THE EFFECT OF ANTIRETROVIRAL THERAPY ON THE INTEGRATED STRESS RESPONSE IN THE CENTRAL NERVOUS SYSTEM: IN VIVO ASSESSMENT IN SIV-INFECTED RHESUS MACAQUES

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Dedication

I dedicate my dissertation work to my family. A special feeling of gratitude to my loving mother Afaf, whose words of encouragement and push for tenacity ring in my ears. My beloved husband Abdulmajeed, who was very supportive and caring even across countries. My sunshine, my daughter, Layali for brightening my life with her joy and innocence. Finally, my father Rashad whose guiding spirit remains ever-present. My sisters, brothers, nieces, and nephews who were always one video-call away.
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Abstract

**Background** Despite the benefits of antiretroviral therapy (ART), a large percentage of people with HIV (PWH) still suffer from some form of neurocognitive impairment. Studies have shown that high degree of oxidative stress and inflammation remain present in the central nervous system of PWH, which can activate the integrated stress response (ISR) pathway. We previously showed that the levels of several markers for ISR activation, such as phosphorylated eukaryotic initiation factor 2α (P-eIF2α), were elevated in neurons and astrocytes in the cortex in autopsy brain tissue of PWH. Phosphorylation of eIF2α is mediated by four kinases, which will result in ISR activation, as reported in several neurodegenerative disorders. In general, ISR is an adaptive pathway; however, chronic ISR activation may contribute to neuronal damage and neurocognitive impairment in PWH. Recently, several studies have reported that certain ART drugs contribute to the persistence of HIV-associated neurocognitive disorders and can induce the ISR. The aim of the present study was to assess ISR activation in neurons, astrocytes, and oligodendrocytes in brain tissue samples of SIV-infected rhesus macaques in a lentiviral model of HIV infection.

**Methods** We examined necropsy brain tissue specimens of 11 rhesus macaques, including SIV-infected/ART-untreated macaques (n = 3), SIV-infected/ART-treated macaques (n = 4), and uninfected/untreated (n = 4) macaques, with the aim to determine if ART aggravated ISR activation in the CNS. Formalin-fixed/paraffin-embedded sections of cortical tissue were
immunofluorescently stained using an antibody to p-eIF2α to assess ISR activation and antibodies against MAP2, GFAP, and ASAP to label neurons, astrocytes, and oligodendrocytes, respectively.

**Results** By semiquantitative analysis of images of the stained specimens we found that p-eIF2α levels in neurons was significantly higher in the SIV-infected/ART-treated group than in the SIV-infected/ART-untreated group. However, we did not observe differences in p-eIF2α levels in astrocytes the gray matter and oligodendrocytes in the white matter among the three groups.

**Conclusion** In our study, we observed a significant increase in ISR activation in neurons in the gray matter of the SIV-infected/ART-treated rhesus macaques compared to the SIV-infected/ART-untreated rhesus macaques, which we did not observe in astrocytes and oligodendrocytes.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover page</td>
<td>1</td>
</tr>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Table of Content</td>
<td>6</td>
</tr>
<tr>
<td><strong>Chapter I THE REVIEW OF THE LITERATURE</strong></td>
<td>8</td>
</tr>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Signs and symptoms of HIV</td>
<td>9</td>
</tr>
<tr>
<td>Clinical Stages of HIV infection</td>
<td>10</td>
</tr>
<tr>
<td>Human Brain</td>
<td>11</td>
</tr>
<tr>
<td>Brain cells</td>
<td>11</td>
</tr>
<tr>
<td>The Era Prior to Antiretroviral Therapy</td>
<td>13</td>
</tr>
<tr>
<td>HIV Associated Neurocognitive Disorders</td>
<td>14</td>
</tr>
<tr>
<td>Antiretroviral Therapy</td>
<td>17</td>
</tr>
<tr>
<td>Integrated Stress Response</td>
<td>20</td>
</tr>
<tr>
<td>Review of the current literature on the effect of ART on the CNS</td>
<td>22</td>
</tr>
<tr>
<td>Study Hypothesis</td>
<td>25</td>
</tr>
<tr>
<td><strong>CHAPTER II THE THESIS</strong></td>
<td>26</td>
</tr>
<tr>
<td>Introduction</td>
<td>27</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>33</td>
</tr>
</tbody>
</table>
CHAPTER I

REVIEW OF THE LITERATURE
Introduction

Human immunodeficiency virus (HIV) is a lentivirus within the family of retroviridae that targets the immune system. HIV affects 38 million people worldwide according to the Centers of Disease Control and Prevention(1, 2). Chronic untreated HIV can lead to acquired immunodeficiency syndrome (AIDS). Currently, no effective cure has been established; however, proper medical care with antiretroviral therapy (ART), which includes multiple drugs, can control HIV symptoms, allowing people with HIV (PWH) to live a long life without infecting their partners and transferring infection to others. Thus, proper treatment has changed HIV diagnosis from a deadly disease to a chronic manageable condition(1).

Symptoms of HIV infection: Summary of the most common signs and symptoms of HIV infection is shown in (Figure 1).

Figure 1. Common signs and symptoms of HIV infection (1).
Clinical Stages of HIV infection

Stage 1: Acute infection, a very contagious stage as there is a large amount HIV in the peripheral blood. During this phase, infected individuals will experience flu-like symptoms. This stage can last for a week or two (3), then individual will no longer have the flu symptoms.

