippocampal DG. Our observation is in agreement with previous studies that reported central administration (i.e., icv) of up to 50μg LPS results in several immunoreactive neurons for activated caspase-3 in the cerebral cortex at 24-72hr post injection (Song et al., 2014). Since significant monocyte activation have been documented as the result of chronic LPS infusion (i.e., 74 days) into the fourth lateral ventricle, particularly in the CA3-CA4 region of the hippocampus (Hauss-Wegrzyniak et al., 2000), it is reasonable to suggest that hippocampal neurons are highly
sensitive to inflammation. In this model, we also observed that neurons of the hippocampal DG were highly susceptible to LPS administration, compared to gp120. Furthermore, in a Parkinson’s disease model administering a single dose of 5μg of LPS into the substantia nigra of rats resulted in significant neurotoxicity and several behavioural abnormalities (Sharma and Nehru, 2015). In the mid-brain of rats significant microglial/astrocyte activation as well as elevated levels of inflammatory cytokine transcripts (i.e., TNFα and IL-1β) up to 21 days post injection were reported. Marked increases in activated caspase-3 expression, suggesting the induction of DNA fragmentation and apoptosis sufficient for the appearance of behavioral abnormalities (e.g., total locomotor activity, catalepsy, rota rod, fault foot placing, beam balance and grid walking) were also reported (Sharma and Nehru, 2015). This is in line with our observations wherein marked increases of activated caspase-3 in neuronal cell bodies of the hippocampus DG following LPS administration was sufficient for the appearance of spatial deficits in learning and memory in rats.

3.2 Study Limitations

There are various explanations for the lack of gp120-mediated behavioural deficits in our model. The primary limitation in our study was the source of gp120; we administered the monomeric purified recombinant protein purchased from ImmunoDX, LLC (Woburn, Massachusetts). While basic quality control parameters are employed by the company, we have observed variable responses between shipment and lots of gp120. This suggests that the level of protein activity was not consistent between experimental replicates in our studies, possibly contributing to inter-rat variability. An alternative is to utilise expression vectors as a means of maintaining a consistent level of gp120 activity. An SV40-derived gene delivery vector that express gp120 in the rat brain has been developed by Louboutin et al., (Louboutin et al., 2009) who has reported various signaling pathways of gp120 and the potential to implement antioxidant compounds as HIV-1 adjuvant therapy (Louboutin et al., 2010a, Louboutin et al., 2010b, 2011). It would be interesting to examine the behavioural phenotype of this animal model in various learning paradigms (e.g., MWM, contextual fear, open field etc.). Secondly, the administration of an isolated viral envelope protein may not have been sufficient. In physiological conditions viral proteins act in concert for successful HIV-1 replication. In addition to gp120, other viral proteins that have been shown to be neurotoxic include Tat (Liu et al., 2013), vpr (Torres and Noel, 2014) and nef (Chompre et al., 2013). HIV-1 tat has recently been shown to induce an inflammatory response in cell systems through the interaction with surface receptor TLR4-MD2-CD14 complex resulting in the activation of the NFkB signalling
pathway (Ben Haij et al., 2015). Since gp120 interacts with the chemokine co-receptors and activates the MAPK pathway (Kaul and Lipton, 2006), co-administering gp120 and Tat likely results in a synergistic effect. In a primary culture of human foetal neurons, significantly higher number of neuronal death and higher levels of intracellular Ca^{2+} concentration were observed when recombinant Tat_{BRU} (60nM) and gp120_{BRU} (30pM) were incubated together (Nath et al., 2000). Thus it is likely that the presence of neurocognitive deficits requires a synergistic effect of various viral components that cannot be replicated by administering an isolated protein in an acute model.

