The Potential Role of Antiretroviral Efavirenz in HIV Associated Neurocognitive Disorders

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The Potential Role of Antiretroviral Efavirenz in HIV Associated Neurocognitive Disorders

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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March 23, 2017

Keywords: HIV associated neurocognitive disorders, efavirenz, beta amyloid, neural stem cell proliferation

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DEDICATION

I dedicate this dissertation to:

My grandmother, Ruth-Ann Schoberg- Mama, you continue to inspire me to be more day in and day out. Without your strong upbringing I would not be where I am today.

My sister, Litz-Ann M. Brown- LAMB1, you believed in me from the beginning, and never allowed me to look back. You truly are more than a sister; you are my friend, my psychologist, my supporter, my mentor, my guidance, my rock. This journey would not be the same without you (double tap).

To my family and friends that put in work & time as my 5 AM to late night supporters/motivators … Thank you everyone no matter where in the world you are. You always continue to be there for me no matter what! I am sure you have learned way more about HIV associated neurocognitive disorders and neuroscience than you ever wanted to.

Finally…. Thank you my Lord and Savior, He has made all of this possible.

   The Lord is my strength and my shield; My heart trusts in Him, and I am helped;

   Therefore my heart exults, And with my song I shall thank Him. (Psalm 28:7)
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<th>Description</th>
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<tbody>
<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>Aβ</td>
<td>Beta Amyloid</td>
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<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADC</td>
<td>AIDS Dementia Complex</td>
</tr>
<tr>
<td>ADLS</td>
<td>Acts of Daily Living</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>ANI</td>
<td>Asymptomatic Neurocognitive Impairment</td>
</tr>
<tr>
<td>ARVs</td>
<td>Antiretrovirals</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ART</td>
<td>Antiretroviral Therapy</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis Signal Regulating Kinase 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AZT</td>
<td>Zidovudine</td>
</tr>
<tr>
<td>BACE-1</td>
<td>β-secretase</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-Associated X Protein</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>cART</td>
<td>Combination Antiretroviral Therapy</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CTF</td>
<td>Carboxyl-Terminal Fragment</td>
</tr>
<tr>
<td>DAT</td>
<td>Dementia of the Alzheimer Type</td>
</tr>
<tr>
<td>DHHS</td>
<td>Department of Health and Human Services</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>DRV</td>
<td>Darunavir</td>
</tr>
<tr>
<td>DTG</td>
<td>Dolutegravir</td>
</tr>
<tr>
<td>EFV</td>
<td>Efavirenz</td>
</tr>
<tr>
<td>eIF2a</td>
<td>Eukaryotic Initiation Factor-2α</td>
</tr>
<tr>
<td>ELV</td>
<td>Elvitegravir</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HAD</td>
<td>HIV Associated Dementia</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV Associated Neurocognitive Disorders</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HIV-E</td>
<td>HIV Encephalitis</td>
</tr>
<tr>
<td>hPreP</td>
<td>Human Presequence Protease</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin-Degrading Enzyme</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>InSTI</td>
<td>Integrase Strand Transfer Inhibitor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>LRP</td>
<td>Lipoprotein Receptor-related Protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial Membrane Potential</td>
</tr>
<tr>
<td>MMP9</td>
<td>Matrix Metalloproteinase 9</td>
</tr>
<tr>
<td>MND</td>
<td>Mild Neurocognitive Impairment</td>
</tr>
<tr>
<td>NEP</td>
<td>Neprilysin</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-Nucleoside Reverse Transcriptase Inhibitor</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural Stem Cell</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Protease Inhibitor</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein Kinase RNA</td>
</tr>
<tr>
<td>p-tau</td>
<td>Phospho-Tau</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycosylation End products</td>
</tr>
<tr>
<td>RAL</td>
<td>Raltegravir</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SGZ</td>
<td>Sub-Granular Zone</td>
</tr>
<tr>
<td>SVZ</td>
<td>Sub-Ventricular Zone</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>t-tau</td>
<td>Total Tau</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
ABSTRACT

The prevalence of milder forms of HIV-associated neurocognitive disorders (HAND) is rising despite combination antiretroviral therapy (cART). Efavirenz (EFV) is among the most commonly used antiretroviral drugs globally, but causes neurological symptoms that may interfere with adherence and reduce tolerability, and may have central nervous system (CNS) effects that contribute in part to HAND in patients on cART. Thus we evaluated a commonly used EFV containing regimen: EFV/zidovudine (AZT)/lamivudine (3TC) in murine N2a cells transfected with the human “Swedish” mutant form of amyloid precursor protein (SweAPP N2a cells) to assess for promotion of amyloid-beta (Aβ) production (Chapter 3). Treatment with EFV or the EFV containing regimen generated significantly increased soluble Aβ, and promoted increased β-secretase-1 (BACE-1) expression while 3TC, AZT, or vehicle control did not significantly alter these endpoints. Further, EFV or the EFV containing regimen promoted significantly more mitochondrial stress in SweAPP N2a cells as compared to 3TC, AZT, or vehicle control. We next tested the EFV containing regimen in Aβ-producing Tg2576 mice combined or singly using clinically relevant doses. EFV or the EFV containing regimen promoted significantly more BACE-1 expression and soluble Aβ generation while 3TC, AZT, or vehicle control did not. Finally, microglial Aβ phagocytosis was significantly reduced by EFV or the EFV containing regimen but not

1 Portions of this abstract have been previously published (Brown LAM, et al., 2014; Jin, J, et al, 2016) and are utilized with permission of the publisher.
by AZT, 3TC, or vehicle control alone. These data suggest the majority of Aβ promoting
effects of this cART regimen are dependent upon EFV as it promotes both increased
production, and decreased clearance of Aβ peptide.

Further (Chapter 4), there is evidence that neural stem cells (NSCs) can migrate
to sites of brain injury such as those caused by inflammation and oxidative stress, which
are pathological features of HAND. Thus, reductions in NSCs may contribute to HAND
pathogenesis. Since the HIV non-nucleoside reverse transcriptase inhibitor EFV has
previously been associated with cognitive deficits and promotion of oxidative stress
pathways, we examined its effect on NSCs in vitro as well as in C57BL/6J mice. Here
we report that EFV induced a decrease in NSC proliferation in vitro as indicated by MTT
assay, as well as BrdU and nestin immunocytochemistry. In addition, EFV decreased
intracellular NSC adenosine triphosphate (ATP) stores and NSC mitochondrial
membrane potential (MMP). Further, we found that EFV promoted increased lactate
dehydrogenase (LDH) release, activation of p38 mitogen-activated protein kinase
(MAPK), and increased Bax expression in cultured NSCs. Moreover, EFV reduced the
quantity of proliferating NSCs in the subventricular zone (SVZ) of C57BL/6 mice as
suggested by BrdU, and increased apoptosis as measured by active caspase-3
immunohistochemistry. If these in vitro and in vivo models translate to the clinical
syndrome, then a pharmacological or cell-based therapy aimed at opposing EFV-
mediated reductions in NSC proliferation may be beneficial to prevent or treat HAND in
patients receiving EFV.
Human Immunodeficiency Virus (HIV)

Human Immunodeficiency Virus (HIV) is the etiological agent causing Acquired Immune Deficiency Syndrome (AIDS). An estimated 35 million people are living with HIV worldwide, with roughly 2.4 million infected each year [1]. HIV is a retrovirus belonging to the lentivirus family, and it uses reverse transcriptase to transcribe RNA into DNA prior to incorporation into the host cell chromosome [2]. The origin of the word *lentus* means slow, referring to the slow course of disease progression associated with HIV infection [2, 3]. This slow progression is what leads to the wasting characteristic of the disease as it causes immune deficiency, neurodegeneration, and death. The complexity of a lentivirus, especially HIV, sets it apart from other viral genomes. Not only does HIV encode for the typical 3 genes of env, gag and pol, but it also has regulatory genes (tat, rev, nef) and auxiliary genes (vif, vpr, vp4) [4, 5]. As aforementioned, HIV affects the world globally; however, there are different types and subtypes most prevalent within a given area. HIV is divided into 2 groups: HIV-1 and HIV-2. HIV-2 is similar to Simian Immunodeficiency Virus, and is restricted to regions within West Africa [6]. HIV-1 however, is the most common type worldwide.
**HIV Replication Cycle**

Entry of the virus into the target cell occurs when the HIV envelope protein gp120/gp41 binds to CD4+ immune cells, in addition to a chemokine co-receptor (CCR5 or CXCR4) [7-9]. Viral entry and fusion aids in uncoating the viral core so as to allow reverse transcription and proviral synthesis [10]. Reverse transcriptase has RNA dependent DNA polymerase, ribonuclease (RNase)-H, and DNA dependent DNA polymerase functionalities, required for HIV viral transcription [11]. A key part of viral processing is the completion of reverse transcription in order to form a pre-integration complex (PIC) composed of cellular and viral components, which migrate to the nucleus. In the nucleus, the HIV integrase begins incorporating the viral DNA with the host DNA [12]. Integration of the HIV DNA is important for HIV mRNA/RNA expression; once integrated into the host genome, transcription can occur through the binding of the HIV regulatory protein Tat to the HIV trans-activation response element (TAR) [13]. Following, new viral RNA and viral proteins move to the cell surface to assemble into new immature virus forms to buds off and release. Assembly and maturation on the inner plasma membrane is the next step of the HIV virus. During maturation, the protease enzyme cleaves the structural polyprotein to form mature Gag proteins, resulting in the production of new infectious virions [14].

**HIV and the Central Nervous System (CNS)**

HIV enters the CNS early post-infection. This viral presence in the CNS can result in cognitive impairment. Although the virus itself cannot infect the neurons, it is the indirect mechanisms that cause the neurocognitive decline [15, 16]. HIV enters the
CNS either by crossing the blood brain barrier (BBB) in infected lymphocytes and monocytes or by entering the cerebrospinal fluid (CSF) on its own resulting in subsequent cognitive impairment. Although the virus itself cannot infect neurons, HIV causes the decrease in neurocognition through various indirect mechanisms [15, 16]. There are two mechanisms by which HIV infects the CNS: productive and restricted infection. Productive infection involves host cells (monocyte-derived macrophages and microglia) aiding in the viral replication and transmission processes [17-20]. Conversely, restricted infection lies latent in a large number of undividing astrocytes as a reservoir [21-23]. The latency of HIV in astrocytes may be a pertinent factor in neuropathology, as even with adherence to a cART regimen, this form of restricted infection may elude the drugs [24]. Moreover, despite their non-proliferative status, latent HIV-infected cells can express viral proteins and neurotoxins [25, 26], and each class of therapeutic agents has displayed difficulty in crossing the BBB in order to efficiently interact with the virus.

**HIV and Alzheimer’s Disease-like Pathology**

The characterization of neurodegenerative disorders is associated with neuronal loss and accumulation of fibrillary materials. These characteristics are found in head trauma, Down Syndrome, and many other instances including Alzheimer’s disease (AD) [27-29]. AD is the most common type of age-associated dementia accounting for 60% to 80% of all cases [30]. AD is characterized by age-dependent β-amyloid/beta amyloid (Aβ) and tau tangle accumulation, which can eventually impair an individual’s ability to carry out acts of daily living (ADLs). It has been hypothesized that AD will significantly increase among persons living with HIV [31]. Furthermore, studies have shown
evidence of AD-like pathologies in HIV demented patients [32-40]. Our study will explore beta amyloid generation accordingly.

**Beta Amyloid (Aβ)**

Aβ (one of the hallmarks of AD) causes the formation of cerebral plaques prone to the frontal cortex and hippocampus. Aβ causes progressive neuronal loss resulting in the deterioration of the brain’s high and basic functionalities. Amyloid precursor protein (APP) is processed via two pathways: non-amyloidogenic (preventing Aβ production) or amyloidogenic (leading to Aβ production). In the non-amyloidogenic pathway APP is initially cleaved by α-secretase yielding soluble (s)APPα and α-carboxyl-terminal fragment (CTF). α-CTF is then cleaved by γ-secretase to form a 23-25 amino acid peptide (p3) precluding formation of Aβ. Conversely, in the amyloidogenic pathway, APP is initially cleaved by β-secretase (BACE)-1, resulting in β-CTF, and sAPPβ. β-CTF is then cleaved by a multi-protein γ-secretase complex to generate Aβ and γ-CTF [41]. Aβ42 represents about 10% of Aβ produced with a greater propensity to form neurotoxic oligomeric and aggregated species. Aβ40, however, is the most common form in the human brain [42, 43].

In addition to the accumulation of Aβ in the brain, the clearance of this protein is a matter to be discussed further in this research. In humans, microglia compose up to 16% (5-12% in rodent) of the cells in CNS and account for one of the main mechanisms by which Aβ is cleared [44]. Microglia express receptors that promote the clearance of Aβ including class A scavenger receptor (SR-A), CD36 and receptor for advanced
glycosylation end products (RAGE) [45, 46]. In addition, microglia secrete proteolytic enzymes that degrade Aβ such as insulin-degrading enzyme (IDE), neprilysin (NEP), matrix metalloproteinase 9 (MMP9), and plasminogen [47, 48]. This suggests the important role of microglial in neuroprotection against Aβ.