Stage 2: Chronic HIV infection, also called asymptomatic HIV infection or clinical latency. HIV is still actively replicating in the body. It is still transmissible, but PWH may not have any symptoms or get sick during this phase. With proper treatment, PWH may never progress to Stage 3 (AIDS). Without treatment, this stage may last a decade or longer, or may progress faster. At the end of this stage, the amount of HIV in the blood (viral load) increases and the PWH progresses to Stage 3 (AIDS).(1)

Stage 3: AIDS, this is the most severe stage of HIV infection and is characterized by reduction of CD4 T cell count to be below 200 cells/ml\(^3\) of blood. People with AIDS can have high viral loads and may easily transmit the virus to others. The immune system is severely damaged in people with AIDS, who are therefore at higher risk of opportunistic infections or other serious illnesses. Without proper treatment, individuals with AIDS usually survive about three years(1).

In this review, we will summarize the effect of current HIV treatment on brain cells.
Human brain

The most complex part of the human body is the brain, which performs and controls the body’s activity and all functions. The human brain controls behavioral, emotional, intellectual, and motor function. Understanding the brain anatomy and physiology can help understand how the human brain functions and what happens in case of dysfunction (4).

Brain cells

Neurons are the primary cells of the brain and the nervous system. Their main function is to transmit nerve impulses across neurons through synapses in a process called action potentials. These impulses are responsible for all human activities. Each neuron consists of three parts: cell body, axon, and dendrites (5). (Figure 2) shows the anatomical structure of the neuron.

![Neuron Diagram](image)

Figure (2) the anatomical structure of the neurons.

Other brain cells include glia cells, which can be further subdivided into microglia, astrocytes, and oligodendrocytes. Microglia are the brain-resident immune cells which are responsible in protecting the brain from mild injuries and clearing out the waste material.
Astrocytes are star-shaped cells that control neurotransmitters and are required to maintain homeostasis within the brain by storing glycogen; they also perform crucial immune functions as they alert the immune system in case of any foreign body invasion to initiate phagocytosis of damaged neurons and promote neurogenesis (6) (Figure 3). Oligodendrocytes also support neuronal axons, especially those traveling for long distances. They also produce the myelin sheath that wraps around the axon to facilitate the saltatory transmission of electrical impulses between neurons (6) (Figure 3).

HIV can only infect certain cell types: CD4+ T cells and myeloid cells (macrophages and microglia). The infection of HIV in microglia and macrophages in the brain is productive, which can result in production of viral proteins and proinflammatory cytokines (21). Even though some viral proteins may be detected in astrocytes, this is considered a non-productive infection. As HIV entry requires a CD4 receptor and a co-receptor, oligodendrocytes and neurons that lack these receptors cannot be infected with HIV. However, infection with HIV can cause damage to these cells through the products released by the HIV-infected macrophages and microglia.
Figure (3) the anatomical structure of the astrocytes and the oligodendrocytes.

**The Era Prior to ART**

Knowing the survival pattern of PWH before the introduction of ART serves as an important baseline to evaluate the success of newly developed treatment strategies. Isingo et al, conducted a household-based demographic surveillance between 1994 and 2006, that allowed them to calculate the number of years the person lived after they were infected with HIV before the introduction of ART (7). They concluded that the estimated survival was 67% at 9 years after infection and that the estimated median survival was 11.5 years. They also found that the age when the infection took place was strongly associated with survival duration. Wekesa et al, examined the temporal trends in characteristics, retention, and mortality outcomes, comparing the era before and after ART introduction in a retrospective study (8). They concluded that there was an increase in the retention in treatment and reduction in the 6-month mortality rate of PWH between the years of 2004 and 2014, which declined from 7.5% to 3.8%, with an increase in the rate of PWH in the asymptomatic disease stage from
8.7% to 43.1% and a decrease in the rate of those with advanced disease from 42.5% to 11.9%, after the use of ART (8).

**HIV-associated neurocognitive disorders**

HIV-associated neurodegenerative disorders (HAND) are a clinical manifestation of HIV infection, notable for disturbances of cognitive, behavioral, motor, and autonomous function. Clinically, the prevalence of the most severe form of HAND, HIV-associated dementia (HAD) is rare in the current ART era. However, both asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND) are more frequently observed. ANI is defined as acquired cognitive impairment that involves at least two ability domains, which does not interfere with daily functions. MND, is characterized by impairment that interferes with daily function, that could be reported by the patient or the people who know them. PWH with HAD have marked deficits in day-to-day functions (9). Even though 70% of PWH with HAND are asymptomatic, they are still two to six-fold increase in the risk of developing symptomatic disease. The prevalence of neurocognitive dysfunctions caused by HIV has increased over time.

HAND is caused by a complex and multi-dimensional immunopathologic process caused by viral infection as well as several host-related factors. Before the introduction of ART, plasma viral load and CD4+ T cell counts were used to predict the development of HAND, which do not serve a similar purpose after the ART introduction. Several studies have identified the following as risk factors for the development of HAND: low educational level, severely
compromised immune system, genetic predisposition, substance misuse, old age, and an association between cardiovascular diseases and HAND development (9) (10) (Figure 4).

Figure 4. Some of the factors contributing to the development of HAND can be summarized in this illustration. Viral infection and the viral byproducts in addition to genetic predisposition as well as substance misuse, cardiovascular diseases, old age and potentially the use of ART.