In addition, there are various strains of gp120 which has been employed in previous studies (i.e., gp120_{ADA}, gp120_{MN}, gp120_{LAV} etc.), as well as high inter-rat variability and rat strain differences which may contribute to the discordant results. In general, HIV is classified into types (HIV-1 or HIV-2), groups (HIV-1 M, N, and O), and then subtypes (or clades) on the basis of genetic relatedness. There are nine genetically distinct subtypes (i.e., A–D, F–H, J, and K) of HIV-1 that give rise to the various strains of HIV-1, and in turn gp120 (Kuritzkes, 2008). These subtypes may impact the degree of infectivity and have been considered as a determinant of disease progression; for example, it has been shown in patients that infections involving HIV-1 subtype D causes a faster decline of plasma CD4+ cells than subtype A (Baeten et al., 2007). In our study, gp120_{ADA} belongs to subtype B. Furthermore, rodent models of human disease may be limited to clinical applicability (Jucker, 2010), thus non-human primates such as macaques could provide a more appropriate model in the case of HIV-associated inflammation and cognitive deficits. Studies have examined various CNS immune markers that are thought to mediate neuroinflammation in pigtailed macaques infected with SIV, a similar virus to HIV which causes immunodeficiency, highlighting potential biomarkers that can be targeted for therapy (Gerngross et al., 2015, Roberts et al., 2010, Williams et al., 2008). However, employing a macaque’s model involves highly specific facilities and means that are not available at our institution. Therefore, murine models remain as a convenient and cost-efficient method of modeling gp120 induced neuroinflammation. Lastly, in this model, the lack of effects in cellular death and behavioural observations may be the result of rapid gp120 clearance from the CNS. It has been shown using radiolabeled gp120_{SF2} (i.e., I-gp120) administered icv in mice that the half-time disappearance of I-gp120 was approximately 12.6min (Cashion et al., 1999). Accordingly, we suggest the consideration of alternative gp120 regimens such as higher dosing, repeated daily administration, or chronic infusion using an osmotic pump. Once a robust model of HIV-1 gp120-induced neurocognitive impairment has been established, the use of anti-inflammatory compounds
or other potential adjuvant therapies can be evaluated using the MWM protocol developed and optimized in this study.

3.3 Future Directions

Our acute rat model mimics biochemical changes during the early period of HIV-1 infection and did not result in neurocognitive deficits. However, using the framework and MWM protocol developed in this study, additional investigations employing alternative methods of gp120 administration could be validated. Developing an acute rodent model may provide a potential tool to evaluate early administration of adjuvant therapy (e.g., anti-inflammatory treatment) in delaying/preventing cognitive dysfunctions in HIV-1 infected patients. Although clinical trials have suggested the use of anti-inflammatory adjuvant therapy, this treatment approach was demonstrated to be ineffective at reversing cognitive impairments. For example minocycline, a second generation tetracycline derivative known to elicit neuroprotective effects, was administered in a clinical trial and did not demonstrate significant effects in decreasing CSF viral load in seropositive patients (Ho et al., 2011) or improving neuropsychological test composite z-scores (Nakasujja et al., 2013, Sacktor et al., 2011). Similarly, chloroquine, a widely used anti-malarial compound with anti-inflammatory properties, did not reveal cognitive improvements in an adult English population (n = 83) from the United Kingdom (Paton et al., 2012), a paediatric population (n = 55) from Thailand (Engchanil et al., 2006) or in an adult Canadian population (n = 19) receiving ARVs (Routy et al., 2014). However in simian immunodeficiency virus (SIV) macaque models, administration of minocycline, early in infection, significantly reduced biochemical markers associated with encephalitis, CNS/CSF viral load, glial activation, brain metabolites and neuronal integrity (Drewes et al., 2014, Meulendyke et al., 2012, Ratai et al., 2010, Zink et al., 2005). In contrast patients enrolled in previous clinical trials have often progressed into advanced cognitive deficits (i.e., chronic infection), thus administering adjuvants (e.g., anti-inflammatory compounds) during early infection may help prevent or delay deficits and potentially lessening the severity of HIV-associated neurocognitive deficits.

3.4 Conclusion

In summary, using an in vivo gp120-induced neuroinflammation model, we have demonstrated significant levels of pro-inflammatory cytokines and oxidative stress marker in the brain. Since administering a single dose of R5 tropic gp120\textsubscript{ADA} did not result in significant caspase-3 activation as evaluated by immunoblotting and immunoperoxidase staining, and no significant spatial
impairments were observed in MWM escape latency, alternative gp120 regimens should be explored such as higher dosing, repeated daily administration, or chronic infusion using a mini-osmotic pump. Once a robust model of HIV-1 gp120-induced neurocognitive impairment has been established, the effect of reversing or delaying neurocognitive deficits using anti-inflammatory compounds (e.g., minocycline, chloroquine), or other potential adjuvant therapies can be evaluated by a spectrum of behavioural tasks including the MWM protocol developed and optimized in this study.
APPENDICES