Though an excess of Aβ can elicit negative effects, it is a natural product of metabolism that can be monitored when production and degradation are balanced. In fact, studies have shown that low physiological levels of Aβ enhance learning and retention. Indeed, Aβ enhances arborization of dendritic-like processes [49], neurite growth [50], and cell survival [49]. Nanomolar doses of Aβ were injected into the hippocampus of mice that later experienced cognitive dysfunction [51-56]. However, when physiological (i.e. picomolar) doses were applied, there was improvement in T-maze foot shock avoidance task, object recognition, Morris water maze and contextual fear memory test for mice [57-60]. Other studies have shown that picomolar doses of Aβ increase long-term potentiation. It is, however, still not clear as to what triggers the amyloidogenic pathway and distribution of Aβ, but different Aβ forms can localize in different areas of the cell (endoplasmic reticulum [ER], mitochondria, endosome, nucleus and Golgi) [61-63]. Additionally, clearance of Aβ has been shown to be important in homeostasis as mentioned previously via enzymes like NEP, IDE, cathepsin, and human presequence protease (hPreP). Notably, all of these are found at low level and with lower activity in AD brain and AD transgenic mouse models [64, 65].
Reactive Oxygen Species (ROS) and Aβ

Mitochondria generate adenosine triphosphate (ATP) by using the electron transport chain (ETC), in order to provide energy to drive protons against their concentration gradient. The force itself driving the proton into the mitochondria includes MMP (charge/electrical gradient), which in turn drives the synthesis of ATP [66]. During aging, there is a decrease in mitochondrial activities and an increase in ROS presence triggering Aβ synthesis [67-69]. In order for cells to function efficiently, they must have healthy mitochondria. Research has shown that increased ROS production is associated with mitochondrial damage, which would result in decreased ATP production. This can lead to neuronal damage, cognitive deficits, and apoptosis.

There is a strong relationship between Aβ and the mitochondria. In fact Aβ has been shown to be associated with and penetrate the mitochondria as intraneuronal soluble proteins before depositing as insoluble, extracellular species [70]. Inside the mitochondria, Aβ has been shown to inhibit mitochondrial function, increase ROS and release cytochrome c [71]. Further studies have shown that ROS increases BACE-1 activity. Tamagno and colleagues showed that oxidative stress induces BACE-1 transcription, thereby promoting the production Aβ. In this study, BACE-1 levels were increased in response to oxidative stress in normal cells, but not in cells lacking presenilins or APP [72]. Clearance and degradation, in addition to production, of Aβ is important in homeostasis and studies have also shown that Aβ degradation and clearance is down-regulated by ROS [73, 74]. Taken together, it becomes imperative to understand further the mechanism by which there is an increase in ROS, and also to
extrapolate a means to understand the mechanism by certain antiretrovirals (ARVs) may induce Aβ accumulation.

Delving deeper into the mechanism, it has been shown that ROS activates pathways leading to BACE-1 expression. Past studies have shown that the phosphorylation of eukaryotic initiation factor-2α (eIF2α) occurs via multiple molecular mechanisms (e.g. protein kinase RNA-activated [PKR]) and halts general translation of proteins, but not BACE-1 translation. This is due to the fact that sustained eIF2α phosphorylation paradoxically causes translational activation of a subset of mRNAs, including BACE-1 [75, 76]. PKR itself can be activated by viral infection or oxidative stress. Therefore EFV could be a strong activator of PKR via ROS production. In addition, it would be advantageous for EFV to induce PKR activation because activated PKR has a strong antiviral activity on HIV-1 expression and production in cell culture and would reduce translation of HIV protein as a protective mechanism. So, this overactivation may indeed lead to BACE-1 increase and Aβ deposition, thus playing a role in HIV associated neurocognitive disorders (HAND) pathogenesis.

EFV is a common ARV prescribed worldwide. Because of its expected long-term use in patients it is important to understand the impact it may have on quality of life. Based on clinical trials that have indicated symptoms from patients related to the CNS and epidemiological studies, which have found Aβ deposits in HIV-infected brains, we will explore the correlation between EFV and Aβ accumulation studies [31-40]. We hypothesize that EFV will increase Aβ production and decrease Aβ clearance. We
developed this hypothesis based on studies that show EFV increases ROS production. ROS production promotes both BACE-1 and can decrease microglial Aβ clearance [72, 77].

Further, mitochondrial function is important to the survival of all cells, and especially so in the neuron whose ability to regenerate/differentiate are limited and still not completely understood. If EFV, a drug prescribed for a life-time, has negative effects on mitochondrial function, it could potentially aid in the development of HAND. We hypothesize that EFV will promote ROS production because it has been shown to inhibit mitochondrial complex I generating ROS in vitro [78]. This can lead to reductions in MMP, and ATP stores. We also expect an increase of BACE-1 enzyme, based on Tamagno et al., who showed that oxidative stress promotes BACE-1 activity [72].

**Neurogenesis**

Neurogenesis previously was a term or concept that was mostly accepted or understood to cease post-natally. It is now understood that adult neurogenesis exists in actuality in the sub-ventricular zone (SVZ) and the sub-granular zone (SGZ) of the dentate gyrus (DG) of the hippocampus [79]. The hippocampus plays a key role in learning and memory and is extremely susceptible to inflammatory insults that affect the brain [79]. Experiments have shown that pro-inflammatory cytokines (like interleukin 6 for example) reduce neural stem cell (NSC) proliferation [80]. Further, high pro-inflammatory cytokine and ROS levels have been shown to induce apoptosis and
damage developing neurons [81-83]. Thus, these negative changes to the hippocampus are associated with neurodegenerative processes seen in AD and HAND.

Neurogenesis incorporates the proliferation and division of NSCs that can give rise to new neurons [84]. The exact mechanism by which neurogenesis is hampered during HAND is yet to be fully understood. However, upon our review, several studies have detected correlations of HIV-1 and its effect on neurogenesis [85]. One such study showed that neural stem cells could be infected by the HIV-1 virus [86], despite the fact that HIV-1 is well known to not infect neurons. In support of this, another study showed that pediatric, post-mortem brain tissue had HIV-1 infection of NSCs [87]. In other studies, post-mortem brain tissue from HAND patients had fewer adult NSCs in the DG than did non-cognitively impaired and non-infected control specimens [88, 89]. This study also showed that HIV-1/gp120 reduced the proliferation NSCs in the hippocampus [90]. This reduction was correlated to the activation of the p38 mitogen-activated protein kinase (MAPK) pathway, which activated arrest of the cell cycle in the G1 phase [88, 90].

p38 MAPK, Bax, and ROS

Eukaryotic cells possess multiple MAPK pathways important for cell regulation. They coordinate activation of gene transcription, protein synthesis, cell cycle machinery, cell death, and differentiation. The activation of MAPK pathways can be activated by several different stimuli acting through diverse receptor families, including hormones and growth factors (i.e. insulin, epidermal growth factor [EGF]), cytokine receptors (e.g.,
growth hormone), G protein-coupled receptors, seven-transmembrane receptors, inflammatory cytokines of the tumor necrosis factor (TNF) family and even environmental stresses such as osmotic shock. This activation of MAPK involves a 3-kinase cascade in which a MAP3K phosphorylates and activates a MAP2K. MAP2K then phosphorylates and activates one or more MAPKs [91, 92].

p38 MAPKs have an initial signaling pathway that starts with the activation of several MAP3Ks. ROS oxidize thioredoxin (Trx) to dissociate it from apoptosis signal regulating kinase 1 (ASK1) for its activation, resulting in the stimulation of the p38 MAPK pathway [93]. As aforementioned, p38 MAPK is activated by inflammatory cytokines and cellular stresses. This activation could play a role in redox signaling. ASK1 has been characterized as an ROS responsive kinase and is a MAP3K of the p38 pathway [94]. ASK1 is activated by various types of stress (ROS, lipopolysaccharide [LPS]), but ROS is the most potent [95]. In general, Trx binds to ASK1 inhibiting its kinase activity [95]. ROS, however, converts Trx to the oxidized form and dissociates Trx from ASK1, inducing ASK1 activity [85, 95]. This relationship was supported when ASK1-deficient cells significantly reduced the activation of the p38 MAPK pathways even when ROS were increased [96]. This led to the notion that ASK1 is an upstream kinase that can be induced by ROS production and activate the downstream p38 MAPK pathway. This activation of p38 MAPK can then reduce neurogenesis [88, 90].

*Bcl*-2-associated X protein (Bax) is a pro-apoptotic member of the Bcl-2 family. The Bcl-2 family of proteins is one of the first groups of proteins to regulate apoptosis.
Their classes of proteins either promote cell survival (Bcl-2, Bcl-X<sub>L</sub>, Bcl-w) or promote cell death (Bax, Bak, Bad). Bax is found in many tissues of the body, and promotes apoptosis. Its deletion has been shown to induce excess neurons [97, 98]. In fact, Bax-deficiency was shown to prolong cerebellar neurogenesis and accelerate medulloblastoma formation. Specifically, Bax<sup>−/−</sup> mice, extended the period of neurogenesis into the third week of postnatal life [98]. In opposition, the overexpression of Bax promotes cell death and blocks the co-expression of pro-survival proteins [99, 100]. Bax is predominantly a soluble cytosolic protein, which, upon apoptosis translocates to the mitochondrial membranes [101, 102]. This induction of apoptosis and translocation to the mitochondria can lead to the loss of MMP and activation of caspase-3 [103-105]. Both p38 MAPK and Bax activation have been linked to the negative regulation of stem cell proliferation. Studies showed that the inhibition of p38MAPK using PD169316 or p38α<sup>−/−</sup> cells is sufficient to induce a high level of neurogenesis spontaneously [106, 107].

**Combination Antiretroviral Therapy (cART)**

HIV treatment had few options prior to 1996. Even though monotherapy was available (zidovudine [AZT]), treatment focused primarily on managing opportunistic AIDS illnesses and pathogens during that time. Inhibitors were then created to target essential proteins/enzymes of HIV [10]. Beginning with monotherapy, antiretroviral therapy (ART) has evolved into a combination of several drugs, now termed combination antiretroviral therapy (cART). cART drastically improved morbidity and mortality rates, suppressing HIV viral load below the levels of detection [108, 109]. With
proper adherence to cART, viral replication can be suppressed for decades [10]. While cART helps patients live longer and healthier lives compared to pre-cART era, there are still factors that limit the extent of treatment and aid in neurodegeneration.

As mentioned, cART is a combination therapy regime with different types of drug targets. cART includes protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer Inhibitors (InSTIs), and fusion inhibitors. These drugs in combination prevent and/or delay the progression to AIDS and may decrease the severity of neuropathogenesis, but cannot prevent the emergence of milder forms of HAND. Our research will focus on NRTIs and NNRTIs, especially the commonly prescribed cART regimen for naïve patients Efavirenz (EFV), AZT, and lamivudine (3TC).

Reverse transcriptase is the target for 2 types of drug classes: NRTIs (analogs for native nucleoside substrates) and NNRTIs (bind to a non-catalytic allosteric pocket on the HIV reverse transcriptase) [10]. The first class of drugs approved by the FDA was NRTIs, which were administered as pro-drugs requiring host cell entry and phosphorylation by kinases prior to causing an antiviral effects. While there are several NRTIs our focus will be AZT and 3TC. AZT was one of the first NRTIs approved for ART, and was often used in the first line of defense as approved by World Health Organization (WHO) guidelines for adults and children 4 weeks and older. It is also used in the prevention of mother to child transmission (MTCT) of HIV [110]. 3TC is also used but must be administered in adults and children 3 months of age and older [111].
Further, it should be understood that NRTIs are often used as the backbone of the cART regimen [112].

NNRTIs differ in the site of action on the enzyme and molecular mechanism when compared to NRTIs. NNRTI binds and induces the formation of a hydrophobic pocket to inhibit reverse transcriptase, changing the conformational shape to reduce polymerase activity. EFV, an NNRTI, was first available in 1998 and can be used for adults and children 3 months and older weighing at least 3.5 kg [113]. The focus of the studies in Chapter 3 and 4 will be EFV.

**HIV Associated Neurocognitive Disorders (HAND)**

Esiri and colleagues observed significant amyloid deposition in the brains of HIV infected patients [32]. Other neuropathological studies have shown that HIV infected patients have increased Aβ deposition in the brain as well [31-40].

These Aβ proteins cause cerebral plaques, a characteristic of the disease. This build up is especially prone in the frontal cortex and hippocampus of the brain. Aβ causes progressive neuronal loss resulting in the deterioration of the brains high and basic functionalities. Along with opportunistic infections commonly seen in HIV patients that are common as a result of their decreased immune systems, in recent years patients infected with HIV have begun to report mild memory problems as well as difficulties in concentration and multitasking even with undetectable viral loads [114, 115]. Neuropathologically, studies have shown that there is a correlation of HIV infection
with increased Aβ deposition to the brain [31-40]

cART and HAND

While cART has increased long-term survival of the HIV infected population, another area of concern has become evident [116]. Over the years, as a result of cART availability, there has been a shift from rapid progression to a more subtle neurocognitive decline. This neurological diagnosis of HAND encompasses three categories based on severity: (1) Asymptomatic Neurocognitive Impairment (ANI), (2) Mild/Minor Neurocognitive Impairment (MNI), and (3) HIV Associated Dementia (HAD). ANI characterizes the least severe form of HAND, while HAD the most severe form. HAND patients are initially assessed based on three main criteria: (1) eliminating co-morbid conditions or infections that may result in dementia, (2) battery testing (evaluated by patients that test below the mean on two cognitive domains), and (3) assessing the impairment on performing tasks associated with ADLs [117]. Even though ANI patients display no significant inability to perform ADLs, they have deficits in at least two of the cognitive areas evaluated by neuropsychological testing. These tests include speed of information processing, sensory-perceptual, motor skills, verbal/language skills, and attention/working memory [117]. MND patients experience the criteria for ANI in addition to mild interruptions in performing ADLs. HAD, the most severe form of HAND, requires a marked cognitive impairment leading to a significant interference with performing ADLs; which is in addition to all the requirements for MND [117]. In the pre-cART era there was an annual incidence of 7% of HAD [118, 119]. Jevtovic and colleagues (2009) found that in the post-cART era there was a decrease in HAD
diagnosis, while more than 50% of persons living with HIV were diagnosed with some form of HAND [120].