More severe forms of HAND can be prevented by controlling viral replication. Viral suppression should slow down or stop the disease progression; therefore, the use of ART should suppress HIV replication, prevent, and treat milder forms of HAND. Randomized controlled trials found that ART improves the cognitive ability in PWH with HAND (9). Studies also showed that the severity of HAND could be reduced by ART usage (11). Figure 5)
demonstrates the differences in the prevalence of different stages of HAND before and after the introduction of ART.

Figure 5. Changes in the prevalence of HAD, MND, and ANI following the implementation of ART (11).

Abbreviations: HAD, HIV-associated neurocognitive disorders; MND, Mild neurocognitive disorder; ANI, Asymptomatic neurocognitive impairment; ART, antiretroviral therapy

Nevertheless, the prevalence of HAND after the introduction of ART does not seem to be decreasing, which suggests the potential effect of ART as a contributing factor for the persistence of HAND development. In our lab, several studies were conducted to investigate the effect of ART in the CNS (12-18).
Antiretroviral Therapy

ART is the current treatment of choice for PWH. Usually, these medications are taken as a combination of 3 or 4 antiretroviral drugs (ARVs). Often referred to as "highly active antiretroviral therapy" or HAART, these drugs suppress HIV replication. ART should be started as soon as the diagnosis is confirmed. ART acts by suppressing viral replication by targeting different stages of the viral replication cycle. ART has led to reduction in mortality and morbidity rates and improved the quality of life in PWH. Another advantage of these drugs is that they aid in prevention the viral transmission by suppressing the viral replication in PWH. This benefit of ART is also defined as “undetectable equals untransmittable”, signaling that people with undetectable HIV cannot transmit it to others. Since the introduction of ARVs in 1996, the HIV-related death incidence has decreased significantly. The current life expectancy of PWH is close that observed in uninfected people (9). Figure 6 shows deaths that were prevented by the use of ART (19). In that study, the authors concluded that in 2022 the number of the expected HIV-related deaths would be 1.44 million deaths without ART. However, the actual death number was 627,547 (20).
**Figure 6.** Representation of the expected HIV-related deaths that were prevented by the use of ART (blue) compared to the actual number of deaths (maroon) (20).

**Figure 7.** HIV replication cycle and the classes of antiretroviral drugs.

Abbreviations: NRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; INSTI, integrase inhibitor
ART, as previously mentioned, targets the viral replication in different stages of the cycle (Figure 7), commencing with the virus entering the cell. HIV requires two receptors for productive infection: the CD4 receptor and a co-receptor, either CCR5 or CXCR4. Once inside the cell, viral RNA in the cytoplasm is reverse-transcribed into DNA, which integrates into the host DNA. This integrated DNA is then utilized for synthesizing viral RNA. Subsequently, viral RNA is encapsulated within viral proteins and released as new virus particles. The ARV classes are the following: nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), and protease inhibitors (PIs). When these drugs are used in combination, they target multiple stages of the viral replication cycle thus, increasing the drug efficacy and decreasing the possibility to develop drug resistance.

The most common side effects of these drugs are nausea and vomiting. PWH on ART may also suffer from diarrhea, difficulty breathing, dry mouth, headache, rash, dizziness, fatigue, and temporary pain at the injection site (in case of injectable ARVs) (21), (13).

Despite the fact that the ART increases the life expectancy of PWH and improves their quality of lives, 15% to 50% of the treated PWH may still develop neurocognitive impairment. However, the mechanisms are still not completely known. Several studies found that some ARVs with high CNS penetration capacity were associated with more frequent neurological symptoms, suggesting that ART may cause neurotoxicity(22-26). Some studies suggest that
ART may also cause oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction. In our lab, we found that certain ARVs exhibit neurotoxic potential through specific cellular pathways, that differ among ARVs that belong to the same class (16), and that integrated stress response (ISR) was one such pathway involved.

Integrated Stress Response

ISR is an adaptive response to cellular stresses such as inflammation, oxidative stress, and viral infections. However, chronic activation can be maladaptive and contribute to neurodegeneration. Eukaryotic initiation factor 2α (eIF2α) is the main mediator of ISR. This protein is involved in cap-dependent protein translation, and its phosphorylation causes global translation attenuation. The phosphorylation of eIF2α can be initiated by four kinases and contributes to downstream signaling cascades that will result in the activation of ISR. These four kinases are protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), heme-regulated inhibitor (HRI), and general control nonderepressible 2 (GCN2) (17) (Figure 8).
Upon phosphorylation, eIF2α initiates two crucial processes: First, it inhibits global protein synthesis, allowing the cell to pause and adapt to the stress. Second, it upregulates specific proteins involved in resolving stress and activates ATF4, a transcription factor that induces genes essential for stress resolution. However, chronic activation of the ISR can have adverse effects, including the upregulation of proapoptotic genes such as caspase 4 and CHOP, which exacerbate the stress response. Chronic ISR activation can contribute to neurodegeneration by inducing neuronal cell injury and death. (17).
Review of the current literature on the effect of ART in the CNS