Appendix A: Cued Fear Conditioning in gp120MN Treated Mice

Cued or contextual fear represents a form of associative learning developed from Burrhus Frederic (B. F.) Skinner’s stimulus-response (SR) theory (Maren et al., 2013). It is important to establish the three main elements associated with fear conditioning: a conditioned response (CR), an unconditioned stimulus (US) and a conditioned stimulus (CS). For example, a dominant defensive fear response in rodents is crouching behaviour that is often accompanied by immobility, shallow breathing increase in heart rate, urination and pilo-erection (Blanchard and Blanchard, 1969). When exposing an animal to a novel environment, the paired presentation of a US (e.g., footshock or air puff) with a CS (e.g., audible sound), will induce a behavioural response (i.e., freezing) suggesting the animal has associated the testing environment (context) and the auditory cue with fear. Repeated paired US-CS presentation conditions the animal to exhibit freezing upon returning to the context or subjected to the auditory cue in a dissimilar context (Rudy et al., 2004). In brief, these behavioural assessments are typically carried out in a foot shock chamber illuminated by a single overhead light with electrifiable stainless steel bar flooring and a speaker mounted on the side wall of the chamber. Baseline freezing behaviour is measured prior to CS-US presentation. The CS (e.g., 65dB at 2900Hz for 30sec) is followed by the US (e.g., mild shock around 2sec, 0.7 mA delivered through the floor of the chamber) which results in a post-shock freezing behaviour that is measured. This cycle of stimulus presentation with the unconditioned stimulus can be presented two or more times for conditioning, but may require optimization depending on the project. Contextual cued fear assessments can be performed following CS-US conditioning by returning subjects to the original training context. Successful condition should increase freezing behaviour as fear was associated with the environment. Auditory cued fear assessments can be performed following CS-US conditioning by placing the subject in a contextually different chamber. Successful conditioning should result in increased freezing behaviour upon presentation of the CS (i.e., 65-dB; 2900-Hz). Figure A-14 illustrates a typical three day protocol assessing contextual fear and auditory-cued fear.
Fear conditioning has been explored in both rats and mice without a clear bias for either species, possibly due to the similar forms of defensive response that can be easily measured to an aversive stimuli such as freezing (Blanchard and Blanchard, 1969, Burton and Johnson, 2012, Goosens and Maren, 2001, Schimanski and Nguyen, 2004). As for sex and strain differences, there appears to be a significant increase in freezing frequency and duration in females compared to male when exposed to frightening stimuli (Archer, 1975). In terms of conditioning, it appears that male rodents are more easily conditioned (i.e., shorter shocks and exposure to context) and exhibit higher levels of contextual freezing than females (Gupta et al., 2001, Maren et al., 1994, Wiltgen et al., 2001). However, Pryce et al., reported that only Fischer rats, but not Wistar or Lewis rats, exhibited more freezing in males than females for both context and tone cued fear, possibly suggesting sex differences could be strain-specific (Pryce et al., 1999).

In adult mice, it has been documented that icv injection of 100ng R5-tropic HIV-1_{SF162} gp120 is able to induce decreased locomotor activity by approximately 52% (Abraham et al., 2008) as well as modestly impair avoidance acquisition and retention (Farr et al., 2002). Far et al., (2002) assessed the effects of adult male CD-1 mice (8-10 weeks) in a T-maze footshock avoidance paradigm after administrating a single high dose of 0-2.5ng of dual/X4-tropic HIV-1 gp120_{SF2} in the right lateral ventricle, which resulted in significantly impaired learning and memory. We proposed a protocol similar to Farr et al. (2002) to evaluate the effect of gp120 in avoidance acquisition and retention using the contextual fear chamber. Anesthetized mice were administered icv a single 10-100ng dose of X4/dual-tropic gp120_{MN} using a 5μl Hamilton syringe (maximum volume 2μl; rate 1μl/minute) to the right lateral ventricle at the following coordinates according to The Allen Reference Atlas:
bregma -0.3mm, lateral 1.0 mm and 2.5mm ventral from the surface of the skull. Wildtype animals (no surgery) and saline treated animals were used as experimental and treatment controls respectively.