HAND in the HIV population can be induced by several factors. One factor may be certain components of cART, a combination of several drugs that have drastically decreased the morbidity and mortality rates as a result of their ability to efficiently suppress the HIV viral load [109]. cART, usually a 3 drug regimen, has contributed to the evolution of HIV from a terminal illness to a chronic disease, enabling individuals to live closer to the age of AD onset. While cART increases patient lifespan, it may contribute to neurocognitive symptomology. In addition, the prospect of ARVs contributing to or inducing HAND is not well studied; however, our lab and other research groups show that certain ARVs induce oxidative stress and neuronal damage in the CNS [121-123]. Data showed negative effects of cART medications on the neuronal Aβ production and clearance by microglial phagocytosis. This experiment suggested that ART increased Aβ generation in SweAPP N2a cells [124].

Others have conducted clinical studies in this area. For example, studies show that significant cognitive symptoms persist in HIV patients despite undetectable plasma viral load [114, 115]. Even though some studies show that the most severe cases of HAND have decreased, there is still a milder form of HAND requiring evaluation by clinicians for patients living with HIV. Furthermore, ARVs have been associated with potentially serious side effects. For example EFV (which will be expanded upon further in the next paragraph), prescribed worldwide since 1998, has clinical CNS side effects
including: cognitive disorders, hallucinations, and insomnia [125-128]. In mice, EFV affected anxiety and cognitive performance as well [129]. In addition, long-term use of EFV has been shown to correlate with the worsening of neurocognitive functioning including speed information processing, verbal fluency, and working memory [125, 130-133]. Our studies will explore EFV effects on AD-like HAND pathology, and will investigate the mechanism by which the effects occur *in vitro* and *in vivo*.

**EFV, Aβ, and Neurogenesis**

Several studies have explored the toxicity of EFV. Patients taking EFV have also been shown to have cognitive disorders even when HIV infection itself is well controlled [125]. Robertson and colleagues further showed neurocognitive improvements after treatment interruption in stable HIV-patients on EFV compared to patients without EFV [132]. The pilot study in our lab showed that some ARVs both alone and in dual combination can increase Aβ production and decrease Aβ microglial phagocytosis *in vitro* [124]. In fact, it has been shown that EFV can lead to the inhibition of mitochondrial complex I altering NADH/NAD⁺ balance and the generation of ROS *in vitro* [78]. Moreover, EFV has been shown to cause mitochondrial dysfunction and increases in ROS [123, 134, 135]. This mitochondrial dysfunction can directly affect p38 and BACE-1, which is significantly activated by ROS [72, 88, 90]. This could suggest the role Bax activation has on MMP, which was shown to be decreased by EFV [123].
Objectives and Hypothesis

Having compiled all data, our main objective was to determine if EFV has a significant effect on Aβ production and neurogenesis, and to identify the underlying mechanism of action, which may enable us to develop a novel therapeutic approach. We hypothesized that exposure to EFV will increase Aβ accumulation and decrease neurogenesis leading to a process that could contribute to HAND.

Chapter 3: Determined the effect of EFV on Aβ production and clearance and explored the mechanism by which it occurs. We measured changes in (1) Aβ production, (2) Aβ clearance, (3) oxidative stress markers (ROS, MMP, and ATP levels), and (4) BACE-1 expression.

Chapter 4: Determined the effect of EFV on neurogenesis. We measured (1) oxidative stress markers (MMP, ATP), (2) proliferation markers (MTT, BrdU, Dcx), and (3) protein expression (p38 MAPK, Bax, Caspase-3).
Fig. 1.1 Hypothesis Overview

We hypothesize that the EFV will increase oxidative stress as several studies have already shown that it inhibits mitochondrial function. This should result in the overall accumulation of Aβ via BACE-1 activation and decrease neurogenesis via p38 MAPK/Bax activation.
References

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CHAPTER TWO
MATERIALS AND METHODS

Cell Culture

**SweAPP N2a Cells**

Murine N2a cells were transfected with the Swedish mutant form of APP (SweAPP). N2a cells were cultured in complete DMEM supplemented with 10% fetal calf serum, and penicillin and streptomycin 100U/ml (Life Technologies, Carlsbad, CA, USA). Cultured media was exchanged approximately every 3-5 days. Treatments were performed in the same media used in the cell culture.

**Rat Neural Stem Cells**

Rat NSCs (EMD Millipore, Billerica, MA USA, cat# SCR021) were cultured in DMEM/F12 media supplemented with serum free Neuro 27 medium (EMD Millipore), human fibroblast growth factor 2 (FGF-2, EMD Millipore), 20 ng/ml, human endothelium growth factor (hEGF, Peprotech, Rocky Hill, NJ, USA), and 20 ng/ml, L-glutamine 2mM (Life Technologies), as well as penicillin and streptomycin 100U/ml (Life Technologies). Culture media were exchanged every 2 days. 10-cm dishes and 96-well plates were pre-coated with 10 µg/ml of poly-L-ornithine (EMD Millipore), followed by laminin (EMD

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2 Portions of this chapter have been previously published (Brown LAM, et al., 2014; Jin, J, et al, 2016) and are utilized with permission of the publisher.
Millipore) at a concentration of 10 µg/ml in PBS solution. NSC treatments were performed during passages 2-6. Treatments were performed in the same medium used for cell culture.

**Mouse Experiments**

*Tg2576*

It is necessary to use an amyloid-depositing mouse model because wild type mice are incapable of forming appreciable mature amyloid plaque during the course of normal aging [1]. For that reason throughout these experiments, cells and mouse models with the Swedish APP gene construct will be used. The purpose for using such a system is to be able to not only have ease in quantifying Aβ production but to also analyze BACE-1 expression and activation. The Swedish APP gene is a double mutation (K670N and M671L) and increases Aβ 6-8 fold. The mouse model used, Tg2756, expresses APP695 with the Swedish double mutation, controlled by the hamster PrP promoter. These mice show Aβ deposition around 9 months [2].

Female Tg2576 mice were purchased at 8 months of age and treated with EFV, AZT, 3TC, and combination in chow for 10 days. The 8-month time point was chosen because it is 1-3 months earlier than the expected Aβ deposition in this model [2]. This will serve as support that ARVs cause early onset of Aβ pathology.
C57BL/6J female mice at the age of eight months old were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and were housed in environmentally controlled conditions (12:12 hour light : dark cycle at 21±1 °C) and provided food and water ad libitum.

**Housing and Maintenance**

All animal work was approved by the University of South Florida Institutional Animal Care and Use Committee (IACUC). They were housed in the Byrd Alzheimer’s Institute under a 12 hour light-dark cycle. Food and water were provided ad libitum.

**Doses and Concentrations**

Tg2576 mice: The dose used in our study for each drug is based on several factors: (1) human clinical therapy [3], (2) body weight, (3) short dosing period of only 10 days, (4) administration method being in chow, and (5) doses used in previous publications [4-7]. The mice were evaluated for changes in cerebral Aβ after 10 days of treatment with each ARV (singly or combined) or with vehicle control. Our experimental groups included oral treatments of: Vehicle (chow no additives), AZT 50 mg/kg, 3TC 40 mg/kg, EFV 15 mg/kg, and combination of drugs (AZT/3TC/EFV).

C57BL/6J Mice: The dose of EFV administered in vivo (20 mg/kg) was based on the body weight of the mice, the dosing period of 30 days as well as our [8] and other [9] previous publications.
Tissue Isolation and Preparation

Administration of EFV and tissue processing of 8-month-old C57BL/6J mice was randomly divided into two groups matched by body weight and gender. One group received EFV via intraperitoneal (i.p.) injection at a dose of 20 mg/kg daily (8 mice) in vehicle control (5% DMSO, 17% Tween 80 in 5% glucose solution) [10] while the other group received vehicle control only via i.p. injection (7 mice). The dose used in the present study (20mg/kg) is in the range used in other studies (10-30 mg/kg) investigating various effects of EFV in rodents [9, 11-13]. The injection period was 4 weeks. On the final week, mice received 50 mg/kg of BrdU (Sigma-Aldrich, St Louis, MO, USA) in phosphate buffered saline (PBS) via i.p. injection daily for 5 consecutive days. 24 hours later, all mice were deeply anesthetized with 1-2 % isoflurane, and transcardially perfused with 20 ml of cold PBS followed by 20 ml of 4% paraformaldehyde (PFA) in PBS. Mouse brain tissues were removed, post-fixed into 4% PFA (pH 7.4) at 4°C overnight, and then neutralized with 30% sucrose in PBS (pH 7.2). Mouse brains were sectioned using a microtome (Leica, Germany) with 40 µm thickness. Sagittal sections from the right hemisphere were used for immunohistochemistry staining.

Protein Analysis

Immunocytochemistry

NSCs were grown in 4-well chambered coverglass (Lab-Tek) at density of $3 \times 10^4$ for 24 hours. After exposure to 5 µM of EFV for 24 hours, 10 µM of BrdU (Sigma-Aldrich) was added at 37°C for 2 hours. Next, 4% formaldehyde in PBS was added to
each well at room temperature for 20 minutes for fixation. NSCs were blocked with 3% donkey serum, incubated with mouse anti-nestin antibody (1:500; Covance, Dedham, MA, USA) at 4°C overnight, and then incubated with secondary Alexa Fluor 594 Donkey anti-mouse IgG at room temperature for 1 hour. NSCs were treated with 2N HCl for 10 minutes at room temperature to denature DNA and were neutralized by citric acid buffer (pH 7.4) for 10 minutes. Cells were blocked with 3% donkey serum incubated with rat anti-BrdU antibody (1:250; Accurate Chemical & Scientific, Westbury, NY, USA) at 4°C overnight, and then incubated with secondary antibody Alexa Fluor 488 Donkey anti-Rat IgG at room temperature for 1 hour. Images were taken using an inverted microscope IX53 and a DP22 color camera (Olympus, Center Valley, PA, USA). The proliferation of NSCs was measured by the percentage of double fluorescence staining of BrdU (green) and nestin (red) over total nestin positive cells.

**Immunohistochemistry**

Sections were blocked with 5% goat serum, 2% BSA in PBS, and 0.1% Triton X-100 followed by hybridization at 4°C overnight with goat anti-doublecortin antibody (Dcx, 1:200; Santa Cruz, Dallas, TX, USA). Sections were then washed, and incubated with the biotinylated anti-Goat IgG (1:200, Vector Laboratories, Burlingame, CA, USA). For BrdU analysis we followed a previous protocol [14]. Briefly, sagittal sections were treated with 50% formamide / 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 hours, rinsed in 2 × SSC, incubated in 2N HCl for 30 min at 37°C, rinsed with borate buffer (pH 8.5) for 10 minutes, and then washed with PBS twice. Sections were incubated with rat anti-BrdU antibody (1:250; Accurate Chemical & Scientific) at 4°C
overnight, and with biotinylated goat anti-rat IgG (1:200), and developed in DAB solution. After mounting the sections onto slides, quantification of positive Dcx and BrdU immunostaining in the subventricular zone (SVZ) was conducted by scanning the slides using an Axioscan Z.1 scanner (Zeiss, NY, USA) to produce 20x digital images. Using the NearCYTE STI computer program (nearcyte.org), a researcher blind to the treatment conditions outlined the region of interest that was then compared to a user-defined pixel color threshold. This file was applied to all of the sections to be analyzed in order to designate positive staining within the determined area. The files were batch processed to generate a ratio of positive stain to total area of for each contour drawn on a slide image. Regarding active caspase-3 analysis, tissue sections were immunostained by free floating method. The primary antibody used was rabbit anti-active caspase-3 antibody (1:250). Vectastain ABC and nickel DAB reagents (Sigma-Aldrich) were used to identify active caspase-3 positive cells. Sections were mounted and imaged manually using an Olympus BX51 microscope with U-CMAD3 camera. The Images were taken by Dp 70 software and analyzed by ImageJ 1.47v. The entire area of the SVZ (50 fields, 0.15 mm² each,) was analyzed from three sagittal brain sections per mouse. Each was separated by a 240 µm interval. The number of positive active caspase-3 positive cells per mm² was quantified.

Western Blot

Tg2576 Mice/SweAPP N2a Cells: For the in vivo studies of Aβ associated pathology, left hemispheres of 8 month old transgenic and wild-type mouse brains were lysed in ice-cold lysis buffer and aliquots were electrophoretically separated using
16.5% Tris–tricine gels. Protein expression for selected proteins (including beta amyloid, BACE-1, Bax, etc) were analyzed using Western blot. After washing tissues or cells with PBS, they were homogenized using 1X lysis buffer, 0.1% protease inhibitor phenylmethylsulphonyl fluoride (PMSF), and protein inhibitor cocktail, and stored at -80°C. Total protein content was estimated using the Bio-Rad protein assay in strict accordance with manufacturer's directions. Equal amounts of protein were loaded per sample for separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed using gels ranging from 8%-16.5% Tris–tricine gels. Electrophoresed proteins were transferred to PVDF membranes (Bio-Rad). Membranes were blocked in Tris-buffered saline (TBS) containing 5% (w/v) non-fat dry milk. Membranes were incubated in primary antibodies were overnight at 4°C. After membranes were washed 3 times for 10 minutes with PBS-T. Appropriate HRP-conjugated IgG secondary antibody as a tracer (i.e. anti-mouse, anti-rabbit) for 1 hour at room temperature was then used. Blots were reprobed with a reference anti-actin antibody, which allows for quantification of total protein. For both in vitro and in vivo studies, blots were developed and then assessed densitometrically using using the Fluor-S Multilmager with Quantity One software from Bio-Rad (Hercules, CA, USA). Immun-Blot polyvinylidene difluoride (PVDF) membranes were purchased from Bio-Rad. Tris-buffered saline was obtained from Bio-Rad and luminol reagent was obtained from Pierce Biotechnology (Rockford, IL, USA).