In a review of the potential mechanisms of neuronal toxicity associated with ARVs (27), the authors categorized the possible effects based on different hypotheses; Table 1 summarizes these mechanisms by which ARVs may injure the CNS (27) (28) (29)(Figure 9).
<table>
<thead>
<tr>
<th>Neurotoxicity</th>
<th>Study</th>
<th>Sample</th>
<th>Observed Effect</th>
<th>Period</th>
<th>Neurotoxic Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct neuronal toxicity in vitro</td>
<td>Robertson et al. 2012</td>
<td>Cultures of rat cortical neurons</td>
<td>Neuronal shrinkage, dendritic pruning, cell death // mitochondrial damage</td>
<td>7 days</td>
<td>EFV, ABC, NVP, ETR, ATV // RTV</td>
</tr>
<tr>
<td>Direct neuronal toxicity in vivo</td>
<td>Hinckley et al. 2016</td>
<td>hiPSC</td>
<td>Mitochondrial membrane potential alteration, ROS, cytotoxicity, and neurite growth</td>
<td>3 days</td>
<td>EFV, RPV, EVG</td>
</tr>
<tr>
<td>Direct neuronal toxicity in vivo</td>
<td>Akay-Espinoza et al. 2017</td>
<td>Primary rat cortical neuronal cell cultures</td>
<td>Oxidative stress and unfolded protein response (UPR) // neuronal death.</td>
<td>6 h // 2 days</td>
<td>SQV, LPV, RTV // EVG</td>
</tr>
<tr>
<td>Direct neuronal toxicity in vivo</td>
<td>Opi et al. 2007</td>
<td>Mongolian gerbils</td>
<td>Oxidative stress on synaptosomes and mitochondria, reduction of Bcl-2, increase in cytochrome-c release, and in caspase-3</td>
<td>6h</td>
<td>ddC</td>
</tr>
<tr>
<td>Direct neuronal toxicity in vivo</td>
<td>Schweinsburg et al. 2005</td>
<td>HIV-infected individuals</td>
<td>Integrity decrease at magnetic resonance spectroscopy measurements (MRS) and decrease in NAA concentrations of frontal white matter, posterior cingulate cortex, and thalamus</td>
<td>18 h</td>
<td>Stavudine, ddi</td>
</tr>
<tr>
<td>Interference with amyloid</td>
<td>Guinta et al. 2011</td>
<td>Cultures of rat cortical neurons</td>
<td>Increase Aβ generation, inhibit microglial phagocytosis of Aβ1–42 extracellular amyloid</td>
<td>Not known</td>
<td>AZT/IDV, AZT/ABC, 3TC/IDV</td>
</tr>
<tr>
<td>Interference with amyloid</td>
<td>Soontornvinyomkit et al. 2018</td>
<td>HIV-infected individuals</td>
<td>Phospho-tau pathology // microgliosis in the putamen</td>
<td>Not known</td>
<td>DRV// RTV</td>
</tr>
<tr>
<td>Astrocytes and BBB disruption</td>
<td>Nooka and Ghorpade 2017</td>
<td>Primary human astrocytes</td>
<td>Upregulation of expression of endoplasmic reticulum stress markers, astrocyte elevated gene-1 (AEG-1), and unfolded protein re- sponses (UPRs).</td>
<td>8 H</td>
<td>3TC, ABC</td>
</tr>
<tr>
<td>Oligodendrocytes and myelin blood</td>
<td>Jensen et al. 2015</td>
<td>Primary mouse oligodendrocyte precursor cell cultures</td>
<td>Prevention of oligodendrocyte differentiation // reversible reduction of myelin proteins // ROS accumulation</td>
<td>24h</td>
<td>LPV, RTV // LPV, RTV // AZT, RTV</td>
</tr>
<tr>
<td>Indirect effect on blood</td>
<td>Bertrand et al. 2016</td>
<td>Human cerebral microvascular cells (hCMEC)</td>
<td>Alteration in claudin-5 expression, increase in endothelial permeability, disruption in BBB integrity</td>
<td>48h</td>
<td>EFV</td>
</tr>
<tr>
<td>Interference with neurotransmitters</td>
<td>Robertson et al. 2012</td>
<td>Cultures of rat cortical neurons</td>
<td>Increased sensitivity to glutamate</td>
<td>2 days</td>
<td>DRV/3TC/ABC</td>
</tr>
<tr>
<td>Autophagy in microglia</td>
<td>Tripathi et al. 2020</td>
<td>Primary rat microglia</td>
<td>Increased formation of autophagosomes as demonstrated by a time-dependent increase of autophagy markers.</td>
<td>24h</td>
<td>—</td>
</tr>
<tr>
<td>Autophagy in microglia</td>
<td>Cheney et. Al.</td>
<td>Primary human astrocytes</td>
<td>Autophagosome biogenesis was inhibited after 24 h as well as after 7 days of daily treatment with the cocktail.</td>
<td>24h / 7 days</td>
<td>P62</td>
</tr>
</tbody>
</table>
Several ARVs have been shown to induce toxicity in neurons and oligodendrocytes. In a study by Stern et. Al, which examined the effect of INSTIs and PIs on rat neuronal cultures, some but not all ARVs caused oxidative stress, ISR and neuronal toxicity \textit{in vitro}. The authors also showed that the attenuation of ISR using trans-ISRIB, which was recently shown to prevent p-eIF2α-mediated ISR activation, was partially protective against neurotoxicity induced by elvitegravir, and INSTI (16). Another study by Roth et. al. (10) investigated the effect of clinically used ARVs on oligodendrocyte precursor cell (OPC) differentiation. They found that elvitegravir inhibited oligodendrocyte maturation while raltegravir did not, confirming a previous study that also found that some PIs also inhibited the maturation of
oligodendrocytes in vitro (30). The investigation also confirmed that elvitegravir caused impairment of oligodendrocyte maturation and remyelination through the ISR, a possible therapeutic target (14).