In two separate experiments using the in-bred strain, C57BL6 mice, we sought to investigate a hippocampal-dependent learning and memory paradigm using the contextual fear chambers. On the first day of training where the animals were exposed to the apparatus for the first time, we expected that the percentage of time spent freezing after a tone-shock pairing should be evidently higher than before tone-shock pairing; this was the general trend observed for all treatment groups except for 25ng HIV-1MN gp120-treated animals (Figure A-15a). Interestingly, for this group, the 0.2mA shock did not appear to induce freezing behaviour; a difference that reached statistical significance. On day two, the trained animals were placed back into the chambers to observe contextual fear memory (Figure A-15b). It is expected that well consolidation during training would induce significant amount of freezing when the animal is placed back in the ‘fearful’ chambers. However, this was not observed; no significant differences in freezing behaviour was detected in all treatment groups (Figure A-15b). Lastly, on day three where trained animals were exposed to a novel chamber and presented with the auditory-tone, we expected to observe a high degree of freezing after the cued stimulus than before (Figure A-15c). A high degree of freezing post-sound indicates that the animals successfully consolidated the fearful experience (i.e. shock) with the cued stimulus (i.e. tone), and thus, showing no impairments in memory. This was observed in the wildtype and saline control. As for gp120-treated animals, there appears to be a trend for memory impairments using HIV-1MN gp120 (Figure A-15). By exhibiting a low freezing time in response to the cued stimulus (i.e., tone), this is indicative that the mice did not successfully sustain the memory. Unfortunately, this trend did not reach statistical significance with the use of a higher number of animals (n = 9-10; figure A-15). In addition, the lack of behavioural deficits measured by contextual fear chambers could be due to the lack of inflammatory response in our mice model.
In other mice models of gp120-induced cognitive deficits, the time to training after injection varies greatly; ranging from 24h-48h (Abraham et al., 2008, Li et al., 2004, Pugh et al., 2000). Therefore, in another set of experiments using a high dose of dual/X4-tropic HIV-1MN gp120 at 100ng, we examined the effects of shortening this delay to 24h (Figure A-16). Possibly due to the insufficient number of animals examined (n = 4), we did not observe any significant differences between groups, possibly due to minimal to no memory impairments.
Figure A-16 Effect of HIV-1MN gp120 icv administration in the right lateral ventricle of C57BL6 mice on contextual-fear conditioned freezing behaviour 24 hours after injection. Behavioural experiment is performed over three days as illustrated: (a) training (1 day after injection), (b) contextual fear (2 days after injection) and (c) auditory-cued fear (3 day after injection). All groups are representative of n = 4. Results are expressed as mean±SEM.

Appendix B: Morris Water Maze Massed Training of gp120 Treated Rats

There has been three previous publications that characterized cognitive deficits, or the lack thereof, using the Morris Water Maze by gp120-administered rats (Bjugstad et al., 2004, Glowa et al., 1992, Tang et al., 2009). We examined the changes in latency time of 8ug HIV-1ADA gp120-treated animals in two separate experiments (Figure A-17). In a three-trial-per-day-four-day training design, we were unable to discern significant differences in spatial acquisition between LPS and gp120 treated groups compared to saline-treated animals (Figure A-17). It appears that all treatment groups, except saline, was able to effectively learn the position of the submerged platform as indicated by decreased latency time (i.e., learning curve) across the four day training (Figure A-17a). After four
consecutive days of training, a probe test was carried out to examine the degree of spatial retention (i.e., memory). It appears all groups spent a similar amount of time in the target quadrant (i.e., the quadrant that formally contained the platform) searching for the platform suggesting no variation in spatial retention (Figure A-17). There was no effect of gp120 administration; no significant difference in percentage of time spent in target quadrant and number of entries into platform zone. The rats of the saline group less frequently crossed into the target zone compared with other groups, however, this trend did not reach statistical significance (Figure A-17). Furthermore, there was no significant preference for the target quadrant in all treatment groups (data not shown). It is important to highlight that we do not have a sufficient statistical power to discern differences at the behavioural level due to a low number of animals per treatment group (n = 6-7).

Figure A-17 Effect of 8ug HIV-1ADA gp120 icv administration in both lateral ventricles of Wistar rat on learning and memory in the Morris Water Maze. Behavioural experiment spans over 5 days as followed: (a) Depiction of learning curve over 4 days of training, 3 trials per day. (b-e) Specific latency times acquired by individual rat on each training day showing variability within groups. Performance during probe test examining spatial memory as (f) percentage of time spent in target quadrant and (d) number of times zone was entered. Results are expressed as mean±SEM. All groups are representative of n = 6-7. Asterisk represents data point significantly different from saline-treated animals (* p <0.05).