NSCs: Following 5 µM EFV or vehicle control treatment, NSCs were harvested and lysed by RIPA buffer in 1 mM PMSF, protease inhibitor cocktail, and phosphatase
inhibitor cocktail (Sigma-Aldrich). Protein concentrations were detected with a BCA assay kit. 30 to 50µg of total proteins were applied to SDS-PAGE (10% polyacrylamide gel) and transferred to PVDF membranes (GE Healthcare Biosciences, Marlborough, MA, USA). Blots were incubated for 1 hour in 0.1% Tween 20 in TBS containing 5% nonfat dry milk. After 3 washes with PBS in 0.1% Tween 20, membranes were incubated for 16 hours with one of the following primary antibodies: phospho-p38 mitogen activated protein kinase (MAPK) antibody (Thr180/Tyr182; Cell Signaling Tech., Danvers, MA, USA), total p38 MAPK antibody (Cell Signaling Tech), or Bax polyclonal antibody (EMD Millipore) at 4°C for 1 hour with horseradish peroxidase-conjugated secondary antibodies (diluted 2000-fold) at room temperature. Target proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA). Blots were stripped and re-probed with anti-β-tubulin III (1:8000, Covance). The resulting protein bands were scanned using an hp Laser Jet 9050 mfp System (hp Inc., Palo Alto, CA, USA), and densitometric analysis of each protein band was performed using ImageJ v1.45 (NIH, Bethesda, MD, USA). To normalize for protein loading, the densitometric analysis of each band was divided by the β–tubulin III band from the same membrane for Bax and total p38MAPK for phospho-p38MAPK.

Aβ ELISA

An ELISA is used to measure the concentration of an analyte in solution. In general, an ELISA occurs in 4 steps coating, blocking, detection and analysis. In this kit, coating adds an antibody specific for NH2 terminus of Aβ. Standards of known Aβ
content, controls, and unknown samples are then pipetted into the wells and co-
incubated with antibody specific for the COOH-terminus of the Aβ sequence. The bound
COOH-terminus antibody was detected by the addition of a horseradish peroxidase.
After removal of all the unbound enzymes, a substrate solution was added, which works
on the enzyme to produce color. Cells were then be plated and stimulated for 18 hours
with EFV, AZT, and 3TC both alone (10 µM) and in combination (10 µM). Cell-free
supernatants were collected and Aβ₁₋₄₀, 4₂ peptides were detected directly from the
conditioned media and quantified in these samples using Aβ₁₋₄₀, 4₂ ELISA kits (Life
Technologies) in accordance with the manufacturer's instructions.

Assays

MMP

The 5,5’,6,6’-tetrachloro-1,1”,3,3”-tetraethylbenzimidazolylcarbo-cyanine iodide
(JC-1) mitochondrial membrane potential (MMP) Detection Kit (Biotium: Fremont, CA,
USA) uses a unique cationic dye to measure MMP changes in cells. The lipophilic dye
enters the negatively charged mitochondria to accumulate. When the critical
concentration is exceeded, the dye aggregates. However it can also localize in the
cytosol in a monomeric form. Therefore, healthy cells can be stained both red and
green. In apoptotic cells the MMP collapses and JC-1 is unable to accumulate in the
mitochondria and remains in the cytoplasm in the monomeric form. Healthy cells are
therefore distinguishable by the fact that it has both red and green staining. JC-1
aggregates in the mitochondrial membranes in non-apoptotic cells are detectable as a
red fluorescence at 590 nm, while it exists in the green fluorescent monomeric form at
530 nm of apoptotic or necrotic cells.

SweAPP N2a: In short, cells were treated for 18 hours with ARVs EFV, AZT, or 3TC alone and in combination. Cell media was removed and replaced with 1X JC-1 for 15 minutes. MMP analysis was performed using a JC-1 (ex: 530/25, em: 590/35) MMP detection kit. The value of red fluorescence divided green fluorescence represented MMP.

NSCs: In brief, NSCs were plated in 96-well plates at a density of 104 cells/well for 24 hours. EFV was applied at concentrations of 0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM. Vehicle control (negative control) and H₂O₂ (100 µM, positive control) were also examined. After 6 hr treatment, JC-1 was added into culture media for 15 minutes at 37°C. JC-1 either accumulated on mitochondrial membranes (non-apoptotic cells, red) or remained in the cytoplasm (apoptotic cells, green) [15]. Media was exchanged for PBS.

2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA)

Cellular ROS generation was analyzed using H₂DCFDA (ex: 485/20, em: 528/20) from the ROS detection kit (Invitrogen: Carlsbad, CA, USA). H₂DCFDA is permeable to cell membranes and oxidized to a fluorescent form by ROS including hydrogen peroxide, hydroxyl radicals and peroxynitrite. In short, cells were plated in a black 96-well plate and the drugs were administered (10µM). The treatment was discarded at 30 and 60 minutes and H₂DCFDA was added and incubated for 30 min. H₂DCFDA was then
removed and ROS production was measured at different time points indicated.

**ATP**

ATP determination was performed using the ATP determination kit (Invitrogen). ATP determination kit is based on the requirement of luciferase for ATP to produce light. In short, reaction buffer containing 1 mM dithiothreitol, 0.5 mM luciferin, and 12.5 µg/ml luciferase was added to treated cells. Unknowns were extrapolated from a generated ATP standard curve. After treatment, cells were lysed by Tris lysis buffer on ice for 30 minutes, and subjected to sonication for 30 seconds at 40% power. Next, 90 µl of the premixed solution, including d-luciferin, firefly luciferase, dithiothreitol, adenosine 5′-triphosphate, and assay buffer was transferred into 96-well plates (Greiner Bio-One, Monroe, NC, USA). Following, 10 µl of cell lysate solution or ATP standard solutions were added into the wells with triplication. Relative luminescence units (RLU) were detected with a microplate reader (Synergy H1, Biotek, Winooski, VT, USA). Percentages of EFV treatment over vehicle control represented intracellular ATP levels.

**SweAPP N2a:** Cells were plated and incubated at 37°C for 24 hours. Cells were then treated with cART drugs (AZT, 3TC, EFV, and in combination) at 10 µM for 48 hours.

**NSCs:** Briefly, NSCs were plated in 12-well plates at a density of 3×10⁵ per well and incubated at 37°C for 24 hours. Then the cells were treated with a series of
concentrations of EFV (0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM), vehicle control, and positive control (H₂O₂, 100µM) for 24 hours.

LDH

Cell death in vitro was measured by lactate dehydrogenase (LDH) assay kit (Thermo Scientific). NSCs were plated in 96-well plates at a density of 10⁴ per well for 24 hours and then treated with EFV (0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM), vehicle control, and cell lysis buffer (positive control, 30 minutes) for 24 hours. 50µL of cell culture media from each treatment were transferred to a flat bottom 96-well plate, and 50µL of reaction mixture (supplied in kit) were added into each well and incubated at room temperature for 30 minutes. After adding stop solution, OD at 490 nm subtracted from the OD at 680 nm represented the LDH value.

MTT

Cellular viability was measured by the Cell Proliferation Kit/MTT assay (Roche, Indianapolis, IN, USA). NADPH-dependent enzymes reduce MTT tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble formazan crystals in living cells. These purple in color crystals can then be solubilized and measured at 570 nm for absorbance levels, with high absorbance levels indicating a higher number of cells.

Briefly, NSCs were plated on pre-coated 96-well plates at a density 10⁴ cells per well and incubated at 37°C in 5% carbon dioxide for 24 hours. After treatment with a
series of concentrations of EFV, vehicle control (ctrl; <0.1% DMSO in PBS), or H$_2$O$_2$
(100 uM; positive control) for 24 hours, 10 uL of MTT solution (5 mg/ml) was added into
each well and incubated at 37$^\circ$ C for 4 hours. Next, 100 uL of solubilization (10% SDS
in 0.01 M HCL (was added to each ell and incubated at 37$^\circ$ C overnight. Optical Density
(OD) was detected at a wavelength of 550 nm via microplate reader (Synergy H1,
BioTek). The percentage of treatment OD divided by the vehicle control OD represented
the NSC proliferation value.

Other Protocols

*Microglial Phagocytosis*

Primary mouse microglia were treated with "aged" Aβ$_{1-42}$ peptide conjugated with
FITC (from BioSource Life Technologies: dissolved in dH$_2$O and pre-incubated for 24 h
at 37$^\circ$C to assist aggregation and form fibrils) with ARVs (AZT, 3TC, EFV) both alone
(10 µM) and in combination (10 µM). The total cellular protein of all groups was
quantified and adjusted using the Bio-Rad BCA protein assay. Extracellular and cell
associated FITC-tagged Aβ was quantified using a SPECTRAmax GEMINI microplate
fluorometer (Molecular Devices Corp.: Sunnyvale, CA, USA) with an Ex/Em= 538/485
nm. Microglial cells were rinsed 3 times in Aβ-free complete medium, and the media
was exchanged with fresh Aβ-free complete medium for 10 minutes both to allow for
removal of non-incorporated Aβ and to promote concentration of the Aβ into
phagosomes. The relative mean fluorescence values for each sample at 37$^\circ$C and 4$^\circ$C
at the indicated time points were determined and calculated as: (mean fluorescence
value for each sample at 37°C - mean fluorescence value for each sample at 4°C) to quantify both extracellular and cell associated FITC-labeled Aβ.
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CHAPTER THREE

EFAVIRENZ PROMOTES Aβ ACCUMULATION

Introduction

There has been considerable growth in patient's receiving combination antiretroviral therapy (cART) in recent years [1]. Up to approximately 50% of long-term HIV-infected patients experience HIV associated neurocognitive disorders (HAND) [2]. Most recently it was shown the non-nucleoside reverse transcriptase Inhibitor (NNRTI) efavirenz (EFV) is associated with cognitive disorders even in asymptomatic HIV-infected patients [3]. A randomized controlled study [4] found subjects receiving EFV-containing regimens showed less improvement from baseline on instruments examining speed of information processing and executive function than patients not on EFV. EFV has substantial rates of central nervous system (CNS) side effects aside of cognitive impairment including sleep and dreaming disturbances and anxiety [5-7] that can interfere with adherence and tolerability as well [8].

Amyloid-beta (Aβ) peptide generation and aggregation as plaques are traditionally known as key events in the development of Alzheimer's Disease (AD; [9-]

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3 Portions of this chapter have been previously published (Brown LAM, et al., 2014) and are utilized with permission of the publisher.
The peptides have been evidenced to be neurotoxic, as they are reported mediators of inflammation [13, 14], and oxidative stress [15]. Aβ peptides are produced via the amyloidogenic pathway of amyloid precursor protein (APP) proteolysis, which involves the actions of β and γ-secretases [12, 16]. Initially, β-secretase (BACE-1) cleaves APP, creating an Aβ-containing carboxyl-terminal fragment known as β-C-terminal fragment (β-CTF) [17]. This proteolysis also generates an amino-terminal, soluble APP-β (sAPP-β) fragment, which is released extracellularly. Intracellularly, β-CTF is then cleaved by a multi-protein γ-secretase complex that results in generation of the Aβ peptide and a smaller γ-CTF [18]. In the human brain Aβ1-40 is the predominant form whereas Aβ1-42 represents about 10% of brain Aβ and has a greater propensity to form neurotoxic oligomeric and aggregated species [19-21].

The rapid, early clinical phase-in of cART required dose de-escalations secondary to toxicities suggested to be related to mitochondrial drug side effects [22]. Mitochondrial dysfunction can result in an elevation of reactive oxygen species (ROS) that in turn promote amyloidogenic APP processing by inducing BACE-1 activity [23]. Such mitochondrial stress has also been reported occurs in patients taking lamivudine (3TC), zidovudine (AZT) and especially EFV [24-26]; a commonly used cART regimen [27-29]. In light of the increasing life-span's imparted by cART, the mitochondrial dysfunction promoted by cART [24, 25, 30-32], and the age associated risk for developing amyloid pathology [33], it is not surprising that a body of epidemiological data suggests significant numbers of long-term HIV survivors are at
elevated risk of developing early brain aging in the form of AD like pathology including Aβ deposition [34-40].

As a result, we hypothesized that Aβ pathology may be produced via the amyloidogenic pathway of APP proteolysis, which involves the actions of BACE-1 [12] and sought to test this with in vitro and in vivo models. Our results indicate that EFV is the primary antiretroviral in this commonly used EFV containing regimen: EFV/3TC/AZT, which is responsible for its promotion of Aβ pathology [27-29].