The overall hypothesis of the current study states that ART induces the ISR in PWH. To study this effect, we used a simian model of lentiviral infection. The current literature in the context of ART and lentiviral infection has studied macaques by looking at viral latency and the ability of the infected cells in the CNS to reestablish productive infection when ARTs are interrupted (31). White et al. (32) studied the effect of ART in the CNS of SIV-infected macaques. However, they investigated the spatial heterogeneity of brain lipids during infection that could possibly impact neurologic function and did not examine possible ART effects in CNS cells (33). Solis-Leal et al. studied viral rebound after the interruption of ART in macaques by investigating lymphoid organs such as spleen and lymph nodes (33). However, none of the studies to date on macaques have investigated the potential activation of ISR by ART. Therefore, we aimed to investigate the potential activation of ISR in neurons, astrocytes, and oligodendrocytes in SIV (+) rhesus macaque brain tissue to determine if ART aggravates or induces the ISR in neurons, astrocytes, and oligodendrocytes. This approach would allow us to gain a more solid and contemporary answer while controlling possible contributing factors.
CHAPTER II

THESIS
Introduction

Human immunodeficiency virus (HIV) targets the immune system, and chronic untreated HIV infection leads to marked decreases in plasma CD4+ T lymphocyte counts to below 200 cells/mm³, which results in the development of acquired immunodeficiency syndrome (AIDS) (6), (4). Currently, there is no effective cure for HIV infection, and once infected, people with HIV (PWH) have it for life. However, proper medical care using antiretroviral therapy (ART) can control HIV symptoms, allowing PWH to live a long life without infecting their partners and transferring HIV. Therefore, ART has transformed a fatal diagnosis to a chronic manageable condition (1, 3).

Currently, there are more than 39 million PWH worldwide (2). Although HIV infection is incurable, ART, which includes a combination of several antiretroviral drugs (ARVs), should be started as soon as the diagnosis is confirmed. These medications act by suppressing viral replication by targeting different stages of the viral life cycle and have led to significant reductions in the mortality and morbidity rates and improved the quality of life of PWH. Since the introduction of ART in 1996, the incidence of HIV-related deaths has decreased significantly. In a study that looked at the HIV-related deaths that were prevented by the use of ART, the authors concluded that in 2022 the number of the expected HIV related deaths to be 1.44 million deaths. However, the actual death number was 627,547 deaths (20). The current life expectancy of PWH is close to that of uninfected people (9). Another advantage of ART is
that they aid in the prevention of viral transmission; this benefit of ART is also defined as “undetectable equals untransmittable.”(34)

**Figure 1.** HIV replication cycle and specific classes of antiretroviral drugs.

Abbreviations: NRTI, nucleotide reverse transcriptase inhibitor; NNRTI, non-nucleotide reverse transcriptase inhibitor; INSTI, integrase inhibitor

ART targets multiple stages of the HIV replication cycle. Figure 1 is an illustration of the HIV replication cycle and the classes of ARVs. Briefly, ARVs are categorized into five groups
according to their mechanism of action: entry inhibitors, nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), and protease inhibitors (PIs).

Despite the fact that the ARVs have been proven to increase the life expectancy of PWH and improve their quality of lives, PWH continue to suffer multiple comorbidities. Importantly, 15-50% of the treated PWH may still develop neurocognitive impairment. HIV-associated neurocognitive disorders (HAND) that affects around 50% of PWH, includes asymptomatic neurocognitive impairment, mild neurocognitive impairment, and HIV-associated dementia. After the introduction of ART in 1996, the life expectancy of PWH has been prolonged and now is comparable to that of non-HIV infected population. However, the incidence of HAND remains similar to that observed before the introduction of ART, although the percentage of PWH with the most severe forms of HAND has decreased significantly (Figure 2) (11). However, PWH suffering from with less severe forms of HAND are at higher risk of developing the more severe forms in the future.
Figure 2. The prevalence of different stages of HAND before and after the introduction of ART.

Abbreviations: HAND, HIV associated neurocognitive disorders; HAD, HIV associated dementia; MND, Mild neurocognitive disorders; ANI, Asymptomatic neurocognitive impairment

HIV can only infect CD4+ T cells and myeloid cells (macrophages and microglia). The infection of HIV in microglia and macrophages in the brain is productive infection that result in the production of new virions as well as proinflammatory cytokines (21). HIV infection requires a CD4 receptor and a co-receptor, which could be CCR5 or CXCR4. Since oligodendrocytes and neurons lack these receptors, HIV cannot infect these cells. However, HIV infection can cause damage to these cells through the products released by the infected and/or activated macrophages and microglia (14).
Figure 3. Illustration of some of the co-morbidities that could be associated with the persistence of HAND in the ART era. These co-morbidities could include genetic predisposition, the HIV infection and the virus by-products, substance misuse, cardiovascular disease, aging and the use of ART. As a response to HAND our brain will activate many coping pathways which could include the oxidative stress, ISR. HAND also can lead to mitochondrial dysfunction.