Appendix C: Enzyme-Linked Immunosorbent Assay (ELISA) in Plasma and CSF

We sought to detect IL-1β, IL-6 and TNFα secretion levels in plasma and CSF samples collected by arterial blood samples and cisterna magna puncture, respectively. Cytokine molecules are measured using commercially available rat IL-1β, IL-6 and TNFα ELISA kits (R&D Systems, MN). The assays were performed according to the manufacturer’s instruction with slight modifications. The detection limit of the assay is typically less than 5pg/ml according to the manufacturer’s data. After adding samples
to the plate, the plate was sealed and incubated at 4°C overnight. The following day, wells were washed HRP-conjugates were added to the wells and again incubated at 4°C overnight. The remaining procedure was carried out according to the manufacturer’s protocol. As outlined by the manufacturer, standard curves were generated using serial dilutions of appropriate rat cytokines and measuring absorbance at 450nm corrected with absorbance at 570nm.

Figure A-18 ELISA analysis of IL-6 secretion in serum collected from Wistar rats treated with 8μg HIV-1_ADA gp120 or 50μg LPS icv administered in both lateral ventricles. Results expressed in mean±SEM of n = 2 per group performed in triplicates from one experiment. Statistical significance will be calculated based on additional replicated experiments.
Figure A-19 ELISA analysis of TNFα secretion in serum collected from Wistar rat treated with 8μg HIV-1ADA gp120 or 50μg LPS icv administered in both lateral ventricles. Results expressed in mean±SEM of n = 2-3 per group performed in duplicates from one experiment.

Figure A-20 ELISA analysis of TNFα secretion after 24hr in the CSF of 8μg HIV-1ADA gp120 or 50μg LPS administered rats. Results are expressed as mean±SEM. Results expressed in mean±SEM of wildtype (n = 1), saline and gp120ADA (n = 4), LPS (n = 1) performed in duplicates from one experiment. Due to the high technical difficulties associated in obtaining CSF samples, only a very small number CSF samples were collected.
Appendix D: Neurofilament Light in Hippocampus and Frontal Cortex

An emerging biomarker that demonstrates a better predictability of subclinical and clinical cognitive impairment is the light subunit of the neurofilament protein (NFL). NFL is a structural protein uniquely expressed in neurons that can be used to estimate the amount of neuronal damage in various CNS disorders (Norgren et al., 2005). In population-based studies, significantly increased NFL levels in the cerebral spinal fluid (CSF) of HIV infected patients have been consistently reported (Abdulle et al., 2007, Jessen Krut et al., 2014, Peluso et al., 2013, Peterson et al., 2014). Furthermore, upon receiving antiretroviral treatment, elevated levels of NFL are reduced (Mellgren et al., 2007). However, in the context of HIV, NFL appears to be a predictive marker predominantly in humans and has not been thoroughly documented in rodent models. In our model, an exploratory study of NFL gene expression in the frontal cortex and hippocampus did not yield significant results. Exploratory analysis of neurofilament light (NFL) gene expression in the frontal cortex and hippocampus did not yield consistent results; elevated levels were detected in the hippocampus and depressed levels were observed in the frontal cortex, this may suggest NFL as a region-specific marker in other CNS disorders. Interestingly, decreased mRNA levels were reported in an axonal hypotrophy rat model proposed in Montreal, Canada (Kuchel et al., 1997). The lack of literature reports of NFL in rats suggests this biomarker may be limited to human investigations.

Figure A-21 Effect of gp120 and LPS treatment on the mRNA levels of neurofilament-L in the a) hippocampus and b) frontal cortex at different time points post injection in adult Wistar rats. Treatments were administered in a total of 8μl bilateral icv (8μg of gp120; 50μg of LPS). Wildtype and saline injected animals were also analysed. Results are expressed as mean±SEM relative to saline controls; all groups n = 3-13. Asterisk represent data point significantly different from saline administered animals at respective time points (*** p < 0.001; ** p < 0.01; * p < 0.05).
Appendix E: Book Chapter as Published in Pharmacology of the Blood-Brain Barrier


List of Publications & Conference Oral/Poster Presentations

Publications


Conference Oral/Poster Presentations


- Amy Chia-Ching Kao, Chiping Wu, Md. Tozammel Hoque, Reina Bendayan. An In Vivo Rat Model of HIV-1 Associated Brain Inflammation and Neurocognitive Deficits. Poster #BS57 at Canadian Association for HIV Research, Toronto, Canada 30 Apr 2015 – 3 May 2015.


- Amy Kao, Reina Bendayan. Potential Role of Anti-inflammatory Drugs in a HIV-1 gp120 brain inoculated neurocognitive deficit rodent model. Presentation at Canadian Institute of Health Research Strategic Training Program in Biotherapeutics 2013 - 2014 Mentor - Trainee Seminar Series, Leslie Dan Faculty of Pharmacy, University of Toronto, 5 Jun 2013.