Results

Epidemiological reports indicate that HAND persists in patients even with good viremic control who take EFV [3]. Previous studies have shown that cART imparts mitochondrial toxicity in the form of elevated ROS [24, 25]. A high ROS microenvironment has been shown to promote the activity of BACE-1, a key enzyme the generation of Aβ in the brain [23]. Brain oligomeric [41] and Aβ1-40,42 [42] have been correlated with cognitive impairment. Since the EFV containing regimen may promote mitochondrial dysfunction [24, 25, 32, 43] which could result in increased BACE-1 activity, we investigated the effect of a commonly used EFV containing cART regimen [27-29] for its ability to upregulate Aβ production via activation of BACE-1 and amyloidogenic APP processing and also for its ability to reduce microglial phagocytosis of Aβ.
**BACE-1 is involved in Aβ generation promoted by the EFV containing cART regimen in cultured SweAPP N2a cells**

Using similar conditions as in our prior investigations [44], SweAPP N2a cells were treated with the EFV containing regimen: 3TC, AZT, EFV or each drug singly at 10 µM in addition to PBS control for 18 hours. Aβ40 and Aβ42 peptides were then measured in conditioned media from these cells by ELISA and Western blot (Fig. 3.1A–C) while BACE-1 expression was measured in cell lysates by Western blot analysis (Fig. 3.1D–E). The EFV containing regimen increased Aβ40 and Aβ42 production in SweAPP N2a cells significantly (p < 0.05). Importantly, we found that EFV alone was more potent than the EFV containing regimen in terms of significantly increasing Aβ40 and Aβ42 production by these cells (p < 0.001). Additionally EFV or the EFV containing regimen increased BACE-1 expression in SweAPP N2a cells significantly (p < 0.001). These data would suggest that 3TC and/or AZT somehow reduce the toxicity of EFV in terms of promoting amyloidogenic APP processing and that EFV is the primary agent promoting Aβ production in SweAPP N2a cells. There is some evidence to indicate that AZT may indeed have a neuroprotective effect [45, 46], which could explain why the EFV containing regimen is less potent in its amyloid producing effects compared to EFV alone.
Figure 3.1: EFV or EFV/3TC/AZT treatment promotes Aβ generation in cultured neuronal cells via BACE-1 activation in vitro.

Aβ species were analyzed in cell lysates from SweAPP N2a cells (A) by ELISA. Data are represented as the mean ± of a percentage of Aβ peptides secreted 24 h after 3TC, AZT, EFV, or 3TC/EFV/AZT administration, relative fold over control. Significant increases in Aβ were observed in EFV or EFV/3TC/AZT treated cells compared to control (***p < 0.001 and **p < 0.05 respectively by ANOVA). (B) Western blot (6E10 antibody) of conditioned media shows increased oligomeric Aβ species vs. s-APP-α (control) in the EFV or EFV/3TC/AZT treated cells (***p < 0.001 and **p < 0.05 respectively). (D) BACE-1 expression in cultured media revealed significant differences between EFV or EFV/3TC/AZT treated cells compared to untreated control (***p < 0.001). β-actin is used for the internal loading control. Results are representative of three independent experiments.
Cerebral amyloidosis in Tg2576 mice is increased by EFV or the EFV containing cART regimen

Brain Aβ deposition is a pathognomonic feature of AD [47], and oligomeric Aβ species are thought to be a driving force in AD-type neurodegeneration [48-51]. They may also play a role in HAND development [35-40]. The transgenic Tg2576 mouse [52] is a widely used model of cerebral amyloidosis, and we purchased them from Taconic (Germantown, NY, USA) at 8 months of age. They were evaluated for changes in cerebral Aβ after 10 days treatment with each antiretroviral singly or combined as well as vehicle control. Western blot analysis of brain homogenates revealed significantly increased Aβ species in both the EFV and EFV containing regimen groups (p < 0.01); again suggesting that EFV accelerates cerebral amyloidosis as opposed to having a cumulative effect with 3TC and AZT. Indeed AZT is most likely behind the reduced potency of the EFV containing regimen compared to EFV alone in terms of Aβ pathology in light of reports that it may be neuroprotective [45, 46, 53]. Additionally EFV or the EFV containing regimen increased BACE-1 expression significantly (p < 0.001) (Fig 3.2).
Figure 3.2: EFV/3TC/AZT increases soluble Aβ levels in Tg2576 mice via BACE-1 activation in vivo. (A) Aβ_{40, 42} peptides were analyzed in brain homogenates from 8 month old Tg2576 mice by ELISA (n=5 mice for each group). One-way ANOVA followed by post hoc comparison revealed significant differences between control (Tg2576 mice treated with PBS) and EFV or EFV/3TC/AZT -treated Tg2576 mice (**p < 0.01 and ***p < 0.001 respectively with n=5 mice/group). (B) Western blot of brain homogenates using anti-Aβ_{1-17} antibody (6E10) shows total APP and bands corresponding to soluble Aβ oligomer species. β-actin was an internal control. A t-test revealed significant differences in soluble Aβ species between EFV and 3TC/AZT/EFV-treated compared to, 3TC or AZT treated Tg2576 mice (**p < 0.01) (D) BACE-1 expression in brain homogenate of Tg2576 mice significantly was increased in EFV or EFV/3TC/AZT -treated Tg2576 mice (**p < 0.001).
EFV promotes mitochondrial stress in SweAPP N2a cells

To determine if EFV or the EFV containing cART regimen could promote mitochondrial stress in an amyloid producing model, SweAPP N2a cells were treated with EFV, 3TC, AZT, or all three antiretrovirals combined in addition to vehicle control for 48 hours. We performed three separate assays to determine general mitochondrial function. These included analyses of cellular ATP production, mitochondrial membrane potential (MMP), and ROS production. EFV or the EFV containing regimen were most potent in reducing mitochondrial function. Mitochondria produce approximately 90% of the total cellular ATP in neurons [54]. We therefore first examined ATP levels in SweAPP N2a cells as a measure of mitochondrial function. Cells treated with EFV or the EFV-containing regimen had greatly decreased ATP levels ($p < 0.001$) although the EFV containing regimen had slightly less ATP depletion than EFV alone. Mitochondria from SweAPP N2a cells treated with EFV or the EFV containing regimen showed significantly reduced MMP compared to 3TC or AZT treated SweAPP N2a cells; mirroring the results with the ATP analysis. The MMP is an indicator of electron transport chain function (Fig. 3.3) [54].
**Figure 3.3: cART treatment of SweAPP N2a cells promotes mitochondrial dysfunction.**

(A) ATP levels are reduced in EFV or EFV/3TC/AZT treated SweAPP N2a neuron cells: SweAPP N2a cells were grown with 10 µM of each medication or all three medications combined for 48 h. We found a significant decrease in ATP levels in cells treated with EFV or 3TC/AZT/EFV (**p < 0.001). (B) MMP is reduced in EFV or EFV/3TC/AZT SweAPP N2a cells: In accord with reduced ATP levels we found a similar reduction in MMP in the EFV or EFV/3TC/AZT treated groups (**p < 0.001)
Mitochondria are the main source of cellular ROS production in the brain, thus the rate of ROS reflects the efficiency of mitochondrial function as well [54] (Fig. 3.4). EFV or the EFV containing regimen caused a large increase in ROS production. AZT and 3TC did not cause a significant rise thus explaining the reduced potency in terms of promoting ROS production of the three drug combination versus EFV alone. From the three cell-based assays that were utilized to monitor different parameters of mitochondrial function, EFV was identified as the most deleterious compound in our screen of this commonly used cART regimen [27-29]. From all three assays we see that AZT and 3TC reduce this effect promoted by EFV.

*Microglial phagocytosis of Aβ1-42 peptides is opposed by EFV*

Amyloid load in the brain is affected not only by production, but also by its clearance from the brain via microglia mediated mechanisms [55]. To determine whether the EFV containing regimen could affect microglial clearance of Aβ and further promote amyloidosis, we performed a phagocytosis assay with primary mouse microglia in the presence of EFV, 3TC, AZT or all three antiretrovirals combined in addition to vehicle control. Following detection of FITC-tagged Aβ1-42 in extracellular and cell associated fractions, we again found that EFV or the EFV containing regimen inhibited microglial phagocytosis/clearance. These two treatments significantly inhibited microglial phagocytosis of Aβ1-42 peptides as determined by high levels of peptide remaining in the cultured media (extracellular) \((p < 0.001 \text{ and } p < 0.05 \text{ respectively})\). In addition, EFV or the EFV containing regimen tested also significantly reduced levels of phagosomal (cell associated) Aβ1-42 \((p < 0.001 \text{ and } p < 0.05 \text{ respectively})\). Also, when
comparing cell associated Aβ₁₋₄₂ levels of EFV compared to the three drug combination and to the levels of these compounds alone, the differences suggest the major reduction in phagocytosis is imparted by EFV and the addition of the other two antiretrovirals of the regimen are not additive in nature. Importantly, when comparing the levels of extracellular Aβ₁₋₄₂ to that of cell associated we can see that the phagocytosis/clearance profiles are relatively congruent for each treatment condition. That is to say, when a given treatment maintains high levels of extracellular Aβ₁₋₄₂, the corresponding cell associated levels are relatively low. Not only does this apparent relationship between extracellular and cell associated Aβ₁₋₄₂ levels confirm the accuracy of the assay, but also furthers the overall significance of the inhibition of microglial phagocytosis by the antiretrovirals (Fig 3.5) [44].

**Statistical Analysis**

All data were normally distributed; therefore, in instances of single mean comparisons, Levene's test for equality of variances followed by t-test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by post-hoc comparison using Bonferroni's method/correction. Alpha levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, IL, USA) was used for all data analysis.
Figure 3.4: cART treatment of SweAPP N2a cells increases reactive oxygen species production.

ROS levels are increased in EFV or EFV/3TC/AZT treated SweAPP N2a cells: EFV-treated neuron cells have significantly higher ROS contents (**p < 0.001) after incubation for 30 and 60 min than untreated N2a cells. Data represents the ROS content in the antiretroviral treatment expressed as % RFU ± standard deviations for each group compared to untreated control neuron cells (100%). (**p < 0.001). Note: 60 min treatment is not shown, but supported data seen at 30 min.
Figure 3.5: EFV/3TC/AZT inhibits microglial phagocytosis of Aβ1-42 peptide.

(A) Primary microglia (1×10⁵ cells/well in 24-well tissue culture plates) were treated with aged FITC tagged Aβ1-42 (50 nM) in complete medium for 60 min with antiretroviral medications (10uM) combined or singly as indicated, or PBS (control). As a control for nonspecifically incorporated Aβ, microglial cells were incubated at 4°C with the same treatment followed by DAPI staining. EFV or 3TC/AZT/EFV inhibited microglia-colocalization by fluorescence microscopy. Green indicates Aβ1-42 positive; blue indicates microglia nuclei. Addition of heat inactivated HIV-1 Tat yielded similar results as vehicle control (data not shown) (B) Cell supernatants and lysates were analyzed for extracellular (top) and cell associated (bottom) FITC-Aβ using a fluorimeter. Data are represented as the relative fold of mean fluorescence change (mean ± SD), calculated as the mean fluorescence for each sample at 37°C divided by mean fluorescence at 4°C (n=6 for each condition presented). One-way ANOVA followed by post-hoc comparison showed a significant difference between EFV (**p < 0.001) or EFV/3TC/AZT (**p < 0.05) but not 3TC or AZT compared to control.
**Discussion**

Here, we elucidate a potential mechanism whereby EFV may have neurotoxic effects *via* promotion of brain Aβ. Our present study has led to the proposed mechanism of neurotoxicity in which EFV promotes an increase in Aβ *in vitro* and *in vivo* on both the production and clearance fronts *via* its inhibition of proper MMP resulting in reduced ATP stores and thus a high ROS environment in the CNS. It is proposed that EFV induced high ROS microenvironments (Fig. 3.4) in the CNS promote BACE-1 APP processing ([23]; Fig. 3.1) and also inhibits microglial phagocytic functions (Fig. 3.5; [56, 57]). These events in turn all promote production of Aβ species.

EFV has been associated with serious adverse reactions, most of which can in part be attributed directly or indirectly to dysfunction of mitochondria [22, 24, 25, 30, 31]. We found that EFV, or the EFV containing regimen consistently and significantly promoted mitochondrial oxidative stress in the form of reduced cellular ATP stores (Fig. 3.3A) and MMP (Fig. 3.3B), as well as increased release of ROS (Fig. 3.4). These observations suggest the mitochondrial stress imparted by this cART regimen is largely dependent upon EFV and that 3TC and/or AZT may have some protective effect. Indeed there is some evidence that the latter antiretroviral may help to preserve cognitive function [45, 46, 53].

Reactive microgliosis can be associated with the formation of microglial phenotypes that are unfavorable to phagocytic activities [57, 58]. ROS are an important
signal for cellular activation and proliferation. Over the long term they can lead to microglial dysfunction, rendering the phagocytes unable to perform their vital clearance functions [57, 58]. This may underlie the reduced microglial phagocytosis of Aβ observed in microglia treated with EFV or the EFV containing regimen (Fig. 3.5).

Several lines of epidemiological evidence signal a role for Aβ in HAND development while some studies have not yet fully implicated over production of the protein as a contributor to HAND. It is known that pathological similarities exist between HAND and AD [35-40]. The latter is more so characterized by extracellular deposits of Aβ1-42 in the form of plaques and aggregations of microtubule-associated tau yielding neurofibrillary tangles (NFT). In contrast, with HIV infection, the plaques are more diffuse [36] rather than neuritic [39].