Abbreviations: ISR, Integrated stress response.

HAND is a multifactorial condition: genetic predisposition, sequela of the HIV infection, and other comorbidities that can affect the CNS, such as substance misuse, cerebrovascular diseases, and old age, contribute to HAND (Figure 3). Several studies also show evidence that ART is a potential risk factor for the persistence of HAND. Studies have demonstrated a high degree of oxidative stress in the brains of PWH, one way to cope with
oxidative stress is the integrated stress response (ISR) (17). In our lab, we previously showed that the levels of several markers for ISR activation, such as phosphorylated eukaryotic initiation factor 2α (p-eIF2α), were elevated in autopsy brain tissue of PWH. Briefly, eIF2α is a translation initiation factor involved in cap-dependent protein translation, which when phosphorylated causes global translation attenuation. Phosphorylation of eIF2α is mediated by 4 kinases, which orchestrate the ISR. ISR activation has been observed in neurodegenerative conditions associated with chronic inflammation and oxidative stress. In general, ISR is an adaptive pathway. However, if chronically activated it can contribute to neuronal damage and death. Recently, studies in our group also showed that certain ARVs drugs might contribute to the persistence of HAND by chronic ISR induction (12-18). Our hypothesis is that ART contributes to ISR activation in neurons, astrocytes, and oligodendrocytes in PWH. This study aimed to assess ISR activation in neurons, astrocytes, and oligodendrocytes in a simian immunodeficiency virus (SIV) model of infection in rhesus macaques, with the overarching aim to determine if ART aggravates or induces p-eIF2α in neurons, astrocytes, and oligodendrocytes. This study was conducted to control all the possible contributing factors and to assess the effect the recommended drug combinations to gain a more solid and contemporary explanation of the effect of ART on different types of the CNS cells.
Materials and Methods

Chemicals and reagents:

The following reagents were used: 4',6-diamidino-2-phenylindole (DAPI); rabbit polyclonal p-eIF2α antibody (Thermo Fisher); mouse monoclonal microtubule-associated protein 2 (MAP2) antibody (Biolegend); rabbit polyclonal glial fibrillary acidic protein (GFAP) antibody (Dako; Carpinteria, CA); aspartoacylase (ASPA) antibody; and mouse monoclonal CD68 antibody (KP-1; Abcam). The following secondary antibodies were from Jackson ImmunoResearch, West Grove, PA: Cy3-conjugated goat anti-mouse, Cy5-conjugated goat anti-rabbit; biotin-conjugated goat anti-rabbit; FITC-conjugated streptavidin. Biotinyl tyramide, TNB buffer, amplification diluent, and HRP-conjugated streptavidin antibody were from Roche. Normal antibody diluent (NAD) and normal goat serum were from Thermo Fisher. Target antigen retrieval solution was from Dako.

Study samples

We used tissue samples of rhesus macaques infected with SIV, a commonly used model of HIV. The specimens, which were kindly provided by Dr. Shilpa Buch at The University of Nebraska, were frontal cortical tissue from rhesus macaques infected with SIV with or without ART. In this study, three groups were included. The control group included four animals treated only with saline, 3 times a day for 6.5 weeks. The second group included animals infected with SIV. In this group, the animals were treated with saline 4 times a day for 9 weeks
in the pre-infection period and followed up for 52 weeks after SIV infection. The progression of infection in this group was monitored via lumber puncture. The third group consisted of four animals that were treated with saline, 3 times a day for 8 weeks, in the pre-infection period. After SIV infection, this group was followed up for 58 weeks while treated with ART. The post-infection regimen for all four animals was tenofovir 30 mg/kg and FTC 30 mg/kg. The post-infection regimen started on week 30 after infection in two animals and on week 12 after infection in two animals. In all four animals, treatment was for 28 weeks, until euthanasia. The progression of infection in this group was also monitored via lumber puncture. At the end of the treatment period, all animals were euthanized. Frontal cortical tissues were prepared from fixed brains and paraffin-embedded to prepare 5-10-µm-thick slides (Table 1).

Table 1. Treatment groups

<table>
<thead>
<tr>
<th>Condition</th>
<th>SIV-uninfected/ART-untreated</th>
<th>SIV-infected/ART-untreated</th>
<th>SIV-infected/ART-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Treatment provided</td>
<td>Treated 3 times a day with saline for 6.5 weeks</td>
<td>Infected with SIV and followed up for 52 weeks</td>
<td>Treatment started by saline 3 times a day for 8 weeks, after SIV infection, the group was followed up for 58 weeks while treatment with ART was provided. The post-infection regimen in all 4 animals was tenofovir 30 mg/kg and FTC 30 mg/kg. The post-infection regimen started on week 30 post-infection in two animals and on week 12 on 2 animals. Animals were treated for 28 weeks until euthanasia</td>
</tr>
</tbody>
</table>
Immunofluorescence staining