Cerebrospinal fluid (CSF) biomarkers can mirror pathogenic cerebral amyloid deposition. Decreased CSF Aβ1-42 and increased CSF tau can differentiate symptomatic AD participants and cognitively normal individuals at high risk for symptomatic AD from cognitively normal individuals at low risk for symptomatic AD [59, 60]. In that regard, at least some HAND patients have CSF Aβ1-42 values comparable to symptomatic AD individuals, that is, reduced [36]. This is salient because reductions in CSF Aβ42 have been found in almost all individuals with increased fibrillar amyloid deposition within the brain as assessed with positron emission tomography (PET) amyloid binding of N-methyl-[11C]2-(4-methylaminophenyl)-6-hydroxybenzothiazole (11C-PiB) [61-63].
Likewise, AIDS dementia complex (ADC) patients had significantly decreased CSF Aβ\(_{1-42}\) and increased total and phospho (t-tau and p-tau respectively) concentrations similar to AD [36]. Achim and colleague’s (2009) reported increased Aβ by autopsy examination, immunoblotting, and microscopy analysis of HIV patients. Specifically, cases with HIV encephalitis (HIV-E) were about twice as likely to have amyloid detected (72%) than HIV+ patients without HIV-E (38%; [35]). In the same year Clifford and colleagues reported Aβ\(_{1-42}\) measurements in CSF of cognitively impaired patients with HIV were similarly reduced as in in patients with mild dementia of the Alzheimer type (DAT). Normal or slightly depressed CSF tau and p-tau measurements distinguished these patients with HAND from patients with DAT [40].

Further analysis as to why there is low CSF Aβ\(_{1-42}\) in patients with HAND is needed. However, there are several reasons which may explain altered Aβ metabolism in HIV disease [40] in addition to the data presented in this report. First, HIV-1 transactivator of transcription (Tat) protein may compete with APP and/or apolipoprotein E (an Aβ chaperone) for binding to the low density lipoprotein receptor related protein (LRP), thus inhibiting LRP mediated clearance of Aβ from brain interstitial fluid to periphery [64]. Second, APP cleavage products (sAPPα and sAPPβ) have been reported to be reduced in the CSF of patients with HAND compared to those with DAT or HIV-negative controls, with sAPPα (a neurotrophic protein) showing a slight decline in the asymptomatic HIV state [65].
In 2010 Ances and colleagues reported cognitively unimpaired HIV+ participants, even with low CSF Aβ_{1-42} (<500 pg/mL), did not have (11)C-PiB parameters suggesting brain fibrillar amyloid deposition. It is suggested this dissimilarity between cognitively unimpaired HIV+ and preclinical AD may reflect differences in Aβ_{1-42} production and/or formation of diffuse plaques [66]. This same group, in 2012, reported symptomatic AD patients were significantly older, had significantly lower CSF Aβ_{1-42}, and had significantly higher CSF tau levels than other groups. Regardless of degree of impairment, HIV patients did not have increased ^{11}C-PiB [67]. Possible reasons for the absence of ^{11}C-PiB in HIV patients are: 1) decreased Aβ_{1-42} production secondary to decreased synaptic activity, 2) increased intraneuronal Aβ_{1-42} deposition that is undetectable by ^{11}C-PiB [35]; and/or 3) increased Aβ_{1-42} brain deposition but in a more diffuse, non-fibrillar form that is undetectable by ^{11}C-PiB [34, 66]. Future longitudinal examinations within older HIV+ participants are required to determine if diffuse or oligomeric forms could with time subsequently become fibrillar (^{11}C-PiB positive) deposits [36, 40]. Our findings reinforce the importance of understanding the effects of cART on amyloid metabolism since EFV could contribute to the neurological complications that are associated with HIV infection seen clinically [3-8, 68].

The current research has several strengths and weaknesses. Regarding the former, we observed consistent findings in both in vitro and in vivo model systems in that EFV or the EFV containing regimen caused increase amyloidogenic APP processing as a function of increased BACE-1 expression and decreased microglial clearance of Aβ. Additionally, we find the level of mitochondrial dysfunction imparted by
each antiretroviral medication correlates consistently with the increased level of BACE-1 expression and Aβ production, and the decreased microglial phagocytosis of Aβ peptide. Second our results coincide with other reports indicating the mitochondrial toxicity of antiretrovirals [24, 25, 30-32], and reports that increased ROS can result in increased BACE-1 activity [23].

This report has limitations as well. First, it describes a mechanism for a subset of HAND cases since not all HIV infected individuals are taking EFV or an EFV containing regimen. It should be noted that in the present study, we did not investigate the plasma or CSF concentrations of antiretrovirals or their metabolites. However, all three drugs seem to have good CNS penetration [22, 69, 70], which could support the neurologic symptoms noted by others [3, 7, 68].

In sum, our present work suggests that EFV promotes an increase in Aβ on both the production and clearance fronts through oxidative stress. We hypothesize that a disrupted MMP with resultant lowered neuronal ATP stores promotes a high level of ROS. In turn, this can both promote BACE-1 activity and impair microglial clearance mechanisms. If this mouse model translates to the clinical syndrome, then a pharmacotherapeutic strategy aimed at opposing the EFV-mediated reduced microglial Aβ clearance and/or EFV-mediated neuronal Aβ over production via BACE-1 should be beneficial to prevent or treat HAND.
References


CHAPTER FOUR

EFAVIRENZ DECREASES NEURAL STEM CELL PROLIFERATION

Introduction

Soon after infection, human immunodeficiency virus-1 (HIV-1) enters the central nervous system (CNS) [1]. Survival times with chronic HIV-1 infection continue to grow, and as a result there is an increasingly large number of patients harboring the virus within the brain. This may be one contributing factor to the substantial prevalence of HIV-associated neurocognitive disorders (HAND) [2]. Past reports have indicated a link between HIV-1 with both reduced proliferation of neural stem cells (NSCs) as well as reduced neurogenesis [3]. Although HIV does not infect mature neurons, the virus has been shown to infect NSCs [4]. It has further been shown that the early HIV-1 infection of NSCs may lead to neurocognitive dysfunction [5, 6]. Importantly, NSCs have an ability for precise migration to widespread areas of pathology in the brain [7-12]. Thus, their reduction may be a contributing factor to HAND, which is marked by chronic inflammatory and oxidative injury in the brain [13-15].

The HIV non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) has been a common component of combination antiretroviral therapy (cART) for HIV infection [16,

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and has also been associated with cognitive side effects in both human clinical studies and murine models [16, 18, 19]. Studies on human glioma and neuroblastoma cell lines as well as rat primary cultures of neurons and astrocytes indicate the involvement of EFV in oxidative stress in the CNS [20]. We performed these experiments to determine whether EFV could affect NSC proliferation in vitro and in vivo. We sought to understand one possible reason why cognitive disorders may occur in patients with well-controlled HIV infection who are on EFV therapy [16, 18].

Results

EFV Reduces Proliferation of NSCs

As shown in Figure 4.1A, EFV decreased cell proliferation in a concentration-dependent manner. At the 5 µM EFV concentration, there were approximately 74% of live cells compared to vehicle control ($p < 0.05$). When EFV concentration was 10 µM, live cells were detected at approximately 36% compared to vehicle control treatment ($p < 0.001$). NSC proliferation had a progressive decline as EFV concentration increased. The threshold for this decline was the 5 µM concentration of EFV. As shown in Figure 4.1B, BrdU incorporation reflected cell proliferation, and nestin was used as an NSC marker [21]. The number of double-fluorescent-labeled cells was significantly decreased by EFV treatment compared with vehicle control. Vehicle control treatments are shown in Figure 4.1B: (a) BrdU, (b) nestin, and (c) merged. EFV treatment (5 µM) is shown in (d) BrdU, (e) nestin, and (f) merged; (g) quantified data expressed as percentage of double-labeled cells show significant decreases in proliferation in EFV-treated NSCs ($p < 0.001$) (Fig. 4.1B).
Figure 4.1. EFV reduces proliferation of NSCs.
Efavirenz (EFV) reduces proliferation of neural stem cells (NSCs). EFV reduces proliferation of NSCs by MTT assay. (A) NSCs were treated with a series of concentrations of EFV, vehicle control (ctrl), and H₂O₂ (positive control; 100 µM) for 24 h. Data are presented as a percentage of EFV treatment over ctrl. Each bar is presented as mean ± SEM (*p < 0.05; ***p < 0.001). (B) EFV inhibits bromodeoxyuridine (BrdU) incorporation into NSCs as evidenced by immunocytochemistry: after treatment consisting of 5 µM of EFV or vehicle control (ctrl) for 24 h, and incubation with BrdU (10 µM) for 2 h, NSCs were fluorescence stained with BrdU (green) and nestin (red). Staining of control treatment is shown in (a) BrdU, (b) nestin, and (c) merged; EFV treatment in (d) BrdU, (e) nestin, and (f) merged. Scale bars: 20 µm (a–f). (g) Quantified data are expressed as the percentage of double-labeled cells. Data are presented as mean ± SEM (***p < 0.001).
EFV Reduces NSC ATP Stores and MMP

NSC viability can also be measured by ATP assay. In order to maintain biophysiologic function, intracellular ATP levels must remain consistent to maintain homeostasis. To determine if EFV could decrease ATP levels in NSCs, as shown in Figure 4.2A, they were treated with a series of concentrations of EFV as well as positive (H₂O₂, 100 µM) and vehicle controls for 24 h. Cell lysates were used to measure ATP levels, and 5 µM of EFV decreased ATP levels to approximately 78% of vehicle control levels (p < 0.05). EFV (10 µM) reduced cellular ATP stores by approximately 57% compared to that of vehicle control (p < 0.01) (Fig. 4.2A). Loss of MMP is an event associated with oxidative stress and the initiation and activation of apoptosis [22, 23].

As shown in Figure 4.2B, NSCs were treated with a series of concentrations of EFV, vehicle control, and positive control (H₂O₂; 100 µM) for 6 h followed by the addition of JC-1. In healthy cells, the JC-1 reagent aggregates on intact mitochondrial membranes and shows red fluorescence, whereas in apoptotic cells, it exists in monomeric form in the cytoplasm showing green fluorescence. The value of red fluorescence (excitation, 550 nm; emission, 600 nm) divided by green fluorescence (excitation, 485 nm; emission, 535 nm) represents MMP. Similar to MTT and ATP assays, the threshold concentration was 5 µM of EFV to observe significant effects (Fig. 4.2B).
Figure 4.2. EFV reduces NSC ATP stores and MMP.
EFV reduces NSC adenosine triphosphate (ATP) stores and mitochondrial membrane potential (MMP). (A) EFV reduces NSC ATP stores: NSCs were plated in 12-well plates with $3 \times 10^5$ cells per well and treated with a series of concentrations of EFV, vehicle control (ctrl), and H$_2$O$_2$ (positive control; 100 µM) for 24 h. The relevant luminescence units (RLU) were measured for each condition. The percentages of treatment over ctrl represents intracellular ATP levels of NSCs. Each bar is presented as mean ± SEM (*p < 0.05; **p < 0.01). (B) EFV reduces MMP of NSCs: after NSCs were treated with a series of concentrations of EFV, vehicle control (ctrl), and H$_2$O$_2$ (positive control; 100 µM) for 6 h, they were incubated with JC-1 to detect MMP. The ratio of red fluorescence (RFU) to green fluorescence (RFU) represents the value of MMP. Data are presented as mean ± SEM (*p < 0.05; **p < 0.01).
EFV Increases NSC Cytotoxicity and Cell Death

Because loss of mitochondrial function as examined by JC-1 can lead to apoptosis, we also examined EFV-promoted cell death by LDH assay. We again treated NSCs with a series of concentrations of EFV, vehicle control, and positive control (cell lysis buffer, 30 min) for 24 h (Fig. 4.3). The cell-cultured media were subjected to LDH assay. The higher the cell death, the more LDH was released into the media via cell membrane damage. EFV promoted toxic responses from NSCs in a concentration-dependent manner. EFV (5 µM) increased LDH release by nearly twofold compared with vehicle control (p < 0.001), while 10 µM of EFV increased LDH release by approximately threefold (p < 0.001).

Effect of EFV on p38 MAPK Phosphorylation and Bax Expression

It has been reported that the phosphorylation of p38 MAPK and Bax activation are central antiproliferation inducers of several cell types including stem cells [24-26]. This was evidenced by a phosphorylation of p38 in EFV stimulated NSCs. We treated these cells with EFV (5 µM; based on the threshold concentration for significant effects on NSC mitochondrial function, proliferation, and cytotoxicity) for 1 h and found that EFV significantly enhanced phospho-p38 (Ph-p38) expression by Western blot (p < 0.01) (Fig. 4.4A). To investigate whether p38 MAPK could be correlated with upregulated Bax expression, NSCs were further treated with the same concentration of EFV for 24 h, which significantly increased Bax expression (p < 0.05) (Fig. 4.4B).
**Figure 4.3. EFV increases NSC cytotoxicity and cell death.**

EFV increases NSC cytotoxicity and cell death. EFV induces increased cell death of NSCs in a dose-dependent manner. Cells were treated with a series of dilutions of EFV, vehicle control (ctrl), or assay-positive control (10% cell lysis buffer) for 24 h, and cell culture media were subjected to an LDH assay. The value of optical density (OD) at 490 nm subtracted from the value of OD at 680 nm represents LDH levels. Data are presented as mean ± SEM (**p < 0.001).
Figure 4.4 EFV increases p38 MAPK phosphorylation and Bax expression.
EFV increases p38 MAPK phosphorylation and Bax expression. (A) EFV elevates the expression of phospho-p38 in NSCs detected by Western blot. NSCs were plated in 24-well plates for 24 h, treated with 5 µM of EFV or vehicle control (ctrl) for 1 h. Cells were then lysed and subjected to Western blot of phospho-p38 (Ph-p38) and total p38. Each bar is presented as mean ± SEM (**p < 0.01). (B) EFV elevates expression of Bax from NSCs. To detect Bax expression, cells were treated for 24 h with 5 µM of EFV or vehicle control (ctrl). Data are presented as mean ± SEM (*p < 0.05).
EFV Administration Impairs NSC Proliferation and Induces Apoptosis in the SVZ In Vivo

To explore NSC proliferation after EFV administration in vivo, C57BL/6J mice at 8 months of age were injected (IP) with 20 mg/kg of EFV or vehicle control for 4 weeks. During the final 5 days, mice additionally received a dose of 50 mg/kg of BrdU daily. Quantitative analysis showed that BrdU expression was significantly decreased in the EFV treatment group in the sub-ventricular zone (SVZ) compared to the control group (p < 0.05) (Fig. 4.5A), indicating an impairment of proliferation. Figure 4.5B exhibits images of Dcx staining of the SVZ in the vehicle control group and the EFV group. There was a strong trend for the EFV-treated group to have a lower Dcx expression level compared to the control group (p=0.08). To uncover a possible underlying effect of EFV that may reduce the number of replicating NSCs in vivo, we measured apoptotic marker active caspase-3. Results indicated that EFV administration increased the number of active caspase-3-expressing cells in the SVZ compared to vehicle control-treated mice (p < 0.05) (Fig. 4.5C).