Paraffin-embedded slides were deparaffinized using histoclear solution, rehydrated with graded ethanol solutions, and incubated in 3% hydrogen peroxide solution in methanol to block peroxidase activity. Antigen retrieval was performed by submerging the slides into target retrieval solution (Dako) and incubating in a hot water bath at 95°C for 60 minutes. Next, the slides were left to cool down for 30 minutes at room temperature (RT) and washed with phosphate-buffered saline (PBS) three times, five minutes each. The slides were processed for autofluorescence blocking using 1 mg/mL sodium borohydride incubation for 5 minutes. Then, the slides were washed three times with PBS and incubated in 10% normal goat serum for 30 minutes. Slides were then incubated in the primary antibody to p-eIF2α (1:500) overnight at 4°C. The next day the slides were washed again for 3 times 5 minutes each with PBS-T, and biotinyl tyramide amplification was performed. First, the slides were incubated in biotinylated goat anti-rabbit antibody (Jackson ImmunoResearch; 1:600) diluted in NAD for 30 minutes at RT. Next, the slides were washed three times in PBS-T and incubated in TNB buffer for at least 30 minutes at RT. The slides were incubated in HRP-conjugated streptavidin antibody at 1:400 in TNB buffer for another 30 minutes, and the slides were washed three times in PBS-T. After incubating the slides in biotinyl-tyramide at 1:100 in amplification diluent for 30 minutes, the slides were washed three times in PBS-T and were incubated in FITC-conjugated streptavidin antibody at 1:200 in TNB buffer for 30 minutes. After washing
with PBS-T, the slides were stained for MAP2 at 1:100 in NAD overnight at 4°C in the humidifier chamber and incubated in the secondary Cy3-conjugated goat anti-mouse antibody at 1:200 with DAPI in NAD for 30 minutes at RT. Following three washes, the slides were blocked with FELTRI buffer for 1 hour at RT and incubated in the primary antibody to ASPA at 1:250 in NAD overnight at 4°C. After washes with PBS-T, the slides were incubated in the secondary Cy5-conjugated goat anti-mouse antibody at 1:200 with DAPI added at 1:1000 in NAD for 30 minutes at RT. After washing with PBS-T 3 times 5 minutes each, the slides were mounted with coverslips using VectaShield vibrance antifade mounting medium.

A second experiment to study the effect of ART on astrocytes and microglia was conducted where the slides were stained for p-eIF2α, as described above, and the primary antibodies to GFAP (1:80) and CD68 (1:100) with Cy3- and Cy5-conjugated secondary antibodies, respectively.

After the completion of the experiments, the stained slides were imaged using the Keyence BZ-X710, an all-in-one florescence microscope, at 20x magnification. At least 10 fields from the white and grey matters were manually selected to avoid overlapping and images were captured using uniform settings. The Keyence quantification software was used to determine the intensity of fluorescence and the area of fluorescence in areas of interest in images, and all data were analyzed using one-way ANOVA.
Results

Neurons

Figure 4. Staining showing p-eIF2α (green), MAP2 (red), staining in merged images, and higher magnification images (focused) in sections from the grey matter of the cortex of the frontal lobe of rhesus macaques.

Magnification: 20X

To examine changes in ISR activation in neurons, we immunofluorescently stained sections of the frontal cortex from our cohort of animals with antibodies against p-eIF2α and MAP2 as well as DAPI. The semiquantitative analysis of the captured images revealed that p-eIF2α immunofluorescence in neurons was more prominent in the SIV-infected group compared to the control group. In addition, the p-eIF2α expression was higher in the SIV-infected/ART-treated group than in the SIV-infected/ART-untreated group (Figure 4). When the
intensity of p-eIF2α that colocalized with MAP2, which labels the neurons, was compared, no significant difference was observed among the three groups (Figure 5).

**Figure 5.** p-eif2α intensity colocalized with MAP2 staining in sections from the grey matter of the frontal lobe of rhesus macaques.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.

We found similar findings when the intensity of p-eIF2α that colocalized with MAP2 was normalized to the MAP2 area (Figure 6).

**Figure 6.** The intensity of P-eif2α normalized to MAP2 area in sections from frontal lobe matter of rhesus macaques.
Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.

However, the quantification of p-eIF2α expression in areas overlapping with MAP2, which was normalized to the number of neurons, revealed a statistically significant increase in p-eIF2α expression in the SIV-infected/ART-treated group than in the SIV-infected/ART-untreated group (Figure 7).

Figure 7. Quantification of p-eIF2α intensity in MAP2-positive areas, normalized to MAP2 cell number in sections from the grey matter of the frontal lobe of rhesus macaques. *** P < 0.001

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.
Astrocytes

**Figure 8.** Illustrates the staining of P-eIF2α (green) to show ISR activation, GFAP (red) to labels the astrocytes, DAPI (blue) a nuclear stating, and merged images of all staining, and a higher magnification sections (focused) in the three different conditions using sections from the grey matter from the frontal lobes of the treated animals. Magnification: 20X

To examine changes in ISR activation in astrocytes, we immunofluorescently stained sections of the frontal cortex from our cohort of animals with antibodies against p-eIF2α and GFAP as well as DAPI. The analysis of the images captured from the gray matter showed no noticeable differences in p-eIF2α expression among the three groups (Figure 8). The intensity of p-eIF2α that colocalized with GFAP, which labels astrocytes, did not significantly differ among the three groups (Figure 9).
Figure 9. p-eif2α intensity colocalized with GFAP staining in sections from the grey matter of the frontal lobe of rhesus macaques.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.

We found similar findings when the intensity of p-eif2α that colocalized with GFAP was normalized to the GFAP area (Figure 10) or the number of GFAP-positive cells (Figure 11).