Statistical Analysis

All statistical analyses were performed with SPSS software (version 18.0; IBM, Armonk, NY, USA). Data are expressed as mean ± SEM, and results were deemed significant when p < 0.05. Variables between groups were determined by independent t-test or one-way analysis of variance (ANOVA). When significance was achieved, analysis of groups was performed using Tukey’s post hoc test.
Figure 4.5. EFV administration impairs NSC proliferation and induces apoptosis in the SVZ in vivo.

EFV administration impairs NSC proliferation and induces apoptosis in the SVZ. (A) Administration of EFV (20 mg/kg) impairs BrdU incorporation in the SVZ of C57BL/6J mice. Mice were administered daily IP injections of EFV or vehicle control (ctrl) for 4 weeks and BrdU administration for 5 consecutive days prior to euthanization. Sagittal sections were stained with BrdU antibody. (a, b) The square marked in (a) represents the vehicle control (ctrl)-treated group and is magnified in (b) (n = 7); (c) and (d) the square marked in (c) represents EFV treated group and is magnified in (d) (n = 8). Scale bar: 20 µm (in micrographs). (e) Quantitative data are represented by the bar graph. Each bar is presented as mean ± SEM (*p < 0.05). (B) Administration of EFV shows a trend to decrease Dcx expression in C57BL/6J mice in the SVZ. Sagittal sections from the same mice were stained by Dcx antibody. (a, b) The square marked in (a) represents the vehicle control (ctrl) group and is magnified in (b) (n = 7). (c, d) The square marked in (c) represents the EFV treatment group and is magnified in (d) (n = 8). Scale bars: 20 µm (in micrographs). (e) Quantitative data are represented by the bar graph. Each bar is presented as mean ± SEM (p = 0.08). (C) Administration of EFV enhances active caspase-3 expression in the SVZ. Sagittal sections from the same mice were stained by active caspase-3 antibody to evaluate apoptosis in the SVZ of C57BL/6J mice. (a, b) The square marked in (a) represents the vehicle control (ctrl) group and is magnified in (b) (n = 7). (c, d) The square in (c) represents the EFV treatment group (n = 8) and is magnified in (d). Scale bars: 20 µm (in micrographs). (e) Quantitative data are represented in the bar graph. Data are presented as mean ± SEM (*p < 0.05).
Discussion

This study examines the effect of EFV on NSC proliferation *in vitro* and *in vivo*. Through *in vitro* experimentation with NSCs, we found that EFV resulted in a concentration-dependent decrease in NSC proliferation beginning at a concentration of 5 µM by MTT assay (Fig. 4.1A). This was further confirmed by immunocytochemistry where EFV significantly reduced the incorporation of BrdU into NSCs as identified by nestin (Fig. 4.1B).

Our findings also indicated a concentration-dependent decrease in intracellular ATP as a result of EFV treatment of NSCs (Fig. 4.2A). Even though studies have shown that NSCs function at a lower metabolic rate when compared to other cells, a decreased level of intracellular NSC ATP can inhibit the overall process of NSC proliferation [27]. Recent studies have shown how various molecules that inhibit the actions of ATP, such as tricyclodecan-9-yl-xanthogenate (D609), decrease the rate of proliferation among NSCs, therefore opposing the process of proliferation [28]. Moreover, *in vitro* results indicated that EFV lowered NSC MMP (Fig. 4.2B), an event associated with not only oxidative stress but also the initiation and activation of apoptosis [22, 23, 29].

LDH is a cytoplasmic enzyme that is released through damaged portions of the cellular membrane into the extracellular space [30]. The LDH assay has been shown to accurately measure cell death *in vitro* [31-34]. Increased levels of LDH among the NSCs treated with EFV (Fig. 4.3), as studied, are indicative of the apoptotic and/or necrotic effect of this drug on NSCs.
It has been reported that the p38 MAPK pathway and Bax are central to the negative regulation of proliferation of several cell types including stem cells [24, 25]. This was shown in our study (Fig. 4.4) by an enhanced phosphorylation of p38 MAPK as well as upregulated Bax expression in EFV-stimulated NSCs. These data are in accordance with the observed reduced proliferation (Fig. 4.1), mitochondrial function (Fig. 4.2), and increased cell death (Fig. 4.3) imparted by EFV. Further, it is in agreement with other reports indicating that the increased reactive oxygen species (ROS), a result of reduced MMP [35], promotes p38 MAPK phosphorylation [36], which in turn promotes Bax activation [26].

In addition to the in vitro treatment of NSCs, administration of EFV in vivo resulted in a decrease in NSC proliferation in C57BL/6J mice (Fig. 4.5). This was suggested by significantly decreased BrdU-positive cells in the SVZ (Fig. 4.5A). To examine whether the decreased BrdU staining that we observed in the SVZ of these mice reflected NSC commitment to the neuronal lineage, we also stained some sections with antibodies against Dcx, a neuronal lineage marker expressed in early stages of neuronal maturation [37, 38]. Here we found a strong trend toward decreased Dcx expression nearing significance (p=0.08) (Fig. 4.5B).

Since our in vitro studies showed increased cell death, cytotoxicity, and Bax expression imparted by EFV, we also stained for active caspase-3, which has previously been linked with loss of MMP [39]. We found a significant increase in active
caspase-3 in the same region (Fig. 4.5C). Thus, our combined data suggest an inhibition of NSC proliferation in vitro and in vivo through induction of apoptosis, in effect leaving fewer cells present in the SVZ to divide as a result of EFV administration.

Regarding the role of EFV in neurodegeneration and survival of adult neurons, oral treatment of rats with EFV for 30 days promoted degenerative changes (decreased cellular population, pyknotic nuclei, and presence of microcysts and edema) in the lateral geniculate body, suggesting a mechanistic action as a neurotoxin that disrupts cellular integrity [40]. Additionally, we demonstrated the ability of EFV to cause signs of neurodegeneration in mice in which pathologies characteristic of Alzheimer's disease (AD), such as increased brain β-secretase (BACE-1) expression and enhanced soluble amyloid-β (Aβ) generation, were observed [41]. Further, we found that EFV-treated SweAPP N2a neurons showed neurodegenerative signs in the form of mitochondrial dysfunction and upregulated BACE-1 expression, which promoted Aβ accumulation in this model of murine N2a cells transfected with human “Swedish” mutant amyloid precursor protein (APP) [41].

There is accumulating data pointing to an interference by EFV with CNS energy homeostasis and subsequent neurodegeneration. One investigation exploring this showed a significant inhibition of creatine kinase (CK) activity in the hippocampus, cerebellum, striatum, and cortex of mice treated with EFV [42]. CK is a catalyst for the transfer of the phosphoryl group from phosphocreatine to adenosine diphosphate (ADP) to regenerate ATP in the brain and other tissues that consume energy at a high rate.
This reduced brain CK activity may partially be responsible for the cognitive impairments seen in EFV-treated mice, as suggested by the fact that knockout mice for CK in the brain demonstrated cognitive impairment [43]. Importantly, results acquired from both animal [44] and human [45] studies have also correlated reduced CK activity with neurodegeneration. Additionally, it has been shown that treatment with EFV (10 or 25 µM) significantly decreased the viability of rat primary cortical neurons [20]. The neurodegenerative potential of the EFV metabolite 8-hydroxyefavirenz has also been shown in primary rat hippocampal neuronal cultures. In this model, 8-hydroxyefavirenz increased entry of extracellular Ca^{2+}, which was mediated largely by L-type voltage-gated calcium channels [46].

The SVZ is an important neurogenic niche, and neurons generated in the SVZ migrate along the rostral migratory stream to reach the olfactory bulb. Importantly, brain injuries stimulate direct migration of new NSCs from the SVZ to the sites of injury [47], which may compensate for some of the neurodegenerative changes promoted by EFV as noted above. It is thus possible that reduction of NSC proliferation in the SVZ due to EFV could contribute to a decline in regenerative actions of these cells, which under non-EFV conditions would oppose the chronic injury induced in the brain by HIV itself, HIV-activated glial cells, and/or the HIV-secreted proteins such as gp120 and Tat [48].

It is important to note that EFV has been linked to cognitive dysfunction in previous studies [16, 18, 19]. For example, Ciccarelli and colleagues found that EFV is associated with cognitive disorders even in asymptomatic HIV-infected patients [18].
Most recently it was also found clinically that long-term EFV treatment was associated with detriments to speed of information processing, verbal fluency, and working memory [16]. We speculate that EFV-mediated reductions of NSC proliferation may underlie some of these reported effects, although no studies to date have examined the exact relationship between reduced NSC proliferation and cognitive dysfunction specifically in EFV-treated HIV patients.

The current research has several strengths and weaknesses. Regarding the former, we observed consistent findings in both *in vitro* and *in vivo* model systems in that EFV causes decreased NSC proliferation. Additionally, we found the level of cellular oxidative stress (reduced ATP stores and MMP) imparted by EFV correlated consistently with reduced NSC proliferation and increased cytotoxicity, as well as p38 MAPK and Bax promotion at the 5 µM concentration. Second, our data coincide with other reports indicating that the process of NSC proliferation is highly sensitive to the redox balance in that cell proliferation is favored in more reduced environments, and cell differentiation is more favored in oxidized environments. As a result, as the levels of oxidative stress increase, the rate of proliferation decreases [49].

This report has limitations as well. First, it describes a mechanism for a subset of HAND cases since not all HIV-infected individuals are on EFV therapy. It should also be noted that, in the present study, we did not investigate the plasma or cerebrospinal fluid (CSF) concentrations of EFV or its metabolites in vivo. However, EFV has good CNS
penetration [50], which could support the neurologic symptoms noted by others [16, 18, 19].

In sum, our present work suggests that EFV promotes a decrease in NSC proliferation, which is correlated with activation of oxidative stress pathways as well as Bax and caspase-3 upregulation. It is in accordance with other reports indicating that oxidative stress promotes p38 MAPK phosphorylation [36], which in turn promotes Bax upregulation [26]. Together these data suggest that EFV is involved in negative regulation of NSC proliferation by reducing the number of existing NSCs that are available to divide and generate newborn cells. Given that oxidative stress correlated with the defects in NSCs, this study also lays the groundwork for future experiments to identify possible antioxidant adjunctive treatments to be given with EFV that would promote protection of NSCs.
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CHAPTER FIVE
FINAL CONSIDERATIONS

Discussion

The Human Immunodeficiency Virus (HIV) pandemic still persists worldwide. What was once seen as a death sentence has now evolved into a chronic disease due to the introduction of combination antiretroviral therapy (cART) in 1996. Since this time, the life expectancy of HIV-positive patients has dramatically increased. As a result more individuals are living longer lives, but there has been an increase in the prevalence of certain forms of HIV associated neurocognitive disorder (HAND) within the HIV positive population. Up to approximately 50% of HIV-infected patients suffer from some form of HAND, yet there is not a clear understanding of the cause of HAND, nor are there any current preventive or therapeutic agents [1].

Clinically, there can be changes in cognition (learning, memory, attention, and executive function), motor coordination (psychomotor slowing, hypertonia) and behavioral changes (apathy) [2-4]. A major study linking Alzheimer’s Disease (AD)-like pathology in HIV-positive patients showed beta amyloid (Aβ) plaques formed in greater amounts in HIV-positive patients who died between 30-69 years of age compared to HIV-negative age-matched patients [5]. In fact, epidemiological data suggests that persons living with HIV are at elevated risk of developing AD-like type brain pathology.
Aβ generation and aggregation are key events in AD, which leads to neurotoxicity and CNS inflammation [9-11].

There has been substantial evidence showing the negative impacts that both the HIV virus and its proteins (tat, gp120) can have on the CNS. However, the potential early onset of AD-like pathology in the HIV population can be induced by several other factors. One such factor may be certain components of cART, a combination of several drugs that have drastically decreased the morbidity and mortality rates as a result of their ability to efficiently suppress the HIV viral load [12, 13]. Despite these results, few research studies have focused on the role these life long cART medications may play in its pathogenesis.

Several factors may contribute to HAND pathology such as: poor cART adherence, emergence of resistant viral species, or residual viral DNA in the central nervous system (CNS) [14]. With the prevalence of milder forms of HAND increasing and the mechanism still unexplained, it is crucial to look at the impact of antiretrovirals (ARVs) on the CNS. Pharmacokinetic studies suggest that many ARVs have limited penetrance into the CNS and that ARV concentrations are low in the cerebrospinal fluid (CSF) and parenchyma. However, it is important to consider that the blood brain barrier (BBB) is compromised by the viral proteins or concomitant factors that could possibly result in higher concentrations of ARVs within the CNS of HIV-positive patients [14]. In addition, efaviranz (EFV) is among the best of all ARVs with a score of 3 out of 4 in the CNS Penetration Effectiveness Rank, which is a measure of ARV penetration into the
CNS [15]. The possible impact of EFV in the CNS of HIV-positive patients is therefore clinically relevant and can be another potential contributor to HAND pathology and its neuropsychiatric symptomology.

While cART increases patient lifespan, it may contribute to CNS toxicity. The prospect of ARVs contributing to or inducing HAND is not well studied, but our lab and other research groups show that certain ARVs induce oxidative stress and neuronal damage in the CNS [14, 16-20]. Furthermore, ARVs are associated with potentially serious side effects. EFV, prescribed worldwide since 1998, has clinical CNS side effects including: neurocognitive decline, hallucinations, psychosis, abnormal dreams, and insomnia [21-24]. In mice, EFV affected anxiety and cognitive performance [25]. Long term use of EFV has also been shown to correlate with the worsening of neurocognitive functioning including speed of information processing, verbal fluency, and working memory [26]. Our studies originality is based on the fact that there is limited research that focuses on the importance of cART drugs in pathology that may promote HAND. The central focus of our study was to determine the effect of the commonly prescribed ARV drug (EFV) and its roles in HAND pathogenesis in terms of oxidative stress, Aβ production/clearance, and neurogenesis.

As aforementioned, a key element of HAND pathology may parallel that of AD. Significant Aβ deposition in the brains of HIV infected patients was observed [5], and it has been suggested that Aβ deposition is a possible pathological feature of HIV infection [6, 27]. Chapter 3 explores the effect of the non-nucleoside reverse
transcriptase inhibitor (NNRTI), EFV, on Aβ accumulation. The major finding and conclusion of this work is that EFV is neurotoxic in vitro and in vivo by increasing Aβ accumulation. As ARVs are often given in a regimen, we first evaluated the 3-drug regimen of zidovudine (AZT), lamivudine (3TC) and EFV. We have shown that EFV treatment confers the most toxicity of the 3-drug regimen prescribed (compared to AZT and 3TC) [20]. This was extrapolated from our data that showed EFV: 1) increased Aβ production; and 2) decreased Aβ clearance. This was then further supported by EFV promotion of oxidative stress in the form of decreased mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP), with increased reactive oxygen species (ROS) production. This work, combined with that of other labs, shows that EFV has significant neurotoxic effects particularly on mitochondrial dysfunction [17, 19, 20]. This increase in ROS production leads to the activation of beta secretase-1 (BACE-1), an enzyme involved in amyloidogenic APP processing, and ultimately promotes neurodegenerative and AD-like pathology (Chapter 3) [28, 29]. Considering that EFV is the main player in toxicity within the cART regimen, this presented the opportunity to explore other neurodegenerative characteristics the drug may have.

As stated earlier HAND patients displayed cognitive dysfunction including, but not limited to, deficits in learning, memory, attention, executive function [2-4]. The hippocampus plays a major role in learning and memory. It also is extremely susceptible to inflammatory insults that affect the brain. Adult neurogenesis has only recently been accepted to occur postnatally. However, the importance of adult neural stem cells (NSCs) has taken the interest of the neuroscience field due to the important roles they
may play in learning, memory, and repair of damaged areas of the brain. Therefore, we wanted to elucidate if EFV toxicity may also have further effects on neurogenesis (Chapter 3). The conclusions drawn from the study on neurogenesis did in fact show negative effects on NSC proliferation [30]. These experiments found that EFV \textit{in vitro} and \textit{in vivo}: 1) reduced proliferation of NSCs; 2) promoted increased mitochondrial dysfunction; and 3) increased cytotoxicity and cell death. Evaluating further, it was shown that there was an increase in p38 phosphorylation and Bax, which suggests a mechanism underlying the observed cell death and reduced NSC proliferation (Chapter 4) [31, 32]. Considering the side effects often reported (e.g., cognitive decline, sleeping disruptions and hallucinations); the impact of EFV on the CNS at the cellular and molecular level is not surprising. This research has nevertheless characterized a possible EFV mediated mechanism that could affect the CNS negatively, especially over the course of long term treatment.

Due to the results of these experiments, it is important to begin looking to the future for improvements in ARV therapy. In April 2015, the Department of Health and Human Services (DHHS) changed the recommended regimen for cART naïve patients [33]. The first line regimen, that included efavirenz (EFV), is now adjusted to consist of a new generation of drugs classified as integrase inhibitors (InSTIs) and protease inhibitors (PIs). The InSTIs include dolutegravir (DTG), elvitegravir (EVG), raltegravir (RAL), while darunavir (DRV) is a PI. One of these InSTIs or the PI is given in different combinations with 2 nucleoside reverse transcriptase inhibitors (NRTIs) to complete the 3 drug cART regimen [33].
InSTIs are a more recent drug class. InSTIs inhibit the strand transfer activity of HIV integrase in the absence of an effect on 3’ end processing [34]. DTG has been available since 2013 through Fast Track approval from the Food and Drug Administration (FDA). It has been approved for cART naïve patients as well as those who may have been on other drug regimens. With limited time to evaluate CNS effects, DTG-containing regimen was shown to increase the risk of insomnia compared to an EFV-containing regimen. Overall however, DTG has been reported to be well tolerated with very few side effects, of which the most common included the aforementioned trouble sleeping, as well as tiredness, and headaches [35-42]. RAL has been available for use since 2007 for patients that were already on some form of cART therapy; however, in 2009, it was made available for cART-naïve patients. In a case report of four patients RAL was said to worsen depression after initiation of treatment [43], while there have also been cases of insomnia induced by RAL [44-46]. ELV was approved by FDA in 2012. Currently there are no reports on CNS effects. However, the most common side effect of ELV is reported to be diarrhea [47, 48].

PIs block proteolysis of the viral polyprotein, which is required to produce infectious virus particles through cleaving the viral precursors. DRV was approved by FDA in 2006. While there have not been a large amount of CNS side effects reported, some of the common side effects reported for patients taking DRV-containing regimens include diarrhea, nausea, rash, headache, stomach pain, and vomiting [49-51].
In general InSTIs are highly effective and have few adverse effects including no significant drug-drug interaction (specifically CYP 3A4) [33]. Even more so, InSTIs have been shown to be better tolerated compared to PIs [33, 52]. Thus it is highly recommended by the DHHS to select an InSTI for most patients. However, DRV does have an advantage. If resistance test results are not readily available prior to treatment or adherence is uncertain, DRV (the PI) has a high genetic barrier to resistance, and low rate of treatment-emergent resistance. DTG has also been shown to be beneficial in this population of individuals [33].

When looking at the increasing numbers of individuals reporting CNS side effects due to EFV, as well as results such as our own work showing the potential negative consequences of EFV on the CNS, it was expected that the regimen would be altered. So far, the research in our lab has focused on the effects of EFV on the CNS and the possibility of it being a contributor to HAND. EFV increased Aβ accumulation (Chapter 3) and decreased neural stem cell (NSC) proliferation (Chapter 4). Furthermore, EFV has been extensively researched for mitochondrial dysfunction. It has been shown to decrease cell viability and increase the release of reactive oxygen species (ROS) [18-20]. In future studies, due to the changes in the first line of regimen in the U.S., we would aim to elucidate whether the new recommended drugs (DTG, RAL, ELV, and DRV) had neurotoxic effects that may lead to Aβ accumulation, increased oxidative stress or decreased NSC proliferation. The direction for this project would include evaluating in vivo studies especially considering the newer drugs have been reported to be more tolerable than EFV.
Unfortunately, while the DHHS has changed their guidelines, the impact of HIV has not changed for developing nations around the world that still have EFV as a first line recommended drug. UNAIDS estimated of the 35 million people infected by HIV at the end of 2013, about 24.7 million live in sub-Saharan Africa; accounting for more than 69% of persons living with HIV. In the US alone, 1.2 million people are infected with HIV as of 2013, which accounts for only 3% of all HIV cases in the world [53]. These statistics are important when looking at the continued changes occurring with cART regimens. Within each region or country there are specific recommendations that govern treatment provided to individuals infected with HIV. In the United States, this is governed by the DHHS. These laws change rapidly as new drugs are created and passed by FDA for treating HIV patients. On a global scale however, the World Health Organization (WHO) sets guidelines in order place in order to explore the needs of those countries that may not be able to have access to newer recommended drugs as they are updated. In most regions affected by HIV, such as sub-Saharan Africa and Asia, they follow the 2013 WHO guidelines [54]. Notably, the WHO guidelines were again revised in 2016, however EFV still remained a first like ARV recommendation [55]. Therefore, further studies to understand other possible negative CNS effects of EFV may be needed.

It is possible that EFV may have an impact on tau hyperphosphorylation, a characteristic seen in both HAND and Alzheimer’s patients. In our review [56], we examined human studies of HIV positive patients measuring p-tau (phosphorylated tau)
or t-tau (total tau) in cerebrospinal fluid (CSF) or via post-mortem brain immunohistochemistry. Several studies show that p-tau, t-tau, or both were increased in patients [7, 57-59]. In a more recent study, Cysique and colleagues showed that increased CSF p-tau levels were associated with current neurocognitive impairment in the majority of their HIV infected study participants [60].

It would be advantageous to explore adjunctive therapies that can reduce the toxicity of EFV for those currently on the drug regimen. cART has limited capability in CNS efficacy, it cannot directly ameliorate HAND pathology, and has in some cases shown negative effects on cognition. Consequently, alternative methods are needed for HIV and HAND treatment. Because the largest population of HIV-positive individuals live in developing countries, researchers are exploring natural antiviral remedies. These remedies would not only be cost effective, but may also display an equal or greater efficacy than the currently approved cART medications. Recent reviews and experiments have analyzed natural extracts (i.e., flavonoids, phenols, and terpenoids) that have the ability to affect the virus at all levels of its life cycle [61-67]. These extracts also show improvements in CNS toxicity in regard to one or more of the following: ROS, NOS, Aβ, tau, neurogenesis and other degenerative pathologies [68-75]. Each of these improvements are also pathological characteristics used to explain the possible underlying mechanisms of HAND development and can be observed despite cART treatment in many persons living with HIV.

As an example, the flavonoid Quercetin is found in Thevetia peruviana (also referred to as yellow oleander or lucky nut): a plant found in Central America and the
Caribbean. Quercetin has been shown to have HIV integrase inhibitory activity [76]. Quercetin also had antioxidant and anti-inflammatory effects, providing possible roles in ischemic brain injuries and neurodegenerative disease through protection against oxidative stress. It ameliorated brain damage and provided neuroprotection in the subarachnoid hemorrhage mouse models [77]. Quercetin was also shown to significantly reduce hyperphosphorylation of tau, Aβ1-40 and Aβ1-42 levels, and a decrease BACE-1-mediated cleavage of APP [78]. With more directed research in this area, it could open new possibilities, not only for the prophylaxis or treatment of HAND pathology but for anti-HIV treatment in general.

The overall limitation of this study is that it may only apply to a subset of HIV patients who would have EFV in their drug regimen. Indeed, we have not explored all drug regimens and the impact on the CNS. Because of the strong previous recommendations of EFV as a part of a first line treatment regimen, a majority of persons living with HIV would have more than likely been prescribed an EFV containing regimen in the course of the HIV therapy.

Conclusion

In the course of our research, I have been able to visit and work in 2 of the high HIV prevalent countries in the world, Malawi (visited in 2016) and Kenya (visited in 2013). To date, there is a high prevalence of adults infected with HIV in both in Malawi and Kenya [79, 80]. While working with either the Development Initiative Network (Malawi) or Centers of Disease Control and Prevention/ Kenya Medical Research
Institute (CDC/KEMRI, Kenya), it was apparent the need not only for our research, but the improvements needed for ARV therapy affecting the CNS as a whole. In both countries there was an under-diagnosis of most neurological diseases due to resource limitations. This includes but is not limited to: 1) the inability to have access to diagnostic tools such as magnetic resonance imaging (MRI) and computerized tomography (CT) scan; 2) lack of specialized personnel (i.e. there are .03 neurologists for 100,000 persons in Kenya in comparison to 2.96 in a developed nation such as the United States [81]), and finally 3) the focus on the more life threatening prognoses (such as death) in high prevalent diseases like malaria, TB and HIV. This is not uncommon for developing countries, in which Malawi and Kenya are ranked among the poorest countries. It is therefore crucial to begin to set in place accessible treatment options for those affected by CNS complications within regions most affected by HIV.

AZT was the first drug approved by the Food and Drug Administration (FDA) to treat HIV in 1987, with a record-breaking approval of less then 4 months via priority review [82]. While the FDA has become more aware of rapid drug approval through various methods such as fast-track and break-through drug therapy protocols, HIV drugs are still rapidly approved due to the need to control viral loads, which ultimately saves lives [83]. However, this may limit the ability to explore more long term effects of these drugs, such as EFV, that may be present after chronic use. This project has therefore added to the knowledge base of the role EFV may have in HAND development, as well as identified mechanisms by which EFV may result in CNS side effects. Our findings suggest there may be a neurotoxic consequence for patients on
EFV therapy. This underlying neurotoxicity stems from mitochondrial dysfunction and ROS production, in turn leading to Aβ accumulation and a reduction in NSC proliferation. The culmination of these results suggests that EFV may have play a role in pathologies that could promote HAND and measures may be needed to improve the future of cART therapy as it relates to neurotoxicity.
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APPENDIX A

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