Figure 10. The intensity of P-eif2α normalized to GFAP area in sections from the grey matter of the cortex of the frontal lobe of rhesus macaques.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy
Figure 1. The p-eIF2α expression overlapping with GFAP, when it was normalized to GFAP + cells number, using sections from the grey matter from the cortex of the frontal lobes of the treated animals.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.
Oligodendrocytes

<table>
<thead>
<tr>
<th>Saline</th>
<th>ASPA</th>
<th>DAPI</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Saline Image]</td>
<td>![ASPA Image]</td>
<td>![DAPI Image]</td>
<td>![Merged Image]</td>
</tr>
<tr>
<td>SV-infected</td>
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<tr>
<td>SV-infected / ART treated</td>
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**Figure 12.** Illustrates the staining of P-eIF2α (green) to show ISR activation, ASPA (white) to label the oligodendrocytes, DAPI (blue) a nuclear staining, and merged images of all staining in the three different conditions using sections from the white matter from the cortex of the frontal lobes of the treated macaques.

Magnification: 20X

When the oligodendrocytes were investigated for ISR activation by observing the phosphorylation of eIF2α using sections from the white matter of the frontal cortex, as shown in the representative images in Figure 12, the expression of p-eIF2α exhibited variability. The quantification of p-eIF2α that colocalized with APSA, which labels the oligodendrocytes, with and without normalization to the cell number or area, confirmed the lack of statistically significant difference among the three groups (Figures 13-15).
Figure 13. The intensity of p-eif2α colocalized with ASPA staining, to investigate the ISR activation in oligodendrocytes in sections from the white matter of the frontal lobe of rhesus macaques.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.

Figure 14. The intensity of P-eif2α normalized to ASPA area in sections from the cortex of the frontal lobe in the shite matter of rhesus macaques.

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy.
Figure 15. The p-eIF2α expression overlapping with ASPA, when it was normalized to ASPA + cells using sections from the white matter from the cortex of the frontal lobes of the treated macaques. Magnification: 20X

Abbreviations: SIV, Simian immunodeficiency virus; ART, Antiretroviral therapy
Discussion

In the pre-ART era, HAND was believed to be a result of HIV-related factors. However, HAND continues to persist after the introduction of ART, especially those including ARVs with great CNS penetration capacity. Recent studies show the potential toxicity of ART in the CNS. Some studies suggested that ART might cause oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction (16). In our lab, we found that certain ARVs had neurotoxic potential through specific cellular pathways that differed even between drugs belonging to the same ARV class(16). The current study explored the hypothesis that ART contributes to the persistence of HAND in PWH through the activation of the ISR. In this study, we compared the effect of ART in the CNS using SIV infection and ART treatment in rhesus macaques. Using immunofluorescence staining to study the effects of ART on specific cells in the CNS, we show that the ISR activation in neurons was more prominent in the SIV-infected/ART-treated group than in the SIV-infected/ART-untreated group and that the observed increase was statistically significant. This result corroborates our previous reports in studies using autopsy brain cortical tissue in PWH on ART (15, 16). However, we did not find an increase in ISR activation in astrocytes or oligodendrocytes in SIV-infected/ART-treated animals.

While we corroborated our previous studies on ISR activation in neurons, we did not observe augmentation of ISR activation in astrocytes or oligodendrocytes in the presence of
ART. This could be due to the small sample size, and future research should include more animals in each group. In addition, we did not have a control group of animals that only received ART. Having this control would give us a clearer observation of the effect of ART without the effect of SIV in the investigated cell types. Moreover, the ART regimen used in the present cohort included two NRTIs, without the presence of INSTIs, which are first-line ARVs; the impact of ARVs on specific cell types of the CNS might be class-dependent. Future investigations that compare the effect of different ARV classes in CNS cells in vivo are warranted to explore this possibility. It is also worth mentioning that we were only able to study the effect of ART on oligodendrocytes in the white matter; due to the small cell size in the gray matter prevented more in-depth analysis.

Our results show a possible association between ART and ISR activation specifically in neurons, which could partly explain the persistence of HAND despite effective ART. Our findings suggest that treatment regimens may also contain adjunctive therapies to help downregulate the phosphorylation of the eIF2α, thus controlling ISR activation. For example, eIF2β controls the phosphorylation of eIF2α, thus downregulating ISR activation. In this context, Calico Labs developed a compound called 2BAct, which could prevent neurological defects caused a chronic ISR via the activation of eIF2β (35). Therefore, future studies should be conducted to investigate the efficacy of such compounds as adjunctive therapy to improve the neurocognitive health of PWH treated with ART.
Conclusion

The introduction of ART has changed the HIV epidemic, but the potential toxicity of ART in the CNS remains a concern. This study supports the hypothesis that ART may be considered as a contributary factor to the persistence of HAND in ART-treated PWH via ISR induction. Our results confirmed the significant increase in ISR activation in frontal cortex neurons in SIV-infected/ART-treated rhesus macaques compared to SIV-infected/ART-untreated and SIV-uninfected/ART-untreated rhesus macaques. We did not find a similar augmentation of ISR activation in astrocytes in the gray matter or oligodendrocytes in the white matter. Despite the study limitations, our findings suggest the use of adjunctive therapies that can attenuate ISR activation to limit neuronal damage and aid in controlling the development of HAND in PWH treat with ART.
Bibliography

6. Types of glia. The University of Queensland.